Laboratory Safety

INTRODUCTION

The following sections do not contain complete listings of all the elements involved in laboratory safety. These precautionary notes serve as a reminder of possible hazards involved in the use of particular operations or substances, especially those items and materials frequently used in AOCS methods. The user of these methods should refer to standard texts on laboratory safety for a more complete treatment of the subject. Follow safety requirements and rules issued by voluntary organizations and government agencies [Occupational Safety and Health Administration (OSHA), in particular] expert in the field of laboratory safety.

EQUIPMENT

1. Blenders, grinders, electrical equipment—Motors on high-speed blenders used to mix flammable solvents with other material should be explosion-proof. Blend toxic or flammable liquids in effective fume-removal device. Accidents involving electrical equipment may result in mechanical injury, e.g., fingers being caught in chopping mill knives or grinders; electrical shock, which may be due to lack of or improper grounding, defective equipment, exposed wiring, or inadequate maintenance; and fire through ignition of flammable vapors by electrical sparks. Ground all electrical equipment to avoid shocks. Installation, maintenance, and repair operations should be performed by qualified electricians.
2. Atomic absorption spectrophotometer—Use effective fume-removal device to remove gaseous effluents from burner. Use specially designed exhausts when nitrous oxide (N_2O) is used as a fuel oxidant. If instrument has drain trap, check to ensure it is filled with water before igniting burner. Explosions of fuel gas accumulated through this drain have been reported.
3. Compressed gas cylinders—Identify contents (by means of attached decal, stencil or tag) of compressed gas cylinders by name of gas contained in the cylinder rather than by color codes. Secure cylinders in upright position by means of strap, chain, or nontip base. Use only correct pressure gages, pressure regulator, and flow regulator for each size of gas cylinder and type of gas, as specified by supplier. Use toxic gases only in effective fume-removal device. When burning gas or performing a reaction, use back flow prevention device in gas line to prevent flame or reaction from being sucked back into cylinder.
4. Distillations, extractions, evaporations—For flammable liquids, perform operations behind safety barrier with hot water, steam, or electric mantel heating. Use effective fume-removal device to remove flammable vapors as they are produced. Set up apparatus on firm supports and secure all connections. Leave ample headroom in flask and add boiling chips before heating is begun. All controls, unless vapor sealed, should be located outside vapor area. While awaiting disposal, store waste flammable solvents in explosion-proof containers. For toxic liquids, use effective fume-removal device to remove toxic vapors as they are produced. Avoid contact with skin and inhalation of vapors. Set up apparatus on firm supports and secure all connections. Store and dispose of toxic solvents in the manner prescribed by the Environmental Protection Agency (EPA) and OSHA.
5. Vacuum—Tape, or shield with safety barrier, any apparatus to be used under vacuum to minimize effects of possible implosion. Vacuum pump drive belts must have effective guards.

ACIDS

Use effective acid-resistant fume-removal device whenever heating acids or performing reactions which liberate acid fumes. When diluting acids, always add acid to water, unless otherwise directed in a method. Keep acids off skin, and protect eyes when working with acids. If acids come in contact with skin or eyes, wash immediately with large amounts of water. Do not store oxidizing acids (perchloric, nitric, sulfuric) near organic materials. Mixing organic materials with these acids, particularly perchloric, could result in an explosion.

ALKALIES

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, like sodium hydroxide and potassium hydroxide, always add pellets to water and not vice versa. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

SOLVENTS

Vapors from some volatile solvents are highly toxic. Several of these solvents are readily absorbed through the skin. Do not let vapors concentrate to a flammable level in the work area, because it is nearly impossible to eliminate all chances of sparks from static electricity, even though electrical equipment is grounded. Use effective fume-removal device to remove solvent vapors as they are liberated.

1. Acetone—Highly flammable. Forms explosive peroxides with oxidizing agents. Use effective fume-removal device. Do not mix with chloroform.

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2. Acetonitrile—Flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.
3. Aniline—An allergen. Toxic if absorbed through the skin. The TLV in air is 2 ppm.
4. Benzene—Benzene is highly toxic and highly flammable. Avoid contact with the skin. Do not breathe vapors. Use effective fume-removal device. Decomposes violently in the presence of strong oxidizing agents. Reacts violently with chlorine. Benzene is a cancer-causing agent.
5. Carbon disulfide—A poison, being harmful either by inhalation, prolonged or repeated skin contact, or by ingestion. Chronic poisoning may ensue from repeated exposure to vapor. It is highly flammable, a dangerous fire and explosion risk, and can be ignited by friction. Extreme precautions should be taken when using this solvent. A fume hood should be used at all times when handling this solvent.
6. Carbon tetrachloride—Carbon tetrachloride is a known carcinogen. It is toxic by ingestion, inhalation, and skin absorption. It is a narcotic. It should not be used to extinguish fires. It decomposes to phosgene gas at high temperature. The TLV is 5 ppm in air. It reacts violently with alkali metals. Fumes may decompose to phosgene gas when heated strongly. Use effective fume-removal device.
7. Chlorobenzene—A moderate fire risk. Explosive limits are 1.8–9.6% in air. Avoid inhalation and skin contact. The TLV is 75 ppm in air. Chlorobenzene and trichlorobenzene are toxic by ingestion and inhalation.
8. Chloroform—Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature, forming phosgene gas when heated to decomposition temperatures. Can react explosively with aluminum, lithium, magnesium, sodium, potassium, disilane, N_2O_4, and sodium hydroxide + methanol. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.
9. Cylcohexane—Highly flammable. Use effective fume-removal device. Can react vigorously with strong oxidizing agents.
10. Diethyl ether—Extremely flammable and a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. Store protected from the light. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. Can react explosively when in contact with chlorine, ozone, lithium aluminum hydride, or strong oxidizing agents. A fume hood should be used at all times when using ethyl ether. Avoid static electricity.
11. Dimethylformamide—A strong irritant to skin and tissue. It is toxic by skin absorption. It is a moderate fire risk. The TLV is 10 ppm in air.
12. Ethanol (ethyl alcohol)—Flammable. Use effective fume-removal device when heating or evaporating.
13. Gossypol—Toxic by ingestion. Avoid contact with particulate matter when working with standards. It is inactivated by heat.
14. Hexane—Hexane is a flammable solvent and a dangerous fire risk. All work should be performed in a fume hood, with no open flames. Always ensure that your hood is functioning before introducing volatile solvents. The TLV for hexane is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.
15. Methanol (methyl alcohol)—Flammable. Toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform, and perchloric acid.
16. Methylene chloride—Methylene chloride is toxic and a carcinogen. The TLC is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.
17. Methyl isobutyl ketone (MIBK)—Flammable and dangerous fire risk. Explosive limits in air are 1.4–7.5%. Avoid inhalation and ingestion. It is absorbed by the skin. The TLV is 50 ppm in air.
18. Petroleum ether—Extremely flammable. Use effective fume-removal device. Avoid static electricity.
19. Pyridine—Flammable and a dangerous fire risk. The explosive limits in air are 1.8–12.4%. It is toxic by ingestion and inhalation. The TLV is 5 ppm in air.
20. Tetrachloroethylene (perchloroethylene)—An irritant to eyes and skin. The TLV is 50 ppm in air.
21. Tetrahydrofuran—Tetrahydrofuran is flammable and a dangerous fire risk. The flammable limits in air are 2–11%. It is toxic by ingestion and inhalation. The TLV in air is 200 ppm.
22. Toluene—Flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.
23. Xylene—Flammable and a dangerous fire risk. The TLV is 100 ppm in air.

CHEMICALS

1. Hydrazine sulfate—Hydrazine sulfate can cause eye, skin, and mucous membrane irritation and liver and kidney damage. This compound is a known carcinogen in laboratory animals, causing lung and liver tumors in rats. It is a suspected human carcinogen. Precautions should be taken in handling this compound—use gloves, eye protection, and respiratory protection. Avoid the inhalation of dust and powder. Dispose of waste material and waste solutions in a proper and safe manner.
2. Lead acetate—Lead acetate is toxic by ingestion, inhalation, and skin absorption.

3. Mercury—Mercury vapors and compounds are extremely toxic and cumulative. Hazardous in contact with ammonia, halogens, and alkali. Regard spills on hot surfaces as extremely hazardous and clean up promptly. Powdered sulfur sprinkled over spilled mercury can assist in cleaning up spills. High degree of personal cleanliness is necessary for persons who use mercury. Handle only in locations that can be readily and completely cleaned up. When mercury evaporation is required, use effective fume-removal device. To avoid environmental contamination, dilute liquid remaining in Kjeldahl digestion flasks to about 300 mL with water, cool to room temperature, and add 50 mL 30% hydrogen peroxide. (If Raney powder method is used, 6 mL of hydrogen peroxide is sufficient.) Warm gently to initiate reaction, let reaction go to completion in warm flask, and separate precipitated mercuric sulfide. Reserve precipitate in closed, labeled container for recovery of mercury or disposal by EPA requirements.
4. Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

REFERENCES

1. *Official Methods of Analysis,* Association of Official Analytical Chemists, 14th edn., 1984, pp. 1010–1015.
2. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* 7th edn., Blackwell Scientific Publications, 1987.
3. *Hawley's Condensed Chemical Dictionary,* 11th edn., revised by N. I. Sax and R. J. Lewis, Jr., 1987.

2013 Table of Contents

Product-Method List by Section

Section A: Vegetable Oil Source Materials	Method number	Latest issue
Cottonseed		
Sampling	Aa 1	–38 (11)
Foreign Matter	Aa 2	–38 (09)
Moisture and Volatile Matter	Aa 3	–38 (09)
Oil Content	Aa 4	–38 (12)
Protein	Aa 5	–38 (89) *
Protein	Aa 5	–91 (11)
Free Fatty Acids	Aa 6	–38 (09)
Residual Lint	Aa 7	–55 (09)
Aflatoxins	Aa 8	–83 (09)
Aflatoxins, Confirmation by TLC	Aa 9	–86 (09)
Aflatoxins in Corn and Peanut by TLC	Aa 9a	–97 (09)
Aflatoxin Standards (preparation)	Aa 10	–88 (09)
Aflatoxin Detection by PHRED	Aa 11	–05 (11)
Peanuts (Groundnuts)		
Sampling	Ab 1	–49 (09)
Moisture and Volatile Matter	Ab 2	–49 (09)
Oil Content	Ab 3	–49 (09)
Protein	Ab 4	–50 (89) *
Protein	Ab 4	–91 (11)
Free Fatty Acids	Ab 5	–49 (09)
Aflatoxins	Ab 6	–68 (09)
Aflatoxins (affinity column)	Ab 7	–91 (09)
Also see	Aa 9	–86 (09)
Also see	Aa 10	–88 (09)
Soybeans		
Sampling	Ac 1	–45 (09)
Moisture and Volatile Matter	Ac 2	–41 (09)
Oil Content	Ac 3	–44 (11)
Protein	Ac 4	–41 (89) *
Protein	Ac 4	–91 (11)
Free Fatty Acids	Ac 5	–41 (13)
Tung Fruit		
Sampling	Ad 1	–48 (09)
Moisture and Volatile Matter	Ad 2	–52 (11)
Oil Content (whole tung fruit)	Ad 3	–52 (09)
Physical Analysis (kernel content)	Ad 4	–52 (09)
Oil Content (tung kernels)	Ad 5	–52 (09)
Oil Content (hulled tung fruit)	Ad 6	–52 (09)
Castor Beans		
Moisture and Volatile Matter	Ae 2	–52 (09)
Oil Content	Ae 3	–52 (09)
Free Fatty Acids	Ae 4	–52 (09)
Flaxseed		
Sampling	Af 1	–54 (93) *
Moisture and Volatile Matter	Af 2	–54 (97) *
Oil Content	Af 3	–54 (95) *
Safflower Seed		
Oil Content	Ag 1	–65 (09)
Copra		
Aflatoxins	Ah 1	–72 (09)

*Surplus method

	Method number	Latest issue
Sunflower Seed		
Sampling	Ai 1	–80 (93) *
Moisture and Volatile Matter	Ai 2	–75 (09)
Oil Content	Ai 3	–75 (99) *
Protein	Ai 4	–75 (89) *
Protein	Ai 4	–91 (11)
Corn		
Aflatoxins	Aj 1	–86 (09)
Zearalenone and α-Zearalenol	Aj 2	–86 (09)
Aflatoxins in Corn	Aj 3	–87 (09)
Also see Aflatoxins (affinity column)	Ab 7	–91 (09)
Oil in Corn Germ	Aj 4	–89 (09)
Fumonisins B_1 and B_2 in Corn by LC	Aj 5	–93 (09)
Fumonisins B_1, B_2, and B_3 in Corn by LC	Aj 5a	–97 (09)
Aflatoxins B_1, B_2 and G_1 in Corn, Cottonseed, Peanuts, and Peanut Butter	Aj 6	–95 (09)
Aflatoxins B_1, B_2, and G_1 in Corn by ELISA	Aj 6a	–97 (09)
Rapeseed		
Glucosinolates	Ak 1	–92 (11)
Chlorophyll	Ak 2	–92 (09)
Oil Content by NMR (and other oilseeds)	Ak 3	–94 (09)
Simultaneous Determination of Oil and Moisture		
Contents of Oilseeds Using Pulsed NMR Spectroscopy	Ak 4	–95 (11)
Oil and Moisture in Oilseed Residues by NMR	Ak 5	–01 (09)
Near Infrared Reflectance	Am 1	–92 (09)
Near Infrared Spectroscopy Instrument Management and Prediction Model Development	Am 1a	–09 (09)
Oil in Oilseeds (FOSFA Method)	Am 2	–93 (11)
Oil in Oilseeds: Supercritical Fluid Extraction Method	Am 3	–96 (09)
Impurities in Oilseeds	Am 4	–02 (09)
Oil Extraction by Filter Bag Technology	Am 5	–04 (09)

Section B: Oilseed By-Products

Cake, Meal, Meats, and Pellets		
Sampling	Ba 1	–38 (09)
Moisture and Volatile Matter (forced draft oven)	Ba 2a	–38 (09)
Moisture and Volatile Matter (vacuum oven)	Ba 2b	–82 (11)
Oil Content	Ba 3	–38 (09)
Protein	Ba 4a	–38 (11) *
Protein, Copper Sulfate Catalyst	Ba 4b	–87 (90) *
Protein, Kjel-Foss Automatic	Ba 4c	–87 (89) *
Protein, Titanium Dioxide + Copper Sulfate Catalyst	Ba 4d	–90 (11)
Protein Nitrogen by Combustion	Ba 4e	–93 (11)
Soybean Meal Protein by Combustion	Ba 4f	–00 (11)
Ash	Ba 5a	–49 (09)
Acid-Insoluble Ash	Ba 5b	–68 (09)
Crude Fiber	Ba 6	–84 (09)
Crude Fiber by Filter Bag Technology	Ba 6a	–05 (09)
Free Gossypol (cottonseed products)	Ba 7	–58 (09)
Free Gossypol (rapid method)	Ba 7b	–96 (11)
Total Gossypol (cottonseed products)	Ba 8	–78 (11)
Gossypol by HPLC	Ba 8a	–99 (11)
Urease Activity (soybean meals, flours, and mill feed)	Ba 9	–58 (09)
Protein Dispersibility Index (PDI) (soybean products)	Ba 10	–65 (09) *
Protein Dispersibility Index (PDI) (Revised)	Ba 10a	–05 (09)
Protein Dispersibility Index (PDI) (Omni Mixer)	Ba 10b	–09 (11)
Nitrogen Solubility Index (NSI) (soybean products)	Ba 11	–65 (09)
Trypsin Inhibitor Activity (soybean products)	Ba 12	–75 (09)
Free Hexane Content in Extracted Meals	Ba 13	–87 (11)
Total Hexane Content in Extracted Meals	Ba 14	–87 (11)
Linters and Hull Fiber		
Sampling	Bb 1	–38 (09)

*Surplus method

	Method number	Latest issue
Oil Content (cottonseed hulls)	Bb 2	–38 (09)
Cellulose Yield	Bb 3	–47 (12)
Soy Flour		
Sampling	Bc 1	–50 (09)
Moisture and Volatile Matter	Bc 2	–49 (09)
Oil Content	Bc 3	–49 (09)
Protein	Bc 4	–49 (89) *
Protein, Titanium Dioxide + Copper Sulfate Catalyst	Bc 4	–91 (11)
Ash	Bc 5	–49 (09)
Crude Fiber	Bc 6	–49 (09)
Screen Test	Bc 7	–51 (09)
Castor Pomace		
Moisture and Volatile Matter	Bd 2	–52 (09)
Oil Content	Bd 3	–52 (09)
Section C: Commercial Fats and Oils		
Sampling	C 1	–47 (09)
Moisture		
Distillation Method	Ca 2a	–45 (09)
and Volatile Matter, Hot Plate Method	Ca 2b	–38 (09)
and Volatile Matter, Air Oven Method	Ca 2c	–25 (09)
and Volatile Matter, Vacuum Oven Method	Ca 2d	–25 (09)
Modified Karl Fischer Method	Ca 2e	–84 (09)
Modified Moisture and Volatiles	Ca 2f	–93 (09)
Insoluble Impurities	Ca 3a	–46 (11)
Hexane Residues in Fats and Oils	Ca 3b	–87 (11)
Volatile Organic Contaminants by GC/MS	Ca 3c	–01 (09)
Sediment in Fats and Oils by Centrifugation	Ca 3d	–02 (11)
Visible Foots in Fats and Oils	Ca 3e	–02 (11)
Soluble Mineral Matter and Fatty Acids Combined as Mineral Soap	Ca 4	–25 (09)
Free Fatty Acids	Ca 5a	–40 (12)
Crude Fatty Acids (total fatty acids plus unsaponifiables)	Ca 5b	–71 (11)
Butyric Acid	Ca 5c	–87 (09) *
Free Fatty Acids in Crude Vegetable Oils	Ca 5d	–01 (11)
Butyric Acid	Ca 5e	–13 (13)
Unsaponifiable Matter		
Except Marine Oils	Ca 6a	–40 (11)
Including Marine Oils	Ca 6b	–53 (11)
Hydrocarbons (mineral oil) in Fats	Ca 6c	–65 (09)
Detection of Chlorinated Solvents		
Beilstein Test	Ca 7	–35 (90) *
Detection of Sulfur		
Coin Test	Ca 8a	–35 (90) *
Silver Benzoate Test	Ca 8b	–35 (90) *
Refining Loss	Ca 9a	–52 (11)
Crude Oils	Ca 9a	–52 (11)
Extracted Soybean Oil	Ca 9a	–52 (11)
Degummed, Hydraulic, and Extracted Soybean Oil	Ca 9a	–52 (11)
Degummed, Expeller Soybean Oil	Ca 9a	–52 (11)
Extracted and Reconstituted Prepressed Cottonseed Oils	Ca 9a	–52 (11)
Neutral Oil and Loss	Ca 9f	–57 (09)
Break Test (crude soybean oil)	Ca 10	–40 (89) *
Ash	Ca 11	–55 (09)
Phosphorus (colorimetric)	Ca 12	–55 (09)
Phosphorus (ISO Method)	Ca 12a	–02 (09)
Phosphorus by AAS	Ca 12b	–92 (09)
Gossypol, Total (crude cottonseed oil and soap stock)	Ca 13	–56 (13)
Glycerol; Total, Free, and Combined	Ca 14	–56 (11)
Glycerol, Free, by HPLC/ELSD	Ca 14b	–96 (09)

*Surplus method

	Method number	Latest issue
Trace Metals by AAS	Ca 15	–75 (09)
Sodium and Calcium by AAS	Ca 15b	–87 (09)
Polyethylene in Fats and Oils	Ca 16	–75 (11)
Trace Elements in Oil by ICP-OES	Ca 17	–01 (09)
Trace Metals, AAS with Graphite Furnace	Ca 18	–79 (09)
Trace Metals, AAS with Graphite Furnace	Ca 18b	–91 (09)
Lead, AAS with Graphite Furnace	Ca 18c	–91 (09)
Cadmium by AAS with Graphite Furnace	Ca 18d	–01 (09)
Phospholipids in Vegetable Oil	Ca 19	–86 (09)
Phosphorus in Oil by ICP-OES	Ca 20	–99 (09)
Detection of Cottonseed Oil, Halphen Test	Cb 1	–25 (09)
Detection of Sesame Oil, Modified Villavecchia Test	Cb 2	–40 (09)
Detection of Teaseed Oil, Fitelson Test	Cb 3	–39 (09)
Identification of Oils, Modified Crismer Test	Cb 4	–35 (09)
Detection of Foreign Fats in Pork Fat	Cb 5	–40 (93) *
Aflatoxins M_1 and M_2 in Dairy Products	Cb 6	–86 (09)
Melting Point		
Capillary Tube Method	Cc 1	–25 (09)
Wiley Method	Cc 2	–38 (91) *
Slip Melting Point of Hard Fats (palm oil), AOCS Standard	Cc 3	–25 (09)
Slip Melting Point, ISO Standard	Cc 3b	–92 (09)
Slip Point (soft fats)	Cc 4	–25 (89) *
Flow Test	Cc 5	–25 (89) *
Cloud Point Test	Cc 6	–25 (09)
Refractive Index	Cc 7	–25 (09)
Bleaching Test		
Cottonseed Oil (refined)	Cc 8a	–52 (12)
Soybean and Sunflower Oil	Cc 8b	–52 (11)
Refined and Bleached Color (tallow and greases for soaps)	Cc 8d	–55 (09)
Modified Bleach Test for Soybean Oil	Cc 8e	–63 (09)
Evaluation of Bleaching Clays and Absorbents	Cc 8f	–91 (09)
Smoke, Flash, and Fire Points; Cleveland Open Cup Method	Cc 9a	–48 (09)
Flash Point, Closed Cup Method	Cc 9b	–55 (11)
Flashpoint by Pensky-Martens Closed Cup Flash Tester	Cc 9c	–95 (09)
Specific Gravity of Oils and Liquid Fats	Cc 10a	–25 (95) *
Specific Gravity of Solid Fats and Waxes	Cc 10b	–25 (09)
Determination of Mass per Unit Volume ("Liter Weight") in Air of Oils and Fats	Cc 10c	–95 (09)
Cold Test	Cc 11	–53 (09)
Titer Test (for oils)	Cc 12	–59 (09)
Color		
FAC Method	Cc 13a	–43 (09)
Wesson Method (AOCS Lovibond)	Cc 13b	–45 (09)
Spectrophotometric Method	Cc 13c	–50 (09)
Chlorophyll Pigments (refined and bleached oils)	Cc 13d	–55 (09)
Lovibond (per ISO standard)	Cc 13e	–92 (09)
Refined and Bleached Saponification Color	Cc 13f	–94 (09)
High Quality Tallow and Coconut Oil Saponification Color	Cc 13g	–94 (09)
Tallow and Coconut Oil Saponification Color	Cc 13h	–94 (09)
Chlorophyll Pigments (crude vegetable oils)	Cc 13i	–96 (13)
Automated Method	Cc 13j	–97 (09)
Chlorophyll Pigments (crude vegetable oils, revised)	Cc 13k	–13 (13)
Congeal Point	Cc 14	–59 (09)
Soap in Oil, Conductivity Method	Cc 15	–60 (89) *
Consistency, Penetrometer Method	Cc 16	–60 (09)
Soap in Oil, Titrimetric Method	Cc 17	–95 (09)
Dropping Point	Cc 18	–80 (09)
Iodine Value (Wijs)	Cd 1	–25 (93) *
Iodine Value, Cyclohexane Method	Cd 1b	–87 (12)
Iodine Value (calculated from GLC)	Cd 1c	–85 (09)

*Surplus method

	Method number	Latest issue
Iodine Value, Cyclohexane-Acetic Acid Method	Cd 1d	–92 (09)
Iodine Value by NIR	Cd 1e	–01 (13)
Thiocyanogen Value	Cd 2	–38 (89) *
Saponification Value (oils and fats)	Cd 3	–25 (13)
Saponification Value, Calculated (oils and fats)	Cd 3a	–94 (09)
Saponification Value (deodorizer distillates and sludges)	Cd 3b	–76 (09)
Saponification Value, Modified Method Using Methanol	Cd 3c	–91 (09)
Acid Value of Fats and Oils	Cd 3d	–63 (09)
Alkalinity of Fats and Oils	Cd 3e	–02 (09)
Acetyl and Hydroxyl Values (by saponification)	Cd 4	–40 (99) *
Reichert-Meissl, Polenske, and Kirschner Values	Cd 5	–40 (09)
Liquid and Solid Fatty Acids	Cd 6	–38 (89) *
Polyunsaturated Acids	Cd 7	–58 (09)
Peroxide Value, Acetic Acid-Chloroform Method	Cd 8	–53 (03) *
Peroxide Value, Acetic Acid-Isooctane Method	Cd 8b	–90 (11)
Oxirane Oxygen	Cd 9	–57 (09)
Solid Fat Index	Cd 10	–57 (97) *
α-Monoglycerides	Cd 11	–57 (11)
Mono– and Diglycerides by Capillary GLC	Cd 11b	–91 (09)
Mono–, Di–, and Triglycerides by Silica Gel Chromatography	Cd 11c	–93 (09)
Mono– and Diglycerides by HPLC-ELSD	Cd 11d	–96 (09)
Fat Stability, Active Oxygen Method (AOM)	Cd 12	–57 (93) *
Fat Stability, Oil Stability Index (OSI)	Cd 12b	–92 (13)
Hydroxyl Value (direct titration)	Cd 13	–60 (09)
trans Isomers, Isolated (FTIR)	Cd 14	–95 (09)
trans Isomers in Partially Hydrogenated Oils by GLC-IR	Cd 14b	–93 (95) *
trans Isomers by Capillary GLC	Cd 14c	–94 (94) *
Also see	Ch 2a	–94 (09)
trans Isomers in Triglycerides by ATR/FTIR	Cd 14d	–99 (13)
Negative Second Derivative Spectroscopic Method for *trans* Fat	Cd 14e	–09 (09)
cis, cis Polyunsaturated Fatty Acids	Cd 15	–78 (09)
Solid Fat Content (SFC) by NMR (Indirect method)	Cd 16	–81 (13)
Solid Fat Content (SFC) by Low-Resolution NMR (Direct method)	Cd 16b	–93 (11)
trans Unsaturation in Margarine	Cd 17	–85 (93) *
p–Anisidine Value	Cd 18	–90 (11)
2–Thiobarbituric Acid (TBA) Value	Cd 19	–90 (09)
Polar Compounds in Frying Fats	Cd 20	–91 (09)
Benzo(a)pyrene in Edible Oils and Fats	Cd 21	–91 (09)
Polymerized Triglycerides by Gel-Permeation HPLC	Cd 22	–91 (09)
Pesticides in Oils and Fats	Cd 23	–93 (09)
Dimethylpolysiloxanes in Fats and Oils	Cd 24	–95 (09)
Dowtherm™ (heat transfer fluid in fats and oils) by GLC	Cd 25	–96 (09)
Dowtherm™ (heat transfer fluid in fats and oils) by HPLC	Cd 25a	–00 (09)
Thermal Heating Fluids in Vegetable Oils by GC	Cd 25b	–07 (09)
Stigmastadienes in Vegetable Oils	Cd 26	–96 (09)
Steroidal Hydrocarbons in Vegetable Oils	Cd 27	–96 (09)
Glycidyl Fatty Acid Esters in Edible Oils	Cd 28	–10 (12)
2- and 3-MCPDs and Glycidol by Acid Transesterification	Cd 29a	–13 (13)
2- and 3-MCPDs and Glycidol by Alkaline-Catalyzed Alcoholysis (low temperature method)	Cd 29b	–13 (13)
2- and 3-MCPDs and Glycidol by Alkaline-Catalyzed Alcoholysis	Cd 29c	–13 (13)
Fatty Acid Composition by GLC	Ce 1	–62 (09) *
Fatty Acid Composition by Capillary GLC	Ce 1a	–13 (13)
Marine Oil Fatty Acid Composition by GLC	Ce 1b	–89 (09)
Fatty Acid Composition by GLC, *cis, cis* and *trans* Isomers	Ce 1c	–89 (95) *
n–3 and n–6 Fatty Acids by Capillary GLC	Ce 1d	–91 (93) *
Fatty Acids by Capillary GLC	Ce 1e	–91 (01) *
cis and *trans* Fatty Acids in Oils and Fats by Capillary GLC	Ce 1f	–96 (09) *
trans Fatty Acids by Silver-Ion Exchange HPLC	Ce 1g	–96 (13)
Fatty Acids by Capillary GC for Nutritional Labeling	Ce 1h	–05 (09)

*Surplus method

	Method number	Latest issue
Fatty Acids in Marine and Other Oils Containing Long Chain PUFAs by GC	Ce 1i	–07 (09)
Fatty Acids in Extracted Fats by Capillary GLC	Ce 1j	–07 (13)
Fatty Acids by GC	Ce 1k	–09 (09) *
Direct Methylation of Lipids in Foods by Alkali Hydrolysis	Ce 2b	–11 (13)
Direct Methylation of Lipids in Foods by Acid-Alkali Hydrolysis	Ce 2c	–11 (13)
Preparation of Methyl Esters of Fatty Acids	Ce 2	–66 (09)
Glycidyl Fatty Acid Esters in Edible Oils	Cd 28	–10 (12)
Tocopherols and Sterols in Soya Residues by GLC	Ce 3	–74 (09)
Erucic Acid in Oils and Fats	Ce 4	–86 (95) *
Erucic Acid in High Erucic Acid Oils and Fats by Packed Column Gas-Liquid Chromatography	Ce 4	–95 (97) *
Triglycerides by Gas Chromatography	Ce 5	–86 (09)
Triglycerides by HPLC	Ce 5b	–89 (11)
Individual Triglycerides by HPLC	Ce 5c	–93 (11)
Antioxidants by HPLC	Ce 6	–86 (09)
Tocopherols (total) in Deodorizer Distillate	Ce 7	–87 (09)
Tocopherols and Tocotrienols in Vegetable Oils and Fats by HPLC	Ce 8	–89 (13)
α-Carotene in Margarines	Ce 9	–01 (09)
Non-Cocoa-Butter Fats by Sterol Degradation Products	Ce 10	–02 (09)
Cocoa Butter Equivalents in Cocoa Butter and Plain Chocolate	Ce 11	–05 (09)
Cocoa Butter Equivalents in Milk Chocolate by GC	Ce 11a	–07 (11)
Feed-Grade Fat Products	Cf 1	–68 (09)
Correlation of Oil Volatiles with Flavor Scores of Edible Oils	Cg 1	–83 (09)
Flavor Panel Evaluation of Vegetable Oils	Cg 2	–83 (09)
Oil Quality and Stability	Cg 3	–91 (09)
Volatiles (VOC) in Fats and Oils by GLC	Cg 4	–94 (09)
Oven Storage Test for Accelerated Aging of Oils	Cg 5	–97 (09)
Accelerated Light Exposure of Edible Vegetable Oils	Cg 6	–01 (09)
Antioxidant Analysis	Cg 7	–05 (09)
Olive Oil, Preparation of Methyl Esters of Long-Chain Fatty Acids	Ch 1	–91 (09)
Olive Oil, Fatty Acids by Capillary GLC	Ch 2	–91 (09)
trans Unsaturated Fatty Acids by Capillary Column GC	Ch 2a	–94 (11)
Olive Oil, Fatty Acids in the 2-Position in Triglycerides	Ch 3	–91 (11)
Olive Oil, Chlorophyll Pigments	Ch 4	–91 (13)
Olive Oil, Specific Extinction	Ch 5	–91 (09)
Olive Oil, Sterol Fraction by TLC and Capillary GLC	Ch 6	–91 (11)
Olive Oil, International Trade Standards	Ch 7	–09 (09)
Olive Oil, Wax Content	Ch 8	–02 (11)
Jojoba Oil, Methods for the Determination of Quality and Purity	Ci 1	–91 (09)
Jojoba Oil, Detection of Triglyceride Adulteration	Ci 2	–91 (09)
Jojoba Oil, Acid Value	Ci 4	–91 (09)
DSC Melting Properties of Fats and Oils	Cj 1	–94 (09)
X-Ray Diffraction Analysis of Fats	Cj 2	–95 (09)
Calibration of Rheological Instruments	Cj 3	–99 (09)
Spreadability	Cj 4	–00 (09)
Biodiesel Feedstock Quality	Ck 1	–07 (09)
Biodiesel Properties by the QTA® System	Ck 2	–09 (13)

Section D: Soap and Synthetic Detergents

Soap and Soap Products

Comparison of AOCS & ASTM Methods	D 1	–99 (09)
Sampling	Da 1	–45 (09)
Moisture and Volatile Matter, Air Oven Method	Da 2a	–48 (09)
Moisture, Distillation Method	Da 2b	–42 (09)
Alcohol-Soluble and Alcohol-Insoluble Matter	Da 3	–48 (11)
Free Acid or Free Alkali (soda soaps)	Da 4a	–48 (11)
Free Alkali and Potassium Carbonate (potash paste soaps)	Da 5	–44 (09)
Water-Insoluble Matter	Da 6	–48 (09)
Total Alkalinity of Alcohol-Insoluble Matter	Da 7	–48 (09)
Total Anhydrous Soap and Combined Alkali	Da 8	–48 (09)
Chlorides	Da 9	–48 (09)

*Surplus method

	Method number	Latest issue
Unsaponified Plus Unsaponifiable Matter	Da 10	–42 (09)*
Unsaponifiable Matter	Da 11	–42 (13)
Rosin, McNicoll Method	Da 12	–48 (09)
Titer Test	Da 13	–48 (09)
Acid Value of Fatty Acids	Da 14	–48 (09)
Iodine Value	Da 15	–48 (09)
Saponification Value	Da 16	–48 (09)
Borax	Da 17	–52 (09)
Alkaline Silicates	Da 18	–48 (09)
Carbonates, Gravimetric Absorption Method	Da 19a	–42 (09)
Carbonates, Volumetric Evolution Method	Da 19b	–42 (09)
Phosphates (gravimetric method)	Da 20a	–48 (09)
Phosphates (titrimetric method)	Da 20b	–57 (11)
Tetrasodium Pyrophosphate	Da 21	–48 (09)
Sulfates	Da 22	–48 (11)
Free Glycerol	Da 23	–56 (11)
Sugars	Da 24	–48 (09)
Starch	Da 25	–48 (09)
Volatile Hydrocarbons	Da 26	–42 (09)
Combined Sodium and Potassium Oxides	Da 27	–48 (09)
Screen Test	Da 28	–39 (09)
Copper	Da 31	–58 (09)
Soap Containing Synthetic Detergents		
Moisture and Volatile Matter	Db 1	–48 (09)
Alcohol-Soluble and Alcohol-Insoluble Matter	Db 2	–48 (09)
Free Acid or Free Alkali	Db 3	–48 (11)
Water-Insoluble Matter	Db 4	–48 (09)
Total Alkalinity of Alcohol-Insoluble Matter	Db 5	–48 (09)
Total Anhydrous Soap	Db 6	–48 (09)
Chlorides (indicator method)	Db 7	–48 (09)
Chlorides, Potentiometric Determination	Db 7b	–55 (09)
Saponification Value	Db 8	–48 (09)
Alkaline Silicates	Db 9	–48 (09)
Fatty Matter	Db 10	–48 (09)
Rosin, McNicoll Method	Db 11	–48 (09)
Fatty Alkyl Sulfates		
Sampling	Dc 1	–59 (11)
Moisture by Distillation Method	Dc 2	–59 (09)
Alcohol-Soluble Matter	Dc 3a	–59 (09)
Alcohol-Insoluble Matter	Dc 3b	–59 (09)
Ester SO_3	Dc 4	–59 (09)
Combined Alcohols	Dc 5	–59 (09)
Alkalinity	Dc 6	–59 (09)
Sodium Sulfate	Dc 7	–59 (09)
Unsulfated Material	Dc 8	–59 (09)
Alkylbenzene Sulfonates		
Sampling	Dd 1	–59 (11)
Moisture by Distillation Method	Dd 2a	–59 (09)
Modified Karl Fischer Reagent	Dd 2b	–59 (89) *
Sodium Alkylbenzene Sulfonate by Ultraviolet Absorption	Dd 3	–60 (09)
Neutral Oil (unsulfonated material)	Dd 4	–60 (09)
Color, Linear Alkylbenzene Sulfonates	Dd 5	–92 (09)

Section E: Glycerin

Sampling	Ea 1	–38 (09)
Ash	Ea 2	–38 (73) *
Alkalinity	Ea 2	–38 (73) *
Acidity	Ea 2	–38 (73) *
Sodium Chloride	Ea 2	–38 (73) *
Total and Organic Residue	Ea 3	–58 (73) *

*Surplus method

	Method number	Latest issue
Glycerol	Ea 6	–51 (12)
Crude Glycerin	Ea 6	–94 (13)
Specific Gravity	Ea 7	–95 (09)
Moisture	Ea 8	–58 (09)
Color (APHA scale)	Ea 9	–65 (09)

Section F: Sulfonated and Sulfated Oils

Moisture, Distillation Method	F 1a	–44 (09)
Moisture and Volatile Matter, Hot Plate Method	F 1b	–44 (09)
Organically Combined Sulfuric Anhydride		
Titration Method	F 2a	–44 (09)
Extraction Titration Method	F 2b	–44 (09)
Ash-Gravimetric Method	F 2c	–44 (09)
Total Desulfated Fatty Matter	F 3	–44 (09)
Total Active Ingredients	F 4	–44 (09)
Unsaponifiable Nonvolatile Matter	F 5	–44 (09)
Inorganic Salts	F 6	–44 (09)
Total Alkalinity	F 7	–44 (09)
Total Ammonia	F 8	–44 (09)
Acidity (sulfonated or sulfated oils, with exceptions)	F 9a	–44 (09)
Acidity (dark-colored oils, with exceptions)	F 9b	–44 (09)
Acidity (in the presence of ammonium or triethanolamine soaps)	F 9c	–44 (09)
Water-Immiscible Organic Solvents	F 10	–44 (09)

Section G: Soap Stocks

Sampling	G 1	–40 (09)
Total Fatty Acids, Wet Extraction Method	G 3	–53 (09)
Oxidized Fatty Acids, Wet Extraction Method	G 3	–53 (09)
Total Fatty Acids	G 4	–40 (09)
Neutral Oil	G 5	–40 (09)
Titer Test	G 6	–40 (09)
pH	G 7	–56 (09)

Section H: Specifications for Reagents, Solvents, and Apparatus

Forced Draft Oven	H 1	–39 (09)
Petroleum Ether	H 2	–41 (09)
Air Oven	H 3	–45 (09)
Vacuum Oven	H 4	–45 (09)
Thermometer Specifications	H 5	–40 (09)
Thermometer Specifications (see H 5-40)	H 6	–40 (09)
Thermometer Specifications (see H 5-40)	H 7	–45 (09)
Thermometer Specifications (see H 5-40)	H 8	–45 (09)
Desiccants	H 9	–87 (09)
Thermometer Specifications (see H 5-40)	H 10	–55 (09)
Thermometer Specifications (see H 5-40)	H 11	–58 (09)
Sodium Hydroxide, Standard Solution	H 12	–52 (09)
Sulfuric Acid, Standard Solution	H 13	–52 (09)
Hydrochloric Acid, Standard Solution	H 14	–52 (09)
Alcoholic Potassium Hydroxide, Standard Solution	H 15	–52 (09)
Commercial Hexane	H 16	–56 (09)
Synthetic Methyl Alcohol	H 17	–58 (09)
Isopropyl Alcohol	H 18	–58 (09)
Industrial Toluene	H 19	–58 (09)
Standard Test Sieves	H 20	–11 (11)

Section J: Lecithin

Moisture	Ja 2a	–46 (09)
Moisture, Karl Fischer Reagent	Ja 2b	–87 (09)
Hexane-Insoluble Matter	Ja 3	–87 (09)

*Surplus method

	Method number	Latest issue
Acetone-Insoluble Matter	Ja 4	–46 (11)
Phosphorus, Total	Ja 5	–55 (89) *
Acid Value	Ja 6	–55 (11)
Phospholipids in Lecithin Concentrates by TLC	Ja 7	–86 (09)
Phospholipids in Lecithin Concentrates by HPLC	Ja 7b	–91 (11)
Lecithin Phospholipids by HPLC-LSD	Ja 7c	–07 (13)
Peroxide Value	Ja 8	–87 (13)
Gardner Color	Ja 9	–87 (09)
Brookfield Viscosity	Ja 10	–87 (09)
Viscosity of Transparent Liquids by Bubble Time Method	Ja 11	–87 (09)
Lecithin Co-Products	Ja 12	–89 (11)
Tocopherols in Lecithin by HPLC	Ja 13	–91 (09)
Iodine Value in Lecithin, Wijs Method	Ja 14	–91 (09)

Section M: Evaluation and Design of Test Methods

Precision of Analytical Methods	M 1	–92 (09)
Writing and Approval of Methods	M 2	–09 (13)
Surplus Status of Methods	M 3	–82 (13)
Collaborative Study Procedures	M 4	–86 (12)
Approved Chemists, Criteria	M 5	–09 (11)
Certified Laboratories, Criteria	M 6	–09 (11)

Section S: Analytical Guidelines for Testing Industrial Oils and Derivatives

Commercial Fatty Acids	S 1	–64 (09)
Drying Oils	S 2	–64 (09)
Epoxidized Oils	S 3	–64 (11)
Fatty Nitrogen Products		
Fatty Amines	S 4a	–64 (09)
Fatty Amidoamines	S 4b	–64 (09)
Fatty Quaternary Ammonium Chlorides	S 4c	–64 (09)
Fatty Diamines	S 4d	–64 (09)
Polymerized Fatty Acids	S 5	–64 (09)

Section T: Test Methods for Industrial Oils and Derivatives

Sampling		
General	Ta 1	–09 (09)
Commercial Fatty Acids	Ta 1	–09 (09)
Epoxidized Oils	Ta 1	–09 (09)
Fatty Nitrogen Products	Ta 1	–09 (09)
Polymerized Fatty Acids	Ta 1	–09 (09)
Dibasic Acids	Ta 1e	–70 (09)
Moisture		
and Volatile Matter Fatty Acids (hot plate method)	Tb 1a	–64 (09)
Modified Karl Fischer Reagent (general)	Tb 2	–64 (97) *
Fatty Nitrogen Compounds (modified Karl Fischer reagent)	Tb 2a	–64 (89) *
Nonvolatiles (solids)		
Drying Oils (hot plate method)	Tc 1a	–64 (09)
Fatty Quaternary Ammonium Chlorides (vacuum oven)	Tc 2a	–64 (09)
Color		
Gardner	Td 1a	–64 (09)
Platinum-Cobalt Scale	Td 1b	–64 (09)
Commercial Fatty Acids	Td 2a	–64 (09)
Fatty Acids (after heating)	Td 3a	–64 (09)
Acid Value		
Commercial Fatty Acids	Te 1a	–64 (09)
Polymerized Fatty Acids	Te 1a	–64 (09)
Drying Oils	Te 2a	–64 (09)
Fatty Quaternary Ammonium Chlorides (including free amine value)	Te 3a	–64 (09)
Dibasic Acids	Te 4a	–70 (09)

*Surplus method

	Method number	Latest issue
Amine Value		
Total Amine Value of Fatty Amines (potentiometric method)	Tf 1a	–64 (09)
Total Amine Value of Fatty Amines (indicator method)	Tf 1b	–64 (09)
Primary, Secondary, and Tertiary Potentiometric Fatty Amines	Tf 2a	–64 (09)
Primary, Secondary, and Tertiary Indicator Fatty Amines	Tf 2b	–64 (09)
Percent Primary, Secondary, and Tertiary Amines in Fatty Acids	Tf 3a	–64 (09)
Composition		
Primary Amine Composition by GLC	Tf 4a	–77 (09)
Composition of Dimer Acids by HPLC	Tf 5	–91 (09)
Iodine Value		
Wijs Method (general)	Tg 1	–64 (12)
Commercial Fatty Acids, Wijs Method	Tg 1a	–64 (12)
Fatty Amines, Modified Wijs Method	Tg 2a	–64 (09)
Fatty Quaternary Ammonium Chlorides, Modified Wijs Method	Tg 3a	–64 (09)
Diene Value		
Drying Oils	Th 1a	–64 (11)
Conjugated Dienoic Acid		
Spectrophotometric Determination (dehydrated castor oil, fatty acids, and their methyl or ethyl esters)	Ti 1a	–64 (09)
Polyunsaturated Acids		
Spectrophotometric Method (commercial fatty acids)	Tj 1a	–64 (93) *
Unsaponifiable Matter		
Drying Oils	Tk 1a	–64 (09)
Commercial Fatty Acids	Tk 1a	–64 (09)
Polymerized Fatty Acids	Tk 1a	–64 (09)
Saponification Value	Tl 1a	–64 (09)
Ash		
Commercial Fatty Acids	Tm 1a	–64 (11)
Drying Oils	Tm 1a	–64 (11)
Fatty Quaternary Ammonium Chlorides	Tm 2a	–64 (09)
Flash and Fire Points		
Cleveland Open Cup (fatty acids)	Tn 1a	–64 (09)
Cleveland Open Cup (drying oils)	Tn 1a	–64 (09)
Closed Cup, Fatty Quaternary Ammonium Chlorides	Tn 2a	–86 (09)
Specific Gravity		
Commercial Fatty Acids	To 1a	–64 (11)
Drying Oils	To 1b	–64 (09)
Refractive Index		
Commercial Fatty Acids	Tp 1a	–64 (09)
Drying Oils	Tp 1a	–64 (09)
Viscosity		
Bubble Time Method (drying oils)	Tq 1a	–64 (11)
Titer		
Commercial Fatty Acids	Tr 1a	–64 (09)
Rosin Acids		
Commercial Fatty Acids	Ts 1a	–64 (09)
Tolerance Test		
Acetone (drying oils)	Tt 1a	–64 (09)
pH		
of Fatty Quaternary Ammonium Chlorides	Tu 1a	–64 (09)
Average Molecular Weight		
Fatty Quaternary Ammonium Chlorides	Tv 1a	–64 (09)
Nonamines		
Fatty Amines and Fatty Diamines	Tw 1a	–64 (09)
Hydroxyl Value of Epoxidized Oils	Tx 1a	–66 (09)
Dicarboxylic Acid Composition of Dimethyl Esters by GLC	Ty 1a	–76 (09)
Activity of Hydrogenation Catalysts	Tz 1a	–78 (09)
Selectivity of Hydrogenation Catalysts	Tz 1b	–79 (11)

*Surplus method

2013 Table of Contents

Alphabetical Index

	Method number	Latest issue
Accelerated Light Exposure of Edible Vegetable Oils	Cg 6	–01 (09)
Acetone–Insoluble Matter in Lecithin	Ja 4	–46 (11)
Acetone Tolerance of Heat–Bodied Drying Oils	Tt 1a	–64 (09)
Acetyl and Hydroxyl Values (by saponification)	Cd 4	–40 (99) *
Acid–Insoluble Ash in Oilseed Meal Products	Ba 5b	–68 (09)
Acid Value		
of Commercial Fatty Acids	Te 1a	–64 (09)
of Dibasic Acids	Te 4a	–70 (09)
of Drying Oils	Te 2a	–64 (09)
of Fats and Oils	Cd 3d	–63 (09)
of Fatty Acids	Da 14	–48 (09)
of Fatty Quaternary Ammonium Chlorides	Te 3a	–64 (09)
of Lecithin	Ja 6	–55 (11)
Acidity		
of Glycerin	Ea 2	–38 (73) *
of Sulfonated and Sulfated Oils, Dark Colored, with Exceptions	F 9b	–44 (09)
of Sulfonated and Sulfated Oils, in the Presence of Ammonium or Triethanolamine Soaps	F 9c	–44 (09)
of Sulfonated and Sulfated Oils, with Exceptions	F 9a	–44 (09)
Active Ingredients in Sulfonated and Sulfated Oils	F 4	–44 (09)
Active Oxygen Method (AOM) for Fat Stability	Cd 12	–57 (93) *
Activity of Hydrogenation Catalysts	Tz 1a	–78 (11)
Aflatoxins		
in Copra and Copra Meal	Ah 1	–72 (09)
in Corn	Aj 1	–86 (09)
in Corn (affinity column)	Ab 7	–91 (09)
in Corn by ELISA	Aj 6a	–97 (09)
in Corn (whole, cracked, meal, germ)	Aj 3	–87 (09)
in Corn and Peanut by TLC	Aa 9a	–97 (09)
in Corn, Zearalenone, and □–Zearalenol	Aj 2	–86 (09)
in Cottonseed, Seed Meal, Meats, Flakes	Aa 8	–83 (09)
in Dairy Products, M_1 and M_2	Cb 6	–86 (09)
in Peanut Butter (affinity column)	Ab 7	–91 (09)
in Peanuts (groundnuts), Raw (affinity column)	Ab 7	–91 (09)
in Peanuts (groundnuts), Raw, Roasted, Meal, Butter	Ab 6	–68 (09)
Aflatoxins B_1, B_2, and G_1 in Corn, Cottonseed, Peanuts, and Peanut Butter	Aj 6	–95 (09)
Aflatoxins, Confirmation by TLC	Aa 9	–86 (09)
Aflatoxin Standards (preparation)	Aa 10	–88 (09)
Aflatoxin Detection by PHRED	Aa 11	–05 (11)
Alcohol-Insoluble Matter in Fatty Alkyl Sulfates	Dc 3b	–59 (09)
Alcohol-Soluble and Alcohol–Insoluble Matter		
in Soap and Soap Products	Da 3	–48 (11)
in Soap Containing Synthetic Detergents	Db 2	–48 (09)
Alcohol-Soluble Matter in Fatty Alkyl Sulfates	Dc 3a	–59 (09)
Alkaline Silicates		
in Soap and Soap Products	Da 18	–48 (09)
in Soap Containing Synthetic Detergents	Db 9	–48 (09)
Alkalinity		
of Fats and Oils	Cd 3e	–02 (09)
of Fatty Alkyl Sulfates	Dc 6	–59 (09)
of Glycerin	Ea 2	–38 (73) *
of Sulfonated and Sulfated Oils	F 7	–44 (09)
Alkalinity of Alcohol–Insoluble Matter		
in Soda Soap and Products	Da 7	–48 (09)
in Soda Soap and Products Containing Synthetic Detergents	Db 5	–48 (09)

*Surplus method

	Method number	Latest issue
Amines, Tests		
for Fatty Quaternary Ammonium Chlorides	Te 3a	–64 (09)
for Primary Amines, by Gas Chromatography	Tf 4a	–77 (09)
for Primary, Secondary, and Tertiary Amines, Percent in Fatty Acids	Tf 3a	–64 (09)
Amine Value		
Fatty Quaternary Ammonium Chlorides	Te 3a	–64 (09)
Primary, Secondary, and Tertiary Indicator Fatty Amines	Tf 2b	–64 (09)
Primary, Secondary, and Tertiary Potentiometric Fatty Amines	Tf 2a	–64 (09)
Total Indicator Fatty Amines	Tf 1b	–64 (09)
Total Potentiometric Fatty Amines	Tf 1a	–64 (09)
Ammonia (total) in Sulfonated and Sulfated Oils	F 8	–44 (09)
Analytical Guidelines for Testing		
of Commercial Fatty Acids	S 1	–64 (09)
of Drying Oils	S 2	–64 (09)
of Epoxidized Oils	S 3	–64 (11)
of Fatty Amidoamines	S 4b	–64 (09)
of Fatty Amines	S 4a	–64 (09)
of Fatty Diamines	S 4d	–64 (09)
of Fatty Quaternary Ammonium Chlorides	S 4c	–64 (09)
for Feed–Grade Fat Products	Cf 1	–68 (09)
of Polymerized Fatty Acids	S 5	–64 (09)
Anhydrous Soap (total)	Db 6	–48 (09)
Anhydrous Soap and Combined Alkali (total)	Da 8	–48 (09)
Anisidine Value	Cd 18	–90 (11)
Antioxidant Analysis	Cg 7	–05 (09)
Antioxidants in Oils by HPLC	Ce 6	–86 (09)
AOCS & ASTM Methods for Soaps and Detergent Analysis	D 1	–99 (09)
Approved Chemists (Criteria)	M 5	–09 (11)
Ash		
in Drying Oils	Tm 1a	–64 (11)
in Fats and Oils	Ca 11	–55 (09)
in Fatty Acids	Tm 1a	–64 (11)
in Fatty Quaternary Ammonium Chlorides	Tm 2a	–64 (09)
in Glycerin	Ea 2	–38 (73) *
in Oilseed Cake and Meals	Ba 5a	–49 (09)
in Oilseed Meal, Acid–Insoluble	Ba 5b	–68 (09)
in Soya Flours/Canola Flours	Bc 5	–49 (09)
Beilstein Test	Ca 7c	–35 (90) *
Benzo(a)pyrene in Edible Oils and Fats	Cd 21	–91 (09)
Biodiesel Feedstocks	Ck 1	–07 (09)
Biodiesel Properties by the QTA* System	Ck 2	–09 (13)
Bleaching Test		
for Cottonseed Oil (refined)	Cc 8a	–52 (12)
for Evaluation of Clays and Absorbents	Cc 8f	–91 (09)
for Soybean Oil (refined)	Cc 8b	–52 (11)
for Soybean Oil (modified test)	Cc 8e	–63 (09)
for Sunflower Oil (refined)	Cc 8b	–52 (11)
Boemer Number for Detection of Foreign Fats in Pork Fat	Cb 5	–40 (93) *
Borax in Soap and Soap Products	Da 17	–52 (09)
Break Test (crude soybean oil)	Ca 10	–40 (89) *
Butyric Acid	Ca 5c	–87 (09) *
Butyric Acid	Ca 5e	–13 (13)
Butyric Acid (Kirschner value) in Butterfat and Coconut Oils	Cd 5	–40 (09)
Cadmium by AAS with Graphite Furnace	Ca 18d	–01 (09)
Carbonates		
by Gravimetric Absorption Method	Da 19a	–42 (09)
by Volumetric Evolution Method	Da 19b	–42 (09)
α-Carotene in Margarines	Ce 9	–01 (09)
Cellulose Yield	Bb 3	–47 (12)
Certified Laboratories (Criteria)	M 6	–09 (11)

*Surplus method

	Method number	Latest issue
Chlorides		
in Glycerin (crude)	Ea 2	–38 (73) *
in Soap and Soap Products	Da 9	–48 (09)
in Soap and Soap Products Containing Synthetic Detergents		
Indicator Method	Db 7	–48 (09)
Potentiometric Method	Db 7b	–55 (09)
Chlorinated Solvents (Beilstein Test)	Ca 7	–35 (90) *
Chlorophyll Pigments (rapeseed)	Ak 2	–92 (09)
Chlorophyll Pigments (refined and bleached oils)	Cc 13d	–55 (09)
Chlorophyll Pigments (crude vegetable oils)	Cc 13i	–96 (13)
Chlorophyll Pigments (crude vegetable oils)	Cc 13k	–13 (13)
Chromium, Trace Amounts		
by AAS	Ca 15	–75 (09)
by AAS with Graphite Furnace	Ca 18	–79 (09)
cis, cis and *trans* Isomers, Fatty Acid Composition by GLC	Ce 1c	–89 (95) *
cis, cis Polyunsaturated Fatty Acids	Cd 15	–78 (09)
Cloud Point Test	Cc 6	–25 (09)
Cocoa Butter Equivalents	Ce 11	–05 (09)
Cocoa Butter Equivalents in Milk Chocolate by GC	Ce 11a	–07 (11)
Cold Test	Cc 11	–53 (09)
Collaborative Study Procedures	M 4	–86 (12)
Color Measurement		
by APHA Scale for Glycerin	Ea 9	–65 (09)
by Color Stability for Fatty Acids (after heating)	Td 3a	–64 (09)
by Combined Alcohols in Fatty Alkyl Sulfates	Dc 5	–59 (09)
by Combined Alkali in Soap and Soap Products	Da 8	–48 (09)
by Combined Sodium and Potassium Oxides in Soap and Soap Products	Da 27	–48 (09)
by FAC Method	Cc 13a	–43 (09)
by Fatty Acids (commercial)	Td 2a	–64 (09)
by Gardner for Industrial Oils	Td 1a	–64 (09)
Color (Automated Method)	Cc 13j	–97 (09)
by Gardner for Lecithin	Ja 9	–87 (09)
by Linear Alkylbenzene Sulfonates (LAS)	Dd 5	–92 (09)
by Lovibond (per ISO Standard)	Cc 13e	–92 (09)
by Lovibond (Wesson)	Cc 13b	–45 (09)
by Platinum–Cobalt Scale for Light-Colored Liquids	Td 1b	–64 (09)
by Spectrophotometric Color Method for Oils	Cc 13c	–50 (09)
by Wesson (Lovibond)	Cc 13b	–45 (09)
Composition of Dibasic Acids by GLC	Ty 1a	–76 (09)
Composition of Fatty Acids by GLC	Ce 1	–62 (09) *
Composition of Primary Amines by GLC	Tf 4a	–77 (09)
Congeal Point	Cc 14	–59 (09)
Conjugated Dienoic Acid in Castor Oil and Acids	Ti 1a	–64 (09)
Consistency, Penetration Method	Cc 16	–60 (09)
Copper		
in Oils by AAS	Ca 15	–75 (09)
in Oils by AAS with Graphite Furnace	Ca 18	–79 (09)
in Soap and Soap Products	Da 31	–58 (09)
Cottonseed Oil, Detection in Oils by Halphen Test	Cb 1	–25 (09)
Crismer Test	Cb 4	–35 (09)
Crude Fatty Acids (total fatty acids plus unsaponifiables)	Ca 5b	–71 (09)
Crude Fiber		
in Grains, Meals, Flours, Feeds, and All Fiber–Bearing Materials		
Extraction by Filter Bag Technology	Ba 6a	–05 (09)
from Which Fat Can Be Extracted	Ba 6	–84 (09)
in Soya Flours	Bc 6	–49 (09)
Desiccants, Specifications	H 9	–87 (09)
Desulfated Fatty Matter, Total	F 3	–44 (09)
Dicarboxylic Acid, Composition by GLC	Ty 1a	–76 (09)
Diene Value of Drying Oils	Th 1a	–64 (11)

*Surplus method

	Method number	Latest issue
Dienoic Acids in Dehydrated Castor Oil and Acids	Ti 1a	–64 (09)
Dilatometric Method for Solid Fat Index	Cd 10	–57 (97) *
Dimer Acids, Composition by HPLC	Tf 5	–91 (09)
Dimethylpolysiloxanes in Fats and Oils	Cd 24	–95 (09)
Dowtherm™ (heat transfer fluid in fats and oils) by GLC	Cd 25	–96 (09)
Dowtherm™ (heat transfer fluid in fats and oils) by HPLC	Cd 25a	–00 (09)
Dropping Point	Cc 18	–80 (09)
DSC Melting Properties of Fats and Oils	Cj 1	–94 (09)
Epoxidized Fatty Materials	Cd 9	–57 (09)
Erucic Acid in High Erucic Acid Oils and Fats by Packed Column Gas–Liquid Chromatography	Ce 4	–95 (97) *
Erucic Acid in Oils and Fats	Ce 4	–86 (95) *
Essential Fatty Acids	Cd 15	–78 (09)
Ester SO_3 in Fatty Alkyl Sulfates	Dc 4	–59 (09)
Fat Stability, Active Oxygen Method (AOM)	Cd 12	–57 (93) *
Fat Stability, Oil Stability Index (OSI)	Cd 12b	–92 (09)
Fatty Acids		
by Capillary GLC	Ce 1e	–91 (01) *
cis, cis and *trans* Isomers by GLC	Ce 1c	–89 (95) *
Combined as Mineral Soaps in Fats	Ca 4	–25 (09)
Composition by GLC of Methyl Esters	Ce 1	–62 (09) *
Composition by Capillary GC for Nutritional Labeling	Ce 1h	–05 (09)
in Marine and Other Oils Containing Long Chain PUFAs by GC	Ce 1i	–07 (09)
Composition of Marine Oils	Ce 1b	–89 (09)
Extracted Fats by Capillary GLC	Ce 1j	–07 (13)
Crude (total fatty acids plus unsaponifiables)	Ca 5b	–71 (11)
Direct Methylation of Lipids in Foods by Acid-Alkali Hydrolysis	Ce 2c	–11 (13)
Direct Methylation of Lipids in Foods by Alkali Hydrolysis	Ce 2b	–11 (13)
Glycidyl Fatty Acid Esters in Edible Oil	Cd 28	–10 (12)
n-3 and n-6 by Capillary GLC	Ce 1d	–91 (93) *
Oxidized in Soap Stocks	G 3	–53 (09)
Separation of Liquid and Solid	Cd 6	–38 (89) *
Total in Coconut and Similar Soap Stock	G 4	–40 (09)
Total in Fats or Hydrolyzed Fats	Ca 5b	–71 (11)
Total in Soap Stocks Other Than Coconut	G 3	–53 (09)
trans Composition of Partially Hydrogenated Oils by GLC–IR	Cd 14b	–93 (95) *
trans Fatty Acids in Oils and Fats by Capillary GLC	Ce 1f	–96 (09) *
trans Fatty Acids (isolated) by FTIR	Cd 14	–95 (09)
trans Fatty Acids by Silver–Ion Exchange HPLC	Ce 1g	–96 (13)
Fatty Matter in Soaps	Db 10	–48 (09)
Fiber (crude)		
in Grains, Meals, Flours, Feeds	Ba 6	–84 (09)
Extraction by Filter Bag Technology	Ba 6a	–05 (09)
in Soya Flours	Bc 6	–49 (09)
Fitelson Test for Teaseed Oil	Cb 3	–39 (09)
Flash Point		
Cleveland Open Cup Method		
for Fats and Oils	Cc 9a	–48 (09)
for Fatty Acids and Drying Oils	Tn 1a	–64 (09)
Pensky–Martens Closed Cup Method		
for Fats and Oils	Cc 9b	–55 (11)
for Fatty Quaternary Ammonium Chlorides	Tn 2a	–86 (09)
Flashpoint by Pensky–Martens Closed Cup Flash Tester	Cc 9c	–95 (09)
Flavor		
Correlation of Volatiles with Flavor	Cg 1	–83 (09)
Panel Evaluation of Vegetable Oils	Cg 2	–83 (09)
Flow Test	Cc 5	–25 (89) *
Foots (Visible) in Fats and Oils	Ca 3e	–02 (11)
Foreign Fats in Pork Fat and Lard	Cb 5	–40 (93) *
Foreign Matter in Cottonseed	Aa 2	–38 (09)

*Surplus method

	Method number	Latest issue
Free Acid or Free Alkali		
in Soda Soap	Da 4a	–48 (11)
in Soda Soap Containing Synthetic Detergent	Db 3	–48 (11)
Free Fatty Acids		
in Castor Beans	Ae 4	–52 (09)
in Cottonseed	Aa 6	–38 (09)
in Crude and Refined Fats and Oils	Ca 5a	–40 (12)
in Crude Vegetable Oils by GC	Ca 5d	–01 (09)
in Peanuts (groundnuts)	Ab 5	–49 (09)
in Soybeans	Ac 5	–41 (09)
Fumonisins B_1 and B_2 in Corn by LC	Aj 5	–93 (09)
in Corn by LC	Aj 5a	–97 (09)
Glucosinolates (rapeseed)	Ak 1	–92 (11)
Glycerin, Total and Organic Residue	Ea 3	–58 (73) *
Glycerine, Free, by HPLC/ELSD	Ca 14b	–96 (09)
Glycerol by Periodate Method		
in Fats and Oils	Ca 14	–56 (11)
in Glycerin	Ea 6	–94 (13)
in Soap and Soap Products (free)	Da 23	–56 (11)
Glycerol, Sodium Periodate Oxidation Method	Ea 6	–51 (12)
Gossypol, Free in Cottonseed Products	Ba 7	–58 (13)
Gossypol, Free, Rapid Method	Ba 7b	–96 (11)
Gossypol by HPLC	Ba 8a	–99 (11)
Gossypol, Total		
in Cottonseed Products	Ba 8	–78 (11)
in Crude Cottonseed Oil and Soap Stock	Ca 13	–56 (13)
Halphen Test for Detecting Cottonseed Oil	Cb 1	–25 (09)
Heat Transfer Fluids		
by GLC	Cd 25	–96 (09)
by HPLC	Cd 25a	–00 (09)
Thermal Heating Fluids in Vegetable Oils by GC	Cd 25b	–07 (09)
Hexane		
Commercial, Specifications	H 16	–56 (09)
Free in Extracted Meals	Ba 13	–87 (11)
Insolubles in Lecithin	Ja 3	–87 (09)
Residues in Fats and Oils	Ca 3b	–87 (11)
Total in Extracted Meals	Ba 14	–87 (11)
Hydrocarbons		
Mineral Oil in Triglycerides	Ca 6c	–65 (09)
Volatile in Soap and Soap Products	Da 26	–42 (09)
Steroidal Hydrocarbons in Vegetable Oils	Cd 27	–96 (09)
Hydrogenation Catalyst		
Activity	Tz 1a	–78 (09)
Selectivity	Tz 1b	–79 (11)
Hydroxyl Value		
of Epoxidized Oils	Tx 1a	–66 (09)
of Fats and Oils (acetyl value)	Cd 4	–40 (99)*
of Fatty Oils and Alcohols	Cd 13	–60 (09)
Impurities in Oilseeds	Am 4	–02 (09)
Inorganic Salts	F 6	–44 (09)
Insoluble Impurities		
in Fats and Oils	Ca 3a	–46 (11)
in Lecithin (hexane insolubles)	Ja 3	–87 (09)
International Trade Methods	Front Matter	
Iodine Value		
by NIR	Cd 1e	–01 (13)
Calculated from GLC	Cd 1c	–85 (09)
Cyclohexane Method	Cd 1b	–87 (12)
Cyclohexane-Acetic Acid Method	Cd 1d	–92 (09)

*Surplus method

	Method number	Latest issue
of Drying Oils	Tg 1	–64 (12)
of Fats and Oils	Cd 1	–25 (93) *
of Fatty Acids	Tg 1a	–64 (12)
of Fatty Amines, Diamines, and Amidoamines	Tg 2a	–64 (09)
of Fatty Quaternary Ammonium Chlorides	Tg 3a	–64 (09)
of Lecithin	Ja 14	–91 (09)
of Soap and Soap Products	Da 15	–48 (09)
Iron, Trace Amounts in Oils		
by AAS	Ca 15	–75 (09)
by AAS with Graphite Furnace	Ca 18	–79 (09)
Isopropyl Alcohol, Specifications	H 18	–58 (09)
Jojoba Oil		
Acid Value	Ci 4	–91 (13)
Detection of Triglyceride Adulteration	Ci 2	–91 (09)
Methods for Determination of Quality and Purity	Ci 1	–91 (09)
Kernel Content of Tung Fruit	Ad 4	–52 (09)
Kirschner Value for Butyric Acid in Butterfat and Coconut Oils	Cd 5	–40 (09)
Lard, Foreign Fat Content	Cb 5	–40 (93) *
Lead in Oils by Graphite Furnace	Ca 18c	–91 (09)
Lecithin Co–Products Analyses	Ja 12	–89 (11)
Lecithin Phospholipids by HPLC–LSD	Ja 7c	–07 (13)
Lint Residue in Cottonseed	Aa 7	–55 (09)
Liquid Fatty Acids in Fats and Oils	Cd 6	–38 (89) *
Manganese in Oils by Graphite Furnace	Ca 18	–79 (09)
Mass per Unit Volume ("Liter Weight") in Air of Oils and Fats	Cc 10c	–95 (09)
Melting Point		
Capillary Tube Method	Cc 1	–25 (09)
Mettler Dropping Point	Cc 18	–80 (09)
Slip Melting Point, AOCS Standard	Cc 3	–25 (09)
Slip Melting Point, ISO Standard	Cc 3b	–92 (09)
Wiley Method	Cc 2	–38 (91) *
Melting Properties of Fats and Oils by DSC	Cj 1	–94 (09)
Metals, Trace		
by AAS for Cr, Cu, Fe, Ni in Oils	Ca 15	–75 (09)
by AAS with Graphite Furnace for Cr, Cu, Fe, Ni, Mn in Oils	Ca 18	–79 (09)
by ICP-OES	Ca 17	–01 (09)
by Spectrophotometry for Copper	Da 31	–58 (09)
Methyl Alcohol, Specifications	H 17	–58 (09)
Methyl Esters		
Preparation	Ce 2	–66 (09)
Methylation of Lipids in Foods by Alkali Hydrolysis	Ce 2b	–11 (13)
Methylation of Lipids in Foods by Acid-Alkali Hydrolysis	Ce 2c	–11 (13)
Mineral Oil in Triglycerides	Ca 6c	–65 (09)
Miscibility of Oils, Crismer Test	Cb 4	–35 (09)
Moisture and Volatile Matter, by Air Oven		
in Castor Beans	Ae 2	–52 (09)
in Castor Pomace	Bd 2	–52 (09)
in Cottonseed	Aa 3	–38 (09)
in Fats and Oils (except coconut and drying oils)	Ca 2c	–25 (09)
in Flaxseed	Af 2	–54 (97) *
in Oilseed Meats, Meal, Cake, and Pellets	Ba 2a	–38 (09)
in Oilseed Protein Concentrates	Ba 2b	–82 (11)
in Peanuts (groundnuts)	Ab 2	–49 (09)
in Soap and Soap Products	Da 2a	–48 (09)
in Soap and Soap Products Containing Synthetic Detergents	Db 1	–48 (09)
in Soya Flours	Bc 2	–49 (09)
in Soybeans	Ac 2	–41 (09)
in Sunflower Seed	Ai 2	–75 (09)
in Tung Fruit (hulls and fruit plus hulls)	Ad 2	–52 (11)

*Surplus method

	Method number	Latest issue
Moisture and Volatile Matter, by Forced Draft Oven		
in Oilseed Cake, Meal, Meats, Pellets	Ba 2a	–38 (09)
Moisture and Volatile Matter, by Hot Plate Method		
Butter, Fats, Margarines, Oils	Ca 2b	–38 (09)
Fatty Acids	Tb 1a	–64 (09)
Modified Moisture and Volatiles	Ca 2f	–93 (09)
Moisture and Volatile Matter, by Vacuum Oven		
in Fats and Oils (except coconut)	Ca 2d	–25 (09)
in Oilseed Cake, Meal, Meats, Pellets	Ba 2b	–82 (11)
Moisture, by Distillation Method		
in Alkylbenzene Sulfonate Products	Dd 2a	–59 (09)
in Fats and Oils	Ca 2a	–45 (09)
in Fatty Alkyl Sulfate Products	Dc 2	–59 (09)
in Lecithin	Ja 2a	–46 (09)
in Soap and Soap Products	Da 2b	–42 (09)
in Sulfonated and Sulfated Oils	F 1a	–44 (09)
Moisture, by Karl Fischer Method		
in Alkylbenzene Sulfonate Products	Dd 2b	–59 (89) *
in Fats and Oils	Ca 2e	–84 (09)
in Fatty Nitrogen Compounds	Tb 2a	–64 (89) *
in Glycerin	Ea 8	–58 (09)
in Industrial Oils and Derivatives	Tb 2	–64 (97) *
in Lecithin	Ja 2b	–87 (09)
Molecular Weight (average) of Fatty Quaternary Ammonium Chlorides	Tv 1a	–64 (09)
Mono- and Diglycerides by Capillary GLC	Cd 11b	–91 (09)
Mono- and Diglycerides by HPLC–ELSD	Cd 11d	–96 (09)
Mono-, Di-, and Triglycerides by Silica Gel Chromatography	Cd 11c	–93 (09)
α-Monoglycerides	Cd 11	–57 (11)
Near Infrared Reflectance	Am 1	–92 (09)
Near Infrared Spectroscopy Instrument Management and Prediction Model Development	Am 1a	–09 (09)
Neutral Oil		
in Alkylbenzene Sulfonates (unsulfonated material)	Dd 4	–60 (09)
in Neutral Oil and Loss for Fats and Oils	Ca 9f	–57 (09)
in Soap Stock (unsaponifiable material)	G 5	–40 (09)
Nitrogen–Ammonia–Protein		
Copper Sulfate Catalyst	Ba 4b	–87 (90) *
Copper Sulfate Plus Titanium Dioxide Catalyst	Ba 4d	–90 (11)
in Cottonseed	Aa 5	–38 (89) *
in Cottonseed	Aa 5	–91 (11)
in Oilseeds, Meats, Meal, Cake, and Pellets	Ba 4a	–38 (11) *
in Peanuts (groundnuts)	Ab 4	–50 (89) *
in Peanuts (groundnuts)	Ab 4	–91 (11)
in Soya Flours	Bc 4	–49 (89) *
in Soya Flours	Bc 4	–91 (11)
in Soybeans	Ac 4	–41 (89) *
in Soybeans	Ac 4	–91 (11)
in Sunflower Seeds	Ai 4	–75 (89) *
in Sunflower Seeds	Ai 4	–91 (11)
Kjel-Foss Automatic	Ba 4c	–87 (89) *
Nitrogen Solubility Index (soybean products)	Ba 11	–65 (09)
Nonamines in Fatty Amines and Diamines	Tw 1a	–64 (09)
Non-Cocoa-Butter Fats	Ce 10	–02 (09)
Nonvolatiles (solids), by Hot Plate Method		
for Drying Oils (solutions)	Tc 1a	–64 (09)
for Quaternary Ammonium Chlorides	Tc 2a	–64 (09)
Oil Content		
by Filter Bag Technology	Am 5	–04 (09)
in Castor Beans	Ae 3	–52 (09)
in Castor Pomace	Bd 3	–52 (09)

*Surplus method

	Method number	Latest issue
in Corn Germ	Aj 4	–89 (09)
in Cottonseed	Aa 4	–38 (12)
in Flaxseed	Af 3	–54 (95) *
in Linters (cottonseed hulls)	Bb 2	–38 (09)
in Oilseed Meats, Cake, and Meal	Ba 3	–38 (09)
in Oilseed Residues by NMR	Ak 5	–01 (09)
in Oilseeds (FOSFA Method)	Am 2	–93 (11)
in Oilseeds: Supercritical Fluid Extraction Method	Am 3	–96 (09)
in Peanuts (groundnuts), Raw or Roasted	Ab 3	–49 (09)
in Rapeseed and other Oilseeds by NMR	Ak 3	–94 (09)
in Safflower Seed	Ag 1	–65 (09)
in Soya Flours	Bc 3	–49 (09)
in Soybeans	Ac 3	–44 (11)
in Sunflower Seed or Dehulled Kernels	Ai 3	–75 (99) *
in Tung Fruit, Hulled	Ad 6	–52 (09)
in Tung Fruit, Kernels	Ad 5	–52 (09)
in Tung Fruit, Whole	Ad 3	–52 (09)
Olive Oil		
Chlorophyll Pigments	Ch 4	–91 (13)
Fatty Acids by Capillary GLC	Ch 2	–91 (09)
trans Unsaturated Fatty Acids by Capillary Column GC	Ch 2a	–94 (11)
Fatty Acids in 2-Position	Ch 3	–91 (11)
Preparation of Methyl Esters	Ch 1	–91 (09)
Specific Extinction	Ch 5	–91 (09)
Sterol Fraction by TLC and Capillary GLC	Ch 6	–91 (11)
International Trade Standards	Ch 7	–09 (09)
Wax Content	Ch 8	–02 (11)
Organic Residue in Glycerin	Ea 3	–58 (73) *
Ovens		
Air, Specifications	H 3	–45 (09)
Forced Draft, Specifications	H 1	–39 (09)
Vacuum, Specifications	H 4	–45 (09)
Oxidized Fatty Acids in Soap Stocks	G 3	–53 (09)
Oxirane Oxygen in Epoxidized Materials	Cd 9	–57 (09)
Peroxide Value		
of Lecithin	Ja 8	–87 (13)
Using Chloroform	Cd 8	–53 (03) *
Using Isooctane	Cd 8b	–90 (11)
Pesticides in Oils and Fats	Cd 23	–93 (09)
Petroleum Ether, Specifications	H 2	–41 (09)
pH		
of Acidulated Soapstocks	G 7	–56 (09)
of Fatty Quaternary Ammonium Chlorides	Tu 1a	–64 (09)
Phosphates in Soap and Soap Products (gravimetric method)	Da 20a	–48 (09)
Phosphates in Soap and Soap Products (titrimetric method)	Da 20b	–57 (11)
Phospholipids		
in Lecithin Concentrates by HPLC	Ja 7b	–91 (11)
in Lecithin Phospholipids by HPLC-LSD	Ja 7c	–07 (13)
in Lecithin Concentrates by TLC	Ja 7	–86 (09)
in Vegetable Oil	Ca 19	–86 (09)
Phosphorus, Atomic Absorption Spectroscopy	Ca 12b	–92 (09)
Phosphorus		
in Lecithin (total)	Ja 5	–55 (89) *
in Oils	Ca 12	–55 (09)
in Oils (ISO Method)	Ca 12a	–02 (09)
in Oil by ICP-OES	Ca 20	–99 (09)
Photometric Index (color) of Commercial Fatty Acids	Td 2a	–64 (09)
Physical Analysis		
Polenske Value	Cd 5	–40 (09)

*Surplus method

	Method number	Latest issue
Tung Fruit (kernel content)	Ad 4	–52 (09)
Polar Compounds in Frying Fats	Cd 20	–91 (09)
Polyethylene in Fats and Oils	Ca 16	–75 (11)
Polymerized Triglycerides by Gel-Permeation HPLC	Cd 22	–91 (09)
Polyunsaturated Acids		
cis, cis Essential Fatty Acids (enzymatic)	Cd 15	–78 (09)
Commercial Fatty Acids, Spectrophotometric Method	Tj 1a	–64 (93) *
in Fats and Oils	Cd 7	–58 (09)
Potassium Carbonate in Potash Soaps	Da 5	–44 (09)
Potassium Oxide in Soap and Soap Products	Da 27	–48 (09)
Precision of Analytical Methods	M 1	–92 (09)
Process Contaminants		
2- and 3-MCPDs and Glycidol by Acid Transesterification	Cd 29a	–13 (13)
2- and 3-MCPDs and Glycidol by Alkaline-Catalyzed Alcoholysis (low temperature method)	Cd 29b	–13 (13)
2- and 3-MCPDs and Glycidol by Alkaline-Catalyzed Alcoholysis	Cd 29c	–13 (13)
Protein Content		
by Combustion	Ba 4e	–93 (09)
by Combustion (soybean meal analysis)	Ba 4f	–00 (11)
in Cottonseed	Aa 5	–91 (11)
in Oilseed Meats, Meal, Cake, and Pellets	Ba 4a	–38 (11) *
in Peanuts (groundnuts)	Ab 4	–91 (11)
in Soya Flours/Canola Flours	Bc 4	–91 (11)
in Soybeans	Ac 4	–91 (11)
Protein Dispersibility Index (PDI) (soybean products)	Ba 10	–65 (09)
Protein Dispersibility Index (PDI) (revised)	Ba 10a	–05 (09)
Protein Dispersibility Index (PDI) (Omni Mixer)	Ba 10b	–09 (11)
in Sunflower Seeds	Ai 4	–91 (11)
Kjel–Foss Automatic Method	Ba 4c	–87 (89) *
with Copper Sulfate Catalyst	Ba 4b	–87 (90) *
with Copper Sulfate Plus Titanium Dioxide Catalyst	Ba 4d	–90 (11)
Refined and Bleached Color (tallow and greases for soaps)	Cc 8d	–55 (09)
Refining Loss		
Degummed, Expeller Soybean Oil	Ca 9a	–52 (11)
Degummed, Hydraulic and Extracted Soybean Oil	Ca 9a	–52 (11)
Extracted and Reconstituted Prepressed Cottonseed Oils	Ca 9a	–52 (11)
Extracted Soybean Oil	Ca 9a	–52 (11)
Vegetable Oils, Crude	Ca 9a	–52 (11)
Refractive Index		
Drying Oils	Tp 1a	–64 (09)
Fats and Oils	Cc 7	–25 (09)
Fatty Acids, Commercial	Tp 1a	–64 (09)
Reichert–Meissl Value	Cd 5	–40 (09)
Residual Lint in Cottonseeds	Aa 7	–55 (09)
Residue, Total and Organic, in Glycerin	Ea 3	–58 (73) *
Rheological Instrument Calibration	Cj 3	–99 (09)
Rosin		
in Soap and Soap Products	Da 12	–48 (09)
in Soap Containing Synthetic Detergents	Db 11	–48 (09)
Rosin Acids in Fatty Acids	Ts 1a	–64 (09)
Sampling		
of Alkylbenzene Sulfonates	Dd 1	–59 (11)
of Cottonseed	Aa 1	–38 (11)
of Cottonseed Linters and Hull Fiber	Bb 1	–38 (09)
of Dibasic Acids	Ta 1e	–70 (09)
of Epoxidized Oils	Ta 1	–09 (09)
of Fats and Oils	C 1	–47 (09)
of Fatty Acids, Commercial	Ta 1	–09 (09)
of Fatty Alkyl Sulfates	Dc 1	–59 (11)
of Fatty Nitrogen Products	Ta 1	–09 (09)

*Surplus method

	Method number	Latest issue
of Flaxseed	Af 1	–54 (97) *
of Glycerin	Ea 1	–38 (09)
of Industrial Oils, General	Ta 1	–09 (09)
of Oilseed Slab, Cake, Pellets, Meals	Ba 1	–38 (09)
of Peanuts (groundnuts), Whole, Shelled	Ab 1	–49 (09)
of Polymerized Fatty Acids	Ta 1	–09 (09)
of Soap and Soap Products	Da 1	–45 (09)
of Soap Stock	G 1	–40 (09)
of Soya Flours	Bc 1	–50 (09)
of Soybeans	Ac 1	–45 (09)
of Sunflower Seed	Ai 1	–80 (93) *
of Tung Fruit	Ad 1	–48 (09)
Saponification Color		
Refined and Bleached	Cc 13f	–94 (09)
High Quality Tallow, Coconut Oils	Cc 13g	–94 (09)
Tallow, Coconut Oils	Cc 13h	–94 (09)
Saponification Value		
of Deodorizer Distillates and Sludges	Cd 3b	–76 (09)
of Drying Oils	Tl 1a	–64 (09)
of Fats and Oils	Cd 3	–25 (13)
of Fats and Oils (Calculated)	Cd 3a	–94 (09)
of Fats and Oils, Modified Using Methanol	Cd 3c	–91 (09)
of Fatty Acids, Commercial	Tl 1a	–64 (09)
of Soap and Soap Products	Da 16	–48 (09)
of Soap Containing Synthetic Detergents	Db 8	–48 (09)
Screen Test		
Soap Powders	Da 28	–39 (09)
Soya Flours	Bc 7	–51 (09)
Sediment in Fats and Oils	Ca 3d	–02 (11)
Selectivity of Hydrogenation Catalysts	Tz 1b	–79 (09)
Sesame Oil Detection, Modified Villavecchia Test	Cb 2	–40 (09)
Simultaneous Determination of Oil and Moisture		
Contents of Oilseeds Using Pulsed Nuclear Magnetic Resonance Spectroscopy	Ak 4	–95 (11)
Slip Melting Point, ISO Standard	Cc 3b	–92 (09)
Slip Point		
Hard Fats (palm oil)	Cc 3	–25 (09)
Soft Fats	Cc 4	–25 (89) *
Smoke, Flash, and Fire Point; Cleveland Open Cup Method	Cc 9a	–48 (09)
Soap in Oil		
by Conductivity Method	Cc 15	–60 (89) *
by Titrimetric Method	Cc 17	–95 (09)
Sodium Alkylbenzene Sulfonate by Ultraviolet Absorption	Dd 3	–60 (09)
Sodium and Calcium by AAS	Ca 15b	–87 (09)
Sodium Chloride in Glycerin	Ea 2	–38 (73) *
Sodium Oxide in Soap and Soap Products	Da 27	–48 (09)
Sodium Sulfate in Fatty Alkyl Sulfates	Dc 7	–59 (09)
Softening Point (slip point of hard fats)	Cc 3	–25 (09)
Solid Fat Content (SFC) by Magnetic Resonance (Direct Method)	Cd 16b	–93 (11)
Solid Fat Content (SFC) by Nuclear Magnetic Resonance (Indirect Method)	Cd 16	–81 (13)
Solid Fat Index (SFI) of Fats and Oils	Cd 10	–57 (95) *
Solid Fatty Acids in Fats and Oils	Cd 6	–38 (89) *
Soluble Mineral Matter and Fatty Acids Combined as Mineral Soap	Ca 4	–25 (09)
Specifications	See Section H	
Specific Gravity:		
Drying Oils	To 1b	–64 (09)
Fatty Acids, Commercial	To 1a	–64 (11)
Glycerin	Ea 7	–95 (09)
Liquid Oils and Fats	Cc 10a	–25 (95) *
Solid Fats and Waxes	Cc 10b	–25 (09)
Spreadability	Cj 4	–00 (09)

*Surplus method

	Method number	Latest issue
Stability and Quality of Fats and Oils (Analytical Guidelines)	Cg 3	–91 (09)
Light Exposure Test for Accelerated Aging of Oils	Cg 6	–01 (09)
Oven Storage Test for Accelerated Aging of Oils	Cg 5	–97 (09)
Stability of Fats and Oils, Active Oxygen Method (AOM)	Cd 12	–57 (93) *
Stability of Fats and Oils, Oil Stability Index (OSI)	Cd 12b	–92 (13)
Standard Solutions		
Alcoholic Potassium Hydroxide	H 15	–52 (09)
Hydrochloric Acid	H 14	–52 (09)
Sodium Hydroxide	H 12	–52 (09)
Sulfuric Acid	H 13	–52 (09)
Standard Test Sieves	H 20	–11 (11)
Starch in Soap and Soap Products	Da 25	–48 (09)
Sterols in Soya Residues by GLC	Ce 3	–74 (09)
Stigmastadienes in Vegetable Oils	Cd 26	–96 (09)
Sugars in Soap and Soap Products	Da 24	–48 (09)
Sulfates in Soap and Soap Products	Da 22	–48 (11)
Sulfur in Olive Oil		
by Coin Test	Ca 8a	–35 (90) *
by Silver Benzoate Test	Ca 8b	–35 (90) *
Sulfuric Anhydride in Sulfonated and Sulfated Oils		
by Ash Gravimetric Method	F 2c	–44 (09)
by Extraction Titration Method	F 2b	–44 (09)
by Titration Method	F 2a	–44 (09)
Surplus Status of Methods	See Index	
Teaseed Oil Detection, Fitelson Test	Cb 3	–39 (09)
Tetrasodium Pyrophosphate	Da 21	–48 (09)
Thermometers, Specifications	H 5	–40 (09)
2-Thiobarbituric Acid (TBA) Value	Cd 19	–90 (09)
Thiocyanogen Value	Cd 2	–38 (89) *
Titer Test		
for Fats and Oils	Cc 12	–59 (09)
for Fatty Acids, Commercial	Tr 1a	–64 (09)
for Soap and Soap Products	Da 13	–48 (09)
for Soap Stocks	G 6	–40 (09)
Tocopherols		
in Deodorizer Distillates, Total	Ce 7	–87 (09)
in Lecithin Concentrates by HPLC	Ja 13	–91 (09)
in Soya Sludge and Residues by GLC	Ce 3	–74 (09)
in Vegetable Oils and Fats by HPLC	Ce 8	–89 (13)
Toluene, Industrial, Specifications	H 19	–58 (09)
Total Fatty Acids		
in Coconut Oil and Palm Oil Soap Stock	G 4	–40 (09)
in Fats or Hydrolyzed Fats	Ca 5b	–71 (11)
in Soap Stocks Other Than Coconut Oil and Palm Oil	G 3	–53 (09)
Trace Metals in Oils		
by AAS (Cr, Cu, Fe, Ni)	Ca 15	–75 (09)
by Graphite Furnace AAS (Cr, Cu, Fe, Ni, Mn)	Ca 18	–79 (09)
by Graphite Furnace AAS Direct (Cu, Fe, Ni)	Ca 18b	–91 (09)
by Graphite Furnace AAS (lead only)	Ca 18c	–91 (09)
by ICP–OES	Ca 17	–01 (09)
trans and *cis,cis* Isomers by GLC	Ce 1c	–89 (95) *
trans Composition of Partially Hydrogenated Oils by GLC–IR	Cd 14b	–93 (95) *
trans Isomers (Isolated) by FTIR	Cd 14	–95 (09)
trans Isomers by Capillary GLC	Cd 14c	–94 (94) *
trans Isomers by Capillary GLC	Ch 2a	–94 (11)
trans Isomers in Triglycerides by ATR/FTIR	Cd 14d	–99 (13)
Triglycerides by GLC	Ce 5	–86 (09)
Triglycerides by HPLC	Ce 5b	–89 (11)
Triglycerides (Individual) by HPLC	Ce 5c	–93 (11)
Trypsin Inhibitor Activity in Soybean and Cotton/Soybean Dry Products	Ba 12	–75 (09)

*Surplus method

	Method number	Latest issue
Tung Fruit, Physical Analysis (kernel content)	Ad 4	–52 (09)
Unsaponifiable Matter		
in Drying Oils	Tk 1a	–64 (09)
in Fats and Oils, Except Marine Oils	Ca 6a	–40 (11)
in Fats and Oils, Including Marine Oils	Ca 6b	–53 (11)
in Fats or Hydrolyzed Fats	Ca 5b	–71 (11)
in Fatty Acids, Commercial	Tk 1a	–64 (09)
in Fatty Acids, Polymerized	Tk 1a	–64 (09)
in Soap and Soap Products	Da 11	–42 (13)
Unsaponifiable Nonvolatile Matter in Sulfated Oils	F 5	–44 (09)
Unsaponified Plus Unsaponifiable Matter in Soap and Soap Products	Da 10	–42 (13) *
Unsulfated Material in Fatty Alkyl Sulfates	Dc 8	–59 (09)
Urease Activity (soybean meals, flours, and mill feed)	Ba 9	–58 (09)
Villavecchia Test for Sesame Oil, Modified	Cb 2	–40 (09)
Viscosity		
Drying Oils, Bubble Time Method	Tq 1a	–64 (11)
Lecithin, Brookfield	Ja 10	–87 (09)
Lecithin, Bubble Time Method	Ja 11	–87 (09)
Transparent Liquids, Bubble Time Method	Ja 11	–87 (09)
Volatile Hydrocarbons in Soap and Soap Products	Da 26	–42 (09)
Volatiles (VOC) in Fats and Oils by GLC	Cg 4	–94 (09)
Volatile Organic Contaminants by GC/MS	Ca 3c	–01 (09)
Water-Immiscible Organic Solvents in Sulfonated and Sulfated Oils	F 10	–44 (09)
Water-Insoluble Matter		
in Soap and Soap Products	Da 6	–48 (09)
in Soap Containing Synthetic Detergents	Db 4	–48 (09)
Wax Content	Ch 8	–02 (11)
Wiley Melting Point Method	Cc 2	–38 (91) *
Writing and Approval of Methods	M 2	–09 (13)
X-Ray Diffraction Analysis of Fats	Cj 2	–95 (09)
Zearalenone and α-Zearalenol in Corn	Aj 2	–86 (09)

*Surplus method

ALPHABETICAL INDEX

Surplus Methods

The methods listed in this section have been declared surplus (i.e., obsolete) by the AOCS Uniform Methods Committee. The surplus methods have been or will be removed from Official Methods in the year stated; however, they are available upon request from AOCS headquarters at the prevailing photocopying rate.

Method number	Topic	Year removed from book
Aa 5-38 (89)	Modified Kjeldahl Method (cottonseed)	1991
Ab 4-50 (89)	Modified Kjeldahl Method (peanuts)	1991
Ac 4-41 (89)	Modified Kjeldahl Method (soybeans)	1991
Af 1-54 (93)	Sampling (flaxseed)	*
Af 2-54 (93)	Moisture and Volatile Matter (flaxseed)	*
Af 3-54 (95)	Oil Content (flaxseed)	*
Ai 1-80 (93)	Sampling (sunflower seed)	*
Ai 3-75 (99)	Oil Content (sunflower seed)	*
Ai 4-75 (89)	Modified Kjeldahl Method (sunflower)	1991
Ba 4a-38 (89)	Modified Kjeldahl Method (oilseed meals)	***
Ba 4b-87 (90)	Modified Kjeldahl Method (copper sulfate)	1991
Ba 4c-87 (89)	Kjel-Foss Automatic Method	1991
Ba 10-65	Protein Dispersibility Index (PDI)	2009
Bc 4-49 (89)	Nitrogen and Protein (soya flour)	1991
Ca 5c-87 (09)	Butyric Acid	2014
Ca 7-35 (90)	Beilstein Test	1991
Ca 8a-35 (90)	Coin Test	1991
Ca 8b-35 (90)	Silver Benzoate Test	1991
Ca 10-40 (89)	Break Test (soybean oil)	1991
Cb 5-40 (93)	Bömer Value	1994
Cc 2-38 (91)	Wiley Melting Point	1994
Cc 4-25 (89)	Slip Point (soft fats)	1992
Cc 5-25 (89)	Flow Test	1992
Cc 10a-25 (95)	Specific Gravity of Oils and Liquid Fats	1995
Cc 15-60 (89)	Soap in Oil (Conductivity)	1993
Cd 1-25 (93)	Wijs Method for Iodine Value	**
Cd 2-38 (89)	Thiocyanogen Value	1991
Cd 4-40 (99)	Hydroxyl Value and Acetyl Value of Fats and Oils	1999
Cd 6-38 (89)	Liquid and Solid Fatty Acids	1991
Cd 8-53 (03)	Peroxide Value—Acetic Acid-Chloroform Method	2003
Cd 10-57 (97)	Solid Fat Index	**
Cd 11-57 (91)	Monoglycerides (Section I)	1991
Cd 12-57 (93)	AOM for Oil Stability	2007
Cd 14-61 (95)	*trans* Fatty Acids by FTIR	1995
Cd 17-85 (93)	*trans* Fatty Acids by GLC	1995
Cd 14b-93 (95)	*trans* Isomers in Partially Hydrogenated Oils by GLC-IR	1999
Cd 14c-94 (94)	*trans* Isomers by Capillary GLC	1999
Ce 1-62 (09)	Fatty Acid Composition by GLC	2014
Ce 1c-89 (95)	Fatty Acid Composition by GLC, cis, cis and trans Isomers	1999
Ce 1d-91 (93)	n-3 and n-6 Fatty Acids by Capillary GLC	1999
Ce 1e-91 (01)	Fatty Acid Composition by Capillary GC	2005
Ce 1f-96 (09)	*cis* and *trans* Fatty Acids in Oils and Fats by Capillary GLC 2013	2013
Ce 1k-09 (09)	Fatty Acids by GC	2012
Ce 4-86 (95)	Erucic Acid by TLC	1995
Ce 4-95 (97)	Erucic Acid by GLC	2003

* This method will be removed when Section A is revised.
**This method was retained for historical and reference purposes only.
*** This method, which uses mercuric oxide, was retained for historical and reference purposes only.

Method number	Topic	Year removed from book
Da 10-42 (09)	Unsaponified Plus Unsaponifiable Matter	2009
Dd 2b-59 (89)	Modified Karl Fischer (alkylbenzene sulfonates)	1989
Ea 2-38 (73)	Acidity (glycerol)	1991
Ea 2-38 (73)	Alkalinity (glycerol)	1991
Ea 2-38 (73)	Ash (glycerol)	1991
Ea 2-38 (73)	Sodium Chloride (glycerol)	1991
Ea 3-58 (73)	Total and Organic Residue	1991
Ja 5-55 (89)	Total Phosphorus in Lecithin	1993
Tb 2-64 (95)	Modified Karl Fischer, General	1991
Tb 2a-64 (89)	Modified Karl Fischer, Fatty Nitrogen Products	1991
Tj 1a-64 (93)	Polyunsaturated Acids, Ultraviolet Spectrophotometric Method	1994

* This method will be removed when Section A is revised.
**This method was retained for historical and reference purposes only.
*** This method, which uses mercuric oxide, was retained for historical and reference purposes only.

AOCS

AOCS Analytical Guidelines

Reapproved 2009

International Trade Methods

Listed below are those methods most frequently used in the international trade of oils and fats. In particular, these methods represent those of the Federation of Oil, Seeds, and Fats Association (FOSFA) International, which are used in approximately 85% of the contracts for oils and fats that are traded on an international basis. If available, the corresponding AOCS method is shown, with its current status in comparison with the FOSFA International Method.

Test Method[a]	FOSFA Method	AOCS Method
1. Acidity-Acid Value (see Free Fatty Acid and/or Mineral Acidity)	ISO 660:1996/AMD 1:2003	Cd 3d-63[b]
2. Anisidine Value	ISO 6885:2006	Cd 18-90[c]
3. Ash	ISO 6884:2008	Ca 11-55[d]
4. Bleach Test-for Palm Oil	BS 684-2.27:1987 Method B	None[d]
5. Carotene	BS 684-2.20:1977	None[d]
6. Cloud Point	BS 684-1.5:1987 Method 2/AMD6051	Cc 6-25[b]
7. Cold Test	AOCS Cc 11-53 (2003)	Cc 11-53[c]
8. Color:		
FAC	AOCS Cc 13a-43 (2000)	Cc 13a-43[c]
Lovibond BS	ISO 15035:1998	Cc 13e-92[c]
Gardner	AOCS Td 1a-64 (2000)	Td 1a-64[c]
Wesson	AOCS Cc 13b-45 (2000)	Cc 13b-45[c]
9. Detection of Sea Water Contamination in Oils	FOSFA Official Method	None[d]
10. Erucic Acid In Vegetable Oils	ISO 5508:1990 and ISO 5509:2000 Erucic Acid Plus Other Isomers Gives Total C22 Monoenes	Ce 4-95[c]
11. Flashpoint Limit Test	ISO 15267:1998	Cc 9c-95[c]
12. Free Fatty Acids or Acid Value	ISO 660:1996/AMD 1:2003	Cd 3d-63[b]
13. Foots by Volume	ISO 19219:2002	None[d]
14. GLC Analysis	ISO 5508:1990	Ce 1b-89
of Methylesters of FA Prep of	ISO 5509:2000	Ce lf-96
Methylesters of Fatty Acids		Ce 2-66[c]
15. Det. Of FA in 2-Position in the Triglyceride of Oils & Fats	ISO 6800:1997	Ch 3-91[c]
16. Hexane Residue	ISO 9832:2002	Ca 3b-87[c]
17. Insoluble Impurities (warm solvent may be used)	ISO 663:2007	Ca 3a-46[b]
18. Iodine Value	ISO 3961:1996	Cd ld-92[c]
19. Liter-Weight in Air	ISO 6883:2007	Cc 10c-95[c]
20. Melting Point (slip point)	ISO 6321:2002	Cc 3b-92 (see Notes, 1)
21. Mineral Acidity	BS 684-2.48:1998	Cd 3d-63[b]
23. Moisture & Volatile Matter	ISO 662:1998	Ca 2c-25[b,d]
24. Organic Dirt/Total Dirt	ISO 663:2007	None[d]
24. Oxidized Fatty Acids	BS 684-2.12:1984	None[d]
25. Oxidative Stability	ISO 6886:2006	Cd 12-57[c]
26. Peroxide Value	ISO 3960:2007	Cd 8-53[c]
27. Phosphoric Acid Test	ISO 150:2006 "Paragraph 9"	None[d]
28. Phosphorus	FOSFA Official Method	Ca 12-55 (see Notes, 2)
29. Phosphorus (B) Direct Graphite Furnace	ISO 10540-2:2003	
30. Polyethylene	ISO 6656:2002	Ca 16-75[c]
31. Rancidity Index (Kreis Test)		None[d]
32. Refined and Bleach Test	AOCS Cc 8d-55 (1997)	Cc 8d-55[c]
33. Refractive Index	ISO 6320:2000/COR 1:2006	Cc 7-25[b]
34. Saponification Value	ISO 3657:2002	Cd 3-25[b]

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35. Soap	ISO 10539:2002	Cc 17-95[c]
36. Solid Fat by Dilatometry	AOCS Cd 10-57 (1997)	Cd 10-57[b]
37. Solid Fat Content-NMR	ISO 8292-1:2008 and ISO 8292-2:2008	Cd 16b-93 (see Notes, 3)
38. Specific Extinction in Ultraviolet Light	ISO 3656:2002	Ch 5-91[b] (see Notes, 4)
39. Sterols (individual)	ISO 12228:1999	Ch 6-91[c]
40. Titer	ISO 935:1988	Cc 12-59[b]
41. Total Fatty Matter	BS 684-2.4:1976	None[d]
42. Trace Metal—Copper, Iron, Nickel	ISO 8294:1994	Ca 18b-91[c]
43. Trace Metal—Lead	ISO 12193:2004	Ca 18c-91[b] (see Notes, 5)
44. Unsaponifiable Matter (Diethyl Ether)	ISO 3596:2000	Ca 6b-53[b]
45. Unsapomfiable Matter (Hexane Method)	ISO 18609:2000	Ca 6a-40
46. Water (by distillation)	ISO 934:1980	Ca 2a-45[b]
47. Water-Insoluble Solvents	BS 684-1.9:1976	F 10-44[b,d]
Other than FOSFA:		
Palm Oil (see Notes, 6):		
Free Fatty Acids	PORIM Method P2.5	Cd 3d-63[b]
Slip Melting Point	PORIM Method P4.2	Cc 3-25[c] (see Notes, 1)
Cloud Point	PORIM Method P4.3	Cc 6-25
Color, Lovibond	PORIM Method P4.1	None
Iodine Value	PORIM Method P3.2	Cd 1-25[b]

[a]Specified in international contracts.
[b]Similar, but not identical to FOSFA or other specified method (opportunity for "harmonization" or adoption of ISO, BS, or other specified method).
[c]Identical to FOSFA or other specified method.
[d]Where no similar or identical AOCS method exists, the AOCS will consider the adoption of the method and note in the Scope of the AOCS version that it may be used in international trade.

NOTES

1. In 1992, the AOCS adopted the ISO slip method as AOCS Official Method, Cc 3b-92; declared AOCS Official Method, Cc 4-25 obsolete; changed the title of AOCS Official Method, Cc 3-25 from "Softening Point" to "Slip Point, AOCS Standard"; and noted the application of Cc 3-25 TO palm oil.
2. Identical with FOSFA method, except for the FOSFA specification to homogenize the whole sample and to carry out the analysis on the unfiltered sample, especially for crude oils with a sediment or haze. The AOCS version is similar, but not identical to IUPAC 2.421 (IUPAC version requires a higher ignition temperature than the AOCS version.)
3. In general, methods are similar; European methods specify different temperatures than US methods.
4. AOCS adopted Italian Standard Method, NGD C40-1976 in 1991, as AOCS Official Method, Ch 5-91.
5. AOCS adopted IUPAC Standard Method, 2.632 in 1991, as AOCS Official Method, Ca 18c-91.
6. PORIM test methods may not completely agree with AOCS methods. FOSFA recommends adoption of ISO/BS methods.

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AOCS

AOCS Official Method Aa 1-38

Revised 2011

Sampling

SCOPE

Applicable to cottonseed (see Notes, 1).

APPARATUS

1. Pneumatic probe sampler—The unit is powered with ⅞ hp, class II, group G motor installed with specially designed cyclone air pump and a cyclone collector tank having bottom release shutter, 123 cm long 30 cm i.d.; rolled aluminum probe tube 2.4 m in length × 6 cm i.d.; bottom of probe tube to be equipped with lightweight steel sawtooth cutter blade and at top with swivel type 90° elbow so that section turns freely the full 360° around top of probe tube; sufficient length (4.6–9 m) of 6 cm i.d. neoprene tubing reinforced with flexible wire. Cyclone collector tank intake nozzle should have 6 cm i.d.
2. Trier—The trier should be a corkscrew type that will take about 1.8–2.7 kg of cottonseed at a single probe. Made of band steel approximately 11 × 4 mm and bent to form an open cylinder about 8 cm in diameter. The pitch of the twist is about 5 cm, and screw portion is long enough to reach the bottom of the loads sampled. A single slight twist will cut the wall so the trier may be readily withdrawn.
3. Spout or chute sampler—This sampler may be fabricated by attaching a 20 × 14 cm elevator bucket to a pole long enough to enable sampler to place the bucket in a level position in the center of stream chute as car or truck is unloaded.
4. Shaker-cleaner—motor-driven device, equipped with a sample reducer. For details of the shaker-cleaner, contact James Falk, United States Department of Agriculture, Agricultural & Marketing Service, Science and Technology Division, Washington, DC, USA (James.Falk@usda.gov).
5. Metal sampling containers—containers with closefitting covers, 88 L.
6. Sample containers—Containers should be moisturetight and of about 1000 g capacity. They are used for sending samples to laboratory.
7. Scale—50 kg capacity, sensitive to ± 0.11 kg.
8. Scale—5 kg capacity, sensitive to ± 15 g.

PROCEDURE

1. Gross sample—Take a gross sample of cottonseed to represent a carload lot or the composite of the lots of several logically combined truckloads (typically representing about 100 tons from a single ginning point). This gross sample shall have a minimum weight of 23 kg and consist of the entire contents taken by the sampler in a minimum of 10 regularly spaced probes. Probes shall be uniformly spaced (typically one probe for each 10-ton sublot) geometrically, as for a loaded car, by time when the lot is in motion as in unloading, or by individual truck weights when random sizes are encountered. Gross samples shall be protected at all times (during probing, handling, compositing and storage) from changes due to exposure to atmospheric conditions by using a suitable moisture-proof container.
2. Carlots before unloading—Make an imaginary division of the carload into four square sections. In or near the center of each, dig a hole about 76 cm deep (a short-handled, five-tine seed fork is helpful). Take about 6.8 kg (15 lb) of seed from the sides and the bottom of each hole. Place immediately into a suitable moisture-proof bag or can.

   Note—If loading conditions permit, either the pneumatic probe or the corkscrew-type trier may be used in the manner specified for trucklots before unloading.
3. Car- or trucklots during unloading—Take samples from the center of the unloading chute with the spout sampler at regular intervals (depending upon the rate of unloading) planned to conform to the general requirements of a minimum of 10 probes for a gross sample with a minimum weight of 23 kg. Protect the sample being accumulated from changes from exposure to atmospheric conditions with a suitable moisture-proof container.
4. Trucklots before unloading—Use either the pneumatic probe sampler or the corkscrew-type trier. The pneumatic probe should be inserted vertically and lowered with an easy up and down motion until the bottom of the load is reached. Avoid forcing the probe into the cottonseed, as this is prone to cause clogging in the tube leading to the cyclone collector. The corkscrew-type trier should be inserted vertically and driven as deeply as possible before being withdrawn. However, particularly if power driven, it should not be rotated after reaching the truck bed to prevent shaking sand and small trash from the sample. With either sampler, plan the probing schedule to meet the general requirements of a minimum weight of 23 kg in the gross sample with a minimum of 10 regularly spaced probes. Allot probes to trucks according to weight, typically one probe for small trucks and two probes for large trucks or multiples thereof. Space probes in a regular geometric pattern (such as front, middle, and back areas with left, central, and right axes), filling out the pattern as it is carried over from truck to truck. Protect the sample being accumulated from changes from exposure to atmospheric conditions with a suitable moisture-proof container.

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CLEANING AND SEPARATION OF LABORATORY SAMPLE

1. Weigh laboratory sample to nearest 0.1 kg, and pass the entire amount over the shaker-cleaner. Collect and weigh all separated foreign matter and calculate the amount. Place 1000 g of the mixed sample in the laboratory sample container.
2. If the shaker-cleaner is not equipped with a sample-reducing device, mix the entire cleaned sample in the MacLennan mixer and place about 1000 g in the laboratory sample container.

Calculate foreign matter as follows:

$$\text{Foreign matter, \%} = \frac{\text{mass, g of foreign matter}}{\text{mass, g of test portion}} \times 100$$

NOTES

1. When cottonseed is purchased on standard grades of the United States Department of Agriculture (USDA), the sampling shall be done only by persons licensed by the Secretary of Agriculture and must be done in accordance with methods approved by the Administrator, Consumer & Marketing Service, USDA.

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AOCS

AOCS Official Method Aa 2-38

Reapproved 2009

Foreign Matter in Laboratory Sample

DEFINITION

All material passing a 3.35 mm sieve no. 6 NIST; any portions of bolls, sticks and leaves from the cotton plant; any noncotton material; and any unginned locks of cotton are removed as foreign material.

SCOPE

Application to laboratory sample of cottonseed (see Notes, 1).

APPARATUS

1. Scale—2000–3000-g capacity and sensitive to ± 1 g.
2. Sieve—no. 6 NIST. The screen cloth should meet ASTM E11-81 specifications with 3.35 mm openings with nominal wire diameter of 1.23 mm.
3. Henry cottonseed mixer—United States Department of Agriculture (USDA)-design mixer, or equivalent (see Notes, 2).
4. Suitable containers with tight-fitting covers—in which portions of the sample for moisture, oil, nitrogen, and free fatty acids determinations can be placed.

PROCEDURE

1. Prior to cleaning and mixing the laboratory sample, small portions of the seed, totaling at least 100 g, shall be withdrawn and placed in a suitable container with a tight-fitting cover. This part of the sample is to be used for the determination of moisture by AOCS Official Method Aa 3-38.
2. Weigh the entire laboratory sample of about 1000 g, pass the sample over the 6 mesh screen (Apparatus, 2), remove as much foreign matter as possible and pick out the remainder by hand after spreading out on a clean, dry surface.
3. Place in an approved mixer and mix until sample is uniform (about 2 min).
4. If a cleaner-mixer is used, entire laboratory sample is placed in the cleaner-mixer where it should be cleaned and mixed for 3 min. Pick out remaining foreign matter by hand.
5. Empty mixed and cleaned sample onto a large piece of paper and divide into four quarters, avoiding remixing. Return quarters 1 and 3 to the original sample container. Place quarters 2 and 4 in the containers designated in Apparatus, 4.

CALCULATIONS

1. Foreign matter, % $= \dfrac{\text{mass, g of foreign matter}}{\text{mass, g of sample}} \times 100$

2. If foreign matter in the laboratory sample is to be reported as part of the total foreign matter in the original sample (about 50 lb), it is calculated as follows:

   % Total foreign matter in original sample (about 50 lb) = FM + [(100 − FM) × fm/100]

   Where—

   FM = % of foreign matter in large sample
   fm = % of foreign matter in laboratory sample

NOTES

1. If foreign matter determination is to be used in the calculation of the "grade" of cottonseed, references should be made to USDA Regulations.
2. Henry cottonseed mixer (USDA design) may be obtained on special order from William Ellis, Memphis, TN, USA (www.wmcellisiron.com) or Mid-Continent Laboratories, Memphis, TN, USA (+1 901 725 1722).

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AOCS

AOCS Official Method Aa 3-38

Reapproved 2009

Moisture and Volatile Matter

DEFINITION

This method determines the moisture and any material that is volatile under the conditions of the test.

SCOPE

Applicable to cottonseed.

APPARATUS

1. Forced-draft oven—AOCS Specification H 1-39.
2. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL capacity WLS 25725-B, 50 mL capacity WLS 25725-D), Thomas Scientific (single, 3840-N10; pack of ten, 3840-N11), or equivalent (see Notes, 1).
3. Desiccator—containing an efficient desiccant with moisture indicator. Silica gel and anhydrous $CaCl_2$ are not suitable desiccants. See AOCS Specification H 9-87.

PROCEDURE

1. Weigh accurately, and as rapidly as possible, duplicate test portions of 5–10 g each (prepared as directed in AOCS Official Method Aa 2-38, Procedure, 1) into tared moisture dishes, picking out and discarding pieces of foreign matter that might affect the accuracy of the determination.
2. Slip the cover on the bottom of the dish, place the uncovered dish in the oven and dry at 130°C for 3 hr.
3. Remove the dishes from the oven, cover immediately, cool in a desiccator to room temperature and weigh.

CALCULATIONS

1. Moisture and volatile matter % $= \dfrac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$

NOTES

1. For test portions with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test portion loss from splattering during heating.
2. Replicate determinations should check within 0.2% moisture and volatile matter.

REFERENCES

1. *Oil & Soap 23:* 285 (1946).

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REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
2. Hydrochloric acid (HCl)—concentrated, sp. gr. 1.19, reagent grade.

PROCEDURE

1. Weigh accurately 4-5 g of the ground test sample into a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the material (see Fig. 3). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
2. Place the wrapped test portion in the Butt extraction tube and assemble the apparatus as shown in Figure 1. Put about 25 mL of petroleum ether into the tared extraction flask before attaching to the tube.
3. Heat in a water bath or on an electric hot plate at such a rate that the solvent will drop from the condenser on the center of the thimble at the rate of at least 150 drops/min.
4. Keep the volume of solvent fairly constant by adding enough to make up for any that may be lost due to evaporation. Continue extraction for 4 hr.
5. Cool and disconnect the extraction flask. Evaporate the ether on a steam bath or in a water bath until no odor of ether remains. A gentle stream of clean, dry air or nitrogen may be used to facilitate removal of the solvent. Cool to room temperature, carefully remove any moisture or dirt from the outside of the flask and weigh. Repeat heating until constant weight is obtained.
6. Determine the moisture in the ground test sample as follows:
   (a) Weigh 5 g into a tared AOCS moisture dish.
   (b) Slip the cover on the bottom of the dish and place dish in a forced-draft oven. Dry at 130°C for 1 hr.
   (c) Remove the dish from the oven and cover immediately. Cool in a desiccator containing an efficient desiccant for 30 min and weigh. *Note*—Calcium chloride is not satisfactory. See AOCS Specification H 9-87.
   (d) Moisture in ground sample, %
   $$= \frac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$$

CALCULATIONS

1. Oil in ground test sample, % $= \dfrac{\text{mass, g of oil}}{\text{mass, g of test sample}} \times 100$

2. The percentage oil is calculated to any desired moisture basis with the following formula:
   $$= \frac{F(100 - \%\ \text{moisture desired})}{100 - \%\ \text{moisture in ground test sample}}$$
   Where—
   F = % oil determined in ground test sample by AOCS Official Method Aa 4-38

PRECISION

1. Two single determinations performed in one laboratory shall not differ by more than 0.42%.
2. Single determinations performed in two different laboratories shall not differ by more than 0.73%.

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that the particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size <0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies, using conventional grinders, screens, and sieves, samples should be prepared so that the particle size ranges from 0.5 mm to 0.9 mm (No. 35 to No. 20 sieve, respectively). Particle size should be verified by screening an *extracted* sample.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occur, it may be advantageous to grind the sample with an equal amount of diatomaceous earth (see AOCS Method Ai 3-75), or to grind the sample with solid carbon dioxide (dry ice).

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If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method is recommended (e.g., AOCS Method Am 2-93).

2. This method was found to give superior results in determining commercially extractable oil and free fatty acid content. These recommendations are based on the results of a study performed in 1998 by Dr. P. Wan, USDA, SRC, and were considered by the Seed and Meal Committee. See technical report.
3. A longer fuming time (up to an additional 30 min) will be required if seed is wet as demonstrated by a second moisture determination (on ground seed).

REFERENCES

1. *J. Am. Oil Chem. Soc. 64*:865 (1987).

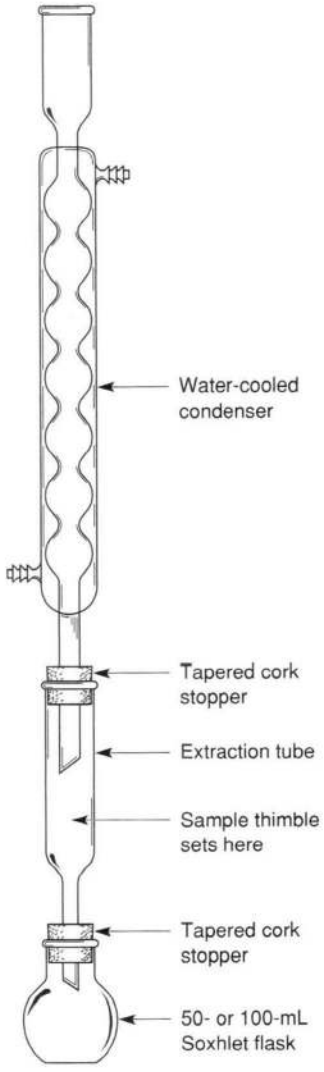

Figure 1. Butt-type extraction.

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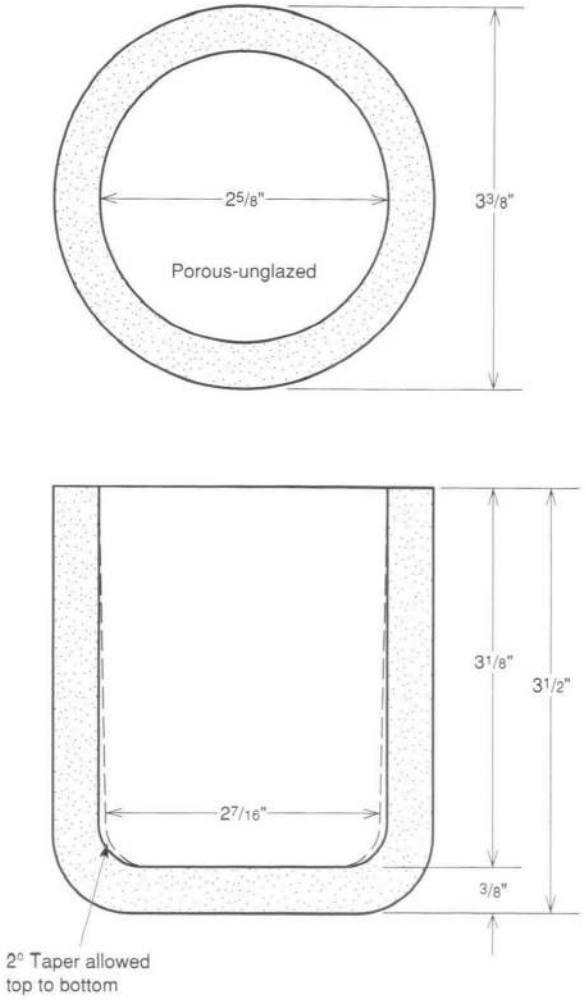

Figure 2. Fuming vessel for cottonseed.

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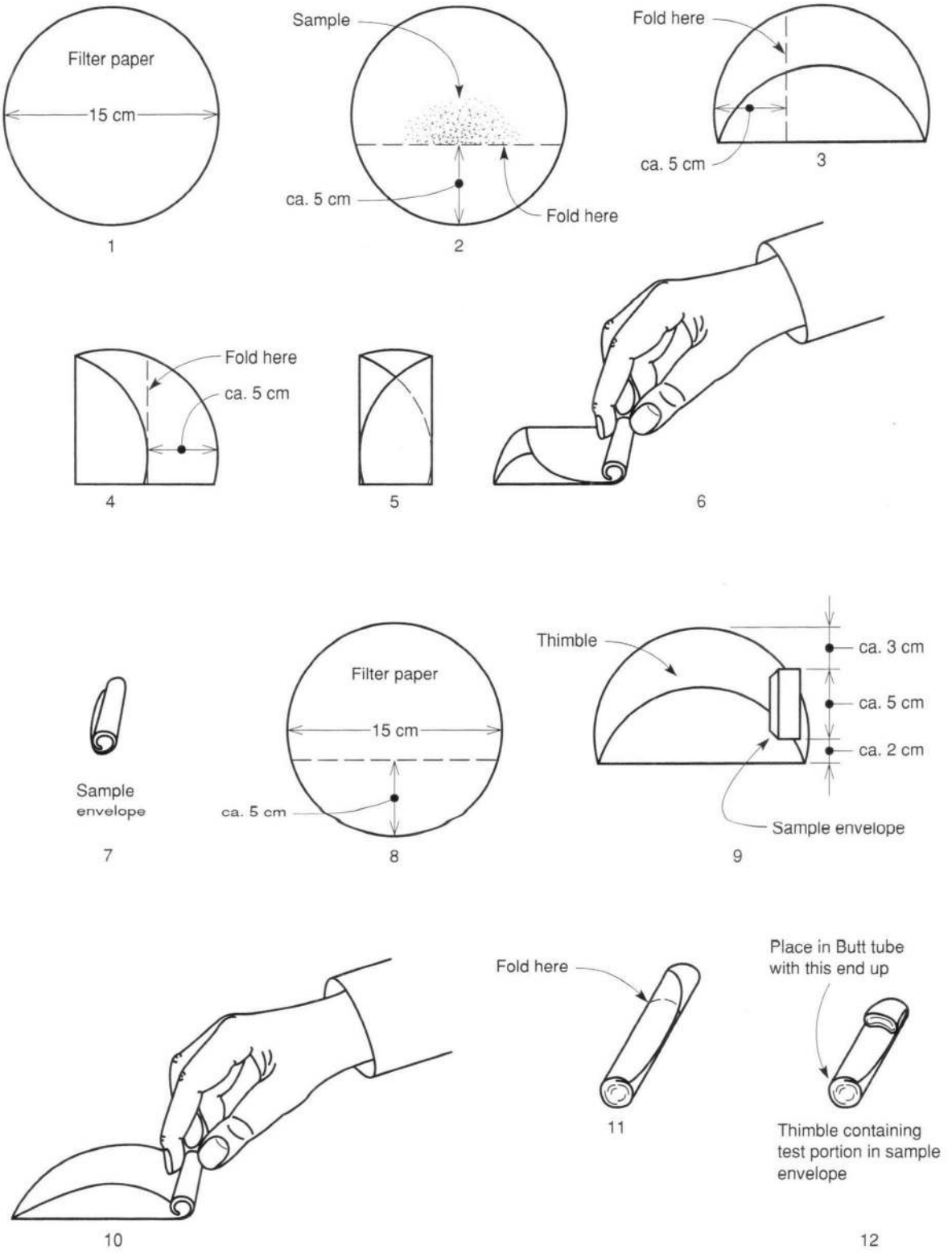

Figure 3. Folding filter paper for oil extraction.

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8. Immediately connect the Kjeldahl flask to the other end of the condenser tube and thoroughly mix the contents by shaking. Apply heat at about a 7.5-min boil rate and distill until at least 150 mL of distillate have been collected.
9. Titrate the contents of the receiving flask with 0.25 M NaOH solution, using 3 or 4 drops of indicator.
10. Conduct a sucrose blank determination of all reagents simultaneously with the test portions and similar in all respects. Correct for blank determined on reagents.
11. The performance of the entire method should be checked frequently by analyzing either National Institute of Standards and Technology (NIST)-certified ammonium dihydrogen phosphate (standard reference material 194) or high-purity lysine hydrochloride.
12. Determine moisture in the ground test sample as directed in AOCS Official Method Ba 2a-38, Procedure section.

CALCULATIONS

1. Nitrogen, % $= \dfrac{\left[\left(MA \times 2 \times mLA\right) - \left(mLBK \times MB\right) - \left(mLB \times MB\right)\right] \times 1400.67}{\text{mg test portion}}$

   Where—

   mLB = volume, mL standard base used
   mLA = volume, mL standard acid used
   mLBK = volume, mL standard base needed to titrate 1 mL of standard acid minus mL standard base needed to titrate reagent blank carried through method and distilled into 1 mL standard acid
   MA = molarity of standard acid
   MB = molarity of standard base

   Note—To determine percent ammonia, substitute 1703.06 for 1400.67 in the equation for the calculation of percent nitrogen.
2. Calculation: N × 6.25 = P
   Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

PRECISION

Tables 1, 2 and 3 list the results of the second collaborative study, March 1991, for the determination of nitrogen in cottonseed and cottonseed meal, using a 0.56 catalyst to acid ratio for $TiO_2/CuSO_4$ catalyst. The project coordinator was James Falk, member of the AOCS Seed and Meal Analysis Technical Committee.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali solutions. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not vice versa. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. This method has been evaluated through interlaboratory comparison of catalysts and has been adopted by the AOCS Uniform Methods Committee as the official replacement for the mercuric oxide catalyzed method, AOCS Official Method Ba 4a-38. An interlaboratory evaluation (References, 1) indicated that AOCS Official Method Ba 4d-90 (using the copper/titanium catalyst mixture) produces results closer in agreement with AOCS Official Method Ba 4a-38 (using mercuric oxide catalyst) than methods using copper sulfate catalyst. As a result of this study, AOCS Official Methods Ba 4a-38 (mercuric oxide), Ba 4b-87 (copper sulfate) and Ba 4c-87 (Kjel-Foss Automatic) were declared surplus (obsolete) in 1991.

   The Association of Official Analytical Chemists (AOAC) collaborative study (References, 2) showed no bias when comparing Cu/Ti catalyst with HgO. The AOCS interlaboratory evaluation (References, 1), based on sample weight, acid, volume, digestion temperature and time, previously specified in AOCS Official Method Ba 4a-38, indicated an average bias of –0.244%, which was statistically significant at the 99% confidence level. Factors that may affect method bias are sample weight and particle size, catalyst composition, use of Alundum, volume of acid used for digestion, digestion temperature and digestion time. In 1991, two additional collaborative studies (cottonseed and cottonseed meal) showed that when 30 mL of sulfuric acid were used, caking of the digest was reduced and results were comparable to the mercuric oxide catalyzed method. As a result, the AOCS versions of this method may specify the use of more than 20 mL of sulfuric acid, but otherwise conform with the AOAC version (References, 3).

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2. Use of steam- and water-resistant rubber tubing and stoppers to connect the glass parts is strongly recommended.
3. Due to large particle size, a larger sample, in the range of 1.4-1.7 g, is recommended for cottonseed meal. This may also require an adjustment in the volume of sulfuric acid used for sample digestion.
4. More than 20 mL of concentrated sulfuric acid is needed for samples high in fat or oil, because more acid is required to effect complete digestion, avoid loss in nitrogen and prevent caking. In recent collaborative studies involving the application of this method to cottonseed meal, it was observed that when 30 mL of H_2SO_4 were used, caking was reduced and results were comparable to the mercuric oxide catalyzed method.
5. Complete conversion of organic nitrogen to ammoniacal nitrogen is essential to obtain accurate and precise results. After the liquid has become clear and colorless, it may be necessary to digest the test portion for approximately an additional 30–50 min. Reagent proportions, heat input and digestion time are critical factors and should not be changed. Prolonged digestion and high temperatures must be avoided, because some ammoniacal nitrogen can be lost by oxidation. Large sample particles should be avoided, because the larger particles will require a longer digestion time.
6. The cooled digest should be liquid, or liquid with a few small crystals. Caking before the addition of water indicates too little residual acid at the end of the digestion period and may result in low nitrogen values.

REFERENCES

1. *INFORM 1:*884 (1990).
2. Kane, P. F., *J. Assoc. Off. Anal. Chem. 70:*907 (1987).
3. *Official Methods of Analysis*, AOAC International, 16th ed., Vol. 1, Gaithersburg, MD, 1995, Chapt. 4, p. 13, Method 988.05.

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Table 1
Summary of collaborative study results for determination of percent nitrogen (dry-weight basis) in delinted cottonseed with 21.2% oil content, using 30 mL of acid for digestion.

	1		9		1 + 9	
Sample	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO
% Nitrogen	4.28	4.29	4.31	4.30	4.30	4.30
SD	0.04	0.06	0.06	0.06	0.04	0.04
CV%	0.97	1.31	1.43	1.43	0.99	0.87
Range	0.13	0.22	0.18	0.20	0.13	0.12

Table 2
Summary of collaborative study results for determination of percent nitrogen (dry-weight basis) in high-protein cottonseed meal, using 30 mL of acid for digestion.

	1		2		1 + 2	
Sample	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO
% Nitrogen	7.82	7.78	7.82	7.80	7.82	7.79
SD	0.09	0.09	0.07	0.05	0.07	0.06
CV%	1.14	1.15	0.90	0.64	0.90	0.73
Range	0.27	0.33	0.19	0.17	0.20	0.17

Table 3
Summary of collaborative study results for determination of percent nitrogen (dry-weight basis) in 52% protein cottonseed meal, using 30 mL of acid for digestion.

	4		8		4 + 8	
Sample	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO
% Nitrogen	8.24	8.26	8.29	8.25	8.26	8.25
SD	0.23	0.09	0.06	0.09	0.14	0.08
CV%	0.97	0.34	0.20	0.30	0.59	0.30
Range	0.27	0.33	0.19	0.17	0.20	0.17

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AOCS

AOCS Official Method Aa 6-38

Reapproved 2009

Free Fatty Acids

DEFINITION

This method determines the free fatty acids in the oil removed from the seed by petroleum ether extraction.

SCOPE

Applicable to cottonseed oil extracted according to method Aa 4–38 or oils obtained from dehulled and ground cottonseed meats (see Notes, 1) following the extraction condition as described in method Aa 4-38.

APPARATUS

1. Oil sample bottle or flask of convenient size.

REAGENTS

1. Isopropyl alcohol (99%)—The alcohol must be neutralized with 0.25 M sodium hydroxide solution to a faint pink color before adding to the test portion.
2. Phenolphthalein indicator solution–1% in 95% alcohol.
3. Sodium hydroxide (NaOH)—0.25 M, accurately standardized (see AOCS Specification H 12–52).

PROCEDURE

1. Weigh 7.05 ± 0.05 g of extracted oil (use oil from Aa 4-38) into a suitable size oil sample bottle or flask. Add 50–75 mL of isopropyl alcohol (neutral alcohol) and 1 mL of indicator. Titrate with 0.25 M NaOH, shaking vigorously until a faint pink color is obtained that persists for at least 1 min (see Notes, 2).

CALCULATIONS

1. Free fatty acids (FFA) calculated (see Notes, 3)—
   % FFA = mL of 0.25 M alkali used
2. $$\% \text{ FFA} = \frac{\text{mL} \times 0.25 \times 282 \times 100}{7.05 \times 1000}$$

   Where 7.05 is the sample weight of oil used in g; mL = mL of NaOH required; 0.25 = molarity of NaOH solution; 282 = equivalent weight of free fatty acids in which results are to be expressed. Free fatty acid is usually expressed as % oleic acid (see Notes, 4).

NOTES

Caution

Both petroleum ether and hexane are extremely flammable.

PRECISION

Avoid static electricity. A fume hood should be used at all times when using these solvents.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Cottonseed kernels or meats can be obtained by dehulling the whole cottonseed with a mechanical device, such as Bauer Mill, Waring Blender, etc. followed by separating the meats from hulls with a 4–6 mesh screen; the dehulled meats should be ground to pass through a 1-mm screen.
2. About 15 mL of petroleum ether or hexane may be added to aid solubilizing the oil in the alcohol, if necessary.
3. The result, expressed as oleic acid, is dependent upon using the sample weight of 7.05 g and 0.25 M sodium hydroxide as specified. Any deviation from either the sample weight or the normality will yield erroneous results.
4. In cases where a low free fatty acid content is presumed, 0.1M NaOH should be substituted and the calculation revised accordingly.

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Table 1
The average, expected between laboratory variation (standard deviation of reproducibility, S_R) for the determination of free fatty acids in oilseeds[a].

Approximate FFA value, %	0.1–1.0	1.0–2.0	2.0–3.0
S_R	0.067	0.106	0.271
RSD (CV, %)	21.07	9.38	11.43
R (95%) = $2.8 \times S_R$	0.19	0.30	0.76

[a]Values obtained from the AOCS Laboratory Proficiency Program.

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AOCS

AOCS Official Method Aa 7-55

Reapproved 2009

Residual Lint

DEFINITION

This method determines the residual lint (cellulose fiber) on the seed.

SCOPE

Applicable to cottonseed. Replaces Aa 7-44.

APPARATUS (see Notes, 1)

1. Infrared oven—The oven is of double wall construction, insulated with 2.54 cm (1 in.) rock wool or fiberglass between the inner and outer walls (Fig. 1). The inner wall should be of a highly reflective metal, such as aluminum or, preferably, stainless steel. The same material also may be used for the outer casing. Width and depth of the oven is contingent upon the number of samples to be treated at one time. Upper and lower heating banks, composed of 125 watt, 110–115 volt infrared lamps spaced 15.2–17.8 cm (6–7 in.) on centers, are opposite in direction and measure 15 cm (13 in.) between the convex surfaces of the opposing lamps. An expanded metal tray, located 15.2 cm (6 in.). from the convex surface of the lower lamps and 17.8 cm (7 in.) from the upper lamps, is supported by channel brackets on both sides. Tray should be marked for placement of dishes so as to be directly between the opposing lamps. Oven temperature is maintained at 118 ± 3°C, by a thermoregulator located just below the tray and outside of the direct lamp beam. An exhaust opening of 3.8 cm (1.5 in.) tubing is located in the top center of the oven and discharged into an exhaust system for the removal of fumes. Six 0.95 cm (⅜ in.) holes should be in the bottom of the oven to permit passage of air. The door on the front of the oven may be hinged at the top and retained in either an open or closed position by tension springs, or other suitable arrangements. Two switches should be installed, the first of which controls both banks of lamps, and the second to control only the lower bank of lamps. An interval timing device may be wired into the circuit or attached to a signal system for convenience.
2. Heat-resistant glass dishes—2.54 cm (1 in.) depth, 5.2 cm (6 in.) diameter (Pyrex™ no. 206, or equivalent).
3. Dish covers—unglazed, porous earthenware, tan or light brown in color on top side (Seedburo Equipment Co., Chicago, IL, USA).
4. Aluminum moisture dishes—5.1 × 1.9 cm (2 × ¾ in.), with covers.
5. Aluminum weighing scoop and tare weight.
6. Mechanical brushing machine—equipped with Tyler 35 mesh (US no. 40) wire screen sieves. The brushes are set at the bottom of a vertical shaft and slightly off-center so that the outer edge of the brush contacts the perimeter of the sieve over which the brush revolves. Sieve shall be US no. 40 mesh and shall be equipped with a 14 gauge piece of copper or galvanized wire that has been bent or shaped into a slight letter "S" across the center of the sieve. It is desirable to have the sides of the sieve 2.5–3.8 cm (1–1.5 in.) higher than the side of a normal sieve so as to prevent seed from being thrown out when the brush begins to revolve. Brushes to be Speed Wash type (Milwaukee Dustless Brush Co., Milwaukee, WI, USA), with bristles 5.7 cm (2¼ in.) long, made from horse hair, and slightly treated with a light plastic. Revolving speed of the brushes shall be a minimum 110 rpm to a maximum 120 rpm. It is important that the slight letter "S" be so placed that the concave shaped part of the wire be in the same direction as the brush moves, causing the seed to be thrown back to the center of the sieve. Time of brushing required is approximately 2 min. Specifications and illustrations may be obtained from Jim Falk, Science and Technology Division, AMS, US Department of Agriculture, Washington, DC 20250.

REAGENTS

1. Dilute hydrochloric acid (HCl) solution—made by adding one volume of concentrated reagent hydrochloric acid (37.0–38.0% HCl) to an equal volume of distilled water (see Notes, *Caution*).

PROCEDURE

1. Use a well-mixed sample free of foreign matter.
2. Weigh 50 g (to nearest ± 0.1 g) of test portion into glass dish, distribute evenly and dry, uncovered, for 15 min at 118 ± 3°C, using top and bottom banks of lamps. Dishes should be placed so that the heat rays shall bear directly on the samples. Test samples containing more than 15% moisture shall be dried an additional 15 min.
3. Toward the end of the drying period, absorb into the inner side of the clay cover 2.0 mL of the diluted solution of HCL (1.0 mL for mechanically delinted seed).
4. At the expiration of the drying period, place the cover on the glass dish with treated side toward the seed. Heat for 20 min at 118 ± 3°C using top bank of lights only. The same oven as may be used for drying (see Notes, 2.)
5. Remove and cool to room temperature without desiccation.
6. Transfer seed to weighing scoop and weigh, recording weight as "A."
7. Remove lint by means of a mechanical brushing machine.

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8. Transfer delinted seed to weighing scoop and weigh, recording weight as "B."
9. Determine moisture in composite test sample of lint removed at intervals, recording moisture as "C."

CALCULATIONS

1. The residual linters (linters on the original seed) are conventionally calculated to an 8.0% moisture basis as follows (when 50 g of sample is used):

$$\% \text{ linters} = 2(A - B) \times \frac{92}{(100 - C)}$$

Where—

A = dried and fumed mass of cottonseed test portion, g
B = delinted seed mass, g
C = percent moisture of removed lint

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. Mechanical brushing machine and infrared oven may be obtained by special order from Mid-Continent Engineering, Memphis, TN, USA.
2. If it is preferred to use the large flat clay pots [inside dimensions approximately 13.2 cm (5 3/16 in.) dia × 4.1 cm (1 5/8 in.) depth; Seedburo Equipment Co.], satisfactory results will be obtained. However, it will be necessary that both upper and lower banks of light be used for both predrying and fuming; otherwise the method remains the same.

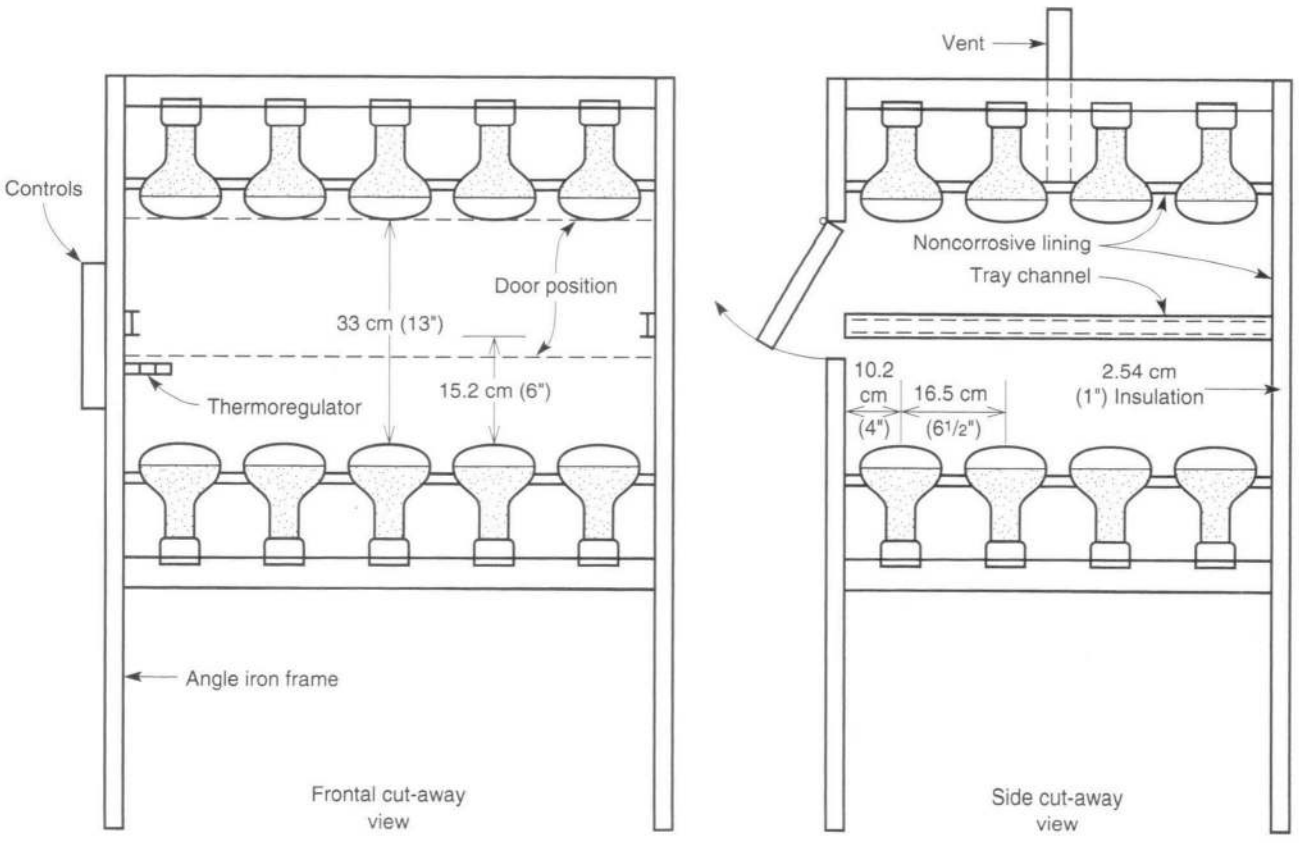

Figure 1. Infrared oven.

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AOCS

AOCS Official Method Aa 8-83

Reapproved 2009

Aflatoxins

DEFINITION

This method determines aflatoxins B_1, B_2, G_1, and G_2 under the conditions of the test (see References, 1–3).

SCOPE

Applicable to cottonseed, cottonseed meats, cottonseed flakes, cottonseed meal, and ammoniated cottonseed meal.

APPARATUS

1. Wiley cutting mill—catalog no. 08-338 (Fisher Scientific, USA), or equivalent.
2. Grinding mill—Bauer no. 148-2 (W.C. Cantrell Co., Ft. Worth, TX, USA), Brinkmann centrifugal grinding mill (CM1; Brinkmann Instruments, Inc., Westbury, NY, USA), Brinkmann rotor beater mill (SR3), or equivalent.
3. Coarse sieves—US nos. 6, 10, 18, and 20.
4. Coarse screen—4/64 in. US no. 6 (3.35 mm dia).
5. Mechanical shaker—Burrell Wrist Action, or equivalent, fitted to hold 500 mL Erlenmeyer flasks fitted with standard taper ground-glass joints.
6. High-speed blender—Waring Model EP-1, explosion-proof, with 1-L jar.
   Note—The type of blender specified in the method is critical. The blender specified should be used.
7. Vials—glass, 5 mL, fitted with either polyethylene friction-fit stoppers, or foil-lined screw caps.
8. Tube-mixer—Vortex, or equivalent.
9. Viewing cabinet—Chromato-Vue Model C-4 or C-6 with four 15 watt longwave UV tubes and contrast filter (Ultraviolet Products, Inc., San Gabriel, CA, USA), or equivalent.
10. Densitometer—Schoeffel SD 3000 (Kratos Analytical Instruments, Inc., Ramsey, NJ, USA), Camag II (Camag Scientific, Wilmington, NC, USA), or equivalent.
11. Thin-layer chromatography equipment—glass plates 20 × 20 cm; applicator for applying 0.25–0.50 mm wet thickness gel layer; mounting board; spotting template; plate holder; desiccating storage cabinet with Drierite or silica gel desiccant; development tank, stainless steel, with trough and glass cover, Desaga-Brinkmann, glass, used without interior liner (no. 25-10-20; Brinkmann Instruments, Inc.), or equivalent; microliter syringe, 10 µL, with 2 cm, 26 gauge tip, 22° point (Hamilton 701-SN, or equivalent); TLC plate-scoring device, SDA 320 (Kratos Analytical Instruments Inc.), or equivalent.
12. Butt tube—available from Sargent-Welch Scientific Co., 2400 Linder Ave., Skokie, IL 60137.
13. Hollow polyethylene stoppers—13 mm top diameter × 7 mm bottom diameter.
14. Liquid chromatograph—with septumless injector; UV absorbance detector, 360–365 nm, 0.005 or 0.01 absorbance units full scale (AUFS) sensitivity; and/or fluorescence detector, 360–365 nm excitation, 400–410 nm cutoff glass filter, or 430 nm cutoff interference filter, containing silica gel packed flowcell (Varian 03-905-857-00, or equivalent) in excitation path.
15. HPLC column—normal phase, silica gel, microparticulate column (10-µm particle size). Waters Associates (Milford, MA, USA) µ-Porasil, 300 mm × 4 mm (i.d.), or Whatman (Maidstone, England) Partisil-10, 250 mm × 4.6 mm (i.d.), are satisfactory. Other types of columns producing essentially complete resolution of aflatoxins in reference standards are satisfactory.
16. Cleanup columns—Econo-columns, 300 mm × 10 mm (i.d.), with 35 µm porous polyethylene frit, nylon stopcock and polypropylene funnel reservoir (Bio-Rad Laboratories, Richmond, CA, USA). Other similarly equipped columns of approximately the same dimensions are suitable.
17. Buchner funnels—2 mL with coarse porosity fritted disc.
18. Filter paper—folded or fluted 18.5 or 24 cm Whatman no. 4 or 2 V paper, or equivalent; glass fiber paper circles, Whatman GS/A (no. 9 cork borer is suitable for cutting circles).

REAGENTS (SEE NOTES, *CAUTION*)

1. Solvents—ACS-grade (stored in glass) acetonitrile, toluene; ACS-grade acetone, diethyl ether (anhydrous, 0.01% ethanol), glacial acetic acid, hexane (bp 68–69°C), toluene; reagent-grade methanol, methylene chloride; HPLC-grade (distilled in glass) acetonitrile, chloroform (0.75% ethanol), cyclohexane, isopropanol, and USP absolute ethanol.
2. Extraction solvents—
   (a) Mix 850 mL acetone, 150 mL distilled water.
   (b) Make 0.1 M HCl by diluting 8.9 mL concentrated HCl to 1000 mL with distilled water.
3. HPLC developing solvent—Prepare water-saturated chloroform by shaking 1 L chloroform with four 100-mL portions of water, about 1 min each time. After each extraction, let phases separate and discard upper aqueous layer. Drain clear lower layer after last extraction into dark glass bottle. Mix water-saturated chloroform/cyclohexane/acetonitrile (25:7.5:1.0), and add either 1.5% absolute alcohol or 2.0% isopropanol.

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4. Lead acetate or optional zinc acetate-aluminum chloride solution—Dissolve 200 g ACS neutral lead acetate trihydrate in distilled water with warming, add 3 mL glacial acetic acid and dilute to 1 L. For optional zinc acetate-aluminum chloride solution, dissolve 200 g zinc acetate and 5 g aluminum chloride hexahydrate in about 900 mL of distilled water and dilute to 1 L.
5. Boiling chips, SiC (Carborundum Co.)—Float off fines and extraneous matter with water, wash with acetone and air dry.
6. Diatomaceous earth (filter aid)—Acid-washed Hyflo Super-Cel (Manville Corp.), or equivalent.
7. Sodium sulfate—anhydrous, granular, ACS grade.
8. Cellulose powder—Whatman CF-11, or equivalent.
9. Thin-layer chromatography (TLC) requirements—
   (a) Silica gel for thin-layer chromatography—Macherey-Nagel GHR (Macherey-Nagel and Co., Düren, Germany; distributed by Brinkmann Instruments, Inc.), Adsorbosils-1 or -5 (distributed by Applied Science Division, State College, PA, USA) and Mallinckrodt Silic AR 4G or 7G (distributed by Mallinckrodt, Paris, KY, USA) all have been found satisfactory.

   Note—Any silica gel that meets the performance requirements noted in the Preliminary TLC section may be substituted.

   (b) Preparation of TLC plates—Weigh 30 g silica gel into 300 mL Erlenmeyer flask; add amount of water recommended by manufacturer. Shake vigorously for about 1 min, and pour into applicator immediately. Coat five 20 × 20 cm glass plates with 0.25 mm wet thickness layer of the silica gel suspension; air dry until gelled (about 10 min). Dry coated plates ≥ 2 hr at 80°C or ≥ 1 hour at 110°C and store in desiccator over indicating silica gel until just before use. Score plates for densitometric quantitation with scoring device (Apparatus, 11). Precoated plates also can be used, e.g., Silica Gel 60 thin-layer chromatography precoated plates, 20 × 20 cm (no. 5721; EM Science, Gibbstown, NJ, USA).
   (c) TLC development solvents—Mix 900 mL chloroform and 100 mL acetone. Mix 960 mL diethyl ether, 30 mL methanol and 1 mL distilled water.
   (d) Spotting and TLC standard solvent—Mix 90 mL toluene and 10 mL acetonitrile. Store in dark glass bottle.
   (e) Confirmatory spray—Add 100 mL sulfuric acid to 300 mL distilled water.
10. Column cleanup—
   (a) Silica Gel 60—no. 7734 (E. Merck, Darmstadt, Germany), 0.063–0.2-mm particle size (80–230 mesh), or equivalent. Dry in about 1 in. thick layer for 2 hr at 105°C, cool in vapor-tight container, add 1.0% water, mix well and equilibrate at least 15 hr. Store in screw-capped bottle in vapor-tight container.
   (b) Wash solvents—Toluene/acetic acid: Mix 900 mL toluene and 100 mL acetic acid. Store in dark glass bottle. Ether/hexane: Mix 675 mL anhydrous diethyl ether and 225 mL hexane. This is sufficient ether/hexane for analysis of 12 samples. If fewer samples are being analyzed, prepare less ether/hexane. Do not store ether/hexane solution (see Notes, *Caution*).
   (c) Aflatoxin elution solvent. Mix 900 mL methylene chloride with 100 mL acetone. Store in dark glass bottle.
11. Aflatoxin standards (see Notes, *Caution*)—
   (a) TLC—Prepare from high-purity crystalline aflatoxins B_1, B_2, G_1, and G_2. Weigh accurately at least 10 mg of each toxin, or obtain an accurately weighed 10 mg sample of each (see Notes, 1). Dissolve each toxin separately in toluene/acetonitrile [Reagents, 9, (d)]. Suitably dilute each with toluene/acetonitrile to contain 100 μg toxin/mL. From these four standard solutions prepare (i) individual toxin reference standards diluted with toluene/acetonitrile to contain 0.5 μg B_1/mL, 0.5 μg G_1/mL, 0.1 μg B_2/mL and 0.1 μg G_2/mL, and (ii) mixed-resolution standard for preliminary TLC (see Preliminary TLC section) diluted with toluene/acetonitrile [Reagents, 9, (d)] to contain 0.5 μg each of B_1 and G_1/mL and 0.01 μg each of B_2 and G_2/mL. Use this mixed standard for quantitative visual estimation and for quantitative densitometric estimation.
   (b) HPLC—Prepare from high-purity crystalline aflatoxins B_1, B_2, G_1, and G_2. Weigh accurately at least 10 mg of each toxin, or obtain an accurately weighed 10 mg sample of each (see Notes, 1). Dissolve each toxin separately in HPLC developing solvent (Reagents, 3). Use HPLC solvent to prepare HPLC standard from each of these solutions to contain 1.0 μg each of B_1 and G_1/mL and 0.3 μg each of B_2 and G_2/mL. Use this HPLC standard for both absorbance and fluorescence estimation. If desired, any appropriate combination of aflatoxins, or individual aflatoxins, of the above concentrations may be used. All diluted standards should be stored in a freezer (preferred) or at refrigerator temperature in 10-mL stoppered glass volumetric flasks, or in 5 mL vials with screw cap fitted with Teflon™ liners (Apparatus, 7).

SAMPLING AND PREPARATION OF SAMPLE

1. Whole seed—Take a minimum 66 kg (30 lb) gross sample with at least 10 uniformly spaced probes using equipment and procedures specified by AOCS Official Method Aa 1-38. The masses of the original sample before and after any predrying and of the kernel fraction shall be determined to allow results to be calculated to a basis of the original seed. The kernels may be obtained by using the equipment and procedures specified in AOCS Official Method Aa 6-38. The entire quantity of kernels shall be ground to US no. 20 mesh (0.85 mm dia) (Apparatus, 3) and intensively mixed before being reduced by quartering or riffling to the laboratory sized sample of at least 1000 g. If decorticating facilities are not available, the entire quantity of seed in the gross sample shall be ground to US no. 10 mesh (2 mm dia) (Apparatus, 3). The linters shall be removed by sieving with convenient screens, with US no. 6 or 3.35 mm perforations. The entire kernel plus hull fraction shall be intensively mixed before being reduced to the laboratory sized sample of at least 1000 g.

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2. Kernels or flakes—Take a minimum 9 kg (20 lb) sample with equipment and procedures of AOCS Official Method Ba 1-38. The entire sample shall be ground to US no. 20 mesh (0.85 mm dia) and intensively mixed before being reduced by quartering or riffling to the laboratory sample of at least 1000 g.
3. Meals and pellets—Use the sampling procedures of AOCS Official Method Ba 1-38. Grind the sample in a Wiley mill (Apparatus, 1), or equivalent, to pass a U.S. no. 18 sieve (1.00 mm dia) (Apparatus, 3) (see Notes, 2).

PROCEDURE

1. Extraction—
   (a) Weigh a 50 g test portion, transfer to blender jar (Apparatus, 6), add 250 mL of extraction solvent [Reagents, 2, (a)] and blend 2 min at slow speed and 3 min at high speed. Alternatively, transfer sample to a 500 mL Erlenmeyer flask fitted with a standard taper ground-glass joint, add 250 mL of extraction solvent [Reagents, 2 (a)], stopper with a glass or a leakproof polyethylene stopper (Apparatus, 13) and shake for 30 min on a mechanical shaker (Apparatus, 5).
   (b) For ammoniated meals or seed, weigh 50 g test portion, transfer to a 500 mL Erlenmeyer flask or blender jar, add 50 mL of 0.1 M HCl [Reagents, 2, (b)], let soak 5 min, add 200 mL acetone (Reagents, 1) and extract by shaking 30 min on a mechanical shaker or by blending 2 min at slow speed and 3 min at high speed (Apparatus, 5, 6).
   (c) Filter through folded or fluted filter paper (Apparatus, 18), collecting at least 100 mL of filtrate.
2. Lead acetate or optional zinc acetate cleanup—
   (a) Measure 100 mL filtrate [Procedure, 1, (c)] into a 250 mL beaker, add 20 mL lead acetate solution (Reagents, 4) and 80 mL distilled water. Stir well, and let stand about 5 min to coagulate precipitate. Add 5 g filter aid (Reagents, 6) and stir well. Optionally, zinc acetate-aluminum chloride (Reagents, 4) may be substituted for lead acetate.
   (b) Filter through folded or fluted filter paper (Apparatus, 18), collecting at least 100 mL of filtrate.
3. Methylene chloride partition—
   (a) Measure 100 mL filtrate [Procedure, 2, (b)] into a 250 mL separatory funnel and add 25 mL methylene chloride. Shake vigorously about 1 min and let phases separate cleanly.
   (b) Prepare Butt tube (Apparatus, 12) with loose plug of glass wool in constriction, cover wool with about 4 cm anhydrous granular sodium sulfate (Reagents, 7) or Whatman CF-11 cellulose powder (Reagents, 8).
   (c) Drain methylene chloride (lower) layer from separatory funnel through Butt tube, collecting in a 150 mL beaker. Repeat partitioning in separatory funnel with another 25 mL methylene chloride and drain through Butt tube into the beaker. Wash Butt tube with additional 25 mL methylene chloride and drain into the beaker.
   (d) Evaporate solvent on steam bath. Remove beaker from steam bath as soon as solvent disappears to prevent overheating of extract. Reserve for column cleanup (next section).
4. Column cleanup—
   (a) Column preparation—Slurry 2 g silica gel [Reagents, 9, (a)] with 10 mL ether/hexane wash solvent [Reagents, 10, (b)] in a 20 mL beaker and pour into cleanup column (Apparatus, 16). Wash beaker with 15 mL ether/hexane wash solvent and add to column. When gel settles, carefully top with 1.5 g layer of sodium sulfate (1-mL beaker filled to top). Drain solvent to top of sodium sulfate layer. (Close stopcock to prevent column drying.)
   (b) Dissolve extract [Procedure, 3, (d)] in 2 mL methylene chloride and pour on column. Wash beaker twice with 1-mL portions of methylene chloride; add washes to column. Drain solvent to top of sodium sulfate, and wash walls of column with 1 mL methylene chloride.
   (c) Add 25 mL toluene/acetic acid wash solvent [Reagents, 10, (b)]. Drain into 100 mL beaker.
   (d) When toluene/acetic acid has drained to top of sodium sulfate, add 50 mL ether/hexane [Reagents, 10, (b)]. Drain into wash beaker [Procedure, 4, (c)], discard washes and replace wash beaker with a clean 100 mL beaker.
   (e) When ether/hexane [Procedure, 4, (d)] has just reached top of column, elute aflatoxins with 60 mL methylene chloride/acetone [Reagents, 10, (c)] collecting eluate in the 100 mL beaker. Evaporate to near dryness on steam bath. (Time for column cleanup, from beginning of first wash to end of elution, should be no longer than 30 min.)
   (f) Dissolve extract in 1 mL methylene chloride and pour through Buchner funnel (Apparatus, 17) with fritted disc overlaid with tightly pressed circle of Whatman GS/A glass fiber paper (Apparatus, 18). Collect filtrate in 5 mL glass screw-cap vial (Apparatus, 7).
   (g) Wash beaker with three 1-mL portions methylene chloride using a fine stream from wash bottle. Transfer each wash to Buchner funnel and allow wash to drain into the vial. (This filtration removes particulate matter that could plug HPLC lines or column filter. Any other system for removing particulate matter without loss of test portion is satisfactory.)
   (h) Evaporate contents of vial to dryness under stream of nitrogen. Reserve for quantitative estimation by HPLC (Quantitative Estimation, 3) or TLC (Quantitative Estimation, 1 or 2). If determinative chromatography is not done the same day, add 0.5–1.0 mL methylene chloride to vial, cap with foil or Teflon™-lined screw cap and store in freezer. Reevaporate when ready to use.

PRELIMINARY TLC

If approximate aflatoxin content is known, preliminary TLC can be eliminated.

1. Dissolve dry extract [Procedure, 4, (h)] in 200 µL of toluene/acetonitrile [Reagents, 9, (d)]. Shake vigorously for 1 min to dissolve, preferably with Vortex mixer (Apparatus, 8).

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2. In subdued incandescent light and as rapidly as possible, spot 2, 5 and two 10 µL spots on imaginary line 4 cm from bottom of TLC plate. Reserve remaining extract for Quantitative Estimation, below.
3. On same plate, spot 2, 5 and 10 µL individual aflatoxin standards [Reagents, 11, (a), (i)]. Spot 5 µL mixed-resolution standard [Reagents, 11, (a), (ii)] on top of one of the two 10 µL test solution origin spots as an internal standard. Also spot at least one separate 5 µL spot of resolution standard [Reagents, 11, (a), (ii)] to determine whether adequate resolution is attained.
4. If Thomas-Mitchell steel tank (Apparatus, 11) is used, pour 50 mL of chloroform/acetone developing solvent [Reagents, 9, (c)] in a trough placed at the rear of a dry tank.
5. If Desaga-Brinkmann glass tank (Apparatus, 11) is used, pour about 150 mL of chloroform/acetone developing solvent [Reagents, 9, (c)] into dry tank.
6. Place the TLC plate in an upright position, with the gel layer facing forward, in the back trough of the Thomas-Mitchell tank, or leaning against the back wall of the Desaga-Brinkmann tank, cover tank and develop in subdued light (35–40 min) at 23–25°C or until aflatoxin reaches an R_f of 0.4–0.7. Use only one plate per tank. Adjust development time to compensate if different temperature is used. Use alternative developing solvent if developing time is > 90 min (see Notes, 3).
7. Remove plate from tank, evaporate solvent at room temperature and illuminate plate from below by placing it flat, coated side up, in viewing cabinet (Apparatus, 9).
8. Observe the fluorescence pattern of the spots of the four individual toxins [Reagents, 11, (a), (i)] and of the four toxins in the mixed-resolution standards [Reagents, 11, (a), (ii)]. In order of decreasing R_f, they are B_1, B_2, G_1, and G_2. Note small color difference (blue fluorescence of B aflatoxins contrasted with slightly green fluorescence of G aflatoxins).
9. If separation of the mixed-resolution standard is poor, repeat TLC using an alternative development solution.
10. Examine chromatograms of test solution aliquots and compare with standards to confirm presence or absence of aflatoxins with R_f and fluorescence pattern similar to standards.
11. From this preliminary plate, estimate the dilution suitable for Quantitative Estimation, below. Record the amount (µL) of test portion extract removed for spotting the preliminary plate. If the dilution is adequate, reserve extract for Quantitative Estimation, below.
12. If a new concentration is required, dry the extract, preferably under a stream of nitrogen, and reserve for Quantitative Estimation, below.
13. Spray plate with fine mist of sulfuric acid [Reagents, 9, (e)] and view under longwave UV. Aflatoxins B_1 and B_2 should now have yellow fluorescence, and G_1 and G_2, yellow-blue fluorescence (see Notes, 4).

QUANTITATIVE ESTIMATION

Use remainder of preliminary TLC extract (Preliminary TLC, 11), or dissolve extract from Preliminary TLC, 12 in the appropriate volume of toluene/acetonitrile [Reagents, 9, (d)] for either visual or densitometric analysis as outlined in Table 1. For HPLC, dissolve dry extract in 200 µL HPLC developing solvent (Reagents, 3).

1. Visual analysis:
   (a) Spot 3, 5 and two 7 µL portions of test portion extract (Preliminary TLC, 11) or dissolved extract (Preliminary TLC, 12) on a TLC plate, along with 2, 3, 4 and 5 µL of the visual aflatoxin standards [Reagents, 11, (a), (i)] corresponding to those aflatoxins observed on the preliminary plate. Spot 5 µL of each standard used on top of one of the two 7 µL sample origin spots as an internal standard.
   (b) Spot at least one 5 µL mixed-resolution standard [Reagents, 11, (a), (ii)] to show whether adequate resolution is attained. Four clearly identifiable spots should be visible when the plate is developed as under Preliminary TLC.
   (c) Examine pattern from test solution spot containing internal standard for aflatoxin spots. R_f values of aflatoxins used as internal standards should be the same as or only slightly different from those of respective standard aflatoxin spots. (Because spots from sample extract are compared directly with standard aflatoxins on the same plate, magnitude of R_f is unimportant. These may vary from plate to plate.)
   (d) Compare test solution spot fluorescence patterns with pattern containing internal standard. Fluorescent spots in test solution thought to be aflatoxins must have R_f values identical to and color similar to aflatoxin standard spots when unknown spot and internal standard spot are superimposed. Spot from the test solution and internal standard combined should be more intense than either sample or standard alone.
   (e) Compare fluorescence intensities of B_1 spots of test solution with those of standard spots and determine which test solution portion matches one of the standards. Interpolate if intensity of test solution is between those of two of standard spots. If spots of smallest portion of test solution are too intense to match standards, dilute test solution and rechromatograph. Compare B_2, G_1, and G_2, if present, in the same manner.
   (f) Calculate concentration of aflatoxin B_1 in µg/kg (ppb), as follows:

$$\mu g/kg\ (ppb) = (S)(Y)(V)/(X)(W)$$

Where—

S = volume, µL aflatoxin B_1 standard spotted equal in intensity to unknown (test solution)

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Y = concentration of aflatoxin B_1 standard, in μg/mL [see Reagents, 11, (a), (i)]
V = final dilution (in μL) of test portion extract
X = volume, μL test portion extract spotted giving fluorescence intensity equal to S (B_1 standard)
W = test portion mass (10 g) represented by final extract [Procedure, 4, (h)]

If final extract dilution does not represent 10 g, calculate correct test portion weight and substitute.

(g) Repeat calculation for aflatoxin B_2, G_1, and G_2, if present, in the same manner as that for B_1, substituting appropriate sample and standard concentrations. Evaporate solvent under a stream of nitrogen. Reserve for densitometric analysis.

2. Densitometric analysis:

(a) Spot on a TLC plate duplicate portions of test portion extract [Preliminary TLC, 12 or Quantitative Estimation, 1, (g)] dissolved in appropriate volume of toluene/acetonitrile [Reagents, 9, (d)] as outlined in Table 1. Spot duplicate 10 μL portions of mixed aflatoxin densitometric standard [Reagents, 11, (a), (ii)], placing spots along imaginary line in center of channel of scored plate 4 cm from bottom [Reagents, 9, (b)]. If B_2 in test portion is considerably higher or lower than B_2 in the standard, it may be necessary to adjust a separate test portion aliquot so that the B_2 in the aliquot spotted has a fluorescence intensity close to B_2 in the standard. Make comparable dilutions for G toxins if present.

(b) Develop plate as outlined under Preliminary TLC, 4–7. If plate is visually inspected before densitometry, use low wattage UV source and minimum exposure time.

(c) Assemble and warm up densitometer apparatus according to manufacturer's instructions; operate in dimly lit room or under suitable protective cover.

(d) Scan plate according to instructions of manufacturer. Irradiate with 365 nm source; observe for emission at 420–460 nm.

(e) Calculate concentration of aflatoxin B_1 in μg/kg (ppb) as follows:

$$\mu g/kg\ (ppb) = (B)(S)(Y)(V)/(Z)(X)(W)$$

Where—

B = average area aflatoxin B_1 peaks in test solution aliquots
S = volume, μL aflatoxin B_1 standard spotted
Y = concentration of aflatoxin B_1 in standard, in μg/mL [Reagents, 11, (a), (ii)]
V = final dilution (in μL) of test portion extract
Z = average area of aflatoxin B_1 peaks in standard aliquots
X = volume, μL test portion extract spotted
W = test portion mass (10 g) represented by final extract (Preliminary TLC, 11 or 12)

(f) Correct aflatoxin concentration values from densitometric measurement for amount of test portion extract removed for preliminary TLC and/or quantitative visual TLC, if either or both were used before densitometric measurement, as follows:

$$\text{Corrected } B_1,\ \mu g/kg\ (ppb) = \frac{\text{Apparent } B_1,\ \mu g \text{ per kg}}{(p/P) + (q/Q)}$$

Where—

p = total μL test portion extract spotted in Preliminary TLC procedure
P = volume, μL of test portion extract prepared in Preliminary TLC
q = total μL test portion extract spotted for visual analysis (if done prior to densitometric analysis)
Q = volume, μL of test portion extract prepared in visual analysis

Use same procedure for B_2, G_1, and G_2, if present.

3. High-performance liquid chromatographic analysis—

(a) Stabilize flow rate at 1.0 mL/min with HPLC developing solvent (Reagents, 3); set UV absorbance detector at 360–365 nm wavelength, detector sensitivity at 0.005 or 0.01 AUFS (Apparatus, 14).

(b) Quantitatively inject 10 μL (10 ng B_1) HPLC standard [Reagents, 11, (b)] for 0.005 AUFS sensitivity, or 20 μL (20 ng B_1) for 0.01 AUFS sensitivity. Record detector response at chart speed of 0.2 in./min (0.5 cm/min) until all four aflatoxins elute (10–11 min).

(c) Dissolve dry test portion extract (equivalent to 10 g original test portion) in 200 μL HPLC developing solvent (Reagents, 3), or if approximate aflatoxin is known, to give 0.5–1.0 μg B_1/mL. Inject 10 μL test portion extract for 0.005 AUFS sensitivity or 20 μL for 0.01 AUFS sensitivity, and record detector response. Although only aflatoxin B_1 and B_2 are normally present in cottonseed products, record response beyond point where G_1 and G_2 elute to confirm their presence or absence. If peaks from test portion injection go off scale, suitably redilute and repeat injection. Use same AUFS sensitivity setting for both standards and test portions.

(d) For fluorescence detection, follow same operating technique used for UV detection [Quantitative Estimation, 3, (a–c)], except set detector for 360–365 nm excitation and 400–410 nm emission (Apparatus, 14). Either inject less volume of both standard and test solution, or suitably attenuate detector to give B_1 peak response 50–60% of full scale for

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standard. If peaks from test solution injection go off scale, suitably redilute and repeat injection. Use same sensitivity setting in both standards and test solutions.

(e) From either integrator area count or peak height, measured from baseline to apex of peak, calculate concentration of aflatoxin B_1 in µg/kg (ppb) as follows:

$$\mu g/kg\ (ppb) = (H)(S)(Y)(V)/(P)(X)(W)$$

Where—

H = area or peak height aflatoxin B_1 test solution peak
S = volume, µL standard aflatoxin B_1 injected
Y = concentration of aflatoxin B_1 in standard in µL/mL
V = final dilution in µL of test portion extract
P = area or peak height of aflatoxin B_1 standard peak
X = volume, µL test solution extract injected
W = test portion mass [10 g in original solution, Procedure, 4, (h), recalculated for amount removed if redilution is necessary].

(f) Correct aflatoxin B_1 concentration values from HPLC measurement for amount of test portion extract removed for Preliminary TLC and/or that removed for TLC quantitation (Quantitative Estimation, 1 and 2). See Quantitative Estimation, 2, (f).

(g) Repeat calculations for aflatoxins B_2, G_1 and G_2, if present, in the same manner as that for B_1, substituting appropriate areas or peak height and standard concentrations.

PRECISION

1. TLC — Within lab: 19.0, B_1; 14.9, B_2
   Between labs: 33.2, B_1; 34.7, B_2
   HPLC—Within lab: 21.9, B_1; 21.3, B_2
   Between labs: 25.6, B_1; 19.0, B_2
2. Values are expressed as coefficient of variation.
3. See Table 4 of References, 4.

NOTES

Caution

Toluene is flammable and a dangerous fire risk. It is toxic by ingestion, inhalation, and skin absorption. The explosive limits in air are 1.27–7% by volume. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Methylene chloride is toxic and a carcinogen. The TLC is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Acetone, cyclohexane, ethanol, hexane, and isopropanol are flammable solvents. They should not be used near an open flame. The use of a fume hood is recommended when using these solvents.

Aflatoxins are very toxic and carcinogenic substances in laboratory animals; results from epidemiologic studies present strong evidence that they may be very hazardous to humans as well. Perform manipulations under hood whenever possible, and take particular precaution, such as use of glove box, when toxins are in dry form because of electrostatic nature and resulting tendency to disperse in working area and air. Swab accidental spills of toxin with 5% sodium hypochlorite bleach containing 5% acetone and then wash thoroughly. See References, 5 and 6 for recommended decontamination procedures for aflatoxins.

NUMBERED NOTES

1. Criteria for purity of aflatoxin primary standards are given in References, 7. Aflatoxin standards are available from Aldrich Chemical Co., W. Milwaukee, WI, USA; Applied Science Division, State College, PA, USA; Calbiochem, La Jolla, CA, USA; Myco Lab Co., Chesterfield, MO, USA; Romer Labs Inc., Washington, MO, USA; Sigma Chemical Co., St. Louis, MO, USA; and Supelco Inc., Bellefonte, PA, USA.

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2. The procedure for the sampling of cottonseed and its products for aflatoxin assay is a paraphrase of conditions in practical application from the *Commercial Feed Regulations of the State of Arizona.* An efficient separation of kernels from the linters, or the hulls plus linters, that are discarded is necessary to prevent a bias toward low results. Intensive mixing is equivalent to 15 min in a double-cone mixer. Provisions for the disposal of the relatively large amounts of ground seed or products, such as denaturization or incineration, will be needed to prevent any contaminated products from entering the food chain.
3. TLC development solvents effective for separation of aflatoxins vary from lab to lab. Use any system that separates B_1, B_2, G_1, and G_2 from each other and also separates interfering substances from the toxins. Alternative solvent systems are listed in Table 2.
4. While G aflatoxins are rarely found in cottonseed, blue fluorescent spots that will not exhibit characteristic color change are sometimes found in the cottonseed extracts at or near the R_f of G_1 or G_2. Test is not confirmatory, but will rule out presumptive aflatoxins that do not show color change.

REFERENCES

1. This method paraphrases *Official Methods of Analysis*, AOAC International, 17th edn., Vol. II, Gaithersburg, MD, 2000, Chapt. 49, p. 24, Method 980.20.
2. *J. Assoc. Off. Anal. Chem. 63:*391 (1980).
3. *Ibid. 66:*357 (1983).
4. *Ibid. 63:*899 (1980).
5. *Ibid. 48:*681 (1965).
6. Davis, W. (ed.), Publication 37, International Agency for Research on Cancer, Lyon, France, 1980.
7. *J. Assoc. Off. Anal. Chem. 53:*92 (1970).

Table 1
Sample dilution and aliquots for TLC visual and densitometric analysis.

Approx. B_1 content from prelim. TLC, µg/kg (ppb)	Visual analysis[a]		Densitometric analysis[a]	
	Dilution of extract, mL	Aliquots on plate, µL	Dilution of extract, mL	Aliquots on plate, µL
0–10	0.125	3, 5, 7	0.125	10, 10
10–25	0.125	3, 5, 7	0.125	5, 5
25–50	0.5	3, 5, 7	0.25	6, 6
50–75	0.75	3, 5, 7	0.50	6, 6
75–125	1.00	3, 5, 7	0.50	5, 5
125–150	1.25	3, 5, 7	0.75	5, 5
150–175	1.50	3, 5, 7	1.00	6, 6
175–200	2.00	3, 5, 7	1.25	6, 6

[a] *For standard containing* 0.5 µg/mL B_1 and G_1; 0.1 µg/mL B_2 and G_2.

Table 2
Alternative developing solvents for aflatoxins B_1, B_2, G_1, and G_2.

Toluene/methanol/acetic acid	95:5:5
Ether/methanol/water	96:3:1
Methylene chloride/chloroform/n-amyl alcohol/formic acid (order of R_f changed to B_1, G_1, B_2, G_2)	80:15:4:1
Chloroform/acetone/water	88:12:1.5
Chloroform/acetone/isopropanol/water	88:12:1.5:1
Chloroform/isopropanol	99:1

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TLC Confirmation of Aflatoxins

DEFINITION

This method identifies aflatoxins B_1 and G_1 by formation of the derivatives B_{2a} and G_{2a} on TLC plates (see References, 1).

SCOPE

Applicable to solutions suitable for TLC of cereal grains, cottonseed, and peanuts, and their products.

APPARATUS

1. Viewing cabinet—Chromato-Vue Model CX-50N with four 15-watt longwave bulbs and contrast filter or high-intensity Blak-Ray lamp 100-B (Fisher Scientific, USA), or equivalent.
2. Thin-layer chromatography equipment—spotting template; plate holder; desiccating storage cabinet with Drierite or silica gel desiccant; developing tank, stainless steel, with glass cover, *or* Desaga-Brinkmann glass, used without interior liner (no. 25-10-20; Brinkmann Instruments, Inc., Westbury, NY, USA); microliter syringe, 10 µL, with 2 cm, 26-gauge tip, 22° point (Hamilton 701-SN, or equivalent); TLC platescoring device, SDA 320 (Kratos Analytical Instruments, Inc., Ramsey, NJ, USA), or equivalent.
3. Hot air blower (Master Appliance Corp., Racine, WI, USA) or hair dryer.

REAGENTS

1. Solvents—ACS-grade (stored in glass) chloroform, acetone, ethyl acetate, toluene, and acetonitrile (see Notes, *Caution*).
2. Precoated silica gel plates—Silica Gel G-HR (66 14 600-6; Brinkmann Instruments, Inc.), precoated Silica Gel 60 (without fluorescent detector) (5763-7; E. Merck, VWR International, West Chester, PA, USA), or equivalent.
3. Solvents for TLC—
   (a) Mix 850 mL chloroform with 150 mL acetone.
   (b) Saturate ethyl acetate with distilled water.
4. Spotting and TLC standard solvent—Mix 90 mL toluene and 10 mL acetonitrile. Store in dark glass bottle.
5. Aflatoxin standards for TLC (see Notes, Caution)—Prepare from high-purity crystalline aflatoxins B_1 and G_1. Weigh accurately at least 10 mg of each toxin, or obtain and accurately weigh 10 mg portion of each (see Notes, 1). Dissolve each toxin separately in toluene/acetonitrile (Reagents, 4). Suitably dilute each with toluene/acetonitrile to contain 100 µg toxin/mL. From these two standard solutions prepare individual toxin reference standards diluted with toluene/acetonitrile to contain 0.5 µg each of B_1/mL and G_1/mL (see Notes, 1).
6. Derivatizing agent—trifluoroacetic acid.
7. Confirmatory spray—Add 100 mL sulfuric acid to 300 mL distilled water.

PROCEDURE

1. Divide silica gel plate (Reagents, 2) in half vertically by scoring line down the middle of the plate with plate-scoring device (Apparatus, 2). Cover one half with a clean glass plate.
2. On uncovered side, spot two 1–10 µL aliquots of unknown test portion extract prepared for TLC containing 0.5–5 ng aflatoxin B_1 and/or G_1. On one of these spots, apply the same amounts of B_1 and G_1 standards (Reagents, 5) as in the sample spot. Also place on plate separate spot containing same amounts of these standard solutions.
3. To extract, extract plus standards, and standards spots, add 1 µL trifluoroacetic acid (Reagents, 6), and allow to react 5 min. Blow warm air (temperature of air at the surface of the plate should be 35–40°C) over plate ≥10 min (Apparatus, 3). All trifluoroacetic acid must be removed to avoid a second solvent front on TLC.
4. Uncover second half of plate and spot as before, but do not add trifluoroacetic acid.
5. Develop plate in chloroform/acetone [Reagents, 3 (a)] or water-saturated ethyl acetate [Reagents, 3 (b)]. Pour solvent directly into tank (Apparatus, 2). Place three small beakers or trough with water in front of plate. Do not equilibrate.
6. After development, examine plate under longwave UV light (Apparatus, 1). Unreacted aflatoxins appear near the top of the plate on half not treated with trifluoroacetic acid. The blue fluorescent derivatives B_{2a} and G_{2a} (water adducts of B_1 and G_1) have R_f values about 25% that of B_1 and G_1. Increase polarity of the developing solvent and repeat with fresh plate if R_f of derivatives is Proof of identity of aflatoxin B_1 and/or G_1 in sample extract is the presence of aflatoxins B_{2a} and G_{2a} in reacted sample extract channel, established by comigration of admixtures. Also examine developed plate under shortwave UV light, which often discloses fluorescing interferences in aflatoxin areas, especially in G_1 areas. Any spot that fluoresces more under shortwave UV light is not aflatoxin.
7. Spray plate with fine mist of sulfuric acid (Reagents, 7) and view under longwave UV (Apparatus, 1). Aflatoxins B_1 and B_{2a} should now have yellow fluorescence, and G_1 and G_{2a} yellow-blue fluorescence (see Notes, 2).

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NOTES

Caution

Toluene is flammable and a dangerous fire risk. It is toxic by ingestion, inhalation, and skin absorption. The explosive limits in air are 1.27–7% by volume. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Both acetone and ethyl acetate are flammable solvents. They should not be used near an open flame. The use of a fume hood is recommended when using these solvents.

Aflatoxins are very toxic and carcinogenic substances in laboratory animals; results from epidemiologic studies present strong evidence that they may be very hazardous to humans as well. Perform manipulations under hood whenever possible, and take particular precautions, such as use of glove box, when toxins are in dry form because of electrostatic nature and resulting tendency to disperse in working area and air. Swab accidental spills of toxin with 5% sodium hypochlorite bleach containing 5% acetone and then wash thoroughly. See References, 2 and 3 for recommended decontamination procedures for aflatoxins.

NUMBERED NOTES

1. Criteria for purity of aflatoxin primary standards are given in References, 4. Aflatoxin standards are available from Aldrich Chemical Co., W. Milwaukee, WI, USA; Applied Science Division, State College, PA, USA; Calbiochem, La Jolla, CA, USA; Myco Lab Co., Chesterfield, MO, USA; Romer Labs, Inc., Washington, MO, USA; Sigma Chemical Co., St. Louis, MO, USA; and Supelco, Inc., Bellefonte, PA, USA.
2. This test only confirms absence of aflatoxins. Many compounds other than aflatoxins may give yellow spots with sulfuric acid. Spots that do not turn yellow are not aflatoxins.

REFERENCES

1. This method paraphrases *Official Methods of Analysis*, AOAC International, 17th ed., Vol. II, Gaithersburg, MD, 2000, Chapt. 49, p. 31, Method 975.37, and *Approved Methods of the American Association of Cereal Chemists*, American Association of Cereal Chemists, 8th ed., 1983, Method 45–25.
2. *J. Assoc. Off. Anal. Chem.* 48:681 (1965).
3. Davis, W. (ed.), Publication 37, International Agency for Research on Cancer, Lyon, France, 1980.
4. *J. Assoc. Off. Anal. Chem. 53:*92 (1970).
5. *J. Assoc Off. Anal. Chem. 58:*110 (1975).

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Determination of Aflatoxins in Corn and Peanuts by Thin-Layer Chromatography Method

DEFINITION

This method detects aflatoxins either visually or densitometrically. Aflatoxins are extracted from samples with methanol/water. Filtrate is diluted with NaCl solution and defatted with hexane. Aflatoxins are partitioned into chloroform which is then removed by evaporation. Aflatoxins are purified by chromatography on 0.5 g silica gel column, and quantitated by thin-layer chromatography (TLC) on silica gel 60 plate with densitometry or visual estimation.

SCOPE

Applicable to corn and raw peanuts. Levels of aflatoxins may be determined densitometrically in the range of 1.5–5.0 ug/g and visually in the range of 7.5–50 ng/g.

APPARATUS

1. Wrist-action shaker—Capable of holding 4 or 8 × 250 mL flasks (Burrell Corp., Pittsburgh, PA, USA, or similar).
2. Silica gel mini-column—6 mL disposable column, packed with 40 µm (60Å) silica gel.
3. Solid phase extraction manifold and vacuum system— equipped with vacuum gauge/flow controller.
4. Vials —glass 8 mL, with foil or Teflon™-lined screw caps.
5. TLC plate—20 × 20 cm glass plate coated with 0.25 mm thick silica gel without fluorescent indicator (e.g., silica gel 60 plates, E. Merck, Gibbstown, NJ, USA).
6. Viewing cabinet—270 × 270 mm base minimum, equipped with 15 W longwave ultraviolet (UV) lamp (e.g., Chromato-Vue, Ultra-Violet Products, Inc., San Gabriel, CA, USA).
7. TLC scanner—capable of scanning in reflectance mode by fluorescence, equipped with high-pressure Hg lamp, monochrometer filters for adjustment to excitation 366 nm, and emission cutoff filter 420 nm (e.g., CAMAG TLC Scanner, Applied Analytical Industries, Wilmington, NC, USA).

REAGENTS

1. Solvents—methanol, hexane, chloroform, anhydrous ethyl ether (100%), dichloromethane, acetone, and isopropanol. ACS grade or equivalent.
2. Aflatoxin standard solution—Prepare in toluene acetonitrile 90:10, v/v, to contain 0.5 µg/mL each B_1 and G_1 and 0.15 µg/mL each B_2 and G_2.

PROCEDURE

1. Test solution preparation—Weigh 50 g (ground to pass 20 mesh) corn or peanuts into 500 mL glass-stoppered Erlenmeyer flask. Add 200 mL methanol/water 85:15, v/v, and secure stopper with masking tape. Shake vigorously by hand until test portions show no clumps. Shake 30 min on wrist-action shaker and filter mixture through medium fluted paper. Collect 40 mL filtrate in 50 mL graduated cylinder. Transfer filtrate to 125 mL separatory funnel. Add 40 mL 10% NaCl solution, mix, and add 25 mL hexane. Shake 1 min. Let phases separate, drain lower (aqueous) phase into second 125 mL separatory funnel, and discard upper phase.

   Extract aflatoxins from aqueous phase with two 25 mL portions $ChCl_3$; shake 1 min each time. Combine $ChCl_3$ fractions in 125-mL Erlenmeyer flask and evaporate to dryness on steam bath.
2. Silica gel column chromatography—Attach silica gel column to extraction system. Condition column by washing with 3 mL hexane, then 3 mL dichloromethane using vacuum (flow rate 6 mL/min) (see Numbered Notes, 1).

   Dissolve test portion residue, prepared in Step 1 (above) in 3 mL dichloromethane and add to column. Let solution drip freely (flow rate ca. 3 mL/min, apply vacuum if needed). Rinse test portion residue container with two 1 mL portions of dichloromethane and add rinses to column. Wash column with 3 mL hexane, 3 mL anhydrous ethyl ether, and then 3 mL dichloromethane. (Use vacuum, flow rate 6 mL/min, or use syringe and adapter to apply pressure to increase solvent flow if necessary.) Do not pull up syringe plunger while it is still attached to the column. Turn off vacuum, remove extraction system cover, and place 8 mL glass vial under each column (test tube rack can be used to hold vials).

   Elute aflatoxins (without vacuum) with two to four 3 mL portions of chloroform-acetone 9:1 (see column suitability, Numbered Notes, 1). Evaporate eluate to dryness on steam bath under stream of nitrogen.

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3. Thin-layer chromatography-Fluorodensitometry Determination—Dissolve test portion extract from step 2 (above) in 250 µL $CHCl_3$. Spot TLC plate with 5 µL $CHCl_3$ test solution in duplicate and 2, 5, 10, and 20 µL aflatoxin standard solution. Randomize standard and test solution spots across plate so duplicate sample spots are not next to each other and standard spots are dispersed evenly. To avoid errors, prepare spotting plan, either on plate or in notebook prior to spotting.

   Develop plate 1 hour with $CHCl_3$-acetone 9:1, v/v. Evaporate solvent 5 min in fume hood followed by 2 min in 50°C forced draft oven.

   Examine plate under longwave UV light to determine presence or absence of aflatoxins.

   Quantitate by fluorodensitometric measurement. Scan sample and aflatoxin reference spots (transmission or reflectance mode, excitation 365 nm and emission cutoff 430 nm). At end of plate scan, rescan 1st or 2nd lane. Scans of same spots should be within +5%; if not, rescan entire plate.

CALCULATIONS

Interpretation of Results—Calculate concentration of aflatoxin B_1 in test portion, using following formula:

$$B_1, \text{ng/g} = \frac{(250 \times R_u)}{(5 \times R_s \times 10)}$$

Where—

250 = volume, µL test portion extract
R_u = average densitometer response for B_1 spots of test portion extract duplicates
5 = volume, µL test portion extract spotted
R_s = calculated average densitometer response/ng for 4 B_1 standard spots
10 = mass, g test portion represented by extract

Calculate concentrations of aflatoxins B_2, G_1, and G_2 similarly.

NOTES

Caution

Wear protective clothing, gloves, and eye wear. See Material Safety Data Sheets for each reagent. Soak all laboratory glassware and plasticware in detergent before washing or disposal. Aflatoxins are extremely carcinogenic to many animals. Neither effects of aflatoxins on humans nor possible routes of entry are presently known.

Grinding of dry samples may result in airborne dust. Even if no toxin is present, there is potential harm from inhalations of mold spores or from allergic response to inhaled dust. Use protective mask and/or dust collector. Prepare samples in area separate from analytical laboratory.

NUMBERED NOTES

1. Check column suitability by adding aflatoxin B_1 standard (3 mL dichloromethane containing 100 ng aflatoxin B_1) to 0.5 g silica gel column. Test column volume ranges at 5 ppb, 20 ppb and 50 ppb. Recovery must be ≥90% by this method.
2. This method was adopted by the Mycotoxin technical committee as a suitable method for use in the AOCS Laboratory Proficiency Program.

REFERENCES

1. *Official Methods of Analysis*, 17th ed., Vol. II, AOAC International, Gaithersburg, MD, 2000, Chapt. 49, p. 16, Method 993.17, Aflatoxins in Corn and Peanuts.
2. *J. AOAC International 77*:637 (1994).

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METHOD PERFORMANCE

Table 1
Method performance for determination of aflatoxins B_1, B_2, G_1, and G_2 in corn and peanuts by thin layer chromatographic method with densitometer estimation.

Product	Aflatoxin	Spike, ng/g	Mean Rec[a] %	S_e	S_R	RSD_r, %	RSD_R, %
Corn	B_1	5	88	1.98	1.98	56.6	56.6
		10	83	2.58	2.58	41.7	41.7
		50	76	14.28	14.25	47.5	47.3
	B_2	3	94	0.64	1.18	26.8	49.3
		15	118	5.37	6.32	45.5	53.6
	G_1	10	102	3.31	4.61	43.6	60.7
		50	91	13.07	15.42	36.1	42.6
	G_2	3	90	1.12	1.44	48.8	62.8
		15	104	4.68	4.68	45.0	45.0
Raw peanuts	B_1	5	78	0.66	0.82	21.3	26.4
		10	83	2.31	2.31	37.3	37.3
		25	84	4.36	4.83	26.1	28.9
	B_2	1.5103	0.38	0.5	38.1	0.3	5
		3	74	0.67	0.67	37.3	37.3
		7.5	123	2.31	2.31	37.2	37.2

[a]Rec = Recovered.

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Aflatoxin Standards

DEFINITION

This method is for the preparation of aflatoxin B_1, B_2, G_1, and G_2 standards.

SCOPE

Standards to be used for aflatoxin quantitation (see Notes, *Caution*).

APPARATUS

1. Vortex VSM-3 mixer—variable speed Vortexer (VWR International, West Chester, PA, USA), or equivalent.
2. Spectrophotometer—capable of measurements from 200–400 nm with 1 cm matched quartz-face cuvettes (see Notes, 1).
3. Analytical balance—with sensitivity of 0.001 mg.
4. Thin-layer chromatography equipment—
   (a) Precoated TLC plates—Silica Gel 60 (without fluorescent indicator), 0.25 mm thick, [MCB Manufacturing Chemicals, Inc. (associate of Merck, Darmstadt, Germany), Cincinnati, OH, USA], or Baker Si 250 (J. T. Baker Chemical Co., Phillipsburg, NJ, USA), or equivalent.
   (b) Glass plates—20 × 20 cm; Desaga-Brinkmann applicator, mounting board, spotting template; microsyringe, 10 µL; desiccating storage cabinet (8-645-6; Fisher Scientific, USA); storage rack (C-4116-3; SGA Scientific, Inc., USA).
5. Developing tank—Developing tank or glass TLC developing chamber for 20 × 20 cm TLC plates.
6. Longwave 15 watt UV lamp (*Caution*—use with UV-absorbing eyeglasses), or Chromato-Vue cabinet equipped with one or two 15 watt lamps, or equivalent.
7. Glass-stoppered flasks—covered with aluminum foil, 100 mL and other convenient sizes.
8. Vials—1 and 2 dram, foil- or Teflon™-lined screw caps (no. 60910-L; Kontes, Vineland, NJ, USA, or equivalent).
9. Microflex tube—1 mL, Kontes K-749000, fitted with Microflex valve (Kontes K-749100).

REAGENTS AND MATERIALS

1. Solvents—ACS reagent-grade (distilled in glass) acetonitrile, acetone, toluene, chloroform, hexane, acetic acid, methanol and anhydrous ethyl ether (see Notes, *Caution*).
   (a) TLC spotting and standard solvent—Mix 90 mL toluene and 10 mL acetonitrile. Store in dark glass bottle.
   (b) TLC developing solvents—Mix 900 mL chloroform and 100 mL acetone. Mix 960 mL diethyl ether, 30 mL methanol and 10 mL distilled water.
2. Primary standards—available from Calbiochem-Behring, San Diego, CA, USA; Myco Lab Co., Chesterfield, MO, USA; Sigma Chemical Co., St. Louis, MO, USA; Supelco Inc., Bellefonte, PA, USA; Eureka Laboratories, Inc., Sacramento, CA, USA.

EVALUATION OF CONCENTRATION OF PRIMARY STANDARDS

1. Preparation of solutions in methanol—
   (a) From crystal form—Weigh (see Notes, 2) approximately 1 mg aflatoxin standard to the nearest 0.001 mg and transfer quantitatively to 100 mL volumetric flask. Dissolve in and dilute to mark with methanol. Calculate concentration of solution in µg/mL.
   (b) Measure absorbance of solution at wavelengths of maximum absorption. Calculate ratios of absorbance for each aflatoxin at wavelengths given in Table 1.
   (c) Calculation of UV molar absorptivities in methanol.

$$\varepsilon = \text{Molar absorptivity} = \frac{A \times MW \times 1000}{\mu g \text{ aflatoxin/mL}}$$

Where—

MW = molecular weight of aflatoxin (B_1 = 312, B_2 = 314, G_1 = 328, G_2 = 330)

A = absorbance

PROCEDURES FOR TLC STANDARDS

1. Preparation of standard solutions—
   (a) For dry film or crystalline form—To container add volume of toluene/acetonitrile (90:10) [see Reagents and Materials, 1, (a)] calculated to give a concentration of 8–10 µg/mL (use label statement of weight as guide). Vigorously agitate solution for 1 min on Vortex mixer and transfer, without rinsing, to a glass-stoppered flask (see Notes, 3–5).

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(b) For standards obtained in solution—Transfer solution to glass-stoppered flask. Dilute, if necessary, to adjust concentration of 8–10 µg/mL using toluene/acetonitrile (90:10) [see Reagents and Materials, 1, (a)].

2. Determination of concentration—
   (a) Record UV spectrum of aflatoxin solution from 330–370 nm against solvent used for solution in reference cuvette.
   (b) Determine concentration by measuring absorption at wavelength of maximum absorption close to 350 nm. Calculate for aflatoxin concentration as

$$1\mu g \text{ aflatoxin/mL} = \frac{A \times MW \times 1000 \times CF}{\text{molar absorptivity } (\varepsilon)}$$

   Where
   MW = aflatoxin molecular weight
   A = absorbance
   CF = the correction factor for particular spectrophotometer used
   See Table 2 for molar absorptivities of aflatoxins in toluene/acetonitrile (90:10).
   (c) Return aflatoxin solution to original flask. Normal exposure to UV light during a measurement results in no observable conversion to photo products.
3. Preparation of resolution standards—Prepare a resolution standard by mixing aflatoxins B_1, B_2, G_1 and G_2 solutions (see Procedures for TLC Standards, 1) to give final concentration of 5 µg/mL each for B_1, G_1 and 0.2 µg/mL each for B_2, G_2.
4. Preparation of spotting standard—After determining concentration of standard solutions from Procedures for TLC Standards, 1, dilute a portion of each standard to spotting concentration of 0.5 µg/mL for aflatoxins B_1, G_1 and 0.2 µg/mL for B_2, G_2, using toluene/acetonitrile (90:10). Prepare 1 mL each time and transfer to microflex tube for convenient storage and dispensing.
5. Determination of chromatographic purity—
   (a) Take portion of standard solution previously diluted to spotting concentration of 0.5 µg/mL for aflatoxins B_1 and G_1 and 0.2 µg/mL for B_2 and G_2.
   (b) Spot successively at 1-cm intervals the following:
   — 5 µL of resolution standard,
   — 5 µL of each aflatoxin spotting,
   — standard on separate lanes,
   — 5µL of each aflatoxin spotting standard + 5 µL *resolution* standard,
   — 50 ng of each aflatoxin (5-6 µL of standard solutions as prepared in Procedures for TLC Standards, 1.
   (c) Develop plate as described in TLC Procedure below.
   (d) After development, spot of individual standard should reveal no other spots except a faint fluorescent spot near the origin.
6. Criteria for purity—
   (a) Chromatographic purity using TLC for ≤ 50 ng per spot.
   (b) Molar absorptivities are within confidence limits in Table 1.
   (c) Absorption peak ratios are within confidence limits in Table 3.
7. Storage of standards, prepared in toluene/acetonitrile (90:10)—
   (a) Before storing, after aliquots have been removed for dilution or spotting, weigh flask containing standard solutions to the nearest mg and record weights for future reference.
   (b) Wrap flask in aluminum foil and store at 0°C.
   (c) When solution is to be used after storage, reweigh flask and record any change.
   (d) Recheck concentration of stored solution by UV detection and recheck purity by TLC each time a portion is taken for dilution to spotting concentration. Aflatoxins stored in methanol are unstable.
   (e) When volume of original solution becomes less than can be employed in the photometer cuvette, use microcells, accurately positioned.
   (f) Any observed change in concentration should correspond with observed loss in weight due to solvent evaporation. If not, discard solution.
   (g) Aflatoxin standards are stable for 1 year when stored at 0°C.

TLC PROCEDURE

1. Preparation of plates—
   (a) Weigh 30 g silica gel into 300 mL glass-stoppered Erlenmeyer and add amount of water recommended by manufacturer.
   (b) Shake vigorously for approximately 1 min and pour onto applicator.
   (c) Immediately coat five 20 × 20 cm plates with 0.25 mm thickness and let stand until gelled (about 10 min).
   (d) Dry coated plates for about 2 hr at 110°C and store in desiccating cabinet until ready to use.
   (e) Scribe line 16 cm from bottom edge as solvent stop, and lines about 0.5 cm from each side, or remove 0.5 cm gel from each side to prevent edge effects.

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(f) Alternatively, use 20 × 20 cm precoated silica gel plates.

2. Developing plates—
   (a) Spot plates as stated in Procedures for TLC Standards, 5.
   (b) Develop plates using 50 mL chloroform/acetone (9:1) in trough in unlined developing tank. Use only one plate per tank.
   (c) Leave plate in tank for 40 min at 23–25°C or until aflatoxins reach R_f 0.4–0.7.
   (d) Adjust developing time to compensate for different temperatures.
   (e) Adjust developing solvent and use freshly activated TLC plates if developing time is >90 min.
   (f) Remove from tank and evaporate solvent at room temperature.
   (g) Illuminate plate from below by placing it flat, coated side up, on longwave UV lamp in darkened room, or view in Chromato-Vue cabinet from above (see Notes, 6).

NOTES

Caution

Toluene is flammable and a dangerous fire risk. It is toxic by ingestion, inhalation, and skin absorption. The explosive limits in air are 1.27–7% by volume. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Ethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using ethyl ether.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Acetone, hexane and methyl alcohol are flammable solvents. They should not be used near an open flame. The use of a fume hood is recommended when using these solvents. Aflatoxins are very toxic and carcinogenic substances in laboratory animals; results from epidemiologic studies present strong evidence that they may be very hazardous to humans as well. Perform manipulations under hood whenever possible, and take particular precautions, such as use of glove box, when toxins are in dry form because of electrostatic nature and resulting tendency to disperse in working area and air. Swab accidental spills of toxin with 5% sodium hypochlorite bleach containing 5% acetone and then wash thoroughly. See References, 1 and 2 for recommended decontamination procedures for aflatoxins.

NUMBERED NOTES

1. Calibrate spectrophotometer as follows: Determine absorbance (A) of the 3 solutions of $K_2Cr_2O_7$ in 0.009 M H_2SO_4 (0.25, 0.125 and 0.0625 mM), at maximum absorption near 350 nm, against 0.009 M H_2SO_4 as solvent blank. Calculate molar absorptivity (e) at each concentration: $\varepsilon = (A \times 1000)/\text{concentration in mM}$. If the three values vary by more than guaranteed accuracy of A scale, check either technique or instrument. Average the three ε values to obtain ε. Determine correction factor (CF) for particular instrument and cuvettes by substituting in equation: $CF = 3160/e$, where 3160 is value for ε of $K_2Cr_2O_7$ solutions. If CF is <0.95 or >1.05, check either technique or instrument to determine and eliminate cause. (Use same set of cuvettes in calibration and determination of purity.)
2. Only weigh dry aflatoxins in facilities equipped to avoid dispersion of electrostatic aflatoxin particles.
3. Sample vials should be wrapped with foil to protect from light and UV radiation.
4. To avoid incorporation of water by condensation, bring all standards to room temperature before use. Do not remove foil from flask until contents reach room temperature.
5. If there is a spill, clean area with 5% sodium hypochlorite solution or bleach containing 5% acetone.
6. Use UV-protective (absorbing) glasses when viewing plates under UV light. Looking directly at UV lamps is harmful to the eyes.

REFERENCES

1. *J. Assoc. Off. Anal. Chem. 48:*681 (1965).
2. Davis, W. (ed.), Publication 37, International Agency for Research on Cancer, Lyon, France, 1980.

OTHER REFERENCES

Info. Bull Tech. Report No. 1, International Union of Pure and Applied Chemistry, June, 1971.
*J. Assoc. Off. Anal. Chem. 53:*92, 96 (1970).
*Ibid. 63:*247, 634 (1980).
Official Methods of Analysis, AOAC International, Gaithersburg, MD, Method 971.22 (17th ed. 2000, 49.2.03).

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Table 1
Molar absorptivities of aflatoxin in methanol and 95% confidence limits from single determination.

Aflatoxin	Wavelength, nm	Molar absorptivity	95% confidence limit
B_1	223	22,100	1,600
	265	12,400	800
	360	21,800	1,100
B_2	222	18,600	1,000
	265	12,100	600
	362	24,000	500
G_1	216	27,400	2,500
	242	9,600	300
	265	9,600	1,200
	362	17,700	700
G_2	214	25,300	2,300
	244	10,500	300
	265	9,000	1,100
	362	19,300	800

Table 2
Absorptivities of aflatoxins in toluene/acetonitrile (90:10).

Aflatoxin	MW	Molar absorptivity
B_1	312	19,300
B_2	314	21,000
G_1	328	16,400
G_2	330	18,300

Table 3
Ratios of major peaks of UV spectra of aflatoxins in methanol and 95% confidence limits ± from single spectrum.

Major peaks compared, nm	Parameter	Aflatoxins			
		B_1	B_2	G_1	G_2
223/265	ratio	1.77	1.54		
	95% CL	0.04	0.05		
214/265	ratio			2.86	2.83
	95% CL			0.15	0.13
242/265	ratio			1.00	1.20
	95% CL			0.02	0.07
362/265	ratio	1.76	1.98	1.84	2.09
	95% CL	0.04	0.08	0.06	0.18

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AOCS Recommended Practice Aa 11-05

Revised 2011

Analysis of Aflatoxin Using HPLC Postcolumn Photochemical Derivatization

DEFINITION

Aflatoxins B_1 and G_1 do not fluoresce under HPLC conditions unless derivatized by chemical or photochemical means. On-line postcolumn derivatization of aflatoxins can increase sensitivity and/or selectivity of responses of the HPLC detector. Photochemical derivatization does not require any chemical additives.

SCOPE

The method is applicable to the analysis of aflatoxin containing extracts when using HPLC.

APPARATUS (see Notes, 1)

Equipment noted is not restrictive: equivalent systems can be substituted.

1. HPLC pump—computing integrator, optional autosampler, and suitable software with a fluorimeter attachment capable of 365 nm excitation and 435 nm emission.
2. Column—C18 reverse-phase, 150 mm × 4.6 mm with 5 µm particle size, (e.g., Beckman Ultrasphere).
3. "PHRED" photochemical reactor—with low-pressure mercury lamp and knitted reactor coils, e.g., KRC 25–25 with a 25 m coil 0.25 mm i.d. (AURA Industries, NY).
4. Silanized vials—4 mL, amber with Teflon-lined screw caps, e.g., Alltech, #72680.
5. Pipet—class A volumetric, 2 mL.

REAGENTS (see Notes, 2,3)

1. Degassed mobile phase—40% methanol in 60% water (40:60, v/v) or a suitable mixture of methanol, acetonitrile, and water giving baseline separation of the aflatoxins.
2. Injection solvent should be the same composition as the mobile phase.
3. Aflatoxin standards—Supelco Inc., Bellefonte, PA, or other suppliers.

FLUORESCENCE DETECTOR CONDITIONS

Set detector parameters in accordance with the manufacturer's recommendations. (Conditions found optimal in one lab for their fluorescence detector: Excitation @ 365 nm; Emission @ 435 nm; Filter Mode, RC Response setting of slow; Digital Filter, 3 s or 5 s; Gain, 10 or above; Attenuation, 1 or as needed.)

STANDARDS

1. Prepare five concentrations of a mixed aflatoxin standard to establish a standard curve. The standards should contain from 0.1 to 1.0 ng of toxin per 20 µL of injection solvent.
2. Evaporate the individual extracts to dryness under nitrogen in silanized vials.
3. Reconstitute each of the extracts in 2.0 mL of injection solvent and dissolve by vortex mixing for at least 2 minutes.

PREPARATION OF TEST SAMPLE

Prepare test sample extracts by any officially recognized aflatoxin extraction method of the AOAC or AOCS, and dry to a film in silanized vials. Test portion extracts may be stored frozen until ready for evaluation.

PROCEDURE

1. Reconstitute test portion extracts with 2.0 mL of injection solvent and dissolve by vortex mixing for at least 2 minutes.
2. Set the mobile phase flow rate to 1 mL/min and inject 20 µL of freshly prepared aflatoxin standard to confirm the equipment is operating within expectations.
3. Inject 20 µL of each test portion extract, record both peak height and peak area data. If the concentration is found to be below 5 ng/g, re-inject 100 µL of the extract. This will increase sensitivity by a factor of 5.
4. Calculate concentrations using the following formula:

$$\text{Aflatoxin ng/g} = A \times (T/I) \times (1/W)$$

where—

A = standard amount response factor (test portion extract response/standard response), expressed as the ng of aflatoxin in microliters of standard or test portion extract injected

T = the final eluate volume

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I = the amount injected in microliters and
W = weight of commodity represented.

5. Use of at least a three-point calibration curve is preferable but a single point calibration can be used if the response has been shown to be linear and enough injections of the standard have been made throughout the run to demonstrate the detector response is within acceptable limits. For example, the 0.5 ng/20 µL standard may be used for the calibration after the curve is verified. The useful life of the ultraviolet bulb is approximately 3000 hours, or until a decrease of response is noticed by an identifiable change of the B_1/B_2 peak area ratio for the aflatoxin standard.

CORRELATION DATA

Figure 1 shows the chromatograms of an aflatoxin standard and a representative test portion extract. This method was demonstrated to have a correlation for the B_1 data of 0.9994 and for the B_2 data of 0.9874 to the current official post-extraction procedures with a probability factor P of <0.0001.

DISPOSAL

For disposal of test portion extracts and standards refer to Notes, 3.

NOTES

Caution

Mycotoxins should be handled as very toxic substances. Perform manipulations in a hood wherever possible, taking particular precautions, such as using a glove box, when toxins are in dry form because of their electrostatic nature and tendency to disperse. Swab any accidental spills and all glassware and waste materials with 5% NaOCl bleach. Use UV glasses if there is exposure to any direct or reflected UV light from the light source.

NUMBERED NOTES

1. Evaluation should be made of any leakage of UV light from source equipment and steps be taken to provide shielding or use of protective glasses during use.
2. Unless otherwise specified, use only analytical grade reagents. While the solvent system indicated for the PHRED system permits the elimination of acetonitrile and can be advantageous for some laboratories wishing to eliminate its use, any combination of the solvents (methanol, water, and acetonitrile) can be used, provided there is adequate separation of the aflatoxins.
3. Use hypochlorite bleach as a cleaning agent to dispose of waste materials. Aflatoxins must be handled with extreme caution as very toxic substances since they are known carcinogens (see Notes, *Caution*).

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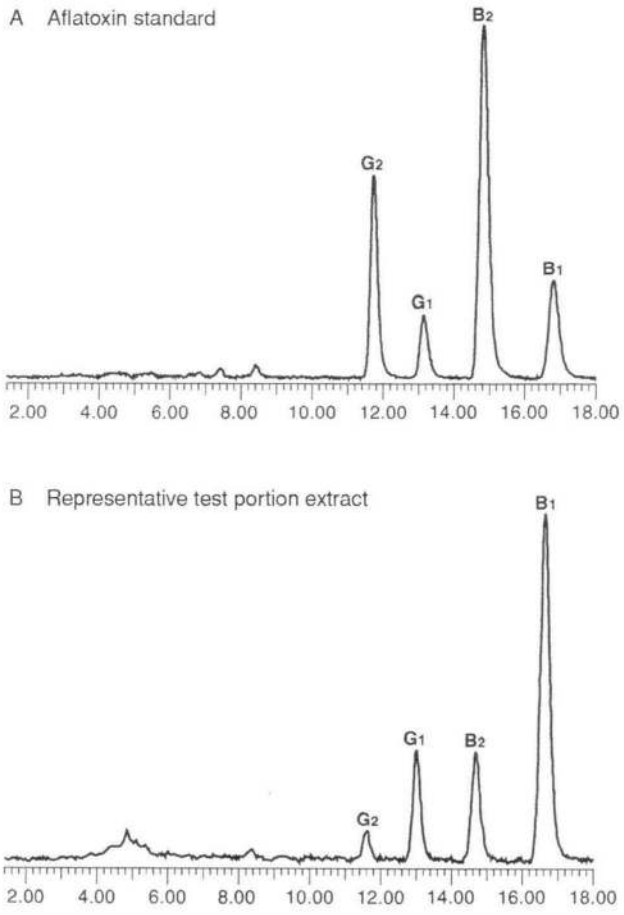

Figure 1. (A) Resolution of an aflatoxin standard. (B) Resolution of an aflatoxin test portion extract.

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AOCS

AOCS Official Method Ab 1-49

Reapproved 2009

Sampling

SCOPE

Applicable to peanuts, whole nuts and shelled stock. Formerly Ab 1-38.

APPARATUS

1. Trier—a pointed metal tube, 5 cm (2 in.) in diameter and 152 cm (60 in.) long with a 3 cm (1.25-in.) slot in 112 cm (44 in.) of its length. A tight-fitting wooden plunger extending to the lower end of the slot is used as the closure when inserting the trier. When the plunger is removed with the slot turned up, the tube will fill along its entire length.
2. Sample riffle—The riffle should have eight 4.5 cm (1.75 in.) slots. Three metal boxes are necessary about 5 cm (2 in.) high, and of such dimensions that the riffle may stand and discharge into these boxes.
3. Spout sampler—Attach a 20 × 13 × 14 cm (8 × 5 × 5.5 in.) elevator bucket to a pole long enough to enable sampler to place bucket in level position near top of seed chute while standing outside and directly in front of car.
4. Metal sample containers—with close-fitting covers, about a 88 L (2.5 US bushel) capacity.
5. Sample containers—
   (a) For whole nuts— 2 L (half-gallon), open-mouth cans equipped with covers suitable for sealing by machine crimping.
   (b) For shelled stock (kernels)—no. 2 open-mouthed cans equipped with covers for sealing by machine crimping.
6. Scales—graduated in kilograms or ounces.

PROCEDURE

1. Carlots before unloading—Proceed as directed in AOCS Official Method Aa 1-38, Procedure, 1, using the peanut trier.
2. Carlots during unloading—Proceed as directed in AOCS Official Method Aa 1-38, Procedure, 2, using the spout sampler.
3. Trucks or wagonloads before unloading—Proceed as directed in AOCS Official Method Aa 1-38, Procedure, 3, using the peanut trier.
4. Sacked peanuts, whole nuts or shelled stock—Withdraw samples from at least 10% of the individual bags with the peanut trier. The bags shall be selected at random from the lot to be sampled and the final sample shall be not less than 23 kg (50 lb), representing a carlot (approximately 25 US tons).
5. All containers shall be filled to capacity and sealed immediately after filling.

CLEANING AND REDUCTION OF LABORATORY SAMPLE

1. Reduce the entire laboratory sample through the riffle to 1.4–1.8 kg (48–64 oz). Weigh the reduced laboratory sample.
2. Remove all the foreign matter from the reduced laboratory sample by hand picking. Weigh this foreign material.
3. Calculate the foreign matter as follows:

$$\text{Foreign matter, \%} = \frac{\text{mass, g of foreign matter}}{\text{mass, g of reduced laboratory sample}} \times 100$$

4. Mix the cleaned sample thoroughly, fill to capacity proper containers as specified in Apparatus, 5 above and seal immediately by machine crimping.

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AOCS

AOCS Official Method Ab 2-49

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Moisture and Volatile Matter

DEFINITION

This method determines the moisture and any material that is volatile under the conditions of the test.

SCOPE

Applicable to peanuts, whole nuts and shelled stock. Formerly Ab 2-47.

APPARATUS

1. Forced-draft oven—AOCS Specification H 1-39.
2. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), Thomas Scientific (single 3840-N10; pack of 11 3840-N11), or equivalent (see Notes, 1).
3. Desiccator—containing an efficient desiccant. Silica gel and anhydrous $CaCl_2$ are not suitable dessicants (see AOCS Specification H 9-87).
4. Sample riffle—The riffle should have eight 4.44 cm (1.75 in) slots. Three metal boxes are necessary, about 5.1 cm (2 in.) high, and of such dimensions that the riffle may stand and discharge into these boxes.
5. Sieve—6 mesh, 30.5–38.1 cm (12–15 in.) diameter.
6. Sample containers—airtight, of convenient size.

PREPARATION OF TEST SAMPLE

1. Reexamine the laboratory sample of foreign matter, which is removed from that portion to be used for analyses. Spread the laboratory sample out on a clean, dry surface and remove all foreign matter by hand picking.
2. Reduce the laboratory sample through the riffle to a size sufficient for the number of tests that will be required.

PROCEDURE

1. Whole nuts—
   (a) Shell a known weight of about 200 g. Keep shells and kernels separate and save all portions.
   (b) Weigh each portion separately and calculate the percentage of each as directed in Calculations.
   (c) Weigh separately 40–50 g of kernels and 20–30 g of shells into tared moisture dishes. Slip the covers on the bottoms of the dishes and dry in forced-draft oven at 130 ± 3°C for 3 hr.
   (d) Remove from the oven, replace cover immediately, cool to room temperature in a desiccator and weigh. Calculate the moisture as directed in Calculations.
2. Shelled stock kernels—
   (a) Proceed exactly as directed in Procedure, 1 (c) and (d), above, using 40–50 g of kernels.

CALCULATIONS

1. Shells, $\% = \dfrac{\text{mass, g of shells}}{\text{mass, g of whole nuts}} \times 100$

2. Kernels, $\% = \dfrac{\text{mass, g of kernels}}{\text{mass, g of whole nuts}} \times 100$

3. Moisture in shells or kernels, $\% = \dfrac{\text{loss in mass, g of shells or kernels}}{\text{mass, g of shells or kernels}} \times 100$

4. Moisture in whole nuts, $\% = AC + BD$

   Where—

   A = % moisture in kernels
   B = % moisture in shells
   C = % kernels
   D = % shells

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NOTES

1. For portions with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to loss from splattering during heating.
2. Replicate determinations should check within 0.2% moisture and volatile matter.

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AOCS

AOCS Official Method Ab 3-49

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Oil

DEFINITION

This method determines the substance extracted by petroleum ether under the conditions of the test.

SCOPE

Applicable to raw and roasted peanut kernels (see Notes, 1). Formerly Ab 3-47.

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in AOCS Official Method Aa 4-38. See Apparatus section and Figure 1 in AOCS Official Method Aa 4-38. Available from Sargent-Welch Scientific Co., Buffalo Grove, IL, USA. Phone 800-727-4368.
2. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, Ahlstrom no. 642, or equivalent.
3. Absorbent cotton—free of petroleum ether extractables.
4. Henry nut slicer—available on special order from Davidson-Kennedy Co., Atlanta, GA, USA.
5. Velocity mixer—Mid-Continent Laboratories, Memphis, TN, USA.
6. Porcelain mortar—about 10.2 cm (4 in.) i.d., with unglazed inside surface and pestle.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PROCEDURE

1. Use about 50 g of kernels, obtained from AOCS Official Method Ab 2-49, Procedure, 1, (a), or from original laboratory sample of kernels. If kernels contain ≥10.0% moisture, dry at 130°C for about 20 min in a forced-draft oven. Drying time will depend on original moisture in the kernels. Ideally, the moisture should be from 6–9%. Dried laboratory samples should be cooled to room temperature before slicing.
2. Pass the kernel through the nut slicer at a feed rate that does not slow down the slicer (see Notes, 2). It is important that a sharp blade be used at all times and that the slice thickness be about 0.18 mm (0.007 in.) The extent of blade projection will have to be adjusted as required for different types of peanuts and for varying moisture content. The nut slicer should produce uniform slices (not chips), without expression of oil from the kernels.
3. Transfer the sliced test portion to the cup of the velocity mixer and mix thoroughly.
4. Weigh accurately duplicate test portions of 2 g each of the sliced material into a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the meats (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
5. Extract the sliced test portion with petroleum ether as directed in AOCS Official Method Aa 4-38, Procedure, 2-5, except that the extraction is halted after 2 hr.
6. Remove the thimble from the Butt tube, allow the petroleum ether to evaporate from the filter paper and test portion at room temperature. Then carefully transfer the test portion to the mortar so there will be no loss. Grind the test portion in the mortar with the pestle for at least 1 min or with 100 vigorous strokes.
7. Return the ground test portion to the same filter paper and continue the extraction as before for another 2 hr.
8. Discontinue the extraction, evaporate the petroleum ether, cool and weigh as directed in AOCS Official Method Aa 4-38, Procedure, 5.
9. Determine the moisture content of the partially dried and sliced test portion as follows:
   (a) Weigh 5 g into a tared AOCS moisture dish.
   (b) Slip the cover on the bottom of the dish and place in a forced-draft oven. Dry at 130°C for 2 hr.
   (c) Remove the dish from the oven and cover immediately. Cool in a desiccator containing an efficient desiccant (see AOCS Specification H 9-87) for 30 min and weigh.

CALCULATIONS

1. $$\text{Moisture in sliced test portion } \% = \frac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$$

2. $$\text{Oil in sliced test portion, } \% = \frac{\text{mass of oil, g}}{\text{mass of test portion, g}} \times 100$$

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3. The percentage oil is calculated to any desired moisture basis with the following formula:

$$\text{Oil, desired moisture basis, \%} = \frac{F\,(100 - \%\ \text{moisture desired})}{100 - \%\ \text{moisture in sliced test portion}}$$

Where—
F = % oil determined in sliced test portion

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that the particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size <0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies, using conventional grinders, screens, and sieves, test portions should be prepared so that the particle size ranges from 0.5 to 0.9 mm (No. 35 to No. 20 sieve, respectively). Particle size should be verified by screening an *extracted* test portion.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occurs, it may be advantageous to grind the laboratory sample with an equal amount of diatomaceous earth (see AOCS Method Ai 3-75), or to grind the laboratory sample with solid carbon dioxide (dry ice).

   If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method is recommended (e.g., AOCS Method Am 2-93).
2. When slicing roasted nuts, the kernels must be fed into the slicer very slowly. The slices will not hold shape and will tend to disintegrate as they are discharged. Mixing should be done at the lowest speed setting of the velocity mixer.

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AOCS

AOCS Official Method Ab 4-91

Revised 2011

Nitrogen-Ammonia-Protein Modified Kjeldahl Method Titanium Dioxide + Copper Sulfate Catalyst

DEFINITION

This method determines total nitrogen content and protein as 6.25 times the nitrogen content of the sample (see Notes, 1).

SCOPE

Applicable to peanuts (groundnuts). Replaced Ab 4-50.

APPARATUS

1. Kjeldahl digestion and distillation apparatus—complete with heat source, traps and block-tin, or equivalent noncorrosive tubing condensers (see Notes, 2).
2. Kjeldahl flasks—800 mL, Pyrex™ or equivalents.
3. Distillate receiving flasks—500 mL, or any convenient size.
4. Use only class A glass burets; calibrate at 10 mL intervals using liquid with density/weight to volume at specified temperature conversions.

REAGENTS

1. Catalyst mixture—containing potassium sulfate, 16.7 g + titanium dioxide, 0.6 g + copper sulfate, 0.01 g + pumice, 0.3 g.
2. Sulfuric acid—ACS sp. gr. 1.84; 95–98% concentrate; nitrogen-free (see Notes, *Caution*).
3. Sodium hydroxide solution—sp. gr. 1.50 (see Notes, *Caution*).
4. Sodium hydroxide solution—0.25 M, accurately standardized (see AOCS Specification H 12-52).
5. Sulfuric acid—0.25 M, accurately standardized (see AOCS Specification H 13-52).
6. Methyl red indicator solution—0.1% in ethyl alcohol, or Alizarin Red S—0.3% in distilled water.
7. Alundum boiling stones—8–14 mesh.
8. Zinc 20 mesh metal—granular. (If the catalyst is used with added pumice, granular zinc metal need not be added as an antibumping agent.)
9. Sucrose—ACS.

PREPARATION OF TEST SAMPLE

1. For peanuts (groundnuts), follow steps 1 and 2 in Procedure, AOCS Official Method Ab 3-49. After test sample preparation, continue with following procedure.

PROCEDURE

1. Weigh 0.250–1.750 g of the ground test sample into the Kjeldahl flask. Add the catalyst mixture (premixed catalyst packs are commercially available and may be used).
2. Add 30 mL of concentrated H_2SO_4 (see Notes, 3) to the sample and catalyst in the digestion flask. Include at least one sample of high-purity lysine hydrochloride in each day's run as a check of the correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.
3. Add a few Alundum boiling stones to the sample flask.
4. To digest the test portion, first adjust heat to bring 250 mL of water at 25°C to rolling boil in 5 min. Place the sample flask on the digestion rack in an inclined position and then heat sample flask at the 5 ` min boil rate until dense, white fumes clear bulb of flask (approximately 5 min). Swirl gently and continue heating an additional 40 min after the liquid has become clear and colorless (see Notes, 4). The Kjeldahl flasks should be rotated a minimum of three times during the digestion.
5. Cool, cautiously add about 300 mL of water and cool to room temperature (see Notes, 5). Add water as soon as possible to reduce amount of caking. If excessive bumping occurs during distillation, increase dilution water from 300 mL to about 350 mL.
6. Accurately transfer a sufficient quantity of the standard acid into receiving flask so there will be an excess of at least 0.5 mL of 0.25 M acid. Add sufficient distilled water to cover the end of the outlet tube and attach to outlet end of condenser tube. The distillate should discharge through a glass tube at the bottom of the receiving flask.
7. Add an additional 0.5–1.0 g Alundum boiling stones to cooled digestion flask. Mix thoroughly and add sufficient alkali solution (Reagents, 3) to make strongly alkaline. Pour the alkali down the side of the Kjeldahl flask so that it does not mix quickly with the acid.

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8. Immediately connect the Kjeldahl flask to the other end of the condenser tube and thoroughly mix the contents by shaking. Apply heat at about a 7.5 min boil rate and distill until at least 150 mL of distillate have been collected.
9. Titrate the contents of the receiving flask with 0.25 M NaOH solution, using 3 or 4 drops of indicator.
10. Conduct a sucrose blank determination of all reagents simultaneously with the samples and similar in all respects. Correct for blank determined on reagents.
11. The performance of the entire method should be checked frequently by analyzing either National Institute of Testing (NIST)-certified ammonium dihydrogen phosphate (standard reference material 194) or high-purity lysine hydrochloride.
12. Determine moisture in the ground test sample as directed in AOCS Official Method Ba 2a-38, Procedure section.

CALCULATIONS

1. Nitrogen, $\% = \dfrac{[(MA \times mLA) - (mLBK \times MB) - (mLB \times MB)] - 1400.67}{\text{mg test portion}}$

   Where—

   mLB = volume, mL standard base used for the test portion
   mLA = volume, mL standard acid used for the test portion
   mLBK= volume, mL standard base needed to titrate 1 mL of standard acid minus mL standard base needed to titrate reagent blank carried through method and distilled into 1 mL standard acid
   MA = molarity of standard acid
   MB = molarity of standard base

   Note—To determine percent ammonia, substitute 1703.06 for 1400.67 in the equation for the calculation of percent nitrogen.
2. Calculation: N × 6.25 = P
   Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

PRECISION

There are no statistics relating the performance of this method to peanuts (groundnuts). The reader is referred to AOCS Official Methods Aa 5-91 and Ba 4d-90 for statistics from the analysis of soybean meal, soy protein concentrate, cottonseed and cottonseed meal.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Alkalies can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali solutions. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not vice versa. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. This method has been evaluated through interlaboratory comparison of catalysts and has been adopted by the committee as the official replacement for the mercuric oxide catalyzed method, AOCS Official Method Ba 4a-38. An interlaboratory evaluation (References, 1) indicated that AOCS Official Method Ba 4d-90 (using the copper/titanium catalyst mixture) produces results closer in agreement with AOCS Official Method Ba 4a-38 (using mercuric oxide catalyst) than methods using copper sulfate catalyst. As a result of this study, AOCS Official Methods Ba 4a-38 (mercuric oxide), Ba 4b-87 (copper sulfate) and Ba 4c-87 (Kjel-Foss Automatic) were declared surplus (obsolete) in 1991.

   The Association of Official Analytical Chemists (AOAC) collaborative study (References, 2) showed no bias when comparing Cu/Ti catalyst with HgO. The AOCS interlaboratory evaluation (References, 1), based on sample weight, acid, volume, digestion temperature and time, previously specified in AOCS Official Method Ba 4a-38, indicated an average bias of –0.244%, which was statistically significant at the 99% confidence level. Factors that may affect method bias are sample weight and particle size, catalyst composition, use of Alundum, volume of acid used for digestion, digestion temperature and digestion time. In 1991, two additional collaborative studies (cottonseed and cottonseed meal) showed that when 30 mL of sulfuric acid were used, caking of the digest was reduced and results were comparable to the mercuric oxide catalyzed method. As a result, the AOCS versions of this method may specify the use of more than 20 mL of sulfuric acid, but otherwise conform with the AOAC version (References, 3).

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2. Use of steam- and water-resistant rubber tubing and stoppers to connect the glass parts is strongly recommended.
3. More than 20 mL of concentrated sulfuric acid is needed for samples high in fat or oil, because more acid is required to effect complete digestion, avoid loss in nitrogen and prevent caking. In recent collaborative studies involving the application of this method to cottonseed meal, it was observed that when 30 mL of H_2SO_4 were used, caking was reduced and results were comparable to the mercuric oxide catalyzed method.
4. Complete conversion of organic nitrogen to ammoniacal nitrogen is essential to obtain accurate and precise results. After the liquid has become clear and colorless, it may be necessary to digest the test portion for approximately an additional 30–50 min. Reagent proportions, heat input and digestion time are critical factors and should not be changed. Prolonged digestion and high temperatures must be avoided, because some ammoniacal nitrogen can be lost by oxidation. Large sample particles should be avoided, because the larger particles will require a longer digestion time.
5. The cooled digest should be liquid, or liquid with a few small crystals. Caking before the addition of water indicates too little residual acid at the end of the digestion period and may result in low nitrogen values.

REFERENCES

1. *INFORM 1*:884 (1990).
2. Kane, P.F., *J. Assoc. Off. Anal. Chem. 70*:907 (1987).
3. *Official Methods of Analysis,* AOAC International, Gaithersburg, MD, (16th ed., 1995, 4.2.03). Method 988.05.

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AOCS

AOCS Official Method Ab 5-49

Reapproved 2009

Free Fatty Acids

DEFINITION

This method determines the free fatty acids in the oil removed from the seed by petroleum ether extraction at room temperature.

SCOPE

Applicable to peanuts. Replaced Ab 5-38.

APPARATUS

1. Henry nut slicer.
2. Cotton—clean, bleached.
3. Butt-type extraction apparatus—assembled exactly as indicated in AOCS Official Method Aa 4-38. See Apparatus section and Figure 1 in AOCS Official Method Aa 4-38. The bottom, constricted part of the tube should be plugged with cotton to support the ground meats (see Notes, 1).
4. Oil sample bottle or flask of convenient size.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution).*
2. Isopropyl alcohol (99%)—The alcohol must be neutralized with sodium hydroxide (NaOH) solution to a faint pink color before adding to the sample.
3. Phenolphthalein indicator solution—1% in 95% alcohol.
4. Sodium hydroxide—0.25 M, accurately standardized (see AOCS Specification H 12-52).

PROCEDURE

1. Remove the foreign matter and reduce the laboratory sample to a convenient size as directed in AOCS Official Method Ab 2-49, Preparation of Test Sample, 1 and 2.
2. Pass 150 g of kernels through the nut slicer. Mix the sliced kernels thoroughly.
3. Place 40–50 g of sliced kernels in the extraction apparatus. Add 50 mL of petroleum ether or hexane and allow all of it to percolate through the test portion into a flask or beaker. Repeat with two 25 mL portions of solvent.
4. Evaporate the solvent from the oil in a water bath under a gentle stream of clean, dry air until free from petroleum ether or hexane.
5. Weigh 7.05 ± 0.05 g of extracted oil into a suitable size oil sample bottle or flask. Add 50–75 mL of neutral alcohol and 1 mL of indicator. Titrate with 0.25 M NaOH, shaking vigorously until a faint pink color is obtained that persists for at least 1 min (see Notes, 2 and 3).

CALCULATIONS

1. Free fatty acids, calculated as oleic, % = mL of 0.25 M alkali used (see Notes, 3).

2. $$\% \text{ FFA} = \frac{\text{mL} \times 0.25 \times 282 \times 100}{7.05 \times 1000}$$

Where—

7.05 g of extracted oil are used
mL = mL of NaOH required
0.25 = molarity of NaOH solution
282 = equivalent weight of FFA in which results are to be expressed. FFA is usually expressed as % oleic acid.

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PRECISION

Table 1
The average, expected between-laboratory variation (standard deviation of reproducibility, S_R) for the determination of free fatty acids in oilseeds.[a]

Approximate FFA value, %	0.1–1.0	1.0–2.0	2.0–3.0
S_R	0.067	0.106	0.271
RSD (CV, %)	21.07	9.38	11.43
R (95%) = 2.8 × S_R	0.19	0.30	0.76

[a] Values obtained from the AOCS Laboratory Proficiency Program.

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. The compactness of the cotton plug and packing of the ground meats in the extraction apparatus should be such that the cold percolation rate will be about 150 drops per minute and that none of the ground test portion will pass through the cotton plug.
2. In Procedure, 5, about 15 mL of petroleum ether may be added to aid solubilizing the oil in the alcohol, if necessary.
3. The result, expressed as oleic acid, is dependent upon using the test portion weight of 7.05 g as specified. Any deviation from the sample weight of 7.05 g will yield erroneous results.

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AOCS

AOCS Official Method Ab 6-68

Reapproved 2009

Aflatoxins

DEFINITION

This method determines aflatoxins B_1, B_2, G_1, and G_2 under the conditions of the test (see References, 1).

SCOPE

Applicable to raw and roasted peanuts, peanut meal, and peanut butter (see Notes, 1).

APPARATUS

1. Centrifuge—IEC International Equipment Co. (Needham Heights, MA, USA), or equivalent with a rotating head accepting 350 mL bottle, achieving greater than 1760 × g (2000 rpm).
2. Centrifuge bottles—250 mL.
3. Chromatographic tubes—with Teflon™ stopcock, reservoir type (250 mL). Use 22 × 300 mm for 50 g samples, and 45 × 600 mm for 1 kg samples.
4. High-speed blender—explosion-proof with 1 L jar. A Waring EP-1, or equivalent may be used. Drill 2.54 cm (1 in.) hole about 1.5 cm from center of lid to permit escape of vapors.
5. Polyethylene stoppers—hollow, 13 mm top dia × 7 mm bottom dia.
6. Thin-layer chromatography apparatus—glass plates, 20 × 20 cm (about 8 × 8 in.); Desaga-Brinkmann applicator, or equivalent; mounting board; spotting template; microsyringe, 10 µL; desiccating storage cabinet (Brinkmann no. 255051, or equivalent); storage rack; stainless steel development tank, about 23 cm long × 9 cm wide × 23 cm deep; trough, or equivalent; longwave 15 watt UV lamp (*Caution*—use with UV-absorbing eyeglasses), or Chromato-Vue cabinet equipped with one or two 15 watt lamps (Ultraviolet Products, Inc., San Gabriel, CA, USA), or equivalent.
7. Tube-mixer—Vortex, or equivalent.
8. Vials—4 dram, foil-lined screw cap.
9. Beakers—stainless steel, 600 mL.

REAGENTS

1. Solvents—reagent- or ACS-grade acetonitrile, acetone, toluene, chloroform, hexane, acetic acid, ethyl alcohol, methyl alcohol, petroleum ether (anhydrous, ≤ 0.01% alcohol) (see Notes, *Caution*).
2. Chloroform/acetone developing solvent—9:1, v/v.
3. Toluene/ethanol/water developing solvent—prepared by shaking toluene/ethanol/water (46:35:19) in a separatory funnel and allowing to stand overnight at ≤ 22°C for phases to separate. Store the two layers in separate, stoppered, glass containers. Alternatively, use toluene/ethanol/water (40:6:3) for the top layer and (40:27:20) for the bottom layer. Prepare enough for several analyses (see References, 2).
4. Toluene/acetonitrile—90:10, v/v.
5. Boiling chips—SiC (Carborundum Co). Float off fines and extraneous matter with H_2O, wash with acetone and dry.
6. Silica gel for chromatography—Any silica gel that meets the test may be used (test each shipment). Macherey-Nagel GHR, Adsorbosil 1 or 5, Mallinckrodt Silic AR 4G or 7G have been found satisfactory.
7. Sodium chloride (NaCl)—reagent grade.
8. Aflatoxin standards—See Notes, 2 for availability. The required standards are prepared as follows (see Notes, *Caution*).
   (a) Mixed B_1, B_2, G_1, and G_2 standard—Prepare a chloroform solution of pure aflatoxins to contain 5.0 µg of B_1, 1.5 µg of B_2, 5.0 µg of G_1, and 1.5 µg of G_2 per mL. Pipette exactly 1.0 mL of the chloroform solution into a 10 mL volumetric flask, and evaporate the solvent under a gentle stream of nitrogen or in a vacuum oven at room temperature. Dissolve in and make to volume with toluene, agitating the flask vigorously to dissolve the aflatoxins. Store at refrigerator temperature in closed jar containing toluene to minimize evaporation losses. Allow container to equilibrate to room temperature before using the standard.
   (b) Mixed B_1 and G_1 standard—Prepare as in Reagents, 8, (a) to contain 0.5 µg of B_1 and 0.4–0.5 µg of G_1 per mL in the diluted standard.
   (c) Individual aflatoxin standards—If desired, individual aflatoxin B_1, B_2, G_1, and G_2 standards may be prepared in toluene solution. Concentration of aflatoxins B_1 and G_1 may be 0.5–1.0 µg/mL, depending on analyst's preference. Concentration of B_2 and G_2 may be 0.1–0.2 µg/mL. Store as outlined in Reagents, 8, (a). A resolution reference standard may be prepared by mixing the individual B_1, B_2, G_1, and G_2 standards at the desired spotting concentration.

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PREPARATION OF TEST SAMPLE

1. Weigh 100 g peanuts or meal, or 50 g peanut butter, into blender jar. Add 250 mL methyl alcohol/H_2O (55:45) and 100 mL hexane to peanut butter. Add 500 mL methyl alcohol/H_2O (55:45) and about 4 g sodium chloride to peanuts or meal.

PROCEDURE

1. Extraction:
   (a) Blend 1 min at high speed.
   (b) Transfer immediately to 250 mL centrifuge bottle and centrifuge 5 min at 2000 rpm. (If time is not important or centrifuge is not available, let mixture stand undisturbed in blender jar as separation will occur within 30 min for peanut butter and raw or roasted peanuts.)
   (c) Pipet 25 mL aqueous methyl alcohol phase into 125 or 250 mL separatory funnel, add 25 mL chloroform, stopper, and shake 30–60 sec. Let layers separate, and drain bottom chloroform layer into 600 mL stainless steel beaker (if available). Do not include any meal with extract.
   (d) Place beaker on steam plate under stream of nitrogen, and add a small amount of H_2O under beaker to improve heat transfer. (100 mL glass beaker is satisfactory, but more time is required for evaporation.)
   (e) Evaporate solvent to between 2 mL and just dryness, or as soon as condensing vapor is no longer visible on beaker tip. Do not leave beaker on hot plate after solvent has evaporated. Transfer extract with careful washing to 4 dram vial and evaporate to dryness in hot H_2O bath. Dissolve extract in 250 µL toluene/acetonitrile (98:2) for spotting on TLC plate.
2. Thin-layer chromatography (TLC):
   Preparation of TLC plates
   (a) Weigh 30 g of Silica Gel GHR (Reagents, 6) into a TS 300 mL Erlenmeyer flask, add 60 mL of distilled water, stopper, and shake vigorously for about 1 min. Pour immediately into applicator, and coat five glass plates (Apparatus, 6) with a 0.25 mm (250 µm) wet thickness layer of the gel. Allow plates to air dry for 10 min, and activate for at least 2 hr at 80°C. Store activated plates in a desiccator over Drierite or silica gel desiccant.
   Preliminary TLC
   (If the approximate aflatoxin content is known, this step may be omitted.)
   (a) Uncap vial containing the test portion extract, add 0.5 mL (500 µL) of toluene, reseal with the polyethylene stopper, cap, place on a Vortex test tube mixer or other similar device, and agitate vigorously for 1 min.
   (b) Puncture the polyethylene stopper to accommodate the syringe needle, and, in subdued incandescent light, spot quickly 2, 5, and 10 µL aliquots of the test portion extract on a TLC plate, placing the spots 1 cm apart along an imaginary line 4 cm from the bottom edge of the plate. In the presence of large amounts of extraneous material in some extracts, the 10 µL aliquot may overload the plate, and cannot be used.
   (c) On the same plate spot 1, 3, and 5 µL aliquots of an aflatoxin standard containing B_1, B_2, G_1, and G_2. Cap the sample extract vial and reserve for Quantitative TLC.
   (d) Draw a line across the top of the plate 2–3 cm from the top edge (as a solvent stop) and a line 0.5 cm in from each side edge.
   (e) Place 50 mL of the chloroform/acetone development solvent (9:1) in the trough of an unlined and unequilibrated development tank. Immediately insert plate from preceding step (d) into trough, placing trough near one side to permit maximum exposure of coated surface to tank volume.
   (f) Seal tank, and develop plate 40 min at 23–25°C or until aflatoxins reach R_f 0.4–0.7. Adjust development time to compensate if a different development tank is used.
   (g) Withdraw the developed plate; air dry in the dark for 15 min.
   (h) Place air-dried plate in viewing cabinet (Apparatus, 6) or over a 15 watt longwave UV lamp in a darkened room.
   Note—If system requires looking directly at longwave UV lamp, protect observer's eyes with UV-absorbing filter (Eastman 2-A, or equivalent).
   (i) Observe pattern of four fluorescent spots in the aflatoxin standard. In order of decreasing R_f they are B_1, B_2, G_1, and G_2. Note small color differences: bluish fluorescence of B aflatoxins as contrasted to slightly green fluorescence of G aflatoxins.
   (j) Examine patterns in the test portion chromatograms for fluorescent spots having R_f close to those of standards and similar appearances (color).
   (k) From this preliminary TLC plate, estimate a suitable dilution of the test portion extract for Quantitative TLC (next section). In the final calculations, take into account quantity of extract used in Preliminary TLC section.
   Quantitative TLC
   (a) If preliminary TLC plate shows a new dilution of the test portion extract is required, evaporate to dryness on a steam bath, preferably under a gentle stream of nitrogen, and redissolve in the calculated volume of toluene/acetonitrile (98:2). Replace hollow polyethylene stopper (Apparatus, 5), cap, and agitate 1 min on tube shaker (Apparatus, 7).
   (b) Spot successively 3.5, 5.0, and two 6.5 µL aliquots of the test portion extract from preceding step (a), and 3.5, 5.0, and 6.5 µL aliquots of the aflatoxin standard or individual aflatoxin standards corresponding to aflatoxins observed on plate in Preliminary TLC section. Spot 5.0 µL of the aflatoxin standard or 5.0 µL of each individual aflatoxin standard on top

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of one of the 6.5 µL test solution aliquot spots. All spots should be of the same size and not larger than about 0.5 cm diameter. At least one origin spot should contain aflatoxins B_1, B_2, G_1, and G_2 to show whether adequate resolution is obtained.

(c) Develop the plates as outlined under Preliminary TLC.

Interpretation of the Chromatogram

(a) Examine the pattern of the aflatoxin standard aliquot(s) containing all four aflatoxins. Four clearly identifiable spots should be visible, as outlined under Preliminary TLC, (i). If four spots are not obtained, TLC must be repeated.

(b) Examine pattern of the 6.5 µL test solution aliquot containing the internal aflatoxin standard. R_f values of aflatoxins used as internal standards should be the same as, or only slightly different from, those of the respective aflatoxin standard(s) spots, and each aflatoxin should appear as a single discrete spot. Because spots from test portion extracts are compared with standards on the same plate, absolute magnitude of R_f values are unimportant. They may vary from plate to plate.

(c) Compare chromatograms of the 3.5, 5.0, and 6.5 µL test solution aliquots with that of the 6.5 µL test solution aliquot containing the aflatoxin internal standard. Fluorescent spots in sample aliquots judged to be aflatoxins must have R_f values identical to, and color similar to, those in the 6.5 µL test solution aliquot containing the aflatoxin standard. The aflatoxin internal standard should be more intense than comparable spots in the test solution aliquots or the standard aliquots alone.

(d) Compare fluorescent intensities of the B_1 spots in the 3.5, 5.0, and 6.5 µL test solution aliquots with those in the aflatoxin standards. Determine which B_1 spot in one of the test solution aliquots matches one of the standard B_1 spots. To aid in estimation, move plate away from lamp to attenuate UV light so that any particular pair of spots can be compared at extinction. Interpolate if the fluorescence of the sample spot is judged to be between that of two of the standard spots. If spots of the smallest test solution aliquot are too intense to match standards, redilute test portion extract and repeat the Quantitative TLC procedure. Similarly, if spots of the largest test solution aliquot are too light to match one of the standards, evaporate the test portion extract to dryness, dilute to a suitable volume, and repeat the Quantitative TLC procedure.

(e) Compare aflatoxins B_2, G_1, and G_2, where applicable, in the same manner outlined in steps (a)-(d) in this section.

(f) If aflatoxin B_2 or G_2 primary standards are not available, assume that B_1 and B_2 have the same fluorescent intensity/mass ratio, and compare B_2 spots in test solution aliquots with the B_1 standard spots for a quantitative estimation of aflatoxin B_2 in the test solution aliquots. Likewise, assume G_2 has same fluorescent intensity/mass ratio as G_1, and compare G_2 spots in test solution aliquots with the G_1 standard spots for a quantitative estimation of G_2 in the test solution aliquots.

(g) Calculate aflatoxin B_1 in the test portion, in µg/kg (ppb), from the equation

$$\text{Aflatoxin } B_1 \text{ (µg/kg)} = \frac{(S)(Y)(V)}{(X)(W)}$$

Where—

S = volume, µL of aflatoxin B_1 standard equal to unknown
Y = concentration of B_1 in standard, in µg/mL
V = volume, µL dilution of test portion extract for Quantitative TLC
X = volume, µL test portion extract giving fluorescent intensity equal to S (B_1 standard)
W = mass, g of original test portion applied to chromatographic column, 10 g if 50 mL of chloroform extract is used

Note—If final extract dilution does not represent 10 g of original test solution, calculate correct sample mass and substitute.

(h) Calculate aflatoxins B_2, G_1, and G_2 in the same manner, substituting the appropriate test solution aliquots and standard concentrations in the equation above. When B_2 and G_2 primary standards are not available, estimate as outlined in (f) in this section.

3. TLC Confirmation of G_1 and G_2:

When aflatoxins G_1 and/or G_2 are detected in test solution aliquots from Quantitative TLC, confirm amount and identity of G_1 and G_2 using the following TLC development system.

(a) Respot the test solution and standard aliquots on a new TLC plate, as outlined under Quantitative TLC.

(b) Put 50 mL of the lower phase from the toluene/ethanol/water development solvent (Reagents, 3) in the bottom of the unlined development tank (Apparatus, 6) and 50 mL of the upper phase in the tank trough. Place trough near one side to permit maximum exposure of coated surface to tank volume.

(c) Without equilibrating tank, place plate in the trough, seal tank, and develop until solvent front reaches scribed line, 12–14 cm beyond origin (30–50 min). Remove plate and air dry in dark for 15 min.

(d) Observe plate in viewing cabinet. In order of decreasing R_f, the aflatoxin standard [Reagents, 8, (a)] gives B_1, B_2, G_1, G_2 in the same order as with chloroform/acetone under Preliminary TLC, but many extraneous fluorescent substances in test solution extracts will have completely different R_f values relative to those of aflatoxins in the two TLC development systems. Aflatoxins G_1 and/or G_2 in test portion extracts should have same R_f as those of respective standards. Make

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a quantitative estimation of G_1 and/or G_2, as outlined in Quantitative TLC and Interpretation of the Chromatogram sections.

PRECISION

1. At 50 (ng/g) total aflatoxin content, within-laboratory precision is ± 5 (ng/g) and between-laboratory precision is ± 10 (ng/g).

NOTES

Caution

Toluene is flammable and a dangerous fire risk. It is toxic by ingestion, inhalation, and skin absorption. The explosive limits in air are 1.27-7% by volume. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Acetone, alcohols, hexane, and petroleum ether are flammable solvents. They should not be used near an open flame. The use of a fume hood is recommended when using these solvents.

Aflatoxins are very toxic and carcinogenic substances in laboratory animals; results from epidemiologic studies present strong evidence that they may be very hazardous to humans as well. Perform manipulations under hood whenever possible, and take particular precautions, such as use of glove box, when toxins are in dry form because of electrostatic nature and resulting tendency to disperse in working area and air. Swab accidental spills of toxin with 5% sodium hypochlorite bleach containing 5% acetone and then wash thoroughly. See References, 3 and 4 for recommended decontamination procedures for aflatoxins.

NUMBERED NOTES

1. Peanut stocks should be adequately sampled by means of an officially sanctioned procedure. Details for sampling peanut stocks and preparation of samples may be obtained from the National Peanut Council, Washington, DC, USA.
2. Criteria for purity of aflatoxin standards are given in References, 5. Aflatoxin standards are available from Aldrich Chemical Co., W. Milwaukee, WI, USA; Applied Science Division, State College, PA, USA; Calbiochem, La Jolla, CA, USA; Calbiochem-Behring, San Diego, CA, USA; Myco Lab Co., Chesterfield, MO, USA; Romer Labs, Inc., Washington, MO, USA; Sigma Chemical Co., St. Louis, MO, USA; Supelco, Inc., Bellefonte, PA, USA; and Eureka Laboratories, Inc., Sacramento, CA, USA.

REFERENCES

1. This method is identical to *Official Methods of Analysis,* AOAC International, 17th ed., Vol. II, Gaithersburg, MD, 2000, Chapt. 49, p. 11, Method 970.45.
2. *J. Am. Oil Chem. Soc. 50:*424 (1973).
3. *J. Assoc. Off. Anal. Chem. 48:*681 (1965).
4. Davis, W. (ed.), Publication 37, International Agency for Research on Cancer, Lyon, France, 1980.
5. *J. Assoc. Off. Anal. Chem. 53:*104 (1970).

OTHER REFERENCES

*J. Assoc. Off. Anal. Chem. 57:*871 (1974).
*Ibid. 66:*355 (1983).

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AOCS

AOCS Official Method Ac 1-45

Reapproved 2009

Sampling

SCOPE

Applicable to soybeans.

APPARATUS (see Notes, 1)

1. Probe—double-tube construction with partitions. Available sizes are 160, 183, 244, and 366 cm. It is important that the probe be capable of reaching the lowest level of the container being sampled.
2. Bag trier—for soybeans a 30 cm trier should be used. (Smaller triers are available, but are not acceptable for sampling soybeans.)
3. Pneumatic probe sampler—unit powered with a ⅞ hp class II, group G motor, installed in a specially designed cyclone air pump and a cyclone collector having a bottom release shutter, 43 cm long, 30 cm i.d., 5 cm outlet and spring-cap 3.8 cm inlet. Two aluminum tubes comprise the probe: outside tube 5 cm o.d. (wall thickness 0.17 cm), inside tube 3.8 cm o.d. (wall thickness 0.12 cm), with the bottom of the probe to be equipped with a cone-tipped section; extensions are available in 0.9-m, 1.2-m and 1.8 m sections. A swivel-type 90° elbow is available so that the section turns freely 360° around the top of the probe. There should be sufficient length of 3.8 cm i.d. flexible seamless vinyl hose to reach from the top of the probe to the cyclone.
4. Grain dockage sieves—
   (a) 0.4 × 1.91 cm slot.
   (b) 0.32 cm round-bottom pan—The slotted sieve is the "scalper" screen and takes off the large dockage which should then be combined with foreign material obtained from the 0.32 cm round-bottom pan.
5. Metal sample containers—with close-fitting covers, about 3 kg capacity.
6. Sample containers—500 g capacity for sending samples to the laboratory.
7. Balance—graduated in grams, sensitive to at least 0.2 g.
8. Boerner divider—no. 34, 36-pocket size. A laboratory rotary divider (approved by the United States Department of Agriculture and the Federal Grain Inspection Service) may be used as an alternative.

PROCEDURE

1. Soybeans in bulk—
   (a) Five or more places shall be probed with the standard (Apparatus, 1) or pneumatic probe sampler (min sample 2500 g).
   (b) Probe in center of car.
   (c) Probe 2–4 feet back from the door post toward the end of the car and approximately 2 feet out from one side of the car.
   (d) Probe from 0.6 to 1.2 m from the same end of the car and approximately 0.6 m from the opposite side of the car as in (c).
   (e) Repeat (c) and (d) above in opposite ends and sides of the car.
2. Soybeans in truckloads or small bins—
   (a) Use same procedure as for soybeans in bulk, above.
3. Soybeans in bags—
   (a) Samples shall be drawn from as many (at least 10–20%) of the bags, selected at random through the lot, as necessary to obtain a representative sample of at least 2500 g.

CLEANING AND REDUCTION OF LABORATORY SAMPLE

1. Thoroughly mix and divide the laboratory sample by use of the Boerner divider and reduce to about 1667 g. Pass the 1667 g of laboratory sample over the grain dockage sieve, but for efficient operation, screen no more than 250 g at one time. Remove broken soybeans, weed seeds and all other foreign material. Weigh all of the foreign material obtained from the total laboratory sample (about 1667 g).
2. Calculate the foreign matter as follows:

$$\text{Foreign matter, \%} = \frac{\text{mass of foreign matter, g}}{\text{total mass of laboratory sample, g}} \times 100$$

3. Reduce the cleaned laboratory sample through the Boerner divider to any size convenient for transmittal to the laboratory.

NOTES

1. One supplier of the sampling equipment noted in this method is Seedburo Equipment Co., Chicago, IL, USA.

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AOCS

AOCS Official Method Ac 2-41

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Moisture and Volatile Matter

DEFINITION

This method determines the moisture and any material that is volatile under the conditions of the test.

SCOPE

Applicable to soybeans.

APPARATUS

1. Forced-draft oven—AOCS Specification H 1-39.
2. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), or equivalent (see Notes, 1).
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87 for alternatives.
4. Boerner sampler or divider—36-pocket size (see Notes, 1).
5. Sieve—20-gauge metal, with round hole perforations 0.32 cm in diameter (see Notes, 2).
6. Airtight sample containers of convenient size.

PREPARATION OF TEST SAMPLE

1. Spread the laboratory sample out on the sieve, shake and remove by hand picking all foreign matter that does not go through the sieve.
2. Reduce the cleaned laboratory sample through the Boerner sampler to 113–227 g. Preserve this test sample in airtight containers and use for all subsequent analyses.

PROCEDURE

1. Weigh 10 g of the whole beans into a tared moisture dish.
2. Dry in the forced-draft oven at 130 ± 3°C for 3 hr. Remove from the oven, cover immediately, cool in a desiccator to room temperature and weigh.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$

NOTES

1. For test samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test portion loss from splattering during heating.
2. Equipment available from the Seedburo Equipment Co., Chicago, IL, USA.

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AOCS Official Method Ab 7-91

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Aflatoxins

DEFINITION

This method quantitatively determines aflatoxins B_1, B_2, G_1, and G_2 under the conditions of the test (see References, 1).

SCOPE

Applicable to corn, raw peanuts, and peanut butter with concentrations ≥10 µg/kg (ppb).

APPARATUS

1. Blender—high speed, explosion proof, with 500 mL blender jar and cover.
2. Filter paper—24 cm, prefolded (Whatman 2V, or equivalent).
3. Glass microfiber filter paper—11 cm diameter (Whatman 934 AH, or equivalent).
4. Affinity column—Afla T-P™ (Vicam, Watertown, MA, USA), or equivalent.
5. Syringe—20 mL, with Luer tip for sample reservoir.
6. Hand pump—20 mL syringe barrel and plunger with polyethylene stopper attached to outlet (Vicam, Watertown, MA).
7. Fluorometer cuvette—12 × 75 mm borosilicate glass culture tube.
8. Fluorometer—360 nm excitation filter and 450 nm emission filter [Vicam VI-Series 4].
9. Automatic dispenser—amber 2 oz (60 mL) bottle with 1.0 mL automatic dispenser [Tri-Continent Scientific (Tri-Continent Scientific, Inc., Grass Valley, CA, USA), or equivalent].
10. Volumetric flasks—2 mL, 50 mL.
11. Erlenmeyer flasks—125 mL, glass-stoppered.
12. LC pump—suitable for flow rate 1 ± 0.005 mL/min.
13. Injection system—syringe-loading injection valve with 50 µL loop, or equivalent.
14. LC column—4.6 mm × 25 cm, 5 µm, C_{18} [Rainin catalog no. 80-225 (Rainin, Woburn, MA, USA), or equivalent].
15. Postcolumn derivatization system—second LC pulseless pump, zero-dead-volume T connector, 610 cm × 0.5 mm i.d. Teflon™ tubing and heating bath or post-column reactor (Rainin catalog nos. F1A920 and F1A910, or equivalent).
16. Fluorescence detector—360 nm excitation filter and > 420 nm cutoff emission filter.
17. Postcolumn photochemical derivatization system, e.g., "PHRED" may be used. See AOCS Recommended Practice Aa 11-05.
18. Vortex mixer.

REAGENTS

Note—See Notes, 1 for proper procedure for cleaning glassware.

1. Solvents—distilled-in-glass methyl alcohol; HPLC-grade methyl alcohol, acetonitrile, and water (see Notes, *Caution*).
2. Extraction solvent—700 mL methanol/300 mL water (7:3, v/v).
3. Bromine developer solution—0.003% bromine in water. Prepare fresh every eight hours. Transfer 5 mL of 0.03% solution (Vicam Aflatext™ developer concentrate) with a volumetric pipette to a 50 mL volumetric flask. Bring to volume with water and mix. Transfer working developer to amber 58 mL bottle with 1.0 mL automatic dispenser.
4. Fluorometer calibration standards—Use 0.05 M H_2SO_4 as blank. Use quinine sulfate dihydrate (Ultrapure, J. T. Baker Chemical Co., Phillipsburg, NJ, USA), 34 mg/mL 0.05 M H_2SO_4, as 20 ng aflatoxin/g standard.
5. HPLC mobile phase—Mix 600 mL H_2O, 200 mL CH_3CN, and 200 mL CH_3OH (3:1:1, v/v/v) and degas.
6. Postcolumn reagent—Dissolve 100 mg iodine in 2 mL CH_3OH. Add 200 mL H_2O, stir 1 hr, filter through 0.45 µm filter. Prepare fresh daily.
7. Aflatoxin standard solutions for LC—
   (a) Mixed aflatoxins stock standard solution—Prepare as in AOCS Official Method Aa 10-88 to contain 500 ng B_1, 125 ng B_2, 250 ng G_1, and 125 ng G_2/mL toluene-CH_3CN (90:10, v/v).
   (b) Working standard solutions—Transfer each quantity indicated (see Table 1) of stock solution [Reagents, 7, (a)] into series of three 2 mL volumetric flasks. Evaporate solutions just to dryness under stream of nitrogen at room temperature. To each flask, add 1 mL CH_3OH, mix, dilute to 2 mL with water, and mix. Prepare fresh daily.

PREPARATION OF TEST SAMPLE

1. Corn—Prepare test sample as in AOCS Official Method Aj 3-87 (Procedure, 1); grind laboratory sample in hammer, disk, or Wiley mill to pass through no. 20 sieve.
2. Raw peanuts—Grind laboratory sample to homogeneous paste in approximately sized Stephan VCM, or equivalent.

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PROCEDURE

1. Extraction—
   (a) Weigh 50 g test portion into blender jar. Add 5 g sodium chloride, 250 mL extraction solvent (Reagents, 2), and blend 2 min at high speed.
   (b) Filter through prefolded paper (Apparatus, 2).
   (c) Pipet 15 mL filtrate into 125 mL glass-stoppered Erlenmeyer flask (Apparatus, 11). Add 30 mL H_2O (Reagents, 1), stopper, and mix.
   (d) Filter diluted extract through glass microfiber filter ≤ 30 min (no more than 30 min) before affinity chromatography. Filtrate should be clear—if not, refilter. Proceed immediately with affinity column chromatography.
2. Affinity column chromatography—
   (a) Secure syringe reservoir to a ring stand. Remove top cap from column. Cut off tip and use cap as connector between column and reservoir.
   (b) Pipet 15 mL second filtrate [Procedure, 1, (d)], equivalent to 1 g test portion, into reservoir.
   (c) Connect air-filled hand pump to reservoir. Remove end cap from column.
   (d) Push extract through column at flow-rate of about 2 drops/sec (6 mL/min).
   (e) Disconnect hand pump and fill pump with air.
   (f) Add 10 mL H_2O to reservoir. Reconnect hand pump and push water through column at flow rate of 6 mL/min.
   (g) Repeat with another 10 mL H_2O. Discard water washings.
   (h) Disconnect hand pump and fill pump with air. Reconnect to reservoir and pass 2–3 mL air through column. Disconnect hand pump.
   (i) Add 1.0 mL HPLC-grade methanol to reservoir. Collect eluate in appropriate container.
   (j) Reconnect hand pump and push methanol through column. Pass an additional 2–3 mL air through column.
   (k) For quantitation by solution fluorometry—Collect methanol eluate in fluorometer cuvette. Immediately proceed with fluorometric determination.
   (l) For quantitation by postcolumn iodine derivatization HPLC—Collect methanol eluate in 2 mL volumetric flask (Apparatus, 10). Dilute volume to 2.0 mL with LC-grade water (Reagents, 1), mix, and proceed with HPLC quantitation.
3. Solution fluorometry—
   (a) Calibration of fluorometer—Refer to Vicam's Fluorometric Protocol Manual for the appropriate calibration procedure for your Series fluorometer. There are four Series fluorometers each with distinct calibration requirements.
   (b) Quantitation—Add 1.0 mL of diluted developer (Reagents, 3) to eluate [Procedure, 2, (k)] in clean cuvette (Apparatus, 7). Vortex-mix for 5 s. If bubbles adhere to wall of cuvette, tap lightly to dislodge. Insert cuvette in fluorometer and wait 60 s. Record reading, which is equivalent to ng total aflatoxin/gram of test portion.
4. HPLC determination with fluorescence detection and postcolumn derivatization—
   (a) Connect LC column outlet to one arm of a stainless steel, low-dead-volume T connector using short piece of 0.01 in. (0.25 mm) i.d. tubing. Connect outlet of second LC pump, which delivers the postcolumn reagent, to second arm of T. Connect one end of 610 cm × 0.5 mm i.d. coil of Teflon™ tubing to third arm of T and connect other end to detector.
   (b) Maintain reaction coil temperature at 70°C with oven or constant temperature bath. Set flow rates as follows:
      —mobile phase (column), 1.0 mL/min
      —postcolumn reagent, 0.3 mL/min
      —total rate through reaction coil, 1.3 mL/min
   (c) Stabilize the entire system by running 10–20 min. If integrator is used, adjust sensitivity controls of fluorescence detector or integrator to give reasonable response (signal/noise = 5:1) for 0.125 ng aflatoxin G2/50 µL. If strip chart recorder is used, adjust fluorescence detector control to give 30–40% scale deflection with 0.125 ng aflatoxin G2/50 µL.
   (d) Inject 50 µL working standard mixture into injector, using 20–30 µL excess to ensure 50 µL loop is completely filled. Aflatoxins elute in order G_2, G_1, B_2, B_1, with retention times of approximately 6, 8, 9, 11 min, respectively, and should be baseline resolved. If necessary, adjust retention times by changing CH_3OH concentration of mobile solvent.
   (e) Inject 50 µL working standard solutions 1, 2, and 3 (Reagents, 7 and Table 1) daily and prepare standard curves for aflatoxins.
   (f) Inject 50 µL extract [Procedure, 2, (l)] into injector. Identify each aflatoxin peak in chromatogram from analysis of test portion by comparing retention times with those of corresponding reference standards.
   (g) Determine quantity of each aflatoxin in eluate injected from corresponding standard curves. Calculate concentration of each aflatoxin in test sample using the following formulas:

$$W = 25\ g \times (15\ mL/125\ mL) \times (15\ mL/45\ mL) = 1\ g$$

$$\text{Aflatoxin, ng/g} = A \times (T_v/I_v) \times (1/W) = A \times 40$$

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Where—

W= mass, g of commodity represented by eluate

A = ng aflatoxin in eluate injected

T_v = final eluate volume (2,000 µL)

I_v = eluate injected (50 µL)

Add concentrations of the four aflatoxins to obtain total aflatoxin concentration.

PRECISION

1. See Tables 2 and 3.
2. See References, 1.

NOTES

Caution

Methanol (methyl alcohol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform, and perchloric acid.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

NUMBERED NOTES

1. Soak all laboratory glassware in 10% solution of household bleach, which generally contains 5.25% NaOCl, before reusing or discarding. See References, 3 for further details on decontamination.

REFERENCES

1. This method paraphrases an Association of Official Analytical Chemists (AOAC) official method, as published in *J. Assoc. Off. Anal. Chem. 74*:81 (1991).
2. *Official Methods of Analysis,* AOAC International, Gaithersburg, MD, Method 968.22, (17th ed., 2000, 49.2.08).
3. *Official Methods of Analysis,* AOAC International, Gaithersburg, MD, Method 991.31 (17th ed., 2000, 49.2.18).
4. Vicam's Fluorometric Protocol Manual (Watertown, MA, USA).

Table 1
Preparation of working standard solutions

Working std soln	Stock soln taken, µL	Amount in working std soln ng/50 µL			
		B_1	B_2	G_1	G_2
1	60	0.750	0.188	0.375	0.188
2	40	0.500	0.125	0.250	0.12
3	20	0.250	0.063	0.125	0.063

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Table 2
Statistical analysis of collaborative results for total aflatoxin by fluorometry from 13 laboratories.[a]

Food	Added, ng/g	Average, ng/g	S_r B_1	RSD_R, %	S_R	RSD_R, %	Outlier labs	Horwitz ratio	Recovered %
Corn	0	2[b]	—	—	1.36	61.91	0	1.60	—
	0	2	—	—	1.11	56.48	1	1.38	—
	10	12	—	—	4.11	33.09	0	1.07	124
	20	21	—	—	3.07	14.44	0	0.51	106
	30	33[c]	5.43	16.57	6.55	20.01	0	0.75	109
	NC[d]	29[c]	5.74	19.71	6.68	22.95	0	0.84	—
Peanut butter	0	2[c]	0.68	30.45	0.81	36.51	0	0.91	—
	10	13[c]	1.54	11.75	1.77	13.57	0	0.43	131
	20	21	—	—	4.38	20.55	0	0.72	107
	30	33	—	—	3.65	10.97	0	0.41	111
Peanuts	0	1[b,c]	0.97	105.52	1.88	117.89	0	2.50	—
	0	1[c]	0.73	87.18	0.89	107.15	1	2.30	—
	10	12	—	—	3.17	27.49	0	0.88	115
	20	21[c]	2.68	12.79	3.22	15.34	0	0.54	105
	30	29	—	—	3.93	13.44	0	0.49	97

[a] S_r, within-laboratory standard deviation; S_R, among-laboratories standard deviation; RSD_r, within-laboratory relative standard deviation; RSD_R, among-laboratories relative standard deviation.
[b] Outlier not removed.
[c] Blind duplicates.
[d] NC, naturally contaminated. Corn has 23 ng/g aflatoxins by the AOAC CB method (References, 2).

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Table 3
Statistical analysis of results for aflatoxin by liquid chromatography postcolumn derivatization from 10 laboratories.[a]

Aflatoxin	Added, ng/g	Average, ng/g	S_r	RSD_R, %	S_R	RSD_R, %	Outlier labs	Horwitz ratio	Recovered %
				Corn					
B_1	0.00	0.52	—	—	0.59	113.16	0	2.27	—
B_2	0.00	0.00	—	—	—	—	0	—	—
G_1	0.00	0.00	—	—	—	—	0	—	—
G_2	0.00	0.00	—	—	—	—	0	—	—
Total	0.00	0.52	—	—	0.59	113.16	0	2.27	—
B_1	5.83	4.47	—	—	2.24	50.07	0	1.39	77
B_2	0.83	0.53	—	—	0.27	51.13	0	1.03	64
G_1	2.50	1.89	—	—	1.08	57.28	0	1.39	76
G_2	0.83	0.30	—	—	0.30	100.62	0	1.85	36
Total	10.00	7.20	—	—	3.66	50.77	0	1.51	72
B_1	11.67	9.88[b]	—	—	1.67	16.85	0	—	—
B_1	11.67	10.00	—	—	0.42	4.21	2	0.13	86
B_2	1.67	1.06	—	—	0.28	26.76	0	0.60	64
G_1	5.00	4.48[b]	—	—	1.03	22.23	0	—	—
G_1	5.00	4.79	—	—	0.34	7.04	1	0.20	96
G_2	1.67	0.81	—	—	0.37	45.25	0	0.97	49
Total	20.00	16.23[b]	—	—	1.15	7.09	0	—	—
Total	20.00	16.66	—	—	0.78	4.68	2	0.16	83
B_1	17.50	15.74[b,c]	3.23	20.55	3.76	23.92	0	—	—
B_1	17.50	14.88	0.86	5.75	1.50	10.09	1	0.26	85
B_2	2.50	1.85[b,c]	0.62	33.56	0.82	44.38	0	—	—
B_2	2.50	1.38[c]	0.35	25.21	0.41	29.74	1	0.33	55
G_1	7.50	7.42[b,c]	1.50	20.26	2.11	28.38	0	—	—
G_1	7.50	7.18[c]	0.68	9.53	0.68	9.53	1	0.35	96
G_2	2.50	1.05[c]	0.20	19.02	0.53	50.59	0	1.04	42
Total	30.00	26.04[b,c]	5.44	20.88	6.80	26.12	—	—	—
Total	30.00	24.49[c]	1.79	7.31	2.86	11.68	1	0.33	81
B_1	NC[d]	18.69[c]	4.63	24.78	4.63	24.78	0	0.80	—
B_2	NC	1.64[c]	0.22	13.72	0.29	17.87	0	0.66	—
Total	NC	20.28[c]	4.35	21.44	4.36	21.48	0	0.80	—

(continued)

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Table 3
(Continued)

Aflatoxin	Added, ng/g	Average, ng/g	S_r	RSD_R, %	S_R	RSD_R, %	Outlier labs	Horwitz ratio	Recovered %
				Peanut butter					
B_1	0.00	0.36[c]	0.33	93.64	0.47	131.44	0	—	—
B_2	0.00	0.02[b,c]	—	—	0.04	210.82	0	—	—
B_2	0.00	0.02[c]	—	—	0.04	198.43	1	—	—
G_1	0.00	0.02[c]	0.04	223.61	0.05	263.52	0	—	—
G_2	0.00	0.00[c]	—	—	—	—	—	—	—
Total	0.00	0.39[c]	0.33	85.81	0.48	123.06	0	—	—
B_1	5.83	5.26[c]	0.78	14.90	1.56	29.61	0	1.05	90
B_2	0.83	0.72[b,c]	0.23	32.27	0.50	69.43	0	—	—
B_2	0.83	0.58[c]	0.12	21.01	0.22	37.61	1	0.64	70
G_1	2.50	2.34[c]	0.55	23.55	0.71	30.59	0	0.95	93
G_2	0.83	0.24	0.19	79.06	0.24	100.73	0	1.77	29
Total	10.00	8.42[c]	1.45	17.22	2.54	30.17	0	0.72	84
B_1	11.67	10.24	—	—	1.98	19.35	0	0.61	88
B_2	1.67	0.93	—	—	0.46	49.68	0	1.09	56
G_1	5.00	4.52	—	—	1.20	26.50	0	0.73	90
G_2	1.67	0.52	—	—	0.39	75.74	0	1.52	31
Total	20.00	16.21	—	—	3.74	23.06	0	0.77	81
B_1	17.50	14.34	—	—	3.00	20.89	0	0.69	82
B_2	2.50	1.62	—	—	0.51	31.72	0	0.75	65
G_1	7.50	6.48	—	—	1.62	25.00	0	0.73	86
G_2	2.50	0.90	—	—	0.43	47.72	0	1.04	36
Total	30.00	23.33	—	—	5.07	21.74	0	0.77	78
				Peanuts					
B_1	0.00	0.03[b,c]	0.07	235.70	0.08	268.53	0	—	—
B_1	0.00	0.00[c]	—	—	—	—	2	—	—
B_2	0.00	0.00[c]	—	—	—	—	0	—	—
G_1	0.00	0.00[c]	—	—	—	—	0	—	—
Total	0.00	0.00[c]	—	—	—	—	0	—	—
B_1	5.83	5.27	—	—	2.06	39.00	0	1.11	90
B_2	0.83	0.58	—	—	0.31	53.79	0	1.10	70
G_1	2.50	2.20[b]	—	—	0.85	38.81	0	—	—
G_1	2.50	2.44	—	—	0.38	15.73	1	0.40	98
G_2	0.83	0.30	—	—	0.22	73.70	0	1.36	36
Total	10.00	8.59	—	—	4.06	47.26	0	1.92	86
B_1	11.67	9.61[b,c]	1.58	16.45	1.89	19.69	0	0.50	82
B_1	11.67	9.71[c]	0.53	5.45	1.62	16.71	1	0.50	83
B_2	1.67	1.07[c]	0.25	23.08	0.41	38.29	0	3.33	64
G_1	5.00	4.54[c]	0.28	6.23	0.66	14.58	0	1.08	91
G_2	1.67	0.65[c]	0.27	42.03	0.50	77.48	0	1.37	39
Total	20.00	15.87[b,c]	2.16	13.61	2.99	18.81	—	—	—
Total	20.00	15.97[c]	0.83	5.20	2.58	16.15	1	0.02	80
B_1	17.50	14.81	—	—	2.87	19.41	0	0.64	85
B_2	2.50	4.03	—	—	5.37	133.31	0	3.63	161
G_1	7.50	6.90	—	—	0.71	10.32	0	0.30	92
G_2	2.50	1.37	—	—	1.80	131.57	0	3.05	55
Total	30.00	27.11	—	—	9.64	35.55	0	1.77	90

[a-d]See Table 2, footnotes *a-d*.

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AOCS

AOCS Official Method Ac 3-44

Revised 2011

Oil

DEFINITION

This method determines the substances extracted from ground soybean seeds by petroleum ether under the conditions of the test. Approximately 0.6–0.7% of the soybean oil extracted is phospholipid (see Notes, 1).

SCOPE

Applicable to soybeans.

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in AOCS Official Method Aa 4-38. See Apparatus section and Figure 1 in Method Aa 4-38.
2. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent.
3. Absorbent cotton—free of petroleum ether extractables.
4. Grinding mill—Mikro, 12,000 rpm (pulley size 4.8 cm), using swing hammers with 1mm round screen (no. 3468; Mikro-Pul, Charlotte, NC, USA). Any grinding mill may be used that will yield ground samples equivalent in fineness to that produced by the Mikro mill.
5. Predrying pans—shallow, flat-bottom metal containers, about 87.5-mm minimum diameter.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Prepare the laboratory sample as noted in AOCS Official Method Ac 2-41, Preparation of Test Sample, 1 and 2.
2. Place 60 g of laboratory sample in a predrying pan. Place laboratory sample and predrying pan in a forced-draft oven for 1 hr at 130 ± 2°C, then let cool to room temperature before grinding. Ideally, the resulting moisture content of the laboratory sample should be in the range of 3–6%, but not higher than 6%. Laboratory samples with very high original moisture content may need additional predrying time.
3. Grind the dried beans through a Mikro-Samplmill with minimum exposure to air. Mix the ground dried beans thoroughly as directed by AOCS Official Method Aa 4-38, Preparation of Test Sample.
4. Keep the ground dried beans in an airtight container at room temperature. This test sample is used for the determination of oil, nitrogen (protein), and second (ground) moisture.

   Note—Weighings for all determinations should be made at the same time.

PROCEDURE

1. Weigh accurately 2 g of the ground test sample into a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the meal (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
2. Place in the Butt tube and extract with petroleum ether as directed in AOCS Official Method Aa 4-38, Procedure, for 5 hr.
3. Keep the volume of solvent fairly constant during the extraction by adding enough to make up for any lost due to evaporation.
4. Cool and disconnect the extraction flask. Evaporate the petroleum ether in a steam bath or in a water bath until no odor of ether remains. A gentle stream of clean, dry air may be used to facilitate removal of the solvent. Cool to room temperature, carefully remove any moisture or dirt from the outside of the flask, and weigh. Repeat heating until constant weight is obtained.
5. Determine the moisture in the ground test sample as follows—
   (a) Weigh 5 g into a tared AOCS moisture dish.
   (b) Slip the cover on the bottom of the dish and place in a forced-draft oven. Dry at 130°C for 1 hr.
   (c) Remove the dish from the oven and cover immediately. Cool to room temperature in a desiccator containing an efficient desiccant (calcium chloride is not satisfactory; see AOCS Specification H 9-87 for alternatives) and weigh.

$$\text{Moisture in ground test sample, \%} = \frac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$$

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CALCULATIONS

1. Oil in ground test sample, $\% = \frac{\text{mass of oil, g}}{\text{mass of test portion, g}} \times 100$

2. The percentage oil is calculated to any desired moisture basis with the following formula:

   Oil, desired moisture basis, $\% = \frac{F(100 - \%\ \text{moisture desired})}{100 - \%\ \text{moisture in ground test sample}}$

   Where—

   F = % oil determined in ground test sample

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size 0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies, using conventional grinders, screens, and sieves, samples should be prepared so that the particle size ranges from 0.5 mm to 0.9 mm (No. 35 to No. 20 sieve, respectively). Particle size should be verified by screening an *extracted* sample.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occur, it may be advantageous to grind the sample with an equal amount of diatomaceous earth (see AOCS method Ai 3-75), or to grind the sample with solid carbon dioxide (dry ice).

   If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method is recommended (e.g., AOCS Method Am 2-93).

REFERENCES

1. *J. Am. Oil Chem. Soc. 64*:865 (1987).

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AOCS

AOCS Official Method Ac 4-91

Revised 2011

Nitrogen-Ammonia-Protein Modified Kjeldahl Method Titanium Dioxide + Copper Sulfate Catalyst

DEFINITION

This method determines total nitrogen content and protein as 6.25 times the nitrogen content of the sample (see Notes, 1).

SCOPE

Applicable to soybeans. Replaces Ac 4-41.

APPARATUS

1. Kjeldahl digestion and distillation apparatus—complete with heat source, traps and block-tin, or equivalent noncorrosive tubing condensers (see Notes, 2).
2. Kjeldahl flasks—800 mL.
3. Distillate receiving flasks—500 mL, or any convenient size.

REAGENTS

1. Catalyst mixture—containing potassium sulfate, 16.7 g + titanium dioxide, 0.6 g + copper sulfate, 0.01 g + pumice, 0.3 g.
2. Sulfuric acid—sp. gr. 1.84 (see Notes, *Caution*).
3. Sodium hydroxide solution—sp. gr. 1.50 (see Notes, *Caution*).
4. Sodium hydroxide solution—0.25 M, accurately standardized (see AOCS Specification H 12-52).
5. Sulfuric acid—0.25 M, accurately standardized (see AOCS Specification H 13-52).
6. Methyl red indicator solution—0.1% in ethyl alcohol, or Alizarin Red S—0.3% in distilled water.
7. Alundum boiling stones—8–14 mesh.
8. Zinc 10-mesh metal—granular. (If the catalyst is used with added pumice, granular zinc metal need not be added as an antibumping agent.)
9. Sucrose—ACS

PREPARATION OF TEST SAMPLE

1. For soybeans, use a test sample prepared as directed in AOCS Official Method Ac 3-44, Preparation of Test Sample, steps 1–3. After sample test preparation, continue with following procedure.

PROCEDURE

1. Weigh 0.250–1.750 g of the ground test sample into the Kjeldahl flask. Add the catalyst mixture (premixed catalyst packs are commercially available and may be used).
2. Add 25 mL of concentrated H_2SO_4 (see Notes, 3) to the test portion and catalyst in the digestion flask. Include at least one sample of high-purity lysine hydrochloride in each day's run as a check of the correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.
3. Add a few Alundum boiling stones to the test portion flask.
4. To digest the test portion, first adjust heat to bring 250 mL of water at 25°C to rolling boil in 5 min. Place the test portion flask on the digestion rack in an inclined position and then heat test portion flask at the 5-min boil rate until dense, white fumes clear bulb of flask (approximately 5 min). Swirl gently and continue heating an additional 50 min after the liquid has become clear and colorless (see Notes, 4). The Kjeldahl flasks should be rotated a minimum of three times during the digestion.
5. Cool, cautiously add about 300 mL of water and cool to room temperature (see Notes, 5). Add water as soon as possible to reduce amount of caking. If excessive bumping occurs during distillation, increase dilution water from 300 mL to about 350 mL.
6. Accurately transfer a sufficient quantity of the standard acid into receiving flask so there will be an excess of at least 0.5 mL of 0.25 M acid. Add sufficient distilled water to cover the end of the outlet tube and attach to outlet end of condenser tube. The distillate should discharge through a glass tube at the bottom of the receiving flask.
7. Add an additional 0.5–1.0 g Alundum boiling stones to cooled digestion flask. Mix thoroughly and add sufficient alkali solution (Reagents, 3) to make strongly alkaline. Pour the alkali down the side of the Kjeldahl flask so that it does not mix quickly with the acid.

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8. Immediately connect the Kjeldahl flask to the other end of the condenser tube and thoroughly mix the contents by shaking. Apply heat at about a 7.5-min boil rate and distill until at least 150 mL of distillate have been collected.
9. Titrate the contents of the receiving flask with 0.25 M NaOH solution, using 3 or 4 drops of indicator.
10. Conduct a sucrose blank determination of all reagents simultaneously with the test portions and similar in all respects. Correct for blank determined on reagents.
11. The performance of the entire method should be checked frequently by analyzing either National Institute of Testing (NIST)-certified ammonium dihydrogen phosphate (standard reference material 194) or high-purity lysine hydrochloride.
12. Determine moisture in the ground test sample as directed in AOCS Official Method Ba 2a-38, Procedure section.

CALCULATIONS

1. $$\text{Nitrogen, \%} = \frac{[(MA \times 2 \times mLA) - (mLBK \times MB) - (mLB \times MB)] \times 1400.67}{\text{mg test portion}}$$

Where—

mLB = volume, mL standard base used for the test portion
mLA = volume, mL standard acid used for the test portion
mLBK = volume, mL standard base needed to titrate 1 mL of standard acid minus mL standard base needed to titrate reagent blank through method and distilled into 1 mL standard acid
MA = molarity of standard acid
MB = molarity of standard base

Note—To determine percent ammonia, substitute 1703.06 for 1400.67 in the equation for the calculation of percent nitrogen.

2. Calculation: N × 6.25 = P
Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

PRECISION

There are no statistics relating the performance of this method to soybeans. Table 1 lists the statistical parameters for soybean meal and soy protein concentrate determined in an Association of Official Analytical Chemists (AOAC) interlaboratory study. The reader is referred to AOCS Official Method Aa 5-91 for statistics from the analysis of cottonseed and cottonseed meal.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Alkalies can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali solutions. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not vice versa. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. This method has been evaluated through interlaboratory comparison of catalysts and has been adopted by the committee as the official replacement for the mercuric oxide catalyzed method, AOCS Official Method Ba 4a-38. An interlaboratory evaluation (References, 1) indicated that AOCS Official Method Ba 4d-90 (using the copper/titanium catalyst mixture) produces results closer in agreement with AOCS Official Method Ba 4a-38 (using mercuric oxide catalyst) than methods using copper sulfate catalyst. As a result of this study, AOCS Official Methods Ba 4a-38 (mercuric oxide), Ba 4b-87 (copper sulfate) and Ba 4c-87 (Kjel-Foss Automatic) were declared surplus (obsolete) in 1991.

   The AOAC collaborative study (References, 2) showed no bias when comparing Cu/Ti catalyst with HgO. The AOCS interlaboratory evaluation (References, 1), based on test portion weight, acid, volume, digestion temperature and time, previously specified in AOCS Official Method Ba 4a-38, indicated an average bias of −0.244%, which was statistically significant at the 99% confidence level. Factors that may affect method bias are test portion weight and particle size, catalyst composition, use of Alundum, volume of acid used for digestion, digestion temperature and digestion time. In 1991, two additional collaborative studies (cottonseed and cottonseed meal) showed that when 30 mL of sulfuric acid were used, caking of the digest was reduced and results were comparable to the mercuric oxide catalyzed method. As a result, the AOCS versions of this method may specify the use of more than 20 mL of sulfuric acid, but otherwise conform with the AOAC version (References, 3).

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2. Use of steam- and water-resistant rubber tubing and stoppers to connect the glass parts is strongly recommended.
3. More than 20 mL of concentrated sulfuric acid is needed for samples high in fat or oil, because more acid is required to effect complete digestion, avoid loss in nitrogen, and prevent caking. In recent studies involving the application of this method to whole soybeans, it was observed that when 25 mL of H_2SO_4 were used and the digestion time increased to 50 min past clearing, the results were comparable to the mercuric oxide catalyzed method, and nicotinic acid recovery increased from 53 to 94%.
4. Complete conversion of organic nitrogen to ammoniacal nitrogen is essential to obtain accurate and precise results. After the liquid has become clear and colorless, it may be necessary to digest the test portion for approximately an additional 30–50 min. Reagent proportions, heat input and digestion time are critical factors and should not be changed. Prolonged digestion and high temperatures must be avoided, because some ammoniacal nitrogen can be lost by oxidation. Large test portion particles should be avoided, because the larger particles will require a longer digestion time.
5. The cooled digest should be liquid, or liquid with a few small crystals. Caking before the addition of water indicates too little residual acid at the end of the digestion period and may result in low nitrogen values.

REFERENCES

1. *INFORM 1:*884 (1990).
2. Kane, P.F., *J. Assoc. Off. Anal. Chem. 70:*907 (1987).
3. *Official Methods of Analysis,* AOAC International, Gaithersburg, MD, Method 988.05 (16th ed. 1995, 4.2.03).

Table 1
Statistical parameters determined from the AOAC interlaboratory study[a].

	Soybean meal				Soy protein concentrate			
	1		2		1		2	
	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO
Labs	13	13	13	13	13	13	13	13
Labs removed	1	1	2	1	2	0	2	1
% protein	48.76	48.58	48.11	48.02	87.13	87.27	88.70	88.69
r, CV%	0.88	1.06	0.60	0.41	0.44	0.75	0.43	0.74
R, CV%	0.88	1.08	0.99	0.96	0.76	0.89	0.58	0.74

[a] *References, 2*

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AOCS

AOCS Official Method Ac 5-41

Revised 2013

Free Fatty Acids

DEFINITION

This method determines the free fatty acids in oil removed from the seed by petroleum ether extraction at room temperature.

SCOPE

Applicable to soybeans.

APPARATUS

1. Grinder—either hand- or power-driven, capable of reducing soybeans to 20 mesh or finer.
2. Cotton—clean and bleached.
3. Gravity-type extraction apparatus—assembled using an extraction tube similar to the butt tube (extraction tube) in AOCS method Aa 4–38, Apparatus 1, Figure 1. The approximate dimensions of this larger tube in mm are body o.d./i.d./length, 40/37/195; overall length, 280; stem o.d. 16. The bottom (where the main body and stem come together) of the tube should be plugged with cotton to support the ground meal (2-2.5 cm of cotton). Support the extraction tube with a stand, clamp, or tripod so a collection bottle can be placed under the stem of the tube. Collection bottle can be a flask or bottle 100-150 mL in size.
4. Oil sample bottle or flask of convenient size.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
2. Isopropyl alcohol, 99%—The alcohol must be neutralized with sodium hydroxide (NaOH) solution to a faint pink color before being added to the sample.
3. Phenolphthalein indicator solution—1% in 95% alcohol.
4. Sodium hydroxide—0.25 M, accurately standardized (see AOCS Specification H 12-52).

PROCEDURE

1. Predry 100 g of a well-mixed test sample, free of foreign matter, for 2 hr at 130 ± 3°C and cool. Grind to 20 mesh or finer and fill the extraction apparatus with sufficient test portion (40–50 g) to yield the required amount of oil.
2. Add 50 mL of petroleum ether or hexane and allow all of it to percolate through the test portion into a flask or beaker. Repeat with two 25-mL portions of solvent.
3. Evaporate the solvent from the oil in a water bath under a gentle stream of clean, dry air until free from petroleum ether or hexane.
4. Weigh 7.05 ± 0.05 g of extracted oil into a suitable size oil sample bottle or flask. Add 50–75 mL of neutral alcohol and 1 mL of indicator. Titrate with 0.25 M NaOH, shaking vigorously until a faint pink color is obtained that persists for at least 1 min (see Notes, 2).

CALCULATIONS (see Notes, 3)

1. Free fatty acids, calculated as oleic, % = mL of 0.25 M alkali used.
2. $$\%\text{EEA} = \frac{\text{mL} \times 0.25 \times 282 \times 100}{7.05 \times 1000}$$

   Where 7.05 is the test portion weight of oil used in g; mL = mL of NaOH required; 0.25 = molarity of NaOH solution; 282 = equivalent weight of free fatty acids in which results are to be expressed. Free fatty acid is usually expressed as % oleic acid.

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PRECISION

Table 1
The average, expected between-laboratory variation (standard deviation of reproducibility, S_R) for the determination of free fatty acids in oilseeds[a].

Approximate FFA value, %	0.1–1.0	1.0–2.0	2.0–3.0
S_R	0.067	0.106	0.271
RSD (CV, %)	21.07	9.38	11.43
R (95%) = $2.8 \times S_R$	0.19	0.30	0.76

[a] Values obtained from the AOCS Laboratory Proficiency Program.

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. The compactness of the cotton plug and packing of the ground meats in the extraction apparatus should be such that the cold percolation rate will be about 150 drops per minute and that none of the ground test portion will pass through the cotton plug.
2. About 15 mL of petroleum ether or hexane may be added to aid in solubilizing the oil in the alcohol, if necessary.
3. The result, expressed as oleic acid, is dependent upon using the test portion weight of 7.05 g as specified. Any deviation from the test portion weight of 7.05 g will yield erroneous results.

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AOCS

AOCS Official Method Ad 1-48

Reapproved 2009

Sampling

SCOPE

Applicable to sampling tung fruit or hulled tung fruit during unloading from truck, wagon or carload lots.

APPARATUS

1. Sampling bucket—may be prepared by attaching a bucket of about 15.2 × 15.2 × 15.2 cm. to a pole of convenient length.
2. Sample container—of convenient size and with a tight-fitting cover. A conventional 23 kg (50 lb) lard or shortening container may be used.
3. Sieve—6 mesh, 30–38 cm. diameter.
4. Scales—capacity 2000–3000 g and sensitive to 1 g.
5. Riffle sampler—of convenient size. One with 12 chutes, about 3.2 cm in width, is satisfactory. Three metal boxes, about 5 cm. high and of such dimensions that the riffle may stand and discharge into these boxes, are necessary.

PROCEDURE

1. Take bucketful from the center of the unloading chute at regular intervals such that the gross sample will fill the sample container.
2. Collect the laboratory samples in the can, and keep tightly covered at all times except when laboratory samples are being placed therein.

CLEANING LABORATORY SAMPLES OF FRUIT

1. Pass the entire laboratory sample over a 6 mesh sieve to remove foreign matter. Remove by hand picking any foreign matter that is not removed by the sieve. Collect and weigh the foreign matter as well as the cleaned laboratory sample.
2. Record the net weight on the identification tag which is submitted with the sample to the laboratory.
3. Calculate the foreign matter as follows:

$$\text{Foreign matter, \%} = \frac{\text{mass, g of foreign matter}}{\text{mass, g of gross sample}} \times 100$$

RIFFLING LABORATORY SAMPLE OF HULLED FRUIT

1. Pass the entire laboratory sample over the riffle. Repeat until two subsamples of about 4 L (1 gal) are obtained.
2. Place laboratory samples in laboratory 4 L (1 gal) cans and close covers tightly.

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AOCS

AOCS Official Method Ad 2-52

Revised 2011

Moisture and Volatile Matter

DEFINITION

This method determines moisture and any material that is volatile under the conditions of the test.

SCOPE

Applicable to tung fruit, hulled tung fruit, hulls and mixtures of hulled tung fruit and hulls.

APPARATUS

1. Forced-draft oven—AOCS Specification H 1-39.
2. Riffle—convenient size. One with eight 4.45 cm chutes is satisfactory. Three metal boxes, about 5 cm high and of such dimensions that the riffle may stand and discharge into these boxes, are necessary.
3. Sieve—6 mesh, 30–38 cm diameter.
4. Wiley sample grinding mill—with 0.64 cm screen. Equip the mill with an auxiliary hopper, fitted above the regular one, to prevent material from being thrown out. Also provide a tight-fitting chute to connect from the bottom of the mill through the cover of a large can into which the ground material is delivered. This is to ensure against loss and drying out of ground laboratory sample.
5. Airtight sample containers for holding ground laboratory samples.
6. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), or equivalent (see Notes, 1).
7. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87 for alternatives.
8. Ground tung hulls—available at some tung mills during processing season.

REMOVAL OF FOREIGN MATTER

1. Tung fruit—
   (a) Weigh the gross sample and compare with original mass recorded on tag to see if there has been any change in moisture.
   (b) Pass the entire gross sample over a 6 mesh sieve to remove foreign matter. Remove by hand picking any foreign matter that is not removed by the sieve. Collect and weigh the foreign matter as well as the cleaned gross sample.
   (c) Calculate the foreign matter as follows:

$$\text{Foreign matter, \%} = \frac{\text{mass, g of foreign matter}}{\text{mass, g of gross sample}} \times 100$$

PREPARATION OF TEST SAMPLE

1. Tung fruit—
   (a) Grind whole tung fruit, picked at random from the gross sample, through the Wiley mill using a 0.64 cm screen. If oil is to be determined on the whole fruit, grind 200–250 tung fruits. If moisture and volatile matter only are to be determined, grind 25 tung fruits.
   (b) Break up any lumps of the ground material and then mix thoroughly.
   (c) Reduce the ground tung fruit, either through a riffle or by quartering of a portion of about 1000 g, and store in airtight container. Quartering is done by dividing a ground and well-mixed tung fruit pile into four approximately equivalent quarters with a spatula. Discard two diagonally opposite quarters. Combine and remix the two remaining quarters and then requarter as before. Continue in this manner until the ground tung fruit is reduced to an appropriate sized test sample.
2. Hulled tung fruit—
   (a) Grind 4 L sample of tung fruit through the Wiley mill using a 0.64 cm screen.
   (b) Mix ground hulled fruit and riffle three times by pouring a thin stream of the ground material evenly over the riffle to yield two test samples of about 150 g each. Place in airtight containers.

PROCEDURE

1. Weigh duplicate 5 g test portions of the ground tung fruit or hulled tung fruit and ground hulls into tared moisture dishes.
2. Slip cover on the bottom of the dish and place the uncovered dish in the oven. Dry at 101°C for 1 hr.
3. Remove the dishes from the oven, cover promptly, cool in a desiccator to room temperature and weigh.
4. Repeat, with heatings of .5 hr intervals, until the loss in mass between successive weighings does not exceed 5 mg, or until a gain in mass is noted. Report as moisture the greatest loss found.

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CALCULATIONS

1. Moisture and volatile matter, $\% = \frac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$

NUMBERED NOTES

1. For test portions with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test portion loss from splattering during heating.

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AOCS

AOCS Official Method Ad 3-52

Reapproved 2009

Oil

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to whole tung fruit.

APPARATUS

1. Bauer mill—no. 148 with plates no. 6912, adjusted to produce a fine meal.
2. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4-38.
3. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent.
4. Absorbent cotton—free of petroleum ether extractables.
5. Airtight sample containers for holding ground samples.
6. Forced-draft oven—AOCS Specification H 1-39.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see *Notes, Caution*).

PREPARATION OF TEST SAMPLE

1. Use a 1 kg portion of ground sample, prepared as directed in AOCS Official Method Ad 2-52, Preparation of test sample.
2. Grind this entire portion through the Bauer mill. Mix carefully by rolling on a large sheet of paper and place in airtight container.

PROCEDURE

1. Weigh accurately 5 g of the ground test sample into a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the meal (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
2. Place wrapped test portion in Butt extraction tubes and assemble the apparatus as shown in Figure 1 in AOCS Official Method Aa 4-38. Place 25–30 mL of petroleum ether in the extraction flask before attaching to the tube.
3. Heat in a water bath or on an electric hot plate at such a rate that the solvent will drop from the condenser at a rate of at least 150 drops/min. Keep the volume of the solvent fairly constant by adding enough to make up for any loss due to evaporation. Extract for 4 hr.
4. Cool and disconnect the extraction flask. Evaporate the solvent from the oil extract in a water bath until no trace of the solvent remains. Evaporation of the solvent should be complete within approximately 20 min. In case of doubt, allow the flask to remain in the water bath for an additional 15 min and rotate the flask slowly. Remove the flask from the water bath, cool to room temperature and weigh.
5. Determine moisture in ground test sample as directed in AOCS Official Method Ad 2-52, Procedure.

CALCULATIONS

1. Oil in whole tung fruit, $\% = \frac{A \times (100 - B)}{D \times (100 - C)} \times 100$

   Where—

   A = mass, g of oil extracted from D
   B = percent moisture from ground portion, AOCS Official Method Ad 2-52, Procedure (this is original moisture)
   C = percent moisture from ground portion, AOCS Official Method Ad 3-52, Procedure (this is second moisture)
   D = mass, g of test portion from AOCS Official Method Ad 3-52, Procedure

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

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NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size <0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies, using conventional grinders, screens, and sieves, test samples should be prepared so that the particle size ranges from 0.5 mm to 0.9 mm (no. 35 to no. 20 sieve, respectively). Particle size should be verified by screening an extracted test portion.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occur, it may be advantageous to grind the laboratory sample with an equal amount of diatomaceous earth (see AOCS method Ai 3-75), or to grind the laboratory sample with solid carbon dioxide (dry ice).

   If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method is recommended (e.g., AOCS Method Am 2-93).

REFERENCES

1. *J. Am. Oil Chem. Soc. 64*:865 (1987).

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AOCS

AOCS Official Method Ad 4-52

Reapproved 2009

Physical Analysis of Tung Fruit

DEFINITION

This method determines the kernel content of tung fruit under the conditions of the test.

SCOPE

Applicable to whole tung fruit.

APPARATUS

1. Balance—capacity ≥ 5000 g and sensitive to ± 1 g.

PROCEDURE

1. Determination of the amount of kernels—Weigh a sample of at least 100 tung fruit. Manually remove the hulls and shells from the kernels and weigh the tung kernels.

$$\text{Kernels in tung fruit, \%} = \frac{\text{mass of kernels, g}}{\text{mass of whole fruit sample, g}} \times 100$$

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AOCS

AOCS Official Method Ad 5-52

Reapproved 2009

Oil

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to tung kernels.

APPARATUS

1. Universal food chopper—no. 71 with 16-tooth blade, or Bauer mill—no. 148 with plates no. 6912 adjusted to produce a fine meal without expressing oil.
2. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4-38.
3. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent.
4. Absorbent cotton—free of petroleum ether extractables.
5. Airtight sample containers for holding ground samples.
6. Forced-draft oven—AOCS Specification H 1-39.
7. Porcelain mortar and pestle—The mortar must be at least 10.2 cm. i.d. at the top. The pestle handle must be large enough to afford a firm hand grip. The inner surface of the mortar can be kept rough by occasionally grinding sand in it.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PROCEDURE

1. Preparation of test sample—
   (a) Grind the kernels separated from at least 100 tung fruit twice in a Universal food chopper or in a Bauer mill (Apparatus, 1). Mix thoroughly.
   (b) Immediately place in sample bottle of convenient size and stopper tightly. If test sample is too large, quarter to desired quantity.
2. Determination of oil in kernels:
   (a) Weigh accurately 5 g of the ground test sample into a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the meal (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
   (b) Place wrapped test portion in Butt extraction tubes and assemble the apparatus as shown in Figure 1 in AOCS Official Method Aa 4-38. Place 25–30 mL of petroleum ether in the extraction flask before attaching to the tube.
   (c) Heat in a water bath or on an electric hot plate at such a rate that the solvent will drop from the condenser into the thimble at a rate of at least 150 drops/min. Keep the volume of the solvent fairly constant by adding enough to make up for any loss due to evaporation. Extract for 4 hr.
   (d) Cool and disconnect the extraction flask and tube, and remove wrapped test portion from tube. Empty the test portion into a mortar, add 1 g fine sand and grind with pestle for 5 min. Rewrap the test portion and continue extraction for an additional 2 hr. Occasionally check the efficiency of extraction by regrinding the test portion for 5 min and extracting for another 2 hr period, collecting the extract in a clean tared flask. The mass of the residue after evaporation of its solvent should be <5 mg.
   (e) Cool and disconnect the extraction flask. Evaporate the solvent from the oil extract in a water bath until no trace of the solvent remains. Evaporation of the solvent should be complete within approximately 20 min. In case of doubt, allow the flask to remain in the water bath for an additional 15 min and rotate the flask slowly. Remove the flask from the water bath, cool to room temperature and weigh.
   (f) Determine moisture in ground test sample as directed in AOCS Official Method Ad 2-52, Procedure.

CALCULATIONS

1. Oil in ground kernels, $\% = \dfrac{\text{mass, g of oil}}{\text{mass, g of test portion}} \times 100$

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2. The percent oil is calculated to any desired moisture basis with the following formula:

$$\text{Oil, desired moisture basis, \%} = \frac{F\,(100 - \%\ \text{moisture desired})}{100 - \%\ \text{of moisture in ground test portion}} \times 100$$

Where—

F = percent oil determined in ground sample

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size <0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies, using conventional grinders, screens, and sieves, test samples should be prepared so that the particle size ranges from 0.5 mm to 0.9 mm (no. 35 to no. 20 sieve, respectively). Particle size should be verified by screening an extracted test sample.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occurs, it may be advantageous to grind the laboratory sample with an equal amount of diatomaceous earth (see AOCS method Ai 3-75), or to grind the laboratory sample with solid carbon dioxide (dry ice).

   If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method is recommended (e.g., AOCS Method Am 2-93).

REFERENCES

1. *J. Am. Oil Chem. Soc. 64*:865 (1987).

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AOCS

AOCS Official Method Ad 6-52

Reapproved 2009

Oil

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to hulled tung fruit.

APPARATUS AND SUPPLIES

1. Bauer mill—no. 148 with plates no. 6912 adjusted to produce a fine meal without expressing oil.
2. Wiley sample grinding mill—with 0.64 cm. screen. Equip the mill with an auxiliary hopper, fitted above the regular one, to prevent material from being thrown out. Also provide a tight-fitting chute to connect from the bottom of the mill through the cover of a large can into which the ground sample is delivered. This is to ensure against loss and drying out of ground sample.
3. Ground-seed mixer—available from Mid-Continent Engineering, Memphis, TN, USA.
4. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4-38.
5. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent.
6. Absorbent cotton—free of petroleum ether extractables.
7. Airtight sample containers for holding ground samples.
8. Forced-draft oven—AOCS Specification H 1-39.
9. Ground tung hulls—available at some tung mills during processing season.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Use a 150 g portion of Wiley-ground laboratory sample prepared as directed in AOCS Official Method Ad 2-52, Preparation of test Sample, 2.
2. Weigh portion of Wiley-ground laboratory sample, add an equal mass of ground hulls and mix thoroughly.
3. Grind mixture of ground hulled tung fruit and hulls through the Bauer mill with plates set about 0.2 mm apart. Mix thoroughly and riffle twice to yield a test portion of about 75 g. Mix in velocity mixer operated at full speed for one full minute.
4. Grind a portion of the ground hulls through the Bauer mill, with plates set about 0.2 mm apart, and place in an airtight container.

PROCEDURE

1. Weigh accurately 5 g of the ground test sample into a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the meal (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
2. Place wrapped test portion in Butt extraction tubes and assemble the apparatus as shown in Figure 1 in AOCS Official Method Aa 4-38. Place 25–30 mL of petroleum ether in the extraction flask before attaching to the tube.
3. Heat in a water bath or on an electric hot plate at such a rate that the solvent will drop from the condenser into the thimble at a rate of at least 150 drops/min. Keep the volume of the solvent fairly constant by adding enough to make up for any loss due to evaporation. Extract for 4 hr.
4. Cool and disconnect the extraction flask. Evaporate the solvent from the oil extract in a water bath until no trace of the solvent remains. Evaporation of the solvent should be complete within approximately 20 min. In case of doubt, allow the flask to remain in the water bath for an additional 15 min and rotate the flask slowly. Remove the flask from the water bath, cool to room temperature and weigh.
5. Determine moisture in:
   (a) Wiley-ground hulled fruit
   (b) ground hulls
   (c) Bauer-ground mixture of hulled fruit and hulls
   (d) Bauer-ground hulls

   as described in AOCS Official Method Ad 2-52, Procedure.

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BLANK DETERMINATION

1. Determine petroleum ether extractable material on duplicate 5 g test portions of the Bauer-ground hulls, exactly as described in the Procedure section.
2. Determine moisture in Bauer-ground hulls as described in AOCS Official Method Ad 2-52, Procedure.

CALCULATIONS

1. Oil in hulled fruit, % $= \left[\left(2 \times \frac{A \times (100 - B)}{D \times (100 - C)}\right) - \frac{E \times (100 - F)}{D' \times (100 - G)}\right] \times 100$

Where—

A = mass, grams extract from Bauer-ground hulled fruit and hulls
B = percent moisture in mixture of Wiley-ground hulled fruit and hulls [Procedure, 5, (a), (b)]
C = percent moisture in Bauer-ground mixture of hulled fruit and hulls [Procedure, 5, (c)]
D = mass, g of Wiley-Bauer-ground test sample
D′ = mass, g of Bauer-ground hull test sample (Blank Determination, 1)
E = grams extract from Bauer-ground hulls
F = percent moisture in ground hulls [Procedure, 5, (b)]
G = percent moisture in Bauer-ground hulls [Procedure, 5, (d)]

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size <0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies using conventional grinders, screens, and sieves, test samples should be prepared so that the particle sizes range from 0.5 mm to 0.9 mm (no. 35 to no. 20 sieve, respectively). Particle size should be verified by screening an extracted test portion.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occurs, it may be advantageous to grind the laboratory sample with an equal amount of diatomaceous earth (see AOCS method Ai 3-75), or to grind the laboratory sample with solid carbon dioxide (dry ice).

   If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method should be used (e.g., AOCS Method Am 2-93).

REFERENCES

1. *J. Am. Oil Chem. Soc. 64*:865 (1987).

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AOCS

AOCS Official Method Ae 2-52

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Moisture and Volatile Matter

DEFINITION

This method determines moisture and any material that is volatile under the conditions of the test.

SCOPE

Applicable to castor seeds (see Notes, *Caution*).

APPARATUS

1. Forced-draft oven—AOCS Specification H 1-39.
2. Sample riffle—The riffle should have eight 4.4 cm slots. Three metal boxes, about 5 cm high and of such dimensions that the riffle may stand and discharge into these boxes, are necessary.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87 for alternatives.
4. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), or equivalent (see Notes, 1).
5. Screen— 20.54 cm diameter, having round hole perforations 0.40 cm in diameter.
6. Airtight sample containers of convenient size.

PREPARATION OF TEST SAMPLE (see Notes, *Caution*)

1. Reexamine the laboratory sample for foreign matter. Place a 300 g portion of the laboratory sample on the 20.54 cm screen and gently shake back and forth to remove fine material passing through the 0.40 cm diameter holes. Hand pick the sample for free and attached whole capsules (hulls), detaching the latter; capsule fragments both filled and unfilled; stems; rocks; etc. Shells (seed coats) remaining on the screen, and cracked and broken seeds are considered part of the sample for analysis. The foreign matter is considered as the total of the material passing the screen and removed by hand picking as described above. It is weighed and reported as a percentage of the original laboratory sample.
2. Reduce the cleaned laboratory sample through the riffle to a size sufficient for the number of tests that will be required.

PROCEDURE

1. Weigh 40–50 g test portions of castor seeds into tared moisture dishes. Slip the covers on the bottom of the dishes and dry in a forced-draft oven at 130 ± 3°C for 4 hr.
2. Remove from the oven, replace covers immediately, cool to room temperature in a desiccator and weigh. Calculate moisture as directed in Calculations.

CALCULATIONS

1. $$\text{Moisture, \%} = \frac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$$

NOTES

Caution

Castor seeds are poisonous due to the presence of ricin, a highly toxic albumin, and ricinine, a highly toxic alkaloid. Neither pressing nor extraction removes them; hence both hazards remain in the pomace. They also contain an allergenic protein polysaccharide (CB-1A) that is among the most powerful known allergens. It is strongly recommended that workers wear rubber gloves when preparing analytical samples, and that they avoid inhaling any of the dust arising from the castor beans by working near an air exhaust or in a well ventilated laboratory hood.

NUMBERED NOTES

1. For analytical samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test portion loss from splattering during heating.

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Oil

DEFINITION

This method determines the substances extracted by commercial hexane under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to castor seeds (see Notes, *Caution*).

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4-38.
2. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent.
3. Absorbent cotton—free of hexane extractables.
4. Universal food chopper—no. 1 with 12 tooth blade, or equivalent.

REAGENTS

1. Commercial hexane—AOCS Specification H 16-56 (see Notes, *Caution*).

PROCEDURE (see Notes, *Caution*)

1. Place 50 g of castor seeds from the cleaned laboratory sample (see AOCS Official Method Ae 2-52, Preparation of Test Sample, 2) in a convenient drying dish and dry at 130 C in a forced-draft oven for not more than 20 min.
2. Cool the cleaned and dried laboratory sample to room temperature and then grind through the food chopper, feeding the beans slowly to prevent expressing any oil (see Notes, 2).
3. Mix the ground test sample thoroughly by rolling on a glazed paper or gently rolling in a large glass jar (see Notes, 2).
4. Weigh accurately duplicate test portions of 5 g each of the ground test sample onto a tared filter paper so that the paper will absorb any oil on the surface of the ground seeds. Fold the paper containing the test portion and enclose in a second filter paper, folded in such a fashion as to prevent escape of the ground seeds (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
5. Extract the ground test portion as directed in AOCS Official Method Aa 4-38, Procedure, 2-4, except that commercial hexane is substituted for petroleum ether, hot plates are used as a source of heat, and the sample is extracted for 24 hr.
6. Discontinue the extraction, cool, and disconnect the extraction flask. Evaporate the commercial hexane on a steam bath for 1 hr after boiling ceases, then place the flask in a forced-draft oven at 101°C for an additional 2 hr. Cool to room temperature, carefully remove any foreign material from the outside of the flask, and weigh. Repeat heating in the forced-draft oven for 30 min intervals until constant weight is obtained.
7. Determine the moisture content of the partially dried and ground test samples as follows—
   (a) Weigh 5 g into a tared moisture dish.
   (b) Slip the cover on the bottom of the dish and place in a forced-draft oven. Dry at 130°C for 2 hr.
   (c) Remove the dish from the oven and cover immediately. Cool in a desiccator containing an efficient desiccant for 30 min and weigh. Calcium chloride in not an efficient desiccant. See AOCS Specification H 9-87 for alternatives.

CALCULATIONS

1. $$\text{Moisture in ground test sample, \%} = \frac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$$

2. $$\text{Oil in ground test sample, \%} = \frac{\text{mass, g of oil}}{\text{mass of test portion, g}} \times 100$$

3. The percentage oil is calculated to any desired moisture basis with the following formula—

$$\text{Oil, desired moisture basis, \%} = \frac{F(100 - \text{\% moisture desired})}{100 - \text{\% moisture in ground test portion}}$$

Where F = % oil determined in ground test sample

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PRECISION

1. Cooperative work to establish the conditions of the test revealed that the standard deviation of the method in a given laboratory on replicates run at the same time is ± 0.484% oil. The standard deviation between laboratories is ± 0.703% oil when duplicate tests are run and averaged in each of the laboratories under comparison.

NOTES

Caution

Castor seeds are poisonous due to the presence of ricin, a highly toxic albumin, and ricinine, a highly toxic alkaloid. Neither pressing nor extraction removes them, hence both hazards remain in the pomace. They also contain an allergenic protein polysaccharide (CB-1A) that is among the most powerful known allergens. It is strongly recommended that workers wear rubber gloves when preparing analytical samples and that they avoid inhaling any of the dust arising from the castor beans by working near an air exhaust or in a well-ventilated laboratory hood.

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. A fume hood should be used at all times when using hexane.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size <0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies, using conventional grinders, screens, and sieves, test samples should be prepared so that the particle size ranges from 0.5 mm to 0.9 mm (No. 35 to No. 20 sieve, respectively). Particle size should be verified by screening an *extracted* test portion.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occur, it may be advantageous to grind the laboratory sample with an equal amount of diatomaceous earth (see AOCS method Ai 3-75), or to grind the laboratory sample with solid carbon dioxide (dry ice).

   If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method is recommended (e.g., AOCS Method Am 2-93).
2. Prior to grinding the laboratory sample through the food chopper, saturate the metal surfaces with oil by passing a quantity of beans through the mill, disassembling, cleaning out the solid material, and reassembling. The glazed paper or jar used for mixing also should be saturated with oil.

REFERENCES

1. *J. Am. Oil Chem. Soc. 64:*865 (1987).

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AOCS Official Method Ae 4-52

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Free Fatty Acids

DEFINITION

This method determines the free fatty acids in oil removed from the seed by methyl alcohol extraction at room temperature.

SCOPE

Applicable to castor seeds (see Notes, *Caution*).

APPARATUS

1. Universal food chopper—no. 1 with 12-tooth blade.
2. Oil sample bottles or flasks of convenient size.
3. Filtration apparatus—of convenient size, consisting of either glass funnel and support or Buchner funnel and suction flask, equipped with S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent filter paper of appropriate size.

REAGENTS

1. Methyl alcohol—reagent grade, having no residual acidity or alkalinity.
2. Isopropyl alcohol, 99%—neutralized with sodium hydroxide solution to a faint pink color before adding to the sample.
3. Phenolphthalein indicator solution—1% in 95% alcohol.
4. Sodium hydroxide (NaOH)—0.25 M, accurately standardized (see AOCS Specification H 12-52).

PROCEDURE (see Notes, *Caution*)

1. Remove the foreign matter and reduce the laboratory sample to a convenient size as directed in AOCS Official Method Ae 2-52, Preparation of Test Sample, 1 and 2.
2. Grind 150 g of seeds through the food chopper. Mix the ground laboratory sample thoroughly.
3. Suspend 20 g of the ground test sample in 100 mL of methyl alcohol at room temperature with occasional agitation for 30 min.
4. Filter by means of either a Buchner funnel or fluted paper. Remove the solvent by heating on a steam bath, applying a vacuum to the flask during the entire period of heating, to remove the last traces of solvent.
5. Weigh 7.05 g of extracted and solvent-free oil into the sample bottle or flask, being careful to avoid transferring the non-oil material which adheres to the bottom of the original flask. Add 50–75 mL of neutral alcohol (see Notes, 1) and 1 mL of indicator. Titrate with 0.25 M NaOH, shaking vigorously until a faint pink color is obtained that persists for at least 1 min.

CALCULATIONS (see Notes, 2)

1. Free fatty acids, calculated as oleic, % = mL of 0.25 M alkali used.
2. $$\% \text{ FFA} = \frac{\text{mL} \times 0.25 \times 282 \times 100}{7.05 \times 1000}$$

Where—

7.05 is the test portion weight of oil used in g;
mL = mL of NaOH required;
0.25 = molarity of NaOH solution;
282 = equivalent weight of free fatty acids in which results are to be expressed.
Free fatty acid is usually expressed as % oleic acid.

NOTES

Caution

Castor seeds are poisonous due to the presence of ricin, a highly toxic albumin, and ricinine, a highly toxic alkaloid. Neither pressing nor extraction removes them, hence both hazards remain in the pomace. They also contain an allergenic protein polysaccharide (CB-1A) that is among the most powerful known allergens. It is strongly recommended that workers wear rubber gloves when preparing analytical samples, and that they avoid inhaling any of the dust arising from the castor beans by working near an air exhaust or in a well-ventilated laboratory hood.

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NUMBERED NOTES

1. About 15 mL of petroleum ether may be added to aid solubilizing the oil in the alcohol, if necessary.
2. The result, expressed as oleic acid, is dependent upon using the test portion weight of 7.05 g as specified. Any deviation from the test portion weight of 7.05 g will yield erroneous results.

REFERENCES

1. Technical Bulletin, *The Processing of Castor Meal For Detoxification & Deallergenation*, The International Castor Oil Association.
2. *Summary*, Technical Report of Division 9, NDRC Vol. 1 Chemical Warfare Agents, and Related Chemical Problems, Part 1, 1946, Office of Scientific Research and Development, Washington, DC.

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AOCS Official Method Af 1-54

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Sampling

SCOPE

Applicable to flaxseed (see Notes, 2).

APPARATUS (SEE NOTES, 1)

1. Trier—double tube, separate compartment grain trier or probe about 60 in. long.
2. Bag trier.
3. Sampling canvas—1.5 × 0.6 m
4. Boerner sample divider—36-pocket size.
5. Waterproof sample sacks.
6. Airtight containers for preserving samples.
7. Spout sampler or "pelican"—for sampling bulk grain from running streams.
8. Carter dockage tester for flaxseed—containing a no. 000 riddle, no. 1 riddle, a top no. 4 slotted sieve (1.63 × 9.53 mm) in the middle, a no. 2 round-hole sieve (1.98 mm diameter), and a no. 7 round-hole sieve (1.79 mm diameter) in the bottom carriage.
9. Hand sieves—1.63 × 9.53 mm perforations, 1.19 × 9.53 mm perforations, 2.12 mm round hole, 1.79 mm round hole, and per 6.45 cm^2.

PROCEDURE

1. Flaxseed in bulk—Probe in seven or more places with a standard trier in various parts of the car. Insert to probe at an angle of 10° with the vertical, with the slots closed and faced up. Open the slots and give the probe several gentle up-and-down movements. Then close the slots and withdraw the probe, taking probe samples as follows:
   (a) Probe in center of car.
   (b) Probe 0.91–1.52 m back from the door post toward the end of the car and approximately 0.61 m out from one side of the car.
   (c) Probe 0.91–1.52 m from the same end of the car and approximately 0.61m from the opposite side of the car as in (b).
   (d) Repeat (b) and (c) above in opposite ends and sides of the car.
   (e) Probe 0.61m from each end of car in center.
   (f) Place contents of each probe on the sampling cloth and mix by lifting alternate edges of the cloth. A minimum sample of 2500 g shall be collected and stored in an airtight container.
2. Flaxseed in truckloads or small bins—Use same procedure as for flaxseed in bulk.
3. Flaxseed in bags—Take samples from the bags by any standard or approved grain trier of sufficient length to reach center of bag. Draw samples from as many individual sacks, selected at random through the lot, as will be necessary to obtain a representative sample of at least 2500 g.
4. Unloading and loading flaxseed—Where a sample is to be taken during a loading or an unloading operation, a pelican sampler device should be used. Subsamples are taken at regular and frequent intervals to ensure a correct and representative sample.

CLEANING AND REDUCTION OF LABORATORY SAMPLE

1. Reduce laboratory sample size to approximately 1000 g by Boerner sampler.
2. Pass the reduced laboratory sample through the Carter dockage tester once.
3. Use the 000 riddle. If this should clog or kick over whole flaxseed kernels, use the no. 1 riddle.
4. When matted lumps of flaxseed clog the riddle or kick over with the coarse dockage, remove the riddle and top sieve no. 4 and proceed to make the determination provided above. In hand picking a portion of the mechanically cleaned flaxseed to complete dockage determination, the matted kernels of flaxseed, even though portions of flax bolls adhere to them, shall be considered as flaxseed.
5. Examine the material that passed over the top sieve (no. 4). If material contains large-sized flaxseed it should be rescreened with a hand sieve having 1.63 × 9.53 mm perforations. If it contains small-sized flaxseed, rescreen with a hand sieve having 1.19 × 9.53 mm perforations, recovering the flaxseed left in this portion.
6. If material that passed over the bottom sieve (no. 7) contains considerable pigeon grass seed or mustard seed, it should be rescreened with a hand sieve having 1.19 × 9.53 mm perforations. The material remaining on top of this sieve is considered dockage. The material that passed through the sieve is returned to the mechanically cleaned sample.

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7. If the flaxseed (mechanically cleaned sample) contains excessive amounts of pigeon grass seed and/or other weed seeds of similar size and shape, place 250 g on a hand sieve having 1.19 × 9.53 mm perforations, work the sample back and forth lengthwise to the perforations until all of the removable material has passed through the sieve. Repeat the operation until the entire sample has been sieved.
8. The flaxseed in the material that has remained on top of the hand sieve should be reclaimed in the following manner. Use the hand sieve having 2.12 mm round-hole perforations (fine-seed sieve), hold the sieve at an angle of from 10 to 20°, place the material on the lower edge of the sieve and strike the lower edge of the sieve with one hand in such a manner as to cause the material to bounce up and down. This will cause the pigeon grass, etc., to pass through the perforations of the sieve. Continue the operation until all of the separable dockage material has passed through the sieve. The material that remains on top of the sieve is returned to the cleaned flaxseed. If the material that passes through the sieve consists of 50% or more of whole or broken kernels of flaxseed, it is returned to the cleaned flaxseed; otherwise, it is added to the dockage.
9. The mechanically cleaned flaxseed, including the flaxseed reclaimed by hand sieving, is passed through the Boerner divider. Analyze a laboratory sample of at least 15 g by handpicking to determine the remaining dockage.
10. Store the cleaned flaxseed laboratory sample in an airtight container.

CALCULATIONS

1. Calculate the foreign matter as follows—
   Foreign matter in original laboratory sample, % = FM + [(100 – FM) × (fm/100)]
   Where—
   FM = percent of foreign matter in large laboratory sample
   fm = percent foreign matter in handpicked laboratory sample

NUMBERED NOTES

1. One supplier of the sampling equipment noted in this method is Seedburo Equipment Co., Chicago, IL, USA.
2. This method has been declared surplus following a vote by the UMC. ISO 542 will be included in future editions when section A is fully remodelled.

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AOCS Official Method Af 2-54

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Moisture and Volatile Matter

DEFINITION

This method determines moisture and any material that is volatile under the conditions of the test.

SCOPE

Applicable to flaxseed.

APPARATUS

1. Forced-draft oven—AOCS Specification H 1-39.
2. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), or equivalent (see Notes, 1).
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87 for alternatives.
4. Boerner sampler or divider—36-pocket size.
5. Airtight sample containers of convenient size.

PREPARATION OF TEST SAMPLE

1. Reduce the mechanically cleaned laboratory sample (AOCS Official Method Af 1-54, Cleaning and Reduction of Laboratory Sample, 10) through the Boerner sampler to about 100 g. Preserve the test sample in an airtight container and use for all subsequent analyses.

PROCEDURE

1. Weigh 10 g of whole flaxseed into a tared moisture dish.
2. Dry in the forced-draft oven at 130 ± 3°C for 3 hr. Remove from the oven, cover immediately, cool in a desiccator to room temperature and weigh.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$

NOTES

1. For test samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test portion loss from splattering during heating.
2. This method closely resembles ISO 665 (1977), *Oilseeds-Determination of Moisture and Volatile Matter,* and the harmonized IUPAC Method 1.121-*Determination of Moisture and Volatile Matter.* The difference is that the temperature of 130°C is specified in the AOCS method and 103°C is specified in the ISO and IUPAC method. This difference in temperature appears large enough to make a difference in results. A collaborative study is under consideration by the AOCS. Please see Table 1: 1995 Flaxseed.

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Table 1
1995 Flaxseed: comparison of methods to determine moisture percentage.[a]

	% Moisture		
	CGC SSVO	AOCS	ISO665
1	7.90	8.50	8.09
2	8.77	9.45	9.06
3	8.12	8.91	8.47
4	8.20	8.81	8.47
5	9.22	10.20	9.70
6	6.20	6.77	6.38
7	6.32	6.90	6.56
8	7.72	8.20	7.79
9	8.08	8.79	8.41
10	6.19	6.96	6.54
Average	7.67	8.35	7.95

[a] Procedures: CGC SSVO, 5 g whole grain, 103°C vacuum oven for 3 h; ISO 665, 5 g whole grain, 103°C air-oven for 3 h then 1 h (until difference is 0.005 g or less); AOCS, 10 g whole grain, 130°C air-oven for 3 h.

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Oil Content

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to flaxseed. This method requires grinding the sample on a special mill which is not available in many laboratories and which is no longer manufactured. It is recommended that AOCS Official Method Am 2-93 be used.

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4-38.
2. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent.
3. Absorbent cotton—free of petroleum ether extractables.
4. Porcelain mortar and pestle—The mortar must be at least 10 cm i.d. at the top. The pestle handle must be large enough to afford a firm hand grip.
5. Roller mill—Allis-Chalmers 6 × 6 in., with cast iron frame, metal feeder housing, bronze bushings, rolls corrugated 28 saw tooth with 1 in. spiral per foot and running sharp to sharp including flat belt pulleys to drive at 2:1 differential from a line shaft below machine (see Notes, 1).
6. Sieve—Tyler no. 20 (US no. 20).
7. Brushes—
   (a) Round—2.54 cm bristle.
   (b) Steel brush—15.24 cm
8. Flaxseed dockage sieve—wire mesh, 4 × 16 mesh per square inch.
9. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), or equivalent (see Notes, 3).

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
2. Sea sand—30 mesh, washed and ignited.

PREPARATION OF TEST SAMPLE

1. Quarter sample (AOCS Official Method Af 2-54, Preparation of Test Sample, 1) until approximately 25 g remains. Hand pick to free of all foreign matter.
2. Pass the flaxseed through the mill with the rolls set loosely so that the seed is thoroughly crushed. Tighten rolls and pass crushed seed through the mill. Loosely set rolls give coarse material having poor extractability and too tight a mill setting gives mushy flaxseed. The ground flaxseed should pass through the no. 20 sieve (except perhaps a few hulls). If a sieve analysis is made to check grind, brush the flaxseed lightly through the sieve using the 2.54 cm bristle brush. Do not shake. Thoroughly clean the rollers with the wire brush after each grind and wipe the scrapers with a cloth.
3. Place all the ground flaxseed, including material cleaned from the rolls, onto the 6.45 cm wire mesh sieve and brush lightly through the sieve using the 2.54 cm bristle brush in order to break up agglomerate tending to destroy uniformity.
4. Mix the ground, screened flaxseed thoroughly by rolling the entire portion back and forth on glazed paper. Transfer to an airtight container. This test sample is used for the determination of oil, nitrogen and second (ground) moisture. Weighings for all determinations should be made at the same time.

PROCEDURE

1. Weigh accurately 3 g of the ground test sample into a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the meal (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
2. Place wrapped test portion in Butt extraction tubes and assemble the apparatus as shown in Figure 1 of AOCS Official Method Aa 4-38. Place 25 mL of petroleum ether into a tared extraction flask before attaching to the tube.
3. Heat in a water bath or on an electric hot plate at such a rate that the solvent will drop from the condenser on the center of the thimble at a rate of at least 150 drops/min. Extract for 2 hr.

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4. Remove thimble from Butt tube; allow petroleum ether to evaporate at room temperature. Transfer material in thimble to a mortar, add 10% by volume of sand and grind. Grind for 1 min or with 100 vigorous strokes.
5. Return the ground test portion to the same filter paper and continue the extraction for 3 hr. Add additional petroleum ether to provide for some loss due to evaporation.
6. Cool and disconnect the flask. Evaporate the solvent on a steam bath or in a water bath until no odor of petroleum ether remains. Cool to room temperature. Carefully remove any moisture or dirt from outside of flask and weigh. Repeat heating until constant weight is obtained.
7. Determine the moisture on ground test sample as follows—
   (a) Weigh 5 g into a tared AOCS moisture dish.
   (b) Slip the cover on bottom of dish and place the dish in a forced-draft oven. Dry at 130 ± 3°C for 1 hr
   (c) Remove from oven and cover immediately. Cool in desiccator to room temperature and weigh.

CALCULATIONS

1. Moisture in ground test sample, $\% = \frac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$

2. Oil in ground test sample, $\% = \frac{\text{mass, g of oil}}{\text{mass of test portion, g}} \times 100$

3. The percent oil is calculated to any desired moisture basis with the following formula:

   Oil, desired moisture basis, $\% = \frac{\text{F (100 – \% moisture desired)}}{\text{100 – \% moisture in ground test sample}}$

   Where—

   F = percent oil determined in ground test sample

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. This method requires regrinding of the flaxseed on a special mill that is not available in many laboratories and is no longer commercially available. AOCS Official Method Am 2-93, Determination of Oil Content in Oilseeds (international trade method), may be used.
2. For test samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test portion loss from splattering during heating.
3. This method has been declared surplus following a vote by the UMC. A single general method to determine Oil Content based on Aa 4-38 will be included in future editions. ISO Method 659 is recommended for further consideration.

REFERENCES

1. *J. Am. Oil Chem. Soc. 64:*865 (1987).

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AOCS

AOCS Official Method Ag 1-65

Reapproved 2009

Oil

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to safflower seed.

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4-38.
2. Filter paper—150 mm; Whatman no. 2, or equivalent.
3. Absorbent cotton—petroleum ether washed.
4. Porcelain mortar and pestle—at least 10 cm top diameter.
5. Iron mortar and pestle—about 0.5 L capacity.
6. Paper extraction thimbles—about 25 × 80 mm.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
2. Sea sand—30 mesh, ether washed and ignited.

PREPARATION OF TEST SAMPLE

1. Split original laboratory sample to 100 g with a Boerner divider (see Notes, 2) and screen over 3.18 mm and 1.79 mm round-hole screens in one operation.
2. Adjust a Bates aspirator to feed 3, slide opening $2\frac{1}{4}$, voltage 60; and aspirate the material remaining on top of the 3.18 mm screen. Hand pick to remove any remaining foreign matter.
3. Hand pick all foreign matter from the 1.79 mm screen, carefully leaving all safflower kernels and pieces of kernels.
4. The total foreign matter will consist of the portions removed by air blast, matter hand picked from both screens and all the material that passed through the 1.79 mm screen.

PROCEDURE

1. Weigh accurately about 10 g whole seed by dipping a spoon into the test sample container.
2. Transfer the charge to the iron mortar and thoroughly pulverize. (A light cover, such as a plastic bag, is used to avoid test portion loss.)
3. Transfer charge to filter paper and fold as illustrated in AOCS Official Method Aa 4-38, Figure 3, steps 1–7 only.
4. Place wrapped test portion into paper thimble and thimble into extraction tube. Connect to tared extraction flask.
5. Wash mortar and pestle carefully with petroleum ether and pour washings through the paper thimble, allowing the filtrate to pass into the receiving flask. Place a cotton plug in the top of the thimble and adjust the solvent volume in the flask to about half full.
6. Attach the condenser and heat in a water bath or on a hot plate, condensing solvent at a rate of about 150 drops/min. Continue extraction for 4 hr.
7. Remove thimble from extraction tube and dry at room temperature. Transfer test portion to porcelain mortar, add 3–4 g sand, and grind with 100 vigorous strokes, or for 1 min.
8. Return test portion to same paper and thimble, wash mortar as in Procedure, step 5. Place thimble back into extraction tube and resume extraction for an additional 4 hr, adding petroleum ether as necessary to maintain volume in flask.
9. Cool and disconnect the flask. Evaporate the solvent on a steam bath or in a water bath until no petroleum ether remains. Carefully remove any moisture or dirt from outside of flask and weigh. Repeat until constant weight is obtained.

CALCULATIONS

1. Oil in prepared test sample, $\% = \dfrac{\text{mass, g of oil}}{\text{mass, g of test portion}} \times 100$
2. If moisture is desired, determine as follows—
   (a) Weigh accurately about 10 g seed into tared AOCS aluminum moisture dishes, minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers (Sargent-Welch no. S-25675, Arthur H. Thomas no. 4522, or equivalent) (see Notes, 3).

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(b) With cover on bottom, heat in a forced-draft oven for 2 hr at 130 ± 3°C.
(c) Remove from oven and cover immediately. Cool in desiccator to room temperature and weigh.
(d) Moisture in prepared test sample, $\% = \frac{\text{mass, g lost}}{\text{mass, g of test portion}} \times 100$

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size <0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies, using conventional grinders, screens, and sieves, test samples should be prepared so that the particle size ranges from 0.5 mm to 0.9 mm (No. 35 to No. 20 sieve, respectively). Particle size should be verified by screening an *extracted* test portion.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occur, it may be advantageous to grind the laboratory sample with an equal amount of diatomaceous earth (see AOCS Method Ai 3-75), or to grind the laboratory sample with solid carbon dioxide (dry ice).

   If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method is recommended (e.g., AOCS Method Am 2-93).
2. One supplier of the sampling equipment noted in this method is Seedburo Equipment Co., Chicago, IL, USA.
3. For test samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test portion loss from splattering during heating.

REFERENCES

1. *J. Am. Oil Chem. Soc. 64*:865 (1987).

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AOCS

AOCS Official Method Ah 1-72

Reapproved 2009

Aflatoxins

DEFINITION

This method determines aflatoxins B_1, B_2, G_1, and G2 under the conditions of the test.

SCOPE

Applicable to copra and copra meal.

APPARATUS

1. High-speed blender—explosion-proof, with 1 qt jar. Drill 3 mm hole 13 mm from lid center to permit escape of vapors.
2. Chromatographic column—19 × 300 mm, fitted with stopcock.
3. Thin-layer chromatography equipment—glass plates, 20 × 20 cm; Desaga-Brinkmann applicator (Brinkmann Instruments, Inc., Westbury, NY, USA), or equivalent; mounting board; spotting template; microsyringe, 10 µL; desiccating storage cabinet (Brinkmann no. 255051, or equivalent); storage rack, with stainless steel trough, or equivalent; UV light source, or longwave 15 watt UV lamp, Chromato-Vue cabinet equipped with one or two 15 watt lamps, or equivalent.
   Note—Use UV-absorbing eyeglasses for eye protection when working with UV light source.
4. Funnel—18.5 cm diameter, long stem with E & D 617 (Whatman no. 2) and Whatman GF/A to fit.
5. Vials—4 dram, screw cap and hollow polyethylene stopper (Kontes no. 60910-4).
6. Glass boiling beads—Wash with water, then wash with acetone and dry.
7. Erlenmeyer flasks—250 and 300 mL, with ground-glass stoppers.
8. Beaker—400 mL.
9. Tube-mixer—Vortex, or equivalent.
10. Pipet—50 mL.

REAGENTS

1. Solvents—acetone, toluene, chloroform ($CHCl_3$), acetonitrile (CH_3CN), diethyl ether (anhydrous, alcohol-free), *n*-hexane, and methyl alcohol; all reagent grade, ACS grade, or equivalent (see Notes, *Caution*).
2. Silica gel—0.050–0.200 mm particle size for column chromatography; passing Association of Official Analytical Chemists (AOAC) test (References, 1) for thin-layer chromatography.
3. Sodium sulfate—granular, ACS grade, or equivalent.
4. Sodium chloride—ACS grade, or equivalent.
5. Aflatoxin standards (see Notes, 1)—Prepare solutions in toluene/CH_3CN (90:10, v/v) by agitating to dissolve, preferably with Vortex shaking machine. Prepare not less than 1 mL at any one time in volumetric flasks. Concentration of aflatoxins B_1 and G_1 may be 0.5–1.0 µg/mL, depending on analyst's preference. Concentration of B_2 and G_2 may be 0.1–0.2 µg/mL, but may vary, depending on ratio expected. Prepare a resolution reference standard (see Notes, 2).
6. Nonabsorbent cotton—extract with chloroform before use.

PREPARATION OF TEST SAMPLE

1. Copra is frozen at –17.8°C (0°F) or below and fed continuously into a hammer mill so that about 10 min is required to grind 4.54 kg of sample.
2. Meal, pellets, or shredded coconut are ground in the blender using Variac to control blender speed.

PROCEDURE

1. Extraction—
   (a) Weigh 50 g test portion into blender jar. Add 4 g sodium chloride, 100 mL H_2O, 250 mL $CHCl_3$, and blend at high speed for 3 min. Decant liquid into 400 mL beaker. Prepare funnel by first folding E & D no. 617 or Whatman no. 2 filter paper, then place a Whatman GF/A glass filter on the inside. Place funnel on 250 mL flask. Decant test solution from beaker into funnel, keeping solids in beaker. Collect 60–70 mL of filtrate. Pipet 50 mL filtrate onto prepared silica gel column.
2. Column chromatography—
   (a) Place cotton wad at bottom of chromatographic column and add $CHCl_3$ until column is two-thirds full. Add 5 ± 0.5 g granular Na_2SO_4 to give even base for silica gel. Then add 10 g silica gel in small increments, mixing after each addition. Stir out any formed bubbles. Allow to settle 15 min.
   (b) Slowly add 15 ± 0.5 g Na_2SO_4. Drain $CHCl_3$ leaving about 2 mm on top of Na_2SO_4. Add 50 mL test solution extract to column and elute at flow-rate 8–12 mL/min throughout.

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Table 1
Comparison of within- and between-laboratory variances of aflatoxin B_1 analysis based on tabulation of S_r^2, S_b^2, and S_d^2 of two pairs of samples.

Pair	Average aflatoxin B_1, µg/kg (ppb)	Precision		Systematic errors, S_b^2	F values		Degrees of freedom
		S_r^2	S_d^2		F calc., S_d^2/S_r^2	F table	
A–C	41	110.25	153.77	21.76	1.39	2.42	14
D–F	31	101.77	322.65	110.44	3.17	2.40	15

Table 2
Comparison of within- and between-laboratory variances of total aflatoxin analysis based on tabulation of S_r^2, S_b^2, and S_d^2 of two pairs of samples.

Pair	Average aflatoxin B_1, µg/kg (ppb)	Precision		Systematic errors, S_b^2	F values	
		S_r^2	S_d^2		F calc., S_d^2/S_r^2	F table
A–C	47	43.21	195.41	76.10	4.52	2.98
D–F	39	171.26	290.39	59.56	1.69	2.98

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AOCS Official Method Ai 1-80

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Sampling

SCOPE

Applicable to sunflower seed.

APPARATUS (see Notes, 1)

1. Triers—double-tube brass, 3.49 cm o.d., heavy bronze point, compartment type and conforming to USDA specifications. The following lengths are for sampling bulk seed:
   (a) Barges and hopper cars—3.0- or 3.6 m triers with 20 compartments.
   (b) Boxcars and trucks—1.5- or 1.8 m trier with 12 compartments.
   (c) Other containers—Use a grain trier that will reach the bottom of the specific container.
2. Pelican grain sampler or Ellis sampler—may be used to obtain a sample at a "free-falling point" when the use of automatic diverter-type sampling equipment is not feasible.
3. Boerner divider (such as no. 34) or a Riffle (Jones sampler)—with 19 mm openings.
4. Balance—graduated in grams, sensitive to ≤ 0.1 g.
5. Airtight, moisture-proof containers or bags.
6. Carter dockage tester—containing a no. 3 sieve in the top sieve carriage, no sieve in the middle sieve carriage and a no. 8 sieve in bottom carriage sieve (double perforation, large buck, triangular).
7. Hand sieves (grain dockage sieves)—33 cm diameter, 6 cm inside depth; top sieve, 4.76 mm diameter round hole; bottom sieve, 3.57 mm triangular hole.

PROCEDURE

1. Sunflower seed in bulk—Seven or more places should be probed with a standard trier. Insert trier in the seed at an angle of about 10° from the vertical, with the slots closed. Open the slots and give the trier several up-and-down movements. Then close the slots and withdraw the trier. Probe samples should be obtained from the following locations:
   (a) Probe in center of car.
   (b) Probe 0.9–1.5 m back from the door post toward the end of the car and approximately 0.6 m out from one side of the car.
   (c) Probe 0.9–1.5 m from the same end of the car and approximately 0.6 m from the opposite side of the car as in (b).
   (d) Repeat (b) and (c) above in opposite ends and sides of the car.
   (e) Probe 0.6 m from each end of car in center.
   A minimum sample of 1000 g or 750 mL shall be collected and stored in airtight, moisture-proof container.
2. Sunflower seed in truckloads or small bins—Use same procedure as for sunflower seed in bulk.
3. Sunflower seed in bags—Take samples from the bags by any standard or approved grain trier of sufficient length to reach center of bag. Draw samples from as many individual sacks, selected at random through the lot, as will be necessary to obtain a representative sample of at least 1000 g.
4. Loading and unloading sunflower seed—When a sample is to be taken during loading or unloading operation, a pelican- or Ellis-type sampler may be used at a free-falling point. Samples are taken at regular and frequent intervals to assure a correct and representative sample.

CLEANING AND REDUCTION OF LABORATORY SAMPLE

1. Hand cleaning method—
   (a) Prior to cleaning and mixing the laboratory sample, about 100 g of seed shall be withdrawn and placed in a suitable container with a tight-fitting cover. This part of the laboratory sample is to be used for the determination of moisture under AOCS Official Method Ai 2–75.
   (b) Reduce laboratory sample size to approximately 600 g with a Boerner divider (record weight W1 for use in calculations).
   (c) Screen half of the laboratory samples, at a time on hand screen, using both a top sieve (with 4.76 mm diameter round hole) and a bottom sieve (with 3.57 mm triangular hole).
   (d) The sieves should be held horizontally, and 30 complete cycles of gentle shaking should be used.
   (e) Weigh all the foreign matter passing through the 3.57 mm triangular sieve from the original 600 g sample (record weight W2 for use in calculations).
   (f) Combine the two sieved portions and split three times through the Boerner divider to obtain about 75 g (record weight W3 for use in calculations). Hand pick all foreign matter from this laboratory sample (record weight W4 for use in calculations). Do not remove kernels or pieces of sunflower seed. Preserve the cleaned laboratory sample in airtight container and use for subsequent analyses.

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(g) Calculations—

$$\text{Total foreign matter (mass)} = W2 + (W1 - W2)\frac{W4}{W3}$$

$$\text{Total foreign matter in original laboratory sample, \%} = \frac{\text{mass, g of total foreign matter}}{\text{mass, g of original laboratory sample}} \times 100$$

2. Machine cleaning method (using Carter dockage tester)—
   (a) Prior to cleaning and mixing the laboratory sample, about 100 g of seed shall be withdrawn and placed in a suitable container with a tight-fitting cover. This part of the laboratory sample is to be used for the determination of moisture under AOCS Official Method Ai 2–75.
   (b) Reduce the laboratory sample to approximately 600 g with a Boerner divider (record weight W1 for use in calculations).
   (c) Adjust the Carter dockage tester as follows: Set air control at $5\frac{1}{2}$. Set feed control at 8. Use no riddle; use no. 3 sieve in the top carriage; use no sieve in middle sieve carriage; and use no. 8 sieve in the bottom sieve carriage.
   (d) Run split laboratory sample through the machine.
   (e) Machine-removed dockage will consist of the material removed by air and the material passing through the no. 8 sieve (record weight W2 for use in calculations).
   (f) Sunflower seed or dehulled kernels passing over or caught in no. 3 sieve are picked out and added to the cleaned seed.
   (g) Split the machine-cleaned seed three times through the Boerner divider to obtain about 75 g (record weight W3 for use in calculations). Hand pick all foreign material from this test sample (record weight W4 for use in calculations). Do not remove kernels or pieces of sunflower seed. Preserve the cleaned laboratory sample in an airtight container and use for subsequent analyses.
   (h) Calculations—

$$\text{Total foreign matter (mass)} = W2 + (W1 - W2)\frac{W4}{W3}$$

$$\text{Total foreign matter in original laboratory sample, \%} = \frac{\text{mass, g of total foreign matter}}{\text{mass, g of original laboratory sample}} \times 100$$

NOTES

1. One supplier of the sampling equipment noted in this method is Seedburo Equipment Co., Chicago, IL, USA.
2. This method has been declared surplus following a vote by the UMC. ISO 542 will be included in future editions when section A is fully remodelled.

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AOCS

AOCS Official Method Ai 2-75

Reapproved 2009

Moisture and Volatile Matter

DEFINITION

This method determines moisture and any material that is volatile under the conditions of the test.

SCOPE

Applicable to sunflower seed.

APPARATUS

1. Forced-draft oven—AOCS Specification H 1–39.
2. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), or equivalent (see Notes, 1).
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9–87 for alternatives.

PROCEDURE

1. Weigh accurately duplicate test portions of 10 g each of sunflower seed [prepared as directed in AOCS Official Method Ai 1-80, Cleaning and Reduction of Test Sample, 1, (a) or 2, (a)] into tared moisture dishes, picking out and discarding large pieces of foreign matter which might affect accuracy of the determination.
2. Slip the covers on the bottoms of the dishes and dry in forced-draft oven at 130 ± 2°C for 3 hr.
3. Remove from the oven, replace covers immediately, cool in a desiccator to room temperature, and weigh.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$

PRECISION

1. Two single determinations performed in one laboratory shall not differ by more than 0.39%.
2. Single determinations performed in two different laboratories shall not differ by more than 0.44%.

NOTES

1. For test samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test portion loss from splattering during heating.
2. This method closely resembles ISO 665 (1977), *Oilseeds—Determination of Moisture and Volatile Matter* and the harmonized IUPAC Method 1.121 *Determination of Moisture and Volatile Matter*. The difference is that the temperature of 130°C is specified in the AOCS method and 103°C is specified in the ISO and IUPAC method. This difference in temperature appears large enough to make a difference in results. A collaborative study is under consideration by the AOCS.

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AOCS

AOCS Official Method Ai 3-75

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Oil Content

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to sunflower seed or dehulled kernels. This method requires some degree of skill to grind the seeds and diatomaceous earth simultaneously with little or no loss of earth. This method gives lower results than the comparable ISO method 659 Oilseeds—Determination of Hexane Extract (or Light Petroleum Extract), called "Oil Content," or the comparable IUPAC method 1.122 Determination of Oil Content in Oil Seeds. For more accurate results, it is recommended that AOCS Official Method Am 2-93 be used.

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4–38.
2. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent.
3. Absorbent cotton—free of petroleum ether extractables.
4. High-speed grinder—Mikro-Samplmill, rated at 12,000 rpm and equipped with 0.035- × 0.5 in. herring-bone screen (see Notes, 2).

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
2. Diatomaceous earth—dried at 130°C overnight before use. It may be kept in moisture-proof containers for several days without redrying; however, it should be dried again after several days.

PROCEDURE

1. Use a portion of the laboratory sample prepared as described in AOCS Official Method Ai 1-80, Cleaning and Reduction of Laboratory Sample, 1, (a) or 2, (a).
2. Weigh approximately 40 g of sunflower seed to the nearest 0.1 g into a large beaker. Add an equal weight of diatomaceous earth. Mix well with a large spatula (see Notes, 3).
3. Grind the mixture immediately with Mikro-Samplmill into a 1-L container. After grinding is complete, remove the container from the mill and place a large rubber stopper (no. 7 or 8) into the container to aid in mixing. Cap and allow the test sample to equilibrate at room temperature. This test sample is used for the determination of oil and nitrogen, and weighings for both determinations should be made at the same time (see Notes, 4).
4. Thoroughly mix test sample by inverting the container several times. The rubber stopper will dislodge any of the ground sample that may cake on the side of the container.
5. Weigh accurately 4 g of the ground mixture to the nearest 0.001 g into a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the meal (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton is placed in the top of the thimble to distribute the solvent as it drops on the test portion.
6. Place in Butt tube and test portion extract with petroleum ether as directed in AOCS Official Method Aa 4-38, Procedure, for 6 hr without interruption for grinding.
7. Cool and disconnect the flask. Evaporate the solvent on a steam bath or in a water bath until no petroleum ether remains. A gentle stream of nitrogen may be used to facilitate removal of solvent. Cool to room temperature. Carefully remove any moisture or dirt from outside of flask and weigh. Repeat heating until constant weight is obtained.

CALCULATIONS

1. Oil in ground test sample, $\% = \dfrac{(\text{mass, g of oil}) \times 2}{\text{mass, g of test portion}} \times 100$
2. The percent oil is calculated to dry-weight basis with the following formula:

$$\text{Oil, \% dry-weight basis} = \frac{\text{\% oil in ground test sample}}{100 - \text{\% moisture in whole seed}} \times 100$$

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3. Record the values obtained to the second decimal place.

PRECISION

1. Two single determinations performed in one laboratory shall not differ by more than 1.93%.
2. Single determinations performed in two different laboratories shall not differ by more than 2.19%
3. See Notes, 5.

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. A review of AOCS oil content methods indicated that particle sizes ranged from 0.16 mm to 1.0 mm, depending on the combination of grinding mill, screen, and sieve specified in each method. Generally, grinding to a particle size <0.16 mm (e.g., using specialized ball mills) will maximize the amount of oil extracted. Based on historical AOCS oil content methodologies, using conventional grinders, screens, and sieves, samples should be prepared so that the particle size ranges from 0.5 mm to 0.9 mm (No. 35 to No. 20 sieve, respectively). Particle size should be verified by screening an extracted sample.

   Grinding conditions should be adjusted so that no oil is expressed and so that no paste is formed. If either or both of these conditions occur, it may be advantageous to grind the laboratory sample with an equal amount of diatomaceous earth (see AOCS Method Ai 3–75), or to grind the laboratory sample with solid carbon dioxide (dry ice).

   If it is necessary to accurately determine oil content for reference purposes, an exhaustive extraction method is recommended (e.g., AOCS Method Am 2–93).
2. Other high-speed grinders, such as Krups 75 or Moulinex model MX 228 grinders, can be used, but may not give as effective grinding or mixing as the Mikro-Samplmill. If these grinders are used, grind a minimum of 24 g of seed with equal weight of diatomaceous earth (two grinds, 12 g seed + 12 g diatomaceous earth each grind). Grind laboratory sample for 30–60 sec, rotating the grinder back and forth and to both sides to ensure that any fine material adhering to grinding lid will be mixed back into the grinding flow of the laboratory sample. Do not grind stationary. Then, using a small spatula, redistribute the contents of the grinder, breaking up any lumps. Grind for an additional 30–60 sec, or until ground material is distinctly dark gray in color. Mix two grinds well.
3. At the same time, also weigh a representative portion of the unground seed for moisture determinations, using AOCS Official Method Ai 2-75. The moisture determination is performed on the uncleaned laboratory sample.
4. Mikro-Samplmill should be cleaned thoroughly between laboratory samples. For quantitative recovery of the sample, removal of feed screw, and herringbone screen for cleanout of lodged diatomaceous earth and ground materials has been found to be necessary. Change screen in Mikro-Samplmill when wear or bending of slot dividers is apparent. Compare to new screen. Sunflower hulls are very abrasive, and screen will have to be changed more often than with other oilseeds.
5. It was reported in References, 1 that the difference between two single determinations in one laboratory can be reduced to 0.6% by grinding sunflower seed to a particle size of ≤ 2.00 mm (an Ultra-Centrifugal mill with 1-mm screen and cyclone attachment from Glen Creston Ltd., London, was used). The AOCS Technical Committee suggests that, when checking the efficiency of grinders to produce particles of ≤ 2.00 mm, the particle size is best determined by screening of an extracted test portion. These reports have not been verified by an AOCS collaborative study.
6. This method has been declared surplus following a vote by the UMC. A single general method to determine Oil Content based on Aa 4-38 will be included in future editions. ISO Method 659 (Am 2–93) is recommended for further consideration.

REFERENCES

1. *J. Am. Oil Chem. Soc. 64*:865 (1987).

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AOCS

AOCS Official Method Ai 4-91
Revised 2011

Nitrogen-Ammonia-Protein Modified Kjeldahl Method Titanium Dioxide + Copper Sulfate Catalyst

DEFINITION
This method determines total nitrogen content and protein as 6.25 times the nitrogen content of the sample (see Notes, 1).

SCOPE
Applicable to sunflower seed. Replaces Ai 4-75.

APPARATUS

1. Kjeldahl digestion and distillation apparatus—complete with heat source, traps and block-tin, or equivalent noncorrosive tubing condensers (see Notes, 2).
2. Kjeldahl flasks—800 mL.
3. Distillate receiving flasks—500 mL, or any convenient size.

REAGENTS

1. Catalyst mixture—containing potassium sulfate, 16.7 g + titanium dioxide, 0.6 g + copper sulfate, 0.01 g + pumice, 0.3 g.
2. Sulfuric acid—sp. gr. 1.84 (see Notes, *Caution*).
3. Sodium hydroxide solution—sp. gr. 1.50 (see Notes, *Caution*).
4. Sodium hydroxide solution—0.25 M, accurately standardized (see AOCS Specification H 12-52).
5. Sulfuric acid—0.25 M, accurately standardized (see AOCS Specification H 13-52).
6. Methyl red indicator solution—0.1% in ethyl alcohol, OR Alizarin Red S—0.3% in distilled water.
7. Alundum boiling stones—8–14 mesh.
8. Zinc metal—granular. (If the catalyst is used with added pumice, granular zinc metal need not be added as an antibumping agent.)

PREPARATION OF TEST SAMPLE

1. Prepare test samples by grinding in a micro-grinder. If the samples are prepared as described in Ai 3-75, the diatomaceous earth causes foaming in the Kjeldahl flasks.

PROCEDURE

1. Weigh 0.250–1.750 g of the ground test sample into the Kjeldahl flask. Add the catalyst mixture (premixed catalyst packs are commercially available and may be used).
2. Add 30 mL of concentrated H_2SO_4 (see Notes, 4) to the test portion and catalyst in the digestion flask. Include at least one test portion of high-purity lysine hydrochloride in each day's run as a check of the correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.
3. Add a few Alundum boiling stones to each flask.
4. To digest the test portion, first adjust heat to bring 250 mL of water at 25°C to rolling boil in 5 min. Place the test portion flask on the digestion rack in an inclined position and then heat test portion flask at the 5-min boil rate until dense, white fumes clear bulb of flask (approximately 5 min). Swirl gently and continue heating an additional 40 min after the liquid has become clear and colorless (see Notes, 5). The Kjeldahl flasks should be rotated a minimum of three times during the digestion.
5. Cool, cautiously add about 300 mL of water and cool to room temperature (see Notes, 6). Add water as soon as possible to reduce amount of caking. If excessive bumping occurs during distillation, increase dilution water from 300 mL to about 350 mL.
6. Accurately transfer a sufficient quantity of the standard acid into receiving flask so there will be an excess of at least 0.5 mL of 0.25 M acid. Add sufficient distilled water to cover the end of the outlet tube and attach to outlet end of condenser tube. The distillate should discharge through a glass tube at the bottom of the receiving flask.
7. Add an additional 0.5–1.0 g Alundum boiling stones to cooled digestion flask. Mix thoroughly and add sufficient alkali solution (Reagents, 3) to make strongly alkaline. Pour the alkali down the side of the Kjeldahl flask so that it does not mix quickly with the acid.

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8. Immediately connect the Kjeldahl flask to the other end of the condenser tube and thoroughly mix the contents by shaking. Apply heat at about a 7.5-min boil rate and distill until at least 150 mL of distillate have been collected.
9. Titrate the contents of the receiving flask with 0.25 M NaOH solution, using 3 or 4 drops of indicator.
10. Conduct a blank determination of all reagents simultaneously with the test portions and similar in all respects. Correct for blank determined on reagents.
11. The performance of the entire method should be frequently checked by analyzing either National Institute of Testing-certified ammonium dihydrogen phosphate (standard reference material 194), or high-purity lysine hydrochloride.
12. Determine moisture in the ground test sample as directed in AOCS Official Method Ba 2a-38, Procedure section.

CALCULATIONS

1. Nitrogen, % = $\frac{[(MA \times 2 \times mLA) - (mLBK \times MB) - (mLB \times MB)] \times 1400.67}{\text{mg test portion}}$

Where—

mLB = volume, mL standard base used for the test portion
mLA = volume, mL standard acid used for that test portion
mLBK = volume, mL standard base needed to titrate 1 mL of standard acid minus mL standard base needed to titrate reagent blank carried through method and distilled into 1 mL standard acid
MA = molarity of standard acid
MB = molarity of standard base

Note—To determine percent ammonia, substitute 1703.06 for 1400.67 in the equation for the calculation of percent nitrogen.

2. Calculation: N × 6.25 = P
Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

PRECISION

There are no statistics relating the performance of this method to sunflower seed. The reader is referred to AOCS Official Methods Aa 5-91 and Ba 4d-90 for statistics from the analysis of soybean meal, soy protein concentrate, cottonseed and cottonseed meal.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Alkalies can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali solutions. Use effective fume removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. This method has been evaluated through interlaboratory comparison of catalysts and has been adopted by the committee as the official replacement for the mercuric oxide catalyzed method, AOCS Official Method Ba 4a-38. An interlaboratory evaluation (References, 1) indicated that AOCS Official Method Ba 4d-90 (using the copper/titanium catalyst mixture) produces results closer in agreement with AOCS Official Method Ba 4a-38 (using mercuric oxide catalyst) than methods using copper sulfate catalyst. As a result of this study, methods Ba 4a-38 (mercuric oxide), Ba 4b-87 (copper sulfate), and Ba 4c-87 (Kjel-Foss Automatic) were declared surplus (obsolete) in 1991.

   The Association of Official Analytical Chemists (AOAC) collaborative study (References, 2) showed no bias when comparing Cu/Ti catalyst with HgO. The AOCS interlaboratory evaluation (References, 1), based on sample weight, acid volume, digestion temperature, and time, previously specified in AOCS Official Method Ba 4a-38, indicated an average bias of –0.244%, which was statistically significant at the 99% confidence level. Factors that may affect method bias are sample weight and particle size, catalyst composition, use of Alundum, volume of acid used for digestion, digestion temperature, and digestion time. In 1991, two additional collaborative studies (cottonseed and cottonseed meal) showed that when 30 mL of sulfuric acid were used, caking of the digest was reduced and results were comparable to the mercuric oxide catalyzed method. As a result, the AOCS versions of this method may specify the use of more than 20 mL of sulfuric acid, but otherwise conform with the AOAC version (References, 3).
2. Use of steam- and water-resistant rubber tubing and stoppers to connect the glass parts is strongly recommended.

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3. The sample prepared according to Ai 3-75 contains diatomaceous earth, which causes foaming during Kjeldahl digestion. If the Kjeldahl method is being used for the determination of protein nitrogen, test samples should be prepared from whole sunflower seed by grinding in a micro-grinder.
4. More than 20 mL of concentrated sulfuric acid is needed for test portions high in fat or oil, because more acid is required to effect complete digestion, avoid loss in nitrogen and prevent caking. In recent collaborative studies involving the application of this method to cottonseed meal, it was observed that when 30 mL of H_2SO_4 were used, caking was reduced and results were comparable to the mercuric oxide catalyzed method.
5. Complete conversion of organic nitrogen to ammoniacal nitrogen is essential to obtain accurate and precise results. After the liquid has become clear and colorless, it may be necessary to digest the test portion for approximately an additional 30–50 min. Reagent proportions, heat input and digestion time are critical factors and should not be changed. Prolonged digestion and high temperatures must be avoided, because some ammoniacal nitrogen can be lost by oxidation. Large test portion particles should be avoided, because the larger particles will require a longer digestion time.
6. The cooled digest should be liquid, or liquid with a few small crystals. Caking before the addition of water indicates too little residual acid at the end of the digestion period and may result in low nitrogen values.

REFERENCES

1. *INFORM 1*:884 (1990).
2. Kane, P. F., *J. Assoc. Off. Anal. Chem.* 70:907 (1987).
3. *Official Methods of Analysis*, AOAC International, Gaithersburg, MD, 1990, p. 70, Method 988.05 (16th ed. 1995, 4.2.03).

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AOCS

AOCS Official Method Aj 1-86

Reapproved 2009

Aflatoxins in Corn

DEFINITION

This method detects total aflatoxins ($B_1 + B_2 + G_1 + G_2$) at concentrations of ≥10 µg/kg under the conditions of the test (see References, 1).

SCOPE

Applicable to white and yellow corn.

APPARATUS

1. Disk mill—Straub 4E (Croyden, PA, USA), or equivalent.
2. Wiley mill—Fisher Scientific no. 08-338, or Raymond hammer mill—MS-237-6 (C-E Process Equipment, Kansas Manufacturing Equipment, Enterprise, KS, USA), or equivalent.
3. Sieve—US no. 20.
4. Blender—high-speed, explosion-proof blender with 1 L jar; Waring model EP-1, or equivalent.
5. Light source—high-intensity longwave UV Blak-Ray B-100 lamp; Chromato-Vue CX-50N viewing cabinet (Fisher Scientific, USA), with four 15 watt longwave tubes and contrast filter; or equivalent.
   Note—Wear UV-absorbing eyeglasses for protection when working with UV light source.
6. Minicolumns—prepared of Pyrex™ tubing, 190 × 5.5–6.0 mm i.d., tapered at one end to about 2 mm i.d.
7. Support rack—to hold minicolumns upright. Test tube rack may be used.
8. Disposable items—culture tubes (20 × 150 mm) with plastic tube closures, 1.0 mL pipets and plastic funnels with 57 mm diameter top.

REAGENTS

1. Toluene—ACS reagent grade, or equivalent (see Notes, *Caution*).
2. Extraction solvent—prepared by mixing 800 mL methanol and 200 mL distilled water.
3. Zinc acetate solution—prepared by dissolving 600 g sodium chloride, 600 g zinc acetate and 15 mL glacial acetic acid (all reagent grade) in 4 L distilled water.
4. Minicolumn developing solvent—prepared by mixing 900 mL chloroform ($CHCl_3$, reagent grade) and 100 mL acetone (reagent grade). See Notes, *Caution*.
5. Florisil—Fisher Scientific no. F101, 100–200 mesh. Dry 2 hr at 110°C and store in vapor-tight container (see Notes, 1).
6. Silica Gel G 60—E. Merck no. 7731, 0.063–0.2 mm particle size (80-230 mesh), or equivalent. Dry 2 hr at 110°C and store in vapor-tight container (see Notes, 1).
7. Neutral alumina—80–200 mesh, Brockman activity 1 [Fisher Scientific no. A950 or J.T. Baker Chemical Co. no. 0540 (Phillipsburg, NJ, USA)], or equivalent. Dry 2 hr at 110°C and store in vapor-tight container (see Notes, 1).
8. Calcium sulfate—anhydrous, granular [Drierite, nonindicating, 20–40 mesh (W.A. Hammond Drierite Co., Xenia, OH, USA), or equivalent].
9. Aflatoxin spiking solution—Concentration is 1 µg aflatoxin B_1/mL, prepared by adding 1 mL solution to 50 g uncontaminated corn, which will be equivalent to 20 µg/kg, before extraction. Carry the extract through all of the steps. The developed minicolumn containing the unknown is compared with minicolumns of extracts of uncontaminated corn and "spiked" corn (see Notes, 2 and *Caution*).

SAMPLING AND PREPARATION OF TEST SAMPLE

1. Take a minimum 5 kg sample of corn kernels representative of an entire lot of corn either by probing (about 15 probes) or by a continuous sampler. The entire quantity of kernels shall be ground to pass a US no. 20 sieve (Apparatus, 3) in a Wiley or Raymond hammer mill (Apparatus, 2). The fine grinding can be facilitated by first cracking the kernels in a Straub disk mill (Apparatus, 1). The entire finely ground test sample is intensively mixed for at least 15 min in a double-cone mixer or in a planetary mixer.

PREPARATION OF THE MINICOLUMNS

1. Tamp small plug of glass wool into tapered end of column (Apparatus, 6).
2. To column, add to height indicated in following order: 5–7 mm calcium sulfate (Reagents, 8), 5–7 mm Florisil (Reagents, 5), 18–20 mm silica gel (Reagents, 6), 8–10 mm neutral alumina (Reagents, 7), and calcium sulfate (Reagents, 8). Tap column very lightly after each addition to settle packings, keeping interfaces as level as possible.
3. After packing, firmly tamp top of glass-wool plug with 5-mm diameter glass rod. Store columns in a desiccator (see Notes, 3).

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PROCEDURE

1. Extraction—
   (a) Weigh a 50 g test sample, transfer to blender jar (Apparatus, 4), add 100 mL of extraction solvent (Reagents, 2), and blend 2 min at high speed.
   (b) Filter 15 mL extract into culture tube (Apparatus, 8).
2. Zinc acetate cleanup—
   (a) To 15 mL filtrate [Procedure, 1, (b)], add 15 mL zinc acetate solution (Reagents, 3).
   (b) Close tube with plastic closure (Apparatus, 8) and shake vigorously for 10 sec (small separatory funnel may be substituted for culture tube).
   (c) Filter 15 mL of contents into second culture tube, add 3 mL toluene (Reagents, 1), close and shake gently 10 sec. Allow layers to separate.
3. Chromatography—
   (a) Pipet 1 mL toluene [upper layer from Procedure, 2, (c)] onto top of minicolumn.
   (b) Let toluene solution [Procedure, 2, (c)] drain into column; then add 3 mL chloroform-acetone (Reagents, 4) and drain.
   (c) Examine column [Procedure, 3, (b)] in darkened room under UV lamp (Apparatus, 5) or in Chromato-Vue cabinet (Apparatus, 5). Look for blue fluorescent band indicative of aflatoxin at top of Florisil layer (about 2.5 mm from bottom of the column). Perform analysis with uncontaminated corn and with corn spiked with aflatoxin at 20 μg/kg (ppb) (Reagents, 9) to obtain control and standard columns for comparison. Some test portions that do not contain aflatoxin will show faint brown, yellow or white fluorescent band at top of the Florisil layer. The band must be blue or bluish if a sample is positive.

REPORTING OF RESULTS

1. Results on a test portion can be reported as less than, equal to, or greater than 20 μg/kg.

NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform. Methylene chloride can be substituted for chloroform in the method.

Acetone and methyl alcohol are flammable solvents. They should not be used near an open flame. The use of a fume hood is recommended when using this solvent.

Aflatoxins are very toxic and carcinogenic substances in laboratory animals; results from epidemiologic studies present strong evidence that they may be very hazardous to humans as well. Perform manipulations under hood whenever possible, and take particular precautions, such as use of a glove box, when toxins are in dry form because of electrostatic nature and resulting tendency to disperse in working areas and air. Swab accidental spills of toxins with 5% sodium hypochlorite bleach containing 5% acetone. Rinse all glassware exposed to toxin with 1% sodium hypochlorite solution containing 5% acetone and then wash thoroughly. See References, 2 and 3 for recommended decontamination procedures for aflatoxins.

NUMBERED NOTES

1. The use of dry adsorbents for minicolumns is critical.
2. Criteria for purity of aflatoxin primary standards are given in References, 4. Aflatoxin standards are available from Aldrich Chemical Co., W. Milwaukee, WI, USA; Applied Science Division, State College, PA, USA; Calbiochem, La Jolla, CA, USA; Myco Lab Co., Chesterfield, MO, USA; Romer Labs, Inc., Washington, MO, USA; Sigma Chemical Co., St. Louis, MO, USA; and Supelco, Inc., Bellefonte, PA, USA.
3. Packed Holaday-Velasco (HV) minicolumns are available from Mycolab Co., P.O. Box 321, Chesterfield, MO, 63107 and Ag Science, Inc., P.O. Box 253, Shellman, GA, 31786.

REFERENCES

1. This method paraphrases *Official Methods of Analysis*, AOAC International, Gaithersburg, MD, Method 979.18 (17th ed. 2000, 49.2.06) and *Approved Methods of the American Association of Cereal Chemists*, American Association of Cereal Chemists, 10th ed., 2000, Method 45–14.
2. *J. Assoc. Off. Anal. Chem. 48*:681 (1965).
3. Davis, W. (ed.), Publication 37, International Agency for Research on Cancer, Lyon, France, 1980.
4. *J. Assoc. Off. Anal. Chem. 53*:93 (1970).

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AOCS

AOCS Official Method Aj 2-86

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Zearalenone and α-Zearalenol in Corn

DEFINITION

This method determines zearalenone and α-zearalenol under the conditions of the test (see Notes, Caution) (see References, 1 and 2).

SCOPE

Applicable to corn.

APPARATUS

1. Disk mill—Straub 4E (Straub Co., Croyden, PA, USA), or equivalent.
2. Raymond hammer mill—15 cm, equipped with screen having 0.32 cm diameter perforations (MS-273-6; C-E Process Equipment, Kansas Mfg. Equipment, Enterprise, KS, USA), or equivalent.
3. Mechanical shaker—Burrell Wrist Action, or equivalent, fitted to hold TS 500 mL Erlenmeyer flasks or 500 mL Erlenmeyer flasks fitted with Teflon™-lined screw caps.
4. Tube-shaking device—Vortex, or equivalent.
5. Vials—glass, 2, 4, and 20 mL, fitted with Teflon™-lined screw caps.
6. Liquid chromatograph—with septumless injector equipped with 20 µL injection loop (Model 600 pump and UK6 injector; Waters Associates, Milford, MA, USA), or equivalent.
7. Liquid chromatographic column—reverse-phase column (10 µm particle size) 250 × 4.6 mm (i.d.) (Zorbax C_8 or C_{18}; Du Pont Co., Instrument Division, Wilmington, DE, USA), or equivalent.
8. Fluorescence detector—variable wavelength, 236 and 274 nm excitation, 418 nm emission cutoff filter (Model FS 970; Kratos Analytical, Ramsey, NJ, USA), or equivalent.
9. Filters—Swinney filter assembly and glass fiber filters (13 mm) (Gelman Sciences, Inc., Ann Arbor, MI, USA), or equivalent filters to remove particulates from samples prior to HPLC.
10. Filter paper—fluted, 24 cm, S & S 588 (Schleicher and Schuell, Inc., Keene, NH, USA), or equivalent.

REAGENTS

1. Solvents—HPLC grade, distilled in glass, consisting of methyl alcohol, acetonitrile, methylene chloride, chloroform (see Notes, *Caution*).
2. HPLC mobile phase—prepared by combining methyl alcohol, acetonitrile and water (1.0:1.6:2.0). Degas by sonication, sparging with helium or vacuum. Stir continuously or sparge with helium during use.
3. Sodium chloride solution—saturated, prepared by saturating distilled water with sodium chloride.
4. Sodium hydroxide solution (2%)—prepared by dissolving 20 g sodium hydroxide in 1 L distilled water (see Notes, *Caution*).
5. Citric acid solution—prepared by dissolving 106 g citric acid monohydrate in 1 L distilled water.
6. Diatomaceous earth filter aid—acid-washed Hyflo Super-Cel (Manville Corp.), or equivalent.
7. Sodium sulfate—anhydrous, granular, ACS reagent grade.
8. Zearalenone and α-zearalenol standards—prepared from high-purity crystalline zearalenone and α-zearalenol (see Notes, 1). Weigh accurately 50 mg of each analyte and dissolve in 200 mL acetonitrile (stock solutions). Prepare working reference standards by transferring 0.1, 0.2, 0.5, 1.0, and 2.0 mL stock solutions to separate vials and evaporate solvent under nitrogen. Add 10.0 mL mobile phase (Reagents, 2) and cap tightly. Protect all standards and solutions from UV light. Working standards are 0.25, 0.50, 1.25, 2.50, and 5.0 µg toxin/mL.

PREPARATION OF TEST SAMPLE

1. Grind representative laboratory sample of corn kernels (2–4 kg) in Raymond hammer mill (Apparatus, 2). Grinding can be facilitated by first cracking the kernels in a Straub disk mill (Apparatus, 1). The entire finely ground test sample is mixed for 15 min in a planetary mixer.

PROCEDURE

1. Extraction—
   (a) Accurately weigh 50 g test portion and transfer to a 500 mL Erlenmeyer flask fitted with a ground-glass stopper or Teflon™-lined screw cap. Add 25 mL diatomaceous earth (Reagents, 6) and 20 mL water. Rotate flask to accomplish some mixing, and add 250 mL chloroform. Stopper tightly and shake 15 min on wrist action shaker (Apparatus, 3).

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(b) Filter through fluted filter paper (Apparatus, 10) and collect 50 mL filtrate (10 g equivalent) in 100 mL graduated cylinder. Collect second 50 mL filtrate and save in case of accidental loss.

2. Acid-base cleanup—
   (a) Transfer 50 mL filtrate to 250 mL separatory funnel, add 10 mL sodium chloride solution (Reagents, 3) and shake vigorously for about 1 min. Allow phases to separate completely. Discard lower layer (chloroform) and as much sediment (if any) as possible. Add 50 mL chloroform, shake about 1 min and again discard chloroform layer. Add 50 mL citric acid solution (Reagents, 5) to separatory funnel and mix by shaking.
3. Methylene chloride partition—
   (a) Add 50 mL methylene chloride to aqueous phase and shake about 1 min to extract zearalenone/zearalenol. Allow phases to separate and drain lower phases through about 2 in (40 g) anhydrous sodium sulfate (Reagents, 7) in glass funnel with glass wool plug. Drain any emulsion at the interface through sodium sulfate. Reextract aqueous phase with second 50–mL portion of methylene chloride and, after phases separate, drain lower phase through sodium sulfate as before. Wash sodium sulfate with 10–15 mL methylene chloride.
   (b) Evaporate combined methylene chloride extracts to near dryness on flask evaporator at ≤ 40°C. Transfer test solution residue to 4 mL Teflon™-lined screw-cap vial (Apparatus, 5) with three to four washes of methylene chloride. Evaporate to dryness under nitrogen, and dissolve in 0.50 mL mobile phase (Reagents, 2). Mix on Vortex mixer (Apparatus, 4) and reserve for HPLC.
4. Filtering for HPLC (optional)—
   (a) Wash filter assembly (Apparatus, 9) with acetone and dry before use. Transfer test solution to syringe barrel with capillary pipet, and slowly push sample through filter into 2 mL vial with Teflon™-lined screw cap (Apparatus, 5). Any other filtration system for removing particulate matter without loss of test solution is satisfactory. Reserve for quantitation by HPLC. Store in refrigerator until ready for determinative chromatography.
5. Quantitative HPLC—
   (a) Precondition column (Apparatus, 7) with several column volume of mobile phase (Reagents, 2). Stabilize flow rate at 1.5 or 2.0 mL/min. Set fluorescence detector (Apparatus, 8) at 236 nm (excitation wavelength) with 418 nm (emission wavelength) cutoff filter. Set range at 1.0 μA; suppression, high; time constant to about 6 sec (0.1 average peak width). Set recorder at 2 min/cm (5 min/in.). Range may be changed to 0.5 μA for low level (< 25 ng) of toxins.
   (b) Quantitatively inject 20 μL of each standard solution (Reagents, 8), and construct standard curve by plotting peak height or area vs. concentration of toxin. Check reproducibility of standard curve daily by making at least two injections of both standards. Minimum concentration standard (Reagents, 8), 0.25 μg/mL, should give a peak height of about 5% full-scale deflection at 0.5 μA range on a 10-mv recorder. The linear range of the fluorescence detector is 5–100 ng toxin at 236 nm. Inject 20 μL of test solution extract [Procedure, 3, (b) or Procedure, 4] under identical conditions as used for standards. If peaks from test solutions go off scale, suitably dilute samples and repeat injection so that test solution peak heights are within the linear range of the detector.
   (c) From either integrator area count or peak height, calculate concentration of zearalenone and α-zearalenol (in ppm) as follows:

$$\text{Zearalenone or } \alpha - \text{zearalenol, } \mu g/g = \frac{(H)(S)(Y)(V)}{(H')(X)(W)}$$

Where—

H = area or peak height of zearalenone/zearalenol test solution peaks
S = volume, μL standard zearalenone/zearalenol injected
Y = concentration of standard in μg/mL
V = final dilution on μL of test solution extract
H′ = area or peak height of zearalenone/zearalenol standard peak
X = volume, μL test solution extract injected
W = test portion weight (g/mL) in final extract [Procedure, 3, (b) or Procedure, 4]

CONFIRMATION OF IDENTITY OF ZEARALENONE AND α-ZEARALENOL

1. Sequentially inject 20 μL zearalenone/α-zearalenol standard and test solution. Determine peak heights at 236 nm and 274 nm, and calculate peak height ratio for standard and sample. Ratio of test solution peak height must agree within ± 5% with ratio of standard peak heights.

PRECISION

1. Table 1 shows percent coefficient of variation within labs and between labs.

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NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Methylene chloride is toxic and a carcinogen. The TLC is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

Methyl alcohol (methanol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fumeremoval device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform and perchloric acid.

Zearalenone and α-zearalenol are estrogenic, with less potency than diethyl stilbesterol. Handle with due regard to their biological activity.

Alkalies can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fumeremoval device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. Criteria for purity of aflatoxin primary standards are given in References, 3. High-purity zearalenone and α-zearalenol standards are available from International Minerals and Chemical Corp., Terre Haute, IN, USA; Makor Chemicals Ltd., Jerusalem, Israel; Myco Lab Co., Chesterfield, MO, USA; Sigma Chemical Co., St. Louis, MO, USA.

REFERENCES

1. This method paraphrases *Official Methods of Analysis,* AOAC International, 16th ed., Vol. II, Gaithersburg, MD, 1995, Chapt. 49, p. 45, Method 985.18.
2. *J. Assoc. Off. Anal. Chem. 68:*381 (1985).
3. *Ibid. 62:*1268 (1979).
4. *Ibid. 68:*958 (1985).

Table 1
Within-laboratory and between-laboratories coefficient of variation (%) for zearalenol and zearalenone.

	Spiked samples[a]	Naturally contaminated samples[a]
Within lab		
α-zearalenol	22.6	52.0
zearalenone	31.4	44.4
Between labs		
α-zearalenol	25.6	42.2
zearalenone	33.8	33.9

[a]Values expressed as percent coefficient of variation.
See References, 4 (particularly Tables 3 and 4).

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AOCS Official Method Aj 3-87

Reapproved 2009

Aflatoxins in Corn

DEFINITION

This thin-layer chromatographic method determines aflatoxins B_1, B_2, G_1, and G_2 under the conditions of the test (see References, 1).

SCOPE

Applicable to corn.

APPARATUS

1. Wiley cutting mill—catalog no. 08-338 (Fisher Scientific, USA), or equivalent, or
2. Hammer mill—C.E. Raymond Co., Houston, TX, USA, or equivalent, or
3. Disk mill—Straub (Straub Co., Craydon, PA, USA), Dickens-Satterwhite (Federal-State Inspecting, Albany, GA, USA), or equivalent.
4. Twin-shell dry blender—Patterson Kelley Co., East Stroudsburg, PA, USA.
5. Sieves—US no. 14 and no. 20.
6. Jones sample splitter.
7. Analytical balance—with sensitivity of 0.001 g.
8. Mechanical shaker—Burrel Wrist Action, or equivalent, fitted to hold 500 mL Erlenmeyer flasks fitted with standard-taper ground-glass joints; or Waring, Model EP-1 explosion-proof, high-speed blender with 1 L (or 1 qt) jar; or equivalent.
9. Hollow polyethylene stoppers—13 mm top diameter, 7 mm bottom diameter to fit vials; linear high-density polyethylene stoppers to fit 500 mL Erlenmeyer flasks (Kimble catalog no. 28160-32; Kimble, Toledo, OH, USA, or equivalent).
10. Funnels—150 mm, fitted with fluted S & S no. 588 paper, or equivalent; or Buchner, 32 cm diameter, fitted with Whatman no. 1 filter paper.
11. Chromatographic tubes—22 × 300 mm, with Teflon™ stopcock, 250 mL (Kimax Div. of Owens Illinois, Toledo, OH, USA).
12. Vials—glass, 5 mL, fitted with either polyethylene friction-fit stoppers or foil-lined screw caps (Kimble).
13. Viewing cabinet—Chromato-Vue Model C-4 or C-6, with four 15 watt longwave bulbs and contrast filter (UV Products, Inc., San Gabriel, CA, USA), or equivalent.
14. Tripleblock heater—Lab-Line no. 2002-1 (Barnstead International, Dubuque, Iowa, USA).
15. Glass manifold—with outlets to allow passage of a stream of nitrogen.
16. Steam bath.
17. Thin-layer chromatography equipment—
    (a) Glass plates—20 × 20 cm.
    (b) Applicator—for applying 0.25–0.50 mm wet thickness gel layer.
    (c) Mounting board.
    (d) Spotting template.
    (e) Plate holder.
    (f) Desiccating storage cabinet—with Drierite or silica gel desiccant.
    (g) Development tank—stainless steel, with trough and glass cover; or Desaga-Brinkmann, no. 25-10-20, glass (use without liner) (Brinkmann Instruments Inc., Westbury, NY, USA); or equivalent.
    (h) Microliter syringe—10 µL, with 2 cm, 26 gauge tip, 22° angle.
    (i) TLC plate-scoring device.
18. Densitometer—Shimadzu model CS-930 dualwavelength thin-layer chromato-scanner, with a power source of 110/220 V, 300 VA 50/60 Hz and equipped with mercury lamps, wavelength range of 200–700 nm, monochromator unit and an integrating calculator (Shimadzu Scientific, Columbia, MD, USA), or equivalent. Use according to manufacturer's specifications.
19. Boiling chips—Silicon Carbide boiling chips, 14 mesh (Crescent Chemical Co., Hauppany, NY, USA). Float off fines and extraneous matter with water, wash with acetone and air dry.
20. Diatomaceous earth filter aid—acid-washed Hyflo SuperCel (Manville Corp., Denver, CO, USA), or equivalent.
21. Sodium sulfate—anhydrous, granular, reagent grade.
22. Vortex mixer—variable speed Vortexer (VWR International, West Chester, PA, USA) or equivalent.
23. UV lamp—100 watt, Blak-Ray UVP B-l00A (VWR International), or equivalent.
24. Protective goggles—Blak-Ray UVP-002-02 (VWR International), or equivalent.

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REAGENTS

1. Solvents—reagent grade, stored in glass acetone, acetonitrile, toluene, diethyl ether (anhydrous, ≤0.01% ethanol), hexane (bp 68–69°C), methanol, methylene chloride, chloroform, sulfuric acid (see Notes, *Caution*).
2. Eluting solvent—Mix 970 mL methylene chloride and 30 mL methanol.
3. TLC spotting and standard solvent—Mix 90 mL toluene and 10 mL acetonitrile. Store in dark glass bottle.
4. TLC developing solvents—
   (a) Mix 900 mL chloroform and 100 mL acetone.
   (b) Mix 960 mL diethyl ether, 30 mL methanol, and 10 mL distilled water (see Notes, 1).
5. Confirmatory spray—Add 100 mL sulfuric acid to 300 mL distilled water (see Notes, *Caution*).
6. Silica gels—
   (a) E. Merck Silica Gel 60, 0.063–0.2 mm for column chromatography (Curtis Matheson Scientific, Inc., Houston, TX, USA), or equivalent. Activate by drying 1 hr at 105°C. Add H_2O, 1 mL/100 g, seal, shake until thoroughly mixed, and store 15 hr in airtight container. Any silica gel that separates aflatoxins may be used for preparation of TLC plates (each shipment should be tested). Macherey-Nagel GHR (Macherey, Nagel & Co., Düren, Germany; distributed by Brinkmann Instruments, Inc.), Applied Science Adsorbosil-Plus (Applied Science Labs, Deerfield, IL, USA) and Mallinckrodt SilicAR 4G or 7G (Mallinckrodt, Paris, KY, USA) have been found satisfactory.
   (b) TLC plate preparation—Weigh 30 g silica gel into 300 mL Erlenmeyer flask; add amount of water recommended by manufacturer. Shake vigorously for about 1 min and pour into applicator immediately. Coat five 20 × 20 cm glass plates with 0.25 mm wet thickness layer of the silica gel suspension; air dry until gelled, about 10 min. Dry coated plates 2 hr at 80°C or 1 hr at 110°C and store in desiccator over indicating silica gel until just before use. Score plates for densitometric quantitation with scoring device [Apparatus, l7, (i)].
   (c) Precoated TLC plates—Silica Gel 60 (without fluorescent indicator), 0.25 mm thick [MCB Manufacturing Chemicals, Inc. (Associate of E. Merck, Darmstadt, Germany) Cincinnati, OH, USA], or Baker Si 250 (J. T. Baker Chemical Co., Phillipsburg, NJ, USA), or equivalent.
7. Aflatoxin standards for TLC—Prepare from high-purity crystalline aflatoxins B_1, B_2, G_1, and G_2. Weigh accurately at least 10 mg of each toxin, or obtain an accurately weighed 10 mg sample of each (see Notes, 2). Dissolve each toxin separately in toluene/acetonitrile (Reagents, 3). Suitably dilute each with toluene/acetonitrile to contain 100 µg toxin/mL. From these four standard solutions prepare:
   (a) Individual toxin reference standards diluted with toluene/acetonitrile to contain 0.5 µg B_1/mL, 0.5 µg G_1/mL, 0.1 µg B_2/mL, 0.1 µg G_2/mL.
   (b) Mixed-resolution standard for preliminary TLC [Procedure, 4, (a) through (n)] diluted with toluene/acetonitrile to contain 0.5 µg each of B_1 and G_1 and 0.1 µg each of B_2 and G_2 per mL. Use this mixed standard for quantitative visual estimation and for quantitative densitometric estimation.

PROCEDURE

1. Test sample preparation—Combine all subsamples of corn into one total lot laboratory sample. Grind entire lot laboratory sample through Wiley, hammer or disk mill (Apparatus, 1–3) to pass no. 14 sieve (Apparatus, 5). Split sample sequentially in sample splitter (Apparatus, 6) until a 1–2 kg portion is obtained for analysis. Retain remainder. Regrind the 1–2 kg analytical portion to completely pass no. 20 sieve (Apparatus, 5). Mix reground portion thoroughly in a tumbler blender (Apparatus, 4). Reriffle the analytical portion in the sample splitter (Apparatus, 6) to obtain the analytical sample. Prepare additional portions from the lot laboratory sample in the same way, if needed.
2. Extraction—
   (a) Weigh 50 g riffled test portion into 500 mL Erlenmeyer flask. Add 25 mL H_2O, 25 g diatomaceous earth (Apparatus, 20) and 250 mL methylene chloride (Reagents, 1). Stopper flask. Shake 30 min on wrist action shaker (Apparatus, 8) and filter through fluted paper. If filtration is slow, use Buchner funnel (Apparatus, 10). See Notes, 3.
   (b) Collect first 50 mL portion of the methylene chloride filtrate.
3. Column chromatography—
   (a) Place ball of glass wool loosely in bottom of glass chromatographic tube (Apparatus, 11). Add about 5 g anhydrous sodium sulfate (a 5 mL beaker almost filled) to give a base for silica gel. Close stopcock. Add methylene chloride until tube is about half full. Add 10 g silica gel [Reagents, 6, (a)]; a 50 mL beaker filled to 20 mL mark is a convenient measure. Wash sides of tube with about 20 mL methylene chloride, and stir to disperse silica gel. Drain some methylene chloride to aid settling, leaving 5–7 cm of methylene chloride above the silica gel. Close stopcock. Slowly add about 15 g sodium sulfate. Drain methylene chloride to the top of the sodium sulfate.
   (b) Place 400 mL beaker under column. Add 50 mL test solution extract [Procedure, 2, (b)] to column. Elute at maximum flow rate with 150 mL hexane followed by elution with 150 mL anhydrous diethyl ether. Discard combined hexane and diethyl ether eluants (collected in 400 mL beaker).
   (c) Place 250 mL beaker under column. Elute aflatoxins with 150 mL methylene chloride/methanol solution (Reagents, 2). Collect this fraction from time of addition until flow stops.

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(d) Add a few boiling chips (Apparatus, 19) to eluant, evaporate nearly to dryness on steam bath or rotary evaporator (Apparatus, 16) and quantitatively transfer residue to vial with methylene chloride. Add one to two boiling chips and evaporate, preferably under a gentle stream of nitrogen.
(e) Seal vial with hollow polyethylene stopper (Apparatus, 9). Save for TLC. If TLC will be done at a later date, store vial(s) in refrigerator.

4. Preliminary TLC (*Note*—If the approximate aflatoxin content is known, this step may be omitted.)
   (a) Uncap vial containing test portion extract, add 200 µL toluene/acetonitrile (Reagents, 3), and reseal with polyethylene stopper. Shake vigorously to dissolve, preferably with a Vortex mixer (Apparatus, 22).
   (b) Puncture polyethylene stopper to accommodate syringe.
   (c) In subdued incandescent light, and as rapidly as possible, spot a 2, a 5, and two 10 µL spots approximately 1 cm apart on an imaginary line 4 cm from bottom edge of the TLC plate [Reagents, 6, (b)]. Keep vial for quantitative analysis.
   (d) On same plate, spot 2, 5, and 10 µL of the aflatoxin standards.
   (e) Spot 5 µL of each standard directly on top of one of the two 10 µL test solution origin spots as an internal standard.
   (f) Spot 5 µL of the resolution reference standard to show whether adequate resolution is attained.
   (g) Place 50 mL of either chloroform/acetone or diethyl ether/methanol/water (Reagents, 4) in trough of unlined developing tank. Use a volume to provide a solvent depth of about 2 cm. Use only one plate per tank, placing trough near one side to permit maximum exposure of coated surface to tank volume. Immediately insert plate into tank and cover. (Other solvent systems may be substituted; see Notes, 1.)
   (h) Develop plate 40 min or until solvent front is within 2.5 cm of top of TLC plate. Adjust developing solvent if time required is >50 min.
   (i) Remove plate from tank; evaporate solvent at room temperature.
   (j) Illuminate plate from below by placing it flat, coated side up, on longwave UV lamp in darkened room (see Notes, *Caution*); view plate in Chromato-Vue cabinet (Apparatus, 13); or illuminate from above.
   (k) Observe pattern of four fluorescent spots of resolution reference standard. In decreasing order the aflatoxins are B_1, B_2, G_1, and G_2. Note the bluish fluorescence of B aflatoxins contrasted with slightly green G aflatoxins.
   (l) Examine patterns from test solution for fluorescent spots having R_f close to and appearances similar to those of standards.
   (m) From this preliminary plate, estimate a suitable dilution for quantitative TLC analysis. Table 1 suggests appropriate volumes for the dilution of extracts.
   (n) In final calculations, take into account the amount of extract used for preliminary TLC.
5. Quantitative TLC—
   (a) If preliminary plate shows that a new concentration of test portion extract is required, evaporate extract to dryness under a stream of nitrogen (or on a steam bath).
   (b) Redissolve in calculated volume (Table 1) of toluene/acetonitrile (Reagents, 3).
   (c) Spot successively a 3.5, a 5.0, and two 6.5 µL portions of test solution extract. All spots should be approximately the same size and ≤0.5 cm in diameter. On same plate, spot 3.5, 5.0, and 6.5 µL of the aflatoxin standards [Reagents, 7, (a)] that were observed on the preliminary plate. Spot 5.0 µL of each standard directly on top of one of the two 6.5 µL test solution origin spots as an internal standard. Spot at least one 5 µL resolution standard [Reagents, 7, (b)] to show whether adequate resolutions are attained.
   (d) Develop plate as in Preliminary TLC [Procedure, 4, (a) through (n)].
   (e) Visual interpretation of chromatogram—Four clearly identifiable spots should be visible in the resolution reference standard. If four spots are not visible, repeat TLC.

Examine pattern from test solution spot containing internal standard for aflatoxin spots. R_f values of aflatoxins used as internal standards should be the same as, or only slightly different from, those of respective standard aflatoxin spots. (Because spots from test solution extract are compared directly with standard aflatoxins on the same plate, magnitude of R_f is unimportant. This may vary from plate to plate.)

Compare test solution fluorescence patterns with patterns containing internal standard. Fluorescent spots in test solution thought to be aflatoxin must have R_f values identical to and color similar to aflatoxin standard spots. Unknown spot and internal standard combined should be more intense than either test solution or standard alone.

Compare fluorescent intensities of B_1 spots of test solution with those of standard spots, and determine which test solution portion matches one of the standards. Interpolate if intensity of test solution spot is between two standard spots. If spots of smallest portion of sample are too intense to match standards, dilute test solution and rechromatograph. Compare B_2, G_1, and G_2, if present, in the same manner (see Notes, 4).

Calculate concentration of aflatoxin B_1 [in µg/kg (ppb)] as follows:

$$B_1, \mu g/kg = \frac{S \times Y \times V}{X \times W}$$

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Where—

S = volume, µL aflatoxin B_1 standard spotted equal in intensity to unknown (test solution spot)
Y = concentration of aflatoxin B_1 standard in µg/mL [Reagents, 7, (a)]
V = final dilution in µL of test solution extract
X = volume, µL test solution extract spotted giving fluorescent intensity equal to S (B_1 standard)
W = g test portion [10 g if 50 mL methylene chloride extract was applied to column as in Procedure, 2, (b)]. If final extract dilution does not represent 10 g, calculate correct test portion weight and substitute.

Repeat calculations for aflatoxin B_2, G_1, and G_2, if present, in the same manner as that for B_1, substituting appropriate sample and standard concentrations.

6. Densitometric quantitation of chromatogram—
   (a) Spot on a TLC plate duplicate portions of test solution extract dissolved in appropriate volume of toluene/acetonitrile as outlined in Table 1. Spot duplicate 10 µL portions of mixed-aflatoxin densitometric standard [Reagents, 7, (b)], placing spots along imaginary line in center of channel of scored plate 4 cm from bottom (Reagents, 6, (b)). If B_2 in test spot has a considerably higher or lower intensity than B_2 in the standard, it may be necessary to adjust a separate test solution aliquot so that the B_2 in the aliquot spotted has a fluorescent intensity close to B_2 in the standard. Make comparable dilutions for G aflatoxins, if present.
   (b) Develop plate as outlined under Preliminary TLC (Procedure, 4). If plate is visually inspected before densitometry, use low wattage UV source and minimum exposure time.
   (c) Assemble and warm up densitometer apparatus according to manufacturer's instructions. Operate in dimly lit room or under suitable protective cover.
   (d) Scan plate according to instruction of manufacturer. Irradiate with 365 nm source; observe for emission at 420–460 nm.
   (e) Calculate concentration of aflatoxin B_1 [in µg/kg] as follows:

$$B_1, \mu g/kg = \frac{B \times S \times Y \times V}{Z \times X \times W}$$

Where—

B = average area aflatoxin B_1 peaks in test solution aliquots
S = volume, µL aflatoxin B_1 standard spotted
Y = concentration of aflatoxin B_1 in standard, in µg/mL [Reagents, 7, (a)]
V = final dilution of test solution extract, in µL
Z = average area of aflatoxin B_1 peaks in standard aliquots
X = volume, µL test solution extract spotted giving fluorescent intensity equal to S (B_1 standard)
W = g test portion weight (10 g) represented by final extract [Procedure, 4, (n)]

   (f) Correct aflatoxin concentration values from densitometric measurement for amount of test portion extract removed for preliminary TLC [Procedure, 4, (c)] and/or quantitative visual TLC [Procedure, 5, (c)], if either or both were used before densitometric measurement, as follows:

$$\text{Corrected } B_1, \mu g/kg = \frac{\text{apparent } B_1, \mu g/kg}{1-(p/P + q/Q)}$$

Where—

p = total µL test solution extract spotted in preliminary TLC [Procedure, 4, (c)]
P = volume µL of test solution extract prepared for preliminary TLC
q = total µL test solution extract spotted for visual analysis (if done prior to densitometric analysis)
Q = volume, µL of test solution extract prepared for visual analysis [Procedure, 5, (c)]

Use same procedure for B_2, G_1, and G_2, if present.

   (g) Repeat calculation for each other aflatoxin observed, substituting appropriate areas and standard concentrations.

PRECISION

1. Between laboratories—Visual 48.7%, B_1; densitometric 47.9%, B_1 (see References, 3).

NOTES

Caution

Mycotoxins should be handled as very toxic substances. Perform manipulations under hood whenever possible, and take particular precautions, such as use of glove box, when toxins are in dry form because of electrostatic nature and resulting tendency to disperse in working areas. Swab accidental spills of toxin with 5% NaOCl bleach. Rinse all glassware exposed to toxins with 1% NaOCl solution and then wash thoroughly. See References, 4 for recommended decontamination procedures for aflatoxins.

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Protect eyes with UV-absorbing filter such as Eastman Kodak Co. 2 A. If a Chromato-Vue cabinet is not used, care should be taken to cover any viewing port with a UV-absorbing filter. If only a darkened room is used, the appropriate safety goggles must be used (Apparatus, 24).

Acetone is a flammable solvent. It should not be used near an open flame. The use of a fume hood is recommended when using this solvent.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Toluene is flammable and a dangerous fire risk. It is toxic by ingestion, inhalation, and skin absorption. The explosive limits in air are 1.27–7% by volume. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

Diethyl ether is extremely flammable and a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. Store protected from the light. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. Can react explosively when in contact with chlorine, ozone, lithium aluminum hydride or strong oxidizing agents. A fume hood should be used at all times when using diethyl ether. Avoid static electricity.

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Methyl alcohol (methanol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform and perchloric acid.

Methylene chloride (dichloromethane) is toxic and a carcinogen. The TLC is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

NUMBERED NOTES

1. TLC development solvents effective for separation of aflatoxins vary from lab to lab. Use any system that separates B_1, B_2, G_1, and G_2 from each other and also separates interfering substances from the toxins. Alternative solvent systems are listed in Table 2.
2. Criteria for purity of aflatoxin primary standards and determination of concentration of diluted working standards for TLC are given in References, 6. Aflatoxin standards are available from Calbiochem, Los Angeles, CA, USA; Supelco, Inc., Bellefonte, PA, USA; Myco Lab Co., Chesterfield, MO, USA; Eureka Laboratories, Inc., Sacramento, CA, USA.
3. Avoid use of suction filtration whenever possible, as the suction tends to speed solvent evaporation.
4. While G aflatoxins are rarely found in corn, blue fluorescent spots are sometimes found in corn extracts, at or near the R_f of G_1 or G_2, that will not exhibit characteristic color change. Test is not confirmatory, but will rule out presumptive aflatoxins that do not show color change.

REFERENCES

1. This method paraphrases *Official Methods of Analysis*, AOAC International, 17th ed., Vol. 2, Gaithersburg, MD, 2000, Chapt. 49, p. 16, Method 972.26.
2. *Ibid.*, Chapt. 49, p. 21, Method 980.20.
3. *J. Assoc. Off. Anal. Chem.* 55:781 (1972).
4. *Ibid.* 48:681 (1965).
5. *Official Methods of Analysis*, AOAC International, 17th ed., Vol. 2, Gaithersburg, MD, 2000, Chapt. 49, p. 5, Method 975.35.
6. *J. Assoc. Off. Anal. Chem.* 53:92 (1970).

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Table 1
Sample dilution and aliquots for TLC visual and densitometric analysis[a].

Approx. B_1 content from prelim., µg/kg (ppb)	Visual analysis[b]		Densitometric analysis[b]	
	Dilution of extract, mL	Aliquots on plate, µL	Dilution of extract, mL	Aliquots of plate, µL
0–10	0.125	3, 5, 7	0.125	10, 10
10–25	0.125	3, 5, 7	0.125	5, 5
25–50	0.50	3, 5, 7	0.25	6, 6
50–75	0.75	3, 5, 7	0.50	6, 6
75–125	1.00	3, 5, 7	0.50	5, 5
125–150	1.25	3, 5, 7	0.75	5, 5
150–175	1.50	3, 5, 7	1.00	6, 6
175–200	2.00	3, 5, 7	1.25	6, 6

[a]From References, 2.
[b]For standard containing 0.5 µg B_1 and G_2 and 0.1 µg B_2 and G_2 per mL.

Table 2
Alternative developing solvents for aflatoxins B_1, B_2, G_1, G_2.[a]

Toluene/methanol/acetic acid	90:5:5
Diethyl ether/methanol/water	96:3:1
Methylene chloride/chloroform/ n-amyl alcohol/formic acid (order of R_f changed to B_1, G_1, B_2, G_2)	80:15:4:1
Chloroform/acetone/water	88:12:1.5
Chloroform/acetone/isopropanol/water	88:12:1.5:1
Chloroform/isopropanol	99:1

[a]From References, 5.

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Oil in Corn Germ

DEFINITION

This method determines the amount of hexane soluble substances in corn germ that are extractable under the test conditions (References, 1).

SCOPE

This method is applicable to whole kernel corn or its milled component parts (see Notes, 1).

APPARATUS

1. Spex mixer/mill—model 8000 equipped with no. 8001 hardened steel cup for grinding and both 6 and 12 mm balls, or equivalent apparatus.

   Note—High oil content samples should be ground using a Brinkmann ZM-1, or equivalent; low oil samples should be ground using a UDY grinder, or equivalent.
2. Filter funnel—Gelman no. 4201 (47 mm) or no. 4230 (47 mm), or equivalent.
3. Filter paper—Whatman no. 7195-004 (a cellulose nitrate 5 µm filter), which is hexane compatible, or equivalent (see Notes, 2).

REAGENTS

1. Hexane—reagent grade or HPLC grade (see Notes, *Caution*).
2. Steam bath—contained in a well-ventilated hood area (see Notes, *Caution*).
3. A mechanical convection airflow oven.

PROCEDURE

1. Grind a 50–100 g laboratory sample to a uniform size (10 mesh) in a grinder or cutting mill (Apparatus, 1).
2. Make certain the laboratory sample is completely mixed, and then accurately weigh, to 0.0001 g, 4–5 g of the test portion into the Spex mill grinding cup.
3. Add one 12 mm and two 6 mm stainless steel balls and 25 mL of hexane to the grinding cup.
4. Seal the cup and mill for 15 min.
5. Place a filter paper in the filter funnel and filter the milled slurry into a dry, clean filtering flask.
6. Wash the residual oil from the Spex grinding cup and cap with three 10 mL portions of hexane.
7. Wash residual oil from the test portion in the funnel with another three 10 mL portions of hexane.
8. Transfer the filtered hexane and oil miscella into a dried, preweighed 150 mL beaker. Rinse the filter flask with a small portion of hexane and add to the extract in the 150 mL beaker.
9. Evaporate the hexane on a steam bath under the hood (see Notes, 3 and 4).
10. Place the beaker in a mechanical convection oven maintained at 100 ± 1°C for 1 hr ± 5 min (see Notes, 4 and 5).
11. Remove the beaker from the oven and transfer to a desiccator. When the beaker has cooled to ambient temperature, weigh the beaker and calculate the dry extract weight.

CALCULATIONS

1. Dry extract mass = (mass, g beaker + extract) − (mass, g beaker)
2. Percent oil (as is) = $\frac{\text{dry extract mass, g}}{\text{mass of test portion, g}} \times 100$

PRECISION

1. Reproducibility—Two single determinations performed in different laboratories should not differ by more that 1.1 percentage points.
2. Collaborative study results (References, 2) are shown in Table 1.

NOTES

Caution

Hexane is a flammable solvent and a dangerous fire risk. All work should be performed in a fume hood, with no open flames. Always ensure that your hood is functioning before introducing volatile solvents. The TLV for hexane is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

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NUMBERED NOTES

1. The material for extraction should be at or below equilibrium moisture. Wet materials should be dried by a method that does not result in fat oxidation.
2. The filter paper noted, or its equivalent, must be used.
3. Care must be taken when evaporating hexane to prevent violent boiling and loss of oil. The flask should be placed on top of the steam bath, so it is in contact with steam vapors, rather than being immersed in the boiling water of the steam bath. A stream of nitrogen may be used to facilitate the removal of hexane and to prevent the oxidation of the oil.
4. Make certain that no hexane remains in the test portion before placing in the oven.
5. Prolonged evaporation or drying of the extract at elevated temperatures may cause fat oxidation and high results. The flask should be heated in the convection oven at the temperature and time specified.

REFERENCES

1. Adopted with permission of the Corn Refiners Association, from *Standard Analytical Methods*, Corn Refiners Association, 6th edn., 1989, Method G-11.
2. *J. Am. Oil Chem. Soc. 66:*1272 (1989).

Table 1
Oil in corn germ collaborative study results[a].

				Oil Content		
Sample	Gluten	Fiber	Corn	Germ #1	Germ #2	Germ #3
1	2.630	2.910	3.450	46.300	44.080	38.550
2	3.050	2.930	3.360	46.950	44.980	39.090
3	2.580	2.920	3.450	46.940	44.730	39.220
4	2.500	3.150	3.810	46.370	43.610	38.640
5	3.210	3.030	3.710	46.280	43.970	38.000
6	3.030	3.210	3.560	46.720	45.130	38.210
7	2.800	2.900	3.500	46.700	44.500	38.900
8	2.800	3.000	3.400	46.600	44.500	39.000
9	3.040	3.020	3.330	46.920	44.160	39.510
10	2.522	3.090	3.420	46.570	44.740	38.700
11	2.550	2.890	3.450	46.840	44.620	38.760
12	2.830	3.030	3.340	46.410	44.200	37.820
13	2.620	2.660	3.330	46.170	45.080	38.210
14	2.760	3.080	3.420	47.020	44.100	38.690
15	2.780	3.160	3.780	46.450	44.670	38.460
Mean			3.49	46.63	44.47	38.65
SD			0.159	0.300	0.441	0.463
R (SD × 2.8)			0.44	0.84	1.23	1.30

[a]Single analysis per laboratory portion; only one lab portion analyzed per collaborator.

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Determination of Fumonisins B_1 and B_2 Corn and Corn-Based Food by Liquid Chromatography

DEFINITION

This method determines fumonisin B_1 and fumonisin B_2 metabolites of *Fusarium moniliforme* in corn and corn products by liquid chromatography (LC). The metabolites are extracted with methanol-water and the extract is added to a disposable strong anion exchange ("SAX") column. After the eluate is evaporated to dryness, the metabolites are derivatized with ortho-phthaldialdehyde and 2-mercaptoethanol to make highly fluorescent derivatives. Reverse-phase LC with fluorescence detection is used for the final separation and determination.

SCOPE

Applicable to corn and corn products, and may be used in the analysis of corn meal and breakfast cereals. For background information on fumonisins and further information on the method, see Notes, 1.

APPARATUS

1. Explosion-proof blender—Waring model EP-1 with 500 mL jar and cover.
2. Strong anion exchange column—Bond-Elut SAX, 3 mL capacity with 500 mg adsorbent (Analytichem, Harbor City, CA, USA).
3. Vacuum apparatus—solid-phase extraction manifold (Supelco, Bellefonte, PA, USA).
4. LC system—model 6000 A pump, U6K injector and μBondapak C18 column, 3.9 × 150 mm (Millipore-Waters, Milford, MA, USA); RF-535 fluorescence detector (Shimadzu Scientific Instruments, Columbia, MO, USA); and SP-4100 integrator (Spectra-Physics, San Jose, CA, USA).
5. Heating module—Reacti-Therm (Pierce Chemical Co., Rockford, IL, USA).

REAGENTS

1. Solvents—acetonitrile, methanol, and acetic acid (all ACS grade) (see Notes, *Caution*).
2. LC mobile phase—acetonitrile/water/acetic acid, 50:50:1, v/v/v.
3. Sodium tetraborate—ACS grade.
4. *ortho*-Phthaldialdehyde (OPA) and 2-mercaptoethanol (Aldrich Chemical Co., Inc., Milwaukee, WI, USA).
5. OPA reagent—Dissolve 40 mg OPA in 1 mL methanol and dilute with 5 mL 0.1 M sodium tetraborate. Add 50 μL 2-mercaptoethanol. Store in dark for <1 week.
6. FB_1 and FB_2 standards—available from Division of Food Science and Technology, CSIR, P.O. Box 395, Pretoria 00001, South Africa.
7. FB_1 and FB_2 standard solutions—Dissolve 1 mg FB_1 in 10 mL acetonitrile/water, 1:1, v/v; dissolve 1 mg FB_2 in 10 mL acetonitrile/water, 1:1, v/v.
8. Mixed standard solution—Transfer 1 mL FB_1 standard solution and 500 μL FB_2 standard solution to a suitable vial; add 8.5 mL acetonitrile/water, 1:1, v/v, to obtain mixed standard solution containing 10 ng FB_1 and 5 ng FB_2/μL.

PROCEDURE

1. Extraction and SAX column chromatography—
   (a) Blend 25 g ground corn with 50 mL methanol/water, 3:1, v/v, at high speed for 2 min. Filter mixture through fluted paper and collect 10 mL extract in graduated cylinder.
   (b) Condition SAX column with 2.5 mL (1 column volume) methanol/water, 3:1, v/v. Maintain flow rate of approximately 2 mL/min and do not allow column to run dry. Add 10 mL extract to column and wash column with 2.5 mL methanol. Change receiver to 16 mL vial and elute FB_1 and FB_2 with 14 mL methanol/acetic acid, 199:1, v/v.
   (c) Evaporate eluate to dryness under stream of nitrogen in heating module (Apparatus, 5) set at 80°C. Dissolve residue in 200 μL methanol to give test solution.
2. Derivatization and LC determination—
   (a) Place 50 μL mixed standard solution or test solution into bottom of 2 mL vial, add 200 μL OPA reagent, mix, and immediately inject 10 μL into liquid chromatograph. Use the following settings: excitation, 335 nm; emission, 440 mn; range, 1; flow rate, 1 mL/min. FB_1 elutes as a sharp peak in 4.9 min; FB_2 elutes in 10.9 min (see Figure 1).

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(b) Determine concentrations of FB_1 and FB_2 in test solution by comparing areas of chromatographic peaks obtained for test solution and standard solution. If peak areas obtained for test solution exceed those obtained for standard solution, dilute test solution and reinject.

CALCULATIONS

1. Calculate concentrations of FB_1 and FB_2 in corn or corn product (test sample) as follows:

$$\text{ng metabolite/g test portion} = \frac{(ABC)}{(DE)}$$

Where—

A = integrator counts from injection of test solution
B = concentration of metabolite (ng/μL) in standard solution injected (*i.e.,* 10 ng FB_1/μL or 5 ng FB_2/μL)
C = final volume of test solution (typically 200 μL)
D = integrator counts from injection of standard solution
E = weight of test portion represented by sample solution concentration (typically 5 g)

To convert ng/g to μg/g, divide by 1000.

PRECISION

1. Naturally occurring levels of FB_1 and FB_2 in control corn analyzed by the described method were found to be 0.23 and 0.06 μg/g, respectively. FB_1 and FB_2 standards were added to this corn to obtain test samples spiked at three levels. Duplicate test portions of these spiked test samples were analyzed, and percent recoveries were calculated after the values obtained for the control were subtracted from the levels found. The results are shown in Table 1 (see Notes, 2).

NOTES

Caution

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Methyl alcohol (methanol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform and perchloric acid.

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

NUMBERED NOTES

1. Background information: *Fusarium moniliforme,* one of the most common molds associated with corn, is known to produce a variety of mycotoxin metabolite, including the recently identified fumonisin B_1 (FB_1) and B_2 (FB_2) (References, 1). The fumonisins have been shown to cause the disease leukoencephalomalacia (LEM) in horses (References, 2) and porcine pulmonary edema (PPE) in swine (References, 3). *F. moniliforme* has been suggested as a cause of rickets-like deformities in chickens and of the increased incidence of human esophageal cancer in Transkei, South Africa and in Lin Xian, The People's Republic of China (References, 4). An increased incidence of LEM and PPE in all regions of the United States was associated with the 1989 corn crop. In most of the presently used methods for the determination of fumonisins in corn, the lower limit of quantitation is around 1 μg/g. To determine the occurrence of FB_1 and FB_2 in corn-based human foods, a method was needed with an even lower limit of quantitation. One liquid chromatographic method (References, 5) that gave satisfactory results was modified by the United States Food and Drug Administration (FDA), Natural Products and Instrumentation Branch, to make the procedure more rapid. The modified method was evaluated, using naturally contaminated corn and spiked controls. This method is based on the modified method described in the FDA Laboratory Information Bulletin (References, 6).
2. The average recoveries from all spiked test samples were 67% for FB_1 and 71% for FB_2. The relative standard deviations were 5% for FB_1 and 3% for FB_2. The method is rapid and easily performed. The estimated limit of quantitation for each metabolite is 10 above the background noise. In study (References, 6), at the limit of quantitation the signal-to-noise ratios are 5:1 for FB_1 and 2:1 for FB_2. The signal-to-noise ratio is higher for the quantitation of FB_1 because the FB_1 peak appears on top of a low shoulder of another peak, a component of the corn matrix, whereas the FB_2 peak appears in an area of the chromatogram where there is baseline resolution from the matrix.

REFERENCES

1. Gelderblom, W.C.A., K. Jaskiewicz, W.F.O. Marasas, P.G. Thiel, R.M. Horak, R. Vleggaar, and N.P.J. Kriek, *Appl. Environ. Microbiol. 54:*1806 (1988).
2. Ross, P.F., P.E. Nelson, J.L. Richard, G.D. Osweiler, L.G. Rice, R.D. Plattner, and T.M. Wilson, *Ibid. 56:*3225 (1990).

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3. Harrison, L.R., B.M. Colvin, J.T. Green, L.E. Newman, and J.R. Cole, *J. Vet. Diagn. Invest. 2:*217 (1990).
4. Sydenham, E.W., W.C.A. Gelderblom, P.G. Thiel, and W.F.O. Marasas, *J. Agric. Food Chem. 38:*285 (1990).
5. Shephard, G.S., E.W. Sydenham, P.G. Thiel, and W.C.A. Gelderblom, *J. Liq. Chromatogr. 13:*2077 (1990).
6. Stack, M.E., and Eppley, R.M., United States Food and Drug Administration, *Laboratory Information Bulletin,* Number 3651 (1992).

OTHER REFERENCES

1. Gay, M.L., M. Stack, J.A.G. Roach, K.D. White, and J.A. Sphon, *Laboratory Information Bulletin,* Number 3655, United States Food and Drug Administration, 1992.
2. Stack, M., and R.M. Eppley, *J. Assoc. Off. Anal. Chem. 75:*834 (1992).

Table 1
Recovery of fumonisins B_1 and B_2 added to corn.[a]

FB_1, µg/g				FB_2, µg/g			
Total added	Net found	FB_1 found	Recovery, %	Total added	Net found	FB_2 found	Recovery, %
0.0	0.23	0.00	—	0.0	0.05	0.00	—
0.0	0.23	0.00	—	0.0	0.07	0.00	—
0.5	0.55	0.32	64.0	0.25	0.23	0.17	67.1
0.5	0.59	0.36	71.5	0.25	0.24	0.18	72.9
1.0	0.86	0.63	63.2	0.5	0.42	0.36	72.6
1.0	0.87	0.64	64.4	0.5	0.42	0.36	72.6
2.0	1.58	1.35	67.4	1.0	0.76	0.70	70.0
2.0	1.66	1.43	71.5	1.0	0.79	0.73	73.3

[a]References, 6.

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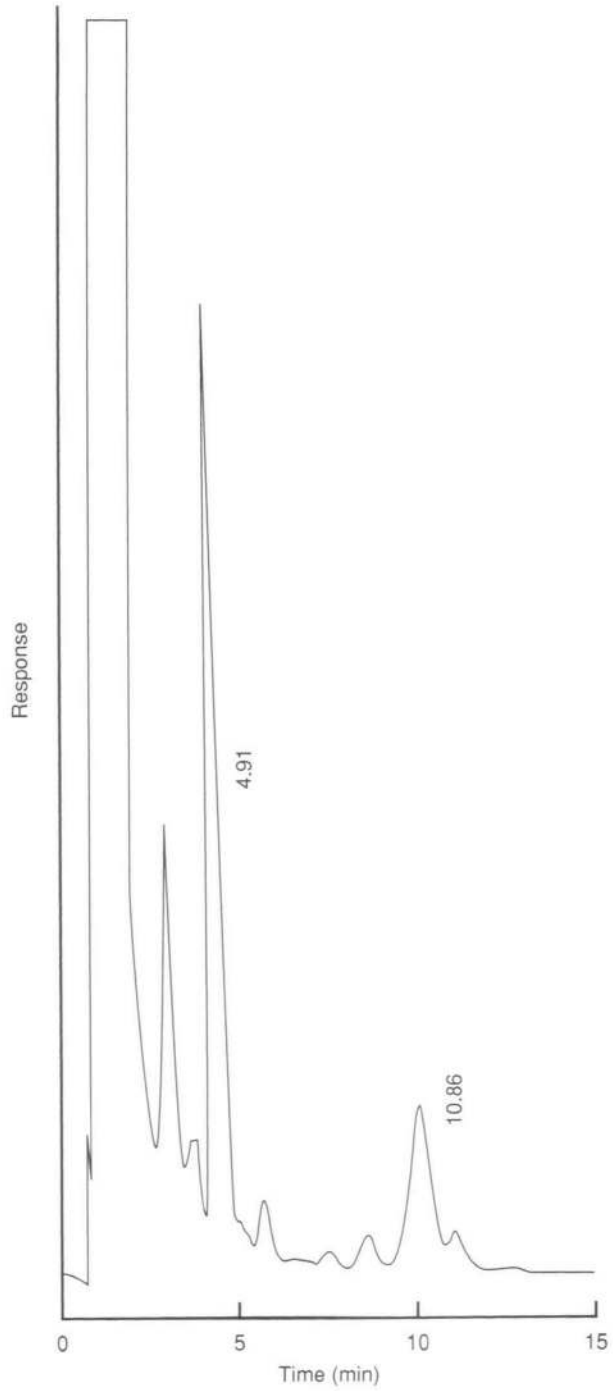

Figure 1. Liquid chromatogram of OPA derivatives of fumonisins B_1 (4.91 min, 138 ng/g) and B_2 (10.86 min, 62 ng/g) extracted from naturally contaminated grits. Mobile phase: acetonitrile/water/acetic acid, 50:50:1, v/v/v; flow rate: 1 mL/min; column: µBondapak C_{18}, 3.9 × 150 mm; fluorescence detector: 335 mn excitation and 440 mn emission.

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Determination of Fumonisins B_1, B_2, and B_3 in Corn by Liquid Chromatography

DEFINITION

This method determines fumonisin B_1, B_2, and B_3 in corn by liquid chromatography. Fumonisins are extracted from corn with methanol-H_2O and purified on a strong anion exchange (SAX) solid-phase extraction cartridge. Fumonisins are eluted with acetic acid-methanol. After evaporation, the residue is dissolved in methanol and reacted with *o*-phthaldialdehyde (OPA)/2-mercaptoethanol to form fluorescent fumonisin derivatives. Reversed-phase liquid chromatography with fluorescence detection is used for the final separation and determination.

SCOPE

Applicable for determination of total fumonisin B_1, B_2, and B_3 in corn at 800–12,800 ng/g.

APPARATUS

1. Tissue homogenizer or explosion-proof blender.
2. Solid-phase extraction (SPE) cartridges—10 mL capacity; containing 500 mg silica-based strong anion exchange (SAX) sorbent. Varian Bond Elut™ SAX Cartridge #12 21–3041 (Varian, Inc., Palo Alto, CA, USA) is suitable. Performance of individual batches of sorbent should be tested.
3. Solid phase extraction manifold and vacuum system.
4. Solvent evaporator—capable of operating at 60°C.
5. Liquid chromatograph (LC)—LC pump capable of delivering 1 mL/min constant flow rate, equipped with injection system calibrated to deliver 10 µL, and a 150 × 4.6 mm i.d. stainless steel column, packed with 5 µm octadecyl silica (ODS) C18 reversed-phase material. Use a suitable reversed-phase guard column and maintain LC column at ambient temperature (23°C).
6. Fluorescence detector—variable wavelength; set at 335 nm (excitation) and 440 nm (emission). Equipped with suitable recorder/integrator.
7. Membrane filter—porosity 0.45 µm.

REAGENTS

1. Methanol, acetonitrile, acetic acid, 2-mercaptoethanol, sodium tetraborate, *o*-phthaldialdehyde (Aldrich Chemical Co.) must be analytical grade, unless otherwise noted.
2. Phosphoric acid—concentration >85%.
3. LC mobile phase—methanol/0.1 M sodium dihydrogen phosphate (77:23, v/v), adjusted to apparent pH 3.3 with phosphoric acid. Filter mobile phase through membrane filter and pump at 1mL/min flow rate. (See Numbered Notes, 1.)
4. OPA reagent—Dissolve 40 mg OPA in 1 mL methanol, and dilute with 5 mL 0.1 M sodium tetraborate. Add 50 µL 2-mercaptoethanol, and mix. Store OPA reagent at room temperature in dark (capped amber or aluminum foil-covered vial). Reagents stable up to one week.
5. Fumonisin standard solutions—Prepare stock solution of individual fumonisin B_1, fumonisin B_2, and fumonisin B_3 at concentrations of 250 µg/mL in acetonitrile/water, 1:1, v/v. Transfer 100 µL aliquots of each stock solution to a clean glass vial and add 200 µL acetonitrile/water, 1:1, v/v, to yield standard working solution containing the three fumonisin analogs at individual concentrations of 50 µg/mL. Fumonisins stock and working standard solutions may be stable up to 6 months when stored at 4°C.

PROCEDURE

1. Extraction and SAX column chromatography—
   (a) Homogenize 50 g test sample (ground so that ca. 90% is retained between 500–250 µm mesh screens) in 100 mL methanol/water, 3:1, v/v, for 3 min at 60% full speed setting. Alternatively, blend at the same speed settings for 5 min to allow for complete extraction.
   (b) Centrifuge mixture 10 min at 500 × g and filter supernatant through fluted filter paper. Filtrate should have apparent pH ca 5.8. If necessary, adjust pH to 5.8–6.5 with 1 M NaOH (only 2–3 drops should be required).
   (c) Condition SAX cartridge in SPE manifold by washing successively with 5 mL methanol, followed by 5 mL methanol/water, 3:1, v/v, followed by 5 mL methanol. Do not allow cartridge to run dry. Apply 10 mL extract to column while maintaining flow rate at approximately 2 mL/min. Wash column with 5 mL methanol/water, 3:1, v/v, followed by

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3 mL methanol. Elute fumonisins with 10 mL acetic acid-methanol, 1:99, v/v, at flow rate <1 mL/min. (*Note*: It is critical that flow rate does not exceed 1 mL/min.) Collect eluate in 20 mL capacity glass vial.

(d) Evaporate solvent to dryness under stream of nitrogen at 60°C, in small vial. Rinse collection vial with 1 mL methanol and add rinsing solvent to small vial, washing sides of vial to concentrate residue at its base. Evaporate additional methanol to dryness to ensure that all acetic acid has evaporated. Dried test portion residues may be retained up to 1 week at 4°C prior to LC analysis. For analysis dissolve dried test portion in 200 µL methanol.

2. Derivatization and LC determination

(a) Preparation of standard derivative—Transfer 25 µL fumonisin standard solution or test solution, to base of small vial. Add 225 µL OPA reagent, mix, and immediately inject 10 µL into LC system. (See Numbered Notes 2, 3.)

(b) Determine concentrations of individual fumonisins by comparison of retention times in extracts with those observed for individual fumonisin standards. If peak areas obtained for test samples exceed those of fumonisin standards, dilute sample extracts with methanol and repeat derivatization with OPA reagent.

CALCULATIONS

Calculate fumonisin present in aliquot F (ng) injected into LC system using peak areas for each fumonisin analog, as follows:

$$F\ (ng) = \left(\frac{P_u}{P_f}\right) \times S$$

Where—

P_u = individual fumonisin peak area of test portion
P_f = individual fumonisin peak area of standard solution
S = amount of individual fumonisin standard injected into LC system; 50 ng/fumonisin analog [based on concentration in fumonisin standard working solution, C(I).]

Calculate concentration of fumonisin present in corn C (ng/g), as follows:

$$C\ (ng/g) = \frac{F \times V_t \times D}{V_i \times W}$$

Where—

V_t = total column of derivatized solution, 250 µL
D = any dilution factor which may have been used
V_i = injection volume, 10 µL
W = sample equivalent weight derivatized, 0.625 g

NOTES

Caution

Fumonisins are hepatotoxic and carcinogenic to rats; effects on humans are not fully known. Wear protective gloves to reduce skin contact with corn extracts. Any laboratory spillages should be washed with a 5% aqueous solution of commercial sodium hypocholorite followed by H_2O. (Dispose of waste solvents according to applicable environmental rules and regulations.)

NUMBERED NOTES

1. LC mobile phase composition may have to be adjusted to conform with individual LC column characteristics.
2. It is critical to adhere to reproducible times between the addition of OPA reagent and injection into the LC system, because fluorescence of OPA-fumonisin progressively decays after 2 min.
3. Detector and recorder response—Adjust sensitivity settings of fluorescence detector, so fumonisin B_1 standard-OPA derivative yields at least 80% recorder response.

REFERENCES

1. *Official Methods of Analysis,* AOAC International, 17th ed., Vol. II, Gaithersburg, MD, 2000, Chapt. 49, p. 44, Method 995.15.

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PRECISION

Table 1
Values taken from AOAC International report on this method.

Fumonisin in analog	Amount added, ng/g	Mean, ng/g	Recovery, %	S_r	S_R	RSD_r, %	RSD_R, %	r[a]	R[b]
B_1	500	405	81.1	28.8	56.2	7.1	13.9	80.6	157.4
	1000	813	81.3	47.4	127.4	5.8	15.7	132.7	356.7
	2000	1621	81.1	124.7	261.1	7.7	16.1	349.2	731.1
	4000	3245	81.1	199.7	494.1	6.2	15.2	559.2	1383.5
	8000	6732	84.2	736.6	1099.7	10.9	16.3	2062.5	3079.2
	NB_{1c}	4246	—	561.7	944.1	13.2	22.2	1572.8	2643.5
B_2	200	152	75.9	12.7	24.7	8.4	16.3	35.6	69.2
	400	313	78.3	26.6	49.4	8.5	15.8	74.5	138.3
	800	618	77.3	73.4	119.7	11.9	19.3	205.5	335.2
	1600	1294	80.9	93.1	222.3	7.2	17.2	260.7	622.4
	3200	2619	81.9	318.4	467.7	12.2	17.9	891.5	1309.6
	NB_{2d}	1234	—	215.4	329.4	17.5	26.7	603.1	922.3
B_3	100	76	75.8	7.7	14.9	10.1	19.6	21.6	41.7
	200	161	80.7	27.7	35.3	17.2	21.9	77.6	98.8
	400	319	79.7	48.9	71.4	15.4	22.4	136.9	199.9
	800	656	82	52.7	138.8	8	21.2	147.6	388.6
	1600	1389	86.6	198.3	270.9	14.3	19.5	554.4	758.5
	NB_{3e}	373	—	63.2	92.7	16.9	24.9	177	259.6

[a] $r = 2.8 \times S_r$
[b] $R = 2.8 \times S_R$
[c] NB_1 = corn naturally contaminated with fumonisin B_1 at a mean concentration of 4246 ng/g.
[d] NB_2 = corn naturally contaminated with fumonisin B_2 at a mean concentration of 1234 ng/g.
[e] NB_3 = corn naturally contaminated with fumonisin B_3 at a mean concentration of 373 ng/g.

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AOCS

AOCS Standard Procedure Aj 6-95

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Total Aflatoxins (B_1, B_2, and G_1) in Corn, Cottonseed, Peanuts, and Peanut Butter

DEFINITION

This method specifies a screening procedure based on an enzyme-linked immunosorbent screening assay (ELISA) for the detection of aflatoxins B_1, B_2, and G_1. The method is based on an international collaborative study conducted jointly by AOAC and IUPAC.

SCOPE

Applicable to screening aflatoxin B_1, B_2, and G_1 contamination in whole cottonseed and peanut butter at ≥20 ng/g and in corn and canned peanuts at ≥30 ng/g. If a false negative rate of <5% is required, perform duplicate ELISA on the same test sample extract. Re-analyze positive samples by official, quantitative method.

Antibodies specific to aflatoxins B_1, B_2, and G_1 are immobilized on a filter, and toxin (aflatoxin B_1) is labeled with an enzyme (horseradish peroxidase). Binding of toxin-enzyme conjugate by immobilized antibodies is inhibited by addition of free toxin present in the test sample. Since a fixed number of antibody reaction sites are available, enzyme activity is proportional to the amount of bound toxin-enzyme conjugate. Antibody-toxin-enzyme complex concentration is inversely proportional to the concentration of free toxin added. Bound enzyme catalyzes oxidation of substrate to form a blue complex. Development of color indicates that the test sample contains aflatoxins at <20 ng/g; no color development indicates that the test sample contains aflatoxins ≥20 ng/g.

REAGENTS

Items 1–10 are available as ImmunoDot Screen (IDS) Cup (International Diagnostic Systems, Inc., P.O. Box 799, St. Joseph, MI, 99085, USA). Reagents from other suppliers can be used provided that the requirements listed are met.

1. Antibody-coated solid support—aflatoxin antibody-coated porous polyethylene membrane, over layer of adsorbent material; support device consisting of high-density polyethylene housing (3.2 cm diameter × 2.5 cm height, capacity 4 mL). Coated cup is specified by manufacturer to be stable for 6 months stored at 4-8°C.
2. Aflatoxin-enzyme conjugate—Aflatoxin B_1-horseradish peroxidase conjugate at toxin-enzyme molar ratio of 10–15:1. Conjugate is specified by manufacturer to be stable for 6 months at 4-8°C.
3. Wash solution—Phosphate-buffered saline solution. Dissolve 0.23 g $NaH_2PO_4 \cdot H_2O$, 1.95 g $K_2HPO_4 \cdot 3H_2O$, 8.70 g NaCl, 0.125 mL Tween 20 (polyoxyethylene[20]sorbitan monolaurate), and 10 mg thimerosal (ethylmercurithiosalicylic acid, Na salt) in 900 mL H_2O; adjust pH to 7.2 and dilute to 1 L.
4. Buffer—0.1% bovine serum albumin in phosphate-buffered saline solution containing 0.05% thimerosal.
5. Substrate solution A—tetramethylbenzidine (TMB) (0.4 g/L H_2O), pH 8.3.
6. Substrate solution B—hydrogen peroxide (0.02% H_2O_2 in 0.13% aqueous citric acid solution), pH 3.0 (Kirkegaard and Perry Laboratories, Inc., 2 Cessna Ct., Gaithersburg, MD 20879).
7. Methanol—reagent grade.
8. Hexane—reagent grade.
9. Chloroform—reagent grade.
10. Standard aflatoxin B_1—approximately 28 µg as dry film.

APPARATUS

Equipment specified is not restrictive; other suitable equipment can be substituted.

1. High-speed blender—with 500 mL jar.
2. Micropipet and tips—recommended range 100–1000 µL; use with disposable polypropylene tips
3. Glass culture (test) tubes—10 × 75 mm; 3 mL.
4. Filters—Whatman No. 4, or equivalent.
5. Timer—graduated in 1 s intervals.
6. Silicon carbide boiling chips—14 mesh.

ANTIBODY SPECIFICITY

Cross-reactivities of polyclonal antibody used in the collaborative study of this method were 100, 70, 75 and <10% for aflatoxins B_1, B_2, G_1, and G_2, respectively.

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SENSITIVITY OF ELISA REAGENT

Calibrate aflatoxin B_1 standard according to AOCS Official Method Aa 10–88.

1. Negative control test sample—Follow procedure in enzyme immunoassay for corn.
2. Threshold-level standard—Used to define lower limit of determination. Dispense 100 µL working standard into test tube. Add 350 µL methanol-buffer (30 + 70), and mix. Follow the procedure in enzyme immunoassay for corn.
3. Positive control test sample—Use working standard solution; follow the procedure for enzyme immunoassay for corn.
4. Negative control test sample should develop a blue color; positive control test sample should have no color development. Threshold standard should show no color development.

GENERAL INSTRUCTIONS

Store all kit components at 4–8°C. Do not freeze. Before use, allow 1 h for cups and reagents to reach room temperature (23–29°C). Use a separate disposable pipet tip for each test solution to avoid cross-contamination. Include one negative control with each group (20 cups) of test samples. Negative control must be functioning properly (must develop a blue color in the center of the cup) for the test to be valid. A positive standard is provided for periodic checking or for use with each group of test samples (must show no color in the center of the cup). The threshold-level standard should also be used and must show no color development. If color develops, repeat the test. Color development in more than 2 tests indicates a defective kit.

Reagents are stable 6 h at room temperature. To ensure shelf life of kit components, promptly refrigerate reagents after use.

Because of difficulty in monitoring 1 min timing intervals, run 1 cup at a time. As proficiency is gained, analysts can run 3 cups successively spaced at convenient time intervals for making observations.

EXTRACTION OF TEST SAMPLE

Note: Test sample extraction and cup testing should be performed during the same day.

1. Corn, peanuts, and whole cottonseed—Weigh 50 g test portion into blender jar. Add 100 mL CH_3OH-H_2O (8 + 2). Blend 3 min at high speed. Filter mixture and recover filtrate. Alternatively, let mixture stand 10–15 min and recover supernatant liquid. *Note:* To obtain results applicable to whole cottonseed when analysis is performed on ground cottonseed meal (about 50% by weight of whole cottonseed), dilute extract in ratio 1:1 with extraction solvent, CH_3OH-H_2O (8 + 2).
2. Peanut butter—Weigh a 50 g test portion into a blender jar. Mix with 250 mL of 55% methanol and 45% water. Add 100 mL hexane and blend for 1 min at high speed. Filter mixture and recover filtrate. Leave for 5 min and remove the lower phase containing methanol-water. Centrifugation may be used to speed up this process.

PREPARATION OF AFLATOXIN B_1 STANDARD SOLUTIONS

1. Stock solution—Dissolve 25 µg dry film aflatoxin B_1 in 2.5 mL methanol (10 ng/µL). Store below –20°C. In absence of acid or base, solution is stable for ca. 6 months in clean container.
2. Working standard—Dispense 250 µL stock solution into clean vial. Add 2250 µL methanol and mix. May be stored 1 month at ca. 5°C (1 ng/µL).
3. Buffer solution of working standard—Prepare <2 h before use. Dispense 5 µL working standard into test tube. Add 300 µL methanol and 700 µL buffer, and agitate vigorously. Proceed as for diluted test extract.

ENZYME IMMUNOASSAY

1. Allow 1 h for all reagents to reach room temperature (20–23°C).
2. Prepare fresh substrate in small culture (test) tubes by mixing 500 µL (10 drops) substrate solution A with 500 µL (10 drops) substrate solution B for each reaction site used. Do not combine substrate solution A with substrate solution B more than 15 min before use.

   Note: Run 1 negative control cup and 1 positive standard cup each day to ensure that all reagents are functional. Threshold-level standard should be run with each set of new reagents [follow steps 4–7]. The negative control cup should be run by applying 100 µL (2 drops) buffer to the center of the cup. For the positive standard cup, apply the working standard to the cup as in 4. For both cups proceed with steps 5–7.
3. Add 100 µL test extract to 200 µL buffer (total volume, 300 µL).
4. Thoroughly mix the diluted test extract and apply 100 µL diluted test extract to center of membrane. Using timer, wait 1 minute.
5. Apply 100 µL (2 drops) enzyme solution to the center of membrane. Using the timer, wait 1 min.
6. Wash with 1.5 mL (30 drops) wash solution added dropwise. If more than 1 reaction site is used, wash successively with 500 µL (10 drops) per cup 3 times.
7. Add the entire contents of the substrate solution 1.0 mL [20 drops Step 2] from each test tube to each reaction site. (Start the timer as soon as the substrate mixture is added to the reaction site.) Wait 1 min and immediately observe site (center of cup) for blue color development (negative) or no color development (positive) (see Interpretation of Results).

INTERPRETATION OF RESULTS

Observe the reaction site (center of the cup) for a blue color or no color development at exactly 1 min after adding the substrate A and B mixture.

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Negative—If the reaction site (center of the cup) turns light blue or darker, test sample contains total aflatoxins B_1, B_2 and G_1 at <20 ng/g.

Positive—If no blue color is observed in the reaction site (center of cup) and reaction site remains completely white (no color change) for at least 1 min, the test sample contains total aflatoxins B_1, B_2 and G_1 at ≥20 ng/g.

Negative control—Negative control cup must develop a blue color in the center of the cup.

Positive control standard—Positive standard cup must remain completely white (no color change) for at least 1 min.

Threshold-level standard—Cup must remain completely white (no color change) for 1 min.

Note: Development at 1 min of any blue color, however faint, means aflatoxin levels are <20 ppb (negative). If a false negative rate <5% is required, duplicate ELISA must be performed on the test sample extract.

CONFIDENCE INTERVALS

The 95% confidence intervals for correct identification of corn samples testing positive for aflatoxin contamination at 20 ng/g level were 83–100% in the collaborative study. See Table 1 for method performance data. Other matrices are described in Table 2.

REFERENCES

1. *JAOAC 72*:957 (1989).
2. *Official Methods of Analysis,* AOAC International, 17th ed., Vol. II, Gaithersburg, MD, 2000, Chapt. 49, p. 15, Method 993.16.
3. *Official Methods of Analysis,* AOAC International, 17th ed., Vol. II, Gaithersburg, MD, 2000, Chapt. 49, p. 7, Methods 990.34.

Table 1
Method performance for immunosorbent assay method for aflatoxins B_1, B_2, and G_1 in corn.

Total Aflatoxins ng/g	Positive Assays at Each Level	% Positive	Lower Bound[a]	Upper Bound[a]	Sensitivity Rate, %	Specificity Rate, %
0	1	3.3	0.1	17.2	—	96.7
10	20	66.7	47.2	82.7	66.7	—
20	29	96.7	82.8	99.9	96.7	—
30	30	100.0	90.5	100.0	100.0	—
NC[b]	30	100.0	90.5	100.0	100.0	—

[a]Lower and upper bounds of % positive assays.
[b]NC = naturally contaminated at 23 ng/g.

Table 2
95% Confidence intervals for correct identification of samples positive for aflatoxin contamination.

Sample	Level, ng/g	95% Conf. Interval, %
Cottonseed	20	79–100
Peanut butter	20	72–99
Raw peanuts	30	78–100

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AOCS

AOCS Standard Procedure Aj 6a-97

Reapproved 2009

Determination of Aflatoxins B_1, B_2, and G_1 in Corn by ELISA

DEFINITION

This method specifies a screening procedure based on an enzyme-linked immunosorbent screening assay (ELISA) for the detection of aflatoxins B_1, B_2, and G_1. The method is based on study conducted by AOAC International.

SCOPE

Applicable to screening aflatoxin B_1, B_2, and G_1 contamination in corn at ≥20 ng/g. If a false negative rate of <5% is required, perform duplicate ELISA on the same test sample extract. Re-analyze positive samples by official, quantitative method.

Antibodies specific to aflatoxins B_1, B_2, and G_1 are immobilized on a filter, and toxin (aflatoxin B_1) is labeled with an enzyme (horseradish peroxidase). Binding of toxin-enzyme conjugate by immobilized antibodies is inhibited by addition of free toxin present in the test sample. Since a fixed number of antibody reaction sites are available, enzyme activity is proportional to the amount of bound toxin-enzyme conjugate. Antibody-toxin-enzyme complex concentration is inversely proportional to the concentration of free toxin added. Bound enzyme catalyzes oxidation of substrate to form a blue complex. Development of color indicates that the test sample contains aflatoxins at <20 ng/g; no color development indicates that the test sample contains aflatoxins ≥20 ng/g.

APPARATUS

Equipment specified is not restrictive; other suitable equipment can be substituted.

1. High-speed blender—with 500 mL jar.
2. Micropipet and tips—recommended range 100–1000 µL; use with disposable polypropylene tips.
3. Glass culture (test) tubes—10 × 75 mm; 3 mL.
4. Filters—Whatman No. 4, or equivalent.
5. Timer—graduated in 1 s intervals.
6. Silicon carbide boiling chips—14 mesh.

REAGENTS

Items below are available as ImmunoDot Screen (IDS) Cup, (International Diagnostic Systems, Inc., St. Joseph, MI, USA). Reagents from other suppliers can be used provided that the requirements listed are met.

1. Antibody-coated solid support—aflatoxin antibody-coated porous polyethylene membrane, with 0.71 cm diameter opening, over layer of adsorbent material; support device consisting of high-density polyethylene housing (3.4 cm diameter × 2.65 cm height) with centered coneshaped ring insert (3.09 cm diameter × 0.9 cm height).
2. Aflatoxin-enzyme conjugate—aflatoxin B_1-horseradish peroxidase conjugate at toxin-enzyme molar ratio of 10-15:1. Conjugate is specified by manufacturer to be stable for 6 months at 4–8°C.
3. Wash solution—Phosphate-buffered saline solution. Dissolve 0.23 g $NaH_2PO_4 \cdot H_2O$, 1.95 g $K_2HPO_4 \cdot 3H_2O$, 8.70 g NaCl, 0.125 mL Tween 20 (polyoxyethylene[20]sorbitan monolaurate), and 10 mg thimerosal (ethylmercurithiosalicylic acid, Na salt), in 900 mL H_2O, adjust pH to 7.2, and dilute to 1 L.
4. Buffer—0.1% bovine serum albumin in phosphate-buffered saline solution containing 0.05% thimerosal.
5. Substrate solution A—Tetramethylbenzidine (TMB) (0.4 g/L H_2O), pH 8.3.
6. Substrate solution B—Hydrogen peroxide (0.02% H_2O_2 in 0.13% aqueous citric acid solution), pH 3.0 (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA).
7. Methanol—reagent grade.
8. Hexane—reagent grade.
9. Chloroform—reagent grade.
10. Standard aflatoxin B_1—approximately 28 µg as dry film.

SPECIFICITY OF ANTIBODIES

Antibodies have the specific ability to bind structurally related compounds, namely, aflatoxins B_1, B_2, and G_1. Determine the specificity of purified rabbit anti-aflatoxin B_1, polyclonal antibodies by direct competitive ELISA method. Coat serially diluted antibodies on microtiter plates. Prepare standard solutions of aflatoxins B_1, B_2, G_1, G_2, and M_1; zearalenone; T-2 toxin; and deoxynivalenol, and add to individual microtiter wells. Then add a solution of aflatoxin B_1 conjugated to horseradish peroxidase

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to each well. Add a substrate solution of tetramethylbenzidine and hydrogen peroxide, and measure the development of color with a scanner. The lowest color development indicates highest reactivity of toxin-antibody reaction. Cross-reactivity to aflatoxin B_1 for the antibody used in the collaborative study of this method was 100, 70, 75, and <10% for aflatoxins B_1, B_2, G_1, and G_2, respectively. All other toxins tested showed no cross-reactivity.

SENSITIVITY OF ELISA REAGENT

Calibrate aflatoxin B_1 standard according to AOCS Official Method Aa 10–88.

1. Negative control test sample—Follow procedure in enzyme immunoassay for corn.
2. Threshold-level standard—Use to define lower limit of determination. Dispense 100 µL working solution into test tube. Add 50 µL methanol-buffer (30 + 70), and mix. Follow the procedure in enzyme immunoassay (steps 4–7 under Enzyme Immunoassay).
3. Positive control test sample—Use working standard solution; follow the procedure for enzyme immunoassay (steps 4–7 under Enzyme Immunoassay).
4. Negative control test sample should develop a blue color; positive control test sample should have no color development. Threshold standard should show no color development.

GENERAL INSTRUCTIONS

Store all kit components at 4–8°C. Do not freeze. Before use, allow 1 h for cups and reagents to reach room temperature (23–29°C). Use a separate disposable pipet tip for each test solution to avoid cross-contamination. Include one negative control with each group (20 cups) of test samples. Negative control must be functioning properly (must develop a blue color in the center of the cup) for the test to be valid. A positive standard is provided for periodic checking or for use with each group of test samples (must show no color in the center of the cup). The threshold-level standard should also be used and must show no color development. If color develops, repeat the test. Color development in more than 2 tests indicates a defective kit.

Reagents are stable 6 h at room temperature. To ensure shelf life of kit components, promptly refrigerate reagents after use.

Because of difficulty in monitoring 1 min timing intervals, run 1 cup at a time. As proficiency is gained, analysts can run 3 cups successively spaced at convenient time intervals for making observations.

EXTRACTION OF TEST SAMPLE

Note: Test sample extraction and cup testing should be performed during the same day.

1. Weigh 50 g test portion into blender jar. Add 100 mL CH_3OH-H_2O (8 + 2). Blend 3 min at high speed. Filter mixture and recover filtrate. Alternatively, let mixture stand 10–15 min and recover supernatant liquid.

PREPARATION OF AFLATOXIN B_1 STANDARD SOLUTIONS

1. Stock solution—Add 3 mL methanol to a vial containing 28 µg aflatoxin B_1 standard (ca. 9 ng/µL). Cap vial, mix contents, and store vial in refrigerator.
2. Working solution—Dispense 300 µL stock standard solution into vial. Add 2400 µL methanol (1 ng/µL), mix, and store solution in refrigerator. Prepare daily. Dispense 10 µL diluted standard (1 ng/µL) into test tube. Add 300 µL methanol and 700 µL buffer. Prepare ≤2 h before use.

ENZYME IMMUNOASSAY

1. Allow 1 h for all reagents to reach room temperature (23–29°C).
2. Prepare fresh substrate in small culture (test) tubes by mixing 500 µL (10 drops) substrate solution A (Reagents, 5) with 500 µL (10 drops) substrate solution B (Reagents, 6) for each cup being used. Do not combine substrate solution A with substrate solution B more than 15 min before use.

   Note: Run 1 negative control cup and 1 positive standard cup each day to ensure that all reagents are functional. Threshold-level standard should be run with each set of new reagents [follow steps 4–7]. The negative control cup should be run by applying 100 µL (2 drops) buffer to the center of the cup. For the positive standard cup, apply 100 µL of the working solution to the cup as in step 4. For both cups proceed with steps 5–7.
3. Add 100 µL test extract to 200 µL buffer (300 µL total).
4. Thoroughly mix the diluted test extract and apply one 100 µL aliquot to the center of each cup. Using the timer, wait 1 min.
5. Apply 100 µL (2 drops) enzyme solution (Reagents, 2) to the center of each cup. Using the timer, wait 1 min.
6. Wash with 1.5 mL (30 drops) wash solution (Reagents, 3) added dropwise. If more than 1 cup is being used, wash successively with 500 µL (10 drops) per cup 3 times.
7. Add the entire contents of the substrate solution 1.0 mL (Enzyme Immunoassay, 2) from each test tube to each cup. (Start the timer as soon as the substrate mixture is added to the cup.) Wait 1 min and immediately observe the disk (center of cup) for blue color development (negative) or no color development (positive) (see Interpretation of Results).

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INTERPRETATION OF RESULTS

Observe the disk (center of the cup) for a blue color or no color development at exactly 1 min after adding the substrate A and B mixture.

Negative—If the disk (center of the cup) turns light blue or darker, test sample contains total aflatoxins B_1, B_2, and G_1 at <20 ng/g.

Positive—If no blue color is observed in the disk (center of cup) and disk remains completely white (no color change) for at least 1 min, the test sample contains total aflatoxins B_1, B_2, and G_1 at ≥20 ng/g.

Negative control—Negative control cup must develop a blue color in the center of the cup.

Positive control standard—Positive standard cup must remain completely white (no color change) for at least 1 min.

Threshold-level standard—Cup must remain completely white (no color change) for 1 min.

Note: Development at 1 min of any blue color, however faint, means aflatoxin levels are <20 ppb (negative). False negative rate may be higher at this cutoff level. If a false negative rate <5% is required, duplicate ELISA must be performed on the test sample extract; otherwise, <30 ppb is considered negative for these commodities.

REFERENCES

1. *JAOAC Int. 77:*655 (1994).
2. *Official Methods of Analysis,* 17th ed., Vol. II, AOAC International, Gaithersburg, MD, 2000, Chapt. 49, p. 15, Method 993.16.

METHOD PERFORMANCE

Table 1

Method performance in immunosorbent assay method for aflatoxins B_1, B_2, and G_1 in corn.

Total aflatoxins ng/g	Positive assays at each level	% Positive	Lower bound[a]	Upper bound[a]	Sensitivity rate, %	Specificity rate, %
0	1	3.3	0.1	17.2	—	96.7
10	20	66.7	47.2	82.7	66.7	—
20	29	96.7	82.8	99.9	96.7	—
30	30	100.0	90.5	100.0	100.0	—
NC[b]	30	100.0	90.5	100.0	100.0	—

[a]Lower and upper bounds of % positive assays.
[b]NC = naturally contaminated at 23 ng/g.

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AOCS Official Method Ak 1-92

Revised 2011

Determination of Glucosinolate Content in Rapeseed and Canola by HPLC

DEFINITION

This method, adopted from Part 1 of ISO 9167 (References, 1), specifies a procedure for the determination of the content of different glucosinolates in rapeseed (colza) by extraction of glucosinolates in a methanol solution, then purification and enzymatic desulfation on ion-exchange resins, followed by determination using reversed-phase high-performance liquid chromatography (HPLC) with gradient elution and ultraviolet detection.

SCOPE

This method does not determine glucosinolates that are substituted on the glucose molecule, but these compounds are of little importance in commercial rapeseed.

APPARATUS

Usual laboratory apparatus and, in particular, the following:

1. High-performance liquid chromatograph—suitable for obtaining an elution gradient and control of the temperature of the column at 30°C, connected to an ultraviolet detector permitting measurements to be made at a wave-length of 229 nm.
2. Chromatography column for HPLC—type C18 or C8, of particle size ≤ 5 µm, for example:
   Lichrosorb RP 18 Column, ≤5 µm (150 mm × 4.6 mm)
   Spherisorb ODS2 Column, ≤ 5 µm (250 mm × 4 mm; 250 mm × 5 mm)
   Novapak C18 Column, 4 µm (150 mm × 4 mm)
   Lichrospher RP8 Column, ≤5 µm (125 mm × 4 mm)
   Nucleosil C18 Column, ≤ 5 µm (200 mm × 4 mm)
   The performance of the column should be checked regularly, preferably using a reference sample of rapeseed glucosinolate (see Notes, 1). In particular, the column shall not degrade 4-hydroxyglucobrassicin, an important but relatively unstable glucosinolate.
   New columns shall be subjected to preliminary conditioning in accordance with the manufacturer's instructions so that reproducible results can be obtained.
3. Double-beam spectrometer—capable of operating in the ultraviolet region of the spectrum and at a controlled temperature of 30°C, equipped with quartz cuvettes 1 cm thick and a recording system.
4. Microgrinder—for example, a coffee mill.
5. Centrifuge—suitable for use with tubes (Apparatus, 6), capable of obtaining a centrifugal acceleration of 5000 × *g*.
6. Polypropylene tubes—6 mL capacity.
7. Water bath, or other heating apparatus—adjustable to 75°C.
8. Glass wool.
9. Pasteur pipets—150 mm long, with suitable stand.

REAGENTS

All the reagents shall be of recognized analytical grade unless otherwise specified. The water used should be suitable for use with an HPLC system.

1. Methanol/water—70% solution, v/v.
2. Sodium acetate—0.02 M at pH 4.0.
3. Sodium acetate—0.2 M solution.
4. Imidazole formate—6 M solution, prepared by dissolving 204 g of imidazole in 113 mL of formic acid in a 500 mL volumetric flask and diluting to the mark with water.
5. Internal standard—use either sinigrin monohydrate (potassium allylglucosinolate monohydrate, M = 415.49) (see Reagents, 6), or for rapeseed (cultivated or self-propagated) in which sinigrin is present naturally, glucotropaeolin (benzylglucosinolate, potassium salt, M = 447.52) (see Reagents, 10).
6. Sinigrin monohydrate.
7. Sinigrin monohydrate—5 mM solution, prepared by dissolving 207.7 mg of potassium allylglucosinolate monohydrate in water in a 100 mL volumetric flask and diluting to the mark with water. The solution thus prepared may be stored in a refrigerator at approximately 4°C for up to a week, or in a freezer at –18°C for a longer period.

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8. Sinigrin monohydrate—20 mM solution, prepared by dissolving 831.0 mg of potassium allylglucosinolate monohydrate in water in a 100 mL volumetric flask and diluting to the mark with water. The solution thus prepared may be stored in a refrigerator at approximately 4°C for up to a week, or in a freezer at –18°C for a longer period.
9. For a purity check of the sinigrin monohydrate standards (Reagents, 7 and 8), use one or more of the following three tests:
   (a) HPLC analysis using the method specified in Procedure, 11;
   (b) analysis of the intact sinigrin by HPLC (ion-pair technique);
   (c) analysis of the desulfated and silylated sinigrin by gas chromatography.

   For each test, the chromatogram shall show only one major peak representing at least 98% of the total peak area.

   Confirm the purity by determining the quantity of glucose released after hydrolysis with myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) (cat. no.T4528; Sigma Chemical Co., St. Louis, MO, USA). Measure the glucose by enzymatic means using a commercially available test kit. Take into account any free glucose present; this is determined in the same way, but without addition of myrosinase. The molar concentration of glucose measured should be at least 98% of the molar concentration of the sinigrin solution tested.
10. Glucotropaeolin—see Notes, 2.
11. Glucotropaeolin—5 mM solution.
12. Glucotropaeolin—20 mM solution.
13. For a purity check of the glucotropaeolin standards (Reagents, 11 and 12), use one or more of the checks noted in Reagents, 9.
14. Response factors—Verify that the response factors of glucotropaeolin, in comparison with sinigrin, correspond to those indicated in Notes, 3.
15. Mobile phases—
   (a) Eluant A—water purified by passing it through an activated charcoal cartridge (e.g., Norganic Millipore system), or water of equivalent purity.
   (b) Eluant B—acetonitrile, HPLC grade, 20% solution, v/v, in purified water. The concentration may be modified in relation to the column used.
16. Ion-exchange resin—use *either* 16 (a) *or* 16 (b):
   (a) DEAE Sepharose C1-6B—suspension available commercially ready for use.
   (b) DEAE Sephadex A25—suspension prepared as follows. Mix 10 g of DEAE Sephadex A25 resin in excess 2 M acetic acid solution. Allow to settle. Add 2 M acetic acid until the volume of the liquid is equal to twice the volume of the sediment.
17. Sulfatase—*Helix pomatia* type H1 (EC 3.1.6.1) (cat. no. S9626; Sigma Chemical Co.), having an activity of >0.5 units of activity per mL of purified sulfatase solution. Purify, test and dilute the sulfatase in accordance with Reagents, 18–21.
18. Prepare ion-exchange columns by cutting five Pasteur pipets (Apparatus, 9) 7 cm above the neck and placing a glass wool plug (Apparatus, 8) in the neck. Place the pipets vertically on the stand and add to each a sufficient quantity of ion-exchange resin (Reagents, 16) such that once the water has drained off, a volume of 500 µL of resin is obtained. Pour 1 mL of the imidazole formate solution (Reagents, 4) into each pipet, and rinse twice with 1 mL portions of water.
19. Purify the sulfatase (Reagents, 17) in the following manner. Weigh, to the nearest 0.1 mg, 25 mg of Helix pomatia type H1; dissolve it in 2.5 mL of water and transfer 500 µL of this solution to each of the columns prepared in Reagents, 18. Wash each column with 1.5 mL of water and discard the effluent. Then add 1.5 mL of a 0.2 M sodium acetate solution (Reagents, 3) and collect the eluates from the five columns in a test tube.

    Concentrate the eluates by filtration using, for example, a Millipore PTGC 11K252 immersion filter, until approximately 100 µL of liquid remains (sulfatase with a molar mass in excess of 5000 is not removed). Add 2.5 mL of water and concentrate once more by filtration until approximately 100 µL of liquid remains. Dilute to 2.5 mL with water and store the purified sulfatase in a freezer at –18°C in small amounts in order to allow defrosting of the amount necessary for immediate use.
20. Test of the sulfatase activity—Prepare a 0.15 mM sinigrin solution, buffered to pH 5.8, by preparing the following solutions in succession:
   (a) Transfer 1 mL of acetic acid to a 500 mL volumetric flask and dilute to the mark with water.
   (b) Transfer 1 mL of ethylene diamine to a 500 mL volumetric flask and dilute to the mark with water.
   (c) Mix 73 mL of solution (a) with 40 mL of solution (b) and adjust the mixture to pH 5.8 using solution (a) or solution (b) as appropriate.
   (d) Transfer 3 mL of the 5 mM sinigrin solution (Reagents, 7) to a 100 mL volumetric flask and dilute to the mark with solution (c).
   (e) Transfer 2 mL of the buffered sinigrin solution [Reagents, 20, (c)] into the reference and measuring cuvettes of a double-beam spectrometer (Apparatus, 3) adjusted to a wavelength of 228 nm with a cuvette temperature of 30°C. At time t = 0, add 50 µL of purified sulfatase (Reagents, 19) to the measuring cuvette and immediately switch on the recorder. Stop the recorder when the absorbance no longer varies (Ae), plot the tangent to the point t = 0 and measure its gradient, $\Delta A/\Delta t$.

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(f) The activity of the sulfatase, expressed in units of activity (i.e., the production of 1 micromole of desulfated sinigrin per min at 30°C and pH 5.8) per mL of sulfatase solution, is equal to

$$\frac{\Delta A}{\Delta t} \times \frac{V}{\Delta E} \times \frac{1000}{50} \times 10^6$$

Where—

$\Delta A/\Delta t$ = the gradient of the tangent to the point t = 0, in absorbance units per minute
V = the volume, in liters, of the reacting medium (i.e., $2.05\ 10^{-3}$ L)
ΔE = (approximately 1500 L mol^{-1} cm^{-1}), the difference between the molar extinction coefficients of sinigrin and of desulfosinigrin at 228 nm, i.e.,

$$\Delta E = \frac{Ae}{lc}$$

Where—

Ae = the difference between the absorbance at equilibrium of the desulfated sinigrin and the absorbance at time t = 0
l = the cuvette thickness, in centimeters (i.e., 1cm)
c = the concentration of desulfated sinigrin at equilibrium, in moles/L, i.e.,

$$c = \frac{0.15 \times 10^{-3} \times 0.95 \times 2}{2.05}$$

$$= 1.39 \times 10^{-4} M$$

0.95 is the yield at equilibrium of the desulfation of the sinigrin.

(g) Alternatively, the activity of the sulfatase may be calculated using the following simplified formula, where the activity is given by the expression

$$\frac{\Delta A \times 5.7}{\Delta t \times Ae}$$

21. Dilution—Using a pipet, transfer 1 mL of purified sulfatase (Reagents, 19) to a 10 mL volumetric flask, dilute to the mark with water and mix. Divide the solution into small quantities and store in a freezer at –18°C to allow defrosting of the amount necessary for immediate use.

SAMPLING

1. Sampling shall be carried out in accordance with accepted standardized techniques.
2. If large nonoleaginous foreign bodies have been separated before the reduction of the laboratory sample, make allowance for this in the calculation.

PREPARATION OF TEST SAMPLE

1. Reduce the laboratory sample in accordance with accepted standardized techniques.
2. Determine the moisture and volatile matter content of the test sample. If the seeds have a moisture and volatile matter content in excess of 10% (w/w), dry them using a current of air at approximately 45°C.
3. If the seeds have been treated, wash them with dichloromethane.
4. Grind the seeds in the microgrinder (Apparatus, 4) for 20 sec. Mix the meal and then grind for an additional 5 sec.

PROCEDURE

1. For the test portion, label two tubes (Apparatus, 6) A and B and transfer 200 ± 0.1 mg of the prepared test sample (Preparation of Test Sample, 4) to each tube.
2. Extract the glucosinolates by placing the tubes in the water bath or other heating apparatus (Apparatus, 7) controlled at 75°C, and leave for 1 min. Add 2 mL of boiling methanol solution (Reagents, 1) and then immediately add to tube A, 200 µL of 5 mM sinigrin solution (Reagents, 7) and to tube B, 200 µL of 20 mM sinigrin solution (Reagents, 8).
3. Continue heating at 75°C for an additional 10 min, shaking the tubes at regular intervals. Mix the contents of each tube and then centrifuge at an acceleration of 5000 × *g* for 3 min. Transfer the supernatant liquid from each tube to two other tubes (Apparatus, 7) labelled A and B; do not discard the supernatant liquids.
4. Add to the two tubes containing the solid residue 2 mL of boiling methanol solution (Reagents, 1) and reheat in the water bath or other heating apparatus (Apparatus, 7) at 75°C for 10 min, shaking the tubes at regular intervals.
5. Centrifuge for 3 min at an acceleration of 5000 × *g*, then add the supernatant liquid from the two tubes to the respective supernatant liquids retained in Procedure, 3.
6. Adjust the volume of the combined extracts to approximately 5 mL with water and mix. These extracts, if stored in the dark in a freezer at –18°C, may be kept for 2 weeks.

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7. Cut the required number of Pasteur pipets (Apparatus, 9) (i.e., one pipet per combined extract) so as to leave a volume of 1.2 mL above the neck and place a glass wool plug (Apparatus, 8) in the neck of each pipet. Place the pipets vertically on the stand.
8. Transfer 0.5 mL of a well-mixed suspension of ion-exchange resin (Reagents, 16) to each pipet and allow to settle. Rinse the pipets with 2 mL of the imidazole formate (Reagents, 4) and then twice with 1 mL portions of water.
9. Carry out the following operations for each combined extract (obtained in Procedure, 6):
   (a) Transfer 1 mL of the extract (Procedure, 6) to a prepared column (Procedure, 8) without disturbing the resin surface and allow to drain. Add two 1 mL portions of the sodium acetate buffer (Reagents, 2), allowing the buffer to drain after each addition.
   (b) Add to the column 75 µL of diluted purified sulfatase solution (Reagents, 21). Leave to act overnight at ambient temperature.
   (c) Place a tube (Apparatus, 6) under the column to collect the eluate.
   (d) Elute the desulfoglucosinolate obtained with two 1 mL portions of water, allowing the water to drain after each addition.
   (e) Mix the eluate well. If not used immediately for chromatography, store the eluate in the dark in a freezer at –18°C.
10. A blank test, if required (see Calculations, 3), should be carried out using the same procedure on a test portion taken from the same test sample, but omitting the sinigrin internal standard solution in order to detect and quantify any sinigrin present in the test portion.
11. Adjustment of the chromatograph (Apparatus, 1)—
    (a) Flow rate and elution gradients of the mobile phase (Reagents, 15) depend on the nature of the column. A typical flow rate is 1.5–2.0 mL/min.
    See Notes, 4 for examples of elution gradients.
    (b) Temperature of the column (Apparatus, 2): 30°C.
    (c) Detection wavelength: 229 nm.
12. For the HPLC analysis—Operating in accordance with the instructions for the apparatus, inject into the chromatograph not more than 50 µL of the desulfoglucosinolate solution obtained in Procedure, 9, (c). Use an elution gradient appropriate to the column employed (see Notes, 4).

EXAMINATION OF CHROMATOGRAMS

1. Take into account only those peaks having an area >1% of the sum total of the peak areas. The order of elution of the peaks with a type C18 column and a suitable elution gradient (see Notes, 4) is generally as shown in Figure 1.

CALCULATIONS

1. Calculation of the content of each glucosinolate—The content of each glucosinolate, expressed in micromoles per gram of dry matter of the product is equal to

$$\frac{Ag}{As} \times \frac{n}{w_t} \times \frac{Kg}{Ks} \times \frac{100}{100 - w_v}$$

Where—

Ag = the peak area, in integrator units, corresponding to desulfoglucosinolate
As = the peak area, in integrator units, corresponding to the internal standard used
Kg = the response factor of desulfoglucosinolate (Calculations, 2)
Ks = the response factor of the internal standard used
w_t = the mass, in grams, of the test portion
n = the quantity, in micromoles, of sinigrin added to the tube (Procedure, 2)
w_v = the moisture and volatile matter content, expressed as a percentage by weight, of the test sample

If it is desired to express the result relative to a specified moisture and volatile matter content (w_s) [e.g., w_s = 9% (w/w)], multiply the glucosinolate result obtained for dry matter (as determined in Calculations, 1) by

$$\frac{100 - w_s}{100}$$

Where—

w_s = specified moisture and volatile matter content

2. Response factors—See Notes, 3 for the response factors that have been adopted.
3. Calculation of the total glucosinolate content—The total glucosinolate content, expressed in micromoles per gram of dry matter of the product, is equal to the sum of the contents of each glucosinolate (the corresponding peak area of which is >1% of the sum total of the peak areas).

   The use of the internal reference standard at two different concentrations (Reagents, 7 and 8) allows for sinigrin contained in the sample or the presence of an unknown compound coeluting with sinigrin.

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Take as the result the arithmetic mean of two determinations provided that the requirements for repeatability (see Precision, 2) are satisfied. (In this case, there is no contamination of the internal standard.)

If the requirements for repeatability are not satisfied, repeat the procedure on two other test portions and perform a blank test (Procedure, 10), omitting the internal standard solution. (The contaminant area is subtracted from the internal standard area to give the true area of the internal reference used in the formula in Calculations, 1.) Take as the result the arithmetic mean of the results of the two new determinations.

PRECISION

1. Results of interlaboratory test—An interlaboratory test, carried out at the international level in 1988, in which 11 laboratories participated, each of which carried out two determinations on each sample, gave the statistical results [evaluated in accordance with ISO 5725-1986 (AOCS Procedure M 1-92)] shown in Table 1.
2. Repeatability—According to the results given in Table 1, the difference between the values of two determinations, carried out in rapid succession (or simultaneously) by the same operator using the same apparatus on the same test sample, shall not exceed 2 µmol/g for glucosinolate contents <20 µmol/g and 4 µmol/g for glucosinolate contents within the range 20 to 35 µmol/g.
3. Reproducibility—According to the results given in Table 1, the difference between the values of the final result obtained by two laboratories using this method for the analysis of the same laboratory sample shall not exceed 4 µmol/g for glucosinolate contents <20 µmol/g and 8 µmol/g for glucosinolate contents with the range 20 to 35 µmol/g.

TEST REPORT

1. The test report shall specify the method used and the result obtained. It shall also mention all operating details not specified in this method, or regarded as optional, together with details of any incidents which may have influenced the result.
2. The test report shall include all information necessary for the complete identification of the sample.

NOTES

1. Reference samples of rapeseed (colza) desulfoglucosinolate may be obtained from the Community Bureau of Reference, Rue de la Loi 200, B-1049 Brussels, Belgium. Rapeseed reference materials, analyzed for content of individual glucosinolates, can be obtained from the Office of Reference Materials, LGC, Queen's Road, Teddington, Middlesex TW11 0LY, United Kingdom.
2. Glucotropaeolin is sometimes difficult to separate from other natural minor glucosinolates.
3. The response factors for various glucosinolates are shown in Table 2. They have been determined experimentally and fixed by consensus between the various laboratories that took part in the collaborative study. They may be subject to revision in the future. The structures of these glucosinolates are shown in Table 3.
4. The following elution gradients are given as examples.
   (a) Lichrosorb RP18 Column, ≤5 µm (150 mm 4.6 mm):
   — 100% of eluant A [Reagents, 15, (a)] and 0% of eluant B [Reagents, 15, (b)] for 1 min
   — a linear elution gradient in 20 min until 0% of eluant A and 100% of eluant B are obtained
   — a linear elution gradient in 5 min until 100% of eluant A and 0% of eluant B are obtained
   — 100% of eluant A and 0% of eluant B for 5 min to establish equilibrium
   (b) Lichrospher RP8 Column, ≤5 µm (125 mm × 4 mm):
   — 100% of eluant A for 2.5 min
   — a linear elution gradient in 18 min until 0% of eluant A and 100% of eluant B are obtained
   — 100% of eluant B for 5 min
   — a linear elution gradient in 2 min of 0% of eluant A and 100% of eluant B until 100% of eluant A and 0% of eluant B are obtained; continue for 5 min to establish equilibrium
   The gradient profiles may be modified to give optimum separations according to the columns used.

REFERENCES

1. *Colza—Determination of glucosinolates content. Part 1: Method by high-performance liquid chromatography,* International Standards Organization, 1991, ISO/DIS 9167–1.

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Table 1
Statistical results of interlaboratory test.

	Rapeseed sample			
	A	B	C	D
Number of laboratories retained after eliminating outliers	11	11	11	11
Mean, μmol/g	20.6	14.1	4.9	25.6
Standard deviation of repeatability, S_r	1.7	0.6	0.3	0.8
Coefficient of variation of repeatability	8.5%	4.4%	6.7%	3.3%
Repeatability, $2.83 \times S_r$	4.9	1.7	0.9	2.4
Standard deviation of reproducibility, S_R	3.4	2.5	1.5	2.4
Coefficient of variation of reproducibility	17%	18%	31%	9.4%
Reproducibility, $2.83 \times S_R$	9.6	7.1	1.4	6.8

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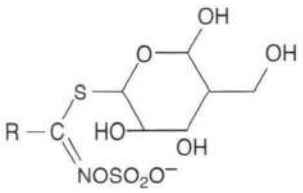
Table 2
Chemical structure of glucosinolates.



R = Functional group

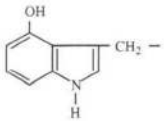
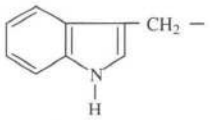
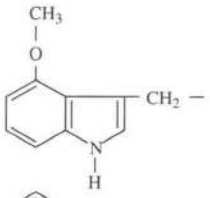
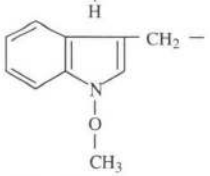
	Common name	Glucosinolate side chain (R)	Structure of side chain (R)
I	Glucoiberin	3-Methylsulphinylpropyl	CH_3– SO–$(CH_2)_3$–
II	Progoitrin	2-Hydroxybut-3-enyl	$CH_2 = CH - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - CH_2 -$
III	Sinigrin	Prop-2-enyl	CH_2=CH–CH_2–
IV	Glucoraphanin	4-Methylsulphinylbutyl	CH_3–SO–$(CH_2)_4$–
V	Gluconapoleiferin	2-Hydroxypent-4-enyl	$CH_2 = CH - CH_2 - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - CH_2 -$
VI	4-Hydroxyglucobrassicin	4-Hydroxy-3-indolylmethyl	
VII	Glucobrassicin	3-Indolylmethyl	
VIII	4-Methylglucobrassicin	4-Methoxy-3-indolylmethyl	
IX	Neoglucobrassicin	1-Methoxy-3-indolylmethyl	
X	Gluconapin	But-3-enyl	CH_2=CH–CH_2–CH_2–
XI	Glucoalyssin	5-Methylsulphinylpentyl	CH_3–SO–$(CH_2)_5$–
XII	Glucotropaeolin	Benzyl	

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Table 3
Response factors for glucosinolates.

Desulfoglucoiberin	1.07
Desulfoprogoitrin	1.09
Desulfoepi-progoitrin	1.09
Desulfosinigrin	1.00
Desulfoglucoraphanin	1.07
Desulfogluconapoleiferin	1.00
Desulfoglucoalyssin	1.07
Desulfogluconapin	1.11
Desulfo-4-hydroxyglucobrassicin	0.28
Desulfoglucobrassicanapin	1.15
Desulfoglucotropaeolin	0.95
Desulfoglucobrassicin	0.29
Desulfogluconasturtiin	0.95
Desulfo-4-methoxyglucobrassicin	0.25
Desulfoneoglucobrassicin	0.20
Other desulfoglucosinolates	1.00

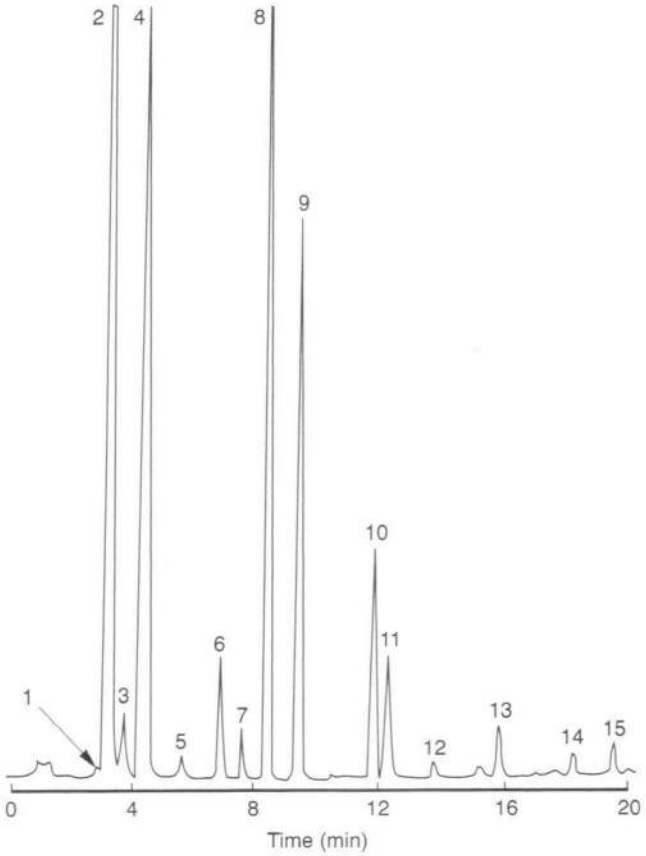

Figure 1. Order of elution peaks: 1, desulfoglucoiberin; 2, desulfoprogoitrin; 3, desulfoepi-progoitrin; 4, desulfosinigrin; 5, desulfoglucoraphanin; 6, desulfogluconapoleiferin; 7, desulfoglucoalyssin; 8, desulfogluconapin; 9, desulfo-4-hydroxyglucobrassicin; 10, desulfoglucobrassicanapin; 11, desulfoglucotropaeolin; 12, desulfoglucobrassicin; 13, desulfogluconasturtin; 14, desulfo-4-methoxyglucobrassicin; 15, desulfoneoglucobrassicin.

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AOCS Official Method Ak 2-92

Reapproved 2009

Determination of Chlorophyll Content in Rapeseed/Canola (Colza) by Spectrometry

DEFINITION

This method, adopted from ISO 10519 (References, 1), defines the chlorophyll content as the total of the substances contributing to the absorption band near 665 nm as determined under the operating conditions specified in this method, measured as chlorophyll a and expressed in milligrams per kilogram (mg/kg) of the product as received. The chlorophyll content of the rapeseed/canola (colza) is determined by extraction of a test portion in a suitable apparatus with a solution of ethanol and isooctane, or 2-propanol, methanol and isooctane, followed by the spectrometric determination of the chlorophyll contained in the extraction solution.

SCOPE

This method specifies a spectrometric method for the determination of the chlorophyll content of rapeseed (colza) used as an industrial raw material.

APPARATUS

Usual laboratory apparatus and, in particular, the following:

1. Analytical balance.
2. Mechanical grinder—blade type, or coffee mill, or equivalent.
3. Mechanical microgrinder (see Figure 1)—stainless steel tubes of approximately 50 mL volume and which can be securely stoppered, stainless steel ball bearings (16 mm in diameter) and an apparatus to shake the securely stoppered tubes horizontally at a frequency of 240 min^{-1}, with a horizontal displacement of 3.5 cm, or, for example, a Dangoumau ball mill (see Notes, 1).
4. Filter paper—medium speed, V-folded.
5. Spectrometer—suitable for carrying out absorbance measurements between 600 and 700 nm, with a spectral band path of 2 nm. If the spectrometer is not able to achieve a spectral band width of 2 nm, it is necessary to carry out a calibration with pure chlorophyll a as described in Notes, 2.
6. Optical cuvettes—having a path length of at least 1.0 cm.
7. Pipets—30 mL capacity, class A, or a repetitive dispenser with a capability of dispensing 30 mL with an accuracy of <1%.
8. Culture tubes—20 mL capacity, with stoppers.

REAGENTS

The reagents used for the preparation of the extraction solvent shall be of recognized analytical grade unless otherwise stated.

1. Extraction solvent—prepared as follows:
   Transfer to a 500 mL beaker either
   (a) 100 mL of anhydrous ethanol, or
   (b) 50 mL of anhydrous 2-propanol and 50 mL of anhydrous methanol.
2. Add to the contents of the beaker 300 mL of anhydrous isooctane (2,2,5-trimethylpentane), or technical n-heptane, or petroleum ether (essentially composed of C7 hydrocarbons, with a boiling range between 90 and 100°C).

PROCEDURE

1. Sampling to obtain the laboratory sample and the reduction of the laboratory sample (as received or after separation of impurities) to the test sample shall be carried out in accordance with accepted standardized techniques and with any contractual agreement.
2. Seeds with a moisture content of >10% (w/w) should be dried for 12 hr at 45°C to reduce the moisture level to 10% (w/w) or less without breaking down chlorophyll pigments.
3. Transfer 50 g of the test sample to the mechanical grinder (Apparatus, 2) and grind to produce a uniform ground seed. If a small grinder such as a coffee mill is used, grind several portions of 10 g and then combine and mix thoroughly the ground portions.
4. For the test portion, weigh, to the nearest 1 mg, 2 g of the test sample (Procedure, 3) into the stainless steel tube (Apparatus, 3), or the extraction vessel of a Dangoumau ball mill (Apparatus, 3).
5. For the extraction of the test portion, add, using a pipet (Apparatus, 7), 30 mL of the extraction solvent (Reagents, 1, 2) to the tube or vessel. If using a tube, add three stainless steel balls to the tube (Figure 1) and shake for 1 hr. For Dangoumau ball mills, add steel balls (at least four medium-sized balls) to the vessel and extract for 20 min.

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6. Allow the extract to settle for 10 min and then decant a sufficient volume of the extract through the filter paper (Apparatus, 4) into a culture tube (Apparatus, 8) to fill the optical cuvette (Apparatus, 6). Stopper the tube as soon as possible to minimize evaporation.

   Note—The presence of more than one phase in the extraction solvent indicates the presence of excessive moisture, either in the sample [which should contain < 10% (w/w) moisture] or in the solvents (which should be anhydrous).
7. Transfer the filtered extract to the cuvette (Apparatus, 6) and determine the absorbance at 665, 705 and 625 nm (the readings at 705 and 625 nm are used to calculate a baseline correction).

CALCULATIONS

1. The chlorophyll content, in milligrams per kilogram (mg/kg) of the product as received, is given by the formula

$$\text{chlorophyll content, mg/kg} = \frac{k \times A_{con} \times V}{m \times 1}$$

Where—

A_{corr} (the corrected absorbance) = A665 – [(A705 + A625)/2]
A665 = absorbance at 665 nm
A705 = absorbance at 705 nm
A625 = absorbance at 625 nm
k = 13
l = path length of the optical cuvette, in cm
m = mass of the test portion, in g
V = volume of solvent added to the tube (Apparatus, 8), in mL. If it is desired to express the chlorophyll content relative to the dry product, take into account in the calculation the water content of the sample, determined in accordance with ISO 665.

PRECISION

1. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should not be greater than the following values:
   (a) chlorophyll content between 10 mg/kg and 20 mg/kg, mg/kg = 2
   (b) chlorophyll content between 20 mg/kg and 30 mg/kg, mg/kg = 3

   Note—Values are not fixed for chlorophyll contents below 10 mg/kg because the industry accepts a content up to 25 mg/kg.
2. Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the following values:
   (a) chlorophyll content between 10 mg/kg and 20 mg/kg, mg/kg = 3
   (b) chlorophyll content between 20 mg/kg and 30 mg/kg, mg/kg = 6

   Note—Values are not fixed for chlorophyll contents below 10 mg/kg because the industry accepts a content up to 25 mg/kg.

TEST REPORT

1. The test report shall specify the method used and the results obtained. It shall also mention all operating conditions not specified in the method, or regarded as optional, together with details of any incidents which may have influenced the results.
2. The test report shall include all information necessary for the complete identification of the sample.

NOTES

Caution

The solvents used in this method are flammable liquids with low flash points. These solvents may defat (dry out) or irritate the skin, eyes and mucous membranes. Consult the appropriate manufacturer's material safety data sheets.

When handling solvents, use protective eye wear, gloves and a static-free (not polyester) laboratory coat. Handle the solvents in a well-ventilated area (preferably a fume hood) away from heat, sparks and open flame. Conduct bulk transfers of the solvents in a fume hood. Take care during transfer, as the liquids are nonconducting and may generate a static charge. Store large volumes of solvents in a cool, ventilated location. Separate from oxidizing material. Isolate from other combustible material.

Diethyl ether may form organic peroxides during storage. All organic peroxides are extremely flammable and unstable (explosive). They are hazardous because of their extreme sensitivity to heat, friction, shock and light, as well as to strong oxidizing and reducing agents. Store diethyl ether away from heat and light in closed vessels, preferably in the container furnished by the supplier. Containers of ether should be labelled with the dates received, opened and of expiration. The contents should be used within 1 month of opening. If a bottle is several years old, or if there is any visible solid around the cap, then no attempt should be made to open it.

Used solvents shall be stored and disposed of in accordance with local regulations.

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NUMBERED NOTES

1. Dangoumau ball mill is an example of a suitable apparatus available commercially. This information is given for the convenience of users of this method and does not constitute an endorsement by AOCS of this apparatus.
2. Calibration for routine operation on spectrometers unable to achieve a spectral bandwidth of 2 nm requires the preparation of solutions of pure chlorophyll a, followed by the determination, using these solutions, of the slope of the calibration curve for the spectrometer.

   Reagents
   (a) Diethyl ether—of recognized analytical grade.
   (b) Chlorophyll a—standard solution (Sigma Chemical, St. Louis, MO, USA). Place about 1 mg of crystalline chlorophyll a of at least 95% purity in a 10 mL volumetric flask, dissolve in the diethyl ether and make up to the mark with diethyl ether.

   Apparatus
   (a) Spectrometer—other than the unit to be calibrated, suitable for carrying out absorbance measurements between 600 and 700 nm and with a spectral band width of < 2 nm. This instrument is required to determine the concentration of chlorophyll a in the standard solution.
   (b) Micropipets—capable of delivering 0.200 mL, 0.400 mL, 0.600 mL, 0.800 mL and 1.000 mL.
   (c) Volumetric flasks—10 mL capacity.
   (d) Working spectrometer—i.e., a spectrometer other than that specified in Notes, 2, Apparatus (a), for which a calibration is desired.

   Calibration
   (a) To determine the chlorophyll a content in the standard solution, transfer 1.0 mL of the chlorophyll a standard solution [Notes, 2, Reagents, (b)] to a 10 mL volumetric flask [Notes, 2, Apparatus, (c)] and dilute to the mark with the diethyl ether [Notes, 2, Reagents, (a)]. Measure the absorbance of this solution using the spectrometer [Notes, 2, Apparatus, (a)] at 660 nm (peak wavelength) and 642.5 nm, using a 1.0 cm cuvette and a spectral band width of 2 nm.
   (b) Calculate the concentration of the chlorophyll a, mg/L, in the standard solution using the formula

   $$\text{Chlorophyll a (mg/L)} = (99.3 \times A660) - (7.8 \times A642.5)$$

   Where—
   A660 = absorbance of the standard chlorophyll a solution at 660 nm
   A642.5 = absorbance of the standard chlorophyll a solution at 642.5 nm

   Note—The factors used for the determination of chlorophyll a (99.3 and 7.8) are derived from the factors published in References, 2. The factors 9.93 and 0.777 from this source are adjusted for a 10 fold dilution.

   Preparation of the Calibration Curve
   (a) To a series of five 10.0 mL volumetric flasks, add 0.200, 0.400, 0.600, 0.800 and 1.000 mL of the standard chlorophyll a solution [Notes, 2, Reagents (b)], the concentration of which was calculated in Notes, 2, Calibration, (b). Fill the flasks to the mark with the extraction solvent (Reagents, 1 and 2).
   Note—The small amounts of diethyl ether in these solutions do not interfere significantly with the determination.
   (b) Measure the corrected absorbance A_{corr} (Calculations, 1) of these calibration solutions and plot this against the concentration of chlorophyll a in the solutions. The slope of the curve thus plotted is the constant k, in mg/L, per corrected absorbance.

REFERENCES

1. *Rapeseed—Determination of chlorophyll content—Spectrometric method,* International Organization for Standardization, Geneva, 1992, ISO 10519:1992(E).
2. *Official Methods of Analysis,* AOAC International, 16th ed., Vol. I, Gaithersburg, MD, 1995, Chapt. 3, p. 27, Method 942.04.

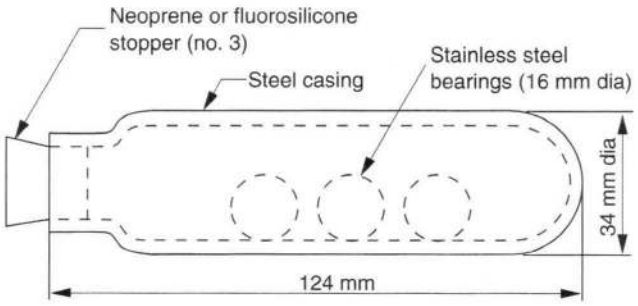

Figure 1. Mechanical microgrinder.

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AOCS Recommended Practice Ak 3-94

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Oil Content of Oilseeds by Nuclear Magnetic Resonance

DEFINITION

This method determines the oil content of rapeseed by continuous-wave, low-resolution nuclear magnetic resonance (NMR). The NMR instrument determines the response of liquid compounds containing hydrogen in seeds that have been previously dried. The oil content is calculated on an as-is basis from the NMR signal by reference to a calibration of NMR response versus oil content determined by solvent extraction.

SCOPE

Applicable to rapeseed, soybean, sunflower seed, and peanut.

APPARATUS

1. Nuclear magnetic resonance instrument—continuous-wave, low-resolution type, installed in a temperature-stable room.
2. Oven—regulated at 103 ± 2°C.
3. Magnet.
4. Nessler tubes—100 mL, calibrated at 40 mL (approximately 50 mm from the bottom of the tube) suitable for use with the NMR instrument.
5. Teflon™ stoppers—to fit Nessler tubes.
6. Rubber bungs—to fit Nessler tubes.
7. Parafilm.
8. Sand—400–100 mesh.
9. Thermometer—readable to 0.1°C.

CALIBRATION OF THE INSTRUMENT

1. Adjust the NMR Spectrometer in accordance with the manufacturer's instructions, then adjust to zero after placing an empty measuring tub in the magnetic field.
2. Into four measuring tubes identical to that used for the adjustment (i.e., also giving a reading of zero when they are placed empty in the magnetic field), weigh to the nearest 0.01 g, 5 g, 10 g, 20 g, and if the apparatus so permits, 30 g of the calibration oil, taking care that no oil adheres to the sides of the tubes above the effective part.
3. Measure the response of the instrument for each of the four tubes, taking R_5, R_{10}, R_{20}, and R_{30} as the mean values of five readings of the integrator on each of the four tubes, during the integration time giving the best repeatability of measurement, using a level of radio frequency energy sufficient to obtain a satisfactory signal in relation to the background noise, but not producing more than approximately 1% saturation. The correct selection of the saturation level may be carried out by the operator if the relation between the radio frequency energy and the saturation is known. If not, the correct adjustment of the level of saturation can be obtained from the manufacturer of the instrument.
4. Draw a straight line representing the value of R_x as a function of the mass of oil placed in the tubes. This line shall pass through the origin. If it does not, contact the manufacturer to adjust the instrument or make the necessary adjustment if the instrument so permits.
5. Check the calibration of the instrument regularly (if possible, once each day). If the instrument and the samples are not thermostatically controlled, check the variation of the gradient of the calibration line by measuring the response of a 20 g or 30 g sample every 30 min.

PROCEDURE

1. Preparation of rapeseed standards—Select six reference samples of rapeseed with oil contents covering the range 40–46% and moisture contents below 10% (Notes, 1). From approximately 1 kg of reference seed, remove any magnetic impurities with a magnet, and take two representative 100 g test samples (Notes, 2). Determine the oil content on one of the 100 g portions by the solvent extraction procedure AOCS Official Method Am 2-93. Weigh the second portion accurately into a 250 mL beaker, transfer approximately 25 g to each of four 100 mL Nessler tubes (Apparatus, 4) and dry seed at 103 ± 2°C for 16 ± 2 hr or overnight. Fit Teflon™ stoppers (Apparatus, 5) to hold seed below 40 mL line, cap with a rubber bung (Apparatus, 6) and seal with Parafilm (Apparatus, 7). Place tubes close to NMR instrument and allow to cool for 2 hr (Notes, 3).
2. Preparation of rapeseed oil standards—Weigh into two Nessler tubes approximately 10.0 g and 11.5 g, respectively (representing 40–45% oil in 25 g seed), of rapeseed oil (extracted previously by AOCS Official Method Am 2-93). To each tube add about 50 g sand (Apparatus, 8) to within 0.5 cm of the 40 mL line. Stopper, cap and seal each tube. Place next to NMR instrument and leave for 1 hr to equilibrate. (These tubes become secondary standards.)

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3. Preparation of rapeseed samples—Pass a magnet through a representative 100 g test sample of seed spread out in an area. If magnetic impurities are removed, weigh and determine their content for the seed test samples.
4. Weigh the seed accurately into four Nessler tubes, dividing into roughly equal (25 g) portions. Dry the seed in an oven at 103 ± 2°C for 16 ± 2 hr or overnight. On removing the tubes from the oven, fit Teflon™ stoppers to hold seed below 40 mL line, cap with a rubber bung and seal with Parafilm. Place tubes in the same room as the NMR instrument and allow to cool for 2 hr (an electric fan may be used to accelerate cooling).

DETERMINATIONS

1. Measurement—Follow manufacturer's instructions to set up the equipment using
   Ref. A concentration of oil standard A
   Ref. B concentration of oil standard B
2. Calibrate the instrument with the oil standards A and B using the assigned concentrations and sample masses for both standards. Note room temperature. For each seed test portion, measure each of the four tubes, entering the seed mass and recording the oil content for each tube. Check room temperature during measurements and if it changes by more than 1°C, recalibrate with the standards. Correct measured oil contents for any changes in temperature up to 1°C; add 0.02% to the oil content for every 0.1°C increase.

CALCULATIONS

1. Calculate the apparent oil content of the seeds using the formula:

$$w_a = \frac{R \times m_x \times 100\%}{R_x \times m_0}$$

Where—

w_a = the apparent oil content, expressed as a percentage by mass, of the seeds
m_0 = the mass, in grams, of the test portion before drying
m_x = the mass, in grams, of crude oil corresponding to the response R_x, read from the calibration line of the instrument
R = the mean value of the responses of the instrument for the test portion, determined in Measurement
R_x = the response of the instrument corresponding to the mass m_x of crude oil, read from the calibration line of the instrument

2. Calculate the response factor of the residue using the formula

$$w_r = \frac{T \times m_x \times 100\%}{R_x \times m_r}$$

Where—

w_r = the response factor, expressed as a percentage by mass, of the residue
m_r = the mass, in grams, of the dried oilseed residue from which the oil has been extracted
T = the mean value of the responses of the instrument for the mass m_r of dried oilseed residue from which the oil has been extracted, determined in Correction for oilseed residue
m_x and R_x = the same meanings as in Expression of Results

Take as the result the arithmetic mean of the results of the two determinations, provided that their difference does not exceed 0.4%. If their difference does exceed 0.4% (m/m), discard the results of the two determinations and repeat the procedure.

3. Calculate the oil content of the seeds using the formula

$$w = \frac{(w_a - w_r)[1 - (W_H/100)]}{1 - (W_r/100)}$$

Where—

w = the oil content of the seeds, expressed as a percentage by mass
w_H = the moisture content of the seeds, expressed as a percentage by mass
w_a and w_r = the same meanings as in Calculation of apparent oil content

Take as the result the arithmetic mean of the results of the two determinations, provided that their difference does not exceed 0.4%. If their difference does exceed 0.4% (m/m), discard the results of the two determinations and repeat the procedure. Express the result to the nearest 0.1%.

REPEATABILITY

The difference between two single test results obtained under repeatability conditions shall not exceed 0.6% (m/m). Reject both results if their absolute difference exceeds 0.6% (m/m) and carry out two new single determinations.

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TEST REPORT

The test report should specify the method used, the results obtained, the name and type of NMR spectrometer used, and, if applicable, all adjustments made. It should also mention all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results.

The test report should include all information necessary for the complete identification of the sample.

NOTES

1. During calibration, seed test samples should be allowed to equilibrate for a few days in the laboratory so that no changes in weight are caused by gain or loss of moisture from the atmosphere.
2. Weighing of test portions taken for NMR measurements and solvent extraction should be made at the same time to avoid any errors due to changes in moisture content.
3. Reference seed test samples (dried) of known oil content (by AOCS Official Method Am 2-93) should be supplied in sealed tubes for calibration purposes. This would help laboratories without facilities for solvent extraction. The precision of such sealed oilseed calibration standards may be a topic of interest for the Bureau of Community Reference, Department of the EEC in Brussels.

REFERENCES

1. ISO 5511, "Oilseeds—Determination of Oil Content—Method using continuous-wave low-resolution nuclear magnetic resonance spectrometry (Rapid Method)."

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AOCS Recommended Practice Ak 4-95

Revised 2011

Simultaneous Determination of Oil and Moisture Contents of Oilseeds Using Pulsed Nuclear Magnetic Resonance Spectrometry

DEFINITION

This International Standard specifies a rapid method for the determination of the oil and moisture contents of commercial oilseeds, using a pulsed nuclear magnetic resonance (pNMR) spectrometer. The method has been tested on rapeseed, soybeans, linseeds, and sunflower seeds (see Notes, 1).

SCOPE

This method applies to rapeseed with a moisture content of less than 10%. For seeds with higher moisture contents, drying is necessary before the oil content can be determined by pulsed NMR. The method requires the following steps: Insertion of the test sample into the magnetic field of a pulsed NMR spectrometer. Application of an alternating electromagnetic field in the form of an intense 90° radiofrequency (RF) pulse that excites all the hydrogen nuclei. Recording of the free induction decay (FID) following the 90° pulse. The maximum amplitude of this signal is proportional to the total number of protons from the water and oil phases of the sample. Application of a second RF pulse, a "so-called 180° pulse," to produce a spin-echo signal when only the signal from the oil phase contributes to the FID (see Notes, 2). Calculation of the difference between the two amplitudes, which is proportional to the moisture content. Automatic conversion of the measured signals, after suitable calibration of the apparatus, into percentages of oil or moisture (see Notes, 3).

APPARATUS

1. Pulsed low-resolution NMR spectrometer—Suitable for measurement of the oil content and moisture content of oilseeds, and having the following characteristics:
   (a) Proton resonance frequency: must be high enough (more than 8 MHz) to achieve a total dead time lower than 45 μsec; if not the water content cannot be measured. Lower frequencies have to be investigated. The magnetic field homogeneity must be good enough for a minimum sample volume of 35 ml, representative of the total amount of the samples. The instrument parameters shall follow the instructions/specifications from the manufacturer.
   (b) Production of 90° and 180° RF pulses for the spinecho method.
   (c) Magnetization time or pulse repetition time: 1 s.
   (d) Excitation time (e) or pulse time (approximate):
   e = 5 μs for the 90° pulse,
   e = 10 μs for the 180° pulse (i.e., twice as long as for the 90° pulse).
   (e) Dead time (t) = 20 μs.
   (f) Total dead time (T_t), comprising the excitation time (e) and the dead time (t), where:
   Tt = 25 μs for 90° pulses,
   Tt = 30 μs for 180° pulses,
   t = 20 μs.
   (g) NMR signal sampling:
   (1) 50 μs after the 90° pulse on the FID,
   (2) 7,000 μs after the 90° pulse on the spin-echo.
   (h) Interval between the 90° and the 180° pulse:
   3,500 μs.
   (i) Electronic bandwidth filter:
   low (10 kHz).
   (j) Number of NMR measurements per sample:
   16 to 25 (i.e., the total time of measurement is between 16 s and 25 s).
   (k) Facility for storage of calibration curves (straight lines, least-squares method) of moisture and oil contents.
   (l) Homogeneity of magnetic field for a desirable sample volume of 35 mL.
   (m) Start system, which automatically starts the measurement after the introduction of the sample tube.
   (n) Temperature-regulated magnet, in order to maintain stability of all of the NMR parameters.

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2. Glass sample tubes—suitable for use with the NMR spectrometer.
3. Analytical balance, electronic—with an accuracy of ±0.01 g, preferably linked to the NMR spectrometer so that the sample mass is recorded directly by the NMR, or linked to a data collection system.
4. Drying oven—capable of being maintained at 103 ± 2°C.
5. Glass or metal dishes—diameter 7–10 cm, and provided with lids.
6. Desiccator—containing an efficient desiccant.

CALIBRATION SAMPLES

Calibration samples shall be homogeneous and free from impurities.

1. Samples for moisture-content calibration—In order to obtain a reliable calibration curve, it is recommended that the moisture content of the calibration samples be between 6 and 10% for all seeds.

   The moisture content of seeds can vary depending on storage conditions. It is therefore necessary to determine it just prior to calibration.
2. Samples for oil-content calibration—Samples of oilseeds shall be of the same species as the test samples and of similar fatty acid compositions (especially for the analysis of rapeseeds, which are rich in erucic acid, or sunflower seeds, which are rich in oleic acid). Oil content shall be determined using the reference method described in ISO 659, or AOCS Am 2-93.

SAMPLING

It is important that the laboratory receive a sample that is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 542 (see References, 4), or AOCS C 1-47.

PREPARATION OF TEST SAMPLE

Prepare the test sample in accordance with ISO 664 (see References, 2). Remove all metallic objects from the prepared test sample (e.g. staples, needles, etc.). Whole seeds shall be homogeneous and, as far as possible, free from impurities.

CALIBRATION PROCEDURE

1. General:
   (a) Use the set-up parameters of the NMR spectrometer (see Apparatus, 1) recommended by the manufacturer and optimize them by preliminary tests. For all calibration and measurement operations, follow the user's manual. Ensure that all operations during calibration and measurement are carried out under the same conditions and, in particular, at the same temperature (±2°C).

   A minimum of three calibration samples is necessary, although more than three samples may be used.

   (b) Enter the parameters for the measurements (i.e., pulse sequence, attenuation, etc.) of the oil or moisture content (as applicable) into the NMR spectrometer following the manufacturer's recommendations, and specify a number under which the calibration curve is to be stored. The total measurement time shall be a minimum of 16 s.

   (c) Set the apparatus to the calibration mode.

   (d) Introduce a portion of the first calibration sample into a tared sample tube (see Apparatus, 2) up to the optimum height specified by the manufacturer and transfer the sample data from the balance to the NMR apparatus (see Notes, 4).

   (e) Enter, as applicable, the value of either the moisture content (as a percentage by mass) or the oil content (as a percentage by mass) into the spectrometer.

   (f) Introduce the sample tube containing the first calibration sample into the measuring head and determine the moisture or oil content.

   (g) Repeat steps (d) through (f) for the other calibration samples (at least three total).

   (h) Calculate automatically or manually the calibration parameters of the calibration curve.

   The correlation coefficient shall normally be greater than 0.95. If this is not the case, check the values obtained using the reference methods, or repeat the calibration procedure using three (or more) other calibration samples (see Notes, 5).

   (i) Complete the oil-content calibration curve by using an empty tube and by repeating steps (d) through (f). This part of the method is not applicable to the moisture-content calibration curve.
2. Calibration procedure for the determination of moisture content:
   (a) Procedure A—Three calibration samples with known moisture contents: To obtain a reliable calibration graph, it is necessary that the moisture contents of the calibration samples span the largest possible range. However, a moisture content of 0 is not permissible. Follow the general procedure specified in 1(a) through 1(f).

   (b) Procedure B—One calibration sample with known moisture content (see Notes, 6).

   (c) Follow the general procedure specified in 1(a) through 1(c).

   (d) Introduce into three tared sample tubes (see Apparatus, 2) portions of the calibration sample to approximate heights of 35 mm, 30 mm, and 25 mm (20 mm minimum) and weigh them. Note the corresponding masses, in grams, as m_1, m_2, and m_3 (see Notes, 7).

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(e) Calculate the relative moisture content, w, as a percentage by mass, of each of the three portions of the calibration sample as follows:

$$w_1 = \frac{m_1 \times W}{m_1}$$

$$w_2 = \frac{m_2 \times W}{m_1}$$

$$w_3 = \frac{m_3 \times W}{m_1}$$

Where—

m_1, m_2, m_3 = mass, in grams, of the three portions of the calibration sample in each of the three tubes, where m_1 is the largest mass;

W = moisture content, as a percentage by mass, of the calibration sample (see References, 3).

(f) Enter into the NMR spectrometer the value, m_1, of the mass of the first portion.

(g) Enter into the NMR spectrometer the value, w_1, of the relative moisture content of the first portion.

(h) Insert the tube containing the first portion of the calibration sample into the measuring head and carry out the determination.

(i) Repeat the operations described in (f) through (h) with the two other portions of the calibration sample, always entering m_1 in step (f), then the respective relative moisture contents w_2 and w_3 in step (g).

(j) Calculate the calibration parameters in accordance with step 1(h).

3. Calibration procedure for the determination of oil content:

(a) Procedure A—Three calibration samples with known oil contents. Use the samples specified in Calibration Samples, 1. Follow the general procedure specified in Calibration Procedure, 1(a) through 1(i).

(b) Procedure B—One calibration sample with known oil content (see Notes, 8). Follow the general procedure specified in Calibration Procedure 1(a).

(c) Introduce into three tared sample tubes (see Apparatus, 2) portions of the calibration sample with known oil content to approximate heights of 35 mm, 30 mm, and 25 mm (20 mm minimum) and weigh. Note the corresponding masses, in grams, as m_1, m_2, and m_3.

(d) Calculate the relative oil content, h, as a percentage by mass, in the three portions of the calibration sample as follows:

$$h_1 = \frac{m_1 \times H}{m_1}$$

$$h_2 = \frac{m_2 \times H}{m_1}$$

$$h_3 = \frac{m_3 \times H}{m_1}$$

Where—

m_1, m_2, m_3 = mass, in grams, of the three portions of the calibration sample,

H = oil content of the calibration sample, determined in accordance with ISO 659 as a percentage by mass (see References, 1).

(e) Follow the general procedure specified in Calibration Procedures 1(b) through 1(h), entering the oil content, in step 1(e) in grams.

(f) Enter into the NMR spectrometer the value, m_1, of the mass of the first portion.

(g) Enter into the NMR spectrometer the value, h_1, of the relative oil content of the first portion.

(h) Insert the tube containing the first portion of the calibration sample into the measuring head and carry out the determination.

(i) Repeat the operations described in (f) through (h) with the two other portions of the calibration sample, always entering m_1 in step (f), then the respective relative oil contents h_2 and h_3 in step (g).

(j) To obtain the zero-point on the calibration curve, enter into the NMR spectrometer the value m_1 of the mass and a relative oil content of 0.01%. Insert an empty tube into the measuring head and carry out the determination.

(k) Calculate the calibration parameters in accordance with Calibration Procedures 1(i).

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PROCEDURE

Carry out at least two determinations on test portions taken from the same test sample.

1. Requirements for the test room and conditioning of the test sample. The temperature of the test room shall be maintained between 17°C and 28°C. Therefore, it is recommended that the test room be temperature controlled. Transfer the test samples (see Preparation of Test Sample) to the test room at least 60 min before the determination to allow them to reach equilibrium temperature.
2. Test portion—Prepare test portions of constant volume using one of the following methods (see Notes, 9).
   (a) Measure a chosen volume of the test sample (see Procedure, 1) and then transfer quantitatively this volume to a tared sample tube (see Apparatus, 2) (e.g., use 40 mL of test sample for sample tubes 40 mm in diameter).
   (b) Transfer a sufficient quantity of the conditioned test sample (see Procedure, 1) to the sample tube to fill it to an optimum height of 30 mm ± 5 mm. (It is recommended that the sample tubes be marked at a height of 30 mm using a felt-tipped pen.) If carrying out measurements on dehulled sunflower seeds, ensure that they are well distributed in the sample tubes (see Notes, 10,11).

DETERMINATION

Important—Check the calibration prior to each series of measurements (or at least once a day), using a calibration sample of known oil and moisture contents.

1. General case:
   (a) Select the program and the instrument parameters for the moisture or oil content, as applicable, and the number of the calibration curve corresponding to the intended test and to the species of seeds.
   (b) Set the apparatus to the measurement mode.
   (c) Weigh the test portion (see Procedure, 2) and transfer the data from the balance to the NMR spectrometer.
   (d) Insert the sample tube containing the test portion into the measuring head and determine the moisture and oil contents.
2. Rapeseeds and sunflower seeds having a moisture content greater than 10% (*m/m*) and soybeans having a moisture content greater than 14% (*m/m*) (see Notes, 12).
   (a) Transfer quantitatively the previously weighed test portion of mass m into dishes (see Apparatus, 5), with the lids removed, and dry in the oven (see Apparatus, 4) set at 103°C for 1 h.
   (b) Remove the dishes from the oven, cover them with a lid, and allow them to cool in the desiccator (see Apparatus, 6). Check that they have reached the measuring temperature (approximately 1 h).
   (c) Transfer the dried test portion quantitatively to a previously weighed sample tube, weigh and deduce the mass, m_0, of the dried test portion.
   (d) Follow the procedure specified in 1(a) and 1(b).
   (e) Enter the value of the original mass, m, of the test portion into the NMR spectrometer.
   (f) Insert the sample tube containing the test portion into the measuring head and determine the moisture content, W_b.
   (g) Correct the reading obtained for the moisture content, W_b, to give the total moisture content W, as a percentage by mass, using the following formula:

$$W = W_b + \frac{m - m_0}{m} \times 100$$

Where—

m = original mass, in grams, of the test portion determined in (a);
m_0 = mass, in grams, of the dried test portion determined in (c);
W_b = moisture content, expressed as a percentage by mass, measured in (f).

EXPRESSION OF RESULTS

Check whether the repeatability requirement (see Precision, 1) is satisfied. If so, take as the result the arithmetic mean of the results of the two or more determinations, If not, reject the results and carry out two additional determinations.

PRECISION

For the results of an interlaboratory test carried out on rapeseeds, see Table 1.

1. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should not be greater than 0.6% for oil and 0.1% for moisture.
2. Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than 1.6% for oil, and 0.7% for moisture.

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NOTES

Caution—Remove metallic objects from the proximity of the NMR spectrometer.

NUMBERED NOTES

1. This method is currently used for soybeans and sunflower seeds; this does not, however, preclude its applicability to other commercial seeds, the oil of which is liquid at the temperature of measurement.
2. The maximum amplitude of this echo signal is proportional to the oil content. It varies with the sample temperature following a complex law. An increase in temperature decreases the measured value of the echo.
3. Simultaneous indications of the oil and moisture contents can be given by some spectrometers equipped with a microprocessor and a specific program.
4. Manual feed of the mass of the calibration sample into the NMR spectrometer is also possible.
5. The calibration curve is stored under the storage number chosen in Calibration Procedure 1(b).
6. In this calibration procedure, the quantity of oil detected by the NMR spectrometer is varied by varying the height to which the sample tubes are filled with the calibration sample.
7. Three calibration points is the minimum for establishing the calibration curve.
8. In this calibration procedure, the quantity of moisture detected by the NMR spectrometer is varied by varying the height to which the sample tubes are filled with the calibration sample.
9. The reproducibility of the method is improved if the NMR measurements are carried out on test portions of constant volume.
10. For seed samples where only small quantities are available, it is possible to use smaller sample tubes with a diameter of 25 mm. A measuring volume of 13 mL, corresponding to about 9 g of rapeseed is required for such tubes. Owing to the short measurement time and the small sample quantity a testing in triplicate is recommended in order to obtain a representative result, and in this case the variability is greater.
11. For a filling height of 30 mm, the following mass ranges are recommended:
    whole rapeseeds: 22–25 g
    whole sunflower seeds: 14–17 g
    whole soybeans: 21–24 g
12. In this case, the pulsed NMR and the spin-echo method do not give a correct value because the excess moisture has a different behavior and contributes together with the oil to the spin-echo signal. Therefore, this excess moisture has to be removed by drying.

REFERENCES

The following standards contain provisions which, through reference in this text, constitute provisions of this Official Method. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this Official Method are encouraged to investigate the possibility of applying the most recent editions of the standards indicated. Members of IEC and ISO maintain registers of currently valid International Standards.

1. ISO 659:1988, *Oilseeds—Determination of hexane extract (or light petroleum extract), called "oil content"* or AOCS Am 2-93.
2. ISO 664:1990, *Oilseeds—Reduction of laboratory sample to test sample.*
3. ISO 542:1990, *Oilseeds—Sampling* or AOCS C 1-47.
4. ISO 5725:1986, AOCS M 1-92, *Precision of test methods—Determination of repeatability and reproducibility for a standard test method by interlaboratory tests.*

Table 1

An interlaboratory test at the international level was carried out by 13 laboratories. The statistical results indicated in the table below were determined in accordance with ISO 5727 (see References, 5).

Interlaboratory test on rapeseed	Oil content		Moisture content	
	Batch 1	Batch 2	Batch 1	Batch 2
Number of laboratories retained after eliminating outliers	13	13	12	12
Mean value of content, % (m/m)	40.83	41.38	7.01	6.01
Standard deviation of repeatability, s_r	0.25	0.15	0.04	0.03
Coefficient of variation of repeatability, %	0.63	0.38	0.60	0.58
Repeatability, 2.83 s_r	0.72	0.45	0.11	0.09
Standard deviation of reproducibility s_R	0.55	0.55	0.24	0.36
Coefficient of variation of reproducibility, %	1.35	1.33	3.41	6.01
Reproducibility, 2.83 s_R	1.56	1.56	0.67	1.02

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Simultaneous Determination of Oil and Moisture Contents of Oilseeds Residues Pulsed Nuclear Magnetic Resonance Spectrometry

DEFINITION

This is a rapid method for the determination of the oil and moisture contents of commercial oilseed residues, using a pulsed nuclear magnetic resonance (pNMR) spectrometer. The method applies to oilseed residues such as flour, plates, or agglomerates, provided the particles are smaller than 2 mm and the water content is not higher than the conversion threshold.

SCOPE

The method requires the following steps: insertion of the test sample into the magnetic field of a pulsed NMR spectrometer. Application of an alternating electromagnetic field in the form of an intense 90° radio frequency (RF) pulse that excites all the hydrogen nuclei. Recording of the free induction decay (FID) following the 90° pulse. The maximum amplitude of this signal is proportional to the total number of protons from the water and oil phases of the sample. Application of a second RF pulse, a so-called "180° pulse," to produce a spin-echo signal when only the signal from the oil phase contributes to the FID (see Notes, 1). Calculation of the difference between the two amplitudes, which is proportional to the moisture content. Automatic conversion of the measured signals, after suitable calibration of the apparatus, into percentages of oil or moisture (see Notes, 2).

APPARATUS

1. Pulsed low-resolution NMR spectrometer—suitable for measurement of the oil content and moisture content of oilseeds. Set the parameters to meet the manufacturer's specifications.
2. Glass sample tubes—suitable for use with the NMR spectrometer.
3. Analytical balance, electronic—with an accuracy of ±0.01 g, preferably linked to the NMR spectrometer so that the sample mass is recorded directly by the NMR, or linked to a data collection system.
4. Drying oven—capable of being maintained at 103 ± 2°C.
5. Glass or metal dishes—diameter 7 cm, with lids.
6. Desiccator—containing an efficient desiccant.
7. Mechanical grinding mill or mortar—easy to clean and capable of grinding the residues to particles smaller than 2 mm, without heating and without modifying the water, volatile matter, and oil contents.

CALIBRATION SAMPLES

Calibration samples shall be homogeneous and free from impurities.

1. Samples for moisture-content calibration—The moisture content of oilseed residues can vary depending on storage conditions. Therefore, it is necessary to determine it using a recognized method just prior to calibration.
2. Samples for oil-content calibration—Use samples of oilseed residues of the same species as the test samples and of similar fatty acid composition (especially for the analysis of rapeseeds, which are rich in erucic acid, or sunflower seeds, which are rich in oleic acid). Determine oil content using a recognized reference method.

PREPARATION OF TEST SAMPLE

Prepare the test sample from a representative sample of the lot to be tested. Remove all metallic objects from the prepared test sample (e.g., staples, needles, etc.). Oilseed residues shall be homogeneous and, as far as possible, free from impurities.

CALIBRATION PROCEDURE

1. General:
   (a) The oil content is low (1 to 3%) compared to the water content (8 to 12%) in many oilseed residues. Procedure B is preferred when the range of products has a very small oil or water content (lower than 2%). Carry out on two samples for 6 points on the calibration curve. When the water content is higher than the oil content, the NMR signal S_1, measured after 50 µs, is very strong compared to the spin-echo signal S_2 of the oil. So, for calibrating and measuring the

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oil content of the oilseed residue, it is recommended to use the value (in decibels) of the NMR signal amplification of the spectrometer optimized for the highest signal, which will consequently be less effective for the lowest signal.

(b) Use the set-up parameters of the NMR spectrometer (see Apparatus, 1) recommended by the manufacturer and optimize them by preliminary tests. For all calibration and measurement operations, follow the user's manual. Ensure that all operations during calibration and measurement are carried out under the same conditions and, in particular, at the same temperature (±2°C). A minimum of three calibration samples is necessary, although more than three samples may be used.

(c) Enter the parameters for the measurements (i.e., pulse sequence, attenuation, etc.) of the oil or moisture content (as applicable) into the NMR spectrometer following the manufacturer's recommendations, and specify a number under which the calibration curve is to be stored. The total measurement time shall be a minimum of 25 s.

(d) Set the apparatus to the calibration mode.

(e) Introduce a portion of the first calibration sample into a tared sample tube (see Apparatus, 2) up to the optimum height specified by the manufacturer and transfer the sample data from the balance to the NMR apparatus (see Notes, 3).

(f) Enter, as applicable, the value of either the moisture content (as a percentage by mass) or the oil content (as a percentage by mass) into the spectrometer.

(g) Introduce the sample tube containing the first calibration sample into the measuring head and determine the moisture or oil content.

(h) Repeat steps (e) through (g) for the other calibration samples (at least three total).

(i) Calculate automatically or manually the calibration parameters of the calibration curve. The correlation coefficient shall normally be greater than 0.95. If this is not the case, check the values obtained using the reference, or repeat the calibration procedure using three (or more) other calibration samples (see Notes, 4).

(j) Complete the oil-content calibration curve by using an empty tube and repeating steps (e) through (g). This part of the method is not applicable to the moisture-content calibration curve.

2. Calibration procedure for the determination of moisture content:

(a) Procedure A—Three calibration samples with known moisture contents: To obtain a reliable calibration curve, it is necessary that the moisture contents of the calibration samples span the largest possible range. However, a moisture content of zero is not permissible. Follow the general procedure specified in 1(b) through 1(i).

(b) Procedure B—one calibration sample with known moisture content (see Notes, 5).

(c) Follow the general procedure specified in 1(b) through 1(d).

(d) Introduce into three tared sample tubes (see Apparatus, 2) portions of the calibration sample with known water content to three different heights, in accordance with the manufacturer's specifications, and weigh them. Note the corresponding masses, in grams, as m_1, m_2, and m_3 (see Notes, 6).

(e) Calculate the relative moisture content, w, as a percentage by mass, of each of the three portions of the calibration sample as follows:

$$w_1 = (m_1 \times W)/ m_1$$
$$w_2 = (m_2 \times W)/ m_1$$
$$w_3 = (m_3 \times W)/ m_1$$

Where—

m_1, m_2, m_3 = mass, in grams, of the three portions of the calibration sample in each of the three tubes, where m_1 is the largest mass

W = moisture content, as a percentage by mass, of the calibration sample

(f) Enter into the NMR spectrometer the value, m_1, of the mass of the first portion.

(g) Enter into the NMR spectrometer the value, w_1, of the relative moisture content of the first portion.

(h) Insert the tube containing the first portion of the calibration sample into the measuring head and carry out the determination.

(i) Repeat the operations described in 2(f) through 2(h) with the two other portions of the calibration sample, always entering m_1 in step 2(f), then the respective relative moisture contents w_2 and w_3 in step 2(g).

(j) Calculate the calibration parameters in accordance with step 1(h).

3. Calibration procedure for the determination of oil content:

(a) Procedure A—Three calibration samples with known oil contents. Use the samples specified in Calibration Samples, 2. Follow the general procedure specified in Calibration Procedure, 1(b) through 1(j).

(b) Procedure B—One calibration sample with known oil content (see Notes, 7). Follow the general procedure specified in Calibration Procedure, 1(b) through 1(d).

(c) Introduce into three tared sample tubes (see Apparatus, 2) portions of the calibration sample with known oil contents to three different heights, and weigh. Note the corresponding masses, in grams, as m_1, m_2, and m_3.

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(d) Calculate the relative oil content, h, as a percentage by mass, in the three portions of the calibration sample as follows:

$$h_1 = (m_1 \times H)/ m_1$$
$$h_2 = (m_2 \times H)/ m_1$$
$$h_3 = (m_3 \times H)/ m_1$$

Where—

m_1, m_2, m_3 = mass, in grams, of the three portions of the calibration sample
H = oil content of the calibration sample.

(e) Follow the general procedure specified in Calibration Procedures, 1(c) through 1(i), entering the oil content, in step 1(f), in grams.
(f) Enter into the NMR spectrometer the value, m_1, of the mass of the first portion.
(g) Enter into the NMR spectrometer the value, h_1, of the relative oil content of the first portion.
(h) Insert the tube containing the first portion of the calibration sample into the measuring head and carry out the determination.
(i) Repeat the operations described in (f) through (h) with the two other portions of the calibration sample, always entering m_1 in step (f), then the respective relative oil contents h_2 and h_3 in step (g).
(j) To obtain the zero-point on the calibration curve, enter into the NMR spectrometer the value m_1 of the mass and relative oil content of 0.01%. Insert an empty tube into the measuring head and carry out the determination.
(k) Calculate the calibration parameters in accordance with Calibration Procedures 1(i).

PROCEDURE

Carry out at least two determinations on test portions taken from the same test sample.

1. Requirements for the test room and conditioning of the test sample. The temperature of the test room shall be maintained between 17 and 28°C. Therefore, it is recommended that the test room be temperature controlled. Transfer the test samples (see Preparation of Test Sample) to the test room at least 60 min before the determination to allow them to reach equilibrium temperature.
2. Test portion—Prepare test portions of constant volume using one of the following methods:
   (a) Measure a chosen volume of the test sample (see Procedure, 1) and then transfer quantitatively this volume to a tared sample tube (see Apparatus, 2) (e.g., use 40 mL of test sample for sample tubes 40 mm in diameter).
   (b) Transfer a sufficient quantity of the conditioned test sample (see Procedure, 1) to the sample tube to fill it to an optimal height of 30 mm ± 5 mm. (It is recommended that the sample tubes be marked at a height of 30 mm using a felt-tipped pen.)
3. Determination—Check the calibration prior to each series of measurements (or at least once a day), using a calibration sample of known oil and moisture contents.
   (a) Select the program and the instrument parameters for the moisture or oil content, as applicable, and the number of the calibration curve corresponding to the intended test and to the species oilseed residues.
   (b) Set the apparatus to the measurement mode.
   (c) Weigh the test portion (see Procedure, 2) and transfer the data from the balance to the NMR spectrometer.
   (d) Insert the sample tube containing the test portion into the measuring head and determine the moisture and oil contents.

EXPRESSION OF RESULTS

The result is the arithmetic mean of the results of two or more determinations.

PRECISION

The results of a preliminary interlaboratory test are given in Table 1. Repeatability and reproducibility values have not been calculated because of the low number of laboratories participating.

NOTES

Caution—Remove metallic objects from the proximity of the NMR spectrometer.

NUMBERED NOTES

1. The maximum amplitude of this echo signal is proportional to the oil content. It varies with the sample temperature following a complex law. An increase in temperature decreases the measured value of the echo.
2. Simultaneous indications of the oil and moisture contents can be given by some spectrometers equipped with a microprocessor and a specific program.
3. Manual feed of the mass of the calibration sample into the NMR spectrometer is possible.
4. The calibration curve is stored under the storage number chosen in Calibration Procedure 1(c).
5. In this calibration procedure, varying the height to which the sample tubes are filled with the calibration sample varies the quantity of moisture detected by the NMR spectrometer.

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6. Three calibration points are the minimum for establishing the calibration curve.
7. In this calibration procedure, varying the height to which the sample tubes are filled with the calibration sample varies the quantity of oil detected by the NMR spectrometer.

REFERENCES

1. ISO 10632, "Oilseed residues—Simultaneous determination of oil and water contents—Method using pulsed nuclear magnetic resonance spectroscopy."

Table 1
Determination of water and oil contents.

		Water content (%)		Oil content (%)	
Sample	Laboratory No.		Mean value		Mean value
Rapeseed	1				
	2	6.0	6.1	19.3	19.5
		6.2		19.7	
	3	5.4	5.5	19.8	20.1
		5.5	20.3		
	4				19.3
Sunflower	1				
(extraction)	2	8.2	8.3	0.9	0.9
		8.3		0.9	
	3	8.0	8.1	1.6	1.8
		8.2		2.0	
	4				1.0
Sunflower	1				
(pressure)	2	6.7	6.8	19.4	19.4
		6.9		19.5	
	3	6.4	6.5	18.4	18.6
		6.5		18.7	
	4				18.7
Soy	1	6.03	5.92	1.05	1.04
		5.80		1.03	
	2	7.9	8.0	2.1	2.1
		8.0		2.1	
	3	8.3	8.2	0.14	0.18
		8.1		0.22	
	4				1.9

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AOCS Standard Procedure Am 1-92

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Determination of Oil, Moisture and Volatile Matter, and Protein by Near-Infrared Reflectance

DEFINITION

This procedure is for the simultaneous determination of oil, moisture and volatile matter, and protein in oilseeds by near-infrared reflectance (NIR).

SCOPE

Determinations may be made either on the cleaned seed or on the sample as received (cleaned seed plus impurities), provided that the impurities do not exceed 2.0% by mass of the sample. The method is valid only within the calibration ranges of the analyzer.

The sample is ground to a specified particle size. The infrared reflectance of the sample is measured by the specified analyzer, which has been accurately calibrated against parameters determined in accordance with other accepted standardized methods (see Notes, 2).

APPARATUS

1. Laboratory hammer mill—225 mm, fitted with 0.8 mm perforated plate screen and clean linen collecting bag (see Notes, 3).
2. Infrared reflectance analyzer—capable of irradiating a sample with near-infrared light, measuring the spectrum of the light reflected from the sample and, by comparison of the absorption at selected wavelengths, computing the required parameters (see Notes, 4).
3. Moisture-proof container—capable of holding the ground sample (see Preparation of Test Sample, 6) without leaving a significant air space.

CALIBRATION OF THE ANALYZER

1. Calibrate the infrared reflectance analyzer in accordance with the manufacturer's instructions. Use at least 40 calibration samples for each parameter, evenly covering the anticipated working range (see Notes, 5).
2. Analyze the calibration samples for reference purposes using accepted standardized methods for oil, moisture, and volatile matter, and protein nitrogen (as determined by a Kjeldahl method). If the respective requirements for precision are not met, reject the result and repeat the analysis (see Notes, 6).
3. The calibrations shall have a multiple correlation coefficient between the reference and the infrared reflectance analysis results of not less than 0.8. The standard error of results about the calibration line shall be not more than 0.3 for oil content, 0.2 for moisture and volatile matter and 0.4 for protein (see Notes, 7).
4. Reliable computer and software should be used to maintain calibration.

SAMPLING

1. The laboratory sample should be taken in accordance with ISO 542, or other accepted standardized sampling technique.

PREPARATION OF TEST SAMPLE

1. Prepare a representative test sample from the laboratory sample in accordance with an accepted standardized sampling technique. If, before the reduction of the laboratory sample, large nonoleaginous foreign bodies have been separated, take this into account in the expression and calculation of results.
2. Determine any impurities.
3. If required (e.g., by contract), clean the sample in accordance with an accepted standardized technique. Otherwise, if the impurities are not below 2.0% by mass of the laboratory sample, clean the laboratory sample so that purities are reduced to below 2.0% by mass of the sample.
4. Fit the 0.8 mm perforated plate screen to the hammer mill and clamp a clean linen collecting bag over the screen.
5. Pour the laboratory sample at a steady rate into the feed tray of the hammer mill so that a sample of 200 g is ground in 1–2 min. Do not exceed this feed rate, otherwise the results may be inaccurate (see Notes, 8).
6. Immediately transfer the ground material (test sample) to the moisture-proof container (Apparatus, 3).
7. Between each grinding, brush the screen and adjacent areas free from any debris. Turn the bag inside out, shake and brush thoroughly to remove any clinging debris.
8. Allow the hammer mill and the sample collecting bag to cool for 10 min after grinding 10 test portions. Thoroughly clean the hammer mill and sample collecting bag after every 20 test portions processed (see Notes, 9).

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PROCEDURE

1. Leave the test portion of the ground material in the moisture-proof container (Preparation of Sample, 6) for at least 1 hr to reach ambient temperature.
2. Fill the glass cell of the analyzer (Apparatus, 2) with the test portion (Procedure, 1) and place it in position. Set the analyzer into operation, and pay particular attention to the instructions in steps 3–6 that follow.
3. Ensure that the quartz cell in the analyzer is kept scrupulously clean and that the window is thoroughly cleaned before each individual measurement. Brush all extraneous ground material or dust from the rest of the cell between each measurement.
4. Perform the measurements immediately after filling the cell. Do not make measurements on material that has been in the instrument for a period of time exceeding one measuring cycle.
5. Keep all test portions awaiting measurement in a cool, dark place, away from sunlight and away from the path of the cooling system of the analyzer.
6. Avoid any jarring of the packed test portion in the cell before making measurements, as bulk density of the material in the cell is of major importance in all infrared reflectance techniques.
7. Carry out two determinations on the same test sample.

PRECISION

1. Repeatability—If the duplicate results differ in absolute terms by more than 0.4% for oil, 0.3% for moisture and volatile matter and 0.6% for protein, repeat the determinations.

EXPRESSION OF RESULTS

1. Provided that the requirements for repeatability (see Precision) are met, record the mean of two determinations (Procedure, 7) for each parameter and report as percentages by mass. If the determination is on cleaned seed and the result is to be expressed on the laboratory sample as received, determine impurities and apply a correction factor.
2. The test report should state the following:
   (a) the method used and the results obtained;
   (b) the make and model of infrared reflectance analyzer;
   (c) whether the determinations were carried out on cleaned samples or samples as received;
   (d) whether the results are expressed on the sample as tested or on the cleaned seed with the results expressed on the material as received;
   (e) any operating conditions not described in this procedure, or regarded as optional, and any circumstances that may have influenced the results;
   (f) all details required for the complete identification of the sample.

NUMBERED NOTES

1. Although this method is based on a specific infrared reflectance analyzer (see Notes, 4), a suitable alternative analyzer may be used provided that it satisfies the accuracy requirements specified in the Calibration of the Analyzer section.
2. In the preparation of this method by the British Standard Food and Agriculture Standards Committee, the oil content was defined as the oil value as determined by the method described and numerically equal to the figure obtained in accordance with the method described in BS 4289, Part 4; the moisture and volatile matter content was defined as the moisture and volatile matter value as determined by the method described and numerically equal to the figure obtained in accordance with the method described in BS 4289, Part 3.
3. A hammer mill does not provide a uniform grind. On ground materials, the UDY mill with a 1-mm screen is recommended for reflectance calibrations and measurements. Generally, calibrations developed on one grinder are not transferable to a different grinder.
4. Technicon Infra-Analyser models 300 and 400 (obtainable from Technicon Instruments Co. Ltd., Hampshire, England) have been found to be suitable.
5. The results may be applied to other analyzers of the same model with checks as described in Notes, 7. Reserve 10 of the calibration samples for reference samples, to be used during test runs to check calibration. Reference samples should be analyzed daily. Reference samples which have been analyzed by acceptable standardized methods should be used routinely.

   Generally, 40 test portions are not enough for a calibration. The NIR instrument can only predict unknown values based on the range of the calibration samples it is shown; 100–300 test portions, representing various factors (age of sample, genetic variations, processing conditions, temperature effects, age of grinder, operator technique, seasonal growing conditions) that affect the IR spectra, must be included in the calibration test portions.
6. The manufacturer's calibration instructions may include a procedure by which some results may be "deleted" from the calibration. It is not recommended that results should be eliminated from the calculations without firm evidence of error.
7. Monthly calibration checks should be performed on 10 samples, comparing the results obtained by IR with those obtained using the methods referred to in Calibration of the Analyzer, 2, to confirm that the grinding mill and infrared apparatus are operating satisfactorily. If necessary, adjustments should be made.
8. A consistent particle size is critical in methods utilizing infrared reflectance. It is essential, therefore, that the grinding procedure described is strictly adhered to and that test portions are prepared in exactly the same manner each time, otherwise

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the results obtained may be inaccurate. The test portion should be reduced to a uniform grind of 100–300 µm. It is critical that there be no moisture loss; therefore, the design of the grinder is quite important (see Notes, 3). Over time, wear on the grinding blades will change the uniformity of the ground laboratory samples and results will be affected. The grinder should be checked frequently for wear; compare reference sample and check sample results obtained from date when grinder was in new condition (or from date when grinding blades were replaced) with current results. Grinder maintenance should be performed once per year or after grinding 2000 samples, whichever comes first.

9. If the hammer mill is used for grinding different types of oilseed, it is essential to thoroughly clean it and the sample collecting bag before it is used for a different type, irrespective of the number of test portions ground.
10. Many near-infrared instruments are now available which allow analysis of intact seeds in either reflective or transmittance mode. AOCS Technical Committees are working toward an AOCS Procedure to cover this application.

REFERENCES

1. This method was adapted from British Standards Institute method BS 4289, Part 6 (1986). BS Method 4289 was prepared under the direction of the Food and Agriculture Standards Committee.

OTHER REFERENCES

*J. Am. Oil Chem. Soc. 58:*819 (1981).
*Ibid. 65:*1627 (1988).
*Ibid. 68:*34 (1991).
*Ibid. 74:*324 (1991).

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AOCS Analytical Guidelines Am 1a-09
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Near Infrared Spectroscopy Instrument Management and Prediction Model Development

DEFINITION

This procedure provides general guidelines for the development and the validation of near infrared (NIR) prediction models and their related instrument management.

SCOPE

This procedure presents general tools for the management of near infrared (NIR) instruments, the development of NIR prediction models, their validation, evaluation, and transfer to other units. NIR spectroscopy is a spectroscopic technique measuring the sample absorption of a beam of NIR light. The absorption spectrum (absorbance value at each wavelength of interest) is the chemical finger print of the sample. For organic matter, the NIR region is mainly populated by overlapping overtone and combination bands of O-H, C-H, and N-H bonds. NIR light absorption occurs when the frequency of the light equals the energy gap needed to raise the molecule excitation level to energy levels $v2$ and higher; this resulting in vibration and rotation of the molecule. The chemical composition of the sample can be determined by using multivariate regression methods to relate the chemical composition of a calibration set with NIR spectra. NIR spectroscopy is inexpensive, quick, non destructive, and very suitable for online application for quality control and screening. However, attention must be paid when developing the calibration model. NIR precision and accuracy are highly dependent on the representativity of the calibration samples, the quality of the reference methods, and statistical tools used to develop the prediction model.

APPARATUS

NIR measurements are performed with reflectance or transmittance instruments. These guidelines apply equally to both configurations.

1. In reflectance, one side of the sample is illuminated and the reflected light is detected. The light generally penetrates in the first few millimeters of samples. This requires samples to be homogeneous to limit scattering differences.
2. In transmittance, the light is sent through the sample contained in a cup or cuvette with a fixed path length.
3. Several measurements of the same sample are often averaged to increase the signal to noise ratio (S/N). S/N is proportional to the square root of the number of subsamples.

INSTRUMENT PERFORMANCE

1. Warm-up Time—Follow manufacturer recommendations to perform checks on the internal functioning of the spectrometer.
2. Environmental conditions—The instrument must be stored and operated within the conditions of temperature and humidity specified by the manufacturer.
3. Instrument Accuracy—Accuracy defines how close each measurements of the same sample are from each other. It should be tested on a minimum of 20 samples and 3 replicates for each sample. Instrument Accuracy is measured by the standard error of instrumental performance (SEIP) and comparisons are made with the reference measurement for each sample with the first replicate for each sample.

$$SEIP = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \bar{y})^2}{n-1}} \quad (1)$$

where

$y_i = x_i - r_i$ represents the difference between the prediction for the first replicate and the reference value for sample i
$\bar{y}$ represents the average of y_i
n represents the number of sample

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4. Repeatability—Also called precision, measures how close a measurement is from the accepted value. It should be tested on a minimum of 20 representative samples and 3 replicates for each sample. Repeatability is determined by calculating the standard deviation (SD) of the replicates across the entire sample population.

$$SD = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{3}\left(P_{ij} - P_i\right)^2}{2n}} \tag{2}$$

where

P_{ij} represents the predicted value for sample i and replicate j
P_i represents the average of the three predicted values for sample i
n represents the number of sample

5. Daily check—In addition to instrument self-checks and in substitution to Accuracy and Repeatability determination, it is possible to implement daily checks on samples that are usually analyzed by the lab (usually in duplicate). This allows a daily evaluation of instrument performances and the evaluation of instrumental variations due to environmental changes and hardware evolution.

CALIBRATION SAMPLES

1. Representativity—Samples selected for calibration should be representative of the population of samples that the prediction model will encounter. The distribution of the sample should be as nearly uniform as possible. Including a type of samples that for one reason or another will not be predicted in the future may add extraneous noise and reduce the calibration performances. Algorithms have been developed to select the most appropriate samples to include in the calibration set. Depending on the application and calibration algorithm, the minimum number of samples will vary. For a grain calibration, a minimum of 100 samples over the entire range of the constituent is usually a standard.
2. Sample presentation—The sample presentation will vary by instrument and application. However, it is necessary to maintain a standardized process for an application in the presentation of the calibration, validation, and routine samples.
3. Temperature—Sample temperature causes wavelength shifts in the NIR spectrum. If various sample temperature conditions will be encountered, include samples over the range of the calibration whose temperature has been adjusted to 3 to 5 temperatures over the future range of use.
4. Timing—Reference measurements should be performed, on the samples kept in the calibration set, as soon as possible after the spectra were collected. Some elements such as moisture and other volatile constituents can vary over time. For other elements not sensible to environmental conditions, analysis can be done later if needed.
5. Approximation of the number of samples

	Use		
Algorithm	**Concept test**	**Use in controlled conditions**	**Use in uncontrolled conditions (temperature changes ...)**
MLR, PCR, PLS	100	300	500
ANN	500	1000	+1000
SVR	50	100	n/a
LWR	300	500	1000

REFERENCE MEASUREMENTS

1. NIR spectroscopy is an indirect method and its prediction error relies on the error of the laboratory. To measure this error, the standard error of the laboratory can be evaluated.

$$SEL = \sqrt{\frac{\sum_{i=1}^{n}\left[\sum_{j=1}^{r}\frac{\left(y_{ij} - \bar{y}_i\right)^2}{(r-1)}\right]}{n}} \tag{3}$$

where

y_{ij} represents the concentration measured for sample i and replicate j
$\overline{y}_i$ represents the average values of the different replicates for sample i
n represents the number of samples
r represents the number of replicates

A representative block of samples (n > 20) should be assayed repeatedly to establish and update SEL.

2. The error of the prediction model will be a function of the SEL. A calibration model can be, at most, as good as the reference method.

DATA PREPROCESSING

1. Preprocessing or pretreatment methods are techniques used to enhance, smooth, correct, and scale raw spectra.
2. The most common preprocessing methods are:

Preprocessing method	Function
Noise and baseline effect	
Offset correction	Remove constant baseline
Weighted least squares baseline correction	Remove varying baseline
Detrend	Remove constant, linear, or curves baseline
Smoothing	Low pass filter removing high frequency noise
Derivative	Remove baseline and enhance peaks
Normalization	
Standard Normal variate (SNV)	Normalize sample to unit standard deviation across variables
Multiplicative Scatter Correction (MSC)	Remove scattering effects
Variable scaling	
Autoscaling	Standardize samples to mean zero and unit standard deviation across samples
Mean centering	Subtract each sample by the mean sample of the set

3. Consult manufacturer for the list with the various preprocessing methods that are compatible with their instrument software.

CALIBRATION DEVELOPMENT

1. For cases where the relationship is linear and the collinearity among wavelength is low, multiple linear regression (MLR) can be used.
2. For highly correlated spectral data, principal component regression (PCR) and partial least squares (PLS) regression methods have been developed.
3. When dealing with non-linear relationships, learning-based methods such as artificial neural networks (ANN) and support vector regression (SVR) methods can be employed.
4. Most instruments can easily handle calibration models developed using MLR, PCR, and PLS. ANN and SVR require an additional prediction engine because they are not included in most chemometrics suite.

VALIDATION OF THE CALIBRATION MODEL

1. Any calibration model must be validated on samples not included in the calibration set. If a totally independent validation set is available (next batch samples, next year samples), the validation parameters will estimate the true model performances. When independent samples are not available, it is possible to keep a part of the calibration set and use it for validation or to employ cross-validation methods. These methods overstate performances and are normally used only for initial concept studies.
2. If sample temperature is of concern, samples at various temperatures should be included in the validation set.

3. Prediction precision—It can be measured either:
   - During calibration—The standard error of calibration (SEC) will provide a first evaluation of the predictive ability of the model. It is calculated by predicting all calibration samples with the model. It generally provides over-optimistic statistics.
   - During cross-validation—The standard error of cross validation (SECV) is calculated by withholding predetermined groups of samples from the calibration set, developing new models from the remaining samples, and finally predicting left apart samples. The operations is repeated by including withholded samples in the calibration set and predicting a second set of samples isolated for validation. Prediction statistics are combined to output cross-validation error. SECV is usually a better indicator of method precision than SEC. Different cross-validation methods can be used based on the nature of the data.
   - During operation—The standard error of prediction (SEP) is calculated by predicting "unknown" samples. SEP is the best estimate of model precision of a model given the variability encountered in validation is present in calibration. SEC/SECV/SEP are calculated as follows:

$$SE = \sqrt{\frac{\sum_{i=1}^{n}\left(y_i - \hat{y}_{ip}\right)^2 - \frac{\left(\sum_{i=1}^{n}\left(y_i - \hat{y}_{ip}\right)\right)^2}{n}}{n-1}} \quad (4)$$

where

$\hat{y}_{ip}$ represents the predicted value of the sample i in prediction scenario
y_i represents the reference value for the sample i
n represents the number of samples

Note that this is a bias adjusted formula. The root mean square error, including the bias is presented later.

Relative predictive determinant (RPD) is another common statistics for precision. RPD is the ratio of the standard deviation of the reference values of the set to predict to the SEC/SECV/SEP, when applied to this same set. RPD values can be interpreted using the following scale.

RPD values	Classification	Application
0.0–2.3	Very poor	Not recommended
2.4–3.0	Poor	Very rough screening
3.1–4.9	Fair	Screening
5.0–6.4	Good	Quality control
6.5–8.0	Very good	Process control
8.1+	Excellent	Any application

4. Prediction accuracy—Bias is often used to characterize the prediction model accuracy in validation situations along with SEP.

$$Bias = \frac{\sum_{i=1}^{n}\left(y_i - \hat{y}_i\right)}{n} \quad (5)$$

where

$\hat{y}_i$ represents the predicted value of the sample i
y_i represents the reference value for the sample i
n represents the number of samples
Provided the primary (destructive) method is used to calibrate the secondary (non-destructive) method, the bias generally should be very close to zero. If not, of course, it's time to recalibrate.

Another statistics combining both precision and accuracy is the root mean scare error of calibration / cross-validation / prediction and is calculated as follows:

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left(y_i - \hat{y}_{ip}\right)^2}{n-1}} \tag{6}$$

Of course, for all of these calculations, it is important to have a sufficiently large data set (replicates over multiple days, multiple analysts, etc.), and when performing calibration and cross-validation, it is important that the sample set be diverse enough to represent the range of values to be expected in practice.

5. Impact of other sources of error. As mentioned earlier, the final error of a calibration model is function of many parameters, the most common being the error of the laboratory, the sampling error, and the instrumental error.

CALIBRATION TRANSFER

1. It is often necessary to use a calibration model, developed on a master unit, on a secondary unit. This is cheaper than developing a model on the secondary unit. Differences in instrumental properties (absorption, S/N, wavelength alignment) can exist and it is necessary to correct for them.
2. Most of the standardization methods require standardization samples run on both master and secondary units Standardization samples must be representative of the constituent range and be well predicted by the calibration. It is possible that a different standardization set exist for different parameters on the same product. A minimum of 20 samples should be used.
3. Optical methods—They are based on the modification of the spectra collected on the secondary unit to match the spectra on the master unit. Methods such as single wavelength standardization, direct standardization, and piecewise direct standardization can be used. They calculate for each wavelength a slope and a bias that are applied to the spectra of the secondary units.
4. Post-regression correction—A slope and a bias are calculated to match predictions of the standardization set on both master and secondary units.
5. Robust models—It is possible to include in the calibration model, spectra collected on the secondary unit. The variability is modeled by the regression algorithm and no further standardization is required. This can be complex for large numbers of secondary units.
6. A post-standardization set of a minimum of 20 samples (independent from the calibration, validation, and standardization sets) should be used to validate the standardization method.

ROUTINE TEST

1. Follow manufacturer specifications for warm-up time and self-check procedure. Use a daily check to develop control charts and manage the evolution of the instrument and sample over time.
2. Perform frequent instrument check performances of accuracy and repeatability to ensure proper functioning.
3. Prepare samples in the same way they were prepared during the calibration process.

OUTLIER DETECTION

1. Outliers are samples that are statistically different from the population of interest. This difference can come from hardware failures, from sample degradation, or from poor reference values.
2. The detection of such samples is typically done with a Hotelling's T^2 test—a multivariate method that tests the membership of an observation to a group—at a given confidence interval, or by detecting samples in the calibration process that present an abnormally large residual value.
3. Obvious outliers must be removed. However, "abnormal" samples can be representative of a new variability that should be included in the calibration set. There is no substitute for an understanding of the physical or biological system.

MOISTURE BASIS

1. Moisture basis—It is the moisture basis at which the constituent concentration was measured in. The moisture basis is often converted to trade standards such as 12% for wheat, 13% for soybean, and 15% for corn. It is also possible to express predictions in dry basis (0%) or "as-is".
2. Native moisture basis—Calibration models will often be developed with constituents converted to a specific moisture basis (direct moisture basis models) or "as-is". As-is predictions will often require conversion to a constant moisture basis based on the measure of the moisture at the time of the NIR analysis. Most instruments can perform this conversion. Direct moisture basis calibrations will provide unacceptable results if the moisture of the sample is not in the range of samples present in the calibration set.

CALIBRATION OF CORRELATED ELEMENTS

1. Often, the parameter to measure will be highly correlated with other constituents that are commonly predicted by NIR.
2. In some cases, using a simple model, based on common predicted parameters to predict the correlated elements, will provide accurate results and be more economically viable than developing an NIR calibration. However, using indirect measurements such as NIR predictions to predict another element with a simple model can quickly become unstable because of the multiplication of error sources.
3. It is possible that some samples will not follow a general pattern of correlation with one or more easily predicted constituents and will be miss-predicted. A strong validation strategy must be employed to ensure the validity of these third level models.

REFERENCE

AACC International. 2000. Approved Methods of the American Association of Cereal Chemists, 10th Ed. Methods 39-00 and 39-01. The Association: St. Paul, MN.

ASTM International. 2005. Standard Practice for Infrared Multivariate Quantitative Analysis, E 1655—05.

National Conference on Weight and Measures. 2002. Grain Moisture Meters and Near Infrared Grain Analyzers, NCWM Publication 14.

U.S.Pharmacopeia. 2006. Near-Infrared Spectrophotometry, method 1119.

Williams P., 2001. Implementation of Near-Infrared Technology. *In* Near-Infrared Technology in the Agricultural and Food Industries, Williams P. and Norris K. (editors), 2nd ed., AACC Inc., St. Paul, MN, USA.

AOCS

AOCS Official Method Am 2-93

Revised 2011

Determination of Oil Content in Oilseeds

DEFINITION

This method determines the oil content of oilseeds used as industrial raw materials by solvent extraction. The oil content is defined as the whole of the substances extracted under operating conditions specified in this method, and expressed as a percentage by weight of the product either as received or of the cleaned seed. On request, it may be expressed relative to the dry weight. This method is based on ISO Method 659. (See Notes, 1)

SCOPE

Applicable to all oilseeds used as industrial raw materials. In addition, the following may be analyzed separately: the pure seeds and impurities (see Notes, 2); peanuts—the pure seeds, the total fines, and the non- and oleaginous impurities. In general, this method is applicable in international trade and in the resolution of trade disputes. For sunflower seeds see Notes, 10.

APPARATUS

1. Analytical balance.
2. Mechanical mill—easy to clean, appropriate to the nature of the oilseeds and allowing the oilseeds to be ground to a uniform particle size without heating or appreciable change in moisture, volatile matter or oil content (see Notes, 3).
3. Mechanical microgrinder—capable of producing a fineness of grinding of oilseeds of less than 160 µm, with the exception of the "shell," particles of which may reach 400 µm (see Notes, 4).
4. Extraction thimble and cotton wool—free from matter soluble in hexane or light petroleum.
5. Extraction apparatus—(fitted with a 200–250 mL flask). Straight through extractors, (e.g., Butt or Smalley type) have been found suitable. Syphoning Soxhlet extractors are not suitable and should not be used (see Notes, 5),
6. Pumice stone—in small particles, or other antibumping granules, previously dried in an oven at 130 ± 2°C and cooled in a desiccator.
7. Apparatus for safely removing solvent from extraction thimble (e.g., in a current of warm air in a properly operating fume hood).
8. Electric heating bath (sand bath, water bath, etc.) or hot plate.
9. Electrically heated oven—with thermostatic control, capable of being maintained in particular at 103 ± 2°C. The oven should be capable of being used at either atmospheric or reduced pressure.
10. Desiccator—containing an efficient desiccant (see AOCS Specification H 9-87).
11. Electrically heated oven—capable of being maintained at 130 ± 2°C.
12. Metal dish—flat-bottomed, 100 mm dia, about 40 mm height.

REAGENTS

1. Technical *n*-hexane—or light petroleum essentially composed of hydrocarbons with 6 carbon atoms, of which less than 5% distills below 50°C and more than 95% distills between 50 and 70°C and which has a bromine value less than 1 (see Notes, *Caution* and 5).

PREPARATION OF THE TEST SAMPLE

1. Take a test sample from the laboratory sample. If large non-oleaginous foreign bodies have been separated before the reduction of the laboratory sample, make allowance for this in the final calculations (Calculations, 5).
2. According to the requirements of the contract, use a test sample as received or after separation of the impurities.
3. The moisture content of the test portion must be less than 10% when extraction of the oil is begun.
4. Use a rapid screening method to assess the moisture content. If it is greater than 10% reduce it to less than 10% by drying the test sample in a dish in an oven maintained at a temperature not greater than 80°C.
5. Store the partially dried material in an airtight jar. Determine the moisture contents (ISO 665) of the partially dried test sample and the original sample.
6. Care must be taken to clean all mills thoroughly before and after each sample has been ground. Any material adhering to the mill must be incorporated with the bulk of the ground material.
7. In all cases the determination of oil must be begun as soon as possible, and not more than 30 min after the test sample has been milled.
8. In the following text "seed" or "kernels" implies both whole seeds or kernels and broken fragments of them.
   Prepare the test samples by grinding separately 600 g of the well-mixed kernels, and all of the shell and dirt separated during the quantitative determination of impurities according to the method given in ISO 658 (see Notes, 7).

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(a) Palm kernels—shell is considered to be part of the impurities, even when attached to the kernels. Shell and dirt must be separated quantitatively from the kernels before analysis.

Shell is hard and difficult to mill. It is almost impossible to obtain a homogeneous sample if kernels and impurities are milled together. If the oil content of the whole sample (tale quale, TQ) is required, analyze the kernels and impurities (shell and dirt) separately and calculate the oil content (Calculations, 6).

(b) Copra (and coconut)—for copra traded on terms other than under FOSFA no. 1 contract, the entire sample should be frozen before milling in the mechanical mill (Apparatus, 2).

The Christy Norris Laboratory Mill with a 6 mm bar screen has been found satisfactory. The length of particles shall be close to 2 mm but should not be greater than 5 mm. Mix the particles carefully and carry out the determination without delay. Care should be taken to avoid condensation of moisture onto the seeds during and after grinding.

(c) Large and medium sized seeds (illipe, sheanuts, sunflower, peanut, soy, etc.)—Except in the case of cottonseed, grind the analysis sample in the mechanical mill (Apparatus, 2), until the major dimension of the particles obtained is not greater than 2 mm. Reject the first particles (about one-twentieth of a sample), collect the rest, mix carefully and carry out the determination without delay. The grinding of very oily seeds can often be improved if the seeds are frozen at 10–20°C but great care should be taken to avoid condensation of moisture onto the seed during and after grinding (see Notes, 8).

(d) Cottonseed—weigh to the nearest 1 mg in the tared metal dish (Apparatus, 12) about 15 g from the analysis sample as received. Place the dish and seeds in the oven (Apparatus, 11) previously heated to 130°C and dry for 2 hr at 130 ± 2°C. Remove the dish from the oven and allow to cool in air for about 30 min. Transfer the whole test portion to a grinder or mill (Apparatus, 2) and grind to break up the seeds and linters. Transfer the ground material to an extraction thimble (Apparatus, 4) and proceed with the determination.

(e) Small seeds (linseed, rape, etc.)—take a representative portion of approximately 100 g from the analysis sample and grind it (Apparatus, 2) so that no whole seeds remain. Ensure that any fine material remaining in the mill is incorporated into the bulk of the ground material and that the whole is thoroughly mixed. Care must be taken that moisture is not lost from the ground test sample. The grinding time and speed (if variable) should be pre-determined for any particular mill and seed. The grinding should not cause segregation of the "meat" and "husks" of the seed. The test sample shall not be oily and at least 95% (m/m) shall pass a 1 mm sieve.

If oil in the seed, free from impurities, is required, remove the impurities by the method given in ISO 658 and prepare at least 30 g of "clean" seed (including broken seed) as it is impractical to prepare 100 g of very small seed such as sesame seed.

PROCEDURE

1. Preparation of flask—weigh to the nearest mg, a flask (Apparatus, 5) containing a few granules of pumice stone (Apparatus, 6), previously dried in an oven and cooled in a desiccator.
2. Test portion—weigh, 9.5–10.5 ± 0.001 g of the test sample (Preparation of the Test Sample). For treatment of impurities, see Notes, 1.
3. Transfer the test portion to the thimble (Apparatus, 4), using a small wad of cotton wool (Apparatus, 4) moistened with solvent (Reagents, 1) to transfer the last traces of ground seed from the weighing container to the thimble. Use this cotton wool to plug the thimble.
4. Solvent extraction—the times stipulated for the three extractions may be varied slightly (e.g., by ± 10 min). On no account should extended extraction times (e.g., overnight) be employed.
5. First extraction—place the thimble containing the test portion in the extraction apparatus (Apparatus, 5). Pour into flask the necessary quantity of solvent. Fit the flask to the extraction apparatus on the electric heating bath or hot plate (Apparatus, 8). Carry out the heating so that the rate of reflux is at least 3 drops per sec (boiling moderately, not violently).

   After extracting for 4 hr, allow to cool. Remove the thimble from the extraction apparatus and place it in a current of air in a properly operating fume hood to expel the greater part of the residual solvent (Apparatus, 7).
6. Second extraction—empty the contents of the thimble into the microgrinder cylinder and grind for 7 min. For most seeds, six 1 cm dia steel balls in a 150 mL cylinder have been found satisfactory. For cottonseed with adherent linters, three 2 cm balls are satisfactory.

   Put the mixture back into the thimble, using a small piece of cotton wool to remove any residual seed particles from the grinding apparatus and adding this to the thimble and putting the latter back into the extraction apparatus; extract again for another 2 hr, using the same flask containing the first extract. Allow to drain and cool, remove the thimble again, eliminate most of the solvent and repeat Step 6.
7. Third extraction—put the mixture back into the thimble, cleaning the grinding apparatus as before, and putting the thimble back into the extraction apparatus. Extract as before for 2 hr.
8. Removal of solvent and weighing of the extract—remove the greater part of the solvent from the flask by distillation on the electric heating bath or the hot plate. Assist the removal of solvent either by blowing air or, preferably, an inert gas (such as nitrogen or carbon dioxide) into the flask for short periods. Remove the last traces of solvent by heating the flask for 30–60 min in the oven (Apparatus, 9) at 103 ± 2°C at atmospheric pressure, or 80°C under vacuum (see Notes, 9).
   (a) In the case of oilseeds rich in volatile acids (copra, palm kernels, etc.) drying of the extract shall be carried out at atmospheric pressure and 80°C maximum.

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(b) In the case of drying or semidrying seeds, it is preferable to remove the residual solvent by drying under reduced pressure.
(c) In the case of nonlauric oils, when a vacuum oven is available, traces of solvent may be removed by heating at 80°C under vacuum.

9. Allow the flask to cool in the desiccator (Apparatus, 10) for at least 1 hr, to ambient temperature and weigh to the nearest 1 mg.
10. Heat again for 10 min under the same conditions, allow to cool and weigh.
11. The difference between the two weighings should not exceed 10 mg. If it does, repeat the operations of heating for 10 min, cooling and weighing until the difference between two successive weighings does not exceed 10 mg. Note the final weight of the flask.
12. If a slight increase in weight is observed, take the previous (lowest) weight.
13. If there is a significant increase in weight (over 5 mg), oxidation of a drying oil may be taking place and further analysis should be carried out, taking precautions to exclude oxygen.
14. Impurities content of the extracted oil—the oil extracted shall be clear; if it is not, determine the impurities content. For this purpose, dissolve the fatty matter in the solvent used for extraction; filter through a filter paper, previously dried at 103 ± 2°C to constant mass; wash the filter paper several times with the same solvent to remove the oil completely and dry again at 103 ± 2°C to constant weight (to cool and weigh the filter paper, use a suitable vessel provided with a lid).
15. Carry out at least two determinations of the same test sample (see Precision)

CALCULATIONS

1. In all cases express the results to two decimal places and take as the result the arithmetic mean of the two determinations, provided that the requirement concerning repeatability (see Precision) is satisfied. Otherwise, repeat the determination on two other test portions. If the difference still exceeds 0.60 g per 100 g of sample, take as the result the arithmetic mean of the four determinations carried out, provided that the maximum difference between the individual results does not exceed 1.50%.
2. Determination of product as received—the oil content, expressed as a percentage by weight of the product as received (tale quale, TQ):

$$\text{Oil content, mass \% as received} = \frac{m_1}{m_0} \times 100$$

m_0 = mass of the test portion (Procedure, 2), g
m_1 = mass of the dried extract (Procedure, 12), g

3. Oil content expressed in relation to the dry matter—on request, the oil content may be expressed as a percentage by weight of the dry matter:

$$\text{Oil content, \% dry mass} = \frac{H_0 \times (100)}{(100 - U)}$$

Where—
H_0 = mass % of oil in the product as received
U = mass % of water and volatile matter, determined according to ISO 771

4. Oil content expressed on the basis of any prescribed moisture content—it may be necessary to convert the oil of a sample, determined at one moisture content, to that at another moisture content, e.g., if a damp test sample has been partially dried before the test sample has been weighed out. In this case:

$$H_2 = H_1 \times \frac{(100 - m_2)}{(100 - m_1)}$$

Where—
H_1 = oil content at moisture content m_1
H_2 = oil content at moisture content m_2
m_1 = moisture content 1, %
m_2 = moisture content 2, %

5. Oil content with separation of non-oleaginous foreign matter

$$\text{Oil content} = H_0 \times \frac{(100 - x)}{100}$$

Where—
H_0 = mass % of oil in material analyzed
x = mass % non-oleaginous foreign bodies previously separated from original product as received

6. Oil content from palm kernels is calculated from separate analyses of the seeds and impurities. This formula serves for calculating the oil content of both seeds and impurities when analyzed separately.

$$\text{Oil content, mass \% as received} = H_1 - \frac{P}{100}(H_1 - H_2)$$

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Where—

H_1 = mass % oil in seeds
H_2 = mass % oil in impurities
P = mass % impurities in sample received

7. Oil content for peanuts as received:

$$\text{Oil content, mass \% as received} = H_1 - \left(\frac{P + I_o + I_n}{100}\right)(H_1 - H_2)$$

Where—

H_1 = mass % oil in pure seeds
H_2 = mass % oil in impurities
I_o = mass % oleaginous impurities
I_n = mass % non-oleaginous impurities
P = mass % total fines

PRECISION

Details of an interlaboratory test of the precision of the method are summarized in Table 4.

1. Repeatability limit—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) shown in Tables 1, 2 or 3.
2. Reproducibility limit—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) shown in Table 1, 2 or 3.

NOTES

Caution

n-Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. It is recommended by the UMC that this method replace Ai 3-75 and Af 3-54.
2. To determine the oil content of the impurities, carry out the analysis in the same manner as for the seeds, with the following modifications: the test portion may be 5–10 g; only one extraction, for a period of 4 hr, is necessary; the small error introduced into the oil content of the product as received is negligible.
3. The following mills have been found to give satisfactory results:
   (a) Christy Norris 8 inch (20.3 cm) Laboratory Mill, Ultra Centrifugal Mill (UCM) (Glen Creston) with perforated plate or bar-type screens, depending on seed type (e.g., 0.8 mm perforated plate, 3 and 6 mm bar screens), (see Preparation of Sample 8, a–c).
   (b) Ultra Centrifugal Mill (UCM) (Glen Creston) with 1 mm screen for sunflower seeds and other screens according to seed type.
   (c) Glen Mills Grinder, manufactured by F. Kurt Retsch BmgH., Rheinische Strasse-36, 5657 Haan-1, Germany. Distributed in the United States by Glen Mills Co., 395 Allwood Drive, Clifton, NJ 07012, USA.
   (d) Other mills may be satisfactory. They must conform to the criteria noted in Apparatus, 2.
4. A Dandoumau Prolabo Analytical Grinder with a 150 mL cylinder and 1 cm, 2 cm, and 3 cm diameter steel balls (approximately 7g, 30g, and 130g, respectively) according to the sample being ground, has been found suitable.

   The following micro grinders have also been found suitable: the Retsch, the EI Retsch, the Planetary Ball Mill, the S1 and S2 Centrifugal Ball Mill, the Batam Mikro Pulverizer Hammer Mill, the IKA Mill, the Fritch Pulverisette 5 Planetary Ball Mill, and the Steam Mill.
5. (a) Various problems have been identified; the temperature of extraction varies.
   (b) When cool, almost pure, solvent syphons onto the hot extract, rapid volatilization can take place, leading to flooding in the condenser and other problems, which can involve loss of oil and poor quantitation.
6. For either solvent, the residue on complete evaporation should not exceed 2 mg/100 mL.
7. The following milling techniques have been found suitable:
   —for pure kernels: grind in a Christy Norris Laboratory Mill with a 6 mm bar screen
   —for shell and dirt: grind for 10 min in a microgrinder using a 3 cm steel ball.
8. The following milling techniques have been found satisfactory:
   —for soy: Christy Norris Laboratory Mill with 0.8 mm perforated plate, or Ultra Centrifugal Mill with 1 mm screen.

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—for sunflower: UCM Mill with 1 mm screen
—for peanut: Christy Norris Laboratory Mill with 3 mm bar screen. With samples containing more than about 45% (m/m) of oil, care should be taken to avoid, as far as possible, the formation of a pasty mass.
—other seeds: 6 mm bar screen

9. In the case of oil seeds rich in volatile acids (copra, palm kernels, etc.) drying of the extract should be carried out at atmospheric pressure, and at 80°C maximum. In the case of drying or semi-drying seeds, it is preferable to remove the residual solvent by drying under reduced pressure. In the case of non-lauric oils, when a vacuum oven is available, traces of solvent may be removed by heating at 80°C under vacuum.
10. FOSFA International has recently introduced an additional determination of moisture content in the preparation of the test sample. A moisture determination is made on the whole seeds and immediately following grinding to allow for a correction to be made for water loss during grinding. Full details of the method and the collaborative study testing this modification may be obtained from FOSFA International, 20 St. Dunstan's Hill, London, EC3R 8NQ, UK.
Tel +44(0)207 283 5511, fax +44(0)207 623 1310, E-mail contact@fosfa.org

REFERENCES

1. FOSFA Official Method; *Determination of oil content in oilseeds.*
2. ISO 664, *Oilseeds—Reduction of contract samples to analysis samples.*
3. ISO 665, *Oilseeds—Determination of moisture and volatile content.*
4. ISO 5725: 1986, *Precision of test methods—Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests (now withdrawn) was used to evaluate the precision data.*

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Table 1
An international collaborative study, carried out by FOSFA International and completed in 1990, in which 33 laboratories participated, each obtaining two test results for each sample of soybeans analyzed, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarized in the following table.

	Sample A	Sample B
Number of laboratories	33	33
Number of accepted results	30	30
Number of laboratories removed	2	2
Mean, % oil	20.60	20.67
Repeatability std. dev., S_r	0.13	0.14
Repeatability relative std. dev., RSD_r	0.64	0.67
Repeatability value, $r = 2.8 \times S_r$	0.37	0.40
Reproducibility std. dev., S_R	0.36	0.39
Reproducibility relative std. dev., RSD_R	1.74	1.87
Reproducibility value, $R = 2.8 \times S_R$	1.01	1.09
R/r	2.7	2.7

Sample key:
A = South American soybeans
B = Duplicate of sample A

Table 2
An international collaborative study, carried out by FOSFA International and completed in 1992, in which 42 laboratories participated, each obtaining two test results for each sample of rapeseed analyzed, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarized in the following table.

	Sample A	Sample B
Number of laboratories	42	42
Number of accepted results	40	40
Number of laboratories removed	1	1
Mean, % oil	42.07	41.98
Repeatability std. dev., Sr	0.14	0.15
Repeatability relative std. dev., RSDr	0.34	0.35
Repeatability value, $r = 2.8 \times S_r$	0.41	0.42
Reproducibility std. dev., S_R	0.54	0.56
Reproducibility relative std. dev., RSD_R	1.31	1.32
Reproducibility value, $R = 2.8 \times S_R$	1.51	1.57
R/r	3.83	3.74

Sample key:
A = Rapeseed
B = Duplicate of sample A

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Table 3
An international collaborative study, carried out by FOSFA International and completed in 1994, in which 22 laboratories participated, each obtaining two test results for each sample of sunflower seed analyzed, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarized in the following table.

	Sample A	Sample B
Number of laboratories	22	22
Number of accepted results	22	22
Number of laboratories removed	0	0
Mean, % oil	45.85	45.91
Repeatability std. dev., S_r	0.18	0.16
Repeatability relative std. dev., RSD_r	0.39	0.35
Repeatability value, $r = 2.8 \times S_r$	0.50	0.44
Reproducibility std. dev., S_R	0.53	0.54
Reproducibility relative std. dev., RSD_R	1.15	1.18
Reproducibility value, $R = 2.8 \times S_R$	1.47	1.51
R/r	2.94	3.43

Sample key:
A = Sunflower seed
B = Duplicate of sample A

Table 4
Statistical results of the interlaboratory test.

	Sample		
Parameter	Rapeseed meal	Soy meal	Sunflower meal
Number of laboratories retained after eliminating outliers	13	13	13
Number of accepted laboratories	9	11	7
Mean oil content [% (m/m)]	4.0	1.4	3.1
Repeatability standard deviation, S_r [% (m/m)]	0.1	0.1	0.0
Repeatability coefficient of variation, CV_r (%)	8.0	20.0	4.3
Repeatability limit, $r = 2.8 \times S_r$ [% (m/m)]	0.3	0.3	0.1
Reproducibility standard deviation, S_R [% (m/m)]	0.2	0.1	0.1
Reproducibility coefficient of variation, CV_r (%)	14.3	28.9	6.1
Reproducibility limit, $R = 2.8 \times S_R$ [% (m/m)]	0.6	0.4	0.2

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Oil in Oilseeds: Supercritical Fluid Extraction Method

DEFINITION

Oil is extracted from the prepared oilseed with a supercritical fluid (liquid carbon dioxide) and deposited directly onto glass wool contained in a collection apparatus. After evaporation of moisture and any residual modifier from the glass wool and collection apparatus, the weight of extracted oil is determined and used to calculate the percent oil in the original sample.

SCOPE

This two-part method is specifically designed to determine either (a) process-scale extractable oil with results comparable to AOCS methods for determining oil content using the Butt extraction apparatus, or (b) total oil content with results comparable to exhaustive extraction methods. The method is applicable to the determination of the oil content of soybeans, safflower, sunflower, cottonseed, and canola. Prepared samples are extracted with either supercritical CO_2 alone or CO_2 + 15% ethyl alcohol as a modifier. Flow rates are given in temperature-independent flow values (see Notes, 1).

APPARATUS

1. Supercritical fluid extractor (SFE)—capable of producing a pressure of 7,500 psi and a temperature of 100°C. The extractor should be equipped with a heated restrictor (adjustable or fixed flow) capable of keeping the temperature of the evolving CO_2 above 40°C. For extractions with CO_2 + 15% ethyl alcohol, an on-line modifier pump is recommended (see Notes, 1).
2. Sample extraction cells—5–10 mL internal volume.
3. Extract collection vessels as appropriate for commercial systems. For in-house designed systems, use 20 × 125 mm culture tubes.
4. Analytical balance—accurate to ± 0.0001 g.
5. Vacuum oven and pump capable of maintaining drying conditions as outlined in AOCS Official Method Ca 2d-25 or a forced-air oven capable of maintaining drying conditions as outlined in AOCS Official Method Ca 2c-25.

REAGENTS

1. Carbon dioxide (CO_2)—welding or industrial grade with dip tube. Purer grades of CO_2 may be used, but are not required.
2. Denatured ethyl alcohol—HPLC grade or equivalent. Moisture content above 1 mg/kg is not recommended. The denaturing solvent type is unimportant.
3. Granular diatomaceous earth.
4. Glass wool—borosilicate glass variety.

PROCEDURE

1. CO_2 only extraction to simulate process extraction—
   (a) Prepare test sample as described in Section A of AOCS Official Methods, using the method specific for the oilseed being analyzed.
   (b) Accurately weigh a 2 g test portion into the extraction cell (Apparatus, 2) with the outlet endcap in place and tap the cell several times on a work surface to pack the test portion into an even plug in the outlet end of the cell. Record the test portion weight to ±0.0001 g. Fill the remaining void in the cell with granular diatomaceous earth (Reagents, 3) and depress the upper surface of the diatomaceous earth approximately 3 mm to tighten the packing. Install the inlet endcap and clean any sample or filler from the outside.
   (c) Take a minimum of 1 g glass wool (Reagents, 4) and pack it into each collection vessel (Apparatus, 3) until the top of the resulting plug is at least 2.5 cm from the bottom of the vessel. Accurately weigh the collection vessel with glass wool to ±0.0001 g and record the weight.

   Note—Caps or septa on the collection vessel are not required for this procedure unless the SFE instrument requires their use.

   (d) Install each collection vessel on the collection reel (for automated systems), or install each collection vessel with the restrictor or the transfer tube leading from the restrictor being inserted as deeply as possible into the glass wool plug (for manual loading systems).

   Note—Some instruments may require adjustment of restrictor depth or additional glass wool to ensure deep positioning of the restrictor or transfer tube within the glass wool.

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(e) Load each extraction cell on the instrument reel (for automated systems), or install each extraction cell into the extractor (for manual loading systems).

Note—Cells should be oriented so that the sample is in the outlet end.

(f) Extract for 30 min with CO_2 under the following conditions: 7,500 psi; 100°C; nominal liquid flow rate of 3.55 g/min (see Notes, 3); restrictor temperature such that evolving CO_2 is at a temperature of at least 40°C but less than 130°C (see Notes, 4).

(g) Remove each collection vessel from the instrument and if necessary (see Notes, 5), dry residual moisture from the oil to constant weight using the temperatures and pressures noted in AOCS Official Method Ca 2d-25 (preferred method), or AOCS Official Method Ca 2c-25 (alternate method). Record the final collection vessel weight to ±0.0001 g.

2. CO_2 + 15% ethyl alcohol extraction to simulate exhaustive extraction—

(a) Prepare test sample as described in Section A of AOCS Official Methods, using the method specific for the oilseed being analyzed.

(b) Accurately weigh a 2 g test portion into the extraction cell (Apparatus, 2) with the outlet endcap in place and tap the cell several times on a work surface to pack the test portion into an even plug in the outlet end of the cell. Record the test portion weight to ±0.0001 g. Fill the remaining void in the cell with granular diatomaceous earth (Reagents, 3) and depress the upper surface of the diatomaceous earth approximately 3 mm to tighten the packing. Install the inlet endcap and clean any test portion or filler from the outside.

(c) Take a minimum of 1 g glass wool (Reagents, 4) and pack it into each collection vessel (Apparatus, 3) until the top of the resulting plug is at least 2.5 cm from the bottom of the vessel. Accurately weigh the collection vessel with glass wool to ± 0.0001 g and record the weight.

Note—Caps or septa on the collection vessel are not required for this procedure unless the SFE instrument requires their use.

(d) Install each collection vessel on the collection reel (for automated systems), or install each collection vessel with the restrictor or the transfer tube from the restrictor being inserted as deeply as possible into the glass wool plug (for manual loading systems).

Note—Some instruments may require adjustment of restrictor depth or additional glass wool to ensure deep positioning of the restrictor or transfer tube within the glass wool.

(e) Load each extraction cell on the instrument reel (for automated systems), or install each extraction cell into the extractor (for manual loading systems).

Note—Cells should be oriented so that the sample is in the outlet end.

(f) Extract for 60 min with CO_2 + 15% ethyl alcohol under the following conditions: 7,500 psi; 100°C; nominal liquid flow rate of 2.22 g/min (see Notes, 3); and restrictor temperature such that evolving CO_2 is at a temperature of at least 40°C but less than 130°C (see Notes, 4).

(g) Remove each collection vessel for the instrument and if necessary (see Notes, 4), dry residual moisture from the oil to constant weight using the temperatures and pressures noted in AOCS Official Method Ca 2d-25 (preferred method), or AOCS Official Method Ca 2c-25 (alternate method). Record the final collection vessel weight to ±0.0001 g.

CALCULATIONS

1. Calculate the percent oil as follows:

$$\text{oil, \%} = \frac{\text{collection vessel weight gain, g}}{\text{test portion mass, g}} \times 100$$

PRECISION

1. Repeatability limit—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than repeatability limit r as shown in Table 1 (CO_2 only extractions) or Table 2 (CO_2 + 15% ethanol modifier extractions).
2. Reproducibility limit—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit R as shown in Table 1 (CO_2 only extractions) or Table 2 (CO_2 + 15% ethanol modifier extractions).

NOTES

Caution

Supercritical fluid extraction utilizes compressed gasses as an extraction fluid. Therefore, it is imperative that proper procedures be followed for the handling and storing of compressed gas cylinders. SFE equipment must be constructed and maintained in proper working order to minimize the chance of any explosive decompression of extraction fluid. Although CO_2 is a nontoxic gas, adequate ventilation in the laboratory must be maintained to avoid the possibility of displacing too much oxygen. Furthermore, adequate ventilation must be maintained when using modifiers to avoid the toxic effects of breathing organic solvent vapors.

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Verification of SFE Equipment Condition and Collection Efficiency / Technique

The two most likely reasons for low percent oil values are major leaks in the SFE instrument or inefficient collection of the extracted oil. Before the initial use of this method, it is highly recommended that replicate 400 mg spikes of soybean oil on granular diatomaceous earth be extracted, using the desired set of conditions and drying procedure, to verify equipment condition, collection efficiency and procedure. Recovery of the oil spike should be >99% and no aerosol of oil should be observed rising above the glass wool during extraction. The operator should then periodically extract oil spikes to test the equipment and collection technique.

NUMBERED NOTES

1. The temperature-independent flow value is given to allow for conversion to the various flow measurement techniques that might be used by various instrument manufacurers. Also, due to the temperature-dependent density of both gaseous and liquid CO_2, errors can be introduced by failing to consider the temperature of the flow measurement. Standardized flow rate units are given in Tables 4 and 5.
2. Static modifier addition techniques and the use of premixed gas cylinders were not studied during the method development phase of this study. However, three laboratories participating in the collaborative study utilized a static modifier addition technique to return % oil values within the standard deviation for the data set. No laboratories returned values using premixed CO_2/modifier cylinders. Analysts desiring to utilize alternate modifier delivery options should validate their results against Am 2-93 on an individual laboratory basis.
3. A liquid flow rate of 2.77–3.55 g/min is permissible.
4. A liquid flow rate of 1.66–2.77 g/min is permissible.
5. The temperature of the expanding CO_2 in the collection vessel can have a direct bearing on the amount of moisture and/or modifier entrained in the extracted oil and the subsequent time required to remove it before the weight can be determined. Overheating can cause loss of the more volatile oils. An expanding CO_2 temperature of 80–100°C is recommended. If the instrument has the capability of heating the collection vessels, a lower expanding CO_2 temperature may be used. Consult your instrument manufacturer for restrictor/collection temperature settings that will minimize moisture (and modifier, if used) removal time. It has been reported that some instruments may not require subsequent removal of moisture or modifier from the extract. This should be verified by drying the extract according to procedure and ensuring that no significant weight loss is observed before the drying step is eliminated.

Table 1

An international interlaboratory study completed by the AOCS Seed and Meal Analysis Technical Committee in 1996, coordinated by Leslie Myer, in which 17 laboratories were requested to provide two test results for each of 10 test samples analyzed by supercritical fluid extraction method using CO_2 alone (without modifier), gave statistical results determined by ISO 5725-1994 and have been summarized in this table.

Collaborative study sample	A/M	E/I	G/R	C/P	K/Q	D/J	B/S	H/N	F/T	L/O
Oilseed[a]	Soy4	Soy7	Sun1	Sun3	Can4	Can3	Saf2	Saf1	Cot1	Cot2
Laboratories after outliers	14	14	15	15	15	15	14	14	14	14
Laboratories removed	3	3	2	2	2	2	3	3	3	3
Accepted values, *n*	28	28	30	30	30	30	28	28	28	28
Mean oil content, *x*	19.2	19.2	38.9	41.6	39.8	37.7	36.6	35.7	19.1	18.4
AOCS method[b]	19.06	19.35	38.74	42.71	43.74	36.81	37.99	36.41	18.17	17.53
Exhaustive extraction[c]	21.67	21.89	41.75	46.01	45.19	42.73	40.64	39.07	20.61	20.00
S_r	0.30	0.90	1.02	1.65	0.45	0.67	1.39	0.86	0.46	0.34
RSD_r	1.55	4.67	2.63	3.97	1.13	1.78	3.79	2.40	2.39	1.85
r	0.83	2.51	2.86	4.62	1.26	1.88	2.25	2.40	1.28	0.95
S_R	0.45	0.91	1.68	2.30	0.90	1.03	1.66	1.08	0.51	0.38
RSD_R	2.35	4.75	4.32	5.54	2.26	2.74	4.55	3.03	2.68	2.05
R	1.27	2.55	4.70	6.45	2.51	2.89	4.65	3.03	1.44	1.06
R/r	1.5	1.0	1.6	1.4	2.0	1.5	2.1	1.3	1.1	1.1

[a] Oilseeds from 1995–1996 AOCS Laboratory Proficiency Program: soy, soybean; sun, sunflower; can, canola; saf, safflower; cot, cottonseed.
[b] Solvent extraction, Butt-tube extraction method, AOCS Official Methods appropriate to oilseed, AOCS Laboratory Proficiency Program results for 1995–1996.
[c] Solvent extraction, AOCS Official Method Am 2–93 (FOSFA International method).

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Table 2

An international interlaboratory study completed by the AOCS Seed and Meal Analysis Technical Committee in 1996, coordinated by Leslie Myer, in which 16 laboratories were requested to provide two test results for each of 10 test samples analyzed by supercritical fluid extraction method using CO_2 + ethyl alcohol modifier, gave statistical results determined by ISO 5725-1994 and have been summarized in this table.

Collaborative study sample	A/M	E/I	G/R	C/P	K/Q	D/J	B/S	H/N	F/T	L/O
Oilseed[a]	Soy4	Soy7	Sun1	Sun3	Can4	Can3	Saf2	Saf1	Cot1	Cot2
Laboratories after outliers	12	13	12	12	12	14	12	12	12	14
Laboratories removed	3	2	3	3	3	1	3	3	3	1
Accepted values, *n*	24	26	23	24	24	28	23	23	23	27
Mean oil content, *x*	20.5	20.4	39.7	43.0	43.5	40.2	38.0	36.7	19.7	19.3
Exhaustive extraction[b]	21.67	21.98	41.75	46.01	45.19	42.73	40.64	39.07	20.61	20.00
AOCS method[c]	19.06	19.35	38.74	42.71	43.47	36.81	37.99	36.41	18.17	17.53
S_r	0.43	0.58	0.44	0.63	0.37	0.86	0.77	0.67	0.18	0.35
RSD_r	2.08	2.85	1.10	1.47	0.85	2.15	2.02	1.81	0.91	1.81
r	1.19	1.63	1.22	1.78	1.04	2.42	2.15	1.86	0.50	0.98
S_R	0.77	0.87	1.65	1.64	1.32	1.43	1.14	1.48	0.52	0.52
RSD_R	3.76	4.25	4.17	3.81	3.02	3.56	3.00	4.04	2.65	2.70
R	2.16	2.43	4.63	4.59	3.68	4.00	3.18	4.15	1.46	1.46
R/r	1.8	1.5	3.8	2.6	3.5	1.7	1.5	2.2	3.0	1.5

[a]Oilseeds from 1995–1996 AOCS Laboratory Proficiency Program: soy, soybean; sun, sunflower; can, canola; saf, safflower; cot, cottonseed.
[b]Solvent extraction, AOCS Official Method Am 2-93 (FOSFA International method).
[c]Solvent extraction, Butt-tube extraction method, AOCS Official Methods appropriate to oilseed, AOCS Laboratory Proficiency Program results for 1995–1996.

Table 3

Comparison of methods: Supercritical fluid extraction (SFE) vs. solvent extraction methods.

Method	SFE CO_2 alone	AOCS Ai 3-75[a]	SFE CO_2 alone	AOCS Ai 3-75[a]	SFE CO_2 + Modifier[b]	AOCS Am 2-93[c]	SFE CO_2 + Modifier[b]	AOCS Am 2-93[c]
Oilseed[d]	Sun1	Sun1	Sun3	Sun3	Sun1	Sun1	Sun3	Sun3
Mean oil content, *x*	38.9	38.46	41.6	42.68	39.7	41.75	43.0	46.01
S_r	1.02	0.10	1.65	0.09	0.44		0.63	
RSD_r	2.63	0.25	3.97	0.20	1.10		1.47	
r	2.86	0.28	4.62	0.25	1.22		1.78	
S_R	1.68	1.24	2.30	0.5	1.65		1.64	
RSD_R	4.32	3.24	5.54	1.17	4.17		3.81	
R	4.70	3.47	6.45	1.40	4.63		4.59	
R/r	1.6	2.8	1.4	5.6	3.8		2.6	
Method	SFE CO_2 alone	AOCS Ac 3-44[a]	SFE CO_2 alone	AOCS Ac 3-44[a]	SFE CO_2 + Modifier[b]	AOCS Am 2-93[c]	SFE CO_2 + Modifier[b]	AOCS Am 2-93[c]
Oilseed[d]	Soy4	Soy4	Soy7	Soy7	Soy4	Soy4	Soy7	Soy7
Mean oil content, *x*	19.2	18.95	19.2	19.27	20.5		20.4	
S_r	0.30	0.03	0.90	0.05	0.43		0.58	
RSD_r	1.55	0.18	4.67	0.27	2.08		2.85	
r	0.83	0.08	2.51	0.14	1.19		1.63	
S_R	0.45	0.36	0.91	0.39	0.77		0.87	
RSD_R	2.35	1.90	4.75	2.03	3.76		4.25	
R	1.27	1.01	2.55	1.09	2.16		2.43	
R/r	1.5	12.6	1.0	7.8	1.8		1.5	

[a]Solvent extraction, Butt tube extraction method, AOCS Official Methods appropriate to oilseed, AOCS Laboratory Proficiency Program results for 1995–1996.
[b]15% ethyl alcohol.
[c]Solvent extraction, AOCS Official Method Am 2-93.
[d]Oilseeds from 1995–1996 AOCS Laboratory Proficiency Program: soy, soybean; sun, sunflower.

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Table 4
Flow conversion table for CO_2 @ ambient pressure: liters per minute (LPM) to g/min (as a function of expanded gas temperature).[a]

Temp (°C)	1.00LPM	1.10LPM	1.20LPM	1.30LPM	1.40LPM	1.50LPM	1.60LPM	1.70LPM	1.80LPM	1.90LPM	2.00LPM
−10	2.04	2.24	2.45	2.65	2.85	3.06	3.26	3.46	3.67	3.87	4.08
−9	2.03	2.23	2.44	2.64	2.84	3.05	3.25	3.45	3.65	3.86	4.06
−8	2.02	2.22	2.43	2.63	2.83	3.03	3.24	3.44	3.64	3.84	4.04
−7	2.01	2.22	2.42	2.62	2.82	3.02	3.22	3.43	3.63	3.83	4.03
−6	2.01	2.21	2.41	2.61	2.81	3.01	3.21	3.41	3.61	3.81	4.01
−5	2.00	2.20	2.40	2.60	2.80	3.00	3.20	3.40	3.60	3.80	4.00
−4	1.99	2.19	2.39	2.59	2.79	2.99	3.19	3.39	3.59	3.79	3.98
−3	1.98	2.18	2.38	2.58	2.78	2.98	3.18	3.37	3.57	3.77	3.97
−2	1.98	2.18	2.37	2.57	2.77	2.97	3.16	3.36	3.56	3.76	3.96
−1	1.97	2.17	2.36	2.56	2.76	2.96	3.15	3.35	3.55	3.74	3.94
0	1.96	2.16	2.36	2.55	2.75	2.94	3.14	3.34	3.53	3.73	3.93
1	1.96	2.15	2.35	2.54	2.74	2.93	3.13	3.33	3.52	3.72	3.91
2	1.95	2.14	2.34	2.53	2.73	2.92	3.12	3.31	3.51	3.70	3.90
3	1.94	2.14	2.33	2.52	2.72	2.91	3.11	3.30	3.50	3.69	3.88
4	1.93	2.13	2.32	2.52	2.71	2.90	3.10	3.29	3.48	3.68	3.87
5	1.93	2.12	2.31	2.51	2.70	2.89	3.08	3.28	3.47	3.66	3.86
6	1.92	2.11	2.31	2.50	2.69	2.88	3.07	3.27	3.46	3.65	3.84
7	1.91	2.11	2.30	2.49	2.68	2.87	3.06	3.25	3.45	3.64	3.83
8	1.91	2.10	2.29	2.48	2.67	2.86	3.05	3.24	3.43	3.62	3.81
9	1.90	2.09	2.28	2.47	2.66	2.85	3.04	3.23	3.42	3.61	3.80
10	1.89	2.08	2.27	2.46	2.65	2.84	3.03	3.22	3.41	3.60	3.79
11	1.89	2.08	2.26	2.45	2.64	2.83	3.02	3.21	3.40	3.59	3.77
12	1.88	2.07	2.26	2.44	2.63	2.82	3.01	3.20	3.38	3.57	3.76
13	1.87	2.06	2.25	2.44	2.62	2.81	3.00	3.19	3.37	3.56	3.75
14	1.87	2.05	2.24	2.43	2.61	2.80	2.99	3.17	3.36	3.55	3.73
15	1.86	2.05	2.23	2.42	2.61	2.79	2.98	3.16	3.35	3.54	3.72
16	1.85	2.04	2.23	2.41	2.60	2.78	2.97	3.15	3.34	3.52	3.71
17	1.85	2.03	2.22	2.40	2.59	2.77	2.96	3.14	3.33	3.51	3.70
18	1.84	2.03	2.21	2.39	2.58	2.76	2.95	3.13	3.32	3.50	3.68
19	1.84	2.02	2.20	2.39	2.57	2.75	2.94	3.12	3.30	3.49	3.67
20	1.83	2.01	2.20	2.38	2.56	2.74	2.93	3.11	3.29	3.48	3.66
21	1.82	2.01	2.19	2.37	2.55	2.73	2.92	3.10	3.28	3.46	3.65
22	1.82	2.00	2.18	2.36	2.54	2.73	2.91	3.09	3.27	3.45	3.63
23	1.81	1.99	2.17	2.35	2.53	2.72	2.90	3.08	3.26	3.44	3.62
24	1.80	1.99	2.17	2.35	2.53	2.71	2.89	3.07	3.25	3.43	3.61
25	1.80	1.98	2.16	2.34	2.52	2.70	2.88	3.06	3.24	3.42	3.60
26	1.79	1.97	2.15	2.33	2.51	2.69	2.87	3.05	3.23	3.41	3.59
27	1.79	1.97	2.14	2.32	2.50	2.68	2.86	3.04	3.22	3.39	3.57
28	1.78	1.96	2.14	2.31	2.49	2.67	2.85	3.03	3.21	3.38	3.56
29	1.77	1.95	2.13	2.31	2.48	2.66	2.84	3.02	3.19	3.37	3.55
30	1.77	1.95	2.12	2.30	2.48	2.65	2.83	3.01	3.18	3.36	3.54
31	1.76	1.94	2.12	2.29	2.47	2.64	2.82	3.00	3.17	3.35	3.53
32	1.76	1.93	2.11	2.28	2.46	2.64	2.81	2.99	3.16	3.34	3.51
33	1.75	1.93	2.10	2.28	2.45	2.63	2.80	2.98	3.15	3.33	3.50
34	1.75	1.92	2.09	2.27	2.44	2.62	2.79	2.97	3.14	3.32	3.49
35	1.74	1.91	2.09	2.26	2.44	2.61	2.78	2.96	3.13	3.31	3.48
36	1.73	1.91	2.08	2.25	2.43	2.60	2.78	2.95	3.12	3.30	3.47
37	1.73	1.90	2.07	2.25	2.42	2.59	2.77	2.94	3.11	3.28	3.46
38	1.72	1.90	2.07	2.24	2.41	2.59	2.76	2.93	3.10	3.27	3.45
39	1.72	1.89	2.06	2.23	2.41	2.58	2.75	2.92	3.09	3.26	3.44
40	1.71	1.88	2.05	2.23	2.40	2.57	2.74	2.91	3.08	3.25	3.42
41	1.71	1.88	2.05	2.22	2.39	2.56	2.73	2.90	3.07	3.24	3.41
42	1.70	1.87	2.04	2.21	2.38	2.55	2.72	2.89	3.06	3.23	3.40
43	1.70	1.87	2.04	2.20	2.37	2.54	2.71	2.88	3.05	3.22	3.39
44	1.69	1.86	2.03	2.20	2.37	2.54	2.71	2.87	3.04	3.21	3.38
45	1.69	1.85	2.02	2.19	2.36	2.53	2.70	2.87	3.03	3.20	3.37
46	1.68	1.85	2.02	2.18	2.35	2.52	2.69	2.86	3.02	3.19	3.36
47	1.67	1.84	2.01	2.18	2.34	2.51	2.68	2.85	3.01	3.18	3.35
48	1.67	1.84	2.00	2.17	2.34	2.50	2.67	2.84	3.01	3.17	3.34
49	1.66	1.83	2.00	2.16	2.33	2.50	2.66	2.83	3.00	3.16	3.33
50	1.66	1.83	1.99	2.16	2.32	2.49	2.66	2.82	2.99	3.15	3.32

[a]Based on ideal gas law equation.

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Table 5
Flow conversion table for liquid CO_2 @ 7500 psi: mL/min to g/min (as a function of pump temperature).[a,b,c]

Pump temp	1.0 mL/min	1.25 mL/min	1.5 mL/min	1.75 mL/min	2.0 mL/min	2.25 mL/min	2.5 mL/min	2.75 mL/min	3.0 mL/min	3.25 mL/min	3.5 mL/min
–10	1.14	1.42	1.71	1.99	2.28	2.56	2.84	3.13	3.41	3.70	3.98
–9	1.13	1.42	1.70	1.99	2.27	2.55	2.84	3.12	3.40	3.69	3.97
–8	1.13	1.41	1.70	1.98	2.26	2.55	2.83	3.11	3.40	3.68	3.96
–7	1.13	1.41	1.69	1.98	2.26	2.54	2.82	3.10	3.39	3.67	3.95
–6	1.13	1.41	1.69	1.97	2.25	2.53	2.82	3.10	3.38	3.66	3.94
–5	1.12	1.40	1.68	1.97	2.25	2.53	2.81	3.09	3.37	3.65	3.93
–4	1.12	1.40	1.68	1.96	2.24	2.52	2.80	3.08	3.36	3.64	3.92
–3	1.12	1.40	1.68	1.96	2.24	2.51	2.79	3.07	3.35	3.63	3.91
–2	1.11	1.39	1.67	1.95	2.23	2.51	2.79	3.07	3.34	3.62	3.90
–1	1.11	1.39	1.67	1.95	2.22	2.50	2.78	3.06	3.34	3.61	3.89
0	1.11	1.39	1.66	1.94	2.22	2.50	2.77	3.05	3.33	3.60	3.88
1	1.11	1.38	1.66	1.94	2.21	2.49	2.77	3.04	3.32	3.59	3.87
2	1.10	1.38	1.65	1.93	2.21	2.48	2.76	3.03	3.31	3.59	3.86
3	1.10	1.38	1.65	1.93	2.20	2.48	2.75	3.03	3.30	3.58	3.85
4	1.10	1.37	1.65	1.92	2.20	2.47	2.74	3.02	3.29	3.57	3.84
5	1.09	1.37	1.64	1.92	2.19	2.46	2.74	3.01	3.28	3.56	3.83
6	1.09	1.36	1.64	1.91	2.18	2.46	2.73	3.00	3.28	3.55	3.82
7	1.09	1.36	1.63	1.91	2.18	2.45	2.72	2.99	3.27	3.54	3.81
8	1.09	1.36	1.63	1.90	2.17	2.44	2.72	2.99	3.26	3.53	3.80
9	1.08	1.35	1.62	1.90	2.17	2.44	2.71	2.98	3.25	3.52	3.79
10	1.08	1.35	1.62	1.89	2.16	2.43	2.70	2.97	3.24	3.51	3.78
11	1.08	1.35	1.62	1.89	2.15	2.42	2.69	2.96	3.23	3.50	3.77
12	1.07	1.34	1.61	1.88	2.15	2.42	2.69	2.96	3.22	3.49	3.76
13	1.07	1.34	1.61	1.88	2.14	2.41	2.68	2.95	3.22	3.48	3.75
14	1.07	1.34	1.60	1.87	2.14	2.41	2.67	2.94	3.21	3.47	3.74
15	1.07	1.33	1.60	1.87	2.13	2.40	2.67	2.93	3.20	3.46	3.73
16	1.06	1.33	1.59	1.86	2.13	2.39	2.66	2.92	3.19	3.46	3.72
17	1.06	1.33	1.59	1.86	2.12	2.39	2.65	2.92	3.18	3.45	3.71
18	1.06	1.32	1.59	1.85	2.11	2.38	2.64	2.91	3.17	3.44	3.70
19	1.05	1.32	1.58	1.85	2.11	2.37	2.64	2.90	3.16	3.43	3.69
20	1.05	1.31	1.58	1.84	2.10	2.37	2.63	2.89	3.16	3.42	3.68
21	1.05	1.31	1.57	1.84	2.10	2.36	2.62	2.88	3.15	3.41	3.67
22	1.05	1.31	1.57	1.83	2.09	2.35	2.62	2.88	3.14	3.40	3.66
23	1.04	1.30	1.56	1.83	2.09	2.35	2.61	2.87	3.13	3.39	3.65
24	1.04	1.30	1.56	1.82	2.08	2.34	2.60	2.86	3.12	3.38	3.64
25	1.04	1.30	1.56	1.82	2.07	2.33	2.59	2.85	3.11	3.37	3.63
26	1.03	1.29	1.55	1.81	2.07	2.33	2.59	2.85	3.10	3.36	3.62
27	1.03	1.29	1.55	1.81	2.06	2.32	2.58	2.84	3.10	3.35	3.61
28	1.03	1.29	1.54	1.80	2.06	2.31	2.57	2.83	3.09	3.34	3.60
29	1.03	1.28	1.54	1.80	2.05	2.31	2.56	2.82	3.08	3.33	3.59
30	1.02	1.28	1.53	1.79	2.05	2.30	2.56	2.81	3.07	3.33	3.58
31	1.02	1.28	1.53	1.79	2.04	2.30	2.55	2.81	3.06	3.32	3.57
32	1.02	1.27	1.53	1.78	2.03	2.29	2.54	2.80	3.05	3.31	3.56
33	1.01	1.27	1.52	1.78	2.03	2.28	2.54	2.79	3.04	3.30	3.55
34	1.01	1.26	1.52	1.77	2.02	2.28	2.53	2.78	3.04	3.29	3.54
35	1.01	1.26	1.51	1.77	2.02	2.27	2.52	2.77	3.03	3.28	3.53
36	1.01	1.26	1.51	1.76	2.01	2.26	2.51	2.77	3.02	3.27	3.52
37	1.00	1.25	1.50	1.76	2.01	2.26	2.51	2.76	3.01	3.26	3.51
38	1.00	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25	3.50
39	1.00	1.25	1.50	1.75	1.99	2.24	2.49	2.74	2.99	3.24	3.49
40	0.99	1.24	1.49	1.74	1.99	2.24	2.49	2.73	2.98	3.23	3.48
41	0.99	1.24	1.49	1.74	1.98	2.23	2.48	2.73	2.97	3.22	3.47
42	0.99	1.24	1.48	1.73	1.98	2.22	2.47	2.72	2.97	3.21	3.46

(Continued)

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Table 5 *(Continued)*

Pump temp	1.0 mL/min	1.25 mL/min	1.5 mL/min	1.75 mL/min	2.0 mL/min	2.25 mL/min	2.5 mL/min	2.75 mL/min	3.0 mL/min	3.25 mL/min	3.5 mL/min
43	0.99	1.23	1.48	1.73	1.97	2.22	2.46	2.71	2.96	3.20	3.45
44	0.98	1.23	1.47	1.72	1.97	2.21	2.46	2.70	2.95	3.19	3.44
45	0.98	1.23	1.47	1.72	1.96	2.21	2.45	2.70	2.94	3.19	3.43
46	0.98	1.22	1.47	1.71	1.95	2.20	2.44	2.69	2.93	3.18	3.42
47	0.97	1.22	1.46	1.71	1.95	2.19	2.44	2.68	2.92	3.17	3.41
48	0.97	1.21	1.46	1.70	1.94	2.19	2.43	2.67	2.91	3.16	3.40
49	0.97	1.21	1.45	1.70	1.94	2.18	2.42	2.66	2.91	3.15	3.39
50	0.97	1.21	1.45	1.69	1.93	2.17	2.41	2.66	2.90	3.14	3.38

[a]Note: Applies to piston displacement of syringe pumps; does not apply to piston displacement of reciprocating pumps.
[b]Based on the work of Pitzer, K.S. et al., *J. Am. Chem Soc. 77* (1955), pp. 3433–3440.
[c](g/mL) = −0.002861 × T (°C) + 0.00001570 × P (psi) + 0.9912.

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AOCS Official Method Am 4-02

Reapproved 2009

Determination of Impurities in Oilseeds

DEFINITION

Oilseed impurities are separated in the method by sieving and sorting, into three categories: fines, non-oleaginous impurities, and oleaginous impurities. The mass of total impurities is determined or, on request, the mass of each category of impurity. Impurities in oilseeds refers to all foreign matter, organic and inorganic, other than seeds of the species under consideration. Fines in oilseeds is defined as particles passing through the sieves of aperture sizes given in Table 1, according to the species being analyzed (see Notes, 1 and 2).

SCOPE

This method determines the impurities content in oilseeds used as primary industrial materials. It also defines the various categories of what are usually understood to be impurities.

APPARATUS

1. Sieves—having round holes with diameters as given in Table 1.
2. Tweezers.
3. Analytical balance—capable of recording to the nearest 0.005 g.
4. Sample dividers—capable of taking 10 g sample aliquots of small seeds and 100 g sample aliquots of sunflower seeds and soybeans.

SAMPLING

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

TEST SAMPLE PREPARATION

1. Prepare the test sample according to approved protocols.

PROCEDURE

Note—If it is required to check whether the repeatability limits (Precision, 2) are met, carry out two single determinations in accordance with steps 2 and 3 below. The determination of impurities content is carried out sufficiently quickly to avoid any appreciable change in the moisture content of the seed.

1. Take as the test portion a complete test sample. For a complete analysis, two or four test samples are necessary (see Precision, 2). Weigh the test portion to the nearest 0.1 g.
2. Separation of fines—Separate the fines quantitatively by sieving the test portion on the sieve (see Apparatus, 1) and collecting them. In the case of groundnuts, collect the total fines thus obtained, which include nonoleaginous fines and fines from the seed. Weigh them to the nearest 0.01 g and determine their oil content by an approved method. Determine also the oil content of the pure seeds in order to calculate the content of non-oleaginous fines.
3. Separation of oleaginous and non-oleaginous impurities—
   a. Copra and medium and larger sized seeds, apart from sunflower seeds and soybeans—
      1) In the material retained by the sieve (see Apparatus, 1), separate, by means of tweezers (Apparatus, 2), the non-oleaginous impurities, if necessary detaching bits of shell adhering to the seeds (as is the case with palm kernels) from the oleaginous impurities.
      2) Weigh together, to the nearest 0.01 g, the nonoleaginous and oleaginous impurities and the fines, except in the case of groundnuts.
      3) If requested, weigh separately, to the nearest 0.01 g, each category of impurity.
      4) If stipulated in the contract, note the nature of the oleaginous impurities in order that this can be recorded in the test report.
   b. Sunflower seeds and soybeans—
      1) In the material retained by the sieve, separate, by means of tweezers, the impurities whose dimensions differ clearly from those of the sunflower seeds or soybeans being examined (large impurities). Class these impurities into two categories (oleaginous and nonoleaginous) and weigh each of them to within 0.01 g.
      2) Using an aliquot portion (minimum 100 g, weighed to the nearest 0.1 g) of partially sorted sunflower seeds or soybeans, separate, by manual sorting, the oleaginous impurities and the non-oleaginous impurities (small impurities). Weigh each of these two fractions to the nearest 0.01 g.
   c. Small seeds—
      1) Transfer the residue from the sieve to a second sieve so as to retain impurities larger than the seeds, or separate these impurities by means of tweezers. To assist the removal of large impurities, a 3.15 mm mesh may be used.

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2) On request, sort this fraction into non-oleaginous impurities and oleaginous impurities.
3) Weigh separately, to the nearest 0.01 g, the fines and the impurities (non-oleaginous and oleaginous) that are larger than the seeds, and also the partially sorted seeds.
4) Using an aliquot portion of the latter fraction of seeds (at least 10 g, weighed to the nearest 0.01 g), separate, by sorting, the small non-oleaginous impurities from the small foreign oleaginous seeds. Weigh these two fractions of impurities, to the nearest 0.005 g, together or, on request, separately (see Notes, 3).

d. Grouping of foreign oilseeds—If required, the foreign oilseeds may be grouped and weighed according to species, in order to show in the test report the mass fraction, in percent, of each species.

EXPRESSION OF RESULTS

1. Method of calculation—Express the results as a mass fraction, in percent, of total impurities. On request, the percentage of each category of impurity may be indicated.
2. When the determination of impurities content has been carried out on the whole test portion [see Procedure, 3(a)], the calculation shall be as follows:
   a. total impurities, I_t, expressed as a mass fraction in percent

$$I_t = \frac{m_4}{m_0} \times 100\% \qquad (1)$$

or

$$I_t = P + I_n + I_0 \qquad (1)=(2)+(3)+(4)$$

   b. fines, P, expressed as a mass fraction in percent

$$P = \frac{m_1}{m_0} \times 100\% \qquad (2)$$

   c. non-oleaginous impurities, I_n, expressed as a mass fraction in percent

$$I_n = \frac{m_2}{m_0} \times 100\% \qquad (3)$$

   d. oleaginous impurities, I_0, expressed as a mass fraction in percent

$$I_0 = \frac{m_3}{m_0} \times 100\% \qquad (4)$$

Where—

m_0 = mass, in grams, of the test portion
m_1, m_2, m_3 = respective masses, in grams, of each category of impurity
m_4 = mass, in grams, of all the impurities, including the fines

3. When only a part of the impurities has been separated from the whole of the test portion and the other from an aliquot portion of the remainder, the calculation may be as follows:
   a. total impurities, I_t, expressed as a mass fraction in percent

$$I_t = \left[\frac{m_4}{m_0} + \frac{(m_0 - m_4)m_5}{m_0 \times m_b} \right] \times 100\% \qquad (5)$$

or

$$I_t = [P + I_n + I_0] \times 100\% \qquad (5)=(6)+(7)+(8)$$

   b. fines, P, expressed as a mass fraction in percent

$$P = \frac{m_1}{m_0} \times 100\% \qquad (6)$$

   c. non-oleaginous impurities, I_n, expressed as a mass fraction in percent

$$I_n = \left[m_{2a} + \left(m_{2b} \times \frac{m_a}{m_a} \right) \times \frac{100\%}{m_0} \right] \qquad (7)$$

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d. oleaginous impurities, I_0, expressed as a mass fraction in percent

$$I_0 = \left[m_{3a} + \left(m_{3b} \times \frac{m_a}{m_b} \right) \times \frac{100\%}{m_0} \right] \qquad (7)$$

Where—

m_0 = mass, in grams, of the initial test portion

m_1 = mass, in grams, of the fines

m_{2a} = mass, in grams, of the fraction of non-oleaginous impurities larger than seeds of the basic species and separated from the whole test portion

m_{2b}= mass, in grams, of the fraction of small nonoleaginous impurities separated from the aliquot portion of the residue obtained after elimination, from the test portion, of fines and impurities larger than seeds of the basic species

m_{3a} = mass, in grams, of the fraction of oleaginous impurities larger than seeds of the basic species and separated from the whole test portion

m_{3b} = mass, in grams, of the fraction of small oleaginous impurities separated from the aliquot portion of the residue obtained after elimination, from the test portion, of fines and impurities larger than seeds of the basic species

m_a = mass, in grams, of the residue obtained after elimination, from the initial test portion, of fines and impurities larger than seeds of the basic species:

$$m_a = m_0 - m_1 - m_{2a} - m_{3a}$$

m_b = mass, in grams, of the aliquot portion of the residue of mass m_a before the small impurities have been separated

m_4 = mass, in grams, of the fines and of the fraction of impurities larger than seeds of the basic species and separated from the whole test portion

m_5 = mass, in grams, of the fraction of impurities separated from the aliquot portion of the residue obtained after elimination, from the test portion, of the fines and the impurities larger than seeds of the basic species

4. In the case of groundnuts, the calculation shall be as follows:

a. total impurities, I_t, expressed as a mass fraction in percent

$$I_t = \left[\frac{m_1}{m_0} \left(1 - \frac{H_2}{H_1} \right) + \frac{m_4}{m_0} \right] \times 100\% \qquad (9)$$

$$I_t = P_s + I_n + I_0 \qquad (9)=(11)+(12)+(13)$$

b. total fines, P, expressed as a mass fraction in percent

$$P = \frac{m_1}{m_0} \times 100\% \qquad (10)$$

c. foreign fines, P_s, expressed as a mass fraction in percent

$$P_s = \frac{m_1}{m_0} \left(1 - \frac{H_2}{H_1} \right) \times 100\% \qquad (11)$$

d. non-oleaginous impurities, I_n, expressed as a mass fraction in percent

$$I_n = \frac{m_2}{m_0} \times 100\% \qquad (12)$$

e. oleaginous impurities, I_0, expressed as a mass fraction in percent

$$I_0 = \frac{m_3}{m_0} \times 100\% \qquad (13)$$

Where—

m_0 = mass, in grams, of the test portion

m_1= mass, in grams, of the fines

m_2 and m_3 = respective masses, in grams, of the non-oleaginous impurities and the oleaginous impurities

m_4 = mass, in grams, of the impurities other than the fines

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H_1 = oil content, expressed as a mass fraction in percent, of the pure seed

H_2 = oil content, expressed as a mass fraction in percent, of the fines

5. Take as the result the arithmetic mean of the two determinations, if the conditions of repeatability are satisfied.
6. Report the results to two decimal places for mass fractions of impurities not exceeding 0.5% and to one decimal place for impurities contents above this limit.

PRECISION

1. Interlaboratory test—Details of an interlaboratory test on the precision of the method are summarized in Table 2. An international collaborative test involving 13 laboratories in 6 countries was carried out on five samples: groundnut kernels; linseed; rapeseed; soybeans; and sunflower seeds. The test was organized by the Leatherhead Food Research Association in 1998 and the results obtained were subjected to statistical analysis in accordance with ISO 5725-1 (3) and ISO 5725-2 (4) to give the precision data shown in Table 2. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.
2. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases be greater than the values given in Table 3. If the difference is greater than the limit indicated in Table 3, obtain two other test portions. Analyze one as before and keep the other for a fourth determination if necessary. In this case, take as the result the arithmetic mean of the result obtained from the third analysis and the nearest result obtained from the previous analyses, provided that the difference does not exceed the allowed limit. Failing this, analyze also the fourth test portion and take as the result the mean of the four determinations.
3. Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5% of cases be greater than the values given in Table 3.

NUMBERED NOTES

1. In the case of groundnuts, meal from the seeds contained in the fines is not regarded as an impurity. Non-oleaginous impurities are non-oleaginous foreign bodies, fragments of stalks, leaves, and all other non-oleaginous parts belonging to the oleaginous seed analyzed, retained by the sieves of aperture sizes given in Table 1.
2. In the case of seeds sold in their shells (i.e., pumpkin or sunflower seeds) the loose shells are regarded as impurities only if their proportion is larger than that of the corresponding kernels present in the same sample. Oleaginous impurities are oilseeds other than those of the species under consideration.
3. Specific methods for the determination of the content of *Sinapis arvensis* (wild mustard) seeds in rapeseeds (*Brassica napus*) and of turnip rape (*Brassica rapa*) have been published (2,3).

REFERENCES

1. *Official Journal of the European Communities,* No. L 300-320. 11. 1975.
2. *Fat Science Technology,* 89, 1987, pp.7–15.
3. ISO 5725-1: 1994, Accuracy (trueness and precision) of measurement methods and results-Part 1:General principles and definitions.
4. ISO 5725-2: 1994, Accuracy (trueness and precision) of measurement methods and results-Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

Table 1
Aperture sizes of sieves.

Nature of product	Aperture diameter, mm
Copra	2.0
Medium and larger sized seeds	1.0
Small seeds	0.5

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Table 2
Precision data.

	Groundnut kernels	Linseed	Rapeseed	Soybeans	Sunflower seeds
Number of laboratories after eliminating outliers	8	13	13	12	12
Number of accepted results	8	13	13	12	12
Mean value, % (mass fraction)	0.00	3.72	1.31	0.77	1.10
Repeatability standard deviation (s_r)	0.004	0.203	0.077	0.065	0.112
Repeatability limit (r)	0.01	0.57	0.21	0.18	0.31
Reproducibility standard deviation (s_R)	0.005	0.923	0.687	0.174	0.348
Reproducibility limit (R)	0.014	2.58	1.92	0.49	0.97

Table 3

Impurities content	Repeatability limit, r	Reproducibility limit, R
Up to and including 0.5	0.2	0.4
Over 0.5 to 1.0 inclusive	0.4	0.8
Over 1.0 to 2.0 inclusive	0.6	1.8
Over 2.0 to 3.0 inclusive	0.8	2.4
Over 3.0 to 4.0 inclusive	1.0	3.0
Over 4.0 to 5.0 inclusive	1.2	3.6
Over 5.0 to 6.0 inclusive	1.4	4.2
Over 6.0	1.6	4.8

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AOCS

AOCS Standard Procedure Am 5-04

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Rapid Determination of Oil/Fat Utilizing High-Temperature Solvent Extraction

DEFINITION

This method determines the substances extracted by petroleum ether under conditions of the test. The compounds extracted, in most samples, are predominantly triacylglycerides. Small amounts of other lipids along with minor components having some solubility in petroleum ether are also extracted.

SCOPE

This method is applicable to extractions of oilseeds (see Numbered Notes, 1), meats, feeds, and foods and is equivalent to the petroleum ether extraction performed by the Butt and Soxhlet extraction apparatus.

APPARATUS

1. Analytical balance—capable of weighing down to 0.1 mg.
2. An extraction instrument capable of performing extractions at elevated temperatures (90°C–100°C) and pressures (40–80 psi) on multiple samples. The apparatus must also be capable of rinsing the sample and evaporating the residual solvent (ANKOM^{XT10} Extractor or ANKOM^{XT20} Fat Analyzer, ANKOM Technology Corp.).
3. Solvent and heat resistant filter bags, capable of being heat sealed closed, retaining 1 micron particles and permitting rapid solvent penetration (XT4 filter bags, ANKOM).
4. Impulse heat sealer.
5. Oven, capable of maintaining a temperature of 100°C ±2°. The oven should have sufficient convection flux to clear the water vapor.
6. Desiccator pouch (Moisture Stop weigh pouch, ANKOM).

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Oilseeds should be prepared according to Section A of AOCS methods using the methods specified for each matrix.
2. Meat should be ground to a uniform consistency with a food processor or similar device.
3. Feed should be ground to a particle size that will pass through a 1 mm sieve and mixed thoroughly (see Numbered Notes, 2).
4. Food should be processed with a food processor or grinder to produce a representative test sample of uniform consistency.

PROCEDURE

1. Weigh filter bag.
2. Weigh one gram (see Numbered Notes, 3) of test sample directly in filter bag and avoid placing the test portion on the upper 4 mm of the bag (wt. of sample).
3. Completely seal the upper edge of the filter bag within 4 mm of the top with the heat sealer encapsulating the test portion.
4. Dry the test portions in the bag at 100°C for 3 hours (see Numbered Notes, 4).
5. Cool test portions in a desiccator pouch to ambient temperature and weigh (wt. of pre-dried sample).
6. Place up to 20 test portions in the bag holder and extract in the instrument at temperatures between 90°C–100°C for 15 to 60 minutes (see Numbered Notes, 5).
7. At the completion of the process remove the test portions from the instrument and dry at 100°C for 30 minutes.
8. Cool test portions in a desiccator pouch to ambient temperature and weigh (wt. of extracted test portion).

CALCULATIONS

$$\% \text{ oil/fat} = \frac{(\text{wt. of pre-dried test portion} - \text{wt. of extracted test portion})}{\text{wt. of test portion}} \times 100$$

PRECISION

Results of the collaborative study in Table 1 indicate the precision (S_r, RSD_r, r) that the analyst should use as a benchmark for evaluation replication in the same laboratory.

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NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when venting petroleum ether.

NUMBERED NOTES

1. Specific data on the accuracy and precision of soybeans, canola, safflower, and corn are found in the data from the collaborative study (Table 1), and similar results have been found with other oilseeds extracted with the Butt apparatus. Since this method does not regrind the test portion after the original extraction and then re-extract, for reference purposes AOCS Method Am 2-93 is recommended.
2. If test sample contains large amounts of H_2O soluble components such as carbohydrates, urea, glycerol, and lactic acid, remove by a water extraction. If H_2O solubles are present, weigh test portion onto filter paper, extract with five 20 mL portions of H_2O allowing each portion to drain. Place the filter paper containing the washed test portion into the filter bag and proceed with pre-drying step.
3. A range of test portion size from about 0.25 g to 3 g can be analyzed with this method depending on the limitation of test sample availability or the need for larger test portions due to the lack of sample homogeneity.
4. Two hours or less should be used for test portions that are highly susceptible to oxidation.
5. Extraction times will be dependent on the matrix and the fineness of grind. Materials composed of animal tissue will require only 15 to 30 minutes. Materials containing plant tissue can range from 15 to 60 minutes.

REFERENCES

1. *Official Methods and Recommended Practices of the American Oil Chemists' Society,* 5th ed., ed. D. Firestone, AOCS Press, Champaign, (1997)
2. *Official Methods of Analysis of AOAC International,* 16th ed., ed. P. Cunniff, AOAC International, Gaithersberg (1997).
3. International Organization for Standardization, Oilseeds—Determination of Hexane Extract (or Light Petroleum extract), called "Oil Content," ISO, Geneve, standard no. 659 (1988).
4. DGF, Deutsche Einheitsmethoden zur Untersuchung von Fetten, Fettprodukten, Tensiden und verwandten Stoffen, Wissenschaftliche Verlagsgesellschaft, Stuttgart (1998).
5. FOSFA International Method: Determination of Oil Content in Oilseeds—Solvent Extract (Reference Method) (1998).
6. Matthaus, B. and L. Bruhl, Comparison of Different Methods for the Determination of the Oil Content in Oilseeds, *J. Am. Oil Chem. Soc. 78:*95–102 (2001)
7. Labconco Goldfisch Fat Extraction Apparatus, model no. 35001, Labconco Corporation, Kansas City, (1990).
8. Randall, E.L., Improved Method for Fat and Oil Analysis by a New Process of Extraction, *J.AOAC 57:*1165–1168 (1974).
9. ANKOM Methods, *Method for Crude Fat Determinations,* ANKOM Technology Corporation, Macedon, NY (2001).
10. Dionex ASE Extraction System, Dionex Corporation, Sunnyvale, CA.
11. AOCS Official Method Am 3-96, Oil in Oilseeds: Supercritical Fluid Extraction Method, *Official Methods and Recommended Practices of the American Oil Chemists' Society,* 5th ed., ed. D. Firestone, AOCS Press, Champaign (1997).
12. Anachemia Chemicals, Rouses Point, NY, Petroleum Ether, Reagent AC-7106.
13. AOCS Official Method Ac 3-44, Oil, *Official Methods and Recommended Practices of the American Oil Chemists' Society,* 5th ed., ed. D. Firestone, AOCS Press, Champaign, (1997).
14. Komarek, R.J., R.G. Jensen, and B.W. Pickett, Quantitative Gravimetric Analysis of Bovine Semen Lipids by Thin-Layer Chromatography, *J. Lipid Res. 5:*268–270, (1964)
15. Youden, W.J., and E.H. Steiner, *Statistical Manual of the Association of Official Analytical Chemists,* AOAC International, Gaithersburg, MD, 8th printing 1997.
16. Frankel, E.N., *Lipid Oxidation,* The Oily Press, Dundee, 1998.

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Table 1

A summary of the statistical analysis of the international collaborative study of the Filter Bag Technique, coordinated by Andrew Komarek and Ronald Komarek (ANKOM Technology Corp. Macedon, NY). Included in the summary is a comparison of the Filter Bag Technique with the results of analysis by AOCS Certified Laboratories using official AOCS or AOAC methods.

Sample ID	oat meal	brownie mix	soybean A	canola	soybean meal	corn A	poultry starter	cattle feed	pig starter	alfalfa	cat food	cookies	bkft cereal	tortilla chips
Number of laboratories	12	12	11	9	12	12	11	10	11	11	12	11	12	12
Number of replicates	24	24	22	18	24	24	22	20	22	22	24	22	24	24
Collaborative average, Oil/Fat %	5.8	8.7	20.9	39.0	1.6	3.3	3.3	3.2	5.6	2.4	6.3	22.7	2.3	19.9
Certified Labs average[a]	5.7	8.7	21.1	39.7	1.6	3.6	3.5	3.0	5.5	2.2	6.2	23.1	2.3	20.0
AOCS Am 2-93 Avg. for Oilseeds			21.9	44.8		3.6								
repeatability														
s(r) = repeatability std dev	0.36	0.20	0.35	0.23	0.14	0.31	0.24	0.18	0.20	0.39	0.27	0.20	0.26	0.39
RSD(r) = repeatability rel. std. dev	6.2	2.3	1.7	0.6	8.5	9.5	7.3	5.6	3.6	16.1	4.2	0.9	11.4	2.0
r = repeatability value	0.99	0.56	0.98	0.65	0.39	0.88	0.68	0.51	0.56	1.08	0.75	0.56	0.72	1.09
Reproducibility														
s(R) = reproducibility std dev	0.54	0.31	0.63	0.68	0.27	0.42	0.42	0.20	0.28	0.50	0.30	0.20	0.36	0.48
RSD(R) = reproducibility rel std. dev	9.4	3.5	3.0	1.7	16.3	12.7	12.6	6.1	5.0	20.7	4.7	0.9	15.7	2.4
R = reproducibility value	1.52	0.86	1.76	1.90	0.75	1.18	1.16	0.55	0.78	1.39	0.83	0.56	1.00	1.35

Sample ID	dog food	crackers	turkey	ham	beef ground	chicken breast	soybean B	safflower	potato chips	hot dog	sausage	corn B	cheese curls	corn silage
Number of laboratories	12	10	12	9	11	11	11	9	11	12	11	12	12	12
Number of replicates	24	20	24	18	22	22	22	18	22	24	22	24	24	24
Collaborative average	6.8	23.8	3.2	11.6	23.8	2.8	19.4	22.5	32.0	39.5	25.7	3.4	30.6	2.3
Certified Labs average[a]	6.9	24.0	3.2	11.3	23.5	2.7	19.7	23.0	32.0	39.0	25.0	3.7	30.5	2.3
AOCS Am 2-93 avg. for oilseeds							20.1	24.5						
Repeatability (r)														
s(r) = repeatability std dev	0.35	0.23	0.21	0.30	0.24	0.33	0.38	0.53	0.48	0.35	0.34	0.39	0.48	0.23
RSD(r) = repeatability rel std dev	5.23	0.96	6.57	2.59	1.01	11.89	1.97	2.36	1.49	0.89	1.33	11.48	1.59	9.87
r = repeatability value	0.99	0.64	0.58	0.84	0.67	0.94	1.07	1.49	1.34	0.98	0.96	1.10	1.36	0.63
Reproducibility (R)														
s(R) = reproducibility std dev	0.35	0.23	0.34	0.30	0.36	0.33	0.62	0.83	0.52	0.59	0.51	0.41	0.69	0.51
RSD(R) = reproducibility rel std dev	5.23	0.96	10.84	2.59	1.49	11.89	3.19	3.69	1.61	1.49	1.98	11.93	2.27	22.45
R = reproducibility value	0.99	0.64	0.96	0.84	0.99	0.94	1.73	2.33	1.45	1.65	1.43	1.14	1.94	1.44

[a]AOCS Official Method Ba 3-38, AOAC 920.39, or equivalent.

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AOCS Official Method Ba 1-38

Reapproved 2009

Sampling

SCOPE

Applicable to oilseed slab and sized cake, pellets and meal.

APPARATUS

1. Laboratory mill—hammer, crusher or attrition type, which will reduce pellets or large pieces of cake to a coarse meal.
2. Riffle type divider—8 × 10 in, see Seedboro Equipment, Chicago, IL.
3. Boerner divider—36 pocket, see Seedboro Equipment, Chicago, IL.
4. Airtight sample containers—1000 g.
5. Bag trier—of sufficient length to reach from the top to the bottom of the bag.
6. Covered sample container—about 10 gal capacity.
7. Automatic mechanical sampler—Sampler shall be designed to cut an increment from the entire cross section of the meal stream at a point where the meal is free falling. (In intermittent operation of cutter, the opening must be sealed when not in motion to prevent fly dust from entering the sample container.) Increments so taken shall be at frequent and regularly spaced intervals to obtain not less than 120 g (4 oz) meal for each 450 kg (100 lb) as loaded. Any automatic sampler, equal or equivalent in performance to those offered by Standard Geary-Jennings with type C modified cutter will be satisfactory.
8. Pneumatic probe sampler—The sampler unit shall be powered with a ⅞ hp class II, group G motor, installed in a specially designed cyclone air pump and a cyclone collector having a bottom release shutter, 43 cm (17 in.) long, 30 cm (12 in.) i.d., 5 cm (2 in.) outlet and spring-cap 3.8 cm (1.5 in.) inlet. Two aluminum tubes comprise the probe: outside tube 5.0 cm (2 in.) o.d. (wall thickness 0.17 cm (0.065 in.)), inside tube 3.2 cm (1.25 in.) o.d. (wall thickness 0.12 cm (0.049 in.)), with the bottom of the probe to be equipped with a cone-tipped section; extensions are available in 0.9, 1.2, and 1.8 cm (3-, 4-, and 6-ft) sections. A swivel-type 90° elbow is available so that the section turns freely 360° around the top of the probe. There should be sufficient length of 3.8 cm (1.5 in.) i.d. flexible seamless vinyl hose to reach from the top of the probe to the cyclone.

PROCEDURE

1. Slab cake in cars or trucks—
   (a) Select 30 full slabs of cake from each car or about 56 tons from each truck. These are selected at random throughout the load, at least as deep as the third layer from the top. Divide each slab by imaginary lines into 15 parts about 6 in. square, and designate A, B, C, and D, as in the following diagram:

A	D	D	D	A
B	C	C	C	B
A	D	D	D	A

   (b) Strike the cake with a heavy bar and break out squares as follows: one piece from position A in each of 8 slabs, one piece from position B in each of 4 slabs, one piece from position C in each of 6 slabs, one piece from position D in each of 12 slabs.
   (c) Grind these 30 pieces in the mill to a coarse meal. Reduce the entire quantity of ground cake through the riffle or Boerner divider until a portion of about 1000 g remains. Place this in an airtight container for the laboratory and protect from moisture change until ready to prepare sample for analysis.
2. Meal, sized (ground) cake and pellets—
   (a) Sample not less than 1 sack for each 2 tons (40 sacks) in the carload or lot, taking 60 g (2 oz) or more from each sack of meal sampled, and 120 g (4 oz) of sized cake or pellets. Final sample shall be not less than 1000 g (2 lb).
   (b) Grind cake and reduce the resultant meal as in Procedure, 1, (c).
3. Meal, bulk shipments at point of origin—
   (a) Examine sampling equipment and container to determine that neither is contaminated, but in clean and sound condition.
   (b) Place automatic sampler in operation before loading is begun.
   (c) After loading has been completed, reduce entire sample through riffle or Boerner divider to three portions of about 500 g (1 lb) each. Place each portion in an airtight container, properly identified, and protect from moisture changes. Provide one portion, identified as an automatically drawn sample, for use of the purchaser.
4. Meal, after loaded in bulk into cars or trucks—
   (a) Assemble sufficient sections of the probe of the pneumatic probe sampler to reach the bottom of the loaded car or truck. With the motor running, insert the probe into the top of the meal and thrust at a uniform rate with a rotating motion through the meal until the bottom is reached. Quickly withdraw probe and insert at the next location.

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(b) The number of probes to be taken shall be sufficient to provide a minimum of 60 g (2 oz) of sample for each 500 kg (1000 lb) of meal loaded. The following probes shall be made: for trucks, a minimum of five probes; for hopper cars, at least one probe from each opening, with a minimum of six probes; for boxcars, a minimum of five probes taken in a roughly symmetrical pattern. One probe shall be in the center of the car. The other probes shall be on lines on both sides of the car about 2 ft from the wall at points 2–4 ft back from the doorposts towards both ends of the car.

(c) Reduce the entire sample collected with the riffle type or Boerner divider to three portions of about 500 g (1 lb) each. Place each portion in a moisture-tight container.

Table 1
Nominal Dimensions of Standard Test Sieves (U.S. Standard Series)

Sieve Designation			Nominal Sieve Opening, inches	Nominal Wire Diameter, mm
International Standard[a] (ISO)		U.S. Standard		
12.5	mm[b]	½ in.[b]	0.500	2.67
11.2	mm	7/16 in.	0.438	2.45
9.5	mm	⅜ in.	0.375	2.27
8.0	mm	5/16 in.	0.312	2.07
6.7	mm	0.265 in.	0.265	1.87
6.3	mm[b]	¼ in.[b]	0.250	1.82
5.6	mm	No. 3 ½	0.223	1.68
4.75	mm	No. 4	0.187	1.54
4.00	mm	No. 5	0.157	1.37
3.35	mm	No. 6	0.132	1.23
2.80	mm	No. 7	0.111	1.10
2.38	mm	No. 8	0.0937	1.00
2.00	mm	No. 10	0.0787	0.900
1.70	mm	No. 12	0.0661	0.810
1.40	mm	No. 14	0.0555	0.725
1.18	mm	No. 16	0.0469	0.650
1.00	mm	No. 18	0.0394	0.580
850	µm[c]	No. 20	0.0331	0.510
710	µm	No. 25	0.0278	0.450
600	µm	No. 30	0.0234	0.390
500	µm	No. 35	0.0197	0.340
425	µm	No. 40	0.0165	0.290
355	µm	No. 45	0.0139	0.247
300	µm	No. 50	0.0117	0.215
250	µm	No. 60	0.0098	0.180
212	µm	No. 70	0.0083	0.152
180	µm	No. 80	0.0070	0.131
150	µm	No. 100	0.0059	0.110
125	µm	No. 120	0.0049	0.091
106	µm	No. 140	0.0041	0.076
90	µm	No. 170	0.0035	0.064
75	µm	No. 200	0.0029	0.053
63	µm	No. 230	0.0025	0.044
53	µm	No. 270	0.0021	0.037

[a]These standard designations correspond to the values for test sieve apertures recommended by the International Organization for Standardization, Geneva, Switzerland.

[b]These sieves are not in the standard series but they have been included because they are in common usage.

[c]1000 µm = 1 mm

Reference

Official Methods of Analysis, Association of Official Analytical Chemists, 16th ed., Vol. 1, Gaithersburg, MD, 1995, p. xvii.

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AOCS

AOCS Official Method Ba 2a-38

Reapproved 2009

Moisture and Volatile Matter

DEFINITION

This method determines the moisture in the products specified, and any material that is volatile under the conditions of the test.

SCOPE

Applicable to cottonseed meats, and meal and ground cake or pellets from cottonseed, soybeans, peanuts, flaxseed, and sunflower seed. For safflower meal see Notes, 1.

APPARATUS

1. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers (see Notes, 2, 3).
2. Forced-draft oven—AOCS Specification H 1-39.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.
4. Riffle type divider 15 × 15 cm (6 × 6 in.) (see Notes, 4).
5. Boerner divider—36 pocket (see Notes, 4).
6. Airtight test sample containers—100-g capacity.

PREPARATION OF TEST SAMPLE

1. Pass the original 1000 g sample through the riffle and reduce to 100 g. Immediately place in airtight container. Weigh moisture test sample immediately.

PROCEDURE

1. Weigh about 5 g (2 g for soybean oil cake or meal and for linseed meal) of the test sample into tared moisture dish.
2. Place the dish in the oven and dry at 130 ± 3°C for 2 h.
3. Remove from the oven, cover immediately, cool in a desiccator to room temperature and weigh.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$

NOTES

1. Moisture content determination for safflower meal is performed according to National Institute of Oilseed Products rules (1.5 hr at 130°C).
2. For test samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to test sample loss from splattering during heating.
3. Suitable equipment may be available from Sargent-Welch.
4. Equipment available from Seedburo Equipment Co., Chicago, IL, USA.
5. Replaces Ba 2-38.

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AOCS

AOCS Recommended Practice Ba 2b-82

Revised 2011

Moisture and Volatile Matter (Vacuum Oven Method)

DEFINITION

This method determines the moisture and any materials that are volatile under the conditions of the test (see References, 1).

SCOPE

Applicable to oilseed protein concentrates. Formerly Ba 2a-82.

APPARATUS

1. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers (see Notes, 1, 2).
2. Vacuum oven—AOCS Specification H 4-45, with air ports throttled and/or baffled to prevent disturbing samples in dishes.
3. Vacuum system—with means to monitor and control pressure within oven.
4. Drying bottles, or tubes to dry air entering oven as sparge or to break vacuum.
5. Desiccator—with efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.
6. Airtight sample containers of convenient size.

PREPARATION OF TEST SAMPLE

1. Mix laboratory sample thoroughly in a closed container by turning container manually or mechanically. Reduce the sample to a suitable size with a minimum of exposure and store in an airtight container.

PROCEDURE

1. Weigh rapidly about 2 ± 0.0001 g test sample into a moisture dish which was previously dried and tared with its cover.
2. Place uncovered dishes with test portions on oven shelf and close oven.
3. Adjust oven pressure to ≤ 25 mm Hg abs, with a sparge air bleed of about two bubbles/sec. Dry to constant weight (about 5 hr) at 98 ± 2°C.
4. Admit dry air into oven to bring to atmospheric pressure.
5. Cover dishes containing dried sample in the oven and transfer to desiccator.
6. As soon as dishes have cooled to room temperature, remove from desiccator and weigh with contents to ± 0.0001 g.

CALCULATIONS

1. Moisture and volatile matter, % = $\dfrac{\text{loss in mass, g}}{\text{mass, g of sample}} \times 100$

NOTES

1. For samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to sample loss from splattering during heating.

REFERENCES

1. This method paraphrases *Official Methods of Analysis,* Association of Official Analytical Chemists, 16th ed., Gaithersburg, MD, 1995, Methods 925.09ff, 926.12 and 934.01, and *Approved Methods of the American Association of Cereal Chemists,* American Association of Cereal Chemists, 8th ed., 1983, Methods 44–32 and 44–40.
2. Suitable equipment may be available from Sargent-Welch or Arthur H. Thomas.

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AOCS

AOCS Official Method Ba 3-38

Reapproved 2009

Oil

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test.

SCOPE

Applicable to cottonseed meats, and cake and meal from cottonseed, soybeans, peanuts, and flaxseed.

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 of AOCS Official Method Aa 4-38. See Figure 1 below.
2. Filter paper—150 mm; S & S no. 597, Whatman no. 2, Reeve Angel no. 211, or equivalent.
3. Absorbent cotton—free of petroleum ether extractables.
4. Porcelain mortar and pestle—The mortar must be at least 4 in. i.d. at the top. The pestle handle must be large enough to afford a firm hand grip. The inner surface of the mortar is kept rough by occasionally grinding with sand.
5. Sieve—US no. 20.
6. Sieve—US no. 30.
7. Laboratory mill—suitable for grinding the samples to a *maximum* particle size of 0.850 mm (US no. 20 sieve); except for linseed meal, for which the samples are ground to a *maximum* particle size of 0.600 mm (US no. 30 sieve).

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Grind the 100-g portion from AOCS Official Method Ba 2a-38, Preparation of Test Sample, through the laboratory mill to a uniform fineness (*maximum* particle size of 0.850 mm, 20 mesh), except for linseed meal (*maximum* particle size of 0.600 mm, 30 mesh). Immediately return to an airtight container. Oil, ground moisture, and protein nitrogen are determined on this portion.

PROCEDURE

1. Meal and ground cake or pellets—
   (a) Weigh 5 g of the ground test sample into a filter paper and enclose in a second filter paper, folded in such a fashion as to prevent escape of the meal (see Fig. 2). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the test portion.
   (b) Place wrapped test portion in the Butt extraction tube and assemble the apparatus as shown in Figure 1. Put about 25 mL of petroleum ether into the tared extraction flask before attaching to the tube.
   (c) Heat in a water bath or on an electric hot plate at such a rate that the solvent will drop from the condenser on the center of the thimble at the rate of at least 150 drops/min.
   (d) Keep the volume of solvent fairly constant by adding enough to make up for any that may be lost due to evaporation. Continue extraction for 3 hr.
   (e) Cool and disconnect the extraction flask. Evaporate the petroleum ether on a steam bath or in a water bath until no odor of solvent remains. A gentle stream of clean, dry nitrogen may be used to facilitate removal of the solvent. Cool to room temperature, carefully remove any moisture or dirt from the outside of the flask and weigh. Repeat heating until constant weight is obtained.
   (f) Determine the moisture in the ground test sample as directed in AOCS Official Method Ba 2a-38.
2. Cottonseed meats—
   (a) Weigh accurately about 2 g of the ground test sample and proceed as directed in Procedure, 1, (a) above, continuing the extraction for 2 hr only.
   (b) Remove the thimble from the Butt tube, allow the solvent to evaporate from the filter paper and test portion at room temperature. Then carefully transfer the test portion to the mortar so there will be no loss. Grind the test portion in the mortar with the pestle for at least 1 min, or with at least 100 vigorous strokes. Use no abrasive.
   (c) Return the reground test portion to the same filter paper and continue the extraction as before for an additional 2 hr. From here on, proceed as directed in Procedure 1, (d), (e), and (f).

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CALCULATIONS

1. Oil, % = $\frac{\text{mass, g of oil}}{\text{mass, g of test portion}} \times 100$
2. The percentage oil may be calculated to any desired moisture basis with the following formula:

   Oil, desired moisture basis, % = $\frac{F(100 - \%\text{ moisture desired})}{100 - \%\text{ moisture in test portion analyzed}} \times 100$

   Where—
   F = percent oil in test portion analyzed

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

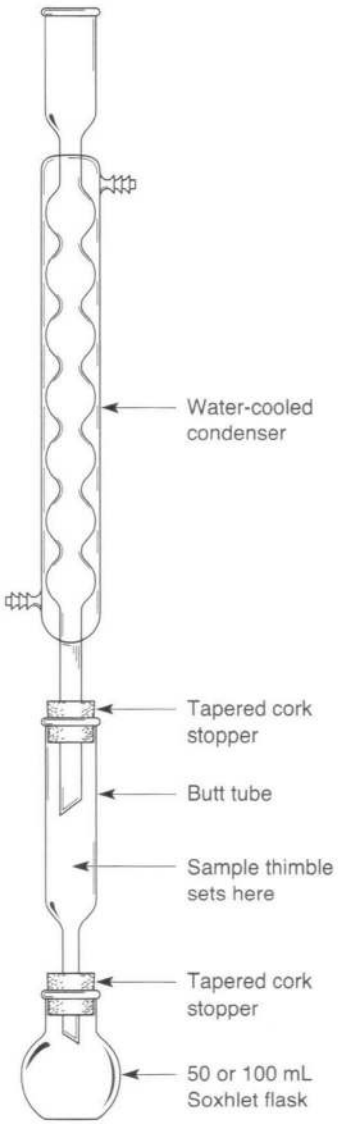

Figure 1. Butt-type extraction apparatus.

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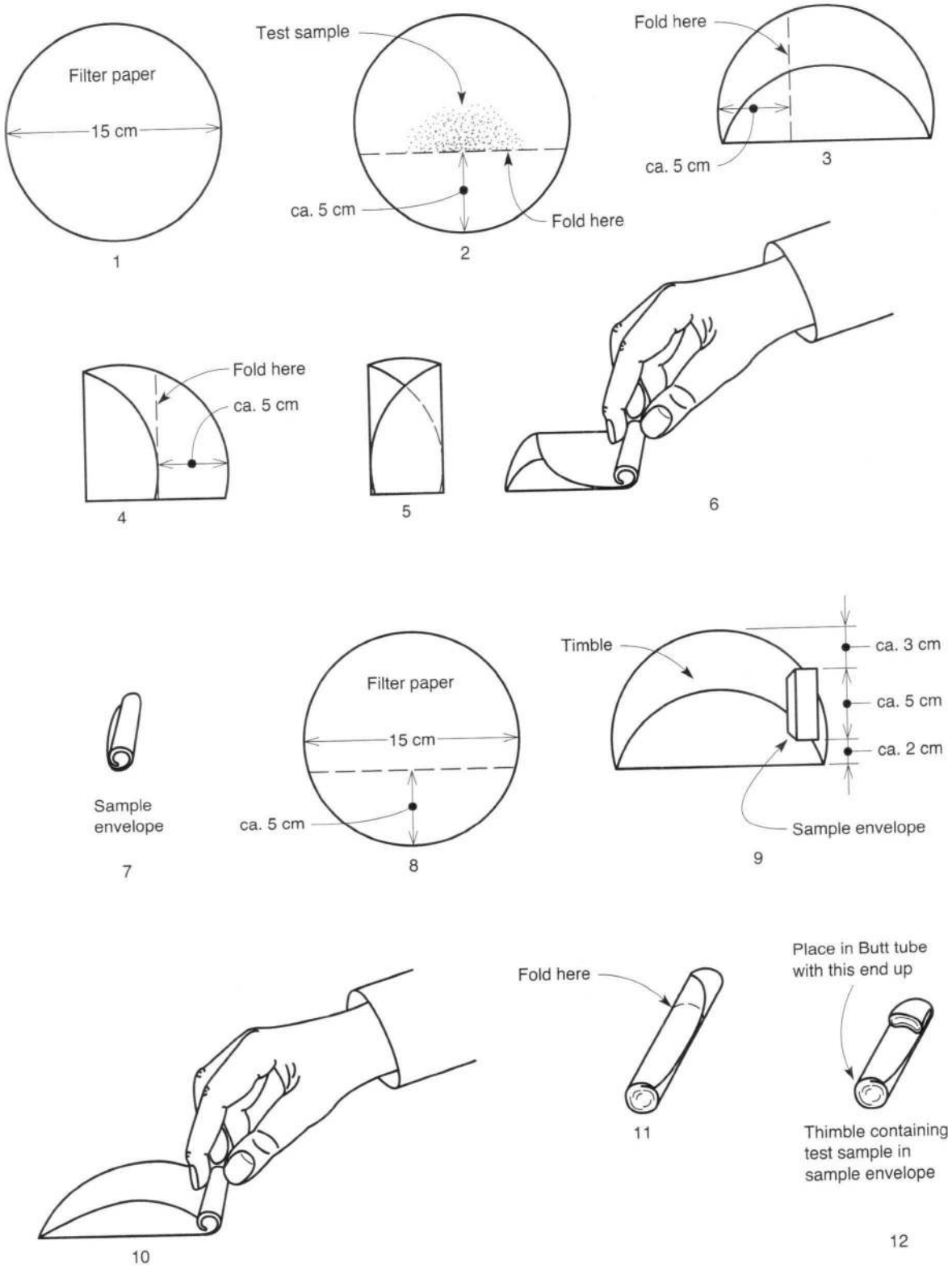

Figure 2. For oil extraction, fold filter paper in the sequence shown, steps 1 through 12.

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AOCS

AOCS Official Method Ba 4a-38

Formerly Ba 4-38, Changed 1989 • Revised 2011

Nitrogen-Ammonia-Protein Modified Kjeldahl Method

DEFINITION

This method determines, as ammonia, the total nitrogen content and protein as 6.25 times the nitrogen content of the sample (see Notes, 1).

SCOPE

Applicable to cottonseed meats, and meal and ground cake or pellets from cottonseed, soybeans, peanuts and flaxseed.

WARNING

This method should not be used because of health and environmental concerns. This method has been replaced by AOCS Official Methods Ba 4d-90 and Ba 4e-93. The method is reproduced here only for historical and reference purposes.

APPARATUS

1. Kjeldahl digestion and distillation apparatus—complete with heat source, traps and block-tin or equivalent noncorrosive tubing condensers (see Notes, 2).
2. Kjeldahl flasks—800 mL.
3. Distillate receiving flasks—500 mL or any convenient size.

REAGENTS

1. Mercuric oxide—reagent grade (see Notes, *Caution*).
2. Sulfuric acid—sp. gr. 1.84 (see Notes, *Caution*).
3. Zinc metal—granular, 20-mesh.
4. Potassium or sodium sulfate—ACS grade.
5. Potassium or sodium sulfide solution—4% in water.
6. Sodium hydroxide solution—sp. gr. 1.50 (see Notes, *Caution*).
7. Sodium hydroxide solution—0.25 N, accurately standardized (see AOCS Specification H 12-52).
8. Sulfuric acid—0.5 N, accurately standardized (see AOCS Specification H 13-52).
9. Methyl red indicator solution—0.1% in ethyl alcohol or Alizarin Red S, 0.3% in distilled water.

SAMPLE PREPARATION

1. Mix the sample thoroughly. A Henry velocity mixer may be used, or mixing may be done by placing the entire ground sample into a ½ gallon Mason fruit jar containing a large rubber stopper. Replace the cover and shake vigorously until thoroughly mixed. Transfer contents to an airtight container. This sample is used for the determination of oil, nitrogen and a second (ground) moisture. Weighings for all determinations should be made at the same time. (Henry velocity mixer available through Mid-Continent Engineering, Memphis, TN, USA.)
2. Weigh 1.7032 g of sample into Kjeldahl flask. If calculations are carried out in terms of nitrogen (N), weigh 1.4008 g. Add 0.7 g of mercuric oxide, 15 g of K_2SO_4 or Na_2SO_4 and 25 mL of H_2SO_4 (see Notes, 3).
3. Place the flask on the digestion rack (in an inclined position) and heat, below the boiling point of the acid, for 5–15 min, or until frothing ceases.
4. Increase the temperature and digest until digestion is complete, conducting the digestion over heating device, adjusted to bring 250 mL H_2O at 25°C in a Kjeldahl flask to rolling boil in 5 min. Digest for at least 30 min after the liquid has become clear and colorless (see Notes, 4).
5. Cool, add about 300 mL of water, a few granules of zinc to prevent bumping, and sufficient sulfide solution (usually about 25 mL) to precipitate all of the mercury.
6. Transfer accurately a sufficient quantity of the standard acid into receiving flask so that there will be an excess of at least 0.5 mL of 0.5 N acid. Add sufficient distilled water to cover the end of the outlet tube and attach to outlet end of condenser tube. The distillate should discharge through a glass tube at the bottom of the receiving flask.
7. Mix thoroughly and add sufficient alkali solution (usually 60 mL) to make strongly alkaline. Pour the alkali down the side of the Kjeldahl flask so that it does not quickly mix with the acid.
8. Connect the Kjeldahl flask to the other end of the condenser tube and mix the contents by shaking. Apply heat and distill until at least 150 mL of distillate have been collected.
9. Titrate the contents of the receiving flask with 0.25 N NaOH solution, using 3 or 4 drops of indicator.

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10. Conduct a blank determination of all reagents, using one gram of sucrose in place of sample, simultaneously with the samples and similar in all respects.
11. Determine moisture in the ground sample as directed in AOCS Official Method Ba 2a-38, Procedure section.

CALCULATIONS

1. Ammonia (NH_3), $\% = \dfrac{(B-S) \times N \times 0.17032 \times 100}{\text{mass, g of sample}}$

2. If factor weight sample and 0.25 N sodium hydroxide are used, the calculation is—

$$\text{Ammonia } (NH_3),\% = \frac{(B-S)}{4}$$

Where—
B = volume, mL of alkali back titration of blank
S = volume, mL of alkali back titration of sample
N = Normality of alkali.

3. Calculate to the desired moisture basis as directed in the Calculation section of AOCS Official Method Ba 3-38, substituting % ammonia (nitrogen or protein) for F—
% ammonia (NH_3) × 5.14 = % protein
4. Calculation: N × 6.25 = P
Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

NOTES

Caution

Mercury and mercury compounds are highly toxic by ingestion, inhalation and skin absorption. The TLV (as mercury) is 0.05 mg/cubic meter of air. See the Laboratory Safety section for a procedure to contain environmental contamination of mercury waste from Kjeldahl digestions. Disposal of any mercury waste must be in accordance with local and EPA regulations.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Alkalies can burn skin, eye and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

This method should not be used because of health and environmental concerns. This method has been replaced by AOCS Official Methods Ba 4d-90 and Ba 4e-93. The method is reproduced here *only* for historical and reference purposes.

NUMBERED NOTES

1. See AOCS Official Method Ba 4b-87 for an alternative procedure which does not require the use of mercury.
2. Use of steam and water-resistant rubber tubing and stoppers to connect the glass parts is strongly recommended.
3. More than 25 mL of concentrated sulfuric acid is needed for samples high in fat or oil (Procedure, paragraph 2) which require more acid to effect complete digestion and at the same time avoid loss in nitrogen. It is suggested that in the analysis of such samples that 30 mL or more of the acid be added.
4. Complete conversion of organic nitrogen to ammoniacal nitrogen is essential to obtain accurate and precise results. Longer digestion than 30 min after the liquid has become clear and colorless may prove necessary even though temperature of heating specified in Procedure, paragraph 4 is maintained during the digestion. Prolonged digestion, however, should be avoided, because some ammoniacal nitrogen can be lost by oxidation. Large sample particles should be avoided, because the larger particles will require a longer digestion time.

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AOCS

AOCS Official Method Ba 4d-90

Revised 2011

Nitrogen-Ammonia-Protein Modified Kjeldahl Method Titanium Dioxide + Copper Sulfate Catalyst

DEFINITION

This method determines total nitrogen content and protein as 6.25 times the nitrogen content of the sample (see Notes, 1).

SCOPE

Applicable to cottonseed meats, and meal and ground cake or pellets from cottonseed, soybeans, peanuts, and flaxseed.

APPARATUS

1. Kjeldahl digestion and distillation apparatus—complete with heat source, traps, and block-tin, or equivalent noncorrosive tubing condensers (see Notes, 2).
2. Kjeldahl flasks—800 mL.
3. Distillate receiving flasks—500 mL, or any convenient size.

REAGENTS

1. Catalyst mixture—containing potassium sulfate, 16.7 g + titanium dioxide, 0.6 g + copper sulfate, 0.01 g + pumice, 0.3 g.
2. Sulfuric acid—sp. gr. 1.84 (see Notes, *Caution*).
3. Sodium hydroxide solution—sp gr 1.50 (see Notes, *Caution*).
4. Sodium hydroxide solution—0.25 M, accurately standardized (see AOCS Specification H 12-52).
5. Sulfuric acid—0.25 M, accurately standardized (see AOCS Specification H 13-52).
6. Methyl red indicator solution—0.1% in ethyl alcohol, or Alizarin Red S—0.3% in distilled water.
7. Alundum boiling stones—8–14 mesh.
8. Zinc metal—granular. (If the catalyst is used with added pumice, granular zinc metal need not be added as an antibumping agent.)

PREPARATION OF TEST SAMPLE

1. Grind the 100 g portion of test sample from AOCS Official Method Ba 2a-38, Preparation of Sample, through a suitable laboratory mill to a uniform fineness of about 20 mesh (about 30 mesh for linseed meal). Mix the sample thoroughly. A Henry velocity mixer (available from Mid-Continent Engineering, Memphis, TN, USA) may be used, or mixing may be done by placing the entire ground sample into a 2 L Mason fruit jar containing a large rubber stopper. Replace the cover and shake vigorously until thoroughly mixed. Transfer the ground test sample to an airtight container. Oil, second (ground) moisture and protein nitrogen are determined on this ground portion. Weighings for all determinations should be made at the same time. If determining ground moisture, immediately proceed with the moisture determination.

PROCEDURE

1. Weigh 0.250–1.750 g of the ground test sample (see Notes, 3) into the Kjeldahl flask. Add the catalyst mixture (premixed catalyst packs are commercially available and may be used).
2. Add 30 mL of concentrated H_2SO_4 (see Notes, 4) to the sample and catalyst in the digestion flask. Include at least one sample of high-purity lysine hydrochloride in each day's run as a check of the correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.
3. Add a few Alundum boiling stones to the sample flask.
4. To digest the sample, first adjust heat to bring 250 mL of water at 25°C to rolling boil in 5 min. Place the sample flask on the digestion rack in an inclined position and then heat sample flask at the 5 min boil rate until dense, white fumes clear bulb of flask (approximately 5 min). Swirl gently and continue heating an additional 40 min after the liquid has become clear and colorless (see Notes, 5). The Kjeldahl flasks should be rotated a minimum of three times during the digestion.
5. Cool, cautiously add about 300 mL of water and cool to room temperature (see Notes, 6). Add water as soon as possible to reduce amount of caking. If excessive bumping occurs during distillation, increase dilution water from 300 mL to about 350 mL.

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6. Accurately transfer a sufficient quantity of the standard acid into receiving flask so that there will be an excess of at least 0.5 mL of 0.25 M sulfuric acid. Add sufficient distilled water to cover the end of the outlet tube and attach to outlet end of condenser tube. The distillate should discharge through a glass tube at the bottom of the receiving flask.
7. Add an additional 0.5–1.0 g Alundum boiling stones to cooled digestion flask. Mix thoroughly and add sufficient alkali solution (Reagents, 3) to make strongly alkaline. Pour the alkali down the side of the Kjeldahl flask so that it does not mix quickly with the acid.
8. Immediately connect the Kjeldahl flask to the other end of the condenser tube and thoroughly mix the contents by shaking. Apply heat at about a 7.5 min boil rate and distill until at least 150 mL of distillate have been collected.
9. Titrate the contents of the receiving flask with 0.25 M NaOH solution, using 3 or 4 drops of indicator.
10. Conduct a blank determination of all reagents simultaneously with the samples and similar in all respects. Correct for blank determined on reagents.
11. The performance of the entire method should be checked frequently by analyzing either National Institute of Standards and Technology (NIST) certified ammonium dihydrogen phosphate (Standard Reference Material (SRM) 194) or high-purity lysine hydrochloride.
12. Determine moisture in the ground sample as directed in AOCS Official Method Ba 2a-38, Procedure section.

CALCULATIONS

1. Nitrogen, % = $\dfrac{[(MA \times 2 \times mLA) - (mLBK \times MB) - (mLB \times MB)] \times 1400.67}{\text{mg test portion}}$

   Where—
   mLB = volume, mL standard base used for the sample
   mLA = volume, mL standard acid used for that sample
   mLBK = volume, mL standard base needed to titrate 1 mL of standard acid minus mL standard base needed to titrate reagent blank carried through method and distilled into 1 mL standard acid
   MA = molarity of standard acid
   MB = molarity of standard base

   Note—To determine percent ammonia, substitute 1703.06 for 1400.67 in the equation for the calculation of percent nitrogen.
2. Calculation: N × 6.25 = P
   Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

PRECISION

1. Table 1 lists the statistical parameters for soybean meal and soy protein concentrate determined from an Association of Official Analytical Chemists (AOAC) interlaboratory study.
2. Tables 2, 3, and 4 list the results of the second AOCS collaborative study, March 1991, for the determination of nitrogen in cottonseed and cottonseed meal, using a 0.56 catalyst-to-acid ratio for $TiO_2/CuSO_4$ catalyst. The project coordinator was James Falk, member of the AOCS Seed and Meal Analysis Technical Committee.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Alkalies can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali solutions. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not vice versa. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. This method has been evaluated through interlaboratory comparison of catalysts and has been adopted by the committee as the official replacement for the mercuric oxide catalyzed method, AOCS Method Ba 4a-38. An interlaboratory evaluation (References, 1) indicated that AOCS Official Method Ba 4d-90 (which uses the copper/titanium catalyst mixture) produces results more closely in agreement with AOCS Official Method Ba 4a-38 (using mercuric oxide catalyst) than methods using copper sulfate catalyst. As a result of this study, Methods Ba 4a-38 (mercuric oxide), Ba 4b-87 (copper sulfate), and Ba 4c-87 (Kjel-Foss Automatic) were declared surplus (obsolete) in 1991.

   The AOAC collaborative study (References, 2) showed no bias when comparing Cu/Ti catalyst with HgO. The AOCS interlaboratory evaluation (References, 1), based on sample weight, acid, volume, digestion temperature and time, previously

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specified in AOCS Official Method Ba 4a-38, indicated an average bias of –0.244%, which was statistically significant at the 99% confidence level. Factors that may affect method bias are sample weight and particle size, catalyst composition, use of Alundum, volume of acid used for digestion, digestion temperature and digestion time. In 1991, two additional collaborative studies (cottonseed and cottonseed meal) showed that when 30 mL of sulfuric acid were used, caking of the digest was reduced and results were comparable to the mercuric oxide catalyzed method. As a result, the AOCS versions of this method may specify the use of more than 20 mL of sulfuric acid, but otherwise conform with the AOAC version (References, 3).

2. Use of steam- and water-resistant rubber tubing and stoppers to connect the glass parts is strongly recommended.
3. Due to large particle size, a larger sample, in the range of 1.4 to 1.7 g, is recommended for cottonseed meal. This may also require an adjustment in the volume of sulfuric acid used for sample digestion.
4. More than 20 mL of concentrated sulfuric acid is needed for samples high in fat or oil, because more acid is required to effect complete digestion, avoid loss in nitrogen and prevent caking. In recent collaborative studies involving the application of this method to cottonseed meal, it was observed that when 30 mL of H_2SO_4 was used, caking was reduced and results were comparable to the mercuric oxide catalyzed method.
5. Complete conversion of organic nitrogen to ammoniacal nitrogen is essential to obtain accurate and precise results. After the liquid has become clear and colorless, it may be necessary to digest the sample for approximately an additional 30–50 min. Reagent proportions, heat input and digestion time are critical factors and should not be changed. Prolonged digestion and high temperatures must be avoided, because some ammoniacal nitrogen can be lost by oxidation. Large sample particles should be avoided, because the larger particles will require a longer digestion time.
6. The cooled digest should be liquid, or liquid with a few small crystals. Caking before the addition of water indicates too little residual acid at the end of the digestion period and may result in low nitrogen values.

REFERENCES

1. *INFORM* 1:884 (1990).
2. Kane, P. F., J. *Assoc. Off. Anal. Chem.* 70:907 (1987).
3. *Official Methods of Analysis*, AOAC International, 16th ed., Vol. 1, Gaithersburg, MD, 1995, Chapt. 4, p.13, Method 988.05.

Table 1
Statistical parameters determined from the AOAC interlaboratory study.[a]

	Soybean meal				Soy protein concentrate			
	1		2		1		2	
	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO
Labs	13	13	13	13	13	13	13	13
Labs removed	1	1	2	1	2	0	2	1
% protein	48.76	48.58	48.11	48.02	87.13	87.27	88.70	88.69
r, CV%	0.88	1.06	0.60	0.41	0.44	0.75	0.43	0.74
R, CV%	0.88	1.08	0.99	0.96	0.76	0.89	0.58	0.74

[a]References, 4.

Table 2
Summary of collaborative study results for determination of percent nitrogen (dry-weight basis) in delinted cottonseed with 21.2% oil content, using 30 mL of acid for digestion.

	1		9		1 + 9	
Sample	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO
% Nitrogen	4.28	4.29	4.31	4.30	4.30	4.30
SD	0.04	0.06	0.06	0.06	0.04	0.04
CV%	0.97	1.31	1.43	1.43	0.99	0.87
Range	0.13	0.22	0.18	0.20	0.13	0.12

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Table 3
Summary of collaborative study results for determination of percent nitrogen (dry-weight basis) in high-protein cottonseed meal, using 30 mL of acid for digestion.

	1		2		1 + 2	
Sample	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO
% Nitrogen	7.82	7.78	7.82	7.80	7.82	7.79
SD	0.09	0.09	0.07	0.05	0.07	0.06
CV%	1.14	1.15	0.90	0.64	0.90	0.73
Range	0.27	0.33	0.19	0.17	0.20	0.17

Table 4
Summary of collaborative study results for determination of percent nitrogen (dry-weight basis) in 52% protein cottonseed meal, using 30 mL of acid for digestion.

	4		8		4 + 8	
Sample	Ti-Cu	HgO	Ti-Cu	HgO	Ti-Cu	HgO
% Nitrogen	8.24	8.26	8.29	8.25	8.26	8.25
SD	0.23	0.09	0.06	0.09	0.14	0.08
CV%	0.97	0.34	0.20	0.30	0.59	0.30
Range	0.27	0.33	0.19	0.17	0.20	0.17

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AOCS Official Method Ba 4e-93

Revised 2011

Generic Combustion Method for Determination of Crude Protein

DEFINITION

This method describes a generic combustion method for the determination of crude protein. Combustion at high temperature in pure oxygen frees nitrogen, which is measured by thermal conductivity detection and then converted to equivalent protein by an appropriate numerical factor. This is an alternative method to the mercury catalyst Kjeldahl method (see Notes, 1) and has two advantages: 1) less time is needed for nitrogen determination, and 2) hazardous and toxic chemicals are not utilized.

SCOPE

This generic method is applicable to determine crude protein in animal feeds, oilseed meals and oilseeds (References, 1, 3). Refer to Ba 4f-00 for the NOPA/AOCS Certified Laboratory referee method.

APPARATUS

1. Any instrument (see Notes, 2) or device designed to measure nitrogen by combustion may be used, if it is equipped to provide the following conditions. Follow manufacturer's recommendations and instructions for the safe operation of the instrument:
   (a) Furnace—to maintain minimum operating temperature of 950°C for pyrolysis of sample in pure (99.9%) oxygen. Some systems may require higher temperature.
   (b) Isolation system—to isolate liberated nitrogen gas from other combustion products for subsequent measurement by thermal conductivity detector. Device for converting NO_2 products to N_2 or measuring nitrogen as NO_2 may be required and included in the design.
   (c) Detection system—to interpret detector response as % nitrogen, w/w. May include features such as calibration with standard material, blank determination and barometric pressure compensation. Any required calibration must be based on theoretical % nitrogen in a pure primary standard organic material, such as EDTA.

SPECIFICATIONS OF EQUIPMENT

1. System equipped as in Apparatus must meet or exceed the following minimum performance specifications:
   (a) System must be capable of measuring nitrogen in materials containing 0.2–20% nitrogen.
   (b) Accuracy of system is demonstrated by making 10 successive determinations of nitrogen in nicotinic acid and 10 successive determinations in lysine-HCL. Means of determinations must be within ±0.15 of the respective theoretical values, with standard deviations ≤ 0.15. Standard tryptophan may be substituted for lysine-HCL.
   (c) Suitable fineness (approximately 0.5 mm) (see Notes, 3) of grind gives a relative standard deviation (RSD) ≤ 2.0% for 10 successive determinations of nitrogen in a mixture of corn grain and soybeans (2 to 1, respectively) that has been ground for analysis.

$$\text{RSD, \%} = \frac{\text{SD}}{\text{mean \% N}} \times 100$$

Fineness (approximately 0.5 mm) required to achieve this precision must be used for all mixed feeds and other nonhomogeneous materials.

CALCULATIONS

1. Calculation: N × 6.25 = P
   Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

PRECISION

1. Overall method performance, based on collaborative study results:

S_r = 0.28
S_R = 0.52
RSD_r = 0.59%
RSD_R = 1.10%

2. For a discussion of the statistical analysis of collaborative study results shown in Tables 1 and 2, see Notes, 4.

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NOTES

1. The performance of the combustion method compares favorably with that for the mercury catalyst Kjeldahl method for determination of crude protein in feeds. Standard deviations by the combustion method covered the full range of possibilities compared to the Kjeldahl method; values were either equivalent, better than, or not as good as for the Kjeldahl method. For the 0.5 mm pairs, values ranged from 0.09–0.58% protein for the Kjeldahl method and from 0.14–0.33% protein for the combustion method, and (S_R) values ranged from 0.23–0.86% protein (Kjeldahl) and from 0.30–0.61% protein (combustion).

   The recovery data on standards demonstrates the ease with which the combustion method recovers nitrogen and emphasizes the difficulties of achieving full recovery with Kjeldahl methods. This has to be considered when comparing the two methods or making recommendations. The average difference between methods in the collaborative study was 0.04% N.
2. The combustion method was written in generic style and includes performance criteria to avoid requiring a particular manufacturer's instrument. Key features of the instrumentation are the focus of the description. Performance guidelines direct application of the method to determination of crude protein. These guidelines center around ensuring accurate measurement and determining the appropriate fineness (approximately 0.5 mm) of grind for nonhomogeneous samples.
3. The question of fineness of grind for samples for the combustion method is best answered by performance criteria demonstrated in this study (References, 1). The 0.5 mm sample grind met the performance guideline and was adequate for the combustion method as represented by the two LECO instruments. The traditional 1.0 mm grind did not perform as well. Sample size, which must be considered, ranged from 90–200 mg, with most weights in the 120–160 mg range. In the future, instruments that could accept a larger sample size should not be restricted to a sample fineness determined at a time when sample size was limited to the 100–200 mg range.
4. The 30 samples analyzed in the AOAC collaborative study among nine laboratories are identified in Table 1. In general, closely matched pairs were obtained by securing different lots of the same product. Collaborators were instructed to assay each sample once by the combustion method and once by AOAC mercury catalyst Kjeldahl method 7.015 (References, 3). The results from the nine laboratories are shown in Table 2.

   Collaborators were also instructed to do the performance tests for the combustion method using the material provided and to report their results. These tests for method accuracy and for establishing sample grind are in the method section on Specification of Equipment. A mixture of corn and soybeans (2 to 1, respectively), ground to 0.5 mm, was used to establish sample grind. The results from these tests were requested to help establish whether the tolerances given were reasonable. The results (References, 1, Table 3) show that the minimum accuracy requirements (average for 10 determinations each: nicotinic acid, 11.38 ± 0.2%, SD 0.25; and lysine-HCL: 15.34 ± 0.3%, SD 0.35) were easily met by most laboratories, which suggests that the tolerances should be made tighter. The sample grind test (RSD ≤ 2.0% for 10 determinations) using a material ground to 0.5 mm was met by all laboratories except Laboratory 5, so it is judged to be reasonable.

   Several collaborators commented that running 10 lysine-HCL samples in succession on the model LECO FP-228 instrument for the accuracy test tended to clog the iron chip filter so it had to be replaced often during the test. The results are quite good in spite of this annoyance. Another material could be used in place of lysine-HCL to avoid this concern.

   Two-sample X-Y plots of the 14 sample pairs were prepared according to Youden (References, 4, pages 10–11) and examined for gross outliers for both methods.

   Of the 14 possible pairs for the Kjeldahl method, nine values reported by Laboratory 3 were gross outliers. These were predominantly in the high (++) quadrant. Laboratory 5 was consistently in the low (– –) quadrant for both methods. Further ranking of the laboratories according to the Cochran method, explained in Steiner (References, 4), confirmed the consistently low results reported by Laboratory 5 for both methods.

   In addition, an analysis of variance of the X-Y differences of the ten 0.5 mm pairs, shows a lab × method interaction when Laboratories 3 and 5 are included in the test, but does not when they are removed. Grand mean of the X-Y differences were 2.46 and 2.65 for nine laboratories vs. seven laboratories, respectively, for the Kjeldahl method, and 2.62 and 2.62, respectively, for the combustion method. Based on these tests, the results from Laboratories 3 and 5 for both methods were excluded from further statistical treatment of the results. The difficulties were predominately associated with the Kjeldahl method.

   The remaining results were processed for precision estimates according to Youden's scheme of closely matched pairs (References, 4). Table 2 in this method shows the 10 pairs representing the 0.5 mm ground materials arranged in order of increasing % protein. Standard deviations for the combustion method were lower than standard deviations for the Kjeldahl method for high-protein materials (pairs 1, 3, 6, and 10) and higher than Kjeldahl results for low-protein materials (pairs 4, 7, and 8). The combustion method does not show the same effect of increasing standard deviation with higher protein level as does the Kjeldahl method. Pooled estimates of standard deviations for the combustion method were calculated. Standard deviations for repeatability (S_r) and reproducibility (S_R) were 0.28 and 0.52, respectively; relative standard deviations (RSD_r and RSD_R) were 0.59 and 1.10%, respectively.

   For the four pairs representing the 1.0 mm ground materials, the standard deviations for the combustion method were generally higher than those of the Kjeldahl method (Table 2). In some cases, the bias component (S_L) could not be estimated and is shown as a < 0 value. This has the effect of lumping all the variations into the repeatability estimate (S_r).

   Each 1.0 mm pair has a complementary 0.5 mm pair, so it is possible to compare precision between methods for the two grinds (References, 1). There is noticeable improvement in precision for the combustion method when the sample is ground finer; this effect was expected.

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Sample means for the 0.5 mm pairs were compared (References, 1). Differences in sample means, combustion-Kjeldahl, range from –0.12 to 0.63, with an average difference of 0.23% protein. Only pair 1, blood meal, shows a higher mean by the Kjeldahl method. The two methods produced results that were not significantly different according to paired t-tests (References, 4) except for pair 5, meat and bone meal, and pair 10, feather meal.

The grand means of the 140 results by each method on the 0.5 mm samples were 47.41% protein by the Kjeldahl method and 47.65% protein by the combustion method. For the 1.0 mm samples, the grand means of the 56 results by each method were 31.50% protein by the Kjeldahl method and 31.82% protein by the combustion method.

Results on the standard material (References, 1) show that recoveries were lower by the Kjeldahl method. Recoveries for five of the nine laboratories were low on nicotinic acid and two had difficulty with lysine-HCL by the Kjeldahl method (data for Laboratories 3 and 5 were removed from the statistical analysis). All results for the combustion method are satisfactory except perhaps the lysine value for Laboratory 5.

REFERENCES

1. This method was adopted from the AOAC: Sweeney, R.A, *JAOAC 72:*770 (1989).
2. Sweeney, R., and P.R. Rexroad, *JAOAC 70:*1028 (1987).
3. *Official Methods of Analysis,* Association of Official Analytical Chemists, 16th ed., Vol. 1, Gaithersburg, MD, Method 990.03, Chapt. 4, p. 18.
4. Youden, W.J., and E.H. Steiner, *Statistical Manual of the AOAC,* AOAC, Arlington, VA. (1975) pp. 74, 22–23, and 55–58.

Table 1
Description of collaborative samples for study of combustion method for determination of crude protein in feeds.

Closely matched pair	Sample no.	Estimated % protein	Grind, mm	Description
1	1	88	0.5	blood meal
	22	88		
2	3	46	0.5	soybean oil meal
	28	44		
3	7	85	0.5	beef finisher, high NPN
	12	81		
4	8	16	0.5	swine grower
	5	15		
5	10	52	0.5	meat and bonemeal
	17	48		
6	16	66	0.5	poultry diet
	26	59		
7	19	11	0.5	swine diet
	29	10		
8	23	21	0.5	corn/soybean mix
	25	20		
9	24	13	0.5	swine finish
	18	13		
10	30	89	0.5	feather meal
	15	81		
11	2	21	1.0	corn/soybean mix
	20	20		
12	4	11	1.0	swine diet
	21	10		
13	9	84	1.0	beef finisher, high NPN
	11	80		
14	27	13	1.0	swine finish
	13	13		
—	6	71	—	nicotinic acid standard
	14	96	—	lysine-HCL standard

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Table 2
Comparison of combustion and mercury catalyst Kjeldahl methods: analysis of pairs ground to 0.5 mm and to 1.0 mm.[a]

Sample pair	Mercury Kjeldahl method Mean % protein						Combustion method Mean % protein				
	X	Y	S_r	S_L	S_R		X	Y	S_r	S_L	S_R
						Samples ground to 0.5 mm					
7	11.16	10.30	0.11	0.13	0.21		11.31	10.54	0.14	0.35	0.52
9	13.65	13.15	0.13	0.24	0.36		13.76	13.27	0.15	0.25	0.39
4	15.69	15.38	0.09	0.19	0.28		16.05	15.59	0.17	0.26	0.41
8	20.40	20.08	0.21	0.07	0.23		20.58	20.35	0.16	0.25	0.39
2	46.70	44.53	0.26	0.29	0.49		46.80	44.59	0.30	0.29	0.51
5	51.68	48.30	0.52	0.06	0.52		52.11	48.93	0.28	0.15	0.35
6	65.78	58.70	0.58	0.29	0.71		65.98	59.15	0.19	0.16	0.30
3	84.64	81.22	0.46	0.51	0.86		85.02	81.67	0.23	0.40	0.61
10	89.31	81.20	0.17	0.48	0.69		89.79	81.34	0.31	0.26	0.47
1	88.33	88.01	0.42	0.48	0.80		88.21	87.93	0.33	0.31	0.55
						Pooled			0.28		0.52
						Pooled RSD_r%			0.59	RSD_R%	1.10
						Samples ground to 1.0 mm					
11	20.37	20.04	0.17	0.21	0.34		20.33	20.37	0.56	0.08	0.58
12	11.09	10.05	0.13	0.22	0.34		11.47	10.31	0.28	0.34	0.56
13	83.89	80.44	0.88	<0	0.79		84.26	80.89	0.86	<0	0.82
14	13.21	12.94	0.36	<0	0.35		13.52	13.44	0.41	0.38	0.68

[a]Data from Laboratories 3 and 5 excluded.

Table 3
An international study of the protein nitrogen combustion method, coordinated by Janet Brown and David Berner of the AOCS during 1993–1994, in which 24 laboratories participated, each analyzing 10 samples of oilseed meal and obtaining two (2) values [except for samples A, B, and C which were submitted in duplicate and for which four (4) values were obtained], gave the statistical results summarized in the following table.

	Samples[a]						
	A	B	C	D	E	F	G
Number of labs after outliers	24	24	24	24	23	24	23
n, determinations	92	91	91	47	45	47	45
Outliers	2	3	4	0	2	0	2
Nitrogen, %—Smalley, combustion[b]	6.61	7.85	8.22	7.86	7.20	3.33	3.35
Nitrogen, %—Smalley, Kjeldahl[b]	6.55	7.77	8.12	7.78	7.13	3.29	3.26
Nitrogen, %—collaborative study, combustion	6.62	7.88	8.25	7.89	7.21	3.34	3.32
Repeatability[c]—							
S_r	0.06	0.05	0.03	0.04	0.03	0.04	0.05
RSD_r	0.85	0.60	0.39	0.46	0.37	1.25	1.47
$r = (2.8 \times S_r)$	0.17	0.14	0.08	0.11	0.08	0.11	0.14
Reproducibility[c]—							
S_R	0.07	0.06	0.07	0.08	0.04	0.11	0.06
RSD_R	1.04	0.81	0.80	0.97	0.60	3.23	1.70
$R = (2.8 \times S_R)$	0.20	0.17	0.20	0.22	0.11	0.31	0.17

[a]Sample key: A = Cottonseed meal. B = Soybean meal. C = Peanut meal. D = Soybean meal. E = Canola meal. F = Safflower meal. G = Safflower meal.
[b]Results from 1993–1994 Smalley Check Sample Program.
[c]Statistical parameters relate only to percent nitrogen values obtained in collaborative study.

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AOCS Official Method Ba 4f-00

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Combustion Method for Determination of Crude Protein in Soybean Meal

DEFINITION

This method describes a generic combustion method for the determination of crude protein in soybean meal. Combustion at high temperature in pure oxygen frees nitrogen, which is measured by thermal conductivity detection and then converted to equivalent protein by an appropriate numerical factor. This is a replacement method for the mercury catalyst and titanium dioxide/copper sulfate Kjeldahl methods and has two advantages: (1) less time is needed for nitrogen determination, and (2) hazardous and toxic chemicals are not utilized.

SCOPE

This method is applicable for the determination of crude protein in soybean meal (References, 1, 3).

APPARATUS

1. Any instrument or device designed to measure nitrogen (N) by combustion may be used, if it is equipped to provide the following conditions. Follow manufacturer's recommendations and instructions for the safe operation and proper maintenance of the instrument:
   (a) Furnace—to maintain a minimum operating temperature of 950°C for pyrolysis of a sample in pure (99.9%) oxygen. Some systems may require a higher temperature.
   (b) Isolation system—to isolate liberated nitrogen gas from other combustion products for subsequent measurement by the thermal conductivity detector. A device for converting NO_2 products to N_2 or measuring nitrogen as NO_2 may be required and included in the design.
   (c) Detection system—to interpret the detector response as % nitrogen, w/w. It may include features such as calibration with standard material, blank determination, and barometric pressure compensation. Any required calibration must be based on theoretical % nitrogen in a pure, standard material such as EDTA.

SPECIFICATIONS OF EQUIPMENT

1. Any system equipped as in Apparatus must meet or exceed the following minimum performance specifications:
   (a) The system must be capable of measuring nitrogen in materials containing 0.2–20% nitrogen.
   (b) Accuracy of the system must be demonstrated by making multiple determinations of nitrogen in two or more types of reference standards. Means of determinations must be within ± 0.025 of the respective theoretical values.
   (c) Suitable fineness of grind should be ≤0.5 mm. For soybean meals this should give a relative standard deviation (RSD) of ≤1.5% for 10 successive determinations of nitrogen content.

REAGENTS

1. Gases—gases will vary based on instrument type and manufacturer. Combustion gases must be of ultra-high purity (≥99.9%).
2. Column packing materials and consumables—these will vary based on instrument configuration, type, and manufacturer. Follow manufacturer specifications for purity requirements on these materials.
3. Primary standard—
   (a) EDTA—NIST traceable grade
4. Secondary standards—
   (a) Aspartic acid—ACS grade
   (b) Glutamic acid—ACS grade
   (c) EDTA—ACS grade

TEST SAMPLE PREPARATION

1. Portions of the laboratory samples must be ground so as to produce a homogenous sample having a grind consistency of ≤0.5 mm.
2. Test samples must be analyzed for moisture content before and after grinding (see Note, 1; References, 2).

INSTRUMENT CALIBRATION

Upon initial use and periodically therafter, instrument calibration against an NIST traceable standard such as EDTA must be performed (see Note, 2). This is to compensate for detector response fluctuations which are caused in part by changes in ambient atmospheric pressure. In performing the calibration, a "nitrogen calibration factor" is obtained. This factor is subsequently used

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when determining the purity of secondary standards, which can then be used to determine the instrument's "nitrogen factor" on a more routine basis.

1. Follow the manufacturer's procedure to verify proper operation of the analyzer.
2. No fewer than 5 successive determinations using EDTA should be made. The nominal weight utilized when analyzing EDTA is 150 ± 3 mg recorded to 0.1 mg (see Notes, 3).
3. Determine the instrument's "nitrogen calibration factor" by dividing the certified value of the EDTA by the average of the 5 determinations.

PROCEDURE

1. Prior to sample analysis, a daily instrument calibration must be performed. Typically, this will consist of analyzing a secondary standard such as aspartic acid in 3 to 5 successive determinations. The nominal weight utilized when analyzing aspartic acid is 170 ± 5 mg (see Notes, 2 and 3).
2. Prior to calibration, a blank and "burn in" sample should be run to purge the system of excess gas.
3. Nitrogen analysis for soybean meals should be carried out using 200 ± 5 mg of tightly pressed or pelleted test portions (see Notes, 3).
4. Specific procedures for sample introduction, analysis, analysis time, etc. will be dictated by instrument configuration, type, and manufacturer.
5. Reference standards, such as aspartic and glutamic acids, should be analyzed at the rate of 1 per every 10 samples.
6. All test portion nitrogen values must be corrected against the overall average of the reference standard values.

CALCULATIONS

1. Correction factor (F *m*) for moisture loss =

$$\frac{100 - \text{moisture before grinding}}{100 - \text{moisture after grinding}}$$

2. Nitrogen calibration factor (F *nc*) for EDTA =

$$\frac{\text{Theoretical \% N content of EDTA}}{\text{Average \% N content of EDTA}}$$

3. Correction factor (F *rs*) for reference standards (RS) =

$$\frac{\text{Theoretical \% N content of RS} \times (\text{F } nc)}{\text{Average \% N content of RS}}$$

4. Crude protein, % = (F *m*) (F *rs*) (6.25) (N)
   Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

PRECISION

For an overall method performance review and a discussion of the statistical analysis of the method, refer to AOCS Official Method, Ba 4e-93.

This method was studied collaboratively in 1999–2000. Precision data are presented in Table 1. Repeatability and reproducibility limits are given by r and R, respectively.

NOTES

1. Due to significant heat buildup in grinding, it is common to see a significant loss of moisture as a result of the grinding process. This must be taken into account when calculating a protein result.
2. In addition to the initial and periodic calibrations, each time system pressure is broken, or if the instrument does not remain in continual use, when new primary or secondary reference materials are put into use, it must be recalibrated prior to the analysis of test portions.
3. The weights taken for test portions and standards may be adjusted in accordance with instrument manufacturer specifications.

REFERENCES

1. This method was adopted in part from the AOAC: Sweeney, R.A., *J. Assn. Off. Anal. Chem. 72*(5):770 (1989).
2. *Official Methods and Recommended Practices of the AOCS*, American Oil Chemists' Society, 5th edn., Champaign, Illinois, Official Method Ac 2-41, 1997.
3. *Official Methods of Analysis*, Association of Official Analytical Chemists, 16th edn., Vol. 1, Gaithersburg, MD, Method 990.03, Chapter 4, p. 18.
4. *Official Methods and Recommended Practices of the AOCS*, American Oil Chemists' Society, 5th edn., Champaign, Illinois, Official Method Ba 4e-93, 1999.

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Table 1
Results of an AOCS collaborative study on soybean meal using a NIST certified reference standard.

Sample	1	2	3	4	5	6	7
Total number of laboratories accepted	14	13	13	13	13	12	14
Total number of replicates	28	26	26	26	26	24	28
Mean of the laboratory values (as % protein)	50.23	49.70	50.59	50.09	49.81	49.44	50.24
Repeatability Standard deviation, s(r)	0.15	0.11	0.15	0.16	0.24	0.15	0.22
Relative standard deviation, RSD(r)%	0.29	0.23	0.29	0.31	0.47	0.31	0.44
Repeatability value, 2.8s(r)	0.41	0.32	0.42	0.44	0.66	0.43	0.62
Reproducibility Standard deviation, s(R)	0.36	0.41	0.36	0.46	0.25	0.25	0.45
Relative standard deviation, RSD(R)%	0.72	0.83	0.70	0.93	0.50	0.51	0.90
Reproducibility value, 2.8s(R)	1.01	1.16	1.00	1.30	0.70	0.70	1.26

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AOCS Official Method Ba 5a-49

Reapproved 2009

Ash

DEFINITION

This method determines as ash the residue remaining after incineration under the conditions specified for the test.

SCOPE

Applicable to cake and meal from cottonseed, soybeans, peanuts, and flaxseed. Formerly Ba 5-47.

APPARATUS

1. Porcelain combustion capsules—Coors no. 170, size no. 3, 25 mL capacity.
2. Electric muffle furnace—with automatic pyrometer control to regulate at a temperature of 600 ± 15°C.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.

PREPARATION OF TEST SAMPLE

1. Use test sample prepared as directed in AOCS Official Method Ba 3-38, Preparation of Test Sample.

PROCEDURE

1. Weigh 2 g of well-mixed test sample into the previously heated and tared combustion capsule, place in muffle furnace heated to 600°C, and maintain at this temperature (± 15°C) for 2 hr.
2. Transfer capsule to a desiccator, cool to room temperature, and weigh immediately thereafter.

CALCULATIONS

1. $$\text{Ash, \%} = \frac{\text{mass, g of ash}}{\text{mass, g of test portion}} \times 100$$

   Report result to nearest 0.1%.

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AOCS

AOCS Official Method Ba 5b-68

Reapproved 2009

Acid-Insoluble Ash

DEFINITION

This method determines as acid-insoluble ash the residue remaining after incineration and acid digestion under the conditions specified for the test.

SCOPE

Applicable to oilseed meal products. Formerly Ba 5a-68.

APPARATUS

1. Crucibles or dishes—50 mL capacity, platinum or porcelain (see Notes, 1).
2. Muffle furnace—with pyrometer control to regulate at a temperature of 600 ± 15°C.
3. Steam bath.
4. Meeker burner.
5. Filter paper—Whatman no. 40.

REAGENTS

1. Hydrochloric acid (HCl)—concentrated reagent grade, sp. gr. 1.19 (see Notes, *Caution*).

PROCEDURE

1. Weigh accurately about 10 g of ground, well-mixed test sample into tared crucible (or dish).
2. Heat carefully with a Meeker burner until the readily combustible material has been burned off.
3. Transfer crucible to the muffle furnace and ignite to constant weight at 600 ± 15°C (see Notes, 2). The weight of the residue at this point can be used to estimate the percentage of total ash in the sample.
4. Add 5 mL of concentrated HCl to residue and heat to boiling. Evaporate the mixture to dryness on a steam bath. Continue heating the dried residue on the steam bath for 30 min.
5. Add 5 mL concentrated HCl to residue and heat to boiling. Add 20 mL of distilled water and heat.
6. Filter the hot solution through a no. 40 Whatman ashless filter paper, quantitatively transferring the precipitate. Wash with a minimum of 150 mL of hot distilled water (see Notes, 3).
7. Return filter paper containing the washed residue to heated and tared crucible and ignite in the muffle furnace for 3 hr at 600 ± 15°C.
8. Cool crucible in a desiccator and weigh.

CALCULATIONS

1. Acid insoluble ash, % (see Notes, 4) $= \dfrac{\text{mass, g of residue after acid treatment}}{\text{mass of test portion, g}} \times 100$

PRECISION

1. Standard deviation within laboratories: 0.017% acid-insoluble ash.
2. Standard deviation between laboratories: 0.018% acid-insoluble ash.
3. Agreement within a laboratory: Two single determinations performed in one laboratory shall not differ by more than 0.05% acid-insoluble ash.
4. Agreement between laboratories: Single determinations performed in two different laboratories shall not differ by more than 0.05% acid-insoluble ash.

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. Platinum crucibles are preferred, but porcelain crucibles may be used, provided they are in good condition.

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2. The time required to ash the sample prior to acid treatment may vary, depending upon the type of vessel used. It takes about 7 hr in the muffle furnace to ash the sample in a 50 mL platinum crucible. Abnormally high values for acid-insoluble ash tend to result when the original meal sample is not thoroughly burned before the acid treatment takes place.
3. It is important to wash the acid-insoluble ash thoroughly to be certain that all soluble material is removed. At least 150 mL of hot water should be used for this purpose.
4. More than 0.5% acid-insoluble ash in linseed meal is considered excessive, according to References, 1.

REFERENCES

1. *Feed Control,* Official Publication, Association of American Feed Control Officials, 1967, definition 39.1.

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AOCS Official Method Ba 6-84

Reapproved 2009

Crude Fiber

DEFINITION

This method determines, as crude fiber, the loss on incineration of the oven-dried residue remaining after digestion of the sample with dilute sulfuric acid and dilute sodium hydroxide, as specified in the conditions outlined in the procedure.

SCOPE

Applicable to grains, meals, flours, feeds, and all fiber-bearing material from which the oil or fat can be extracted leaving a workable residue. This is a joint AOCS-Association of Official Analytical Chemists (AOAC) method (References, 1). Replaces Ba 6-49 and Ba 6-61.

REAGENTS

1. Sulfuric acid solution—containing 1.25 g H_2SO_4 per 100 mL (1.25% or 0.127 M) (see Notes, *Caution*).
2. Sodium hydroxide solution—containing 1.25 g NaOH per 100 mL (1.25% or 0.313 M) (see Notes, *Caution*).
3. Ceramic fiber—Prepare by blending about 150 g of ceramic fiber with 250 mL distilled water in a Waring blender at high speed for 5 min. Store in plastic jar. Individual mats may be prepared by slowly pouring 75–100 mL of mixed slurry into a Buchner funnel (Figure 1), without suction and allowing it to settle before turning on the vacuum. Optimum mat size for 70 mm funnel is 3–4 g; for 55 mm funnel, 2–2.5 g. Alternate preparation: Stack US no. 6 mesh sieve on top of no. 10 mesh sieve. Place suitable amount of ceramic fiber on no. 6 mesh sieve and rub across sieve until all of the fiber has passed through the openings. Discard small amount left on no. 6 sieve. Shake material on no. 10 mesh sieve for about 1 min, and discard fines that pass through the sieve. Save and store the fiber remaining on the no. 10 mesh sieve. Any method of preparation is acceptable if it results in the making of a satisfactory mat in the Buchner funnel. Discarding the fines will reduce the magnitude of the blank determination.
4. Glass wool—Pyrex™ (Owens-Corning no. 3950), or equivalent.
5. Methyl alcohol 95%, isopropyl alcohol or ethyl alcohol 95%.
6. Diethyl ether—ACS grade, or petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
7. Dow-Corning Antifoam A Emulsion—diluted 1:4 with water. This is also available in a solvent-dilutable form as Antifoam A Compound.
8. Bumping chips or granules—broken alundum crucibles or alundum granules, or equivalent (see Notes, 2).

APPARATUS

1. Crude fiber digestion apparatus—with condenser to fit a 600 mL beaker and a hot plate adjustable to a temperature that will bring 200 mL of distilled water at 25°C to a rolling boil in 15 ± 2 min (suitable equipment is available from Precision Scientific Co., Chicago, IL, USA).
2. 600 mL Beaker—designed to fit digestion apparatus.
3. Ashing dishes—Silica, Vitrosil 70 × 15 mm, porcelain Coors no. 60197, or equivalent.
4. Air oven—maintained at 130 ± 2°C.
5. Electric muffle furnace—with rheostat control and pyrometer that will maintain a temperature of 600 ± 15°C.
6. Desiccator—with efficient desiccant. Calcium chloride is not satisfactory. Drierite 4–8 mesh is satisfactory. See AOCS Specification H 9-87.
7. Filtering device—consisting of a no. 200 mesh US series or equivalent stainless steel screen and constructed to afford easy and efficient washing of the digested residues (see Fig. 1 and Notes, 3 and 4). When extremely fine material is analyzed, it is necessary to precoat this filtering device to avoid loss. In this case, the additional "blank" which may be occasioned by extra ceramic fiber should be determined (see Notes, 5).
8. Suction filtering apparatus—to accommodate the California State modified Buchner funnel. This includes suction flask attached to a trap in line with a water aspirator or other suction line, equipped with a valve to break the suction.
9. Apparatus designed to preheat alkali, acid, and wash water to 100°C. Suggested design consists of a tank composed of sheet copper, containing three coils made of 12.5 ft long, $\frac{3}{8}$-in. copper tubing. Inlets and outlets are soldered where tubing passes through tank walls. Fill with water and fit with reflux condenser to reduce evaporation. Two 750 watt hot plates will maintain boiling. Use Tygon tubing to connect the coils to reservoirs of distilled water, 1.25% acid, and 1.25% alkali, with gum tubing for delivery from the outlets. The capacity is adequate for 60 fiber analyses in 8 hr. See Figure 2.

PREPARATION OF TEST SAMPLE

1. Reduce a 1000 g laboratory sample using a divider or other suitable means to about 100 g and place in a sealed, moisture-proof container. Determine the moisture immediately.
2. Grind the remainder of the 100 g test sample obtained above through a laboratory mill to a uniform fineness.

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Note—Laboratory experiments have shown that the Weber mill (screen size .033–.040 in.), the micro screen (1/25–1/16 in.), and the Wiley mill (1 mm screen) will give grinds of comparative fineness. While other mills may be suitable, it is recommended that one of the mills mentioned above be used for preparing the sample for crude fiber determination. Most materials will lose moisture during grinding, and the moisture should be determined on the ground sample as the portion is taken for the crude fiber determination.

PROCEDURE USING CERAMIC FIBER (see Notes, 5)

1. Extract 2 g of the ground test sample with diethyl or petroleum ether to remove fat. Transfer to a 600 mL beaker, being careful to avoid fiber contamination from paper or brush used in the process.
   Note—If fat content of test portion is below 1%, the extraction step can be eliminated.
2. Add about 3 g of prepared ceramic fiber for 70 mm Buchner funnel or about 2 g of prepared ceramic fiber for 55 mm Buchner funnel, 200 mL of boiling H_2SO_4 (0.255 N), and 1 drop of diluted antifoam agent. Add alundum chips or granules to reduce bumping.
   Note—This antifoam agent will give high results if used in excess. Use only when necessary to control foaming.
3. Place the beaker and contents on the digestion apparatus with the hot plate at the temperature specified in Apparatus, 1. Digest at boiling temperature for exactly 30 min, rotating the beaker periodically to keep any solid matter from adhering to the sides.
4. Remove at the end of the 30 min period and filter, using the California State modified Buchner funnel, according to the following procedure:
   (a) With the Buchner funnel mounted on the suction flask and the suction on, pour the entire contents of the digestion beaker into the funnel. Rinse the beaker with 50–75 mL of boiling water and wash through the funnel. Repeat with three more 50 mL washings, and allow the vacuum to dry the funnel.
   (b) Remove the mat and digest by snapping the bottom part of the funnel against the top while covering the stem with the thumb or forefinger and place the mat and digest in the original digestion beaker. Rinse funnel with 200 mL of boiling 1.25% alkali and digest for exactly 30 min at temperature specified in Apparatus, 1.
   (c) At the end of the alkali digestion, immediately filter contents of beaker through a Gooch crucible, equipped with a glass-fiber filter disk to fit over the bottom of crucible or a 5 mm thick mat of ceramic fiber. Rinse beaker twice with 25 mL portions of 0.255 N H_2SO_4. Then rinse beaker with three 50 mL portions of distilled water. Finally, pour 25 mL of alcohol into the crucible and allow the vacuum to dry the filter. Remove crucible.
5. Dry crucible and contents at 130°C for 1 hr. Cool in a desiccator and weigh. Incinerate for 30 min at 600 ± 15°C. Cool in a desiccator and weigh again.

PROCEDURE USING GLASS WOOL (see Notes, 5)

1. Extract 2 g of the ground test sample with diethyl or petroleum ether to remove fat. Transfer to a 600 mL beaker, being careful to avoid fiber contamination from paper or brush used in the process.
   Note—If fat content of test portion is below 1%, the extraction step can be eliminated.
2. Add 200 mL of boiling 1.25% H_2SO_4 and 1 drop of the diluted antifoam agent. Add alundum chips or granules to reduce bumping, if desired.
   Note—This antifoam agent will give high results if used in excess. Use only when necessary to control foaming.
3. Place the beaker and contents on the digestion apparatus with the hot plate at the temperature specified in Apparatus, 1. Digest at boiling temperature for exactly 30 min, rotating the beaker periodically to keep any solid matter from adhering to the sides.
4. Remove at the end of the 30 min period and filter, using the California State modified Buchner funnel, according to the following procedure:
   (a) Prepare the Buchner funnel (55 mm i.d.) by cutting a round piece of glass wool of sufficient size to completely line the bottom and sides of the funnel. The glass wool pad should be shaped to the inside of the funnel. Then apply suction, using a stream of water, to form a seal between funnel and glass wool.
   (b) Care must be taken not to separate the glass fibers in the mat. This is necessary for maximum retention of the sample on the glass wool.
   (c) Glass wool no. 3950 is packed in rolls. Carefully unroll as needed. Each large layer consists of six thin layers. Normal meals, feeds, grains, and feed ingredients will be retained by a three-layer lining (see Notes, 6).
5. With the Buchner funnel mounted on the suction flask and suction applied, pour the entire contents of the digestion beaker into the funnel. Rinse the beaker with 50–75 mL of boiling water and wash through the funnel. Repeat with three more 50 mL washings and allow the vacuum to dry the funnel.
6. Transfer the glass fiber mat and digest from the funnel into the original digestion beaker. Using a stream of 1.25% alkali, rinse funnel into beaker. Add additional alkali to bring the volume to 200 mL, and digest for 30 min at the temperature specified in Apparatus, 1. Digest at boiling point for exactly 30 min, rotating the beaker periodically to keep any solid matter from adhering to the sides.
   Note—The glass fiber mat must be immersed in the solution at all times; gentle swirling of the solution at times will prevent entrapment of air and aid in keeping material in contact with the solution.

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7. Prepare a glass wool fiber mat about 10 mm thick in the Gooch crucible. Then transfer the glass fiber mat and digest to the Gooch crucible. A convenient way to do this is to pick up the wet fibers using large tweezers and twist the material into a narrow or composite mass, making it possible to place in the crucible without touching the outer edge. Then compress the materials as necessary with glass rod and pour. With the Gooch crucible mounted on a suction flask and suction applied, pour the entire contents of the digestion beaker through the Gooch crucible.
8. Rinse the beaker and glass rod using 25 mL of boiling 1.25% H_2SO_4, followed with three 50 mL washes with hot distilled water. Add all washes to the Gooch crucible. Finally, wash the crucible with 25 mL of alcohol, allow the vacuum to dry the filter mat and remove the crucible.
9. Dry the crucible and contents at 130°C for 1 hr. Cool in a desiccator and weigh. Incinerate for 30 min at 600 ± 15°C. Cool in a desiccator and weigh again.

CALCULATIONS

1. Crude fiber in ground test sample, $\% = \frac{(A - B) \times 100}{W}$

   Where—
   A = the loss in mass from incineration of sample
   B = the loss in mass from incineration of blank
   W = mass, g of test portion

2. Crude fiber, $\% = \frac{(100 - \%\text{ moisture desired})}{(100 - \%\text{ moisture in ground test sample})}$

PRECISION

1. Using ceramic fiber (References, 2)—mean, 14.24; average range, 0.24; average maximum spread, 2.24; pooled standard deviation, 0.62; S_r, 0.28; S_b, 0.55.
2. Using glass wool—
   (a) Within-lab SD: SD = 6% of the mean crude fiber (CF) value, but not less than 0.35% CF.
   (b) Between-lab SD (single determinations): SD = 7% of the mean CF value, but not less than 0.4% CF.
   (c) Between-lab SD (replicate determinations): SD = 5% of the mean CF value, but not less than 0.3% CF.
   Note—The SD varies with the size of the numerical value of the CF, but at low values reaches a minimum.
   Example: At 10.0% CF the SD for within-lab determinations would be 6% of 10.0, or 0.6%. However, at 2.0% CF the SD would be 0.35%.
3. Using asbestos (see Notes, 7)—Precision of the method was established using AOCS Official Method M 1-59 (declared surplus in 1989), modified by eliminating duplicates. Four preground samples, ranging from 2 to 12% crude fiber were submitted to twelve collaborators; 384 results were obtained and calculated on a 95% confidence limit basis:
   (a) Agreement within laboratories: Two single determinations performed in one laboratory using the Oklahoma Filter Screen shall not differ by more than 0.48%. Two single determinations performed in one laboratory using the California State modified Buchner funnel shall not differ by more than 0.68%.
   (b) Agreement between laboratories: Single determinations performed in two different laboratories using either filtering device shall not differ by more than 0.79%.
   (c) These precision data do not apply to products with high fiber, such as alfalfa meal, or products that present filtering problems, such as yeast.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

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NUMBERED NOTES

1. This equipment may be available from Porter-Warner, Rock Hill, SC, USA, or E. J. Bartell Co., Renton, WA, USA (the product available from E. J. Bartell Co. has the trade name "Cerafiber").
2. R.R. alundum 90 mesh manufactured by the Norton Co., Worcester, MA, USA.
3. Stainless steel, type 304, is recommended. Available from W. S. Tyler, Inc., Mentor, OH, USA.
4. The two-piece polyethylene funnel is similar to Fisher no. 10-362-B or -C, or Nalge no. 4280, 55 or 70 mm. The funnel is supplied without the 200 mesh screen. This can be sealed easily to the filtering surface of the funnel using a small tip soldering iron.
5. A blank determination must be run using all reagents, but without any sample.
6. Materials that will pass through a 200 mesh sieve will require all six layers of glass wool.
7. Due to the hazard associated with its use, asbestos was eliminated from this method in 1989. The precision data were retained for historical and informational purposes only.

REFERENCES

1. *Official Methods of Analysis,* AOAC International, 16th ed., Gaithersburg, MD, 1995, Chapt. 4, p. 26, Method 962.09.
2. *J. Assoc. Off. Anal. Chem. 65:*265 (1982).

OTHER REFERENCES

*J. Assoc. Off. Anal. Chem. 42:*222 (1959).
*Ibid. 43:*335 (1960).
*Ibid. 44:*567 (1961).
*Ibid. 45:*578 (1962).

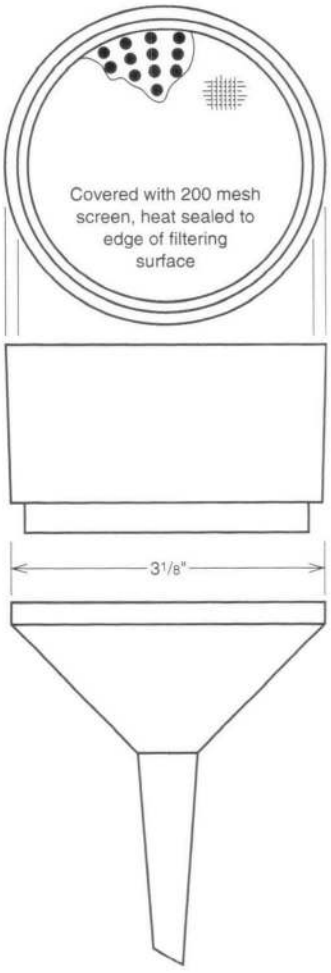

Figure 1. California State modified Buchner funnel, two-piece polyethylene (55- or 70-mm dia).

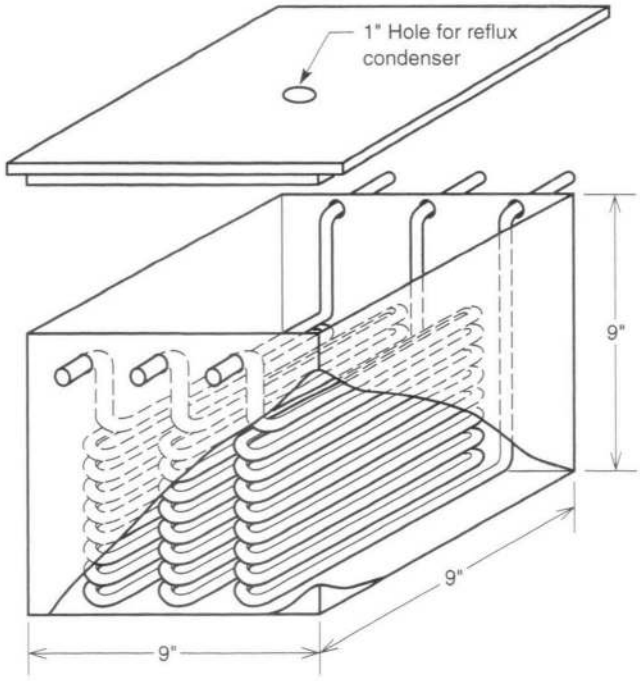

Figure 2. Continuous heater for distilled water, 1.25% alkali and 1.25% acid.

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AOCS

AOCS Standard Procedure Ba 6a-05

Reapproved 2009

Crude Fiber Analysis in Feeds by Filter Bag Technique

DEFINITION

This method determines crude fiber which is the organic residue remaining after digesting with 0.127 M H_2SO_4 and 0.313 M NaOH. The compounds removed are predominantly protein, sugar, starch, lipids, and portions of both the structural carbohydrates and lignin.

SCOPE

This method is applicable to all feed materials such as grains, meals, pet foods, mixed feeds, forages, and the following oilseeds: corn and soybeans.

APPARATUS

1. Analytical balance—capable of weighing down to 0.1 mg.
2. Oven—capable of maintaining a temperature of 102 ± 2°C.
3. Electric muffle furnace—with rheostat control and pyrometer that will maintain a temperature of 600 ± 15°C.
4. Digestion instrument—capable of performing the digestion at 100 ± 0.5°C and maintaining a pressure of 10–25 psi. The instrument must also be capable of creating a similar flow around each sample to ensure uniformity of extraction. (ANKOM^{200}/ANKOM^{2000}, 65 rpm agitation, ANKOM Technology).
5. Filter bags—constructed from chemically inert and heat resistant filter media, capable of being heat sealed closed and able to retain 25 micron particles while permitting rapid solution penetration (F 57 or F 58, ANKOM Technology, see Notes, 1).
6. Heat sealer—sufficient for sealing the filter bags closed to ensure complete closure.
7. Desiccator pouch—collapsible sealable pouch with desiccant inside that enables the removal of air from around the filter bags (MoistureStop weigh pouch, ANKOM Technology).

REAGENTS

1. Sulfuric acid solution—0.127 ± 0.005 M. 1.25 g H_2SO_4/100 mL. Concentration must be checked by titration (see Notes, *Caution*).
2. Sodium hydroxide solution—0.313 ± 0.005 M. 1.25 g NaOH/100 mL. Concentration must be checked by titration (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

Grind portions of the laboratory sample through a centrifugal mill with a 2-mm screen or cutter type (Wiley) mill with a 1-mm screen. Samples ground finer may show particle loss from the filter bags and result in low values.

PROCEDURE

1. Use a solvent-resistant marker to label the filter bags. Weigh filter bag (W_1) and zero balance (see Notes, 2).
2. Weigh 0.95–1.00 g of the test sample (W_2) directly in filter bag. Avoid placing the material on the upper 4 mm of the bag.
3. Completely seal the upper edge of the filter bag within 4 mm of the top with the heat sealer encapsulating the sample (see Notes, 3).
4. Weigh one blank bag and include in run to determine blank bag correction (C_1).
5. Extract fat from test portions by placing all bags into a 350 mL container. Add enough petroleum ether to cover bags and soak for 10 min. Pour off solvent and allow bags to air-dry. Spread sample uniformly inside the filter bag by shaking and flicking the bag to eliminate clumping.
6. Place a maximum of 24 bags into the Bag Suspender. All nine trays are used regardless of the number of bags being processed. Place three bags per tray and then stack trays on center post with each level rotated 120 degrees. Insert the Bag Suspender with bags into the fiber analyzer vessel and place the Bag Suspender weight on top of the empty 9th tray to keep it submerged (see Notes, 4).
7. When processing 24 sample bags, pour 1900–2000 mL of ambient temperature acid (Reagents, 1) solution over the bag suspender in the vessel. If processing fewer than 20 bags add 100 mL/bag of the acid solution (minimum of 1500 mL).
8. Turn Agitate and Heat on and confirm that Bag Suspender is agitating properly. Close and completely seal the lid of vessel. Extract samples for a total of 40 min.
9. At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid (see Notes, 5).

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10. After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900 mL of (50–85°C) rinse water and agitate for 5 min. The lid may be sealed with the Heat on or left open with the Heat off. Repeat hot water rinse (total of two rinses).
11. When processing 24 sample bags, pour 1900–2000 mL of ambient temperature base (0.313 M NaOH) solution over the Bag Suspender in the vessel. If processing fewer than 20 bags add 100 mL/bag of the base solution (minimum of 1500 mL).
12. Turn Agitate and Heat on and confirm that Bag Suspender is agitating properly. Close and completely seal the lid of vessel. Extract samples for a total of 40 min.
13. At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid (see Notes, 6).
14. After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900 mL of (50–85°C) rinse water and agitate for 5 min. The lid may be sealed with the Heat on or left open with the Heat off. Repeat hot water rinse (total of three rinses).
15. Remove filter bags from Bag Suspender and gently press out excess water. Place bags in a 250 mL beaker and add acetone to cover bags. Allow bags to soak 3–5 min then remove and lightly press out excess acetone.
16. Spread bags out and let air-dry. When the acetone is completely evaporated, dry the bags in an oven at 102 ± 2°C (most ovens will complete drying within 2–4 hrs).
17. Remove bags from oven, place directly into a collapsible desiccant pouch and flatten to remove air; do not use a conventional desiccator container. Allow to cool to ambient temperature and weigh bags.
18. Ash the entire bag/sample in pre-weighed crucible for 2 hrs at 600 ± 15°C, cool in desiccator and weigh to calculate loss of weight of organic matter (W_3).

CALCULATIONS

$$\% \text{ Crude fiber} = \frac{[W_3 - (W_1 \times C_1)] \times 100}{W_2}$$

Where—

W_1 = bag tare weight
W_2 = test portion weight
W_3 = weight of Organic Matter (loss of weight on ignition of bag and fiber)
C_1 = ash corrected blank bag factor (loss of weight on ignition of blank bag/original blank bag)

PRECISION

Results of the collaborative study (see Table 1) indicate the precision that the analyst should use as a benchmark for evaluation of replication in the same laboratory.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. Always add acid to water and not the reverse.

Sodium hydroxide can severely burn skin, eyes and respiratory tract. Protective clothing should be worn when working with this alkali Always add caustic material to water and not the reverse.

Petroleum ether and acetone are extremely flammable. Avoid static electricity. A fume hood should be used at all times when using petroleum ether or acetone.

NUMBERED NOTES

1. F57 filter bags may produce up to 0.5% units lower results on finely ground feed samples. Finely ground samples are samples with fiber particles of less than 25 microns.
2. Do not pre-dry filter bags; any moisture will be accounted for by the blank bag correction.
3. Use sufficient heat to completely seal the filter bag and allow enough cool time before removing the bag from the heat sealer.
4. Prior to inserting the Bag Suspender, if the vessel temperature is warm from a previous run, add cool water and exhaust.
5. The solution in the vessel is under pressure. The exhaust valve needs to be opened to release the pressure and solution prior to opening the lid.

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Table 1
Results of a collaborative study carried out in 2005.

	Whole Corn	Cattle Feed	Alfalfa	Whole Soy	Poultry Starter	Calf Starter	Swine Feed	Horse Feed	Soy Meal	Pig Starter	Dog Food
Number of laboratories	11	10	11	10	11	11	9	10	11	11	10
Number of replicates	22	20	22	20	22	22	18	20	22	22	20
Mean	1.69	14.44	22.62	9.6	4.65	10.73	17.72	6.21	3.7	2.83	1.25
Reference method value[a]	2.05	14.23	22.67	9.57	4.4	10.7	17.4	6.43	3.73	2.85	1.45
Repeatability											
s_r	0.16	0.44	0.36	0.32	0.26	0.28	0.18	0.1	0.2	0.09	0.23
RSD_r	9.6	3.1	1.6	3.3	5.5	2.6	1.1	1.6	5.3	3.3	18.1
r	0.46	1.23	1	0.88	0.72	0.8	0.51	0.27	0.55	0.26	0.6
Reproducibility											
S_R	0.19	0.44	0.67	0.48	0.27	0.33	0.28	0.27	0.22	0.17	0.31
RSD_R	11.4	3.1	2.9	5	5.8	3.1	1.6	4.3	6	6	24.5
R	0.54	1.23	1.86	1.34	0.75	0.94	0.78	0.75	0.62	0.48	0.86

[a]Official Method Ba 6–84/AOAC 962.09

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AOCS

AOCS Official Method Ba 7-58

Revised 2013

Free Gossypol

DEFINITION

The term free gossypol defines gossypol and gossypol derivatives in cottonseed products that are soluble in aqueous acetone under the conditions of the method.

SCOPE

Application to cottonseed, cottonseed meats, cottonseed slab and sized cake, and cottonseed meals of normal commercial production. Application to chemically treated meals should be verified before use (see Notes, 1).

APPARATUS

1. Mechanical shaker—equipped to hold 250 mL Erlenmeyer flasks and to provide vigorous agitation; Burrell Wrist Action shaker, or equivalent.
2. Spectrophotometer—isolating a band at 440 nm and equipped with cuvettes of 1 cm light path is preferred.
3. Grinding mill—Bauer Bros. no. 148 laboratory mill with no. 6912 plate, 36,700 rpm.
4. Grinding mill—Wiley, with 1 mm screen.
5. Solid glass beads—about 6 mm in diameter.
6. Erlenmeyer flasks—250 mL capacity, fitted with leakproof glass or polyethylene stoppers (Kimble no. 28160, size 27, or equivalent).
7. Pipets—volumetric, class A.
8. Filter paper—medium retention, 11 cm diameter circles (S & S no. 597, Whatman no. 2, or equivalent).
9. Volumetric flasks—25, 200, and 250 mL, class A.
10. Water bath—for operation at 100°C, equipped with clamps for supporting 25 mL volumetric flasks. Alternatively, rustproof metal washers slipped over the necks of the flasks may be used for stability. Operation of the bath in a well-ventilated hood is recommended.
11. Forced draft oven.

REAGENTS

1. Solvents (see Notes, *Caution*)—
   (a) Aqueous acetone—prepared by mixing 700 mL reagent-grade acetone and 300 mL of distilled water.
   (b) Aqueous isopropyl alcohol (2-propanol)—prepared by mixing 800 mL isopropyl alcohol and 200 mL of distilled water.
   (c) Aniline—prepared by distilling reagent-grade aniline over a small amount of zinc dust, using an efficient water-cooled condenser, and discarding the first and last 10% of the distillate. Store in a stoppered, brown glass bottle in refrigerator. Redistill when the reagent blank (Calibration, 6) exceeds 0.022 absorbance (95% transmittance).
2. Thiourea solution—prepared by dissolving 10 g of reagent-grade thiourea in distilled water and diluting to 100 mL.
3. Hydrochloric acid (1.2 M)—prepared by diluting 106 mL of concentrated hydrochloric acid (35–37% HCl) (see Notes, *Caution*) to 1 L with distilled water.
4. Gossypol—primary standard quality gossypol or gossypol acetic acid (89.61% gossypol by weight) should be used for calibration. Gossypol and gossypol acetic acid standards are available from Atomergic Chemetals Corp., Plainview, NY, USA; Sigma Chemicals, St. Louis, MO, USA; Chemical Dynamics Corp., South Plainfield, NJ, USA; Aldrich Chemical Co., Milwaukee, WI, USA. For determination of purity, accurately weigh 2 mg of gossypol or gossypol acetic acid, using a semimicro or micro balance, into a 100 mL volumetric flask. Add about 40 mL of spectral-grade cyclohexane, and warm on a steam bath, with swirling, to dissolve the compound. Cool to room temperature, and dilute to volume with cyclohexane. Using a calibrated spectrophotometer and matched 1.000 cm standard, or far ultraviolet, silica cells, determine the absorbance of the gossypol solution against the cyclohexane solvent at 358 nm. Calculate the absorptivity as follows:

$$a = \frac{A}{(c)(l)}$$

Where—

a = absorptivity
A = absorbance
c = concentration, in g/L
l = light path, 1.000 cm

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The absorptivity of highest purity primary standard gossypol should be 39.9 ± 0.2, and that of highest purity primary standard gossypol acetic acid 35.8 ± 0.2. Absorptivity values in the range of 39.1–39.9 for gossypol, and 35.1–35.8 for gossypol acetic acid, (98–100% purity), denote primary standards satisfactory for calibration.

5. Standard gossypol solution—when preparing solution it is important to mix well after each addition. Accurately weigh 25 mg of primary standard gossypol, or 27.9 mg of primary standard gossypol acetic acid, and transfer quantitatively to a 250 mL volumetric flask, using 100 mL of reagent-grade acetone. Add 1.0 mL glacial acetic acid, 75 mL of distilled water, dilute to volume with acetone and mix well. Pipet 45 mL of the above solution into a 200 mL volumetric flask, add 100 mL acetone, 45 mL distilled water, dilute to volume with acetone and mix well. The latter standard gossypol solution contains 0.025 mg of gossypol/mL if exactly 25 mg of gossypol or 27.9 mg of gossypol acetic acid were weighed. It is stable for 24 hr when protected from the light.

CALIBRATION

1. Pipet duplicate 1-, 2-, 3-, 4-, 5-, 7-, 8-, and 10 mL aliquots of the standard gossypol solution (0.025 mg/mL) (Reagents, 5) into 25 mL volumetric flasks.
2. To one set of aliquots, designated as "A," add 2 drops of 10% aqueous thiourea, 1 drop of 1.2 N HCl and dilute to volume with aqueous isopropyl alcohol solution.
3. Determine the absorbance of aliquots A at 440 nm, using aqueous isopropyl alcohol to set the instrument at 0 absorbance (100% transmittance).
4. To the other set of standard gossypol aliquots, designated as "B," add 2 drops of 10% aqueous thiourea, 2 drops of 1.2 M HCl and 2 mL of redistilled aniline. Prepare a reagent blank containing 10 mL of aqueous acetone, 2 drops of 10% aqueous thiourea and 2 mL of aniline (do not add any 1.2 M HCl to the reagent blank).
5. Heat the standards and the reagent blank in a boiling water bath (100°C) for 30 min, remove, cool to room temperature, and dilute to volume with aqueous isopropyl alcohol solution.
6. With the instrument set at 0 absorbance (100% transmittance) with aqueous isopropyl alcohol, determine the absorbance of the reagent blank taking care that a clean cuvette is used for the blank. If the reagent blank exceeds 0.022 absorbance units (below 95% transmittance), the analysis must be repeated using freshly distilled aniline. Determine the absorbance of aliquot B at 440 nm, using the reagent blank to set the instrument at 0 absorbance (100% transmittance).
7. Calculate the corrected absorbance for each standard gossypol aliquot as follows:

$$\text{Corrected absorbance} = (\text{absorbance of B} - \text{absorbance of A})$$

8. Calculate a linear regression calibration factor, F, using the actual milligrams of gossypol in each final standard solution (Reagents, 5) and its corresponding corrected absorbance (Calibration, 7) in the following equation:

$$\text{mg gossypol} = F \times \text{corrected absorbance}$$

The resulting r^2 should be at least 0.999 with the slope F being the calibration factor.

9. The mg of gossypol in the sample aliquots (Procedure, 11) is then found by multiplying the corrected absorbance of the sample aliquot (Procedure, 10) by the calibration factor (Calibration, 8).

PREPARATION OF TEST SAMPLE

1. Cottonseed—Dehull about 50 g of laboratory sample, prepared as directed in AOCS Official Method Aa 2-38, using a Bauer mill with the plates separated so that the seed are just broken. Remove the meats from the hulls and lint by screening on a 4-6 mesh screen. Grind the meats in a Wiley mill to pass a 2 mm screen. Do not preheat cottonseed, and avoid heating during grinding. Dry meats in a forced draft oven at 82°C for 4 hr. Cool meats before grinding or store in moisture-proof containers.
2. Slab and sized cake and meals—Prepare a test sample as directed in AOCS Official Method Ba 1-38. Grind about 50 g in a Wiley mill to pass a 1 mm screen.

TEST PORTION SIZE

1. The test portion weight and aliquot for analysis will depend on the free gossypol content of the cottonseed material. Table 1 is intended as a guide. Although most accurate values are obtained when the test portion weight is ≤1 g, it is necessary to increase the test portion size for very low free gossypol meals.

PROCEDURE

1. Transfer the accurately weighed test portion (see Sample Size and Table 1) to a 250 mL Erlenmeyer flask, and cover the bottom of the flask with glass beads.
2. Add 50 mL of aqueous acetone (see Sample Size and Table 1) by pipet, stopper the flask with a leakproof glass or polyethylene stopper (Apparatus, 6), and shake vigorously on a mechanical shaker for 1 hr.
3. Filter through dry filter paper of medium retention (Apparatus, 8), discarding the first 5 mL of filtrate and collecting filtrate in a small flask. Place a watch glass over the funnel to reduce evaporation during filtration.
4. Pipet appropriate duplicate aliquots of the filtrate (see Sample Size and Table 1) into 25 mL volumetric flasks.

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5. To one sample aliquot, designated as solution "C," add 2 drops (0.10 mL) of 10% aqueous thiourea (Reagents, 2), 1 drop (0.05 mL) of 1.2 M HCl (Reagents, 3), and dilute to volume with aqueous isopropyl alcohol (Reagents, 1, (b)). Determine absorbance (according to Calibration, 3).
6. To the other sample aliquot, designated as solution "D," add 2 drops of 10% aqueous thiourea (Reagents, 2), 1 drop of 1.2 M HCl (Reagents, 3), and 2 mL of redistilled aniline (Reagents, 1, (c)). A rapid delivery pipet may be used for dispensing aniline.
7. Prepare a reagent blank containing a volume of aqueous acetone solution (Reagents, 1, (a)) equal to that of the sample aliquot, and add 2 drops of 10% aqueous thiourea (do not add any 1.2 N HCl) and 2 mL of aniline.
8. Heat the sample aliquot D (Procedure, 6) and the reagent blank (Procedure, 7) in a boiling water bath (100°C) for 30 min.
9. Remove the solutions from the bath, add about 10 mL of aqueous isopropyl alcohol to effect homogenous solution, and cool to room temperature in an appropriate water bath. Dilute to volume with aqueous isopropyl alcohol. Determine absorbance (according to Calibration, 6).
10. Calculate the corrected absorbance of the sample aliquot as follows:

$$\text{Corrected absorbance} = (\text{absorbance of D} - \text{absorbance of C})$$

Note—If the readings on solutions C and D above were taken in terms of transmittance, convert to absorbance, using the following equation:

$$\text{Absorbance} = 2 - \text{logarithm transmittance}$$

11. From the corrected absorbance of the sample aliquot determined in Procedure, 10, determine the mg of gossypol in the sample aliquot by use of the calibration factor (see Calibration, 8).

$$\text{Gossypol, mg} = \text{corrected absorbance} \times \text{factor}$$

CALCULATIONS

1. Calculate the free gossypol content as follows:

$$\text{Free gossypol, \%} = \frac{5(G)}{(W)(V)}$$

Where—
G = mg gossypol in the aliquot (Procedure, 11)
W = mass of test portion, g
V = volume, mL of aliquot used (Procedure, 4)

NOTES

Caution

Acetone, isopropyl alcohol, and cyclohexane are flammable solvents. They should not be used near an open flame. The use of a properly operating fume hood is recommended when using these solvents.

Aniline is an allergen and is toxic if absorbed through the skin. The TLV in air is 2 ppm. Protective clothing and a properly operating fume hood should be used when using aniline.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended.

NUMBERED NOTES

1. This method may not be applicable to feeds containing whole, unprocessed cottonseed. Components in the feed interfere with this method and may give false positive results. Previously the AOCS Technical Committee had alternate methodology under review, see Ba 7b–96, Ba 8–78, Ba 8a–99. For feed samples containing whole, untreated cottonseed, the analyst may want to try alternate published HPLC methods.

Table 1
Test portion size for gossypol analysis.

Type of sample	Expected free gossypol content, %	Test portion mass, g	Aliquot size, mL
Meats	0.5-1.5	0.25	2
Meal	0.2-0.4	0.50	2
Meal	0.1-0.2	1.00	2
Meal	0.05-0.10	1.00	5
Meal	0.02-0.05	1.00	10
Meal	0.01-0.02	2.00	10
Meal	Below 0.01	5.00	10

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AOCS

AOCS Recommended Practice Ba 7b-96

Revised 2011

Rapid Method for Estimating Free Gossypol in Cottonseed, Meats, Collets, and Extracted Meals

DEFINITION

This method (References, 1) for estimating free gossypol (FG) decreases sample-determination time from over 2 hr to about 25 min per sample. With auto pipetters and bottle-top dispensers, six sample determinations can be completed in approximately 50 min. The method consists of adding water and acetone separately to a fixed sample weight, mixing, filtering, diluting with 65% acetone, and reading absorbance on a spectrophotometer.

SCOPE

Applicable to full-fat cottonseed, cottonseed meats, or expanded collets and solvent-extracted cottonseed meal that contain FG within the ranges of 0.02–0.25% and 0.9–1.8%.

APPARATUS

1. Forced-draft oven.
2. Wiley mill—20 mesh.
3. Spectrophotometer.
4. Matched set of semi-micro, quartz, spectrophotometric cells—Spectrocell SM - 3010, 10 mm, 1.4 mL (Spectrocell Corporation, Oreland, PA), or equivalent.

REAGENTS (see Notes, *Caution*)

1. Potassium dichromate—crystalline, primary standard grade.
2. Water—from Milli -Q water system (Millipore, Marlborough, MA).
3. Acetone—spectrophotometric grade.

TEST SAMPLE PREPARATION

1. Dry all full-fat seed, meats, and collet samples for 1 hr at 45°C in forced-draft oven (Apparatus, 1) before fine grinding using mill (Apparatus, 2). This will minimize pigment-gland rupture and subsequent binding of FG during grinding.
2. Fine grind mill-run cottonseed meats and meal samples using mill (Apparatus, 2).

CALIBRATION

1. Prepare a standard $K_2Cr_2O_7$ (Reagents, 1) solution, 0.0514 g in 250 mL water (Reagents, 2), to calibrate wavelength on the spectrophotometer. At a wavelength of 430 nm, the absorbance should approximate 0.360.
2. Determine the free gossypol, using the official AOCS Ba 7-58 in a set of 6 samples of the type to be analyzed, i.e., seed, meats, collets, in order to obtain a calibration factor, F, for that particular series (e.g. F_{seed}, F_{meats}, $F_{collets}$).
3. Calculate a linear regression calibration factor, F (slope) and b (intercept) for each series of materials using the % free gossypol (Calibration, 2) and its corresponding absorbance (Procedure, 4) in the following equation:

$$\% \text{ free gossypol} = F \times (\text{absorbance}) + b$$

PROCEDURE

1. Accurately weigh 5.0 g ± 0.001 (for 0.02–0.25% FG) or 1.0 g ± 0.001 (for 0.9–1.8% FG) of the fine ground test sample (Test Sample Preparation, 2) into a 150 mL beaker. Pipet 15 mL water into the beaker. Swirl the resulting slurry to rupture pigment glands. Cover slurry with a watch glass and let slurry stand 5 min.
2. Add 35 mL acetone (Reagents, 3) to the slurry, making the equivalent of a 70% aqueous acetone solution. Swirl slurry, cover and again allow to stand 5 min.
3. Filter slurry through fluted Whatman #2 filter paper into another 150 mL beaker. After filtering for approximately 5 min, pipet 1 mL filtrate into a 10 mL volumetric flask. Fill flask to mark with acetone-water (65:35, v/v).
4. Read sample absorbance at a wavelength of 430 nm against a 65% aqueous acetone background.
5. Periodically adjust wavelength setting on the spectrophotometer by using the fixed absorbance reading of standard potassium dichromate solution (Calibration, 1).

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CALCULATIONS

1. Determine FG by inserting the sample's absorbance reading into the appropriate linear least-squares calibration equation corresponding to the material being analyzed.
2. To determine % FG in a sample by the quick method, standardization or calibration equations must first be obtained.

METHOD PERFORMANCE

Absorbance was plotted against the AOCS FG method (Method Ba 7-58, see Reference 2) for samples that contained FG between 0.02 and 0.9%. Quadratic least-squares regression for 31 samples had a correlation coefficient of r_2 = 0.986 and a standard error of estimated FG of 0.032% (see References, 1).

NOTES

Caution

Acetone is highly flammable and forms explosive peroxides with oxidizing agents. It should not be used near an open flame. The use of a fume hood is recommended when using acetone. Do not mix with chloroform.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

REFERENCES

1. This method paraphrases a method developed by R.J. Hron, Sr., M.S. Kuk, and P.J. Wan and published in *J. Am. Oil Chem. Soc. 73:*199 (1996).
2. *Official Methods and Recommended Practices of the American Oil Chemists' Society,* 5th ed., American Oil Chemists' Society, Champaign, IL, 1997.

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AOCS

AOCS Official Method Ba 8-78

Revised 2011

Total Gossypol

DEFINITION

Total gossypol defines gossypol and gossypol derivatives, both free and bound, in cottonseed products that are capable of reacting with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, which then reacts with aniline to form dianilinogossypol under the conditions of the method. Gossypol, gossypol analogs, and gossypol derivatives having an available aldehyde moiety are measured by the method (see Notes, 1)

SCOPE

Applicable to decorticated, glanded and glandless cottonseed, cottonseed flour, cooked cottonseed meats, cottonseed press cake, cottonseed meal, crude cottonseed oil, and cottonseed soap stock (see Notes, 2). Replaces Ba 8-55.

APPARATUS

1. Disk mill—Bauer Bros. no. 148, or equivalent, with plates separated to just break the seed.
2. Cutting mill—Wiley, with 1 and 2 mm screens.
3. Water bath—for operation at 95–100°C, with clamps for supporting 50 and 25 mL volumetric flasks. Metal washers can be placed over the neck of flasks for support.
4. Spectrophotometer—for operation at 440 nm, with 1 cm light path cuvettes. A colorimeter can be used if equipped with a filter with maximum transmission in range of 440–460 nm (see Notes, 3).
5. Volumetric flasks—25, 50 and 100 mL.
6. Pipets—volumetric, class A, 1, 2, 4, 5 and 10 mL.
7. Filter paper —medium retention, 11 cm diameter (Whatman no. 2, S & S no. 597, or equivalent); creped paper for oil filtration (Eaton and Dikeman 617, Reeve Angel 230, S & S 478, or equivalent).

REAGENTS

1. Solvents, reagent grade (see Notes, *Caution*)—isopropyl alcohol (2-propanol); *n*-hexane (boiling range 68–69°C); dimethylformamide (N,N-dimethylformamide); 3-amino-1-propanol (propanolamine), free of color; glacial acetic acid; and aniline. The aniline should be redistilled over zinc dust using water-cooled condenser. Discard first and last 10% of distillate, and store in brown bottle in refrigerator when not in use. Redistill when reagent blank (Procedure, 8) absorbance exceeds 0.022.
2. Isopropyl alcohol-hexane mixture—60 volumes of isopropyl alcohol and 40 volumes of *n*-hexane.
3. Complexing reagent—prepared by pipetting 2 mL of 3-amino-1-propanol and 10 mL glacial acetic acid into a 100 mL volumetric flask, cooling to room temperature and diluting to volume with dimethylformamide. Prepare reagent weekly and store in refrigerator when not in use.
4. Gossypol or gossypol acetic acid—primary standard quality. Gossypol and gossypol acetic acid standards are available from Atomergic Chemetals Corp., Plainview, NY, USA; Sigma Chemical Co., St. Louis, MO, USA; Chemical Dynamics Corp., South Plainfield, NJ, USA; Aldrich Chemical Co., Milwaukee, WI, USA. Determine purity as in AOCS Official Method Ba 7-58 (Reagents, 4). Absorptivity of 39.1–39.9 for gossypol and 35.1–35.8 for gossypol acetic acid indicate satisfactory purity in range of 98–100%. Gossypol acetic acid contains 89.62% gossypol by weight, or 0.8962 mg gossypol per mg of gossypol acetic acid.
5. Standard gossypol solution—prepared by weighing 25 mg of primary standard gossypol, or 27.9 mg of gossypol acetic acid into a 50 mL volumetric flask. Dissolve in and make up to volume with the complexing reagent (Reagents, 3). Solution is stable up to 1 week if stored in refrigerator. If exact amounts of either gossypol or gossypol acetic acid were weighed as indicated, either solution contains 0.50 mg gossypol/mL. Multiply milligrams gossypol acetic acid by 0.8962 to obtain milligrams of gossypol.

CALIBRATION (see Numbered Notes, 6)

1. Pipet duplicate 1, 2, 4, 6, 8 and 10 mL aliquots of the standard gossypol solution (Reagents, 5) into 50 mL volumetric flasks. To each standard add sufficient volume of complexing reagent (Reagents, 3) to make volume 10 mL. Use 10 mL of complexing reagent as a blank.
2. Heat flasks in a water bath (95–100°C) for 30 min, cool to room temperature, dilute to volume with the isopropyl alcohol-hexane solution and mix well.
3. Pipet duplicate 2 mL aliquots of each standard and the reagent blank into 25 mL volumetric flasks.
4. Dilute one set of standard aliquots and the reagent blank to volume with the isopropyl alcohol-hexane solution, and reserve as reference solutions for absorbance measurements.

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5. Add 2 mL aniline to the other set of standard aliquots and the blank, heat in water bath (95–100°C) for 30 min, cool to room temperature, dilute to volume with the isopropyl alcohol-hexane solution, and mix well. Allow to stand for 1 hr at room temperature before determining absorbance.
6. Determine absorbance of reagent blank using a spectrophotometer at 440 nm (Apparatus, 4) as in Procedure, 8, and of the standard aliquots as in Procedure, 9. Subtract absorbance of reagent blank from absorbance of each standard to obtain corrected absorbance for use in following step.
7. Calculate a linear regression calibration factor, F, using the actual milligrams of gossypol in each final standard solution (Calibration, 5) and its corresponding corrected absorbance (Calibration, 6) in the following equation.

$$\text{mg gossypol} = F\ (\text{corrected absorbance})$$

The resulting r^2 should be at least 0.999 with the slope F being the calibration factor.

PREPARATION OF TEST SAMPLE

1. Cottonseed—Dehull about 50 g sample as in AOCS Official Method Aa 2-38, using Bauer mill with plates separated so seeds are just broken. Screen on 4–6 mesh sieve to remove meats from hulls and lint. Grind meats through 2 mm screen in a Wiley mill, avoiding over-heating meats and expressing oil.
2. Cooked meats—Reduce moisture to 5% or below by air equilibration, or by blowing air over meats. Grind through 2 mm screen in a Wiley mill.
3. Press cake and meal—Prepare sample as in AOCS Official Method Ba 1-38 and grind 50 g through 1 mm screen in a Wiley mill.
4. Crude oil—Heat portions of the laboratory sample to 50°C, mix well and filter through paper suitable for oil filtration (Apparatus, 7). Analyze filtered sample within 1 day, or store at –20°C (0°F) if analysis is delayed. Gossypol in oil undergoes changes if stored at or above room temperature.
5. Soapstock—Heat to 50°C and mix well before withdrawing analytical sample. Store under same conditions as for crude oil.

TEST PORTION SIZE

1. Test portion weight and aliquot used for aniline reaction depend on expected total gossypol content. Ideally, the test portion should contain from 0.5 to 5.0 mg of gossypol, and the aliquot for the aniline reaction should contain about 0.1 mg gossypol. Table 1 is a guide, providing milligrams of gossypol for aniline reaction within the range used for calibration.

PROCEDURE

1. Weigh test portion (see Test Portion Size and Table 1) on analytical balance accurate to ± 0.001 g and transfer to a 50 mL volumetric flask. A small piece of aluminum foil or weighing paper is useful for weighing and transferring sample to flask. Add 10 mL complexing reagent (Reagents, 3) by pipet, washing down any material adhering to neck of flask. A rapid delivery pipet may be used (see Notes, 4).
2. Prepare reagent blank containing 10 mL of complexing reagent in a 50 mL volumetric flask.
3. Heat test portion and blank in water bath (95–100°C) for 30 min, cool to room temperature, dilute to volume with isopropyl alcohol-hexane mixture (Reagents, 2) and mix well.
4. Filter test portion extract through 11 cm medium-retention paper (Apparatus, 7) into a 50 mL glass-stoppered Erlenmeyer flask, discarding first 5 mL of filtrate.
5. Pipet duplicate aliquots of extract (see Test Portion Size and Table 1) into 25 mL volumetric flasks. Pipet duplicate blank aliquots of same volume as test portion aliquot into 25 mL volumetric flasks.
6. Dilute one set of test and blank aliquots to volume with the isopropyl alcohol-hexane mixture and reserve as reference solutions for absorbance measurement.
7. Add 2 mL aniline, by pipet, to the other set of test and reagent blank aliquots and heat in a water bath (95–100°C) for 30 min. Cool to room temperature, dilute to volume with the isopropyl alcohol-hexane mixture, and mix well. Allow to stand for 1 hr at room temperature after dilution (see Notes, 5).
8. With a spectrophotometer (Apparatus, 4) at 440 nm, determine absorbance of reagent blank reacted with aniline (Procedure, 7), using blank aliquot without aniline (Procedure, 6) as reference solution.
9. Determine absorbance of test aliquot reacted with aniline (Procedure, 7), using diluted sample aliquot without aniline (Procedure, 6) as reference solution. Subtract absorbance of reagent blank (Procedure, 8) from that of test aliquot reacted with aniline to obtain corrected absorbance (see Notes 3, 5).
10. From corrected absorbance of test aliquot (Procedure, 9), determine milligrams of gossypol in sample aliquot by multiplying absorbance by the calibration factor (F) (Calibration, 7).

CALCULATIONS

1. Calculate percentage total gossypol in sample as follows:

$$\text{Total gossypol, \%} = \frac{5 \times G}{W \times V}$$

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Where—

G = mg gossypol in test aliquot (Procedure, 10)
W = mass of test portion in grams
V = volume, mL of test aliquot used for analysis (Procedure, 9)

2. If gossypol concentration is desired in parts per million (mg/kg), use the following equation:

$$\text{Total gossypol, mg/g} = \frac{5 \times G}{W \times V} \times 10{,}000$$

PRECISION

1. A collaborative study established the following precision estimates for the method. As the percentage gossypol decreases, precision (coefficient of variation, CV) becomes poorer. Precision in the 0.50–0.10% range was estimated to be 3.5–7.0%; for the 0.06–0.01% range the precision was estimated to be 10.0–17.0%. See Table 2 for precision values related to gossypol determination.

NOTES

Caution

Isopropyl alcohol and n-hexane are flammable solvents. They should not be used near an open flame. The use of a properly operating fume hood is recommended when using these solvents. The TLV for hexane is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

Aniline is an allergen and is toxic if absorbed through the skin. The TLV in air is 2 ppm. Protective clothing and a properly operating fume hood should be used when using aniline.

Dimethylformamide is a strong irritant to skin and tissue. It is toxic by skin absorption. It is a moderate fire risk. The TLV is 10 ppm in air.

3-amino-1-propanol (propanolamine) is a tissue irritant. Avoid breathing vapors and contact with the skin.

Glacial acetic acid is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

NUMBERED NOTES

1. See References, 1 for details and verification of the method.
2. This method may not be applicable to feeds containing whole, unprocessed cottonseed. Components in the feed interfere with this method and may give false positive results. For feed samples containing whole, untreated cottonseed, the analyst may want to try alternate published HPLC methods.
3. Absorption maximum of the dianilinogossypol reaction product is at 440 nm but may vary with the wavelength accuracy of the instrument. All absorbance measurements should be made at the actual maximum for the spectrophotometer used.
4. Extracts of sample treated with the complexing reagent (Procedure, 1-4) are exceptionally stable and, if necessary, can be stored under refrigeration for several days before conducting the aniline spectrophotometric reaction.
5. After the aniline reaction (Procedure, 7), extracts of samples and standards exhibit slight increase in absorbance up to 1 hr, and are stable for 1–5 hrs. For purposes such as plant control, absorbance measurements can be made immediately after aniline reaction and dilution, provided that the calibration factors (Calibration, 5–7) are determined under the same conditions. A variation of about 10 min in determining absorbance will result in about 2% relative error in total gossypol.
6. Once a given spectrophotometer is calibrated, calibration factors are valid for long-time use and need not be repeated except for occasional check of instrument performance. Matched cuvettes for the absorbance measurements should be used, or suitable absorbance corrections applied to unmatched cuvettes, using the isopropyl alcohol-hexane mixture for cuvette comparison.

REFERENCES

1. *J. Am. Oil Chem. Soc. 35*:93 (1958).

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Table 1
Test portion size for gossypol analysis.

Expected total %	gossypol mg/kg	Test portion weight, g	Aliquot, mL
0.002–0.01	20–100	5.0	10
0.01–0.05	100–500	5.0	5
0.05–0.10	500–1,000	5.0	2
0.10–0.20	1,000–2,000	2.0	2
0.20–0.40	2,000–4,000	1.0	2
0.40–0.60	4,000–6,000	0.75	2
0.60–1.0	6,000–10,000	0.50	2
1.0–2.0		0.20	2
2.0–4.0		0.20	1

Table 2
Precision values for gossypol determination.

Total gossypol,%	SD	CV, %
0.970	0.0102	1.1
1.200	0.0146	1.2
1.376	0.0117	0.9
0.208	0.0062	3.0

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AOCS Recommended Practice Ba 8a-99

Revised 2011

Determination of (+)-, (–)-, and Total Gossypol in Cottonseed, Cottonseed Meats, and Meal by HPLC

DEFINITION

This method describes a procedure for the quantitative determination of both free and bound, (total), gossypol and methoxy-gossypol in glanded and glandless cottonseed, cottonseed meats, and cottonseed meal samples, as the sum of the (+) and (–)-isomers, using HPLC.

SCOPE

Applicable to decorticated cottonseed, cottonseed meats, and ground extracted meal samples. The samples are treated with a complexing reagent containing optically active aminopropanol to produce, with heat, (+)- and (–)-gossypol-aminopropanol complexes. Mobile solution is added to the sample and the mixture filtered. An aliquot of the filtrate is further diluted with mobile phase and the isomers separated by reverse phase elution by HPLC and detected at 254 nm.

APPARATUS

1. High-performance liquid chromatograph is operated isocratically with a mobile phase flow rate of 1.0 mL/min. Fitted with a Phenomenex, Inc. (Torrance, CA) Prodigy, 5 µ, ODS-3, 150 × 3.2 mm reverse phase column (part no. 00F-4097-RO) or equivalent and a Rheodyne, L.P. (Rohnet Park, CA) 7125 sample injector equipped with a 20 µL sample loop. An equivalent autosampler system can be used instead of the single sample injector. A UV detector set at 254 nm can be used to measure peak area on a valley-to-valley base-line basis as shown in the attached chromatograms (Fig. 1). The sample is analyzed as (+) and (–)- gossypol-aminopropanol complexes, not as pure gossypol.
2. Isotemp drybath with 20 mm blocks or water bath, for operation at 95–100°C with clamps or support structure for 20 × 125 mm sample tubes.
3. Industrial glassware—20 × 125 mm glass tubes with natural polypropylene screw caps.
4. Kimble glassware (or equivalent)—19 × 65 mm amber glass, screw-top vials.
5. Glass tube rack.
6. Volumetric flasks—10, 25, 100 mL.
7. Stoppered graduated cylinders—1,000 mL.
8. Glass beakers—50 and 100 mL.
9. Filter funnels.
10. Filter paper 10 cm, Whatman #2 or equivalent.
11. Pipets, measuring, 1, 5, 10 and 25 mL.

REAGENTS

1. Solvents are:
   (a) HPLC grade acetonitrile (ACN)
   (b) HPLC grade water
   (c) Spectrophotometric grade N, N -dimethylformamide (DMF)
   (d) Spectrophotometric grade D-alaninol ([R]-[–]-2-amino-1-propanol)
   (e) ACS reagent grade phosphoric (H_3PO_4) acid (85.7%)
   (f) ACS reagent grade glacial acetic acid
2. Reagent grade, crystalline potassium phosphate monobasic (KH_2PO_4).
3. Complexing reagent—prepared by pipetting 2 mL of 2-amino-1-propanol (2-AP) and 10 mL glacial acetic acid into a 100 mL volumetric flask, cooling to room temperature, and diluting to volume with DMF. Store complexing reagent up to one week in refrigerator when not in use.
4. HPLC mobile phase solution (pH 3.0) is 78% (v/v) ACN and 22% (v/v) 10 mM KH_2PO_4 buffer. It is prepared in a stoppered 1,000 mL graduated cylinder by mixing 780 mL of acetonitrile with 220 mL of a buffer solution containing 1.36 g KH_2PO_4 in 1,000 mL of HPLC grade water and adjusted to pH 3.0 with H_3PO_4.
   Note: The addition of 1.44 mL of H_3PO_4 should adjust the buffer solution to pH 3.0.

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STANDARD STOCK SOLUTION PREPARATION

1. Gossypol acetic acid (GAA) is primary standard quality, exits as a racemate, and is considered to contain 89.62% gossypol by weight, or 0.4481 mg each, of (+) and (–)-gossypol isomers per mg of gossypol acetic acid.
2. Stock standard gossypol solution is prepared by very accurately weighing 38.0 mg of GAA into a 25 mL volumetric flask, bringing to volume with complexing agent (Reagents, 3), and mixing well by inversion until all GAA is in solution. Final gossypol concentration of standard stock solution is 681.1 g/mL (+) and 681.1 g/mL(–)-gossypol. Solution is not stable and should be used the same day.

PREPARATION OF TEST SAMPLES

1. Dehull about 50 g of the mixed seed laboratory sample as in AOCS Official Method Aa 2-38) using a Bauer mill with plates separated so that seeds are just broken. Screen on 4–6 mesh sieve to remove meats from hulls and lint. Oven-dry meats (no hulls) for 4 h at 82°C, then cool to room temperature. Grind a 10–20 g portion of the cottonseed meats or extracted meal sample using a Retsch centrifugal grinding mill ZMI, equipped with a 1.0 mm screen.
2. Store ground samples in freezer and bring to room temperature before analyzing.

TEST PORTION ANALYSIS

1. Accurately weigh out 0.2 g test portions of ground, glanded kernels, meats, and meals or 1.0 g for all glandless materials into a 20 × 125 mm sample tube.
2. Pipette 4 mL of 2-AP complexing reagent (Reagents, 3) into the tube, cap, and carefully mix until sample is completely wetted with reagent. Mix gently to prevent splashing up the sides of the tube.
3. Place tubes in a dry bath, or water bath, pre-heated to 95–100°C and heat for 30 min. Remove from heat, mix for 30 sec on a vortex mixer, and let samples cool for 30 min.
4. Pipette 16 mL of mobile solution (Reagents, 4) into each tube, recap tubes, and mix by inverting 20 times. Do not let test solutions sit after dilution.
5. Filter each test solution through Whatman #2 or its equivalent fluted filter paper into a 100 mL beaker. For glanded seed, meats, and meals, pipette a 5 mL aliquot into a 10 mL volumetric flask and fill flask to mark with mobile solution. Do not dilute glandless seed and meal test solutions. Store each filtrate in 12 mL amber screw-cap vials prior to injection into the chromatograph.
6. Flush the liquid chromatograph 20 µL sample loop with approximately 1 mL of mobile phase, while the injector is in the "inject" position, prior to each sample run. Then turn injector to the "load" position and inject approximately 500 µL of sample in order to flush and fill the sample loop. Turn the sample injector lever in order to initiate the sample run.
7. After injection of the sample, the first major peak on the chromatogram after the initial solvent peak is the (+)-gossypol-aminopropanol complex, which elutes in about 2.5 min. The (–)-gossypol-aminopropanol complex elutes in approximately 5.0 min. The (+)- and (–)- methoxy-gossypol-aminopropanol complex peaks sometimes are evident, especially in the case of Pima seed, and they elute at about 2.0 min and 3.6 min respectively. Total elapsed time per sample should not exceed 6 min. Fig. 1 shows typical Upland seed (A), Pima seed (B), and extracted meal (C) results.
8. Integrate the (+) and (–)-gossypol-aminopropanol complex peak areas on a valley-to-valley basis and insert the values into the following (+) and (–) percent (%) gossypol equations:

$$\% \text{ (+)-gossypol} = K\{m_{(+)} [\text{(+)-peak area}] + b_{(+)}\}$$
$$\% \text{ (–)-gossypol} = K\{m_{(-)} [\text{(–)-peak area}] + b_{(-)}\}$$

Constants $m_{(+)}$, $m_{(-)}$, $b_{(+)}$, and $b_{(-)}$ are derived from the calibration equations for each isomer (Calibration, 6). K is a constant that converts mg gossypol/test portion to percent (%). K = 0.5 for glanded seed, meat, and meal samples where sample weight is fixed at 0.2 g, and K = 0.05 for glandless samples where sample weight is fixed at 1.0 g. The percent (+) and (–) values are added together to obtain the percent (%) total gossypol in each test sample. For glanded seed report (+), (–), and total gossypol values to the nearest 0.01%. For all others report (+), (–), and total gossypol values to the nearest 0.001%.

Note 1: Because a methoxy-gossypol standard is not readily available, in the case of Pima seed and meal samples, the (+)- and (–)-methoxy-gossypol aminopropanol complex peak areas are considered to be equivalent to and are therefore added to the corresponding (+)- and (–)-gossypol-aminopropanol complex peak areas.

Note 2: A few additional small reaction peaks usually appear in meal chromatograms (Fig. 1, C) and although their spectra are similar to gossypol, their molecular structures are presently unknown and their areas are therefore not included in the calculations.

CALIBRATION

1. For a normal range % gossypol in seed (0.3–2.7%), pipette out six standard stock solution (C.2) aliquots, as per Table 1, into 20 × 125 mm sample tubes.
   Accurately pipette additional 2-AP complexing agents (Reagents, 3) to each tube to obtain a total of 4 mL.
2. Place tubes in a dry bath or water bath pre-heated to 95–100°C and heat slurry test portions for 30 min. Remove from heat, mix for 30 seconds on a vortex mixer, and allow tubes to cool for 30 min.

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3. Pipette exactly 16 mL of mobile phase solution (Reagents, 4) into each tube, recap tubes, and mix by inverting 20 times. Do not let samples sit after dilution. Table 1 indicates the concentrations of (+), (–)-gossypol in each of the standard working solution samples.
4. Filter each standard solution through Whatman #2 or its equivalent fluted filter paper into a 50 mL beaker. Pipette a 5 mL aliquot of each standard into a 10 mL volumetric flask and fill flasks to mark with mobile solution (Reagents, 4). Store each filtrate in 12 mL amber, screw-cap vials prior to injection into the chromatograph.
5. Flush the liquid chromatograph 20 µL sample loop with approximately 1 mL of mobile phase, while the injector is in the "inject" position, prior to each working standard solution run. Then turn the injector to the "load" position and inject approximately 500 µL of working standard solution sample in order to thoroughly flush and fill sample loop.
6. Inject each working standard solution sample into the liquid chromatograph by turning the sample injector lever. Each standard solution run should be approximately 6 min. Determine peak area on a valley-to-valley baseline basis. The (+) and (–)-gossypol-amino-propanol isomer peaks elute at approximately 2.5 and 5.0 min, respectively. Individual peak areas are plotted (X) vs. mg gossypol isomer (Y) to obtain standard Y = mX + b straight line standardization equations using linear regression to calculate the constant m and b values for each isomer. The calibration procedure should be done in duplicate using all fresh solutions and the area data used to calculate averaged m and b constant values.

$$\text{mg pure (+)-gossypol} = m_{(+)}\,[\text{(+)-peak area}] + b_{(+)}$$
$$\text{mg pure (–)-gossypol} = m_{(-)}\,[\text{(–)-peak area}] + b_{(-)}$$

REFERENCES

1. Kim, H. L., M. C. Calhoun, and R. D. Stipanovic, Accumulation of Gossypol Enantiomers in Ovine *Tissues, Comp. Biochem. Physiol. 113B:*417–420 (1996).

Table 1
Preparation procedures for standard working solutions.

Tube number	Standard stock solution (mL)	Complexing reagent (2-AP) (mL)	Mobile phase solution (mL)	(+) Gossypol[a] (mg)	(–) Gossypol[a] (mg)
1	0.5	3.5	16	0.3406	0.3406
2	1.0	3.0	16	0.6811	0.6811
3	2.0	2.0	16	1.3622	1.3622
4	3.0	1.0	16	2.0433	2.0433
5	3.5	0.5	16	2.3839	2.3839
6	4.0	0.0	16	2.7244	2.7244

[a]Actual concentrations can be calculated once accurate weight and purity of gossypol acetic acid (GAA) used to make up the standard stock solution (1.3622 mg/mL total gossypol) is known. In this case the GAA supplied is considered to be 89.62% pure racemic gossypol.

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Table 2
Precision data table of total gossypol[a]

Strain/variety	9/4/99	9/8/98	10/5/98	10/6/98	10/7/98	SIT 9/9/98	SIT 9/10/98	SIT 9/11/98	SIT 9/15/98	SIT 9/16/98	Average All	SD All	Average No SIT	SD No SIT	Average SIT	SD SIT
Pima	0.94	0.919	0.892	0.906	0.908	0.922	0.923	0.928	0.939	0.922	0.921	0.015	0.916	0.022	0.927	0.006
	1.02	0.998	0.966	0.982	0.985	0.999	0.999	1.008	1.014	0.966	0.994	0.018	0.993	0.024	0.997	0.017
LAHG063	1.53	1.532	1.511	1.509	1.519	1.520	1.571	1.573	1.550	1.497	1.531	0.025	1.521	0.010	1.542	0.030
DES119	0.86	0.823	0.820	0.845	0.838	0.858	0.845	0.843	0.873	0.853	0.846	0.016	0.839	0.021	0.554	0.011
ST474	0.99	0.984	0.958	0.951	0.952	0.957	0.982	0.965	0.958	0.959	0.966	0.014	0.972	0.015	0.964	0.009
LAHG660	1.61	1.552	1.600	1.580	1.562	1.574	1.590	1.606	1.614	1.548	1.584	0.024	1.587	0.027	1.586	0.024
LA91008	1.37	1.340	1.362	1.341	1.354	1.378		1.383	1.396	1.373	1.366	0.017	1.353	0.013	1.383	0.009
DP50	0.93	0.559	0.883	0.909	0.884	0.934	0.907	0.935	0.922	0.898	0.909	0.020	0.903	0.022	0.919	0.015
Average SD												0.010		0.010		0.009
Total	6.96	6.778	6.738	6.776	6.740	6.842	6.894	6.930	6.931	6.721	T%SD	0.087	0.088	0.084	0.078	0.078
SD of SD												0.010		0.010		0.010
Average total													6.799		6.864	
Percent positive isomer																
1	47.0	46.7	46.9	46.9	46.7	47.0	46.7	46.7	46.8	47.0	46.8	0.13	46.9		16.8	0.14
1	46.7	46.4	46.6	46.6	46.4	46.6	46.4	46.4	46.5	46.7	46.5	0.12	46.6		46.5	1.12
2	69.5	69.4	69.4	69.4	69.5	69.3	69.3	69.5	69.3	69.3	69.4	0.08	69.4		69.3	0.08
4	61.7	61.2	62.0	62.0	62.1	61.9	62.2	61.8	61.9	61.7	61.9	0.27	61.7		61.9	0.17
5	65.9	65.9	66.0	65.9	65.9	65.2	66.3	65.9	66.0	65.9	65.9	0.26	65.9		65.9	0.36
6	69.8	69.7	69.7	69.7	69.8	69.9	69.4	69.7	69.9	69.8	69.7	0.14	69.7		69.7	0.19
7	68.9	69.0	68.5	68.6	68.5	68.6	68.7	68.5	68.8	68.6	68.7	0.17	68.8		68.6	0.10
8	67.4	67.7	67.7	67.9	67.5	67.5	67.6	67.3	67.5	67.5	67.6	0.18	67.7		67.5	0.16
Total	496.9	496.0	496.8	497.0	496.4	496.0	496.8	495.8	496.7	496.5	Avg. SD	0.17				0.17

[a]Seed samples from Delta Research and Extension Center, Mississippi State University, Stoneville, MS.

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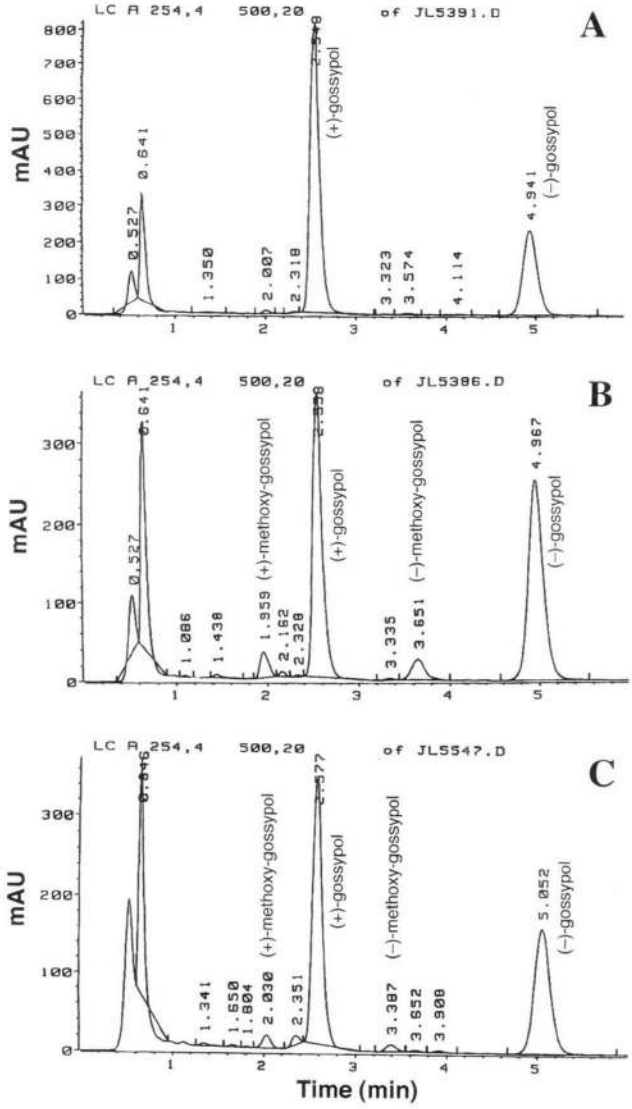

Figure 1. (A) cottonseed: Upland; (B) cottonseed: Pima; (C) cottonseed Meal.

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AOCS Official Method Ba 9-58

Reapproved 2009

Urease Activity

DEFINITION

This method determines the presence of residual urease in soybean products under the conditions of the test (References, 1).

SCOPE

Applicable to soybean meals, soy flour and to soybean mill feeds, except when urea has been added.

APPARATUS

1. Water bath—capable of being maintained at a temperature of 30 ± 0.5°C.
2. pH Meter—equipped with glass and calomel electrodes and capable of testing 5 mL of solutions. It should be a precision instrument with a temperature compensator having a sensitivity of ± 0.02 pH units or better. Follow manufacturer's instructions for the operation, standardization, and determination of pH. Calibrate the meter with standard buffers with values at or near the range at which measurements are to be made (see Notes, 1).
3. Test tubes—20 mm × 150 mm, fitted with rubber stoppers.

REAGENTS

1. Phosphate buffer solution (0.05 M)—Dissolve 3.403 g of reagent-grade monobasic potassium phosphate (KH_2PO_4) in approximately 100 mL of freshly distilled water. Dissolve 4.355 g of reagent-grade dibasic potassium phosphate (K_2HPO_4) in approximately 100 mL of water. Combine the two solutions and make to exactly 1000 mL. If reagents are pure, pH should be at 7.0. If it is not, adjust to 7.0 with a solution of an acid or base before using. The useful life of the buffer solution, prepared as described, is less than 90 days.
2. Buffered urea solution—Dissolve 15 g urea (reagent grade) in 500 mL of the phosphate buffer solution. Add 5 mL of toluene (see Notes, Caution) to serve as a preservative and to prevent mold formation. Adjust the pH of the urea solution to 7.0 as in Reagents, 1.

PREPARATION OF TEST SAMPLE

1. Grind the laboratory sample as finely as possible without raising the temperature, and mix completely. At least 60% of the grind should pass a US no. 40 standard sieve (particle size 0.425 mm or less). Soy flour requires no grinding, but make certain it is well mixed.

PROCEDURE

1. Weigh 0.200 g (± 0.001 g) of the test sample into a test tube and add 10 mL of the buffered urea solution. Stopper, mix, and place in water bath at 30°C. Do not invert the tube during the process of mixing.
2. Prepare a blank by weighing 0.200 g (± 0.001 g) test sample into a test tube and add 10 mL of the phosphate buffer solution. Stopper, mix, and place in water bath at 30°C. Allow a time interval of 5 min between the preparation of the test and the blank portions. Agitate the contents of each tube at 5-min intervals.
3. Remove the test and blank portions from the water bath after 30 min. Transfer the supernatant liquids to a 5.0 mL beaker, maintaining the 5 min interval between the test and blank. Determine the pH of the supernatant liquids at exactly 5 min after removal from the bath (see Notes, 1).

CALCULATIONS

1. A difference between the pH of the test sample and the pH of the blank is an indication and an index of urease activity.

NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

NUMBERED NOTES

1. Care must be exercised to prevent contamination of all glassware or electrodes. Should the pH instrument fail to deliver a prompt and stable reading, investigate. Frequently, the flow of electrolyte through the porous fibers in the calomel electrode may be retarded by a coating of the soluble fraction from soybean.

REFERENCES

1. This method is a modification of the procedure of Caskey, C.D., and F.C. Knapp, *Ind. Eng. Chem., Anal. Ed. 16*:640 (1944).

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AOCS Official Method Ba 10-65
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Protein Dispersibility Index (PDI)

DEFINITION

This method determines the dispersible protein in soybean products under the conditions of the test. In contrast to the alternate slow-stir method for Nitrogen Solubility Index (NSI), AOCS Official Method Ba 11-65, the faster stirring technique used in this method will give generally higher results. Results are dependent upon the exact equipment used.

SCOPE

Applicable to ground soybeans, whole or ground full-fat or extracted flakes, full-fat and defatted soy flours and grits, and soybean meal.

NOTE

Models of equipment listed in this method are no longer commercially available; any modifications should be approached with caution.

APPARATUS

1. Hamilton Beach Drinkmaster blender (800-851-8900) (Model 600WP, 727W, or 65250)—modified to accommodate Waring blender cup (see Notes, 1, 2).
2. Blade assembly (Cenco-Pinto)—The blades may be fabricated from two pieces of 2.25 in. stainless steel, which are ½ to ⅜ in. in the middle and ¼ in. at the tip. The tips on one blade are bent up at a 90° angle and the tips on the other blade are bent down at a 30° angle. The two blades are positioned at a 90° angle to each other. The cutting edges should be in the direction of rotation.

   Note —The blade assembly supplied with the Hamilton Beach Drinkmaster may be used; however, this may give slightly different results when compared to the Cenco-Pinto blade assembly noted.
3. Waring blender cup—1 L capacity, bottom sealed with no. 3 stopper.
4. Glassware—300 mL volumetric flask, 15 mL pipet, 600 mL beaker.
5. Centrifuge—with 50 mL tubes, or equivalent, capable of delivering 1400 relative centrifugal force at the tip.
6. An analytical balance with ±0.1 g accuracy is essential.
7. Timer—with alarm.
8. Variable transformer—e.g., Variac; for controlling the speed of the blender.
9. Standard Kjeldahl equipment—as noted in the Apparatus section of AOCS Official Methods Aa 5-91 and Ba 4d-90.
10. Tachometer—capable of measuring up to 10,000 rpm.
11. Voltmeter (optional).

REAGENTS

1. Distilled water—neutral pH.
2. Standard reagents for Kjeldahl nitrogen determination—as noted in the Reagents section of AOCS Official Methods Aa 5-91 and Ba 4d-90.

PREPARATION OF SAMPLE

1. Use sample as received, with no additional preparation.

STANDARDIZATION OF BLENDER

1. Hamilton Beach Drinkmaster, modified—
   (a) Connect the blender to the variable transformer (Apparatus, 8).
   (b) Measure 300 mL of distilled water into the blender cup, and place in position on the mixer.
   (c) Remove chrome cap that covers the top of the drive shaft. Using the proper tip, place tachometer in position on the rotating shaft.
   (d) With the switch in high position, gradually increase the transformer setting until the shaft shows 8500 rpm on the tachometer. Note voltmeter reading (if voltmeter option is used) and transformer setting. Use the same setting(s) for blending of sample.

   Note—Standardization of the blender should be done before each series of tests to eliminate errors due to fluctuation in line voltage.

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PROCEDURE

1. Hamilton Beach Drinkmaster blender—
   (a) Weigh 20 ± 0.1 g of sample.
   (b) Fill a 300 mL volumetric flask with distilled water at 25 ± 1°C. Pour 50 mL of the water into the blender cup.
   Note—Water-dispersible protein is related to temperature. The blender cup should be at 25 ± 1°C.
   (c) Transfer the weighed sample quantitatively to the blender cup. Stir with a spatula to form a paste. Add remainder of water to rinse spatula and blender cup walls. Place cup in position for blending.
   (d) Using the setting(s) determined in Standardization of Blender, 1, (d), blend the sample for 10 min at 8500 rpm.
   (e) Remove the blender cup and pour the slurry into a 600 mL beaker. After the slurry has been separated, decant or pipet a portion of the upper layer into a 50 mL centrifuge tube and centrifuge for 10 min at 2700 rpm.
   (f) Pipet 15 mL of supernatant liquid into a Kjeldahl flask and determine Kjeldahl nitrogen by using the Procedure section in either AOCS Official Method Aa 5-91 or AOCS Official Method Ba 4d-90.
   Note—15 mL of the supernate is equivalent to 1.0 g of sample in the Kjeldahl methods.

CALCULATIONS

1. % water-dispersible protein =

$$\frac{(B - S) \times N \times 0.014 \times 100 \times 6.25}{\text{mass, g of sample}/20}$$

Where—

B = volume, mL of alkali back titration of blank in Kjeldahl method
S = volume, mL of alkali back titration of sample in Kjeldahl method
N = normality of alkali used in Kjeldahl method
Note—Factor of 6.25 converts Kjeldahl nitrogen to protein.

2. Protein dispersibility index (PDI)

$$= \frac{\%\text{ water-dispersible protein} \times 100}{\%\text{ total protein}}$$

Note—Requires a Kjeldahl protein determination on the whole sample, using the same Kjeldahl method used in Calculations, 1.

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 4.375.
2. Agreement between laboratories: Two single determinations performed in different laboratories should not differ by more than 9.664 (see Notes, 1).

NOTES

1. In 1989, the option of using the Sorvall Omni-Mixer was deleted from this method. Based on experience at several laboratories, the use of the Sorvall Omni-Mixer resulted in a ±10% variation in results, compared to using the Hamilton Beach Drinkmaster blender. The laboratories noted better standard deviation (approximately half the values noted in the Precision statement in this method) when using the Hamilton Beach Drinkmaster.
2. Hamilton Beach Drinkmaster (model 727W or 65250) modification for protein dispersibility index testing—Parts (one each)
   (a) Set of blades (see Figure 1 for fabrication, 1 set = 2 pieces).
   (b) Upper mixer bracket (see Figure 2 for fabrication, 1 piece).
   (c) Lower mixer bracket (see Figure 3 for fabrication, 1 piece).
   (d) Waring 2 qt mixing cup (glass).
   (e) No. 3 rubber stopper.
   (f) Wire nut.

   Procedure
   (a) Remove current blade assembly (will unscrew, reverse threads).
   (b) Remove current mounting brackets (upper and lower) and bracket screws.
   (c) Remove four cover screws from the blender top.
   (d) Remove (or loosen) two lower screws just under front edge of blender.
   (e) Tilt back top cover (leaving speed control wires attached).
   (f) Loosen two front cover screws (just inside top/front, two Phillips screws).
   (g) Remove motor mounting bolts (three bolts with springs on them).
   (h) Lift motor assembly and tilt back to expose remaining cover screw (front/top).
   (i) Remove last cover screw (on front of blender at the top of the backplate, just above safety switch).
   (j) Carefully slide stainless steel backplate forward to remove it.

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(k) Remove the safety switch and cut the two wires going to it.
(l) Strip .25 in. of insulation from cut ends of wire and splice with wire nut.
(m) Put top bolt from safety switch assembly back in place.
(n) Carefully slide stainless steel backplate in place.
(o) Tighten or replace top (2) and bottom (2) backplate screws.
(p) Attach top blender cup bracket and tighten screw.
(q) Tip motor assembly back down in place, and replace/tighten three motor bolts (with springs on top).
(r) Replace top cover and cover screws, and tighten in place.
(s) Place bottom bracket in place. Use original bracket screw in top hole; use the bottom screw from safety switch in the bottom hole.
(t) Invert two blade pieces and put together at 90° angles (one points up and other points down).
(u) Using screw supplied with alternate blade (comes with blender, in box with paperwork), mount blades to motor shaft.

Note—Due to the design and low horsepower of this blender, a contact or reflective tape tachometer cannot readily be used. A tachometer that works from motor vibration (Vibra-Tac) can be obtained from Martin Engineering Company, Neponset, IL, USA. There are two types available: Vibra-Tac (slow), 200 – 2000 rpm; Vibra -Tac (fast), 2000–21,000 rpm (usable for standard PDI test).

REFERENCES

For method development and history see *J. Am. Oil Chem. Soc. 37*:165 (1960).

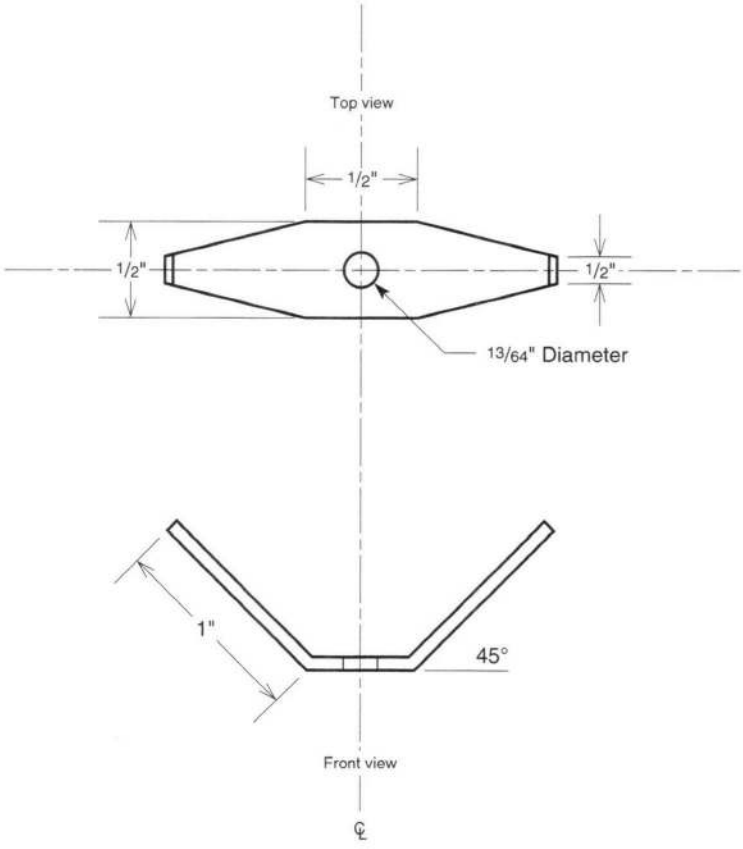

Figure 1. PDI mixer blade, two blades per set.

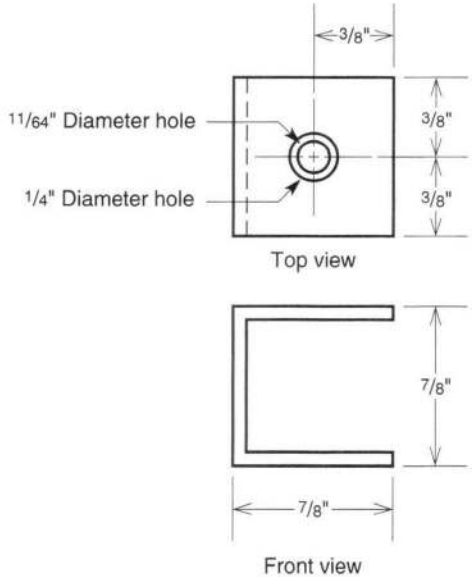

Figure 2. Upper mixer bracket.

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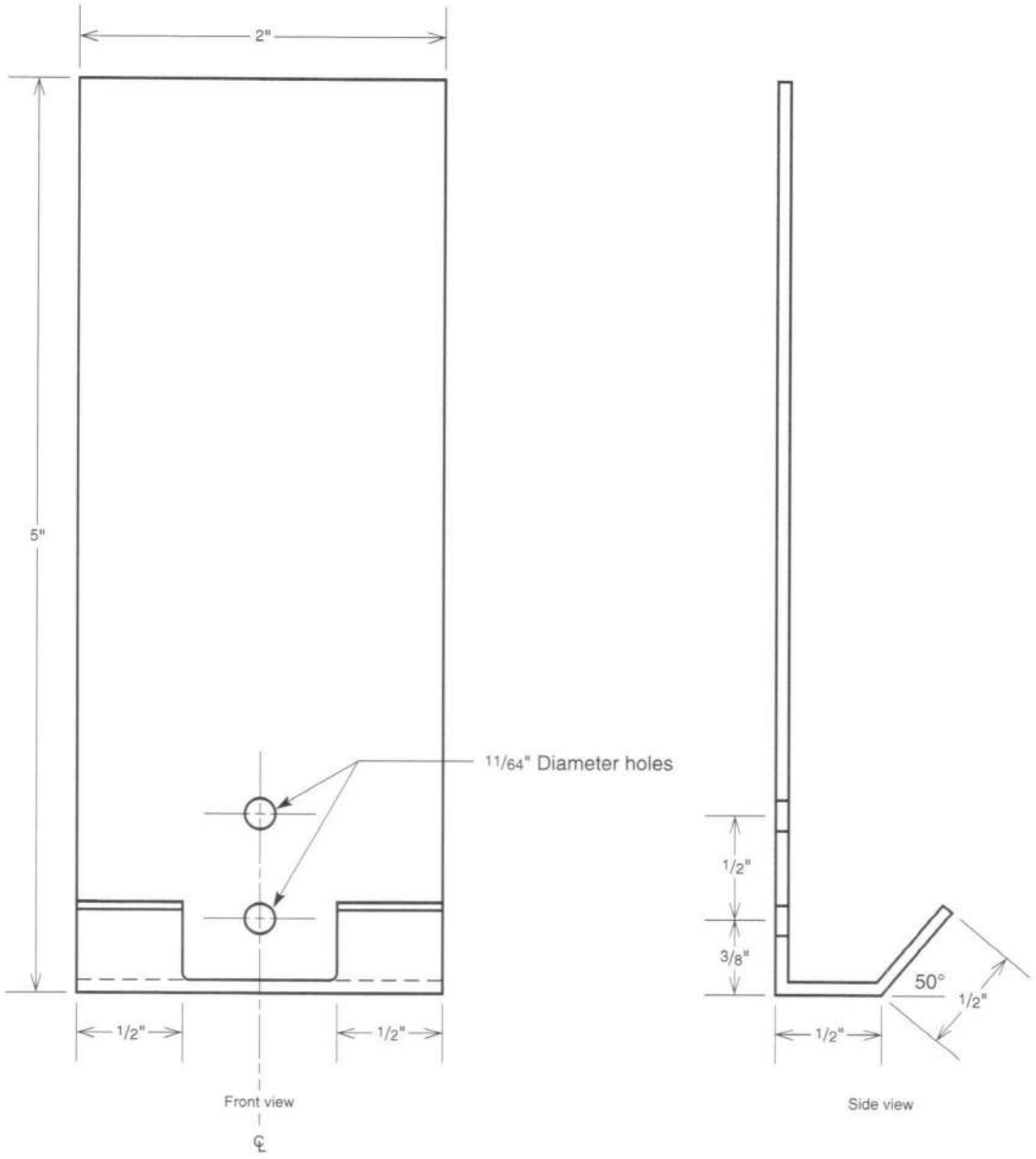

Figure 3. Lower mixer bracket.

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AOCS Recommended Practice Ba 10a-05
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Protein Dispersibility Index (PDI)

DEFINITION

This method determines the dispersible protein in soybean products under the conditions of the test. In contrast to the alternate slow speed stir method for Nitrogen Solubility Index (NSI), AOCS Recommended Practice Ba 11-65, the faster stirring technique used in this method will give generally higher results than those obtained by the slow speed stir method. Results are dependent upon the exact equipment used.

SCOPE

Applicable to ground soybeans, whole or ground full-fat or extracted flakes, full-fat and defatted soy flours and grits, and soybean meal.

NOTE

This equipment is no longer manufactured in a form which allows this modification.

APPARATUS

1. Hamilton Beach Commercial (Model 936) blender—modified to accommodate Waring blender cup (see Notes, 1).
2. PDI blade assembly kit (see Notes, 2). The tips on one blade are bent down at a 30° angle. The opposing two blades are positioned at a 90° angle to each other. The cutting edges should be in the direction of rotation (see Figure 1 for a detailed description). *Note:* The blade assembly supplied with the Hamilton Beach Commercial blender should not be used, since it may give different results when compared to the PDI blade assembly kit noted. Other variations in blade design and shaft length will give other results. Care should be used when comparing results.
3. Waring Blender cup—1 L capacity, with bottom sealed with appropriate sized rubber stopper.
4. Glassware—300 mL volumetric flask, 15 mL pipet, 600 mL beaker.
5. Centrifuge—capable of speeds in the region of 2,700 rpm, equipped with buckets to hold 50 mL centrifuge tubes, capable of withstanding 1,400 × g (relative centrifuge force) at the tip (check the centrifuge manual for the correct speed setting).
6. Analytical balance—with ± 0.1 g accuracy.
7. Timer with alarm
8. Variable transformer—for controlling the speed of the blender.
9. Standard Kjeldahl equipment as noted in the apparatus section of AOCS Official Method Ba 4d-90, or Ba 4e-93—combustion method.
10. Tachometer—capable of measuring up to 10,000 rpm.
11. Voltmeter (optional).

REAGENTS

1. Distilled water—neutral pH.
2. Standard reagents for Kjeldahl nitrogen determination—as noted in method Ba 4d-90 or Ba 4e-93—combustion method.

PREPARATION OF TEST SAMPLE

1. Use sample as received, with no additional preparation.

STANDARDIZATION OF BLENDER

1. Hamilton Beach Commercial blender (Model 936; Notes, 3)
   a. Connect the blender to the variable transformer (Apparatus, 8). Conect transformer to timer (Apparatus, 7).
   b. Measure 300 mL of distilled water into the blender cup, and place in position on the mixer.
   c. Place a small self-adhesive strip of reflective tape in upper part of blender shaft.
   d. With the switch in medium position, gradually increase the transformer setting until the shaft shows 8,500 rpm on the strobe tachometer. Note voltmeter reading (if voltmeter option is used) and transformer setting. Use the same setting(s) for blending of sample.

   Note: Standardization of the blender should be done before each series of tests to eliminate errors due to fluctuation in line voltage.

PROCEDURE

Preparation of water-dispersible protein solution

1. Hamilton Beach Commercial blender (Model 936)
   a. Weigh 20 g ± 0.2 g of test sample.
   b. Fill a 300 mL volumetric flask with distilled water at 25°C ± 1°C. Pour 50 mL of the water into the blender cup.

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Note: Water-dispersible protein is related to temperature. The blender cup should be at 25°C ± 1°C; during blending the temperature increase should not exceed 4°C ± 1°C.

c. Transfer the weighed test portion quantitatively to the blender cup. Stir with a spatula to form a paste. Add remainder of water to rinse the spatula and blender cup walls. Place the cup in position for blending.
d. Using the setting(s) determined in the Standardization of Blender procedure (d), blend the test portion for 10 min at 8,500 rpm.
e. Remove the blender cup and pour the slurry into a 600 mL beaker. After the slurry has been allowed to separate, decant or pipet a portion of the upper layer into a 50 mL centrifuge tube and centrifuge for 10 min at 1,400 g.

DETERMINATION OF NITROGEN CONTENT

1. Pipet 15 mL of supernatant liquid into a Kjeldahl nitrogen digestion tube and determine nitrogen content as described in Ba 4d 90. Alternatively, nitrogen may be determined by combustion according to Ba 4e-93.
2. Determine the nitrogen content of a 1 g sample of the starting sample (use this volume for the calculation of % total protein).

   Note: 15 mL of the supernatant is equivalent to 1.0 g of test portion in the Kjeldahl method. When using Ba 4e-93 for determination of nitrogen the volume of the liquid sample (water dispersible proteins) must be equivalent to the 15 mL aliquot used in the Kjeldahl method.

CALCULATIONS

1. Using the Kjeldahl method—

$$\% \text{ Water Dispersible Protein} = \frac{(B - S) \times M \times 0.014 \times 100 \times 6.25}{\text{wt of test portion} \times 0.05}$$

2. $$\% \text{ Total Protein} = \frac{(B - S) \times M \times 0.014 \times 100 \times 6.25}{\text{wt of test portion (g)}}$$

where—

B = mL of alkali back titration of blank Kjeldahl method
S = mL of alkali back titration of sample Kjeldahl method
M = Molarity of alkali used in Kjeldahl method

Note: Factor of 6.25 converts Kjeldahl nitrogen to protein.

Protein dispersibility index (PDI) =

$$\frac{\% \text{ water dispersible protein} \times 100}{\% \text{ total protein}}$$

Note: Requires a protein determination on the whole test portion, using the same protein method used in Calculations, 1.

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 4.4.
2. Agreement between laboratories—Two single determinations performed in different laboratories should not differ by more than 9.6 (see Notes, 1).

NOTES

1. A Hamilton Beach 936 Unit, modified exactly according to above description and equipped with two Waring blender cups was available from VOS Instrumenten B.V., Schimminck 10, NL-5301 KR Zaltbommel, the Netherlands (Tel: +31 418 575 080; Fax: +31 418 684 900; E-mail: info@vosinstrumenten.nl).
2. PDI blade assembly kit is available from PCM Company, 3536 N. 27th Street, Decatur, IL; (217) 875-2460; pcm50 @insightbb.com
3. Hamilton Beach Commercial blender Model 936 was available from Ryan Equipment & Supply, 805 N. Morgan Street, Decatur, IL 62521; (217) 422-1786; ryaneq@midwest.net
4. Procedure: blender, modification
   a. Remove the top and bottom brackets that come with the machine.
   b. Attach the top bracket (with supplied nut shown in the adaptor assembly drawing), and slide the switch bar in the upper position. This locks the switch in the "on" position.
   c. Attach the bottom brackets as shown in Figure 1. Insert supplied bolt at top of new bracket slot. Use the original bracket bolt in lower part of new bracket slot.
   d. Remove original blades from the mixer shaft and substitute the PDI blade assembly kit.
   e. The tips of bracket D may be covered with rubber tubing as a cushion for the glass cup.

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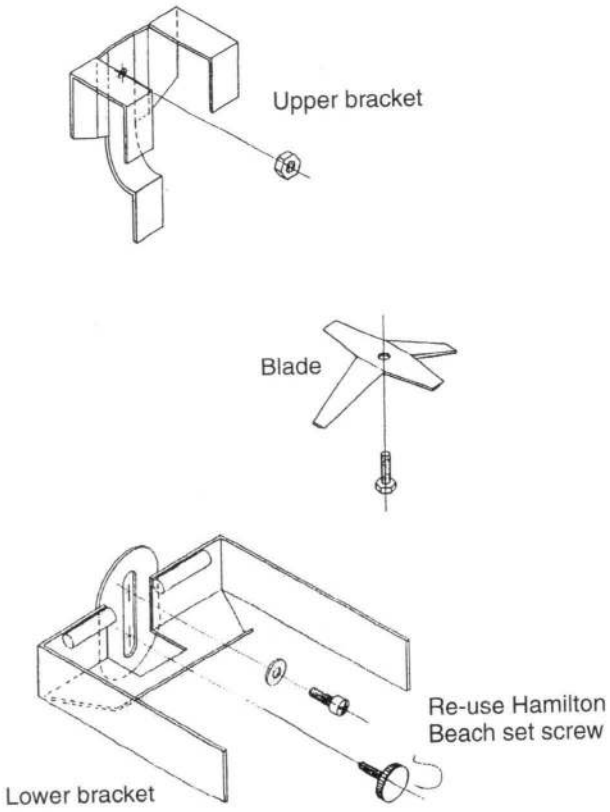

Figure 1. PDI blade assembly kit.

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AOCS Standard Procedure Ba 10b-09

Revised 2011

Protein Dispersibility Index (PDI)

DEFINITION

This method determines the dispersible protein in soybean products under the conditions of the test. In contrast to the alternate slow-stir method for Nitrogen Solubility Index (NSI), AOCS Official Method Ba 11-65, because of the faster stirring technique used in this method, will give generally higher results.

SCOPE

Applicable to ground soybeans, whole or ground full-fat or extracted flakes, full-fat and defatted soy flours and grits, and soybean meal. This method has been revised to reflect changes in available mixers detailed in previous versions of this method. The results obtained using this method are highly dependent upon the equipment used and should be instituted appropriately (See Notes, 1).

APPARATUS

1. Vertical mixer with variable speed motor and enclosed blade assembly. Suitable equipment may include the Omni-Mixer, Omni-Macro, and Omni-Max with 473 ml glass bottle and blade assemblies. 2 in. (5 cm) rotor knife assemblies are preferred over edge knife assemblies.
2. Glassware—250 mL graduated cylinder, 15 ml pipet, 500 mL beaker.
3. Centrifuge—with 50 mL tubes, or equivalent, capable of delivering 1400 × g.
4. Analytical balance with ± 0.1 g accuracy.
5. Timer.
6. Kjeldahl or Dumas equipment.
7. Tachometer—capable of measuring up to 10,000 rpm.

REAGENTS

1. Distilled water—neutral pH.
2. Standard reagents for Kjeldahl determination if required.

PREPARATION OF TEST SAMPLE

1. Use portions of the laboratory sample as received, with no additional preparation.

STANDARDIZATION OF BLENDER

1. Measure 150 ml of distilled water into the mixer cup and place in position on the mixer.
2. Remove cap which covers the top of the drive shaft. Using the proper tip, place tachometer in position on rotating shaft.
3. Gradually increase the built-in rheostat setting until the shaft shows 8,500 rpm on the tachometer. Note rheostat setting and use for blending of samples.
4. Standardize the blender before each series of tests.

PROCEDURE

1. Weigh a 10 g ± 0.1 g test portion of soy product. Add 150 ml of distilled water to a graduated cylinder. Pour about 50 mL of the water into the mixer cup. (Water dispersible protein is related to temperature so the mixer cup should be at room temperature.) Transfer the weighed test portion quantitatively to the mixer cup. Stir with a spatula to form a paste. Add remainder of water in increments, with stirring, to form a smooth slurry, using last of water to rinse spatula and mixer cup walls. Place cup in position for blending.
2. Blend at a speed of 8,500 rpm for 10 min.
3. Remove mixer cup and pour the slurry into 400 mL beaker. After the slurry has separated, decant a portion into a 50 mL centrifuge tube and centrifuge 10 min at 2,700 rpm.
4. Determine the protein content of the supernatant by either Kjeldahl or Dumas procedures (15 mL = 1.0 g test portion).

CALCULATION

1. Protein Dispersibility Index (PDI) = $\frac{\%\ \text{water dispersible protein} \times 100}{\%\ \text{total protein}}$

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PRECISION

This method has not been collaboratively studied. Results obtained may be extremely variable depending upon equipment and conditions of application. Users should determine the within run and within laboratory variability according to accepted protocols.

NUMBERED NOTES

1. This method was originally replaced by Ba 10-65 and Ba 10a-05 when a particular piece of apparatus was chosen as the standard mixer. Changes in model and design have now made those versions obsolete because the recommended modifications are no longer possible. The present method specifies a readily accessible piece of scientific equipment with a broad distributorship (www.omni-inc.com).

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AOCS Official Method Ba 11-65

Reapproved 2009

Nitrogen Solubility Index (NSI)

DEFINITION

This method determines the dispersible nitrogen in soybean products under the conditions of the test. In contrast to the alternate fast-stir method for Protein Dispersibility Index (PDI), AOCS Official Method Ba 10-65, the slower stirring technique used in this method will give generally lower results.

SCOPE

Applicable to ground soybeans, whole or ground full-fat and defatted soy flours and grits, and soybean meal.

APPARATUS

1. Beaker—400 mL.
2. Graduated cylinder—200 mL.
3. Glass stirring rod.
4. Volumetric flask—250 mL.
5. Stirring apparatus—adjustable up to 120 rpm, equipped with a stirring blade having the shape of a segment of a circle, the extreme diameter of the blade being approximately 50 mm. This type of stirrer is commercially available from most laboratory supply companies (e.g., Sargent-Welch, Fisher, Kontes, etc.).

   Note—Most of the commercial electrical stirring devices can be adjusted to 120 rpm with a variable transformer. Cone drive-type stirrers are particularly adapted for slow-speed stirring.
6. Balance—± 0.01 g accuracy.
7. Water bath—maintained at a temperature of 30°C.
8. Wash bottle.
9. Centrifuge—with 50 mL round-bottom centrifuge tubes.
10. Standard Kjeldahl equipment—as noted in Apparatus section of AOCS Official Method Aa 5-91 (titanium dioxide + copper sulfate catalyst).
11. Small glass funnels.
12. Beakers—200 mL.
13. Pipet—25 mL.

REAGENTS

1. Distilled water.
2. Standard reagents for Kjeldahl nitrogen determination, as noted in the Reagents section of AOCS Official Method Aa 5-91.
3. Silicone antifoam agent—Dow Corning AF, Hodag F-2, FD-82, or equivalent.
4. Glass fiber.

PREPARATION OF TEST SAMPLE

1. If the test sample is not in form of a fine flour, grind it so at least 95% will pass through a 100 mesh screen. Use an impact-type laboratory mill such as a Mikro-Pulverizer or Mikro-Samplmill (available from Mikro-Pul, Summit, NJ, USA), equipped with a no. 3428 Mikro-Screen (tweed type). To obtain sufficient impact, rotor speed should be at least 10,000 rpm; however, do not exceed 16,000 rpm.
2. For a full-fat sample, use a screen such as Mikro-Pul no. 027RD26G, or equivalent, that will grind the material to a fine flour so at least 80% of the material will go through an 80 mesh screen and 90% will go through a 60 mesh screen.
3. In order to avoid heat denaturation during grinding, mix an equal amount of dry ice with the sample prior to grinding. At least 25 g of sample should be used for the mill.

   Note—This grinding step is optional (see Notes, 1).

PROCEDURE

1. Weigh 5 g of the test sample into a 500 mL beaker. Measure 200 mL of distilled water at 30°C. Add a small portion of the water at a time and disperse it thoroughly with a stirring rod. Stir in the remainder of the water, using the last of it to wash off the stirring rod.
2. Stir the mixture at 120 rpm with the mechanical stirrer for 120 min at 30°C with the beaker immersed in the 30°C water bath. Transfer the mixture to a 250 mL volumetric flask by carefully washing out the contents of the beaker into the flask. Add 1 or 2 drops of antifoam, dilute to mark with distilled water and mix the contents of the flask thoroughly.

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3. Allow to stand for a few minutes and decant off about 40 mL into a 50 mL centrifuge tube. Centrifuge 10 min at 1500 rpm and decant supernatant through a funnel containing a plug of glass fiber (be careful not to transfer any of the centrifuged solids to the filter). Collect the clear filtrate in a 100 mL beaker.
4. Pipet 25 mL of the clear liquid into a Kjeldahl flask; then proceed according to standard practice for determining protein nitrogen, as noted in the Procedure section of AOCS Official Method Aa 5-91.
5. Determine total nitrogen in the test portion using AOCS Official Method Aa 5-91.

CALCULATIONS

1. Water-soluble nitrogen, $\% = \frac{(B - S) \times M \times 0.14}{\text{mass of test portion, g}} \times 100$

   Where—

   B = volume, mL of alkali back titration of blank in AOCS Official Method Aa 5-91
   S = volume, mL of alkali back titration of sample in AOCS Official Method Aa 5-91
   M = molarity of alkali used in AOCS Official Method Aa 5-91

2. Nitrogen solubility index (NSI) $= \frac{\%\text{ water-soluble nitrogen}}{\%\text{ total nitrogen}^a} \times 100$

   [a]Requires a Kjeldahl protein nitrogen determination on the whole sample, using AOCS Official Method Aa 5-91.

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 3.735.
2. Two single determinations performed in different laboratories should not differ by more than 7.224.

NOTES

1. NSI may be reported on an "as is" basis or on a "ground sample" basis, and this should be so specified: "Nitrogen Solubility Index (NSI), as is" or "Nitrogen Solubility Index (NSI), ground sample."

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AOCS Official Method Ba 12-75

Reapproved 2009

Trypsin Inhibitor Activity

DEFINITION

This method determines the total and residual trypsin inhibitors in food and feed products under the conditions of the test.

SCOPE

Applicable to soybean meals and flours (raw and toasted), to soy protein concentrates and isolates, and to corn-soy mixtures.

APPARATUS

1. Water bath—controlled at 37 ± 0.5°C.
2. Spectrophotometer—functional at 410 nm, with 1-cm square cuvettes, or colorimeter calibrated to furnish equivalent absorbance values.
3. Test tubes—18 × 150 mm.
4. Vortex stirrer.
5. Magnetic stirrer.
6. Grinding mill—capable of reducing sample to 100 mesh or smaller without loss of material or temperatures in excess of 40°C.

REAGENTS

1. Tris buffer (pH 8.2, 0.05 M)—Dissolve 6.05 g tris (hydroxymethyl) -aminomethane and 2.94 g $CaCl_2 \cdot 2H_2O$ in 900 mL CO_2-free water, adjust to pH 8.2, and dilute to 1 L. Bring to 37°C immediately before using. See Numbered Notes, 2.
2. BAPA solution—Dissolve 40 mg benzoyl-DL-arginine-p-nitroanalide hydrochloride (BAPA) in 1 mL dimethyl sulfoxide. Dilute to 100 mL with tris buffer. Prepare fresh daily. Maintain at 37°C for use.
3. Trypsin solution—Dissolve 4 mg accurately weighed, twice-crystallized, salt-free trypsin in 200 mL 0.001 N hydrochloric acid.
4. Acetic acid solution—Mix 30 mL glacial acetic acid and 70 mL water (see Notes, *Caution*).
5. Sodium hydroxide solution—0.01 M.

PREPARATION OF TEST SAMPLE

1. Avoid subjecting laboratory sample to temperatures in excess of 37–40°C at any time.
2. Grind laboratory sample as finely as possible without heating, preferably 100 mesh or smaller. If the material cannot be ground without excessive heating, the sample may be ground with a small quantity of dry ice (solid CO_2).
3. Laboratory samples with more than 2% fat should be defatted with petroleum ether or hexane (see Notes, *Caution*), with both extraction and desolventizing at approximately 25°C, but not greater than 25°C.

PROCEDURE

1. Weigh 1.00 g of test sample into a suitable beaker containing a magnetic stirring bar. Add 50.0 mL 0.01 M sodium hydroxide solution. Slowly agitate suspension 3 hr at room temperature. At the end of 3 hr determine the pH, which should be between 8.4 and 10.0.
2. Dilute an aliquot of suspension with distilled water so the absorbance of the 1 mL assay is between 0.4 and 0.6 times the absorbance of the 0 mL assay (blank), to minimize the relative standard deviation. When it is not possible to estimate the expected trypsin inhibitor units/gram (TIU/g) (see Notes, 1), more than one dilution should be made. The dilution factor is the final volume divided by amount of aliquot taken.
3. Add 0, 0.6, 1.0, 1.4, and 1.8 mL of the diluted suspension to duplicate sets of test tubes. Add sufficient water to bring the volume in each test tube to 2.0 mL.
4. Add 2 mL trypsin solution to each tube, quickly mix on the Vortex stirrer, and immediately place in the 37°C water bath.
5. Prepare a timer for a 10 min interval. Observe the same sequence in starting and stopping the reaction. To each tube in turn, add 5 mL BAPA solution, mix on Vortex stirrer, return to 37°C bath, and immediately begin timing the 10 min interval.
6. Immediately after the 10 min interval, stop the reaction in each tube, using the original sequence, by adding 1 mL acetic acid solution and mixing on the Vortex stirrer.
7. Prepare a blank. Pipet 2 mL dilute suspension into a test tube. Add 5 mL BAPA solution and mix on Vortex stirrer. Place in water bath at 37°C for 10 min. Add 1 mL acetic acid solution and mix on Vortex stirrer. Add 2 mL trypsin solution and mix.
8. (a) Filter the contents of each tube through no. 2 or no. 3 Whatman filter paper, or equivalent, or
   (b) Centrifuge all 10 tubes for 5 minutes at 10,000 rpm in a laboratory centrifuge and decant the clear supernate. 9. Determine the absorbance of each assay against the blank at 410 nm.

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CALCULATIONS

1. Trypsin inhibitor units per milliliters diluted sample suspension as assayed (see Notes, 1):

$$\text{TIU/mL} = \frac{100 \times [(\text{absorbance of B}) - (\text{absorbance of S})]}{\text{Y}}$$

Where—

B = blank (0 mL assay)
S = test solution
Y = number of mL of diluted test portion suspension used in assay (S)

2. Calculate TIU/mL for each of the assays. Plot these results against Y mL. If a linear relationship is obtained, extrapolate to 0 mL to obtain the final TIU/mL. Otherwise, use the simple mean of the TIU/mL obtained.
3. $\text{TIU/g} = \frac{\text{TIU}}{\text{mL}} \times \text{dilution factor} \times 50$

PRECISION

1. See Table 1 for estimates of variation in relative standard deviation (RSD) for trypsin inhibitor assays.

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Acetic acid in the pure state (glacial acetic acid) is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

NUMBERED NOTES

1. One trypsin unit is arbitrarily defined as an increase of 0.01 absorbance units at 410 nm per 10 mL of the reaction mixture under the conditions used in the method.
2. Tris [tris (hydroxymethyl) -amino methane] buffer mixture may be obtained from Sigma as part #T5378.

Table 1
Variation estimates of trypsin inhibitor assay studies.

Source of variation	RSD, %
Deviation about assay lines[a]	5
Collaborators with common extract[b]	2
Collaborators with separate extracts[c]	5

[a]Error involved in converting the change in trypsin units to TIU/mL from the milliliter of soy used for assay, such as 0.6, 1.0, 1.4, and 1.8 mL. The relative standard deviation from the standard trypsin curve is < 5%.
[b]On-the-spot assay by all collaborators on the same extract.
[c]Extracts and assays made in laboratories of individual collaborator.

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AOCS Official Method Ba 13-87

Revised 2011

"Free" Hexane Content in Extracted Meals

DEFINITION

Extraction of oilseeds with hydrocarbon-based solvents results in a residue (extracted meal) containing residual volatile hydrocarbons. The "free" hexane content in extracted meals is the quantity of volatile hydrocarbons desorbed by heating the sample at 80°C in a closed vessel after addition of an internal standard. After determination of a calibration factor, hydrocarbons in the headspace are determined by gas chromatography, using packed or capillary columns. Results are expressed as hexane in milligrams per kilogram (ppm).

SCOPE

This method is applicable to the determination of "free" volatile hydrocarbons, expressed in terms of hexane, remaining in oilseed residues after extraction with hydrocarbon-based solvents. This method is equivalent to International Union of Pure and Applied Chemistry (IUPAC) standard method 1.172 (References, 1 and 2).

APPARATUS

1. The gas chromatograph should have, at a minimum, the following characteristics—
   (a) Thermostated column oven capable of maintaining the desired column temperature within ±1°C.
   (b) Sample inlet system, separately thermostated, that can be maintained at a minimum temperature of 100°C. If a capillary column is used, the inlet system must be capable of a 1:100 split injection. For serial analysis a headspace gas chromatograph with automatic sample injection and tempering bath has been shown to be satisfactory.
   (c) Flame ionization detector, separately thermostated, that can be maintained at a minimum temperature of 100°C.
2. Electronic integrator.
3. Chromatographic column—A capillary column with the following minimum requirements may be used for this analysis—The column should be constructed of glass or fused silica, approximately 30 m long and 0.3 mm i.d. Stationary phase: methyl polysiloxane (film thickness 0.2 µm).
4. Syringe—10 µL capacity.
5. Syringe—1000 µL capacity, gas-tight (preferably with valve).
6. Septum vials—20 mL capacity.
7. Septa and aluminum caps suitable for septum vials, together with crimping pliers. The septa must be resistant to oils and solvents (butyl rubber or red rubber is recommended).
8. Metallic foil—e.g., aluminum foil.
9. Tongs—suitable for holding septum vials.
10. Heating bath—with clamps for holding septum vials, thermostatically regulated, and capable of maintaining a temperature of 80°C. For continuous operation, glycerol is recommended as the heating liquid.

REAGENTS

1. Gases—
   (a) Carrier—helium (preferred for better resolution) or nitrogen, minimum purity 99.99 mol%, dried, and containing a maximum of 10 mg O_2/kg.
   (b) Flame ionization detector—hydrogen, minimum purity 99.95 mol%; air or O_2, dry (dew point – 59°C maximum) and hydrocarbon free (<2 ppm hydrocarbons equivalent to CH_4).
2. Technical hexane or a light petroleum—with a composition similar to that used in industrial extraction, or failing these, *n*-hexane (see Notes, *Caution*). For calibration, technical extraction hexane is preferred.
3. *n*-Heptane (internal standard)—analytical reagent grade. (Cyclohexane is also suitable for use as an internal standard, provided that the solvent used for the extraction or calibration has a negligible content of cyclohexane.) (See Notes, *Caution.*)
4. Oilseed meal—solvent free. The meal is to be used for calibration and should be of a similar nature as the sample. It should be free of extraction solvent (< 0.01%). If it contains a greater amount of solvent, this should be reduced by leaving the meal in thin layers in the open air for several hours.

   Note—Commercial oilseed residues usually have a water content of 12–14%. For samples having a different water content, the calibration has to be made with an oilseed residue that has the same water content as the sample.

SAMPLING AND LABORATORY SAMPLE STORAGE

1. It is essential that loss of solvent from the sample be prevented. The laboratory sample should be in a completely sealed container and stored at 4°C. Plastic containers should not be used. Analyses should be carried out as soon as the container is opened.

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GC OPERATING CONDITIONS

1. Carrier gas flow depends on the carrier gas and the type of column being used for the analysis and should be optimized accordingly. Helium is recommended, but nitrogen may be used. Some loss of resolution between peaks may be experienced with nitrogen.
2. The flow of hydrogen and air or oxygen to the flame ionization detector should be optimized according to the manufacturer's recommendation for the instrument being used.
3. Injector and detector temperatures should be set at about 120°C.
4. The column should be maintained at 40°C.

PROCEDURE

1. Determination of the calibration factor—Weigh, to the nearest 0.1 g, 5 g of solvent-free meal (Reagents, 4) into each of five septum vials (Apparatus, 7). Seal each vial with a septum and cap (Apparatus, 8). By means of the 10 µL syringe (Apparatus, 5), add technical hexane (Reagents, 2) to four of five vials according to the following table:

Vial number	1	2	3	4
µL Technical hexane to add	1	2	4	7

Do not add any technical hexane to vial 5.

Allow the sample vials to stand at room temperature for 24 hr. In the case of shorter contact times, it cannot be certain that the extraction solvent is absorbed completely by the meal and that the absorption-desorption equilibrium of the solvent between meal and air has been attained. Using a syringe (Apparatus, 5), add through the septum exactly 5 µL of internal standard (Reagents, 3) to each of the five vials.

At 15 min intervals, place one of the vials in the heating bath. The heating medium should come up to the neck of each vial. Heat at 80°C for exactly 60 min.

Warm the gas-tight syringe (Apparatus, 6) to 60°C.

Without removing the vial from the heating bath, use the gas-tight syringe and withdraw through the septum 1 mL of the gaseous phase from the headspace. Inject immediately into the gas chromatograph.

For each of the four vials containing added technical hexane, a calibration factor, F, may be determined by the formula:

$$F = \frac{C_S \times A_i}{(A_H - A_B - A_i) \times C_i}$$

Where—

A_H = total peak area of solvent hydrocarbons, including the area of internal standard present in the oilseed residue in the vial. For identification purposes, a typical chromatogram of the technical solvent composition should be obtained. Hydrocarbons that usually make up the technical solvent are 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane, etc. Do not include peaks due to the oxidation products, some of which may be present in significant amounts.

A_B = peak area of the solvent hydrocarbons present in the oilseed residue to which technical hexane has not been added (vial 5) less the peak area of the internal standard

A_i = peak area corresponding to the internal standard

C_i = quantity of internal standard added, expressed in mg/kg (ppm) of oilseed residue

Note—For an addition of 5 µL to 5 g of sample, C_i = 680 mg for n-heptane and C_i = 780 mg for cyclohexane.

C_S = quantity of technical hexane added to the oilseed residue present in the vial, expressed in mg/kg (ppm) of meal, which is given by the following table:

Vial number	1	2	3	4
Technical hexane added (mg/kg) (ppm)	134	268	536	938

If n-hexane is used, C_S is given by the following table:

Vial number	1	2	3	4
n-Hexane added (mg/kg) (ppm)	132	264	528	924

Calculate the mean calibration factor from the results obtained for the four vials. The mean calibration factor should be 1.0 ± 0.1, and the differences between the individual results should be small.

2. Test sample analysis—Weigh, to the nearest 0.1 g, 5 g of the test sample into a septum vial (Apparatus, 7) as quickly as possible and close immediately with a septum and cap (Apparatus, 8). Using a syringe (Apparatus, 5), add through the septum exactly 5 µL of internal standard (Reagents, 3). Immerse the vial up to the neck in the heating bath (Apparatus, 11) at 80°C for exactly 60 min. Warm the gas-tight syringe (Apparatus, 6) to 60°C. After tempering at 80°C for exactly 60 min, use the gastight syringe and take from the vial, without removing it from the bath, 1000 µL of the headspace above the test portion. Immediately inject into the gas chromatograph.
3. Number of determinations—Carry out two determinations in rapid succession on each test portion.

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RESULTS

1. The "free" hexane content, W, expressed in milligrams per kilogram (ppm), is given by the formula:

$$W = \frac{(A_H - A_i) \times F \times C_i}{A_i}$$

Where—

A_H = total peak area of solvent hydrocarbons, including the area of the internal standard. Hydrocarbons that usually make up the technical solvents are 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane, etc. A typical chromatogram of these solvents was shown by the calibration standards. Do not include peaks due to the oxidation products. Some of these products may be present in significant amount.

A_i = peak area corresponding to internal standard in the test portion

C_i = quantity of internal standard added in mg/100 g

Note—For an addition of 5 µL of heptane per 5 g of sample, C_i = 680 mg/1000 g; C_i = 780 mg if cyclohexane is used.

F = calibration factor obtained in Procedure, 1

Report as the final result the mean of the results of the two determinations, provided the requirements for repeatability are met. If the requirements for repeatability are not met, discard the results and carry out two additional determinations on the sample.

PRECISION

1. The results of one interlaboratory test organized at an international level (for different samples) gave the statistical results shown in Table 1.
2. Repeatability—When the mean of the duplicate determinations lies within the range of the mean values cited in Table 1, the difference between the results of the two determinations, carried out in rapid succession by the same operator, using the same apparatus for the analysis of the same test sample, should not be greater than the repeatability value (r), which can be obtained from Table 1, for the level of hexane in the sample examined.
3. Reproducibility—When the means of the duplicate determinations, obtained in two different laboratories using this standard method for the analysis of the same laboratory sample, lie within the range of the mean values cited in Table 1, the difference between the mean results obtained by those laboratories should not be greater than the reproducibility value (R), which can be a obtained from Table 1, for the level of hexane in the sample examined.

NOTES

Caution

The solvents used in this method—hexane, petroleum ether, *n*-heptane, and cyclohexane—are all highly flammable. They should not be used near open flame. Procedures requiring the use of these solvents should be carried out in a properly operating fume hood. For hexane the TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th ed., Blackwell Scientific Publications, 1987, IUPAC Method 1.172.
2. Collaborative study results published in *Pure Appl. Chem.* 59 (10):1407 (1987).

Table 1
Statistical analysis of results for "free" hexane (1984 collaborative study; duplicate determinations).

	Sample		
	Fresh rapeseed meal	Fresh rapeseed meal	Stored sunflower seed meal
Moisture	8.8%	12.1%[a]	9%
Number of laboratories	13	13	12
Number of laboratories after elimination of outliers	11	11	12
Number of results	26	26	24
Mean value, mg/kg (ppm)	624	599	178
Repeatability standard deviation (S_r)	17	33	12
Repeatability coefficient of variation (%)	2.7	5.5	6.7
Repeatability value r (Sr × 2.83)	48	94	34
Reproducibility standard deviation (SR)	129	125	55
Reproducibility coefficient of variation (%)	21	21	31
Reproducibility value R (S_R × 2.83)	364	363	156

[a] Sample 1 (moisture 8.8%) adjusted to moisture level of 12.1%.

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AOCS Official Method Ba 14-87

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Total Hexane Content in Extracted Meals

DEFINITION

Extraction of oilseeds with hydrocarbon-based solvents results in a residue (extracted meal) containing residual volatile hydrocarbons. The total residual hexane content in extracted meals is the quantity of volatile hydrocarbons desorbed by heating the sample at 110°C with water in a closed vessel. After developing a calibration curve, hydrocarbons are determined in the headspace above the sample by gas chromatography, using capillary or packed columns. Results are expressed as hexane in milligrams per kilogram (ppm) of oilseed residue.

SCOPE

This method is applicable to the determination of the total amount of volatile hydrocarbons, expressed in terms of hexane, remaining in oilseed residues after extraction of the seed with hydrocarbon-based solvents. This method is equivalent to International Union of Pure and Applied Chemistry (IUPAC) standard method 1.171 (References, 1 and 2).

APPARATUS

1. The gas chromatograph should have, at a minimum, the following characteristics—
   (a) Thermostated column oven capable of maintaining the desired column temperature within ± 1°C.
   (b) Sample inlet system, separately thermostated, that can be maintained at a minimum temperature of 100°C. If a capillary column is used, the inlet system must be capable of a 1:100 split injection. For serial analysis a headspace gas chromatograph with automatic sample injection and tempering bath has been shown to be satisfactory.
   (c) Flame ionization detector, separately thermostated, that can be maintained at a minimum temperature of 100°C.
2. Electronic integrator.
3. Chromatographic column—A capillary column with the following minimum requirements may be used for this analysis—The column should be constructed of glass or fused silica, approximately 30 m long and 0.3 mm i.d. Stationary phase: methyl polysiloxane (film thickness 0.2 µm).
4. Syringe—10 µL capacity.
5. Syringe—1000 µL capacity, gas-tight (preferably with valve).
6. Septum vials—50–60 mL capacity, all with the same volume to within 2%.
7. Septa and aluminum caps suitable for septum vials, together with crimping pliers. The septa must be resistant to oils and solvents (butyl rubber or red rubber is recommended).
8. Tongs—suitable for holding septum vials.
9. Electric oven—regulated at 110°C.

REAGENTS

1. Gases—
   (a) Carrier—helium (preferred for better resolution) or nitrogen, minimum purity 99.99 mol%, dried and containing a maximum of 10 mg O_2/kg.
   (b) Flame ionization detector—hydrogen, minimum purity 99.95 mol%; air or O_2, dry (dew point –59°C maximum) and hydrocarbon free (< 2 ppm hydrocarbons equivalent to CH_4).
2. Technical hexane or a light petroleum—with a composition similar to that used in industrial extraction, or failing these, *n*-hexane. For calibration, technical extraction hexane is preferred.

SAMPLING AND LABORATORY SAMPLE STORAGE

1. It is essential that loss of solvent from the sample be prevented. The laboratory sample shall be in a completely sealed container and stored at –20°C or below, for example in a deep freezer. Plastic containers should not be used. The determination of residual hydrocarbons should be carried out as soon as the container, kept at +4°C for 24 hr before opening, has been opened.

GC OPERATING CONDITIONS

1. Carrier gas flow depends on the carrier gas and the type of column being used for the analysis and should be optimized accordingly. Helium is recommended, but nitrogen may be used. Some loss of resolution between peaks may be experienced with nitrogen.
2. The flow of hydrogen and air or oxygen to the flame ionization detector should be optimized according to the manufacturer's recommendation for the instrument being used.
3. Injector and detector temperatures should be set at about 120°C.
4. The column should be maintained at 40°C.

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PROCEDURE

1. Preparation of calibration curve—Three calibration standards, for example, with 2, 5 and, 10 µL of solvent (Reagents, 2) are usually sufficient for constructing a calibration curve. They correspond, respectively, to 268, 670, and 1340 mg/kg (ppm) of technical hexane in a test portion of 5 g of meal. If using n-hexane, they correspond to 264, 660, and 1320 mg/kg (ppm), respectively. Add 6 mL of water to a septum vial (Apparatus, 7). This volume is determined by the fact that the test portion (5 g) hydrated with water (2.5 mL) will occupy a volume of 6 mL. Using a 10 µL syringe (Apparatus, 5), accurately and rapidly add a specific volume of solvent, corresponding to one of the prescribed calibration standards, to the vial. Seal with a septum and aluminum cap and crimp with pliers (Apparatus, 8). Place vial in the oven (Apparatus, 10) at 110°C for 10 min. Warm the gas-tight syringe (Apparatus, 6) to 60°C. Remove the vial from the oven and allow to cool for 2 min. Using the warmed gas-tight syringe (Apparatus, 6), take exactly 0.5 mL of the headspace and inject immediately into the gas chromatograph (Apparatus, 1). Determine the sum of the peak areas for the solvent peaks. Hydrocarbons that usually make up the technical solvents are 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane, etc.

   Repeat this operation for two additional quantities of solvent as described above. Determine the peak areas for the solvents in each case.

   Plot the calibration curve, expressing the relation between the sum of the solvent peak areas and the mass of solvent introduced into the vials. It may be assumed that 1 µL of solvent has a mass of 670 µg in the case of technical hexane and 659 µg in the case of *n*-hexane.
2. Test sample analysis—Weigh rapidly, to the nearest 0.1 g, 5 g of the test sample into a septum vial (Apparatus, 7). Add 2.5 mL of distilled water. Seal with a septum and aluminum cap and crimp with pliers (Apparatus, 8). Place the vial in the oven (Apparatus, 10) at 110°C for exactly 90 min. It is important that each vial remain in the oven for the same length of time. Warm the gastight syringe (Apparatus, 6) to 60°C. Remove the vial from the oven and allow to cool for 2 min. Agitate the vial by inverting. Using the warmed gas-tight syringe (Apparatus, 6), take exactly 0.5 mL of the headspace and inject immediately into the gas chromatograph (Apparatus, 1). Determine the sum of the peak areas for the solvent peaks. Hydrocarbons that usually make up the technical solvents are 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane, etc. A typical chromatogram of these solvents was shown by the calibration standards. Do not include peaks due to the oxidation products. Some of these may be present in significant amounts. Determine from the calibration curve the corresponding quantity, m, of solvents present in the vial.
3. Number of determinations—Carry out two determinations in rapid succession on each sample.

RESULTS

1. The total hexane content, expressed in milligrams per kilogram (ppm), is given by the formula:

$$\frac{m_1}{m_0}$$

   Where—

   m_0 = mass, in g, of test portion

   m_1 = mass, in µg, of solvents present in the flask

   Report as the final result the mean of the results of the two determinations, provided the requirements for repeatability are met. If the requirements for repeatability are not met, discard the results and carry out two additional determinations on the sample.

PRECISION

1. The results of one interlaboratory test organized at an international level (for different samples) gave the statistical results shown in Table 1.
2. Repeatability—When the mean of the duplicate determinations lies within the range of the mean values cited in Table 1, the difference between the results of the two determinations, carried out in rapid succession by the same operator, using the same apparatus for the analysis of the same test sample, should not be greater than the repeatability value (r), which can be obtained from Table 1, for the level of hexane in the sample examined.
3. Reproducibility—When the means of the duplicate determinations, obtained in two different laboratories using this standard method for the analysis of the same laboratory sample, lie within the range of the mean values cited in Table 1, the difference between the mean results obtained by those laboratories should not be greater than the reproducibility value (R), which can be obtained from Table 1, for the level of hexane in the sample examined.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th ed., Blackwell Scientific Publications, 1987, IUPAC Method 1.171.
2. Collaborative study results published in *Pure Appl. Chem. 59* (10):1407 (1987).

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Table 1
Statistical analysis of results for total hexane (1984 collaborative study; determinations).

	Sample		
	Rapeseed residue	Soybean residue	Homogenized rapeseed residue
Number of laboratories	15	15	15
Number of results	30	30	30
Mean value, mg/kg (ppm)	452	378	341
Repeatability standard deviation (S_r)	35	19	29
Repeatability coefficient of variation (%)	7.8	5.1	8.6
Repeatability value r ($S_r \times 2.83$)	100	54	83
Reproducibility standard deviation (S_R)	109	129	108
Reproducibility coefficient of variation (%)	24	34	32
Reproducibility value R ($S_R \times 2.83$)	308	365	305

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AOCS

AOCS Official Method Bb 1-38

Reapproved 2009

Sampling

SCOPE

Applicable to cottonseed linters and cottonseed hull fibers.

APPARATUS

1. Waterproof container—20 L capacity, with close-fitting cover.
2. Airtight container—1 L capacity, mason fruit jar with rubber gasket or no. 3 canning can sealed with sealer.

PROCEDURE

1. The sample must be taken in a dry location. If raining, sample inside the car or building.
2. Cut the wrapping and withdraw 25 g from each bale. Select the sample from different positions in the different bales. The sample is pulled from underneath the first layer. If the bale is too tightly compressed to do this, remove and discard a piece of the outer portion of the bale.
3. Place the sample in a 20 L container and press the sample down after each portion is added. The container must be closed, as nearly airtight as possible, immediately after each addition. The final sample should weigh 2–3 kg per carlot of product.
4. If the sample cannot be analyzed promptly, a separate 100 g portion of the original sample is placed in an airtight quart container. This sample is used for the moisture determination in case the larger portion should change, so the cellulose yield may be calculated to the moisture basis of the original material.

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AOCS

AOCS Official Method Bb 2-38

Reapproved 2009

Oil

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to cottonseed hulls.

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4-38.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PROCEDURE

1. Grind a 100 g portion of well-mixed hulls with whole seed and meat particles removed, in the laboratory mill to a uniform fineness (*maximum* particle size of 0.850 mm, 20 mesh).
2. Weigh 5 g of the ground test sample into a filter paper and enclose in a second filter paper, folded in such a fashion as to prevent escape of the meal (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the sample.
3. Place wrapped test portion in the Butt extraction tube and assemble the apparatus as shown in Figure 1 in AOCS Official Method Aa 4-38. Put about 25 mL of petroleum ether into the tared extraction flask before attaching to the tube.
4. Heat in a water bath or on an electric hot plate at such a rate that the solvent will drop from the condenser on the center of the thimble at the rate of at least 150 drops/min.
5. Keep the volume of solvent fairly constant by adding enough to make up for any that may be lost due to evaporation. Continue extraction for 3 hr.
6. Cool and disconnect the extraction flask. Evaporate the petroleum ether on a steam bath or in a water bath until no odor of solvent remains. A gentle stream of clean, dry nitrogen may be used to facilitate removal of the solvent. Cool to room temperature, carefully remove any moisture or dirt from the outside of the flask, and weigh. Repeat heating until constant weight is obtained.
7. If necessary, determine the moisture in the ground test sample as directed in AOCS Official Method Ba 2a-38.

CALCULATIONS

1. $$\text{Oil, \%} = \frac{\text{mass, g of oil}}{\text{mass, g of test portion}} \times 100$$
2. The percentage oil may be calculated to any desired moisture basis with the following formula:

$$\text{Oil, desired moisture basis, \%} = \frac{\text{F (100 – \% moisture desired)}}{\text{100 – \% moisture in test portion analyzed}}$$

Where—

F = percentage oil in test portion analyzed

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. The grinding procedure specified in this method should be strictly followed as indicated. For a discussion on how particle size influences the amount of oil extracted from oilseeds, see References, 1.

REFERENCES

1. *J. Am. Oil Chem. Soc. 64*:865 (1987).

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AOCS

AOCS Official Method Bb 3-47

Revised 2012

Cellulose Yield Pressure-Cook Method

DEFINITION

This method determines the yield of cellulose from cottonseed linters, specifically that amount of dry cellulose remaining after linters are pressure cooked and washed by this procedure. Values are expressed as a percentage of the "as received" sample.

SCOPE

Applicable to cottonseed linters and cottonseed hull fiber.

APPARATUS

1. Forced-draft oven—AOCS Specification H 1-39, maintained at 107.5 ± 2.5°C.
2. Mechanical lint mixer—Mixer must thoroughly mix the sample and throw hull pepper, dust, and foreign material back into the sample during mixing. Two sizes of mixers are available: Figure 1 is the size for samples of 2–3 kg; Figure 2 is for samples in the 1 kg range.
3. Mechanical washer—Mechanical washer is designed to permit the suspension of the sample in water with subjection to the beating action of small jets of water. Figure 3 shows the setup and critical dimensions of the washer. A suitable flowmeter installation is shown in Figure 4. If desired, a separate flowmeter for each washer may be used. Flowmeter should be sized to maintain the specified 16 h per minute of wash water. The washer spray tubes are important in the performance of the washer. These tubes are made from ⅛ in. standard-weight brass pipe with 44 holes of 1/32 in. diameter. Size of the holes in the spray tubes is critical in obtaining the specified flow rate and pressure. The holes are drilled in six equally spaced rows around the circumference and within a distance of 6.25 in. from the plugged end. Bottom row has 13 holes, and the four intermediate rows have six holes each. The washer may be purchased from William Ellis (Memphis, TN, USA).
4. Pressure cooker—Cooker or autoclave must maintain an internal steam pressure of 105 ± 1 lb per sq. in. (equivalent to 172°C).
5. Digestion vessels—stainless steel pots with a capacity of 1000 mL. One pot, a stainless steel metal specimen container (catalog item M228AA2; SDL Atlas, Rock Hill, SC, USA), is acceptable when modified as follows:
   (a) Containers shall be cut down at the top so that the overall dimensions will be 3.5 in. in diameter × 6.5 in. high.
   (b) Eliminate the straight restricted mouth at top which will leave a straight wall.
   (c) Omit positioning knob on side.
   (d) Eliminate gaskets and set cover clamp proportionally lower to compensate for same.
   (e) Use type 316 stainless steel in both the cover and main body of the container.
   (f) Drill six 1/32 in. diameter holes in the cover at equal spacing on a 1 in. radius (2 in. diameter circle).
   (g) Cover to fit within 1/64 in. maximum.

   The old 1000 mL iron containers (glue pots), originally specified for this test, are still acceptable.
6. Metal moisture dishes—capacity about 25 cubic in., with tight-fitting covers.
7. Metal yield sample dishes—capacity about 25 cubic in., with tight-fitting covers.
8. Analytical balance—capable of weighing to ± 0.01 g.

REAGENTS

1. Oleic acid—commercial grade. Refined tall oil in an equivalent amount is a satisfactory substitute.
2. Sodium hydroxide solution—1.0% NaOH by weight, accurately standardized (see Notes, *Caution*). See AOCS Specification H 12-52.
3. Cooking solution—prepared by adding 0.5 mL of commercial oleic acid to 525 mL of the 1% alkali solution.

PROCEDURE

1. Sample should be 2–3 kg for car- or trucklot shipments (see AOCS Official Method Bb 1-38 for sampling procedure). Smaller samples can be processed for special test results (not representing lot shipments).
2. Place lot sample of lint or hull fiber in the large mixer (Figure 2) and rotate for 3–5 min. Three minutes are sufficient for second cut and hull fiber. Five minutes are necessary for mill run. For samples in the 1 kg range the small mixer (Figure 3) should be used. After mixing, the sample is ready for the moisture and yield aliquots (which must be run simultaneously when running the cellulose yield test). See Notes, 1, pertaining to conditions when special moisture tests for lot shipments are necessary.
3. For moisture test, weigh about 25 g of sample into tared metal dish (Appartus, 6). Dry in oven at 107.5 ± 2.5°C for 4 hr. Remove from oven, replace cover, cool to room temperature and weigh.

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4. For yield sample, weigh accurately 35 g of the sample and place in digestion vessel. Add 525 mL of the cooking solution (Reagents, 3). Press the sample down as the alkali is added. It is essential that all of the sample be wet. Stir the sample thoroughly with a steel or stainless steel rod, fasten lids securely, and place in autoclave.
5. Fasten the lid of the autoclave securely. Vent by blowing steam through vessel for a short interval to remove air. Gradually increase the steam pressure up to 105 lb. Steam-up should be controlled within a 30–45 min range. Observe the temperature (or pressure) closely during the entire digestion period. Continue the digestion for 3 hr after a temperature of 172°C has been reached and then reduce the steam pressure gradually (blow-down time should be about 15 min).

   Caution—Do not allow the pressure to drop during digestion, because this may result in the loss of liquor, causing worthless results.
6. Remove the test sample from the autoclave and add sufficient water to fill the digester container. Pour the mixture directly into the lower half of the lint washer. Rinse the sample container with enough water to ensure a complete transfer of all fiber and pour into the washer. Fill lower half of washer with water, approximately 2300 mL. Attach the upper portion of the cylinder, turn the screened end of the washer down by hand, and allow the water to drain. After draining, the position of the washing cylinder is reversed and the closed cylinder is then filled with water through the perforated tube. The cylinder is then reversed and the water allowed to drain through the sieve end. Start washer after last draining and open water valve. Observe the time at which the water is turned on. Maintain the water pressure at a constant level between 21 and 22 lb/sq in. at a rate of 3.9–4.0 gal/min. Wash for 5 min. Wash water temperature should be maintained between 16–30°C. Flowmeter calibration should be checked at regular intervals.
7. Close the water valve and stop the washer when the screened end of the washer reaches the bottom of its rotation. The cylinder should be slightly off vertical to ensure complete drainage. When the flow of drainage is almost stopped, open the water valve again for an instant to wash any adhering fibers from the walls of the cylinder. Remove the lower half of the washer containing the sample. Carefully remove the sample from the screen and compress it to remove as much water as possible.
8. Place the test portion in a tared metal yield sample dish (Appartus, 7). Dry in forced-draft oven at 105–110°C for 15–18 hr. Remove from the oven, replace cover, cool to room temperature, and weigh.

CALCULATIONS

1. $$\text{Moisture, \%} = \frac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$$
2. $$\text{Cellulose yield (dry cellulose) received basis, \%} = \frac{\text{mass, g of dry residue}}{\text{mass, g of test portion}} \times 100$$
3. The cellulose yield may be calculated to any desired moisture basis with the following formula:

   $$\text{Cellulose yield, desired moisture basis, \%} = \frac{A(100 - \text{\% moisture desired})}{100 - \text{\% moisture in test portion analyzed}}$$

   Where—

   A = percentage dry cellulose yield determined from Calculations, 2

REPORTING

1. Report cellulose yield to one decimal place on an "as received" moisture basis and report moisture to one decimal place.
2. Yield values calculated to any moisture basis other than "as received" must be described as derived results at a specified moisture level.
3. All recheck analyses must be calculated on the "as received" moisture basis that was determined at the original test date.

PRECISION

1. AOCS Procedure M 1-59 (declared surplus in 1989) was used to obtain precision data in a 3 year program with 10 cooperating laboratories. Precision of the method varies with yield level as shown in Table 1.

NOTES

Caution

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. Special moisture tests for lot shipments—If the conditions of temperature and humidity in the laboratory are such that the moisture may change during preparation, a moisture sample must be taken immediately upon receipt of the sample *before mixing*. This must be ascertained in each laboratory periodically by determining moisture before and after mixing.

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Also, as specified in the sampling instructions (AOCS Official Method Bb 1-38), if the laboratory sample will not be analyzed promptly, a separate 100 g portion of the original sample should be placed in an airtight quart container. This sample is used for the moisture determination in case the larger portion changes; then the cellulose yield may be calculated to the moisture basis of the original material.

Table 1
Precision data for cellulose yield.

Yield level	78%	73%	68%
Sr within labs	0.4	0.6	1.1
Sr × 2.83[a] within labs	1.1	1.7	3.0
SR between labs	0.6	0.7	1.4
SR × 2.83[b] between labs	1.7	1.9	3.9

[a]Two single determinations performed within a single laboratory shall not differ by more than this tabulated value.
[b]Single determination performed in two different laboratories shall not differ by more than this tabulated value.

It has been suggested that samples should be analyzed in duplicate, rather than a single analysis, to provide a better statistical basis for determining yield and price.

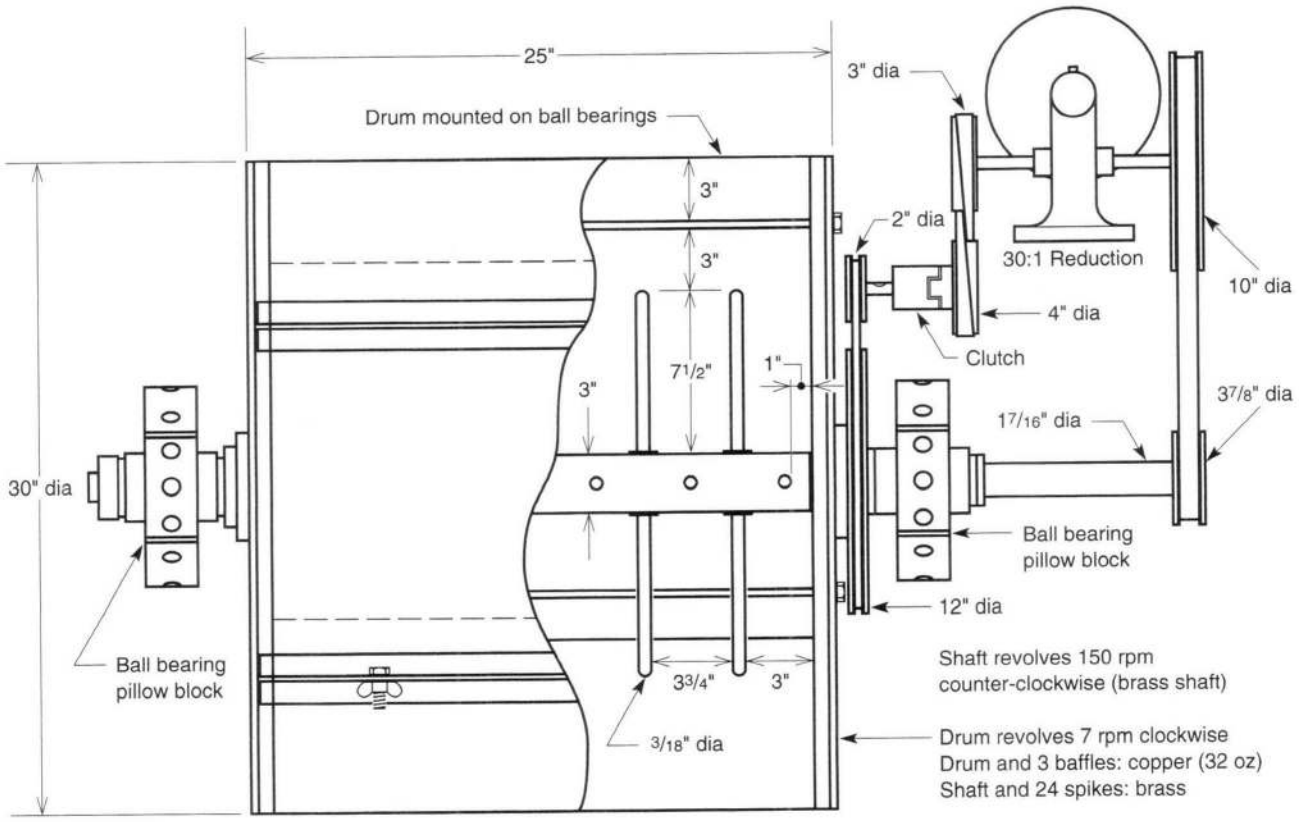

Figure 1. Mechanical lint mixer for cellulose yield (2–3 kg capacity).

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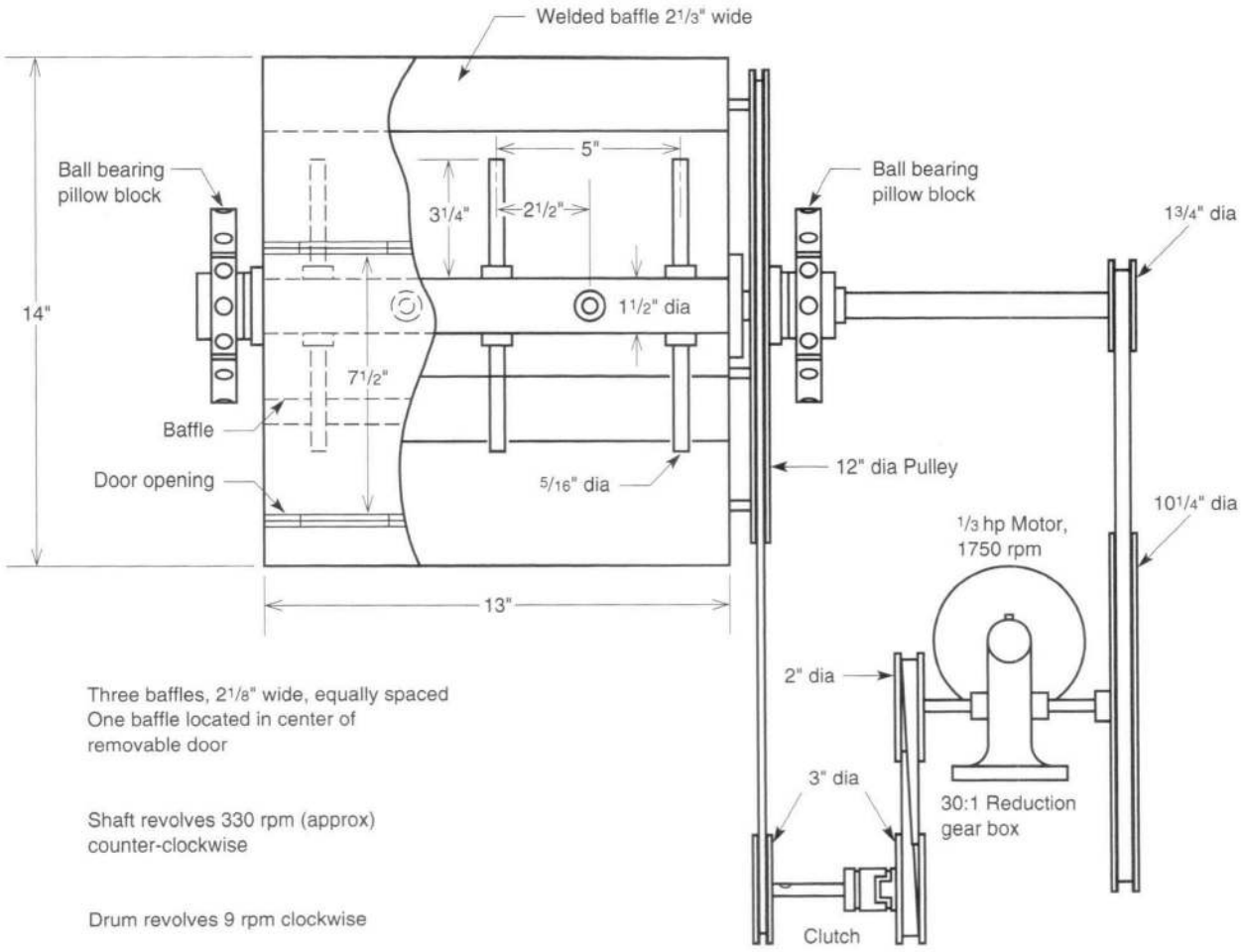

Figure 2. Small lint mixer for cellulose yield (1 kg capacity).

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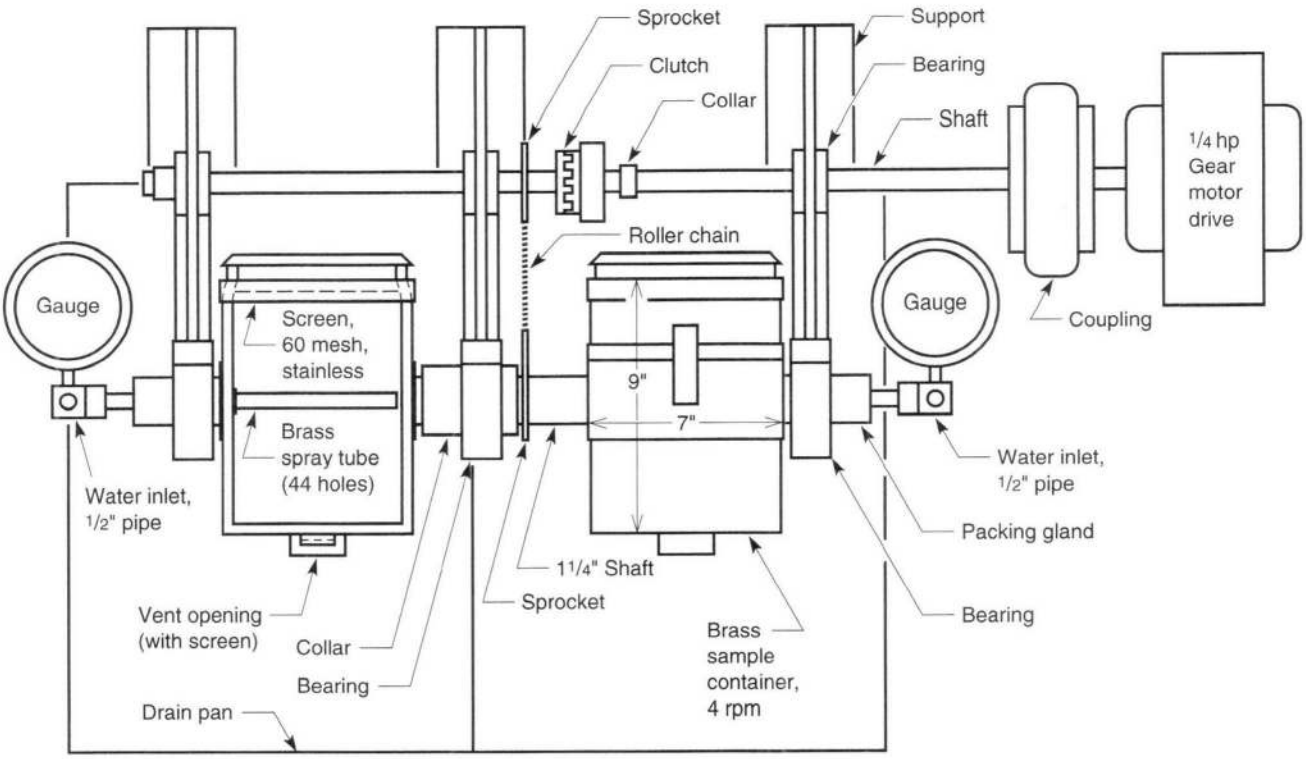

Figure 3. Mechanical yield washer.

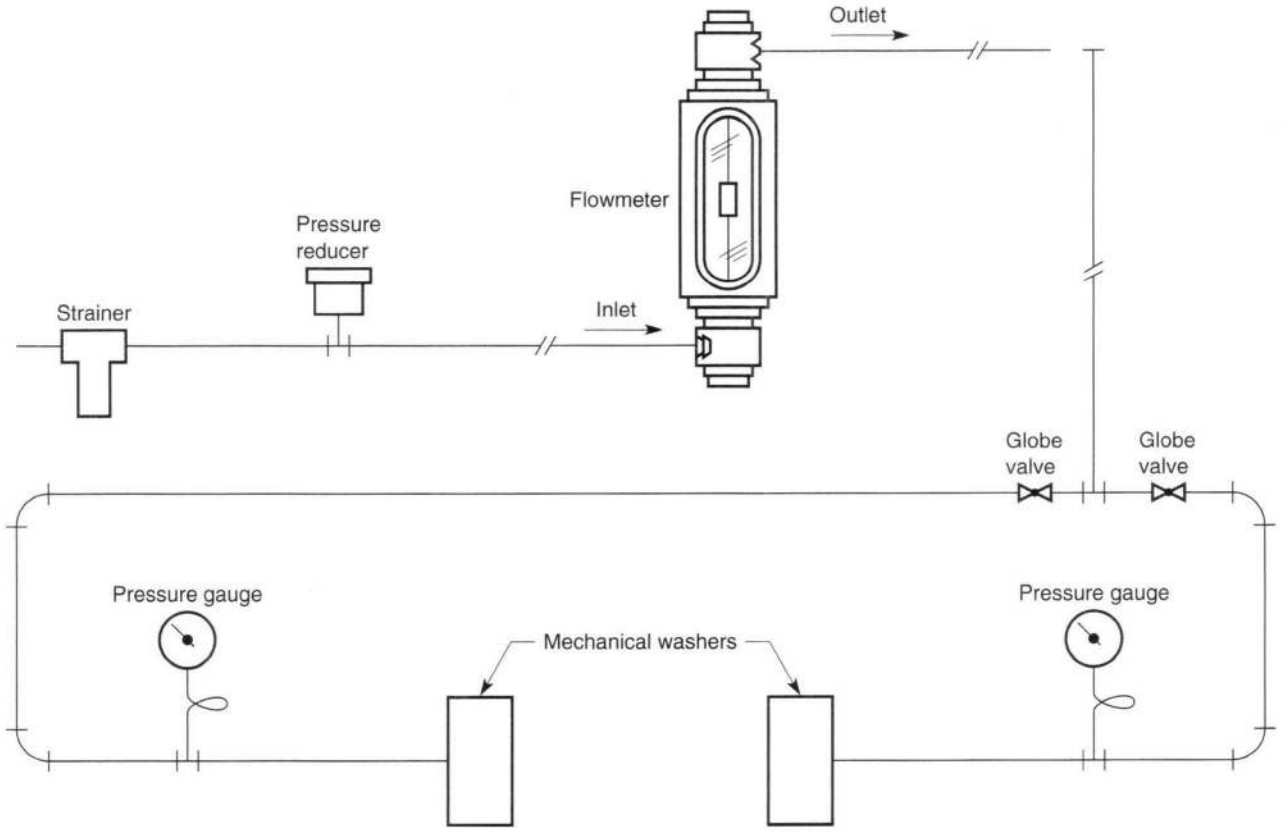

Figure 4. Installation of flowmeter on mechanical washer.

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AOCS

AOCS Official Method Bc 1-50

Reapproved 2009

Sampling

SCOPE

Applicable to sampling soybean flour during production or at storage or transit points. Formerly Bc 1–49

APPARTUS

1. Small scoop of conventional design—50 g capacity, to be used for production line sampling.
2. Closed container—for accumulation of production line samples. A convenient design is shown in Figure 1. The material of construction should be 26–28 gauge nonrusting metal, and the container should be equipped with a tight-fitting lid which is replaced by the hinged cover during the actual sampling period.
3. Airtight sample containers—about 500 g capacity.
4. Airtight sample container—about 2 L capacity.
5. Trier—for sampling loaded bags, constructed of stainless steel and equipped with a special cutting lip. The details of the trier are as follows:

Overall length	79 cm (31 in.)
Inside diameter of body tube	1.6 cm (⅝ in.)
Wall thickness	0.8 mm (1/32 in.)
Length of slot	51 cm (20 in.)
Width of slots	6.3 mm (¼ in.)

Right side of slit is slightly depressed and left side slightly raised, thus forming a cutting lip. A 6.5 cm (2.5 in.) tapered point is sealed off from the body tube at one end, and a 12.5 cm concentric wooden handle is attached at the end opposite the tapered point (see Fig. 2).

PROCEDURE

1. Production line sampling—
   (a) Using the scoop, remove 40–50 g of flour from the bag at the filling line and place in container through hinged cover.
   (b) Take samples at periodic intervals, regulated so that not less than 10% of the bags in the lot will be sampled. If the lot is composed of <100 bags, sample a minimum of 10 bags.
   (c) As soon as the lot has been sampled, remove the cover with the hinged spout and replace with a tight-fitting cover. Mix thoroughly by agitation and/or tumbling to give a homogeneous mass.
   (d) Fill a sufficient number of 16 oz airtight containers, using the scoop and taking the portions from various points in the gross sample.
   (e) Fill the containers to within .25 in. of the top. Close tightly and immediately.
2. Procedure for loaded bags—
   (a) The number of bags to be sampled shall be equivalent to the square root of the number of bags in the lot, but never less than 10, and uniformly distributed throughout the entire lot. See Table 1.
   (b) Insert the trier diagonally downward through the bag with the slot of the trier down. Rotate the trier clockwise for about one turn and then withdraw with the sample.
   (c) Collect the samples in the clean, dry container (2 L). Immediately after all bags have been sampled, mix by agitation and/or tumbling to give a homogeneous mass.
   (d) Fill a sufficient number of 500 g airtight containers, using the scoop and taking the portions from various points in the gross sample.
   (e) Fill the containers to within 0.5 cm of the top. Close tightly and immediately.

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Table 1
Bags to be sampled vs. total bags in lot.

Number of bags in lot	Number of bags to be sampled	Number of bags in lot	Number of bags to be sampled
1–10	all	400–440	21
10–100	10	440–485	22
100–120	11	485–530	23
120–145	12	530–575	24
145–170	13	575–625	25
170–200	14	625–680	26
200–230	15	680–735	27
230–260	16	735–790	28
260–290	17	790–845	29
290–325	18	845–900	30
325–360	19	900–1000	31
360–400	20		

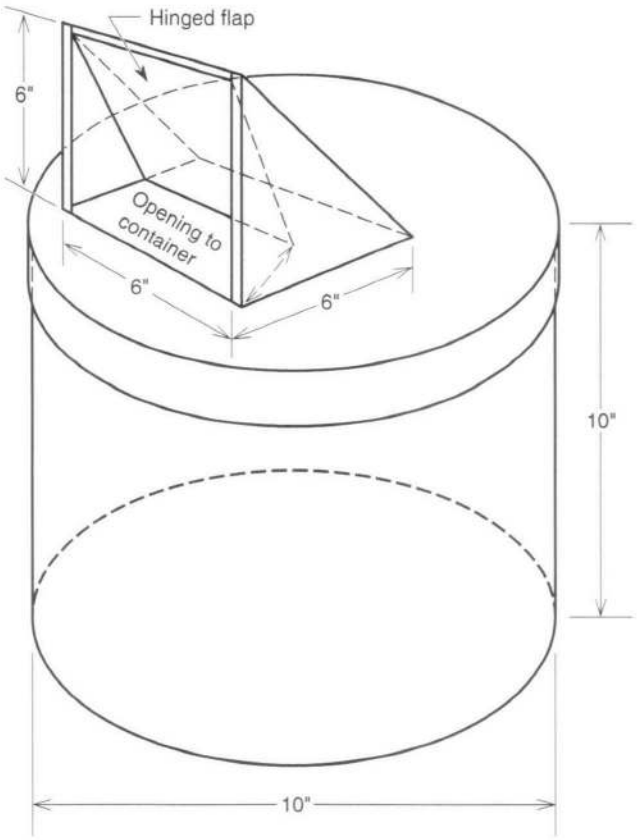

Figure 1. Sample container for soybean flour (with spouted cover in place)

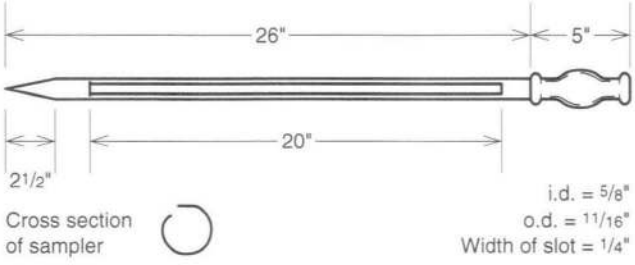

Figure 2. Trier for sampling flour.

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AOCS

AOCS Official Method Bc 2-49

Reapproved 2009

Moisture and Volatile Matter

DEFINITION

This method determines the moisture and any materials that are volatile under the conditions of the test.

SCOPE

Applicable to high-fat, low-fat, and defatted soybean flours. Formerly Bc 2-47.

APPARATUS

1. Forced-draft oven—AOCS Specification H 1-39.
2. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch no. S-25675, Arthur H. Thomas no. 4522, or equivalent (see Notes, 1).
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.
4. Airtight sample containers of convenient size.

PREPARATION OF TEST SAMPLE

1. Mix laboratory sample thoroughly in a closed container by turning the container manually or mechanically. Reduce the sample to suitable size and store in an airtight container.

PROCEDURE

1. Weigh accurately, and as rapidly as possible, about 5 g of test sample into tared moisture dish.
2. Slip the cover on the bottom of the dish, place the uncovered dish in the oven and dry at 130 ± 3°C for 2 hr (see Notes, 2).
3. Remove the dish from the oven, cover immediately, cool in a desiccator to room temperature, and weigh.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$

NOTES

1. For test portions with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to sample loss from splattering during heating.
2. Drying time begins when the oven temperature recovers to 130°C.

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AOCS

AOCS Official Method Bc 3-49

Reapproved 2009

Oil

DEFINITION

This method determines the substances extracted by petroleum ether under the conditions of the test.

SCOPE

Applicable to high-fat, low-fat, and defatted soybean flours. Formerly Bc 3-47.

APPARATUS

1. Butt-type extraction apparatus—assembled exactly as indicated in Apparatus, 1 and Figure 1 of AOCS Official Method Aa 4-38.
2. Filter paper—150 mm; S & S no. 597, Reeve Angel no. 211, Whatman no. 2, or equivalent.
3. Absorbent cotton—free of petroleum ether extractables.
4. Airtight sample containers of convenient size.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Mix laboratory sample thoroughly in a closed container by turning the container manually or mechanically. Reduce the laboratory sample to suitable size and store in an airtight container.

PROCEDURE

1. Weigh about 2 g of high-fat flour, or 5 g of low-fat or defatted flour sample, on a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the meal (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the sample.
2. Place wrapped test portion in the Butt extraction tube and assemble the apparatus as shown in Figure 1 of AOCS Official Method Aa 4-38. Place about 25 mL of petroleum ether into the tared extraction flask before attaching to tube.
3. Heat in a water bath or on an electric hot plate at such a rate that the solvent will drop from the condenser on the center of the thimble at the rate of at least 150 drops/min.
4. Keep the volume of solvent fairly constant by adding enough to make up for any that may be lost due to evaporation. Continue the extraction for 5 hr.
5. Cool and disconnect the extraction flask. Evaporate the petroleum ether on a steam bath or in a water bath until no odor of solvent remains. A gentle stream of clean, dry nitrogen may be used to facilitate removal of the solvent. Cool to room temperature, carefully remove any moisture or dirt from the outside of the flask, and weigh. Repeat heating until constant weight is obtained.

CALCULATIONS

1. $$\text{Oil, \%} = \frac{\text{mass, g of oil}}{\text{mass, g of test portion}} \times 100$$

2. The percentage oil may be calculated to any desired moisture basis with the following formula:

$$\text{Oil, desired moisture basis, \%} = \frac{\text{F (100 – \% moisture desired)}}{\text{100 – \% moisture in test portion analyzed}}$$

Where—

F = percentage oil in test portion analyzed

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

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AOCS

AOCS Official Method Bc 4-91

Revised 2011

Nitrogen-Ammonia-Protein Modified Kjeldahl Method Titanium Dioxide + Copper Sulfate Catalyst

DEFINITION

This method determines total nitrogen content and protein as 6.25 times the nitrogen content of the sample (see Notes, 1).

SCOPE

Applicable to high-fat, low-fat, and defatted soybean and canola flours. Replaces Bc 4-49.

APPARATUS

1. Kjeldahl digestion and distillation apparatus—complete with heat source, traps, and block-tin, or equivalent noncorrosive tubing condensers (see Numbered Notes, 2).
2. Kjeldahl flasks—800 mL.
3. Distillate receiving flasks—500 mL, or any convenient size.

REAGENTS

1. Catalyst mixture—containing potassium sulfate, 16.7 g + titanium dioxide, 0.6 g + copper sulfate, 0.01 g + pumice, 0.3 g.
2. Sulfuric acid—sp. gr. 1.84 (see Notes, *Caution*).
3. Sodium hydroxide solution—sp. gr. 1.50 (see Notes, *Caution*).
4. Sodium hydroxide solution—0.25 M, accurately standardized (see AOCS Specification H 12-52).
5. Sulfuric acid—0.25 M, accurately standardized (see AOCS Specification H 13-52).
6. Methyl red indicator solution—0.1% in ethyl alcohol, or Alizarin Red S—0.3% in distilled water.
7. Alundum boiling stones—8–14 mesh.
8. Zinc metal—granular. (If the catalyst is used with added pumice, granular zinc metal need not be added as an antibumping agent.)

PREPARATION OF TEST SAMPLE

1. For soybean and canola flours, mix the laboratory sample thoroughly in a closed container by turning the container manually or mechanically. Reduce the sample to suitable size and store in an airtight container. After test sample preparation, continue with the following procedure.

PROCEDURE

1. Weigh 0.250–1.750 g of the ground test sample into the Kjeldahl flask. Add the catalyst mixture (premixed catalyst packs are commercially available and may be used).
2. Add 30 mL of concentrated H_2SO_4 (see Numbered Notes, 3) to the sample and catalyst in the digestion flask. Include at least one sample of high-purity lysine hydrochloride in each day's run as a check of the correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.
3. Add a few Alundum boiling stones to the sample flask.
4. To digest the test portion, first adjust heat to bring 250 mL of water at 25°C to rolling boil in 5 min. Place the flask on the digestion rack in an inclined position and then heat sample flask at the 5 min boil rate until dense, white fumes clear bulb of flask (approximately 5 min). Swirl gently and continue heating an additional 40 min after the liquid has become clear and colorless (see Numbered Notes, 4). The Kjeldahl flasks should be rotated a minimum of three times during the digestion.
5. Cool, cautiously add about 300 mL of water, and cool to room temperature (see Numbered Notes, 5). Add water as soon as possible to reduce amount of caking. If excessive bumping occurs during distillation, increase dilution water from 300 mL to about 350 mL.
6. Accurately transfer a sufficient quantity of the standard acid into receiving flask so there will be an excess of at least 0.5 mL of 0.5 M acid. Add sufficient distilled water to cover the end of the outlet tube and attach to outlet end of condenser tube. The distillate should discharge through a glass tube at the bottom of the receiving flask.
7. Add an additional 0.5–1.0 g Alundum boiling stones to cooled digestion flask. Mix thoroughly and add sufficient alkali solution (Reagents, 3) to make strongly alkaline. Pour the alkali down the side of the Kjeldahl flask so that it does not mix quickly with the acid.

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8. Immediately connect the Kjeldahl flask to the other end of the condenser tube and thoroughly mix the contents by shaking. Apply heat at about a 7.5 min boil rate and distill until at least 150 mL of distillate have been collected.
9. Titrate the contents of the receiving flask with 0.25 M NaOH solution, using 3 or 4 drops of indicator.
10. Conduct a blank determination of all reagents simultaneously with the samples and similar in all respects. Correct for blank determined on reagents.
11. The performance of the entire method should be checked frequently by analyzing either National Institute of Standards and Testing-certified ammonium dihydrogen phosphate (standard reference material 194) or highpurity lysine hydrochloride.
12. Determine moisture in the ground test sample as directed in AOCS Official Method Ba 2a-38, Procedure section.

CALCULATIONS

1. Nitrogen, % = $\frac{[(MA \times 2 \times mLA) - (mLBK \times MB) - (mLB \times MB)] \times 1400.67}{\text{mg test portion}}$

   Where—

   mLB = volume, mL standard base used for the sample
   mLA = volume, mL standard acid used for the sample
   mLBK = volume, mL standard base needed to titrate 1 mL of standard acid minus mL standard base needed to titrate reagent blank carried through method and distilled into 1 mL standard acid
   MA = molarity of standard acid
   MB = molarity of standard base

   Note—To determine percent ammonia, substitute 1703.06 for 1400.67 in the equation for the calculation of percent nitrogen.
2. Calculation: N × 6.25 = P

   Nitrogen content × 6.25 is the accepted conversion factor for calculating protein content of oilseeds and oilseed byproducts.

PRECISION

There are no statistics relating the performance of this method to soybean flours. The reader is referred to AOCS Official Methods Aa 5-91 and Ba 4d-90 for statistics from the analysis of soybean meal, soy protein concentrate, cottonseed, and cottonseed meal.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse. Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali solutions. Use effective fume removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. This method has been evaluated through interlaboratory comparison of catalysts and has been adopted by the committee as the official replacement for the mercuric oxide catalyzed method, AOCS Method Ba 4a-38. An interlaboratory evaluation (References, 1) indicated that AOCS Official Method Ba 4d-90 (using the copper/titanium catalyst mixture) produces results closer in agreement with AOCS Official Method Ba 4a-38 (using mercuric oxide catalyst) than methods using copper sulfate catalyst. As a result of this study, methods Ba 4a-38 (mercuric oxide), Ba 4b-87 (copper sulfate) and Ba 4c-87 (Kjel-Foss Automatic) were declared surplus (obsolete) in 1991.

   The Association of Official Analytical Chemists (AOAC) collaborative study (References, 2) showed no bias when comparing Cu/Ti catalyst with HgO. The AOCS interlaboratory evaluation (References, 1), based on sample weight, acid, volume, digestion temperature, and time, previously specified in AOCS Official Method Ba 4a-38, indicated an average bias of –0.244%, which was statistically significant at the 99% confidence level. Factors that may affect method bias are sample weight and particle size, catalyst composition, use of Alundum, volume of acid used for digestion, digestion temperature, and digestion time. In 1991, two additional collaborative studies (cottonseed and cottonseed meal) showed that when 30 mL of sulfuric acid were used, caking of the digest was reduced and results were comparable to the mercuric oxide catalyzed method. As a result, the AOCS versions of this method may specify the use of more than 20 mL of sulfuric acid, but otherwise conform with the AOAC version (References, 3).

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2. Use of steam- and water-resistant rubber tubing and stoppers to connect the glass parts is strongly recommended.
3. More than 20 mL of concentrated sulfuric acid is needed for samples high in fat or oil, because more acid is required to effect complete digestion, avoid loss in nitrogen, and prevent caking. In recent collaborative studies involving the application of this method to cottonseed meal, it was observed that when 30 mL of H_2SO_4 were used, caking was reduced and results were comparable to the mercuric oxide catalyzed method.
4. Complete conversion of organic nitrogen to ammoniacal nitrogen is essential to obtain accurate and precise results. After the liquid has become clear and colorless, it may be necessary to digest the sample for approximately an additional 30–50 min. Reagent proportions, heat input, and digestion time are critical factors and should not be changed. Prolonged digestion and high temperatures must be avoided, because some ammoniacal nitrogen can be lost by oxidation. Large sample particles should be avoided, because the larger particles will require a longer digestion time.
5. The cooled digest should be liquid, or liquid with a few small crystals. Caking before the addition of water indicates too little residual acid at the end of the digestion period and may result in low nitrogen values.

REFERENCES

1. *INFORM 1:*884 (1990).
2. Kane, P. F., *J. Assoc. Off. Anal. Chem. 70:*907 (1987).
3. *Official Methods of Analysis,* AOAC International, 16th edn., Vol. 1, Gaithersburg, MD, 1995, Chap. 4, p. 13, Method 988.05.

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AOCS

AOCS Official Method Bc 5-49

Reapproved 2009

Ash

DEFINITION

This method determines as ash the residue remaining after incineration under the conditions specified for the test.

SCOPE

Applicable to high-fat, low-fat, and defatted soybean or canola flours. Formerly Bc 4-47.

APPARATUS

1. Porcelain combustion capsules—Coors no. 170, size no. 3, 25 mL capacity.
2. Electric muffle furnace—with automatic pyrometer control to regulate at a temperature of 600 ± 15°C.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87
4. Airtight sample containers of convenient size.

PREPARATION OF TEST SAMPLE

1. Mix laboratory sample thoroughly in a closed container by turning the container manually or mechanically. Reduce the laboratory sample to suitable size and store in an airtight container.

PROCEDURE

1. Weigh about 2 g of well-mixed test sample into the previously heated and tared combustion capsule, place in muffle furnace previously heated to 600°C, and maintain at this temperature (±15°C) for 2 hr.
2. Transfer capsule to a desiccator, cool to room temperature, and weigh immediately thereafter.

CALCULATIONS

1. $$\text{Ash, \%} = \frac{\text{mass of ash, g}}{\text{mass of test portion, g}} \times 100$$

   Report to nearest 0.1%.

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AOCS

AOCS Official Method Bc 6-49

Reapproved 2009

Crude Fiber in Soybean Flours

DEFINITION

This method determines as crude fiber the loss of incineration of the dried residue remaining after digesting the sample with dilute sulfuric acid and sodium hydroxide as specified for the test.

SCOPE

Applicable to high-fat, low-fat, and defatted soybean flours. Formerly Bc 6-47. Refer to Ba 6-84.

APPARATUS

1. The apparatus is exactly as described in AOCS Official Method Ba 6-84, Apparatus section.

REAGENTS

1. The reagents are exactly as described in AOCS Official Method Ba 6-84, Reagents section.

PREPARATION OF TEST SAMPLE

1. Mix laboratory sample thoroughly in a closed container by turning the container manually or mechanically. Reduce the laboratory sample to suitable size and store in an airtight container.

PROCEDURE

1. Proceed as directed in AOCS Official Method Ba 6-84.

CALCULATIONS

1. Crude fiber, $\% = \dfrac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$

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AOCS Official Method Bc 7-51

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Screen Test

DEFINITION

This method determines the quantity of material coarser than the screen used under the conditions of the test (see Notes, 1).

SCOPE

Applicable to high-fat, low-fat, and defatted and reconstituted soybean flours.

APPARATUS

1. Metal crucible—5 cm high, 5 cm top diameter, 2.5 cm bottom diameter, fitted with US standard (or Tyler equivalent) wire screen, 200, 325, or other mesh, as the need may be. The crucible may be made of sheet copper with the screen soldered to the bottom opening.
2. Crucible holder.
3. Filter flask—250 mL.
4. Compressed air supply, tubing, and tee—as shown in Figure 1.

REAGENTS

1. Carbon tetrachloride (CCl_4)—reagent grade (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Mix laboratory sample thoroughly in a closed container by turning the container manually or mechanically. Reduce the laboratory sample to suitable size and store in an airtight container.

PROCEDURE

1. Weigh 1.000 g test portion into crucible.
2. Open air valve to give maximum agitation without causing sample to be forced out or over the edge of the crucible (20–30 mL/sec).
3. Place crucible tightly in holder in filter flask and close air line to flask, using a clamp.
4. Add 15 mL CCl_4 (see Notes, *Caution*) around sides of crucible by means of burette, graduated pressure bottle, or graduated cylinder (see Notes, 2).
5. Place finger over air-control tube as air line to flask is opened. When flour is well mixed due to aeration (less than 0.5 mL solvent should have passed through screen at this point), release air-control tube for fraction of a second. Continue manipulating air-control tube with finger so air pressure is on bottom of the crucible 2 sec and released for a fraction of a second, until only about 1–2 mL of solvent remains in the crucible. At this point, remove finger from air-control tube, and shake or rotate crucible and flask until the remaining solvent has drained into filter flask.
6. Repeat above steps using successively one 10 mL and four 5 mL portions of solvent (see Notes, 3 and 4).
7. Remove crucible from holder and wash residue three times with 1.5 mL of solvent.
8. Wipe sides and bottom of crucible with a towel. Let solvent evaporate in air or oven for a few minutes, and transfer retained particles to small tared pan. Heat in oven 0.5 hr at 110°C or 15 min at 135°C and weigh. If desirable, weighing may be made directly in crucible.

CALCULATIONS

1. % Coarser than specified screen size = grams retained × 100

NOTES

Caution

Carbon tetrachloride is a known carcinogen. It is toxic by ingestion, inhalation, and skin absorption, causing irritation, headaches, and nausea on mild exposure. More severe exposure can lead to liver and kidney damage. It is a narcotic. It should not be used to extinguish fires. It decomposes to phosgene gas at high temperature. The TLV is 5 ppm in air. A fume hood should be used at all times when using carbon tetrachloride.

NUMBERED NOTES

1. The method depends on
   (a) forcing air up through the bottom of the screen to clean the screen and obtain complete mixing of solvent and flour, and
   (b) permitting only small increments of the solvent and flour to pass through the screen at one time so that no cake is built up in the screen.

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2. At no time should solvent be applied directly onto the screen.
3. After adding first 5 mL of wash liquid, any clump of flour floating on top of the solvent should be broken up with small spatula. The spatula may be rinsed off when adding the second 5 mL wash portion.
4. When necessary to use sieve such that more than 15% of the sample remains on the sieve, two 10 mL washings instead of one should be used.

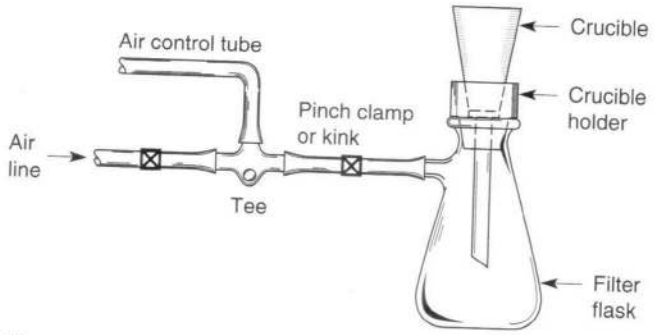

Figure 1. Apparatus for the screen test.

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Oil

DEFINITION

This method determines the substances extracted by commercial hexane under the conditions of the test (see Notes, 1 and References, 1).

SCOPE

Applicable to castor pomace (see Notes, *Caution*).

APPARTUS

1. Butt-type extraction appartus—assembled exactly as indicated in Appartus, 1 and Figure 1 of AOCS Official Method Aa 4-38.
2. Filter paper—150 mm; S & S no. 597, Whatman no. 2, Reeve Angel no. 211, or equivalent.
3. Absorbent cotton—free of commercial hexane extractables.
4. Sieve—US no. 20.
5. Laboratory mill—suitable for grinding the samples to a maximum particle size of US no. 20 sieve.

REAGENTS

1. Commercial hexane—AOCS Specification H 16-56 (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Using the laboratory mill, grind 100 g of the laboratory sample that remains after the determination of moisture and volatiles in castor pomace (AOCS Official Method Bd 2-52) (see Notes, *Caution*). Return immediately to an airtight container. Oil and ground moisture are determined on this portion.

PROCEDURE

1. Weigh about 5 g of the ground test sample onto a filter paper and enclose in a second filter paper folded in such a fashion as to prevent escape of the pomace (see Fig. 3 in AOCS Official Method Aa 4-38). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the sample.
2. Place wrapped sample in the Butt extraction tube and assemble the appartus as shown in Figure 1 of AOCS Official Method Aa 4-38. Place about 25 mL of commercial hexane into the tared extraction flask before attaching to the tube.
3. Heat on an electric hot plate at such a rate that the solvent will drop from the condenser on the center of the thimble at the rate of at least 150 drops/min.
4. Keep the volume of solvent fairly constant by adding enough to make up for any that may be lost due to evaporation. Continue the extraction for 4 hr.
5. Cool and disconnect the extraction flask. Evaporate the commercial hexane on a steam bath for 1 hr after boiling ceases; then place the flask in a forced-draft oven at 101°C for an additional 2 hr.

   Caution—Make certain that all traces of solvent are gone from the extraction flask before placing in the oven. A gentle stream of clean, dry nitrogen may be used to facilitate the removal of solvent.

   Cool to room temperature, carefully remove any foreign material from the outside of the flask, and weigh. Repeat heating in the forced-draft oven for 30 min intervals until constant weight is obtained.
6. Determine the moisture in the ground test sample as directed in the method for moisture and volatiles in castor pomace,AOCS Official Method Bd 2-52.

CALCULATIONS

1. $$\text{Oil, \%} = \frac{\text{mass, g of oil}}{\text{mass, g of test portion}} \times 100$$

2. The percentage oil may be calculated to any desired moisture basis with the following formula:

$$\text{Oil, desired moisture basis, \%} = \frac{F\,(100 - \%\ \text{moisture desired})}{100 - \%\ \text{moisture in test portion analyzed}}$$

Where—

F = percentage oil in test portion analyzed

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NOTES

Caution

Castor meal or pomace contains a highly toxic albumin, ricin, a toxic alkaloid, ricinine, and an allergenic protein polysaccharide (CB-1A) that is among the most powerful known allergens. Therefore, it is strongly recommended that workers wear rubber gloves when preparing and handling analytical samples and that they avoid inhaling any of the dust arising from such samples by working near an exhaust or in a well-ventilated laboratory hood.

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

NUMBERED NOTES

1. Particle size affects the amount of oil extracted. The grinding procedure specified in this method should be strictly followed as indicated. For a discussion on how particle size influences the amount of oil extracted from oilseeds, see References, 1.

REFERENCES

1. J. Am. Oil Chem. Soc. 64:865 (1987).
2. Technical Bulletin, The Processing of Castor Meal for Detoxification and Deallergenation, The International Castor Oil Association.
3. Summary, Technical Report of Division 9, NDRC Vol. 1 Chemical Warfare Agents, and Related Chemical Problems, Part 1, 1946, Office of Scientific Research and Development, Washington, D.C.

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AOCS Official Method Bd 2-52

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Moisture and Volatile Matter

DEFINITION

This method determines the moisture and any material that is volatile under the conditions of the test.

SCOPE

Applicable to castor pomace (see Notes, *Caution*).

APPARTUS

1. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch no. S-25675, Arthur H. Thomas no. 4522, or equivalent (see Notes, 1).
2. Forced-draft oven—AOCS Specification H 1-39.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.
4. Riffle type divider, 15 × 15 cm (see Notes, 2).

PREPARATION OF TEST SAMPLE

1. Reduce the original 1000 g laboratory sample through the riffle (see Notes, *Caution*) to about 100 g and immediately place in an airtight container. Weigh moisture sample immediately.

PROCEDURE

1. Weigh about 5 g of the test sample into tared moisture dish.
2. Place the dish in the oven and dry at 130 ± 3°C for 2 hr.
3. Remove from the oven, cover immediately, cool in a desiccator to room temperature, and weigh.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass, g of test portion}} \times 100$

NOTES

Caution

Castor seeds contain an alkaloid, ricinine. They also contain a protease to which some individuals are allergic. It is strongly recommended that workers wear rubber gloves when preparing analytical samples, and that they avoid inhaling any of the dust arising from such samples by working near an air exhaust or in a well-ventilated laboratory hood.

NUMBERED NOTES

1. For samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes may give erroneously high results due to sample loss from splattering during heating.
2. Equipment available from Seedburo Equipment Co., Chicago, IL, USA.

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AOCS Official Method C 1-47

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Sampling

SCOPE

Applicable to the sampling of animal and vegetable fats, and crude and refined vegetable and marine oils.

APPARATUS

1. Core sampler for oils and liquid fats (official trier of the National Cottonseed Products Association) (Fig. 1)—a metal tube of 5.0 cm (2 in.) diameter throughout. The length must be sufficient to take a cross section through the entire depth of oil, about 3 meters (10 ft) for tank cars. One end of the trier is fitted with a tight valve which allows an unrestricted opening 5.0 cm (2 in.) in diameter when fully opened and is free from leaks when closed. The valve is opened and closed by means of a rod from the top of the trier. The trier is so constructed as to take a sample within 6.4 mm (0.25 in.) (or less) of the bottom of the tank. The sampler is suitable for tank cars.
2. Bomb or zone sampler for oils and liquid fats (Fig. 2)—a tightly closed cylindrical compartment so constructed that a sample can be taken from any specified section of the tank. The sampler must permit taking a sample from within 13 mm (0.5 in.) (or less) of the bottom of the tank. The valve or valves must be tight-fitting so the sampler can be withdrawn without loss or transfer of contents. The valves should be readily opened by hand and manipulated either automatically or by an attached cord. The device must be readily cleanable.
3. Trier for solid fats—a half-round metal tube 13–25 mm (0.5–1 in.) in diameter. The length may be 61–213 cm (2–7 ft), depending upon the size of package to be sampled. One end is tapered to a point, the taper to be not more than 2.5 cm (1 in.) long. The other end is attached to a D- or T-shaped handle. Copper, brass, or bronze must not be used in the construction of this tube.

   Note—A trier for tank cars when the contents are solid may be similar but about 5.0 cm (2 in.) in diameter and 2–2.5 meters (7–8 ft) long.
4. Oil thief—a glass tube 9.5–13 mm (⅜–½ in.) i.d. The length may be convenient for the size of packages to be sampled. One end is constricted to about 6.5 mm by means of a short taper no longer than 2.5 cm. The other end is constricted sufficiently so it can be used as a finger valve.

   Note—If the material to be sampled is semisolid, the constriction at the lower end must be enlarged sufficiently to permit the material to flow into the tube.
5. Sample containers as necessary (see Notes, 1).

SIZE AND NUMBER OF SAMPLES

1. The general procedure is to draw a number of portions from the bulk quantity or a number of portions from all or several packages, composite these, mix thoroughly and distribute representative portions into suitably sized airtight containers for the laboratory sample. The number of laboratory samples to prepare depends on the circumstances. In all cases involving commercial transactions, a minimum of three (preferably four) is required. This permits one for the buyer, one for the seller and one or two in reserve for possible arbitration (referee analysis).
2. A gross sample in the proportion of not less than 50 lb (about 23 kg) per 60,000 lb (25,000 kg) cargo is required of all tank cars, and a minimum of 50 lb (about 23 kg) is required for all other bulk oil quantities, such as in ships or shore tanks.
3. A gross sample in the proportion of not less than 20 lb (9 kg) for each 100 barrels or equivalent quantity is required when drums, tierces, barrels and other packages are sampled.
4. When sampling drums, tierces and all other packages, each package is to be sampled unless the concerned parties agree to the following schedule:

Number of packages in shipment	Number of packages to be sampled*
1–10	1–3
10–25	2–4
25–50	3–6
50–75	6–8
75–100	8–10

*In no case should less than 10% of the packages be sampled.

5. In the case of all bulk oil shipments, whether in drums, tank cars, ships or shore tanks, the minimum size for each laboratory sample is about 4 L (1 gal).
6. In the case of tallows, greases and other inedible fats, the minimum size for each laboratory sample is about 1.3 kg (3 lb).

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7. In the case of edible fats and oils, the minimum size for each laboratory sample is about l kg (2 lb) for fats and 1 L (1 qt) for oils. If refining or bleaching tests are required, the minimum quantity is about 4 L (1 gal).

GENERAL PRINCIPLES

1. It is impossible to write directions for sampling fats and oils that will encompass all conditions and circumstances that may confront the individual charged with the responsibility of taking the sample. There are many instances in which the experience and judgment of that individual must prevail. There are, however, certain general rules which must always govern if the sample is to be representative.
2. The best sample of bulk oil quantities can be taken if the product to be sampled is completely liquid and thoroughly mixed. In such cases, a core sample or even a sample dipped from the tank while undergoing vigorous agitation will be representative.
3. Settled material, in which the water and solid impurities are likely to be concentrated at the bottom, is difficult to sample and reconstitute in proportional quantities. The contour of the tank must be taken into account. If the bottom of the tank is smaller than the middle or top, or vice versa, a core sample is not adequate. To overcome this, the number of portions from each section [e.g., each 30 cm (1 ft) level] should be inversely proportional to the cubical capacity of each section. For example, if the bottom 1 ft section is one-fourth of the middle 1 ft section, then one 1 ft sectional sample should be drawn from the bottom level and four 1 ft sectional samples from the middle section. These portions are then composited into one sample and mixed.
4. All samples must be completely labeled for identification.

PROCEDURE

1. Continuous-flow method (Fig. 3) for sampling tank and tank car during loading or unloading—
   (a) If the conditions are suitable, this is a satisfactory method of sampling. This method is applicable only if the product is completely liquid and free flowing and does not contain any material that may plug the bleeder line.
   (b) The ⅜ in. (1 cm) bleeder line is ⅜ in. (1 cm) standard pipe with a slight downward slope, located in a vertical section of the pumping line through which the product is continuously flowing upward to the individual tank or tank car being sampled. The sample line should be located as far away from elbows or tees as possible, should penetrate to the center of the pumping line, should be cut beveled at the end looking downward, and should discharge into a sample tank or drum as illustrated in Figure 3. The sample line should not have a petcock.
   (c) The metal sample tank or drum is of approximately 50 gal (185 L) capacity. Just above the bottom of the drum a ⅜ in. (1 cm) draw-off line equipped with petcock is installed and is used for obtaining the required sample(s) from the gross sample. To facilitate complete draining and easy cleaning, the bottom of the drum should be replaced by a securely welded inverted-cone bottom having an apex angle of approximately 120°, the other two angles with the horizontal being about 30° each. To prevent loss of solvent by evaporation, a suitable metal cover, with slots or holes to permit insertion of sampling pipe and mixer shaft, should be placed over the sample tank during the sampling and mixing operation.
   (d) Prior to the start of the pumping period, the sampling equipment should be examined and the draw-off lines closed. During the pumping period it should be made certain that a continuous flow of oil is being obtained. When the filling of the tank or tank car has been completed, the mechanical mixer is started and the gross sample is mixed thoroughly in order to obtain uniform distribution of moisture, oil, and impurities. After thorough mixing, with the agitator still running, the draw-off line is opened and three 1 gal (3.7 L) samples are withdrawn into new and dry 1 gal (3.7 L) containers to a level about 5 cm (2 in.) from the top. The sample containers are immediately closed and properly labeled.
   (e) Surplus oil remaining in the sample tank is returned to oil storage, or to the tank car if the official weight was obtained prior to sampling, through the 3 in. (7.5 cm) line connected to the apex of the conical bottom. After draining, the tank is thoroughly cleaned by flushing, washing, and drying, or other suitable means, depending upon conditions, and the cleanings withdrawn through the clean-out line. The cleaning procedure should be such that there will be no contamination of the next sample drawn.

      Note—Where multiple loading or unloading facilities are in use, a separate sample tank should be available for each unit.

   (f) In locations where the liquid pressure in the 3 in. (7.5 cm) line is too high to retain all of the sample in the 50 gal (185 L) drum, an intermittent sample may be taken. For this purpose, install a 3/8 in. (1 cm) electric solenoid valve as shown in Figure 3. It may be a slide gate valve which opens fully and does not have any area to retain solids. The valve is operated by a timer. With a 90 psig (6200 Pa) and normal pumping rate, the valve is open 30 sec and closed 25 sec to secure 50 gal (185 L) per 60,000 lb oil (25000 kg). The valve should be operated to open and close at least one time every minute.
2. Grab method for sampling tanks or tank cars during loading or unloading—
   (a) Use a dipper, or any other convenient container, and withdraw about 1 lb (454 g) from the discharge end of the pipe at regular intervals as the product is entering the tank.
   (b) Collect, mix and distribute as directed in Procedure, 1, (d).

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3. Loaded tanks or tank cars, liquid contents (official method of the National Cottonseed Products Association)—
   (a) Lower the official oil trier vertically through the oil at a uniform rate with the bottom valve completely open so that 10–15 sec will be required to reach the bottom of the car (see Notes, 2). Close the bottom valve and withdraw the tube.
   (b) Take several portions in this manner and then proceed as directed in Procedure, 1, (d).
4. Loaded tanks or tank cars, solid contents—
   (a) Solid material cannot be correctly sampled in tanks or tank cars. If possible, the material should be liquified (see Notes, 3) and then sampled as directed in Procedure, 3.
   (b) When necessary to sample solid material (see Notes, 3), use the designated trier and withdraw several portions from the car taken vertically and obliquely toward the ends of the car. The trier should pass through the stock until it touches the sides of the car so a complete core will be taken. Soften (but do not melt) and mix all portions thoroughly before distributing into laboratory sample containers.
5. Ship and shore tanks, liquid contents, using a bomb or zone sampler—
   (a) Tanks constructed so that the sampler cannot be lowered vertically to the lowest part of the tank cannot be correctly sampled for moisture and settlings with any sampler. In order to sample such material properly, the contents should be pumped to another tank into which the sampler can be lowered to the lowest part of the tank. The sampling may then be done by the petcock method during pumping or with a suitable sampler after the transfer.
   (b) If the tanks are suitably constructed, lower the sampler to the lowest point in the tank. As soon as the sampler is completely full, withdraw and empty into a suitable container. If any foreign material (water, dirt, etc.) is encountered, take samples at consecutive 2.5 cm (1 in.) levels until there is no further evidence of these impurities. Above this level withdraw samples at consecutive 30 cm (1 ft) levels, if the sampler is a 30 cm (1 ft) sampler, until the top level of the oil has been reached. The samples are composited in the proportion that each represents to the total depth of oil in the tank. For example, if 12 portions are taken at 2.5 cm (1 in.) levels and the total depth is 6 m (20 ft), combine in the ratio of 1 of the portions taken at the 2.5 cm (1 in.) levels to 19 of the portions taken at higher levels.
   (c) Combine 11 portions and mix them thoroughly together before distribution into laboratory sample containers.
6. Ship and shore tanks, liquid contents, using a core sampler—
   (a) Use a sampler as described in Apparatus, 1, except of sufficient length to take a cross section through the entire depth of oil. This method is impractical if the tanks to be sampled are so deep as to make the sampler unwieldy. In such cases, the sectional sampler must be used.
   (b) Proceed as directed in Procedure, 3.
7. Barrels, tierces, casks, and drums; liquid or semisolid contents—
   (a) Roll the container to mix the contents, and insert the oil thief slowly (see Notes, 2 and 3) through the bung or, preferably, through an end opening or hole drilled at one end. If possible, the sample should be drawn from end to end.
   (b) As soon as the thief is fully inserted, close the upper constriction with a finger and transfer the sample into a suitable container. Take several portions in this manner from this and other packages as directed in Size and Number of Samples, 4. Mix thoroughly before distribution into laboratory sample containers.
8. Barrels, tierces, casks, and drums; solid contents—
   (a) Remove the bung or end opening, or drill a hole through an end or side with an auger large enough in diameter to accommodate the trier (see Notes, 3). If possible, the sample should be drawn from end to end.
   (b) Insert the trier through the opening, push it through to the opposite end or side, turn it in a complete circle and withdraw with the sample. Collect several such portions from this and other packages as directed in Size and Number of Samples, 4. Soften (but do not melt) and mix thoroughly before distribution into laboratory sample containers.
9. Barrels, tierces, casks, and bags; very hard materials—
   (a) If the material is in the form of flakes or small pieces, take grab samples of uniform and proportional size from packages as directed in Size and Number of Samples, 4. If the material is in the form of large pieces, these should be broken up before taking the grab sample. Mix thoroughly, quarter and distribute into laboratory sample containers (see Notes, 3).
10. Packages of odd sizes and mixed lots—
   (a) The procedure for taking samples and the number of packages to be sampled is exactly as described in Procedure, 7, 8, and 9.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive splattering. Always add the acid to water, never the reverse.

NUMBERED NOTES

1. If the stability or keeping quality of the product is involved, all equipment and containers must be scrupulously clean. The containers may be new (unused) tin cans or glass jars. Metal, such as copper, bronze, and brass, must under no circumstances be allowed to come in contact with the sample. Glass containers are cleaned with an appropriate cleaning solution,

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thoroughly rinsed with distilled water to remove all traces of cleaning solution, and dried by heat. Glass jars with rubber gaskets are satisfactory, but all parts must be cleaned as described above. Jars with plastic or enameled tops, or covers containing paper liners, are not recommended.

Prepare the cleaning solution by placing 5–10 g of potassium dichromate ($K_2Cr_2O_7$) in a 1-L Erlenmeyer flask with about 50 mL of water. Warm the flask in a hot water bath to dissolve as much dichromate as possible. Slowly and carefully add concentrated H_2SO_4 (see Notes, Caution) until the volume is about 200 mL. Allow the hot solution to stand for about 5 min; then dilute to 1 L with concentrated H_2SO_4.

Remove sample from the package with a stainless steel trier [butter type, 46-92 cm long (18-36 in.)] that has been previously well cleaned with soap and water, thoroughly rinsed with distilled water and completely dried by heat or with a new paper towel. Samples are collected so none of the shortening will be taken less than 5 cm (2 in.) from the well of the container or from the surface of the sample.

Samples are packed and transported to the destination laboratory in such a way that they will be protected from the light and arrive in a solid state. Samples that have been melted or partially melted at any time are not satisfactory.

2. As the trier is lowered into the oil, the rate must be slow enough so that the level of oil inside and outside of the trier remains the same. Otherwise, an unduly large portion will be drawn from the bottom, which is likely to contain a considerable concentration of moisture and settlings.
3. If the physical structure of the product is involved, such as the consistency of shortening or the graininess of certain types of animal fats and hydrogenated vegetable fats, the material must not be softened, melted, or sampled with any of the devices mentioned herein. In such cases, the cover of the container must be removed and a large section (if shortening) cut out with a large spatula without disturbing the surface. If the product is grainy, use a large dipper to remove the sample with as little disturbance as possible.
4. The address for Refinery Supply Co. is 6901 E. 12th St., Tulsa, OK 74112, USA. Phone (918) 836-4681.
5. The address for Zone Devices is 3449 Ocean View Blvd., Glendale, CA 91208 USA. Phone (818) 249-5887.

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Figure 1. Core sampler for oils and liquid fats (see Notes, 4).

Figure 2. Liquid zone sampler (see Notes, 5).

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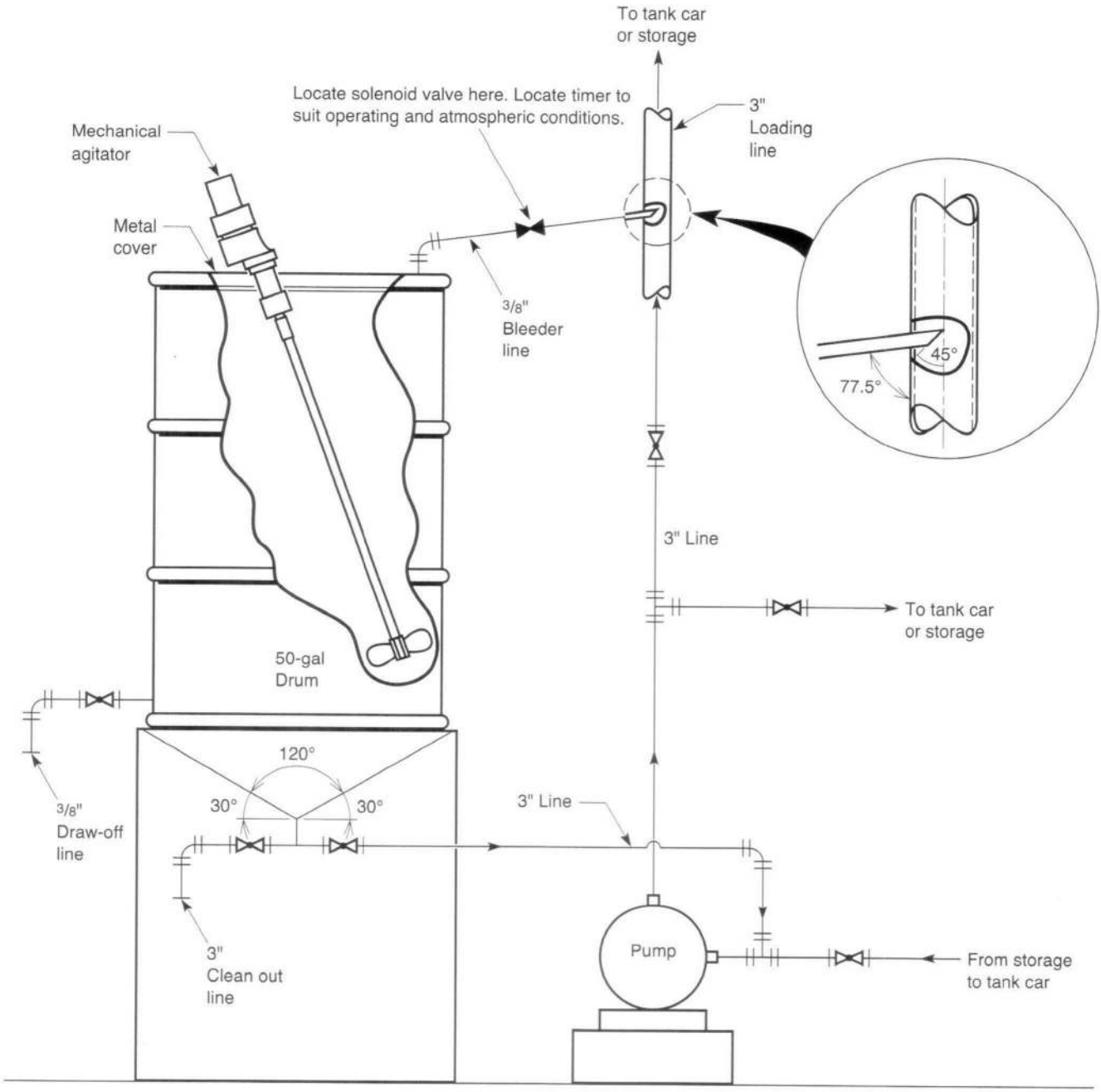

Figure 3. Schematic of sampling device.

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AOCS Official Method Ca 2a-45

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Moisture Distillation Method

DEFINITION

This method determines the moisture by distillation with an immiscible solvent.

SCOPE

This method is applicable to all normal fats and oils, including emulsions, for the determination of moisture only, as differentiated from moisture and volatile matter. It is not applicable to samples containing water-miscible volatile substances or to samples containing added monoglycerides.

APPARATUS

1. Glass distillation apparatus—with ground-glass connections, constructed and assembled in accordance with the specifications shown in Figure 1.
   (a) Flask—short-neck, round-bottom type, 500–1000 mL.
   (b) Water-cooled condenser—the lower tip of which should be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established.
   (c) Trap—constructed of well-annealed glass and graduated to contain 5 mL at 20°C. It shall be subdivided into 0.1 mL divisions with each mL line numbered and the 5 mL line at the top. The error at any indicated capacity shall not exceed 0.05 mL.
   (d) Distillation may be better controlled by wrapping the tube that leads from the flask to the receiver with glass-fiber tape. It is advantageous to wrap the flask also, especially if there are drafts in the vicinity of the distillation apparatus.
   (e) Calibrate the receiver by the following procedure: Add an accurately measured portion, 1 ± 0.01 mL, of distilled water to 100 mL of toluene in the distillation flask. Conduct the distillation as described in Procedure, 2–5 and calculate as directed. Cool the apparatus, add another 1 mL of water and repeat the distillation. Continue in this manner up to the capacity of the receiving tube.
2. Pipet—measuring type, capacity 1.0 mL, graduated in 0.01 mL.
3. Heat source—either a gas flame, or an electric heater provided with a suitable means of control.
4. Copper or nichrome wire—long enough to extend through the condenser with one end twisted into a spiral. The diameter of the spiral is such that it will fit snugly within the receiver, yet can be moved up and down.

REAGENTS

1. Toluene—reagent grade (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Because water tends to settle in samples that have softened or melted, care must be taken to mix samples thoroughly so as to distribute the water uniformly. Soften with gentle heat (do not melt) and mix thoroughly with an efficient mixer.

PROCEDURE

Note—The entire apparatus should be cleaned with potassium dichromate-sulfuric acid solution to minimize the adherence of water droplets to the sides of the condenser and receiver. Rinse thoroughly and dry completely before using.

1. The size of the test portion is determined by the amount of moisture present:

Moisture range	Size of test portion
<1%	200 g
1–5%	100 g
Larger amounts of moisture	Smaller size test portion

2. Place the specified size of sample in a distillation flask, add at least an equal volume of solvent (never <100 mL) and swirl to mix. Assemble the apparatus and fill the receiver with solvent by pouring through the condenser until the solvent begins to overflow into the distillation flask. Insert a loose cotton plug in the top of the condenser to prevent condensation of atmospheric moisture within the tube.
3. Heat the flask so the distillation rate will be about 100 drops/min. When the greater part of the water has distilled, increase the distillation rate to about 200 drops/min and continue until the water level in the receiver remains unchanged for 30 min.
4. Discontinue heating, dislodge any drops of water that may be adhering to the inside of the condenser tube or receiver with a wire spiral and wash with about 5 mL of toluene.

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5. Immerse the receiver in water at 25°C for at least 15 min, or until the toluene layer is clear, and then read the volume of water.

CALCULATIONS

1. Moisture, $\% = \frac{(\text{volume of water, mL} \times 0.997}{\text{mass of test portion, g}} \times 100$

NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

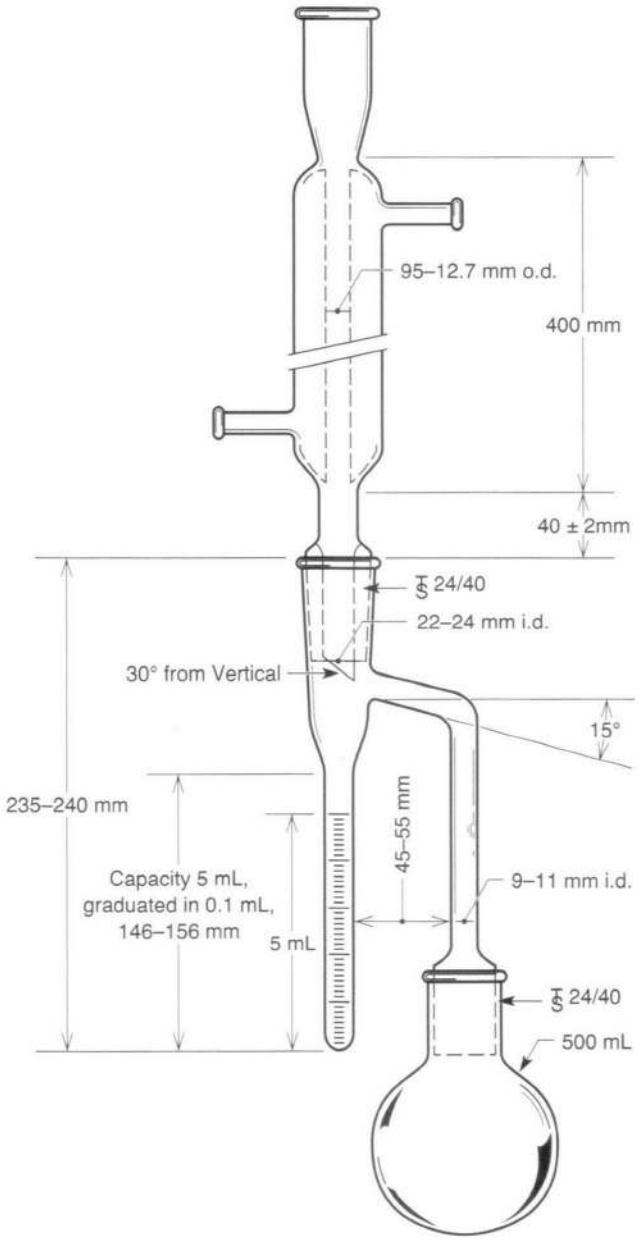

Figure 1. Moisture distillation apparatus.

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AOCS Official Method Ca 2b-38

Reapproved 2009

Moisture and Volatile Matter Hot Plate Method

DEFINITION

This method determines the moisture and any other material volatile under the conditions of the test.

SCOPE

Applicable to all of the ordinary fats and oils, including emulsions, such as butter, oleomargarine, and high-acid coconut oil. It is not applicable to certain abnormal samples, such as solvent-extracted fats and oils, which may contain residues from solvents with fairly high boiling points or to samples containing added monoglycerides.

APPARATUS

1. Electric hot plate—The surface should have a high polish; otherwise, cover with a heat-resistant pad to prevent wear on the moisture dish.
2. Glass beakers—100–150 mL, or other similar and convenient dish.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.

PREPARATION OF TEST SAMPLE

1. Because water tends to settle in samples that have softened or melted, care must be taken to mix laboratory samples thoroughly so as to distribute the water uniformly. Soften with gentle heat (do not melt) and mix thoroughly with an efficient mixer.

PROCEDURE

1. Weigh accurately 5–20 g of well-mixed test sample into a tared beaker that has been dried and cooled previously in a desiccator.
2. Heat the test portion on the hot plate, rotating the beaker gently by hand to avoid spattering which may result from too rapid ebullition of moisture.
3. The approach of the end point may be judged by the cessation of the rising bubbles of steam as well as by the absence of foam. Another good method of judging the end point is to place a clean, dry watch glass on top of the beaker. The evolution of steam is indicated by condensation on the watch glass. The temperature of the sample is at no time to exceed 130°C, except at the end of the test.
4. When the apparent end point has been reached, heat momentarily to the point of incipient smoking, but use caution not to overheat.
5. Cool to room temperature in a desiccator and weigh.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$

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AOCS

AOCS Official Method Ca 2c-25

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Moisture and Volatile Matter Air Oven Method

DEFINITION

This method determines the moisture and any other material volatile under the conditions of the test.

SCOPE

Applicable to animal and vegetable fats, but not to drying or semidrying oils or oils of the coconut group. It is not applicable to fats containing added monoglycerides.

APPARATUS

1. Air oven—AOCS Specification H 3-45.
2. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), or equivalent (see Notes, 1).
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.

PREPARATION OF TEST SAMPLE

1. Because water tends to settle in samples that have softened or melted, care must be taken to mix laboratory samples thoroughly so as to distribute the water uniformly. Soften with gentle heat (do not melt) and mix thoroughly with an efficient mixer.

PROCEDURE

1. Weigh accurately 5 g of test sample into a tared moisture dish that has been dried and cooled previously in a desiccator.
2. Place in air oven and dry for 30 min at 130 ± 1°C. Remove from the oven, cool to room temperature in a desiccator and weigh.
3. Repeat Procedure, 2 until the loss in weight does not exceed 0.05% per 30 min drying period.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$

NOTES

1. For samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes is not permitted. Erroneously high results may be obtained due to oil loss from splattering during heating.

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AOCS

AOCS Official Method Ca 2d-25

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Moisture and Volatile Matter Vacuum Oven Method

DEFINITION

This method determines the moisture and any other material volatile under the conditions of the test.

SCOPE

Applicable to all normal fats and oils, except those of the coconut oil group containing 1 percent or more of free fatty acids. For those fats, use the hot plate method, AOCS Official Method Ca 2b-38. Method Ca 2d-25 is not applicable to fats containing added monoglycerides.

APPARATUS

1. Vacuum oven—AOCS Specification H 4-45.
2. Aluminum moisture dishes—minimum 30 gauge, approximately 50 × 20 mm, with tight-fitting slip-over covers; Sargent-Welch (20 mL WLS 25725-B, 50 mL WLS 25725-D), or equivalent (see Notes, 1).
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.

PREPARATION OF TEST SAMPLE

1. Because water tends to settle in samples that have softened or melted, care must be taken to mix laboratory samples thoroughly so as to distribute the water uniformly. Soften with gentle heat (do not melt) and mix thoroughly with an efficient mixer.

PROCEDURE

1. Weigh accurately 5 g of well-mixed test sample into a tared moisture dish that has been dried and cooled previously in a desiccator.
2. Dry to constant weight in a vacuum oven at not less than 20°C and not more than 25°C above the boiling point of water at the operating pressure, which shall not be more than 100 mm of mercury (see Table 1).
3. Remove from the oven, cool to room temperature in a desiccator and weigh.
4. Constant weight is attained when the loss is not more than 0.05% in successive 1 hr drying periods.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$

NOTES

1. For samples with high moisture and volatile content, the use of lightweight, coverless, disposable weighing dishes is not permitted. Erroneously high results may be obtained due to oil loss from splattering during heating.

Table 1
Permissible oven temperature.

Internal oven pressure, mm	Permissible oven temperature, °C	
	Minimum	Maximum
100	72	77
90	70	75
80	67	72
70	65	70
60	62	68
50	58	63
40	54	59

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AOCS

AOCS Official Method Ca 2e-84

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Moisture Karl Fischer Reagent

DEFINITION

This method determines the actual water content of fats and oils by titration with Fischer reagent, which reacts quantitatively with water.

SCOPE

Applicable to fats and oils that do not react with, and are soluble in, the reagents and that do not contain impurities leading to secondary reactions. Such impurities are alkaline compounds and peroxides, which react with the reagent and therefore show high results (see References, 1). Replaces Ca 2e-55.

APPARATUS

1. Karl Fischer titration assembly—manual or automatic, with stirrer.

REAGENTS

Note—Karl Fischer reagent (see Notes, 1) is available from most chemical supply houses. While the reagent may be prepared as indicated below, due to the hazards associated with the solvents used in the reagent preparation, it is recommended that the Karl Fischer reagent be purchased commercially. See Notes, Caution regarding the hazards associated with the solvents used in the reagent preparation.

1. Methanol—not containing more than 0.05% (wt/wt) of water. If the reagent contains more than this quantity of water, dry it by distillation from magnesium turnings activated with iodine. Collect the distillate in a receiver protected from atmospheric moisture by means of a guard tube fitted with desiccant (Reagents, 8).
2. 2-Methoxyethanol (ethylene glycol monomethyl ether)—not containing more than 0.05% (wt/wt) of water. If the reagent contains more than this quantity of water, dry it by distillation, rejecting the first portion of distillate, which contains the water.
3. Pyridine—not containing more than 0.05% (wt/wt) of water. If the reagent contains more than this quantity of water, dry it by distillation, rejecting the first portion of distillate, which contains the water.
4. Sample solvent—either a mixture containing 4 parts by volume of the methanol (Reagents, 1) and 1 part by volume of the pyridine (Reagents, 3) or, preferably, for determinations with compounds containing carbonyl groups, a mixture containing 4 parts by volume of the 2-methoxyethanol (Reagents, 2) and 1 part by volume of the pyridine (Reagents, 3). In special cases, other solvents may be used, e.g., acetic acid, pyridine, or a mixture containing 1 part by volume of the methanol (Reagents, 1) and 3 parts by volume of chloroform.
5. Sulfur dioxide—anhydrous liquified gas in cylinder.
6. Iodine—reagent grade.
7. Karl Fischer reagent—stabilized single solution, available commercially or prepared as follows:
   (a) Place 670 mL of the methanol (Reagents, 1) or the 2-methoxyethanol (Reagents, 2) in a dry brown glass flask, fitted with a ground-glass stopper and having a capacity of slightly greater than 1 L.
   (b) Add about 85 g of iodine. Stopper the flask and shake it occasionally until the iodine is completely dissolved. Then add approximately 270 mL of the pyridine (Reagents, 3), stopper the flask again and mix thoroughly. Using the method described below, dissolve 65 g of sulfur dioxide in this solution, cooling to ensure that the temperature does not exceed 20°C.

      Note—As the reaction is exothermic, it is necessary to cool the flask from the beginning and to maintain it at about 0°C, e.g., by immersing it in an ice bath or in crushed carbon dioxide.
   (c) Replace the ground-glass stopper by an attachment for introducing sulfur dioxide, consisting of a cork bearing a thermometer and a glass inlet tube (6 × 8 mm), reaching to within 10 mm of the bottom of the flask, and a small capillary tube for connecting to the atmosphere.
   (d) Place the whole assembly with the ice bath on a balance and weigh to the nearest 1 g.
   (e) Connect the inlet tube to a cylinder of sulfur dioxide by means of a flexible connection and a drying tube filled with the desiccant (Reagents, 8); gently open the tap on the cylinder. Adjust the rate of flow of the sulfur dioxide so that all the gas is absorbed without the liquid showing any sign of rising in the inlet tube. Maintain the equilibrium of the balance by gradually increasing the tare, and ensure that the temperature of the liquid does not exceed 20°C. Close the tap on the cylinder as soon as the increase in weight reaches 65 g.
   (f) Immediately remove the flexible connection and reweigh the flask and its inlet attachment. The weight of dissolved sulfur dioxide should be between 60 and 70 g. A slight excess is not harmful. Stopper the flask, mix the solution, and leave for at least 24 hr before use. In fact, as the result of imperfectly understood reactions that occur in the fresh reagent, the water equivalent of the reagent decreases rapidly to begin with and then much more slowly. This water equivalent

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is between 3.5 and 4.5 mg/mL. It must be determined daily if methanol has been used, but may be determined less frequently if 2-methoxyethanol has been used. It is possible to prepare Karl Fischer reagent having a lower water content by diluting the solution prepared as described above with the sample solvent (Reagents, 4).

(g) Store the reagent in the dark and protected from atmospheric moisture. It preferably should be stored in the reagent bottle.

8. Aluminum sodium silicate—anhydrous, in the form of granules of 1.7 mm diameter, for use as a desiccant. The granules may be regenerated by washing with water and drying at 350°C for at least 48 hr. Alternatively, activated silica gel may be used as a desiccant.
9. Chloroform—reagent grade.

DETERMINATION OF THE WATER EQUIVALENT OF THE KARL FISCHER REAGENT

1. Accurately weigh (to the nearest 0.0001 g) a drop of water, approximately 50 mg, and transfer to the titration vessel. For this calibration of the reagent, 250 mg of crystalline sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) may also be used instead of a drop of water.
2. Add the stirring rod by means of a pair of tweezers and connect the titration vessel with the apparatus.
3. Add 20 mL of methanol (Reagents, 1) by means of a pipet or a burette. Close the aperture and dissolve by stirring gently.
   Note—The amount of solvent used must allow the electrode to dip 2–3 mm.
4. Titrate with the Karl Fischer reagent up to the electrometric end point. This point is reached when the deflection remains constant for 30 sec after dropwise adding the Karl Fischer reagent. Carry out a blank test.
5. For direct reading apparatus, the calibration factor is inserted into the equipment in accordance with manufacturer's instruction, and a blank determination is not required.

PROCEDURE

1. Weigh (to the nearest 0.01 g) 5–25 g of the test sample into the titration vessel, but make certain that it does not contain more than 100 mg of water. Dilute the Karl Fischer solution in case of a low water content and/or poor solubility of the sample in order to get a sufficiently high titration.
2. Put into the titration vessel the stirring rod by means of a pair of tweezers and connect it with the apparatus. Add, if necessary, exactly 10 mL chloroform (Reagents, 9) or another suitable solvent and dissolve the sample by stirring. Often solid fats do not dissolve completely. These are first dissolved in 10 mL of dry chloroform and then 10 mL of methanol are added.
3. The amount of solvent used must allow the electrode to dip 2–3 mm.
4. Add 10 mL of methanol and titrate with the Karl Fischer reagent up to the electrometric end point.
5. Carry out a blank test following the same procedure and using the same quantity of all of the reagents as used for the determination but omitting the test portion. This is not required for direct reading instruments.

CALCULATIONS

1. The water equivalent, T, of the Karl Fischer reagent in mg H_2O/mL is given by the formula

$$T = \frac{m_1}{V_0 - V_1}$$

Where—

m_1 = mass, in mg, of the water added
V_0 = volume, in mL, of the Karl Fischer reagent used for the determination of the water equivalent of the Karl Fischer reagent
V_1 = volume, in mL, of the Karl Fischer reagent used for the blank test

2. The water content of the test sample is given by the formula

$$\text{Percent } H_2O \text{ (wt/wt)} = \frac{(V_2 - V_3) \times T}{m_2} \times 100$$

Where—

m_2 = mass, in mg, of the test portion
V_2 = volume, in mL, of the Karl Fischer reagent used for the determination (Procedure, 4)
V_3 = volume, in mL, of the Karl Fischer reagent used for the blank test (Procedure, 5)

3. Take as a result the arithmetic mean of two determinations.

PRECISION

1. The difference between the results of two different determinations carried out simultaneously or in rapid succession by the same operator on the same sample shall not exceed 0.6% relative error.

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NOTES

Caution

Methanol is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform, and perchloric acid.

Pyridine is flammable and a dangerous fire risk. The explosive limits in air are 1.8–12.4%. It is toxic by ingestion and inhalation. The TLV is 5 ppm in air. The danger from crude pyridine is greater than from pure pyridine, the associated homologs and impurities being even more toxic than pyridine itself.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

NUMBERED NOTES

1. Because of its toxicity, pyridine is an undesirable constituent of the Karl Fischer reagent. Both one- and two-component Karl Fischer reagents, based on aliphatic amines and heterocyclic compounds and containing no pyridine, are available commercially.

REFERENCES

1. Reference for the collaborative study is Bernetti, R., S. J. Kochan, and J. J. Pienkowski, *J. Assoc. Off. Anal. Chem. 67*:299 (1984).

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AOCS Recommended Practice Ca 2f-93
Reapproved 2009

Determination of Moisture and Volatile Matter in Fats and Oils Modified Method

DEFINITION

This method determines moisture and volatile matter in fats and oils by the addition of acetone to the fat or oil, followed by heating at 100°C. The moisture and volatile matter are removed during the evaporation of the acetone and are determined by the loss in weight of the original sample (see Notes, 1).

SCOPE

Applicable to crude fats and oils. This moisture and volatile matter method can be completed in one hour without the use of elaborate equipment, permitting a rapid determination of the commercial value of crude fats and oils.

APPARATUS

1. Electric hot plate—with a magnetic stirring device.
2. Water pump aspirator—producing a vacuum equivalent to 100 mm absolute pressure.
3. Filter flask—Pyrex™, 125 mL.
4. Magnetic stirring bar—2 cm long.
5. Rubber vacuum tubing—withstanding the specified vacuum.
6. Porcelain dish—containing glycerol as heat transfer medium.
7. Thermometer—0–150°C.
8. Desiccator—containing an efficient desiccant (see AOCS Specification H 9-87).
9. Graduated cylinder—10 mL.

REAGENTS

1. Acetone—see Notes, *Caution*.

PROCEDURE

1. Accurately weigh 15-20 g of well-mixed test sample into the tared filter flask (Apparatus, 3), containing the magnetic stirring bar as part of the tared weight. If the sample is not liquid, it may be heated, but not more than 10°C higher than the melting point of the sample.
2. Add 5 mL acetone (see Notes, 2), using a graduated cylinder. The flask is stoppered and placed in the glycerol bath, which is heated by means of the electric hot plate (Apparatus, 1). With continuous stirring, the flask is placed under vacuum, heated to (but not exceeding) 100°C in about 10 min, and held at 100°C for 20 min. The flask is removed from the glycerol bath and cooled to room temperature in a water bath while maintaining the vacuum.
3. After carefully releasing the vacuum, the flask is dried, placed in the desiccator for a few minutes, and weighed.

CALCULATIONS (see Notes, 3)

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$

NOTES

Caution

Acetone is highly flammable. It forms explosive peroxides with oxidizing agents. Use effective fume-removal device. Do not mix with chloroform.

NUMBERED NOTES

1. The moisture and volatile matter content of fats and oils can be determined according to AOCS Official Method Ca 2b-38 (hot-plate method), AOCS Official Method Ca 2d-25 (vacuum oven method) and the IUPAC sandbath method. The last two methods are rather time-consuming, whereas the hot-plate method has been found to give varying results because of the requirement to heat the sample to the point of incipient smoking.
2. The addition of acetone serves to avoid the splattering of the fat or oil during evaporation of the moisture. Acetone, being a solvent for both oil and water, was found to be better in this application than alcohols and hydrocarbons.

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3. The efficiency of this method was tested by adding weighed amounts (0.5–1%) of water to a dried sample and repeating the procedure. The reproducibility and the accuracy of the procedure were found to be satisfactory. The difference between the amount of water added and found was below 1%.

REFERENCES

Hartman, L. and F. H. Jablonka, *J. Am. Oil Chem. Soc. 69:*1276 (1992).

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AOCS

AOCS Official Method Ca 3a-46

Revised 2011

Insoluble Impurities

DEFINITION

This method determines dirt, meal, and other foreign substances insoluble in kerosene and petroleum ether.

SCOPE

Applicable to all normal fats and oils. Replaces Ca 3-25 and Ca 3-46.

APPARATUS

1. Gooch crucible—prepared with a glass-fiber filter without organic filler (Reeve Angel 934 AH or Whatman GF/C). Wash the filter with water, alcohol, and ether. Dry to constant weight at 101 ± 1°C. Cool in a desiccator to room temperature and weigh.
2. Filter flask of convenient size and Gooch crucible adapter.

REAGENTS (see Notes, *Caution*)

1. Petroleum ether—AOCS Specification H 2-41.
2. Kerosene—refined petroleum distillate with a flash point not below 23°C (75°F), as determined by the American Society for Testing and Materials Standard Method D56, using the tag closed tester. The kerosene should be filtered through a Gooch crucible, prepared as in Apparatus, 1, before using.

PREPARATION OF TEST SAMPLE

1. Laboratory samples must be mixed thoroughly. If necessary, soften with gentle heat (do not melt) and mix thoroughly with an efficient mixer.

PROCEDURE

1. Use the residue from the moisture and volatile matter determination (AOCS Official Methods Ca 2b-38 or Ca 2d-25), or a test portion prepared in the same manner (see Notes, 1).
2. Add 50 mL of kerosene to the residue and heat in a water bath to dissolve the fat.
3. Filter through the prepared Gooch crucible with the aid of a vacuum. Wash with five 10 mL portions of hot kerosene (see Notes, 2), allowing each portion to drain before adding the next.
4. Wash thoroughly with petroleum ether to remove all of the kerosene. Dry the crucible and contents to a constant weight at 101 ± 1°C, cool to room temperature in a desiccator, and weigh.

CALCULATIONS

1. Insoluble impurities, $\% = \dfrac{\text{gain in mass of crucible}}{\text{mass of test portion taken for moisture}} \times 100$

NOTES

Caution

Petroleum ether and kerosene are flammable solvents. A fume hood should be used at all times when working with these solvents.

NUMBERED NOTES

1. Samples (e.g., certain feed stocks) with a higher than usual percentage of insoluble impurities may be difficult, if not impossible, to filter. A study among five laboratories indicated that, for such samples, a 2 g subsample (rather than a 5 g subsample as specified in AOCS Official Methods Ca 2b-38 and Ca 2d-25) can be used without greatly affecting the precision of the method.
2. The assay is empirical and highly dependent on the temperature of the hot kerosene. When using this method in trade, both parties shall use the same temperature.

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AOCS

AOCS Official Method Ca 3b-87

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Hexane Residues in Fats and Oils

DEFINITION

The residual hexane content is the quantity of volatile hydrocarbons remaining in the fats and oils following processing involving the use of solvents. The volatile hydrocarbons are desorbed by heating the sample at 80°C in a closed vessel after addition of an internal standard. After determination of a calibration factor, hydrocarbons in the headspace are determined by gas chromatography using a capillary column. Results are expressed as hexane in milligrams per kilogram (mg/kg) (ppm).

SCOPE

This method is applicable to the determination of "free" volatile hydrocarbons, expressed in terms of hexane, remaining in animal and vegetable fats and oils after extraction with hydrocarbon-based solvents. It is suitable for the determination of quantities of hexane between 10 and 1500 mg/kg (ppm) in fat or oil. This method is equivalent to International Union of Pure and Applied Chemistry Standard Method (IUPAC) 2.607 (References, 1 and 2).

APPARATUS

1. The gas chromatograph should have, at a minimum, the following characteristics—
   (a) Thermostated column oven capable of maintaining the desired column temperature within ± 1°C.
   (b) Sample inlet system, separately thermostated, that can be maintained at a minimum temperature of 100°C. The inlet system must be capable of a 1:100 split injection. For serial analysis a headspace gas chromatograph with automatic sample injection and tempering bath has been shown to be satisfactory.
   (c) Flame ionization detector, separately thermostated, that can be maintained at a minimum temperature of 100°C.
2. Electronic integrator.
3. Chromatographic column—The column should be constructed of glass or fused silica, approximately 30 m long and 0.3 mm i.d. Stationary phase: methyl polysiloxane (film thickness 0.2 µm).
4. Syringe—1 µL capacity.
5. Syringe—10 µL capacity.
6. Syringe—1000 µL capacity, gas-tight (preferably with valve).
7. Septum vials—20 mL capacity.
8. Septa and aluminum caps suitable for septum vials, together with crimping pliers. The septa must be resistant to oils and solvents (butyl rubber or red rubber is recommended).
9. Tongs—suitable for holding septum vials.
10. Heating bath—with clamps for holding septum vials, thermostatically regulated and capable of maintaining a temperature of 80°C. For continuous operation, glycerol is recommended as the heating liquid.
11. Shaking machine.

REAGENTS

1. Gases—
   (a) Carrier—helium (preferred for better resolution) or nitrogen, minimum purity 99.99 mol%, dried and containing a maximum of 10 mg O_2/kg.
   (b) Flame ionization detector—hydrogen, minimum purity 99.95 mol%; air or O_2, dry (dew point –59°C maximum) and hydrocarbon free (< 2 ppm hydrocarbons equivalent to CH_4).
2. Technical hexane or a light petroleum—with a composition similar to that used in industrial extraction, or failing these, *n*-hexane (see Notes, *Caution*). For calibration, technical extraction hexane is preferred.
3. *n*-Heptane (internal standard)—analytical reagent grade. (See Notes, *Caution*.)
4. Vegetable oil—solvent free, freshly refined and deodorized. The oil is to be used for calibration and should be of a similar nature as the sample. It should be free of extraction solvent (< 0.001%). If it contains a greater amount of solvent, this should be reduced by laboratory deodorization of the oil.

SAMPLING AND SAMPLE STORAGE

1. It is essential that loss of solvent from the sample be prevented. The laboratory sample should be in a completely sealed container and stored at 4°C. Plastic containers should not be used. Sample analyses should be carried out immediately when the sample container is opened.

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GC OPERATING CONDITIONS

1. Carrier gas flow depends on the carrier gas and the type of column being used for the analysis and should be optimized accordingly. Helium is recommended, but nitrogen may be used. Some loss of resolution between peaks may be experienced with nitrogen.
2. The flow of hydrogen and air or oxygen to the flame ionization detector should be optimized according to the manufacturer's recommendations for the instrument being used.
3. Injector and detector temperatures should be set at about 120°C.
4. The column should be maintained at 40°C.

PROCEDURE

1. Determination of the calibration factor—Weigh, to the nearest 0.01 g, 5 g of solvent-free vegetable oil (Reagents, 4) into each of seven septum vials (Apparatus, 8). Seal each vial with a septum and cap (Apparatus, 9). By means of a syringe (Apparatus, 5 or 6), add technical hexane (Reagents, 2) to six of the seven vials (the vial with no added solvent is the blank) according to the following table:

Vial number	1	2	3	4	5	6	7
µL/5 g	0	0.5	1	2	4	7	10
mg/1000 g	0	67	134	268	536	938	1340

One vial remains without the addition of solvent.

If n-hexane is used as the calibrating solvent, the following table applies:

Vial number	1	2	3	4	5	6	7
µL/5 g	0	0.5	1	2	4	7	10
mg/1000 g	0	66	132	264	528	924	1320

Shake vigorously the six vials containing the solvent in the shaking machine at room temperature for 1 hr. Using a syringe (Apparatus, 6), add through the septum exactly 5 µL of internal standard (Reagents, 3) to each of the seven vials.

Successively immerse the vials up to the neck in the heating bath (Apparatus, 11) at 80°C at intervals of approximately 15 min. This time interval depends on the duration of the GC analysis, which is complete upon the elution of the internal standard (*n*-heptane). The samples must be placed in the heating bath at intervals such that each sample is tempered for exactly 60 min.

Warm the gas-tight syringe (Apparatus, 7) to 60°C.

After tempering at 80°C for exactly 60 minutes and without removing the vial from the heating bath, use the gas-tight syringe and withdraw through the septum, 1000 µL (1 mL) of the headspace above the oil. Inject immediately into the gas chromatograph.

For each of the vials containing added solvent, a calibration factor, F, may be determined by the formula:

$$F = \frac{C_S \times A_i}{(A_H - A_B - A_i) \times C_i}$$

Where—

A_H = total peak area of solvent hydrocarbons, including the area of internal standard present in the spiked oil. For identification purposes, a typical chromatogram of the solvent composition should be obtained. Hydrocarbons that usually make up the technical hexane are: 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane, etc. Do not include peaks due to the oxidation products, some of which may be present in significant amounts. See Figure 1.

A_B = peak area of the solvent hydrocarbons present in the oil to which solvent has not been added (blank), less the peak area of the internal standard

A_i = peak area corresponding to the internal standard in the spiked samples

C_i = quantity of internal standard added, expressed in mg/kg (ppm) of oil

Note—For an addition of 5 µL to 5 g of sample, C_i = 680 mg for *n*-heptane.

C_S = quantity of technical hexane added to the oil present in the vial, expressed in mg/kg (ppm) of oil Express the results to the third decimal place.

Calibration factors for the six standards should be approximately the same. The mean calibration factor should be about 0.45.

The factor (F) so evaluated can be used for determining vial quantities of hexane <60 mg/kg (ppm). If the value of F found for the vial containing 0.5 µL of hexane is significantly below the mean value, this deviation is probably due to the difficulty of introducing exactly 0.5 µL and this determination must be either eliminated or repeated.

For quantities of hexane between 10 and 20 mg/kg (ppm), it is better to prepare calibration standards by adding 2 µL of internal standard rather than 0.5 µL. In this case, C_i for *n*-heptane = 272 mg/kg (ppm).

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2. Sample analysis—Weigh, to the nearest 0.01 g, 5 g of the test sample into a septum vial (Apparatus, 8) as quickly as possible and close immediately with a septum and cap (Apparatus, 9). Using a syringe (Apparatus, 6), add through the septum exactly 5 µL of internal standard (Reagents, 3). Shake vigorously by hand for about 1 minute and then immerse the vial up to the neck in the heating bath (Apparatus, 11) at 80°C for exactly 60 min. Warm the gas-tight syringe (Apparatus, 7) to 60°C. After tempering at 80°C for exactly 60 min, use the gas-tight syringe and take from the vial, without removing it from the bath, 1000 µL (1 mL) of the headspace above the sample. Immediately inject into the gas chromatograph.
3. Number of determinations—Carry out two determinations in rapid succession on each sample.

RESULTS

1. The residual solvent content, W, expressed in mg/kg (ppm), is given by the formula:

$$W = \frac{(A_H - A_i) \times F \times C_i}{A_i}$$

Where—

A_H = total peak area of solvent hydrocarbons, including the area of the internal standard. Hydrocarbons that usually make up the technical solvents are 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane, etc. A typical chromatogram of these solvents was shown by the calibration standards. Do not include peaks due to the oxidation products. Some of these products may be present in significant amounts.

A_i = peak area corresponding to internal standard in the sample

C_i = quantity of internal standard added in mg/kg (ppm)

Note—For an addition of 5 µL of heptane per 5 g of sample, C_i = 680 mg/kg (ppm).

F = calibration factor obtained in Procedure, 1

Report as the final result the mean of the results of the two determinations, provided the requirements for repeatability are met. If the requirements for repeatability are not met, discard the results and carry out two additional determinations on the sample.

PRECISION

1. Interlaboratory tests—Details of an interlaboratory test on the precision of the method are given in Table 1. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given. An international collaborative test involving 21 laboratories in 8 countries was carried out using the method given in this International Standard. The collaborative test was organized by the Federation of Oils, Seeds and Fats Associations (FOSFA International) in 1994. The results obtained were subjected to statistical analysis in accordance with ISO 5725 [ISO 5725:1986 (now withdrawn) was used to obtain the precision data] to give the precision data as shown in Table 1. The collaborative test was carried out on spiked samples of soybean and sunflower seed oils, as follows:
   samples A to D: sunflower seed oil;
   samples E to H: soybean oil.
2. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases be greater than the repeatability limit r given in Table 1.
3. Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5% of cases be greater than the reproducibility limit R given in Table 2. Reproducibility limit values R cited in Table 1 apply in the particular case when the results of single determinations obtained by two laboratories are being compared. When following the method described and it is desired to compare the final results (which have been derived from the means of duplicate determinations) obtained by two laboratories, the values for R should be converted to the 95% probability critical difference values, C_{95}, applicable to the means of two determinations using the following formula:

$$C_{95} = \sqrt{(R^2 - r^2/2)}$$

NOTES

Caution

The solvents used in this method—hexane, petroleum ether, and *n*-heptane—are all highly flammable. They should not be used near open flame. Procedures requiring the use of these solvents should be carried out in a properly operating fume hood. For hexane the TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

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REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.607.
2. Collaborative study results published in *Pure Appl. Chem. 59* (11):1561 (1987).

Table 1

Summary of statistical results: expected means of the samples provided to analysts.

	Sample							
	A	B	C	D	E	F	G	H
No. of laboratories originally instructed	21	21	21	21	21	21	21	21
No. of laboratories returning results	18	18	18	18	17	18	18	18
No. of laboratories with acceptable results	11	14	14	14	12	15	15	15
Overall mean (mg/kg)	1.55	392.6	1059.1	757.5	1.65	374.5	1043.4	808.8
Repeatability standard deviation, s_r (mg/kg)	0.09	10.06	20.43	24.9	0.39	8.31	25.8	18.9
Repeatability limit, r (mg/kg)	0.26	28.2	57.2	69.8	1.09	23.3	72.2	52.9
Reproducibility standard deviation, s_R (mg/kg)	0.58	22.1	73.3	55.9	0.65	26.9	55.9	48.2
Reproducibility limit, R (mg/kg)	1.62	61.8	205.2	156.4	1.83	75.4	156.6	135.1
Ratio R/r	6.1	2.2	3.6	2.2	1.7	3.2	2.2	2.6
Range of accepted results	1 to 2.8	342.4 to 428.2	881 to 1214.1	588 to 873.7	0 to 3.1	326.2 to 447	896 to 1153.6	693 to 888.0

Table 2

Comparison of expected and actual means and repeatability limits: the actual means obtained and the repeatability limits found by the collaborative test.

Type of oil	Sample	Expected mean from homogeneity test	Overall mean from collaborative test	Interpolated repeatability limit	Repeatability limit from collaborative test
Sunflower seed oil	A	1.30	1.55	1.8	0.26
	B	384.8	392.6	74.8	28.2
	C	1100	1059.1	202	57.2
	D	787	757.5	152	69.8
Soybean oil	E	1.17	1.65	1.8	1.09
	F	361.4	374.5	70.4	23.3
	G	1028	1043.4	198	72.2
	H	779.6	808.8	151	52.9

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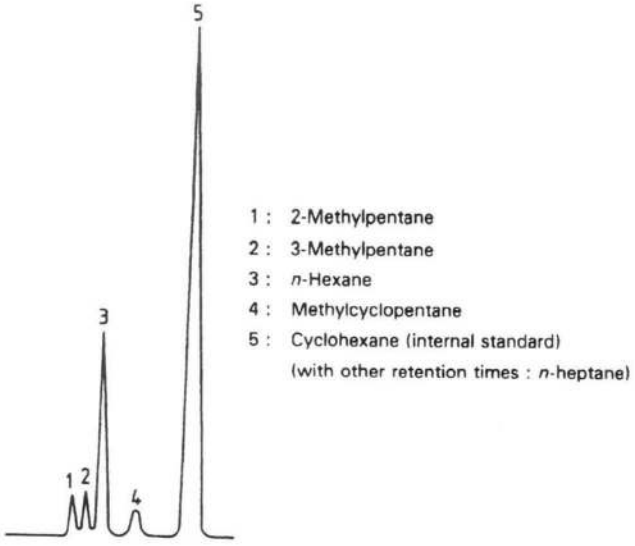

Figure 1. Example of a gas chromatogram of hexane hydrocarbons.

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Detection of a Volatile Organic Contaminant by GC-MS

DEFINITION

This method describes the determination of a volatile organic contaminant in edible oils. It is used to identify volatile industrial chemicals in crude and refined edible oils that are suspected of contamination, and the concentration of the contaminant. A deuterated reference compound with a GC retention time similar to that of the suspected contaminant is added to the oil at a concentration close to that of the suspected contaminant. A sample of the oil is then introduced into a thermal cold-trapping inlet of a GC-MS apparatus. Volatile components evaporate from the oil and are held in the cold trap. The trap is then flash-heated to 160°C and the chemicals released from the trap are swept into the GC-MS for analysis.

SCOPE

This method is suitable for identification of the following compound classes: saturated halogenated hydrocarbons; unsaturated halogenated hydrocarbons; esters; aldehydes; alcohols; amines; ketones; ethers; cyclic and aromatic compounds; nitrogen compounds; acrylates; etc. The method has been evaluated for the 1–0 mg/kg range and is not applicable to very volatile or nonvolatile chemicals. The concentration of chemicals that may react with the edible oil or one of its natural components cannot be determined using this method.

APPARATUS

1. Capillary gas chromatograph
2. Mass spectrometer
3. Thermal desorption cold-trapping device—Chrompack purge and trap system is an example of a suitable product available commercially.
4. Capillary column—50 m length with OV101 (or equivalent) stationary phase, 0.5 μm film thickness, and 0.32 mm i.d.
5. Gas chromatograph-mass spectrometer, operating under the following conditions:
   (a) Thermal desorption cold-trapping temperature program—at injection, thermal cold trap oven 160°C, and thermal cold trap below –40°C. The trap is held at a temperature below –40°C for 5 min and is then flash-heated to 160°C. The GC temperature program is started simultaneously with the flash heating of the trap. The trap is held at 160°C for 3 min.
   (b) GC temperature program—initial temperature 50°C for 5 min; increase at 7.5°C per min; final temperature 250°C for 5 min.
   (c) MS conditions—scan 250 a.m.u. to 20 a.m.u. at 1 s/decade; source 70 ev. 200°C, 100 μA, 4 kV.
   (d) Gas flows—column 1 mL/min helium; split during cold trapping 5 mL/min

REAGENTS

1. Standard—corresponding to suspected contaminating compound (99% pure).
2. Methanol
3. Fully deuterated internal standards of benzene, ethyl benzene, and naphthalene (99% pure).
4. Helium
5. Refined, bleached, deodorized (RBD) groundnut oil, or similar stable vegetable liquid oil known to be free of industrial chemicals related to the analytes in question.

PROCEDURE

1. Preparation of standard and blank
   (a) Use a RBD vegetable oil, e.g., groundnut, (Reagents, 5) as a carrier and as a blank.
   (b) Mix the standard (Reagents, 1) in a concentrated form (e.g., 100 mg/kg) in the carrier oil (Procedure, 1). This is the stock solution. Dilute this stock solution with carrier oil (Procedure, 1) to the required concentration (usually in the range 1–10 mg/kg). The standard solution should be a concentration similar to the contaminant in the sample.
   (c) Dilute the suspect oil, if necessary, with the carrier oil (Reagents, 5) so that the concentration of the suspected contaminant is likely to be in the range 1–10 mg/kg.
2. Preparation of internal standards—Prepare a solution of deuterated benzene, ethyl benzene, or naphthalene (Reagents, 3) in methanol (Reagents, 2) at a concentration of 0.1 g/L (0.1 μg/μL) as needed. The internal standard chosen should be the one having the GLC retention time closest to that of the analyte.

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3. Preparation of analytical sample—Accurately weigh 1.00 g of oil. Add 5.0 µL of internal standard solution in methanol (Reagents, 2), selected from deuterated benzene, deuterated ethyl benzene, or deuterated naphthalene according to Procedure, 2. Mix well on a vortex mixer.
4. Determination by GC-MS analysis—Pack an empty thermal cold-trapping (TCT) tube with clean glass wool to 3–4 cm. Place approximately 10 mg of test sample in the tube. If the observed contaminant concentration exceeds 10 mg/kg, repeat the determination with 2.5 mg of test sample. If the concentration is still outside the 1–10 mg/kg concentration range, accurately dilute the test portion with the blank oil (Procedure, 1) until the appropriate range is reached. Pre-cool the cold trap to below –20°C, and continue cooling to below –40°C. Put the prepared tube in the TCT oven and start the TCT and GC-MS programs (Apparatus, 5).
5. Identification—Identification requires a good match of both spectra and GC retention times. Compare the GC retention time and mass spectrum of the analyte with those of the standard.

CALCULATIONS

The area of the analyte ion divided by the area of the internal standard ion is the ratio R:

R_{SAM} = R in sample

R_{STD} = R in standard

The concentration of analyte is C

C_{SAM} = concentration in sample

C_{STD} = concentration in standard

The concentration of internal standard is C_{rs}

$R = (K \times C)/C_{rs}$ where K is a constant

$R_{SAM} = (K \times C_{sam})/C_{rs}$

Since C_{rs} is a constant by definition

$R_{SAM} = K' \times C_{SAM}$

Where $K' = K/C_{rs}$

Similarly

$R_{STD} = K' \times C_{STD}$

$(C_{SAM} \times K')/(C_{STD} \times K') = R_{SAM}/C_{STD}$

$C_{sam} = (R_{SAM} \times C_{STD})/R_{STD}$

2. Expression of results—Carry out duplicate determinations. Report the identity of the contaminant and the mean of the two duplicate values obtained for its concentration provided the repeatability limit in Precision, 2 is satisfied. If not, repeat the determination on two additional test portions. If the difference still exceeds 0.6 mg/kg, take the arithmetic mean of the four determinations, provided the maximum difference between individual results does not exceed 1.0 mg/kg oil. Report the result to one decimal place.

PRECISION

1. Interlaboratory tests—Details of interlaboratory tests on precision are summarized in Tables 1 through 4. The values derived from these interlaboratory tests may not be applicable to concentration ranges and matrices other than those given.
2. Repeatability value—The difference between the values obtained from two single determinations, carried out in rapid succession by the same operator, using the same apparatus for the analysis of the same test sample, should not exceed 0.4 mg/kg.
3. Reproducibility value—The difference between the values for the final result, obtained by two (or more) laboratories using this standard method for the analysis of the same laboratory sample should not be greater than 1.0 mg/kg. This method has been ring-tested with 1,1,1-trichloroethane, 2-ethylhexyl acrylate, dicylopentadiene, tetrachloroethylene, N,N-ethylmethyl aniline, N,N-dimethylaniline, hexane-2-ol benzene, cumene, and ethyl acrylate.

REFERENCES

Animal and vegetable fats and oils—Detection of a volatile organic contaminant by GC-MS, ISO 15303.2.

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Table 1
Samples for the first test on edible oil cargoes[1].

Sample code	Supposed list of previous cargoes[2]	Spiked concentration in oil (mg/kg)
A	Dichloromethane 1,2-Dibromoethane 1,1,1-TRICHLOROETHANE	1.12
B	2-ETHYL HEXYLACRYLATE Methyl acrylate Dipentene	1.25
C	Methyl t-butyl ether DICYCLOPENTADIENE Furfural	1.29
D	Trichloroethylene TETRACHLOROETHYLENE 2-Furaldehyde	1.03
E	N,N-ETHYLMETHYLANILINE 5-Ethyl-2-methylpyridine N,N-Dimethyl aniline	1.04
F	Pyridine N,N-DIMETHYLANILINE Nitrotoluene	1.10
G	Butanol HEXAN-2-OL Di-isobutyl ketone	1.04
H	Cumene Pyridine BENZENE	1.04

[1]First interlaboratory trial. Twenty-one laboratories from 10 countries were invited to participate, with 7 laboratories from 5 countries submitting results. In order to simulate actual shipping practices in which the nature of the last cargoes, and that of the potential contaminant is known, the analysts were told that the potential contaminant could be one of three compounds.
[2]Chemicals written in capital letters are those actually contained in the oil, but this information was not given to the analysts until after the test.

Table 2
Samples for the second test on edible oil cargoes[1].

Sample code	Supposed list of previous cargoes[2]	Spiked concentration in oil (mg/kg)
I	2-Ethyl hexyl acrylate ETHYL ACRYLATE Vinyl acetate	1.03
J	N,N-Dimethylaniline Epichlorhydrin 1,3-Dichloropropene	
K	CUMENE Tetrachloroethylene N,N-Ethylmethylaniline	1.25

[1]Second interlaboratory trial. Sixteen laboratories from 7 countries were invited to participate, with 9 laboratories from 5 countries submitting results. In order to simulate actual shipping practices in which the nature of the last cargoes, and that of the potential contaminant is known, the analysts were told that the potential contaminant could be one of three compounds.
[2]Chemicals written in capital letters are those actually contained in the oil, but this information was not given to the analysts until after the test.

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Table 3
Statistical analysis of interlaboratory trial 1.

Sample	A	B	C	D	E	F	G	H
No. of laboratories	7	7	7	7	7	7	7	7
Mean observed concentration (mg/kg)	1.11	1.32	1.53	1.05	1.58	1.18	0.83	0.91
Spiked concentration (mg/kg)	1.12	1.25	1.29	1.03	1.04	1.10	1.04	1.04
No. of accepted results for the repeatability determination	2	2	3	2	3	2	2	3
Repeatability standard deviation, S_r (mg/kg)	0.15	0.08	0.12	0.08	0.13	0.04	0.11	0.04
Repeatability coefficient of variation (%)	13.7	5.6	15.9	6.6	19.4	3.5	14.3	4.0
No. of accepted results for the reproducibility determination	7	6	7	6	5	6	6	7
Reproducibility standard deviation, S_R (mg/kg)	0.64	0.57	1.09	0.29	1.80	0.43	0.44	0.18
Reproducibility coefficient of variation (%)	58.2	42.2	79.0	29.7	135	36.8	53.0	19.6
Repeatability limit (r) $2.83 \times S_r$	0.43	0.23	0.62	0.21	0.51	0.11	0.31	0.11
Reproducibility limit (R) $2.83 \times S_R$	1.82	1.61	3.09	0.81	5.09	1.22	1.25	0.51
Range of results (mg/kg)	0.58–2.50	0.58–2.10	0.57–3.50	0.68–1.30	0.40–4.73	0.4–1.7	0.17–1.37	0.6–1.10

NOTE: Although it is not statistically accurate to take a mean of values for r and R when these values are obtained from different samples, it was considered to be an acceptable compromise in estimating the precision of the method overall in the present case. It can be calculated from this table that the mean repeatability limit (r) is 0.36 and the mean reproducibility limit (R) is 2.38.

Table 4
Statistical analysis of interlaboratory trial 2.

Sample (mz) Internal standard (mz)	I (55) Benzene (84)	K (105) Ethyl benzene (98)	K (105) Ethyl benzene (116)	K (120) Ethyl benzene (98)	K (120) Ethyl benzene (116)
No. of laboratories	9	9	8	8	8
No. of accepted results	7	8	7	6	7
Mean (mg/kg)	1.19	0.94	0.99	0.92	0.97
Repeatability standard deviation, S_r (mg/kg)	0.48	0.07	0.08	0.04	0.08
Repeatability coefficient of variation (%)	40.0	7.5	8.5	4.15	8.7
Reproducibility standard deviation, S_R (mg/kg)	0.50	0.50	0.51	0.52	0.52
Reproducibility coefficient of variation (%)	42	53.25	51.2	56.8	53.5
Repeatability limit (r) $2.83 \times S_r$	1.3	0.2	0.24	0.11	0.24
Reproducibility limit (R) $2.83 \times S_R$	1.4	1.4	1.4	1.47	1.47
Range of results (mg/kg)	0.55–2.75	0.16–1.69	0.15–1.66	0.1–1.47	0.09–1.67

NOTE: Although it is not statistically accurate to take a mean of values for r and R when these values are obtained from different samples, in the present case it was considered to be an acceptable compromise in estimating the precision of the method overall. It can be calculated from this table that the mean repeatability limit (r) is 0.61 and the mean reproducibility limit (R) is 1.43.

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AOCS Official Method Ca 3d-02

Revised 2011

Determination of Sediment in Crude Fats and Oils—Centrifuge Method

DEFINITION

A homogenized test sample is subjected to centrifuging as specified. The amount of separated material, called sediment (part of the insoluble matter in a crude fat or oil which can be centrifugally separated and is the total amount of the unclear layer of components collected at the bottom of the measuring tube after centrifuging) is volumetrically measured in a calibrated centrifuge tube.

SCOPE

This method determines sediment that can be separated from crude fats and oils by centrifugal force. The method is applicable to crude oils and to oils with a sediment content of 0.03 mL per 100 g to 15 mL per 100 g, obtained by means of extraction and/or crushing. The method is not applicable to fats which are not liquid at a temperature of 20°C. This method is identical to International Organization of Standardization (ISO) 15301.

APPARATUS

Usual laboratory apparatus and, in particular, the following:

1. Centrifuge tubes, 100 mL capacity, pear- or cone-shaped, made from thoroughly annealed glass and fitted with a stopper (see Figures 1 and 2) (see Notes, 2).
2. Buckets, for centrifuge tubes (Apparatus,1), resistant to fats and oils.
3. Centrifuge, suitable for the centrifuge tubes (Apparatus, 1) placed in the buckets (Apparatus, 2), capable of controlling the rotational frequency so as to give a radial acceleration at the narrow part of the tubes of 700 to 800 times. See Notes, 3 for the calculation of the rotational frequency of the centrifuge. In rooms which are not air-conditioned, use a centrifuge capable of maintaining its temperature between 20°C and 25°C.
4. Balance, capable of weighing to the nearest 0.01 g.

SAMPLING

1. It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.
2. A recommended sampling method is given in AOCS Official Method C 1-47 or ISO 5555 (1).
3. Store laboratory samples in glass or polyethylene terephthalate (PET) bottles.

PREPARATION OF TEST SAMPLE

1. Prepare the test sample in accordance with ISO 661.
2. Bring the test sample, if necessary, to a temperature of between 20°C and 25°C.
3. Redisperse any sediment in the oil from the bottom of the sample bottle to ensure a sufficiently homogeneous and representative sample. Immediately proceed in accordance with Procedure.

PROCEDURE

1. Weigh two centrifuge tubes (Apparatus, 1) to the nearest 0.1 g.
2. Transfer the prepared test sample (see Test sample preparation, 1-3) to each of the centrifuge tubes.
3. Weigh the tubes and place them in the buckets (Apparatus, 2) of the centrifuge (Apparatus, 3). Adjust speed to give a radial acceleration at the narrow part of tubes of 700-800 × g.
4. Centrifuge for 1 h ± 5 s.
5. Read sediment volumes up to and including 1.5 mL to the nearest 0.03 mL. Read sediment volumes greater than 1.5 mL to the nearest 0.5 mL.
6. When using a cone-shaped tube, it may be more difficult to read the volume of the unclear layer; read the sediment volumes as accurately as possible.
7. If in a pear-shaped tube the separation is not complete (clear layer in the neck of the narrow tube section of the tube), the sediment reading should be corrected for this volume.
8. Record the × g force (rpm) used or the swing diameter and the rpm of the centrifuge (Notes, 3).
9. Record the temperature before and after centrifuging.

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EXPRESSION OF RESULTS

Calculate the sediment content of the test sample using the equation:

$$w = \frac{V \times 100}{(m_1 - m_2)}$$

Where—

w = the numerical value of the sediment content of the test sample, in milliliters per 100g

V = the numerical value of the sediment volume, in milliliters

m_1 = the numerical value of the mass of the centrifuge tube with the test portion, in grams

m_2 = the numerical value of the mass of the centrifuge tube, in grams

Calculate the mean of the results for the two tubes and report the results to the nearest 1 mL per 100 g.

PRECISION

1. Details of interlaboratory tests on the precision of the method are given in Tables 1 and 2. The values derived from these tests may not be applicable to concentration ranges and matrices other than those given. The precision of the method was established by two interlaboratory tests organized by the Netherlands Oils, Fats and Oilseeds Trade Association (NOFOTA) in cooperation with the Federation of Oils, Seeds and Fats Associations (FOSFA International) in 1996 and 1997/1998 and carried out in accordance with International Organization of Standardization 5725-2 (3). In the first test 12 laboratories participated. Six (spiked) samples of crude sunflower seed oil were investigated. In the second test 9 laboratories participated. Four (spiked) samples of crude sunflower seed oil were investigated. See Tables 1 and 2 for a summary of the statistical results of the tests.

Comparison with the 96 h method

In the two interlaboratory tests, the centrifuge method was compared with the method which leaves the test sample to stand at a controlled temperature for 96 h. Applying regression analysis to the results of both interlaboratory tests for the relation between the two methods, the following equation was established (with a correlation coefficient of 0.96):

$$w_2 = 2.5w_1 - 0.5$$

w_1 = the numerical value of the sediment content obtained with the 96 h method, in milliliters per 100 mL

w_2 = the numerical value of the sediment content obtained with the centrifuge method, in milliliters per 100 mL

This indicates that the centrifuge method may be considered as a good alternative to the 96 h method, where a rapid control method is required for levels of sediment exceeding 0.5 mL per 100 mL.

2. Repeatability
   The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases exceed the repeatability limit given in or derived from Table 3.
3. Reproducibility
   The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5% of cases exceed the R reproducibility limit given in or derived from Table 3.

NOTES

1. The sediment contains, for example, phospholipids, impurities, dirt, etc. dispersed in a water-containing phase, and can be quantified according to this method. Any white crystalline components deposited on top of and within the dark layer of insoluble materials are regarded as part of the sediment.
2. Reading the volume of the unclear layer may be more difficult in a cone-shaped tube than in a pear-shaped tube.
3. The relationship between centrifugation parameters is given by the equation:

$$n = 1{,}337 \frac{a_r}{d}$$

Where—

n = the numerical value of the rotational frequency, in min^{-1};

a_r = the relative radial acceleration (for example $a_r = 700$ or $a_r = 800$);

d = the numerical value of the diameter of swing, measured between the tips of opposite tubes when tubes are in a rotating position, in millimeters.

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REFERENCES

1. ISO 5555:1991, Animal and vegetable fats and oils—Sampling.
2. ISO 5725-1:1994, Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions.
3. ISO 5725-2:1994, Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

Table 1
Statistical results of the interlaboratory test organized in 1996.

Parameter	Sample					
Number of laboratories retained after eliminating outliers	12	12	12	11	10	11
Mean sediment content, mL per 100 g	0.75	1.36	0.54	1.62	2.07	2.61
Repeatability standard deviation (s_r), mL per 100 g	0.04	0.06	0.04	0.06	0.03	0.07
Repeatability coefficient of variation, %	4.76	4.29	6.85	3.60	1.59	2.78
Repeatability limit (r) [$r = 2.8 \times s_r$], mL per 100 g	0.10	0.16	0.10	0.16	0.09	0.20
Reproducibility standard deviation (s_R), mL per 100 g	0.26	0.22	0.26	0.28	0.26	0.35
Reproducibility coefficient of variation, %	34.6	16.0	47.9	17.2	12.6	13.5
Reproducibility limit (R) [$R = 2.8 \times s_R$], mL per 100 g	0.72	0.61	0.72	0.78	0.73	0.99

Table 2
Statistical results of the interlaboratory test organized in 1997/1998.

Parameter	Sample			
Number of laboratories retained after eliminating outliers	7	8	8	7
Mean sediment content, mL per 100 g	0.07	1.28	1.20	2.39
Repeatability standard deviation (s_r), mL per 100 g	0.00	0.04	0.04	0.00
Repeatability coefficient of variation, %	0.00	2.77	2.95	0.00
Repeatability limit (r) [$r = 2.8 \times s_r$], mL per 100 g	0.00	0.10	0.10	0.00
Reproducibility standard deviation (s_R), mL per 100 g	0.07	0.12	0.08	0.35
Reproducibility coefficient of variation, %	97.8	9.1	7.0	14.8
Reproducibility limit (R) [$R = 2.8 \times s_R$], mL per 100 g	0.20	0.33	0.24	0.99

Table 3
Repeatability limit (r) and reproducibility limit (R).

Sediment content	r	R
mL per 100 g	mL per 100 g	mL per 100 g
< 1	0.1	0.7
1 to 3	0.2	1.0

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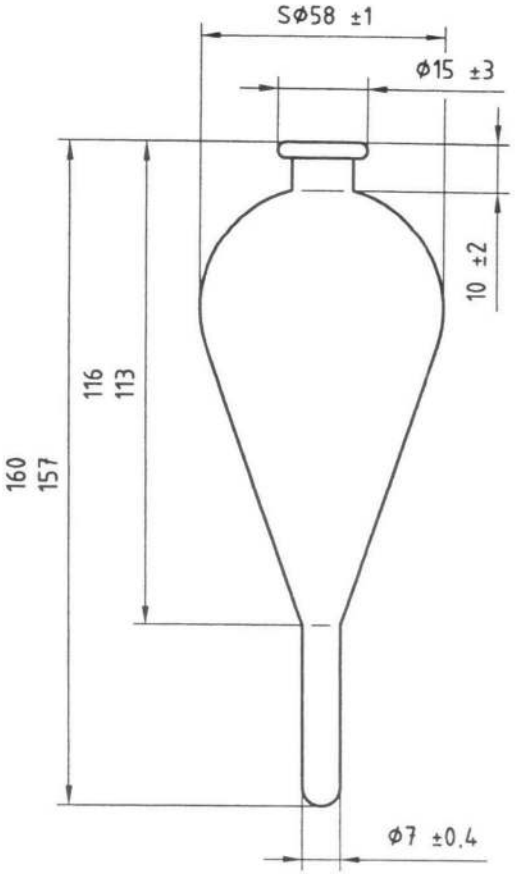

Figure 1. Pear-shaped sediment tube.

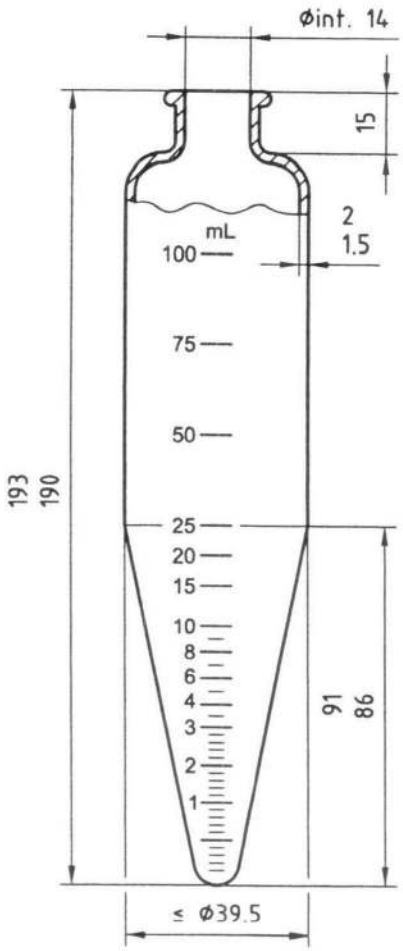

Figure 2. Cone-shaped sediment tube.

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AOCS Official Method Ca 3e-02

Revised 2011

Determination of Visible Foots in Crude Fats and Oils

DEFINITION

A homogenized test portion of crude fat or oil is allowed to stand at a controlled temperature for a period of 96 h. The volume of separated material, called "visible foots," is read off from the graduated vessel. "Visible foots" refers to the insoluble matter in fats and oils, together with occluded oil, which settles out from oil or fat within 96 h at the temperature specified in this method (see Notes, 1 and 2).

SCOPE

This method specifies a method for the determination in crude fats or oils of visible matter which can be separated by gravity.

APPARATUS

Usual laboratory equipment and, in particular, the following.

1. Sediment tube, pear-shaped, used when the sediment is ≤1.5 mL per 100 mL. See Figure 1.
2. Sediment tube, cone-shaped, used when the sediment is <1.5 mL per 100 mL. See Figure 2.

SAMPLING

A recommended sampling method is given in AOCS Official Method C 1-47 or ISO 5555 (1). It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transportation, sampling, or storage. Samples shall be stored in glass or polyethylene terephthalate (PET) bottles.

PREPARATION OF TEST SAMPLE

Warm the fat or oil until it is fully liquid and mix (i.e., shaking the sample by hand) until any sediment from the bottom of the sample is redispersed in the fat or oil to ensure a sufficient homogeneous and representative sample.

PROCEDURE

1. Number of determinations—Carry out the test in duplicate.
2. Preparation of test sample—Warm the fat or oil until it is fully liquid and shake the sample by hand until any sediment is redispersed in the fat or oil to ensure a homogeneous and representative sample.
3. Preparation of test portion—Cool the prepared test sample, with constant stirring, until the temperature is 20°C or 10°C above the melting point, whichever is the higher.
4. Determination—Fill the sediment tube (Apparatus, 1 or Apparatus, 2) to the 100 mL graduation mark, as quickly as possible, with the homogeneous fat or oil at the temperature of measurement. Allow it to stand in a vertical position for 96 h at 20°C or 10°C above the slip melting point, whichever is the higher. During the period of the test, the tube should remain undisturbed. Read the volume of the "foots" at the bottom of the tube after 96 h, to the nearest 0.1 mL.

EXPRESSION OF RESULTS

Report the value of visible foots as a percentage by volume, at T°C (where T°C is the temperature at which the test was carried out). State which sediment tube was used (Apparatus, 1 or Apparatus, 2).

Calculate the mean of the results of the two tubes and report the results to the nearest—
0.1 mL per 100 mL for results <1 mL per 100 mL, 0.5 mL per 100 mL for results from 1 mL to 3 mL per 100 mL,
1.0 mL per 100 mL for results >3 mL per 100 mL.

PRECISION

1. Interlaboratory tests—Tables 1 and 2 give the precision data for international interlaboratory tests carried out in 1996 and 1997/1998. The values derived from this test may not be applicable to concentration ranges and matrices other than those given.

Regression analysis for AOCS Official Methods Ca 3d-02 and Ca 3e-02—A comparison was made of the results found using the method given in Ca 3d-02 and the present method. Using the analysis results obtained from both collaborative ring tests, the relationship between the two methods is shown in the following equation (with a correlation coefficient of 0.96):

$$V = 2.5C - 0.5$$

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Where—

V = the volume of foots found with the Ca 3e-02 method (96 h test);
C = the volume of sediment/foots found with the Ca 3d-02 method (centrifuge test).

This indicates that the centrifuge method may be considered as a good alternative to the visible foots method, where a rapid control method is required for levels of sediment >0.5 mL per 100 mg.

2. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases be greater than:

Level of foots found (mL per 100 mL)	<1	1 to 3	>3
Repeatability limit, r	0.1	0.3 to 0.5	1.0

3. Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5% of cases be greater than:

Level of foots found (mL per 100 mL)	<1	1 to 3	>3
Reproducibility limit, R	1.0	3.0	5.0

NOTES

1. "Foots" is a term that was originally used to describe those impurities that precipitate from raw linseed oil during storage and subsequently settle to the bottom (foot) of a storage tank.
2. Visible foots are quantified by storage of a sample of the homogenized fat or oil for a period of 96 h at 20°C or 10°C above the melting point, whichever is the higher.

REFERENCES

1. ISO 5555, Animal and vegetable fats and oils — sampling.
2. ISO 5725-1:1994, Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions.
3. ISO 5725-2:1994, Accuracy (trueness and precision) measurement methods and results—Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.
4. ISO 15301:2001, Animal and vegetable fats and oils—Determination of sediment in crude fats and oils—Centrifuge method.

Table 1
Visible foots (1996 study).

	Sample no.					
	1	2	3	4	5	6
No. of participating laboratories	12	13	12	13	13	13
No. of acceptable results	11	10	9	12	12	10
Mean value	1.39	2.49	0.42	3.96	5.72	6.73
Repeatability standard deviation, s_r	0.18	0.08	0.03	0.18	0.14	0.23
Coefficient of variation of repeatability, %	12.64	3.24	6.22	4.52	2.50	3.47
Repeatability limit, r	0.49	0.23	0.07	0.50	0.40	0.65
Reproducibility standard deviation, s_R	1.10	0.48	0.62	1.15	1.05	0.85
Coefficient of variation of reproducibility, %	79.4	19.3	146	29.0	18.4	12.7
Reproducibility limit, R	3.09	1.34	1.73	3.21	2.95	2.39

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Table 2
Visible foots (1997/98 study).

	Sample no.			
	1	2	3	4
No. of participating laboratories	9	9	9	9
No. of acceptable results	8	9	9	9
Mean value	0.07	2.46	2.41	4.76
Repeatability standard deviation, S_r	0	0.07	0.02	0.26
Coefficient of variation of repeatability, %	0	3.04	0.98	5.53
Repeatability limit, r	0	0.21	0.07	0.74
Reproducibility standard deviation, S_R	0.05	0.90	0.90	1.90
Coefficient of variation of reproducibility, %	66.6	36.5	37.5	40.3
Reproducibility limit, R	0.30	2.51	2.53	5.38

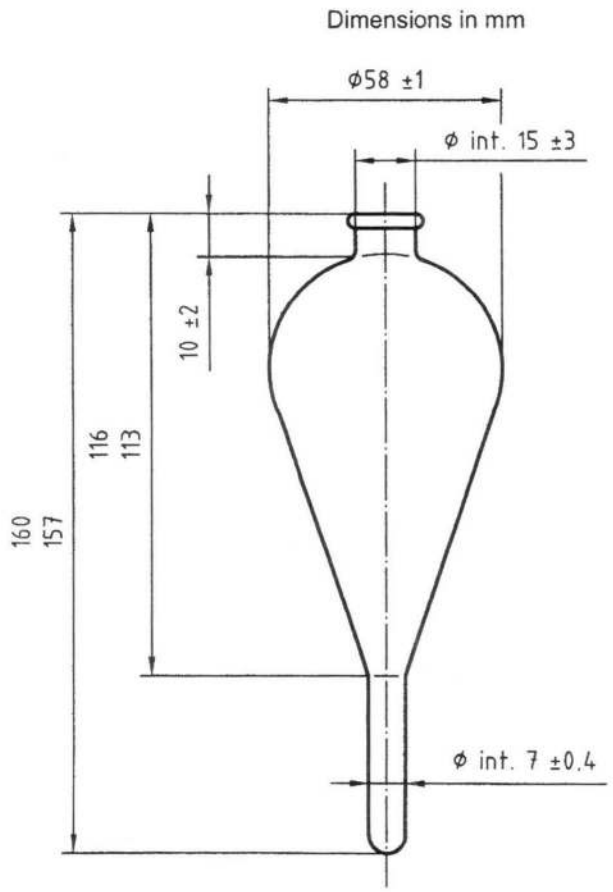

Figure 1. Pear-shaped sediment tube.

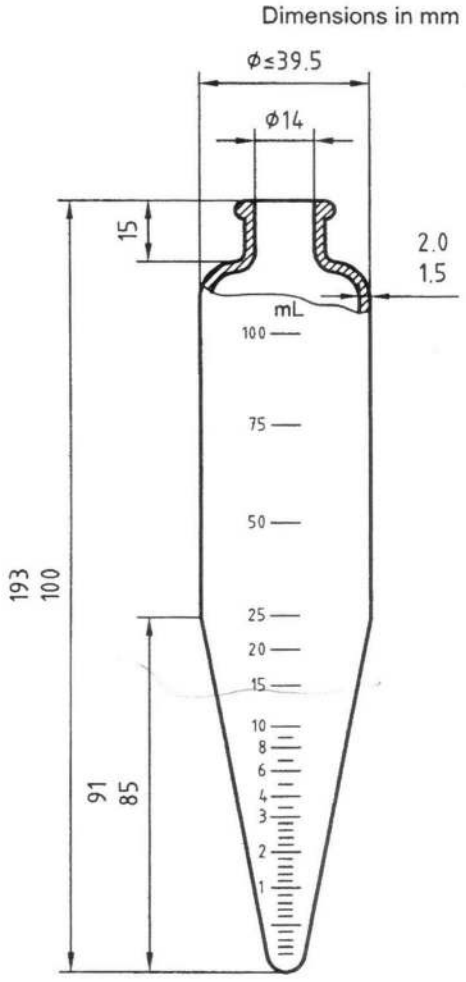

Figure 2. Cone-shaped sediment tube.

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Table 1
Free fatty acid range, alcohol volume and strength of alkali[a]

FFA range (%)	Test portion (g)	Alcohol (mL)	Strength of alkali
0.00 to 0.2	56.4 ± 0.2	50	0.1 M
0.2 to 1.0	28.2 ± 0.2	50	0.1 M
1.0 to 30.0	7.05 ± 0.05	75	0.25 M
30.0 to 50.0	7.05 ± 0.05	100	0.25 or 1.0 M
50.0 to 100	3.525 ± 0.001	100	1.0 M

[a]FFA, free fatty acid; N, normality.

Table 2
The average, expected between-laboratory variation (standard deviation of reproducibility, S_R) for the determination of free fatty acids in refined, bleached and deodorized oils[a]

Approximate FFA value (%)	0–0.05	0.05–0.1	0.1–1.0	1.0–2.0
SR	0.007	0.010	0.046	0.073
RSD (CV, %)	33.93	12.73	9.90	4.75
R (95%) = 2.8 × S_R	0.02	0.03	0.13	0.20

[a]Values obtained from the AOCS Laboratory Proficiency Program.

Table 3
The average, expected between-laboratory variation (standard deviation of reproducibility, S_R) for the determination of free fatty acids in crude oils[a]

Approximate FFA value (%)	0.1–1.0	1.0–2.0
SR	0.077	0.156
RSD (CV, %)	14.57	9.84
R (95%) = 2.8 × S_R	0.22	0.44

[a]Values obtained from the AOCS Laboratory Proficiency Program.

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AOCS Official Method Ca 5b-71

Revised 2011

Crude Fatty Acids

DEFINITION

This method determines the total hexane-soluble fatty acids and unsaponifiable matter in a test sample, whether present in neutral or hydrolyzed form. A procedure is included for removal of hexane-soluble unsaponifiable matter as a separate fraction when desired (see Notes, 1).

SCOPE

Applicable to all oils or hydrolyzed fats, except coconut, palm kernel, and similar oils. This is a joint AOCS-AOAC International method (References, 1).

APPARATUS

1. Liquid-liquid extractor—assembled as noted in Figure 1, complete with magnetic stirring disk, condenser, and refluxing flask. Any similar extraction apparatus that gives equivalent results may be used. A supplier of similar liquid-liquid extractors is Kontes, Vineland, NJ, USA.
2. Combination hot plate-magnetic stirrer—capable of providing magnetic stirring at 25–80°C.
3. Heating mantle—controlled by a variable transformer, or other suitable device, for refluxing hexane.
4. Rotary evaporator—which provides for the release of vacuum with nitrogen or helium.
5. Water bath—maintained at 80°C.
6. Nitrogen gas (low oxygen content) or helium gas.
7. Disposable dropping pipets.
8. Beakers—50 mL.
9. Graduated cylinders—10, 50, and 100 mL.
10. Boiling chips—20 mesh.

REAGENTS

1. Potassium hydroxide (KOH) solution, 50%—prepared by dissolving 50 g of reagent-grade KOH in distilled water and diluting to 100 mL. Mix well (see Notes, *Caution*).
2. Potassium hydroxide (KOH) solution, 0.5 M.
3. Sodium hydroxide (NaOH) solution, 0.02 M.
4. Methyl orange indicator solution—0.1% in distilled water.
5. Phenolphthalein indicator solution, 1% in 95% ethyl alcohol.
6. Hydrochloric acid (HCl) solution, 6 M—prepared by mixing 1 volume of concentrated, reagent-grade HCl (sp gr 1.19) with 1 volume of distilled water (see Notes, *Caution*).
7. Commercial hexane—AOCS Specification H 16-56, Phillips *n*-hexane is suitable (see Notes, *Caution*).
8. Ethyl alcohol, 95%—USSD formulas 30 or 3A are permitted (see Notes, *Caution*).

PROCEDURE

Total Fatty Acids plus Unsaponifiable Matter

1. Warm the test sample to liquify, but do not heat to more than 10°C over the melting point. Stir the melted sample until homogeneous, transfer about 5 g to a 50 mL beaker, cool and weigh (with disposable dropping pipet in beaker). Transfer, by means of the dropping pipet, 2.0–2.5 g ± 1.0 mg to the reaction vessel which contains the magnetic stirring disk (see Figure 1).
2. Add 50 mL of 95% ethyl alcohol and 3 mL of 50% KOH solution to the test portion in the reaction vessel and heat just to boiling, with gentle stirring, for 30 min. Adjust to original volume with 95% ethyl alcohol, then add 50 mL of distilled water, warming if necessary to dissolve the soap.
3. Acidify the freshly saponified sample by slowly adding 6 M HCl (about 5 mL will be required) to the reaction vessel. Use methyl orange indicator to be certain that the mixture is strongly acidic.
4. Fill the inner tube with hexane, immediately insert it into position in the reaction vessel to prevent backflow of the aqueous solution and attach the condenser.
5. Add 100 mL of hexane to a tared 300 mL round-bottom flask (weighed to the nearest milligram), insert a boiling chip, attach to the side arm, and begin refluxing (heat is applied to the round-bottom flask by means of a heating mantle controlled with a suitable variable transformer) with the magnetic stirrer rotating at minimal speed. While watching to see that emulsions do not rise in the reaction vessel, gradually increase the rotation to about 500 rpm and increase the reflux rate to 200–250 drops/min. Continue the refluxing with stirring for 2.0 hr.

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6. Cool the round-bottom flask (if the solution is cloudy, filter the contents through filter paper on a sintered-glass crucible with the aid of vacuum and make certain the filtrate is returned to the tared round-bottom flask) and then remove the solvent either (a) on a steam bath using a gentle stream of nitrogen, or (b) on a rotary evaporator, applying vacuum slowly for 1 min, then applying full vacuum for 20 min with the flask immersed in a water bath at 80°C.
7. Cool, release the vacuum with nitrogen, dry the outer surface of the round-bottom flask, and weigh to the nearest milligram. Repeat either procedure (a) or procedure (b) in step 6, to assure constant weight. Successive weighings should agree within ± 0.001 g.

Hexane-Soluble Unsaponifiable Matter

1. After completion of step 2 in the previous procedure, omit step 3 and proceed with steps 4 and 5.
2. After the 2.0 hr reflux period, cool the round-bottom flask and transfer the hexane solution to a 250 mL separatory funnel, using a rinse of 10 mL of hexane.
3. Rotate the combined hexane extracts gently with 20 mL of water. Violent agitation at this step may result in emulsions that are difficult to break. Allow the layers to separate completely and draw off the lower aqueous layer. Wash the hexane layer two more times, using 20 mL of water each time, shaking gently each time and discarding the lower aqueous layer after separation.
4. Wash the combined extracts in the separatory funnel three times, using 20 mL portions of 0.5 M KOH, shaking vigorously. Follow each alkali washing by washing with 20 mL of water. If an emulsion forms during this washing procedure, allow to separate as much as possible, discard the clear aqueous layer, and proceed with the next step, leaving any emulsion in the separatory funnel with the hexane layer. After the third washing with 0.5 M KOH, wash the hexane with successive 20 mL portions of water until the washings are no longer alkaline to phenolphthalein.
5. Transfer the hexane extract to a tared beaker, rinsing the separatory funnel and its pouring edge with hexane and adding the rinsings to the solution in the beaker. Evaporate to dryness in a water bath, using a gentle stream of clean, dry nitrogen. When almost all of the hexane has been evaporated, add 2–3 mL of acetone and remove all traces of solvent with the aid of a stream of nitrogen. Complete the drying to constant weight in a vacuum oven at 75–80°C and an internal pressure of not more than 200 mm of mercury. Cool in a desiccator and weigh. The result becomes "B" in the calculations.
6. After weighing, take up the residue in 2 mL of hexane and then add 10 mL of 95% alcohol, containing phenolphthalein indicator and previously neutralized to the phenolphthalein end point. Titrate with 0.02 M NaOH to the same final color. Correct the weight of the residue (see Notes, 2) for free fatty acid content, using the following relationship: 1 mL of 0.02 M NaOH is equivalent to 0.0056 g of oleic acid. The grams of fatty acid determined by this titration become "C" in the calculations. A reagent blank correction should be determined.

CALCULATIONS

1. Crude fatty acids, $\% = \dfrac{A}{\text{mass of test portion, g}} \times 100$

   Where—

   A = weight of total fatty acids plus unsaponifiable matter
2. Total fatty acids, % = % crude fatty acids – % unsaponifiable matter
3. Unsaponifiable matter, $\% = \dfrac{B - C}{\text{mass of test portion, g}} \times 100$

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 1.30.
2. Single determinations performed in two different laboratories should not differ by more than 2.55.

NOTES

Caution

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add the pellets to the water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Ethyl alcohol is flammable. Use a fume hood when heating or evaporating this solvent.

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NUMBERED NOTES

1. See the scope of AOCS Official Method Ca 6a-40 for limitations of hydrocarbon solvent in extracting unsaponifiable matter.
2. The titration correction for extractable free fatty acids and other extractable unsaponifiable impurities (both reported as oleic acid) will tend to increase as the crude nature of the sample increases. For example, highenergy fats (used in animal feeds), feed fats, tall oil, and foots would be expected to give a higher free fatty acid titration than relatively pure refined, bleached and deodorized (RBD) oil.

REFERENCES

1. *Official Methods of Analysis,* AOAC International, 16th edn., Vol. II, Gaithersburg, MD, 1995, Chap. 41, p. 10, Method 972.28.

OTHER REFERENCES

*J. Assoc. Off. Anal. Chem. 55:*846 (1972).

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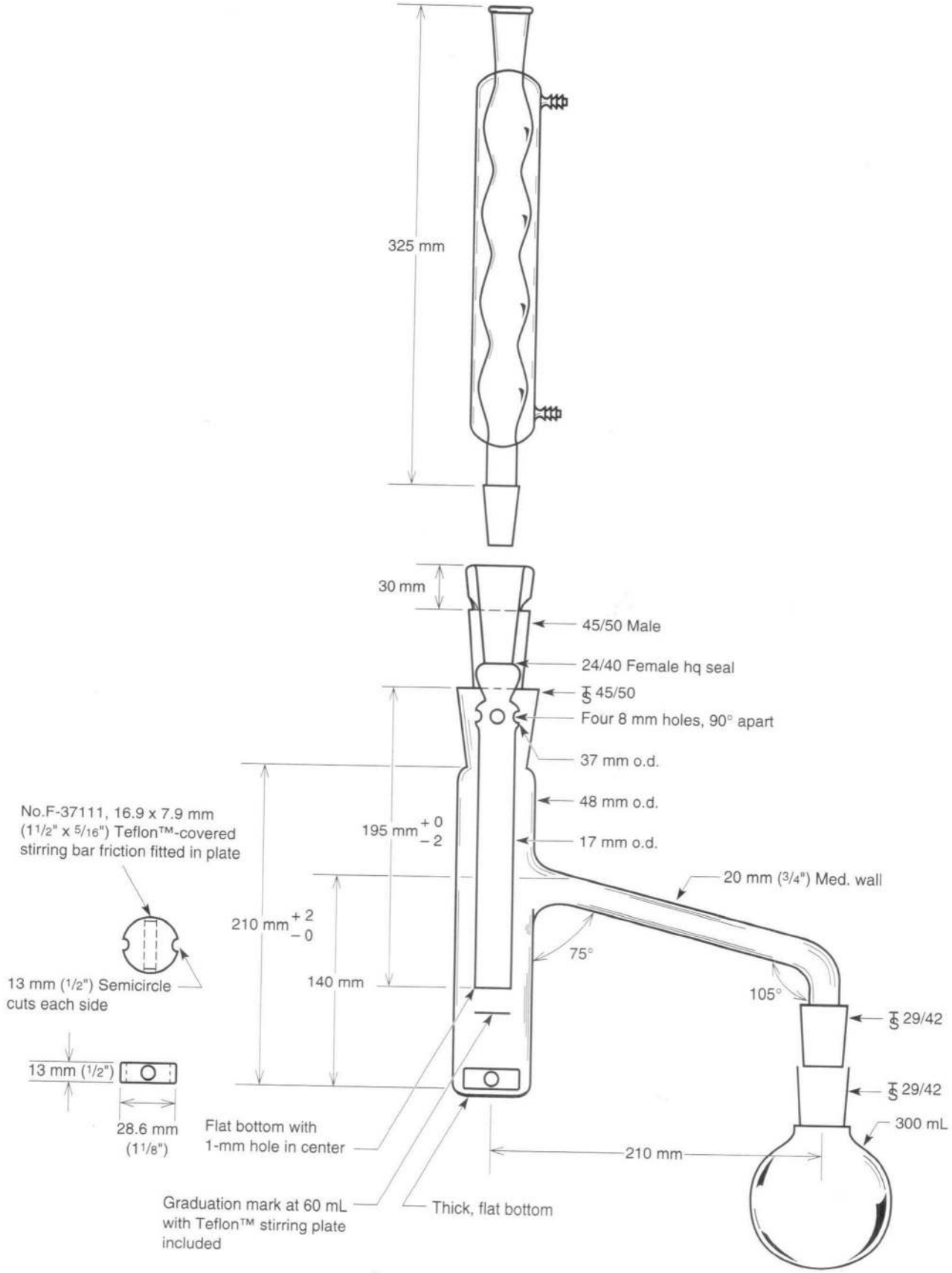

Figure 1. Liquid-liquid extractor.

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AOCS Official Method Ca 5c-87

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Butyric Acid

DEFINITION

The butyric acid content of milkfat, butterfat, or mixtures of fats containing milkfat and butterfat is the quantity, expressed as a percentage by weight, of butyric acid determined in the sample by the following method. Saponification of the fat with potassium hydroxide (KOH) solution is followed by acidification with phosphoric acid to liberate the fatty acids. Water-soluble fatty acids are separated by filtration and direct determination of butyric acid is carried out by gas-liquid chromatography in the presence of an internal standard.

SCOPE

This method is applicable to the determination of the butyric acid content of milkfat or butterfat, or mixtures of fats containing milkfat or butterfat. This method is equivalent to International Union of Pure and Applied Chemistry standard method 2.310 (References, 1, 2).

APPARATUS

1. Gas chromatograph, which should have, at a minimum, the following characteristics—
   (a) Thermostated column oven capable of maintaining the desired column temperature within ± 1°C.
   (b) Sample inlet system, on-column or all glass. If on-column injection is not used, the injection port should be separately thermostated and capable of being maintained at a minimum temperature of 175°C.
   (c) Flame ionization detector, separately thermostated, that can be maintained 25–50°C above the column oven temperature.
2. Electronic integrator.
3. Chromatographic column—The column should be constructed of glass, approximately 2 m long and 3 mm i.d., to fit the gas chromatograph (Apparatus, 1). It should be filled with a packing consisting of 10–15% stationary phase suitable for free fatty acid analysis (FFAP and SP-1220 with 1% phosphoric acid are suitable) on an 80–100 mesh, acid-washed, silanized support (Chromosorb W is suitable). The column should be conditioned at approximately 180°C for at least 48 hr prior to use.
4. Syringe—1 µL capacity (a 5 or 10 µL syringe is acceptable).
5. Beaker—50 mL.
6. Test tubes—with ground-glass stoppers, 10 mL.
7. Pipets—2–5 mL, graduated.
8. Fast filter paper.
9. Glass beads.
10. Watch glasses.
11. Water bath—maintained at boiling temperature.

REAGENTS

1. Gases—
   (a) Carrier—helium (preferred for better resolution) or nitrogen, minimum purity 99.95 mol%, dried and containing a maximum of 10 mg O/kg.
   (b) Flame ionization detector—hydrogen, minimum purity 99.95 mol%; air, dry (dew point –59°C maximum) and hydrocarbon free (< 2 ppm hydrocarbons equivalent to CH_4).
2. Potassium hydroxide (KOH) solution—approximately 0.5 N in ethanol, prepared by dissolving 4.5 g KOH in 100 mL of 95% ethanol (see Notes, *Caution*).
3. *ortho*-Phosphoric acid—5%, w/v, aqueous solution.
4. *n*-Butyric acid—reference standard, 0.4 mg/mL aqueous solution, prepared by dissolving 400 mg *n*-butyric acid in 100 mL water and diluting 10 mL of this solution to 100 mL. This solution must be freshly prepared for each analysis.
5. *n*-Valeric acid—internal standard, 0.25 mg/mL aqueous solution, prepared by dissolving 250 mg *n*-valeric acid in 100 mL water and diluting 10 mL of this solution to 100 mL. This solution must be freshly prepared for each analysis.

GC OPERATING CONDITIONS

1. Carrier gas flow depends on the carrier gas and column being used for the analysis and should be optimized accordingly (a retention time of about 5 min for butyric acid is desirable). Helium is recommended, but nitrogen may be used.
2. The flow of hydrogen and air or oxygen to the flame ionization detector should be optimized according to the manufacturer's recommendations for the instrument being used.

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3. The analysis temperature should be approximately 130–135°C. If on-column injection is not used, the injection port should be maintained at not less than 175°C. The flame ionization detector temperature should be maintained at least 25–50°C above the analysis temperature.
4. If baseline separation of the butyric acid and *n*-valeric acid peaks is not obtained, it may be found that the resolution on columns containing a phosphoric acid stationary phase can be improved by the injection of 1 μL quantities of 2.5% (w/v) phosphoric acid solution onto the column while it is heated at the analysis temperature.
5. If tailing of peaks is observed, even after conditioning of the column, this can sometimes be reduced or eliminated by injecting 2 μL of trimethylchlorosilane (TMCS) onto the column.

PROCEDURE

1. Construction of a calibration curve—
   (a) By means of graduated pipets (Apparatus, 8), transfer to individual test tubes (Apparatus, 7) 0.2, 0.5, 1.0, 2.0, 3.5, and 5.0 mL of butyric acid reference standard solution (Reagents, 4). To each test tube add by pipet (Apparatus, 8) 2.0 mL of *n*-valeric acid solution (Reagents, 5) and, respectively, 4.8, 4.5, 4.0, 3.0, 1.5, and 0 mL water. Stopper the test tubes and gently mix the solutions. The solutions in the test tubes contain, respectively, 0.08, 0.2, 0.4, 0.8, 1.4, and 2.0 mg of butyric acid and 0.5 mg of *n*-valeric acid.
   (b) Stabilize the column for at least 30 min at the analysis temperature.
   (c) Using a microsyringe (Apparatus, 5) inject approximately l μL of the prepared standard solutions in turn.
   (d) Measure the heights of the butyric acid and *n*-valeric acid peaks to the nearest 0.5 mm.
   (e) Calculate the peak height ratio (butyric acid/valeric acid) for each standard and plot against the corresponding weight of butyric acid. The attenuator on the gas chromatograph should be adjusted so that the height of the butyric acid peak for the highest butyric acid standard is about 80% full-scale recorder reading.
2. Determination of the butyric acid content—
   (a) Weigh accurately about 100 mg of test sample into a beaker (Apparatus, 6). Add, by pipet (Apparatus, 8), 3 mL of the ethanolic potassium hydroxide solution (Reagents, 2) and a few glass beads (Apparatus, 10). Cover the beaker with a watch glass (Apparatus, 11) and place in a boiling water bath (Apparatus, 12). Heat for at least 10 min, or until fat globules are no longer visible on the surface. Remove the watch glass and continue heating until the ethanol has completely evaporated. Allow the beaker to cool.
   (b) Add, by pipet (Apparatus, 8), 5.0 mL of water. Cover with a watch glass (Apparatus, 11) and swirl gently to completely dissolve the soap. It may be necessary to warm the mixture gently to achieve complete solution of the soaps.
   (c) Add, by pipet (Apparatus, 8), 5.0 mL of the phosphoric acid solution (Reagents, 3). Swirl gently to coagulate the precipitated fatty acids. Filter through a small, fluted, fast filter paper (Apparatus, 9). By pipet, transfer 5.0 mL of the filtrate to a test tube. Add, by pipet, 2.0 mL of the *n*-valeric acid internal standard solution (Reagents, 5). Stopper and mix.
   (d) Stabilize the column for at least 30 min at the analysis temperature.
   (e) By means of a microsyringe (Apparatus, 5), inject about 1 μL of the final solution onto the column. The syringe should be rinsed thoroughly with water between samples and, at the completion of analysis, it should be rinsed with a diluted soap solution in order to minimize any corrosion due to the phosphoric acid.
   (f) Measure, to the nearest 0.5 mm, the peak heights of butyric acid from the sample and the *n*-valeric acid added as an internal standard.
   Note—Peaks from caproic acid and caprylic acid may appear after the *n*-valeric acid in the chromatogram and interfere with subsequent analyses, if care is not taken to ensure that these acids have eluted before another sample solution is injected.
   (g) After a series of sample injections, it is recommended that injections of one or more of the solutions of butyric acid/*n*-valeric acid standards be made and the calibration curve be checked against the corresponding butyric acid/*n*-valeric acid peak height ratios obtained from the standard solutions.
3. Number of determinations—Carry out two determinations in rapid succession.

CALCULATIONS

1. Calculate the peak height ratio of butyric acid/*n*-valeric acid obtained from the analysis of the sample and read from the calibration curve the weight of butyric acid equivalent to the peak height ratio. The butyric acid content expressed as a percentage (w/w) of the sample is given by:

$$\frac{W(b) \times 200}{W(s)}$$

Where—
W(b) = the mass, in mg, of butyric acid read from the calibration curve
W(s) = the mass, in mg, of the test portion

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2. Report as the final result the mean of the results of the two determinations, provided the requirements for repeatability are met. If the requirements for repeatability are not met, discard the results and carry out an additional two determinations on the sample.

PRECISION

1. Repeatability—When the mean of the duplicate determinations lies between any two of the mean values shown in Table 1, the difference between the results of the two determinations, carried out in rapid succession by the same operator, using the same apparatus for the analysis of identical test material, should not be greater than the value indicated in Table 1 for the repeatability value (r) which corresponds to the higher of the two mean values.
2. Reproducibility—
   (a) When the means of the duplicate determinations, obtained in two different laboratories using this standard method for the analysis of identical test material, lie between any two of the mean values shown in Table 1, the difference between the mean results obtained by those laboratories should not be greater than the reproducibility value (R) which corresponds to the higher of the two mean values.
   (b) Table 1 summarizes the statistical analysis of data derived from the collaborative study carried out for the evaluation of this method (see References, 1).

NOTES

Caution

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dust or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

REFERENCES

1. Pocklington, W.D., and Hautfenne, A., *Pure Appl. Chem. 58* (10):1419 (1986).
2. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.310.

Table 1
Statistical analysis of results for butyric acid.

Level[a]	A	B	C	D
Number of laboratories	13	7	11	13
Number of accepted results	22	14	22	22
Mean value (%, w/w)	0.185	0.354	1.79	3.46
Repeatability standard deviation	0.008	0.015	0.044	0.102
Repeatability coefficient of variation, %	4.5	4.4	2.4	2.95
Repeatability value r (95) ISO 5725	0.02	0.04	0.12	0.29
Reproducibility standard deviation	0.024	0.056	0.155	0.242
Reproducibility coefficient of variation, %	12.9	15.9	8.7	7.0
Reproducibility value R (95) ISO 5725	0.07	0.16	0.44	0.69

[a]Levels A, B, C and D represent levels of butyric acid to be found in fats containing about 5, 10, 50, and 100% w/w butterfat, respectively.

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AOCS Recommended Practice Ca 5d-01

Revised 2011

Free Fatty Acids in Crude Vegetable Oils by Capillary Gas Chromatography

DEFINITION

Oil samples are silylated in a suitable solvent. Silylation is a very mild derivatization technique that primarily affects hydroxyl and carboxyl moieties. Fatty acids are derivatized to increase their volatility without glyceride hydrolysis. Derivatized fatty acids are then separated by gas chromatography and detected by flame ionization. The weight of each fatty acid is determined by using an internal standard and measuring the relative detector response for each fatty acid.

SCOPE

This method is applicable to most crude vegetable oils and other oil-bearing materials that contain free fatty acids.

APPARATUS

The gas chromatograph should have the following characteristics:

1. Split or split/splitless injector—operated in the split mode, independently heated, and capable of operating at 350-400°C.
2. Phenyl (5%)-methyl (95%) capillary column stable to 375°C (or 400°C on a temperature ramp). Recommended column size is 15 m × 0.25 mm i.d. × 0.1 mm coating thickness; however, other configurations may be acceptable depending on application.
3. Independently heated flame ionization detector—capable of operating at 350–400°C, and a method to record and integrate detector output.
4. Gases—high-purity hydrogen or helium for the carrier gas. Hydrogen, oxygen (air is acceptable), and nitrogen or helium (as make-up gas) for the flame ionization detector.

REAGENTS

1. Silylation-grade pyridine
2. Hexameyldisilazane (HMDS)
3. Trifloroacetic acid (TFA)
4. Cholesterol methyl ether (CAS 1174-92-1) as internal standard.

PROCEDURE

1. Approximately 100 mg of crude oil (1–5% free fatty acid) is accurately weighed into a sealable vial, followed by 1 mL of silylation-grade pyridine containing a known amount of internal standard (IS), 1 mL of hexamethyldisilazane (HMDS), and 100 mL of trifloroacetic acid (TFA). It is convenient to prepare the IS in pyridine and weight it into the vial as part of the solvent addition. Cholesterol methyl ether (CAS 1174–92-1) has been used successfully as an IS that does not coelute with most other crude oil components. However, other compounds are equally applicable after coelution is checked.
2. Solutions are capped and heated at 60°C for 30–45 min before chromatography. A single liquid phase should exist after heating (Notes, 1). Solutions are typically stable for at least 24–48 h.
3. One half to two microliters of derivatized test portion is injected into the GC inlet. The column temperature program is set to allow separation of the fatty acids and to allow elution of higher molecular weight glycerides. The following temperature program has yielded reasonable results: 100°C for 3 min, ramp to 150°C at 10°C/min, ramp to 250°C at 5°C/min, ramp to 360°C at 10°C/min, and hold at 360°C for 10 to 15 min. A starting temperature of 75°C is preferable for eluting C_8-C_{12} fatty acids. For peanut oil samples, a final temperature of 370°C is beneficial to eluting triglycerides containing longer chain fatty acids C_{20}-C_{24}. A split ratio of 1:50 and a 1 mL/min column flow rate are suitable starting conditions. The injector is operated typically at 340–360°C, and it is beneficial to place a silylated glass wool plug in the inlet liner to retain any non-volatile components. The detector is operated at 340–360°C.

CALCULATIONS

1. Fatty acid peaks are identified by comparing elution times with the elution times for silylated fatty acids standards (see Notes, below). Some fatty acid pairs may be difficult to separate, e.g., linolenic and oleic acids coelute on the 5% phenyl/95% methyl column. (The response factors for these coeluting pairs are often similar. Consequently, it is not unreasonable to consider that the sum of these acids is determined.)
2. The concentration of a fatty acid (C_{FA}) is determined from the mass of the fatty acid in the sample and the mass of sample weighed into the vial (Eq. 1). The mass of the unknown acid (M_{FA}) is determined by internal standardization (see Notes, 2)

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from the mass of IS solution added to the preparation (M_{ISS}), the IS concentration of this solution (C_{IS}), the IS area (A_{IS}), the fatty acid area (A_{FA}), and the relative response factor (R_{FA}) (Eq. 2).

$$C_{FA}\,(\%) = 100 \times M_{FA} / M_{oil} \qquad [1]$$

$$M_{FA} = A_{FA} \times C_{IS} \times M_{ISS} / A_{IS} \times R_{FA} \qquad [2]$$

DETERMINATION OF RESPONSE FACTORS

A response factor is needed for each silylated fatty acid of interest. These can be determined by preparing a solution of the IS in pyridine (mass of IS/mass of solution) and a solution of the fatty acid in pyridine (mass of fatty acid/mass of solution) (see Notes, 3). Both the fatty acid and IS solutions are weighted into a series of vials in different ratios; HMDS and TFA are added in proportions similar to those described above, and the solution is heated as described. After cooling the silylated standards, each sample is analyzed and the peak areas of the IS and fatty acid of interest are determined. The ratio of the masses (fatty acid/IS) and areas (fatty acid/IS) are calculated and plotted with the mass ratio on the coordinate and the area ratio on the abscissa. The slope of this plot is the response factor. Note that it is often possible to determine the response factors for several non-coeluting fatty acids simultaneously. (See Notes 4 and 5.)

PRECISION

The procedure above has been used to analyze the ten different crude vegetable oils containing various free fatty acid levels from 0.5–9.0%. The response factors for the important C_{16} and C_{18} fatty acids were similar and varied by less than 2%. The lower carbon acids had slightly lower response factors. A typical chromatogram of cottonseed oil derived with the conditions above is shown in Figure 1.

Values determined by GC and by titration for a series of crude oil samples are given in Table 1. For this series of samples, the method correlated well with determinations made by titration (Pearson R = 0.997). Values measured by the method tended to be 5-10% lower than values determined by titration. Coefficient of variance for repeated determinations of a soybean sample by the method was <2.5% (n = 6), which was comparable to the coefficient of variance for repeated determinations on the same sample by titration.

NOTES

1. Initial mixture of the crude oil sample and reagents may be cloudy or heterogeneous. A single liquid phase should result after heating at 60°C for 30–45 minutes.
2. For a review of internal standardization, see M.A. Kaiser and F.J. Debbrecht, "Qualitative and Quantitative Analysis by Gas Chromatography," Chap. 4, in *Modern Practices in Gas Chromatography,* edited by R.L. Grob, John Wiley and Sons, New York, pp. 151–211.
3. The exact concentration of the IS solution is not needed if the same IS solution is used both for determining the response factors and analyzing the samples.
4. Once the relative detector response is determined to be linear, only a single point is needed to estimate the response factor.
5. Because silica from the silyl compounds can build up on the surfaces of the detector, detector responses can change over time and should be redetermined periodically. The detector surfaces should be cleaned periodically.

REFERENCES

1. Wan, P.J., and M.K. Dowd, Free Fatty Acid Determination by Trimethylsilylation and Gas Chromatography.
2. Dowd, M.K., Gas Chromatographic Characterization of Soapstocks from Vegetable Oil Refining, *J. Chromatogr.* A 816:185–193 (1998).
3. Wan, P.J., and M.K. Dowd, Comparative Study of the Extraction and Measurement of Cottonseed Free Fatty Acids, *J. Am. Oil Chem. Soc.* 77:23–27 (2000).

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Table 1
Free fatty acid (FFA, %) in crude vegetable oil determined by capillary gas chromatography and titration.

	Results by capillary gas chromatography				
Oil sample	Replicate	Mean FFA (%)	Std. dev.	Relative std. dev.	FFA (%) by titration
Canola	3	0.640	0.003	0.47	1.10
Coconut	3	2.961	0.064	2.16	3.64
Corn	3	1.263	0.005	0.40	1.80
Cottonseed-C	3	1.261	0.016	1.27	1.90
Cottonseed-1	3	2.376	0.159	6.69	2.70
Cottonseed-D	3	1.842	0.016	0.87	2.20
Peanut	3	2.035	0.011	0.54	2.23
Rice bran	3	4.310	0.019	0.44	6.20
Safflower	3	0.601	0.006	1.00	0.87
Soybean-1	3	0.875	0.008	0.91	0.40
Soybean-2	3	0.731	0.014	1.92	1.06
Soybean-3	3	1.374	0.039	2.84	1.94
Sunflower	3	0.883	0.012	1.36	1.30
Wheat germ	3	8.251	0.063	0.76	9.05

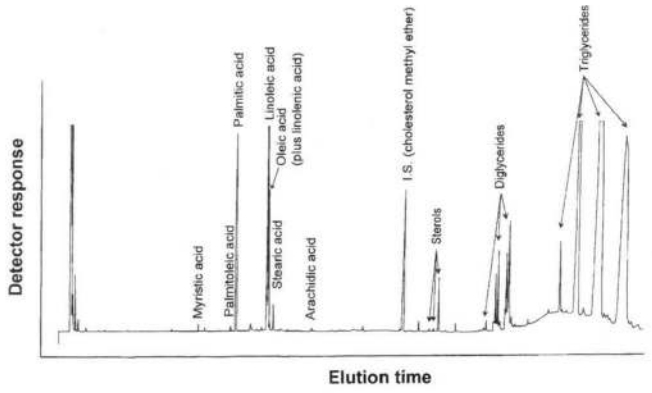

Figure 1. Chromatogram of silylated crude cottonseed oil.

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AOCS Recommended Practice Ca 5e-13

Approved 2013

Butyric Acid

DEFINITION

The butyric acid content of milkfat, butterfat, or mixtures of fats containing milkfat and butterfat is the quantity, expressed as a percentage by weight, of butyric acid determined in the sample by the following method. Saponification of the fat with potassium hydroxide (KOH) solution is followed by acidification with phosphoric acid to liberate the fatty acids. Water-soluble fatty acids are separated by filtration and direct determination of butyric acid is carried out by gas-liquid chromatography in the presence of an internal standard.

SCOPE

This method is applicable to the determination of the butyric acid content of milkfat or butterfat, or mixtures of fats containing milkfat or butterfat.

APPARATUS

1. *Gas Chromatograph*—Any gas chromatograph having a flame ionization detector (FID) and a split/splitless injector system suitable for use with a fused silica capillary column may be used, provided the system has sufficient sensitivity, linearity, and range to obtain a minimum peak height response for a 0.01% peak of five times the height of the signal background noise, while not exceeding the full scale of either the detector or the electronic integration for the highest peak. The split injection system shall not discriminate over the boiling range of the samples analyzed. The system shall be capable of operating at the conditions given in the Instrument Parameters.

   Instrument Parameters

   Column 30m × 0.25 mm ID SPB-1000 (bonded FFAP like phase) fused silica capillary, internally coated to a 0.25-µm thickness. Alternative column dimensions and stationary phases may be used provided that suitable separation can be assured through validation experiments.
   Carrier gas: helium
   Flow, linear velocity set at 155°C, 25cm/second
   Split ratio 50:1
   Detector gases—As prescribed by the instrument manufacturer.
   Sample size, 1.0µL
   Temperatures
   Injector, 250°C
   Detector, 250°C
   Column: 155°C Isothermal (15 minutes or until after caproic acid elutes from the column)
2. Electronic integration with tangent capabilities is recommended.
3. Chromatographic column—30 meters × 0.25mm ID × 0.25µm df SPB-1000 (or bonded FFAP equivalent phase) fused silica capillary column. The column should be conditioned at approximately 200°C for at least 10 minutes prior to use.
4. Syringe—1 µL capacity (a 5 or 10 µL syringe is acceptable).
5. Beaker—50 mL.
6. Test tubes—with ground-glass stoppers, 10 mL.
7. Pipets—2–5 mL, graduated.
8. Fast filter paper.
9. Glass beads.
10. Watch glasses.
11. Water bath—maintained at boiling temperature.

REAGENTS

1. Gases—
   (a) Carrier—helium (preferred for better resolution) or nitrogen, minimum purity 99.99 mol%, dried and containing a maximum of 10 mg O/kg.
   (b) Flame ionization detector—hydrogen, minimum purity 99.99 mol%; air, dry (dew point –59°C maximum) and hydrocarbon free (< 2 ppm hydrocarbons equivalent to CH_4).

   Flame Support Gas—Air, total, hydrocarbon less than 2 ppm.
   Make Up Gas—Nitrogen, total, hydrocarbon less than 2 ppm.
2. Potassium hydroxide (KOH) solution—approximately 0.5 N in ethanol, prepared by dissolving 4.5 g KOH in 100 mL of 95% ethanol (see Notes, *Caution*).

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3. *ortho*-Phosphoric acid—5%, w/v, aqueous solution.
4. *n*-Butyric acid—reference standard, 0.4 mg/mL aqueous solution, prepared by dissolving 400 mg *n*-butyric acid in 100 mL water and diluting 10 mL of this solution to 100 mL. This solution must be freshly prepared for each analysis.
5. *n*-Valeric acid—internal standard, 0.25 mg/mL aqueous solution, prepared by dissolving 250 mg *n*-valeric acid in 100 mL water and diluting 10 mL of this solution to 100 mL. This solution must be freshly prepared for each analysis.

GC OPERATING CONDITIONS

1. Carrier gas flow depends on the carrier gas and column being used for the analysis and should be optimized accordingly (a retention time of about 5 min for butyric acid is desirable). Helium is recommended, but nitrogen may be used.
2. The flow of hydrogen and air or oxygen to the FID should be optimized according to the manufacturer's recommendations for the instrument being used.
3. The analysis temperature should be approximately 155°C. A split injection is used to introduce the sample into the column.
4. Baseline separation of the butyric acid and *n*-valeric acid peaks must be obtained with sharp symmetrical shaped peaks.

PROCEDURE

1. Construction of a calibration curve—
   (a) By means of graduated pipets (Apparatus, 8), transfer to individual test tubes (Apparatus, 7) 0.2, 0.5, 1.0, 2.0, 3.5, and 5.0 mL of butyric acid reference standard solution (Reagents, 4). To each test tube add by pipet (Apparatus, 8) 2.0 mL of n-valeric acid solution (Reagents, 5) and, respectively, 4.8, 4.5, 4.0, 3.0, 1.5, and 0 mL water. Stopper the test tubes and gently mix the solutions. The solutions in the test tubes contain, respectively, 0.08, 0.2, 0.4, 0.8, 1.4, and 2.0 mg of butyric acid and 0.5 mg of *n*-valeric acid.
   (b) Stabilize the column for at least 30 min at the analysis temperature.
   (c) Using a microsyringe (Apparatus, 5) inject approximately l µL of the prepared standard solutions in turn.
   (d) Measure the heights of the butyric acid and *n*-valeric acid peaks to the nearest 0.5 mm.
   (e) Calculate the peak height ratio (butyric acid/*n*-valeric acid) for each standard and plot against the corresponding weight of butyric acid. The attenuator on the gas chromatograph should be adjusted so that the height of the butyric acid peak for the highest butyric acid standard is about 80% full-scale recorder reading.
2. Determination of the butyric acid content—
   (a) Weigh accurately about 100 mg of test sample into a beaker (Apparatus, 6). Add, by pipet (Apparatus, 8), 3 mL of the ethanolic potassium hydroxide solution (Reagents, 2) and a few glass beads (Apparatus, 10). Cover the beaker with a watch glass (Apparatus, 11) and place in a boiling water bath (Apparatus, 12). Heat for at least 10 min, or until fat globules are no longer visible on the surface. Remove the watch glass and continue heating until the ethanol has completely evaporated. Allow the beaker to cool.
   (b) Add, by pipet (Apparatus, 8), 5.0 mL of water. Cover with a watch glass (Apparatus, 11) and swirl gently to completely dissolve the soap. It may be necessary to warm the mixture gently to achieve complete solution of the soaps.
   (c) Add, by pipet (Apparatus, 8), 5.0 mL of the phosphoric acid solution (Reagents, 3). Swirl gently to coagulate the precipitated fatty acids. Filter through a small, fluted, fast filter paper (Apparatus, 9). By pipet, transfer 5.0 mL of the filtrate to a test tube. Add, by pipet, 2.0 mL of the *n*-valeric acid internal standard solution (Reagents, 5). Stopper and mix.
   (d) Stabilize the column for at least 30 min at the analysis temperature.
   (e) By means of a microsyringe (Apparatus, 5), inject about 1 µL of the final solution onto the column. The syringe should be rinsed thoroughly with water between samples and, at the completion of analysis, it should be rinsed with a diluted soap solution in order to minimize any corrosion due to the phosphoric acid.
   (f) Measure, to the nearest 0.5 mm, the peak heights of butyric acid from the sample and the *n*-valeric acid added as an internal standard.
   Note—Peaks from caproic acid and caprylic acid may appear after the *n*-valeric acid in the chromatogram and interfere with subsequent analyses, if care is not taken to ensure that these acids have eluted before another sample solution is injected.
   (g) After a series of sample injections, it is recommended that injections of one or more of the solutions of butyric acid/*n*-valeric acid standards be made and the calibration curve be checked against the corresponding butyric acid/*n*-valeric acid peak height ratios obtained from the standard solutions.
3. Number of determinations—Carry out two determinations in rapid succession.

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CALCULATIONS

1. Calculate the peak height ratio of butyric acid/*n*-valeric acid obtained from the analysis of the sample and read from the calibration curve the weight of butyric acid equivalent to the peak height ratio. The butyric acid content expressed as a percentage (w/w) of the sample is given by:

$$\frac{W(b) \times 200}{W(s)}$$

Where—

W(b) = the mass, in mg, of butyric acid read from the calibration curve
W(s) = the mass, in mg, of the test portion

2. Report as the final result the mean of the results of the two determinations.

METHOD PERFORMANCE

Precision of the method has not been established.

NOTES

Caution

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dust or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

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AOCS Official Method Ca 6a-40

Revised 2011

Unsaponifiable Matter

DEFINITION

Unsaponifiable matter includes those substances frequently found dissolved in fats and oils, which cannot be saponified by the usual caustic treatment, but are soluble in ordinary fat and oil solvents. Included in this group of compounds are higher aliphatic alcohols, sterols, pigments, and hydrocarbons.

SCOPE

Applicable to normal animal and vegetable fats and oils. This method is not suitable for fats and oils containing an excessive amount of unsaponifiable matter, such as marine oils. This method does not apply to feed-grade fats.

APPARATUS

1. Extraction cylinder—graduated, with glass stopper, capacity about 200 mL (see Notes, 1).
2. Erlenmeyer flasks or Soxhlet flasks—100 to 200 mL capacity.
3. Separatory funnels—500 mL.
4. Glass siphon—See Procedure, 3 and Notes, 1.

REAGENTS

1. Ethyl alcohol 95%—USSD formulas 30 and 3A are permitted (see Notes, *Caution*).
2. Aqueous potassium hydroxide (KOH)—50% by weight, prepared by dissolving 60 g of reagent-grade KOH in 40 mL of distilled water with cooling (see Notes, *Caution*).
3. Petroleum ether—see AOCS Specification H 2-41 (see Notes, *Caution*).
4. Sodium hydroxide (NaOH) solution, 0.02 M—accurately standardized. See AOCS Specification H 12-52.
5. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol.

PROCEDURE

1. Accurately weigh about 5 g ± 0.1 mg of well-mixed test sample into an Erlenmeyer flask or Soxhlet flask. Add 30 mL of 95% ethyl alcohol and 5 mL of 50% KOH solution. Boil gently, but steadily, under reflux for 1 hr, or until completely saponified. Complete saponification is essential.
2. Transfer to the extraction cylinder (see Notes, 1) and wash to the 40 mL mark with 95% ethyl alcohol. Complete the transfer with warm and then cold distilled water until the total volume is 80 mL. Wash out the flask with a small volume (5 mL) of petroleum ether and add to the cylinder. Cool the contents of the cylinder to room temperature (20–25°C) and then add 50 mL of petroleum ether.
3. Insert the stopper, shake vigorously for at least 1 min, and allow to settle until both layers are clear. Use a glass siphon to remove the upper layer as completely as possible without including any of the lower portion (see Notes, 1).
4. The petroleum ether fractions are combined in a 500 mL separatory funnel.
5. Repeat the extraction at least six times, using 50 mL portions of petroleum ether each time and shaking vigorously with each extraction (see Notes, 2).
6. Wash the combined extracts in the separatory funnel three times, using 25 mL portions of 10% ethyl alcohol in distilled water, shaking vigorously and drawing off the aqueous alcohol layer after each extraction. Avoid removing any of the petroleum ether layer.

   Continue to wash with the 10% ethyl alcohol solution until the wash solution no longer gives a pink color after the addition of one drop of phenolphthalein solution (Reagents, 5) (see Notes, 3).
7. Transfer the petroleum ether extract to a tared beaker and evaporate to dryness on a water bath, using a gentle stream of clean, dry nitrogen. After all of the solvent has evaporated, complete the drying to constant weight in a vacuum oven at 75–80°C and an internal pressure of not more than 200 mm of mercury. Cool in a desiccator and weigh. The result becomes "A" in the calculation.
8. After weighing, take up the residue in 50 mL of warm (50°C) 95% alcohol, containing phenolphthalein indicator previously neutralized to the phenolphthalein end point. Titrate with 0.02 M NaOH to the same final color. Correct the weight of the residue (see Notes, 3) for free fatty acid content, using the following relationship:

   1 mL of 0.02 M NaOH is equivalent to 0.0056 g of oleic acid.

   The grams of fatty acid determined by this titration become "B" in the calculation for determining percent unsaponifiable matter (see Notes, 3). A reagent blank correction should also be determined.
9. Correct for any reagent blank by conducting the unsaponifiable matter procedure without any fat or oil present. The blank determined by this procedure becomes "C" in the calculations.

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CALCULATIONS

1. Unsaponifiable matter, $\% = \frac{A - (B + C)}{\text{mass of test portion, g}} \times 100$

   Where—

   A = mass of residue, g

   B = mass of fatty acids, g

   C = mass of blank, g

PRECISION

1. There are no statistics from a collaborative study of AOCS Official Method Ca 6a–40. The following values for reproducibility standard deviation (S_R), percent relative standard deviation (RSD%), and reproducibility (R) were obtained from 1985–1986 Smalley Check Sample Program results for unsaponifiable values using AOCS Official Method Ca 6a–40.

Sample	No. of analyses	Outliers %	Mean, unsap.	S_R	RSD%	R
1	49	2	0.37	0.128	34.23	0.36
2	51	3	0.36	0.097	27.25	0.28
3	47	0	0.38	0.108	28.13	0.31
4	47	2	0.40	0.088	22.06	0.25
5	47	2	0.39	0.075	19.48	0.21

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1-6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Alternately, a 500 mL separatory funnel may be substituted for the extraction cylinder, eliminating the need for the siphon. If a separatory funnel is substituted, draw off the lower aqueous layer into another separatory funnel, retaining the petroleum ether extract in the first funnel. Repeat the petroleum ether extraction of the aqueous phase as noted in Procedure, 5, combining all of the petroleum ether extracts in the first funnel.
2. There are some cases in which seven extractions may not be sufficient. This is best judged by making another extraction and separately evaporating this extract as noted in Procedure, 7. There should be no unsaponifiable matter in this extract. If there is, dissolve in a small volume of petroleum ether and add back to the combined extracts. Continue with the extractions until no unsaponifiable matter remains in the extract.
3. The titration correction for extractable free fatty acids and other extractable unsaponifiable impurities (both reported as oleic acid) will tend to increase as the crude nature of the sample increases. For example, highenergy fats (used in animal feeds), feed fats, tall oil, and foots, would be expected to give a higher free fatty acid titration than relatively pure refined, bleached, and deodorized (RBD) oil.

   Historically, in the AOCS method, the correction for extractable free fatty acids has been determined by titration of the unsaponifiable residue. There is an indication that the quantity of free fatty acids determined in this manner may be lower than the actual quantity present, due to the fact that some of the free fatty acids may have been converted to soaps and therefore would not be titrated. This can be verified by determining the total free fatty acids in the unsaponifiable residue by GLC such as AOCS Recommended Practice Cd 5d-01. If soaps are present, they may be eliminated by repeated washing of the combined extracts (Procedure, 6) with water until neutral washings are obtained.

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AOCS Official Method Ca 6b-53

Revised 2011

Unsaponifiable Matter

DEFINITION

Unsaponifiable matter includes those substances frequently found dissolved in fats and oils that cannot be saponified by the usual caustic treatment, but are soluble in ordinary fat and oil solvents. Included in this group of compounds are higher aliphatic alcohols, sterols, pigments, and hydrocarbons.

SCOPE

Applicable to fats and oils containing higher levels of unsaponifiable matter than usually found in normal tallows and greases. This method is especially suited for marine oils, but is also applicable to vegetable oil deodorizer distillates and sludges. This method does not apply to feed-grade fats.

APPARATUS

1. Extraction cylinder—graduated, with glass stopper, capacity about 200 mL (see Notes, 1).
2. Erlenmeyer flasks—narrow mouth, 250 mL capacity, with *S* 24/40 outer joint.
3. Separatory funnels—250 mL.
4. Glass siphon—see Procedure, 3 and Notes, 1.
5. Condenser with *S* 24/40 joint to fit Erlenmeyer flasks. Either water- or air-cooled condensers may be used.
6. Beakers—250 mL.
7. Erlenmeyer flasks, or flat-bottom extraction flasks—50 mL.

REAGENTS

1. Ethyl alcohol, 95%—USSD formulas 30 and 3A are permitted (see Notes, *Caution*).
2. Aqueous potassium hydroxide (KOH), 50% by weight—prepared by dissolving 60 g of reagent-grade KOH in 40 mL of distilled water with cooling (see Notes, *Caution*).
3. Aqueous potassium hydroxide (KOH) solution, 0.5 M, prepared by dissolving 30 g of reagent grade KOH in water, cooling and diluting to 1 liter (see Notes, *Caution*).
4. Sodium hydroxide (NaOH) solution, 0.02 M—accurately standardized. See AOCS Specification H 12-52.
5. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol.
6. Diethyl ether—reagent grade, free from peroxides (see Notes, *Caution*).
7. Acetone—reagent grade (see Notes, *Caution*).

PROCEDURE

1. Accurately weigh about 2.0–2.5 g ± 0.1 mg of well-mixed test sample into a 250 mL Erlenmeyer flask with ground-glass joint. Add 25 mL of 95% ethyl alcohol and 1.5 mL of 50% KOH solution. Boil gently but steadily under reflux, with occasional swirling, for 30 min or until completely saponified. Complete saponification is essential. No loss of alcohol should occur during saponification.
2. Transfer while warm to the extraction cylinder (see Notes, 1), using a total of 50 mL of water. Wash the flask with 50 mL of diethyl ether and add to the cylinder. Cool the contents of the cylinder to room temperature (20–25°C).
3. Insert the stopper and shake vigorously for at least 1 min, and allow to settle until both layers are clear. Use a glass siphon to remove the upper layer as completely as possible without including any of the lower portion (see Notes, 1). Transfer the diethyl ether layer to a 250 mL separatory funnel.
4. Repeat the extraction two more times, using 50 mL portions of diethyl ether each time and shaking vigorously with each extraction (see Notes, 2).
5. Rotate the combined diethyl ether extracts gently with 20 mL of water. Violent agitation at this step may result in emulsions that are difficult to break. Allow the layers to separate completely and draw off the lower aqueous layer. Wash the diethyl ether layer two more times, using 20 mL of water each time, shaking gently each time and discarding the lower aqueous layer after separation.
6. Wash the combined extracts in the separatory funnel three times, using 20 mL portions of 0.5 M KOH, shaking vigorously. Follow each alkali washing by washing with 20 mL of water. If an emulsion forms during this washing procedure, allow to separate as much as possible, discard the clear aqueous layer, and proceed with the next step, leaving any emulsion in the separatory funnel with the diethyl ether layer. After the third washing with 0.5 M KOH, wash the diethyl ether with successive 20 mL portions of water until the washings are no longer alkaline to phenolphthalein.
7. Transfer the diethyl ether extract to a tared beaker, rinsing the separatory funnel and its pouring edge with diethyl ether and adding the rinsings to the solution in the beaker. Evaporate to dryness in a water bath, using a gentle stream of clean, dry nitrogen. When almost all of the diethyl ether has been evaporated, add 2–3 mL of acetone and remove all traces of solvent

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with the aid of a stream of nitrogen. Complete the drying to constant weight in a vacuum oven at 75–80°C and an internal pressure of not more than 200 mm of mercury. Cool in a desiccator and weigh. The result becomes "A" in the calculations.

8. After weighing, take up the residue in 2 mL of diethyl ether and then add 10 mL of 95% alcohol, containing phenolphthalein indicator and previously neutralized to the phenolphthalein end point. Titrate with 0.02 M NaOH to the same final color. Correct the weight of the residue (see Notes, 3) for free fatty acid content, using the following relationship: 1 mL of 0.02 M NaOH is equivalent to 0.0056 g of oleic acid. The grams of fatty acid determined by this titration become "B" in the calculations. A reagent blank correction should be determined.
9. Correct for any reagent blank by conducting the unsaponifiable matter procedure without any fat or oil present. The blank determined by this procedure becomes "C" in the calculations.

CALCULATIONS

1. Unsaponifiable matter, $\% = \frac{A - (B + C)}{\text{mass of test portion, g}} \times 100$

Where—

A = mass of residue, g

B = mass of fatty acid, g

C = mass of blank, g

PRECISION

For vegetable oil deodorizer distillates and sludges—

1. Two single determinations performed in the same laboratory should not differ by more than 2.6%.
2. Two single determinations performed in different laboratories should not differ by more than 3.8%.

For other fats and oils see Numbered Notes, 4.

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Acetone is highly flammable. It may form explosive peroxides with oxidizing agents. Use effective fume-removal device. Do not mix with chloroform.

NUMBERED NOTES

1. Alternately, a 500 mL separatory funnel may be substituted for the extraction cylinder, eliminating the need for the siphon. If a separatory funnel is substituted, draw off the lower aqueous layer into another separatory funnel, retaining the diethyl ether extract in the first funnel. Repeat the diethyl ether extraction of the aqueous phase, as noted in Procedure, 4, combining all of the diethyl ether extracts in the first funnel.
2. Some fats high in unsaponifiable matter, especially those of marine origin, may require more than three extractions for complete removal of the unsaponifiable matter. This is best judged by making another extraction and separately evaporating this extract as noted in Procedure, 7. There should be no unsaponifiable matter in this extract. If there is, dissolve in a small volume of diethyl ether and add back to the combined extracts. Continue with the extractions until no unsaponifiable matter remains in the extract.
3. The titration correction for extractable free fatty acids and other extractable unsaponifiable impurities (both reported as oleic acid) will tend to increase as the crude nature of the sample increases. For example, highenergy fats (used in animal feeds), feed fats, tall oil, and foots would be expected to give a higher free fatty acid titration than relatively pure refined, bleached and deodorized (RBD) oil.
4. ISO 3596 recommends the use of samples up to 5.0 g and adjusting solution volumes to 50 mL KOH/ethanol and doubling the volumes of diethyl ether and washing solutions. Precision values using these conditions are presented in Tables 1-3.

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Table 1
Test organized by FOSFA International in June 1995.

	Soybean oil[a]	
	A	B
No. of participating laboratories after eliminating outliers[b]	49	50
Mean value, % (by mass)	0.58	0.69
Repeatability standard deviation, s_r, %	0.025	0.027
Repeatability limit r ($2.8 \times s_r$), %	0.07	0.08
Coefficient of variation of repeatability, %	4.3	3.9
Reproducibility standard deviation, s_R, %	0.22	0.24
Reproducibility limit R ($2.8 \times s_R$), %	0.62	0.67
Coefficient of variation of reproducibility, %	37.9	34.7

[a]Sample A is refined, bleached, deodorized soybean oil, Sample B is dried, crude water-degummed soybean oil.
[b]Collaborative test involving 51 laboratories in 16 countries.

Table 2
Test organized by the FOSFA International.

	Fish oil
No. of participating laboratories after eliminating outliers[a]	37
Mean value, % (by mass)	0.81
Repeatability standard deviation, s_r, %	0.02
Repeatability limit r ($2.8 \times s_r$), %	0.06
Coefficient of variation of repeatability, %	2.46
Reproducibility standard deviation, s_R, %	0.29
Reproducibility limit R ($2.8 \times s_R$), %	0.81
Coefficient of variation of reproducibility, %	35.8

[a]Collaborative test involving 43 laboratories in 17 countries.

Table 3
Test organized by IUPAC between 1976 and 1997.

	Refined soybean oil	Refined tallow	Crude rapeseed oil
No. of participating laboratories after eliminating outliers[a]	10	10	10
Mean value, % (by mass)	0.630	0.253	1.432
Repeatability standard deviation, s_r, %	0.032	0.089	5.0
Repeatability limit r ($2.8 \times s_r$), %	0.024	0.067	9.3
Coefficient of variation of repeatability, %	0.068	0.19	24.7
Reproducibility standard deviation, s_R, %	0.140	0.397	22.3
Reproducibility limit R ($2.8 \times s_R$), %	0.154	0.435	60.9
Coefficient of variation of reproducibility, %	0.137	0.389	9.6

[a]International collaborative test involving 10 laboratories.

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AOCS Official Method Ca 6c-65

Reapproved 2009

Hydrocarbons (Mineral Oil)

DEFINITION

The sample in solvent is passed through a chromatographic column and the hydrocarbons recovered from the effluent by removal of solvent.

SCOPE

Applicable to the determination of saturated hydrocarbons in glycerides. This is a joint AOCS-Association of Official Analytical Chemists (AOAC) method (References, 1 and 2).

APPARATUS

1. Flasks—Erlenmeyer, 125 and 500 mL.
2. Flasks—Soxhlet, 250 mL.
3. Glass wool.
4. Chromatographic column—with Teflon™ stopcock, 25–35 mm diameter and 400 mm length.

REAGENTS

1. Aluminum oxide—activated alumina, grade F-20, 80–200 mesh. Available as Alumina N.O.L. from Midland Scientific, Inc., Omaha, NE, USA.

   Note—Dry the alumina for a minimum of 4 hr at 200°C before using. Store in sealed bottles in a desiccator. The alumina must have a moisture content of 0–3% to retain glycerides.
2. Sodium sulfate—anhydrous, reagent grade.
3. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PROCEDURE

1. Weigh 10 ± 0.01 g of melted, well-mixed test sample into a 125 mL Erlenmeyer flask (see Notes, 1).
2. Dissolve in 50–100 mL of petroleum ether, warming gently, if necessary.
3. Prepare a column by tamping a plug of glass wool into the bottom of the chromatographic column in such a manner that some of the glass wool is in the constricted portion above the stopcock. Fill the column about two-thirds full with petroleum ether and add 200 g of alumina through a powder funnel. Tap the side of the column to aid in packing. Cover with about 1.5 cm of anhydrous sodium sulfate. Fix the elution rate at 80–90 drops/min (3.0–3.5 mL) using the stopcock for control.
4. When the solvent level is reduced to about 1 cm above the packing, add the sample solution.
5. Allow the solvent to flow until about 1 cm of head remains.
6. Rinse the sample flask with 50 mL of petroleum ether and add to the column. Repeat three times, adding each washing to the column when the solvent head is reduced to about 1 cm and washing the sides of the column during each transfer.
7. Continue adding petroleum ether to the column until a total of 400 mL has passed through the column.

   Note—An inverted volumetric flask can serve as a convenient reservoir for this final addition of solvent.
8. Collect the eluate in a 500 mL Erlenmeyer flask.
9. Place on a steam bath and reduce the volume to 50–75 mL. A stirring rod placed in the flask will help prevent superheating and subsequent boiling over. A gentle stream of clean, dry nitrogen will aid in solvent removal.
10. Transfer to a tared 250 mL Soxhlet flask, rinsing with three 20 mL portions of petroleum ether to effect the transfer.
11. Remove the remaining solvent by evaporation on a warm surface employing a gentle stream of nitrogen and keeping the solution below the boiling point.
12. Cool to room temperature in a desiccator, and weigh the residue on an analytical balance.
13. Repeat steps 11 and 12, with additional heating periods of 20 min, until change in residue weight is less than 0.5 mg (see Notes, 2).
14. Conduct a blank determination, following steps 2 through 13.

CALCULATIONS

1. $$\text{Hydrocarbons, \%} = \frac{A - B}{C} \times 100$$

   Where—

   A = mass of sample residue, g

   B = mass of blank residue, g

   C = mass of test portion, g

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PRECISION

1. Collaborative studies have shown that the 95% confidence limits noted in Table 1 may be expected.

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. This procedure can be adapted to smaller quantities, but recoveries of hydrocarbon and retention of glycerides must be evaluated under the new test conditions.
2. Further identification of residue is possible by comparison of spectra with those of pure hydrocarbons.

REFERENCES

1. *Official Methods of Analysis,* AOAC International, 16th ed., Gaithersburg, MD, 1990, p. 980, Method 966.17.
2. *J. Am. Oil Chem. Soc. 42:*525A (1965).

Table 1
Within-lab and between-labs variation that can be expected for various hydrocarbon levels.

	Hydrocarbon content, % at levels of			
	0.00	0.05	0.5	5.0
Two single determinations performed in one laboratory should not differ more than:	0.06	0.09	0.14	0.33
Single determinations performed in different laboratories should not differ more than:	0.06	0.10	0.14	0.40

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AOCS Official Method Ca 9a-52

Revised 2011

Refining Loss

DEFINITION

This method determines the loss of free fatty acids, oil, and impurities when the sample is treated with alkali solutions under the specific conditions of the test.

SCOPE

Applicable to crude peanut oil, crude coconut oil, crude corn oil, crude sunflower oil, crude soybean oil (expeller and hydraulic), extracted soybean oil, degummed hydraulic and extracted of soybean oil, degummed expeller-type soybean oil, and crude cottonseed oil (expeller and hydraulic). Also applicable to solvent-extracted crude cottonseed oil and to the reconstituted whole mixture of crude cottonseed oil from prepressing and subsequent solvent processing of cottonseed. This method is not applicable to crude cottonseed oil extracted from prepressed cake by solvent. Includes Ca 9b–52, Ca 9c–52, Ca 9d–52, and Ca 9e–52.

APPARATUS

1. Balance—torsion type, or equivalent, capacity 1000 g and sensitive to 0.1 g.
2. Balance weights—0.1-500 g.
3. Refining cups—stainless steel, seamless or enameled iron with handle, 10–10.5 cm (4–4.5 in.) diameter, 10–10.5 cm (4–4.5 in.) deep, total capacity about 900 mL. Refining cups are available from AOCS Headquarters.
4. Refining apparatus—constructed according to AOCS specifications and built on order by the Barrow-Agee Laboratories, Memphis, TN, USA.
   (a) The bath may be constructed to hold 6, 12, 18, or 24 cups.
   (b) The bath is provided with cup supports arranged so as to hold the cups in a fixed position.
   (c) The bath is provided with drain and hot and cold water. It may be heated with steam, gas, or electricity. The size of drain and accommodations for heating must be such that the change from cold to hot temperature can be made in 1 min. Separate baths may be used for the hot and cold water, if desired.
   (d) The bath is provided with mechanical stirring arrangement which will provide agitation for the samples at 70 ± 5 rpm and 250 ± 10 rpm.
   (e) The water in the bath must be continually agitated to ensure uniform temperature, and the level of water in the bath must be as high as the level of oil and alkali within the refining cups.
   (f) T-shaped paddles constructed of stainless steel or nickel-plated copper or brass (heavily coated) with 2.5 × 9 cm (1 × 3.5 in.) blades set at right angles to the shaft of the paddles are required for stirring the contents of the refining cups (see Fig. 1).
   (g) The cup supports should be rigid enough so that the bottom of each paddle is 63 mm (0.25 in.) above the bottom of the refining cups at all times during the test and under all variations of loading.
   (h) The paddles are driven through gears by a motor of sufficient capacity to drive all paddles simultaneously at specified speeds under maximum load.
5. Cooling bath—air or water, of sufficient size and cooling capacity (for soybean oils).
6. Filter paper—32 cm (12.5 in.); Reeve Angel no. 871, Eaton & Dikeman no. 2V or S & S no. 596.
7. Diatomaceous earth—Manville Hyflo Supercel, or equivalent.

REAGENTS

1. Sodium hydroxide, 0.25 M—accurately standardized. See AOCS Specification H 12-52.
2. Sodium hydroxide (NaOH) solutions (see Notes, *Caution*)—These must be of accurately known NaOH content, free from carbonate and other impurities, and prepared as follows: Add 750 g of distilled water to 1000 g of pure, dry, solid NaOH (see Notes, *Caution*). Heat on the steam bath with occasional stirring for at least 3 hr. Cool and allow to settle for 24–48 hr, keeping the vessel tightly covered to exclude carbon dioxide (CO_2). During cooling, a portion of the NaOH which was dissolved in the hot solution will crystallize and precipitate. If the crystals do not separate, the solution was not supersaturated and may not be satisfactory. If properly prepared as described, the solution will contain no measurable quantity of carbonate or other impurities. Allow to settle until clear and then carefully decant. Filter through filter paper if the solution is not perfectly clear and protect from the air during filtration to prevent the absorption of CO_2. This solution is then diluted with previously boiled and cooled distilled water to the desired concentrations (see Table 1). The actual strength of each solution used for the defining test must be determined by weighing and titrating with standard acid. Specific gravity or Baumé (Bé) tests are not sufficiently accurate.

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PREPARATION OF TEST SAMPLE

1. The laboratory sample container must be shaken vigorously and the sample thoroughly mixed in order to incorporate and uniformly distribute meal or other sediment. For most oils, heat to 20°C if the oil is cold; heat soybean oil to 50°C and coconut oil to 38°C until completely melted before shaking. Inspect the inside of the can to be sure that no sediment remains clinging to the sides or bottom. If any sediment is found, remove it completely (cut the can open if necessary) and incorporate thoroughly with the oil.

   Note—The uniform incorporation and distribution of settlings and suspended matter is very significant in determining the accuracy of the final refining loss.

DETERMINATION OF FREE FATTY ACIDS

1. Determine the free fatty acids as directed in AOCS Official Method Ca 5a-40.

PROCEDURE—GENERAL

1. Refer to Table 2, Tabular Statement of Refining Conditions, for specific details such as temperature, time intervals, etc. Refer to specific procedures for different oils.

   Note—Test portions must not be allowed to come in contact with copper or brass, due to the effect of these metals on color.
2. Weigh 500 g of thoroughly mixed test sample into tared refining cup and allow to settle to permit the air to escape. Remove any persisting foam before adjusting the final weight. Place in the refining machine, fill the water bath to the specified height and adjust the temperature of water and oil to the designated cold temperature (see Table 2).
3. Refer to Tables 3–10 to determine the correct amount of alkali to be added and the number of cups to run, depending upon the kind and type of oil and free fatty acid content.

   Note—The percentages indicated in the tables are multiplied by 5 to obtain the actual weight to be added to each refining cup. The designated amounts may be weighed into 50 mL beakers and kept covered with watch glasses until ready to add to the sample. If beakers are used, compensation should be made for the lye that does not drain completely from the beakers.
4. With the agitators running at high speed, add the alkali to each cup, draining as completely and quickly as possible. Continue high-speed agitation and maintain the cold temperature within the required limits for the various oils as directed in Table 2.
5. Change the agitation to slow speed and increase the bath to the hot temperature. This temperature change should be completed in about 1 min. Continue the agitation as directed in Table 2. At the end of the stirring period, the oil sample itself must be at the specified temperature. Adjust the temperature of the water bath to obtain the correct final oil temperature.
6. Discontinue agitation, remove paddles and return as much of the sample clinging to the paddles as possible. Hold the samples at the hot temperature for 1 hr. Decrease the bath to the cold temperature and hold for an additional 30 min. After the 30 min holding time, drain the bath and allow the samples to remain at ambient temperature for at least 12 hr. Unless the oil has been maintained at the cold temperature (see Table 2), cool again to this temperature for 30 min before pouring off.
7. Weigh the cup and contents and deduct this weight from the total weight at the start to obtain the evaporation loss.
8. Decant the refined oil into another tared refining cup and drain the soap stock for 30 min by inverting the soap stock cup into the refined oil cup. If the soap stock is too soft to drain, allow the cup to stand and pour off oil several times. Any floating foots (sediment) decanted with the oil is recovered and returned to the main body of soap stock. The oil may be poured through a small strainer to collect the foots, providing the screen is such that the oil loss is negligible.
9. Weigh the soap stock immediately to prevent any moisture loss. Melt the soap stock in a water bath at 75 ± 2°C without stirring for 30 min, and then cool in cold water (see Table 2) for 15 min or until thoroughly chilled. Decant all possible oil into a tared beaker and proceed as follows:
   (a) All crude oils except soybean—Repeat remelting until not more than 1.5 g is removed and record the weight (to 0.1 g) of all the oil obtained. If the oil is difficult to remove due to soft soap stock or for any other reason, use a pipet.
   (b) Crude soybean oils—Repeat remelting until not more than 1.0 g of oil is recovered on two successive remelts and record the weight (to 0.1 g) of all the oil obtained. If the oil is difficult to remove due to soft soap stock or for any other reason, use a pipet. Remelting should not be continued beyond the recovery of not more than 1.0 g on successive remelts.
   (c) Degummed oils—refining may produce a poorly coalescing and difficult to separate soap stock. Carefully decant, as described in Procedure, 8, to prevent loss of soap stock and to remove as much oil as possible. After the first melt, coagulate the soap stock by swirling the cup, without splashing, at 100 ± 20 rpm, with a simultaneous gentle rocking motion. Emulsification can occur if this is continued for too long a period or at too fast a rate. Make subsequent remelts as described.
10. Weigh the retained oil.
11. If a bleach test is required, filter the oil through one of the approved papers into a clean, dry container. The bleach test is determined on the filtered sample as directed in AOCS Official Method Cc 8a–52 or Cc 8b–52.
12. For the determination of color it is essential that the filtered oil be absolutely clear. Add 0.5 g of the diatomaceous earth (Apparatus, 7) per 300 g of oil, mix for 2.5 min at 250 ± 10 rpm at a temperature of 25°C, and then filter through an

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approved paper into a clean, dry container. Determine the color of the filtered sample as directed in AOCS Official Method Cc 13b–45 or Cc 13c–50.

ADDITIONAL PROCEDURES

1. Coconut Oil—Add 0.1% dry sodium chloride (NaCl) for each 1.0% free fatty acid present, before adding the alkali.
2. Degummed Hydraulic and Extracted Soybean Oil—Use an excess of 0.1–0.2 g of caustic (NaOH) above the theoretical amount required to neutralize the FFA in 100 g oil. Refer to Table 9 for the correct amount.
3. Degummed Expeller-Type Soybean Oil—Use an excess of 0.15–0.20 g of caustic (NaOH) above the theoretical amount required to neutralize the FFA in 100 g oil. Refer to Table 10 for the correct amount.
4. Solvent-Extracted Cottonseed Oil—Solvent-extracted cottonseed oil or the oil mixture resulting from prepressing and subsequent solvent extraction of the cottonseed may be processed using either the slow-breaking or regular method depending on the producer's designation.

CALCULATIONS

Calculate the refining loss by methods 1 and 2, below, and report the average of these results, which should agree within 0.25%.

1. Refining loss, $\% = \dfrac{\text{mass, g of crude oil} - \text{mass, g of refined oil}}{5}$
2. Refining loss, $\% = \dfrac{(A + B) - C}{5}$

Where—

A = mass, g of soap stock

B = evaporation loss

C = mass, g of alkali solution used. Include the mass, g of NaCl in the case of coconut oil.

LYE REQUIREMENTS (TABLES 3–10)

1. The maximum amount of actual NaOH for hydraulic or hot-pressed cottonseed oil, peanut oil, corn oil, and hydraulic and expeller soybean oil is calculated by the following formula:

$$\text{Grams NaOH/100 g of oil} = \frac{\%\ \text{FFA}}{5.2} + 0.54 \qquad [1]$$

2. The maximum amount of actual NaOH for expeller or cold-pressed cottonseed oil is calculated by the following formula:

$$\text{Grams NaOH/100 g of oil} = \frac{\%\ \text{FFA}}{4.365} + 0.77 \qquad [2]$$

3. The maximum weight of alkali solution required per 100 g of sample is calculated by the following formula:

$$\frac{100\ A}{B}$$

Where—

A = maximum NaOH calculated as noted in formulas 1 and 2 of this section

B = percentage NaOH of alkali solution to be used from Table 1

4. Example—Given a test portion of expeller cottonseed oil with a free fatty acid content of 2.2% and substituting in formula 2 in this section:

$$\text{Maximum NaOH} = \frac{2.2}{4.365} + 0.77 = 1.27\text{g}$$

Three refinings are required (Table 3, line 9):

(a) $\dfrac{1.27}{11.06} \times 80 = 9.2\%$ of 16° Bé, or 46.0 g/500 g of sample

(b) $\dfrac{1.27}{14.36} \times 80 = 7.1\%$ of 20° Bé, or 35.5 g/500 g of sample

(c) $\dfrac{1.27}{14.36} \times 100 = 8.9\%$ of 20° Bé, or 44.5 g/500 g of sample

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PRECISION

1 Reproducibility—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit R as shown in Table 11, which on average is 3.00%.
2. Repeatability—No information is available on the expected differences obtained by the same operator in the same laboratory.

NOTES

Caution

Sodium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as sodium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Table 1
Alkali concentration.

Nominal degrees Baumé at 15°C	Actual NaOH content by weight, %	Permissible variations	
		Minimum, %	Maximum, %
10	6.57	6.50	6.64
12	8.00	7.92	8.08
14	9.50	9.40	9.60
16	11.06	10.95	11.17
18	12.68	12.55	12.81
20	14.36	14.22	14.50
22	16.09	15.93	16.25
24	17.87	17.69	18.05
26	19.70	19.50	19.90
28	21.58	21.36	21.80
30	23.50	23.26	23.74

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Table 2
Tabular statement of refining conditions.

	Cottonseed				Soybean					
	Hydraulic[a]	Expeller	Slow break	Peanut	Expeller or Hydraulic	Extracted	Degummed Hydraulic or Extracted	Degummed expeller	Coconut	Corn
Refining:										
Temperature of cold bath, °C	20–24	20–24	20–24	20–24	20–24	20–24	20–24	20–24	30–35	20–24
rpm in cold bath, ± 10	250	250	250	250	250	250	250	250	250	250
Time in cold bath, min	15	45	45	30	90	90	90	90	5	15
Temperature of hot bath, °C	63–67	63–67	63–67	63–67	63–67	63–67	63–67	63–67	50–53	63–67
rpm in hot bath, ± 5	70	70	70	70	70	70	70	70	70	70
Time in hot bath, min	12	12	20	12	12	20	20	20	5	12
Final oil temperature, °C	60–65	60–65	60–65	60–65	60–65	60–65	60–65	60–65	45–50	60–65
Settling time in hot bath, min	60	60	60	60	60	60	60	60	60	60
Temperature of cooling bath, °C	20–24	20–24	20–24	20–24	5–10	5–10	5–10	5–10	26–30	20–24
Time in cooling bath, min	30	30	30	30	30	30	30	30	30	30
Additional settling time, minimum hr	12	12	12	12	12	12	12	12	12	12
Chill soap stock, min	30	30	30	30	30	30	30	30	30	30
Drain soap stock, min	30	30	30	30	30	30	30	30	30	30
Clarification:										
Official clay, g	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Temperature, °C	25	25	25	25	25	25	25	25	25	25
rpm ± 10	250	250	250	250	250	250	250	250	250	250
Time, min	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Remelting:										
Temperature of bath, °C	73–77	73–77	73–77	73–77	73–77	73–77	73–77	73–77	48–52	73–77
Agitation	None	None	None	None	None	None	None	None	None	None
Time in hot bath, min	30	30	30	30	30	30	30	30	30	30
Temperature of cooling bath, °C	20–24	20–24	20–24	20–24	5–10	5–10	5–10	5–10	26–30	ice water
Time in cooling bath, min	15, or until firm	15, or until firm	15, or until firm	15, or until firm	15, or until firm	15, or until firm	15, or until firm	15, or until firm	15, or until firm	15, or until firm
Drain, min	15	15	15	15	15	15	15	15	15	15

[a]Also suitable for solvent extracted, and mixture obtained from prepressing and subsequent solvent extraction.

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Table 3
Lye requirements—strength and number of refinings.

Oil	FFA Type	No. of range	Strength of Alkali Solutions, refinings	Bé
Cottonseed	Hydraulic	0–1.5	2	80% of maximum of 12° 80% of maximum of 14°
Cottonseed	Hydraulic	1.6–3.0	3	80% of maximum of 12° 80% of maximum of 16° Maximum of 16°
Cottonseed	Hydraulic	3.1–4.0	2	Maximum of 14° Maximum of 18°
Cottonseed	Hydraulic	4.1–5.0	2	Maximum of 16° Maximum of 20°
Cottonseed	Hydraulic	5.1–7.5	2	Maximum of 18° Maximum of 22°
Cottonseed	Hydraulic	7.6–10.0	2	Maximum of 20° Maximum of 24°
Cottonseed	Hydraulic	10.1–15.0	2	Maximum of 20° Maximum of 26°
Cottonseed	Hydraulic	15.1–20.0	2	Maximum of 22° Maximum of 28°
Cottonseed	Expeller	0–3.0	3	80% of maximum of 16° 80% of maximum of 20° Maximum of 20°
Cottonseed	Expeller	3.1–5.0	2	Maximum of 16° Maximum of 20°
Cottonseed	Expeller	5.1–10.0	2	Maximum of 20° Maximum of 26°
Cottonseed	Expeller	Above 10.0	2	Maximum of 20° Maximum of 30°
Cottonseed Cottonseed	Solvent extracted Prepressed and solvent extracted	as hydraulic		
Peanut		0–3.0	3	60% of maximum of 12° 60% of maximum of 16° 80% of maximum of 16°
Peanut		Above 3.0	3	60% of maximum of 16° 80% of maximum of 16° 80% of maximum of 20°
Soybean	Expeller and hydraulic	All	2	66.6% of maximum of 12° Maximum of 12°
Soybean	Extracted	All	2	87.5% of maximum 14° 66.6% of maximum 14°
Soybean	Degummed hydraulic or extracted	All	2	Maximum of 12°[a] Maximum of 12°
Soybean	Degummed expeller	All	2	Maximum of 12°[a] Maximum of 12°
Corn		All	2	50% of maximum of 16° 66.6% of maximum of 16°
Coconut		All	1	(1.1 × %FFA) = grams of 20° caustic/100 g of oil

[a]See Procedures for addition of excess NaOH.

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Table 4
Weight of lye in grams per 100 grams of oil—hydraulic or hot-pressed cottonseed oil.

FFA	Max NaOH 80%	12° 80%	14° 80%
0.5	0.51	6.4	5.4
0.6	0.52	6.6	5.5
0.7	0.54	6.7	5.7
0.8	0.56	6.9	5.8
0.9	0.57	7.1	6.0
1.0	0.59	7.3	6.2
1.1	0.60	7.5	6.3
1.2	0.62	7.7	6.5
1.3	0.63	7.9	6.6
1.4	0.65	8.1	6.8
1.5	0.66	8.3	7.0

FFA	Max NaOH	12° 80%	16° 80%	16° Max
1.6	0.85	8.5	6.1	7.7
1.7	0.87	8.7	6.3	7.8
1.8	0.89	8.9	6.4	8.0
1.9	0.91	9.1	6.5	8.2
2.0	0.92	9.2	6.7	8.4
2.1	0.94	9.4	6.8	8.5
2.2	0.96	9.6	7.0	8.7
2.3	0.98	9.8	7.1	8.9
2.4	1.00	10.0	7.2	9.1
2.5	1.02	10.2	7.4	9.2
2.6	1.04	10.4	7.5	9.4
2.7	1.06	10.6	7.7	9.6
2.8	1.08	10.8	7.8	9.7
2.9	1.10	11.0	7.9	9.9
3.0	1.12	11.2	8.1	10.1

FFA	Max NaOH	14° Max	18° Max
3.1	1.14	12.0	9.0
3.2	1.16	12.2	9.1
3.3	1.18	12.4	9.3
3.4	1.19	12.6	9.4
3.5	1.21	12.8	9.6
3.6	1.23	13.0	9.7
3.7	1.25	13.2	9.9
3.8	1.27	13.4	10.0
3.9	1.29	13.6	10.2
4.0	1.31	13.8	10.3

FFA	Max NaOH	16° Max	20° Max
4.1	1.33	12.0	9.2
4.2	1.35	12.2	9.4
4.3	1.37	12.4	9.5
4.4	1.39	12.5	9.7
4.5	1.41	12.7	9.8
4.6	1.42	12.9	9.9
4.7	1.44	13.1	10.1
4.8	1.46	13.2	10.2
4.9	1.48	13.4	10.3
5.0	1.50	13.6	10.5

FFA	Max NaOH	18° Max	22° Max
5.1	1.52	12.0	9.5
5.2	1.54	12.1	9.6
5.3	1.56	12.3	9.7
5.4	1.58	12.4	9.8
5.5	1.60	12.6	9.9
5.6	1.62	12.8	10.0
5.7	1.64	12.9	10.2
5.8	1.66	13.1	10.3
5.9	1.68	13.2	10.4
6.0	1.69	13.4	10.5
6.1	1.71	13.5	10.6
6.2	1.73	13.7	10.8
6.3	1.75	13.8	10.9
6.4	1.77	14.0	11.0
6.5	1.79	14.1	11.1
6.6	1.81	14.3	11.2
6.7	1.83	14.4	11.4
6.8	1.85	14.6	11.5
6.9	1.87	14.7	11.6
7.0	1.89	14.9	11.7
7.1	1.91	15.0	11.8
7.2	1.92	15.2	12.0
7.3	1.94	15.3	12.1
7.4	1.96	15.5	12.2
7.5	1.98	15.6	12.3

FFA	Max NaOH	20° Max	24° Max
7.6	2.00	13.9	11.2
7.7	2.02	14.1	11.3
7.8	2.04	14.2	11.4
7.9	2.06	14.3	11.5
8.0	2.08	14.5	11.6
8.1	2.10	14.6	11.7
8.2	2.12	14.7	11.8
8.3	2.14	14.9	12.0
8.4	2.16	15.0	12.1
8.5	2.17	15.1	12.2
8.6	2.19	15.3	12.3
8.7	2.21	15.4	12.4
8.8	2.23	15.5	12.5
8.9	2.25	15.7	12.6
9.0	2.27	15.8	12.7
9.1	2.29	15.9	12.8
9.2	2.31	16.1	12.9
9.3	2.33	16.2	13.0
9.4	2.35	16.3	13.1
9.5	2.37	16.5	13.2
9.6	2.39	16.6	13.4

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Table 4 *(Continued)*

FFA	Max NaOH	20° Max	24° Max
9.7	2.40	16.8	13.5
9.8	2.42	16.9	13.6
9.9	2.44	17.0	13.7
10.0	2.46	17.2	13.8

FFA	Max NaOH	20° Max	26° Max
10.1	2.48	17.3	12.6
10.2	2.50	17.4	12.7
10.3	2.52	17.6	12.8
10.4	2.54	17.7	12.9
10.5	2.56	17.8	13.0
10.6	2.58	18.0	13.1
10.7	2.60	18.1	13.2
10.8	2.62	18.2	13.3
10.9	2.64	18.4	13.4
11.0	2.66	18.5	13.5
11.1	2.67	18.6	13.6
11.2	2.69	18.8	13.7
11.3	2.71	18.9	13.8
11.4	2.73	19.0	13.9
11.5	2.75	19.2	14.0
11.6	2.77	19.3	14.1
11.7	2.79	19.4	14.2
11.8	2.81	19.6	14.3
11.9	2.83	19.7	14.4
12.0	2.85	19.8	14.5
12.1	2.87	20.0	14.6
12.2	2.89	20.1	14.7
12.3	2.91	20.2	14.7
12.4	2.92	20.4	14.8
12.5	2.94	20.5	14.9
12.6	2.96	20.6	15.0
12.7	2.98	20.8	15.1
12.8	3.00	20.9	15.2
12.9	3.02	21.0	15.3
13.0	3.04	21.2	15.4
13.1	3.06	21.3	15.5
13.2	3.08	21.4	15.6
13.3	3.10	21.6	15.7
13.4	3.12	21.7	15.8
13.5	3.14	21.8	15.9
13.6	3.16	22.0	16.0
13.7	3.17	22.1	16.1
13.8	3.19	22.2	16.2
13.9	3.21	22.4	16.3
14.0	3.23	22.5	16.4
14.1	3.25	22.6	16.5
14.2	3.27	22.8	16.6
14.3	3.29	22.9	16.7
14.4	3.31	23.0	16.8
14.5	3.33	23.2	16.9
14.6	3.35	23.3	17.0
14.7	3.37	23.4	17.1
14.8	3.39	23.6	17.2
14.9	3.41	23.7	17.3
15.0	3.42	23.8	17.4

FFA	Max NaOH	22° Max	28° Max
15.1	3.44	21.4	16.0
15.2	3.46	21.5	16.0
15.3	3.48	21.6	16.1
15.4	3.50	21.8	16.2
15.5	3.52	21.9	16.3
15.6	3.54	22.0	16.4
15.7	3.56	22.1	16.5
15.8	3.58	22.2	16.6
15.9	3.60	22.4	16.7
16.0	3.62	22.5	16.8
16.1	3.64	22.6	16.8
16.2	3.66	22.7	16.9
16.3	3.67	22.8	17.0
16.4	3.69	23.0	17.1
16.5	3.71	23.1	17.2
16.6	3.73	23.2	17.3
16.7	3.75	23.3	17.4
16.8	3.77	23.4	17.5
16.9	3.79	23.6	17.6
17.0	3.81	23.7	17.7
17.1	3.83	23.8	17.7
17.2	3.85	23.9	17.8
17.3	3.87	24.0	17.9
17.4	3.89	24.2	18.0
17.5	3.91	24.3	18.1
17.6	3.92	24.4	18.2
17.7	3.94	24.5	18.3
17.8	3.96	24.6	18.4
17.9	3.98	24.8	18.5
18.0	4.00	24.9	18.5
18.1	4.02	25.0	18.6
18.2	4.04	25.1	18.7
18.3	4.06	25.2	18.8
18.4	4.08	25.3	18.9
18.5	4.10	25.5	19.0
18.6	4.12	25.6	19.1
18.7	4.14	25.7	19.2
18.8	4.16	25.8	19.3
18.9	4.17	25.9	19.3
19.0	4.19	26.1	19.4
19.1	4.21	26.2	19.5
19.2	4.23	26.3	19.6
19.3	4.25	26.4	19.7
19.4	4.27	26.5	19.8
19.5	4.29	26.7	19.9
19.6	4.31	26.8	20.0
19.7	4.33	26.9	20.1
19.8	4.35	27.0	20.1
19.9	4.37	27.1	20.2
20.0	4.39	27.3	20.3

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Table 5
Weight of lye in grams per 100 grams of oil—expeller or cold-pressed cottonseed oil.

FFA	Max NaOH	16° 80%	20° 80%	20° Max
0.5	0.88	6.4	4.9	6.2
0.6	0.91	6.6	5.1	6.3
0.7	0.93	6.7	5.2	6.5
0.8	0.95	6.9	5.3	6.6
0.9	0.98	7.1	5.4	6.8
1.0	1.00	7.2	5.6	7.0
1.1	1.02	7.4	5.7	7.1
1.2	1.04	7.6	5.8	7.3
1.3	1.07	7.7	5.9	7.4
1.4	1.09	7.9	6.1	7.6
1.5	1.11	8.1	6.2	7.8
1.6	1.14	8.2	6.3	7.9
1.7	1.16	8.4	6.5	8.1
1.8	1.18	8.6	6.6	8.2
1.9	1.21	8.7	6.7	8.4
2.0	1.23	8.9	6.8	8.5
2.1	1.25	9.0	7.0	8.7
2.2	1.27	9.2	7.1	8.9
2.3	1.30	9.4	7.2	9.0
2.4	1.32	9.5	7.4	9.2
2.5	1.34	9.7	7.5	9.3
2.6	1.37	9.9	7.6	9.5
2.7	1.39	10.0	7.7	9.7
2.8	1.41	10.2	7.9	9.8
2.9	1.43	10.4	8.0	10.0
3.0	1.46	10.5	8.1	10.1

FFA	Max NaOH	16° Max	20° Max
3.1	1.48	13.4	10.3
3.2	1.50	13.6	10.5
3.3	1.53	13.8	10.6
3.4	1.55	14.0	10.8
3.5	1.57	14.2	10.9
3.6	1.59	14.4	11.1
3.7	1.62	14.6	11.3
3.8	1.64	14.8	11.4
3.9	1.66	15.0	11.6
4.0	1.69	15.2	11.7
4.1	1.71	15.5	11.9
4.2	1.73	15.7	12.1
4.3	1.76	15.9	12.2
4.4	1.78	16.1	12.4
4.5	1.80	16.3	12.5
4.6	1.82	16.5	12.7
4.7	1.85	16.7	12.9
4.8	1.87	16.9	13.0
4.9	1.89	17.1	13.2
5.0	1.92	17.3	13.3

FFA	Max NaOH	20° Max	26° Max
5.1	1.94	13.5	9.9
5.2	1.96	13.6	10.0
5.3	1.98	13.8	10.1
5.4	2.01	14.0	10.2
5.5	2.03	14.1	10.3
5.6	2.05	14.3	10.4
5.7	2.08	14.4	10.5
5.8	2.10	14.6	10.7
5.9	2.12	14.8	10.8
6.0	2.14	14.9	10.9
6.1	2.17	15.1	11.0
6.2	2.19	15.2	11.1
6.3	2.21	15.4	11.2
6.4	2.24	15.6	11.4
6.5	2.26	15.7	11.5
6.6	2.28	15.9	11.6
6.7	2.30	16.0	11.7
6.8	2.33	16.2	11.8
6.9	2.35	16.4	11.9
7.0	2.37	16.5	12.0
7.1	2.40	16.7	12.2
7.2	2.42	16.8	12.3
7.3	2.44	17.0	12.4
7.4	2.47	17.2	12.5
7.5	2.49	17.3	12.6
7.6	2.51	17.5	12.7
7.7	2.53	17.6	12.9
7.8	2.56	17.8	13.0
7.9	2.58	18.0	13.1
8.0	2.60	18.1	13.2
8.1	2.63	18.3	13.3
8.2	2.65	18.4	13.4
8.3	2.67	18.6	13.6
8.4	2.69	18.7	13.7
8.5	2.72	18.9	13.8
8.6	2.74	19.1	13.9
8.7	2.76	19.2	14.0
8.8	2.79	19.4	14.1
8.9	2.81	19.5	14.3
9.0	2.83	19.7	14.4
9.1	2.85	19.9	14.5
9.2	2.88	20.0	14.6
9.3	2.90	20.2	14.7
9.4	2.92	20.3	14.8
9.5	2.95	20.5	15.0
9.6	2.97	20.7	15.1
9.7	2.99	20.8	15.2
9.8	3.02	21.0	15.3
9.9	3.04	21.1	15.4
10.0	3.06	21.3	15.5

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Table 6
Weight of lye in grams per 100 grams of oil—peanut oil.

FFA	12 60%	16° 60%	16° 80%	20° 80%
0.1	4.2	3.0	4.0	
0.2	4.3	3.1	4.2	
0.3	4.5	3.2	4.3	
0.4	4.6	3.3	4.5	
0.5	4.8	3.5	4.6	
0.6	4.9	3.6	4.7	
0.7	5.1	3.7	4.9	
0.8	5.2	3.8	5.0	
0.9	5.3	3.9	5.2	
1.0	5.5	4.0	5.3	
1.1	5.6	4.1	5.4	
1.2	5.8	4.2	5.6	
1.3	5.9	4.3	5.7	
1.4	6.1	4.4	5.9	
1.5	6.2	4.5	6.0	
1.6	6.4	4.6	6.1	
1.7	6.5	4.7	6.3	
1.8	6.6	4.8	6.4	
1.9	6.8	4.9	6.5	
2.0	6.9	5.0	6.7	
2.1	7.1	5.1	6.8	
2.2	7.2	5.2	7.0	
2.3	7.4	5.3	7.1	
2.4	7.5	5.4	7.2	
2.5	7.7	5.5	7.4	
2.6	7.8	5.6	7.5	
2.7	7.9	5.7	7.7	
2.8	8.1	5.9	7.8	
2.9	8.2	6.0	7.9	
3.0	8.4	6.1	8.1	
3.1		6.2	8.2	6.3
3.2		6.3	8.4	6.4
3.3		6.4	8.5	6.5
3.4		6.5	8.6	6.7
3.6		6.7	8.9	6.9
3.7		6.8	9.1	7.0
3.8		6.9	9.2	7.1
3.9		7.0	9.3	7.2
3.5		6.6	8.8	6.8
4.0		7.1	9.5	7.3
4.1		7.2	9.6	7.4
4.2		7.3	9.7	7.5
4.3		7.4	9.9	7.6
4.4		7.5	10.0	7.7
4.5		7.6	10.2	7.8
4.6		7.7	10.3	7.9
4.7		7.8	10.4	8.0
4.8		7.9	10.6	8.2
4.9		8.0	10.7	8.3
5.0		8.1	10.9	8.4
5.1		8.2	11.0	8.5
5.2		8.4	11.1	8.6
5.3		8.5	11.3	8.7
5.4		8.6	11.4	8.8
5.5		8.7	11.6	8.9
5.6		8.8	11.7	9.0
5.7		8.9	11.8	9.1
5.8		9.0	12.0	9.2
5.9		9.1	12.1	9.3
6.0		9.2	12.3	9.4
6.1		9.3	12.4	9.5
6.2		9.4	12.5	9.7
6.3		9.5	12.7	9.8
6.4		9.6	12.8	9.9
6.5		9.7	12.9	10.0
6.6		9.8	13.1	10.1
6.7		9.9	13.2	10.2
6.8		10.0	13.4	10.3
6.9		10.1	13.5	10.4
7.0		10.2	13.6	10.5
7.1		10.3	13.8	10.6
7.2		10.4	13.9	10.7
7.3		10.5	14.1	10.8
7.4		10.6	14.2	10.9
7.5		10.8	14.3	11.0
7.6		10.9	14.5	11.2
7.7		11.0	14.6	11.3
7.8		11.1	14.8	11.4
7.9		11.2	14.9	11.5
8.0		11.3	15.0	11.6
8.1		11.4	15.2	11.7
8.2		11.5	15.3	11.8
8.3		11.6	15.5	11.9
8.4		11.7	15.6	12.0
8.5		11.8	15.7	12.1
8.6		11.9	15.9	12.2
8.7		12.0	16.0	12.3
8.8		12.1	16.1	12.4
8.9		12.2	16.3	12.5
9.0		12.3	16.4	12.7
9.1		12.4	16.6	12.8
9.2		12.5	16.7	12.9
9.3		12.6	16.8	13.0
9.4		12.7	17.0	13.1
9.5		12.8	17.1	13.2
9.6		12.9	17.3	13.3
9.7		13.0	17.4	13.4
9.8		13.2	17.5	13.5
9.9		13.3	17.7	13.6
10.0		13.4	17.8	13.7

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Table 7
Weight of lye in grams per 100 grams of oil—expeller and hydraulic soybean oil.

FFA	12° Max	12° 66.6%
0.1	7.0	4.7
0.2	7.2	4.8
0.3	7.5	5.0
0.4	7.7	5.1
0.5	8.0	5.3
0.6	8.2	5.5
0.7	8.4	5.6
0.8	8.7	5.8
0.9	8.9	5.9
1.0	9.2	6.1
1.1	9.4	6.3
1.2	9.6	6.4
1.3	9.9	6.6
1.4	10.1	6.7
1.5	10.4	6.9
1.6	10.6	7.1
1.7	10.8	7.2
1.8	11.1	7.4
1.9	11.3	7.5
2.0	11.6	7.7
2.1	11.8	7.9
2.2	12.0	8.0
2.3	12.3	8.2
2.4	12.5	8.3
2.5	12.8	8.5
2.6	13.0	8.7
2.7	13.2	8.8
2.8	13.5	9.0
2.9	13.7	9.1
3.0	14.0	9.3
3.1	14.2	9.5
3.2	14.4	9.6
3.3	14.7	9.8
3.4	14.9	9.9
3.5	15.2	10.1
3.6	15.4	10.3
3.7	15.6	10.4
3.8	15.9	10.6
3.9	16.1	10.8
4.0	16.4	10.9
4.1	16.6	11.1
4.2	16.8	11.2
4.3	17.1	11.4
4.4	17.3	11.6
4.5	17.6	11.7
4.6	17.8	11.9
4.7	18.0	12.0
4.8	18.3	12.2
4.9	18.5	12.4
5.0	18.8	12.5

Table 8
Weight of caustic solution (NaOH) in grams per 100 grams of extracted soybean oil.

FFA, %	14° Bé 87.5%	14° Bé 66.6%
0.1	5.2	3.9
0.2	5.3	4.1
0.3	5.5	4.2
0.4	5.7	4.3
0.5	5.9	4.5
0.6	6.0	4.6
0.7	6.2	4.7
0.8	6.4	4.9
0.9	6.6	5.0
1.0	6.7	5.1
1.1	6.9	5.3
1.2	7.1	5.4
1.3	7.3	5.5
1.4	7.5	5.7
1.5	7.7	5.8
1.6	7.8	5.9
1.7	8.0	6.1
1.8	8.2	6.2
1.9	8.3	6.4
2.0	8.5	6.5
2.1	8.7	6.6
2.2	8.9	6.8
2.3	9.0	6.9
2.4	9.2	7.0
2.5	9.4	7.2
2.6	9.6	7.3
2.7	9.8	7.4
2.8	9.9	7.6
2.9	10.1	7.7
3.0	10.3	7.8
3.1	10.5	8.0
3.2	10.6	8.1
3.3	10.8	8.2
3.4	11.0	8.4
3.5	11.2	8.5
3.6	11.4	8.6
3.7	11.5	8.8
3.8	11.7	8.9
3.9	11.9	9.1
4.0	12.1	9.2
4.1	12.2	9.3
4.2	12.4	9.5
4.3	12.6	9.6
4.4	12.8	9.7
4.5	12.9	9.9
4.6	13.1	10.0
4.7	13.3	10.1
4.8	13.5	10.3
4.9	13.7	10.4
5.0	13.8	10.5

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Table 9
Weight of lye in grams per 100 grams of degummed soybean oil.

	0.1 Gram excess		0.2 Gram excess	
% FFA	Actual NaOH, g	12° Bé NaOH, g	Actual NaOH, g	12° Bé NaOH, g
0.1	0.114	1.4	0.214	2.7
0.2	0.128	1.6	0.228	2.9
0.3	0.143	1.8	0.243	3.0
0.4	0.157	2.0	0.257	3.2
0.5	0.171	2.1	0.271	3.4
0.6	0.185	2.3	0.285	3.6
0.7	0.199	2.5	0.299	3.7
0.8	0.214	2.7	0.314	3.9
0.9	0.228	2.9	0.328	4.1
1.0	0.242	3.0	0.342	4.3
1.1	0.256	3.2	0.356	4.5
1.2	0.270	3.4	0.370	4.6
1.3	0.284	3.6	0.384	4.8
1.4	0.299	3.7	0.399	5.0
1.5	0.313	3.9	0.413	5.2
1.6	0.327	4.1	0.427	5.3
1.7	0.341	4.3	0.441	5.5
1.8	0.355	4.4	0.455	5.7
1.9	0.370	4.6	0.470	5.9
2.0	0.384	4.8	0.484	6.1
2.1	0.398	5.0	0.498	6.2
2.2	0.412	5.2	0.512	6.4
2.3	0.426	5.3	0.526	6.6
2.4	0.441	5.5	0.541	6.8
2.5	0.455	5.7	0.555	6.9
2.6	0.469	5.9	0.569	7.1
2.7	0.483	6.0	0.583	7.3
2.8	0.497	6.2	0.597	7.5
2.9	0.511	6.4	0.611	7.6
3.0	0.526	6.6	0.626	7.8

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Table 10
Weight of lye in grams per 100 grams of degummed, expeller-type soybean oil.

	0.15 Gram excess		0.20 Gram excess	
% FFA	Actual NaOH, g	12° Bé NaOH, g	Actual NaOH, g	12° Bé NaOH, g
0.1	0.164	2.1	0.214	2.7
0.2	0.178	2.2	0.228	2.9
0.3	0.193	2.4	0.243	3.0
0.4	0.207	2.6	0.257	3.2
0.5	0.221	2.8	0.271	3.4
0.6	0.235	2.9	0.285	3.6
0.7	0.249	3.1	0.299	3.7
0.8	0.263	3.3	0.314	3.9
0.9	0.278	3.5	0.328	4.1
1.0	0.292	3.7	0.342	4.3
1.1	0.306	3.8	0.356	4.5
1.2	0.320	4.0	0.370	4.6
1.3	0.334	4.2	0.384	4.8
1.4	0.349	4.4	0.399	5.0
1.5	0.363	4.5	0.413	5.2
1.6	0.377	4.7	0.427	5.3
1.7	0.391	4.9	0.441	5.5
1.8	0.405	5.1	0.455	5.7
1.9	0.420	5.2	0.470	5.9
2.0	0.434	5.4	0.484	6.1
2.1	0.448	5.6	0.498	6.2
2.2	0.462	5.8	0.512	6.4
2.3	0.476	6.0	0.526	6.6
2.4	0.491	6.1	0.541	6.8
2.5	0.505	6.3	0.555	6.9
2.6	0.519	6.5	0.569	7.1
2.7	0.534	6.7	0.583	7.5
2.8	0.548	6.8	0.597	7.5
2.9	0.562	7.0	0.611	7.6
3.0	0.576	7.2	0.626	7.8

Table 11
Statistical results evaluated in accordance with ISO 5725-1986, obtained from Smalley Laboratory Proficiency Program results for cottonseed oil obtained from participating laboratories in 1985–1986.

Sample	1	2	3	4
Labs after outliers	34	33	34	32
Labs removed	1	0	0	1
Refining loss, %	22.05	15.41	16.08	17.55
Repeatability std. dev., S_r	NA	NA	NA	NA
Repeatability relative std. dev., RSD_r	NA	NA	NA	NA
Repeatability value, $r = (2.8 \times S_r)$	NA	NA	NA	NA
Reproducibility std. dev., S_R	1.244	0.652	1.692	0.661
Reproducibility relative std. dev., RSD_R	5.64	4.23	10.52	3.77
Reproducibility value, $R = (2.8 \times S_R)$	3.51	1.85	4.79	1.87

Notes
NA = Not available.

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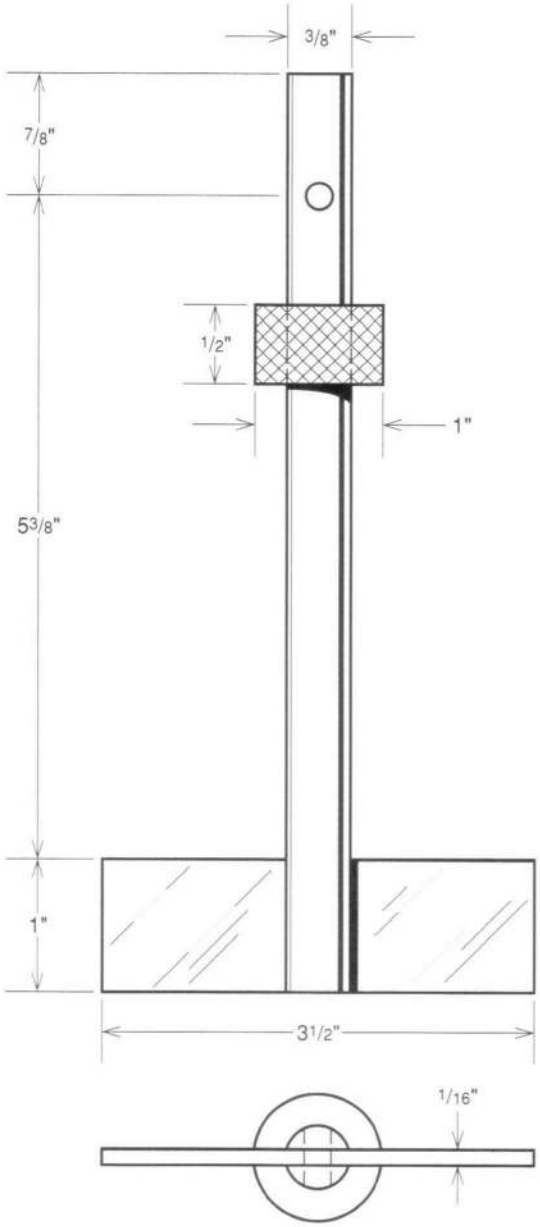

Figure 1. Paddle for refining apparatus.

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AOCS

AOCS Official Method Ca 9f-57

Reapproved 2009

Neutral Oil and Loss

DEFINITION

The total neutral oil of natural fats and oils consisting essentially of triglycerides and unsaponifiable matter is determined by this method. The free fatty acids and miscellaneous nonfat substances are removed by passing through a column of activated alumina. The loss is the difference between total neutral oil and 100%.

SCOPE

This method has been satisfactorily applied to cottonseed, soybean, peanut, linseed, coconut, and sunflower oils. Application of this method to other oils has not been investigated, but it is probably applicable to practically all natural animal and vegetable fats and oils.

APPARATUS

1. Chromatographic equipment—
   (a) Chromatographic column.
   (b) Velasco flask—20 mL, with base and extension tube (see Notes, 1).
   (c) Solvent reservoir—175 mL.
   This equipment should conform to the specifications shown in Figures 1 and 2. Equipment meeting these specifications may be obtained from Ace Glass, Inc. (item no. 5869), Hahn Laboratories or Glenco Scientific Co.
2. Soxhlet flask—250 mL.
3. Separatory funnel—125 mL, with glass stopcock (Teflon™ stopcocks are not suitable).
4. Wash bottles—polyethylene, with fine tip, capacity about 250 mL.
5. Funnels.
6. Stirring rods—about 5 × 150 mm.
7. Forced-draft oven—AOCS Specification H 1-39.

REAGENTS

1. Solvent mixture, diethyl ether-methanol—prepared by mixing 25 mL of methanol (ACS grade) with 975 mL of anhydrous diethyl ether (ACS grade) (see Notes, *Caution*).
2. n-Hexane—commercial grade, AOCS specification H 16-56, or better (see Notes, *Caution*).
3. Aluminum oxide—activated alumina, grade F-20 (see Notes, 2). The alumina as received must be further processed so that 100% will pass a 100-mesh US standard sieve and a minimum of 85% held on a 200-mesh sieve. The moisture content is adjusted to grade 4 on the Brockmann-Schodder scale by addition of water. The water content is determined by the loss on ignition of about 1 g of alumina, accurately weighed, in a tared, covered platinum or porcelain crucible for 2 hr at 600°C. The loss on ignition is regulated to 11 ± 1%.

PREPARATION OF TEST SAMPLE

1. The laboratory sample container must be shaken vigorously and the sample mixed thoroughly in order to incorporate and uniformly distribute meal or other sediment. In order to achieve this, the oil must be brought to a temperature of at least 20°C (50°C for soybean oil and 38°C, until completely melted, for coconut oil) before shaking. Inspect the inside of the container to be sure that no sediment remains clinging to the sides or bottom. If any sediment is found, remove it completely (cut can open if necessary) and incorporate thoroughly with the oil. The uniform incorporation and distribution of settlings and suspended matter are very significant in determining the accuracy of the result of the analysis.

PREPARATION OF ALUMINA COLUMN

1. Fill the glass chromatographic column to about two-thirds capacity with diethyl ether-methanol solvent mixture. Add 20 g (± 1 g) of alumina to the column in slurry form. The slurry may be added from a beaker, or in the following manner:
   (a) Add the weighed alumina, with the aid of a small polyethylene funnel, to a 125 mL separatory funnel (glass stopcock) containing about 50 mL of the diethyl ether-methanol solvent mixture. Dispense the alumina into the glass column by opening the separatory funnel stopcock (see Notes, 3). Maintain a high solvent level in the column by adjusting the flow of solvent from the funnel and column. Wash down the sides of the glass column and standard joint with diethyl ether-methanol solvent mixture. Tap the glass column to pack and level the alumina.
   (b) Drain the column until the diethyl ether-methanol solvent mixture is almost level with the top of the alumina. Add hexane to the column until the level rises to about 0.5 in. from the bottom of the standard joint.

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PROCEDURE

1. Place a tared 250 mL Soxhlet flask under the delivery tube of the column; to prevent splashing, place a glass rod in the flask and position it to receive the column flow.
2. Weigh a test portion of approximately 5 g to an accuracy of ± 0.0002 g into a Velasco flask. Remove the flask from its weighing base and attach the extension tube to the inner joint of the flask using hexane to wet the joint and a slight twisting pressure to seal the joint (see Notes, 4).
3. Place the flask with extension tube on the glass column, wetting the joint with hexane. Wash down the neck and sides of the flask with the diethyl ether-methanol solvent mixture from a polyethylene wash bottle. Carefully direct the fine stream from the bottle in the center of the oil pool to dilute and mix the oil. Continue dilution until the level rises almost to the top of the siphon arm. Give the flask a final wash down with a minimum of solvent so the level is just above the siphon arm.
4. Add 125 mL of the diethyl ether-methanol solvent mixture to the reservoir. Place the reservoir on the flask using solvent mixture to wet the joint. Partially open the reservoir and column stopcocks; adjust the column flow rate to about 4-6 mL/min with the column stop-cock. The level of solution in the flask should be a little above the siphon arm. This level can be adjusted by closing the column stopcock and turning the reservoir stopcock to deliver more solvent into the flask. If the solvent does not flow from reservoir to flask, tap the reservoir to start the flow. The column should now operate without any further attention. Allow the column to drain, then wash down any traces of oil from the delivery tube of the column into the Soxhlet flask.
5. Evaporate the solvent solution on a steam bath or in a water bath (contained in a properly operating fume hood) with the aid of a gentle stream (2-5 L/min) of clean, dry nitrogen. A jet of suitable size to deliver the predetermined volume of nitrogen/min should be inserted against the inside of the neck of the flask to a depth equal to approximately two-thirds the length of the neck and at a slight angle (see Notes, 5). After the last traces of solvent have just disappeared, remove the sample from steam bath and immediately place it in a 105°C forced-draft oven for 1 hr. Remove from the oven, cool and weigh the flask and contents.

CALCULATIONS

1. Neutral oil, $\% = \dfrac{\text{mass, g of residue}}{\text{mass, g of test portion}} \times 100$
2. Loss, % = 100 – % neutral oil

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 0.15.
2. Single determinations performed in two different laboratories should not differ by more than 0.30.

NOTES

Caution

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution.

Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Methanol (methyl alcohol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform and perchloric acid.

n-Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

NUMBERED NOTES

1. The length of the extension tube depends upon the manufacturer's chromatographic column. It is advisable to order extension tubes of 100 mm in length and to cut them to fit the particular column. There should be a distance of about 5–15 mm between the top of the alumina column and the tip of the extension tube.
2. Available as Alumina N.O.L. from Midland Scientific, Inc., Omaha, NE, USA, and Alumina (F20) from Barrow Agee Laboratories, Memphis, TN, USA.
3. The clogging of the fritted disk in the chromatographic column is minimized by dispensing the alumina slurry from a separatory funnel because the fine alumina dust remains suspended in the separatory funnel.
4. To ensure a good seal in the extension tube joint, rub some graphite from a soft lead pencil into the standard taper of the extension tube. Attach the tube to the inner joint of the flask and revolve the tube to spread an even coat of graphite on both pieces.

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5. In addition to speeding up the evaporation of solvent, the nitrogen prevents loss of neutral oil due to (a) creeping over the top of the flask and (b) oxidation of the sample. The use of air rather than nitrogen may cause oxidation, thus giving unrealistic yields of neutral oil. A controlled volume of air, in the range specified, should not contribute to detectable oxidation under the conditions of test. However, if air is used, the aeration should be discontinued before the removal of the last traces of solvent.

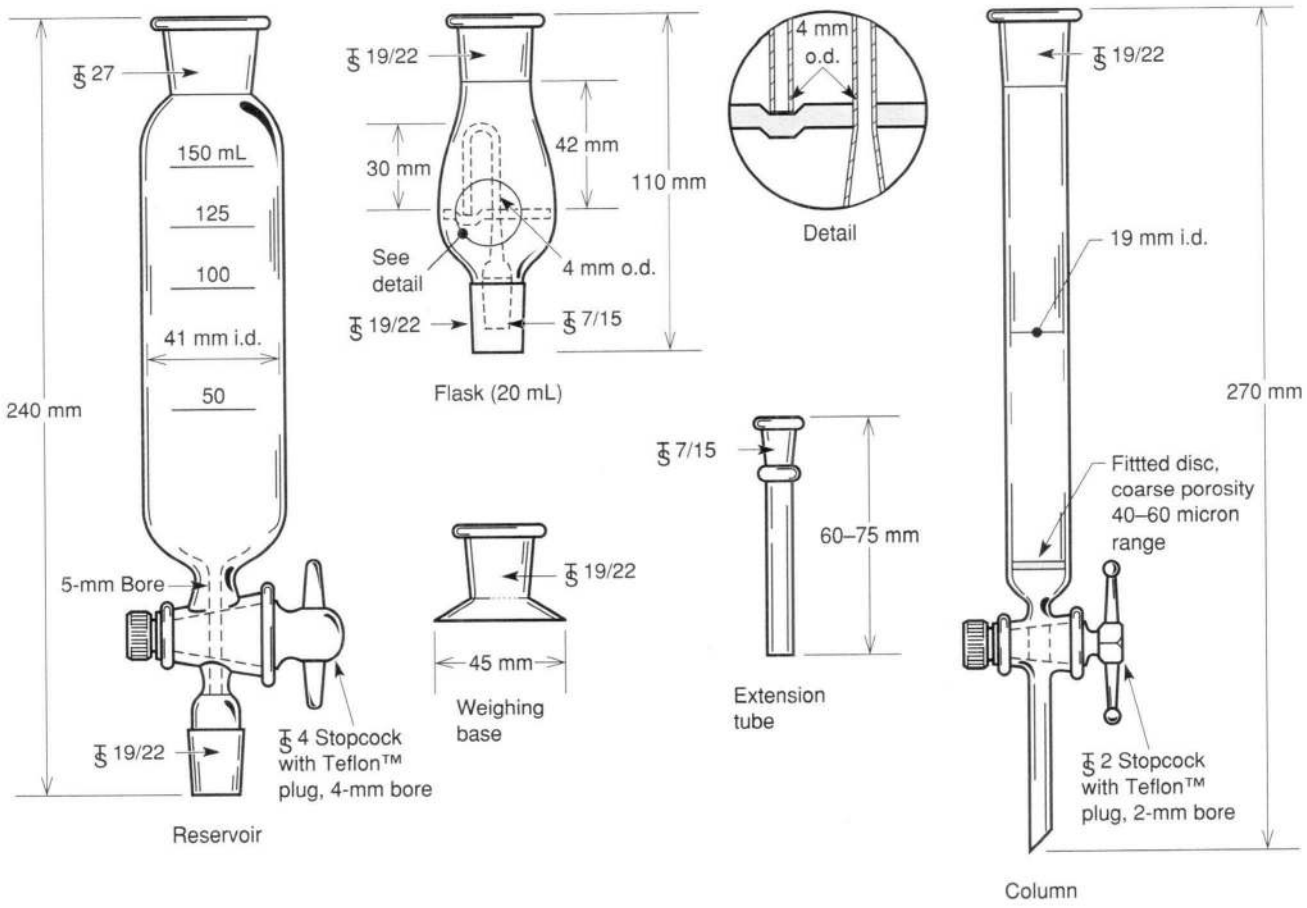

Figure 1. Chromatographic equipment.

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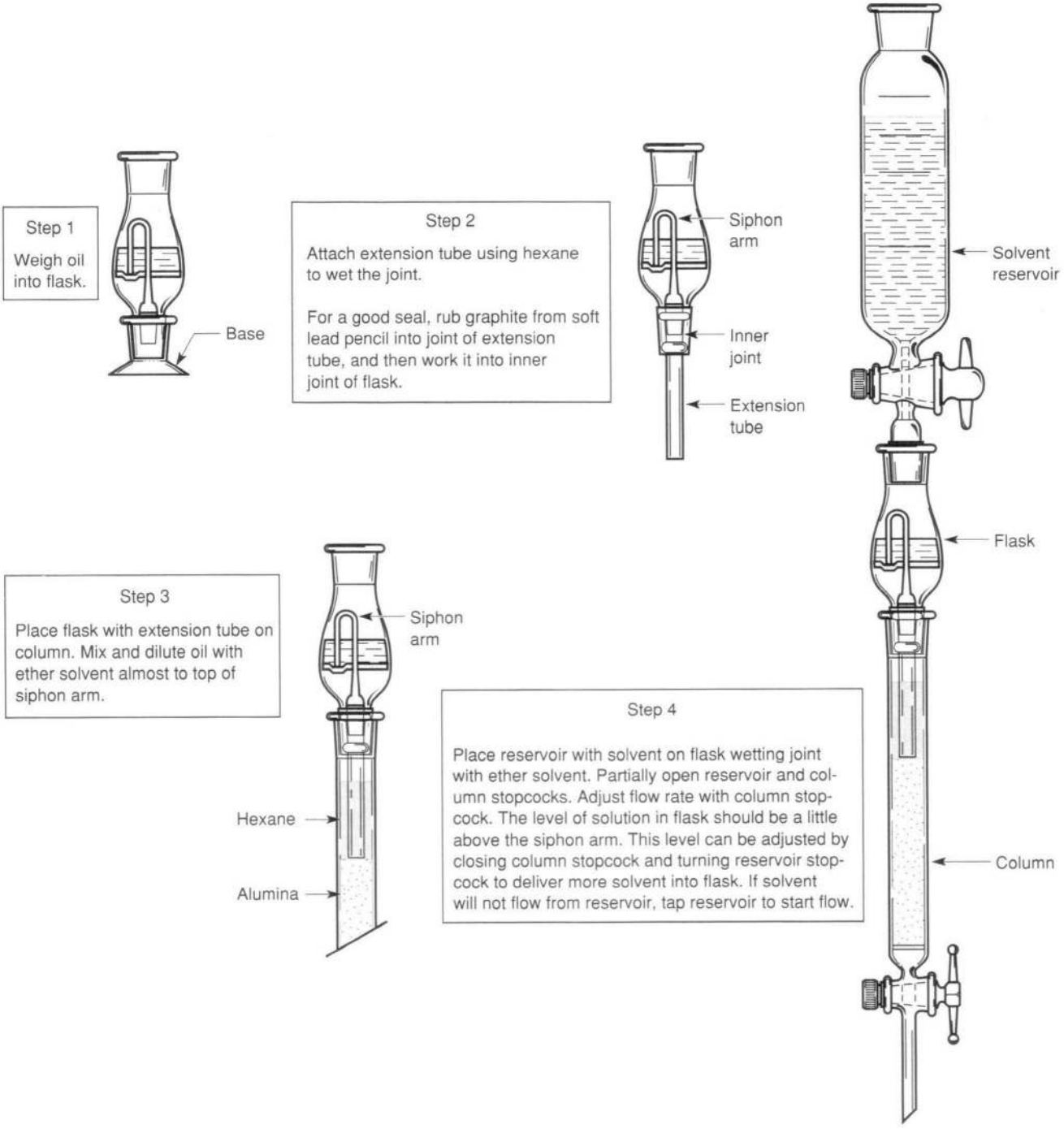

Figure 2. Chromatographic equipment set-up.

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AOCS

AOCS Official Method Ca 11-55

Reapproved 2009

Ash

DEFINITION

This method determines the residue remaining after incineration under the specific conditions of the test.

SCOPE

Applicable to animal fats and vegetable and marine oils. Not applicable to bodied oils containing lead or zinc. Formerly Ca 11-46.

APPARATUS

1. Platinum dish, or fused-quartz crucibles—100 mL capacity.

PREPARATION OF TEST SAMPLE

1. Laboratory samples must be thoroughly mixed. If necessary, soften with gentle heat (do not melt) and mix with an efficient mixer.

PROCEDURE

1. Heat the platinum dish to redness, cool slightly, place in a desiccator, cool to room temperature, and weigh.
2. Add about 50 ± 0.1 g of test sample and heat gently on a gas burner until the test portion can be ignited at the surface (see Notes, *Caution*). Reduce the size of the flame until the heat is just enough to keep the sample burning.
3. When sufficient test portion has burned to permit addition of more, remove the heat source, allow to cool until the fat ceases burning, and then add a second weighed portion of the test sample. A total of about 75 g should be used.
4. Continue heating the material to a black char and transfer to an electric muffle furnace. Heat at 550–650°C for 1 h.
5. Remove from the furnace, cool slightly, place in a desiccator and cool to room temperature. Weigh and repeat heating in the furnace (Procedure, 4) to constant weight.

CALCULATIONS

1. $$\text{Ash, \%} = \frac{\text{mass, g of residue}}{\text{mass, g of test portion}} \times 100$$

NOTES

Caution

The ignition and burning of the sample should be carried out in a properly operating fume hood.

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AOCS

AOCS Official Method Ca 12-55

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Phosphorus

DEFINITION

This method determines phosphorus or the equivalent phosphatide content by ashing the sample in the presence of zinc oxide, followed by the spectrophotometric measurement of phosphorus as a blue phosphomolybdic acid complex.

SCOPE

Applicable to crude, degummed and refined vegetable oils. Replaces Ca 12-53.

APPARATUS

1. Vycor crucibles, or equivalent—capable of withstanding a muffle furnace temperature of at least 600°C, 50 mL.
2. Watch glasses—75 mm diameter.
3. Electric hot plate—with rheostat control.
4. Muffle furnace—with pyrometer and suitable controls to maintain a temperature of 550–600°C.
5. Funnel—glass, short stem, 50 mm diameter.
6. Filter paper—ashless, 90 mm diameter, Whatman no. 42, or equivalent.
7. Wash bottle—1 liter, for use with hot water.
8. Volumetric flasks—50, 100, 250, and 500 mL, with glass stoppers.
9. Transfer pipets—2, 5, 10, and 25 mL.
10. Pipet—Mohr type, 10 mL, with 0.1 mL subdivisions.
11. Spectrophotometer—capable of measuring absorbance at 650 nm with a repeatability of 0.5%.
12. Cuvettes—1.000 ± 0.005 cm, for use in the visible region of the spectrum.

REAGENTS

1. Hydrochloric acid (HCl)—concentrated, sp gr 1.19 (see Notes, *Caution*).
2. Zinc oxide—commercial grade or reagent grade.
3. Potassium hydroxide (KOH) pellets—reagent grade (see Notes, *Caution*).
4. Sulfuric acid (H_2SO_4)—concentrated, sp gr 1.84 (see Notes, *Caution*).
5. Sodium molybdate—reagent grade.
6. Hydrazine sulfate—reagent grade (see Notes, *Caution*).
7. Potassium dihydrogen phosphate—reagent grade, dried at 101°C for 2 hr before use.

SOLUTIONS

1. Sodium molybdate—Carefully add 140 mL of concentrated H_2SO_4 to 300 mL of distilled water. Cool to room temperature and add 12.5 g of sodium molybdate. Dilute to 500 mL with distilled water, mix thoroughly, and allow the solution to stand for at least 24 hr before use.
2. Hydrazine sulfate, 0.015%—Dissolve 0.150 g of hydrazine sulfate in 1 liter of distilled water.
3. Potassium hydroxide, 50% solution—Dissolve 50 g of KOH in 50 mL of distilled water with cooling (see Notes, *Caution*).
4. Standard phosphate solution—
   (a) Standard stock solution—prepared by dissolving 1.0967 g of dry potassium dihydrogen phosphate (Reagents, 7) in distilled water and diluting to 250 mL in a volumetric flask. This solution contains 1 mg of phosphorus per mL.
   (b) Standard working solution—prepared by pipetting 5 mL of the standard stock solution into a 500 mL volumetric flask and diluting to volume with distilled water. This solution contains 0.01 mg of phosphorus per mL.

PROCEDURE

1. Weigh 3.0–3.2 ± 0.001 g of test sample into a Vycor crucible. Add 0.5 g of zinc oxide.
2. Heat slowly on a hot plate until the sample thickens, then gradually increase the heat until the mass is completely charred.
3. Place the crucible in a muffle furnace at 550–600°C and hold for 2 hr. Remove and cool to room temperature.
4. Add 5 mL of distilled water and 5 mL of concentrated HCl to the ash.
5. Cover the crucible with a watch glass and heat to gentle boil for 5 min.
6. Filter the solution into a 100 mL volumetric flask. Wash the inside of the watch glass and the sides of the crucible with about 5 mL of hot distilled water, using a wash bottle with a fine stream of water. Wash the crucible and the filter paper with four additional 5 mL portions of hot distilled water.
7. Cool the solution to room temperature and neutralize to a faint turbidity by the dropwise addition of 50% KOH solution. Add concentrated HCl dropwise until the zinc oxide precipitate is just dissolved, then add 2 additional drops. Dilute to volume with distilled water and mix thoroughly.

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8. Pipet 10 mL of this solution into a clean, dry 50 mL volumetric flask (see Notes, 1).
9. Add 8.0 mL of hydrazine sulfate solution and 2.0 mL of sodium molybdate solution, in the order given.
10. Stopper and invert three or four times. Loosen the stopper and heat for 10 ± 0.5 min in a vigorously boiling water bath.
11. Remove from the bath, cool to 25 ± 5°C in a water bath, dilute to volume and mix thoroughly (see Notes, 2).
12. Transfer the solution to a clean, dry cuvette. Measure the absorbance at 650 nm with the instrument adjusted to read 0% absorbance (100% transmittance) for a cuvette containing distilled water (see Notes, 1).
13. Prepare a reagent blank with no added oil by following the procedure as described in Procedure, 1-12.
14. Measure the phosphorus content of the sample and the blank by comparison with the standard curve, obtained as follows:
Preparation of Standard Curve
    (a) Pipet 0.0, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mL of the standard working solution [Solutions, 4, (b)] into 50 mL volumetric flasks. Dilute each to 10 mL with distilled water, using a measuring pipet. Proceed as directed in Procedure, 9-12. The aliquots respond, respectively, to 0.0, 0.01, 0.02, 0.04, 0.06, 0.08, and 0.10 mg of phosphorus.
    (b) Plot the absorbance of each standard against its phosphorus content in milligrams on linear graph paper.

CALCULATIONS

1. Phosphorus, $\% = \frac{10\,(A - B)}{W \times V}$

    Where—

    A = phosphorus content of sample aliquot in mg
    B = phosphorus content of blank aliquot in mg
    W = mass of test portion in g
    V = volume of aliquot taken in Procedure, 8.
2. Equivalent phosphatides, % = % phosphorus × 30 (see Notes, 4)

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors.

When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive splattering. Always add the acid to water, never the reverse.

Hydrazine sulfate can cause eye, skin, and mucous membrane irritation, and liver and kidney damage. This compound is a known carcinogen in laboratory animals, causing lung and liver tumors in rats. It is a suspected human carcinogen. Precautions in handling this compound include use of gloves, eye protection, and respiratory protection. Avoid the inhalation of dust and powder. Dispose of waste material and waste solutions in a proper and safe manner.

NUMBERED NOTES

1. If the absorbance of the colored solution measured in Procedure, 12 is too high (>0.9), take a smaller size aliquot of the sample solution than specified in Procedure, 8 (e.g., 2.0 mL), dilute to 10 mL with distilled water using a measuring pipet and continue as directed in Procedure, 9-12.
2. Samples of high phosphorus content may still give absorbencies >0.9. If this is the case, pipet 10 mL of the test solution prepared in Procedure, 7 into a 100 mL volumetric flask and dilute to volume with distilled water. Carry out the color development sequence noted in 8–12, using an appropriate size aliquot diluted to 10 mL with distilled water. Multiply the phosphorus content obtained, using the equation in Calculations, by the dilution factor (10, if following the procedure described in this paragraph).
3. No unreasonable delay should be incurred between developing the color in Procedure, 11 and measuring the absorbance in Procedure, 14.
4. This is an approximation for converting percent phosphorus to percent phosphatides in soybean oil.

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AOCS Official Method Ca 12a-02

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Colorimetric Determination of Phosphorus Content in Fats and Oils

DEFINITION

The test portion is carbonized in the presence of magnesium hydroxycarbonate and then ashed. The ash is dissolved in dilute hydrochloric acid after which the phosphorus is determined colorimetrically by the molybdenum blue method.

SCOPE

This method specifies a colorimetric method for the determination of the phosphorus content of animal and vegetable oils and fats (see Notes, 1).

APPARATUS

Usual laboratory apparatus and, in particular, the following:

1. Test tubes, of 25 mL capacity, made of borosilicate glass, with stoppers and standard tapered necks, and graduated at 5 mL-intervals or less. The reproducibility of the 15 mL graduation should be checked, as well as the resistance of the calibration marks to heating at 550°C.
2. Block or muffle furnace, thermostatically controlled for temperatures up to 400°C.
3. Ashing oven or muffle furnace, suitable for temperatures up to 700°C.
4. Tube rack, resistant to high temperatures, preferably of corrosion resistant steel, for use in the muffle furnace. The rack should hold the tubes at such an angle that the open ends are about 3 cm above the bottom of the tubes.
5. Spectrophotometer, suitable for measurements at 720 nm, using 1 cm and 4 cm cells.
6. Spectrophotometer cells, of path length 1 cm, and 4 cm, and suitable for measurements at 720 nm.

REAGENTS

Use only reagents of recognized analytical grade, unless otherwise stated.

1. Magnesium hydroxycarbonate—$[(MgCO_3)n{\cdot}Mg(OH)_2] \cdot H_2O$ with a MgO content of 40% to 46% (by mass). Magnesium carbonate, hydrated, basic, $[(MgCO_3)_4 \cdot Mg(OH)_2]{\cdot}5H_2O$ is suitable.
2. Hydrochloric acid = 2 M.
3. Sodium hydroxide solution—c(NaOH) = 5 M.
4. Reducing solution—Weigh out 0.500 g metol $[(HOC_6H_4NHCH_3)2{\cdot}H_2SO_4]$, 2.5 g sodium bisulfite heptahydrate $(Na_2SO_3{\cdot}7H_2O)$, and 58.5 g sodium metabisulfite $(Na_2S_2O_5)$. Transfer the weighed out materials to a 1 L volumetric flask, dissolve in water, make up to the mark, and mix. Keep the solution in a well-sealed brown bottle.
5. Sulfate-molybdate reagent—Dissolve 25.0 g of ammonium molybdate tetrahydrate $[(NH_4)6Mo_7O_{24}{\cdot}4H_2O]$ in 250 mL of sulfuric acid solution = 5 M (prepared by diluting 278 mL sulfuric acid, $c(H_2SO_4 = 18$ mol/L, with water to 1 liter). Transfer the solution to a 1 L volumetric flask, make up to the mark with water, and mix. Store the solution in a brown bottle. WARNING: Care must be taken when diluting concentrated sulfuric acid.
6. Sodium acetate solution—Dissolve 340 g of sodium acetate trihydrate $(CH_3COONa{\cdot}3H_2O)$ in water, transfer to a 1 L volumetric flask, make up to the mark with water, and mix. Store the solution in a brown bottle.
7. Standard phosphate solution for calibration—
   a. Stock solution (phosphorus content ca 100 µg/mL)—Weigh to 0.1 mg, about 440 mg of potassium dihydrogen phosphate (KH_2PO_4). Dissolve it in water, transfer quantitatively to a 1 L volumetric flask, make up to the mark with water, and mix. Calculate the phosphorus content of the solution by the formula:

$$P_b = \frac{m_s \times M_p}{V \times M_s}$$

Where—

P_b = the phosphorus content of stock solution in micrograms per milliliter (µg/mL)
m_s = the mass of potassium dihydrogen phosphate in milligrams
M_p = the molar mass of phosphorus in grams (M_p = 31.03 g)
V = the volume of stock solution in flask in liters (V = 1)
M_s = the molar mass of potassium dihydrogen phosphate in grams (M_s = 136.09 g)

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b. Standard phosphate solution 1, (phosphorus content ca 10 µg/mL). Pipette 25 mL of stock solution [Reagents, 7(b)] into a 250 mL volumetric flask, make up to the mark with water, and mix. Calculate the phosphorus content of this solution by the formula:

$$P_{s1} = 0.1\ P_b$$

Where—

P_{s1} = the phosphorus content of standard phosphate solution 1, in micrograms per milliliter (µg/mL)
P_b = the phosphorus content of the stock solution [stock solution (phosphorus content ca 100 µg/mL)] in micrograms per milliliter (µg/mL)

c. Standard phosphate solution 2, (phosphorus content ca 50 µg/mL). Pipette 50 mL of stock solution (Reagents, 1) into a 100 mL volumetric flask, make up to the mark with water and mix. Calculate the phosphorus content of this solution by the formula:

$$P_{s2} = 0.1\ P_b$$

Where—

P_{s2} = the phosphorus content of standard phosphate solution 2, in micrograms per milliliter (µg/mL)
P_b = the phosphorus content of the stock solution [Reagents, 7(a)] in micrograms per milliliter (µg/mL)

SAMPLING

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage. A recommended sampling method is given in AOCS Official Method C 1-47 or ISO 5555. Store samples in glass or polyethylene terephthalate (PET) bottles.

PREPARATION OF TEST SAMPLE

If the laboratory sample is not completely molten at room temperature, heat it to a maximum of 10°C above the melting point. If the sample is not clear when liquid, homogenize it carefully immediately before weighing out the test portions. It is essential that any sediment, which may be rich in phosphorus, is incorporated homogeneously into the sample.

PROCEDURE

1. Determination of the calibration factor
   a. For phosphorus contents of 0 mg/kg to 125 mg/kg (in the oil)—
      1. Weigh 30 mg of magnesium hydroxycarbonate (Reagents, 1) into each of a series of 7 test tubes (Apparatus, 1), and using a microburette or pipette add to the test tubes 0 mL (blank); 0.25 mL; 0.5 mL; 1.0 mL; 1.5 mL; 2.0 mL; and 2.5 mL of standard phosphate solution 1 [Reagents, 7(b)] so that the test tubes contain quantities of phosphorus ranging from 0 µg to ca 25 µg, equivalent to the amount of phosphorus in 0.2 g of an oil containing between 0 mg/kg to about 125 mg/kg phosphorus (see Notes, 2).
      2. Add 2 mL of hydrochloric acid (Reagents, 2) to each test tube and wait until a clear solution is obtained. Then add 0.5 mL of sodium hydroxide solution (Reagents, 3) to each test tube and mix.
      3. Using a pipette or burette add 5 mL of reducing solution (Reagents, 4) to each test tube and mix.
      4. In a similar manner, add 2.5 mL of sulfatemolybdate reagent (Reagents, 5) to each test tube and mix. Stopper the test tubes and let them stand for 20 minutes in a dark place.
      5. Fill the test tubes to the 15 mL mark with sodium acetate solution (Reagents, 6) and mix.
      6. Measure the absorbance of the solutions against the blank, in a 1 cm cell, at 720 nm (see Notes, 3).
      7. Calculate the calibration factor in accordance with Procedure, 1 (c). Alternatively compute the equation of the regression line for the calibration.
   b. For phosphorus contents of 125 mg/kg to 500 mg/kg (in the oil)—
      1. Weigh 30 mg of magnesium hydroxycarbonate (Reagents, 1) into each of a series of 6 test tubes, and using a microburette or pipette add to the test tubes 0 mL (blank); 0.5 mL; 0.8 mL; 1.2 mL; 1.6 mL; and 2.0 mL of standard phosphate solution 2 [Reagents, 7 (c); phosphorus content ca 50 µg/mL] so that the test tubes contain quantities of phosphorus ranging from about 25 µg to 100 µg, equivalent to phosphorus contents of about 125 mg/kg to 500 mg/kg in the oil. The absorbance of the highest standard, in a 1 cm cell, should be approximately 0.8.
      2. Add 2 mL of hydrochloric acid (Reagents, 2) to each test tube and wait until a clear solution is obtained. Then add 0.6 mL of sodium hydroxide solution (Reagents, 3) to each test tube and mix.
      3. Using a pipette or burette add 5 mL of reducing solution (Reagents, 4) to each test tube and mix.
      4. In a similar manner add 2.5 mL of sulfate-molybdate reagent (Reagents, 5) to each test tube and mix. Stopper the tubes and let them stand for 20 minutes in a dark place.
      5. Fill the test tubes to the 15 mL mark with sodium acetate solution (Reagents, 6) and mix.

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6. Measure the absorbance of the solutions against the blank, in a 4 cm cell, at 720 nm (see Notes, 3).
7. Calculate the calibration factor in accordance with Procedure 1(c). Alternatively compute the equation of the regression line for the calibration.

c. Calculation of the calibration factor: For each solution i of the series measured for phosphorus contents of 0 mg/kg to 125 mg/kg (in the oil) and for phosphorus contents of 125 mg/kg to 500 mg/kg (in the oil) calculate the calibration factor using the formula:

$$f_i = \frac{(V_i \times P_i)}{A_i}$$

Where—

f_i = the calibration factor for solution i of the series, in micrograms
V_i = the volume of standard phosphate solution in solution i, in milliliters
P_s = the phosphorus content of the phosphate solution used, in micrograms per milliliters (µg/mL)
A_i = the absorbance measured for solution i

Use the average of the factors f_i as the calibration factor f for the calculation in (Calculation).

2. Ashing of oil test portion—
   a. Weigh approximately 30 mg of magnesium hydroxycarbonate (Reagents, 1) into a test tube (Apparatus, 1) and weigh the test tube, with the magnesium hydroxycarbonate, to 0.1 mg.
   b. Using a Pasteur pipette add approximately 0.2 g (10 to 15 drops) of the oil sample (6), taking care that all the material falls to the bottom of the tube and mixes with the magnesium hydroxycarbonate. No drops should be allowed to fall or splash onto the side walls of the test tube.
   c. Re-weigh the test tube to 0.1 mg.
   d. Prepare a blank test tube containing magnesium hydroxycarbonate only.
   e. Place the test tubes in the heating block or in the tube rack (Apparatus, 4) in the cold muffle furnace (Apparatus, 2). Heat the test tubes to 350°C until the sample is carbonized to a dry black mass (1 hour to 2 hours).
   f. After carbonization, increase the temperature to 550°C and heat the sample at this temperature until the ash is completely white (about 2 hours).
   g. Remove the test tubes (and tube rack) and allow the test tubes to cool.
3. Colorimetric determination—
   a. Dissolve the residue from the ashing procedure in 2 mL of hydrochloric acid (Reagents, 2) by warming carefully until the liquid boils.
   b. Allow the test tubes to cool and neutralize the contents by adding 0.6 mL of sodium hydroxide solution (Reagents, 3) to each, then add, using a measuring pipette or burette, 5 mL of reducing solution (Reagents, 4) and mix.
   c. In a similar manner add 2.5 mL of sulfate-molybdate reagent (Reagents, 5) and mix.
   d. Stopper the test tubes and allow them to stand in a dark place for 20 minutes.
   e. Fill the test tubes to the 15 mL mark with sodium acetate solution (Reagents, 6) and mix.
   f. Measure the absorbance of the solution against the blank in a 4 cm cell at 720 nm (see Notes, 6).
   g. If the measure of absorbance is higher than that of the highest standard (about 0.8) it lies outside the calibration range and the measurements must be repeated, using a 1 cm cell.

CALCULATION

Calculate the phosphorus content using the formula:

$$w_p = \frac{(f \times A)}{m}$$

Where—

w_p = the phosphorus content of the test portion, in milligrams per kilograms (mg/kg)
f = the average calibration factor calculated as in Procedure, 1 (c), in micrograms
A = the absorbance measured as in Reagents, 3
m = is the mass of the test portion, in grams

Alternatively, if the equation of a regression line is used for the calculation of the phosphorus content of the test portion then:

$$w_p = \frac{m_p}{m}$$

Where—

w_p = the phosphorus content of the oil, in milligrams per kilograms (mg/kg)
m_p = the phosphorus content of the test portion (Reagents, 2), in micrograms
m = the mass of the test portion (Reagents, 2), in grams

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PRECISION

1. Interlaboratory test—Details of interlaboratory tests on the precision of the method are summarized in Table 1. The values derived from these interlaboratory tests may not be applicable to concentration ranges and matrices other than those given. Two international tests were carried out on samples of soybean oil using the colorimetric method.
   a. Results of the first interlaboratory test (1995)—The first interlaboratory test was organized by the Federation of Oils, Seeds and Fats Associations Ltd. (FOSFA) involving 8 laboratories in 4 countries. It was carried out in July 1995. The results obtained were subjected to statistical analysis in accordance with ISO 5725:1986 (3); the precision data are shown in Table 1.
   b. Results of the second interlaboratory test (1999)—The second interlaboratory test was organized by FOSFA International and the American Oil Chemists' Society (AOCS), involving 13 laboratories. The results obtained were subjected to statistical analysis in accordance with ISO 5725-1:1994 and ISO 5725-2:1994; the precision data are shown in Table 2.

      The same samples were also tested using atomic absorption spectrometry (AAS), and inductively coupled plasma (ICP) optical emission spectrometry. Statistical results related to AAS and ICP methods are presented in Ca 12b-92 and Ca 20-99, respectively.
2. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time will in not more that 5% of cases be greater than the repeatability limit (r), deduced by linear interpolation from Table 3.
3. Reproducibility—When the values of two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, lie within the range of the values in Table 3, the absolute difference between the two test results will in not more than 5% of cases be greater than the reproducibility limit (R), deduced by linear interpolation from Table 3.

NUMBERED NOTES

1. This method is not suitable for determining the phosphorus content of commercial lecithin as this requires an ashing temperature of 800°C.
2. The absorbance of the highest standard, containing 2.5 mL of standard phosphate solution 1, under these conditions should be approximately 0.8.
3. Alternatively, measure the absorbance of all the solutions against water, as a check on the blank, and then, unless the equation of a regression line is to be computed, correct all the measurements for the blank value.

REFERENCES

1. ISO 5555:1991, Animal and vegetable fats and oils—sampling.
2. ISO 661:1989, Animal and vegetable fats and oils—Preparation of test samples.
3. ISO 5725:1986, Precision of test methods—Determination of repeatability and reproducibility for a standard test method by interlaboratory tests (withdrawn in 1994).
4. ISO 5725-1:1994, Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions.
5. ISO 5725-2:1994, Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.
6. ISO/DIS 10540-2:2001, Animal and vegetable fats and oils—Determination of phosphorus content—Part 2: Method using graphite furnace atomic absorption spectrometry.
7. ISO/DIS 10540-3:2001, Animal and vegetable fats and oils—Determination of phosphorus content—Part 3: Method using inductively coupled plasma (ICP) optical emission spectroscopy.

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Table 1
Statistical results of the first interlaboratory test (1995).

	Sample[a]	
Parameter	A	B
Number of participating laboratories after eliminating outliers	8	8
Mean value, mg/kg	10.51	318.31
Repeatability standard deviation, s_r, mg/kg	0.68	4.41
Coefficient of variation of repeatability, %	6.47	1.39
Repeatability limit r ($r = 2.8\ s_r$), mg/kg	1.92	12.34
Reproducibility standard deviation, s_R, mg/kg	2.12	17.93
Coefficient of variation of reproducibility, %	20.17	5.63
Reproducibility limit R ($R = 2.8\ s_R$), mg/kg	5.96	50.21

[a]Sample A: RBD soybean oil.
Sample B: Crude water degummed soybean oil.

Table 2
Results of the second interlaboratory test (1999).

	Sample[a]							
Parameter	A	B	C	D	E	F	G	H
Number of participating laboratories after eliminating outliers	7	7	7	7	7	7	7	7
Mean value, mg/kg	473.1	305.0	131.6	101.4	56.6	32.0	13.8	273.8
Repeatability standard deviation, s_r, mg/kg	14.2	5.1	3.5	4.3	3.0	2.8	1.1	2.4
Coefficient of variation of repeatability, %	3.0	1.7	2.7	4.2	5.3	8.6	8.2	2.9
Repeatability limit r ($r = 2.8\ s_r$), mg/kg	39.8	14.3	9.8	12.0	8.4	7.9	3.2	6.7
Reproducibility standard deviation, s_R, mg/kg	56.4	59.2	31.0	14.6	6.5	3.3	2.2	56.4
Coefficient of variation of reproducibility, %	11.9	19.4	23.6	14.4	11.5	10.4	16.2	20.7
Reproducibility limit R ($R = 2.8\ s_R$), g/kg	158.0	165.7	86.8	40.9	18.2	9.3	6.3	158.0

[a]Samples of a soybean oil containing different levels of phosphorus.

Table 3
Repeatability and reproducibility limits at different phosphorus contents.

Phosphorus content	r	R
mg/kg	mg/kg	mg/kg
10	2	6
50	8	18
100	12	41
300	13	105
400	34	135

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AOCS Official Method Ca 12b-92

Reapproved 2009

Phosphorus by Direct Graphite Furnace Atomic Absorption Spectrometry

DEFINITION

This method, based on International Union of Pure and Applied Chemistry (IUPAC) Method 2.423 (see References, 1), determines the total phosphorus present in oils and fats, expressing the value as mg/kg (parts per million, ppm). The method requires the vaporization of the oil or fat in a suitable graphite furnace, with or without a L'vov platform, connected to an atomic absorption spectrometer, previously calibrated using standard solutions of soy lecithin and the measurement of the phosphorus content from the observed absorption at a wavelength of 213.6 nm.

SCOPE

This method is for the determination of trace amounts (mg/kg) of phosphorus in all types of crude or refined edible oils and fats. Spectral interference by other elements is not to be expected, provided the measurements are carried out at the wavelength specific for phosphorus (213.6 nm). Replaces Ca 12b-87.

APPARATUS

1. Test tubes—10 mL.
2. Micropipet—20 µL.
3. Pipet tips.
4. Electric oven—regulated at 60 ± 2°C.
5. Atomic absorption spectrometer—equipped with either "peak height" mode and printer, or "continuous" mode and pen recorder (full-scale response in 0.2 sec), together with the appropriate electrode-less discharge lamp (or hollow cathode lamp) and deuterium background corrector (or Zeeman atomic absorption spectrometer).
6. Graphite furnace atomizer—placed in the atomic absorption spectrometer (Apparatus, 5), equipped with a control unit for temperature programming.
7. Graphite tube—normal (uncoated).
8. Platform—pyrolytic, in combination with uncoated graphite tube (see Numbered Notes, 1).

REAGENTS

1. Cyclohexane—analytical grade (see Notes, *Caution*).
2. Lanthanum organometallic standard—such as lanthanum-2,4-pentadionate (see Numbered Notes, 2). A suitable standard (Conostan, 5000 mg/kg) is available from Conoco, Ponca City, OK, USA.
3. Matrix modifier (see Numbered Notes, 2)—0.05% (m/v) Lanthanum solution is prepared by dissolving 5 g lanthanum standard (Reagents, 2) in 50 mL cyclohexane (Reagents, 1), [see Procedure, 1, (c) for use of matrix modifier].
4. Blank oil—refined liquid edible oil with a phosphorus content not greater than 1 mg/kg (see Numbered Notes, 3).
5. Lecithin—a well-defined lecithin containing 2% phosphorus, e.g., soy lecithin (see Numbered Notes, 3).
6. Standard stock solution—A stock solution of 400 mg P/kg is prepared by dissolving 1 g of lecithin (Reagents, 5) in 4 g cyclohexane (Reagents, 1) and 45 g of blank oil (Reagents, 4).
7. Standard working solutions—10 mg P/kg, 20 mg P/kg, and 40 mg P/kg are prepared by diluting the 400 mg/kg stock solution (Reagents, 6) with blank oil (Reagents, 4).
8. Argon—minimum purity 99.99%.

PROCEDURE

1. Treatment of test samples, blank, and standards—
   (a) Place all test samples, blank oil, and standard working solutions in the oven (Apparatus, 4), regulated at 60 ± 2°C.
   (b) Shake test samples vigorously.
   (c) Weigh 1.00 g test portion in a 10 mL test tube together with 1.00 g matrix modifier (Reagents, 3) and mix thoroughly (see Numbered Notes, 4).
   (d) Apply steps (b) and (c) to the three standard working solutions (Reagents, 7) and the blank oil (Reagents, 4).
2. Preparation of apparatus—
   (a) In accordance with the manufacturer's instructions supplied with the spectrometer, switch on the atomic absorption spectrometer system and adjust the lamp current, slit width, wavelength, and amplification. The required wavelength is 213.6 nm.

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(b) Optimize the position of the graphite furnace atomizer (Apparatus, 6) in the atomic absorption spectrometer (Apparatus, 5), and set the required program on the control unit of the furnace. If available, place platform in graphite tube.
(c) Pretreat the pipet tip (Apparatus, 3) before each injection by pipetting and then discarding 20 µL cyclohexane.
(d) Inject 20 µL of the standard working solution with a content of 40 mg P/kg (Reagents, 7) with the micropipet (Apparatus, 2) into the graphite furnace, initiate the temperature program and record the absorption.
(e) Repeat the previous step until the absorption is constant (see Notes, 5).

3. Program for the graphite furnace atomizer (see Numbered Notes, 6):

Step no.	Temp, °C	Ramp time, sec	Hold time, sec	Initial gas flow, mL/min.
1	600	40	20	300
2	1600	50	40	300
3	2800	0	5	0
4	2800	1	3	50

DETERMINATION

1. Measurement of the graphite tube blank—Record the absorption, if any, of the graphite tube as such and autozero this absorption (see Notes, 5).
2. Measurement of the blank—Inject 20 µL of the blank solution prepared according to Procedure, 4 into the graphite furnace, initiate the temperature program and record the absorption.
3. Measurement of the working standards—Inject 20 µL of the three standard solutions prepared according to Procedure, 1, (d) into the graphite furnace and record the absorptions.
4. Measurement of test solutions—Inject 20 µL of the test solution prepared according to Procedure, 1, (c) into the graphite furnace, initiate the temperature program and record the absorption.

CALCULATIONS

1. Measure the peak height on the recorder chart, or take the reading of the display or printer.
2. Draw a calibration curve by plotting the absorption of the three standards (Determination, 2), corrected for the blank (Determination, 1), against their respective phosphorus content (see Notes, 3).
3. Measure the absorption of the sample and correct for the blank.
4. Read the phosphorus content of the sample from the calibration curve.
5. Express the results as mg/kg (to two significant figures).

PRECISION

1. Repeatability limit—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) as calculated from the formulas in Table 1.
2. Reproducibility limit—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as calculated from the formulas in Table 1.
3. Trueness (bias)—The bias of the method was demonstrated in the collaborative study of the method (see Table 2) to be negligible when used for the determination of concentrations of phosphorus in the range 10-30 mg/kg.
4. Sensitivity—The sensitivity of the method is demonstrated by the low values for r and R at the low absorption levels studied (see Table 2). The limit of detection is 0.1 mg/kg; the limit of determination is 1 mg/kg.
5. An interlaboratory test carried out at the international level in 1989 by the IUPAC Commission on Oils, Fats and Derivatives, in which 21 laboratories participated, each obtaining two test results for each sample, gave the statistical results [evaluated in accordance with International Organization for Standardization (ISO) 5725–1986] summarized in Table 2 (References, 2).
6. The second interlaboratory test were organized by FOSFA International and the American Oil Chemists Society (AOCS), involving 6 laboratories. It was carried out in 1999. The results obtained were subjected to statistical analysis in accordance with ISO 5725-1:1994 and ISO 5725–2:1994, the precision data are shown in Table 3.

NOTES

Caution

Cyclohexane is flammable and a dangerous fire risk. It is moderately toxic by inhalation and skin contact. The TLV in air is 300 ppm.

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NUMBERED NOTES

1. Both atomization off the wall and atomization off the platform can be used. It is not necessary to change the temperature program for the graphite furnace.
2. The amount of phosphorus found depends on the types of phosphatide present in the oil. The addition of lanthanum proved to be essential to find the total amount of phosphorus. The lanthanum standard is specified to enhance the phosphorus signal and stabilize the phosphorus so it is not lost. Instead of lanthanum, calcium may be used. Conostan metallo organic standards, including lanthanum, are available from Interchim, France and Conoco, Ponca City, OK, USA.
3. The concentration of phosphorus in the blank oil and in the lecithin is determined by an accepted digestion/colorimetric method, IUPAC 2.421, AOCS Official Method Ca 12-55, or ISO 10540-part 1. As an alternative to lecithin, a phosphatide of known purity, e.g., phosphatidyl serine, could be used.
4. If the expected or found concentration of phosphorus is higher than 40 mg/kg, dilute the sample with blank oil (Reagents, 4). In that case, multiply the observed concentration (Determination, 4) with the correct dilution factor.
5. With a new graphite tube, three to four "determinations" using one and the same working standard have to be carried out in order to obtain an acceptable state of equilibrium.
6. For those having a Varian apparatus, the following temperature program should be used:

Step no.	Temp, °C	Time, sec	Gas flow, L/min
1	120	20	3.0
2	120	20	3.0
3	500	30	3.0
4	500	10	3.0
5	1600	50	3.0
6	1600	20	3.0
7	1600	2	0.0
8	2800	1	0.0
9	2800	2	0.0
10	2800	5	3.0
11	40	20	3.0

REFERENCES

1. AOCS Official Method Ca 12b-92 replaces AOCS Recommended Practice Ca 12b-87, Phosphorus by Atomic Absorption Spectroscopy. Method Ca 12b-92 was adopted from *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.423 and is the basis of ISO 10540-part 2.
2. Collaborative study results published in *Pure Appl. Chem. 63:*1191 (1991).

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Table 1
Repeatability (r) and reproducibility (R) limits.

P in edible oil:	$r = 0.19 \times m$	$R = 0.30 \times m$
Where—		
m = corresponding mean concentration value		

Table 2
Statistical and other data derived from the results of the interlaboratory test.

Sample:	Sunflower oil		
Batch:	A (high)	B (medium)	C (low)
Number of laboratories retained after eliminating outliers	17	17	17
Number of outliers, laboratories	2	1	1
Number of accepted results	32	34	34
Mean value, mg/kg	29.55	20.00	10.18
True or accepted value, mg/kg	28.50	19.00	9.00
Repeatability standard deviation, s_r in mg/kg	2.30	1.68	0.91
Repeatability relative standard deviation, RSD_r, %	7.8	8.4	9.0
Repeatability limit, r, $2.8 \times s_r$	6.45	4.71	2.56
Reproducibility standard deviation, s_R in mg/kg	3.51	2.33	1.24
Reproducibility relative standard deviation, RSD_R, %	11.9	11.7	12.2
Reproducibility limit, R, $2.8 \times s_R$	9.82	.48	6.633

Table 3
The second interlaboratory test were organized by FOSFA International and the American Oil Chemists' Society (AOCS), involving 6 laboratories (see Notes, 12).

	Sample[a]						
Parameter	A	B	C	D	E	F	G
Number of participating laboratories after eliminating outliers	5	5	5	5	5	5	5
Mean value, mg/kg	388.1	285.4	118.1	93.2	51.2	27.1	13.4
Repeatability standard deviation, s_r, mg/kg	3.3	15.6	1.9	12.0	1.1	1.0	0.3
Coefficient of variation of repeatability, %	1.4	5.5	1.6	12.8	2.1	3.5	2.2
Repeatability limit r ($r = 2.8 \times s_r$), mg/kg	14.7	43.5	5.2	33.5	3.0	2.7	0.8
Reproducibility standard deviation, s_R, mg/kg	127.5	77.5	39.4	22.1	13.7	5.4	3.8
Coefficient of variation of reproducibility, %	32.9	27.2	33.4	23.7	26.7	19.4	28.6
Reproducibility limit R ($R = 2.8 \times s_R$), mg/kg	356.9	216.9	110.4	61.8	38.3	15.0	10.8

[a]Samples of soybean oil containing different levels of phosphorus.

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AOCS

AOCS Official Method Ca 13-56

Revised 2013

Total Gossypol in Oils

DEFINITION

Total gossypol defines gossypol and gossypol derivatives, both free and bound, in cottonseed products that are capable of reacting with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, which then reacts with aniline to form dianilinogossypol under the conditions of the test method. Gossypol, gossypol analogs and gossypol derivatives having an available aldehyde moiety are measured by the method (see Notes, 1).

SCOPE

Applicable to crude cottonseed oil and cottonseed soap stock (see Notes, 2).

APPARATUS

1. Water bath—for operation at 95–100°C, with clamps for supporting 50 and 25 mL volumetric flasks. Metal washers may be placed over the necks of the flasks for support.
2. Spectrophotometer—for operation at 440 nm with 1 cm light path cuvettes.
3. Volumetric flasks—25, 50, and 100 mL.
4. Pipets—class A volumetric, 1, 2, 4, 5, and 10 mL.
5. Filter paper—medium retentivity, 11 cm in diameter.

REAGENTS

1. Solvents, reagent grade (see Notes, *Caution*)—isopropyl alcohol (2-propanol); *n*-hexane (boiling range 68–69°C); dimethylformamide (N,N-dimethylformamide); 3-amino-1-propanol (propanolamine), free of color; glacial acetic acid; and aniline. The aniline should be redistilled over zinc dust using water-cooled condenser. Discard first and last 10% of distillate, and store in brown bottle in refrigerator when not in use. Redistill when reagent blank (Procedure, 8) absorbance exceeds 0.022.
2. Isopropyl alcohol-hexane mixture—60 volumes of isopropyl alcohol and 40 volumes of n-hexane.
3. Complexing reagent—prepared by pipetting 2 mL of 3-amino-1-propanol and 10 mL glacial acetic acid into a 100 mL volumetric flask, cooling to room temperature and diluting to volume with dimethylformamide. Prepare reagent weekly and store in refrigerator when not in use.
4. Gossypol or gossypol acetic acid—primary standard quality. Gossypol and gossypol acetic acid standards are available from Atomergic Chemetals Corp., Plainview, NY, USA; Sigma Chemical Co., St. Louis, MO, USA; Chemical Dynamics Corp., South Plainfield, NJ, USA; Aldrich Chemical Co., Milwaukee, WI, USA. Determine purity as in AOCS Official Method Ba 7-58 (Reagents, 4). Absorptivity of 39.1–39.9 for gossypol and 35.1–35.8 for gossypol acetic acid indicate satisfactory purity in range of 98–100%. Gossypol acetic acid contains 89.62% gossypol by weight, or 0.8962 mg gossypol per mg of gossypol acetic acid.
5. Standard gossypol solution—prepared by weighing 25 mg of primary standard gossypol, or 27.9 mg of gossypol acetic acid into a 50 mL volumetric flask. Dissolve in and make up to volume with the complexing reagent (Reagents, 3). Solution is stable up to 1 week if stored in refrigerator. If exact amounts of either gossypol or gossypol acetic acid were weighed as indicated, either solution contains 0.50 mg gossypol/mL. Multiply milligrams gossypol acetic acid by 0.8962 to obtain milligrams of gossypol.

CALIBRATION (SEE NOTES, 3)

1. Pipet duplicate 1, 2, 4, 6, 8 and 10 mL aliquots of the standard gossypol solution (Reagents, 5) into 50 mL volumetric flasks. To each standard add sufficient volume of complexing reagent (Reagents, 3) to make volume 10 mL. Use 10 mL of complexing reagent as a blank.
2. Heat flasks in a water bath (95–100°C) for 30 min, cool to room temperature, dilute to volume with the isopropyl alcohol-hexane solution and mix well.
3. Pipet duplicate 2 mL aliquots of each standard and the reagent blank into 25 mL volumetric flasks.
4. Dilute one set of standard aliquots and the reagent blank to volume with the isopropyl alcohol-hexane solution, and reserve as reference solutions for absorbance measurements.
5. Add 2 mL aniline to the other set of standard aliquots and the blank, heat in water bath (95–100°C) for 30 min, cool to room temperature, dilute to volume with the isopropyl alcohol-hexane solution, and mix well. Allow to stand for 1 hr at room temperature before determining absorbance.

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6. Determine absorbance of reagent blank using a spectrophotometer at 440 nm (Apparatus, 4) as in Procedure, 8, and of the standard aliquots as in Procedure, 9. Subtract absorbance of reagent blank from absorbance of each standard to obtain corrected absorbance for use in following step.
7. Calculate a linear regression calibration factor, F, using the actual milligrams of gossypol in each final standard solution (Calibration, 5) and its corresponding corrected absorbance (Calibration, 6) in the following equation.

$$\text{mg gossypol} = F\ (\text{corrected absorbance})$$

The resulting r^2 should be at least 0.999 with the slope F being the calibration factor.

PREPARATION OF TEST SAMPLE

1. Cottonseed—Dehull about 50 g sample, free from foreign matter as in AOCS Official Method Aa 2-38, using Bauer mill with plates separated so seeds are just broken. Screen on 4–6 mesh sieve to remove meats from hulls and lint. Grind meats through 2 mm screen in a Wiley mill, avoiding over-heating meats and expressing oil.
2. Cooked meats—Reduce moisture to 5% or below by air equilibration, or by blowing air over meats. Grind through 2 mm screen in a Wiley mill.
3. Press cake and meal—Prepare sample as in AOCS Official Method Ba 1-38 and grind 50 g through 1 mm screen in a Wiley mill.
4. Crude oil—Heat portions of the laboratory sample to 50°C, mix well and filter through paper suitable for oil filtration (Apparatus, 7). Analyze filtered sample within 1 day, or store at –20°C (0°F) if analysis is delayed. Gossypol in oil undergoes changes if stored at or above room temperature.
5. Soapstock—Heat to 50°C and mix well before withdrawing analytical sample. Store under same conditions as for crude oil.

TEST PORTION SIZE

1. Test portion weight and aliquot used for aniline reaction depend on expected total gossypol content. Ideally, the test portion should contain from 0.5 to 5.0 mg of gossypol, and the aliquot for the aniline reaction should contain about 0.1 mg gossypol. Table 1 is a guide, providing milligrams of gossypol for aniline reaction within the range used for calibration.

PROCEDURE

1. Weigh test portion (see Test Portion Size and Table 1) on analytical balance accurate to ± 0.001 g and transfer to a 50 mL volumetric flask. A small piece of aluminum foil or weighing paper is useful for weighing and transferring sample to flask. Add 10 mL complexing reagent (Reagents, 3) by pipet, washing down any material adhering to neck of flask. A rapid delivery pipet may be used (see Notes, 4).
2. Prepare reagent blank containing 10 mL of complexing reagent in a 50 mL volumetric flask.
3. Heat test portion and blank in water bath (95–100°C) for 30 min, cool to room temperature, dilute to volume with isopropyl alcohol-hexane mixture (Reagents, 2) and mix well.
4. Filter test portion extract through 11 cm medium-retention paper (Apparatus, 7) into a 50 mL glass-stoppered Erlenmeyer flask, discarding first 5 mL of filtrate.
5. Pipet duplicate aliquots of extract (see Test Portion Size and Table 1) into 25 mL volumetric flasks. Pipet duplicate blank aliquots of same volume as test portion aliquot into 25 mL volumetric flasks.
6. Dilute one set of test and blank aliquots to volume with the isopropyl alcohol-hexane mixture and reserve as reference solutions for absorbance measurement.
7. Add 2 mL aniline, by pipet, to the other set of test and reagent blank aliquots and heat in a water bath (95–100°C) for 30 min. Cool to room temperature, dilute to volume with the isopropyl alcohol-hexane mixture, and mix well. Allow to stand for 1 hr at room temperature after dilution (see Notes, 6).
8. With a spectrophotometer (Apparatus, 4) at 440 nm, determine absorbance of reagent blank reacted with aniline (Procedure, 7), using blank aliquot without aniline (Procedure, 6) as reference solution.
9. Determine absorbance of test aliquot reacted with aniline (Procedure, 7), using diluted sample aliquot without aniline (Procedure, 6) as reference solution. Subtract absorbance of reagent blank (Procedure, 8) from that of test aliquot reacted with aniline to obtain corrected absorbance (see Notes 5, 6).
10. From corrected absorbance of test aliquot (Procedure, 9), determine milligrams of gossypol in sample aliquot by multiplying absorbance by the calibration factor (F) (Calibration, 7).

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CALCULATIONS

1. Calculate percentage total gossypol in sample as follows:

$$\text{Total gossypol, \%} = \frac{5 \times G}{W \times V}$$

Where—

G = mg gossypol in test aliquot (Procedure, 10)
W = mass of test portion in grams
V = volume, mL of test aliquot used for analysis (Procedure, 9)

2. If gossypol concentration is desired in parts per million (mg/kg), use the following equation:

$$\text{Total gossypol, \%} = \frac{5 \times G}{W \times V} \times 10{,}000$$

PRECISION

1. A collaborative study established the following precision estimates for the method. As the percentage gossypol decreases, precision (coefficient of variation, CV) becomes poorer. Precision in the 0.50–0.10% range was estimated to be 3.5–7.0%; for the 0.06–0.01% range the precision was estimated to be 10.0–17.0%. See Table 2 for precision values related to gossypol determination.

NOTES

Caution

Isopropyl alcohol and n-hexane are flammable solvents. They should not be used near an open flame. The use of a properly operating fume hood is recommended when using these solvents. The TLV for hexane is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

Aniline is an allergen and is toxic if absorbed through the skin. The TLV in air is 2 ppm. Protective clothing and a properly operating fume hood should be used when using aniline.

Dimethylformamide is a strong irritant to skin and tissue. It is toxic by skin absorption. It is a moderate fire risk. The TLV is 10 ppm in air.

3-amino-1-propanol (propanolamine) is a tissue irritant. Avoid breathing vapors and contact with the skin.

Glacial acetic acid is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

NUMBERED NOTES

1. See References, 1 for details and verification of the method.
2. This method may not be applicable to feeds containing whole, unprocessed cottonseed. Components in the feed interfere with this method and may give false positive results. For feed samples containing whole, untreated cottonseed, the analyst may want to try alternate published HPLC methods.
3. Once a given spectrophotometer is calibrated, calibration factors are valid for long-time use and need not be repeated except for occasional check of instrument performance. Matched cuvettes for the absorbance measurements should be used, or suitable absorbance corrections applied to unmatched cuvettes, using the isopropyl alcohol-hexane mixture for cuvette comparison.
4. Extracts of sample treated with the complexing reagent (Procedure, 1-4) are exceptionally stable and, if necessary, can be stored under refrigeration for several days before conducting the aniline spectrophotometric reaction.
5. Absorption maximum of the dianilinogossypol reaction product is at 440 nm but may vary with the wavelength accuracy of the instrument. All absorbance measurements should be made at the actual maximum for the spectrophotometer used.
6. After the aniline reaction (Procedure, 7), extracts of samples and standards exhibit slight increase in absorbance up to 1 hr, and are stable for 1–5 hrs. For purposes such as plant control, absorbance measurements can be made immediately after aniline reaction and dilution, provided that the calibration factors (Calibration, 5–7) are determined under the same conditions. A variation of about 10 min in determining absorbance will result in about 2% relative error in total gossypol.

REFERENCES

1. *J. Am. Oil Chem. Soc. 35*:93 (1958).

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Table 1
Test portion size for gossypol analysis.

Expected total %	gossypol mg/kg	Test portion weight, g	Aliquot, mL
0.002–0.01	20–100	5.0	10
0.01–0.05	100–500	5.0	5
0.05–0.10	500–1,000	5.0	2
0.10–0.20	1,000–2,000	2.0	2
0.20–0.40	2,000–4,000	1.0	2
0.40–0.60	4,000–6,000	0.75	2
0.60–1.0	6,000–10,000	0.50	2
1.0–2.0		0.20	2
2.0–4.0		0.20	1

Table 2
Precision values for gossypol determination.

Total gossypol,%	SD	CV, %
0.970	0.0102	1.1
1.200	0.0146	1.2
1.376	0.0117	0.9
0.208	0.0062	3.0

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AOCS

AOCS Official Method Ca 14-56

Revised 2011

Total, Free and Combined Glycerol Iodometric-Periodic Acid Method

DEFINITION

This method determines total, free, and combined glycerol in fats and oils. The total glycerol is determined after saponification of the sample, the free glycerol directly on the sample as taken and the combined glycerol by difference.

SCOPE

Applicable to the determination of total and free glycerol in fats and oils.

APPARATUS

1. Burette—50 mL, accurately calibrated.
2. Meniscus magnifier—suitable to permit reading the burette to 0.01 mL.
3. Flask—1 L volumetric with glass stopper.
4. Pipets—volumetric, 10, 25, 50, and 100 mL. The 25 and 50 mL must conform to National Institute of Standards and Testing tolerances and be accurately calibrated to deliver 25 and 50 mL.
5. Beakers—400 mL, with watch glasses to serve as covers.
6. Variable-speed electric stirrer—with glass stirrer.
7. Graduated cylinders—100 and 1000 mL.
8. Erlenmeyer flasks—Pyrex™, 250 or 300 mL, and air condensers 65 cm long. The flasks and condensers should have 24/40 ground-glass joints.

REAGENTS

1. Periodic acid ($HIO_4 \cdot 2H_2O$)—reagent grade, purchased from a chemical supplier (see Notes, *Caution*).
2. Sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$—reagent grade.
3. Potassium iodide (KI)—reagent grade.
4. Glacial acetic acid—reagent grade, 99.5% (see Notes, *Caution*).
5. Starch solution—prepared as noted in Solutions, 4; tested for sensitivity as follows: Place 5 mL of starch solution in 100 mL of water and add 0.05 mL of freshly prepared 0.1 N KI solution and one drop of a 50 ppm chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution to 1000 mL. The deep blue color produced must be discharged by 0.05 mL of 0.1 N sodium thiosulfate.
6. Chloroform ($CHCl_3$)—reagent grade (see Notes, *Caution* and Recommendations). Blank tests with periodic acid with and without chloroform must agree within 0.5 mL. If they do not, a fresh supply of chloroform must be obtained.
7. Potassium dichromate—reagent grade. The potassium dichromate is finely ground and dried to constant weight at 105–110°C before using.
8. Hydrochloric acid (HCl)—reagent grade, concentrated, sp. gr. 1.19 (see Notes, *Caution*).
9. Potassium hydroxide (KOH)—reagent-grade pellets (see Notes, *Caution*).
10. Ethyl alcohol, 95%—USSD formulas 30 or 3A are permitted (see Notes, *Caution*).

SOLUTIONS

1. Periodic acid solution—Dissolve 5.4 g of periodic acid in 100 mL distilled water and then add 1900 mL of glacial acetic acid and mix thoroughly. Store the solution in a dark, glass-stoppered bottle or store in the dark in a clear, glass-stoppered bottle.

   Note—Only glass-stoppered bottles should be used. Do not use cork or rubber stoppers under any circumstances.
2. Sodium thiosulfate solution, 0.1 N—prepared by dissolving 24.8 g of sodium thiosulfate in distilled water and diluting to 1 L. The solution is standardized as follows: Pipet 25 mL of the standard dichromate solution (Solutions, 5) into a 400 mL beaker. Add 5 mL of concentrated HCl, 10 mL of KI solution (Solutions, 3) and rotate to mix (a magnetic stirrer and stirring bar may be used). Allow to stand for 5 min without mixing and then add 100 mL of distilled water. Titrate with the sodium thiosulfate solution, stirring continuously until the yellow color has almost disappeared. Add 1–2 mL of starch solution and continue the titration, adding the thiosulfate solution slowly, until the blue color just disappears. The strength of the thiosulfate solution is expressed in terms of normality:

$$\text{Normality of } Na_2S_2O_3 = \frac{2.5}{\text{mL } Na_2S_2O_3 \text{ required}}$$

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3. Potassium iodide (KI) solution—prepared by dissolving 150 g of KI in distilled water and diluting to 1 L. Protect this solution from the light.
4. Starch indicator solution—prepared by making a homogeneous paste of 10 g soluble starch (see Notes, 1) in cold distilled water. Add this paste to 1 L of rapidly boiling distilled water, stir rapidly for a few seconds and cool. Salicylic acid (1.25 g/L) may be added to preserve the starch. The solution should be stored in a refrigerator (4-10°C) when not in use. Fresh indicator must be prepared when the end point of the titration fails to be sharp, or if the starch solution fails the sensitivity test (see Reagents, 5).
5. Standard potassium dichromate solution, 0.1 N—prepared by dissolving 4.9035 g of finely ground and dried potassium dichromate in distilled water in a 1 L volumetric flask and making up to volume at 25°C.
6. Alcoholic KOH solution—prepared by dissolving 40 g of KOH in 1 L of 95% ethyl alcohol. Filter solution before using if it is cloudy.

PROCEDURE FOR TOTAL GLYCEROL

1. Weigh duplicate test portions accurately to ± 0.1 mg into an Erlenmeyer flask. The proper sample size is indicated in Table 1.
2. Add the indicated amount of alcoholic KOH (Table 1), connect the air condenser and gently boil for 30 min.
3. Add the indicated amount (Table 1) of chloroform (see Notes, *Caution*), measured from a burette to within ± 0.2 mL to a 1 L volumetric flask. Then add with a graduate cylinder 25 mL of glacial acetic acid (see Notes, 2).
4. Remove the saponification flask from the hot plate or steam bath, wash down the condenser with a little distilled water; disconnect the condenser and transfer the sample quantitatively to the volumetric flask, washing the flask, using approximately 500 mL of distilled water.
5. Stopper and shake the flask vigorously for 30–60 s.
6. Add distilled water to the mark, stopper, mix thoroughly by inverting, and set aside until the aqueous and chloroform layers separate.
7. Pipet 50 mL of periodic acid reagent into a series of 400 mL beakers, and prepare two blanks by adding 50 mL of distilled water to each.
8. Pipet 50 mL of the aqueous layer obtained in step 6 into a 400 mL beaker containing 50 mL of the periodic acid reagent and shake gently to effect thorough mixing. Cover with a watch glass and allow to stand for 30 min (see Notes, 2).
   Note—If the aqueous solution contains suspended matter, filter before pipetting the sample for the test.
9. Add 20 mL of KI solution, mix by gentle shaking, and allow to stand 1 min (but never more than 5 min) before titrating. Do not allow to stand in bright light or direct sunlight.
10. Dilute the sample to approximately 200 mL with distilled water and titrate with the standardized sodium thiosulfate solution. Continue the titration until the brown iodine color has almost disappeared. Add 2 mL of starch indicator solution and continue the titration until the blue iodine-starch complex color just disappears.
11. Read the burette to nearest 0.01 mL.
12. The blank determinations are handled in exactly the same way as the samples; see Procedure for Total Glycerol, 9 and 10.
13. If the titration of the sample (Procedure for Total Glycerol, 11) is less than 0.8 of the titration of the blank (Procedure for Total Glycerol, 12), then
    (a) Repeat the test using smaller portions (50, 25, 10, and 5 mL) in Procedure for Total Glycerol, 8, until the titration of the sample is more than 0.8 of the blank, or
    (b) If 10 mL (or less) of the sample solution is necessary to bring the titration within the limit noted in Procedure for Total Glycerol, 13, (a), repeat the test starting at the beginning (Procedure for Total Glycerol, 8) with a sample of 2 g or less.
14. If the titration of the blank (Procedure for Total Glycerol, 12) minus the titration of the sample is less than 4 mL, then
    (a) Repeat the test using 100 mL in Procedure for Total Glycerol, 8. If the test portion taken for the test is still too small, repeat the test beginning with step 1 of Procedure for Total Glycerol, using twice as much sample, or
    (b) If doubling the sample size exceeds 10 g, use only 10 g.

PROCEDURE FOR FREE GLYCEROL

1. Weigh approximately 10 ± 0.01 g of test sample into a weighing dish.
2. Melt the sample by warming on a hot plate and wash into a 1 L volumetric flask with 90 mL of chloroform, measured from a burette to within ± 0.2 mL.
3. Add approximately 500 mL of distilled water, stopper, and shake the flask vigorously for 30–60 sec.
4. Add distilled water to the mark, stopper, mix thoroughly by inverting, and set aside until the aqueous and chloroform layers separate.
5. Pipet 50 mL of periodic acid reagent into a series of 400 mL beakers, and prepare two blanks by adding 100 mL of distilled water to each.
6. Pipet 100 mL of the aqueous layer obtained in step 4 into a 400 mL beaker containing 50 mL of the periodic acid reagent and shake gently to effect thorough mixing. Cover with a watch glass and allow to stand for 30 min (see Notes, 2).
   Note—If the aqueous solution contains suspended matter, filter before pipetting the sample for the test.

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7. Add 20 mL of KI solution, mix by gentle shaking, and allow to stand 1 minute (but never more than 5 min) before titrating. Do not allow to stand in bright light or direct sunlight.
8. Dilute the sample to approximately 200 mL with distilled water and titrate with the standardized sodium thiosulfate solution. Continue the titration until the brown iodine color has almost disappeared. Add 2 mL of starch indicator solution and continue the titration until the blue iodine-starch complex color just disappears.
9. Read the burette to nearest 0.01 mL.
10. The blank determinations are handled in exactly the same way as the samples; see Procedure for Free Glycerol, 7 and 8.
11. If the titration of the sample (Procedure for Free Glycerol, 9) is less than 0.8 of the titration of the blank (Procedure for Free Glycerol, 10), then repeat the test using a smaller sample size.

CALCULATIONS

1. Report the total glycerol to the nearest 0.1%.

$$\text{Total glycerol, \%} = \frac{(B - S) \times N \times 2.302}{W}$$

Where—

S = volume of titrant, mL of sample
B = volume of titrant, mL of blank
N = normality of sodium thiosulfate solution
W = weight of test portion taken for analysis in Procedure for Total Glycerol in which

$$W = \frac{\text{mass of test portion}^a \times \text{mL of test portion}^b}{900}$$

[a]From Procedure for Total Glycerol, 1
[b]From Procedure for Total Glycerol, 8

2. Percentage free glycerol is calculated the same as for total glycerol, using the values obtained as directed in Procedure for Free Glycerol.
3. Combined glycerol in fat or oil, % = (total glycerol in fat or oil, %) – (free glycerol in fat or oil, %).

PRECISION

1. Collaborative studies have shown that the 95% confidence limits in Table 2 may be expected.

NOTES

Caution

Periodic acid is an oxidizing agent and is dangerous in contact with organic materials. It is a strong irritant. It decomposes at 130°C. Do not use cork or rubber stoppers on storage bottles.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature, forming phosgene gas when heated to decomposition temperatures. Can react explosively with aluminum, lithium, magnesium, sodium, potassium, disilane, N_2O_4 and a mixture of sodium hydroxide + methanol. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular, lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

RECOMMENDATIONS

1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride and methylene chloride. Cyclohexane

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and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

NUMBERED NOTES

1. "Potato Starch for Iodometry" is recommended, because this starch produces a deep blue color in the presence of the iodonium ion. "Soluble Starch" is not recommended because a consistent deep blue color may not be developed when some soluble starches interact with the iodonium ion. The following are suitable starches: Soluble Starch for Iodometry, Fisher S516–100; Soluble Potato Starch, Sigma S-2630; Soluble Potato Starch for Iodometry, J. T. Baker 4006-04.
2. Samples may be allowed to stand 1.5 hr at room temperature before titrating, but never longer. Do not allow samples to stand in bright light or direct sunlight.

Table 1
Proper sample size for the estimated percent of total glycerol.

Total glycerol, %	Approximate sample size, g	Weighing accuracy, g	Alcoholic potassium hydroxide, mL	$CHCl_3$ to be added, mL
10–40	2	± 0.001	50	99 ± 0.2
5–20	4	± 0.003	50	96 ± 0.2
2–8	10	± 0.01	100	91 ± 0.2

Table 2
Expected variation in analytical results at the 95% confidence limit.

	Total and combined glycerol at level of		Free glycerol at level of	
	10.0	0.2	0.5	0.05
Duplicate determinations made on the same day by an analyst should not differ by more than approximately	0.10	0.10	0.01	0.01
Single determinations made in two different laboratories should not differ by more than approximately	0.30	0.17	0.17	0.03
Averages of duplicate determinations made in two different laboratories should not differ by more than approximately	0.28	0.14	0.17	0.03

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AOCS Recommended Practice Ca 14b-96
Reapproved 2009

Quantification of Free Glycerin in Selected Glycerides and Fatty Acid Methyl Esters by HPLC with Laser Light-Scattering Detection

DEFINITION:
This method enables quantification of free glycerin in 15 min without need for sample derivatization or use of strong oxidants and chloroform as required by the periodate method.

SCOPE
Applicable to glycerides and fatty acid methyl esters.

APPARATUS

1. Pump—Waters 501 isocratic, or equivalent.
2. Injection syringe—Waters 715 Wisp, or equivalent, 100 µL.
3. Column—4.6 mm i.d. × 25 cm axially compressed 8 micron CN with guard, ambient.
4. Detector—Varex MarkII or IIA, or equivalent, with nitrogen flow at 55 mL/min and heater set for 50°C.
5. Switching valve—Valco 2 port position valve with electronic actuation, or equivalent, set up as in Table 1.
6. Data system—Waters 845 Vax Station, or equivalent.

REAGENTS

1. Solvent—Isooctane-ethanol, 90:10 v/v (special 3A denatured with isopropyl alcohol).
2. Glycerin—Emery 918 (USP grade).

PROCEDURE

1. Sample preparation—Prepare 800 mg of test sample in a 10 mL volumetric flask. Dilute with mobile phase (Reagents, 1).
2. Flow—1.0 mL/min.
3. Calibration—Glycerin-free glycerides and methyl esters are spiked with 0.3–3.0% levels of glycerin (Reagents, 2) (see Figure 1).

CALCULATION

1. Use a regression equation, where the sample weight and area counts of the glycerin are entered, to calculate the results.

METHOD PERFORMANCE

1. Glycerin response—2nd order with a correlation coefficient of 0.995 or better (see Figure 2).
2. Limits of quantification—24 µg, 0.3–3.0% glycerin.

REFERENCES

1. This method paraphrases a method presented to the Fatty Acid and Derivatives Committee at the 85th AOCS Meeting and Expo, Atlanta, GA, May 8–12, 1994, by Jeff Barnhorst, Henkel Corporation, Emery Group.

Table 1
Valve setup

Time	Action
0.0 min	Waste
10.5 min	Detector
14.5 min	Waste

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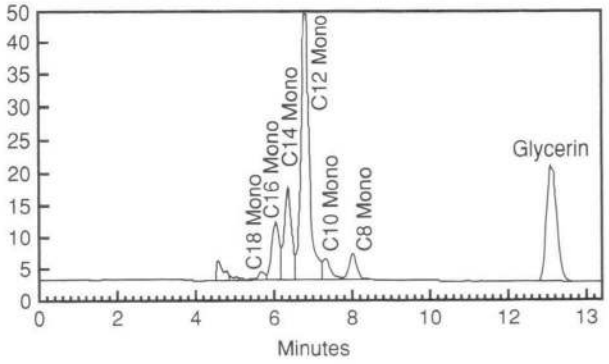

Figure 1. Methyl ester sample with column switching valve.

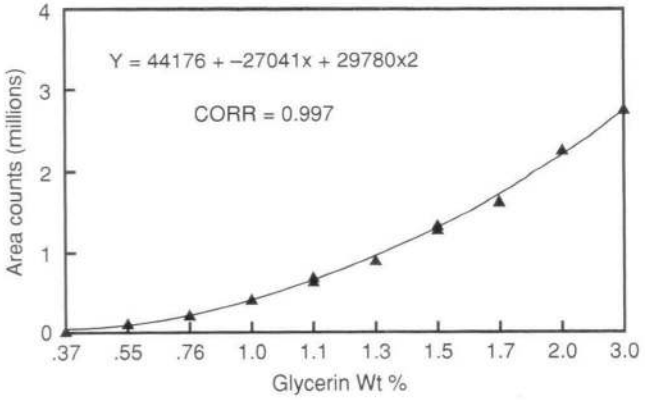

Figure 2. Glycerin response.

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AOCS Official Method Ca 15-75

Reapproved 2009

Analyses for Chromium, Copper, Iron and Nickel in Vegetable Oils by Atomic Absorption Spectrophotometry

DEFINITION

Vegetable oils dissolved in methyl isobutyl ketone (MIBK) are analyzed for metals by direct aspiration. Calibration is based on MIBK solutions of organometallic reference compounds solubilized in purified vegetable oil.

SCOPE

This method is intended for the analysis of crude or partially refined vegetable oils that contain at least 2.0 ppm of each measured metal in the form of oil-soluble derivatives. Hardened oils and animal fats have limited solubilities in MIBK and, even at reduced solute concentrations, they readily foul the burner. For these reasons this method is not recommended for such samples (see Notes, 1).

AOCS Method Ca 18-79 has a lower detection limit (0.1 mg/kg, or 0.1 ppm, for an individual metal) as opposed to 2 mg/kg, or 2 ppm, for AOCS Method Ca 15-75. Therefore, when lower detection limits are required, as in the case of contractual trade methods, AOCS Method Ca 18-79 should be used.

APPARATUS

1. Atomic absorption spectrophotometer—air-acetylene laminar-flow burner head and appropriate accessories.
2. Desiccator—containing fresh phosphorus pentoxide.
3. Weighing bottles—10–15 mL.
4. Hot plate—with rheostat temperature control.
5. Oven.
6. Beakers—25 and 100 mL.
7. Volumetric flasks—25, 50, and 250 mL.
8. Polyethylene bottles—60 and 120 g.
9. Pipets—graduated, to deliver 5 and 50 mL.
10. Balance—top loading, 500 g capacity with ±0.01 g sensitivity.
11. Balance—analytical, 200 g capacity with ±0.0001 g sensitivity.

REAGENTS

1. Organometallic standards—National Institute of Testing (NIST); Conoco, (Ponca City, OK, USA) or equivalent.
   (a) tris(1-phenyl-1,3-butanediono)chromium III.
   (b) bis(1-phenyl-1,3-butanediono)copper II.
   (c) tris(1-phenyl-1,3-butanediono)iron III.
   (d) Nickel cyclohexanebutyrate.
2. 4-Methyl-2-pentanone (methyl isobutyl ketone, MIBK)—reagent grade.
3. Xylene—reagent grade.
4. 2-Ethylhexanoic acid—reagent grade.
5. 2-Ethylhexylamine—reagent grade.
6. Purified vegetable oil (base oil)—Corn or soybean oil containing not more than 0.02 ppm Cr, Cu, Ni, or Fe can be prepared by distilling a low-ash refined oil on a rotating-disk molecular still, rejecting the first and last 15% fractions. .
7. Fresh desiccant—Calcium chloride is not recommended. See AOCS Specification H 9-87.

PREPARATION OF STANDARDS

All standards should be prepared in a fume hood and operators should wear gloves.

1. Chromium stock standard, 100µg/g—Dry about 0.1 g tris(1-phenyl-1,3-butanediono)chromium III 1 hr at 110°C. Weigh 51.6 mg into a 25 mL beaker, add 3 mL xylene, add 3 mL 2-ethylhexanoic acid, and warm on hot plate without refluxing, swirling occasionally until salt dissolves. Let cool to ambient. Transfer solution to a tared 60 g polyethylene bottle, rinse beaker with five 5 mL portions of base oil, bring the contents to 50 mL with base oil, cap with nitrogen, mix, and store in a cool location. Standard solution should be stable for at least 6 months.
2. Copper stock standard, 100µg/g—Dry about 0.05 g bis(1-phenyl-1,3-butanediono) copper II 30 min at 110°C. Weigh 30.3 mg into a 25 mL beaker, add 2 mL xylene, add 2 mL 2-ethylhexylamine, and warm on hot plate without refluxing,

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swirling occasionally until salt dissolves. Let cool to ambient. Transfer solution to a tared 60 g polyethylene bottle, rinse beaker with five 5 mL portions of base oil, bring the contents to 50 mL with base oil, cap with nitrogen, mix, and store in a cool location. Standard solution should be stable for at least 6 months.

3. Iron stock standard, 100µg/g—Dry about 0.1 g tris(1-phenyl-1,3-butanediono)iron III for 1 hr at 100°C. Weigh 48.6 mg into a 25 mL beaker, add 3 mL xylene, add 3 mL 2-ethylhexanoic acid, and warm on hot plate without refluxing, swirling occasionally until salt dissolves. Let cool to ambient. Transfer solution to a tared 60 g polyethylene bottle, rinse beaker with five 5 mL portions of base oil, bring the contents to 50 mL with base oil, cap with nitrogen, mix, and store in a cool location. Standard solution should be stable for at least 6 months.
4. Nickel stock standard, 10µ g/g—Desiccate about 0.05 nickel cyclohexane butyrate (13.89% Ni) for 48 hr over phosphorus pentoxide. Quickly weigh 36.0 mg into a 25 mL beaker, add 3 mL xylene, add 3 mL 2-ethylhexanoic acid, and warm on hot plate without refluxing, swirling occasionally until salt dissolves. Let cool to ambient. Transfer solution to a tared 60 g polyethylene bottle, rinse beaker with five 5 mL portions of base oil, bring the contents to 50 mL with base oil, cap with nitrogen, mix, and store in a cool location. Standard solution should be stable for at least 6 months.
5. Mixed stock standard, 20µg/g Cr, Cu, Fe, and Ni—Weigh carefully by additions into a tared 120 g bottle 20 ± 0.02 g each of the 100 µg/g Cr, Cu, Fe, and Ni stock standards, and 20 ± 0.02 g base oil. Purge with nitrogen, cap, and mix.
6. Mixed working standards—Weigh 50 ± 0.1 g base oil into a 100 mL beaker, transfer to 250 mL volumetric flask with MIBK, dilute to volume, and mix (20% wt/vol base oil in MIBK). Weigh accurately (± 5 mg) 5.00, 3.75, 2.50, 1.25, and 0.625 g mixed stock standard into 50 mL volumetric flasks. In order, add by pipet 25.0, 31.3, 37.5, 43.8, and 46.9 mL of the 20% base oil in MIBK solution. Dilute to volume with MIBK and mix. These solutions contain 2.0, 1.5, 1.0, 0.5, and 0.25 µg/mL Cr, Cu, Ni, and Fe. Retain the remaining 20% base oil solution for zeroing the spectrophotometer and, if necessary, diluting the sample oil.

PROCEDURE

1. Weigh accurately 5.0 ± 0.0005 g of test sample oil into a 25 mL volumetric flask, dilute with MIBK and mix.
2. Set up the instrument for operation with an air-acelene-organic solvent flame according to the manufacturer's recommendations (see Notes, 2).
3. Set the monochromator to the recommended wavelength (see Table 1) and allow the instrument to stabilize.
4. Light the burner and allow to warm to equilibrium (approx. 3 min).
5. While aspirating the 20% base oil solution, reduce acetylene flow until the yellow incandescence of the flame just disappears. Next, optimize the fuel-air ratio for the determination (Notes, 3) by varying the acetylene flow while aspirating one of the mixed standards until a maximum absorption is obtained. Flush the burner by aspirating MIBK for 15 sec (Notes, 4) and then zero the instrument while aspirating the 20% base oil solution.
6. Record absorptions for the five mixed working standards in order of increasing concentration. Record the absorption of the sample solution and then aspirate the 20% base oil solution to check the zero point (Notes, 5). If the sample absorption exceeds the highest standard, prepare a new sample solution by weighing a suitable ratio of sample oil to base oil to produce a 20% oil solution that absorbs within the calibration range and repeat the absorption measurements.
7. From the standard absorptions, construct a calibration curve to be used to determine the concentration (µg/mL) of metal in the sample solution.
8. Repeat Procedure, 3–7 for each analysis, using the appropriate source lamp and conditions.

CALCULATIONS

1. Metal, µg/g (ppm) = $C \times \frac{25}{W}$

   Where—

   C = metal in µg/mL from calibration graph
   W= mass of sample, g in 25 mL of sample solution

PRECISION

1. Table 2 gives the standard deviations for each metal analysis. Eight collaborators analyzed two samples of soybean oil. Test solutions were retained and absorption measurements were repeated on the following day.

NOTES

Caution—Xylene is flammable and a dangerous fire risk. The TLV is 100 ppm in air.

Methyl isobutyl ketone (MIBK) is flammable and a dangerous fire risk. Explosive limits in air are 1.4–7.5%. Avoid inhalation and ingestion. It is absorbed by the skin. The TLV is 50 ppm in air.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

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NUMBERED NOTES

1. Mixed solvent systems, i.e., chloroform-MIBK, may reduce fat solubility problem and lessen burner fouling, but it will be necessary to prepare the standards in the same solvent system. However, the use of chloroform is not recommended due to the potential of forming phosgene gas under the test conditions.
2. If a Boling burner head is used, be sure that it is designed to withstand the highly oxidizing conditions required for the nickel determination. Boling burners of an early design could be permanently warped under these conditions.
3. Chromium requires a rich (yellow) flame, copper and iron a lean (blue) flame, and nickel a very lean flame.
4. The MIBK flush removes contaminated oil and minimizes fouling of the burner head by oil buildup.
5. With correct fuel adjustment, a linear calibration curve that intercepts the origin can usually be obtained.

Table 1
Recommended wavelengths.

Element	Wavelength, nm
Cr	357.9
Cu	324.8
Fe	248.3
Ni	232.0

Table 2
Precision.

		Standard deviations		
Element	Level, µg/g	Day 1	Day 2	Average
Cr	2.5	0.0536	0.1155	0.084
	8.5	0.2654	0.3007	0.283
Cu	3.5	0.1137	0.1344	0.124
	9.5	0.1574	0.1940	0.176
Fe	2.0	0.0627	0.1640	0.113
	8.0	0.4990	0.2875	0.393
Ni	3.0	0.3599	0.3239	0.342
	9.0	0.3159	0.2797	0.298

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AOCS Recommended Practice Ca 15b-87
Reapproved 2009

Sodium and Calcium by Atomic Absorption Spectrophotometry

DEFINITION

Sodium (sodium soap) and calcium (calcium soap) present in a fat or oil sample are acidified and then extracted into a known volume. The sodium and calcium concentrations are measured using flame AAS and can be directly measured after calibration of the instrument.

SCOPE

The method is applicable to unemulsified fats and oils.

APPARATUS

1. Atomic absorption spectrometer—Perkin-Elmer 306, or equivalent, equipped for flame analysis.
2. Sodium hollow cathode lamp—Perkin-Elmer 303-6065, or equivalent.
3. Calcium hollow cathode lamp—Perkin-Elmer 303-6017, or equivalent.
4. Screw-cap culture tubes—16 × 125 mm (Corning #9825, or equivalent).
5. Disposable glass pipets.
6. Volumetric pipets—1, 4, 5, 10, 20, and 25 mL, class A.
7. Volumetric flasks—50 and 1000 mL, class A.

REAGENTS

1. Sodium standard solution—aqueous, 1000 µg/g (Fisher SO-S-139, or equivalent). See Notes, 1.
2. Calcium standard solution—aqueous, 1000 µg/g (Fisher SC-S-191, or equivalent). See Notes, 1.
3. Deionized water.
4. Potassium chloride (KCl)—reagent grade.
5. Methylene chloride—reagent grade (see Notes, *Caution*).
6. 0.2 M Hydrochloric acid (HCl) standard volumetric solution—prepared by diluting the contents of two 0.1 M HCl volumetric concentrates (Sargent-Welch Acculate®, SC 10064, or equivalent) to 100 mL with deionized water. Alternately, the appropriate volume of concentrated HCl, suitable for trace metal analysis, can be diluted to 1000 mL with deionized water.
7. 0.2 M HCl wash solution—prepared as noted in Reagents, 6.
8. 0.2 M HCl/KCl solution—prepared by diluting the contents of two 0.1 M HCl concentrates (see Reagents, 6) and two 0.1 M KCl volumetric concentrates (Sargent-Welch Acculate®, SC 16942, or equivalent) to 1000 mL with deionized water. Alternately, the appropriate volume of concentrated HCl, suitable for trace metal analysis, and the appropriate weight of reagent-grade KCl can be diluted to 1000 mL with deionized water.
9. Sodium working solution (5.00 µg/g Na)—Pipet 5 mL of the 1000 ppm sodium standard solution into a 1000 mL volumetric flask and dilute to volume with deionized water.
10. Calcium working solution (25.0 µg/g Ca)—Pipet 25 mL of the 1000 ppm calcium standard solution into a 1000 mL volumetric flask and dilute to volume with deionized water.

PROCEDURE

1. Instrument setup (sodium)—
   Sodium lamp—10 ma
   Wavelength—589 nm
   Slit—0.7 mm
   Background correction—off
   Flame—air/acetylene (lean, blue)
2. Instrument setup (calcium)—
   Calcium lamp—12 ma
   Wavelength—422.7 nm
   Slit—0.7 mm
   Background correction—off
   Flame—air/acetylene (reducing, rich, slightly yellow)
3. Preparation of standard and sample—
   (a) Wash all glassware, first with dilute HCl (0.2 M) and then with copious amounts of deionized water.

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(b) Weigh 4.0 g [see Procedure, 4, (e) or Procedure, 5, (e) below] of sample to the nearest mg into an acid-washed screw-top culture tube.
(c) Add 3.0 mL of methylene chloride to each tube.
(d) Pipet 4.00 mL of 0.2 M HCl/KCl solution into each tube.
(e) Cap and shake for 2 min.
(f) Centrifuge for 30 sec.
(g) Prepare 0.5, 1.0, and 2.0 µg/g sodium standards by pipetting 5, 10, and 20 mL, respectively, of the sodium working solution (Reagents, 9) into 50 mL volumetric flasks. These are then diluted to volume using the same matrix as the samples (0.2 M HCl/KCl).
(h) Prepare 0.5, 2.5, and 5.0 µg/g calcium standards by pipetting 1, 5, and 10 mL, respectively, of the calcium working solution (Reagents, 10) into 50 mL volumetric flasks. These are then diluted to volume using the same matrix as the samples (0.2 M HCl/KCl).

4. Analysis for sodium—
(a) Light the flame and optimize the nebulizer, burner and flame settings while aspirating a 2.0 µg/g sodium standard.
(b) Zero the instrument using the 0.2 M HCl/KCl blank.
(c) Check the absorbances of the 0.5, 1.0, and 2.0 µg/g sodium standards for response and linearity.
(d) Switch to the autoconcentration mode and calibrate at 0 (zero), 0.5 and 2.0 µg/g sodium.
(e) Aspirate the aqueous layer of each sample tube and read the sodium concentrations directly from the instrument. For accuracy, analysis of samples containing >3 µg/g sodium should be repeated, using an adjusted sample size. Recommended test portion sizes are as follows:

Sodium content µg/g	Test portion, g
0.10 ≤ 3.0	4.0
3 ≤ 10	1.0
10 ≤ 20	0.5
> 20	0.2

5. Analysis for calcium—
(a) Light the flame and optimize the nebulizer, burner and flame settings while aspirating 5.0 µg/g calcium standard.
(b) Zero the instrument using the 0.2 M HCl/KCl blank.
(c) Check the absorbances of the 0.5, 2.5, and 5.0 µg/g calcium standards for response and linearity.
(d) Switch to the autoconcentration mode and calibrate at 0 (zero), 0.5 and 5.0 µg/g calcium.
(e) Aspirate the aqueous layer of each sample tube and read the calcium concentration directly from the instrument. For accuracy, analysis of samples containing >5 µg/g calcium should be repeated, using an adjusted sample size. Recommended test portion sizes are as follows:

Sodium content µg/g	Test portion, g
0.10 ≤ 5.0	4.0
5 ≤ 10	1.0
10 ≤ 20	0.5
> 20	0.2

CALCULATIONS

1. Sodium content is calculated using the formulas

$$\frac{\text{µg/g Na (reading)} \times 4}{\text{mass of test portion, g}} = \text{µg/g Na}$$

µg/g sodium oleate = µg/g Na × 13.2

2. Calcium content is calculated using the formulas

$$\frac{\text{µg/g Ca (reading)} \times 4}{\text{mass of test portion, g}} = \text{µg/g Ca}$$

µg/g calcium oleate = µg/g Ca × 15

3. Soap is calculated using the formula
µg/g soap = µg/g calcium oleate + µg/g sodium oleate

NOTES

Caution

Methylene chloride is toxic and is a carcinogen. The TLV is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

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Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalationa and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. The calcium and sodium standards are stable for one week.

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AOCS

AOCS Recommended Practice Ca 16-75

Revised 2011

Polyethylene in Fats and Oils

DEFINITION

This method determines, by a gravimetric procedure, polyethylene and other plastic polymers present as contaminants in fats and oils.

SCOPE

This method is applicable to animal fats, other fats, and vegetable oils containing polyethylene. For rendered fats, protein could be an interference. Soaps may interfere, causing high results; however, the initial precipitation from an acid solution is designed to minimize the interference due to soaps. The probable limitation of the method for normal polyethylene is 50 mg/kg (50 ppm). The effect of the presence of biodegradable plastic materials in fats and oils is yet to be determined.

APPARATUS

1. Beakers—600 and 400 mL.
2. Funnels—
   (a) Buchner with California State modification—7.0 cm i.d., or equivalent. May be obtained from Labconco, Kansas City, MO, USA.
   (b) Porcelain—size 1, 56 mm i.d.
3. Vacuum filtration flasks—1 L and 500 mL.
4. Metal retaining rings—
   (a) Size 1, 7.0 cm o.d. × 5.0 cm high × 3.0 mm wall thickness.
   (b) Size 2, 5.4 cm o.d. × 4.0 cm high × 2.0 mm wall thickness.
5. Filter paper—
   (a) Glass fiber—7.0 and 5.5 cm diameter; Reeve Angel no. 934-AH, Whatman type GFA, or equivalent. Predry at 105°C and store in a desiccator.
   (b) Whatman no. 4—5.5 cm diameter, or equivalent.
6. Wash bottles—Guth Universal, 500 mL, hand operated, with Kool-Grip neck; or equivalent.
7. Hot plate.
8. Drying oven—105°C.
9. Cold water bath—10°C maximum.
10. Stirrer—high speed.
11. Magnetic stirrer.

REAGENTS

1. Chloroform—reagent grade (see Notes, *Caution*).
2. Tetrachloroethylene—reagent grade (see Notes, *Caution*).
3. Filter aid—Manville Hyflo Supercel, or equivalent.
4. Sulfuric acid/ethanol solution (10% solution, v/v)— prepared by mixing 1 volume of concentrated sulfuric acid, sp. gr. 1.84, with 9 volumes of 95% ethanol (see Notes, 1).
5. Methanol—reagent grade (see Notes, *Caution*).

PROCEDURE

1. Heat the sample to a temperature of 60–70°C in a water bath or on a steam bath. Stir the sample with the highspeed stirrer for 3–4 min to break up and distribute any large particles of plastic polymer throughout the sample.
2. Weigh 50 ± 0.5 g of the sample into a 600 mL beaker (see Notes, 2).
3. Add 75 mL of the sulfuric acid/ethanol solution to the weighed portion of the sample. Cover the beaker and stir, using a magnetic stirrer, for 5 min at a temperature of 60–70°C. Cool to a temperature below 35°C, add 270 mL of chloroform and mix until the sample is dissolved.

   Note—The solution may not be clear. If this is the case, add 1.0 g of the filter aid suspended in 30 mL chloroform (to avoid formation of lumps), but do not heat.
4. Attach the California State modified Buchner funnel to a 1 L filtering flask. Place two pieces of 7.0 cm glassfiber filter paper (see Notes, 3) and the size 1 retainer ring in the funnel. Wet the filter paper with chloroform prior to filtration. Apply gentle suction and pour the mixture rapidly into the funnel, taking care to keep it from going dry.
5. Wash the beaker, retainer ring, and the paper thoroughly with about 50 mL of chloroform from the wash bottle. Pull air through the filter for 2 min to dry. Discard the filtrate.

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6. Bring 75 mL of tetrachloroethylene to a brisk boil (see Notes, *Caution*) in a 400 mL beaker on a hot plate. Using gloves and tongs, remove the beaker from the hot plate, transfer the filter paper from Procedure, 5 to the beaker, and stir for 2 min with a glass stirring rod.
7. Attach the porcelain Buchner funnel, to which a 5–6 cm length of rubber tubing has been attached to the stem to prevent filtrate from being drawn into the side delivery tube, to a clean (see Notes, 4) 500 mL filtration flask. Place a disk of 5.5 cm Whatman no. 4 filter paper, two disks of 5.5 cm glass-fiber paper and the size 2 retainer ring in the funnel in the order given.
8. Replace the 400 mL beaker from Procedure, 6 onto the hot plate and bring to a gentle boil (see Notes, *Caution*).
9. Wet filter paper with tetrachloroethylene prior to filtration and apply gentle suction to the filtration flask. Rapidly pour the hot tetrachloroethylene solution through the filter. Using a wash bottle, rinse the beaker, retainer ring and paper four times with 10-15 mL portions of near boiling tetrachloroethylene (see Notes, 5). Disconnect the funnel from the filtration flask.
10. To the contents of the filtration flask, add 175 mL of precooled methanol (5–10°C, with a maximum of 10°C; see Notes, 6). Swirl to dispense thoroughly and let stand for 10 min in cold water bath to completely precipitate the polyethylene.
11. Weigh accurately two pieces of 7.0 cm moisture-free glass-fiber filter paper and place them along with the size 1 retainer ring in the California State modified Buchner funnel. Place the funnel on a 1 L filtration flask and, with gentle suction, filter the solution from Procedure, 10. Using a wash bottle, wash the flask, retainer ring and paper four times with 20 mL portions of cold methanol.
12. Pull air through the glass-fiber paper for 2 min. Carefully remove the paper from the funnel and dry in an oven at 105°C to constant weight. Ten min drying time should be sufficient. Cool in a desiccator and reweigh the paper and contents to constant weight.

CALCULATIONS

1. Calculate the mg/kg (ppm) polyethylene in the samples as follows:

$$\text{Polyethylene, mg/kg} = \frac{\text{mass of precipitate, g}}{\text{mass of test portion, g}} \times 10^6$$

PRECISION

1. The probable accuracy (based on the previous version of this AOCS method) is ± 10 mg/kg for values in the range of 50 to 500 ppm (see Notes, 7). International Union of Pure and Applied Chemistry Method 2.606, noted a standard deviation of 20 mg/kg (ppm) at a level of 200 mg/kg (ppm) of polyethylene. The probable reproducibility of the method (based on International Organization for Standardization/British Standards Institution Method BS 3919) is a coefficient of variation of about 15% up to 100 mg/kg (ppm) and about 10% in the range of 100–400 mg/kg (ppm).
2. Details of an interlaboratory test on the precision of the method are summarized in Table 1 (1). The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given. These values indicate a higher degree of variation than previously calculated. See Notes, 7.

NOTES

Caution

A fume hood must be used for the entire analysis.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLC is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Tetrachloroethylene (perchloroethylene) is an irritant to eyes and skin. The TLC is 50 ppm in air.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

Methyl alcohol (methanol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform and perchloric acid.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to the water, never the reverse.

NUMBERED NOTES

1. 95% ethyl alcohol (ethanol) may be replaced by industrial methyl alcohol.
2. For samples expected to contain more than 400 mg/kg (ppm) plastic polymer, the sample weight may be reduced to 25 g.
3. The glass-fiber paper is very rapid and extremely retentive, but it is delicate and must be handled with care.

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4. The filtering flask (500 mL) used in Procedure, 7-10 should be thoroughly cleaned of any residual polyethylene film after each completed analysis. A strong, hot caustic solution is adequate for this purpose. Observe proper caution in handling strong caustic solutions.
5. Procedure, 9 requires complete and thorough washing. The tetrachloroethylene must be kept at near boiling at all times.
6. The methanol used in Procedure, 10 and 11 should be precooled below 10°C by refrigeration or cold water bath to ensure complete and rapid precipitation.
7. The probable accuracy is thought to be due to the inherently nonuniform nature of materials in the samples. It represents the approximate agreement that is attainable. It is recommended that the analyst becomes familiar with the procedure on trial samples to be able to recognize or anticipate difficulties.

REFERENCES

1. ISO 6656 Animal and Vegetable Fats and Oils—Determination of polyethylene-type polymers.

Table 1
Interlaboratory test results.

	Tallow 1	Tallow 2
Number of laboratories retained after eliminating outliers	10	9
Mean, mg/kg	41	184
Standard deviation of repeatability (s_r), mg/kg	8	15
Coefficient of variation of repeatability, %	20	8.4
Repeatability limit (r) ($2.83 \times s_r$), mg/kg	23	24
Standard deviation of reproducibility (s_R), mg/kg	14	33
Coefficient of variation of reproducibility, %	34	18
Reproducibility limit (R) ($2.83 \times s_R$), mg/kg	39	92

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AOCS Recommended Practice Ca 17-01
Reapproved 2009

Determination of Trace Elements (Calcium, Copper, Iron, Magnesium, Nickel, Silicon, Sodium, Lead, and Cadmium) in Oil by Inductively Coupled Plasma Optical Emission Spectroscopy

DEFINITION

Solvent-diluted vegetable oils are analyzed for the elements above by direct aspiration.

SCOPE

This procedure describes a method to quantify calcium, copper, iron, magnesium, nickel, silicon, lead, sodium, and cadmium in oil. Almost all forms of vegetable oils may be analyzed, from crude oil to degummed, refined, bleached, deodorized, and hardened, and nearly all types of lecithins and phosphatides, depending on dilution solvent. This procedure is suitable only when the elements are present in a solubilized form. When they are present as fine particles (bleaching earth, catalysts, or rust), inductively coupled plasma optimal emission spectroscopy (ICP-OES) analysis results in poor recovery due to nebulization and atomization problems. The only suitable non-ashing direct method for these samples is graphite furnace AAS.

PRINCIPLE

Liquid samples are nebulized and carried into the excitation source by a flowing gas. Atoms are quantitated by measuring the specific emission lines produced by atoms decaying from high energy levels.

APPARATUS

1. Inductively coupled plasma optical emission spectrometer (ICP-OES) (see Numbered Notes, 1)
2. Analytical balance—(4-place accuracy)
3. Oven—capable of maintaining a temperature of 60 ± 2°C
4. Disposable plastic vials
5. Disposable pipet tips
6. Tilt table mixer
7. Volumetric flasks—100 mL capacity

REAGENTS

1. 1-Butanol
2. Kerosene or xylene (see Numbered Notes, 2)
3. Standard elements present in solution as organic material. Multi-element standards may be used. Suitable materials may be obtained from either SPEX-Certiprep, 203 Norcross Ave., Metuchen, NJ 08840 (908-549-7144) or Accu-Standard (Constan brand standards), 25 Science Park, New Haven, CT 06511 (800-442-5290).
4. Base 20 Oil or Base 75 Oil from Accu-Standard may be used to check the blank oil used and for the dilution of standard solutions as needed.

PROCEDURE

1. General
   a. Dilute all samples, standards, and blanks 1:1 with 1-butanol (or kerosene/xylene) to reduce the viscosity of the oil for better nebulization (See Notes, 3). 1-Butanol is preferred because it has better moisture tolerance and allows a higher flow rate with higher pressure than kerosene, without putting out the torch. The higher flow rate provides for improved detection limits. The increased moisture tolerance permits the analysis of crude oils and lecithins without phase separation. Some samples are more soluble in 1-butanol than others.
   b. Melt solid samples at 10°C above their melting point prior to dilution. Keep the diluted sample warm and monitor it throughout the analysis to ensure that it remains in solution. The relative maximum temperature for the analysis of hardened fats is 60°C.

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c. Precise conditions will vary from instrument to instrument (see Notes, 1). Operate the instrument according to the manufacturer's directions and specifications.
d. Ignite the instrument and allow it to warm. It is profiled on an internal Hg lamp.
e. Element detection limit and emission line used are:

Element	Detection limit	Major emission line
Calcium	>0.05 ppm	317.9 nm 393.3 nm
Copper	>0.05 ppm	324.7 nm
Iron	>0.05 ppm	259.9 nm
Magnesium	>0.05 ppm	285.2 nm
Nickel	>0.05 ppm	231.6 nm
Silicon	>0.10 ppm	251.6 nm
Sodium	>1.00 ppm	588.9 nm
Lead	>0.5 ppm	220.4 nm
Cadmium	>0.2 ppm	226.5 nm 214.4 nm

f. Standardize the instrument as described and scan all samples in triplicate (see Notes, 4)

2. Preparation of standards
   a. Blank—Use a refined and bleached soya bean or other oil which has been shown to be free of trace elements. Blank oil is diluted 1:1 as described in Procedure, 1a. Use base oil (Reagents, 4) as an absolute reference blank to determine that the blank oil is free of trace elements.
   b. Standards—Prepare the standards from commercially available single- or multi-element organic-based standards. Accurately weigh the standard and add enough blank oil to total 50.00 g. Add 50.00 g of solvent (1-butanol, kerosene or xylene) to achieve a 1:1 dilution.

      A single standard concentration will work; however, up to 4 multi-level, multi-element standards will provide a better calibration for linearity and accuracy. Levels should include 1, 2.5, 5, and 10 mg/kg standards, depending on the range of values expected.

      If an internal standard is used, weigh it as part of the elements or incorporate it as part of the dilution solvent to yield 10 mg/kg to match the amount added to the sample (see Notes, 5).

STANDARDIZATION

1. Run the blank oil standard and base oils at the specified wavelength for the element(s) of interest.
2. Run the standard solutions at the chosen wavelength(s).
3. Scan blanks, samples, and standards in triplicate for trace element(s) and then average.
4. Run standards and the blank every ten samples or fewer and restandardize the instrument as needed. For accuracy use a narrow range of standardization (0–10 mg/kg of each element). Test samples should be diluted to keep the trace element(s) content within the range of standardization.

PREPARATION OF TEST PORTIONS

1. Weigh 2.5 g (± 0.02 g) of test sample into an auto-sampler tube and use an automatic pipette to dilute the sample with 2.5 g of 1-butanol (kerosene or xylene). Cap the tube and invert 40–50 times on a mixing table.
2. Dilute 0.2 g lecithins (up to 100% AI) to 5.0 g with blank soybean oil and then to 10 g with 1-butanol. Mix the samples on a tilt table mixer for 1 hr. Dilute 1:10 with 1:1 blank oil/1-butanol to give a total dilution of 1:250.

CALCULATIONS

Most instrument programs have a computation feature. Area counts from known samples are inserted into the linear regression formula and from this relationship, concentrations may be determined. Be sure to include the correct dilution factor. Most programs are able to accommodate up to 4 multi-level, multi-element standards.

PRECISION

Table 1 shows the results of a collaborative study carried out for nickel, iron, and copper using AOCS Laboratory Proficiency Samples.

NUMBERED NOTES

1. The conditions for analysis will vary from instrument to instrument from different manufacturers and will further vary within identical instruments from the same manufacturer depending on the type of nebulizer and pumping system used.
2. If kerosene or xylene is used, all instrumental operating conditions (such as pump flow rate, etc.) will change from those set for 1-butanol. Therefore the analysis must be standardized and all analyses run with all standards, blank, and samples with kerosene or xylene.
3. Oils suspected to have high levels of calcium or sodium should be diluted 1:5 or 1:10.

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4. Calibration drift has been noted and is often due to carbon buildup on the injector tip.
5. Yttrium has a number of wavelengths which may be used for analysis. The wavelength chosen should match closely with the wavelength of the element analyzed. 224.3 nm is one wavelength used routinely. Scandium and cobalt also have been used.

Table 1
ICP analysis of high levels of trace element(s) in oil: summary of analyses from 11 laboratories. The precision of the method has been established for Cu, Fe, and Ni in soybean oil by an international study conducted in 1999 by AOCS. Data are analyzed according to ISO 5725:1994 guidelines.

	Copper				Iron				Nickel			
Sample	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10	S-11	S-12
Laboratories instructed	11	11	11	11	11	11	11	11	11	11	11	11
Number of laboratories included	9	8	8	9	11	11	11	10	9	10	8	10
Total number of replicates	18	16	16	18	22	22	22	20	18	20	16	20
Mean of the laboratory values, g/g	2.05	9.91	8.14	9.94	2.05	2.07	8.29	10.08	4.94	5.64	7.64	2.10
Repeatability standard deviation (S_r)	0.05	0.10	0.12	0.10	0.06	0.05	0.25	0.19	0.09	0.23	0.37	0.05
Repeatability relative standard deviation (RSD_r), %	2.29	1.03	1.48	1.03	3.10	2.49	3.06	1.91	1.86	4.06	4.88	2.62
Repeatability value ($2.8 \times S_r$)	0.13	0.29	0.34	0.29	0.18	0.14	0.71	0.54	0.26	0.64	1.04	0.15
Reproducibility standard deviation (S_R)	0.06	0.13	0.31	0.43	0.20	0.19	0.56	0.41	0.25	0.41	0.46	0.20
Reproducibility relative standard deviation (RSD_R), %	2.85	1.32	3.81	4.37	9.62	9.38	6.70	4.10	4.97	7.18	5.98	9.51
Reproducibility value ($2.8 \times S_R$)	0.16	0.37	0.87	1.22	0.55	0.54	1.56	1.16	0.69	1.13	1.28	0.56

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AOCS

AOCS Official Method Ca 18-79

Reapproved 2009

Analysis for Chromium, Copper, Iron, Nickel, and Manganese in Triglyceride Oils by Atomic Absorption Spectrophotometry Using a Graphite Furnace

DEFINITION

Vegetable and animal oils dissolved in methyl isobutyl ketone (MIBK) or MIBK + HNO_3 are analyzed for metals by direct injection into a graphite tube furnace. Calibration is based on MIBK or MIBK + HNO_3 solutions of organometallic reference compounds.

SCOPE

This method is intended for the analysis of crude and refined vegetable oils and animal fats that contain trace metals that may be present in the form of oil-soluble adducts. The detection limit of this method is approximately 0.1 mg/kg (0.1 ppm). This method has a lower detection limit than AOCS Method Ca 15-75, which has a detection limit of approximately 2 mg/kg (2 ppm). Therefore, when lower detection limits may be required, as in the case of contractual trade methods, AOCS Method Ca 18-79 should be used.

APPARATUS

1. Atomic absorption spectrophotometer—with Zeeman or Smith-Hieftje background correction and graphite tube furnace.
2. Vacuum desiccator—containing a suitable *fresh* desiccant (see AOCS Specification H 9-87).
3. Weighing bottles—10–15 mL capacity.
4. Hot plate—with rheostat temperature control.
5. Oven.
6. Beakers—25 and 100 mL.
7. Pipets—graduated, to deliver 5, 10, and 20 mL.
8. Eppendorf pipets—5, 10, 20, 30, 40, and 50 µL, with disposable tips.
9. Balance—analytical, 200 g capacity with ±0.0001 g sensitivity.

REAGENTS

1. Organometallic standards—National Institute of Testing (NIST); Conoco (Ponca City, OK), USA, or equivalent.
   (a) tris(1-phenyl-1,3-butanediono)chromium III.
   (b) bis(1-phenyl-1,3-butanediono)copper II.
   (c) tris(1-phenyl-1,3-butanediono)iron III.
   (d) Nickel cyclohexanebutyrate.
   (e) Manganous cyclohexanebutyrate.
2. 4-methyl-2-pentanone (methyl isobutyl ketone, MIBK)—reagent grade (see Notes, *Caution*).
3. Xylene—reagent grade (see Notes, *Caution*).
4. 2-Ethylhexanoic acid—practical grade.
5. 2-Ethylhexylamine—practical grade.
6. Nitric acid—Spectral or Ultrex grade (see Notes, *Caution*).

PREPARATION OF STANDARDS

All standards should be prepared in a fume hood and operators should wear gloves.

1. Chromium stock standard, 100 µg/mL—Dry about 2 g tris(1-phenyl-1,3-butanediono)chromium III 1 hr at 110°C. Weigh 103.1 mg into a 25 mL beaker, add 5 mL xylene and 5 mL 2-ethylhexanoic acid, and gently warm on hot plate without refluxing, swirling occasionally until the salt dissolves. Cool to ambient temperature. Transfer the solution to a 100 mL volumetric flask, rinsing the beaker with five 10 mL portions of MIBK, then dilute to exactly 100 mL, mix, and store at 20 ± 2°C. This standard stock solution should be stable for at least 6 months.
2. Copper stock standard, 100 µg/mL—Dry about 0.13 g bis(1-phenyl-1,3-butanediono)copper II 30 min at 110°C. Weigh 60.6 mg into a 25 mL beaker, add 4 mL xylene and 4 mL ethylhexylamine, and gently warm on hot plate without refluxing, swirling occasionally until the salt dissolves. Cool to ambient temperature. Transfer the solution to a 100 mL volumetric

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flask, rinsing the beaker with five 10 mL portions of MIBK, then dilute to exactly 100 mL, mix, and store at 20 ± 2°C. This standard stock solution should be stable for at least 6 months.

3. Iron stock standard, 100 µg/mL—Dry about 0.2 g tris(1-phenyl-1,3-butanediono)iron III for 1 hr at 110°C. Weigh 97.1 mg into a 25 mL beaker, add 5 mL xylene and 5 mL 2-ethylhexanoic acid, and gently warm on hot plate without refluxing, swirling occasionally until the salt dissolves. Cool to ambient temperature. Transfer the solution to a 100 mL volumetric flask, rinsing the beaker with five 10 mL portions of MIBK, then dilute to exactly 100 mL, mix, and store at 20 ± 2°C. This standard stock solution should be stable for at least 6 months.
4. Nickel stock standard, 100 µg/mL—Desiccate about 0.15 g nickel cyclohexanebutyrate for 48 hr over suitable fresh desiccant. Rapidly weigh 67.6 mg into a 25 mL beaker, add 5 mL xylene and 5 mL 2-ethylhexanoic acid, and gently warm on hot plate without refluxing, swirling occasionally until the salt dissolves. Cool to ambient temperature. Transfer the solution to a 100 mL volumetric flask, rinsing the beaker with five 10 mL portions of MIBK, then dilute to exactly 100 mL, mix, and store at 20 ± 2°C. This standard stock solution should be stable for at least 6 months.
5. Manganese stock standard, 100 µg/mL—Desiccate about 0.15 g manganese cyclohexanebutyrate for 48 hr over suitable fresh desiccant. Rapidly weigh 71.4 mg into a 25 mL beaker, add 5 mL xylene and 5 mL 2-ethylhexanoic acid, and gently warm on hot plate without refluxing, swirling occasionally until the salt dissolves. Cool to ambient temperature. Transfer the solution to a 100 mL volumetric flask, rinsing the beaker with five 10 mL portions of MIBK, then dilute to exactly 100 mL, mix, and store at 20 ± 2°C. This standard stock solution should be stable for at least 6 months.

WORKING STANDARDS

1. Fe—Pipet 10 µL of 100 ppm stock standard into a 25 mL volumetric flask. Add 15 mL MIBK, 12 drops concentrated HNO_3, swirl, dilute with MIBK to a final volume of exactly 25 mL, and mix completely.
2. Cu—Pipet 10 µL of 100 ppm stock standard into a 25 mL volumetric flask, dilute with MIBK to a final volume of exactly 25 mL, and mix completely.
3. Ni—Pipet 10 µL of 100 ppm stock standard into a 25 mL volumetric flask. Add 15 mL MIBK, 600 µL concentrated HNO_3, swirl, dilute with MIBK to a final volume of exactly 25 mL, and mix completely.
4. Mn—Pipet 10 µL of 100 ppm stock standard into a 25 mL volumetric flask, dilute with MIBK to a final volume of exactly 25 mL, and mix completely.
5. Cr—Pipet 10 µL of 100 ppm stock standard into a 25 mL volumetric flask, dilute with MIBK to a final volume of exactly 25 mL, and mix completely.

PROCEDURE

1. Accurately weigh 1.0 g ± 0.1 mg of oil into a 10 mL volumetric flask, dilute to volume with appropriate solvent system [see
   (a) through (e) immediately following] and mix (see Notes, 1).
   (a) Fe-MIBK + 250 µL concd HNO_3/10 mL MIBK
   (b) Ni-MIBK + 250 µL concd HNO_3/10 mL MIBK
   (c) Cu-MIBK
   (d) Mn-MIBK
   (e) Cr-MIBK
2. Set up instrument for operation with the following graphite furnace conditions:

Dry	30 sec	100°C
Char	20 sec	800°C
Atomize	20 sec	2300°C[a]
Argon purge gas	5–6 L/min	

[a]2300°C is the upper limit for P-E HGA-2000 with grooved graphite tubes. If either HGA-2100 or HGA-2200 is available, atomization at 2500°C is preferred.

3. Following the manufacturer's instructions, set up the instrument, and adjust to the recommended wavelength (Table 1).
4. Record absorbance readings for the standard curve of interest by preparing at least a five-point curve. By varying injection volume, a single working standard solution can be used to establish the entire curve. Inject 10, 20, 30, 40, and 50 µL volumes of working standard solution in which the absorbance of at least three determinations at each injection volume agree within ± 0.005 absorbance units. Plot the curve for the metal of interest using the average absorbance as the Y axis and concentration in µg as the X axis.
5. Sample analysis—
   (a) Inject 20 µL of test solution as a first estimate. If an unacceptable absorbance value is obtained (either a low absorbance value or a high absorbance value that exceeds the working linear range of the standard curve), the sample injection volume must be adjusted accordingly (see Notes, 2). With P-E grooved graphite tubes, individual injection volumes must not exceed 50 µL.

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(b) For samples with unusually low metal content (see Notes, 3), multiple sample injections are permissible, if, after each injection, the sample is allowed to proceed through the dry and char cycle, and is then interrupted for each additional injection until the final aliquot is injected, in which case the dry/char/atomize cycle is not interrupted, but allowed to continue through atomization (see Notes, 4).

(c) The absorbance value is then compared to the standard curve and the µg value for the sample is obtained from the X axis.

6. Repeat Procedure, 3-5 for each analysis using the appropriate source lamp (as noted in Table 1) and conditions.

CALCULATIONS

1. $$\text{Metal, ppm} = \frac{(1000)(C)(SV)(F)^*}{(IV)(SM)}$$

Where—

C = metal in mg from calibration graph

SV = final volume of sample flask

IV = volume, mL of test solution injected into furnace

SM = test sample mass in grams

F = dilution factor*, where F = volume of dilution flask/aliquot taken

*Use dilution factor only when dilution is made because initial sample preparation is too concentrated.

PRECISION

1. Several laboratories participated in the collaborative study of the graphite furnace technique used to determine copper, iron, nickel, chromium, and manganese at levels below 1 µg/g and mostly at 0.2 µg/g and under. Results showed a relative standard deviation (RSD) of 15 and 30% at levels of 0.2 µg/g and 0.1 µg/g, respectively. At the 0.05 µg/g level, RSD values of 50% and above were frequently observed.

NOTES

Caution

Methyl isobutyl ketone (MIBK) is flammable and a dangerous fire risk. Explosive limits in air are 1.4–7.5%. Avoid inhalation and ingestion. It is absorbed by the skin. The TLV is 50 ppm in air.

Xylene is flammable and a dangerous fire risk. The TLV is 100 ppm in air.

Nitric acid—Use effective acid-resistant fume-removal device whenever heating acids or performing reactions that liberate acid fumes. When diluting acids, always add acid to water unless otherwise directed in a method. Keep acids off skin and protect eyes when working with acids. If acids come in contact with skin or eyes, wash immediately with large amounts of water. Do not store oxidizing acids (perchloric, nitric, sulfuric) near organic materials. Mixing organic materials with these acids, particularly perchloric, could result in an explosion.

NUMBERED NOTES

1. Achieving a 10% wt/vol MIBK solution for C_{16} and C_{18} fatty acid triglycerides is readily accomplished down to an iodine value (IV) of 65–70. Below this IV, solubility decreases to approximately 2% wt/vol for triglycerides with an IV of 0-10. Lauric stearins, however, are soluble in MIBK at 7–8% wt/vol.
2. The following is a guide to the range of linear response to the metals of interest:

Fe	2.4 ng
Cu	8.0 ng
Ni	11.0 ng
Mn	2.0 ng
Cr	2.0 ng

3. For a sample with either an unusually low metal content or a troublesome sample matrix, the "standard method of addition" technique is recommended.
4. It is recommended that the purge gas *not* be interrupted during the atomization cycle. Past experience indicates that during the final stage of triglyceride decomposition when moving from the char to the atomization mode, a last surge of smoke is liberated from the sample. The background corrector is capable of compensating the magnitude of the smoke scatter effect. However, when operating in a purge gas interrupt mode during atomization, a nonuniform and poorly reproducible smoke pattern results in an apparent scatter of sample response. Operating in "purge gas on" mode during atomization may cause some loss in sensitivity, but with "purge gas on" it transforms or mixes the smoke into a uniform gray field that is reproducibly corrected.

REFERENCES

1. Olejko, J.T., *J. Am. Oil Chem. Soc. 53*:480 (1976).

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Table 1
Recommended wavelengths.

Element	Wavelength, nm
Fe	248.3
Cu	324.8
Ni	232.0
Mn	279.5
Cr	357.9

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AOCS Official Method Ca 18b-91

Reapproved 2009

Determination of Copper, Iron, and Nickel by Direct Graphite Furnace Atomic Absorption Spectrophotometry

DEFINITION

This method is for the determination of trace amounts (µg/kg) of copper, iron, and nickel. The method requires the vaporization of the oil or fat in a suitable graphite furnace connected to an atomic absorption spectrometer (previously calibrated using standard solutions of organocompounds of copper, iron, and nickel), followed by the measurement of the metal content from the observed absorptions at selected wavelengths. The elements are analyzed sequentially (see References, 1 and 2).

SCOPE

Applicable to the determination of copper, iron, and nickel in all types of crude or refined oils and fats, including contaminants from bleaching earth and/or catalysts. This method is equivalent to International Union of Pure and Applied Chemistry standard method 2.631 (References,1 and 2).

APPARATUS

1. Polyethylene- or polypropylene-capped bottles—20 and 50 mL, metal free (see Notes, 1).
2. Micropipets—20 and 50 µL.
3. Pipet tips.
4. Atomic absorption spectrometer—The spectrometer should be located in a dust-free atmosphere.

   Note—Zeeman or Smith-Hieftje background correction technique is considered superior to the deuterium arc technique.
5. Graphite furnace atomizer.
6. Graphite tubes—for use in a furnace atomizer (Apparatus, 5).
7. Chromatographic columns—diameter/height ratio 1:10 (see Notes, 2).
8. Electric oven—regulated at 60 ± 2°C and suitable for heating at 150°C.
9. Water bath.
10. Balance—analytical, 200 gram capacity with ±0.001 gram sensitivity.

REAGENTS

1. *n*-Heptane—analytical grade (see Notes, *Caution*).
2. Light petroleum—b.p. 40–60°C, analytical grade (see Notes, *Caution*).
3. Sunflower oil—refined, or a similar stable liquid oil with a low metal content (see Notes, 2).
4. Aluminum oxide—chromatographic grade (see Notes, 3).
5. Nitric acid—2 M, free from traces of iron, nickel and copper.
6. Niobium nitrate [$Nb(NO_3)_5$]—aqueous solution 1000 mg/L (see Notes, 4).
7. Standard stock solutions of Fe (10 mg/kg), Ni (10 mg/kg), and Cu (2 mg/kg)—prepared by appropriate dilution of organometallic standards with sunflower oil (Reagents, 3) (see Notes, 5).
8. Standard working solutions—prepared daily by diluting the stock solutions (Reagents, 7) with the low metal content oil (Reagents, 3) to give the following concentrations:

   Copper: 0.05, 0.1, 0.2 mg/kg

   Iron: 0.25, 0.5, 1.0 mg/kg

   Nickel: 0.25, 0.5, 1.0 mg/kg
9. Argon—minimum purity 99.99% (see Notes, 6).

PROCEDURE

1. Treatment of samples and solutions—
   (a) Place all samples and working solutions in the oven (Apparatus, 8) during the period of determination.
   (b) Shake samples vigorously before analysis.
   (c) If the metal content of a crude oil is known to be outside the range specified for the standards (Reagents, 8), dilute the sample with the low metal content oil (Reagents, 3) to bring the metal content within that range.

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2. Preparation of apparatus—
   (a) Prepare the atomic absorption spectrometer (Apparatus, 4) in accordance with the instructions of the manufacturer. The required wavelengths (nm) are:
   Copper: 324.7
   Iron: 302.1
   Nickel: 232.0
   (b) Optimize the position of the graphite furnace atomizer (Apparatus, 5) in the atomic absorption spectrometer (Apparatus, 4), and set the required programs for the furnace as follows:

	Step	Temp, °C	Ramp time, sec	Hold time, sec	Gas flow, mL/min
For Cu	1	900	50	30	300
	2	2700	1	5	50
For Fe, Ni	1	1200	50	30	300
	2	2700	1	5	50

If it is not possible to program the graphite furnace exactly as above, use a comparable program suitable for the equipment (see Notes, 7).
   (c) Use a normal graphite tube (see Notes, 8).
   (d) Pretreat the pipet tip (Apparatus, 3) by pipetting (using a micropipet; Apparatus, 2) and then discarding 20 µL heptane. The film of heptane remaining on the wall of the tip facilitates a reproducible transfer of the oil sample. The tip must be pretreated before each injection of an oil sample.

DETERMINATION

1. Graphite tube blank—Record the absorption, if any, of the graphite tube (Apparatus, 6) as such and autozero this absorption.
2. Liquid oil blank—By means of a micropipet (Apparatus, 2), inject 20 µL low metal content oil (Reagents, 3) into the graphite furnace (Apparatus, 5), initiate the temperature program and record the absorption.
3. Standardization of apparatus—By means of a micropipet (Apparatus, 2), inject 20 µL of the three standard working solutions of the metal under investigation (Reagents, 8) into the graphite furnace (Apparatus, 5) and record the absorptions.
4. Test sample analysis—
   (a) Oils (liquid)—By means of a micropipet (Apparatus, 2), inject 20 µL of the oil into the graphite furnace (Apparatus, 5), initiate the temperature program and record the absorption.
   (b) Fats (solid)—(mp 40°C and higher) Introduce an extra temperature programming step: hold time 20 sec, temperature 60°C, internal gas flow 0 mL/min. Initiate the temperature program. Within the first program step introduce, by means of a micropipet (Apparatus, 2), 20 µL of the melted fat into the graphite furnace (Apparatus, 5), allow the tip to remain in the injection opening to liquify the fat and then inject. Record the absorption (see Notes, 9).
5. Number of determinations—Carry out two determinations in rapid succession.

CALCULATIONS

1. Measure the peak height.
2. Draw a calibration curve by plotting the absorption of the three standards (Determination, 3), corrected for the blank (Determination, 2), against their respective metal contents (see Notes, 10).
3. Read the metal content of the sample from the relevant calibration curve.
4. Report as the final result the mean of the results of the two determinations, provided the requirements for repeatability (Precision, 1) are met. If the requirements for repeatability are not met, discard the results and carry out two additional determinations on the test sample.

PRECISION

1. Repeatability value—The difference between the values obtained from two single determinations, carried out in rapid succession by the same operator, using the same apparatus for the analysis of the same test sample, should not be greater than the repeatability value (r) as calculated from the formulas in Table 1, which express the precision in relation to the determined mean value.
2. Reproducibility value—The difference between the values for the final result, obtained by two (or more) laboratories using this standard method for the analysis of the same laboratory sample, should not be greater than the reproducibility value (R) as calculated from the formulas in Table 1, which express the precision in relation to the determined mean value.

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NOTES

Caution

Heptane and light petroleum are flammable and a dangerous fire risk. Vapors may cause lung irritation and may produce neurotoxic effects. A fume hood should be used at all times when using these solvents.

NUMBERED NOTES

1. The polyethylene- or polypropylene-capped bottles are made metal free as follows: Clean the bottles thoroughly with warm nitric acid (Reagents, 5), rinse with distilled water, and dry the bottles in a drying oven (Apparatus, 8) at about 80°C.
2. Low metal content oil is obtained as follows: Activate aluminum oxide (Reagents, 4) by heating in an oven (Apparatus, 8) at 150°C for 14 hr. Prepare a chromatographic column (Apparatus, 7), using twice the mass of activated aluminum oxide as the mass of the oil to be purified. Dissolve 1 part of the oil (Reagents, 3) by mass in 3 parts by volume of light petroleum (Reagents, 2). Add the oil solution to the column and elute with 5 parts by volume of light petroleum. Evaporate the light petroleum in the eluate on a heated water bath (Apparatus, 9), using a gentle stream of nitrogen (2-5 L/min). Under vacuum, remove the final traces of light petroleum from the purified oil.
3. Merck product no. 1077 is suitable.
4. Solution (code 88083) is available from Alfa Division, Danvers, MA, USA.
5. Suitable standards are available from Conoco, Ponca City, OK, USA (Conostan, 5000 mg/kg), or Merck, D- 6100 Darmstadt, Germany (metal in standard oil, 1000 mg/kg).
6. If argon is not available, nitrogen may be used as purge gas. At temperatures above 2300°C, nitrogen forms toxic cyanogen gas; therefore, continuous ventilation in the furnace area must be provided.
7. If in this case the background correction fails, dilute blank, standards, and samples with an organic fat solvent, *e.g.*, heptane, (Reagents, 1) to a maximum of 1:2 (m/m) and work at ambient temperature.
8. Separate graphite tubes should be used for each metal. The graphite tube for the determination of iron has to be coated with niobium to ensure that the total amount of iron is determined. With an uncoated tube the result will vary according to the type of iron compound present in the oil. A suitable coating procedure is as follows: By means of a micropipet (Apparatus, 2), inject 100 µL niobium nitrate solution (Reagents, 6) into the furnace. Start the temperature program to dry at 100°C for 60 sec, and then atomize at 2700°C for 5 sec. Repeat this procedure until 300 µL of niobium nitrate solution has been injected. Atomize at 2700°C until constant absorbance is attained (to remove any iron contamination).
9. The normal, minimum limit of detection can be improved by either a greater scale expansion or by repeated injections of the sample at the end of the ashing operation, then allowing the program to proceed to completion. If the metal content is too high (*i.e.,* exceeds the calibration curve), measure the absorption after a further dilution of the sample with sunflower oil.

   Better precision can be obtained with the use of an autosampler, with sample tray held at 60°C. At 60°C, delivery of different amounts of sample is minimized, because all edible oils have nearly the same density at 60°C. While solvents may be used for sample dilution, their use is discouraged; direct injection is preferred. 10. With the use of sophisticated equipment, autocalibration can be applied.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.631.
2. *Pure Appl. Chem. 60:*893 (1988).

Table 1
Repeatability (r) and reproducibility (R) values.

Metal	Substrate	r	R
Cu	oil	0.0102 + 0.140 m	0.0085 + 0.358 m
	fat	0.0076 + 0.109 m	0.0028 + 0.492 m
Fe	oil	0.077 + 0.081 m	0.040 + 0.480 m
	fat	0.026 + 0.196 m	0.031 + 0.543 m
Ni	oil	0.056 + 0.127 m	0.027 + 0.442 m

m = corresponding mean concentration value.

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Determination of Lead by Direct Graphite Furnace Atomic Absorption Spectrophotometry

DEFINITION

This method is used for the determination of trace amounts (µg/kg) of lead. The method requires the vaporization of the oil or fat in a suitable graphite furnace, with or without a platform, connected to an atomic absorption spectrometer (previously calibrated using standard solutions of an organocompound of lead), followed by the measurement of the metal content from the observed absorption at a wavelength of 283.3 nm (see References, 1 and 2).

SCOPE

Applicable to the determination of lead in all types of crude or refined edible oils and fats. This method is equivalent to International Union of Pure and Applied Chemistry standard method 2.632 (References, 1 and 2).

APPARATUS

1. Polyethylene- or polypropylene-capped bottles—20 mL, metal free (see Notes, 1).
2. Micropipet—20 µL.
3. Pipet tips.
4. Electric oven—regulated at 60 ± 2°C.
5. Atomic absorption spectrometer.

   Note—Zeeman or Smith-Hieftje background correction technique is considered superior to the deuterium arc technique.
6. Graphite furnace atomizer—placed in the atomic absorption spectrometer (Apparatus, 5), equipped with a control unit for temperature programming.
7. Graphite tube—normal (uncoated).
8. Platform—pyrolytic, in combination with uncoated or pyrolytically coated graphite tube (see Notes, 2).
9. Volumetric flask—100 mL size.

REAGENTS

1. Cyclohexane—analytical grade (see Notes, *Caution*).
2. Lecithin—well defined, containing 2% phosphorus.
3. Matrix modifier—2% (m/v) lecithin solution prepared by dissolving 2 g lecithin (Reagents, 2) in 100 mL cyclohexane (Reagents, 1).
4. Blank oil—refined; any edible oil is suitable. Store in a metal-free polyethylene bottle. The lead content of oil should not be greater than 1 µg/kg.
5. Standard stock solution of Pb (10 mg/kg)—prepared by appropriate dilution of an organometallic standard (see Notes, 3) with the blank oil (Reagents, 4).
6. Standard working solutions—prepared daily by diluting the stock solution (Reagents, 5) with the blank oil (Reagents, 4) to give the following concentrations of Pb: 0.020 mg/kg, 0.050 mg/kg, and 0.100 mg/kg.
7. Argon—minimum purity 99.99%.

PROCEDURE

1. Treatment of test samples, blank, and standards—
   (a) Place all test samples and standard working solutions in the oven (Apparatus, 4), regulated at 60 ± 2°C.
   (b) Shake test samples vigorously.
   (c) Weigh 5.00 g test sample in a 20 mL bottle (Apparatus, 1) together with 5.00 g matrix modifier (Reagents, 3) and mix thoroughly.
   (d) Repeat steps (b) and (c) for the three standard working solutions (Reagents, 6) and the blank oil (Reagents, 4).
2. Preparation of apparatus—
   (a) Prepare the atomic absorption spectrometer in accordance with the manufacturer's instructions. The required wavelength is 283.3 nm.

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(b) Optimize the position of the graphite furnace atomizer (Apparatus, 6) in the atomic absorption spectrometer (Apparatus, 5), and set the required program on the control unit of the furnace. If available, place platform in graphite tube (see Notes, 2).
(c) Before each injection, pretreat the pipet tip (Apparatus, 3) by pipetting and then discarding 20 µL of cyclohexane.

3. Programs for the graphite furnace atomizer are given in Table 1 (for Varian spectrometer, see Notes, 4).

DETERMINATION

1. Measurement of the blank—Inject 20 µL of the blank solution prepared according to Procedure, 1 into the graphite furnace, initiate the temperature program and record the absorption.
2. Measurement of standards—Inject 20 µL of the three standard solutions prepared according to Procedure, 1 into the graphite furnace and record the absorptions.
3. Measurement of test solutions—Inject 20 µL of the test sample solution prepared according to Procedure, 1 into the graphite furnace, initiate the temperature program and record the absorption.

CALCULATIONS

1. Measure the peak height on the chart recorder, or take the reading of the display or printer.
2. Draw a calibration curve by plotting the absorption of the three standards (Determination, 2), corrected for the blank (Determination, 1), against their respective metal content (see Notes, 4, 5).
3. Read the metal content of the test sample from the calibration curve.
4. Report as the final result the mean of the results of two single determinations, provided the requirements for repeatability (see Precision, 1) are met. If the requirements for repeatability are not met, discard the results and repeat two determinations on the test sample.

PRECISION

1. Repeatability value—The differences between the values obtained from two single determinations, carried out in rapid succession by the same operator, using the same apparatus for the analysis of the same test sample, should not be greater than the repeatability value (r) as calculated from the formulas in Table 2, which express the precision in relation to the determined mean value (see Notes, 6).
2. Reproducibility value—The difference between the values for the final result, obtained by two (or more) laboratories using this standard method for the analysis of the same laboratory sample, should not be greater than the reproducibility value (R) as calculated from the formulas in Table 2 which express the precision in relation to the determined mean value (Table 3).

NOTES

Caution

Cyclohexane is flammable and a dangerous fire risk. It is moderately toxic by inhalation and skin contact. The TLV in air is 300 ppm.

NUMBERED NOTES

1. The polyethylene- or polypropylene-capped bottles are made metal free in the following way: Clean the bottles thoroughly with warm nitric acid, rinse with distilled water, and dry the bottles in a drying oven at about 80°C.
2. Both atomization off the wall and atomization off the platform can be used. The accuracy and the sensitivity off the platform is two times higher than off the wall.
3. A suitable standard (Conostan, 5000 mg/kg) is available from Conoco, Ponca City, OK, USA.
4. For those having a Varian spectrophotometer, the modifications shown in Table 4 should be made.
5. With the use of sophisticated equipment, autocalibration can be applied.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.632.
2. *Pure Appl. Chem. 60*:893 (1988).
3. Hendrikse, P.W., F.J. Slikkerveer, A. Folkersma, and A. Dieffenbacher, *Pure and Appl. Chem. 63*: 1183–1192 (1991). Report on the collaborative study.

OTHER REFERENCES

Anal. Chem. 54:67A (1982).
At. Spectrosc. 1:30 (1980).
J. Assoc. Off. Anal. Chem. 72:34 (1989)

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Table 1
Programs for the graphite furnace atomizer.

(a) Uncoated tube (Apparatus, 7), off the wall, maximum power heating, gas stop.

Step	Temp, °C	Ramp time, sec	Hold time, sec	Gas flow, mL/min
1	100	10	20	300
2	650	60	40	300
3	1900	0	5	0
4	2700	1	3	50

(b) Pyro-coated tube (Apparatus, 8) with platform, maximum power heating, gas stop.

Step	Temp, °C	Ramp time, sec	Hold time, sec	Gas flow, mL/min
1	200	10	20	300
2	650	60	40	300
3	1700	0	5	0
4	2700	1	3	50

Table 2
Repeatability (r) and reproducibility (R) values.

Pb in edible oil	r = 0.19 m	R = 0.30 m
Pb in cocoa butter	r = 0.15 m	R = 0.68 m

m = corresponding mean concentration value.

Table 3
The interlaboratory test carried out at the international level in 1991 by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Oils, Fats and Derivatives, in which 20 laboratories participated, each obtaining two test results for each sample, gave the statistical results [evaluated in accordance with International Organization for Standardization (ISO) 5725-1986] summarized in the following table.

Statistic	Level 1		Level 2		Level 3	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
Edible Oil (Soybean Oil)						
No. Labs	14	16	16	14	16	16
Pb (mg/kg)[a]	0.080	0.090	0.047	0.054	0.018	0.023
Mean	0.083	0.086	0.046	0.057	0.018	0.022
S_r	0.005	0.006	0.003	0.004	0.002	0.002
S_R	0.007	0.017	0.010	0.005	0.005	0.006
RSD_r	6.1	7.3	5.9	6.7	11.3	11.2
RSD_R	9.0	19.3	22.6	8.5	30.2	28.2
rb	0.014	0.018	0.008	0.011	0.006	0.010
R_c	0.021	0.046	0.029	0.013	0.015	0.020
Cocoa Butter						
No. Labs	15	16	16	15	15	16
Pb (mg/kg)[a]	0.080	0.090	0.047	0.054	0.018	0.023
Mean	0.084	0.090	0.052	0.058	0.024	0.029
S_r	0.004	0.003	0.004	0.002	0.001	0.001
S_R	0.018	0.019	0.012	0.013	0.007	0.008
RSD_r	5.3	3.7	7.0	3.5	4.9	5.0
RSD_R	21.1	20.8	22.3	22.8	29.7	26.9
rb	0.012	0.009	0.010	0.006	0.003	0.004
R_c	0.050	0.053	0.032	0.037	0.020	0.022

[a]Actual values.
[b]$2.8 \times S_r$.
[c]$2.8 \times S_R$.

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Table 4
Modifications for Varian spectrophotometer.

(a) Program for uncoated tube (Apparatus, 7), off the wall.

Step	Temp, °C	Time, sec	Gas flow, L/min	Gas type	Read command
1	100	20.0	3.0	Normal	No
2	100	40.0	3.0	Normal	No
3	550	60.0	3.0	Normal	No
4	550	40.0	3.0	Normal	No
5	550	1.0	0.0	Normal	No
6	2500	1.0	0.0	Normal	Yes
7	2500	5.0	0.0	Normal	Yes
8	2500	3.0	3.0	Normal	No

Use 10 µL for injection.

(b) Program for pyro-coated tube (Apparatus, 8), with platform.

Step	Temp, °C	Time, sec	Gas flow, L/min	Gas type	Read command
1	200	10.0	3.0	Normal	No
2	200	20.0	3.0	Normal	No
3	650	60.0	3.0	Normal	No
4	650	40.0	3.0	Normal	No
5	650	1.0	0.0	Normal	No
6	2000	0.7	0.0	Normal	Yes
7	2000	5.0	0.0	Normal	Yes
8	2000	3.0	3.0	Normal	No

Use 10 µL for injection.

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Determination of Cadmium Content by Direct Graphite Furnace Atomic Absorption Spectrophotometry

DEFINITION

The oil or fat is incinerated and atomized in a suitable graphite tube furnace with a platform connected to an atomic absorption spectrophotometer, previously calibrated using standard solutions of an organo-compound of cadmium. The metal content is determined from the observed absorption at a wavelength of 228.8 nm. Palladium is added as a matrix modifier in order to prevent loss of cadmium during the thermal pretreatment.

SCOPE

Applicable to the determination of trace amounts (µg/kg) of cadmium in all types of crude or refined edible oils and fats. This method is equivalent to International Organization for Standardization Method 15774:2000 (References, 1).

APPARATUS

1. Polyethylene- or polypropylene-capped bottles—20 mL and 50 mL, metal free (see Numbered Notes, 1).
2. Micropipet—10 µL and 20 µL.
3. Pipette tips.
4. Volumetric flask—100 mL.
5. Electric oven—regulated at 60 ± 2°C.
6. Atomic absorption spectrometer—equipped with so-called "peak area" mode and "autocalibrate" mode, an appropriate electrode-less discharge lamp (or hollow cathode lamp) and deuterium background corrector (or Zeeman atomic absorption spectrometer).
7. Graphite furnace atomizer—placed in the atomic absorption spectrometer (Apparatus 6), equipped with a control unit for temperature programming.
8. Graphite tube—normal (uncoated).
9. Platform—pyrolytic.
10. Autosampler—for graphite furnace atomizer (optional), with polyethylene cups (see Notes, 2).

REAGENTS

1. Cyclohexane—analytical grade (see Notes, *Caution*).
2. Hydrochloric acid.
3. Palladium chloride.
4. Matrix modifier—0.1% (m/v) palladium solution prepared by dissolving 0.167 g of palladium chloride (Reagents, 4) in 50 mL water in a 100 mL volumetric flask (Apparatus, 4), adding 1 mL hydrochloric acid (Reagents, 2) and making up the volume with water.
5. Blank oil—refined; any edible oil is suitable. Store in a metal-free bottle. The cadmium content of the oil should not be greater than 0.2 µg/kg.
6. Standard stock solution of Cd (10 mg/kg)—prepared by diluting 200 mg of an organometallic standard (Conostan, 5000 mg/kg is available from Conoco, Ponca City, OK, USA) with 100 g of blank oil (Reagents, 5).
7. Standard working solutions—prepared daily by diluting the stock solution (Reagents, 6) with the blank oil (Reagents, 5) to give the following concentrations of Cd: 2.5 µg/kg, 5.0 µg/kg, and 10 µg/kg.
8. Argon—minimum purity 99.99%.

PROCEDURE

1. Treatment of test samples, blank, and standards—
   (a) Place all test samples, standard working solutions (Reagents, 7), and blank oil (Reagents, 5) in the oven (Apparatus, 5), regulated at 60 ± 2°C. If an autosampler is used, dilute 1:1 (m/v) all solutions with an appropriate solvent (Reagents, 1). Homogenize well before pouring the solution into the sample cups of the autosampler.
   (b) Shake test samples vigorously. If the metal content of a test sample is known to be outside the given range (Reagents, 7), dilute by mass with blank oil (Reagents, 5).

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2. Preparation of apparatus—
   (a) Switch on the atomic absorption spectrometer and the background correction system. In accordance with the manufacturer's instructions supplied with the spectrometer, adjust the lamp current, slit width, wavelength, and amplification. The required wavelength is 228.8 nm.
   (b) Optimize the position of the graphite furnace atomizer (Apparatus, 7) in the atomic absorption spectrometer (Apparatus, 6) and set the required program on the control unit of the furnace. Place the platform (Apparatus, 9) in the graphite tube (Apparatus, 8).
   (c) Before each injection, pretreat the pipette tip (Apparatus, 3) by pipetting and then discarding 10 µL of cyclohexane (Reagents, 2).
3. A program for the graphite furnace atomizer is given in Table 1.

DETERMINATION

1. Graphite tube blank—record the absorption, if any, of the graphite tube (Apparatus, 8) and autozero this absorption.
2. Blank oil—By means of micropipettor (Apparatus, 2) or an autosampler (Apparatus, 10), inject 20 µL of the matrix modifier (Reagents, 4) into the graphite furnace (Apparatus, 7) and initiate the modifier temperature program (step 1; see Table 1). Inject 10 µL of the blank oil (Reagents, 5) into the graphite furnace (Apparatus 7), initiate the temperature program (steps 1-4), and record the absorption (for the autosampler, see Notes, 2).
3. Calibration of apparatus—Use micropipettor (Apparatus, 2) to inject 20 µL of the matrix modifier (Reagents, 4) into the graphite furnace (Apparatus, 7) and initiate the modifier temperature program (step 1). Inject 10 µL of the first of the three standard working solutions, prepared according to Reagents, 8, into the graphite furnace. Initiate the temperature programs (steps 1–4). Continue with the second and third standard working solutions successively. Calibrate the spectrometer according to the operating procedure of the apparatus used (for autosampler, see Notes, 2). Plot the analytical curve.
4. Oils (liquid)—Use micropipettor (Apparatus, 2) to inject 20 µL of the matrix modifier (Reagents, 4) into the graphite furnace (Apparatus, 7) and initiate the modifier temperature program (step 1). Inject 10 µL of the oil into the graphite furnace, initiate the temperature programs (steps 1–4), and record the concentration according to the operating procedure of the apparatus used (for autosampler, see Numbered Notes, 2).
5. Fats (solid) with melting point 40°C and higher— Introduce an extra temperature-programming step with temperature 60°C, hold time 20 s, and with an internal gas flow of 0 mL/min. Use micropipettor (Apparatus, 2) to inject 20 µL of the matrix modifier (Reagents, 4) into the graphite furnace (Apparatus, 7) and initiate the modifier temperature program (step 1). In the first program step (step 0; Table 1), inject 10 µL of the melted fat into the graphite furnace by allowing the tip to remain in the injection opening to liquefy the fat before injecting it. Record the concentration according to the operating procedure of the apparatus used (for autosampler, see Notes, 2).
6. Number of determinations—Carry out two determinations in rapid succession.

CALCULATIONS

1. Measure the peak height on the chart recorder, or take the reading of the display or printer.
2. Draw a calibration curve by plotting the absorption of the three standards (Determination, 3), corrected for the blank (Determination, 2) against their respective metal content (see Reagents, 7).
3. Read the metal content of the sample from the calibration curve.
4. Report as the final result the mean of the results of two single determinations, provided the requirements for repeatability are met (see Precision, 2). If the requirements for repeatability are not met, discard the results and repeat two determinations on the test sample.

PRECISION

1. Interlaboratory test—Details of an interlaboratory test on the precision of the method are listed in Table 2. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.
2. Repeatability—The differences between the values obtained from two single determinations, carried out in rapid succession by the same operator, using the same apparatus for the analysis of the same test sample, should not be greater than the 1.1 µg/kg when the mean value lies between 2 and 8 µg/kg for oil samples, or 1.7 µg/kg when the mean value lies between 3 and 7 µg/kg for fat samples.
3. Reproducibility—The absolute difference between the values for the final result, obtained by two (or more) laboratories using this standard method for analysis of the same laboratory sample, should not be greater than 2.9 µg/kg when the values of the two results lie between 2 and 8 µg/kg for oil samples; or 2.6 µg/kg when the values of the two results lie between 3 and 7 µg/kg for fat samples.

NOTES

Caution

Cyclohexane is flammable and a dangerous fire risk. It is moderately toxic by inhalation and skin contact. The TLV in air is 300 ppm.

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NUMBERED NOTES

1. The polyethylene- or polypropylene-capped bottles are made metal-free in the following way: Clean the bottles thoroughly with warm nitric acid, rinse with distilled water, and dry the bottles in a drying oven at about 80°C.
2. To use an autosampler equipped with a heating device, regulated at 60°C ± 10°C, fill sample cups with blank oil (Reagents, 6), standard working solutions (Reagents, 8), and oil samples. Fats with a melting point of 40°C and higher should be diluted 1:1 by mass with blank oil (Reagents, 6). Inject 15 µL of test solution.
3. To use an autosampler without a heating device, dilute 1:1 (m/v) the blank oil (Reagents, 6), the standard working solutions (Reagents, 7), and oil or fat samples with an organic solvent [e.g., cyclohexane (Reagents, 2)] at ambient temperature. Inject 20 µL of sample.

REFERENCES

1. ISO 15774:2000 (E).
2. *Pure Appl. Chem.*, Vol. 71, No. 2, 361-368 (1999).

Table 1
Programs for the graphite furnace atomizer.

Step	Temperature (°C)	Ramp time (sec)	Hold time (sec)	Internal gas flow (mL/min)
Injection of matrix modifier				
1	200	30	30	300
Injection of oil sample				
0[a]	60	0	20	0
1	200	30	30	300
2	650	60	40	300
3	1600	0	5	0
4	2700	1	3	50

[a]Extra temperature programming step only for fat samples with melting points over 40°C.

Table 2
Results of an international collaborative test carried out in 1994 by the IUPAC Commission on Oils, Fats and Derivatives.

	Sunflower oil		Lard	
	A	B	A	B
Number of laboratories retained after eliminating outliers	6	7	6	7
Number of outliers (laboratories)	2	1	2	1
Mean value, µg/kg	7.87	1.58	7.17	2.7
True value, µg/kg	8.0	2.0	7.0	3.0
Repeatability standard deviation, s_r, µg/kg	0.31	0.39	0.61	0.23
Repeatability coefficient of variation, %	3.9	24.9	8.6	8.5
Repeatability limit, r ($2.8 \times s_r$), µg/kg	0.87	1.11	1.74	0.65
Reproducibility standard deviation, s_R, µg/kg	0.99	0.55	0.92	0.59
Reproducibility coefficient of variation, %	13.2	34.8	12.9	22.0
Reproducibility limit, R ($2.8 \times s_R$), µg/kg	2.94	1.55	2.61	1.68

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AOCS Recommended Practice Ca 19-86
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Phospholipids in Vegetable Oils Nephelometric Method

DEFINITION

The nephelometric method measures turbidity in oil-acetone mixtures due to phospholipids. The turbidity is correlated to the phosphorus level.

SCOPE

This method is applicable to crude, degummed, once-refined, bleached, and deodorized vegetable oils. The presence of soap in oil samples may give erroneous results (see Notes, 1).

APPARATUS

1. Lab turbidimeter—model 2100N (Hach Company, Loveland, CO, USA, 800-227-4224), or equivalent.
2. Turbidimeter sample cells—2.5 × 9.6 cm (Hach catalog no. 20849-00), or equivalent.
3. Gelex secondary turb standard set. Hach catalog no. 225-26-00.
4. Microwave oven or hot plate.
5. Fluted filter paper—12 cm, Whatman no. 2, or equivalent.
6. Filter funnel—6 cm diameter.
7. Pyrex™ beakers—150 mL.
8. Volumetric flasks—50 mL.
9. Stablcal Calibration Kit—Hach catalog no. 26621-00.

REAGENTS

1. Acetone—spectral grade (see Notes, *Caution*).

PROCEDURE

1. Turn on the power switch of the turbidimeter and allow it to warm up at least 15 min before use.
2. Calibrate the turbidimeter according to the manufacturer's instructions, and perform a blank determination on the acetone (see Notes, 2).
3. Heat the oil sample to about 50°C, using a microwave oven or hot plate (see Notes, 3).
4. Pass the sample through dry, fluted filter paper.
5. Weigh (see Notes, 4) an appropriate amount of the oil sample (0.33, 1.67, or 8.35 g, depending on the phosphorus level and the oil type) into a 50 mL volumetric flask.
6. Add acetone to the 50 mL mark.
7. Stopper, mix well, and pour the mixed sample into the turbidimetric cell (see Notes, 5) to the required level (about 30 mL).
8. Cap the sample cell and shake by hand for about 10 sec.
9. Wipe the sample cell clean with a tissue and place it in the turbidimeter (see Notes, 6 and 7).
10. Select the correct turbidity range: either 2, 20, or 200 nephelometric turbidity units (NTU).
11. Record the turbidity reading after exactly 5 min (see Notes, 8).
12. Subtract the NTU value for the acetone blank from the reading obtained in step 11.

NEPHELOMETRIC PHOSPHORUS DETERMINATION

1. The phosphorus level in mg/L (ppm) for a given oil type can be determined nephelometrically either by estimating phosphorus directly from a phosphorus vs. NTU curve, or by calculating phosphorus from the corresponding equation.

PREPARATION OF CORRELATION GRAPH AND EQUATION FOR CURVE

1. For a given type of vegetable oil, obtain approximately 10–15 samples of either crude, degummed, once-refined, bleached, or deodorized oils.
2. Determine the turbidity of each sample, using sample sizes indicated in Table 1. If the sample is different from corn or soybean oil, sample size must be chosen to give NTU values similar to those shown in Table 1.
3. Determine phosphorus level of each sample, using AOCS Official Method Ca 12-55.
4. Plot phosphorus in mg/L (ppm) vs. turbidity (NTU values) for each sample.
5. Calculate the equation for the data set using least-squares analysis.

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EQUATIONS FOR CURVES AND SAMPLE SIZES

1. See Table 1.

CALCULATION EXAMPLE

1. For crude soybean oil, the best-fit equation is
   $P = (5.89 \times NTU) + 316.4$
   Where—
   P = phosphorus, mg/L (ppm)
   NTU = turbidity
   The phosphorus levels of the other oil types are calculated similarly, except using the appropriate equation.

NOTES

1. High soap levels (50–100 mg/L or greater) in vegetable oil may give erroneous values.
2. The turbidity of acceptable acetone for this method is 0.5 NTU or less.
3. Heating the oil sample promotes faster filtration. Highly hydrogenated oil samples may need heating in excess of 50°C to assure complete melting. Some oil samples filtered in the refining process may not require further filtration before analysis. Oil sample moisture level should not exceed 0.5%.
4. Samples should be weighed to the nearest 0.01 g for best accuracy.
5. The interior and exterior of the sample cell and cap must be cleaned with only low-NTU acetone prior to each analysis.
6. The temperature of the oil-acetone mixture prior to analysis should be 25°C.
7. The sample cell must be properly aligned, according to the manufacturer's instructions, when placed in the turbidimeter.
8. If after 5 min the turbidity reading has not stabilized, repeat the entire procedure.

REFERENCES

*J. Am. Oil Chem. Soc. 63(5):*667 (1986).

Table 1
Guide for measurement of phosphorus in soybean and corn oils at various levels of refinement.

Oil type	Equation for curve	Recommended test portion size, g
Soybean		
Crude	$P = (5.89 \times NTU) + 316.4$	0.33
Degummed	$P = (5.32 \times NTU) + 3.38$	1.67
Once-refined	$P = (8.26 \times NTU) - 4.49$	1.67
Bleached	$P = (1.27 \times NTU) - 0.225$	8.35
Deodorized	$P = (1.72 \times NTU) - 0.528$	8.35
Corn		
Crude	$P = (5.62 \times NTU) + 97.2$	0.33
Degummed	$P = (3.69 \times NTU) - 2.77$	1.67
Once-refined	$P = (1.42 \times NTU) - 2.21$	1.67
Bleached	$P = (2.60 \times NTU) - 1.05$	8.35
Deodorized	$P = (0.99 \times NTU) + 0.027$	8.35

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AOCS Official Method Ca 20-99

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Analysis for Phosphorus in Oil by Inductively Coupled Plasma Optical Emission Spectroscopy

DEFINITION

This method describes a procedure for the quantification of phosphorus in oil using inductively coupled plasma optical emission spectroscopy (ICP-OES).

SCOPE

Depending on the dilution solvent, nearly all forms of vegetable oils may be analyzed—from crude oil to degummed, refined, bleached, deodorized, and hardened—and nearly all types of lecithins and phosphatides. This procedure is suitable only when the elements are present in a solubilized form. When they are present as fine particles such as bleaching earth, catalysts, or rust, ICP-OES analysis results in poor recovery due to nebulization and atomization problems. The only suitable non-ashing direct method for these samples is graphite furnace AAS.

APPARATUS

1. Inductively coupled plasma optical emission spectrometer (General Notes, 1)
2. Analytical balance (4-place accuracy)
3. Oven
4. Disposable plastic vials
5. Disposable pipet tips
6. Tilt table mixer
7. Volumetric flasks

REAGENTS

1. 1-Butanol
2. Kerosene or xylene (General Notes, 2)
3. Standard elements as organic soluble:
   (a) SPEX, 203 Norcross Ave., Metuchen, NJ 08840
   (b) Constan brand standards available from: Accu Standard, 25 Science Park, New Haven, CT 06511
4. Base 20 oil or Base 75 oil from Accu-Standard may be used to check the blank oil used and for the dilution of the standard solutions as needed.

PRINCIPLE

Solvent-diluted vegetable oils are analyzed for phosphorus by direct aspiration. Liquid samples are nebulized and carried into the excitation source by a flowing gas. Atoms are quantitated by measuring the specific emission lines produced by atoms decaying from high energy levels.

PROCEDURE

1. Sampling—All test samples, standards, and blanks are diluted 1:1 with 1-butanol (or kerosene/xylene) to reduce the viscosity of the oil for better nebulization. 1-Butanol is preferred because it has better moisture tolerance and allows a higher flow rate with higher pressure than kerosene without putting out the torch. The higher flow rate provides for improved detection limits. The increased moisture tolerance permits the analysis of crude oils and lecithins without phase separation.
   (a) Hardened fats are first melted and mixed prior to dilution. The test solution is then kept warm and monitored throughout the analysis to insure it remains in solution. The relative maximum melting point of hardened fats analyzable is 60°C. However some are more soluble in 1-butanol than others.
   (b) 2.5 g (±0.02 g) of test sample is weighed into an autosampler tube and diluted with 2.5 g of 1-butanol (kerosene or xylene) delivered from an autopipetor, capped and inverted 40–50 times.
   (c) Lecithins up to 100% AI (0.2 g) are diluted to 5.0 g with blank soybean oil and then to 10 g with 1-butanol. The test solution is then mixed on a tilt table mixer for one hour, then diluted 1:10 with 1:1 blank oil/1-butanol (total dilution 1:250).

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2. Internal standard—If an internal standard is desired (see Standardization below), it should be incorporated as part of the dilution step above. Typically, the resultant dilution should contain 10 mg/kg yttrium. Thus, under the dilution sequence for sampling, Procedure 1, (a), the 2.5 g of 1-butanol added as diluent should contain 20 mg/kg yielding 10 mg/kg yttrium in the 1:1 dilution to accomplish this. Operate your instrument according to the manufacturer's directions and specifications (see General Notes, 1).
3. The instrument is ignited and allowed to warm. It is profiled on an internal Hg lamp.
4. Blank—Standardization is performed first on a blank oil (typically refined and bleached soybean or other oil which has been shown to be free of trace elements) and run at the specified wavelength for phosphorus. Base 20 or Base 75 oil is used as an absolute reference blank to determine that the blank oil used is free of elements. A blank oil is diluted 1:1 as described in the sampling procedure and analyzed directly.
5. Standardization—A single standard, prepared from a commercially available single element organic-based standard, is run at the phosphorus wavelength. Standards are prepared by weighing the standard and adding enough blank oil to total 50.00 g. 50.00 g of solvent is then added to achieve a 1:1 dilution. One standard concentration will work; however, up to 4 standards will provide a better calibration for linearity and accuracy. Levels should include 2.5, 5, and 10 mg/kg standards. If an internal standard is used, it may be weighed as part of the elements or incorporated as part of the dilution solvent to yield 10 mg/kg to match that amount added to the sample.
6. All test samples, blanks, and standards are scanned in triplicate for phosphorus and are averaged.
7. Standards and a blank are run every ten samples or less and the instrument is restandardized as needed. The range of standardization is very narrow for improved accuracy: 0–25 mg/kg for phosphorus. However, the linearity is somewhat greater. A wider range of standards is required for calibration if high levels of phosphorus are expected.

CALCULATIONS

Computation is performed within the instrumental program and relates to area counts from known samples plugged into the linear regression formula calculated from the area and concentration numbers from the blank and standard. Be sure to include the correct dilution factor. Most ICP software packages provide for this calibration.

Element detection limit (DL) and emission line used:

phosphorus	>0.5 mg/kg	178.287 nm
		(213.6 or 214.9 nm may be used as an alternative)

PRECISION

1. Phosphorus collaborative study results are shown in Tables 1 and 2.
2. Precise conditions will vary from instrument to instrument.
3. For the collaborative study, many instruments were used with many permutations (Table 3).

GENERAL NOTES

1. The conditions for analysis will vary from instrument to instrument from different manufacturers and will further vary within identical instruments from the same manufacturer depending on the type of nebulizer and pumping system used.

   For a Thermo-Jarrell Ash IRIS simultaneous ICP-ES:

Power	1350 watts
Auxiliary flow	1.5 L/min
Nebulizer flow	20.06 psi
Pump flow rate	1.85 mL/min

   For an ARL 3410 Mini-torch ICP, a pump flow of 2.5 mL/min is used.

   For a Unicam 7000 ICP:

Power	1.2 KW
Uptake time	99 sec
Integration time	3 × 1 sec
Nebulizer flow	40 psi
Pump flow rate	1.5 mL/min
Auxiliary flow	0.6 L/min
Coolant flow	16 L/min

   Other instruments may be used for this analysis. See Precision section (Table 3).
2. If kerosene or xylene is used, all instrumental operating conditions such as pump flow rate, etc., will change from those set for 1-butanol. Therefore the analysis must be standardized and all analyses run with all standards, blanks, and samples with kerosene or xylene.

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3. Yttrium has a number of wavelengths which may be used for analysis. The wavelength used should be chosen to match closely with the element analyzed, e.g., 224.3 nm is one wavelength frequently used. Scandium and cobalt have been used.
4. Calibration drift has been noted and often is due to carbon buildup on the injector tip.

REFERENCES

1. This method was written from a number of several in-house analyses from various companies.
2. ISO 10540-3 Animal and vegetable fats and oils—Determination of phosphorus content—Part 3: Method using inductively coupled plasma optical emission spectroscopy (ICP-OES).

Table 1
Phosphorus collaborative study results.

Samples	1	2	3	4	5	6	7	8
Returned results	15	15	15	15	15	15	15	15
Total labs	13	13	13	13	13	13	13	13
Total replicates	26	26	26	26	26	26	26	26
Overall mean, mg/kg	497	335	147	101	56	29	12	305
SD repeatability, s_r	9.2	7.8	1.9	1.3	1.0	0.4	0.3	6.2
Repeatability relative to SD, RSD_r	1.9	2.3	1.3	1.3	1.7	1.4	2.5	2.0
Repeatability value, r ($2.8 \times s_r$)	25.8	21.9	5.4	3.8	2.7	1.2	0.8	17.4
Reproducibility, s_R	48.4	39.3	22.3	12.1	4.7	1.7	1.3	36.3
Reproducibility relative to SD, RSD_R	9.7	11.8	15.9	11.9	8.4	6.0	11.0	11.9
Reproducibility value, R ($2.8 \times s_{R)}$	136	110	62	34	13	4.9	3.7	102

Table 2
The results of a 1999 international collaborative study to determine precision data for the determination of phosphorus in oil at low concentrations.

Samples	1	2	3	4	5
Number of laboratories	8	8	9	8	9
Mean, mg/kg	7.51	3.40	1.60	3.42	14.66
Repeatability					
Sr	0.22	0.05	0.08	0.05	0.96
RSD_r	2.93	1.59	5.07	1.33	6.53
r	0.62	0.15	0.23	0.13	2.68
Reproducibility					
S_R	0.59	0.44	0.29	0.42	0.96
RSD_R	7.86	13.03	18.24	12.22	6.53
R	1.65	1.24	0.81	1.17	2.68

Table 3
Instrument configurations.

Instrument	Grating configuration	Nebulizer
Unicam 7000	Radial	V-groove
Leeman PS1000	Radial	V-groove
Thermo-JA	Axial	Meinhard TR 30-K3
Spectroflame Modula OES	Radial	Cross flow
Spectroflame	Radial	Meinhard
P-E Plasma 400	Axial	High solids
ARL 3410	Radial	Glass concentric
Thermo-JA Iris	Radial	Modified lichte
P-E Optima 3000 DV	Axial	External mount gemcone

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Detection of Cottonseed Oil Halphen Test

DEFINITION

This method detects, qualitatively, the presence of cottonseed oil in vegetable or animal fats or oils.

SCOPE

Kapok oil reacts with an intensity equal to or greater than that of cottonseed oil, and fats from animals that have been fed cottonseed meal may give a positive test.

APPARATUS

1. Test tubes—250 × 25 mm.
2. Oil or saturated brine bath—capable of being regulated at 110–115°C.

REAGENTS

1. Prepare a solution of 1% sulfur in carbon disulfide (see Notes, *Caution*) and then add an equal volume of amyl alcohol.

PROCEDURE

1. Mix 10 mL of liquid test sample in a 250 × 25 mm test tube with an equal volume of the reagent. Shake and heat gently in hot water (70–80°C) for a few minutes, with occasional shaking, until the carbon disulfide is boiled off (see Notes, *Caution*) and the test sample stops foaming.
2. Place the tube in the 110–115°C bath and hold for 1 to 2 hr. A red color at the end of this period indicates the presence of cottonseed oil (see Notes, 1-3).
3. If appreciable quantities of cottonseed oil are present, a positive reaction will be obtained in 1 hr or less in a water bath at or near the boiling temperature. For small amounts, a 2 hr time interval may be necessary at a temperature of 110–115°C.

NOTES

Caution

Carbon disulfide is a poison, being harmful by inhalation, prolonged or repeated skin contact, and ingestion. Chronic poisoning may ensue from repeated exposure to vapor. It is highly flammable, a dangerous fire and explosion risk and can be ignited by friction. Extreme precautions should be taken when using this solvent. A fume hood should be used at all times when handling this solvent.

NUMBERED NOTES

1. The depth of color is to a certain extent proportional to the amount of cottonseed oil present so that by comparing with test portions containing known quantities of cottonseed oil, some idea of the amount can be obtained. However, at best this is only a rough approximation and subject to many doubts.
2. Hydrogenation and heating reduce the intensity of the reaction and may destroy it entirely.
3. Different lots of cottonseed oil may react with different intensities.

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Detection of Sesame Oil Modified Villavecchia Test

DEFINITION

This method detects, qualitatively, the presence of sesame oil in animal and vegetable fats and oils (References, 1).

SCOPE

Applicable to normal animal and vegetable fats and oils.

APPARATUS

1. Test tube—25 × 200 mm, or 25 × 250 mm.

REAGENTS

1. Hydrochloric acid—reagent grade, sp. gr. 1.19 (see Notes, *Caution*).
2. Color forming reagent—prepared by adding 2 mL of reagent-grade furfural to 100 mL of 95% ethyl alcohol (USSD formulas 30 and 3A are permitted).

PROCEDURE

1. Mix 10 mL of the liquid test sample with an equal volume of HCl in a test tube. Add to this mixture 0.1 mL (see Notes, 1) of the reagent. Shake well for 15 sec and allow to stand until the emulsion breaks.
2. Observe the color of the lower layer as soon as possible after the emulsion is broken. If no pink to crimson color appears, the test is negative (see Notes, 2 and 3).
3. If any color is observed in the lower layer, add 10 mL of distilled water, shake again, and observe the color as soon as the layers are separated. If the color persists, sesame oil is present. If the color disappears, sesame oil is not present (see Notes, 4).

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and is a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. The sensitivity of the Villavecchia test to small quantities of sesame oil may be improved by increasing the amount of reagent up to 1 mL. However, doing this hastens the rate of development as well as the amount of noncharacteristic colors that are formed. Therefore, if greater amounts of the reagent are used, relatively greater care must be taken in the observation of the final color.
2. Observe the color as soon as possible so that the pink color, if present, may be observed before it is masked by the development of other noncharacteristic colors.
3. The depth of color is, to a certain extent, proportional to the amount of sesame oil present, so that by comparing with test portions containing a known amount of sesame oil, some idea of the amount present in the test portion can be obtained. However, this is only an approximation.
4. The test is applicable to hydrogenated as well as unhydrogenated sesame oil, although not with the same degree of sensitivity. An AOCS technical committee has found that 0.5%, and sometimes as little as 0.25%, of sesame oil can be detected.

REFERENCES

1. Adopted from *Official Methods of Analysis,* AOAC International, 16th ed., Vol. II, Gaithersburg, MD, 1995, Chapt. 41, p. 38, Method 893.01.

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Detection of Teaseed Oil Fitelson Test (Modified Lieberman-Burchard Test)

DEFINITION

This method determines the presence of teaseed oil in olive oil.

SCOPE

Applicable only to mixtures of olive oil and teaseed oil.

APPARATUS

1. Clear glass tubes—19 mm i.d., 154 mm overall length. The color tubes (AOCS Official Method Cc 13b-45) are very suitable for this purpose. Ordinary test tubes of similar size may be used, provided they are of clear glass and well matched.
2. Pipets—Mohr type, to deliver exactly 0.2, 0.8, and 1.5 mL.
3. Glass tubing—about 4 mm o.d., 2 mm i.d., calibrated to deliver 7 drops of oil equivalent to about 0.22 g.

REAGENTS

1. Chloroform—reagent grade (see Notes, *Caution*).
2. Sulfuric acid—sp gr 1.84, reagent grade (see Notes, *Caution*).
3. Acetic anhydride—reagent grade (see Notes, *Caution*).
4. Diethyl ether—anhydrous, reagent grade (see Notes, *Caution*).

PROCEDURE

1. Place in a tube exactly 0.8 mL of acetic anhydride, 1.5 mL of chloroform, and 0.2 mL of sulfuric acid. Mix and cool in an ice water bath to 5°C.
2. Add 7 drops of the test sample to be tested. If any cloudiness appears, add acetic anhydride, drop by drop, shaking after each addition until the turbidity disappears. Maintain at 5°C for 5 min.
3. Add 10 mL of cold (5°C) diethyl ether, stopper with cork, and mix immediately by inverting the test tube. Replace in ice water bath and observe the color. Pure teaseed oil gives an intense red color within 1 min, which reaches a maximum and then fades away.

   Note—Some olive oils pass through a faint pink color.
4. The depth of the red color produced by mixtures of teaseed and olive oils is closely proportional to the amount of teaseed oil present (see Notes, 1 and 2). An estimate of the amount of teaseed oil may be obtained by comparing the test sample with mixtures of known composition prepared and tested simultaneously in an identical manner.

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature, forming phosgene gas when heated to decomposition temperatures. Can react explosively with aluminum, lithium, magnesium, sodium, potassium, disilane, and sodium hydroxide + methanol. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive splattering. Always add the acid to water, never the reverse.

Acetic anhydride is a strong irritant and is corrosive. It can cause burns and eye damage. Eyes and hands should be protected when working with this compound. Work should be carried out in a properly operating fume hood.

Diethyl ether is extremely flammable and a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. Store protected from the light. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85-48%. The TLV is 400 ppm in air. Can react explosively when in contact with chlorine, ozone, lithium aluminum hydride, or strong oxidizing agents. A fume hood should be used at all times when using diethyl ether. Avoid static electricity.

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NUMBERED NOTES

1. This test will give reliable results with test samples of edible olive oils containing more than 10% teaseed oil, but inedible grades sometimes mask the end point so that results are only approximate. Any color indicating less than 10% teaseed oil is uncertain and speculative, although not necessarily erroneous.
2. The dark color of some olive oils tends to mask the characteristic red color. To overcome this, proceed as follows: Saponify a mixture of 5 g of test sample and 5 g white mineral oil with an excess of alcoholic KOH (or 5 mL of 50% KOH in about 30 mL of alcohol). Brisk boiling for 10–15 min usually suffices. Pour solution into a separatory funnel, add equal volume of distilled water, shake, and allow the layers to separate. Draw off and reject the lower (soap solution) layer. Wash the oily layer several times with distilled water to remove the soap. Dry the test sample by shaking or stirring with an excess of anhydrous $NaSO_4$ and allow to stand for several minutes. Filter and proceed as directed in Procedure, 1-4. Standards should be prepared simultaneously and in an identical manner (containing mineral oil) for comparison.

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Identification of Oils
Crismer Test (Fryer and Weston Modification)

DEFINITION

This test determines the miscibility of the test sample with a standard reagent. The values obtained are characteristic within certain limits for each kind of oil (see Notes, 1).

SCOPE

Applicable to normal animal and vegetable fats and oils.

APPARATUS

1. Thermometer—AOCS Specification H 7-45, –2–80°C.
2. Test tube—13 × 127 mm (5 × 0.5 in.), etched to indicate 2 and 4 mL capacities.
3. Corks—to fit the test tubes with center hole for thermometer and side notch for small stirrer.
4. Stirrer—small glass rod or copper wire with horizontal loop at one end.
5. Beaker—400 mL.

REAGENTS

1. Sweet almond oil—USP grade.
2. Ethyl alcohol—ACS grade.
3. Amyl alcohol—reagent grade.
4. Sodium sulfate—anhydrous, reagent grade.

PROCEDURE

1. Mix equal volumes of the ethyl and amyl alcohol, and determine the temperature at which turbidity appears with almond oil as directed in Procedure, 2-7. Adjust the alcohol reagent, if necessary, by the careful addition of distilled water to the reagent until the mixture, after correction for acidity, becomes turbid at 70°C. This standardized reagent of amyl-ethyl alcohol is used for tests on unknowns.
2. Dry a sufficient quantity of the test sample by shaking or stirring with an excess of anhydrous Na_2SO_4 and allowing to stand for several minutes. Filter and heat a portion of this test sample in a test tube in boiling water for 5 min and then fill one of the calibrated test tubes to the 2 mL mark. Add sufficient standardized amyl-ethyl alcohol reagent to bring to the 4 mL mark.
3. Place the test tube in a 400 mL beaker that is about half-full of water. Insert the cork holding the thermometer and stirrer and adjust the location of the thermometer so that the bulb is covered by the liquid and suspended about equidistant from the sides and bottom.
4. Heat the water and test sample slowly, moving the stirrer up and down, until the mixture is clear. Continue heating until the temperature is about 5°C above the point of clarification.
5. Raise the stirrer from the liquid without removing the cork, discontinue heating, and allow to cool. Observe the temperature at the first point of definite cloudiness.
6. Repeat the determination with fresh test sample and reagent. Duplicate determinations are expected to agree within 0.5°C.
7. Determine free fatty acids in the test sample as directed in AOCS Official Method Ca 5a-40, calculating the acidity as oleic acid. Refer to Table 1 to determine the correction to be made depending upon the free fatty acid content.

CALCULATIONS

1. Crismer value, corrected = turbidity observed (°C) + (% acidity × correction factor)

EXAMPLE

Olive Oil	
Turbidity observed	69.6
% Acidity	0.63
Correction (0.63 × 2.07)	1.28
Crismer value (corrected)	70.9

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NOTES

Caution

Ethyl alcohol is flammable. Use effective fume-removal device when heating or evaporating.

NUMBERED NOTES

1. The corrected Crismer values vary more or less within a narrow range for a given kind of pure oil. The committee has found the range thus determined for olive oil to be from 68.5 to 71.5.

Table 1
Correction for acidity of oils.

Type of oil	Typical oils	Correction factor for each 1% acidity
Marine	Whale	1.95
Drying	Linseed, sunflower, soybean	2.05
Semi-drying	Cottonseed, sesame, corn	2.03
	Rape	1.61
Non-drying	Almond, olive, peanut	2.07
	Coconut, palm kernel	2.01
	Palm	1.72
	Butterfat	1.54
	Lard	2.13

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Aflatoxins M_1 and M_2 in Dairy Products

DEFINITION

This method determines aflatoxins M_1 and M_2 under the conditions of the test (see Notes, 1).

SCOPE

Applicable to liquid milks, powdered milks, and cheeses.

APPARATUS

1. Blender—explosion-proof, Waring Model EP-1 or equivalent, fitted with a 1 liter blending jar and cover, available from Waring Products Division, Route 44, New Hartford, CT 06057.
2. Cleanup columns—Econo-columns, glass, 500 mm × 10 mm i.d. with 35 µm porous polyethylene frit and nylon Luer stopcocks, available from Bio-Rad Laboratories, Richmond, CA. Other equivalent glass columns are suitable.
3. Solvent evaporating equipment—
   (a) Vacuum evaporator, Buchi Rotavapor-R, Brinkman Instruments, Westbury, NY, or equivalent.
   (b) Heating block, Reacti-Therm heating and evaporating module, Pierce Chemical Co, Rockford, IL.
4. Test tube shaker—Vortex Model K-500, Scientific Industries, Springfield, MA, or equivalent.
5. Ultraviolet (UV) viewing cabinet.
6. Vials—glass 1 to 1.5 dram capacity, with Teflon-lined screw caps, American Scientific Products, McGaw Park, IL (vials and caps); A. H. Thomas, Philadelphia, PA (Teflon liners).
7. Densitometer, fluorescence measuring spectodensitometer.
8. Thin layer chromatographic equipment—
   (a) Plate scoring device.
   (b) Glass or stainless steel developing tank with cover.
   (c) Microliter syringes, 10 and 100 µL, Hamilton Model 701 and 710, respectively, or equivalent.
   (d) Commercial pre-coated silica gel plates, 0.25 mm dry thickness, 20 cm × 20 cm, available from Machery Nagel, Merck, Brinkmann Instruments, or equivalent.
9. Filter paper—24 cm, available as S & S No. 588, or other equivalent fast flow, high wet-strength paper.
10. Separatory funnels, 250 mL.
11. Erlenmeyer flasks, 125 mL.

REAGENTS

1. Solvents (see Notes, *Caution*)—Reagent grade acetone, acetonitrile, benzene, chloroform (containing less than 0.75% ethanol), diethyl ether (anhydrous, containing less than 0.01% ethanol), glacial acetic acid, hexane (bp 68–69°C), isopropanol, methanol, and toluene.
2. Salt solution, prepared by dissolving an excess (360–400 g) of reagent grade sodium chloride in 1000 mL of hot, distilled water and allowing to stand overnight to ensure saturation.
3. Silica gel for column cleanup, Merck silica gel No. 60, 0.063–0.200 mm particle size. Stir for one hour in methanol, filter, and treat with chloroform in the same manner. Filter the chloroform treated silica gel, air dry overnight in a shallow pan contained in a properly operating fume hood, heat at 105°C for one hr in a convection oven, add 1% water, place in a tightly closed container, and equilibrate for at least 15 hrs.
4. Washing solvents—
   (a) 100 mL glacial acetic acid + 900 mL toluene.
   (b) 100 mL acetonitrile + 300 mL diethyl ether + 600 mL hexane.
5. Aflatoxin elution solvent—
   (a) Mix 200 mL acetone + 800 mL chloroform.
6. Sodium sulfate, anhydrous and granular.
7. Diatomaceous earth (filter aid), Manville Hyflo Super-Cel or equivalent.
8. Aflatoxin M_1-M_2 standard solution (see Notes, *Caution* and Numbered Notes, 2). Dissolve pure, crystalline aflatoxins M_1 and M_2 in separate glass containers with warm acetonitrile to prepare concentrated stock solutions containing 100 to 200 µg/mL of aflatoxins. Pure commercial solutions or films may be substituted. Determine actual concentration of each stock solution spectrophotometrically. Prepare working standard solution to contain 0.50 µg of aflatoxin M_1/mL and 0.10 µg of aflatoxin M_2/mL in acetonitrile/benzene, 1:9, v/v, for either visual or densitometric analysis. Store all standard solutions in 1 dram vials with Teflon™ lined screwcaps at ≤ 0°C to minimize solvent evaporation.
9. Solvent mixtures for thin layer chromatography (TLC)—
   (a) Mix 900 mL benzene + 100 mL acetonitrile.
   (b) Mix 30 mL isopropanol + 100 mL acetone + 870 mL chloroform.

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(c) Mix 10 mL water + 40 mL methanol + 950 mL diethyl ether.

PREPARATION OF TEST SAMPLE

1. Fluid milk—Allow to reach ambient temperature before use.
2. Powdered milk—Dissolve powder in 10-fold volume of water to reconstitute the milk (eg., 5 g of powder in 50 mL of water). Stir occasionally and allow to sit 8 hr to ensure complete solution.
3. Cheese—Cut into small slices or cubes.

PROCEDURE

Note—Due to the toxic nature of the solvents used in the test sample extraction and column cleanup steps, these operations should be carried out in a properly operating fume hood.

1. Milk Extraction—
   (a) Shake 50 mL of fluid milk (Preparation of Test Sample, 1) or reconstituted powdered milk (Preparation of Test Sample, 2), 10 mL salt solution (Reagents, 2), and 120 mL chloroform in a 250 mL separatory funnel for 60 seconds.
   (b) Allow the layers to separate and drain chloroform (bottom layer) into a 125 mL Erlenmeyer flask containing about 10 g of sodium sulfate (Reagents, 6).
   (c) Stir mixture occasionally for 3 min and filter the chloroform layer through filter paper (Apparatus, 9) into a 100 mL graduated cylinder. Record the volume and save extract for column cleanup step.
2. Cheese Extraction—
   (a) Blend 15 g of cheese (Preparation of Test Sample, 3) with 1 mL salt solution (Reagents, 2), 5 g diatomaceous earth Reagents, 7), and 100 mL chloroform for 60 seconds.
   (b) Filter mixture through filter paper (Apparatus, 9) in a 125 mL Erlenmeyer flask containing 10 g of sodium sulfate (Reagents, 6).
   (c) Close the top of the filter paper and compress the entire filter to expel the maximum amount of filtrate.
   (d) Stir the cheese extract occasionally for 3 minutes and filter the chloroform extract through filter paper (Apparatus, 9) into a 100 mL graduated cylinder. Record the volume and save extract for column cleanup step.
3. Chromatography, Column Preparation—
   (a) Equip cleanup column (Apparatus, 2) with fritted disk or glass wool pad to provide a base for the silica gel.
   (b) Pour column half full with chloroform and add 2.0 g silica gel (Reagents, 3) to column. Add additional 3–4 mL chloroform and slurry silica with stainless steel rod.
   (c) Drain chloroform to settle silica gel, but do not allow chloroform to drain past the top of the silica gel. Rinse the sides of the column with chloroform.
   (d) Retain about 3 mL chloroform on top of the silica gel and add 2 g sodium sulfate (Reagents, 6) to cap the column.
   (e) Rinse column sides with chloroform and drain chloroform until solvent level is about 1 cm above the top of the sodium sulfate.
4. Chromatography, Test Sample Cleanup—
   (a) Add dairy product extract (Procedure 1, c or 2, d) in small volumes to the column and drain by gravity until the entire test sample extract has been added.
   (b) If the flow rate slows, carefully stir the sodium sulfate layer with stainless steel rod. Rinse graduated cylinder and the sides of the column with chloroform and drain through the column to the top of the sodium sulfate.
   (c) Wash column with 25 mL of the acetic acidtoluene solution (Reagents, 4, a) and drain to the top of the column.
   (d) Wash column with 25 mL hexane and drain to the top of the column.
   (e) Wash column with 25 mL acetonitrile-diethyl ether-hexane solution (Reagents, 4, b) and drain to the top of the column.
   (f) Discard all column eluates.
   (g) Elute the aflatoxin with 60 mL acetone-chloroform solution (Reagents, 5, a).
   (h) Evaporate the acetone-chloroform eluate to *near* dryness in vacuo or on a steam bath and transfer residue quantitatively to a 1 or 1.5 dram vial (Apparatus, 6), using chloroform rinses to effect the transfer.
   (i) Evaporate chloroform rinses to dryness in a heating block (Appartus, 3, b) at 50°C under a gentle stream of nitrogen. Cap the vial and reserve for thin layer chromatography.

THIN LAYER CHROMATOGRAPHY (TLC)

1. Test Sample Preparation—Add 100 µL benzene-acetonitrile (Reagents, 9, a) to dry residue in vial (Procedure, 4, i). Cap vial and shake vigorously on a Vortex mixer for one minute. Test sample volume is sufficient for visual or densitometric analysis, but not for both. After TLC, store remaining test sample extract in cold (< 0°C) for confirmation of identity.
2. Milk Samples, One-dimensional TLC, Visual Analysis—
   (a) Score the 20 cm × 20 cm TLC plate (Apparatus, 8) vertically into 1 cm wide strips. Mark the origin 1.5 cm from the bottom edge of the plate. Spot two 20 µL aliquots of test sample extract (prepared as noted in TLC, Test Sample Preparation, 1) and 2 µL, 4 µL, 6 µL, 8 µL, 10 µL aliquots of the M_1–M_2 standard solution (Reagents, 8). A second 10 µL aliquot of

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the standard solution is spotted on top of one of the 20 µL test sample aliquots as an internal standard. Deliver aliquots in small increments to obtain small origin spots.

(b) Develop the TLC plate in an unlined, unequilibrated stainless steel tank (Apparatus, 8), using isopropanol-acetone-chloroform solvent (Reagents, 9, b). After the solvent front has migrated a distance of no more than 10 cm from the origin, remove the plate from the tank and air dry in a fume hood *in the dark.*

(c) After the plate has dried, examine under longwave UV light (Apparatus, 5). Compare chromatogram of test sample with those of standards for the presence or absence of a single bluish fluorescent spot with R_f and fluorescence pattern similar to those in the standards.

(d) If aflatoxin M_1 (and M_2) is judged to be present in the test portion aliquot without the internal standard, match fluorescence with one of the standard spots; interpolate between standards if necessary. If M_1 in the test portion spot is more intense than the 10 µL standard spot, dilute and repeat the TLC procedure, steps a through c.

(e) Calculate the concentration of aflatoxin M_1 (and M_2) from the formula—

$$\text{aflatoxin } M_1 \text{ [µg/L or µg/kg (ppb)]} = \frac{(S)(Y)(V)}{(X)(M)}$$

Where—

S = mL M1 standard equal to test portion
Y = concentration (mg/mL) aflatoxin M_1 standard
V = mL of final dilution of test portion extract for TLC
X = mL test portion extract equal to standard
M = mass or volume of milk product represented by final extract for TLC, i.e.,

$$\frac{\text{(volume or mass test portion)(filtrate volume)}}{\text{120 (for milk) or 100 (for cheese)}}$$

3. Milk Samples, Densitometric Analysis—

(a) Prepare TLC plate as described in Thin Layer Chromatography, 2, a.

(b) Spot three 20 µL test portion extract aliquots (Thin Layer Chromatography, 1) and 5 µL, 5 µL, 10 µL, and 10 µL of aflatoxin M_1-M_2 standard with one of the 10 µL aliquots spotted on one of the 20 µL test portion aliquots. Deliver aliquots in small increments to ensure small origin spots.

(c) Develop and examine the plate as described under Thin Layer Chromatography 2, a through c.

(d) If aflatoxin M_1 is judged to be present, scan the plate according to the manufacturer's recommended procedure. Excitation = 365 nm; Emission = 420 nm.

(e) Calculate the concentration of aflatoxin M_1 (and M_2) from the formula:

$$\text{aflatoxin M1 [µg/L or µg/kg (ppb)]} = \frac{(A_x)(C_s)(V_s)(D)}{(A_s)(V_x)(M)}$$

Where—

A_x = average area count for M_1 test portion spot
C_s = M_1 concentration of standard, µg/mL
V_s = µL of standard spotted
D = test portion dilution, µL
A_s = average area count for M standard spot
V_x = µL of test portion spotted
M = mass or volume of milk product represented by final extract for TLC, i.e.,

$$\frac{\text{(volume or mass test portion)(filtrate volume)}}{\text{120 (for milk) or 100 (for cheese)}}$$

4. Cheese Samples, Two-dimensional TCL—

(a) Score 20 cm × 20 cm (or 10 cm × 10 cm) TLC plate (Apparatus, 8, d).

(b) Spot 20 µL aliquot of test portion extract (Thin Layer Chromatography, 1) on origin (A in Figure 1) and either 6 µL, 2 µL, 4 µL, and 6 µL of M_1 standard for visual determination or 10 µL, 5 µL, 5 µL and 10 µL of M_1 standard for densitometric determination on B, C, D and E (see Figure 1), respectively.

(c) Develop plate in water-methanol-diethyl ether solvent (Reagents, 9, c) in Direction 1 (Figure 1). Remove the plate when the solvent front reaches the scored line, air dry 2 minutes in a fume hood, heat in forced air oven at 50°C for 2 minutes, and allow plate to cool.

(d) Develop plate in isopropanol-acetone-chloroform (Reagents, 9, b) in Direction 2 (Figure 1).

(e) Remove plate when solvent front reaches the scored line and air dry.

(f) Examine and quantitate visually (Thin Layer Chromatography, 2, c) or densitometrically (Thin Layer Chromatography, 3, d).

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PRECISION

	M_1, Milk, %CV	M_1, Cheese, %CV
Within lab	21.6–26.4	19.2–35.4
Between labs	29.7–36.2	37.8–51.7

J. Assoc. Off. Anal. Chem. 63: 907–921 (1980), page 918, Table II.

NOTES

Caution

Mycotoxins—Mycotoxins should be handled with extreme care because they are highly toxic substances. Perform manipulations under a properly operating fume hood. Take particular precautions, such as the use of a glove box, when toxins are in dry form, because of their electrostatic nature and resulting tendency to disperse in working areas. Swab accidental spills of toxin with 5% NaOCl bleach. Rinse all glassware exposed to toxins with 1% NaOCl bleach solution and then wash thoroughly with warm water. See *J. Assoc. Off. Anal. Chem. 48:* 681 (1965) for recommended decontamination procedures for aflatoxins.

Solvents—The solvents used in this method are flammable (acetone, acetonitrile, benzene, diethyl ether, hexane, isopropanol, methanol, and toluene); toxic (acetone, acetonitrile, glacial acetic acid, hexane, isopropanol, methanol, and toluene); carcinogenic (benzene, chloroform). A properly operating fume hood should be used at all times when using these solvents. Avoid open flame and static electricity when using flammable solvents. Consult the Laboratory Safety section of this book for additional details on the nature of these solvents.

NUMBERED NOTES

1. This method paraphrases AOAC Method 26.A10–26.A14 of the Thirteenth Edition of the *Official Methods of Analysis* published by the Association of Official Analytical Chemists.
2. Criteria for purity of aflatoxin primary standards and determination of concentration of diluted working standards for TLC are given in Method 26.006–26.013 (same reference as Note 1). Aflatoxin M_1 standards are available from Eureka Laboratories, Inc., 215 N. 25th Street, Sacramento, CA 95816; Calbiochem, 10933 N. Torrey Pines, LaJolla, CA 92037; and Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.

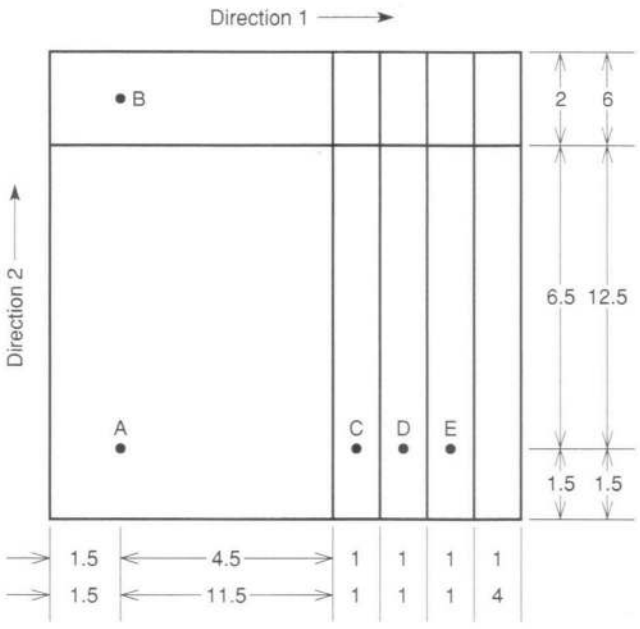

Figure 1. Plate scoring technique for two-dimensional TLC of cheese extract.

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Melting Point Capillary-Tube Method

DEFINITION

The natural fats and oils, that is, those of animal and vegetable origin, are mixtures of glycerides and a number of other minor components (pigments, sterols, tocopherols, etc.). They do not exhibit either a definite or a sharp melting point. Therefore, the term "melting point" does not imply the same characteristics that it does with pure substances of a definite crystalline nature. Fats pass through a stage of gradual softening before they become totally liquid. The melting point then must be defined by the specific conditions of the method by which it is determined and, in this case, it is the temperature at which the test sample becomes completely clear and liquid.

SCOPE

Applicable to all normal animal and vegetable fats.

APPARATUS

1. Melting-point tubes—capillary glass tubing, i.d. 1 mm, o.d. 2 mm maximum, convenient length 50–80 mm. Available from Kontes, Vineland, NJ, USA.
2. Thermometer—range –2–68°C or –2–80°C (AOCS Specification H 6-40 or H 7-45, respectively), which should be calibrated against a National Institute of Testing traceable thermometer.
3. Glass beaker—600 mL.
4. Heat source—electric hot plate with rheostat control (see Notes, 1).

PROCEDURE

1. Melt the test sample, and filter through filter paper to remove any impurities and the last traces of moisture. It is essential that the test portion sample be absolutely dry.
2. Dip at least three clean capillary tubes in the completely liquid test portion so that the test portion rises about 10 mm high in the tubes. Fuse the end of the tube (where the test portion is located) in a small flame, taking care not to burn the fat.
3. Place the tube in a beaker and hold in a refrigerator (see Notes, 2) at 4–10°C for 16 hr (may be done overnight).
4. Remove the tubes from the refrigerator, and attach with a rubber band or by any suitable means to the thermometer so that the lower ends of the melting point tubes are even with the bottom of the mercury bulb of the thermometer.
5. Suspend the thermometer in a 600 mL beaker that is about half full of clear distilled water. The bottom of the thermometer is immersed in the water to the immersion mark.
6. Adjust the starting bath temperature to 8–10°C below the melting point of the test portion at the beginning of the test. Agitate the water bath with a small stream of air or other suitable means, and apply gentle heat to increase the bath temperature at the rate of 0.5°C/min.
7. Fats usually pass through an opalescent stage before melting completely. The heating is continued until the tubes are completely clear throughout. Observe the temperature at which each tube becomes clear and calculate the average of all tubes. Results should agree within 0.5°C. Report this average as the melting point.

NOTES

1. A water bath with thermostat may be used for closer temperature control.
2. The test portion must be completely liquid when the tubes are placed in the refrigerator. It is good practice to pass the ends of the tubes containing the test portion momentarily through the flame just before they are taken to the refrigerator.

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Slip Melting Point
AOCS Standard Open Tube Melting Point

DEFINITION

The slip point is an index of the temperature at which fat softens and becomes sufficiently fluid to slip in an open capillary tube.

SCOPE

This method is used in the characterization of palm oil in international trade. This method is applicable to palm oil and hard fats, including palm kernel oil, coconut oil, stearin, hydrogenated fats, and hard tallows. It is less satisfactory for lard, soft tallows, and animal fats. It is unsatisfactory for lard compounds, mixtures of hard and soft fats, and emulsions.

APPARATUS

1. Melting point tubes—capillary glass tubing, 1 mm i.d., 2 mm o.d. maximum, convenient length 50–80 mm. Available from Kontes, Vineland, NJ, USA.
2. Thermometer—range –2–68°C or –2–80°C (AOCS Specification H 6-40 or H 7-45, respectively), which should be calibrated against a National Institute of Standard & Testing (NIST)-traceable thermometer.
3. Glass beaker—600 mL.
4. Heat source—electric hot plate with rheostat control (see Notes, 1).

PROCEDURE

1. Melt the test sample, and filter through filter paper to remove any impurities and the last traces of moisture. It is essential that the test portion be absolutely dry.
2. Dip at least 3 *clean* capillary tubes in the completely liquid test portion so that the test portion rises about 10 mm high in the tubes. Chill the test portion at once by holding the ends of the tubes that contain the test portion against a piece of ice until the fat solidifies.
3. Place the tubes in a beaker and hold in a refrigerator (see Notes, 2) at 4–10°C (40–50°F) for 16 hr (may be done overnight) (see Notes, 3).
4. Remove the tubes from the refrigerator and attach with a rubber band, or by any suitable means, to the thermometer so that the lower ends of the melting point tubes are even with the bottom of the mercury bulb of the thermometer.
5. Suspend the thermometer in a 600 mL beaker of clear distilled water. The bottom of the thermometer is immersed in the water to the immersion mark.
6. Adjust the starting bath temperature to 8–10°C below the slip point of the test portion. Agitate the water bath with a small stream of air, or other suitable means, and apply heat so as to increase the bath temperature at the rate of 1°C/min, slowing the rate to 0.5°C/min as the slip point is approached.
7. Continue heating until the fat column rises in each tube. Observe the temperature at which each column rises, and calculate the average temperature of all tubes. Report this as the slip point.

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PRECISION

Table 1
The interlaboratory study completed by PORIM in 1980, in which 13 laboratories participated, each obtaining two test results for each individual test portion analyzed, gave the statistical results summarized in the following table.[a]

	Test Samples[b]		
Parameter	A	B	C
Mean slip point, °C	35.3	20.2	52.2
S_r, repeatability	0.30	0.17	0.20
C_V of repeatability, %	0.8	0.8	0.4
Repeatability value, $r = 2.8 \times S_r$	0.8	0.5	0.5
S_R, reproducibility	0.87	0.68	0.59
C_V of reproducibility, %	2.5	3.4	1.1
Reproducibility value, $R = 2.8 \times S_R$	2.4	1.9	1.6

[a] References, 1.
[b] Test sample key: A. palm oil (two duplicate test samples); B. palm olein (two duplicate test samples); C. palm stearin (two duplicate test samples).

NOTES

1. A water bath with thermostat may be used for closer temperature control.
2. Palm oil and palm oil products should be tempered at 10 ± 1°C for 16 hr.
3. For a rapid method for quality control purposes, the 16 hr may be reduced to 2 hr or 1 hr. The result shall be reported as "slip point, 2 hours" or "slip point, 1 hour." For most fats, the slip point at 2 hr is about 0.2°C lower than the standard slip point at 16 hr. For palm olein the difference is greater, about 1.2°C.

REFERENCES

1. *J. Am. Oil Chem. Soc. 59*(5):244 (1982).

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Slip Melting Point ISO Standard

DEFINITION

The slip melting point in open capillary tubes is defined as the temperature at which a column of fat in an open capillary tube begins to rise under the conditions specified in this method. The method requires the immersion of a capillary tube, containing a column of the fat which has been crystallized under controlled conditions, to a specified depth in water, the temperature of which is increased at a specified rate. The temperature at which the column fat is observed to start rising in the capillary tube is recorded as the slip melting point.

SCOPE

This method, based on the International Standard ISO 6321 (References, 1), specifies two methods for the determination of the melting point in open capillary tubes, commonly known as the slip point, of animal and vegetable fats and oils. This method is used in the international trading of oils and fats (see Notes, 1).

Method A is only applicable to animal and vegetable fats and oils that are solid at ambient temperature and that do not exhibit pronounced polymorphism.

Method B is applicable to all animal and vegetable fats and oils that are solid at ambient temperature. Method B should be used for fats whose polymorphic behavior is unknown (see Notes, 2 and 3).

APPARATUS

Usual laboratory apparatus and, in particular, the following:

1. Capillary tubes—having uniform walls and that are open at both ends, of internal diameter 1.0–1.2 mm, external diameter 1.3–1.6 mm, wall thickness 0.15–0.2 mm, length 50–60 mm. Check the dimensions of the capillary tubes using a test gauge such as that shown in Figure 1.

   Before use, clean the tubes thoroughly by washing successively with a mixture of chromic acid, water and acetone, and then dry them in an oven. It is recommended that new tubes be used.
2. Thermometer—graduated in divisions of 0.1°C, calibrated vs. a standard thermometer over the range of expected melting points.
3. Stirrer—electrical.
4. Cooling bath—filled with brine or other nonfreezing liquid, thermostatically maintained at a temperature of −10 to −12°C, or filled with a mixture of flaked ice and salt (in the proportions 2 to 1 by mass) at a temperature of −10 to −12°C.
5. Heating apparatus—consisting of the following elements:
   (a) Water jacket—made of glass, provided with inlet and outlet tubes, and having the shape and dimensions shown in Figure 2.
   (b) Water heater—capable of delivering a slow stream of water, the temperature of which can be controlled to increase at a rate of between 0.5 and 4°C/min through the water jacket described in Apparatus, 5, (a).
   Note—An example of a suitable heating apparatus is shown in Figure 3 (see Notes, 4).

SAMPLING

1. Carry out sampling in accordance with ISO 5555 (References, 2). The specifications are given in the following summary:
   (a) All sampling operations must be performed by an operator with clean hands or wearing gloves, using clean and dry sampling apparatus and containers, flushed with test sample to remove all traces of the previous sample.
   (b) After removing all extraneous material from the outside of sampling instruments, heat fats in bulk storage at a rate not to exceed 5°C per day, using heating coils with a large area in relation to the volume of fat, with the temperature of the coils kept as low as possible to avoid local overheating [steam at a maximum pressure of 150 kPa (1.5 bar), gauge reading 128°C; or hot water only if the coils are self-draining]. Avoid contamination of the fat by steam or water. Avoid heating the fat more than 10°C over its melting point.

PREPARATION OF TEST SAMPLE

1. Prepare the test sample in accordance with ISO 661 (References, 3). The specifications are given in the following summary. The laboratory test sample must be prepared in the following manner—
   (a) Liquid sample, clear and without sediment—Render the laboratory sample as homogeneous as possible by shaking the closed container.

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(b) Liquid sample, turbid or with sediment, for the determination of moisture and volatile matter, insoluble impurities, mass per unit volume and/or any other determination requiring the use of unfiltered samples, or if the determination is affected by heat—Shake the container vigorously (holding the laboratory sample) until the sediment is completely separated from the walls of the container. Immediately pour the sample into another container, and check that no sediment remains adhering to the walls of the original container; if sediment remains, remove it completely (if necessary, cutting open the container), and incorporate in the body of the sample.

(c) Liquid sample for all other determinations—Place the container holding the laboratory sample in a drying oven controlled at 50°C, leave it until the sample has reached 50°C and then render the sample as homogeneous as possible by shaking the closed container. If, after heating and mixing, the sample is not completely clear, filter the oil, carrying out the filtration inside the oven at 50°C or by means of a heated filter funnel. Do not leave the sample in the oven longer than necessary, in order to avoid any modification of the fatty matter by oxidation or polymerization. The filtrate must be perfectly clear.

(d) Solid test sample for the determination of moisture and volatile matter, insoluble impurities, mass per unit volume and/or any other determination requiring the use of unfiltered samples, or if the determination is affected by heat—Gently warm the test sample until it is just mixable, and mix thoroughly in order to render it as homogeneous as possible.

(e) Solid test sample for all other determinations—Melt the laboratory sample by keeping it in the drying oven, controlled at a temperature not more than 10°C above the melting temperature of the particular fat or oil. If, after heating, the test sample is perfectly clear, render the test sample as homogeneous as possible by shaking the closed container. If it is turbid or contains a sediment, filter it at the chosen temperature, either inside the oven or by means of a heated filter funnel. The filtrate must be perfectly clear.

(f) Drying—If the mixed test sample still contains moisture (especially in the case of acid oils, fatty acids and solid fats), it should be dried for those determinations in which the results may be affected by the presence of moisture (e.g., iodine value), taking all necessary precautions to avoid its oxidation. For this purpose, keep part of the thoroughly mixed test sample [from steps (a), (b), (c), (d) or (e), as appropriate] in the drying oven for as short a period of time as possible, at a temperature not more than 10°C above the melting point, preferably under nitrogen, after having added anhydrous sodium sulfate in proportion of 1–2 g per 10 g of oil or fat. Never dry at a temperature in excess of 50°C.

Vigorously stir the heated test sample together with the anhydrous sodium sulfate, then filter. If the fat or oil solidifies on cooling, carry out the filtration in the drying oven or by means of a heated filter funnel at an appropriate temperature which should not exceed 50°C.

(g) Storage—The laboratory sample to be stored shall be placed in an inert and airtight container, which is sealed securely, stored under refrigeration (at a maximum of 10°C) and protected from light. Laboratory samples may be stored for three months.

PROCEDURE, METHOD A

1. Preparation of the capillary tubes for Method A—
   (a) Melt a portion of the test sample as rapidly as possible to at least 5°C, but not more than 10°C, above the temperature at which it is completely melted.
   (b) Dip two capillary tubes (Apparatus, 1) into the melted test sample until columns of fat 10 ± 2 mm long are obtained. Immediately after filling the tubes, wipe them quickly with absorbent tissue to remove any fat adhering to the outer surfaces of the tubes. Immediately place (for a few sec) the filled capillary tubes against a beaker filled with ice so that the fat solidifies.
   (c) Place the tubes in the cooling bath (Apparatus, 4) for 5 min.
   (d) Continue as directed in Determination section.

PROCEDURE, METHOD B

1. Preparation of the capillary tubes for Method B—
   (a) Melt a portion of the test sample as rapidly as possible to at least 5°C, but not more than 10°C, above the temperature at which it is completely melted.
   (b) Cool the melted test sample, with occasional stirring, until its temperature is 32–34°C. Then, stir continuously with the stirrer (Apparatus, 3), allowing the fat to cool until the first signs of cloudiness appear.
   (c) Continue stirring by hand until the fat has a pasty consistency, and then transfer the fat to a 100 mL beaker at 17 ± 2°C.
   (d) Store the fat at this temperature for a minimum of 24 hr.
   (e) Push four capillary tubes (Apparatus, 1) into the conditioned fat until a plug of fat 10 ± 2 mm long is obtained in the tube. Wipe the tubes quickly with absorbent tissue to remove any fat adhering to the outer surfaces of the tubes. Store the tubes at 17 ± 2°C until required.
   (f) Continue as directed in Determination section.

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DETERMINATION

1. Avoiding transfer of body heat to the fat, attach two capillary tubes prepared for Method A or for Method B to the thermometer (Apparatus, 2) using small rubber bands (or by any other suitable means, e.g., a rubber ring) so that the columns of fat are located at the lower ends of the tubes and lie adjacent to the bulb of the thermometer (see Fig. 2).
2. Fill the water jacket [Apparatus, 5, (a)] and the water heater [Apparatus, 5, (b)] with previously boiled water cooled to 15°C. Clamp or suspend the thermometer and capillary tubes centrally in the water jacket so that the lower ends of the capillary tubes are 30 mm below the surface of the water (see Fig. 2).
3. Operate the heating apparatus (Apparatus, 5) so that a slow stream of water passes through the water jacket, regulating the heating so that the rise in temperature of the water, as measured by the thermometer in the water jacket, is about 3–4°C/min for Method A and 1°C/min for Method B.
4. For each of the two capillary tubes, note the temperature value indicated by the thermometer immediately as the fat starts to rise in the tubes.
5. For Method A, note the arithmetic mean of the two readings obtained, and take this arithmetic mean as the result of one determination.
6. For Method B, repeat the operations described in Determination, 1-3, using the remaining two capillary tubes [Procedure, Method B, 1, (e)], decreasing the rate of temperature rise to 0.5°C/min when the water temperature is within 5°C of the mean reading obtained in Determination, 5. For each of the two capillary tubes, note the temperature value indicated by the thermometer immediately as the fat starts to rise in the tubes. Record the arithmetic mean of the two readings obtained and take this as the result of one determination.

CALCULATIONS

1. Carry out two determinations on the same test sample, i.e., obtain two mean readings for Method A and two final mean readings for Method B.
2. Take as the result the arithmetic mean of the two determinations.
3. Express the result to the nearest 0.1°C.

PRECISION

1. Two interlaboratory tests, carried out at the international level in 1982 and 1986 by ISO/TC 34/SC 11, in which 20 laboratories [each of which carried out three determinations on each test portion (columns 2, 3 and 8 in Table 1)] and 15 laboratories [each of which carried out three determinations on each test portion (columns 4–7 in Table 1)] participated, gave the statistical results (evaluated in accordance with ISO 5725, References, 4) shown in Table 1.
2. Repeatability—The difference between the values of two determinations, carried out in rapid succession (or simultaneously) by the same operator using the same apparatus on the same test sample, must not exceed 0.5°C for Method A and 1.0°C for Method B.

TEST REPORT

1. The test report must specify the method used and the result obtained. It must also state all operating details not specified in this method, or regarded as optional, together with details of any incidents which may have influenced the result.
2. The test report must include all information necessary for the complete identification of the test sample.

NUMBERED NOTES

1. The Federation of Oils, Seeds and Fats Associations (FOSFA) International specifies this method in contracts on the international trading of oils and fats.
2. When applied to fats with pronounced polymorphism, Method A will give different and less satisfactory results than Method B.
3. Fats that exhibit pronounced polymorphism are principally cocoa butter and fats containing appreciable quantities of 2-unsaturated, 1,3-saturated triacylglycerol.
4. Other types of heating apparatus, such as a water bath with magnetic stirrer, capable of being controlled to produce the specified temperature rise, may also be used.

REFERENCES

1. Adopted from Animal and vegetable fats and oils—Determination of melting point in open capillary tubes (slip point), International Standards Organization, 1991, ISO 6321.
2. Animal and vegetable fats and oils—Sampling, International Standards Organization, 1983, ISO 5555.
3. Animal and vegetable fats and oils—Preparation of test sample, International Standards Organization, 1980, ISO 661.
4. Precision of test methods—Determination of repeatability and reproducibility for a standard test method by interlaboratory tests, International Standards Organization, 1986, ISO 5725.
5. The determination of melting point in open capillary tubes (slip point) for palm oil products. 1989, MS 817.
6. AOCS Official Method Cc 3-25, Slip melting point AOCS standard open tube melting point.

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Table 1
Statistical results of interlaboratory tests.

	Method A		Method B				
	PKO	HSBO	CB	PO	HCO	HPO	HPO
Laboratories after outliers	18	18	14	14	8		
Mean, °C	27.6	35.4	31.4	36.3	37.1	45.5	47.5
SD of repeatability, s_r	0.15	0.14	0.29	0.35	0.30	0.13	0.15
Coefficient of variation of repeatability, %	0.5	0.4	0.9	1.0	0.8	0.3	0.3
Repeatability, $2.8 \times s_r$	0.4	0.4	0.8	1.0	0.8	0.4	0.4
SD of reproducibility, s_R	0.31	0.75	2.0	2.5	0.9	0.5	0.77
Coefficient of variation of reproducibility, %	1.1	2.1	6.4	6.9	2.5	1.1	1.7
Reproducibility, $2.8 \times s_R$	0.9	2.1	5.7	7.1	2.6	1.4	2.2

PKO = palm kernel oil
HSBO = hydrogenated soybean oil
CB = cocoa butter
PO = palm oil
HCO = hydrogenated coconut oil
HPO = hydrogenated palm oil

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Table 2
Comparison of methods using palm oil test samples.

		Slip point	ISO 6321	
Test Samples		MS 817,[a] AOCS Cc 3-25[b]	Method A	Method B
Palm oil, RBD[c]	1	36.8	38.2	36.5
	2	35.3	37.4	35.5
	3	35.2	37.7	35.5
	4	36.6	38.0	36.5
	5	35.6	37.5	35.5
Palmolein, RBD	1	22.3	24.4	25.5
	2	22.2	24.4	25.5
	3	22.5	24.3	25.5
	4	22.5	24.2	24.9
	5	22.3	24.2	24.9
Palm stearin, RBD	1	51.6	51.8	51.5
	2	52.8	51.8	52.8
	3	46.0	44.8	45.0
	4	52.3	52.8	45.3
	5	51.0	51.0	51.5
Crude palm oil	1	35.8	35.6	—
	2	35.3	36.6	—
	3	35.8	36.4	—
	4	35.0	35.8	—
	5	35.8	36.8	—
Crude palm kernel oil	1	27.8	27.7	27.6
	2	26.6	27.8	27.6
	3	26.7	26.7	27.0
	4	26.8	26.7	27.0
	5	27.0	27.5	27.4
Palm kernel oil, RBD	1	27.8	27.8	28.2
	2	27.8	27.6	27.6
	3	27.7	27.5	28.0
	4	27.8	27.2	28.0
	5	27.6	27.3	27.8
Palm kernel olein, RBD	1	26.2	25.8	26.0
	2	23.4	23.3	23.8
	3	23.5	23.4	23.8
	4	23.4	23.4	23.8
	5	24.6	24.4	24.5
Palm kernel stearin, RBD	1	32.2	32.2	33.0
	2	32.2	32.8	33.0
	3	39.3	38.5	39.4
	4	33.3	33.0	33.2
	5	32.3	33.6	33.2

[a] Malaysian Standard MS 817 [5].
[b] AOCS Official Method Cc 3-25 [6].
[c] RBD: refined, bleached, deodorized.

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Table 3
Statistical results for palm oil test samples

	Palm oil	Palm olein	Palm stearin
No. of laboratories retained after eliminating outliers	10	11	11
Mean (°C)	37.4	20.5	52.1
Standard deviation of repeatability, s_r (°C)	0.23	0.15	0.09
Coefficient of variation of repeatability (%)	0.6	0.7	0.2
Repeatability limit, r ($2.8 \times s_r$) (°C)	0.64	0.42	0.25
Standard deviation of reproducibility, s_R (°C)	0.78	0.98	0.54
Coefficient of variation of reproducibility (%)	2.1	4.8	1.0
Reproducibility limit, R ($2.8 \times s_R$) (°C)	2.2	2.7	1.5

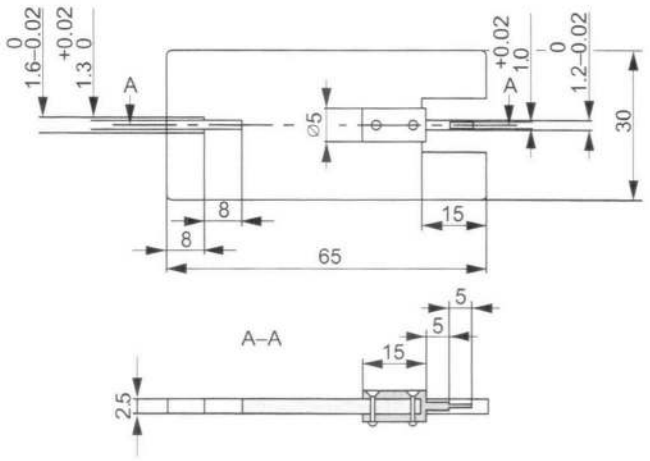

Figure 1. Test gauge for capillary tubes. Dimensions in millimeters.

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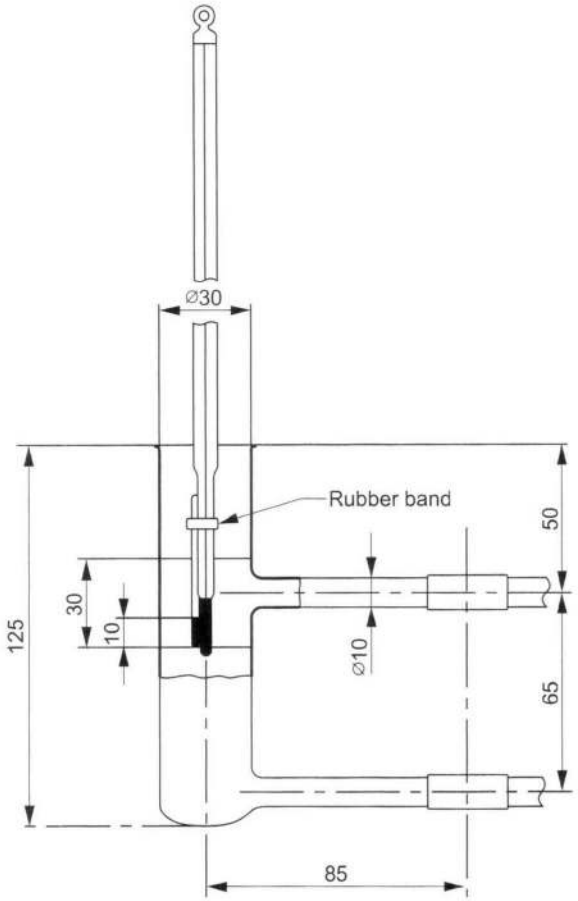

Figure 2. Water jacket. Dimensions in millimeters.

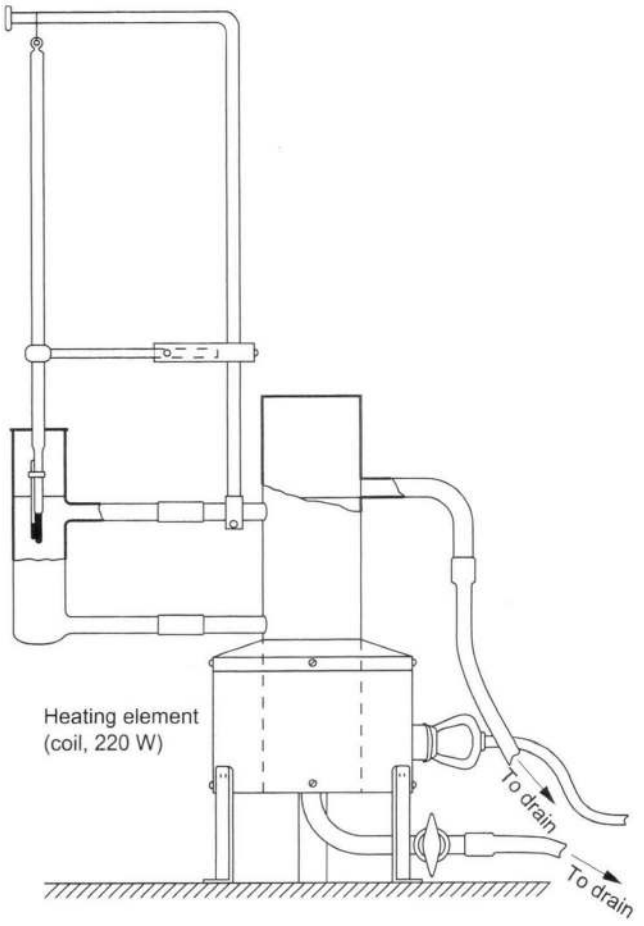

Figure 3. Example of heating apparatus (heating by natural convection).

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Cloud Point Test

DEFINITION

The cloud point is that temperature at which, under the conditions of this test, a cloud is induced in the test sample caused by the first stage of crystallization.

SCOPE

Applicable to all normal animal and vegetable fats.

APPARATUS

1. Oil sample bottle—115 mL (4 oz) or 20–30 mm i.d. suitable test tube.
2. Thermometer—range −2-68°C, AOCS Specification H 6-40.
3. Water bath—made up of chipped ice and water; or chipped ice, salt and water, depending on the temperature required. The temperature of the cloud point bath shall not be less than 2°C, nor more than 5°C below the cloud point. Either a beaker or insulated container is convenient for the test.

PROCEDURE

1. The test sample must be completely dry before making the test. If the test portion contains traces of moisture, it should be filtered through suitable filter paper. Heat 60–75 g of the test portion to 10–15°C above the sample melting point (see Notes, 1) just before making the test. Pour 45 mL of the heated fat into the oil sample bottle.
2. Begin to cool the bottle and contents in the water bath, stirring enough to keep the temperature uniform. When the test portion has reache d a temperature about 10°C above the cloud point, begin stirring steadily and rapidly in a circular motion so as to prevent supercooling and solidification of fat crystals on the sides or bottom of the bottle.
3. From this point on, do not remove the thermometer from the test portion; doing so may introduce air bubbles, which will interfere with the test. The test bottle is maintained in such a position that the upper level of the test portion in the bottle is level with the water in the bath.
4. Remove the bottle from the bath and inspect regularly. The cloud point is that temperature at which the graduation marks on the thermometer immersed in the oil are no longer visible when viewed horizontally through the bottle and the test portion.

NOTES

1. It is essential that the test portion be heated to 10–15°C above the sample melting point to destroy any crystal nuclei. Some samples may need to be seeded with small crystals just prior to the expended cloud point to avoid supercooling.

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Refractive Index

DEFINITIONS

The refractive index of a substance is the ratio of the speed of light in a vacuum to the speed of light in the substance. For practical measurements, including this method, the scales of standard instruments indicate refractive indices with respect to air, rather than vacuum. The index of refraction of oils is characteristic within certain limits for each kind of oil. It is related to the degree of unsaturation, but it is affected by other factors, such as free fatty acid content, oxidation, and heat treatment.

SCOPE

Applicable to all normal oils and liquid fats.

APPARATUS

1. Refractometer—Any standard refractometer equipped with the Abbé, Butyro or any other standard scale. A dipping-type refractometer is satisfactory. The temperature of the refractometer must be controlled to within at least ± 0.1°C, and for this purpose it is preferable to use a thermostatically controlled water bath and motor-driven pump to circulate water through the instrument. The relationship between indices of refraction (n_D), obtained with the Abbé refractometer, and Butyro scale readings is given in Table 1.
2. Light source—If the refractometer is equipped with a compensator, a tungsten lamp or daylight bulb is permissible. Otherwise, a monochromatic light, such as a sodium vapor lamp, is required. In practice, the selected wavelength is the mean of the sodium D lines (589.6 nm).

REAGENTS

1. Toluene (see Notes, *Caution*), or some other fat solvent, such as hexane, light petroleum, or acetone, for cleaning the prisms. Lens tissue or cotton is recommended for cleaning the prisms in order to avoid injury to them.
2. Ethyl laurate of suitable quality and of known refractive index.

PROCEDURE

1. Melt the test sample, if it is not already liquid, and filter through filter paper to remove any impurities and the last traces of moisture. The test sample must be completely dry.
2. Check the repeatability requirement of the method by carrying out two single determinations as described below. Compare the results with the values in Table 2.
3. The instrument is standardized, following the manufacturer's instructions, with ethyl laurate or with a glass prism of known refractive index. Distilled water, which has a refractive index of 1.3330 at 20.0°C, is satisfactory in some cases.
4. Measure the refractive index of the test sample at one of the following temperatures (see Notes, 1):
   -20°C Completely liquid at 20°C
   -40°C Completely liquid at 40°C, but not at 20°C
   -50°C Completely liquid at 50°C, but not at 40°C
   -60°C Completely liquid at 60°C, but not at 50°C
   -80°C or above for other fats and oils.
5. Be sure the prisms are clean and completely dry. Place several drops of the test sample on the lower prism. Close the prisms and tighten firmly with the screwhead. Allow to stand for 1–2 min or until the test portion comes to the temperature of the instrument.
6. Adjust the instrument and light to obtain the most distinct reading possible, then determine the refractive index to the nearest 0.0001 and record the temperature of the prism. Take at least three separate readings and calculate the average of all readings.

PRECISION

1. Interlaboratory test—Details of an interlaboratory test on the precision method are summarized in Table 2. The values derived from this test may not be applicable to concentration ranges and matrices other than those given.
2. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will, in not more than 5% of cases, be greater than the repeatability limit r given in Table 2.
3. Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will, in not more than 5% of cases, be greater than the reproducibility limit R given in Table 2.

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NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

NUMBERED NOTES

1. Temperature corrections may be made for slight temperature differences (<3°C) using the following calculations:
   (a) Butyro refractometer—
   $R = R' + K(T' - T)$

   Where—
   R = the reading reduced to temperature T
   R′ = the reading at T′°C.
   T = the standard temperature
   T' = the temperature at which the reading R′ is made
   K = 0.55 for fats and 0.58 for oils
   (b) Abbé refractometer—
   The formula is identical to that given for the Butyro refractometer except that K is:
   0.00035 at T = 20°C
   0.00036 at T = 40, 50, 60°C
   0.00037 at T = 80°C and above.

REFERENCES

1. Elsdon, G.D., *Edible Oils and Fats,* Ernest Benn Limited, London, 1926.

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Table 1
Butyro refractometer readings and indices of refraction (n_D).[a]

	Fourth decimal of n_D									
n_D	0	1	2	3	4	5	6	7	8	9
	Butyro scale readings									
1.422	0.0	0.1	0.2	0.4	0.5	0.6	0.7	0.9	1.0	1.1
1.423	1.2	1.4	1.5	1.6	1.7	1.9	2.0	2.1	2.2	2.4
1.424	2.5	2.6	2.7	2.8	3.0	3.1	3.2	3.3	3.5	3.6
1.425	3.7	3.8	4.0	4.1	4.2	4.3	4.5	4.6	4.7	4.8
1.426	5.0	5.1	5.2	5.4	5.5	5.6	5.7	5.9	6.0	6.1
1.427	6.2	6.4	6.5	6.6	6.8	6.9	7.0	7.1	7.2	7.4
1.428	7.5	7.6	7.7	7.9	8.0	8.1	8.2	8.4	8.5	8.6
1.429	8.7	8.9	9.0	9.1	9.2	9.4	9.5	9.6	9.8	9.9
1.430	10.0	10.1	10.3	10.4	10.5	10.6	10.7	10.9	11.0	11.1
1.431	11.3	11.4	11.5	11.6	11.8	11.9	12.0	12.2	12.3	12.4
1.432	12.5	12.7	12.8	12.9	13.0	13.2	13.3	13.5	13.6	13.7
1.433	13.8	14.0	14.1	14.2	14.4	14.5	14.6	14.7	14.9	15.0
1.434	15.1	15.3	15.4	15.5	15.6	15.8	15.9	16.0	16.2	16.3
1.435	16.4	16.6	16.7	16.8	17.0	17.1	17.2	17.4	17.5	17.6
1.436	17.8	17.9	18.0	18.2	18.3	18.4	18.5	18.7	18.8	18.9
1.437	19.1	19.2	19.3	19.5	19.6	19.7	19.8	20.0	20.1	20.2
1.438	20.4	20.5	20.6	20.8	20.9	21.1	21.2	21.3	21.4	21.6
1.439	21.7	21.8	22.0	22.1	22.2	22.4	22.5	22.6	22.7	22.9
1.440	23.0	23.2	23.3	23.4	23.5	23.7	23.8	23.9	24.1	24.2
1.441	24.3	24.5	24.6	24.7	24.8	25.0	25.1	25.2	25.4	25.5
1.442	25.6	25.8	25.9	26.1	26.2	26.3	26.5	26.6	26.7	26.9
1.443	27.0	27.1	27.3	27.4	27.5	27.7	27.8	27.9	28.1	28.2
1.444	28.3	28.5	28.6	28.7	28.9	29.0	29.2	29.3	29.4	29.6
1.445	29.7	29.9	30.0	30.1	30.3	30.4	30.6	30.7	30.8	30.9
1.446	31.1	31.2	31.4	31.5	31.6	31.8	31.9	32.1	32.2	32.3
1.447	32.5	32.6	32.8	32.9	33.0	33.2	33.3	33.5	33.6	33.7
1.448	33.9	34.0	34.2	34.3	34.4	34.6	34.7	34.9	35.0	35.1
1.449	35.3	35.4	35.6	35.7	35.8	36.0	36.1	36.3	36.4	36.5
1.450	36.7	36.8	37.0	37.1	37.2	37.4	37.5	37.7	37.8	37.9
1.451	38.1	38.2	38.3	38.5	38.6	38.7	38.9	39.0	39.2	39.3
1.452	39.5	39.6	39.7	39.9	40.0	40.1	40.3	40.4	40.6	40.7
1.453	40.9	41.0	41.1	41.3	41.4	41.5	41.7	41.8	42.0	42.1
1.454	42.3	42.4	42.5	42.7	42.8	43.0	43.1	43.3	43.4	43.6
1.455	43.7	43.9	44.0	44.2	44.3	44.4	44.6	44.7	44.9	45.0
1.456	45.2	45.3	45.5	45.6	45.7	45.9	46.0	46.2	46.3	46.4
1.457	46.6	46.7	46.9	47.0	47.2	47.3	47.5	47.6	47.7	47.9
1.458	48.0	48.2	48.3	48.5	48.6	48.8	48.9	49.1	49.2	49.4
1.459	49.5	49.7	49.8	50.0	50.1	50.2	50.4	50.5	50.7	50.8
1.460	51.0	51.1	51.3	51.4	51.6	51.7	51.9	52.0	52.2	52.3
1.461	52.5	52.7	52.8	53.0	53.1	53.3	53.4	53.6	53.7	53.9
1.462	54.0	54.2	54.3	54.5	54.6	54.8	55.0	55.1	55.3	55.4
1.463	55.6	55.7	55.9	56.0	56.2	56.3	56.5	56.6	56.8	56.9
1.464	57.1	57.3	57.4	57.6	57.7	57.9	58.0	58.2	58.3	58.5
1.465	58.6	58.8	58.9	59.1	59.2	59.4	59.5	59.7	59.8	60.0
1.466	60.2	60.3	60.5	60.6	60.8	60.9	61.1	61.2	61.4	61.5
1.467	61.7	61.8	62.0	62.2	62.3	62.5	62.6	62.8	62.9	63.1
1.468	63.2	63.4	63.5	63.7	63.8	64.0	64.2	64.3	64.5	64.7
1.469	64.8	65.0	65.1	65.3	65.4	65.6	65.7	65.9	66.1	66.2

(Continued)

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Table 1 *(continued)*

	Fourth decimal of n_D									
n_D	0	1	2	3	4	5	6	7	8	9
	Butyro scale readings									
1.470	66.4	66.5	66.7	66.8	67.0	67.2	67.3	67.5	67.7	67.8
1.471	68.0	68.1	68.3	68.4	68.6	68.7	68.9	69.1	69.2	69.4
1.472	69.5	69.7	69.9	70.0	70.2	70.3	70.5	70.7	70.8	71.0
1.473	71.1	71.3	71.4	71.6	71.8	71.9	72.1	72.2	72.4	72.5
1.474	72.7	72.9	73.0	73.2	73.3	73.5	73.7	73.8	74.0	74.1
1.475	74.3	74.5	74.6	74.8	75.0	75.1	75.3	75.5	75.6	75.8
1.476	76.0	76.1	76.3	76.5	76.7	76.8	77.0	77.2	77.3	77.5
1.477	77.7	77.9	78.1	78.2	78.4	78.6	78.7	78.9	79.1	79.2
1.478	79.4	79.6	79.8	80.0	80.1	80.3	80.5	80.6	80.8	81.0
1.479	81.2	81.3	81.5	81.7	81.9	82.0	82.2	82.4	82.5	82.7
1.480	82.9	83.1	83.2	83.4	83.6	83.8	83.9	84.1	84.3	84.5
1.481	84.6	84.8	85.0	85.2	85.3	85.5	85.7	85.9	86.0	86.2
1.482	86.4	86.6	86.7	86.9	87.1	87.3	87.5	87.6	87.7	88.0
1.483	88.2	88.3	88.5	88.7	88.9	89.1	89.2	89.4	89.6	89.8
1.484	90.0	90.2	90.3	90.5	90.7	90.9	91.1	91.2	91.4	91.6
1.485	91.8	92.0	92.1	92.3	92.5	92.7	92.9	93.0	93.2	93.4
1.486	93.6	93.8	94.0	94.1	94.3	94.5	94.7	94.8	95.0	95.2
1.487	95.4	95.6	95.8	96.0	96.1	96.3	96.5	96.7	96.9	97.0
1.488	97.2	97.4	97.6	97.8	98.0	98.1	98.3	98.5	98.7	98.9
1.489	99.1	99.2	99.4	99.6	99.8	100.0	—	—	—	—

[a] From References, 1.

Table 2
Statistical analysis of collaborative study results according to ISO 5725-1 and ISO 5725-2 (1994).

Test samples	Rapeseed oil	Sunflower oil	Modified linseed oil	Modified castor oil	Castor oil
Number of laboratories	9	9	9	9	9
Number of observations	45	45	45	45	45
Mean (mg/kg)	1.47324	1.45751	1.48233	1.48391	1.47930
Repeatability					
s_r	0.00006	0.00006	0.00006	0.00005	0.00005
RSD_r	0.01	0.01	0.01	0.01	0.01
r	0.00017	0.00017	0.00017	0.00015	0.00013
Reproducibility					
S_R	0.00027	0.00030	0.00033	0.00040	0.0003
RSD_R	0.05	0.06	0.06	0.08	0.07
R	0.00075	0.00084	0.00094	0.00112	0.00098

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AOCS

AOCS Official Method Cc 8a-52

Revised 2012

Bleaching Test

DEFINITION

This method determines the color of the test sample after treatment with a specified bleaching earth under the conditions of this test.

SCOPE

Applicable to refined cottonseed oil. Formerly Cc 8a-47.

APPARATUS

1. Balances—
   (a) For the oil-torsion type, or equivalent—capacity 1000 g and sensitive to 0.1 g.
   (b) For the clay—balance sensitive to 0.05 g.
2. Balance weights—0.01–500 g.
3. Refining cups—stainless steel or unchipped, enameled iron, seamless, with handle, 4–4.5 in. in diameter, 4–4.5 in. deep, total capacity 900 mL. Refining cups are available at AOCS headquarters.
4. Bleaching apparatus—may be similar in design and construction to the refining apparatus described in AOCS Official Method Ca 9a-52 b. The essential features of the bleaching apparatus are:
   (a) T-shaped paddles constructed of stainless steel, with 0.5 × 3.5 in. blade. The blade must be fitted to the shaft in such a way that oil or grime cannot accumulate in a crack or space between the two parts.
   (b) The paddles are driven at 250 ± 10 rpm by any convenient means.
   (c) A heat source is required that will permit heating test portions to 120°C in 5 ± 1 min and maintain them at not less than 115°C. Either gas or electric heat may be used.
   (d) The support for the cups must be so arranged that they will not rotate or change position during the test, and the agitator shaft must be centered, with the bottom of the paddle at a distance of 0.25 in. above the bottom of the cup. The design of this support must also be such that the area at which heat is applied is essentially the entire bottom surface of the cup. The concentration of intense heat within small portions of this area must be avoided.
   (e) Thermometer clamps arranged so that a thermometer is held rigidly with the bulb completely immersed in the oil while agitating, but without interfering with the motion of the paddle.
5. *Natural* bleaching earth.
6. Filter paper—32 cm; Eaton & Dikeman no. 513, Reeve Angel no. 871, Whatman no. 2V, S & S no. 596, or equivalent. Folded filter papers, such as E & D no. 617, Reeve Angel no. 230, or equivalent, are optional. They may be used to filter the refined oil for use in running the bleach test. The purpose is to speed up the filtering rate. The use of these two "fast" filter papers is not permitted when the refined oil test portion is to be used directly for color determination.
7. Colorimeter, color glasses and color tubes—as specified in AOCS Official Method Cc 13b-45, or a spectrophotometer, as specified in AOCS Official Method Cc 13c-50.
8. Laboratory thermometer—0–150°C, calibrated for 3 in immersion.
9. Funnels—stemless and seamless; of aluminum, stainless steel or glass (60° cone with 5–6 in. top diameter and ⅜–½ in opening).

PROCEDURE

1. Filter the oil, using one of the filter papers noted in Apparatus, 6 (or equivalent), into a clean and dry container.
2. Weigh 300 g of test portion into refining cup. Add *natural* bleaching earth in the amount specified on the label of the can.
3. Begin stirring at 250 ± 10 rpm and heat immediately to 120 ± 2°C, taking no longer than 5 ± 1 min. Continue stirring at the same rate for 5 min. Do not allow the temperature to drop below 115°C during this period, even if reheating is necessary.
4. Immediately pour all the oil and earth onto a dry filter paper. Allow a minimum of 60 mL to pass through the filter before collecting a sufficient amount for the color reading.
5. Determine the color as directed in AOCS Official Method Cc 13b-45, AOCS Official Method Cc 13c-50, or AOCS Official Method Cc 13e-92, as appropriate or as required.

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AOCS

AOCS Official Method Cc 8b-52

Revised 2011

Bleaching Test

DEFINITION

This method determines the color of the test sample after treatment with a specified bleaching earth under the conditions of this test.

SCOPE

Applicable to refined soybean oil and refined sunflower oil. Formerly Cc 8b-49.

APPARATUS

1. Balances—
   (a) For the oil-torsion type, or equivalent—capacity 1000 g and sensitive to 0.1 g.
   (b) For the clay—balance sensitive to 0.05 g.
2. Balance weights—0.01–500 g.
3. Refining cups—stainless steel or unchipped, enameled iron, seamless, with handle, 10.6–11.4 cm in diameter, 10.6–11.4 cm deep, total capacity 900 mL. Refining cups are available at AOCS headquarters.
4. Bleaching apparatus—may be similar in design and construction to the refining apparatus described in AOCS Official Method Ca 9a-52. The essential features of the bleaching apparatus are:
   (a) T-shaped paddles constructed of stainless steel, with a 1.27 × 8.9 cm blade. The blade must be fitted to the shaft in such a way that oil or grime cannot accumulate in a crack or space between the two parts.
   (b) The paddles are driven at 250 ± 10 rpm by any convenient means.
   (c) A heat source is required that will permit heating test samples to 120°C in 5 ± 1 min and maintain them at not less than 115°C. Either gas or electric heat may be used.
   (d) The support for the cups must be so arranged that they will not rotate or change position during the test, and the agitator shaft must be centered, with the bottom of the paddle at a distance of .64 cm above the bottom of the cup. The design of this support must also be such that the area at which heat is applied is essentially the entire bottom surface of the cup. The concentration of intense heat within small portions of this area must be avoided.
   (e) Thermometer clamps arranged so that a thermometer is held rigidly with the bulb completely immersed in the oil while agitating, but without interfering with the motion of the paddle.
5. *Natural* bleaching earth.
6. Filter paper—32 cm; Eaton & Dikeman no. 513, Reeve Angel no. 871, Whatman no. 2V, S & S no. 596, or equivalent. Folded filter papers, such as E & D no. 617, Reeve Angel no. 230, or equivalent, are optional.
7. Colorimeter, color glasses and color tubes—as specified in AOCS Official Method Cc 13b-45, or a spectrophotometer, as specified in AOCS Official Method Cc 13c-50.
8. Laboratory thermometer—0–150°C, calibrated for 7.62 cm immersion.
9. Funnels—stemless and seamless; of aluminum, stainless steel or glass (60° cone with 5–6 in. top diameter and .952–1.27 cm opening).
10. *Activated* bleaching earth (for Procedure, 2).

PROCEDURE

1. For normal colored oils—
   (a) Filter the oil, using one of the filter papers noted in AOCS Official Method Cc 8a-52 (Apparatus, 6), into a clean and dry container. Folded filter papers, such as Eaton & Dikeman no. 617, Reeve Angel no. 230, or equivalent, are optional. They may be used to filter the refined oil for use in running the bleach test. The purpose is to speed up the filtering rate. The use of these two "fast" filter papers is not permitted when the refined oil sample is to be used directly for color determination.
   (b) Weigh 300 g of test sample into refining cup. Add *natural* bleaching earth in the amount specified on the label of the can.
   (c) Commence stirring at 250 ± 10 rpm and immediately heat to 120 ± 2°C, requiring 5 ± 1 min. Continue stirring at the same rate for 5 min. Do not allow the temperature to drop below 115°C during this period, even if reheating is necessary.
   (d) Immediately pour all the oil and earth onto a dry filter paper. Allow a minimum of 60 mL to pass. Then, collect a sufficient amount for the color reading.
   (e) Determine the color as directed in AOCS Official Method Cc 13b-45 or Official Method Cc 13c-50.
2. For green oils with high chlorophyll content—
   (a) The procedure is identical to that described in Procedure, 1, except that 4% (12.0 g) of the *activated* bleaching earth is used instead of the *natural* bleaching earth.

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AOCS

AOCS Official Method Cc 8d-55

Reapproved 2009

Refined and Bleached Color

DEFINITION

This method determines the color of the test sample after treatment with alkali and a specified bleaching earth.

SCOPE

Applicable to tallows and greases intended for soap production. Formerly Cc 8d-53.

APPARATUS

1. Refining apparatus and supplies as described in AOCS Official Method Ca 9a-52, Apparatus, 1–3.
2. Bleaching apparatus—may be similar in design and construction to the refining apparatus described in AOCS Official Method Ca 9a-52, Apparatus, 4. The essential features of the bleaching apparatus are
   (a) T-shaped paddles constructed of stainless steel, with 0.5 × 3.5 in. blade. The blade must be fitted to the shaft in such a way that oil or grime cannot accumulate in a crack or space between the two parts.
   (b) The paddles are driven at 250 ± 10 rpm by any convenient means.
   (c) A heat source is required that will permit heating test samples to 110°C in 5 ± 1 min and maintain them at not less than 105°C. Either gas or electrical heat may be used.
3. *Activated* bleaching earth
4. Filter paper—qualitative, coarse porosity, to provide fast rate of filtration, 250 mm.
5. Filter paper—qualitative, medium porosity, to provide medium rate of filtration, 250 mm.
6. Colorimeter, color glasses and color tubes—as specified in AOCS Official Method Cc 13b–45.
7. Hot water funnel or other suitable funnel heater—adjusted to a temperature such that the test sample will remain completely liquid during filtration, but not higher than 25°C above the melting point of the test sample.
8. Funnels—125- or 150-mm diameter.
9. Laboratory thermometer—0–150°C.

REAGENTS

1. Sodium hydroxide (NaOH) solution, 0.25 M—accurately standardized. See AOCS Specification H 12-52.
2. Sodium hydroxide—20° Baumé (Bé), (14.07-14.65% actual NaOH by weight), prepared as directed in AOCS Official Method Ca 9a-52, Reagents, 2 (see Notes, *Caution*).
3. Specially denatured alcohol—USSD formulas 30 or 3A. Add 0.05% phenolphthalein and a sufficient amount of 0.25 M NaOH solution to give the alcohol a faint pink color. This adjustment should be made just prior to using the alcohol.

PREPARATION OF TEST SAMPLE

1. Melt the test sample if it is not completely liquid and shake or stir thoroughly to obtain a uniform mixture. Avoid local overheating and do not heat more than 10°C above the melting point.

DETERMINATION OF FREE FATTY ACIDS (FFA)

1. Weigh 7.05 g of well-mixed test sample into a 250 mL Erlenmeyer flask and add 50 mL of neutral alcohol (Reagents, 3) containing 0.05% phenolphthalein.
2. Warm in a water bath and titrate with 0.25 M NaOH until a permanent pink color is obtained, and which persists after 30 sec of vigorous shaking.
3. Free fatty acids as oleic, % = mL of 0.25 M NaOH solution used, provided exactly 0.25 M NaOH and 7.05 g of test sample are used.

PROCEDURE

1. Weigh 500 g of test portion into refining cup. Place in the refining machine, fill the water bath to the specified height and heat to 51–55°C.
2. Find the percent excess NaOH required (see Table 1) corresponding to the percent FFA determined. Substitute the values for percent excess NaOH and percent FFA in the formulas [Procedure, 4, (a) and (b)] to determine the total amount of alkali solution to be added.
3. Start stirring at 250 rpm and add the specified amount of 20° Bé alkali solution calculated in Procedure, 2. Continue agitation for 5 min after addition of the alkali.

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4. (a) $\frac{\%\ \text{FFA in fat}}{7.05} = \%\ \text{NaOH to neutralize FFA} = A$

   (b) $\frac{5 \times (A + \%\ \text{excess NaOH required from Table 1})}{0.1436}$ = grams of 20° Bé alkali solution to add to 500 g of test portion

5. After the 5-min period noted in Procedure, 3, change the speed of stirring to 70 rpm and immediately increase the bath temperature to 61–64°C. Stir under these conditions for 10-15 min. Sometimes it may be necessary to add a small amount of water to the mixture to hasten coagulation and to ensure complete precipitation.
6. When the foots appear to be ready to settle, discontinue stirring and allow to stand until the foots have settled and the fat is clear. The condition of the foots can be determined by removing a small portion of the mixture from the cup and observing the tendency to settle.
7. Filter a sufficient quantity with the filter paper noted in Apparatus, 4, using a heated filter funnel. Then weigh 300 ± 1.0 g of the filtered test portion into a refining cup (see Notes, 1). Heat to 105–110°C and then add 3% (9 g) of AOCS activated bleaching earth.
8. Stir at 250 rpm for 5 min, maintaining the temperature at 105–110°C.
9. Immediately filter through the filter paper noted in Apparatus, 5, using a heated filter funnel. As soon as the test portion filters clearly, start collecting the test portion in a Lovibond color tube and fill to the 5.25 in. (133.35-mm) mark. Read the color as directed in AOCS Official Method Cc 13b-45.

NOTES

Caution

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials such as sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. Refining cups are available at AOCS headquarters.

Table 1
Percent excess NaOH required in refining, depending on free fatty acid (FFA) content.

FFA in fat, %	Excess NaOH required, %
0.1–4.00	0.2
4.01–6.00	0.3
6.01–8.00	0.5
8.01–15.00	0.6

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AOCS

AOCS Official Method Cc 8e-63

Reapproved 2009

Modified Bleach Test for Soybean Oil

DEFINITION

This method determines the color of the test sample after treatment with a specified bleaching earth under the conditions of the test.

SCOPE

Applicable to soybean oil.

APPARATUS

1. Same as specified in AOCS Official Methods Ca 5a–40, Ca 9a-52, and Cc 8b–52.

REAGENTS

1. Same as specified in AOCS Official Methods Ca 5a–40, Ca 9a-52, and Cc 8b–52.

PROCEDURE

1. Determine the free fatty acid content of the test sample as directed in AOCS Official Method Ca 5a–40.
2. Refine 500 g of the test sample as follows.
   (a) Place the test sample in the refining apparatus at 20–24°C and agitate at 250 rpm.
   (b) Add the caustic solution as specified in AOCS Official Method Ca 9a–52 for soybean oil, with a free fatty acid level as determined in Procedure, 1, using the highest dosage of caustic as determined in Table 1.
   (c) Agitate for 15 min then decrease the agitation rate to 70 ± 5 rpm. Heat the test portion to 65°C and agitate for 15 min at 65°C.
   (d) Allow the oil to stand at 60–65°C for at least 1 hr to permit the soaps to settle. Longer settling times may be used, but normally no longer than 2 hr should be required.
   (e) Remove from the refining apparatus and decant the clear oil into a second refining cup, being careful to exclude any soaps that have settled. Filter the decanted oil through filter paper and bleach as outlined in AOCS Official Method Cc 8b-52.

Table 1.
Weight of caustic (sodium hydroxide) in grams per 100 grams of oil.

FFA	12° Max	FFA	12° Max	FFA	12° Max	FFA	12° Max
0.1	7.0	1.4	10.1	2.7	13.2	.04	16.4
0.2	7.2	1.5	10.4	2.8	13.5	.14	16.6
0.3	7.5	1.6	10.6	2.9	13.7	4.2	16.8
0.4	7.7	1.7	10.8	3.0	14.0	4.3	17.1
0.5	8.0	1.8	11.1	3.1	14.2	4.4	17.3
0.6	8.2	1.9	11.3	3.2	14.7	4.5	17.6
0.7	8.4	2.0	11.6	3.3	14.7	4.6	17.8
0.8	8.7	2.1	11.8	3.4	14.9	4.7	18.0
0.9	8.9	2.2	12.0	3.5	15.2	4.8	18.3
1.0	9.2	2.3	12.3	3.6	19.4	4.0	18.5
1.1	9.4	2.1	12.5	3.7	15.6	5.0	18.8
1.2	9.6	2.5	12.8	3.8	15.9	—	—
1.3	9.9	2.6	13.0	3.9	16.1	—	—

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AOCS

AOCS Analytical Guidelines Cc 8f-91

Reapproved 2009

Evaluation of Bleaching Clays and Adsorbents

SCOPE

This method describes the procedures recommended for the evaluation of bleaching clays and adsorbents. The listed methods are recommended for the evaluation of test oils before and after conducting a bleaching test. The method contains recommendations for experimental apparatus and procedures for conducting bleaching tests (see Notes, 1).

EVALUATION METHODS

Table 1
Recommended methods for the evaluation of test oils before and after conducting a bleaching test.

Test	AOCS Official Method or Recommended Practice
Color	
FAC	Cc13a-43
Gardner for industrial oil	Td 1a-64
Lovibond (Wesson)	Cc13b-45
Spectrophotometric Method	Cc13c-50
Chlorophyll	Cc13d-55
Phosphorus	
Ashing/Spectrophotometry	Ca 12-55
Atomic Absorption Spectroscopy	Ca12b-92
Nephelometry	Ca 19-86
Soap	
Titrimetric	Cc 17-95
Peroxide Value	
Acetic Acid/Chloroform	
Acetic Acid/Isooctane	Cd 8b-90
Free Fatty Acid	
Crude and refined fats and oils	Ca 5a-40
Stability	
Oil Stability Index	Cd 12b-92
Moisture	
Air Oven Method	Ca 2c-25
Vacuum Oven Method	Ca 2d-25
Karl Fischer titration	Ca 2e-84

DESCRIPTION OF EVALUATION METHODS

1. Color—The methods describe several approved colorimeters to determine the color of a test sample by comparison with known standards. Guidelines for red to yellow ratios are given for various feedstocks to help reduce abnormalities created by different hues when using instruments based on tinted glass.
2. Chlorophyll—The method spectrophotometrically determines the chlorophyll content in mg/kg (ppm) in refined and bleached oils. The method cannot be used on hydrogenated and deodorized oils. Some Lovibond automated colorimeters are equipped to measure chlorophyll as part of the color determination.
3. Phosphorus—The most accurate method uses atomic absorption spectroscopy (AAS) to determine phosphorus content in mg/kg (ppm) directly and quantitatively. Alternate methods listed in Table 1 include colorimetric and nephelometric techniques. Quantitative measurements are obtainable from the colorimetric method. The nephelometric technique may be influenced by other components, but is satisfactory for relative qualitative comparisons.
4. Soap—The presence of soap, as sodium oleate, is determined by titration with a dilute HCl solution.
5. Peroxide value—The methods determine the presence of all substances, in terms of milliequivalents of peroxide per 1000 grams of test portion, which oxidize potassium iodide under the conditions of the test. The test is applicable to all normal fats and oils, but is highly empirical. The procedure and precautions must be followed closely.

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6. Free fatty acid—The method determines the acid content of nearly all fatty chemicals via base neutralization to a phenolphthalein end point. The calculations may be adjusted to represent the dominant fatty chain length, lauric, palmitic and oleic acids. The titration will be affected by the presence of free mineral acids which, if present, will interfere with the true quantitative measurement of free fatty acid content.
7. Fat stability—The OSI method measures the amount of time required for a test portion of fat to reach a predetermined peroxide value, or state of oxidation. The test is highly empirical and results have not been universally well correlated with accepted definitions or other measurements of rancidity. The test is subject to a wide variety of errors and can be very time consuming. It is recommended that this test be conducted by those groups who have established relationships between the OSI results and oil quality (see Notes, 2).

BLEACHING TEST APPARATUS

1. Three-necked glass reactor—between 250 mL and 1000 mL in size. The size is determined by the quantity of oil required for analytical evaluation.
2. Stirring paddle—vacuum tight, appropriately sized, fitted to an adjustable-speed mechanical stirring motor. Magnetic stirring bars are not recommended.
3. Thermometer and automated temperature control system—connected to an electrical heating mantle that will allow the temperature to be controlled within ± 5°C maximum, with ± 2°C preferred.
4. Mechanical vacuum system—able to control and maintain a vacuum between 20 and 100 mm Hg absolute pressure.
5. Nitrogen—or other suitable inert gas source.
6. Jacketed Buchner funnel—either porcelain or stainless steel, sized appropriately for the oil quantity to be filtered in one filling only.
7. Filter paper—designed to retain particles between 2.5 and 5.0 µm and sized to fit the Buchner funnel.

PROCEDURE

Note—See Notes, 3 for guidelines for variation from the following recommended procedure.

1. Determine from the list of AOCS official methods and recommended practices (Table 1) which quality parameters are to be used to evaluate the performance of the treatment material. Calculate the quantity of test oil required to do each of the analytical tests. At least three times this quantity should be allotted for each bleaching test. Determine the number of bleaching tests to be run. Each bleaching condition parameter should be done at several levels. Select at least one set of conditions to be done in duplicate, for a measure of reproducibility. The total of these will determine the minimum quantity of oil required for each treatment sample to be tested.
2. Obtain a quantity of test oil in excess of the minimum required. Analyze this oil for the initial values of the quality parameters. For the treatment material to be evaluated, run the bleaching tests under the preselected conditions. Analyze each of the bleached samples for each of the quality parameters (see the Results section for data analysis). Proceed as follows.
3. Charge known quantity of test oil to reaction vessel, not to exceed one-half the total volume of the vessel. This will allow foaming to occur without loss of material.
4. Heat oil to 60 ± 5°C under predetermined vacuum set point, with agitation. Dry and deaerate for 15 min. Break vacuum with nitrogen.
5. Add known quantity of treatment material as percent weight of oil. Each bleaching condition parameter should be done using several levels of bleaching clay.
6. Return to vacuum set point, set agitation, heat to predetermined temperature as quickly as possible (less than 5 min).
7. Start timer when temperature reaches set point. Hold for 30 min at constant temperature, vacuum, and agitation.
8. After 30 min, cool rapidly to 70°C and break vacuum with nitrogen.
9. Filter test portion through temperature-controlled vacuum filter. Seal test portion under nitrogen until analyzed.

RESULTS

1. Graph the initial values and postbleached values for each treatment sample vs. a set of conditions. This yields the contours for comparison of effects. Depending on the type of experimental design employed, these contours will allow the experimenter to determine which treatment material is best suited to the production process, or what production parameters should be changed to best benefit the quality of the product. Regardless of the results generated by this method, the final conclusion cannot be made until the tests are made in the production area. Factors such as variation in feedstock quality, scale, and normal production variation cannot be fully accounted for in laboratory studies. The results will help justify changes in raw materials or conditions and provide starting parameters for production trials.

NOTES

1. This method was developed by the AOCS Bleaching Methods Committee.
2. Guidelines for variation from recommended procedure—The proposed method, as noted in the Procedure section, is a compilation of parameters generally used in the industry. The best measure of how well laboratory treatments will work in a production facility will be obtained by modifying the procedure so that conditions are as close to the production conditions as possible. Modification of the proposed method is recommended so that the results will be translatable in the production

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facility. Furthermore, the general procedure can be used to optimize the industrial bleaching process by varying certain parameters and noting the effect on oil quality. These parameters include:

(a) Quantity of treatment material—One of the purposes of this method is to determine the amount of treatment material needed to obtain the desired oil quality. Typical loading levels vary from 0.25 to 5.0% by weight of oil. Consultation with suppliers is recommended to determine initial concentration levels.

(b) Predrying of the oil—If driers are not used in the production refining and bleaching process, do not perform step 2 of the recommended procedure. Some treatments are recommended to be used with wet oil. Consultation with these suppliers is recommended for best initial guidelines.

(c) Vacuum—Systems and vacuum levels vary from site to site, or may not be used at all. Adjust the vacuum to match existing conditions, or run under open atmosphere if required. Atmospheric bleaching may cause excessive oxidation and is not recommended for edible oil systems.

(d) Temperature—Bleaching temperatures may vary between 95 and 120°C. The temperature may be adjusted to match existing conditions in production. It is generally accepted that temperatures outside this range will be either ineffective or detrimental.

(e) Contact time—The amount of contact time required is a function of treatment type, temperature, agitation, vacuum, and initial oil quality. Poor-quality oils may require more than the recommended 30 min, while high-quality oils may require less.

(f) Agitation—Set the agitation such that the clay is uniformly suspended in the oil, and so the oil surface is not being whipped. A small vortex is recommended. Precise measurement of rpm may be obtained using either a contact or strobe tachometer.

(g) Filtration—A pressurized filtration system may also be used. These systems can use an inert gas as the pressurizing agent, minimizing contact with air and improving the rate of filtration.

(h) Nitrogen or inert gas—Although the production system may not have an inert gas system, they are usually designed to minimize contact with air, unless bleaching is done under atmosphere. It is recommended that the tests be conducted under an inert gas in order to preserve the benefits of the test in the final analysis.

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AOCS

AOCS Official Method Cc 9a-48

Reapproved 2009

Smoke, Flash and Fire Points Cleveland Open Cup Method

DEFINITION

These methods determine the temperature at which the test sample will smoke, flash, or burn.

SCOPE

Applicable to animal, vegetable, and marine fats and oils. Flash point not applicable to test samples that flash below 150°C (300°F). Formerly Cc 9-40.

APPARATUS

1. Cabinet—constructed of the materials and in accordance with the dimensions shown in Figure 1.
2. Thermometer—AOCS Specification H 5–40.
3. Cleveland open flash cup—American Society for Testing and Materials Designation D 92–72, constructed of brass and conforming to the dimensional requirements prescribed in Table 1. The beveled edge of the cup is at an angle of about 45°. There may be a fillet of approximately 3.97 mm ($\frac{5}{32}$ in.) in radius inside the bottom of the cup.
4. Heating plate and cup support—constructed of brass, cast iron, wrought iron, or steel; 6.35 mm ($\frac{1}{4}$ in.) thick and 152.4 mm (6 in.) in diameter. There is a plane depression 0.79 mm ($\frac{1}{32}$ in.) deep in the center of the plate with diameter just sufficient to fit the cup and centered with a circular opening cut through the plate, 55.0 mm ($2\ \frac{3}{16}$ in.) in diameter.
5. Heat source—electric heater with rheostat control.
6. Metal flame test burner.

PROCEDURE

1. Smoke point—
   (a) Fill the cup (see Notes, 1) with the oil or melted fat test sample so that the top of the meniscus is exactly at the filling line, and adjust the position of the apparatus so that the beam of light is directed across the center of the cup. Suspend or secure the thermometer in a vertical position in the center of the dish with the bottom of the bulb 6.35 mm from the bottom of the cup.
   (b) Heat the test portion rapidly to within 42°C (75°F) of the smoke point. Thereafter, regulate the heat so that the temperature of the test portion increases at the rate of 5–6°C (9-11°F)/min. The smoke point is the temperature indicated by the thermometer when the test portion gives off a thin, continuous stream of bluish smoke. In some cases, a slight puff appears before the test portion begins to smoke continuously. This is disregarded.
2. Flash point—
   (a) The flash may be conducted without the cabinet, but in a room or compartment free from air drafts and darkened sufficiently so that the flash is readily discernible. Avoid breathing over the surface of the test portion.
   (b) Fill the cup with the oil or melted fat test sample so that the top of the meniscus is exactly at the filling line. Suspend or secure the thermometer in a vertical position with the bottom of the bulb 6.35 mm from the bottom of the cup and in a position halfway between the center and back of the cup.
   (c) Heat the test portion at a rate not to exceed a 16°C (30°F) rise per minute to within 55°C (100°F) of the flash point. Thereafter, regulate the rate of heating so that the temperature of the test portion increases 5–6°C (9–11°F)/min.
   (d) Apply the test flame [which should be about 3 mm ($\frac{1}{8}$ in.) in diameter] as the temperature reaches each successive 2°C (5°F) mark. Pass the flame in a straight line or on the circumference of a circle having a radius of at least 6 in. (about 150 mm) across the center of the cup and at right angles to the diameter passing through the thermometer. The test flame should, while passing across the surface of the test portion, be in the same plane as the upper edge of the cup. The time for the passage of the test flame across the cup should be no more than 1 sec.
   (e) The flash point is the temperature indicated by the thermometer when a flash appears at any point on the surface of the test portion. The true flash must not be confused with a bluish halo that sometimes surrounds the test flame.
3. Fire point—
   (a) The fire point may be conducted without the cabinet, but in a room or compartment free from air drafts and darkened sufficiently so that the flame is readily discernible. Avoid breathing over the surface of the test portion.
   (b) Continue the heating *after* the flash point determination, as directed in Procedure, 2, (c)-(e) until the fire point is reached.
   (c) The fire point is the temperature indicated by the thermometer when application of the test flame causes burning for a period of at least 5 sec.

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NOTES

1. It is imperative that the apparatus, especially the cup, be scrupulously clean and free from any substance that may cause smoke to appear ahead of the true smoke point.

Table 1
Dimensional requirements for Cleveland open flash cup.

	Millimeters			Inches		
	Minimum	Normal	Maximum	Minimum	Normal	Maximum
Inside diameter immediately below filling mark	62.7	63.4	64.3	2 15/32	2 1/2	2 17/32
Outside diameter below flange	67.5	68.3	69.1	2 21/32	2 11/16	2 23/32
Inside height from center of bottom to rim	32.5	33.3	34.1	1 9/32	1 5/16	1 11/32
Thickness of bottom	2.8	3.2	3.6	7/64	1/8	9/64
Distance from rim to filling mark	9.1	9.5	9.9	23/64	3/8	25/64
Distance from lower surface flange to bottom of cup	31.0	31.8	32.6	1 7/32	1 1/4	1 9/32
Vertical distance from upper surface flange to rim	2.8	3.2	3.6	7/64	1/8	9/64
Thickness of rim	2.0	2.4	2.8	5/64	3/32	7/64
Width of lower surface of flange	14.3	15.1	15.9	9/16	19/32	5/8

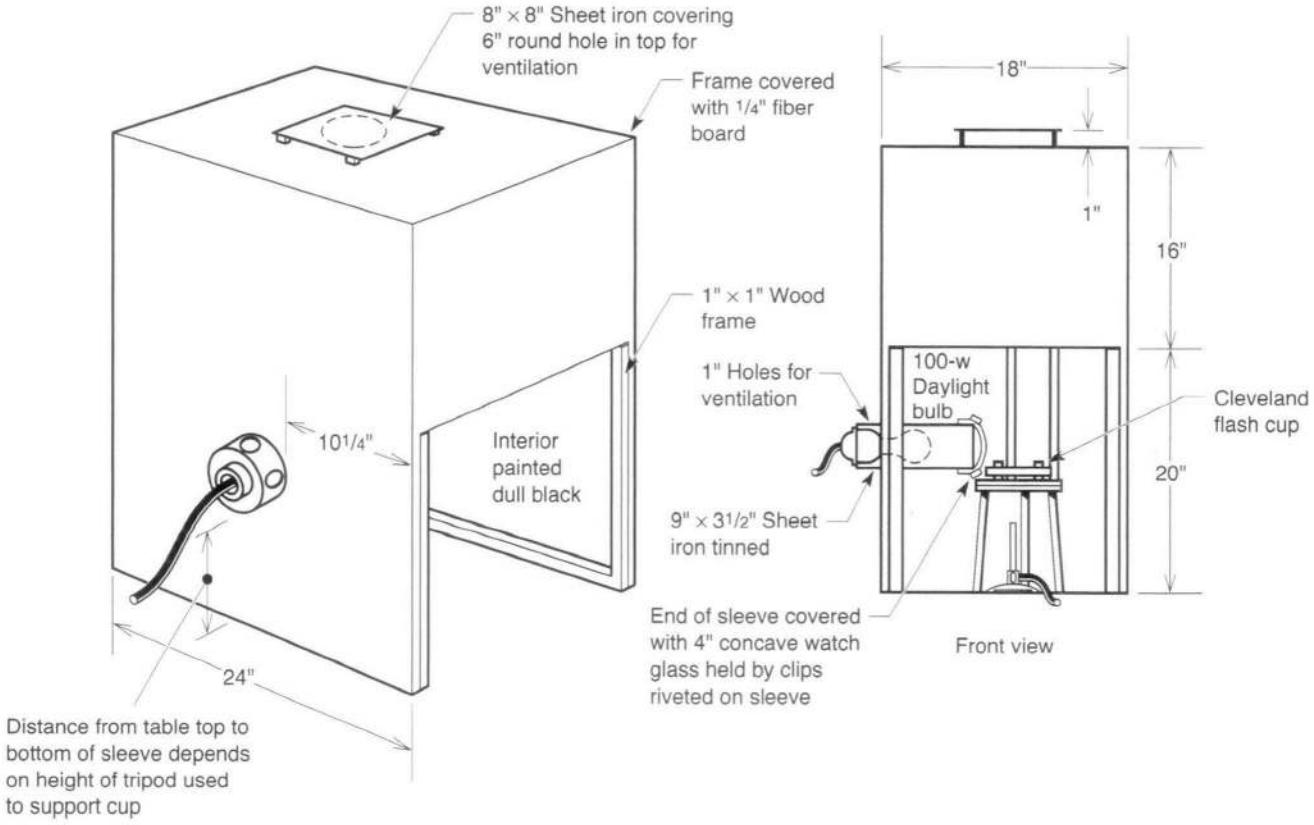

Figure 1

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AOCS

AOCS Official Method Cc 9b-55

Revised 2011

Flash Point Closed Cup Method

DEFINITION

This method determines the temperature at which the test sample will flash when a test flame is applied under the conditions specified for the test. (Modified Closed Cup Method, ASTM Designation D 93-80)

SCOPE

Applicable to animal, vegetable, and marine fats and oils. The fats and oils may or may not contain small amounts of volatile inflammable solvents (see Notes, 1).

APPARATUS

1. Pensky-Martens closed cup flash test, American Society for Testing and Materials Designation D 93–80—either hand or motor stirring is allowable, but the latter is preferred. The specified low-range ASTM Pensky-Martens and high-range ASTM Pensky-Martens centigrade and Fahrenheit thermometers are replaced by a single centigrade or Fahrenheit thermometer as described below.
2. Thermometers—AOCS Specification H 10–55, closed cup flash point thermometers.
3. Laboratory centrifuge—sling type of sufficient size to hold 120-mL bottles. Size 1 centrifuge (International Equipment Co., Boston, MA, USA) has been found to be satisfactory for this purpose.

REAGENTS

1. Anhydrous cupric sulfate (Cu_2SO_4)—reagent grade.

PROCEDURE

1. To an adequately weighed amount of the oil (see Notes, 2) to be examined, add 5% anhydrous cupric sulfate if the moisture content of the test portion exceeds 0.1 % (see Notes, 3). If the dehydration step is necessary, agitate the oil/cupric sulfate mixture vigorously for 1 min in a closed container and allow to stand for 0.5 hr. Centrifuge until a sufficient amount of clear oil is obtained to make the flash point determination.
2. Fill the cup with the oil or fat test portion so that the top of the meniscus is exactly at the filling line, place the lid on the cup and properly engage the locating devices. Insert the thermometer and suspend so that the bottom of the bulb is exactly 4.45 cm (1.75 in.) below the level of the rim of the cup, which corresponds to the level of the lower surface of the portion of the lid inside the rim.
3. Light the test flame and adjust so that it is the size of a bead approximately 4 mm ($^5/_{32}$ in.) in diameter.
4. Heat the test portion so that the temperature increases not less than 5°C (9°F) nor more than 6°C (11°F) per minute. During the heating, adjust the stirring device at one to two revolutions per second.
5. At every 5°C (10°F) rise in temperature, discontinue stirring and apply the test flame by operating the device which controls the shutter and lowers the test flame into the shutter opening. Lower the test flame in 0.5 sec and leave in a lowered position for 1 sec, then quickly return to the raised or high position. As soon as the test flame has been returned to the high position, resume stirring.
6. The flash point is the temperature indicated on the thermometer at the time of the flame application that causes a distinct flash in the interior of the cup. The true flash must not be confused with the bluish halo that sometimes surrounds the test flame.

NOTES

1. This method can be used for test samples having flash points >150°C. In industrial applications, the temperature range is generally limited by the thermometer scale. Most compounds having a flash point >200°C are categorized as such. This is done primarily to satisfy the United States Department of Transportation definition of noncombustible material.
2. If the fat is a solid at room temperature, it should be liquefied by heating at a temperature not to exceed 5°C (10°F) over its melting point. The flash point determination will then start at this elevated temperature.
3. If dehydration is necessary, it should be conducted on a liquefied test sample. The dehydration step with copper sulfate may be omitted if it is known that the moisture content of the oil to be tested is <0.1%. High moisture content causes excessive foaming and extinction of the flame.

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AOCS

AOCS Official Method Cc 9c-95

Reapproved 2009

Determination of the Flashpoint by Pensky-Martens Closed Cup Flash Tester

DEFINITION

The method determines whether a test sample of oil or fat at a specific temperature will flash when a test flame is applied to the test sample under the conditions (described in the method) of the test.

SCOPE

Applicable to animal, vegetable, and marine fats and oils, to determine if a cargo of edible oil or fat is hazardous to the point of being explosive due to residual technical hexane or other solvents being present. The fats and oils may or may not contain small amounts of volatile inflammable solvents. When determining if a cargo of edible oil or fat is hazardous to the point of being explosive, this method should be used rather than AOCS Official Method Cc 9b-55. This revised flashpoint method is based on an international collaborative study carried out by the Federation of Oils, Seeds and Fats Associations Limited (FOSFA) International during 1995, and it is recommended for use in international trade.

The test sample is heated at a slow constant rate with continual stirring. Once the specified temperature is reached and stabilized, a small flame is directed into the cup. The test sample is deemed to have flashed when a large flame appears and instantaneously propagates itself over the surface of the test sample; a halo should be ignored.

APPARATUS

1. Pensky-Martens Closed Cup Flash Tester (see Numbered Notes, 1).
2. Thermometers—conforming to the requirements of Specification ASTM EL (having a range of 10–200°C). Alternatively, an IP thermometer 101°C (having a range of 20–150°C).
3. Laboratory centrifuge—a swing type of sufficient size to hold 120 mL centrifuge tubes.
4. Centrifuge tubes—120 mL with stoppers.

REAGENTS

1. Anhydrous sodium sulphate—analytical grade.

SAMPLING

It is important that a laboratory receives a laboratory sample that is truly representative and has not been damaged or changed during transportation and storage.

Sampling is not part of the method specified in this procedure. Recommended sampling methods are given in ISO 5555 (1991) and AOCS C1-47.

Laboratory samples should not be stored in plastic (polyethylene, polypropylene, etc.) bottles since volatile materials may diffuse through the walls of the bottle.

PREPARATION OF TEST SAMPLE

Prepare the test sample in accordance with ISO 661.

PROCEDURE

1. Preparation of test portion.
   (a) If the fat is a solid at room temperature it should be liquefied by warming slowly to a temperature not exceeding 5°C over its melting point. The flashpoint determination will then start at this elevated temperature.
   (b) Carefully transfer (see Notes, 2) approximately 90 g of fat or oil to the stoppered tube and add 5 g of anhydrous sodium sulphate. Shake the mixture vigorously for 1 min with the stopper secured and allow it to stand for 30 min, if necessary at an elevated temperature (see Procedure, 1, a).
   (c) Centrifuge the treated oil as prepared in (see Procedure, 1, b) at 2,500 rpm for 3 min or until a sufficient amount of clear oil is available to make the flashpoint determination. Maximum time of centrifuging—5 min.
2. Determination:
   (a) Fill the cup with the liquefied oil or fat test sample (see Procedure, 1, c) so that the top of the meniscus is exactly at the filling line marked in the cup. Place the lid on the cup and engage the locating devices. Insert the thermometer (see Apparatus, 2) and suspend it so that the bulk is 4.445 cm below the level of the rim of the cup, which corresponds to the level of the lower surface of the portion of the lid inside the rim.

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(b) Light the test flame and adjust it so that it is the size of a bead (4 mm) in diameter.
(c) Heat the test sample so that the temperature increases not less than 5°C and not more than 6°C per minute. During the heating, turn the stirring device one to two revolutions per second.
(d) At the specified temperature (usually 121°C) discontinue stirring and apply the test flame by operating the device that controls the shutter and lowers the test flame into the shutter opening. Lower the test flame within 0.5 sec and observe whether this causes a distinct flash in the interior of the cup. The true flash must not be confused with the bluish halo that sometimes surrounds the test flame.
(e) It is not permitted to open the cup more than once during each determination because solvent vapor, although present in too low a concentration to cause a flash, might escape. This would lead to an incorrect result at the measuring temperature.
(f) If information is needed about the precise level of he flashpoint, the whole procedure should be repeated at temperatures other than 121°C. Fresh portions of the test samples should be used for each temperature determination.
(g) Carry out the test in duplicate.

TEST REPORT

1. Report "flash" or "no flash" at the specified temperature and the method and apparatus used
2. If different results are obtained, disregard the no flash result.

NUMBERED NOTES

1. Standard specification for Pensky-Martens Closed Cup Flash Tester, see AOCS Official Method Cc 9b-55, ASTM D 93-80 or ISO 2719:1988.
2. When preparing the test sample, the oil should be taken straight from the test sample container and directly transferred to the centrifuge tubes. Pouring or decanting the oil into another container, e.g., a beaker, for transfer to the centrifuge tube has been shown to cause a loss of hexane. The test sample should be poured down the side of the centrifuge tube and the flash tester cup to avoid agitation that will result in the loss of hexane.

REFERENCE

1. ISO/TC 34/SC11 N547.

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AOCS

AOCS Official Method Cc 10b-25

Reapproved 2009

Specific Gravity of Solid Fats and Waxes

DEFINITION

This method determines the ratio of the mass of a unit volume of the test sample at 25°C to the mass of a unit volume of water at 25°C.

SCOPE

Applicable to solid fats and waxes.

APPARATUS

1. Westphal balance.
2. Small camel hair brush.
3. Hot water funnel.
4. Beaker—tall form, 100 mL.
5. Burette—50 mL.
6. Laboratory forceps.

REAGENTS

1. Ethyl alcohol (see Notes, *Caution*).

PROCEDURE

1. Prepare seven solutions (50–100 mL each) of alcohol and water with densities ranging from 0.940 to 1.000 at 25°C, varying by 0.01 specific gravity units. Use the Westphal balance to determine the specific gravity. Store in glass-stoppered bottles, appropriately numbered, from 1 to 7, in order of increasing specific gravity.
2. Melt 5 g of the test sample, making certain the melted test portion is clear, free of moisture and air bubbles. If the test portion is turbid and/or contains moisture, filter through dry filter paper with the aid of a hot water funnel. Allow the test portion to cool slowly to room temperature.
3. Cut a small pellet (3–5 mm in diameter) from the solidified test portion and brush over with a camel hair brush (wet with distilled water) to avoid the development of air bubbles on the surface.
4. Pour alcohol solution 1 into a beaker, adjust to 25 ± 0.1°C and immerse the pellet with the aid of forceps. If the pellet sinks, repeat with solution 2, and so on, until a solution is found in which the pellet just floats.
5. When the solution is found in which the pellet floats, slowly add alcohol at 25°C from a burette, stirring gently with a glass stirring rod to prevent the formation of air bubbles around the test portion pellet. Continue adding the alcohol until the pellet just sinks. If the correct amount of alcohol is exceeded, distilled water may be added dropwise and the end point again determined.
6. Determine the specific gravity of the solution generated in Procedure, 5 with the Westphal balance, making certain the solution is at 25 ± 0.1°C. The specific gravity of this solution is identical with the specific gravity of the test portion.
7. The result is confirmed by adding one or more pellets to the same solution. If the test portion floats, it should slowly sink upon the addition of a drop of alcohol. If the test portion sinks, slowly add water dropwise until it just rises, and then determine the specific gravity of the solution.

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

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AOCS

AOCS Official Method Cc 10c-95

Reapproved 2009

Determination of Mass per Unit Volume ("Liter Weight") in Air of Oils and Fats

DEFINITION

This method determines the mass per unit volume ("liter weight") of oils and fats in order to convert volume to mass or mass to volume, and involves the measurement of the mass, at the required temperature, of a volume of the oil or fat contained in a pycnometer that has been calibrated at the same temperature. The mass per unit volume ("liter weight") of an oil or fat is defined as the ratio of the mass of the oil or fat to its volume at a given temperature and is expressed in grams per milliliter (g/mL) or kilograms per liter (kg/L).

SCOPE

This method is applicable to animal and vegetable fats and oils that do not deposit crystals at the temperature of determination. The determination may be made directly on test samples that are liquid at ambient temperature, or after complete melting of other test samples, preferably at 40, 50, or 60°C. If necessary, a higher temperature may be used.

In principle, this method is the same as AOCS Official Method Cc 10a-25, Specific Gravity of Oils and Liquid Fats; however, AOCS method Cc 10a-25 specifies temperatures of 25°C and 60°C, whereas method Cc 10c-95 is more flexible in terms of temperature.

This method, identical to ISO 6883 (1987), is used in the international trade of oils and fats.

APPARATUS

1. Pycnometer—50 mL capacity, fitted with a calibrated thermometer graduated in divisions of 0.1°C and with a side-arm and cap (Fig. 1). The pycnometer preferably should be made of borosilicate glass, but if this is not available, then one made of soda glass may be used.

   Note—The cap is required only when the determination is carried out at a temperature below ambient. The top of the cap is perforated and leads into an expansion tube.

   Alternatively, the type 3 (Gay-Lussac) pycnometer may be used; however, the use of pycnometer with thermometer is preferred.

   Calibrate the pycnometer at least annually at the temperature of determination as follows:

   (a) Weigh, to the nearest 0.1 mg, the empty pycnometer with the thermometer or stopper.

   (b) Fill the pycnometer with recently distilled water or deairated water of equivalent purity at the approximate temperature of calibration and replace the thermometer or stopper, taking care not to include air bubbles. Place the filled pycnometer in the water bath or oven at a temperature that does not vary by more than 1°C from the temperature required for the determination until the contents have reached a stable temperature (approximately 1 hr). Allow the water to overflow and wipe the surplus from the top of the outlet. Record the temperature of the pycnometer to the nearest 0.1°C. Remove the pycnometer from the water bath, wiping it carefully with lint-free material, or from the oven. Allow its temperature to reach ambient temperature. Weigh the full pycnometer.

   (c) The volume, V_T, in mL, of the pycnometer is equal to

$$V_T = \frac{m_1 - m_0}{\rho H_2O,\ T_0}[1 + \alpha(T - T_0)]$$

   Where—

   m_0 = mass of the empty pycnometer, g
   m_1 = mass of the pycnometer filled with water, g
   α = mean coefficient of cubic expansion of glass (equal to 0.000010 K^{-1} for borosilicate glass, or 0.000025 K^{-1} for soda glass)
   T = temperature used in the determination (see Scope), °C
   T_0 = temperature at which the pycnometer was calibrated, °C
   $\rho H_2O,\ T_0$ = density of water in air at T°C, g/mL (see Table 1)

2. Water bath or oven, capable of being maintained at the temperature chosen for the determination.

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TEST SAMPLE PREPARATION

1. Prepare the test sample in accordance with ISO 661, but do not filter it. It is essential that the fat or oil be completely liquid when the determination is made. If the test sample is not liquid at room temperature, heat it to a temperature no more than 10°C above its melting point.

PROCEDURE

1. Fats that are liquid at ambient temperature—
   (a) Weigh the empty pycnometer with the thermometer or stopper, to the nearest 0.1 mg.
   (b) Fill the pycnometer with the test sample and replace the thermometer or stopper, taking care not to include air bubbles. Place the filled pycnometer in the water bath or oven maintained at the temperature, $T_0 \pm 1$°C, required for the determination until the contents reach this temperature.

   Allow the test sample to overflow and wipe the surplus from the top of the outlet. Record the temperature, T, of the pycnometer to the nearest 0.1°C.
   (c) Remove the pycnometer from the water bath, wiping it carefully with lint-free material, or from the oven. Allow its temperature to reach ambient. Weigh the full pycnometer.
2. Fats that are solid at ambient temperature—Melt the test sample at a temperature approximately 10°C above its melting point. Follow Procedure 1, (a)–(c), allowing the full pycnometer to cool before weighing.
3. Number of determinations—Carry out two determinations on the same test sample.

CALCULATIONS

1. The mass per unit volume, ρ_T, expressed in g/mL or kg/L, of the fat or oil at T°C is equal to

$$\frac{m_2 - m_0}{V_T}$$

Where—

M_0 = mass of the empty pycnometer, g
M_2 = mass of the pycnometer filled with fat or oil, g
V_T = volume of the pycnometer at temperature T, mL

If the conditions of repeatability are fulfilled, take as the result the arithmetic mean of the two determinations, and express it to four decimal places.

2. Temperature correction—If the volume of the fat or oil is measured at a temperature $T \pm 1$°C of temperature T_x, relate the result to the temperature T_x using the following equations:

ρ at $T_x = \rho$ at $T + 0.00068(T - T_x)$ if $T > T_x$
ρ at $T_x = \rho$ at $T - 0.00068(T_x - T)$ if $T < T_x$

Note—The correction coefficient 0.00068 is an approximate mean coefficient of fats or oils, but if the actual coefficient for the test sample is known, it is recommended that this be used in the interest of greater accuracy.

Note—The formulas used for the correction can also be used to convert the mass per unit volume determined at one temperature to that at another temperature provided that the temperature difference is not more than 5°C and the test sample is liquid at both temperatures.

PRECISION

1. Interlaboratory tests—International collaborative tests have been carried out on the method. The tests on refined bleached and deodorized (RBD) palm olein, crude coconut oil and crude rapeseed oil were organized by FOSFA International.
2. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will, in not more than 5% of cases, be greater than .0002 g/mL.
3. Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will, in not more than 5% of cases, be greater than 0.007 g/mL.

TEST REPORT

1. The test report shall show the method used, the measurement temperature and the results obtained. It shall also mention any operating details not specified in this method, or regarded as optional, together with details of any incidents likely to have influenced the results.
2. The test report shall include all the information necessary for the complete identification of the test sample.

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REFERENCES

1. This method was adopted from the International Organization for Standardization method 6883 (1987), "Animal and vegetable fats and oils—Determination of mass per unit volume ('litre weight') in air" and is identical with International Union of Pure and Applied Chemistry (IUPAC) standard method 2.101.

Table 1
Density of water in air at temperatures from 15–65°C.

Temperature °C	Density of water g/mL	Temperature °C	Density of water g/mL
15	0.99805	41	0.99079
16	0.99789	42	0.99039
17	0.99772	43	0.98999
18	0.99754	44	0.98958
19	0.99735	45	0.98917
20	0.99715	46	0.98874
21	0.99694	47	0.98832
22	0.99672	48	0.98788
23	0.99649	49	0.98744
24	0.99624	50	0.98699
25	0.99599	51	0.98654
26	0.99573	52	0.98607
27	0.99546	53	0.98561
28	0.99518	54	0.98513
29	0.99490	55	0.98465
30	0.99460	56	0.98416
31	0.99429	57	0.98367
32	0.99398	58	0.98317
33	0.99365	59	0.98267
34	0.99332	60	0.98217
35	0.99298	61	0.98165
36	0.99264	62	0.98113
37	0.99228	63	0.98060
38	0.99192	64	0.98006
39	0.99155	65	0.97952
40	0.99117		

Table 2
Precision data.

	RBD palm olein		Crude coconut oil		Crude rapeseed oil	
Type of oil	1995	1995	1997	1997	1999	1999
No. of laboratories returning results	53	53	36	36	62	62
No. of laboratories with acceptable results	40	40	33	33	51	51
Mean value, g/mL	0.8906	0.8906	0.9078	0.9079	0.9054	0.9050
Repeatability limit, *r*, g/mL	0.0002	0.0002	0.0001	0.0002	0.0003	0.0002
Reproducibility limit, *R*, g/mL	0.0011	0.0012	0.0065	0.0065	0.0082	0.0062

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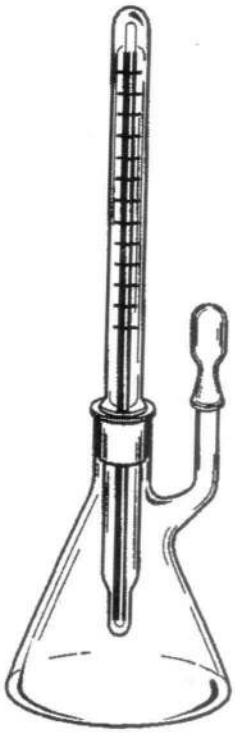

Figure 1. Specific gravity bottle (pycnometer).

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AOCS Official Method Cc 11-53

Reapproved 2009

Cold Test

DEFINITION

This method measures the resistance of the test sample to crystallization and is commonly used as an index of the winterization and stearin removal processes.

SCOPE

Applicable to all normal, refined and dry animal and vegetable oils. Replaces Cc 11-42.

APPARATUS

1. Oil sample bottles—115 mL (4 oz). These must be completely clean, dry, and free from dust particles.
2. Water bath—maintained at 25°C.
3. Chipped ice and water bath—at 0°C. Prepare by filling a suitable container of 2–5-kg capacity (an insulated container may be used) with finely chipped ice. Add cold water sufficient to fill to the top of the test sample bottle when it is immersed in the ice bath (see Notes, 1).

PROCEDURE

1. Filter a sufficient quantity of the test sample (200–300 mL) through a filter paper and then heat the filtered portion. Stir the test sample continuously while heating and remove from the heat source immediately when the temperature reaches 130°C (see Notes, 2).
2. Fill an oil sample bottle completely full with the test sample and insert a cork tightly. Adjust the test sample and bottle to 25°C in a water bath and then seal with paraffin.
3. Immerse the bottle containing the test portion in the ice water bath so that the entire bottle is covered with water and ice.
4. Replenish the ice as often as necessary to keep the bath solidly packed, otherwise the bath temperature will rise above 0°C. It is essential that the bath be maintained at 0°C.
5. At the end of 5.5 hr, remove the bottle from the bath and examine closely for fat crystals or cloudiness. Do not mistake small and finely dispersed air bubbles for crystals. To pass the test, the test portion must be completely clear and brilliant (see Notes, 3 and 4).

NOTES

1. The AOCS Technical Committee has found that a mechanical cooling bath is satisfactory, provided baffles are present in the bath to dampen the harmonic effects of the mechanical cooling system.
2. The purpose of the preliminary heat treatment is to remove traces of moisture and to destroy any crystal nuclei that may persist. Either will interfere with the test, causing cloudiness or premature crystallization.
3. If it is desired, the test may be continued by reexamination of the test portion at hourly intervals after the first 5.5-hr examination. However, in so doing, the test portion should be returned to the bath as promptly as possible after each inspection so that the temperature will not increase to any significant degree.
4. Viewing the test portion against a dark background that is backlit is helpful. The test portion should be about 2 meters away from the dark background.

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AOCS

AOCS Official Method Cc 12-59

Reapproved 2009

Titer Test

DEFINITION

This method determines the solidification point of a mixture of fatty acids from a saponified test sample of either fat or oil.

SCOPE

Applicable to normal animal fats, vegetable oils, and marine oils. Replaced Cc 12-41.

APPARATUS

1. For expected titers above 35°C, use Griffin low-form beakers, 2 L capacity and a wide-mouth bottle, capacity 450 mL, 190 mm high with neck of i.d. 38 mm. Assemble as shown in Figure 1.
2. For expected titers below 35°C, a glass beaker may be used in place of the stainless steel beaker, if desired, to observe the test portion during testing. In this case, the 5800 mL and 8000 mL stainless steel beakers with the ground cork insulation between them may be replaced with a single 8000 mL glass beaker. Assemble as shown in Figure 2.
3. Titer test tubes—length 100 mm, diameter 25 mm, with or without rim. These titer test tubes may have an etched mark extending around the tube at a distance of 57 mm from the bottom to show the height to which the tube is to be filled (see Notes, 1).
4. Saponification vessel—may be a flask or beaker, of convenient capacity (about 500 mL). The form of this vessel is not important as long as it is satisfactory for saponification (alkali resistant).
5. Stirrer—2–3 mm o.d., with one end bent in the form of a loop 19 mm in outside diameter. Glass, nichrome, stainless steel or monel wire may be used. The upper end can be formed to accommodate hand stirring, or it may be attached to a mechanical stirrer (see Notes, 2).
6. Laboratory thermometer—0–150°C.
7. Titer test thermometer—see AOCS Specification H 6–40, –2–68°C. This thermometer should be calibrated against a National Institute of Testing-certified thermometer.
8. Filter paper-qualitative, rapid filtering grade.

REAGENTS

1. Glycerol-caustic solution—prepared by dissolving (with warming) 250 g of reagent-grade potassium hydroxide (KOH) pellets (see Notes, 3 and *Caution*) in 1250 g of reagent-grade glycerin. To avoid foaming, do not heat above 135–145°C.
2. Dilute sulfuric acid (H_2SO_4), 30% solution—prepared by mixing 16 mL of concentrated H_2SO_4 (sp. gr. 1.84) with 70 mL of distilled water (see Notes, *Caution*).
3. Ethylene glycol ("antifreeze").
4. Dry ice (solid carbon dioxide, CO_2).
5. Methyl orange indicator solution—1.0% in distilled water.

PROCEDURE

1. Preparation of fatty acid test sample—
   (a) Weigh about 110 g of the glycerol-caustic solution into the saponification vessel. With stirring, heat to 150°C. Add about 50 mL of the liquid test sample (either oil or melted fat) and reheat to 140–150°C. In some cases (feed-grade fats, test samples with high levels of impurities), additional glycerol-caustic solution may be necessary to ensure complete saponification.
   (b) Continue stirring until saponification is complete (see Notes, 4).
   Note—Do not heat test sample over 150°C under any circumstances.
   (c) Cool slightly, add 200–300 mL of distilled water, stir the mixture well and heat until the soaps are dissolved. Add carefully while stirring, 50 mL of dilute H_2SO_4. Boil until the fatty acids are completely melted and clear. Additional water may be added either before or during the boiling, if necessary. A hot plate with thermostat control should be used to prevent scorching on the side of the dish.
   (d) Remove the aqueous layer containing the H_2SO_4. This may be done using a glass siphon or separatory funnel. Again add water and boil for 2–3 min, or until the fatty acids are entirely melted and clear. Acids of high melting point fats are sometimes slow to melt and clear. Inspect the fatty acid layer without stirring or agitation to be certain that all has melted.
   (e) Remove the water again and, if necessary, repeat the washing as directed in Procedure, 1, (d) until the wash water is neutral to methyl orange indicator.

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(f) Transfer the fatty acids to a filter paper so as not to include any water. The filter paper may be supported on a funnel. If necessary, use a hot water funnel, because the fatty acid mixture must remain completely melted during filtration.

(g) Heat the filtered fatty acid mixture on a hot plate to 130°C to remove traces of moisture, and fill a titer test tube to a height of exactly 57 mm from the bottom.

Note—The test portion may not be held at 130°C nor reheated to this temperature more than once. If excessive moisture is present, allow the water to settle, decant the fatty acids and then filter again and reheat to 130°C. The fatty acids must be completely dry for the test.

2. Solidification of the fatty acids—

(a) For expected titer above 35°C, fill the water bath to the designated level and adjust the temperature 15–20°C below the expected titer point.

(b) For expected titer below 35°C, fill the bath with ethylene glycol to the designated level. Insert the stainless steel screen and place dry ice just beneath the ethylene glycol level. Crunch the dry ice to about 1 cm cubes and add slowly to the ethylene glycol to reach the desired temperature. Maintain the bath temperature 15–20°C below the titer point of the test portion.

(c) Place the titer tube containing the fatty acid mixture in the assembly as shown in the figures. Insert the titer thermometer to the immersion mark and equidistant from the sides of the titer tube.

(d) Stir, with stirring rod in a vertical manner at a rate of 100 complete up-and-down motions per minute (see Notes, 2). The stirrer moves through a vertical distance of about 38 mm. The agitation is started while the temperature is at least 10°C above the titer point.

(e) Stir at the directed rate until the temperature remains constant for 30 sec, or begins to rise in less than a 30-sec interval. Discontinue stirring immediately, remove the stirrer (or raise it out of the test portion) and observe the increase in temperature. The titer point is defined at the highest temperature indicated by the thermometer during the rise.

PRECISION

1. Duplicate determinations, carried out on the same fatty acid mixture [as obtained in Procedure, 1, (f) and (g)], are usually expected to agree within ± 0.2°C.

NOTES

Caution

Potassium hydroxide, like all alkalies, can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive splattering. Always add the acid to water, never the reverse.

NUMBERED NOTES

1. Titer test tubes may be obtained from Fisher Scientific, USA; catalog no. 14-963A.
2. Stirring may be performed mechanically by attaching a small motor with a suitable speed-reducing mechanism to the stirring rod.
3. Sodium hydroxide (NaOH) may not be substituted for potassium hydroxide (KOH) in the glycerol method.
4. The AOCS Technical Committee investigated a number of tests for judging the completeness of the saponification procedure, but none was found that was reliable under all circumstances. Familiarity with the changes that take place in the appearance and character of the mixture usually enables one to determine the proper end point. The initial stage of saponification is usually indicated by a change in the appearance of the mixture and is often accompanied by an increase in the viscosity of the mixture. After this occurs, the test portion thins out after the reaction is complete and assumes a homogeneous appearance. The most common characteristic is that of soap bubbles forming and rising from the surface. There are cases in which none of these criteria can be depended upon, so considerable care must be exercised at all times to ensure complete saponification.

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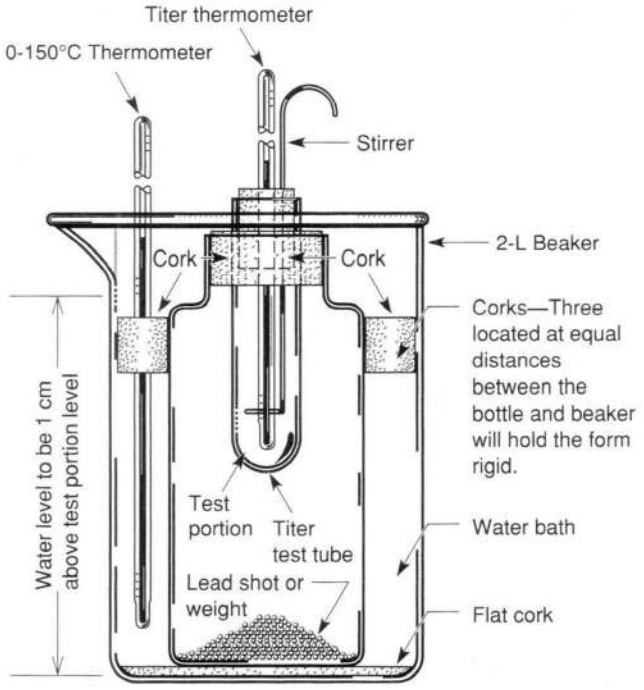

Figure 1. Titer assembly for expected titers above 35°C.

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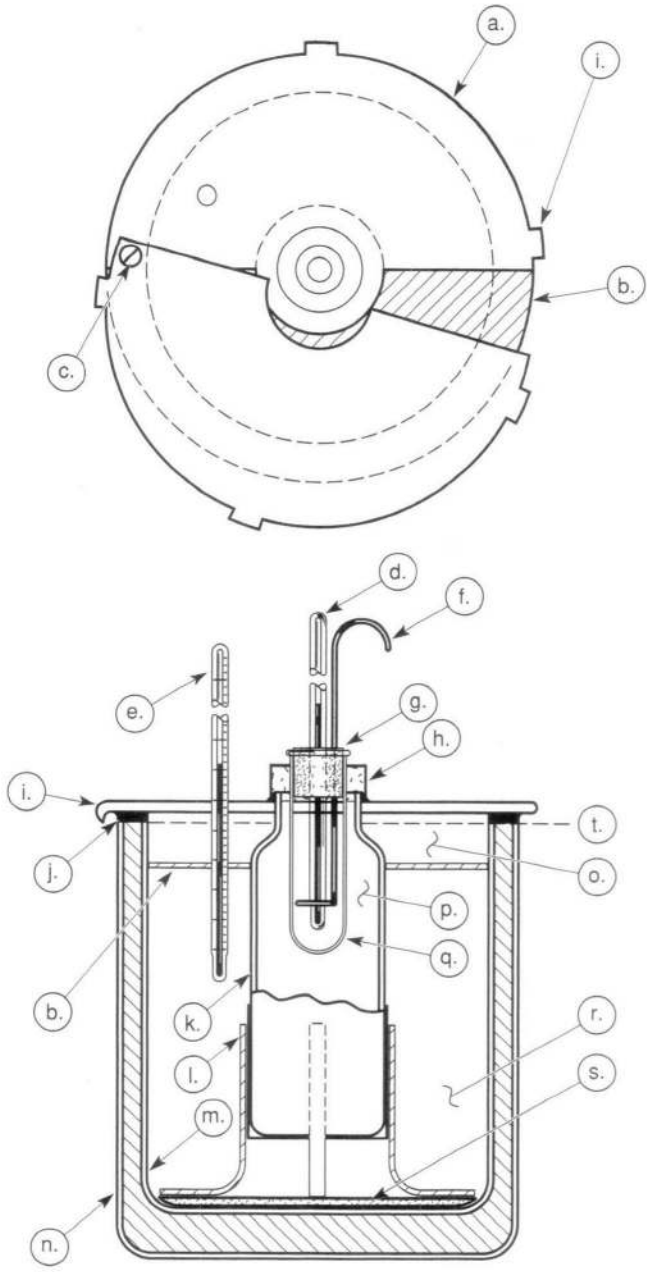

a. Tight-fitting stainless steel cover, two parts, with 10-mm lap at joint. Provide lugs to fit beaker rim.
b. Stainless steel screen to hold dry ice
c. Swivel joint
d. Titer thermometer
e. Thermometer, –35 to 51°C
f. Stirrer
g. Cork
h. Wood bushing
i. Lugs to fit rim
j. Poured solder seal
k. Wide mouth bottle: capacity, 450 mL; height, 190 mm; inside diameter of neck, 38 mm
l. Stainless steel bottle support
m. 5800-mL stainless seel beaker: height, 225 mm; diameter, 200 mm
n. 8000-mL stainless steel beaker: height, 250 mm; diameter, 225 mm
o. Dry ice
p. Test portion
q. Titer test tube
r. Glycol bath
s. Ground cork insulation
t. Glycol level 1 cm above test portion level

Figure 2. Titer assembly for expected titers below 35°C.

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AOCS

AOCS Official Method Cc 13a-43

Reapproved 2009

Color

FAC Standard Color

DEFINITION

This method determines the color of fats and oils by comparison with permanent color standards.

SCOPE

Applicable to animal fats and all fats and oils too dark to be read by the Wesson method.

APPARATUS

1. Color tubes of clear glass—10.5±0.25 mm i.d., 12.25 ±0.25 mm o.d., 100 mm long. Available from Tintometer Company (see Notes, 1).
2. Comparator block—constructed of wood, metal, or plastic and painted dull black; dimensions as shown in Figure 1.
3. Light source—daylight-type fluorescent light.
4. Filter paper—Whatman no. 1, or equivalent.
5. FAC standard color set—This set consists of 26 permanent color standards from 1 to 45 in odd numbers. These are divided into five groups, as shown in Table 1.
6. The FAC color standards are arranged in four disks in the following order:
   Disk 1: 1, 5, 9, 11A, 11B, 11C, 13, 17, 21
   Disk 2: 3, 7, 11, 11A, 11B, 11C, 15, 19, 23
   Disk 3: 25, 29, 33, 37, 41, 45
   Disk 4: 27, 31, 35, 39, 43

PROCEDURE

1. The test sample must be entirely liquid, but should not be heated any higher than 10°C above melting point.
2. Filter the test sample through an appropriate filter paper, never using more than two papers.
3. Pour the melted and filtered test portion into one of the color tubes. Place this tube in the comparator block and insert a pair of color disks that cover the range of color of the test portions.
4. Hold the block containing the standards and test portion up to the light in a vertical position and in such a way that the standards and test portion are viewed simultaneously.
5. The test portion should be reported as not darker than the standard that it matches or not darker than the darker of the two standards between which it falls. Any test portion darker than 45 is reported as darker than 45.
6. The visual relationship in intensity between the FAC color standards is shown in Table 2.

NOTES

1. Details of suppliers of FAC permanent color standards mounted in discs and comparator blocks may be obtained from The Tintometer Limited, Waterloo Road, Salisbury, Wiltshire, SPI 2JY, UK. Tel: +44 1722 327242, Fax: +44 1722 412322, E-mail: sales@tintometer.com
2. The x, y, and z coordinates for the FAC color scale were obtained by first visually matching the FAC standard solutions against Lovibond glass standards. The color matching was originally carried out during the mid 1950s, but, because of difficulties caused by variations in different sets of standard solutions, it was finally agreed to match against a "special" set of solutions numbered "1908." This took place in 1959. The glass standards which resulted from the 1959 matching were scanned by a SP600 Pye Unicam spectrophotometer to obtain transmission data which in turn were used to calculate the CIE values. The glass standards were then placed into discs which were accepted as a master set by the AOCS Fat Analysis Committee (FAC) in October 1960.

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Table 1
FAC standard color set.

Light colored fats	Predominantly yellow fats	Dark fats (red cast)	Very dark fats (predominantly green)	Very dark fats (predominantly red)
1	11	13	21	31
3	11A	15	23	33
5	11B	17	25	35
7	11C	19	27	37
9	—	—	29	39
—	—	—	—	41
—	—	—	—	43
—	—	—	—	45

Table 2
Visual relationship in intensity between FAC color standards.

F.A.C. Standard no.	Standards listed below are equal to or lighter than the corresponding standards in the left-hand column.
1	1
3	1,3
5	1,3,5
7	1,3,5,7
9	1,3,5,7,9
11	1,3,5,7,9,11
11A	1,3,5,7,9,11,13,11A
11B	1,3,5,7,9,11,13,15,11A,11B
11C	1,3,5,7,9,11,13,15,17,19,11A,11B,11C
17	1,3,5,7,9,11,13,15,17,11A,11B
19	1,3,5,7,9,11,13,15,17,19,11A,11B,11C
21	1,3,5,7,9,11,13,15,17,19,21,31,33,11A,11B,11C
23	1,3,5,7,9,11,13,15,17,19,21,23,31,33,35,11A,11B,11C
25	1,3,5,7,9,11,13,15,17,19,21,23,25,31,33,35,37,11A,11B,11C
27	1,3,5,7,9,11,13,15,17,19,21,23,25,27,31,33,35,37,39,11A,11B,11C
29	1,3,5,7,9,11,13,15,17,19,21,23,25,27,29,31,33,35,37,39,41,43,11A,11B,11C
31	1,3,5,7,9,11,13,15,17,19,31,11A,11B,11C
33	1,3,5,7,9,11,13,15,17,19,21,31,33,11A,11B,11C
35	1,3,5,7,9,11,13,15,17,19,21,23,31,33,35,11A,11B,11C
37	1,3,5,7,9,11,13,15,17,19,21,23,25,31,33,35,37,11A,11B,11C
39	1,3,5,7,9,11,13,15,17,19,21,23,25,27,31,33,35,37,39,11A,11B,11C
41	1,3,5,7,9,11,13,15,17,19,21,23,25,27,31,33,35,37,39,41,11A,11B,11C
43	1,3,5,7,9,11,13,15,17,19,21,23,25,27,29,31,33,35,37,39,41,43,11A,11B,11C
45	1,3,5,7,9,11,13,15,17,19,21,23,25,27,29,31,33,35,37,39,41,43,45,11A,11B,11C

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Table 3
Trichromatic coefficients of the prototype set of permanent glass FAC standards (see Notes, 2).

F.A.C. standard no.	x	y	z	T
1	0.3387	0.3676	0.2935	0.83
3	0.3651	0.3985	0.2361	0.79
5	0.3812	0.4015	0.2107	0.70
7	0.3946	0.4165	0.1889	0.66
9	0.4146	0.4466	0.1388	0.67
11	0.4442	0.4740	0.0820	0.58
11A	0.4848	0.4908	0.0246	0.63
11B	0.5111	0.4682	0.0206	0.50
11C	0.5276	0.4591	0.0133	0.44
13	0.4505	0.4688	0.0808	0.50
15	0.4702	0.4613	0.0686	0.42
17	0.4900	0.4696	0.0406	0.36
19	0.5076	0.4645	0.0281	0.26
21	0.5538	0.4411	0.0052	0.104
23	0.5694	0.4263	0.0044	0.075
25	0.5842	0.4137	0.0019	0.054
27	0.5838	0.4147	0.0015	0.044
29	0.6057	0.3938	0.0006	0.015
31	0.5568	0.4277	0.0156	0.15
33	0.5736	0.4195	0.0068	0.162
35	0.5876	0.4083	0.0039	0.078
37	0.5919	0.4050	0.0030	0.078
39	0.6064	0.3917	0.0020	0.064
41	0.6149	0.3843	0.0008	0.028
43	0.6256	0.3741	0.0005	0.0135
45	0.6421	0.3578	0.0000	0.0042

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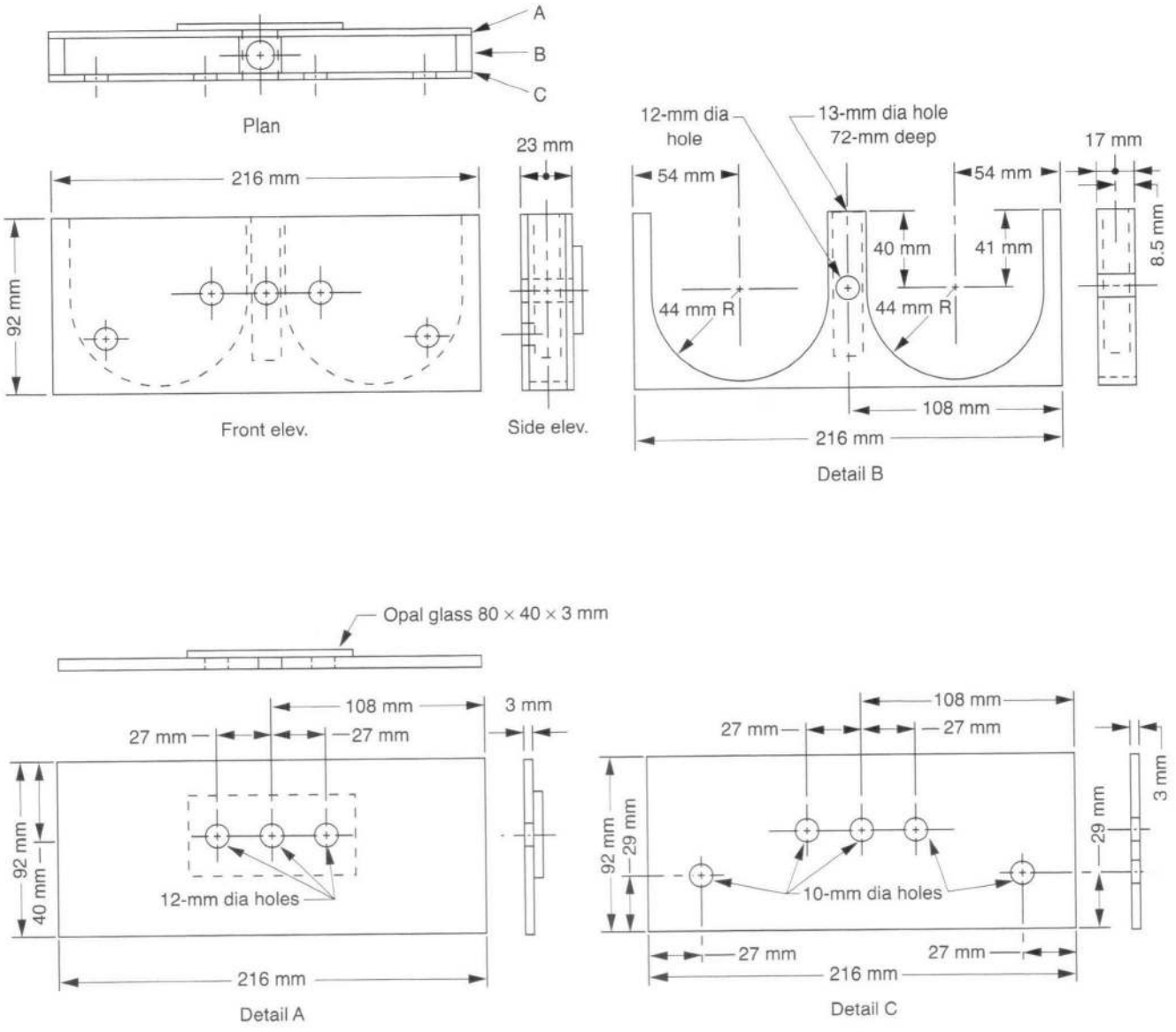

Figure 1. Comparator block assembly. Material: wood, metal, or plastic; paint flat black.

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AOCS

AOCS Official Method Cc 13b-45

Reapproved 2009

Color

Wesson Method Using Color Glasses Calibrated in Accordance with the AOCS-Tintometer Color Scale

DEFINITION

This method determines color by comparison with glasses of known color characteristics.

SCOPE

Applicable to all normal fats and oils, providing no turbidity is present in the test sample. In international trade, the British Standard Lovibond color, AOCS Official Method Cc 13e-92, may be preferred or required.

APPARATUS

1. Colorimeter specifications—The instrument consists of a light-proof box with a dull black interior, illuminated by a 100-watt blue frosted electric lightbulb.

   A block of magnesia is placed in the instrument at the proper angle to reflect the light from the electric bulb vertically upward through the color tube and color glasses. The block must be free from oil and dirt and positioned to give equal illumination to both sides of the viewing field.

   An eye piece finished with a dull black interior is fitted over (outside) the rectangular top of the tube holder so that the light passes through the color tube and color glasses. Eye pieces with split fields are *not* permitted.

   The tube holder (2.54 cm i.d. or 1 in.) is fitted with 30.2 mm ($1^3/_{16}$ in.) i.d. rings at the bottom. One ring is to retain the color tube containing the oil test sample, and the other is to permit an equal amount of light to reach the color glasses.
2. Colorimeters—The following colorimeters have been found to be acceptable for use in this method:
   (a) The Wesson colorimeter, constructed as shown in Figure 1.
   (b) The Stevenson colorimeter conforms to the specifications noted in Apparatus, 1 for the Wesson colorimeter and may be used in this method (see References, 1).
   (c) The Lovibond Tintometer model AF 710 (Notes, 1) conforms to the specifications noted in Apparatus, 1 and may be used in this method when operated according to the manufacturer's instructions. Any subsequent improved model(s), using the same optical principles as the AF 710, may also be used in this method.
3. Color cabinet—The colorimeter is maintained in a booth or cabinet, not less than 102 cm wide × 76 cm deep (40 in. wide × 30 in. deep). The booth or cabinet is closed so that no external light can enter. The inside of the booth is painted a dull, neutral gray of Munsell value 4/.

   The booth or cabinet is indirectly illuminated by means of a bulb and fixture mounted 122 cm (48 in.) above the colorimeter in such a way that no direct light rays strike the colorimeter or the eye of the observer. The level of illumination of the booth (at the top of the box of the colorimeter) should be not less than 1 nor more than 5 foot candles.
4. Color glasses—Glasses must conform to the AOCS-Tintometer Color Scale, within tolerances as follows:

Value of glasses	Tolerance, ±
0.1 to 0.9 red, inclusive	0.05
1.0 to 5.0 red, inclusive	0.10
6.0 to 10.0 red, inclusive	0.20
Above 10.0 red	4% of declared value

Glasses with valid calibrations to the N" Priest-Gibson Scale conform to the AOCS-Tintometer Color Scale. Glasses are supplied and guaranteed by the Tintometer Company (USA) and Tintometer, Ltd. (UK and Europe), to conform to the AOCS-Tintometer Color Scale. Such glasses are identified by the letter "T" engraved on the glass with the color value. Red glasses of the Lovibond Color Scale Series 200 do not conform.

Standard-type glasses of the AOCS-Tintometer Color Scale are deposited with Tintometer, Ltd., Salisbury, England and the National Institute of Testing, Washington, D.C., USA. In the case of disputes, arrangements for checking glasses may be made with the manufacturer or the National Institute of Testing.

The minimum standard set shall consist of the following numbers of red and yellow glasses:

Red*: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 7.6, 8.0, 9.0, 10.0, 11.0, 12.0, 16.0, 20.0

Yellow: 1.0, 2.0, 3.0, 5.0, 10.0, 20.0, 35.0, 50.0, 70.0

*Glasses above 1.0 red need not have the exact value shown, but should cover the same range.

Note—Keep the color glasses clean and free from any oil film. Handle carefully and protect against scratches. It is especially important that every color glass used be clean at the time of use.

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5. Color tubes—
   (a) Description—Color tubes should be made of clear, colorless glass with a smooth, flat, polished bottom and have dimensions as follows: length, 154 mm; overall inside diameter, 19 mm; outside diameter, 22 mm.
   (b) Markings—The color tubes should have two etched marks: one to indicate an oil column of 133.35 mm (5.25 in.), and another to indicate an oil column of 25.4 mm (1 in.).
   (c) Suppliers—In the USA, suppliers of these color tubes include Fisher Scientific and Wilkens-Anderson Co., Chicago, IL.
6. Filter paper—qualitative, medium porosity, to provide medium rate of filtration, 250 mm.

PROCEDURE

1. Crude, raw and refined oil test samples must be treated with 0.5 g of diatomaceous earth (filter aid) per 300 g of oil. Add the diatomaceous earth to the oil and agitate for 2.5 min at 250 rpm at room temperature (or at no more than 10°C above the melting point of the test sample, if it is necessary to maintain the test sample in the liquid state), and filter the test sample through an appropriate filter paper (Apparatus, 6). Oils that have been bleached in the laboratory in accordance with AOCS Official Methods Cc 8a-52, Cc 8b-52 or CC 8d-55 normally are sufficiently clear for the color determination. Suspended material, even if of colloidal size, will cause light scattering. If the test portion is not absolutely clear, treat with diatomaceous earth (filter aid) as noted, and filter before proceeding with the color determination.
2. Adjust the temperature of the test portion to 25–35°C, and fill the color tube to the desired mark. If the test portion is not completely liquid at 25–35°C, heat to a temperature of not more than 10°C above the melting point of the test portion.
3. Place the tube containing the test portion in the colorimeter and place along side of it such red and yellow glasses [see paragraphs (a-e) immediately following and Notes, 2] as are necessary to match the color of the oil, observing the test portion of the oil and the glasses through the eyepiece.
   (a) Crude and raw oil color—For crude oils of the coconut type, read the color using the proper ratio of yellow to red listed below:

   Up to 3.9 red use 6 yellow to 1 red
   4.0 to 4.9 red use 25 yellow to 1 red
   5.0 to 5.9 red use 30 yellow to 1 red
   6.0 to 6.9 red use 35 yellow to 1 red
   7.0 to 7.9 red use 40 yellow to 1 red
   8.0 to 10.9 red use 50 yellow to 1 red
   11.0 to 14.9 red use 70 yellow to 1 red
   15.0 to 19.9 red use 100 yellow to 1 red
   Above 20.0 red use 150 yellow to 1 red

   If the above ratios fail to give a satisfactory match, this fact should be noted and a second reading made using the amount of yellow color required for a good match. *Report both readings.*
   (b) Raw inedible oils—For raw inedible oils (such as tallows, greases, fatty acids, etc.), use the ratios of yellow to red listed below:

   Up to 3.5 red use 10 yellow to 1 red
   3.5 to 5.0 red use 35 yellow to 1 red
   Above 5.0 red use 70 yellow to 1 red

   (c) Dark oils—If the color of the oil or fat test sample exceeds 40.0 red when using the regular 133.35 mm column, fill another tube to the 25.4 mm mark and read the color under the same conditions as described for the longer column. Report the height of the column used, because it is assumed that any color result in which the column height is not designated has been determined on a 133.35 mm column.
   (d) Refined oils—For determining refined oil color, use only one yellow glass; 35 yellow for refined cottonseed and refined peanut oils, 70 yellow for refined soybean oil. Use not more than two red glasses up to and including 13.0 red, and not more than three red glasses above 13.0 red.
   (e) Refined and bleached oils—For refined and bleached oils, the ratios of yellow to red to be used in determining color are as follows, except where trading rules specify the yellow and/or red to be used in classifying particular grades:

   Cottonseed, peanut and corn oils—

   Up to 3.5 red use 10 yellow to 1 red
   Above 3.5 red use 35 yellow to 1 red

   Coconut and palm kernel oils—

   Up to 3.9 red use 6 yellow to 1 red
   Above 3.9 red use 10 yellow to 1 red

   Soybean oil—

   Up to 3.5 red use 10 yellow to 1 red
   Above 3.5 red use 70 yellow to 1 red

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Tallows, greases, fatty acids, etc.—
Up to 3.5 red use 10 yellow to 1 red
3.5 to 5.0 red use 35 yellow to 1 red
Above 5.0 red use 70 yellow to 1 red

REPORTING

1. When any color result is reported, it is usually assumed that the column height of the oil on which the color of the test portion is determined is 133.35 mm (5.25 in.). Therefore, if the color of a test portion is determined on a column of oil other than 133.35 mm (5.25 in.), the height of the column *must* be reported with the color value.

NOTES

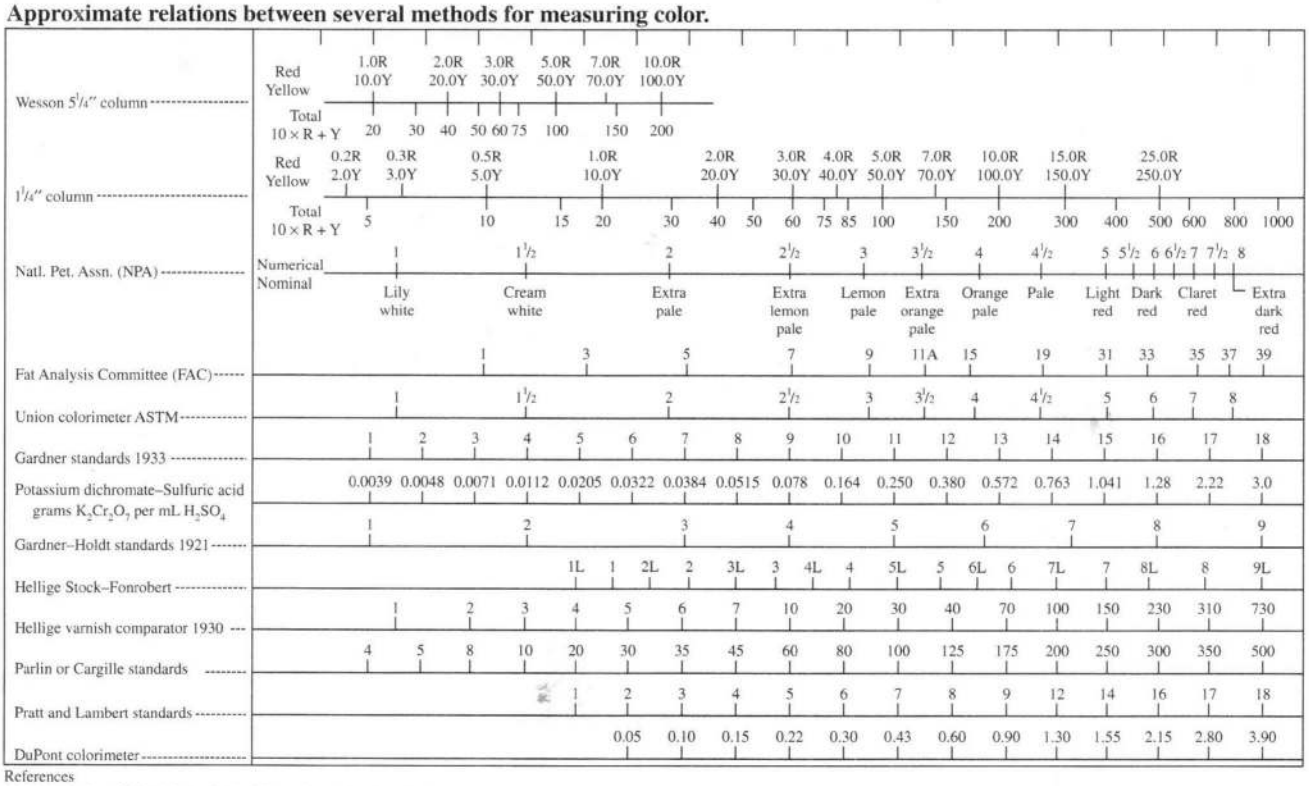
1. Details of suppliers of suitable apparatus may be obtained from The Tintometer Limited, Wiltshire, SPI 2JY, UK. Tel: +44 1722 327242, Fax: +44 1722 412322, E-mail: sales@tintometer.com
2. Some oils are at times subject to abnormalities in the composition of their pigment content, leading to off colors (or "hues") in the oils. The hues that occur usually cannot be matched, even approximately, using the fixed yellow or yellow-to-red ratios noted in this method. In such cases, if a standard combination is specified, report whether the oil is lighter or darker than the standard. If a standard combination is not specified, report the red and yellow glasses whose color most closely resembles the color of the test portion.
3. A comparison of different color scales is presented in Table 1. This information is presented for comparison purposes only.

REFERENCES

1. *Oil and Soap 13:*18 (1936).

Table 1.

Approximate relations between several methods for measuring color.



References

1. Mehlenbacher, V. C., *The Analysis of Fat and Oils*, 1960, p. 443.
2. *J. Am. Oil Chem. Soc. 30:*259 (1953).

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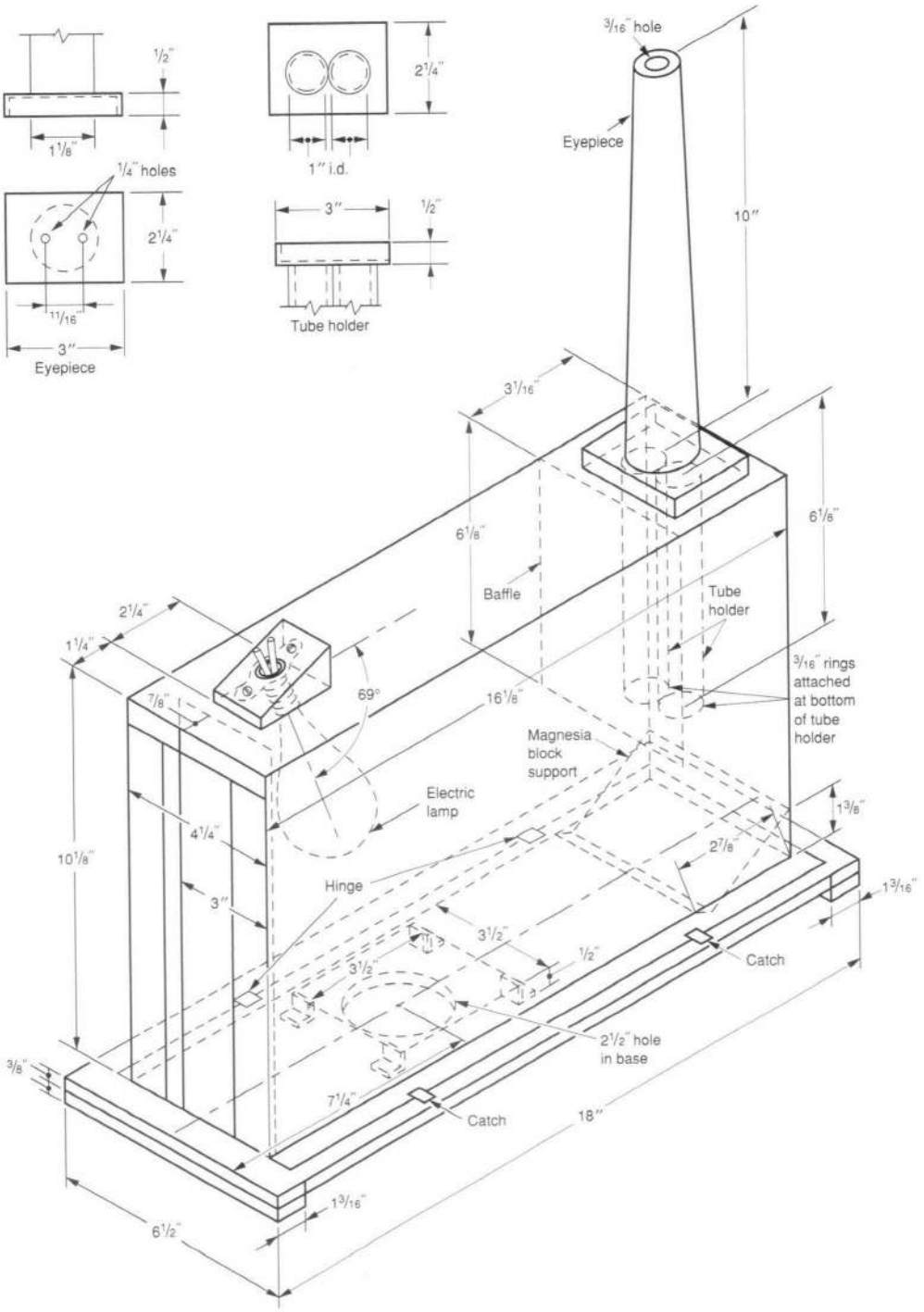

Figure 1. Color measurement apparatus.

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Color Spectrophotometric Method

DEFINITION

This method designates the color of fats and oils with a single number (see Notes, 1).

SCOPE

Applicable to cottonseed, soybean, and peanut oils (see Notes, 2). This method requires revision to accommodate the use of contemporary double-beam spectrophotometers. (Method in Temporary Surplus status pending revision.)

APPARATUS

1. Spectrophotometer—capable of adjustment to give the following readings on a standard nickel sulfate solution at 25–30°C, after setting the zero point of the instrument and after adjusting the 100% transmittance point (0 absorbance) against methylene chloride (CH_2Cl_2) in a cuvette, having the specifications noted in Apparatus, 2.

nm	Transmittance
400	< 4.0%
460	26.2 ± 2.0%
510	73.9 ± 1.0%
550	54.8 ± 1.0%
620	5.2 ± 0.5%
670	1.1 ± 0.5%
700	< 2.0%

2. Matched glass cylindrical cuvettes—approximately 21.8 mm i.d., approximately 24.5 mm o.d. (see Notes, 3). All cuvettes to be used with a given instrument should be checked with methylene chloride and nickel sulfate solution at 550 nm and should give the same transmittance within ± 0.6%. The cuvettes should be kept clean and free from scratches.
3. Filter paper—qualitative, medium porosity, to provide medium rate of filtration, 250 mm.

REAGENTS

1. Methylene chloride (CH_2Cl_2)—reagent grade (see Notes, 4 and *Caution*). The transmittance should not differ from distilled water by more than 0.5% at 400 nm.
2. Nickel sulfate ($NiSO_4 \cdot 6H_2O$)—reagent grade.
3. Hydrochloric acid (HCl)—reagent grade, concentrated, sp gr 1.19 (see Notes, *Caution*).
4. Standardizing nickel sulfate solution—prepared by dissolving 200 g of reagent-grade $NiSO_4 \cdot 6H_2O$, reagent grade, in distilled water, adding 10 mL of concentrated HCl and diluting to exactly 1000 mL with distilled water in a volumetric flask. The temperature of the solution should be between 25 and 30°C. The nickel content of the solution must fall between 4.40 and 4.46 g of nickel/100 mL at 25–30°C.
5. Diatomaceous earth (filter aid)—Manville HyFlo Supercel, or equivalent.

CALIBRATION OF SPECTROPHOTOMETER

1. Turn on the spectrophotometer and allow a suitable warmup period before standardizing or making any adjustments.
2. After the initial warm-up period, adjust the instrument so that an exact zero reading is obtained.
3. Set the wavelength to 460 nm. Recheck the zero reading of the instrument, insert a cuvette filled with CH_2Cl_2 in the instrument and set the 100% transmittance point exactly.
4. Fill a cuvette with the standardizing $NiSO_4$ solution and measure the transmittance of the solution. The reading must fall between 24.2 and 28.2.
5. In a like manner, set the instrument at 550 nm and measure the transmittance of the solution. The reading must fall between 53.8 and 55.8.
6. If the 460 nm measurement is above 26.2, the 550 nm measurement should be above 54.8; if the 460 measurement is below 26.2, the 550 measurement should be below 54.8; otherwise, adjust the wavelength knob underneath the instrument until both measurements are in the same direction above or below the median values established, but within the specific limits.
7. Set the instrument at 510 nm and measure the transmittance of the $NiSO_4$ solution. The 510 nm reading must fall between 72.9 and 74.9.
8. Determine the other specified values. All must fall within the limits noted in the table in Apparatus, 1.

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PROCEDURE

1. The refined oil test sample must be treated with 0.5 g of diatomaceous earth (filter aid) per 300 g of oil. Add the diatomaceous earth to the oil, agitate for 2.5 min at 250 rpm at room temperature (or at not more than 10°C above the melting point of the fat) and filter.

   Note—Test samples other than refined oil must be absolutely clear. Suspended material, even if colloidal in size, will cause light scattering. If the test portion is not absolutely clear, filter through one of the specified filter papers at a temperature of 10–15°C above the melting point of the fat. If not perfectly clear, treat with diatomaceous earth as outlined above.
2. Adjust the temperature of the oil to 25–30°C, fill the cuvette, using a sufficient amount of oil of ensure a full column in the light beam.
3. Place the filled cuvette in the instrument and measure the absorbance, A, to the nearest 0.001 at 460, 550, 620, and 670 nm.

CALCULATIONS

1. Photometric color index = $1.29(A_{460}) + 69.7(A_{550}) + 41.2(A_{620}) - 56.4(A_{670})$

NOTES

Caution

Methylene chloride is toxic and a carcinogen. The TLC is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. Collaborative study results reported by AOCS Technical Committee on Color (see References, 1). Also see AOCS Technical Committee report in References, 2. The collaborative study results showed a correlation coefficient of 0.993 between spectrophotometric color and Lovibond color.
2. The color of other fats and oils that have not yet been studied can probably be designated by this method.
3. The cuvettes are test tubes, which may be obtained from Kontes, USA.
4. Methylisobutylketone (MIBK) may be a suitable substitute for methylene chloride.

REFERENCES

1. *J. Am. Oil Chem. Soc. 27:*233 (1950).
2. *Ibid. 25:*271 (1948).

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Chlorophyll Pigments

DEFINITION

This method is used to determine mg/kg (ppm) of chlorophyll-related pigments (predominantly pheophytin a) in oils from spectrophotometric absorption measurements at 630, 670, and 710 nm (see Notes, 1).

SCOPE

Applicable to expelled, refined and bleached oils. This method is not applicable to hydrogenated oils, deodorized oils and finished products, because in these processed oils, the absorption maximum does not occur at 670 nm (see Notes, 2 and 3).

APPARATUS

1. Spectrophotometer.
2. Cuvettes—50 mm long, 20 mm wide, and 40 mm deep. These cuvettes must be matched and differ from each other by not more than 0.5% transmittance when checked with distilled water at 400 nm. Any suitable matched cuvettes with 50 mm light path may be used. Cuvettes with shorter light path, carefully matched, should be available for oils with an absorbance >0.8 at the wavelengths used.

REAGENTS

1. Methylene chloride (CH_2Cl_2) or methylisobutylketone (MIBK)—reagent grade (see Notes, *Caution*). The transmittance should not differ from that of distilled water by more than 0.5% at 400 nm.

ADJUSTMENT OF SPECTROPHOTOMETER

1. Before using, the spectrophotometer should be checked carefully following the instructions given in the operating manual obtained with the instrument. The wave-length setting of the instrument should be checked with the blue sensitive phototube in the instrument. With the instrument turned on and with no cuvettes in the cuvette compartment, the maximum sensitivity obtainable with the instrument should occur at approximately 540 nm. It is advisable to check the instrument occasionally against a mercury vapor lamp using 579.1, 546.1, 435.8, and 404.7 nm as check points. Any mercury vapor lamp can be focused to pass through the instrument in place of the regular incandescent lamp normally used. Transmission peaks should occur at the specified wavelengths.

PROCEDURE

1. Turn on the spectrophotometer.
2. Place a 50 mm cuvette containing the CH_2Cl_2 or MIBK in the cuvette compartment. Zero the instrument.
3. Adjust the temperature of the oil to 25 ± 2°C. The oil test sample must be clear and free of moisture, haze, and particulate matter. Fill a second 50 mm cuvette with the oil test sample and place in the cuvette compartment of the spectrophotometer.
4. Measure the absorption of the oil test sample. Adjust the wavelength setting to the next desired wavelength with either CH_2Cl_2 or MIBK in the light path. Adjust the instrument to give a zero reading. Place the test portion in the light path and measure the absorbance. Make all desired readings following this procedure.
5. All absorbance values should preferably fall between 0.3 and 0.8. Use the maximum cuvette size to give the desired values. On bleached oils, absorbencies of less than 0.3 will be obtained.

CALCULATIONS

1. Concentration of chlorophyll pigments—

$$\text{mg/kg (ppm) } C = \frac{A_{670} - (A_{630} + A_{710})/2}{F \times L}$$

Where—

C = chlorophyll pigments
A = absorbance
L = cuvette length, cm
F = factor specific to each spectrophotometer (see Notes, 3)

NOTES

Caution

Methylene chloride is toxic and a carcinogen. The TLC is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

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NUMBERED NOTES

1. This method is not directly applicable to processed and heated oils, because in such oils part or all of the chlorophyll-related pigments may be converted to products that do not exhibit an absorbance maximum at 670 nm.
2. Several variations of this method have been proposed; they include the following:
   (a) Treatment with acetic acid (to convert any remaining chlorophyll to pheophytin), and determining the total pheophytin content, to yield more accurate results. (The pheophytin content is related to the original chlorophyll content.)
   (b) Individually determine chlorophyll and pheophytin by HPLC, using appropriate chlorophyll and pheophytin standards.
   (c) Determine the actual absorbance maximum of the test portion and substitute this value in the equation used in the calculation for mg/kg (ppm) chlorophyll.
   (d) Determine the chlorophyll pigments spectrophoto-metrically as pheophytin a as follows (References, 1 and 2):

$$C = \frac{345.3 \times (A_{670} - 0.5 \times A_{630} - 0.5\ A_{710})}{F \times L}$$

   Where—
   C = concentration of pigments in mg/kg (ppm) oil
   A = absorbance at respective wavelengths
   L = thickness of spectrophotometer cuvette
3. Factors may be determined for spectrophotometer models currently available, using chlorophyll standards. However, due to the instability of chlorophyll, if this approach is taken, the analyst is advised to check the composition of the chlorophyll standards by an appropriate method, e.g., HPLC. The true composition of the standards as so determined may then be used to calibrate the spectrophotometer.

REFERENCES

1. *La Revista Italiana delle Sostanze Grasse 67*:521 (1990).
2. IUPAC Commission on Oils and Fats, Working Report, International Union of Pure and Applied Chemistry, July 9, 1990, p. M-10.

OTHER REFERENCES

Fette, Seifen, Anstrichm. 86 (8):304 (1984).
J. Am. Oil Chem. Soc. 62 (1):113 (1985).
Rev. Fr. Corps Gras 32 (6–7):257 (1985).

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Color

Lovibond Method Using Color Glasses Calibrated in Accordance with the Lovibond Tintometer Color Scale

DEFINITION

The method determines color by matching the color of the light transmitted through a specific depth of liquid fat or oil to the color of the light originating from the same source, transmitted through glass color standards.

SCOPE

Applicable to all normal animal and vegetable fats and oils, providing no turbidity is present in the test sample. This method is harmonized with ISO 15305. This method is the accepted international standard for the measurement of color in animal and vegetable fats and oils.

APPARATUS

1. Usual laboratory apparatus.
2. Colorimeter—maintained in good, clean condition in accordance with the manufacturer's recommendations (see Notes, 1).
3. Lighting cabinet—consisting of two 60-watt pearl (not coated) lamps, operated at the correct main voltage and each illuminating the test portion and white reference field at 45 degrees. The lamps shall be positioned at either side of the viewing tube. (See Notes, 2.)

   Note—The lamps shall not be used for longer than 100 hours. A suitable logging system shall be employed to register lamp life. Lamps shall be changed in pairs.

   The viewing tube shall have a field of view subtending 2 degrees at the eye and contain a daylight correction filter.

   The lighting cabinet shall be such that enables the test portion and white reference field to be viewed at 90 degrees to normal. The lighting cabinet shall be inspected at frequent intervals for any dirt particles, which shall be removed, and for aging of the paint. The cabinet shall be repainted with a matte white paint when the color becomes darker than Munsell Notation 5Y 9/1 (obtainable from Tintometer Ltd.).
4. Color racks—consisting of red, yellow, blue, and neutral racks with color readings as follows, and fitted with colorless compensating slides:

Red	0.1–0.9, 1.0–9.0, 10.0 and 70.0
Yellow	0.1–0.9, 1.0–9.0, 10.0–70.0
Blue	0.1–0.9, 1.0–9.0, 10.0–40.0
Neutral	0.1–0.9, 1.0–3.0

5. Spillage tray (where required).
6. Glass cells—made of optical glass with optical path lengths as follows (see Notes, 2 and 3):

   1.6 mm ($\frac{1}{16}$ in.), 3.2 mm ($\frac{1}{8}$ in.), 6.4 mm ($\frac{1}{4}$ in.)

   12.7 mm ($\frac{1}{2}$ in.), 25.4 mm (1 in.), 76.2 mm (3 in.),

   133.4 mm ($5\frac{1}{4}$ in.)

SELECTION OF OPERATORS

1. All operators shall satisfy the requirements of a color vision test and shall be retested at five yearly intervals (see Notes, 5).
2. Tinted or light-sensitive spectacles or lenses shall not be worn (see Notes, 6).

SAMPLING

1. The test sample shall be taken in accordance with acceptable sampling techniques, e.g., as outlined in ISO 5555 or AOCS C 1–47.

PREPARATION OF TEST SAMPLE

1. Prepare the test sample in accordance with standard procedures. It is essential that the fat or oil is completely liquid, clear, and bright when the determination is made. Heating shall be avoided if it is likely to cause a color change. If the test sample is not liquid at room temperature, heat it to a temperature about 10°C above melting point.

PROCEDURE

1. It is essential that the determination be carried out in subdued ambient light, i.e., not facing a window or in direct sunlight.

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2. Pour the prepared test sample into a cell (Apparatus, 6) of sufficient optical path length to give color readings within the ranges given in Apparatus, 4. Ensure that the cell is thoroughly clean and dry and, if necessary, prewarmed so that no solid matter separates from the test sample during the determination.
3. Place the cell containing the test portion in the lighting cabinet close to the viewing tube (Apparatus, 3). Immediately determine the color of the test portion, initially by using the color racks in the ratio of 10 yellow to 1 red. Correct until an accurate color match is obtained, without necessarily maintaining the ratio of 10 yellow to 1 red and using the minimum number of blue or neutral units to obtain the match, but not more than 9.0 blue or 3.0 neutral.

   Note—To ensure that the number of glass surfaces in both test portion and color filter fields are the same, the racks holding the color filters are fitted with compensating slides.
4. Because the onset of eye fatigue is rapid, the operator shall rest the eyes after each 30-sec period of matching. The test must be carried out by two trained operators.
5. If the requirements noted in the Repeatability section and Table 1 are not satisfied, a third trained operator must carry out the test. The mean of the two closest readings (of three) should be taken.
6. Record the size of cell used and the red, yellow, blue, or neutral readings forming the color match.

EXPRESSION OF RESULTS

1. Take the mean of the results obtained by the two trained operators. If the requirements of repeatability (see Repeatability section and Table 1) are not met, the mean of the two closest readings (of three) should be taken (see Procedure, 5).
2. Express the results in terms of the following:
   (a) the number of red, yellow, and blue or neutral readings needed to obtain the match;
   (b) the length of cell used.
3. Color measurements taken in one cell length shall not be used to calculate the color values for another cell length.

PRECISION

The details of an interlaboratory test are given in Table 1. The values presented may not be applicable to matrices other than those presented and may not be representative for other concentrations.

REPEATABILITY

The difference between two test results on the same material, in the same laboratory under the same conditions, should not exceed the repeatability value, *r*.

REPRODUCIBILITY

The difference between two test results on the same material, under the same conditions in different laboratories, should not exceed the reproducibility value, *R*.

TEST REPORT

1. The test report shall show the method used and the result obtained. It shall also mention any operating conditions not specified in this standard or regarded as optional, as well as any circumstances that may have influenced the results.
2. The report shall include all details required for the complete identification of the test portion.

NUMBERED NOTES

1. The universal Lovibond Tintometer Model F/C and Model F (BS684) (or earlier/subsequent equivalents), have been found suitable. Details of suppliers may be obtained from The Tintometer Limited, Waterloo Road, Salisbury, Wiltshire, SPI 2JY, UK. Tel: +44 1722 327242; Fax: +44 1722 412322. E mail: sales@tintometer.com

   The Lovibond Schofield Tintometer, Wesson Colorimeter and the AOCS Tintometer have not been found suitable for use in this method.
2. Tintometer AF 905/E, AF 900/C and Model E are constructed to meet these specifications.
3. In practice, including international trade, the imperial units are used at present.
4. Glass cells should be made of good quality optical glass with a high standard finish.
5. Preferably, the color vision test should be carried out by a qualified optician.
6. Operators who normally wear eyeglasses or contact lenses may continue to wear them.

REFERENCES

1. Animal and Vegetable Fats and Oils—Determination of Lovibond Colour, ISO 15305.

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Table 1
The results of an international collaborative test held by FOSFA International (values in Lovibond units).

	Palm oil (RBD)		Crude palm kernel oil	
Color scale: 133.4 mm cell	Red	Yellow	Red	Yellow
n	9	9	9	9
Mean (in Lovibond units)	2.30	21.60	5.00	47.7
Repeatability				
Sr	0.07	1.22	0.25	2.35
r	0.20	3.42	0.71	6.58
Reproducibility				
SR	0.29	1.80	0.76	4.34
R	0.81	5.04	2.12	12.26

Table 2
Repeatability and reproducibility limits.

Color scale	Level	R	R
Red: 133.4 mm cell	2	0.2	0.8
(5 $^{1}/_{4}$ inch)	5	0.7	2
Yellow: 133.4 mm cell	20	3	5
(5 $^{1}/_{4}$ inch)	50	6	12

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AOCS Recommended Practice Cc 13f-94

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Refined and Bleached Color and Saponification Color

DEFINITION

This recommended practice determines the color of the test sample after treatment with alkali and a specified bleaching earth (Part I) and after saponification of the refined and bleached test sample (Part II).

SCOPE

Applicable to tallows and greases intended for soap production. Part I determines bleached and refined color and Part II determines saponification color.

APPARATUS

1. Hot water funnel or other suitable funnel heater—adjustable to such temperatures that the test sample will remain completely liquid during filtration.
2. Water bath—adjustable to a temperature range of 52–68°C.
3. Lovibond Tintometer—AOCS Wesson Method Cc 13b-45.
4. Beakers—1000 and 500 mL.
5. Magnetic stirrer.
6. Hot plate.
7. Cuvette—5¼ in.
8. Erlenmeyer flask—250 mL.

REAGENTS

1. Potassium hydroxide solution (KOH)—50% aqueous (wt%)—prepared by dissolving 115 g of C.P. potassium hydroxide pellets (87% pure) Baker "analyzed" reagent or equivalent, in 100 g of distilled water.
2. Sodium hydroxide (NaOH)—20° Bé at 15.56°C, free from carbonate and other impurities. The actual strength must be determined by weighing and titrating with standard acid.
3. Reagent alcohol.
4. AOCS Official Activated Bleaching Earth—tested and approved by the AOCS.

PREPARATION OF TEST SAMPLE

1. Melt the test sample, if it is not completely liquid, and shake or stir thoroughly to obtain a uniform mixture. Avoid local overheating and do not heat to more than 10°C above the melting point.

PROCEDURE

Part I—Refined and Bleached Color

1. Weigh 500 g of the thoroughly mixed test sample of crude tallow or grease into a 1000-mL beaker and heat in a water bath to 51–55°C.
2. Find the % excess NaOH in Table 1 corresponding to the % free fatty acid (FFA). Substitute these two values in the formulae to determine the amount of alkali solution to use (See Notes, 1).

$$\frac{\%\ \text{FFA in fat as oleic}}{7.05} = \%\ \text{NaOH to neutralize free fatty acids} = A$$

$$\frac{(A + \%\ \text{Excess NaOH required from Table 1}) \times 500}{\text{Actual \% NaOH in 20 Bé NaOH}} = \text{g of 20° Bé NaOH solution to add to 500 g of test portion}$$

3. Start stirring at 250 rpm and add the specified amount of 20° Bé alkali solution, as calculated above, and continue agitation after addition of the alkali for 5 min (See Notes, 2).
4. Change the stirring speed to 70 rpm and increase the bath temperature immediately to 60–64°C. Stir under these conditions for 10–15 min. It may sometimes be necessary to add a small amount of water to the mixture of tallow and foots to hasten coagulation and ensure complete precipitation.
5. When the foots appear to be ready to settle, discontinue stirring and allow to stand until the foots have settled and the fat is clear. The condition of the foots can be determined by removing a small portion of the mixture from the beaker with a spoon and observing the tendency to settle.

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6. Filter a sufficient quantity through a 60 mesh screen or any rapid filter paper [(E & D No. 617 (25 cm) or Reeve-Angel No. 230, 24 cm) or equivalent] using a funnel heater and then weigh 300 ± 1.0 g of filtered test portion into a 500 mL beaker (See Notes, 3).
7. Heat the tallow or grease on a hot plate to 105–110°C. Add 3% (9 g) of AOCS Official Activated Bleaching Earth.
8. Stir at 250 rpm for 5 min, maintaining the temperature at 105–110°C.
9. Filter immediately through a Whatman #4 filter paper or equivalent using a funnel heater. As soon as the test portion filters clearly, transfer a sufficient volume to a 5¼ in. cuvette and read the Lovibond Color. See Table 2.

Part II—Saponification Color

1. Weigh 50.0 ± 0.1 g of the filtered, bleached and refined test sample from Part I, Procedures, 9, into a 250 mL Pyrex™ Erlenmeyer flask.
2. Before developing the color of the test portion, run a reagent blank using the same volume of reagents but omitting the test portion. If the blank is not colorless, it is necessary to run the test portion using purified reagent alcohol.
3. Add, using suitable pipets, 50 mL of reagent alcohol and 20.0 mL of a 50% aqueous KOH solution to the test portion in the flask.
4. In a water bath, heat the contents of the flask to 68°C, with continuous swirling until the oil is saponified. The completion of saponification can be estimated by noting the complete solution of the oil and the clearing of the solution. The test portion completely saponifies in 3–5 min.
5. Transfer a sufficient volume of the saponified solution to a 5¼ in. cuvette and read the Lovibond color (AOCS Method Cc 13b-45). The color must be read immediately, some test portions darken on standing. Report the numbers of the yellow and red glasses which match the color of the oil; or if a standard combination is specified (as prime, for example) report whether the oil is prime or off as compared with the standard.

NOTES

1. Excess NaOH is necessary to ensure satisfactory separation of the foots. Experience indicates that the % of actual NaOH excess calculated on the basis of the fat weight must be increased with increasing % FFA in the stock. In general, satisfactory foots separation results when NaOH in excess of the amount required to react with FFA (calculated as Oleic Acid) as in Table 1 is added.
2. To obtain uniform results, it is recommended that all laboratories purchase a tachometer for testing and regulating the speed of the stirrers. This should be done at least once a month.
3. When the foots do not separate to permit decantation of the clear oil, it is recommended that only sufficient test portion (100 g) be filtered off for the bleaching step using the proportional amount of bleaching earth (3 g).

REFERENCES

1. O'Daniel, L. and Parsons, L.B., *Oil and Soap 20:72* (1943).
2. *AOCS Official Methods*, Cc 8d-55, (1993) "Refined and Bleached Color."

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Table 1
Percent Excess NaOH corresponding to FFA.

% FFA in Fats Required as Oleic Acid	% Excess 20° Bé NaOH
0.10–4.00	0.2
4.01–6.00	0.3
6.01–8.00	0.5
8.01–15.00	0.6

Table 2
Lovibond color.

The ratio of yellow to red in determining color should be as follows, except where rules specify the yellow and/or red to be used in determining given grades:

Cottonseed Oil	
(Refined):	35 yellow with necessary red to obtain best match.
(Bleached):	10 yellow to 1 red.
Coconut Oil	
(Refined or bleached):	6 yellow to 1 red when red < 3.0.
Palm Kernel Oil	
(Refined or bleached):	6 yellow to 1 red when red < 3.0.
Peanut Oil	
(Refined or bleached):	10 yellow to 1 red when red < 3.5.
Soybean Oil	
(Refined or bleached):	10 yellow when red > 3.5. 70 yellow when red > 3.5.
Tallows, Greases, Fatty Acids, etc.:	10 yellow to 1 red when red < 3.5. 35 yellow where red is 3.5–5.0. 70 yellow where red > 5.0.

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AOCS Recommended Practice Cc 13g-94
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High-Quality Tallows and Coconut Oils, Saponification Color

DEFINITION
The color of the test sample is determined after saponification of the test sample with a caustic.
SCOPE
Applicable to high-quality tallows and coconut oils.

APPARATUS

1. Water bath—adjustable to a temperature range of 51–68°C.
2. Alkali resistant Erlenmeyer flask—250 mL.
3. Pipets—50, 30, and 1.5 mL.
4. Cuvette—5¼ in.
5. Reflux condenser.

REAGENTS

1. Potassium hydroxide solution (KOH)—50% aqueous.
2. Reagent alcohol.

PREPARATION OF TEST SAMPLE

1. Melt the test sample if it is not completely liquid and stir thoroughly to obtain a uniform mixture. Avoid local overheating and do not heat to more than 10°C above the melting point.
2. If the test sample is not absolutely clear, filter the test sample to remove impurities and moisture.

PROCEDURE

1. Accurately weigh 50.1 ± 0.1 g of test sample into an alkaliresistant 250 mL Erlenmeyer flask provided with a reflux condenser. An all glass apparatus provided with standard taper joints is recommended.
2. Add, using suitable pipets, 50 mL of reagent alcohol and 31.5 mL of the 50% aqueous KOH solution to the test sample in the flask.
3. In a water bath, heat the contents of the flask to 68°C with continuous swirling until the oil is saponified. The completion of saponification can be estimated by noting the complete solution of the oil and the clearing of the solution.
4. Transfer a sufficient volume of the saponification solution to a 5¼ in. cuvette and read the Lovibond color (AOCS Method, Cc 13b-45).
5. Run a blank (Procedure 1–4) using the same volume of reagents, but omitting the test portion. If the blank is not colorless, it is necessary to rerun the test portion using purified reagent alcohol in Procedures, 2.

REFERENCES

1. *AOCS Official Method* Cc 13f-94 "Tallows and Greases—Refined and Bleached Color and Saponfication Color."

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AOCS Recommended Practice Cc 13h-94
Reapproved 2009

Tallows and Coconut Oils, Saponification Color

DEFINITION

The color of the test sample is determined after saponification of the test sample with a caustic. This method includes the use of the Automatic Lovibond Tintometer.

SCOPE

Applicable to tallows and coconut oils.

APPARATUS

1. Water bath—adjustable to a temperature range of 51–68°C.
2. Alkali resistant Erlenmeyer flask—250 mL.
3. Pipets—50 and 20 mL.
4. Cuvettes—5¼ in.

REAGENTS

1. Potassium hydroxide solution (KOH)—50% aqueous.
2. Reagent alcohol.

PREPARATION OF TEST SAMPLE

1. Melt the test sample if it is not completely liquid and stir thoroughly to obtain a uniform mixture. Avoid local overheating and do not heat to more than 10°C above the melting point.
2. If the test sample is not absolutely clear, filter the test sample to remove impurities and moisture.

PROCEDURE

1. Weigh 50.0 ± 0.1 g of test sample into a 250 mL Erlenmeyer flask.
2. Before developing the color of the test portion, run a reagent blank, using the same volume of reagents, but omitting the test portion. If the blank is not colorless, it is necessary to purify the reagent alcohol.
3. Add, using suitable pipets, 50 mL of reagent alcohol and 20.0 mL of the 50% aqueous KOH solution to the test portion in the flask.
4. In a water bath, heat the contents of the flask to 68°C with continuous swirling until the oil is saponified. The completion of saponification can be estimated by noting the complete solution of the oil and the clearing of the solution. The test portion completely saponifies in 3–5 min.
5. Transfer a sufficient volume of the saponified solution to a 5¼ in. cuvette and read the Lovibond color (AOCS Method Cc 1d-92). See Table 1. The color must be read immediately. Some test portions darken on standing. Report the numbers of yellow and red glasses which match the color of the oil; or, if a standard combination is specified (as prime, for example), report whether the oil is prime or off as compared with the standard.

REFERENCES

1. O'Daniel, L. and Parsons, L.B., *Oil and Soap 20:*72 (1943).

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Table 1
Lovibond Color.

The ratio of yellow to red in determining color shall be as follows, except where rules specify the yellow and/or red to be used in determining given grades:

Cottonseed Oil (Refined):	35 yellow with necessary red to obtain best match.
(Bleached):	10 yellow to 1 red.
Coconut Oil (Refined or bleached):	6 yellow to 1 red when red < 3.0.
Palm Kernel Oil (Refined or bleached):	6 yellow to 1 red when red < 3.0.
Peanut Oil (Refined or bleached):	10 yellow to 1 red when red ≥ 3.0.
Soybean Oil:	10 yellow to 1 red when red < 3.5.
Corn Oil:	70 yellow when red > 3.5.
Tallows, Greases, Fatty Acids, etc.:	10 yellow to 1 red when red < 3.5. 35 yellow when red is 3.5–5.0. 70 yellow when red > 5.0.

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AOCS

AOCS Official Method Cc 13i-96

Revised 2011

Determination of Chlorophyll Pigments in Crude Vegetable Oils

DEFINITION

The content of chlorophyll pigments in vegetable oils is expressed as mg of pheophytin a in 1 kg of oil.

SCOPE

This method determines total chlorophyll pigments, expressed as pheophytin a, in crude vegetable oils. The method is suitable for the determination of quantities of chlorophyll pigments higher than 1 mg/kg (1 ppm). The chlorophyll pigments are determined by measuring the absorbance at 670 nm, correcting the result for the background absorption, and calculating the content with the use of the absorptivity of pheophytin *a,* which is the main chlorophyll pigment in crude vegetable oils.

APPARATUS

1. Spectrophotometer allowing the measurement in the range of 630–710 nm.
2. Glass spectrophotometer cells, with light path of 5 mm and 10 mm.
3. Glass funnel—diameter 40–60 mm.

PROCEDURE

1. The test sample is heated to a temperature no more than 5°C higher than the melting point of the test sample, if the test sample is solid at room temperature. The liquid test sample is mixed thoroughly, and if it is turbid, it is filtered immediately prior to the analysis, using a filter paper of medium porosity.
2. The test sample is measured at 630 nm, 670 nm, and 710 nm, in a 5 mm or 10 mm spectrophotometer cell (Apparatus, 2) against air rather than a reference cell.

CALCULATION OF RESULTS

1. The content of chlorophyll pigments is expressed in mg of pheophytin a, which is calculated as follows:

$$C = 345.3 \times \frac{(A_{670} - 0.5 \times A_{630} - 0.5 \times A_{710})}{L}$$

Where—

C = content of chlorophyll pigments as mg of pheophytin a in 1 kg of oil
A = absorbance at the respective wavelength (nm)
L = light path of the spectrophotometer cell (mm)

PRECISION

1. Repeatability, expressed by the coefficient of variation, RSD_r is—0.4 at high concentration of chlorophyll pigments; 1.0 at moderate concentration of chlorophyll pigments; and 1.4 at low concentration of chlorophyll pigments. See Tables 1–3.
2. Reproducibility, expressed by the coefficient of variation, RSD_R, is—1.3 at high concentration of chlorophyll pigments; 3.5 at moderate concentration of chlorophyll pigments; and 6.0 at low concentration of chlorophyll pigments. (See Tables 1–3.)

REFERENCE

1. Results of the IUPAC Commission on Oils, Fats and Derivatives collaborative study for the determination of chlorophyll pigments in crude vegetable oils, *Pure & Applied Chemistry 67*(10):1781 (1995).

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Table 1
Results of a 1990 collaborative study on the determination of chlorophyll pigments in rapeseed oil.

Test Sample	1	2	3	4	5	6
Total number of laboratories accepted	12	12	12	12	12	12
Mean of the laboratory values (mg/kg)	34.54	11.30	4.02	33.95	15.37	5.88
Repeatability						
Standard deviation, s_r	2.24	2.99	0.90	1.27	1.14	0.97
Relative standard deviation, RSD_r%	6.49	26.45	22.29	3.75	7.43	16.46
Repeatability value, $2.8 \times s_r$	6.34	8.46	2.54	3.60	3.23	2.74
Reproducibility						
Standard deviation, s_R	3.39	4.02	1.78	3.09	2.47	1.66
Relative standard deviation, RSD_R%	9.81	35.57	44.20	9.40	16.06	28.33
Reproducibility value, $2.8 \times s_R$	9.58	11.38	5.03	9.03	6.99	4.71

Table 2
Results of a 1991 collaborative study on the determination of chlorophyll pigments in rapeseed oil.

Test Sample	1	2	3	4	5	6
Total number of laboratories accepted	10	10	7	7	10	9
Mean of the laboratory values (mg/kg)	46.56	51.23	0.20	0.32	17.01	20.93
Repeatability						
Standard deviation, s_r	0.38	0.25	0.01	0.03	0.72	0.21
Relative standard deviation, RSDr%	0.82	0.49	5.04	9.72	4.26	0.98
Repeatability value, $2.8 \times s_r$	1.09	0.72	0.03	0.09	2.05	0.58
Reproducibility						
Standard deviation, s_R	4.01	4.02	0.11	0.13	2.29	0.63
Relative standard deviation, RSD_R%	8.62	7.84	57.69	40.73	13.47	3.01
Reproducibility value, $2.8 \times s_R$	11.36	11.37	0.32	0.13	48.6	1.78

Table 3
Results of a 1992 collaborative study on the determination of chlorophyll pigments in vegetable oil.

Test Sample	1	2	3	4	5	6
Total number of laboratories accepted	12	12	12	11	11	12
Mean of the laboratory values (mg/kg)	54.49	52.39	28.15	25.40	7.28	6.71
Repeatability						
Standard deviation, s_r	0.23	0.10	0.11	0.06	0.06	0.07
Relative standard deviation, RSD_r%	0.66	0.29	0.31	0.18	0.17	0.19
Repeatability value, $2.8 \times s_r$	0.43	0.20	0.39	0.25	0.84	1.00
Reproducibility						
Standard deviation, s_R	0.85	0.69	0.50	1.93	0.28	0.18
Relative standard deviation, RSD_R%	2.39	1.42	1.42	5.40	0.80	0.51
Reproducibility value, $2.8 \times s_R$	1.55	1.31	1.78	7.60	3.90	2.71

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AOCS

AOCS Official Method Cc 13j-97

Reapproved 2009

Color

DEFINITION

This method determines color of refined and refined-bleached-deodorized (RBD) oils and washed-dried filtered and deodorized tallow, providing no turbidity is present in the test sample. Automated method giving results in the AOCS-Tintometer Color (Wesson method) or the Lovibond Color Scales.

SCOPE

This method has been developed for use on refined oils only.

APPARATUS

1. Automated colorimeter—capable of measuring AOCS-Tintometer Color and Lovibond color.
2. Reference standards—one set of three (See Notes, 1).
3. 133.5 mm (5.25 in.) and 25.4 mm (1 in.) optical cells.
4. Soft lint-free wipes.
5. Glass cleaner.
6. Routine laboratory equipment.

PROCEDURE

1. Crude, raw and refined oil test samples must be treated with 0.5 g of diatomaceous earth (filter aid) per 300 g of oil. Add the diatomaceous earth to the oil and agitate for 2.5 min at 250 rpm at room temperature (or at no more than 10°C above the melting point of the test sample), and filter the test sample through an appropriate filter paper. Oils that have been bleached in the laboratory in accordance with AOCS Official Methods Cc 8a-52, Cc 8b-52 or Cc 8d-55 normally are sufficiently clear for the color determination. Suspended material, even if of colloidal size, will cause light scattering. If the test portion is not absolutely clear, treat with diatomaceous earth (filter aid), and filter before proceeding with the color determination.
2. Turn on the colorimeter and perform the required set-up (see O&M manual for details).
3. Set color scale to that for AOCS-Tintometer Color. Set the path length as required (normally 133.5 mm (5.25 in.)). Set heater program to 30°C and switch heater on. Clean all sample cells with glass cleaner and soft, lintfree wipes before each test.
4. Adjust the temperature of the test portion to 30°C, and fill the 133.5 mm (5.25 in.) cell three-quarters full. Similarly with the 25.4 mm (1 in.) cell when required. If the test portion is not completely liquid at 25–35°C, heat to a temperature of not more than 10°C above the melting point of the test portion and maintain that temperature during measurement.
5. Follow manufacturer's instructions to obtain readings.
6. For alternate values, switch to the Lovibond Color scale and record readings.
7. When any color result is reported, it is usually assumed that cell path length used to measure the color of the test portion was 133.5 mm (5.25 in.). Therefore, if the color of a test portion is determined using a cell size other than 133.5 mm (5.25 in.), the cell path length must be reported with the color value.

PRECISION

This method was developed by comparing the performance of the Lovibond PFX990 with the visual methods of color determination Cc 13b-45 (Wesson method) and Cc 13e-92 Lovibond method). In an international collaborative study on oil color 18 test samples of oil were analyzed by 30 laboratories from 14 countries. A summary of this study is published (References, 2). A complete copy of the final report titled: "1996 International Collaborative Study—Oil Color Automated Method versus Visual Measurements" may be purchased from the AOCS Technical Department in Champaign, IL. Precision data from the study are summarized in Tables 1–4.

NOTES

1. The following automatic colorimeters have been found suitable: Lovibond PFX990 or PFX995 (heated version). For automated measurement of AOCS-Tintometer Color or Lovibond color the Lovibond PFX880 series has also been found suitable.
2. Reference and calibration standards are available for different color ranges. They are recommended for routine operation of Lovibond automatic colorimeters and may be required to permit laboratories to conform to their requirements of GLP and ISO 9001.
3. For supplier details, contact: The Tintometer Ltd,Waterloo Road, Salisbury, SPI 2JY, UK. Tel: +44 1722 327242; Fax: +44 1722 412322 E-mail: sales@tintometer.com

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REFERENCES

1. Wan, P.J., and D.R. Pakarinen, *J. Am. Oil Chem. Soc. 72*:455–458 (1995).
2. Wan, P.J., T.W. Hurley, J.D. Guy, and D.L. Berner, *J. Am. Oil Chem. Soc. 74* (6):731–738(1997).

Table 1
Least square linear regression for red color: *Y* = intercept + slope (*X*).

Dependent variable *Y*	Independent variable *X*	R-Square	Intercept	Standard error	Slope	Standard error
For refined oils[a]						
AOCS-NA[b]	Visual-NA	0.983	-0.574	0.511	1.305	0.070
Lovibond-EU[c]	Visual-EU	0.986	0.266	0.377	1.021	0.050
Visual-EU	Visual-NA	0.975	0.216	0.485	1.003	0.066
AOCS-EU	AOCS-NA	0.987	0.484	0.384	0.900	0.042
Lovibond-EU	Lovibond-NA	0.965	0.698	0.573	0.863	0.068
Lovibond-EU	AOCS-NA	0.962	0.984	0.578	0.779	0.064
Visual-NA	AOCS-(NA + EU)	0.987	0.320	0.216	0.791	0.024
Visual-EU	Lovibond-(NA + EU)	0.946	0.279	0.463	0.883	0.056
For refined, bleached, deodorized oils (RBD)[d]						
AOCS-NA	Visual-NA	0.94	0.557	0.135	0.910	0.087
Lovibond-EU	Visual-EU	0.986	0.029	0.087	1.155	0.052
Visual-EU	Visual-NA	0.975	0.350	0.083	0.881	0.053
AOCS-EU	AOCS-NA	0.994	-0.023	0.055	1.013	0.029
Lovibond-EU	Lovibond-NA	0.996	-0.129	0.052	1.097	0.028
Lovibond-EU	AOCS-NA	0.982	-0.151	0.107	1.095	0.056
Visual-NA	AOCS-(NA + EU)	0.933	-0.479	0.130	1.020	0.068
Visual-EU	Lovibond-(NA + EU)	0.983	-0.054	0.056	0.891	0.029
For refined oils and refined, bleached, deodorized oils combined[a,d]						
AOCS-NA	Visual-NA	0.984	0.013	0.208	1.235	0.041
Lovibond-EU	Visual-EU	0.992	0.218	0.130	1.028	0.024
Visual-EU	Visual-NA	0.985	0.213	0.163	1.001	0.032
AOCS-EU	AOCS-NA	0.991	0.230	0.145	0.931	0.023
Lovibond-EU	Lovibond-NA	0.978	0.307	0.208	0.901	0.035
Lovibond-EU	AOCS-NA	0.974	0.462	0.225	0.825	0.035
Visual-NA	AOCS-(NA + EU)	0.988	-0.046	0.100	0.825	0.016
Visual-EU	Lovibond-(NA + EU)	0.970	0.002	0.165	0.909	0.028
For standard color glasses						
AOCS-NA	Visual-NA	0.999	0.255	0.118	0.984	0.028
Visual-NA	Known-NA	1.000	-0.243	0.043	1.051	0.010
AOCS-NA	Known-NA	1.000	0.015	0.081	1.034	0.019
Lovibond-EU	Visual-EU	1.000	-0.028	0.041	1.089	0.010
Visual-EU	Known-EU	1.000	0.025	0.040	0.982	0.010
Lovibond-EU	Known-EU	1.000	0.000	0.085	1.070	0.021
Visual-EU	Visual-NA	1.000	0.229	0.000	0.903	0.000
AOCS-EU	AOCS-NA	1.000	0.007	0.039	1.017	0.009
Lovibond-EU	Lovibond-NA	1.000	-0.018	0.001	1.034	0.000
Lovibond-EU	AOCS-NA	1.000	-0.031	0.083	0.999	0.019

[a]Red color data of refined canola oil were excluded from these regression analyses.
[b]NA, laboratories from North America.
[c]EU, laboratories outside of North America.
[d]Lovibond-EU-Red and Visual-EU-Red for RBD sunflower oil tested by labs outside of North America were excluded from the regression analysis for the variables with suffix of EU due to unreasonably large deviation.

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Table 2
Least square linear regression for yellow color: *Y* = intercept + slope (*X*).

Dependent variable *Y*	Independent variable *X*	R-Square	Intercept	Standard error	Slope	Standard error
For refined oils[a]						
AOCS-NA[b]	Visual-NA	0.506	40.864	9.166	0.448	0.167
Lovibond-EU[c]	Visual-EU	0.722	19.357	10.756	0.761	0.178
Visual-EU	Visual-NA	0.463	37.193	9.521	0.427	0.174
AOCS-EU	AOCS-NA	1.000	−1.679	0.521	1.025	0.008
Lovibond-EU	Lovibond-NA	0.985	−4.536	3.288	1.060	0.050
Lovibond-EU	AOCS-NA	0.984	7.835	2.0806	0.885	0.043
Visual-NA	AOCS-(NA + EU)	0.504	−19.679	17.937	1.113	0.276
Visual-EU	Lovibond-(NA + EU)	0.723	−4.231	9.940	0.980	0.152
For refined, bleached, deodorized oils (RBD)						
AOCS-NA	Visual-NA	0.973	−1.868	1.341	1.405	0.088
Lovibond-EU	Visual-EU	0.999	−1.328	0.291	1.186	0.015
Visual-EU	Visual-NA	0.961	−0.367	1.471	1.271	0.097
AOCS-EU	AOCS-NA	0.999	−0.354	0.250	1.017	0.012
Lovibond-EU	Lovibond-NA	0.995	−0.117	0.592	0.960	0.026
Lovibond-EU	AOCS-NA	0.997	0.164	0.482	1.079	0.024
Visual-NA	AOCS-(NA + EU)	0.968	1.750	0.640	0.685	0.031
Visual-EU	Lovibond-(NA + EU)	0.996	1.090	0.279	0.824	0.012
For refined oils and refined, bleached, deodorized oils combined						
AOCS-NA	Visual-NA	0.842	6.727	4.480	1.029	0.111
Lovibond-EU	Visual-EU	0.969	0.881	2.137	1.065	0.048
Visual-EU	Visual-NA	0.836	7.218	4.176	0.937	0.104
AOCS-EU	AOCS-NA	1.000	−0.285	0.192	1.004	0.004
Lovibond-EU	Lovibond-NA	0.998	−0.672	0.608	1.000	0.012
Lovibond-EU	AOCS-NA	0.996	1.370	0.738	0.988	0.015
Visual-NA	AOCS-(NA + EU)	0.841	−0.419	2.924	0.816	0.061
Visual-EU	Lovibond-(NA + EU)	0.968	0.035	1.394	0.909	0.028
For standard color glasses						
AOCS-NA	Visual-NA	0.990	−1.157	2.402	0.989	0.099
Visual-NA	Known-NA	0.992	1.154	2.102	1.032	0.093
AOCS-NA	Known-NA	1.000	−0.173	0.234	1.030	0.010
Lovibond-EU	Visual-EU	0.999	−0.777	0.977	1.042	0.033
Visual-EU	Known-EU	1.000	−0.367	0.443	0.993	0.015
Lovibond-EU	Known-EU	0.998	−1.149	1.448	1.034	0.047
Visual-EU	Visual-NA	0.987	−1.896	3.668	1.290	0.151
AOCS-EU	AOCS-NA	1.000	0.000	0.000	1.000	0.000
Lovibond-EU	Lovibond-NA	1.000	0.000	0.000	1.000	0.000
Lovibond-EU	AOCS-NA	0.998	−1.207	1.518	1.362	0.065

[a]Yellow color of refined oils was near or exceeded the maximum scale. Therefore, the correlations for this group are poor and meaningless.
[b]NA, laboratories from North America.
[c]EU, laboratories outside of North America.

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Table 3
Means of red values—1996 International Oil Color Study.

Oil type	Refined or RBD oil[a]	Automated AOCS-EU[b]	Automated AOCS-NA[c]	Automated Lovibond-EU	Automated Lovibond-NA	Visual-EU	Visual-NA	Known red values EU	Known red values NA
Canola	Refined	8.4	10.2	7.8	9.5	12.8	14.7		
Corn	Refined	11.0	10.8	10.4	10.2	9.6	8.9		
Cottonseed	Refined	15.2	17.3	13.0	15.9	11.7	12.7		
Palm olein	Refined	3.2	3.1	3.1	3.1	3.1	2.8		
Peanut	Refined	2.9	2.9	3.1	3.1	2.9	2.7		
Soybean	Refined	10.4	10.3	9.8	9.5	10.0	9.0		
Soybean—duplicate	Refined	10.4	10.3	9.9	9.7	10.0	9.0		
Sunflower	Refined	3.7	3.4	3.5	3.5	2.7	2.9		
Tallow	Wash, dry, filtered	1.9	1.9	1.8	1.8	1.4	1.5		
Canola	RBD	1.3	1.3	1.2	1.2	0.9	0.8		
Corn	RBD	1.8	1.7	1.9	1.9	1.5	1.2		
Cottonseed	RBD	3.3	3.2	3.5	3.2	2.9	2.9		
Palm	RBD	2.9	3.0	3.1	3.0	2.8	2.9		
Peanut	RBD	1.0	1.0	0.9	0.9	0.9	0.6		
Soybean	RBD	0.9	0.9	0.9	0.9	0.8	0.5		
Soybean—duplicate	RBD	0.9	0.9	0.9	0.9	0.7	0.5		
Sunflower	RBD	0.9	1.0	0.9	1.0	0.8	0.6		
Tallow	Deodorized	2.3	2.3	2.1	2.1	1.8	1.3		
Glass standard-1		0.5	0.5	0.5	0.5	0.5	0.3	0.5	0.5
Glass standard-2		3.8	3.7	3.5	3.5	3.3	3.4	3.3	3.5
Glass standard-3		6.7	6.6	6.6	6.4	6.1	6.5	6.2	6.4

[a]RBD, refined, bleached, deodorized.
[b]EU, laboratories outside of North America.
[c]NA, laboratories from North America.

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Table 4
Means of yellow values—1996 International Oil Color Study.

Oil type	Refined or RBD oil[a]	Automated AOCS-EU[b]	Automated AOCS-NA[c]	Automated Lovibond-EU	Automated Lovibond-NA	Visual-EU	Visual-NA	Known yellow values EU	Known yellow values NA
Canola	Refined	70.0	70.0	70.0	70.0	55.0	63.7		
Corn	Refined	70.0	70.0	70.0	70.0	52.4	57.6		
Cottonseed	Refined	70.0	70.0	70.7	70.0	72.0	71.4		
Palm olein	Refined	50.0	49.8	54.2	53.9	47.2	29.8		
Peanut	Refined	70.0	70.0	70.0	69.9	67.4	43.3		
Soybean	Refined	70.0	70.0	70.0	70.0	70.2	71.8		
Soybean—duplicate	Refined	70.0	70.0	70.0	70.0	68.9	72.1		
Sunflower	Refined	70.0	70.0	66.8	70.4	64.4	30.4		
Tallow	Wash, dry, filtered	33.7	34.9	37.4	40.4	34.5	21.9		
Canola	RBD	8.9	9.4	10.4	11.4	9.5	8.6		
Corn	RBD	11.5	11.4	13.3	13.2	13.2	10.1		
Cottonseed	RBD	34.2	35.0	35.8	40.1	31.9	29.6		
Palm	RBD	46.7	45.5	50.7	51.0	43.3	30.2		
Peanut	RBD	4.1	4.3	4.7	4.9	4.8	4.0		
Soybean	RBD	5.3	5.5	6.1	6.3	6.0	5.4		
Soybean—duplicate	RBD	5.3	5.2	6.0	6.0	6.1	5.0		
Sunflower	RBD	4.2	4.6	4.8	5.4	5.1	4.7		
Tallow	Deodorized	5.4	5.7	6.3	6.7	6.6	4.5		
Glass standard-1		1.0	1.0	1.1	1.1	1.2	0.9	1.3	1.0
Glass standard-2		13.0	13.0	15.1	15.1	16.1	16.5	17.0	13.0
Glass standard-3		38.0	38.0	51.0	51.0	49.4	38.7	50.0	37.0

[a]RBD, refined, bleached, deodorized.
[b]EU, laboratories outside of North America.
[c]NA, laboratories from North America.

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AOCS Official Method Cc 13k-13

Approved 2013

Spectrophotometric Determination of Chlorophyll and Chlorophyll Pigments in Vegetable Oil

DEFINITION

This method is used to determine mg/kg (ppm) of chlorophyll in cold pressed oil (e.g. virgin olive oil) and chlorophyll-related pigments (predominantly pheophytin *a*) in crude heated oils from spectrophotometric absorption measurements at 630, 670 and 710 nm.

SCOPE

This method is applicable to cold pressed and expelled oils. This method is not applicable to hydrogenated oils, deodorized oils and finished products because in these processed oils the absorption maximum does not occur at 670 nm.

APPARATUS

1. Spectrophotometer allowing the measurement in the range of 630 to 710 nm.
2. Glass cuvettes of various light paths are allowed, 5 mm, 10 mm and 50 mm. Cuvettes must be matched and differ from each other by not more than 0.5% transmittance when checked with distilled water at 400 nm.
3. Glass funnel of a diameter of 40 to 60 mm and medium porosity filter paper.

REAGENTS

1. Chlorophyll *a* of at least 95% purity (See Notes, 1).

PROCEDURE (SEE NOTES, 2 AND 3)

1. Heat the test sample to a temperature no more than 5°C higher than the melting point of the test sample, if the test sample is solid at room temperature. Mix the liquid test sample thoroughly; and if it is turbid, filter it immediately prior to the analysis using a filter paper of medium porosity.
2. Measure the test sample at 630 nm, 670 nm and 710 nm, in a 5 mm, 10 mm or 50 mm spectrophotometer cell against air rather than a reference cell.

CALCULATIONS

The content of chlorophyll pigments is expressed in mg/kg (ppm) of chlorophyll *a* or pheophytin *a* according to the formula as follows:

For chlorophyll as chlorophyll a (equation 1):

$$C = k \times \frac{Abs_{670} - 0.5\left(Abs_{630} + Abs_{710}\right)}{L}$$

Where—

k factor (k = 1/F) being determined for chlorophyll for each spectrophotometer using chlorophyll standards with the formula:

F = $Abs/C_{std}L$ with:

L = light path (cm)

C_{std} = standard concentration (mg/L)

Abs = measured absorption

C = content of chlorophyll *a* in mg/kg of oil

Abs = absorbance at the respective wavelength (unit)

L = light path of the spectrophotometer cell (cm)

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For chlorophyll-pigment as pheophytin a (equation 2):

The k factor is different for chlorophyll and pheophytin. In the case of processed and heated oils, part or all of the chlorophyll-related prigments may be converted to products that do not exhibit an absorbance maximum at 670 nm.

$$C = k \times \frac{Abs_{670} - 0.5\left(Abs_{630} + Abs_{710}\right)}{L}$$

Where—

k factor (k = 1/F) being determined for pheophytins by treating the standard chlorophyll solution with an acid according to Perkins and Roberts (1962) or using 34.53 according to (Holasová *et al.*, 1990).

C = content of chlorophyll-pigments as pheophytin in mg/kg of oil

Abs = absorbance at the respective wavelength (unit)

L = light path of the spectrophotometer cell (cm)

To obtain more accurate results:

1. The processed or heated oil could be treated with acetic acid according to Perkins and Roberts (1962) to convert any remaining chlorophyll to pheophytin, determining the total pheophytin content of the processed oil. The pheophytin is directly related to the original chlorophyll content.
2. Chlorophyll and pheophytin relative contents can be individually determined by HPLC using appropriate chlorophyll and pheophytin standards
3. The actual absorbance maximum of the test portion can be determined and substituted in the equation used in the calculation for mg/kg (ppm) chlorophyll.

PRECISION (See Notes, 4)

1. Repeatability, expressed by the coefficient of variation, RSD_r is—0.4 at high concentration of chlorophyll pigments; 1.0 at moderate concentration of chlorophyll pigments; and 1.4 at low concentration of chlorophyll pigments. (See Tables 1–3.)
2. Reproducibility, expressed by the coefficient of variation, RSD_R, is—1.3 at high concentration of chlorophyll pigments; 3.5 at moderate concentration of chlorophyll pigments; and 6.0 at low concentration of chlorophyll pigments. (See Tables, 1–3.)

NUMBERED NOTES

1. Chlorophyll *a* and pheophytin *a* are commercially available standards. Chlorophyll *a* can be converted to pheophytin *a* through treatment with acid (e.g. formic acid) and subsequently converted to pyropheophytin *a* by the application of heat (see Reference, 6).
2. For sample with high chlorophyll pigment concentrations, absorbance readings must be so that Beer-Lambert law linear relation between absorbance reading and compound concentration is still applicable.
3. For pheophytin determination, the actual absorbance maximum of the test portion can be determined and substituted for 670 nm in equation 2.
4. Results reported by IUPAC Commission on Oils, Fats and Derivatives collaborative study for the determination of chlorophyll pigments in crude vegetable oils, *Pure & Applied Chemistry*, 67(10), 1781-1787.

REFERENCES

1. Daun, J.K. and Thorsteinson, C.T. (1989) J. Am. Oil Chem. Soc., 66 (8), 1124–1128.
2. Holasová, M., Parízková, H. and Pokorný, J. (1990) La Rivista Italina delle Sostanze Grasse, LXVII, 521–522.
3. Perkins, H.J. and Roberts, D.W.A. (1962) Biochim. Biophys. Acta, 58, 486–498.
4. Pokorný, J., Kalinová, L. and Dysseler, P. (1995) Pure and Appl. Chem., 67(10), 1781–1787.
5. Stilmann, R.C. (1955) J. Am. Oil Chem. Soc., 32, 503–505.
6. Aitzetmüller, K. (1989) Fat Sci. Technol., 91(3), 99–105.

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Table 1
Results of a 1990 collaborative study on the determination of chlorophyll pigments in rapeseed oil.

Sample	1	2	3	4	5	6
Total number of laboratories accepted	12	12	12	12	12	12
Mean of the laboratory values (mg/kg)	34.54	11.30	4.02	33.95	15.37	5.88
Repeatability						
Standard deviation, s_r	2.24	2.99	0.90	1.27	1.14	0.97
Relative standard deviation, RSD_r%	6.49	26.45	22.29	3.75	7.43	16.46
Repeatability value, $2.8 \times s_r$	6.34	8.46	2.54	3.60	3.23	2.74
Reproducibility						
Standard deviation, s_R	3.39	4.02	1.78	3.09	2.47	1.66
Relative standard deviation, RSD_R%	9.81	35.57	44.20	9.40	16.06	28.33
Reproducibility value, $2.8 \times s_R$	9.58	11.38	5.03	9.03	6.99	4.71

Table 2
Results of a 1991 collaborative study on the determination of chlorophyll pigments in rapeseed oil.

Sample	1	2	3	4	5	6
Total number of laboratories accepted	10	10	7	7	10	9
Mean of the laboratory values (mg/kg)	46.56	51.23	0.20	0.32	17.01	20.93
Repeatability						
Standard deviation, s_r	0.38	0.25	0.01	0.03	0.72	0.21
Relative standard deviation, RSD_r%	0.82	0.49	5.04	9.72	4.26	0.98
Repeatability value, $2.8 \times s_r$	1.09	0.72	0.03	0.09	2.05	0.58
Reproducibility						
Standard deviation, s_R	4.01	4.02	0.11	0.13	2.29	0.63
Relative standard deviation, RSD_R%	8.62	7.84	57.69	40.73	13.47	3.01
Reproducibility value, $2.8 \times s_R$	11.36	11.37	0.32	0.13	0.48	1.78

Table 3
Results of a 1992 collaborative study on the determination of chlorophyll pigments in vegetable oil.

Sample	1	2	3	4	5	6
Total number of laboratories accepted	12	11	12	8	11	12
Mean of the laboratory values (mg/kg)	54.49	52.51	28.15	25.94	7.20	6.71
Repeatability						
Standard deviation, s_r	0.23	0.10	0.11	0.08	0.10	0.07
Relative standard deviation, RSD_r%	0.66	0.29	0.31	0.23	0.28	0.19
Repeatability value, $2.8 \times s_r$	0.43	0.16	0.39	0.31	1.36	1.00
Reproducibility						
Standard deviation, s_R	0.85	0.63	0.50	0.26	0.46	0.18
Relative standard deviation, RSD_R%	2.39	1.79	1.42	0.74	1.31	0.51
Reproducibility value, $2.8 \times s_R$	1.55	1.20	1.78	1.00	3.43	2.71

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Congeal Point

DEFINITION

The congeal point is a measure of the solidification temperature of fat under the conditions prescribed by the test method. The congeal point differs from the titer point in that the congeal point is based on triglyceride composition, while the titer point is based on fatty acid composition.

SCOPE

Applicable to fats and oils and fat mixtures that congeal or solidify within the temperature range of 25–45°C (see Notes, 1).

APPARATUS

1. Thermometer—titer, AOCS Specification H 6-40. This thermometer should be calibrated against a National Institute of Testing-certified thermometer.
2. Thermometer—0–150°C, 1.0° subdivisions.
3. Beakers—
   (a) Electrolytic (tall form)—180 mL.
   (b) Glass—2000 mL.
   (c) Stainless steel—500 mL, approximately 125 mm high with 75 mm inside diameter.
   (d) Stainless steel—4000 mL.
4. Lamp—100 watt, without reflector.
5. Hot plate—with variable control.
6. Filter paper—Eaton & Dickerman 615, or equivalent.
7. Cooling baths as illustrated in Figure 1—
   (a) Bath A for cooling the test sample to a definite cloud point.
   (b) Bath B for maintaining the test sample while solidification proceeds.

ASSEMBLY OF APPARATUS

1. The initial cooling bath (A in Figure 1) consists of a 2000 mL glass beaker filled with clean water and maintained with either ice or cold water at—
   (a) 15 ± 0.5°C for congeal points below 35°C.
   (b) 20 ± 0.5°C for congeal points at 35°C and above.
2. The second cooling bath (B in Figure 1) consists of a 4000 mL stainless steel beaker filled with water and maintained with either ice or cold water at 20°C. A 500 mL stainless steel beaker is immersed in this water bath, centered and supported with either a wooden or plastic ring. Place some lead weights in the bottom of the 500 mL beaker to prevent it from floating. Provide a cover for the air bath with a hole in the center such that it will also serve as a support for the 180 mL tall-form beaker.
3. Mount the lamp so that it is positioned behind the cooling bath (A) at a distance of about 2.5 and 5.0 cm below the water level. The lamp should be in line with the center of the beaker and at eye level.
4. Provide a cork stopper to fit the tall-form beaker. The stopper should be drilled through the center to accommo-date the titer thermometer. The stopper must be movable up and down along the stem of the titer thermometer.

PREPARATION OF TEST SAMPLE

1. Melt the test sample to 10°C above its melting point, but not any higher, and mix thoroughly. If any water is present, allow it to settle and decant the fat. Filter the test portion through filter paper to remove insoluble matter and traces of moisture.

PROCEDURE

1. Weigh 90 ± 1 g of the test sample into a 180 mL tall-form beaker and heat rapidly with constant stirring to 130°C. Hold at this temperature until all evidence of moisture is removed. The test portion must be completely dry.
2. Allow the beaker and test portion to stand at room temperature until the temperature of the test portion reaches 65°C. At this point, immerse the beaker in the first cooling bath (A), so that the oil level is about 6 mm below the water level.
3. Stir the test portion continuously at the minimum rate of 250 rpm with the titer thermometer until it reaches the cloud point. Rub the thermometer continuously around the sides and bottom of the beaker to minimize any accumulation of crystals.

   Note—Avoid cooling too rapidly. Immerse the beaker in the first cooling bath (A) about 75% of the time until the first crystals appear and then reduce the immersion time to about 50%. The immersion time may be reduced below 50% if crystal growth is rapid enough so that uniform dispersion cannot be maintained at the indicated rate of stirring (250 rpm).

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4. Continue cooling and stirring until the thermometer, when viewed with the eye along a horizontal line with the light, is visible when held vertically in the center of the beaker, but is not visible when held vertically along the back wall of the beaker.
5. At this point, immediately remove the beaker from bath A, quickly wipe dry and slip the cork down so the titer thermometer is suspended in oil up to the immersion mark. Immediately place the beaker in bath B.
   Note—Do not disturb the test portion after it has been placed in bath B.
6. Record the temperature of the test portion each minute until it reaches a maximum and then begins to descend. The maximum temperature attained is the congeal point.

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 1.52.
2. Single determinations performed in two different laboratories should not differ by more than 2.38.

NOTES

1. A temperature range of 25 to 45°C is sufficient to include most of the ordinary commercial products to which this method is applicable. In certain limited cases which fall outside of this range, the following generalizations may be helpful.
   (a) The cooling bath (A) temperature should be at least 3–5°C below the cloud point of the test portion and the maximum spread between the bath and clouding temperatures should not exceed 25°C.
   (b) The congeal point bath (B) should be at least 5°C below the congeal temperature of the fat, and the maximum spread between the bath temperature and the congeal point should not exceed 25°C.

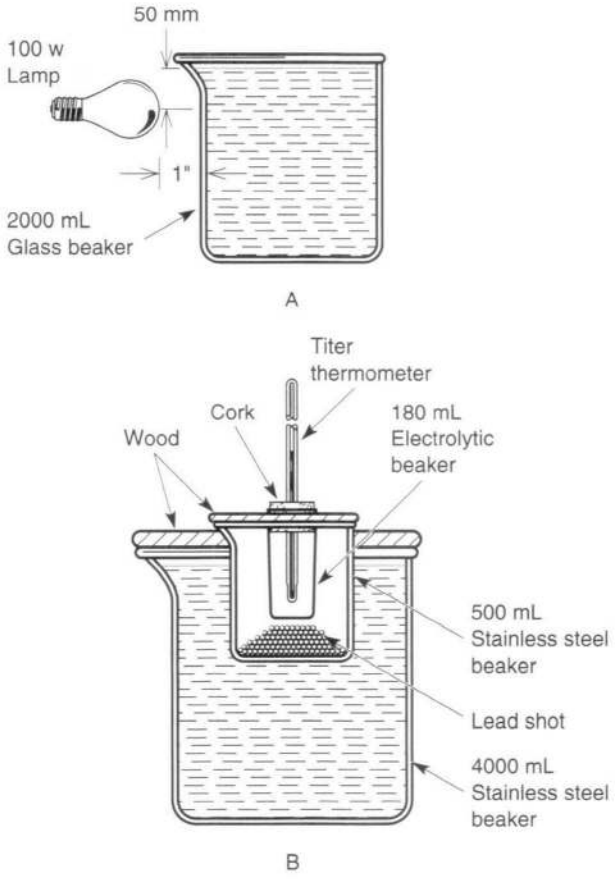

Figure 1. Cooling baths used in the congeal point determination.

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Consistency Penetrometer Method

DEFINITION

The penetrometer method provides an arbitrary measure of the firmness of plasticized fats by measuring the distance a given weight of defined shape will penetrate the fat in a certain period of time. The firmness of fat is related to the composition and character of the fat, to the temperature of the test sample at the time of measurement, and to the previous history of the test sample.

SCOPE

This method is applicable to plastic fats and solid fat emulsions such as shortenings, margarine, butter, and similar products.

APPARATUS

1. The penetrometer is a mechanical device which provides in one unit a support to grip and release the penetrating device (shank and cone), a platform to support the test sample, spirit level and adjustments to maintain the penetrometer in a level position, and a gauge graduated to allow reading the depth of penetration in 0.1 mm units. The penetrometer conforms to American Society for Testing and Materials (ASTM) Designations D5, D217 and D937 (see Notes, 1).
2. The penetration cone should conform in dimensions to the specifications shown in Figure 1 (see Notes, 2). This cone (weight 45 g) should be turned from good quality aluminum and must be free from casting flaws. The total weight of the cone assembly and shank is 92.5 g and must be so maintained. The dimensions are also critical. The shank of the cone should conform to specifications given in ASTM Designation D5 and weigh 47.5 g.
3. Thermometers—convenient range, e.g., 0–110°C, which should be calibrated against a National Institute of Testing-certified thermometer.
4. Holder for margarine test samples—a frame so constructed that it will hold three quarter-pound prints side by side and firmly together. The frame need be confined only on two sides and the prints slipped between.
5. Timer.
6. Aluminum scraper—about 70 × 30 × 3 mm, with a 45° bevel along the 70-mm edge.

PREPARATION OF TEST SAMPLE

1. Freshly manufactured shortenings and other plasticized fats must be allowed a tempering period for development of the normal crystal pattern and equilibration of the liquid and solid fractions. The optimum temperature and time required to reach this condition may vary with different products and package size, but a minimum of 48 hr at 25 ± 1°C will suffice for most common plastic fats. Margarine should be tempered for 48 hr at 10 ± 0.5°C. In the absence of special buyer or seller specifications, these conditions must apply.
2. When the product to be tested is contained in small containers, such as 454 g (1 lb) packages, the test may be applied directly to the fat in the container. If the shortening is contained in larger packages to which the penetrometer cannot be conveniently applied, remove a portion of the fat from the container in some suitable form, such as a cone, cylinder, or cube of convenient size. It is essential that the portion of the test sample to which the test is to be applied be undisturbed to avoid distortion of the crystal pattern.
3. In the event that the fat is to be tested at some temperature other than that at which it is tempered, or if the history of the test sample is unknown, the test portion must be conditioned for at least 48 hr at the specified penetration temperature.
4. All test samples of shortening and margarine should remain at the respective conditioning temperatures until they are removed for penetration testing. Penetration measurements are best determined at the temperature at which the test sample was conditioned, and the penetrometer should be at the same temperature.
5. For margarine testing, place three quarter-pound prints side by side within the special frame (Apparatus, 4) and condition as noted in Preparation of Test Sample, 1. Use the middle print for penetration tests.

PROCEDURE

1. If the surface of the test portion is perfectly smooth, the penetration test may be applied as is. If the surface is not smooth, remove a thin layer of the test portion with aluminum scraper (Apparatus, 6) so as to leave a smooth, plane surface. The test portion and scraper should be maintained at the tempering temperature during this operation.
2. Adjust the penetrometer to a level position and make certain that the cone is thoroughly clean and free from any residual test portion. The cone should drop freely when released. Make certain that the dial pointer is adjusted to zero when the cone is raised to the upper stop.
3. Place the test portion on the platform of the penetrometer in such a position that the cone contacts the product at least 2.5 cm from the edge of the test portion. In the case of quarter-pound prints of margarine, the cone should contact the center of the test portion.

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4. Lower the entire head of the penetrometer so that the tip of the cone just touches the test portion. Release the cone and allow it to descend for exactly 5 sec. The length of time the cone is allowed to penetrate the test portion is critical and must be observed with a stop-watch or other suitable timer.
5. After the 5-sec interval, immediately lower the indicator rack which causes the dial pointer to turn and then observe the reading on the dial (penetration distance). Additional tests on the test portion must be made at least 1 in. apart, and the cone must be cleaned preceding each measurement. A minimum of three determinations should be made per test portion, and the results averaged.
6. Determine the temperature of the test portion. Report the penetration to 0.1 mm at the temperature of measurement. If the temperature of the test portion is not within ± 0.5°C of the conditioning temperature, a correction of 5 points/degrees may be made. Otherwise, additional conditioning is needed.

PRECISION

1. The coefficient of variation within a laboratory is 3.5% and between laboratories is 4.0%.

NOTES

1. Suitable penetrometers are available from Fisher Scientific and Sargent-Welch, including penetrometers with magnetic (rather than manual) release control.
2. The dimensions of the penetrometer cone available with the instrument should be checked to make certain it conforms to the specifications noted in Figure 1. If cones of the proper dimensions are not available commercially, they will have to be specially constructed. One supplier is Kohler Instruments, Bohemia, NY, USA.

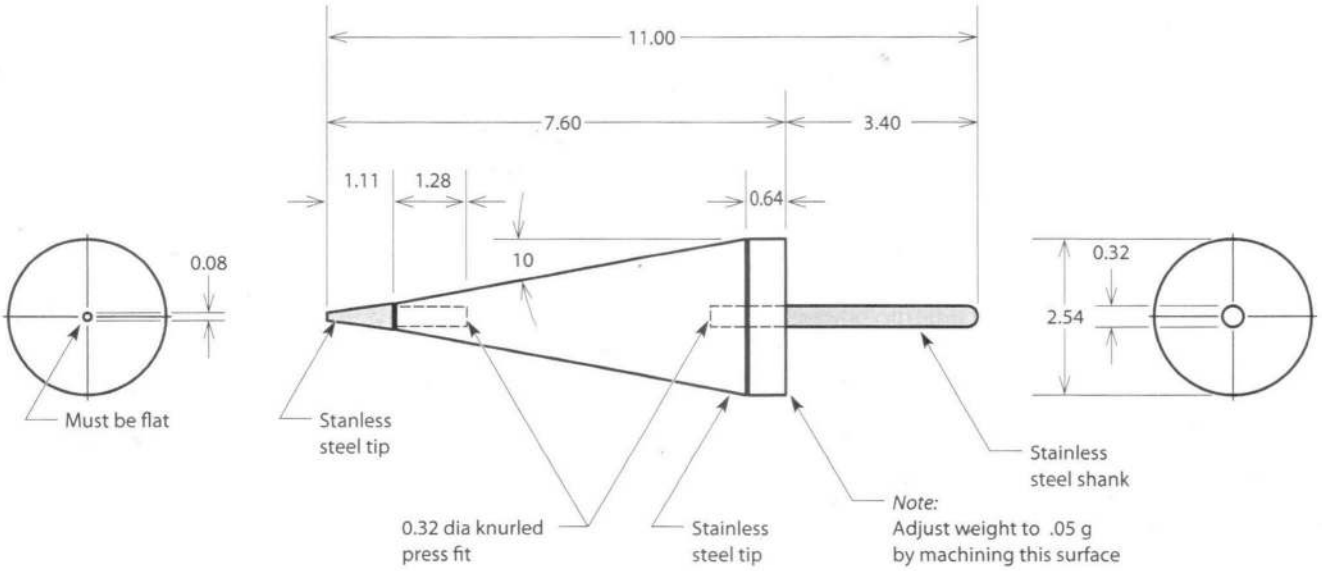

Figure 1. Penetrometer cone (all dimensions in cm).

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AOCS Recommended Practice Cc 17-95

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Soap in Oil Titrimetric Method

DEFINITION

The titrimetric method determines the alkalinity of the test sample as sodium oleate.

SCOPE

Applicable only to refined vegetable oils (see References, 1). Formerly Cc 17-79.

APPARATUS

1. Test tubes—approximately 150 × 40 mm of borosilicate glass fitted with ground glass stoppers and flattened at the base.
2. Microburet—5 mL.
3. Steam bath—hot water bath may also be used.

REAGENTS

1. Acetone—containing 2% water, prepared by adding 20 mL distilled water to 980 mL of reagent-grade acetone (see Notes, *Caution*).
2. Hydrochloric acid (HCl)—approximately 0.01 M, accurately standardized. See AOCS Specification H 14–52.
3. Bromophenol blue indicator solution—1.0 % in water.
4. Sodium hydroxide (NaOH)—approximately 0.01 M.

PROCEDURE

1. Just prior to the analysis, prepare the test solution by adding 0.5 mL of the bromophenol blue indicator solution (Reagents, 3) to each 100 mL of the aqueous acetone solution (Reagents, 1) and titrating with 0.01 M HCl (Reagents, 2) or 0.01 M NaOH (Reagents, 4) until the test solution is just yellow in color.
2. Weigh 40 g (Notes, 1) of the oil or fat to be tested into a test tube (Apparatus, 1) which has been well rinsed with the test solution (Procedure, 1).
3. Add 1 mL of water to the test sample, warm on a steam bath (or in water bath) and shake vigorously. Add 50 mL of the test solution (Procedure, 1), and after warming, shake the tube well and allow the contents to separate until two distinct layers are formed. *Note*—If soap is present in the oil or fat, the upper layer will be colored green to blue.
4. Slowly add 0.01 M HCl (Reagents, 2) from the microburet until the color just changes from green/blue to yellow. Repeat the warming, shaking and addition of 0.01 M HCl until the yellow color of the upper layer remains permanent. Record the total volume of acid required as mL_s.
5. A blank correction should be determined on soap-free oil, using Procedure, steps 1–4. Record the volume of acid required for the blank as mL_b.

CALCULATIONS

1. ppm soap as sodium oleate $= \dfrac{(mL_s - mL_b) \times M \times 304{,}400}{\text{test portion mass, g}}$

Where—

mL_s = volume, mL HCl obtained in Procedure, 4
mL_b = volume, mL HCl obtained in Procedure, 5
M = molarity of HCl

NOTES

Caution

Acetone is highly flammable. Forms explosive peroxides with oxidizing agents. Use effective fume-removal device. Do not mix with chloroform.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. The method as written is suitable for the determination of soap at concentrations of up to 0.05% in oils and fats. At higher concentrations it is better to analyze 4 g of test portion and use 0.01 M HCl.

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REFERENCES

1. A study among seven industrial organizations indicated that this method is suitable only for refined oils. *Yukagaku (Japan) 39:*1056 (1990).
2. This method is identical with Codex Alimentarius method CAC/RM 13-1969 and similar to British Standard 648:1958.
3. Wolff, J.P. *Oléagineux*, p. 197 (April, 1948).

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AOCS Standard Procedure Cc 18-80

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Dropping Point

DEFINITION

The dropping point of a fat or oil is the temperature at which the test sample will become fluid to flow under the conditions of the test.

SCOPE

Applicable to hydrogenated and nonhydrogenated fats and oils that solidify sufficiently when held at -5°C or lower for the allotted time.

APPARATUS

1. Mettler FP90 control unit and FP83HT dropping point furnace or their equivalent—Mettler Instrument Corp., Hightstown, NJ, USA (see Notes, 1).
2. Sample cups—drip hole diameter 2.8 mm, Mettler no. 18712, or equivalent.
3. Sample cup holder—Mettler no. 18711, or equivalent.
4. Sample collecting sleeve—Mettler no. 18713, or equivalent.
5. Freezer—capable of maintaining test samples at a temperature of -5°C or lower (see Notes, 2).

STANDARDIZATION

Lauric acid is used as the standard and is doped with 0.5% powdered carbon. Place a portion of the carbon-doped lauric acid in a small clean beaker and melt on a warm hot plate between 60–70°C. After melting, swirl well to get a uniform suspension of the carbon. Place the test sample into a single melting cup as described in the Test Sample Conditioning section. Place the test sample in the freezer at a temperature of -5°C or lower for exactly 15 minutes. Preheat the dropping point furnace to a temperature of exactly 39°C. Place the test sample in the furnace and hold for exactly one minute prior to initiating the heating program. (For the FP-45 furnace the operator must clock with a stopwatch the one-minute delay before starting. For the FP-80 furnace there is a 30-second hold time built in; therefore the analyst must time a 30-second delay before initiating the run button. For the PF-90 furnace, the one-minute hold time is built in.) Set the heating rate to 1.0°C and complete the analysis.

The collaborative study done in 1995 involved 23 labs with 26 instruments (78 total data points). Minimum dropping point: 44.90°C. Maximum dropping point: 47.30°C. Average 45.84°C. Standard Deviation 0.52°C.

Lauric acid: NuChek Prep Inc., Cat. Number N-12-A; Powdered carbon: Westvaco Nuchar SN-20.

TEST SAMPLE CONDITIONING

1. Place a sample cup in the freezer on a clean smooth surface at least 10 min prior to filling. The sample cup must be absolutely clean and free from any residual fat or oil.
2. Melt the test sample and mix thoroughly.
3. Fill the test sample cup in the freezer to the top with the melted test portion.
4. Allow the cup to stand in the freezer for 15 min after filling.

PROCEDURE

1. Preheat the dropping point furnace to a temperature of at least 5°C below the dropping point of the test portion (see Notes, 3).
2. Set the heating rate of the dropping point apparatus at 1°C.
3. Insert the sample cup into the cartridge and remove from the freezer, avoiding repeated handling of the filled sample cup.
4. Place the sample holder immediately into the dropping point furnace, making certain that it is seated properly.
5. Activate the heater.
6. Read and record the dropping point temperature from the display board.

PRECISION

1. Table 1 lists the expected precision at a confidence level of 95% for Mettler dropping point values above and below 33°C.

NUMBERED NOTES

1. Based on a collaborative study carried out by the AOCS Commercial Oils and Fats Analysis Committee in 1993, the following recommendations are made regarding the operation and standarization of the apparatus:
   (a) Diode voltage should be between 8 and 12 volts. Low diode voltage (<8) may result in the failure of the photo detector to detect the initial drop of clear materials—in particular, standard reference materials such as lauric acid.

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(b) When using clear standard reference materials, such as lauric acid, finely powdered carbon (0.5% by weight) should be added to the reference material to improve detector response. The powdered carbon will not affect the melting point. When powdered carbon is used, the sample cup should be cleaned thoroughly.

(c) A test should be considered valid only if the following two criteria are met: it is verified that some test portion has indeed fallen into the collection sleeve, and at least half of the original test sample still remains in the sample cup. Any test not meeting these two requirements should be considered invalid and the test repeated. Continuous failure of the tests could indicate a malfunctioning instrument, and the manufacturer should be contacted.

2. Optimum precision is obtained from test portions with dropping points below 33°C when a freezer temperature of -20°C or lower is used.
3. In cases where the dropping point of the test portions requires the furnace temperature to be set below room temperature, the furnace may be placed in a styrofoam box with dry ice, a refrigerator or other suitable cooling device.

Table 1
Expected precision of Mettler dropping point determinations.

At a confidence level of 95% in the range of values of:	Below 33°C	Above 33°C
Two single determinations on the same test sample performed in one laboratory should not differ by more than	1.5°C	0.7°C
The average of duplicate determinations on the same test sample performed by two or more laboratories should not differ for the mean value by more than	±1.6°C	±1.0°C

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AOCS Official Method Cd 1-25

Withdrawn (Historical Interest Only)

Iodine Value of Fats and Oils Wijs Method

DEFINITION

The iodine value is a measure of the unsaturation of fats and oils and is expressed in terms of the number of centigrams of iodine absorbed per gram of sample (% iodine absorbed).

SCOPE

Applicable to all normal fats and oils that do not contain conjugated double bonds (see Notes, 1). Applicable to all normal fats and oils with samples having iodine values between 0–15.

APPARATUS

1. Glass-stoppered iodine flasks—500 mL.
2. Glass-stoppered volumetric flasks—1000 mL, for preparing standard solutions.
3. Pipet—25 mL, for accurately dispensing 25.0 mL of Wijs solution.
4. Volumetric dispenser—20 mL, 1 mL adjustability, for 10% potassium iodide (KI) solution.
5. Volumetric dispenser—2 mL, 1 mL adjustability, for starch solution.
6. Volumetric dispenser—50 mL, 1 mL adjustability, for distilled water.
7. Repeater pipet—with filling flask, 20 mL, for cyclohexane.
8. Analytical balance—accurate to ±0.0001 g.
9. Magnetic stirrer.
10. Filter paper—Whatman no. 41H, or equivalent.
11. Beakers—50 mL.
12. Hot air oven.
13. Timer.

REAGENTS

1. Wijs solution—see Notes, 2 and *Caution.*
2. Potassium iodide (KI)—reagent grade.
3. Carbon tetrachloride—reagent grade (see Notes, *Caution* and Recommendations). The absence of oxidizable matter in this reagent is verified by shaking 10 mL of the reagent with 1 mL of saturated aqueous potassium dichromate solution and 2 mL of concentrated sulfuric acid; no green coloration should appear.
4. Soluble starch solution—recently prepared, tested for sensitivity (see Notes, 3). Make a paste with 1 g of natural, soluble starch (see Notes, 4) and a small amount of cold distilled water. Add, while stirring, to 100 mL of boiling water.

   Test for sensitivity—Place 5 mL of starch solution in 100 mL of water and add 0.05 mL of *freshly prepared* 0.1 N KI solution and one drop of a 50 ppm chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution to 1000 mL. The deep blue color produced must be discharged by 0.05 mL of 0.1 N sodium thiosulfate.
5. Potassium dichromate—primary standard grade. The potassium dichromate is finely ground and dried to constant weight at about 110°C before using.

   Note—A standard sample of potassium dichromate with a certificate of analysis may be obtained from the National Bureau of Standards in Washington, D.C., USA. This sample, or equivalent, is strongly recommended for the primary standard for this method. Treat as directed in the certificate of analysis accompanying the sample.
6. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)—reagent grade.

SOLUTIONS

1. Potassium iodide (KI) solution—100 g/L (10% solution), prepared by dissolving 100 g of reagent grade KI in 1000 mL of deionized water.
2. Starch indicator solution—prepared and tested as noted in Reagents, 4. Salicylic acid (1.25 g/L) may be added to preserve the indicator. If long storage is required, the solution must be kept in a refrigerator at 4–10°C (40–50°F). Fresh indicator must be prepared when the end point of the titration from blue to colorless fails to be sharp. If stored under refrigeration, the starch solution should be stable for about 2–3 weeks.
3. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) solution—0.1 N (see Notes, 5), accurately standardized vs. potassium dichromate primary standard as follows:
   (a) Sodium thiosulfate solution 0.1 N, prepared by dissolving 24.9 g of sodium thiosulfate in distilled water and diluting to 1 L.
   (b) Weigh 0.16–0.22 g of finely ground and dried potassium dichromate into a 500 mL flask or bottle by difference from a weighing bottle. Dissolve in 25 mL of water, add 5 mL of concentrated hydrochloric acid, 20 mL of potassium iodide solution, and rotate to mix. Allow to stand for 5 min, and then add 100 mL of distilled water. Titrate with sodium

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thiosulfate solution, shaking continuously until the yellow color has almost disappeared. Add 1–2 mL of starch indicator and continue the titration, adding the thiosulfate solution slowly until the blue color just disappears. The strength of the sodium thiosulfate solution is expressed in terms of its normality.

Normality of $Na_2S_2O_3$ solution = 20.394 × mass of $K_2Cr_2O_7$ in g/mL of sodium thiosulfate

4. Wijs solution—see Notes, 2 and *Caution*.

PROCEDURE

1. Melt the sample, if it is not already liquid (the temperature during melting should not exceed the melting point of the sample by more than 10°C), and filter through two pieces of filter paper to remove any solid impurities and the last traces of moisture. The filtration may be performed in an air oven at 80–85°C, but should be completed within 5 min ± 30 sec. The sample must be absolutely dry.

   Note—All glassware must be absolutely clean and completely dry.
2. After filtration, allow the filtered sample to achieve a temperature of 68–71 ± 1°C before weighing the sample.
3. Once the sample has achieved a temperature of 68–71 ± 1°C, immediately weigh the sample into a 500 mL iodine flask, using the weights and weighing accuracy noted in Table 1 (see Notes, 6).
4. Add 15 mL of carbon tetrachloride on top of the sample and swirl to ensure that the sample is completely dissolved.
5. Dispense 25 mL of Wijs solution using the pipet (Apparatus, 3) into flask containing the sample, stopper the flask, and swirl to ensure an intimate mixture. Immediately set the timer for 30 min.
6. Immediately store the flasks in the dark for the required reaction time at a temperature of 25 ± 5°C.
7. Remove the flasks from storage and add 20 mL of KI solution, followed by 100 mL of distilled water (see Notes, 8 and 9).
8. Titrate with 0.1 N $Na_2S_2O_3$ solution, adding it gradually and with constant and vigorous shaking (see Notes, 10). Continue the titration until the yellow color has almost disappeared. Add 1–2 mL of starch indicator solution and continue the titration until the blue color just disappears.
9. Prepare and conduct at least one blank determination with each group of samples simultaneously and similar in all respects to the samples.

CALCULATIONS

1. The iodine value = $\dfrac{(B - S) \times N \times 12.69}{\text{mass, g of sample}}$

Where—

B = volume of titrant, mL of blank

S = volume of titrant, mL of sample

N = normality of $Na_2S_2O_3$ solution

NOTES

Caution

Wijs solution causes severe burns, and the vapors can cause lung and eye damage. Use of a fume hood is recommended. Wijs solution without carbon tetrachloride is available commercially.

Carbon tetrachloride is a known carcinogen. It is toxic by ingestion, inhalation, and skin absorption. It is a narcotic. It should not be used to extinguish fires. It decomposes to phosgene gas at high temperature. The TLV is 5 ppm in air. A fume hood should be used at all times when using carbon tetrachloride.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and is a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Chlorine is a poisonous gas. The TLV is 1 ppm in air. It is a strong oxidizing agent and should not be allowed to come in contact with organic materials, hydrogen, powdered metals, and reducing agents. A fume hood should be used at all times when using chlorine.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

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RECOMMENDATIONS

The most satisfactory replacement found to date for carbon tetrachloride has been cyclohexane + acetic acid, 1:1, v/v (see AOCS Cd 1d-92). Because of environmental concerns, 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113) is not recommended. Acetic acid alone and a mixture of cyclohexane + acetic acid (4:1, v/v), respectively, have been shown to be unsatisfactory replacements for carbon tetrachloride.

NUMBERED NOTES

1. When the iodine value is determined on materials having conjugated systems, the result is not a measure of total unsaturation, but rather is an empirical value indicative of the amount of unsaturation present. Reproducible results are obtained which afford a comparison of total unsaturation. When the iodine value is required on fatty acids, the preparation and separation are performed as directed in AOCS Official Method Cd 6-38.
2. Because the preparation of the Wijs solution is time-consuming and involves the use of both hazardous and toxic chemicals, this solution may be purchased from a chemical supplier. Solutions are available which contain no carbon tetrachloride, and such solutions should be used. All Wijs solutions are sensitive to temperature, moisture, and light. Store in a cool and dark place, and never allow to come to a temperature above 25–30°C. The laboratory preparation of Wijs solution is noted for reference purposes:

   Reagents

   (a) Glacial acetic acid—reagent grade (see Notes, *Caution*). The permanganate test should be applied to ensure that this specification is met.

   Permanganate test—Dilute 2 mL of the acid with 10 mL of distilled water and add 0.1 mL of 0.1 N $KMnO_4$. The pink color must not be entirely discharged within 2 hr.

   Alternate test—The absence of oxidizable matter in the reagent may be verified by shaking 10 mL of the reagent with 1 mL of saturated aqueous potassium dichromate solution and 2 mL of concentrated sulfuric acid; no green coloration should appear.

   (b) Chlorine—99.8% (see Notes, *Caution*). Satisfactory commercial grades are available in cylinders, but the gas must be dried by passing through sulfuric acid (sp. gr. 1.84) before introducing into the iodine solution.

   (c) Iodine—reagent grade.

   (d) Hydrochloric acid (HCl)—reagent grade, concentrated, sp. gr. 1.19 (see Notes, *Caution*).

   (e) Sulfuric acid (H_2SO_4)—reagent grade, concentrated, sp. gr. 1.84 (see Notes, *Caution*).

   (f) Iodine monochloride—reagent grade.

LABORATORY PREPARATION

(a) Dissolve 13.0 g of iodine in 1 L of glacial acetic acid. Gentle heat may be necessary to promote solution. Cool, remove a small quantity (100–200 mL), and set aside in a cool place for future use. Pass dry chlorine gas into the iodine solution until the original titration is not quite double. A characteristic color change takes place in the Wijs solution when the desired amount of chlorine has been added. This may be used to assist in judging the end point. A convenient procedure is to add a small excess of chlorine and immediately bring back to the desired titration by addition of some of the original iodine solution which was taken out at the beginning. The original solution and finished Wijs solution are both titrated with $Na_2S_2O_3$ solution as directed in Procedure, 6 and 7.

(b) The Wijs solution may be prepared from commercial iodine monochloride as follows:

Stock solution—Add 317 ± 0.1 g of iodine monochloride to 1 L of glacial acetic acid and filter through Whatman no. 41H filter paper, or equivalent, into a clean and dry actinic glass bottle. Filter rapidly to prevent contamination with moisture and store in a cool place. Discard the solution if a precipitate forms on standing.

Wijs solution—Using a graduate, pour 117.0 ± 0.1 mL of the stock solution into a standard 5 lb bottle of glacial acetic acid and mix well by shaking.

(c) The I/Cl ratio of the Wijs solution should be within the limits of 1.10 ± 0.1. The procedure for determining the ratio is as follows:

Iodine content—Pour 150 mL of saturated chlorine water into a 500 mL Erlenmeyer flask and add a few glass beads. Pipet 5 mL of the Wijs solution into the flask containing the saturated chlorine water. Shake, and heat to boiling. Boil briskly for 10 min, cool, and add 30 mL of 2% sulfuric acid and 15 mL of 15% potassium iodide solution. Mix well and titrate immediately with 0.1 N sodium thiosulfate solution to a starch end point.

Total halogen content—Pour 150 mL of recently boiled distilled water into a clean, dry 500 mL Erlenmeyer flask. Add 15 mL of 15% potassium iodide solution. Pipet 20 mL of Wijs solution into the flask and mix well. Titrate with 0.1 N sodium thiosulfate solution to a starch end point.

Calculation of halogen ratio—

$$\text{Halogen ratio } R = \frac{2A}{3B - 2A}$$

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Where—

A = volume of titrant, mL of iodine content as mL sodium thiosulfate

B = volume of titrant, mL of total halogen content as mL sodium thiosulfate

3. 1% starch solution may be purchased from a chemical supplier.
4. "Potato Starch for Iodometry" is recommended, because this starch produces a deep blue color in the presence of the iodonium ion. "Soluble Starch" is not recommended because a consistent deep blue color may not be developed when some soluble starches interact with the iodonium ion. The following are suitable starches: Soluble Starch for Iodometry, Fisher S516-100; Soluble Potato Starch, Sigma S-2630; Soluble Potato Starch for Iodometry, J.T. Baker 4006-04.
5. The sodium thiosulfate solution may be purchased from a chemical supplier. However, it still must be accurately standardized in the laboratory.
6. The weight of the sample must be such that there will be an excess of Wijs solution of 50–60% of the amount added; i.e., 100–150% of the amount absorbed. In the case of tung oil, weigh 0.1–0.2 g of sample, using an excess of 55 ± 3% Wijs solution. In the case of dehydrated castor oils and dehydrated castor oil fatty acids, weigh 0.11–0.13 g.
7. The indicated reaction times are those specified in the International Union of Pure and Applied Chemistry (IUPAC) Iodine Value Method 2.205 (References, 1) and were the reaction times used in the IUPAC/ISO validation study of the cyclohexane + acetic acid method (AOCS Official Method Cd 1d-92). Previous AOCS versions of iodine value methods specified a reaction time of 0.5 hr, regardless of the iodine value, but noted that "a longer reaction time may be necessary for oils with high iodine value." The longer reaction times appear to be particularly critical when cyclohexane is used as a replacement for carbon tetrachloride.
8. If the reaction is not terminated within 3 min after the reaction time, the sample must be discarded.
9. The sample must be titrated within 30 min of reaction termination, after which the analysis is invalid.
10. Mechanical stirring is recommended for agitation during the addition of thiosulfate.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.205.

Table 1
Sample weights.

Iodine value	Mass of sample 100% excess	Mass of sample 150% excess	Weighing accuracy
	g	g	g
<3	10	10	± 0.001
3	10.576	8.4613	0.005
5	6.346	5.0770	0.0005
10	3.1730	2.5384	0.0002
20	1.5865	1.2720	0.0002
40	.7935	.6346	0.0002
60	.5288	.4231	0.0002
80	.3966	.3173	0.0001
100	.3173	.2538	0.0001
120	.2644	.2115	0.0001
140	.2266	.1813	0.0001
160	.1983	.1587	0.0001
180	.1762	.1410	0.0001
200	.1586	.1269	0.0001

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AOCS Recommended Practice Cd 1b-87

Revised 2012

Iodine Value of Fats and Oils Cyclohexane Method

DEFINITION

The iodine value is a measure of the unsaturation of fats and oils and is expressed in terms of the number of centigrams of iodine absorbed per gram of test sample (% iodine absorbed).

SCOPE

Applicable to all normal fats and oils with iodine values in the range of 15 to 70 that do not contain conjugated double bonds (see Notes, 1).

APPARATUS

1. Glass-stoppered iodine flasks—500 mL.
2. Glass-stoppered volumetric flasks—1000 mL, for preparing standard solutions.
3. Volumetric dispenser—25 mL, 1 mL adjustability, for Wijs solution.
4. Volumetric dispenser—25 mL, 1 mL adjustability, for 15% potassium iodide (KI) solution.
5. Volumetric dispenser—2 mL, 1 mL adjustability, for starch solution.
6. Volumetric dispenser—50 mL, 1 mL adjustability, for distilled water.
7. Repeater pipet—with filling flask, 20 mL, for cyclohexane.
8. Analytical balance—accurate to 0.0001 g.
9. Magnetic stirrer.
10. Filter paper—Whatman #541, or equivalent.
11. Beakers—50 mL.
12. Hot air oven.
13. Timer.

REAGENTS

1. Wijs solution—accurately standardized (see Notes, 2 and *Caution*).
2. Potassium iodide (KI) solution—15%, prepared by dissolving 15 g of reagent-grade KI in 100 mL of distilled water.
3. Cyclohexane—reagent grade (see Notes, 3 and 4 and *Caution*).
4. Soluble starch—tested for sensitivity (see Notes, 5). Make a paste with 1 g of starch (see Notes, 6) and a small amount of cold distilled water. Add, while stirring, 200 mL of boiling water.

   Test for sensitivity—Place 5 mL of starch solution in 100 mL of water and add 0.05 mL of *freshly prepared* 0.1 M KI solution and one drop of a 50 ppm chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution to 1000 mL. The deep blue color produced must be discharged by 0.05 mL of 0.1 M sodium thiosulfate.
5. Potassium dichromate—reagent grade. The potassium dichromate is finely ground and dried to constant weight at about 110°C before using (see Notes, *Caution*).
6. Hydrochloric acid—reagent grade, concentrated, sp gr 1.19 (see Notes, *Caution*).
7. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)—0.1 M, accurately standardized, prepared from reagent-grade $Na_2S_2O_3 \cdot 5H_2O$ (see Notes, 7).

   Standardization of sodium thiosulfate—Weigh 0.16–0.22 g of finely ground and dried potassium dichromate into a 500 mL flask or bottle by difference from a weighing bottle. Dissolve in 25 mL of water, add 5 mL of concentrated hydrochloric acid, 20 mL of potassium iodide solution, and rotate to mix. Allow to stand for 5 min, and then add 100 mL of distilled water. Titrate with sodium thiosulfate solution, shaking continuously until the yellow color has *almost* disappeared. Add 1–2 mL of starch indicator and continue the titration, adding the thiosulfate solution slowly until the blue color just disappears. The strength of the sodium thiosulfate solution is expressed in terms of its molarity.

$$\text{Molarity of } Na_2S_2O_3 \text{ solution} = \frac{20.394 \times \text{wt of } K_2Cr_2O_7, \text{ g}}{\text{volume, mL of sodium thiosulfate}}$$

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PROCEDURE

1. Melt the test sample, if it is not already liquid (the temperature during melting should not exceed the melting point of the test sample by more than 10°C), and filter through two pieces of filter paper to remove any solid impurities and the last traces of moisture. The filtration may be performed in an air oven at 80–85°C, but should be completed within 5 min ± 30 sec. The test sample must be absolutely dry.

   Note—All glassware must be absolutely clean and completely dry.
2. After filtration, allow the filtered test portion to achieve a temperature of 68–71 ± 1°C before weighing the test portion.
3. Once the test portion has achieved a temperature of 68–71 ± 1°C, immediately weigh the test portion into a 500 mL iodine flask, using the weights and weighing accuracy noted in Table 1 (see Notes, 8).
4. Add 20 mL of cyclohexane on top of the test portion and swirl to ensure that the test portion is completely dissolved (see Notes, 4).
5. Dispense 25 mL of Wijs solution into flask containing the test portion, stopper the flask and swirl to ensure an intimate mixture. Immediately set the timer for 1.0 or 2.0 hr, depending on the iodine value of the test portion: IV < 150, 1.0 hr; IV ≥ 150, 2.0 hr (see Notes, 4).
6. Store the flasks in the dark at a temperature of 25 ± 5°C (see Notes, 9).
7. Prepare and conduct at least one blank determination with each group of test portion simultaneously and similar in all respects to the test portion.
8. Remove the flasks from storage and add 20 mL of KI solution, followed by 100 mL of distilled water (see Notes, 10).
9. Titrate with 0.1 M $Na_2S_2O_3$ solution, adding it gradually and with constant and vigorous shaking (see Notes, 11). Continue the titration until the yellow color has *almost* disappeared. Add 1–2 mL of starch indicator solution and continue the titration until the blue color has just disappeared.

CALCULATIONS

1. $$\text{The iodine value} = \frac{(B - S) \times M \times 12.69}{\text{mass, g of test portion}}$$

   Where—

   B = volume of titrant, mL of blank
   S = volume of titrant, mL of test portion
   M = molarity of $Na_2S_2O_3$ solution

PRECISION

1. See Table 2.

NOTES

Caution

Cyclohexane is flammable and a dangerous fire risk. It is moderately toxic by inhalation and skin contact. The TLV in air is 300 ppm.

Wijs solution causes severe burns, and the vapors can cause lung and eye damage. Use of a fume hood is recommended. Wijs solution without carbon tetrachloride is available commercially.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and is a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular, lung cancer. It is a strong oxidizing agent and a dangerous fire risk when in contact with organic chemicals.

NUMBERED NOTES

1. When the iodine value is determined on materials having conjugated systems, the result is not a measure of total unsaturation, but rather is an empirical value indicative of the amount of unsaturation present. Reproducible results are obtained which afford a comparison of total unsaturation.
2. Because the preparation of the Wijs solution is time-consuming and involves the use of both hazardous and toxic chemicals, this solution may be purchased from a chemical supplier. Solutions are available which contain no carbon tetrachloride, and such solutions should be used. All Wijs solutions are sensitive to temperature, moisture and light. Store in a cool and dark place, and never allow to come to a temperature above 25–30°C.
3. Fresh cyclohexane should be used. Erratic results may be obtained if old cyclohexane is used.
4. The AOCS study reported in *J. Am. Oil Chem. Soc.* (References, 1) used cyclohexane alone. When using cyclohexane alone, the 30 min reaction time specified in the former AOCS version of the Wijs method is insufficient for test portions having an IV >100, especially fish oils, as reported in References, 1. A study completed by FOSFA International in 1992 (References, 2), showed that the ISO 3961 (1989) and AOCS Recommended Practice Cd 1b-87 (1993) methods for determining IV do

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not give rise to values that are statistically different, and that the use of cyclohexane (alone) in place of carbon tetrachloride as the solvent does not affect the observed IV for titration of triglyceride oils having iodine values between 18 and 52 (the range of iodine values encountered in the study). Variable results were obtained in the application of the method to fish oils; however, further studies carried out by the International Association of Fish Meal Manufacturers indicated that reproducible IV results could be obtained if the reaction time of the Wijs reagent and fish oil was extended.

5. 1% starch solution may be purchased from a chemical supplier.
6. "Potato Starch for Iodometry" is recommended, because this starch produces a deep blue color in the presence of the iodonium ion. "Soluble Starch" is not recommended because a consistent deep blue color may not be developed when some soluble starches interact with the iodonium ion. The following are suitable starches: Soluble Starch for Iodometry, Fisher S516-100; Soluble Potato Starch, Sigma S-2630; Soluble Potato Starch for Iodometry, J.T. Baker 4006-04.
7. The sodium thiosulfate solution may be purchased from a chemical supplier. However, it still must be accurately standardized in the laboratory.
8. The weight of the test portion must be such that there will be an excess of Wijs solution of 50–60% of the amount added; i.e., 100–150% of the amount absorbed.
9. If the reaction is not terminated within 3 min past the reaction time, the test portion must be discarded.
10. The test portion must be titrated within 30 min of reaction termination, after which the analysis is invalid.
11. Mechanical stirring is recommended for agitation during the addition of thiosulfate.

REFERENCES

1. AOCS collaborative study results using cyclohexane alone appear in *J. Am. Oil Chem. Soc. 65*:745 (1988).
2. *INFORM 3*:1246 (1992).

Table 1
Test sample weights.

Iodine value	Test sample weight		Weighing accuracy
	100% excess	150% excess	
	g	*g*	*g*
<3	10	10	± 0.001
3	10.576	8.4613	0.005
5	6.346	5.0770	0.0005
10	3.1730	2.5384	0.0002
20	1.5865	1.2720	0.0002
40	.7935	.6346	0.0002
60	.5288	.4231	0.0002
80	.3966	.3173	0.0001
100	.3173	.2538	0.0001
120	.2644	.2115	0.0001
140	.2266	.1813	0.0001
160	.1983	.1587	0.0001
180	.1762	.1410	0.0001
200	.1586	.1269	0.0001

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Table 2
The interlaboratory study completed by FOSFA International in 1989, in which 37 laboratories participated, each obtaining four test results for each test portion analyzed by AOCS Recommended Practice Cd 1b-87 (cyclohexane) and four test results for each test portion analyzed by ISO method 3961 (carbon tetrachloride), gave the statistical results (evaluated in accordance with ISO 5725–1986) summarized in the following table.

	Test Samples[a]			
Name of statistic	P30-A	P30-B	P30-A	P30-B
No. of laboratories	37	37	37	37
No. of laboratory returns	29	28	35	35
No. of accepted results	29	27	34	33
Mean	52.09	18.17	52.79	18.18
Repeatability standard deviation	0.11	0.05	0.20	0.08
Repeatability coefficient of variation (%)	0.20	0.28	0.39	0.44
Reproducibility standard deviation	0.32	0.14	0.49	0.16
Reproducibility coefficient of variation (%)	0.61	0.77	0.94	0.89
Repeatability (r)	0.30	0.15	0.57	0.22
Reproducibility (R)	0.90	0.40	1.39	0.46
Range of accepted results	51.4–52.89	17.8–18.43	50.7–53.3	17.88–18.55
R/r	3.01	2.72	2.45	2.05

[a]Test Sample key: P30-A, RBD palm oil; P30-B, crude palm kernel oil.

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AOCS Recommended Practice Cd 1c-85

Reapproved 2009

Calculated Iodine Value

DEFINITION

This method determines the iodine value for edible oils directly from fatty acid compositions (see Notes, 1 and 2).

SCOPE

Applicable to triglycerides and free fatty acids and their hydrogenated products (see Notes, 3).

APPARATUS

1. Gas chromatograph—to provide fatty acid composition by accepted validated methodology, e.g., AOCS Official Method Ce 1-62, AOCS Official Method Ce 1c-89, etc.

PROCEDURE

1. Proceed as directed in the accepted validated method of choice.
2. Determine the fatty acid composition of the oil or fatty acid mixture.

CALCULATIONS (see Notes, 4)

Iodine values may be calculated according to the potential number of iodine atoms added to each fatty acid or triglyceride according to Table 1. Examples of calculations are given below.

1. Triglycerides, iodine value = (% hexadecenoic acid × 0.950) + (% octadecenoic acid × 0.860) + (% octadecadienoic acid × 1.732) + (% octadecatrienoic acid × 2.616) + (% eicosenoic acid × 0.785) + (% docosenoic acid × 0.723).
2. Free fatty acids, iodine value = (% hexadecenoic acid × 0.9976) + (% octadecenoic acid × 0.8986) + (% octadecadienoic acid × 1.810) + (% octadecatrienoic acid × 2.735) + (% eicosenoic acid × 0.8175) + (% docosenoic acid × 0.7497).

NOTES

1. While this procedure provides an iodine value, it is not intended to be a rapid method, but rather gives two results from one analysis.
2. For oils with an unsaponifiable content greater than 0.5% (e.g., fish oils), the calculation tends to be low.
3. The calculation tends to be low for materials with low iodine value.
4. Calculated IV based on GC fatty acid composition of non-triglyceride lipid materials such as partial esters of glycerol, partial esters of sorbitol/sorbitan/isosorbide esters, partial esters of polyoxyethylene sorbitol/sorbitan/isosorbide or glycerol, etc. will provide the calculated IV of only the fatty acids used to prepare the partial esters. To obtain the actual IV of partial esters with nonfatty acid polyol diluents, the chlorinated Wijs Reagent IV method should be used. IV values of partial esters via the Wijs method are lower than those obtained by GC because of the dilution effect of the polyol material.

Table 1
Conversion factors for triglycerides and free fatty acids

	MW Triglyceride	I	I M.W. 126.9045	Factor Triglyceride	MW Fatty Acid	I	I M.W. 126.9045	Factor Fatty Acid
C12:1	633.01	6	761.4270	1.2029	198.3500	2	253.8090	1.2796
C14:1	717.18	6	761.4270	1.0617	226.3800	2	253.8090	1.1212
C15:1 *trans*	759.16	6	761.4270	1.0030	240.4000	2	253.8090	1.0558
C15:1	759.16	6	761.4270	1.0030	240.4000	2	253.8090	1.0558
C16:1 *trans*	801.34	6	761.4270	0.9502	254.4300	2	253.8090	0.9976
C16:1	801.34	6	761.4270	0.9502	254.4300	2	253.8090	0.9976
C17:1 *trans*	843.42	6	761.4270	0.9028	268.4800	2	253.8090	0.9454
C17:1	843.42	6	761.4270	0.9028	268.4800	2	253.8090	0.9454
C18:1 *trans*	885.50	6	761.4270	0.8599	282.4800	2	253.8090	0.8985
C18:1	885.50	6	761.4270	0.8599	282.4800	2	253.8090	0.8985
C18:2 *trans*	879.50	12	1522.8540	1.7315	280.4800	4	507.6180	1.8098
C18:2	879.50	12	1522.8540	1.7315	280.4800	4	507.6180	1.8098
C18:2 CLA	879.50	12	1522.8540	1.7315	280.4800	6	507.6180	1.8098
C18:3 *trans*	873.50	18	2284.2810	2.6151	278.4800	6	761.4270	2.7342
C18:3	873.50	18	2284.2810	2.6151	278.4800	6	761.4270	2.7342
C20:1	969.66	6	761.4270	0.7853	310.5400	2	253.8090	0.8173

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AOCS

AOCS Official Method Cd 1d-92

Reapproved 2009

Iodine Value of Fats and Oils Cyclohexane-Acetic Acid Method

DEFINITION

The iodine value is a measure of the unsaturation of fats and oils and is expressed in terms of the number of centigrams of iodine absorbed per gram of test sample (% iodine absorbed).

SCOPE

Applicable to all normal fats and oils with iodine values in the range of 18–165 that do not contain conjugated double bonds (see Notes, 1).

APPARATUS

1. Glass-stoppered iodine flasks—500 mL.
2. Glass-stoppered volumetric flasks—1000 mL, for preparing standard solutions.
3. Pipet—25 mL, for accurately dispensing 25.0 mL of Wijs solution.
4. Volumetric dispenser—20 mL, 1 mL adjustability, for 10% potassium iodide (KI) solution.
5. Volumetric dispenser—2 mL, 1 mL adjustability, for starch solution.
6. Volumetric dispenser—50 mL, 1 mL adjustability, for distilled water.
7. Repeater pipet—with filling flask, 20 mL, for cyclohexane.
8. Analytical balance—accurate to ± 0.0001 g.
9. Magnetic stirrer.
10. Filter paper—Whatman no. 541, or equivalent.
11. Beakers—50 mL.
12. Hot air oven.
13. Timer.

REAGENTS

1. Wijs solution—see Notes, 2 and *Caution*.
2. Potassium iodide (KI) solution—100 g/L (10% solution), prepared by dissolving 100 g of reagent grade KI in 1000 mL of deionized water.
3. Cyclohexane—reagent grade (see Notes, 3 and *Caution*).
4. Glacial acetic acid—reagent grade (see Notes, *Caution*).
5. Reagent for diluting test sample—prepared by mixing cyclohexane and glacial acetic acid, 1:1, v/v. The absence of oxidizable matter in the reagent is verified by shaking 10 mL of the reagent with 1 mL of saturated aqueous potassium dichromate solution and 2 mL of concentrated sulfuric acid; no green coloration should appear.
6. Hydrochloric acid—reagent grade (see Notes, *Caution*).
7. Soluble starch solution—recently prepared, tested for sensitivity (see Notes, 4). Make a paste with 1 g of natural, soluble starch (see Notes, 5) and a small amount of cold distilled water. Add, while stirring, to 100 mL of boiling water.

   Test for sensitivity—Place 5 mL of starch solution in 100 mL of water and add 0.05 mL of freshly prepared 0.1 M KI solution and one drop of a 50 ppm chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution to 1000 mL. The deep blue color produced must be discharged by 0.05 mL of 0.1 N sodium thiosulfate.
8. Potassium dichromate—reagent grade. The potassium dichromate is finely ground and dried to constant weight at about 110°C before using (see Notes, *Caution*).
9. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)—0.1 M, accurately standardized, prepared from reagent-grade $Na_2S_2O_3 \cdot 5H_2O$ (see Notes, 6).

   Standardization of sodium thiosulfate—Weigh 0.16–0.22 g of finely ground and dried potassium dichromate into a 500 mL flask or bottle by difference from a weighing bottle. Dissolve in 25 mL of water, add 5 mL of concentrated hydrochloric acid, 20 mL of potassium iodide solution and rotate to mix. Allow to stand for 5 min, and then add 100 mL of distilled water. Titrate with sodium thiosulfate solution, shaking continuously until the yellow color has almost disappeared. Add 1–2 mL of starch indicator and continue the titration, adding the thiosulfate solution slowly until the blue color just disappears. The strength of the sodium thiosulfate solution is expressed in terms of its molarity.

$$\text{Molarity of } Na_2S_2O_3 \text{ solution} = \frac{20.394 \times \text{mass of } K_2Cr_2O_7, \text{ g}}{\text{volume, mL of sodium thiosulfate}}$$

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PROCEDURE

1. Melt the test sample, if it is not already liquid (the temperature during melting should not exceed the melting point of the test sample by more than 10°C), and filter through two pieces of filter paper to remove any solid impurities and the last traces of moisture. The filtration may be performed in an air oven at 80–85°C, but should be completed within 5 min ± 30 sec. The test sample must be absolutely dry.

   Note—All glassware must be absolutely clean and completely dry.
2. After filtration, allow the filtered test portion to achieve a temperature of 68–71 ± 1°C before weighing the test portion.
3. Once the test portion has achieved a temperature of 68–71 ± 1°C, immediately weigh the test portion into a 500 mL iodine flask, using the weights and weighing accuracy noted in Table 1 (see Notes, 7).
4. Add 15 mL of cyclohexane + glacial acetic acid (Reagents, 5) on top of the test portion and swirl to ensure that the test portion is completely dissolved.
5. Dispense 25 mL of Wijs solution using the pipet (Apparatus, 3) into the flask containing the test portion, stopper the flask, and swirl to ensure an intimate mixture. Immediately set the timer for 1.0 or 2.0 hr, depending on the iodine value of the test portion IV < 150, 1.0 hr; IV ≥ 150, 2.0 hr (see Notes, 8).
6. Immediately store the flasks in the dark for the required reaction time at a temperature of 25 ± 5°C.
7. Remove the flasks from storage and add 20 mL of KI solution, followed by 100 mL of distilled water (see Notes, 9 and 10).
8. Titrate with 0.1 M $Na_2S_2O_3$ solution, adding it gradually and with constant and vigorous shaking (see Notes, 11). Continue the titration until the yellow color has almost disappeared. Add 1–2 mL of starch indicator solution and continue the titration until the blue color just disappears.
9. Prepare and conduct at least one blank determination with each group of test portions simultaneously and similar in all respects to the test portion.

CALCULATIONS

1. $$\text{Iodine value} = \frac{(B - S) \times M \times 12.69}{\text{mass of test portion, g}}$$

   Where—

   B = volume of titrant, mL of blank
   S = volume of titrant, mL of test portion
   M = molarity of $Na_2S_2O_3$ solution

PRECISION

1. International Union of Pure and Applied Chemistry (IUPAC)/International Organization for Standardization (ISO) collaborative studies gave the statistical results shown in Table 2.

NOTES

Caution

Wijs solution causes severe burns, and the vapors can cause lung and eye damage. Use of a fume hood is recommended. Wijs solution without carbon tetrachloride is available commercially.

Cyclohexane is flammable and a dangerous fire risk. It is moderately toxic by inhalation and skin contact. The TLV in air is 300 ppm.

Glacial acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and is a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular, lung cancer. It is a strong oxidizing agent and a dangerous fire risk when in contact with organic chemicals.

NUMBERED NOTES

1. When the iodine value is determined on materials having conjugated systems, the result is not a measure of total unsaturation, but rather is an empirical value indicative of the amount of unsaturation present. Reproducible results are obtained that afford a comparison of total unsaturation. When the iodine value is required on fatty acids, see Method Tg 1a-64.
2. Because the preparation of the Wijs solution is time-consuming and involves the use of both hazardous and toxic chemicals, this solution may be purchased from a chemical supplier. Solutions are available which contain no carbon tetrachloride, and such solutions should be used. All Wijs solutions are sensitive to temperature, moisture and light. Store in a cool and dark place, and never allow to come to a temperature above 25–30°C. The laboratory preparation of Wijs solution is noted in AOCS Official Method Cd 1-25.
3. Fresh cyclohexane should be used. Erratic results may be obtained if old cyclohexane is used. See Reagents, 5 for a test for the presence of oxidizable substances.

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4. 1% starch solution may be purchased from a chemical supplier.
5. "Potato Starch for Iodometry" is recommended, because this starch produces a deep blue color in the presence of the iodonium ion. "Soluble Starch" is not recommended because a consistent deep blue color may not be developed when some soluble starches interact with the iodonium ion. The following are suitable starches: Soluble Starch for Iodometry, Fisher S516-100; Soluble Potato Starch, Sigma S-2630; Soluble Potato Starch for Iodometry, J.T. Baker 4006–04.
6. The sodium thiosulfate solution may be purchased from a chemical supplier. However, it still must be accurately standardized in the laboratory.
7. The weight of the test portion must be such that there will be an excess of Wijs solution of 50–60% of the amount added, i.e., 100–150% of the amount absorbed.
8. The indicated reaction times are those specified in the IUPAC Iodine Value Method 2.205 (References, 1) and were the reaction times used in the IUPAC/ISO validation study of the cyclohexane + acetic acid method. Previous AOCS versions of iodine value methods specified a reaction time of 0.5 hr, regardless of the iodine value, but noted that "a longer reaction time may be necessary for oils with high iodine value." The longer reaction times appear to be particularly critical when cyclohexane is used as a replacement for carbon tetrachloride (References, 2).
9. If the reaction is not terminated within 3 min after the reaction time, the test portion must be discarded.
10. The test portion must be titrated within 30 min of reaction termination, after which the analysis is invalid.
11. Mechanical stirring is recommended for agitation during the addition of thiosulfate.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.205.
2. AOCS collaborative study results using cyclohexane alone appear in *J. Am. Oil Chem. Soc. 65:*745 (1988).
3. IUPAC collaborative study results using cyclohexane + glacial acetic acid (1:1) appear in *Pure Appl. Chem. 62:*2339 (1990) and were reported in *INFORM 1:*484 (1990).

Table 1
Test Sample weights

Iodine Value expected	Mass, g, ± 0.001
<5	3.000
5–20	1.000
21–50	0.400
51–100	0.200
101–150	0.130
151–200	0.100

Table 2
Statistical analysis of results obtained in IUPAC/ISO collaborative study (11 laboratories) of Wijs iodine value with carbon tetrachloride and cyclohexane + glacial acetic acid (1:1) solvents[a].

Test Sample	Mean value		r		R		RSD_r		RSD_R		SD_r		SD_R	
(oil)	CTC	CHX	CTC	CHX	CTC	CHX	CTC	CHX	CTC	CHX	CTC	CHX	CTC	CHX
Sunflower	133.6	132.9	3.00	0	7.20	4.80	1.07	1.29	2.57	1.71	1.43	1.71	3.44	2.28
Refined palm	53.1	53.0	0.65	0.82	1.43	1.90	0.23	0.29	0.51	0.68	0.12	0.16	0.27	0.36
Crude fish	109.1	108.5	1.67	1.40	4.35	2.80	0.60	0.50	1.55	1.00	0.65	0.54	1.69	1.09
Tung	164.5	163.1	3.47	2.39	5.27	4.32	1.24	0.85	1.88	1.54	2.04	1.39	3.10	2.52
Tallow (beef)	47.2	46.9	1.37	1.33	2.75	2.10	0.49	0.48	0.98	0.75	0.23	0.22	0.46	0.35
Crude palm	52.5	52.6	1.36	2.15	2.12	2.89	0.49	0.77	0.76	1.03	0.26	0.40	0.40	0.54
Used frying	37.7	37.7	0.97	1.38	1.18	2.48	0.35	0.49	0.42	0.89	0.13	0.19	0.16	0.33
Palm kernel	18.2	18.3	0.17	0.14	0.51	0.64	0.06	0.05	0.18	0.23	0.01	0.01	0.03	0.04
Olive	82.3	82.2	0.75	1.75	1.86	2.62	0.27	0.63	0.66	0.94	0.22	0.51	0.55	0.77
HSBO1[b]	102.6	102.3	1.40	2.20	4.80	5.10	0.50	0.79	1.71	1.82	0.51	0.80	1.76	1.86
HSBO2[b]	74.7	74.8	1.70	1.50	3.70	2.10	0.61	0.54	1.32	0.75	0.45	0.40	0.99	0.56
HFO[b]	73.0	72.8	1.70	1.60	2.50	2.30	0.61	0.57	0.89	0.82	0.44	0.42	0.65	0.60

[a]See References, 3. RSD_r, relative standard deviation, repeatability; RSD_R, relative standard deviation, reproducibility; SD_r, standard deviation, repeatability; SD_R, standard deviation, reproducibility; CTC, carbon tetrachloride; CHX, cyclohexane + glacial acetic acid (1:1); HSBO, hydrogenated soybean oil; HFO, hydrogenated fish oil.

[b]Additional study involving 17 laboratories.

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AOCS Standard Procedure Cd 1e-01
Revised 2013

Determination of Iodine Value by Pre-calibrated FT-NIR with Disposable Vials

DEFINITION

The method determines the iodine value (IV) for triglyceride-based oils and fats using pre-calibrated Fourier transform near infrared (FT-NIR) spectrometers with neat sampling in disposable glass vials. Near infrared spectra in the C-H 2nd overtone stretching region are used to determine IV. The ratio of band intensities correlates well with IV while not being perturbed by other chemical and physical properties in the test samples. A partial-least-squares (PLS) algorithm is used to model frequencies of the test samples from 7560 cm^{-1} to 9100 cm^{-1}. Using a comprehensive training set, a robust calibration has been developed that can be used to predict IV in any triglyceride based oil and fat without any further calibration effort. Outlier monitoring is used to prevent erroneous prediction of test samples not suitable for analysis. Furthermore, to insure proper prediction of IV, the FT-NIR must meet the specifications of spectrometer reproducibility stated in the method. Neat test samples are placed in disposable glass vials and maintained at 75°C ± 1°C. The elevated temperature ensures that all test samples are in liquid form. The near IR beam is transmitted through several millimeters of test sample across the diameter of the vial. This gives adequate absorbance in the C-H 2nd overtone region. Test samples with catalyst are filtered prior to analysis.

SCOPE

Calibration development in the NIR requires knowledge of chemometric principles. This method avoids calibration development steps by employing pre-calibrated FT-NIR analyzers. The calibration installed in the software of each FT-NIR analyzer is the result of a comprehensive calibration development program using the Wijs method for reference values. It has undergone extensive validation and has been subjected to round robin testing. The spectrometer specifications and operating parameters required for proper function of the calibration are provided in this method, which permits users and manufacturers to develop and maintain analyzers with the same statistical performance as described here. This also permits transfer of the calibration to other FT-NIR devices. The participants in the round robin were required only to scan test samples and predict the results with the calibration provided. The round robin results from this study were obtained with triglyceride based oils and fats obtained from the AOCS Laboratory Proficiency Program having a IV from 0.2–142.

Three standardization protocols are used. The first, (and most important), requires the use of theoretically calculated certified standards for accurate validation of IV. Since these certified standards are limited to discrete regions, standards obtained from the AOCS Laboratory Proficiency Program may be utilized to inhibit interpolation and extrapolation effects. Consequently, even the AOCS Program may be limited in the range and type of test samples available; thus the last validation protocol is a practical approach based upon using local process test samples and cross-checking the results with the local Wijs method. For this, the reproducibility of the official Wijs method must be fully evaluated. Simple diagnostic and outlier detection protocols, which are commonly used by manufacturers, will prevent invalid results from the spectrometer or test sample, respectively. When used, this method must be validated with check test samples to monitor its short- and long-term stability.

APPARATUS

1. FT-NIR spectrometer with 16 cm^{-1} resolution or equivalent and capable of scanning the region from 6000 cm^{-1} to 10,000 cm^{-1}. It is important to ensure linearity of detector response. For this it is recommended to use any one of a number of available near IR photovoltaic detectors such as indium arsenide (InAs), indium gallium arsenide (InGaAs), or deuterated triglycine sulfate (DTGS).
2. A practice used to define the performance of Fourier transform infrared spectrometers is defined by ASTM[1]. Instrument specifications are defined as: wavenumber repeatability ± 0.002 cm^{-1} (± 2σ) and wavenumber reproducibility ± 0.04 cm^{-1} (± 2σ) at 7300 cm^{-1} at a resolution of 2 cm^{-1}. Spectral response repeatability (100% line deviation) within 0.1% in the 8000 to 4500 cm^{-1} range for two consecutive measurements at a resolution of 16 cm^{-1}. The signal to random noise in the absorbance spectra is critical only for analysis results that need to be highly repeatable, such as the IV values for fully saturated oils and fats (IV<5). For a repeatability of 0.1 IV units, the noise absorbance must be <25 micro absorbance root mean square (rms) from 10,000 to 4500 cm^{-1} at a resolution of 16 cm^{-1}. These criteria establish a benchmark for instrument performance, which largely influence calibration results.

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3. To ensure a global calibration strategy, all manufactured units must reproduce the absorbance spectrum of a stable reference sample. For the round robin reported here, all FT-NIRs were validated with a test sample of high purity toluene in a 0.5 mm cell at 28 ± 1°C. The absorbance reproducibility was maintained to <0.001 A rms. in the region 4100–6000 cm^{-1} at a resolution of 16 cm^{-1}. The manufacturer must also have a protocol to test the equivalent of this specification frequently, and particularly after repairing an analyzer.
4. Data collection should allow conversion of the spectra onto the absorbance scale.
5. In order to make sampling easy and with minimal operator influence on the analysis, the sampling interface should be able to accommodate 8 mm o.d. (6.6 mm i.d.) HPLC autosampler vials thermally equilibrated to 75°C ± 1°C. Dimensional variation in commercial vials is ± 0.25 mm i.d. and ± 0.4 mm o.d.
6. A validated calibration software package should be used to develop all multivariate equations. This software should allow the generation of partial-least-squares (PLS) equations. This software also needs to provide discrimination parameters used for calibration selection and outlier detection.
7. The software for routine analysis should provide straightforward implementation of the calibrations and accommodate the use of sophisticated analysis procedures. It is recommended that this software be validated in accordance with GAMP (Good Automated Manufacturing Practice) guidelines.

CALIBRATION STANDARDS

1. Natural oils and fats consist of complex mixtures. This makes the preparation of calibration standards impractical. Therefore, the multivariate calibration must be developed from actual process test samples[2]. Spectra are measured on test samples for which the IV values are calculated from the AOCS Official Method Cd 1d-92. For a robust calibration, the calibration set must represent the entire variation observed during routine sampling conditions, such as temperature and constituent range. The concentration range of the calibration samples should attempt to exceed the lowest and highest values encountered during normal analysis conditions to prevent extrapolation. Table 1 below describes the type of test samples incorporated into the global IV calibration used for the round robin testing in this study. Over 1200 test samples were used in the calibration from 0 to 190 IV. Most products were subjected to a wide variety of refining methods, i.e., hydrogenation, interesterification, fractionation, etc.
2. Since the FT-NIR uncertainty can be no better than the reference method for which it is based upon, the reference method reproducibility must be evaluated. In this case, it is only necessary to demonstrate that the reference measurement is being practiced in accordance with the procedure described in the AOCS Official Method Cd 1d-92, and that the repeatability obtained is statistically comparable to that published in the method. Data from established quality control procedures are used to demonstrate that the repeatability of the reference method is within the official method specifications. If such data are not available, then repeatability should be obtained on at least 10 test samples in duplicate across the concentration range of interest. The standard deviation should be calculated and compared to that expected based on the published repeatability (see Notes, 1).

STANDARDIZATION PROTOCOLS AND LONG-TERM STABILITY

1. The repeatability of the calibration should be evaluated using duplicate test sample testing across the entire range of the calibration and calculated with the PSD equation (see Notes, 1). The precision results should be comparable to the round robin statistical results reported in this study.
2. The uncertainty of the measurement should be evaluated (and biased if necessary) with theoretically calculated standardization samples (see Notes, 2). Table 2 below provides a list of theoretically calculated standards.
3. Since the pure standards in Table 2 are limited to discrete regions, pre-evaluated process test samples may be used to provide a boxcar distribution of standards across the range to help evaluate uncertainty. It is recommended that these test samples be obtained from the AOCS Laboratory Proficiency Program (see Notes, 3), which have undergone round robin testing. These standards can range in reproducibility error (from their Wijs round robin results) from 0.06–1.74.
4. Under conditions where a representative test sample set is not available from the AOCS Program, local process test samples may be used as standards to evaluate the uncertainty. It is necessary to fully evaluate the official method on which these values are based before using this last protocol (see Notes, 1).
5 Check samples can be used to evaluate the long-term stability of the method. They must be chemically stable when stored and sampled. At least three check samples representing the low, medium, and high regions of the calibration are recommended for analysis. After analysis, check samples should be discarded as repetitive heating of some samples may induce oxidative effects. Since some check samples are solid at room temperature, it is recommended that at least one years supply are transferred to vials and stored in a freezer.

PREPARATION OF TEST SAMPLES

1. Liquid test samples are mixed and transferred to 8 mm vials using disposable pipettes. Solid test samples must be heated beyond their melting point, stirred, and transferred. Microwave heating may produce localized heating; therefore, water-bath heating is recommended.

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PROCEDURE

1. Set up the NIR instrument according to the manufacturer's recommendations and carry forward all instrument diagnostics to ensure that the instrument is performing to the specifications established by this procedure (defined in Apparatus, 2 and 3).
2. Adjust the resolution to 16 cm^{-1} or equivalent covering the range from 7500–9200 cm^{-1}. The number of scans influences the signal-to-noise ratio. 128 scans at 16 cm^{-1} resolution should correspond to less than 2 min analysis time. The conditions should be identical for the training set and unknown test samples.
3. Obtain an open beam, air background spectrum through the sampling device without the vial. Air background spectra are typically collected every 30 to 120 minutes. The reference scan waiting period is dependent on temperature and humidity fluctuations. Collecting background spectra every 10 min over a 24-hr period validates this. Spectral subtraction of the first collected spectrum from all other air background spectra can be used to visually observe changes due to instrument drift or environmental influences.
4. Remove any air bubbles from the test sample vial and insert into the sampling accessory. Check that thermal equilibrium has been attained at 75°C ± 1°C, or if your test sample has a higher melting point, adjust your temperature controller slightly over this value. Collect the test sample spectrum. Ratio the test sample spectrum against the open beam and air background spectrum and convert to absorbance.

CALCULATIONS AND CHEMOMETRICS

Detailed multivariate analysis is beyond the scope of this method; however, a summary of the practices for infrared, multivariate, quantitative analysis is described by the ASTM[2]. This is a standard guide for implementing multivariate calibrations with infrared spectrometers in determining the physical and chemical characteristics of materials. Sample selection theory and validation of the reference methods are described. Procedures for collecting and treating data for developing FT-NIR calibrations are outlined with definitions, terms, and calibration techniques. The criteria for validating the performance of calibration models are also provided. Most commercial multivariate software packages have the utilities to perform most of the data preprocessing steps and multivariate calculations described.

This portion of the method will describe the wavelength regions and preprocessing steps necessary to design the method, which are further described in detail elsewhere[4,5].

1. *Wavelength regions*—The curve in Figure 1 shows FT-NIR Absorbance spectra of three oils from 7560–9100 cm^{-1} with varying IV.
2. *Preprocessing*—Before multivariate methods such as PLS are applied, several preprocessing steps are applied to the data. Mean centering is used to enhance subtle differences in the data. The spectra are meancentered by calculating the average spectrum in the training set and then subtracting this from each individual spectrum. In addition, the mean IV of the concentration set is calculated and subtracted from each individual training set test sample. A two-point baseline correction applied at 9100 and 7560 cm^{-1} will correct for additive shifts in the baseline caused by scattering.

   A normalization step is used to eliminate intensity changes related to pathlength differences in the disposable vials. This routine essentially divides the absorbance by the sum of absorbance at many wavelengths. For IV determination, the sum of absorbance is defined as the total area under the curve from 9100–7560 cm^{-1}. Each spectrum in the training set is normalized. The preprocessing steps instituted on the calibration set must be identical for routine sampling conditions.
3. *PLS equations*—PLS equations are developed in the regions from 7560 cm^{-1} to 9100 cm^{-1}.
4. *Discrimination functions*—Discrimination functions are used to eliminate various test sample outliers in calibration development or during routine scanning conditions. These functions may be also serve as pattern recognition tools for calibration selection. The definition and principles of these functions are beyond the scope of this method; however, the functions used for the round robin results in this method are described elsewhere[4,5].

ROUND ROBIN RESULTS

An international interlaboratory study was completed by the AOCS Edible Oil Committee in 2000. Thirteen laboratories participated, each obtaining two test results for each test sample analyzed[5]. All labs utilized the same Global IV calibration placed on different FT-NIRs; all instruments were ABB Bomem MB 154 (FT-NIR/FT-IR) or MB 160 (FT-NIR) models (see Notes, 4). All test samples were measured in disposable glass sample vials at 75°C. The statistical results (evaluated in accordance with ISO 5725-1994) are summarized in the following tables.

REPRODUCIBILITY

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit R as shown in Tables 3 and 4.

REPEATABILITY

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment with short intervals of time, should not be greater than the repeatability limit r as shown in Tables 3 and 4.

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PURE STANDARD RESULTS

Table 5 shows the prediction results for pure standards, which may be used to adjust the slope or bias of the global calibration. The predicted result for the trilinolenin standard is extrapolated since the Global IV utilized here extended only to 190 IV. The calibration coefficients should only be adjusted based on standards that are representative of the calibration.

UNCERTAINTY

A NIST technical note is used to describe the guidelines for expressing uncertainty in analytical methods. The FT-NIR uncertainty (SEP) is calculated from the average of predicted results from the round robin and from the pure standards. The round robin reference results are based on Wijs round robin testing obtained from the AOCS Laboratory Proficiency Program. The reproducibility of these test samples across the entire range of IV varied from 0.06–1.74, which is shown in Tables 2 and 3 with each known value.

Figure 2 shows the scatter plot of the FT-NIR prediction results for the entire range from 0.2 to 140 IV. The SEP was calculated as 0.55 with an R^2 of 0.99.

NUMBERED NOTES

1. The pooled standard deviation (PSD) to determine laboratory repeatability is defined as:

$$\text{pooled standard deviation} = \sqrt{\frac{\sum(\Delta^2)}{N-1}}$$

   Where—
   Δ is defined as the difference in duplicate measurements from the same method on identical test material. However, the sum total of duplicate tests may assume different products, different operators on different days and different equipment. N is the total number of duplicate measurements.
2. Nu-Check-Prep, Inc. P.O. Box 295, Elysian, MN 56028, fax (507) 267-4790.
3. Test Samples for testing Official AOCS Methods are available from the AOCS Reference Sample Program, P.O. Box 17190, Urbana, IL 61820 USA, fax (217) 351-8091.
4. ABB Bomem FT-NIRs, ABB Bomem, 585 Est Charest Ave, Bureau 300, Quebec City, Quebec, Canada G1K 9H4. Phone (800) 858-3847, fax (418) 877-2834.

REFERENCES

1. Standard Practice for Describing and Measuring Performance of Fourier Transform Infrared (FT-IR) Spectrometers: Level Zero and Level One Tests, ASTM E 1421-91.
2. Standard Practices for Infrared, Multivariate, Quantitative Analysis, ASTM E 1655-97.
3. Taylor, B.N., and Kuyatt, C.E., Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results, NIST Technical Note 1297, (1994).
4. Li, H., F.R. van de Voort, J. Sedman, A.A. Ismail, R. Cox, C. Simard, and H. Buijs, Discrimination of Edible Oil Products and Quantitative Determination of Their Iodine Value by Fourier Transform Near-Infrared Spectroscopy, *J. Am. Oil Chem. Soc. 77*:29–36 (2000).
5. R. Cox, J. Lebrasseur, E. Michiels, H. Buijs, H. Li, F.R. van de Voort, A.A. Ishmail, and J. Sedman, Determination of Iodine Value with a Fourier Transform-Near Infrared Based Global Calibration Using Disposable Vials: An International Collaborative Study, *J. Am. Oil Chem. Soc. 77*:1229–1234 (2000).

Table 1
List of oils and fats used in the global IV calibration used in the round robin.

Almond	Corn	Milk fat	Safflower
Canola	Edible tallow	Olive	Soybean
Castor	Fish	Palm crude	Sunflower
Coconut	Lard	Palm kernel	Walnut
Cocoa butter	Linseed	Palm olein	
Cottonseed	Margarine blend	Palm stearin	

Table 2
Pure standards.

Standard	IV
C18:1cis Triolein	86.01
C18:2cis Trinlinolein	173.1
C18:3cis Trilinolenin	261.1
C12 Trilaurin	0
C14 Trimyristin	0
C16 Tripalmitin	0
C18 Trinstrearin	0

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Table 3

	1	2	3	4	5	6	7	8
Labs after outliers	13	12	11	13	12	13	11	12
Labs removed	0	1	2	0	1	0	2	1
Mean value (IV)	105.12	69.27	79.15	75.23	51.11	57.58	141.26	126.20
Known value (IV)	104.30 ± 1.40	69.12 ± 1.00	78.33 ± 1.57	74.8 ± 1.00	51.21 ± 0.46	57.45 ± 0.52	140.99 ± 0.73	126.08 ± 0.77
S_r	0.30	0.18	0.19	0.18	0.19	0.21	0.04	0.10
RSD_r	0.28	0.26	0.24	0.24	0.37	0.36	0.03	0.08
$r = (2.8 \times S_r)$	0.83	0.50	0.53	0.50	0.53	0.59	0.12	0.29
S_R	1.02	0.24	0.21	0.31	0.34	0.31	0.19	0.34
RSD_R	0.97	0.35	0.27	0.42	0.67	0.54	0.14	0.27
$R = (2.8 \times S_R)$	2.87	0.68	0.59	0.88	0.96	0.87	0.54	0.95

Table 4

	9	10	11	12	13	14	15	16
Labs after outliers	13	11	13	13	12	12	12	12
Labs removed	0	2	0	0	1	1	1	1
Mean value (IV)	142.11	95.12	46.92	24.18	8.83	1.15	4.42	0.36
Known value (IV)	142.37 ± 1.74	96.100 ± 0.3	46.550 ± 0.3	24.500 ± 0.2	8.750 ± 0.1	0.800 ± 0.06	4.400 ± 0.1	0.200 ± 0.06
S_r	0.08	0.11	0.12	0.18	0.13	0.13	0.09	0.09
RSD_r	0.06	0.12	0.25	0.76	1.42	11.77	2.02	23.89
$r = (2.8 \times Sr)$	0.22	0.32	0.32	0.51	0.35	0.38	0.25	0.24
S_R	0.34	0.44	0.30	0.33	0.16	0.44	0.16	0.19
RSD_R	0.24	0.46	0.64	1.34	1.76	37.98	3.66	52.35
$R = (2.8 \times S_R)$	0.96	1.23	0.84	0.91	0.43	1.22	0.45	0.53

Reference Sample key for Table 3 and Table 4

1 = AOCS reference sample edible fat
2 = AOCS reference sample edible fat
3 = AOCS reference sample edible fat
4 = AOCS reference sample edible fat
5 = AOCS reference sample palm oil
6 = AOCS reference sample palm oil
7 = AOCS reference sample crude safflower oil
8 = AOCS reference sample crude corn oil
9 = AOCS reference sample crude safflower oil
10 = Private lab oleic safflower oil
11 = Private lab hydrogenated palm
12 = Private lab palm kernel olein
13 = Private lab coconut oil
14 = Private lab hydrogenated walnut
15 = Private lab hydrogenated soybean oil
16 = Private lab hydrogenated palm kernal oil

Table 5
Prediction of pure standards.

Standard	Theoretical IV	Predicted IV from global calibration
C18:1cis Triolein	86.01	85.9
C18:2cis Trinlinolein	173.1	172.79
C18:3cis Trilinolenin	261.1	262.62
C12 Trilaurin	0	0.03
C14 Trimyristin	0	0.65
C16 Tripalmitin	0	1.07
C18 Trinstrearin	0	1.22

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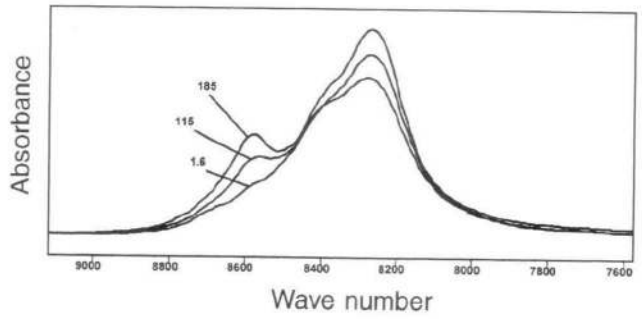

Figure 1. 3 spectra with varying IV

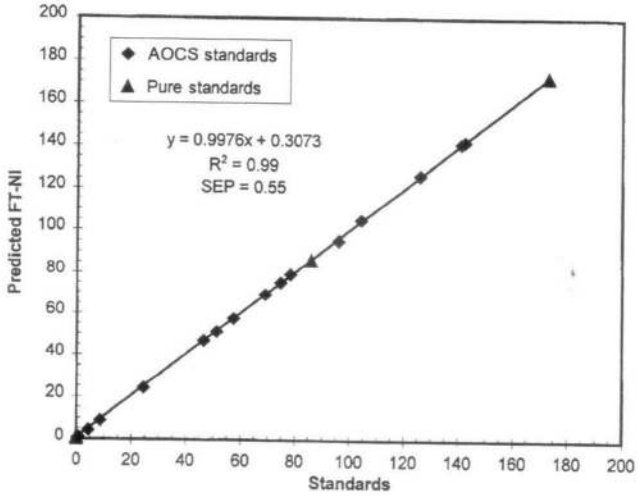

Figure 2. Scatter plot for pure standards and AOCS standards.

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AOCS

AOCS Official Method Cd 3-25

Revised 2013

Saponification Value

DEFINITION

The saponification value is the amount of alkali necessary to saponify a definite quantity of the test sample. It is expressed as the number of milligrams of potassium hydroxide (KOH) required to saponify 1 gram of the test sample.

SCOPE

Applicable to all normal fats and oils (see Notes, 1).

APPARATUS

1. Erlenmeyer flasks—alkali resistent, 250 or 300 mL, Ŧ24/40 ground-glass joint.
2. Condensers—with Ŧ24/40 ground-glass joint to fit Erlenmeyer flasks.
3. Water bath, or a hot plate with variable heat control.

REAGENTS

1. Hydrochloric acid, 0.5 M—accurately standardized. See AOCS Specification H 14-52.
2. Alcoholic potassium hydroxide (KOH)—may be purchased or prepared by placing 5–10 g of reagent-grade KOH (see Notes, *Caution*) pellets in a 2-L flask, adding 1–1.5 L of 95% ethyl alcohol (see Notes, *Caution*) and boiling in a water bath under reflux for 30–60 min. Distill and collect alcohol in a clean, glass-stoppered receiver. Dissolve 40 g of potassium hydroxide, low in carbonate, in 1 L of the distilled alcohol (see Notes, *Caution*) with cooling to keep the temperature at or below 15°C. This solution should remain clear (see Notes, 2-3).
3. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol (see Notes, 4).

PROCEDURE

1. Melt the test sample (see Notes, 5) if it is not already liquid and filter through dry filter paper to remove any impurities and moisture. The test sample must be completely dry.
2. Weigh a test portion of such size that the back titration is 45–55% of the blank. This usually requires a test portion of 1.0–2.2 g if 25 mL of KOH are used (Table 1) or 4–5 g if 50 mL of KOH are used. Add the alcoholic KOH with a pipet and allow the pipet to drain for approximately 10 s (see Notes, 6).
3. Prepare and conduct blank determinations concurrently with the test portion omitting the fat or oil.
4. Connect the condenser and boil gently, but steadily, until the test portion is completely saponified. This usually requires at least 30 min for normal test portions (see Notes, 7). Make certain that the vapor ring in the condenser does not rise to the top of the condenser, or loss may occur.
5. After the flask and condenser have cooled somewhat, but not sufficiently to form a gel, wash the inside of the condenser with a small quantity of distilled water. Disconnect the condenser, add about 1 mL of phenolphthalein indicator and titrate with 0.5 M HCl until the pink color just disappears. Record the volume of 0.5 M HCl required for the titration.

CALCULATIONS

1. Saponification value $= \dfrac{(B - S) \times (M)}{W} \times 56.1$

   Where—

   B = volume of 0.5 M HCl required to titrate blank, mL
   S = volume of 0.5 M HCl required to titrate test portion, mL
   M = molarity of HCl solution
   W = mass of test portion in grams

PRECISION

Repeatability and reproducibility values from an international collaborative study are given in Table 2.

NOTES

Caution

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials such as potassium hydroxide, always add pellets to water

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and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. When the saponification value is required on the fatty acids, the preparation and separation are performed as directed in AOCS Official Method Tl 1a-64.
2. Ethanol may be stabilized by the addition of 5 g aluminum pellets to the reflux flask prior to refluxing and distillation. Decant the clear supernatant from the precipitated potassium carbonate formed after dissolving the potassium hydroxide.
3. An alternative method to stabilize alcoholic KOH is the addition of 4 g aluminum tert-butylate and allowing it to stand for several days. Decant the clear supernatant from the precipitated potassium carbonate formed after dissolving the potassium hydroxide.
4. If the starting solution is strongly colored, 0.5–1 mL of alkali blue 6B solution can be used in place of phenolphthalein as the indicator. Note that alkali blue 6B has a pKa range that differs from phenolphthalein and the resulting pKa of the titrated test portion could vary similarly.
5. Test samples must be well mixed and entirely liquid before weighing; however do not heat the sample more than 10°C over the melting point.
6. For consistent results, a 25 or 50 mL class A volumetric pipet calibrated "to deliver" should be used. The pipet should be allowed to drain for about 10 s. And the same pipet should be used for all analyses.
7. Some test portions, particularly those difficult to saponify, may require 1–6 hr refluxing. This can only be determined by trial. Clarity and homogeneity of the test solution are partial indicators of complete saponification, but they are not necessarily absolute criteria.

Table 1
Mass of test portion.

Expected saponification value	Mass of test portion
150 to 200	2.2 g–1.8 g
200 to 250	1.7 g–1.4 g
250 to 300	1.3 g–1.2 g
> 300	1.1 g–1.0 g

Table 2
Results of a 2000 collaborative study conducted by DIN on the determination of saponification value.

Test Sample	Rapeseed oil	Palm oil	Coconut oil	60% Coconut oil + 40% MCT oil	MCT oil
Total number of laboratories accepted	19	17	20	18	16
Mean of the laboratory values (mg/g)	190.20	199.50	256.80	287.50	334.10
Repeatability					
Standard deviation, s_r	0.71	0.56	0.72	0.71	1.41
Relative standard deviation, RSD_r%	0.4	0.3	0.3	0.2	0.4
Repeatability value, $2.8 \times s_r$	2.01	1.56	2.02	1.99	3.95
Reproducibility					
Standard deviation, s_R	1.77	2.04	4.17	2.36	2.86
Relative standard deviation, RSD_R%	0.9	1.0	1.6	0.8	0.9
Reproducibility value, $2.8 \times s_R$	4.97	5.72	11.67	6.61	8.00

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AOCS

AOCS Recommended Practice Cd 3a-94

Reapproved 2009

Calculated Saponification Value

DEFINITION

The saponification value (SV) is the amount of alkali necessary to saponify a definite quantity of the test sample. It is expressed as the mg of potassium hydroxide (KOH) required to saponify 1 g of the test sample.

SCOPE

The saponification value is calculated from the normalized fatty acid composition determined by gas-liquid chromatography (Notes, 1). The method may be applied to all normal fats and oils, including linseed oil, provided they contain low concentrations of free fatty acids (≤ 0.1%) and mono- and diglycerides (≤ 0.1%). To meet this specification a freshly deodorized oil would have to be chromatographically fractionated over 5% water-deactivated silica gel to isolate the pure triglyceride fraction. The method is not directly applicable to fats and oils having a large unsaponifiable fraction.

PROCEDURE

1. Determine the normalized fatty acid composition (FAC) of the test sample by accepted, validated methodology, e.g., AOCS Official Methods Ce 1-62, Cd 1c-89, or Ce 1e-91.

CALCULATIONS

1. Determine the fractional molecular weight of each fatty acid in the test sample by multiplying the fatty acid percentage (divided by 100) by its molecular weight.
2. Saponification value, $SV = \dfrac{3 \times 56.1 \times 1000}{[(\text{m m wt} \times 3) + 92.09] - (3 \times 18)}$

   Where—

   m m wt = mean molecular weight (sum of fractional weights, determined as noted in Calculations, 1, of all fatty acids in the test sample)

   56.1 = molecular weight of KOH

   92.09 = molecular weight of glycerol

EXAMPLE

1. For soybean oil with a FAC of $C_{16:0}$ (10.6%), $C_{18:0}$ (4.2%), $C_{18:1}$ (22.7%), $C_{18:2}$ (54.5%), $C_{18:3}$ (8.0%), the m m wt of the fatty acids is 278.36 and,

   Saponification value, $SV = \dfrac{3 \times 56.1 \times 1000}{[(278.36 \times 3) + 92.09] - (3 \times 18)} = \dfrac{168{,}300}{873.17} = 192.8$

NOTES

The method has not been applied to fatty acid composition data based on mass/mass.

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AOCS

AOCS Official Method Cd 3b-76

Reapproved 2009

Saponification Value

DEFINITION

The saponification value is the amount of alkali necessary to saponify a definite quantity of the test sample. It is expressed as the number of milligrams of potassium hydroxide (KOH) required to saponify 1 gram of the test sample.

SCOPE

Applicable to vegetable oil deodorizer distillates and sludges.

APPARATUS

1. Erlenmeyer flasks—alkali resistant, 250 or 300 mL, with Ŧ24/40 ground-glass joint.
2. Condensers—with Ŧ24/40 ground-glass joint to fit Erlenmeyer flasks.
3. Water bath, or a hot plate with variable heat control.
4. Distillation filter–24 with Ŧ ground-glass joint, fitted with water-cooled condenser, for refluxing and distilling 95% ethyl alcohol as noted in Reagents, 2.

REAGENTS

1. Hydrochloric acid, 0.5 M—accurately standardized. See AOCS Specification H 14-52 (see Notes, *Caution*).
2. Alcoholic potassium hydroxide (KOH)—reagent grade ethanol containing 5% water.
3. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol.
4. Toluene—reagent grade (see Notes, *Caution*).

PROCEDURE

1. Heat the test sample (see Notes, 1) at 100°C until liquid and then mix thoroughly.
2. Accurately weigh about 2 g ± 0.1 mg of the melted test sample into the Erlenmeyer flask.
3. Add 25 mL of toluene and heat gently to dissolve the test sample.
4. Add 50 mL of the alcoholic KOH with a pipet and allow the pipet to drain for a definite period of time (see Notes, 2).
5. Prepare and conduct blank determinations simultaneously with the test sample and similar in all respects, except omitting the fat or oil.
6. Add several boiling chips to the flask and connect the condenser. Boil gently, but steadily, until the test sample is completely saponified. This usually requires about 1 hr for normal test samples. Make certain that the vapor ring in the condenser does not rise to the top of the condenser, or loss may occur.
7. After the flask and condenser have cooled somewhat, but not sufficiently to form a gel, wash down the inside of the condenser with a small quantity of distilled water. Disconnect the condenser, add about 1 mL of phenolphthalein indicator and titrate with 0.5 M HCl until the pink color just disappears. Record the volume of 0.5 M HCl required for the titration (see Notes, 3).

CALCULATIONS

1. Saponification value $= \frac{(B - S) \times (M)}{W} \times 56.1$

Where—

B = volume, mL 0.5 M HCl required to titrate blank
S = volume, mL 0.5 M HCl required to titrate test portion
M = molarity of HCl solution
W = mass of test portion in grams

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 3.2 units.
2. Two single determinations performed in different laboratories should not differ by more than 6.4 units.

NOTES

Caution

Potassium hydroxide, like all alkalies, can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to water

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and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm air. A fume hood should be used at all times when using toluene.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting this acid, always add the acid to the water, never the reverse.

If alcohol of less than reagent grade is used, follow the purification procedure listed. Prepare by placing 5–10 g of reagent-grade KOH (see Notes, *Caution*) pellets in a 2 L flask, adding 1–1.5 L of 95% ethyl alcohol (see Notes, *Caution*) and boiling in a water bath under reflux for 30–60 min. Distill and collect the alcohol in a clean, glass-stoppered receiver. Dissolve 40 g of potassium hydroxide, low in carbonate, in 1 L of the distilled alcohol (see Notes, *Caution*) with cooling to keep the temperature at or below 15°C. This solution should remain clear.

NUMBERED NOTES

1. When the saponification value is required on the fatty acids, the preparation and separation are performed as directed in AOCS Official Method Tl 1a-64.
2. For consistent results, a 50 mL class A volumetric pipet calibrated "to deliver" should be used. The pipet should be allowed to drain for the time specified.
3. Some test portions may yield a solution too dark in color to permit satisfactory end point detection. To overcome this problem, use a smaller test portion size (about 1 g).

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AOCS

AOCS Official Method Cd 3c-91

Reapproved 2009

Saponification Value Modified Method Using Methanol

DEFINITION

The saponification value is the amount of alkali necessary to saponify a definite quantity of the test sample. It is expressed as the number of milligrams of potassium hydroxide (KOH) required to saponify 1 gram of the test sample (see Notes, 1).

SCOPE

Applicable to fats and oils and fatty derivatives. While it is reasonably accurate and fairly easy to run, AOCS Official Method Cd 3-25 does have definite drawbacks (see Notes, 2). The problems are most evident to individuals and laboratories that do not perform the analysis routinely. A few improvements have been suggested for difficult materials (References, 1 and 2). The saponification solution must be carefully prepared and is best if made fresh. It tends to turn brown (References, 3) and give inconsistent results within several days because the solution media is ethanol, which contains small amounts of acetaldehyde that self-condense or polymerize. Preparing the solution is inconvenient if only a few determinations are required.

APPARATUS

1. Erlenmeyer flasks-20/40 Ʇ joint, 250 mL.
2. Air condensers—minimum 650 mm in length.
3. Hot plate—with variable heat control.
4. Burettes—two 50 mL for the acid and base solutions (see Notes, 3).

REAGENTS

1. Hydrochloric acid, 0.5 M—see Notes, 4.
2. Methanolic potassium hydroxide, 1.0 M—see Notes, 4 and 5.
3. Phenolphthalein solution—about 1% in ethanol.

PROCEDURE

1. Weigh the amount of test sample indicated in Table 1 to ± 0.001 g. Accurately add 20 mL of the 1.0 M KOH via the 50 mL burette (see Notes, 3).
2. Prepare and conduct blank determinations simultaneously with the test sample and similar in all respects.
3. Connect the air condensers and boil gently, but steadily, for 1.5–2 hr (see Notes, 6).

   Note—The vapor ring in the condenser must not rise to the top of the condenser; if this occurs there may be some loss of test sample.
4. After the flask and condenser have cooled somewhat, but not sufficiently to gel, disconnect the condenser, add about 1 mL of indicator solution (Reagents, 3), and titrate with 0.5 M HCl until the pink color has just disappeared.

CALCULATIONS

1. Saponification value $= \dfrac{28.05(B - S)}{\text{mass of test portion, g}}$

   Where—

   B = volume of titrant, mL of blank

   S = volume of titrant, mL of test portion

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. After various trials with 1.0 M KOH in methanol and 0.1 M KOH in methanol, using different test portion sizes and reaction times, the results shown in Table 2 were obtained by the procedure described in this method. The normally available 1.0 M KOH, which is also used for hydroxyl value determination, is an ideal "saponification value solution." The

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C-26 Guerbet alcohol and the C-8/10 alcohol were obtained from Exxon and Procter & Gamble, respectively. The meadowfoam oil was obtained from the Meadowfoam Growers Association. The oleyl oleate and synthetic jojoba alkyl esters were prepared by standard techniques.

2. The major difference between AOCS Official Method Cd 3-25 and this method is that the normal saponification value solution is about 0.7 M in ethanol in method Cd 3-25, while the solution used in this method is 1.0 M in methanol. A larger mole ratio of potassium hydroxide to ester and a longer reaction time are recommended to ensure complete reaction. Smaller volumes and weights are used so blank titrations can be conveniently made with a 50 mL burette. Table 3 summarizes the key differences: (i) The new method compares well to Official Method Cd 3-25 except in the case of the Guerbet C-26 alcohol and (ii) the highly hindered nature of the ester is likely the reason for the difference.

   In summary, in a 2–2.5 hr period, an accurate saponification value can be obtained with little effort.
3. Use of a digital burette makes the addition of base simple and accurate, although a standard burette may be used. An alternative would be to drain the 1.0 M KOH burette into another flask and pipet via standard technique.
4. The 0.5 M HCl and the 1.0 M methanolic KOH were purchased from Ricco Chemical and were used as is. The normality of the HCl solution should be confirmed by standardization.
5. The 1.0 M KOH in methanol solution appears usable for at least three months, provided the solution is protected from carbon dioxide and blanks are determined with each analysis.
6. Some test portions, particularly those difficult to saponify, may require more than 2 hr digestion. This can only be determined by trial. Clarity and homogeneity of the test solution are partial indicators of complete saponification, but they are not necessarily absolute criteria.

REFERENCES

1. *Nippon Koshohin Kasakkaishi, 8*(2):120 (1984).
2. *Ibid. 10*(4):269 (1986).
3. *INFORM 1*(6):543 (1990).

Table 1
Appropriate test sample weight.

Expected saponification value	Mass needed, g
0–20	15
20–50	6–15
50–100	3–6
100–150	2–3
above 150	2

Table 2
Comparison of saponification value analyses. Five materials were chosen for which the saponification value is important—two alkyl esters (oleyl oleate and synthetic jojoba), a high molecular weight alcohol (C-26 Guerbet alcohol, Exxal 26), a low molecular weight alcohol (C-8/10 alcohol, Proctor & Gamble 810), and a triglyceride oil (refined meadowfoam oil). See Notes, 1.

Raw material	Official method	New method
Oleyl oleate	92.1[a]	92.6[b]
Synthetic jojoba	94.8[a]	94.4[b]
Exxal 26 (C-26 Guerbet alcohol)	12.8[a]	15.6[b]
Meadowfoam oil	168.9[c]	166.5[c]
P & G 810 alcohol	0.5[d]	0.5[d]

[a]Average of three runs done on different days.
[b]Average of four runs done on different days.
[c]Average of two runs done on different days.
[d]One analysis.

Table 3
Comparison of saponification methods.

Item	Official Method	New Method
Alcohol	Ethanol	Methanol
Molarity	about 0.7	1.0
Amount used, mL	50	20
KOH moles/reactant moles	2/1	3/1
Reaction time, hr	1	1.5
Typical test portions weight, g	5	2

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AOCS Official Method Cd 3d-63

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Acid Value

DEFINITION

The acid value is the number of milligrams of potassium hydroxide necessary to neutralize the free acids in 1 gram of test sample. With test samples that contain virtually no free acids other than fatty acids, the acid value may be directly converted by means of a suitable factor to percent free fatty acids.

SCOPE

Applicable to crude and refined animal, vegetable, and marine fats and oils, and various products derived from them. Formerly Cd 3a-63.

APPARATUS

1. Erlenmeyer flasks—250 or 300 mL.
2. Magnetic stirring device.
3. Burette—10 mL, graduated in 0.05 mL divisions with a tip drawn to a fine opening and extending at least 10 cm below the stopcock.
4. Analytical balance—accurate to 0.0001 g.

REAGENTS

1. Potassium hydroxide (KOH), 0.1 M—Reagent grade KOH having a carbonate specification of 0.5% max, or 0.1 M KOH with NIST traceable standardization to ±1 part in 1000 in water, methanol, or ethanol.
2. Solvent mixture consisting of equal parts by volume of isopropyl alcohol (AOCS Specification H 18-58) and toluene (AOCS Specification H 19-58). See Notes, *Caution*. The mixture must give a distinct and sharp end point with phenolphthalein in the titration as noted in Procedure, 5.
3. Phenolphthalein indicator solution—1.0% in isopropyl alcohol.

PROCEDURE

1. Add indicator solution to the required amount of solvent in ratio of 2 mL to 125 mL and neutralize with alkali to a faint but permanent pink color.
2. Determine the test portion size from the following table:

Acid value	Mass, g of test portion (±10%), g	Weighing accuracy, ±g
0–1	20	0.05
1–4	10	0.02
4–15	2.5	0.01
15–75	0.5	0.001
75 and over	0.1	0.0002

3. Weigh the specified amount of a well-mixed liquid test portion into an Erlenmeyer flask.
4. Add 125 mL of the neutralized solvent mixture. Be sure that the test portion is completely dissolved before titrating. Warming may be necessary in some cases.
5. Shake the test portions vigorously while titrating with standard alkali to the first permanent pink color of the same intensity as that of the neutralized solvent before the latter was added to the test portion. The color must persist for 30 s.
6. Perform a blank titration using 125 mL of the neutralized solvent mixture.

CALCULATIONS

1. Acid value, mg KOH/g of test portion $= \dfrac{(A - B) \times M \times 56.1}{W}$

where—

A = volume, mL of standard alkali used in the titration
B = volume, mL of standard alkali used in titrating the blank
M = molarity of standard alkali
W = mass, grams of test portion

To express in terms of free fatty acids as percent lauric, oleic, or palmitic, divide the acid value by 2.81, 1.99, or 2.19, respectively.

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PRECISION

1. Single determinations performed in two different laboratories should not differ by more than 0.22 for values less than 4 nor by more than 0.36 for values in the range 4–20.

ALTERNATE PROCEDURE FOR HIGHLY COLORED TEST SAMPLES

APPARATUS

1. Glass electrode—calomel electrode pH meter for electrometric titration. A sleeve-type calomel electrode should be used (see Numbered Notes, 2).
2. Variable-speed mechanical stirrer—with glass stirring paddle.
3. Burette—10 mL, graduated in 0.05 mL divisions with a tip drawn to a fine opening and extending at least 10 cm below the stopcock.
4. Beakers—250 mL.
5. Stand and mountings for electrodes, stirrer, and burette.
6. Magnetic stirring device.

REAGENTS

1. Same as for the phenolphthalein titrimetric procedure, except that the standard alkali should be standardized by electrometric titration of pure potassium acid phthalate and no indicator solution is necessary.

PROCEDURE

1. Determine the test portion size from Procedure, 2 and weigh the test portion into a 250 mL beaker.
2. Add 125 mL of solvent mixture.
3. Mount the beaker in the titration assembly so that the electrodes are half immersed. Start the stirrer and operate at speeds that will give vigorous agitation without splattering. Immerse the tip of the burette to 1 cm below the surface of the test portion.
4. Titrate with suitable increments of alkali. After each addition of alkali, wait until the meter reading is essentially constant (usually within 2 min), then record burette and meter readings graphically. Limit increments of alkali so that changes in meter readings are 0.5 pH units (0.03 volts) or less; when inflections in titration curve occur, add alkali in 0.05 mL portions.
5. Remove titrated solution, rinse electrodes with isopropyl alcohol, and immerse in distilled water.
6. Perform a blank titration, using 125 mL of solvent mixture.

CALCULATIONS

1. Acid value, mg KOH/g of test portion = $\frac{(A - B) \times M \times 56.1}{W}$

where—

A = volume, mL of standard alkali used in titrating to middle of inflection in titration curve of test portion
B = volume, mL of standard alkali used in titrating to same pH meter reading for the blank
M = molarity of standard alkali
W = mass, grams of test portion

To express in terms of free fatty acids as percent oleic, lauric, oleic or palmitic, divide the acid value by 2.81, 1.99, or 2.19, respectively.

PRECISION

Table 1 shows the precision data from interlaboratory studies evaluated according to 150 5725:1994.

NOTES

Caution

Isopropyl alcohol is flammable and a dangerous fire risk. The explosive limits in air are 2–12%. It is toxic by ingestion and inhalation. The TLV in air is 400 ppm.

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm air. A fume hood should be used at all times when using toluene.

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NUMBERED NOTES

1. A standard methanolic potassium hydroxide (0.1 M) solution (see AOCS Specification H 15-52) may be used as an alternate titrant in place of the standard aqueous solution. The methanolic potassium hydroxide is reported to provide a complete solvent system, having a distinct, clear end point. See References, 1 regarding the ruggedness of the acid value method.
2. The pH meter should be standardized to pH 4.0 with standard buffer solution. Immediately before using, wipe the electrodes thoroughly with clean cloth or tissue and soak for several minutes in distilled water. At weekly intervals, or more often if necessary, clean the glass electrode in a suitable cleaning solution. Also, drain calomel electrode and refill with fresh potassium chloride (KCl) electrolyte at weekly intervals. Both electrodes should be stored in distilled water when not in use.

REFERENCES

1. *J. Assoc. Off. Anal. Chem. 59*:658 (1976).

Table 1
Precision data from interlaboratory studies.

Oil	Palm kernel	Sunflower	Coconut	Palm oil	Palm oil	Palm kernel	Palm kernel	Lampante olive oil	Lampante olive oil
Number of laboratories	23	23	25	12	27	41	41	28	28
Number of acceptable results	22	22	25	10/11	27	39	40	26	24
Mean acidity values, %	7.26	0.83	1.49	3.11	4.09	6.46	1.72	3.80	19.55
Repeatability, r	0.07	0.025	0.025	0.03	0.06	0.07	0.06	0.07	0.26
Reproducibility, R	0.24	0.075	0.075	0.45	0.18	0.23	0.20	0.33	1.67

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Determination of Alkalinity in Fats and Oils

DEFINITION

This method determines the alkalinity (proportion of alkali) in a fat or oil by titration with hydrochloric acid.

SCOPE

The alkalinity of animal and vegetable fats and oils is determined without distinguishing between the various constituents. The method is not applicable to dry melted animal fats, nor to oils and fats with an acidity greater than 60% (mass fraction). Use AOCS Official Method Cd 3d-63 (see Notes, 1) for test samples in this range. This method is identical to ISO 10539 (2002).

APPARATUS

Usual laboratory apparatus and, in particular, the following.

1. Borosilicate glass apparatus, for aqueous acetone (Reagents, 2).
2. Conical flasks, with wide necks, and of capacities 400 mL to 500 mL.

REAGENTS

Use only reagents of recognized analytical grade, unless otherwise stated.

1. Water.
2. Aqueous acetone—Mix 970 mL of acetone, 20 mL of water, and 10 mL of bromophenol blue solution (2 g/L in acetone). Add sodium hydroxide solution [c(NaOH) = 0.1 mol/L] to give a blue color, then add hydrochloric acid [c(HCl) = 0.1 mol/L] until a yellowish green color appears.
3. Hydrochloric acid standard volumetric solution, c(HCl) = 0.01 mol/L, standardized not more than 7 days before use.

SAMPLING

It is important that the laboratory receive a test sample which is truly representative and has not been damaged or changed during transport or storage. A recommended sampling method is given in AOCS Official Method C 1-47.

PROCEDURE

1. Preparation of the apparatus—Rinse each conical flask (Apparatus, 2) and stirrer to be used with successive 20 mL amounts of aqueous acetone (Reagents, 2) until the aqueous acetone does not change color. Allow the flasks and stirrer to dry.
2. Test portion—From the test sample weigh, to the nearest 0.1 g, a test portion of up to 40 g (see Table 1) according to the expected result and color of the test sample. Melt a solid test portion at below the boiling point of acetone. Add 100 mL of the acetone (Reagents, 2) at 40°C and stir. Allow to settle until the solution splits into two layers; in the presence of soap, the upper layer is blue-colored.
3. Determination—Titrate the warm (30°C to 40°C) solution with the hydrochloric acid (Reagents, 3) while stirring until the color of the indicator changes from blue (or blue green) to the yellowish green of the acetone (Reagents, 2) added. Allow the mixture to settle in order to observe the color clearly (see Notes, 2).
4. Number of determinations—Carry out two determinations on the same test sample, treating the replications for small test portions as one determination (see Table 1).

EXPRESSION OF RESULTS

Alkalinity may be expressed as a mass, in milligrams, of sodium oleate per kilogram of fat or oil, or as a percentage (mass fraction) of sodium hydroxide:

a) the alkalinity in terms of sodium hydroxide is given by the expression

$$A = \frac{4Vc}{m}$$

b) the alkalinity in terms of sodium oleate is given by the expression

$$S = 304000\frac{Vc}{m}$$

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Where—

A = the alkalinity expressed as a percentage (mass fraction) of sodium hydroxide
S = the alkalinity expressed as milligrams of sodium oleate per kilogram of sample
m = the mass of the test portion, in grams
c = the exact concentration of the standard volumetric hydrochloric acid solution used, in moles per liter
V = the volume of hydrochloric acid standard volumetric solution used to titrate the test portion, in milliliters

Take as the result the mean of two determinations, provided that the requirement for repeatability (see Precision, 2) is met.

PRECISION

1. Interlaboratory test— An international collaborative test has been carried out on the method given in this International Standard. The test, involving fifteen laboratories in six countries, was organized by AFNOR and the results obtained were subjected to statistical analysis in accordance with Internal Organization for Standardization (ISO) 5725 [1,2] to give the precision data reported in Table 2. The values derived from this interlaboratory test may not be applicable to concentration ranges other than those given.
2. Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases exceed the repeatability limit r given in Table 2 for the relevant level of the mean value determined.
3. Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5% of cases exceed the reproducibility limit R given in Table 2 for the relevant level of the mean value determined.

NUMBERED NOTES

1. Fats and oils can contain alkaline constituents either naturally (e.g., calcium soaps from bones) or accidentally (e.g., sodium soaps in imperfectly refined fats and oils).
2. An explosion-resistant hotplate with magnetic stirrer and burette support is particularly useful.

REFERENCES

1. ISO 5725-1:1994, Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions.
2. ISO 5725-2:1994, Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

Table 1
Mass of test portion.

Color of test sample	Expected result (mg sodium oleate/kg of test portion)	Mass (g)
Light	up to 500	40
Light	over 500 to 1 000	10
Light	over 1 000	4
Dark	not relevant	2
Very dark	not relevant	1

For test portions of less than 10 g, take replicate portions totaling at least 5 g for titration and sum the masses and titers to give the result for one determination.

Table 2
Precision data from Interlaboratory test.

	Sample D	Sample E	Sample H
Number of laboratories	15	15	15
Number of laboratories retained after eliminating outliers	14	11	12
Mean alkalinity (mg/kg)	8.3	216.3	12.5
Repeatability standard deviation, s_r (mg/kg)	0.7	0.6	0.5
Repeatability coefficient of variation (%)	8.1	0.3	3.6
Repeatability limit, r (mg/kg)	1.9	1.8	1.3
Reproducibility standard deviation, s_R (mg/kg)	2.3	8.4	9.5
Reproducibility coefficient of variation (%)	27.1	3.9	76.1
Reproducibility limit, R (mg/kg)	6.4	23.7	27.0

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Reichert-Meissl, Polenske, and Kirschner Values, Modified AOAC Methods

DEFINITION

The Reichert-Meissl value is a measure of soluble volatile fatty acids (chiefly butyric and caproic) and the Polenske value is an index of the insoluble volatile fatty acids (mostly caprylic, capric, and lauric). The Kirschner value is a measure of butyric acid. All are expressed in terms of the mL of 0.1 M NaOH required to neutralize the fatty acids obtained from a 5 g test sample under the specific conditions of the method.

SCOPE

The methods are applicable to normal fats and oils but are generally applied to butterfat, oils of the coconut group, and similar products.

NOTE

These methods are highly empirical and the specifications of procedure and apparatus must be followed in exact detail.

APPARATUS

1. Graduated cylinder—25 mL.
2. Glass distillation apparatus of the dimensions and assembly shown in Figure 1.

REAGENTS

1. Sodium hydroxide (NaOH) solution—50% NaOH by weight (see Notes, *Caution*). This solution must be free from carbonates and kept protected from carbon dioxide. It is advisable to allow the solution to settle and use only the clear liquid.
2. Sulfuric acid (H_2SO_4) solution—prepared by adding 200 mL of concentrated H_2SO_4, sp. gr. 1.84, to distilled water and diluting to 1 L (see Notes, *Caution*).
3. Phenolphthalein indicator solution—1% in 95% alcohol.
4. Barium hydroxide [$Ba(OH)_2$] solution, 0.1 M—accurately standardized.
5. Glycerol-soda solution—add 20 mL of the 50% sodium hydroxide solution to 180 mL either USP grade or reagent grade glycerol.
6. Boiling chips that have been cleaned in distilled water. Store in distilled water until used.
7. Sodium hydroxide (NaOH) solution, 0.1 M—accurately standardized. See AOCS Specification H 12-52.
8. Aluminum wire.
9. Ethyl alcohol—95%, SDA Formulas 30 and 3A are permitted (see Notes, *Caution*).
10. Silver sulfate—reagent grade.

PROCEDURE FOR THE REICHERT-MEISSL VALUE

1. Melt the test sample, if it is not already liquid, and filter through filter paper to remove any impurities and moisture.
2. Weigh accurately 5 ± 0.0001 g (see Notes,1) into a 300 mL distillation flask. Add 20 mL of glycerol-soda solution and heat until completely saponified. This is indicated by the mixture becoming perfectly clear. Shake the flask gently if any foaming occurs.
3. Add 135 mL of recently boiled distilled water, drop by drop at first to prevent foaming. Then add 6 mL of dilute H_2SO_4 and a few boiling chips.
4. Connect the flask to the distillation apparatus and distill without previous melting of the fatty acids. Rest the distillation flask on a fireproof support having a 50 mm dia hole in the center. Regulate the flame so as to collect 110 mL of distillate in as close to 30 min as possible. The distillate must drip into the receiver at a temperature no higher than 20°C.
5. When 110 mL have been collected, remove the flame and substitute a 25 mL cylinder for the receiving flask to collect any drops that may fall after the flame has been removed.
6. Mix the contents of the receiver with gentle shaking and immerse almost entirely in water at 15°C for 15 min.
7. Filter the 110 mL of distillate through 90 mm dry filter paper. Titrate 100 mL with 0.1 M NaOH, using 0.5 mL of phenolphthalein indicator to the appearance of a pink color which persists for 2–3 min.
8. Prepare and conduct a blank determination similar in all respects to the test sample.

CALCULATIONS

The Reichert-Meissl value = 1.1 × (titration of test portion – titration of blank)

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PROCEDURE FOR THE POLENSKE VALUE

1. Remove the remainder of the water-soluble acids from the water-insoluble acids on the filter paper by washing with three successive 15 mL portions of distilled water, each having been previously passed through the condenser, the 25 mL cylinder, and the 110 mL receiving flask. These water washings are discarded.
2. Dissolve the insoluble acids by repeating the washing procedure described in the preceding paragraph, using three 15 mL portions of 95% alcohol which has been neutralized with NaOH solution to the phenolphthalein end point.
3. Combine the alcohol washings and titrate with 0.1 M NaOH using 0.5 mL of phenolphthalein indicator, to the appearance of a pink color which will persist for 2–3 min.

CALCULATION

The Polenske value = volume, mL of 0.1 M NaOH solution required

PROCEDURE FOR THE KIRSCHNER VALUE

1. Neutralize 100 mL of the Reichert-Meissl distillate with 0.05 M $Ba(OH)_2$ solution, very accurately, to a faint pink color using 0.5 mL of phenolphthalein indicator. Conduct the titration in a closed flask to prevent the absorption of CO_2.
2. Add 0.3 g of Ag_2SO_4 in a finely powdered form. Allow the mixture to stand for 1 hr with frequent shaking and then filter.
3. Collect 100 mL of the filtrate, place in a 300 mL distillation flask, add 35 mL of distilled water and 10 mL of dilute H_2SO_4. Add several boiling chips to prevent bumping. Attach to distillation apparatus and distill at the rate of 110 mL in about 20 min.
4. After 110 mL of distillate has been collected, filter this total amount and titrate 100 mL with 0.1 M NaOH using 0.5 mL of phenolphthalein indicator to the appearance of a pink color which persists 2–3 min.
5. Prepare and conduct a blank determination similar in all respects to the test portion.

CALCULATION

$$\text{The Kirschner value} = \frac{A \times 121 \times (100 + B)}{10{,}000}$$

Where—

A = titration of test portion – titration of blank

B = mL of 0.05 M $Ba(OH)_2$ required to neutralize the original 100 mL of Reichert-Meissl distillate.

NOTES

Caution

Sodium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

NUMBERED NOTES

1. Less than 5 g test sample should be taken with certain fats or derivatives, e.g., acetylated monoglycerides, that require more alkali for complete saponification than available in the prescribed amount of glycerol-soda solution. A diluent oil with a Reichert-Meissl value of less than 0.2, e.g., cottonseed oil, is added to the test sample flask to provide a total of 5 g of test sample plus diluent oil.

CALCULATION

$$\text{The Reichert-Meissl value} = 1.1 \times (\text{titration of test portion} - \text{titration of blank}) \times \frac{5}{\text{mass of test portion, g}}$$

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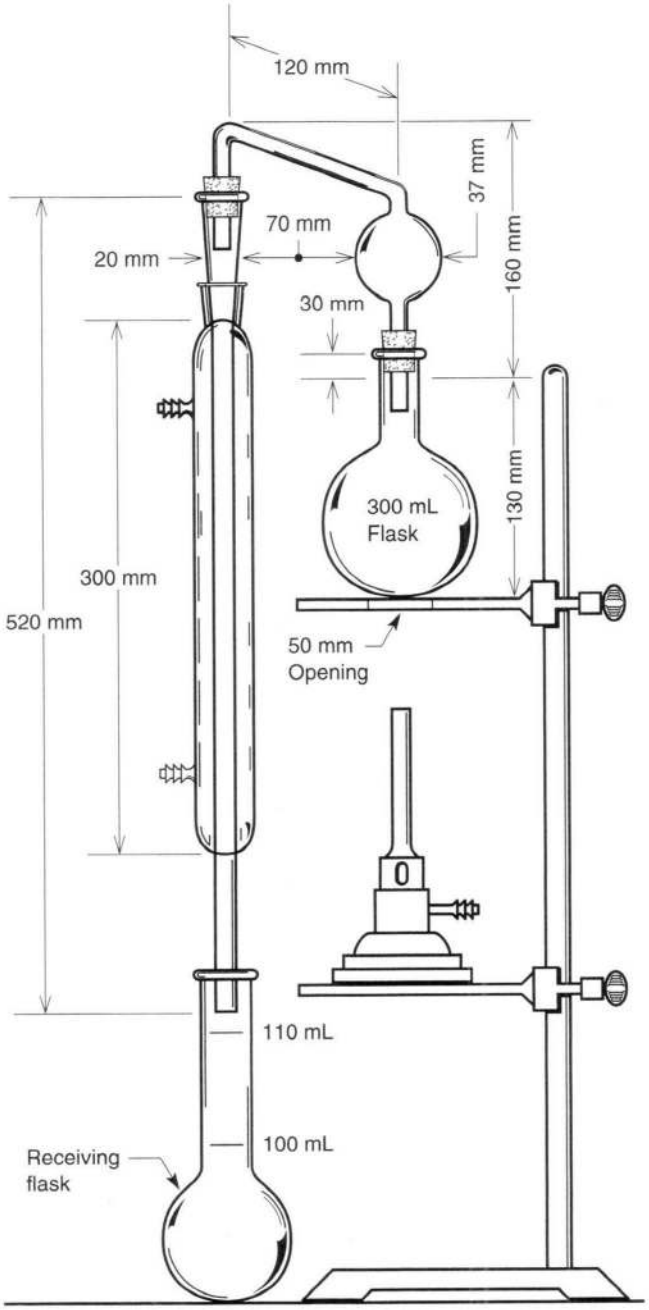

Figure 1. Reichert-Meissl distillation apparatus.

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Polyunsaturated Acids, Ultraviolet Spectrophotometric Method

DEFINITION

The conjugated constituents of a fat or oil contain one single bond between two double bonds and the non-conjugated constituents contain two or more single bonds between two double bonds. The conjugated constituents are determined by measuring the ultraviolet absorption in a purified solvent. The non-conjugated polyunsaturated constituents are partially conjugated by heating in a potassium hydroxide-glycol solution and then the absorption of the conjugated constituents is measured in an ultraviolet spectrophotometer. The percentages of conjugated diene, triene, tetraene, and pentaene acids and of linoleic, linolenic, arachidonic, and pentaenoic acids are calculated from these measurements.

SCOPE

This method is applicable to the determination of polyunsaturated acids from dienoic through pentaenoic in animal and vegetable fats containing only the natural of *cis*-isomers of the non-conjugated polyunsaturated acids, only small amounts of preformed conjugated material and only small amounts of pigments whose absorption may undergo considerable change during the alkali isomerization. The method is not applicable or is applicable only with specific precautions, to modified drying oils, hydrogenated oils, or other fats containing *trans*-isomers of the unsaturated fatty acids; to fish oils or similar fats containing acids more highly unsaturated than pentaenoic; to crude oils or unusual test samples containing pigments whose absorption undergoes changes during the alkali procedure, causing interferences which are unknown and cannot be determined or to fats and oils containing large quantities of preformed conjugated fatty acids (see Notes, 2).

APPARATUS

1. Ultraviolet spectrophotometer—covering a spectral range of 220–360 nm with wavelength scale readable to 0.1 nm, capable of holding 1.000–10.000 cm cuvette.
2. Absorption cuvettes—quartz, matched pairs, of pathlengths 1.000–10.000 ± 0.005 cm. When filled with water or isooctane, the paired cuvettes must match within 0.01 absorbance units.
3. Constant temperature bath—maintained at 180.0 ± 0.5°C, capacity sufficient to immerse 25 × 2.5 cm Pyrex™ test tubes to a depth of 11.5 cm. For the bath liquid, use bath wax.
4. Test tubes—Pyrex™, 25 × 2.5 cm, with glass-distributing head to fit (see Fig. 1). The 7 mm tubing in the center of the head has both ends open and two small holes about 2.5 cm and 4 cm from the bottom.
5. Manifold, glass—about 15 × 2.5 cm, with outlets for connecting capillary tubes.
6. Capillary tubes—1 mm i.d. and about 5 cm long, connected to the outlets with rubber tubing.
7. Nitrogen cylinder containing less than 0.01% O_2.
8. Manometer—6 mm glass tubing bent in the shape of a U-tube, height about 38 cm. Fill the manometer approximately half full with water containing 1 drop of methyl orange solution and 1 drop of H_2SO_4, sp. gr. 1.84.

   The preferred flow rate is 50–100 mL/min. When adjustment has been made, mark the level of the liquid in the manometer with a glass-marking pencil.
9. Distilling flask—3 L, containing 2 necks with ground glass joints and glass stoppers to fit.
10. Reflux tube—with ground glass joint to fit the 3 L distilling flask.
11. Condenser assembly—consisting of the following units equipped with ground glass joints: trap to fit distilling flask, condenser and connecting tube bent at 75° angle to attach the trap to the condenser.
12. Water bath or electric heating mantle (controlled by variable transformer)—for heating 3 L distilling flask.
13. Filter tube—80 cm × 4.5 cm, with glass stopcock sealed at one end.
14. Cork stopper—covered with aluminum foil to fit loosely in the top of the filter tube.
15. Flask—Pyrex™, 1 L, round bottom type with ground glass joint and both solid and hollow glass stoppers to fit. A short outlet tube and an inlet tube reaching to the bottom of the flask are sealed into the hollow glass stopper.
16. Oil bath or electric heating mantle—maintained at 100–190°C for heating the 1 L round bottom flask.
17. Cups—Pyrex™, 1 mL, dia 14 mm, height 10 mm, Fisher "Petticups" are satisfactory.
18. Graduated cylinder—100 mL.
19. Beakers—150 mL and 3 L.
20. Glass stirring rod—dia about 5 mm, length 30 cm, with lower end curved so the 1 mL glass cup can be moved up and down in the 25 × 2.5 cm test tube.

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21. Erlenmeyer flasks—2 L.
22. Volumetric flask—1 L.
23. Volumetric flasks—glass-stoppered, 100 mL.
24. Thermometer—0-200°C for the oil bath (see Apparatus, 16).

REAGENTS

1. Zinc powder (dust)—reagent quality.
2. Potassium hydroxide (KOH)—reagent grade (see Notes, *Caution*).
3. Synthetic methanol—absolute, reagent grade (see Notes, *Caution*). Check the absorbance of 1 cm layer of methanol against distilled water at 220 nm and through the range of wavelengths used in the conjugated and non-conjugated acid analysis. The absorbance at 220 nm compared with distilled water set at zero absorbance must be less than 0.4 and the curve should be smooth in the range 262–322 nm; otherwise purify as follows and recheck the absorbance.

   Note—To purify the methanol, place 2 L of methanol from a new drum or from glass bottles into a 3 L distilling flask, double neck type. Add 10 g KOH, analytical reagent grade, and 25 g of zinc powder. Place a glass stopper in one outlet of the flask and reflux tube in the other. Reflux on the steam bath for 3 hr. Remove from the steam bath. Replace the reflux tube with a trap, 75° connecting tube and condenser. Place the flask in a water bath or electric heating mantle and heat enough to distill the methanol. Collect the distillate in a 2 L Erlenmeyer flask. Store in a glass-stoppered bottle. Ethanol is permitted if of comparable purity (as obtained or purified).
4. Silica gel—40–60 mesh, Code 11-08, Davison Chemical Company, Baltimore, MD, USA, or equivalent.
5. Glass wool.
6. Isooctane (2,2,4-trimethyl pentane), National Institute of Testing certified grade, Rohm and Haas Company, Philadelphia, PA, USA, Spectro grade, Phillips Petroleum Company, Bartlesville, OK, USA. Hexane or cyclohexane is permitted if absorbance requirements are met (see Notes, *Caution*).
   (a) Place about 9 cm of glass wool above the stopcock at the lower end of the 80–4.5 cm filter tube. Add about 30 cm of silica gel. Fasten the tube vertically to a ring stand and place a 2 L Erlenmeyer flask under the stopcock. Pour isooctane slowly into the tube, filling about three-fourths full. Insert cork stopper covered with aluminum foil loosely in top of tube and allow the isooctane to filter through the silica gel. Renew the silica gel in the tube as often as necessary to yield isooctane conforming to the absorbance limit given in Reagents, 6, (b).

   Note—A constant flow can be maintained by filling the tube nearly full with silica and then supporting an inverted, 1 L volumetric flask so the mouth of the flask is below the surface of the liquid in the tube.
   (b) Check the absorbance of 1 cm layer of the isooctane against distilled water through the range of wavelengths used in the conjugated and non-conjugated acid analysis. The absorbance compared with distilled water set at zero absorbance must be not more than 0.070 at all wavelengths and the resultant absorbance vs. wavelength curve must be smooth; otherwise filter again and recheck the absorbance.
7. Ethylene glycol—Eastman no. 133 or equivalent, Carbide and Carbon product is acceptable.
8. Hydrochloric acid, 1 M—accurately standardized. See AOCS Specification H 14-52.
9. Phenolphthalein indicator solution—1.0% in 95% alcohol.
10. Potassium hydroxide-glycol solution—6.6% KOH for 25 min isomerization.
    (a) Weigh about 750.0 g of ethylene glycol into a 1 L round bottom Pyrex™ flask. Close with the hollow ground glass stopper which contains a short outlet tube and an inlet tube reaching to the bottom of the flask.
    (b) Connect the inlet tube to an oxygen-free nitrogen supply and bubble the gas through the liquid to exclude all air and to agitate the liquid slightly.
    (c) Place an oil bath maintained at 100–150°C around the flask. Raise the bath temperature to 190°C and hold at 190°C for 10 min. Lower the bath and allow the bath temperature to drop to 120°C.
    (d) When the bath temperature reaches 120°C, carefully add 60 g of KOH reagent grade pellets to the glycol and swirl the flask until the alkali is completely dissolved with nitrogen sweeping vigorously (50–100 mL of nitrogen/min) through the flask.
    (e) Remove the hollow glass stopper and close with a solid glass stopper. Keep the solution in a refrigerator at about 4°C under nitrogen during storage.
    (f) Neutralize about 90 mL of methanol with 1 M HCl to phenolphthalein end point. Add 10.00 g of the KOH-glycol solution, mix thoroughly, and add 0.5 mL of phenolphthalein indicator solution. Titrate with standardized HCl until the pink color has just disappeared.

$$\text{KOH, \%} = \frac{\text{HCl, mL} \times \text{molarity} \times 5.61}{\text{test solution mass, g}}$$

    (g) If the KOH is not 6.5%–6.6%, dry some glycol as directed in Reagents, 10, a–c, inclusive and adjust to the indicated percentage of KOH by adding dry glycol.

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11. Potassium hydroxide solution—21% KOH for 15 minute isomerization (see Notes, *Caution*).
    (a) Prepare solution as specified in Reagents, 10, (a)–(f), inclusive, except that 210 g of KOH reagent grade pellets are used instead of 60 g.
    (b) If the % KOH is not 21 ± 0.1, dry some glycol as directed in Reagents, 10, (a) to (c), inclusive, and adjust to this % KOH by adding dry glycol.
12. Nitrogen gas—
    (a) If the nitrogen gas contains more than 0.01% O_2, set up a purification apparatus consisting of a coil of copper gauze in a combustion tube held in a 30 cm long combustion chamber maintained at 300–325°C with tube inlet connected to the nitrogen cylinder, and pass the gas through the heated combustion tube and through a wash bottle containing glass beads and H_2SO_4, sp. gr. 1.84.
    (b) When the coil becomes coated with oxide, pass hydrogen over the coil, allowing the excess gas to escape from the T-tube outlet at the end of the combustion tube. Be sure to close the tube leading to the wash bottles. When the coil is reduced to metallic copper, flush out the combustion tube thoroughly with nitrogen before attaching a sample tube.
13. Copper gauze—reagent quality.
14. Sulfuric acid (H_2SO_4)—concentrated, reagent grade, sp. gr. 1.84 (see Notes, *Caution*).
15. Methyl orange solution—0.1% in distilled water.

PREPARATION OF TEST SAMPLE

Melt the test sample carefully on the steam bath. Do not heat more than 10°C over the melting point of the test sample. Stir thoroughly to ensure a representative test portion. If the test portion is not clear, or if it contains water, filter through filter paper. The test portion must be absolutely dry.

PROCEDURE

1. For conjugated polyunsaturated acid—
    (a) Weigh into a 1 mL Pyrex™ cup, to the nearest mg, sufficient test portion to give an absorbance reading of 0.2 or more (about 200 mg). Place about 75 mL of purified isooctane in a 150 mL beaker. Hold the cup just above the solvent and allow it to drop to the bottom of the beaker.

        Note—Hexane or cyclohexane, which has been purified in a manner identical to the isooctane, is permitted.

    (b) Rotate the beaker and warm the contents if necessary to completely dissolve the test portion. Cool to room temperature and quantitatively transfer the solution to a 100 mL glass stoppered volumetric flask. Dilute to volume with purified solvent and mix thoroughly.
    (c) Measure the absorbance of the solution in an ultraviolet photoelectric spectrophotometer, following the manufacturer's instructions for operating. Use a matched cuvette containing solvent only for the blank cuvette.
    (d) Take absorbance readings, diluting the original solution and/or using other cuvette lengths, if necessary, so that the observed absorbance is between 0.2–0.8. "Normal" sensitivity, i.e., the sensitivity control set at 3 counterclockwise turns from its clockwise limit, should be used for all readings except at 262, 268, and 274 nm where the sensitivity control should be set to permit readings at slit widths between 0.8–0.9 mm. Spectrophotometric readings are taken on both sides of the analytical wavelengths (underscored below) to ascertain that a maximum is present. A component is considered absent if a maximum is not found in the characteristic region and no further calculations are made in this region. Wavelengths (nm) involved in the equation are—

Dienoic		233	
Trienoic	262	268	27
Tetraenoic	308	315	32
Pentaenoic		346	

    However, readings are not required at all wavelengths for all analyses. For readings required for various types of test samples, see the Spectrophotometric Readings and Calculations Required section below.
    (e) Take absorbance readings at 346, 322, 315, 308, 274, 268, 262, and 233 nm, diluting the original solution and/or using other cuvette lengths if necessary so that the observed densities are between 0.2–0.8.
    (f) Record the cuvette length, g of test portion per L of the final dilution used for the measurement and the absorbance reading for each wavelength.
2. For non-conjugated polyunsaturated acids (6.6% KOH), 25 min isomerization—
    (a) Weigh about 100 mg of test portion to the nearest 0.5 mg into 1 mL Pyrex™ glass cup. Weigh 11.0 ± 0.1 g of the 6.6% KOH-glycol solution into a 25 × 2.5 cm Pyrex™ test tube.
    (b) Prepare and conduct at least two blanks simultaneously with the test portion.
    (c) Adjust the combustion furnace to 300 ± 25°C and flush out purification apparatus and manifold with the purified nitrogen.
    (d) Cover the test tube with a distributing head and connect to a capillary tube on the manifold. Start the flow of nitrogen and adjust to the mark on the manometer which should indicate a minimum of 50–100 mL of nitrogen passing through the tube per minute. Allow the nitrogen to sweep through the test tube containing the KOH-glycol solution for about

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1 min to remove air, then immerse it to a depth of 10.5 cm in the bath maintained at 180 ± 0.5°C. The temperature should be checked by an accurate thermometer standardized at frequent intervals.

(e) After 20 min of heating, remove the distributing head and drop the 1 mL glass cup containing the weighed test portion into the test tube. Note the exact time when the cup is dropped into the tube. Replace the distributing head. Prepare the KOH-glycol blank in a similar manner, using a clean 1 mL glass cup.

(f) Keep the distributing head in place. Remove the test tube from the bath, swirl vigorously for a few seconds, and return to the bath. At the end of 1 min of heating in the bath, remove the test tube and examine the solution. If the solution is clear, return the tube to the bath. If the solution is not clear, indicating incomplete saponification, swirl the test tube two or three times, and then return to the bath. At intervals of 1 min of heating, repeat the swirling step until saponification is complete. Make sure the bath temperature is maintained at 180.0 ± 0.5°C.

(g) Exactly 25 min after dropping the test portion into the test tube, remove the tube from the bath, wipe clean, and place in a 3 L beaker to cool. Continue to pass nitrogen over the solution while it is cooling. Add cold water to the beaker to shorten the cooling period.

(h) When the solution has cooled to room temperature, remove the head from the test tube and wash the lower tubing on the head with about 20 mL of purified methanol, collecting the washings in the test tube. Pour the methanol from a beaker; do not use a wash bottle.

Note—Ethanol which conforms to the methanol specifications is permitted. If the ethanol requires purification, proceed as directed for methanol.

(i) Insert a glass stirring rod, 30 cm long, with curved end at the bottom into the test tube and move the 1 mL glass cup up and down until the KOH-glycol and alcohol are completely mixed. Quantitatively transfer the contents of the test tube to a 100 mL glass-stoppered volumetric flask. Dilute the volume with the purified methanol and mix thoroughly.

(j) Proceed as directed in Procedure, 2, (c)–(f), inclusive, using the KOH-glycol blank solution in the blank cuvette. When further dilutions of the test solution are required, be sure to make similar dilutions of the KOH-glycol blank solution. If the blanks do not check, repeat the test increasing the flow of nitrogen.

3. For non-conjugated polyunsaturated acids (21% KOH), 15 min isomerization—

(a) Proceed as directed in Procedure, 2, (a)-(j), inclusive, with the following exceptions. Use about 80 mg of test portion and the 21 ± 0.1% KOH-glycol solution (Procedure, 2, (a); and isomerize exactly 15 min [Procedure, 2, (g)]).

SELECTION OF PROCEDURE

Selection of Procedure, 2 (6.6% KOH) or Procedure, 3 (21% KOH) for determination of non-conjugated polyunsaturated acids depends upon the nature of the test sample to be analyzed. As a general rule the 6.6% KOH procedure is preferred when test samples contain only linoleic and linolenic acids and the 21% KOH procedure is preferred when the test samples contain linoleic, linolenic, and arachidonic acids. However, when linoleic, linolenic, arachidonic, and pentaenoic acids are present in the test sample, it is essential to use the 21% KOH procedure.

SPECTROPHOTOMETRIC READINGS AND CALCULATIONS REQUIRED

The spectrophotometric readings required and the calculations to be performed in any analysis depend upon the nature of the specific test sample. Two general rules are to be observed:

1. If a polyunsaturated fatty acid constituent is known to be absent or its absence is confirmed during the course of the analysis (i.e., no maximum detected at the analytical wavelength [see Procedure, 1, d]), no spectrophoto-metric readings are required in the region of absorption of this particular constituent and no absorptivities at this region need be included in the equations used to calculate other constituents. For example, cottonseed oil is known to contain no polyunsaturated constituents more highly unsaturated than dienoic (linoleic acid). Hence, in the analysis of this oil, spectrophotometric measurements are required only at 233 nm and the equation to calculate linoleic acid content requires absorptivities only at this wavelength. Linseed oil contains no arachidonic or more highly unsaturated fatty acid. In the analysis of this oil, spectrophotometric readings are required only at the diene and triene regions and the equations to calculate the linoleic and linolenic acid content require absorptivities at these two wavelength positions only.
2. Corrections for background absorption are essential when measuring very small traces of fatty acids in oils, fats, and similar substances. However, when the fatty acid is present in more than a trace these background corrections are not required and their use may lead to dangerously erroneous results. A convenient criterion is suggested—when the absorptivity of any polyunsaturated constituent after isomerization is, at its analytical wavelength, greater than 1.0, no background corrections are to be made for any acid under any circumstances.

CALCULATIONS

1. Absorptivity for conjugated constituents—

(a) Calculate the absorptivity, a, for each wavelength recorded in Procedure, 1, d, using subscripts 233, 268, 315, and 346 to designate each individual a.

$$\text{Absorptivity} = a = \frac{A}{b \times c}$$

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Where—

A = observed absorbance

b = cuvette length in cm

c = g of test portion per L of the final dilution used for the absorbance measurement

In the equations which follow, subscripts 2, 3, 4, and 5, refer to diene, triene, tetraene, and pentaene constituents, respectively.

(b) Absorptivity at 233 nm corrected for absorption by acid or ester groups—

$a_2 = a_{233} - a_0$

$a_0 = 0.07$ for esters, 0.03 for soaps and fatty acids

(c) Absorptivity at 268 nm corrected for background absorption = $a_3 = 2.8[a_{268} - \frac{1}{2}(a_{262} + a_{274})]$

(d) Absorptivity at 315 nm corrected for background absorption = $a_4 = 2.5[a_{315} - \frac{1}{2}(a_{308} + a_{322})]$

(e) Absorptivity at 346 nm = $a_5 = a_{346}$

2. Conjugated acids—

Note—If the quantities within the brackets under Calculations, 1, c or d are zero or negative, no characteristic absorption maxima are present and the corresponding constituent is reported as absent. As preformed constituents are usually present in small quantities, background absorption corrections are usually required. If large quantities of preformed constituents are present, this method is not applicable (see Scope). However, no background corrections are to be applied to readings in the pentaenoic region, 346 nm.

(a) Conjugated diene, % = $C_2 = 0.91a_2$

(b) Conjugated triene, % = $C_3 = 0.47a_3$

(c) Conjugated tetraene, % = $C_4 = 0.45a_4$

(d) Conjugated pentaene, % = $C_5 = 0.39a_5$

3. Absorptivities for non-conjugated constituents (6.6% KOH), 25 min isomerization—

(a) Calculate the absorptivity, á, for each wavelength in Procedure, 1, d:

$$\text{Absorptivity} = \acute{a} = \frac{A}{b \times c}$$

A, b, and c are defined under Calculations, 1, a.

(b) Absorptivity at 233 nm corrected for conjugated diene acids originally present is:

$\acute{a}_2 = \acute{a}_{233} - a_2 - 0.03$

(c) Absorptivity at 268 nm corrected for background absorption and for undestroyed conjugated triene is:

$\acute{a}_3 = 4.03[\acute{a}_{268} - \frac{1}{2}(\acute{a}_{262} + \acute{a}_{274})] - a_3$

(d) Absorptivity at 315 nm corrected for background absorption and for undestroyed conjugated tetraene is:

$\acute{a}_4 = 2.06[\acute{a}_{315} - \frac{1}{2}(\acute{a}_{308} + \acute{a}_{322})] - a_4$

4. Non-conjugated acids (6.6% KOH), 25 min isomerization-

(a) Without background corrections

Linoleic acid, % = $X = 1.086\ \acute{a}_2 - 1.324(\acute{a}_{268} - a_{268}) + 0.040(\acute{a}_{315} - a_{315})$

Linolenic acid, % = $Y = 1.980(\acute{a}_{268} - a_{268}) - 4.92\ (\acute{a}_{315} - a_{315})$

Arachidonic acid, % = $Z = 4.69(\acute{a}_{315} - a_{315})$

(b) When background corrections are required

Linoleic acid, % = $X = 1.086\acute{a}_2 - 1.324\acute{a}_3 + 0.40\ \acute{a}_4$

Linolenic acid, % = $Y = 1.980\acute{a}_3 - 4.92\acute{a}_4$

Arachidonic acid, % = $Z = 4.69\acute{a}_4$

5. Absorptivities for non-conjugated constituents (21% KOH), 15 min isomerization—

(a) Calculate absorptivities á for each wavelength 233, 268, 315, and 346 nm. Remember that spectrophotometric readings are to be taken on both sides of these analytical wavelengths to ascertain that a maximum is present. If no maximum is found the component is to be reported as zero without further measurement or calculation.

(b) Absorptivity at 233 nm = $\acute{a}_2 = \acute{a}_{233} - a_{233}$

(c) Absorptivity at 268 nm = $\acute{a}_3 = \acute{a}_{268} - a_{268}$

(d) Absorptivity at 315 nm = $\acute{a}_4 = \acute{a}_{315} - a_{315}$

(e) Absorptivity at 346 nm = $\acute{a}_5 = \acute{a}_{346} - a_{346}$

6. Non-conjugated acids (21% KOH), 15 min isomerization-

Note—The spectrophotometric method of analysis will not differentiate between acids that have the same number of double bonds but different chain length, e.g., between C_{20} and C_{22} pentaenes. The first two sets of equations below are for test samples containing C_{20} pentaene acid and for test samples containing C_{22} pentaene acid, respectively. If chain length is unknown, the assumption is made that these pentaene acids are present in equal quantities, and the third set of equations given below is to be used. This assumption, although not necessarily true for all fats, is in agreement with the approximate proportions found in beef suprarenal lipids.

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7. Equations for acids in test sample—
   (a) Test samples containing C_{20} pentaene acid
   Linoleic acid, % = X = $1.09Á_2 - 0.57á_3 - 0.26á_4 + 0.002á_5$
   Linolenic acid, % = Y = $1.10á_3 - 0.88á_4 + 0.31á_5$
   Arachidonic acid, % = Z = $1.65á_4 - 1.55á_5$
   Pentaenoic acids, % = P = $1.14á_5$
   (b) Test samples containing C_{22} pentaene acid
   Linoleic acid, % = X = $1.09Á_2 - 0.57á_3 - 0.26á_4 - 0.12á_5$
   Linolenic acid, % = Y = $1.10á_3 - 0.88á_4 - 0.31á_5$
   Arachidonic acid, % = Z = $1.65á_4 - 1.86á_5$
   Pentaenoic acids, % = P = $1.98á_5$
   (c) Test samples containing pentaene acids of unknown chain length (calculated as 50% C_{20} and 50% C_{22} pentaenoic acids)
   Linoleic acid, % = X = $1.09Á_2 - 0.57á_3 - 0.26á_4 - 0.03á_5$
   Linolenic acid, % = Y = $1.10á_3 - 0.88á_4 + 0.19á_5$
   Arachidonic acid, % = Z = $1.65á_4 - 1.67á_5$
   Pentaenoic acids, % = P = $1.45á_5$
8. Total composition—
   (a) Total conjugated polyunsaturated acids, % = $C_2 + C_3 + C_4 + C_5$
   (b) Total non-conjugated polyunsaturated acids, % = X + Y + Z + P
   (c) Oleic acid, % = $$\frac{\text{(IV of test portion} - [1.811(C_2 + X) + 2.737(C_3 + Y) + 3.337(C_4 + Z) + 4.014^1(C_5 + P)])}{0.899}$$
   [1]Corresponding constant for test samples containing all C_{20} pentaene acids is 4.197; for test samples containing all C_{22} pentaene acids, the constant is 3.841.
   (d) Saturated acids, % total fatty acid– (% oleic acid + % conjugated acid + % non-conjugated acid)
   Note—The % total fatty acid of most naturally occurring oils is 95.6.
   (e) To calculate to a fatty acid basis, multiply the percentage value by

$$\frac{100}{\%\text{ total fatty acid}}$$

NOTES

Caution

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume removal device or gas mask to protect respiratory tract against alkali dusts or vapors.

When working with extremely caustic materials like potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

Methyl alcohol (methanol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform, and perchloric acid.

Isooctane, hexane, and cyclohexane are extremely flammable. Use effective fume removal device. Avoid static electricity. Hexane is a flammable solvent and a dangerous fire risk. All work should be performed in a fume hood, with no open flames. Always ensure that your hood is functioning before introducing volatile solvents. The TLV for hexane is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/m^3 for a time weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

NUMBERED NOTES

1. Nomenclature—Nomenclature used throughout this method is that suggested by the Joint Committee on Nomenclature in Applied Spectroscopy, *Anal. Chem. 24:*1349 (1952).
2. Recent observations [*JAOCS 68:*693 (1991)] indicate that this method is not applicable to sesame oil and mixtures thereof. This observation has not been investigated by the AOCS Committee.

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REFERENCES

Official Methods of Analysis, AOAC International, 16th ed., Vol. 11, Gaithersburg, MD, 1995, Chapt. 41, p. 13, Method 957.13
*Anal. Chem. 24:*1349 (1952)
*JAOAC 40:*487 (1957)
*Ibid. 42:*42 and 354 (1959)
*Ibid. 68:*693 (1991)

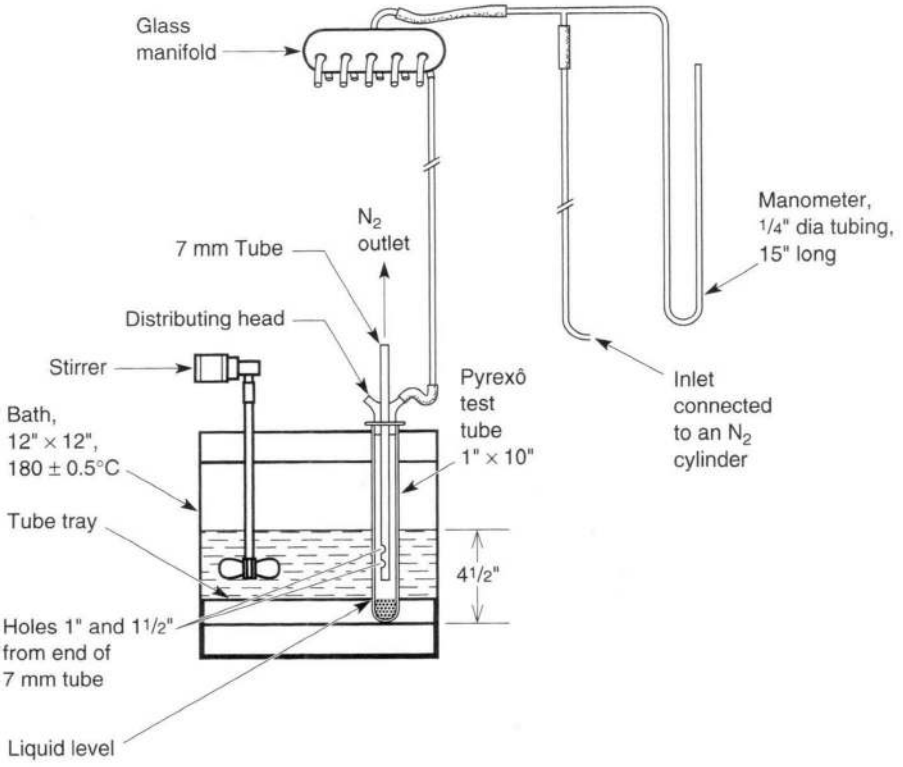

Figure 1. Schematic for arrangement of sample tubes and manifold.

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AOCS

AOCS Official Method Cd 8b-90

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Peroxide Value Acetic Acid-Isooctane Method

DEFINITION

This method determines all substances, in terms of milliequivalents of peroxide per 1000 grams of test sample, that oxidize potassium iodide under the conditions of the test. The substances are generally assumed to be peroxides or other similar products of fat oxidation.

SCOPE

Applicable to all normal fats and oils, including margarine. This method is highly empirical, and any variation in the test procedure may result in erratic results. This method gives erratic results at peroxide values ≥70.

APPARATUS

1. Pipet—0.5 mL, or other suitable volumetric apparatus capable of dispensing 0.5 mL of saturated potassium iodide (KI) solution.
2. Erlenmeyer flasks—with glass stoppers, 250 mL.
3. Burette—25 mL or 50 mL, class A, graduated in 0.1 mL divisions.
4. Timer.
5. Balance—top loading, 500 g capacity with ±0.01 gram sensitivity.

REAGENTS

1. Acetic acid-isooctane solution—3:2, v/v, prepared by mixing 3 volumes of reagent-grade glacial acetic acid (see Notes, *Caution*) with 2 volumes of reagent-grade isooctane (see Notes, *Caution*).
2. Potassium iodide (KI) solution—saturated, prepared fresh each day analysis is performed by dissolving an excess of KI in recently boiled distilled water. Make certain the solution remains saturated during use, as indicated by the presence of undissolved KI crystals. Store in the dark when not in use. Test the saturated KI solution by adding 2 drops of starch solution to 0.5 mL of the KI solution in 30 mL of the acetic acid-isooctane solution. If a blue color is formed that requires more than 1 drop of 0.1 M sodium thiosulfate solution to discharge, discard the KI solution and prepare a fresh solution.
3. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) solution—0.1 M, accurately standardized vs. potassium dichromate primary standard as follows:
   (a) Sodium thiosulfate solution 0.1 M, prepared by dissolving 24.9 g of sodium thiosulfate in distilled water and diluting to 1 L.
   (b) The potassium dichromate (see Notes, *Caution*) primary standard should be finely ground, dried at 105°C for 2 hr and cooled in a desiccator. Weigh 0.16–0.22 g of potassium dichromate into a 500 mL flask or bottle by difference from a weighing bottle. Dissolve in 25 mL of water, add 5 mL of concentrated hydrochloric acid (35–37%), 20 mL of potassium iodide solution (15% solution, 15 g KI in 100 mL water), and rotate to mix. Allow to stand for 5 min and then add 100 mL of distilled water. Titrate with sodium thiosulfate solution, shaking continuously until yellow color has almost disappeared. Add 1–2 mL of starch indicator and continue the titration, adding the thiosulfate solution slowly until the blue color just disappears. The strength of the sodium thiosulfate solution is expressed in terms of its molarity.

$$\text{Molarity of } Na_2S_2O_3 \text{ solution} = \frac{20.394 \times \text{mass of } K_2Cr_2O_7, \text{g}}{\text{mL of sodium thiosulfate}}$$

4. Sodium thiosulfate solution—0.01 M, accurately standardized. This solution may be prepared by accurately pipetting 100 mL of 0.1 M sodium thiosulfate into a 1000 mL volumetric flask and accurately diluting to volume with recently boiled distilled water.
5. Starch indicator solution—tested for sensitivity, prepared by making a paste with 1 g of starch (see Notes, 1) and a small amount of cold distilled water. Add, while stirring, to 200 mL of boiling water and boil for a few seconds. Immediately remove from heat and cool. Salicylic acid (1.25 g/L) may be added to preserve the indicator. If long storage is required, the solution must be kept in a refrigerator at 4–10°C. Fresh indicator must be prepared when the end point of the titration from blue to colorless fails to be sharp. If stored under refrigeration, the starch solution should be stable for about 2–3 weeks.

   Test for sensitivity—Place 5 mL of starch solution in 100 mL of water and add 0.05 mL of freshly prepared 0.1 M KI solution and one drop of a 50 ppm chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution to 1000 mL. The deep blue color produced must be discharged by 0.05 mL of 0.1 M sodium thiosulfate.
6. Sodium dodecyl sulfate (SDS)—≥98% [Aldrich Chemical (W. Milwaukee, WI, USA) or Mallinckrodt (Paris, KY, USA)]. Prepare 10% solution by dissolving 10 g SDS in 100 mL water.

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PROCEDURE FOR FATS AND OILS

1. Weigh the test portion (Table 1) into a 250 mL Erlenmeyer flask with glass stopper and add 50 mL of the 3:2 acetic acid-isooctane solution. Swirl to dissolve the test portion. Add 0.5 mL of saturated KI solution using a suitable volumetric pipet.
2. Allow the solution to stand for exactly 1 min, thoroughly shaking the solution at least three times during the 1 min, and then immediately add 30 mL of distilled water.
3. Titrate with 0.1 M sodium thiosulfate, adding it gradually and with constant and vigorous agitation (see Notes, 2). Continue the titration until the yellow iodine color has almost disappeared. Add 0.5 mL of 10% SDS (Reagents, 6), and then add about 0.5 mL of starch indicator solution. Continue the titration with constant agitation, especially near the end point, to liberate all of the iodine from the solvent layer. Add the thiosulfate solution dropwise until the blue color just disappears (see Notes, 3 and 4).
4. Conduct a blank determination of the reagents daily. The blank titration must not exceed 0.1 mL of the 0.1 M sodium thiosulfate solution.

PROCEDURE FOR MARGARINE

1. Melt the test portion by heating with constant stirring on a hot plate set at low heat, or by heating in an air oven at 60–70°C. Avoid excess heating and particularly prolonged exposure of the oil to temperatures above 40°C.
2. When completely melted, remove the test portion from the hot plate or oven and allow to settle in a warm place until the aqueous portion and most of the milk solids have settled to the bottom.
3. Decant the oil into a clean beaker and filter through a Whatman no. 4 paper (or equivalent) into another clean beaker. Do not reheat for filtration unless absolutely necessary. The test portion must be clear and brilliant.
4. Proceed as directed in Procedure for Fats and Oils, paragraphs 1–4.

CALCULATIONS

1. Peroxide value (milliequivalents peroxide/1000 g test portion) $= \dfrac{(S - B) \times \mathrm{M} \times 1000}{\text{mass of test portion, g}}$

Where—

B = volume of titrant, mL of blank
S = volume of titrant, mL of test portion
M = Molarity of sodium thiosulfate solution

PRECISION

The details of interlaboratory tests are given in Tables 2–4. The values presented may not be applicable to matrices other than those presented and may not be representative for other concentrations.

1. Repeatability—The difference between two test results on the same material, in the same laboratory under the same conditions, should not exceed the repeatability value, r.
2. Reproducibility—The difference between two test results on the same material, under the same conditions in different laboratories, should not exceed the reproducibility value, R.

NOTES

Caution

Isooctane is flammable and a fire risk. Explosive limits in air are 1.1–6.0%. It is toxic by ingestion and inhalation. A properly operating fume hood should be used when working with this solvent.

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcino-genicity of chromium [+6], in particular, lung cancer. It is a strong oxidizing agent and a dangerous fire risk when in contact with organic chemicals.

NUMBERED NOTES

1. "Potato Starch for Iodometry" is recommended, because this starch produces a deep blue color in the presence of the iodonium ion. "Soluble Starch" is not recommended because a consistent deep blue color may not be developed when some soluble starches interact with the iodonium ion. The following are suitable starches: Soluble Starch for Iodometry, Fisher S516–100; Soluble Potato Starch, Sigma S–2630; Soluble Potato Starch for Iodometry, J.T. Baker 4006–04.
2. There is a 15–30 sec delay in neutralizing the starch indicator for peroxide values 70 meq/kg and higher. This delay is due to the tendency of isooctane to float on the surface of an aqueous medium, and the time necessary to adequately mix the solvent in large volumes of aqueous titrant, thereby liberating the last traces of iodine. Based on collaborative study results (References,1,2), the recommendation is to use 0.1 M titrant for peroxide value ranges (10–150 meq/kg). Erratic results reported for this method, especially at higher peroxide values, appear to be related to the isooctane floating on the surface of the aqueous layer (References, 3). Rapid mechanical stirring (*e.g.*, with magnetic stirrer) and/or use of a surfactant, such as sodium lauryl sulfate (Reagents, 6), is highly recommended.

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3. If the titration is less than 0.5 mL using 0.1 M sodium thiosulfate, repeat the determination using 0.01 M sodium thiosulfate, using vigorous agitation and/or surfactant for the reason stated in Notes, 2. Analysts may use 0.001 M sodium thiosulfate if full validation protocols are followed.
4. The test should be carried out in diffuse daylight or in artificial light shielded from a direct light source (References, 4).

REFERENCES

1. Brooks, D.D., and D.L. Berner, Isooctane as an Alternative Solvent for Peroxide Value Determination. Study I, poster presentation at AOCS Annual Meeting, Baltimore, MD, April 23, 1990.
2. Collaborative study results published in *INFORM 1*:884 (1990).
3. Brooks, D.D., S.K. Brophy, B. Hayden, and G.R. Goss, Alternative Solvents for Peroxide Value Determination, poster presentation at AOCS Annual Meeting, Cincinnati, OH, May 10, 1989.
4. *J. Assoc. Off. Anal. Chem. 75*:507 (1992).
5. The International Standards Organization (ISO) successfully completed an international collaborative study of this method in 1996. The results are presented in ISO 3960.

Table 1
Mass of test portion and accuracy of weighing.

Expected peroxide value (meq/kg)	Mass of test portion (g)	Weighing accuracy (g)
0 to 12	5.0 to 2.0	± 0.01
12 to 20	2.0 to 1.2	± 0.01
20 to 30	1.2 to 0.8	± 0.01
30 to 50	0.8 to 0.5	± 0.001
50 to 90	0.5 to 0.3	± 0.001

Table 2
The results of international collaborative tests held between 1993 and 1999 (see References, 5).

	Coconut oil	Linola oil	Lard	Tallow	Beef fat	Olive oil	Palm stearin
n	14	15	13	11	11	16	16
Mean	1.34	3.11	2.89	14.0	12.2	24.10	9.27
Repeatability							
sr	0.07	0.16	0.18	0.79	0.36	0.98	0.73
RSD_r	5.38	5.14	6.34	5.61	2.93	4.08	7.86
r	0.20	0.45	0.51	2.20	1.00	2.75	2.04
Reproducibility							
sR	0.35	0.99	0.31	4.07	4.14	3.92	2.43
RSD_R	26.04	31.76	10.65	29.08	33.96	16.26	26.18
R	0.99	2.77	0.86	11.40	11.60	10.98	6.80

Table 3
Test on olive oil.

	Refined olive oil	Olive oil	Virgin olive oil
Mean value, meq/kg	13.11	7.66	12.27
Relative repeatability standard deviation, %	11.53	6.05	6.50
Repeatability limit, r, meq/kg	4.23	1.30	2.23
Relative reproducibility standard deviation, %	12.48	13.24	13.52
	4.58	2.84	4.65

Table 4
Test on lard, tallow, and beef fat.

	Lard	Tallow	Beef fat
Number of laboratories	11	11	11
Number of acceptable results	11	11	11
Mean value, meq/kg	4.9	6.7	5.4
Repeatability limit, r, meq/kg	0.4	1.1	0.5
Reproducibility limit, R, meq/kg	2.4	5.7	5.8

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AOCS

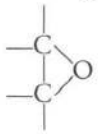
AOCS Official Method Cd 9-57

Reapproved 2009

Oxirane Oxygen

DEFINITION

This method determines oxirane oxygen which is the oxygen contained in the following grouping.



Under the prescribed conditions of this test method, the oxygen is titrated directly with hydrogen bromide in acetic acid.

SCOPE

Applicable to epoxidized fatty materials and epoxy compounds in general (see Notes, 1).

APPARATUS

1. Burette assembly—with either gravity feed or reservoir bottle. The assembly should be an enclosed system for titration to avoid the loss of hydrogen bromide. Attach the titration flask to the burette tip with a one-hole rubber stopper. The hole in the stopper should be formed so the burette tip fits snugly in the stopper with a small side opening to permit air to escape from the flask during titration.
2. Erlenmeyer flasks—50 mL.
3. A magnetic stirrer—any suitable type with round magnetic stirring bars covered with Teflon™ protective coating, or equivalent.

REAGENTS

1. Glacial acetic acid—reagent grade, free of acetic anhydride.
2. Hydrogen bromide gas—anhydrous, available in small cylinders, or 30–32% hydrogen bromide in acetic acid (see Notes, *Caution*).
3. Crystal violet—gentian violet.
4. Potassium acid phthalate—primary standard grade, dried for 2 hr at 120°C and allowed to cool to room temperature in an efficient desiccator prior to use.
5. Chlorobenzene—reagent grade (see Notes, *Caution*).

SOLUTIONS

1. Crystal violet indicator solution—prepared by dissolving 0.1 g of crystal violet (gentian violet) in 100 mL of glacial acetic acid.
2. Hydrogen bromide (HBr)—0.1 M solution in acetic acid, prepared by one of the following procedures:
   (a) Bubble hydrogen bromide gas through glacial acetic acid to approximately 0.1 M. A suitable balance should be used to estimate the amount of hydrogen bromide added.
   Note—This procedure should be carried out in a properly operating fume hood.
   (b) Dilute 30–32% (approximately 4 M) hydrogen bromide in acetic acid with glacial acetic acid to approximately 0.1 M.
   (c) Standardization—Accurately weigh about 0.4 ±0. 0001 g of potassium acid phthalate into a 50 mL Erlenmeyer flask. Dissolve in 10 mL of glacial acetic acid, warming slightly to dissolve. Titrate the solution at room temperature with hydrogen bromide in acetic acid, using no more than 0.1 mL (5 drops from a fine dropping pipet) of crystal violet indicator. The standardization should be performed in duplicate with a difference not exceeding 0.0004 M. The HBr must be standardized on the same day that test samples are analyzed (see Notes, 2).

$$\text{Molarity of HBr} = \frac{\text{mass of potassium acid phthalate, g}}{0.2042 \times \text{mL HBr solution}}$$

PROCEDURE

1. Weigh 0.3–0.5 ± 0.0001 g of test sample into a 50 mL Erlenmeyer flask. Dissolve the test sample in 10 mL of glacial acetic acid or chlorobenzene (in the case of epoxy resins use chlorobenzene). Add the coated stirring bar and no more than 0.1 mL (5 drops from a fine dropping pipet) of crystal violet indicator.
2. Place the rubber stopper in position and lower the tip of the burette until it will discharge just above the surface of the solution. This is important to avoid loss of HBr.
3. Stir and titrate the test portion (rapidly at first) with the 0.1 M HBr solution to a blue-green end point that persists for 30 sec. Control the rate of the magnetic stirrer to avoid splashing.

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CALCULATIONS

$$\text{Oxirane oxygen, \%} = \frac{\text{mL HBr to titrate test portion} \times \text{M} \times 1.60}{\text{mass of test portion, g}}$$

Where—
M = Molarity of HBr solution

PRECISION

The average variance of components calculated from the collaborative study data obtained by the AOCS Technical Committee indicate the following 95% probability limits—

1. The difference between duplicate determinations made within one laboratory should not exceed 0.08.
2. The difference between the average of duplicate determinations made in different laboratories should not exceed 0.19.

NOTES

Caution

Hydrogen bromide gas and hydrogen bromide are toxic by inhalation and strong irritants to eyes and skin. Use a properly operating fume hood when working with these compounds.

Chlorobenzene is a moderate fire risk. Explosive limits in air are 1.8–9.6%. Avoid inhalation and skin contact. The TLV in air is 75 ppm. Use a properly operating fume hood when working with this solvent.

NUMBERED NOTES

1. This method is not applicable to oils containing α- and β-unsaturated ketones, cyclopropenes, conjugated dieneols, oxidized fats, and soaps.
2. Long standing has a deleterious effect on hydrogen bromide solutions and should be avoided. The hydrogen bromide solution should be standardized each time prior to use.

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AOCS

AOCS Official Method Cd 11-57

Revised 2011

α-Monoglycerides

DEFINITION

This method determines α-monoglycerides from the periodic acid consumed in the oxidation of the adjacent hydroxyl groups. β-Monoglycerides are not oxidized by periodic acid because the hydroxyl groups are not adjacent.

SCOPE

This method is applicable to fats, oils, monoglycerides, and blends. This method is not applicable when the test sample contains, in addition to monoglycerides, chloroform-soluble polyhydric substances with two or more adjacent hydroxyl groups. Note: At α-monoglyceride levels below 15%, there may be interfering oxidation products in the test sample which will contribute to an analytical result that is higher than the actual α-monoglyceride content. In such cases, the analyst may consider the use of the GLC method, AOCS Official Method Cd 11b-91. This is a joint AOCS-Association of Official Analytical Chemists (AOAC) method (References, 1).

APPARATUS

1. Burettes—50 and 100 mL, accurately calibrated.
2. Meniscus magnifier—suitable to permit reading the burette to 0.01 mL.
3. Flasks—volumetric, 1000 mL.
4. Flasks—volumetric, with glass stoppers, 100 mL.
5. Pipets—volumetric, 10, 25, and 50 mL. The 25 and 50 mL pipets must conform to NIST tolerances and be accurately calibrated to deliver 25 and 50 mL, respectively.
6. Beakers—400 mL, with watch glasses to serve as covers.
7. Stirrer—variable-speed electric stirrer with glass stirrer, or magnetic stirrer with coated magnetic stirring bar.
8. Graduated cylinders—100 and 1000 mL.
9. Erlenmeyer flasks—with glass stoppers, 500 mL.
10. Balance—analytical, 200 g capacity, with +0.0001 g sensitivity.
11. Balance—top loading, 500 g capacity with +0.01 g sensitivity.

REAGENTS

1. Periodic acid (H_5IO_6)—reagent grade, tested for quality by adding 50 mL of the periodic acid solution (Solutions, 1) to 0.5–0.6 g of glycerine in 50 mL of distilled water. Allow to stand 30 min and proceed as described in Procedure, paragraphs 9–11. Titration of the solution containing glycerol, divided by the titration of the blank, will be between 0.75 and 0.76 when the periodic acid is satisfactory.
2. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)—reagent grade.
3. Potassium iodide (KI)—reagent grade.
4. Glacial acetic acid—reagent grade, 99.5% (see Notes, *Caution*).
5. Starch solution, 1% potato starch—tested for sensitivity as follows: Place 5 mL of starch solution in 100 mL of water and add 0.05 mL of freshly prepared 0.1 N KI solution and one drop of a 50 ppm chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution to 1000 mL. The deep blue color produced must be discharged by 0.05 mL of 0.1 M sodium thiosulfate.
6. Chloroform ($CHCl_3$)—reagent grade (see Notes, *Caution*). Blank tests run on periodic acid with and without 50 mL of chloroform must check with 0.5 mL. If they do not, obtain a fresh supply of chloroform.
7. Potassium dichromate—either primary standard grade or reagent grade. The potassium dichromate must be finely ground and dried to constant weight at 105–110°C before using.

   Note—A standard sample of potassium dichromate with a certificate of analysis may be obtained from the NIST in Gaithersburg, MD, USA. This sample is strongly recommended as the primary standard for this method. Treat as directed in the certificate of analysis accompanying the sample.
8. Hydrochloric acid—concentrated, reagent grade, sp. gr. 1.19 (see Notes, *Caution*).

SOLUTIONS

1. Periodic acid solution—prepared by dissolving 5.4 g of periodic acid in 100 mL distilled water and then adding 1900 mL of glacial acetic acid and mixing thoroughly. Store the solution in a dark, glass-stoppered bottle (see Notes, 1).
2. Sodium thiosulfate solution—0.1 N, prepared by dissolving 24.8 g of sodium thiosulfate in distilled water and diluting to 1 L. The solution is standardized by pipetting 25 mL of the standard dichromate solution into a 400 mL beaker. Add 5 mL

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of hydrochloric acid, 10 mL of potassium iodide solution (Solutions, 3) and rotate to mix. Allow to stand for 5 min and then add 100 mL of distilled water. Titrate with sodium thiosulfate solution, stirring continuously until the yellow color has almost disappeared. Add 1–2 mL of starch indicator solution and continue titration, adding the thiosulfate solution slowly, until the blue color just disappears. The strength of the thiosulfate is expressed in terms of its normality.

$$\text{Normality of } Na_2S_2O_3 \text{ solution} = \frac{2.5}{\text{mL sodium thiosulfate required}}$$

3. Potassium iodide (KI) solution—prepared by dissolving 150 g of KI in distilled water and diluting to 1 L.
4. Starch indicator solution—prepared by making a homogenous paste of 2 g of potato starch (see Notes, 2) in cold distilled water and adding the paste with rapid stirring to a sufficient volume of boiling water to give a final volume of 200 mL. Boil for a few seconds and remove from the heat. Salicylic acid (1.25 g/L) may be added to preserve the starch solution. If long storage is required, the solution must be kept in a refrigerator at 4–10°C (40–50°F). Fresh indicator must be prepared when either end point of the titration from blue to colorless fails to be sharp or when the starch fails to meet the sensitivity test (Reagents, 5).
5. Standard potassium dichromate solution—0.1 M. Dissolve 4.9035 g of finely ground and dried potassium dichromate in distilled water in a 1000 mL volumetric flask and make up to volume at 25°C.
6. Acetic acid solution—prepared by mixing 5 mL of glacial acetic acid with 95 mL of distilled water.

PREPARATION OF TEST SAMPLE

1. Solid test samples in flake form—Mix without melting and take portion for test.
2. Solid test samples not in flake form—Melt at not more than 10°C above the melting point of the test sample, mix thoroughly, and take a subsample for analysis. Do not test test samples that contain so much free glycerol that it separates when the test sample solidifies.
3. Semisolid and liquid test samples—Liquefy by heating at not more than 10°C above melting point of the test sample, mix thoroughly, and take a subsample for analysis. Do not test test samples that contain so much glycerol that it separates from the test sample when cooled to room temperature.

PROCEDURE

1. Weigh accurately onto a weighing dish the proper size test sample as indicated in Table 1.
2. Dissolve the test sample, and transfer to a 100 mL glass-stoppered volumetric flask, being careful not to lose any of the test sample. Use small portions of chloroform to quantitatively transfer the test sample.
3. Make up the volume with chloroform and mix thoroughly.
4. Pour the contents of the volumetric flask into a 500 mL glass-stoppered Erlenmeyer flask.
5. Add 100 mL of water with a graduated cylinder (use a pipet if free glycerol is to be determined). If emulsions form, causing a poor separation in the next step, use 100 mL of the 5% acetic acid solution (Solutions, 6) in place of 100 mL of water.
6. Stopper and shake vigorously for 1 min, set aside, and allow to stand until the aqueous phase and the chloroform phase separate and the chloroform layer is clear or only slightly cloudy. This usually takes 1–3 hr.
7. Pipet 50 mL of periodic acid reagent into a series of 400 mL beakers. Prepare three for blanks. Add 50 mL of chloroform to two of the blanks and 50 mL of water to the other. The blanks are handled exactly like the test portion (Procedure, 9 and 10). The titrations of the water and chloroform blanks are used as a check on the chloroform (see Reagents, 6).
8. Pipet (see Notes, 3) 50 mL of the chloroform sample solution into a 400 mL beaker containing 50 mL of periodic acid reagent (Solutions, 1), and shake gently to effect thorough mixing. Cover with a watch glass and allow to stand 30 min.

   Note—If the room temperature should exceed 35°C (95°F), both the reagent and chloroform sample solution must be cooled to 35°C (95°F) or below before pipetting.
9. Add 20 mL of KI solution, mix by gentle shaking, and allow to stand at least 1 min, but never more than 5 min, before titrating. Do not allow to stand in strong sunlight.
10. Add 100 mL of distilled water and titrate with 0.1 N $Na_2S_2O_3$ solution. Use the variable-speed electric stirrer or the magnetic stirrer to keep the solution thoroughly mixed. Continue the titration until the iodine color almost disappears from the aqueous layer. Add 2 mL of starch indicator solution and continue the titration to the disappearance of iodine from the chloroform layer and the disappearance of the blue iodo-starch color from the aqueous layer. Vigorous agitation is essential for complete removal of iodine from the chloroform layer.
11. Read the burette to ± 0.01 mL.
12. The blanks are handled exactly like the test portion (Procedure, 9 and 10).
13. If titration of the test portion (Procedure, 11) is less than 0.8 of the titration of the blank (Procedure, 12):
    (a) Repeat the test, using smaller portions (25, 10, or 5 mL in Procedure, 8) until the titration of the test portion is more than 0.8 times that of the blank, or
    (b) If 10 mL (or less) of the sample solution is necessary to bring the titration within limit required by 13 (a), repeat, starting at the beginning with a smaller test portion, referring to Procedure, 1 to find proper amount of test portion to weigh.

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Note—The titration of the test portion must be more than 0.8 that of the blank to assure an adequate excess of periodic acid.

14. If the titration of the blank (Procedure, 12) minus the titration of the test portion is less than 4 mL:
    (a) Repeat the test in the interest of greater accuracy, beginning with paragraph 1 of the Procedure and using twice as much test portion, *or*
    (b) If doubling the test portion size exceeds 10 g, use only 10 g.

CALCULATIONS

1. The following calculations assume that the monoglyceride is monostearin.

$$\text{Monoglyceride, \%} = \frac{(B - S) \times N \times 17.927}{W}$$

Where—

B = volume of titrant, mL of blank containing 50 mL chloroform
S = volume of titrant, mL of test solution
N = normality of $Na_2S_2O_3$ solution
W = mass of test portion, represented by aliquot pipetted for test in Procedure, 8
17.927 = molecular weight (MW) of monostearin divided by 20

2. The monoglyceride may be calculated on the basis of a monoester other than monostearin by dividing the molecular weight of the monoglyceride by 20 and substituting this value for 17.927 in the formula above. If a better estimate of the molecular weight of the monoglyceride is desired, proceed as follows:
    (a) Separate the fatty acids as described in AOCS Official Method Cc 12-59.
    (b) Determine acid number of the fatty acids as directed in AOCS Official Method Da 14-48.
    (c) Average MW of fatty acids = $\frac{56108}{\text{acid value}} = M$
    (d) MW of monoglyceride = (M + 92.09) –18.02

PRECISION

1. Collaborative studies have shown that the 95% confidence limits shown in Table 2 may be expected.

NOTES

Caution

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence for the carcinogenicity of chromium [+6] in humans, in particular, lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

NUMBERED NOTES

1. Rubber or cork stoppers must never be used where periodic acid can come in contact with them.
2. "Potato Starch for Iodometry" is recommended, because this starch produces a deep blue color in the presence of the iodonium ion. "Soluble Starch" is not recommended because a consistent deep blue color may not be developed when some soluble starches interact with the iodonium ion. The following are suitable starches: Soluble Starch for Iodometry, Fisher S516-100; Soluble Potato Starch, Sigma S-2630; Soluble Potato Starch for Iodometry, J.T. Baker 4006-04.
3. Pipet chloroform sample solution with care and in such manner that none of the aqueous layer is included.

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REFERENCES

1. *Official Methods of Analysis,* AOAC International, 16th edn., Gaithersburg, MD, 1994, Method 966.18.
2. *J. Am. Oil Chem. Soc. 34:*301 (1957).
3. *J. Assoc. Off. Anal. Chem. 49:*816 (1966).
4. *Ibid. 55:*352 (1972).

Table 1
Monoglyceride test sample size.

Monoglyceride, %	Approximate size of test portion, g	Weighing accuracy, ±g
100	0.30	0.0002
75	0.40	0.0002
50	0.60	0.0003
40	0.70	0.0005
30	1.00	0.001
20	1.50	0.001
15	2.50	0.002

Table 2
Expected variation of results based on 95% confidence limits.

α-Monoglyceride at level of	40%	90%
Duplicate determinations made on same day by an analyst should not differ by more than approximately	0.5	1.2
Single determinations made in two different laboratories should not differ by more than approximately	1.2	3.2
Averages of duplicate determinations made in two different laboratories should not differ by more than approximately	1.1	3.1

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AOCS Official Method Cd 11b-91

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Determination of Mono- and Diglycerides by Capillary Gas Chromatography

DEFINITION

This method is for the determination of mono- and diglycerides by capillary GLC. Mono- and diglycerides are converted with bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorsilane (TMCS) in pyridine into more volatile trimethylsilylether derivatives and determined by capillary GLC.

SCOPE

This method is applicable to mono- and diglyceride concentrates and mono- and diglycerides in fats and oils (see References, 1). Other emulsifiers and components of fats and oils, such as glycerol, fatty acids, sterols, etc., may be converted into the trimethylsilylether derivatives and analyzed by the same GLC procedure. For the identification of the components, coupled GC/MS may be advantageous.

APPARATUS

1. Gas chromatograph—with split injection or on-column injection (see Notes, 1), oven temperature programming and flame-ionization detector.
2. Recording potentiometer.
3. Electronic integrator.
4. Column—capillary, glass or fused silica, surface fully deactivated by silylation agent (see Notes, 2), 15–25 m, 0.25–0.35 mm i.d., coating SE-54 (or other phase with similar polarity), film thickness 0.1–0.2 μm (see Notes, 3).
5. Operating conditions:
   —split injection (split ratio 1:10–1:50)
   —direct injection (splitless, hold for 1 min)
   —injection port 320°C (or for on-column injection 60°C)
   —column initial 80°C (or for on-column 60°C)
   —program rate 10°C/min
   —final temperature 360°C, hold 15 min (see Notes, 3)
   —detector 350°C
   —carrier gas flow 5 mL He/min (at 80°C)
   —injection volume 1–5 μL
6. An automatic sampler is advantageous (see Notes, 4).
7. Screw-cap vials—2.5 mL; or crimp-top vials for auto sampler (*e.g.,* 2.0 mL), with Teflon™-faced septa (see Notes, 3).
8. Heating device for vials—70°C.
9. Balance—analytical, 200-g capacity with ± 0.0001 g sensitivity.

REAGENTS

1. *N,N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).
2. Trimethylchlorsilane (TMCS).
3. Pyridine—analytical reagent grade, kept over KOH (see Notes, *Caution*).
4. n-Tetradecane—analytical reagent grade (minimum 99%).
5. n-Hexane—analytical reagent grade (see Notes, *Caution*).
6. Reference materials—glycerol, palmitic acid, 1-palmitoyl glycerol, 1-stearoyl glycerol, 1,2-dipalmitoyl glycerol, 1,3-dipalmitoyl glycerol, 1,2-distearoyl glycerol.
7. Internal standard solution—prepared by accurately weighing approximately 100 mg internal standard n-tetradecane into 10-mL volumetric flask and diluting to volume with pyridine.
8. Reference solution—prepared by accurately weighing approximately 100 mg of reference substance (*e.g.,* glycerol, fatty acid, mono- and diacyl glycerol) and accurately weighing approximately 100 mg of n-tetradecane into the same 10 mL volumetric flask and diluting to volume with pyridine; or weigh approximately 100 mg of a mixture containing several (*e.g.,* 5) reference materials and n-tetradecane, each component being present in about the same quantity, into a 2 mL volumetric flask and dilute to volume with pyridine (see Notes, 5).

PROCEDURE

1. Test solution—Accurately weigh approximately 10 mg of homogenized test sample of emulsifier concentrates, or 50 mg of oils and fats containing emulsifiers, into a 2.5 mL screw-cap vial with Teflon™-faced septa. Add 0.2 mL BSTFA and 0.1 mL

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TMCS, and then 0.1 mL of internal standard solution (Reagents, 7) containing 1 mg n-tetradecane to the test sample (see Notes, 4). Moisture must be strictly excluded. Close vial and shake vigorously. Heat the reaction mixture in heating device at 70°C for approximately 20 min. Inject 1–5 µL of the reaction mixture into the gas chromatograph showing a stable base line. Avoid delay of GC analysis. The reaction is carried out two times, and two injections are made per reaction (see Notes, 1 and 2).

2. Reference solution—Add 0.10 mL of reference solution (Reagents, 8) into vials and add the silylating agents, 0.2 mL BSTFA and 0.1 mL TMCS (no internal standard solution is added). Heat the reaction mixture and inject into the gas chromatograph as described above (see Notes, 4). Use a concentration range of reference standards similar to the range of the components to be quantified in the test solution. A plot of response vs. concentration of reference substances may be useful to check linearity (see Notes, 1 and 2).
3. Response factors—Check response factors periodically. Response factors should be above approximately 0.5. Lower response factors indicate some loss or decomposition. Use a concentration range of 0.5–10 mg/mL of components in both the reference and test solutions. See Calculations, 1, for calculation of response factors.

IDENTIFICATION

1. Analyze reference solution under the same operation conditions as test solution. Identify peaks by comparison of retention time with known substances, or apply coupled GC/MS. See Figure 1.

CALCULATIONS AND EXPRESSION OF RESULTS

1. Response factor—Calculate response factor of the reference substance vs. internal standard using the reference solution chromatogram. The value of the response factor is given by the formula:

$$R_x = (m_{is}/m_x) \times (A_x/A_{is})$$

Where—

R_x = response factor of reference substance x
m_{is} = mass of internal standard, in mg
m_x = mass of reference substance x, in mg
A_x = peak area of reference substance x
A_{is} = peak area of internal standard

2. Calculation of test portion component content—Calculate percentage of mass content of component x in the test portion by the formula:

$$m'_x\,(\%) = 1/R_x \times (m'_{is}/m'_s) \times (A'_x/A'_{is}) \times 100\%$$

Where—

m'_x = percentage of mass of component x in test portion
R_x = response factor of component x in test portion
m'_{is} = mass of internal standard in test portion, in mg
m'_s = mass of test portion, in mg
A'_x = peak area of component x in test portion
A'_{is} = peak area of internal standard in test portion

3. Typical chromatograms—See Figure 1 for typical chromatograms of reference standards and mono- and diglycerides. The silylation procedure, column specifications, operating conditions and peak identification relating to Figure 1 are as follows:
   (a) Silylation—Test sample size: 10 mg; reagents: 0.1 mL pyridine containing 1.0 mg n-tetradecane, 0.2 mL BSTFA, 0.1 mL TMCS; reaction time: 20 min at 70°C.
   (b) Column—Fused silica capillary, 25 m × 0.31 mm (i.d.); film thickness, 0.17 µm, consisting of 5% phenylmethyl silicon, Ultra #2 (Hewlett-Packard, Palo Alto, CA, USA).
   (c) Operating conditions—Injector 320°C, column initial 80°C, program 10°C/min, final 360°C, hold 15 min, detector 350°C; carrier gas, helium at 5 mL/min (at 80°C) (see Apparatus, 5).
   (d) Peak identification—IS (internal standard), tetradecane; 1, glycerol; 2, diglycerol; 3, hexadecanoic acid; 4, octadecanoate acid; 5, glycerol 1-tetradecanoate; 6, glycerol 2-hexadecanoate; 7, glycerol 1-hexadecanoate; 8, glycerol 2-octadecanoate; 9, glycerol 1-octadecanoate; 10, glycerol 1-icosanoate; 11, glycerol 1-docosanoate; 12, glycerol 1-tetradecanoate-3-hexadecanoate; 13, glycerol 1,2-dihexadecanoate; 14, glycerol 1,3-dihexadecanoate; 15, glycerol 1-hexadecanoate-2-octadecanoate; 16, glycerol 1-hexadecanoate-3-octadecanoate; 17, glycerol 1,2-dioctadecanoate; 18, glycerol 1,3-dioctadecanoate; 19, triglyceride C48; 20, triglyceride C50; 21, triglyceride C52; 22, triglyceride C54.

PRECISION

1. Repeatability—When the mean of the values obtained from two single determinations, carried out in rapid succession by the same operator using the same apparatus under the same conditions for the analysis of the same test sample, lies within the range of the mean values cited in Tables 1, 2 and 3, the difference between the two values obtained should not be greater than the repeatability value (r), which can generally be deduced by linear interpolation from Tables 1, 2 and 3 (References, 2).

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2. Reproducibility—When the values for the final result, obtained by operators in different laboratories, using different apparatus under different conditions for the analysis of the same laboratory sample, lie within the range of mean values cited in Tables 1, 2 and 3, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility value (R), which can generally be deduced by linear interpolation from Tables 1 and 2.

NOTES

Caution

Pyridine is flammable and a dangerous fire risk. The explosive limits in air are 1.8–12.4%. It is toxic by ingestion and inhalation. The TLV is 5 ppm in air. The danger from crude pyridine is greater than from pure pyridine, the associated homologs and impurities being even more toxic than pyridine.

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng per cubic meter for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

NUMBERED NOTES

1. For on-column injection, or direct injection, dilute 50 µL of reaction mixture (Procedure, 1 and 2) with 1 mL hexane and inject (1 µL). In order to lengthen lifetime of the columns, when applying on-column injections, a precolumn is useful. On-column injection gives better response factors.
2. Use length of columns required to separate mono- or diglycerides. Individual unsaturated mono- and diglycerides may not be separated from the saturated or less-unsaturated mono- or diglycerides. TLC on silica gel impregnated with boric acid, immediately prior to derivatization, can be used to resolve 2-mono-glycerides from 1-monoglycerides (References, 3).
3. The final column temperature of 360°C is not compatible with the maximum operating temperature of 320°C to 325°C recommended for SE-54. The detector should be 10°C to 20°C higher than the maximum column temperature. Therefore, it is recommended to use a maximum column temperature of 325°C to 330°C (apparently this will not affect the separation of the mono- and diglycerides), with a detector temperature of 335°C to 350°C. These proposed changes have not been validated by collaborative study.
4. For automatic samplers with 2-mL crimp-top vials, it is convenient to double the amount of test sample and reagents.
5. One or more reference solutions (Reagents, 8) can also be prepared without adding n-tetradecane to the solution. Silylation of the reference solutions (Procedure, 2) is then carried out as described for the test solution (Procedure, 1) after addition of the internal standard solution (Reagents, 7) and silylating reagents.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 6.002 (ex. 2.326).
2. *Pure Appl. Chem. 63:*1153 (1991).
3. Christie, W.W., *Lipid Analysis,* 2nd edn., Pergamon Press, New York, 1982, pp. 101, 159.

Table 1
Statistical analysis of results for mono- and diglyceride concentrates (expressed as percent of mass of test portion).

	1-Myristate	1-Palmitate	1-Stearate	1,3-Dipalmitate	1-Palmitate-3-stearate	1,3-Distearate
No. of laboratories	8	8	8	8	8	8
No. of results	16	16	16	16	16	16
No. of laboratories retained after elimination of outliers[a]	7	8	8	7	7	8
No. of accepted results	14	16	16	14	14	16
Mean value, %	1.7	27.2	60.1	0.2	0.8	1.1
Repeatability standard deviation (S_r)	0.05	0.9	2.1	0.01	0.05	0.07
Repeatability co-efficient of variation (RSD_r)	3.0	3.3	3.5	4.6	6.0	6.8
Repeatability value (r) [$2.83 \times S_r$]	0.14	2.61	5.91	0.031	0.14	0.20
Reproducibility standard deviation (S_R)	0.1	2.4	6.4	0.06	0.1	0.3
Reproducibility co-efficient of variation (RSD_R)	5.7	8.9	10.7	30.0	17.8	24.8
Reproducibility value (R) [$2.83 \times S_R$]	0.3	6.8	18.1	0.2	0.4	0.8

[a]Eliminated using Dixon and Cochran test methods.

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Table 2
Statistical analysis of results for mono- and diglycerides in oil (expressed as percent of mass of test portion).

	1-Palmitate	1-Stearate	1,2-Dipalmitate	1,3-Dipalmitate	1,2-Distearate
No. of laboratories	8	8	8	8	8
No. of results	16	16	16	16	16
No. of laboratories retained after elimination of outliers[a]	8	8	8	8	8
No. of accepted results	16	16	16	16	16
Mean value, %	0.96	0.98	0.97	0.93	0.97
Repeatability standard deviation (S_r)	0.03	0.03	0.04	0.02	0.06
Repeatability co-efficient of variation (RSD_r)	3.3	3.4	4.0	2.5	6.2
Repeatability value (r) [$2.83 \times S_r$]	0.08	0.08	0.11	0.06	0.17
Reproducibility standard deviation (S_R)	0.12	0.14	0.24	0.19	0.19
Reproducibility co-efficient of variation (RSD_R)	12.0	13.8	24.4	20.2	19.8
Reproducibility value (R) [$2.83 \times S_R$]	0.34	0.40	0.68	0.54	0.54

[a]Eliminated by the Dixon and Cochran test methods.

Table 3
Statistical analysis of results for mono- and diglycerides in sunflower oil (blind duplicates, expressed as percent of mass of test portion).

	1-Palmitate	1-Stearate	1,2-Dipalmitate	1,3-Dipalmitate	1,2-Distearate
No. of laboratories	8	8	8	8	8
No. of results	30	30	30	30	30
No. of laboratories retained after elimination of outliers	7	8	7	7	7
No. of accepted results	26	30	26	26	30
Mean value, %	0.75	1.4	1.1	1.3	2.6
Repeatability standard deviation (S_r)	0.07	0.24	0.09	0.07	0.30
Repeatability co-efficient of variation (RSD_r)	10.0	17.0	8.4	5.3	11.8
Repeatability value (r) [$2.83 \times S_r$]	0.21	0.67	0.25	0.48	0.86
Reproducibility standard deviation (S_R)	0.14	0.33	0.11	0.12	0.94
Reproducibility co-efficient of variation (RSDR)	18.5	23.4	10.4	9.2	36.3
Reproducibility value (R) [2.83 × SR]	0.39	0.92	0.32	0.28	2.6

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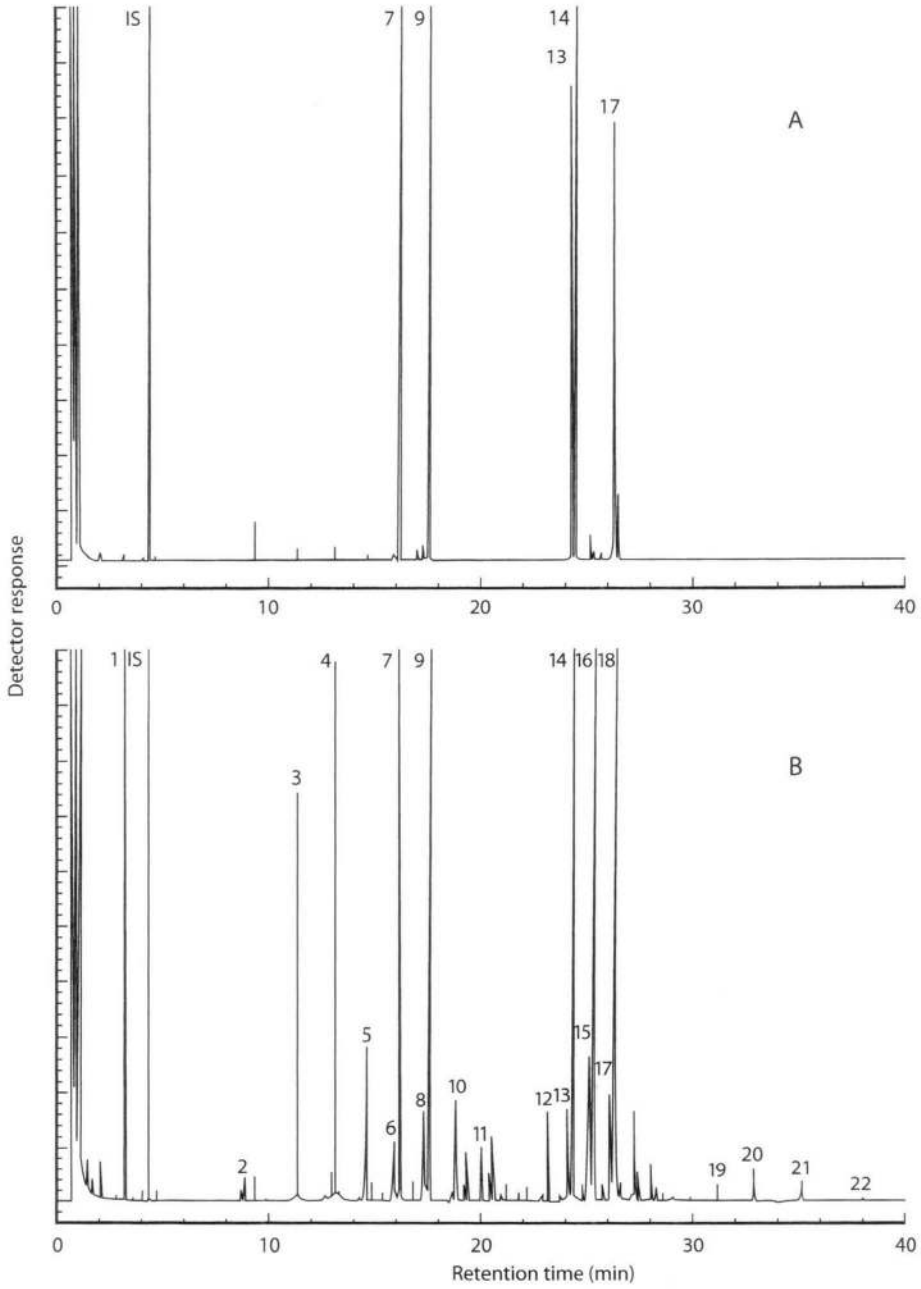

Figure 1. Typical chromatograms of trimethylsilylether derivatives of mono- and diglycerides: A, reference standards and B, mono- and diglyceride emulsifier. The silylation procedure, column specifications, operating conditions, and peak identification are as follows: (a) *Silylation*—Test sample size, 10 mg; reagents, 0.1 mL pyridine containing 1.0 mg n-tetradecane, 0.2 mL BSTFA, 0.1 mL TMCS; reaction time, 30 min at 70°C. (b) *Column*—25 m × 0.31 mm i.d. fused silica; 0.17 µm film thickness (5% phenylmethyl silicon, Ultra #2, Hewlett-Packard). (c) *Operating conditions*—Injector, 320°C; hold 15 min; detector, 350°C; carrier gas, He, 5 mL/min, 80°C. (d) *Peak identification*—IS, n-tetradecane (internal standard); 1, glycerol; 2, diglycerol; 3, hexadecanoic acid; 4, octadecanoic acid; 5, glycerol 1-tetradecanoate; 6, glycerol 2-hexadecanoate; 7, glycerol 1-hexadecanoate; 8, glycerol 2-octadecanoate; 9, glycerol 1-octadecanoate; 10, glycerol 1-icosanoate; 11, glycerol 1-docosanoate; 12, glycerol 1-tetradecanoate-3-hexadecanoate; 13, glycerol 1,2-dihexadecanoate; 14, glycerol 1,3-dihexadecanoate; 15, glycerol 1-hexadecanoate-2-octadecanoate; 16, glycerol 1-hexadecanoate-3-octadecanoate; 17, glycerol 1,2-dioctadecanoate; 18, glycerol 1,3-dioctadecanoate; 19, triglyceride C48; 20, triglyceride C50; 21, triglyceride C52; and 22, triglyceride C54.

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Quantitative Separation of Monoglycerides, Diglycerides, and Triglycerides by Silica Gel Column Chromatography

DEFINITION

This method determines glyceride mixtures by solid-liquid adsorption chromatography (SLAC), using silica gel as the adsorbent. Triglycerides are eluted with 10% diethyl ether in petroleum ether, diglycerides with 25% diethyl ether in petroleum ether, and monoglycerides with 100% diethyl ether. Free fatty acids present are found predominantly in the triglyceride fraction; the remainder in the diglyceride fraction. Thin-layer chromatography (TLC) is used to verify the purity of the fractions and/or the effectiveness of the separation.

SCOPE

This method is applicable to emulsifiers and shortenings.

APPARATUS

1. Chromatographic column assembly—25 × 2 cm column with Teflon™ stopcock and solvent reservoir, 250 mL, with glass joint, fitting the top of the column.
2. Receiving flasks—four 250 mL.
3. Variable-thickness thin-layer chromatographic spreader (see Notes, 1).
4. Glass plates—Pyrex™, 5 × 20.5 × 0.32 cm (see Notes, 1).
5. Cylindrical thin-layer developing chamber—6.5 cm diameter × 24 cm high, with cap (see Notes, 1).

REAGENTS

1. Silica gel—Davison grade 923, adjusted to 5% moisture.
2. Silica gel—GF-254 for thin-layer chromatography, E. Merck (Brinkmann Instruments, Inc., distributor).
3. Chloroform—reagent grade (see Notes, *Caution*).
4. Petroleum ether—reagent grade (see Notes, *Caution*).
5. Diethyl ether—anhydrous, reagent grade (see Notes, *Caution*).
6. Ethyl alcohol—SDA Formula 3A (see Notes, Caution).
7. Acetone—reagent grade (see Notes, *Caution*).
8. Sulfuric acid—concentrated (sp. gr. 1.84), reagent grade, diluted to give a 70:30, v/v, sulfuric acid/water solution (see Notes, *Caution*).
9. Chromium trioxide—technical grade.

PROCEDURE

1. Prepare the column with 30 g silica gel (Reagents, 1) slurried in 50–60 mL petroleum ether (Reagents, 4).
2. Accurately weigh 0.7–0.9 g of test sample into a 30 mL beaker. Add 3 mL chloroform (Reagents, 3) to dissolve the test sample and quantitatively transfer it to the top of the column. Rinse beaker with up to 3 mL of chloroform, adding the rinsings to the column.
3. Elute the test sample at a flow rate of 2 mL/min, using 250 mL of solvent for each fraction, as noted below in this section for Fractions I through IV. If the separation of methyl esters from triglycerides is desired, 200 mL of a solvent system of 5% diethyl ether (Reagents, 5) in petroleum ether (Reagents, 4), v/v, should be used prior to the elution of the triglycerides in Fraction I.

   Use the following solvent systems to elute the indicated fraction:
   (a) Fraction I (triglycerides)—250 mL 10% diethyl ether (Reagents, 5) in petroleum ether (Reagents, 4).
   (b) Fraction II (diglycerides)—250 mL 25% diethyl ether (Reagents, 5) in petroleum ether (Reagents, 4).
   (c) Fraction III (monoglycerides)—250 mL 100% diethyl ether (Reagents, 5).
   Note—If the elution of glycerol and/or other polar material is desired, a fourth fraction is collected:
   (d) Fraction IV (glycerol and/or polar material)—200 mL 100% ethyl alcohol (Reagents, 6).
4. Collect the effluents separately for each fraction and evaporate to dryness in tared 250 mL flasks on a steam bath under a stream of nitrogen. A boiling chip should be placed into each flask before the tare weight is determined in order to prevent bumping.
5. Weigh the fraction and repeat drying until a constant weight is obtained.

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CALCULATIONS

1. If confirmatory tests by TLC indicate that the fractionation was effective, the weight percent of each fraction is determined as follows:

   (a) Fraction I (triglycerides) $= \dfrac{\text{mass of Fraction I, g} \times 100}{\text{mass of test portion, g}}$

   (b) Fraction II (diglycerides) $= \dfrac{\text{mass of Fraction II, g} \times 100}{\text{mass of test portion, g}}$

   (c) Fraction III (monoglycerides) $= \dfrac{\text{mass of Fraction III, g} \times 100}{\text{mass of test portion, g}}$

   (d) Fraction IV (glycerol and/or polar material) $= \dfrac{\text{mass of Fraction IV, g} \times 100}{\text{mass of test portion, g}}$

2. Conversion of methyl ester isolated to percent free fatty acid:

   $$\text{Weight \% free fatty acid} = \frac{100 \times \text{mass of methyl ester, g} \times \text{GF}}{\text{mass of test portion, g}}$$

   Where—

   GF = gravimetric factor
   = 0.935 for free fatty acid expressed as lauric
   = 0.942 for free fatty acid expressed as myristic
   = 0.948 for free fatty acid expressed as palmitic
   = 0.953 for free fatty acid expressed as oleic

THIN-LAYER CHROMATOGRAPHIC VERIFICATION

Note—If commercially available TLC plates (see Notes, 1) are used, omit steps 1 through 3 and proceed directly to step 4.

1. Plate preparation: Twenty-two plates (5 × 20.5 cm) are placed in a thin-layer chromatographic spreader bed. The variable-thickness spreader blade is adjusted for 0.25 mm adsorbent application thickness.
2. Adsorbent preparation: Slurry 30 g of silica gel GF-254 (Reagents, 2) with 60 mL of water. Shake vigorously for 1 min. Quickly pour slurry into the spreader and coat plates.
3. Air dry the coated plates at room temperature.
4. Activate plates by heating at 110–120°C for 30 min (or follow manufacturer's directions). Store plates in closed container over silica gel containing a moisture indicator.
5. Spot 30–60 µg of each fraction from a 30–40 mg/mL $CHCL_3$ (Reagents, 3) test solution approximately 2 cm up from the bottom edge of the plate; also spot an appropriate standard (*e.g.*, Dur-Em 104, Dur-Em 187, commercial 40% monoglyceride emulsifier, etc.) next to fraction samples.
6. Place TLC plate in the developing chamber after a saturated atmosphere (approximately 15 min chamber saturation time with closed chamber) is obtained with the following solvent system:
   92% petroleum ether (Skelly Solve F) (Reagents, 4),
   8% acetone (Reagents, 7).
7. Remove plate from the chamber after the solvent front has moved 16 cm from the origin of spotting and air dry plate in a suitable, operating fume hood.
8. Spray plate with a 70% aqueous sulfuric acid solution (Reagents, 8) saturated with chromium trioxide (Reagents, 9) and place on a hot plate (at 200°C) until charring is complete (white fumes cease).
9. Verify composition of fractions by comparing spots of fractions with spots of standards of known composition.

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

Acetone is highly flammable; it forms explosive peroxides with oxidizing agents. Use effective fume-removal device. Do not mix with chloroform.

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Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive splattering. Always add the acid to water, never the reverse.

NUMBERED NOTES

1. Commercially available plates may be used.

REFERENCES

1. Distler, E., and F.J. Baur, *JAOAC 49:*812 (1966).
2. Quinlin, P., and H.J. Weiser, *JAOCS 35:*325 (1958).
3. This method was submitted by AOCS Uniform Methods Committee member Robert Manning in 1992.

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AOCS Official Method Cd 11d-96
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Mono- and Diglycerides Determination by HPLC-ELSD

DEFINITION

This standard describes a method for the quantitative determination of monoacylglycerides and diacylglycerides. Neutral lipid classes are separated with normal-phase high-performance liquid chromatography (HPLC), and the monoglycerides and diglycerides present are determined with an evaporative light scattering detector (ELSD). Test samples are dissolved in a solvent mixture consisting of *n*-hexane/2-propanol (90:10, v/v) and analyzed without derivatization. Because the response of the ELSD is not linear, a log(response)/log(mass) calibration curve must be established for expected analytes based on ELSD response to calibration standards identical to the test sample components. The method quantitation and detection limits (S/N = 2) are approximately 0.25 mg and 0.06 mg, respectively.

SCOPE

Applicable to both oils and emulsifiers. (Phytosterols may interfere with the determination of 1,3-diacylglycerides in vegetable oils.)

APPARATUS

1. HPLC—The procedure was developed using a Hewlett-Packard 1050 (with the Hewlett-Packard ChemStation computer interfaced with the HPLC, which is not essential to the method); however, any HPLC with the following minimum requirements could be used:
   (a) Column—150 mm × 4.6 mm, with 10 µ Chromegasphere SI-60 stationary phase (ES Industries, Marlton, NJ) or equivalent.
   (b) Column temperature—maintained at 40°C with a column heater.
   (c) Dual channel gradient, programmed as noted in Table 1.
   (d) Mobile phase flow rate—2 mL/min, with an initial pressure of 27 bar (20, 251 torr).
2. ELSD—The procedure was developed using a Varex ELSD II (Varex Corp., Rockville, MD); however, any suitable ELSD with the following minimum requirements could be used:
   (a) Drift tube temperature—maintained at 90°C.
   (b) Nitrogen carrier gas flow rate maintained at 30 mm (approximately 1.2 L/min) on the flow meter of the ELSD.
3. Volumetric flasks, 50 mL, 100 mL.
4. Injection syringe, capable of accurately injecting 20 µL of either standard solutions or test solutions.

REAGENTS (see Notes, Caution)

1. HPLC-grade solvents—
   (a) *n*-Hexane.
   (b) 2-Propanol (isopropanol).
   (c) Ethyl acetate.
2. Reagent-grade solvents—formic acid (88%), 2-propanol, *n*-hexane.
3. Solvent solutions for HPLC—
   (a) Formic acid/2-propanol (10:90, v/v).
   (b) Channel A mobile phase—*n*-hexane [Reagents, 1(a)].
   (c) Channel B mobile phase—*n*-hexane [Reagents, 1(a)]/2-propanol [Reagents, 1(b)]/ethyl acetate [Reagents, 1(c)]/10% formic acid [Reagents, 3(a)] (80:10:10:1, v/v/v/v); for gradient program for mobile phase, see Table 1.
   (d) Column rinse (to remove formic acid when column is not in use)—*n*-hexane [Reagents, 1(a)]/2-propanol [Reagents, 1(b)]/ethyl acetate [Reagents, 1(c)] (80:10:10, v/v/v).
4. Solvent solution for test sample preparation—*n*-hexane [Reagents, 1(a)]/2-propanol [Reagents, 1(b)] (90:10, v/v).

PROCEDURE

1. Test sample preparation (see Notes, 1)
   Vegetable oils:
   (a) Weigh approximately 2 g (see Notes, 2) of vegetable oil, to within 0.1 mg, into a 50 mL volumetric flask [Apparatus, 3].
   (b) Dissolve test sample in *n*-hexane/2-propanol solvent mixture (Reagents, 4) and bring to 50 mL volume with solvent mixture (Reagents, 4).

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Monoglyceride emulsifiers:

(a) Weigh approximately 100 mg of test sample, to within 0.1 mg, into a 50 mL volumetric flask [Apparatus, 3].
(b) Dissolve test sample in *n*-hexane/2-propanol solvent mixture (Reagents, 4) and bring to 50 mL volume with the solvent mixture (Reagents, 4).
(c) Prior to HPLC analysis, dilute the 50 mL test solution to a final volume of approximately 0.5 mg/mL using the solvent mixture (Reagents, 4).

2. Calibration

Monoacylglycerides and diacylglycerides:

The responses of the ELSD to 1,3-distearin, 1,3-dipalmitin, and 1,3-dilinolein (and other diglycerides as needed) and to 1-monostearin, 1-monopalmitin, and 1-monoolein (and other monoglycerides as needed) must be determined using a series of solutions prepared from individual standards. Standards should be of 99%+ purity and may be obtained from Nu-Check-Prep, Elysian, MN.

(a) Accurately weigh approximately 0.1 g (see Notes, 3) of each standard (1-monopalmitin and 1,3-dipalmitin) into a 100 mL volumetric flask [Apparatus, 3(b)], dissolve it in a portion of the *n*-hexane/2-propanol solvent mixture (Reagents, 4) (see Notes, 2 and 4), and dilute to 100 mL with the solvent mixture (Reagents, 4) to provide a stock standard solution.
(b) From the stock standard solution (approximately 1 mg/mL), prepare 1:2, 1:5, and 1:10 dilutions with the hexane/2-propanol solvent mixture (Reagents, 4) to obtain approximately 0.5, 0.2, and 0.1 mg/mL working standard solutions. Use the 1.0, 0.5, 0.2, and 0.1 mg/mL working standard solutions to establish the calibration curve for monoacylglycerides and diacylglycerides.
(c) Analyze the four working standard solutions according to Procedure, 3, and plot the log of the peak area (log [response]) against the log of the concentration (log [mass, µg]) of monoglyceride (1-monopalmitin) and diacylglyceride (1,3-dipalmitin) to obtain calibration curves. Use both linear and quadratic equations to calculate the results (see Notes, 5). A typical response pattern for working standards is shown in Figure 1; ELSD response curves for the working standards are shown in Figures 2 and 3.

Monoglyceride emulsifiers:

Use the calibration curve of 1-monopalmitin from Procedure, 2(a) and (b) and both linear and quadratic equations to calculate the results. If a new calibration curve is needed for monoacylglyceride emulsifier samples (B series in this study), prepare the working standards from the same stock solution of Procedure 2(a). Analyze the working standard solutions according to Procedure, 3, and plot the log of the peak area against the log of the concentration of 1-monopalmitin to obtain a calibration curve.

3. Analysis procedure

(a) Inject 20 µL of solution (working standard or test sample) onto the HPLC column, using the conditions noted in Apparatus, 1(a) to 1(d), with mobile phases Reagents, 3(b) and 3(c) programmed to provide the gradient noted in Table 1.
(b) When not in use, the column must be rinsed with the mixture of *n*-hexane/2-propanol/ethyl acetate [Reagents, 3(d)] to remove formic acid.

RESULTS

The 1,3-diacylglycerides are resolved from the 1,2-diacylglyceride positional isomers, although some 1,3-diacylglycerides of low molecular weight interfere with the 1,2-diacylglycerides of high molecular weight. For monoglycerides, the separation between 1-acylglycerides or 3-acylglycerides and 2-acylglycerides is optimized only between those pairs with identical fatty acyl groups.

1. The ELSD is a universal detector whose response is a function of the mass of the analyte. As shown in Figures 2 and 3, an approximate linear response may be obtained by a log/log plot of ELSD response vs. mass of the analyte, with $r_2 = 0.996$.
2. Figures 4 and 5 show results for the analysis of olive and peanut oils, which were approximately two years old. The olive and peanut oils appeared to contain free fatty acids (peak C), 1,3-diacylglycerides (peak D) and 1,2-diacylglycerides (peak F), but not detectable monoacylglycerides.
3. Figure 5 also shows the chromatogram of the peanut oil spiked with 5% (m/m) of a commercial monoacylglyceride emulsifier. Phytosterols may interfere with the determination of 1,3-diacylglycerides in vegetable oils.
4. This method may be expected to give a small relative standard deviation (<2%) between duplicates and between days. To illustrate the accuracy of this method, monoglyceride results from the analysis of a commercial monoglyceride emulsifier by HPLC-ELSD, GLC, and SFC methods are compared in Table 2.

PRECISION

1. *Repeatability limit*—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than repeatability limit r as shown in Tables 3 through 6.

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2. *Reproducibility limit*—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit R as shown in Tables 3 through 6.

NOTES

Caution

Hexane is a flammable solvent and a dangerous fire risk. All work should be performed in a fume hood, with no open flames. Always ensure that your hood is functioning before introducing volatile solvents. The TLV for hexane is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/M_3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

Isopropanol (isopropyl alcohol) is flammable and a dangerous fire risk. The explosive limits in air are 2–12%. It is toxic by ingestion and inhalation. The TLV in air is 400 ppm. The use of a fume hood is recommended when using isopropanol.

NUMBERED NOTES

1. In the collaborative study the test sample preparation procedures were used with the different test samples as follows:
   (a) Vegetable oils—P.
   (b) Monoglyceride emulsifiers—B-1 and B-2.
   (c) Collaborative study test samples—A-1, A-2, A-3, A-4, A-5, A-6, A-7, and A-8.
2. The maximum concentration of vegetable oil that may be dissolved in the solvent mixture (Reagents, 4) is 4 g/100 mL (4%, m/v).
3. Record the weight of the standards to 0.1 mg (0.0001 g).
4. Sonication may be necessary.
5. Report only the results from 1,3 diacylglycerides. Ignore the 1,2-diacylglycerides (retention time >>6 min).

REFERENCES

1. *IUPAC Commission on Oils, Fats and Derivatives* (1995). Wq 1/94, Project 52–94.
2. Liu *et al. JAOCS 70:*343 (1993).

Table 1
The mobile phase gradient program used for neutral lipids separation on a 150 × 4.6 mm, 10 | Chromegasphere SI-60 column. Column temperature, 40°C. Channel A, hexane; Channel B, hexane/2-propanol/ethyl acetate/10% formic acid (80:10:10:1, v/v/v/v).

Time (min)	Flow (mL/min)	% of each channel A	B
0	2	98	2
8	2	65	35
8.5	2	2	98
15	2	2	98
15.1	2	98	2
19	2	98	2

Table 2
The comparison of monoglyceride results of a commercial emulsifier as determined by the HPLC-ELSD, GC, and SFC methods

	Total monoglycerides, g/100 g		
	HPLC-ELSD	GC/derivatized[a]	SFC/underivatized[a]
Lot 1840			
Mean	92.5	92.3	93.4
%RSD (n)	1.1 (4)	1.3 (4)	3.5 (11)
Lot 6022			
Mean	94.7	94.0	95.9
%RSD (n)	1.5 (4)	1.8 (4)	3.4 (12)

[a]Data are from Lee et al., *J. Assoc. Anal. Chem. 74:*533 (1991).

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Table 3

For the determination of monoacylglycerides, the interlaboratory study completed by the IUPAC Commission VI.3 in 1995, in which 11 laboratories participated, each analyzing 8 test samples (4 sets of blind duplicates) and obtaining two (2) values for each test sample, gave the results summarized in the following table. Results were determined by linear equation.

Test Samples	1_a	2_b	3_c	4_d
Labs before outliers	11	11	11	11
Labs after outliers	9	8	9	9
Accepted results	34	28	34	34
Mono. (%)	0.011	0.025	0.054	0.088
Mono., known (%)	0.0102	0.0204	0.0511	0.0919
Repeatability:				
S_r	0.001	0.002	0.003	0.003
RSD_r	10.92	7.43	5.66	3.39
r	0.003	0.006	0.008	0.008
Reproducibility:				
S_R	0.001	0.008	0.010	0.017
RSD_R	12.95	32.01	18.60	19.75
R	0.003	0.023	0.028	0.049

[a]Combined results for duplicate test samples A1/A7
[b]Combined results for duplicate test samples A2/A5
[c]Combined results for duplicate test samples A3/A8
[d]Combined results for duplicate test samples A4/A6

Table 4

For the determination of monoacylglycerides, the interlaboratory study completed by the IUPAC Commission VI.3 in 1995, in which 11 laboratories participated, each analyzing 8 test samples (4 sets of blind duplicates) and obtaining two (2) values for each test sample, gave the results summarized in the following table. Results were determined by quadratic equation.

Test Samples	1_a	2_b	3_c	4_d
Labs before outliers	11	11	11	11
Labs after outliers	7	7	8	8
Accepted results	28	26	32	32
Mono. (%)	0.011	0.021	0.056	0.092
Mono., known (%)	0.0102	0.0204	0.0511	0.0919
Repeatability:				
S_r	0.001	0.001	0.003	0.003
RSD_r	8.95	2.31	5.16	3.16
r	0.003	0.001	0.008	0.007
Reproducibility:				
S_R	0.001	0.002	0.008	0.016
RSD_R	10.41	8.99	13.28	17.23
R	0.003	0.005	0.021	0.045

[a]Combined results for duplicate test samples A1/A7
[b]Combined results for duplicate test samples A2/A5
[c]Combined results for duplicate test samples A3/A8
[d]Combined results for duplicate test samples A4/A6

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Table 5
For the determination of diacylglycerides, the interlaboratory study completed by the IUPAC Commission VI.3 in 1995, in which 11 laboratories participated, each analyzing 8 test samples (4 sets of blind duplicates) and obtaining two (2) values for each test sample, gave the results summarized in the following table. Results were determined by linear equation.

Test Samples	1_a	2_b	3_c	4_d
Labs before outliers	11	11	11	11
Labs after outliers	6	7	6	7
Accepted results	24	24	24	26
Di. (%)	0.126	0.092	0.055	0.042
Di., known (%)	0.1270	0.0873	0.0564	0.0461
Repeatability:				
S_r	0.006	0.003	0.003	0.001
RSD_r	4.87	2.73	5.77	2.18
r	0.018	0.007	0.008	0.003
Reproducibility:				
S_R	0.019	0.013	0.006	0.006
RSD_R	14.82	14.70	10.12	14.36
R	0.053	0.038	0.016	0.017

[a]Combined results for duplicate test samples A1/A7
[b]Combined results for duplicate test samples A2/A5
[c]Combined results for duplicate test samples A3/A8
[d]Combined results for duplicate test samples A4/A6

Table 6
For the determination of diacylglycerides, the interlaboratory study completed by the IUPAC Commission VI.3 in 1995, in which 11 laboratories participated, each analyzing 8 test samples (4 sets of blind duplicates) and obtaining two (2) values for each test sample, gave the results summarized in the following table. Results were determined by quadratic equation.

Test Samples	1_a	2_b	3_c	4_d
Labs before outliers	11	11	11	11
Labs after outliers	6	7	7	7
Accepted results	24	28	28	28
Di. (%)	0.133	0.081	0.057	0.056
Di., known (%)	0.1270	0.0873	0.0564	0.0461
Repeatability:				
S_r	0.007	0.002	0.003	0.001
RSD_r	5.19	2.71	5.24	1.87
r	0.020	0.006	0.008	0.003
Reproducibility:				
S_R	0.017	0.027	0.008	0.026
RSD_R	12.93	33.87	14.51	46.54
R	0.049	0.078	0.023	0.073

[a]Combined results for duplicate test samples A1/A7
[b]Combined results for duplicate test samples A2/A5
[c]Combined results for duplicate test samples A3/A8
[d]Combined results for duplicate test samples A4/A6

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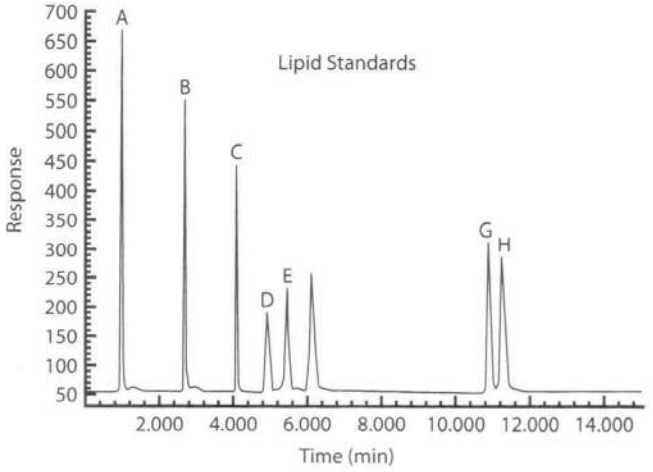

Figure 1. Chromatogram of various lipid standards: (A) cholesteryl myristate, (B) tripalmitin, (C) palmitic acid, (D) 1,3-dipalmitin, (E) cholesterol, (F) 1,2-dipalmitin, (G) 1-monopalmitin, and (H) 2-monopalmitin. The concentration of each standard is in the neighborhood of 1 mg/mL. The HPLC parameters can be seen in Table 1. The Varex II ELSD parameters are: drift tube temperature, 90°C; nitrogen gas flow, 30 mm.

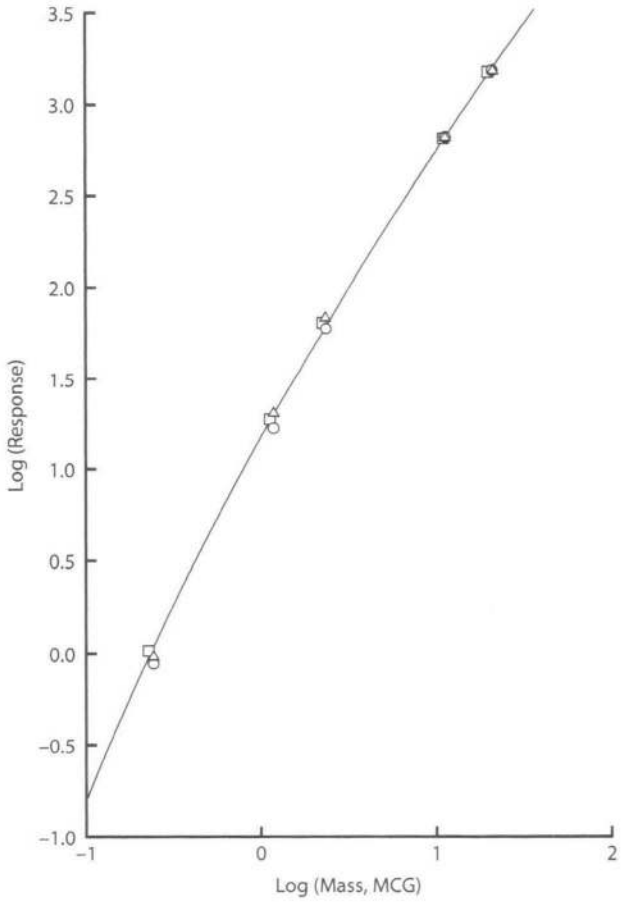

Figure 3. The response curves of 1-monopalmitin (○), 1-monostearin (△), and 1-monoolein (□) with a Varex ELSD using the HPLC and the ELSD parameters as described in Figure 1.

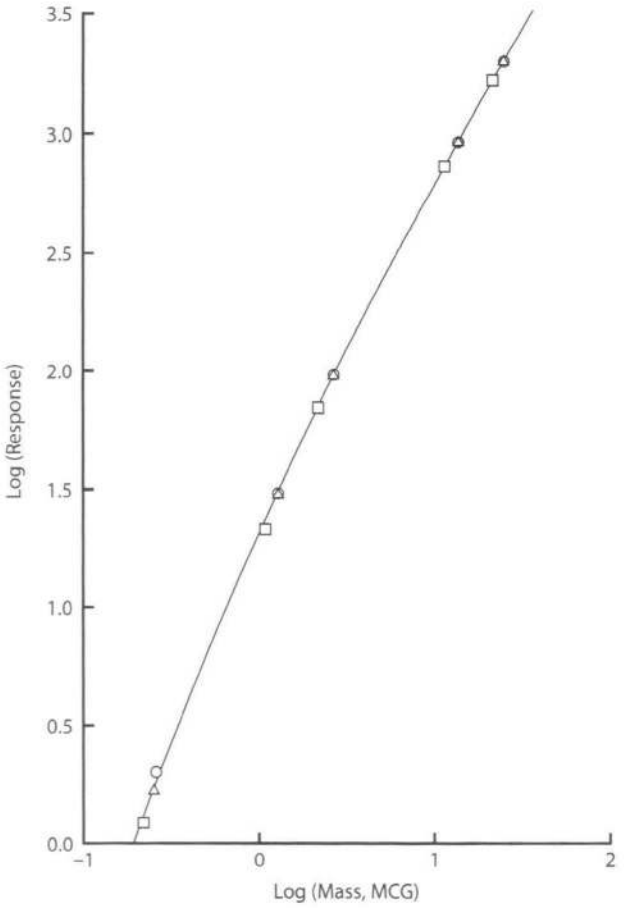

Figure 2. The response curves of 1,3-dipalmitin (○), 1,3-stearin (△), and 1,3-dilinolein (□) with a Varex ELSD using the HPLC and the ELSD parameters as described in Figure 1.

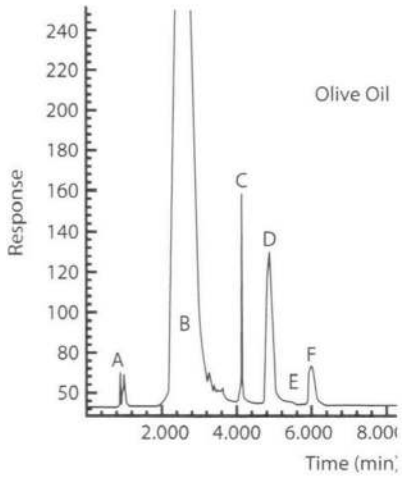

Figure 4. The chromatogram of an olive oil obtained with the HPLC and the ELSD parameters as described in Figure 1. Peaks are identified by retention time only: (A) steryl esters, (B) triglycerides, (C) free fatty acids, (D) 1,3-diacylglycerols, (E) minor 1,3-diacylglycerols and/or phytosterols, and (F) 1,2-diacylglycerols.

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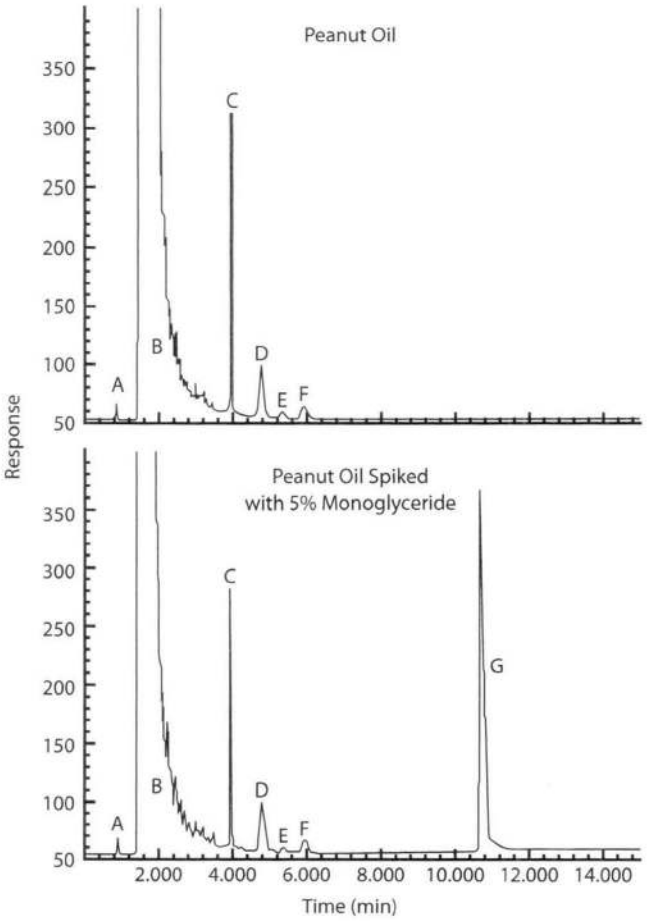

Figure 5. The HPLC-ELSD chromatogram of a peanut oil with and without the addition of a monoglyceride emulsifier. Peaks are identified by retention time only: (A) steryl esters, (B) triglycerides, (C) free fatty acids, (D) 1,3-diacylglycerols, (E) minor 1,3-diacylglycerols and/or phytosterols, (F) 1,2-diacylglycerols, and (G) monoglycerides. Refer to Figure 1 for the HPLC and the ELSD parameters.

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AOCS

AOCS Official Method Cd 12b-92

Revised 2013

Oil Stability Index (OSI)

DEFINITION

All oils and fats have a resistance to oxidation which depends on the degree of saturation, natural or added antioxidants, prooxidants, or prior abuse. Oxidation is slow until this resistance is overcome, at which point oxidation accelerates and becomes very rapid. The length of time before this rapid acceleration of oxidation is the measure of the resistance to oxidation and is commonly referred to as the "induction period."

In this method for determining the induction period, a stream of purified air is passed through a test sample of oil or fat which is held in a thermostated bath. The effluent air from the oil or fat test sample is then bubbled through a vessel containing deionized water. The conductivity of the water is continually monitored. The effluent air contains volatile organic acids, swept from the oxidizing oil, that increase the conductivity of the water as oxidation proceeds. Formic acid is the predominant organic acid formed (see References, 1). The conductivity of the water is monitored by a computer or strip chart recorder. The Oil Stability Index (OSI) is defined as the point of maximum change of the rate of oxidation, or mathematically as the maximum of the second derivative of the conductivity with respect to time (see Fig. 1). This time-based end point may be determined by a computer that can calculate the maximum of the second derivative with respect to time, or by a slope-change algorithm, which is similar to detecting the onset of peaks for integration of GLC chromatograms. The end point may be approximated by using other methods. One commonly used approximation is a graphic method in which tangents are drawn manually (see Fig. 2). The OSI may be run at temperatures of 100, 110, 120, 130, and 140°C. Because by its nature this analysis has this temperature flexibility, all OSI results should specify the OSI time, with the analysis temperature reported immediately after (for example, "OSI 11.7 hours at 110°C").

SCOPE

This method is applicable in general to all fats and oils and has been subjected to a collaborative study (see References, 2) covering a broad range of test sample types. It can be used to analyze crude oils, or other oils that are prone to foaming, if one drop of silicone antifoam is added prior to analysis. It may be used for other types of oils outside the range of test samples tested in the collaborative study (see Notes, 1 and References, 3). This analysis is an automated replacement for the Active Oxygen Method (AOM) for fat stability, AOCS Official Method Cd 12-57.

GENERAL PRECAUTIONS

1. Trace-metal contamination of the glassware will cause accelerated oxidation. Because of the difficulty in removing the final traces of chromate from a glass surface, chromate cleaning solutions should not be used. Only detergents without surface-active agents should be used for cleaning. Traces of surface-active agents remain adhered to a glass surface even after the most thorough rinsing with deionized water. Water used for rinsing should be checked as a potential source of trace-metal contamination.
2. Improper temperature control is the most likely source of error. The temperature must be calibrated by checking the actual temperature of a test sample in the bath. The temperature must be maintained within at least ± 0.1°C. The temperature of the water in the effluent trap must not exceed 25°C, which minimizes the loss of formic acid.

APPARATUS

1. Two commercial instruments embody the equipment needed for this analysis:
   (a) Oxidative stability instrument—from Omnion Scientific Instruments, a division of ULTRA Scientific, Smith Street, North Kingstown, RI 02852.
   (b) Rancimat—model 617 from Brinkmann Instruments, Inc., subsidiary of Sybron Corporation, Cantiague Road, Westbury, NY 11590, USA.
2. Constant temperature bath—to maintain all test samples at the desired temperature to within ± 0.1°C.
3. Air distributing manifold—constructed of stainless steel, plastic, aluminum, or glass. Airflow between sample channels should be calibrated to permit the same airflow to within 10%. This may be accomplished by using either matched capillary tubes or needle valves and flow meters.

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4. Source of low pressure, clean, oil-free, low CO_2, compressed dry air (see Notes, 2)—5.5 pounds per square inch (PSI) is sufficient to supply air to 24 sample tubes. The air may be purified with the system as follows:
   (a) Air inlet tube from compressed air source equipped with stainless steel needle valve or pressure regulator.
   (b) A Balston Model 75-45 Purge Gas Dryer for FTIR and a Balston A963-000 Trace Hydrocarbon remover. Balston, Inc., Naperville, IL 60563, or its equivalent.
   (c) Bottled air may be used, provided that it has low CO_2 levels (<2 ppm CO_2).
5. Thermometer—accurately calibrated, able to indicate the temperature to within ± 0.1°C. For OSI operation, a precision thermometer calibrated against an NIST traceable thermometer is required. Brooklyn Thermometer, Farmingdale, NY provides such a thermometer with a temperature range of 95–150°C in 0.1°C finest readable interval, 76 mm immersion. The stock # is 3551 RM. Brooklyn Thermometer will provide NIST traceable calibration for an additional charge. For temperature calibration of the Rancimat Analyzer, an NIST traceable calibrated platinum resistance (RTD) digital thermometer is required with calibration points at 100, 110, 120, 130, and 140°C. Omega Engineering, Inc., Stanford, CT, provides such a thermometer. A custom 3 wire 2 mm × 7 mm sensor probe is required for calibration of test sample temperature with air flowing through the test sample.
6. Gas flow meter—either a rotameter type or a soap bubble type is satisfactory. Airflow should be measured at each tube under the conditions of the test and should be adjusted to 2.5 ± 0.1 mL/sec. Alltech provides a digital flow meter, Model 7080, suitable for air flow measurement.
7. Sample reaction tubes—soda glass 25 mm × 200 mm. Each tube requires a two-hole stopper and aeration tube. An all-glass assembly, either Martin M 7920, or Brinkmann EA1087, or equivalent, is satisfactory.
8. Conductivity measurement tube and probe—The tube should be capable of holding 50 mL water such that a depth of 5 cm or more covers the tip of the aeration tube to provide contact time with the water. This is necessary for the volatile organic acids to dissolve. The conductivity probe should supply a full-scale sensitivity of 300–500 uS-cm^{-1}.
9. Instrumentation for detecting conductivity and means for recording and evaluating the end point—A computer or multichannel strip chart recorder may be used to monitor the conductivity of each probe in the instrument.
10. Tube-cleaning bath—consisting of a stainless steel pan or sink, heated with either steam coils (*note*: do not use copper tubing) or gas, and of sufficient area to permit laying the required number of aeration and test tubes on the bottom.
11. Test tube brush—fan tip, nylon.
12. Tongs—stainless steel or nickel plated, suitable for handling tubes in hot detergent solution.
13. Hood.

REAGENTS

1. Water—deionized or distilled (conductivity < 5 µS-cm^{-1}).
2. 1,1,1-Trichloroethane, purified grade (see Notes, *Caution*).
3. Acetone—ACS Grade (see Notes, *Caution*).
4. Detergent—for cleaning glassware without leaving contaminating residue. Contrad 70, Liquinox, Citranox RBS (Fluka Ltd., Buchs, Switzerland), and Micro are satisfactory.
5. Silicone antifoam—Dow Corning A. One drop is added to oils that have a tendency to foam.

CLEANING OF TEST SAMPLE REACTION TUBES

The following procedures are either for instruments that do not utilize disposable glassware, or for precautionary cleaning of disposable glassware.

1. Initial cleaning—The initial cleaning should be performed immediately after the preceding determination. Melt and drain off as much of the fat from sample tube as possible. Wash off the remaining fat with a suitable solvent. 1,1,1-Trichloroethane is a satisfactory fat and oil solvent.
2. Tube cleaning—Prepare a 1% solution of detergent (Reagents, 4) and heat almost to boiling in the cleaning bath. Rinse each tube with the hot detergent solution, brushing briefly with the nylon brush (Apparatus, 11). Then place the tubes in the hot detergent solution contained in the cleaning bath (Apparatus, 10) in such a manner that no air bubbles are trapped within. Boil vigorously for 30 min. Brush each tube vigorously with a nylon brush, and rinse in the hot detergent solution. Rinse thoroughly with tap water, followed by deionized water, and place upright in a test tube rack. Fill with deionized water and soak at least 1 hr. At the end of the soaking period, rinse the tubes once again with fresh deionized water, drain on clean filter paper, and dry. Store in a dust-free location.

   Tube cleaning, alternative procedure—Add excess potassium hydroxide (KOH) to an isopropyl alcohol/water solution (90:10, v/v) and mix until saturation has occurred. Half fill the tubes with this solution, and heat in a boiling water bath for several hours with occasional shaking. Finally, rinse with deionized water. If traces of oil remain, the saponification must be repeated. When the tubes have been cleaned in this manner, they should be immersed in a 1% solution of detergent at >80°C for 1 hr. Finally, rinse all glassware with deionized water and dry.
3. Conductivity tube and probe cleaning—To clean the conductivity tubes and probes, first rinse the reaction products with denatured ethanol. The hot detergent solution should then be used as a soak or, with the aid of a brush, to scrub conductivity probes that have the rigidity to withstand such treatment. Rinse repeatedly with deionized water, and soak the tubes

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and probes in deionized water for at least 1 hr. When ready to reuse, fill the tubes with deionized water and connect the probe to the conductivity meter. After 30 min, if the water conductivity is under 25 $\mu S\text{-}cm^{-1}$ and is not changing, then the conductivity tube is ready for reuse. If the water conductivity in the tube is too high (i.e., >25 $\mu S\text{-}cm^{-1}$), the probe must be recleaned.

SAMPLING

1. Because this procedure is a measure of oil stability, anything that might detrimentally affect the stability of the test sample must be avoided. Test Samples should be kept cool and in the dark. Where packaged fats are involved, the test sample should consist of an unopened package, if possible. If this is not possible, test samples must be removed from large containers or processing equipment with clean sampling devices of stainless steel, aluminum, nickel, or glass. Test samples of solid fat should be taken at least 5 cm from the walls of large containers and 2.5 cm from the walls of small containers. If liquid oil is poured from a container, the pouring spout or lip should first be cleaned thoroughly using a clean cloth moistened with acetone. After removal from packages or processing equipment, test samples should be transported and stored only in clean glass or plastic containers. Test samples should be protected from contact with heat, light, and air as much as possible. Test samples should be stored with little or no headspace; if headspace is unavoidable, purge the headspace with nitrogen.

TEMPERATURE CALIBRATION

Note—Because variations in temperature are potentially the most serious source of error, temperature calibration of the instrument is critical.

1. The oil sample should be added to the reaction tube to the level equivalent to 5 g for OSI and Rancimat instruments without an insert, and 2.5 g for Rancimat instruments fitted with disposable inserts. The airflow is then adjusted as directed (Apparatus, 6), and a thermometer (Apparatus, 5) is suspended in the oil such that it does not touch the walls of the reaction tube.
2. Allow the oil temperature to equilibrate for 15 min before reading the thermometer. When noting the temperature, do not move the thermometer up or down.
3. Adjust the block temperature controller to compensate for any differences. Block temperature *must* be within ± 0.1°C of the target temperature (e.g., for 110°C in the range of 109.9–110.1°C; for 130°C in the range of 129.9–130.1°C).
4. After adjustments have been made, wait until the controller is showing the new temperature, and then allow the oil to equilibrate for 15 min. Record the temperature and repeat calibration steps 2–4 until the target temperature is reached.
5. Check the temperature periodically to ensure accuracy.

PROCEDURE

1. Fill the conductivity tubes with 50 mL deionized water and attach the probes. Verify that the water conductivity in the tube is 25 $\mu S\text{-}cm^{-1}$ or less and that the conductivity is constant.
2. Unless already completely liquid, the test sample should be melted at a temperature not more than 10°C above its melting point. The test sample size is 5.0 ± 0.2 g for OSI and Rancimat instruments without an insert, or 2.5 ± 0.2 g for Rancimat instruments fitted with disposable inserts. Carefully place the test sample directly into the bottom of the reaction tube. It has been found that better precision is obtained if the test sample is not allowed to coat the side of the tube. Avoid, as noted in the sampling procedure, contaminating the test sample during transfer.
3. In the collaborative studies, all determinations were performed at 110 and 130°C. The temperature should be selected so as not to permit the OSI time to be less than 4 hr or more than 15 hours. Times less than 4 hr result in a wider variation of end point determination. Sample temperature should be checked periodically to ensure the temperature controllers have not drifted.
4. Connect the tubing from the air manifold to the conductivity measurement tube, and adjust the aeration tubes to within 5 mm of the bottom of both the reaction and the conductivity tubes. Measure the airflow, adjusting to 2.5 ± 0.2 mL/sec (see Note, 3). An exhaust hood is beneficial for the removal of obnoxious volatiles resulting from oxidation reactions.
5. A computer or multichannel strip chart recorder should be used to monitor the conductivity of each probe in the instrument. A plot of water conductivity vs. time obtained from the recorder is then analyzed, and the OSI inflection point is determined either by a microprocessor-computed slope/change algorithm or a maximum of the second derivative, or by the tangential method (see Fig. 2), etc.

PRECISION

1. A collaborative study, in which the stability of rapeseed oil and palm oil was determined at 100°C by 11 laboratories in Norway and the UK, was reported by Woestenburg and Zaalberg (References, 4) to have given an interlaboratory reproducibility coefficient of variation (RSD_R) of 9.1% and an instrumental repeatability coefficient of variation of 3.3%.
2. The OSI method was subjected to a collaborative study among 15 laboratories, using all currently available commercial instruments noted in this method. The overall average coefficient of variation was 10.2% for test samples having from 7 to 80 hr of stability at 110°C. Summaries of the statistical analysis of the results of the collaborative study carried out by the

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AOCS Active Oxygen Method (AOM) Alternatives Committee are shown in Table 1 for 110°C and in Table 2 for 130°C. Generally, a larger variation was noted for lower temperatures and longer induction periods.

3. Repeatability limit—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) as calculated from the formulas in Tables 1 and 2.
4. Reproducibility limit—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as calculated from the formulas in Tables 1 and 2.
5. Table 1 contains the statistical analysis of the interlaboratory study completed by the AOCS AOM Alternatives Committee in 1991, in which 15 laboratories participated, each obtaining four test results for each test sample analyzed at 110°C [statistical results evaluated in accordance with International Organization for Standardization (ISO) 5725-1986].
6. Table 2 contains the statistical analysis of the interlaboratory study carried out by the AOCS AOM Alternatives Committee in 1991, in which 14 laboratories participated, each obtaining four test results for each test sample analyzed at 130°C (statistical results evaluated in accordance with ISO 5725-1986).

NOTES

Caution

Trichloroethane is an irritant to eyes and skin. The TLV is 350 ppm in air.

Acetone is highly flammable and forms explosive peroxides with oxidizing agents. Use effective fume-removal device. Do not mix with chloroform. The explosive limits in air are 2.6–12.8%. The TLV is 750 ppm. It is narcotic in high concentrations and moderately toxic by ingestion and inhalation.

NUMBERED NOTES

1. The OSI analysis may be used to analyze many other types of oils and fats; however, some of these were not included in the collaborative study. Extremely stable fats may be analyzed by accelerating the rate of oxidation by raising the temperature at which the oil is oxidized (e.g., to 140°C). The analysis of these high-stability oils and fats can be performed if the conductivity cell water loss is kept to a minimum by cooling, or is kept at constant volume by the periodic addition of fresh water to make up for any loss. The stability of free fatty acids has been found to be too low to permit analysis at 110°C, but the analysis could be performed at a lower temperature. Fish oils and linseed oils have been successfully analyzed at 80°C, but in general have a long, sloping oxidation curve rather than a sharply defined rapid rise. Margarines and mayonnaises may be tested after separation and drying of the oil with anhydrous magnesium sulfate ($MgSO_4$) prior to analysis.
2. The presence or absence of moisture in the air may have an effect on the oxidative stability of certain antioxidants (References, 5). As noted in Reference 5, when analyzing fats containing moisture-susceptible antioxidants, it is advisable to place a drying tower containing a suitable drying agent in the air train.
3. Precision decreases if the oil coats the side of the reaction tube. If the required airflow causes splattering of the test sample onto the walls of the reaction tube, it may be helpful to decrease the size of the bubbler orifice (References, 6).

REFERENCES

1. deMan, J.M., F. Tie, and L. deMan, *J. Am. Oil Chem. Soc. 64:*993 (1987).
2. Jebe, T., M.T. Matlock, and R.T. Sleeter, *J. Am. Oil Chem. 70:*1055 (1993).
3. Matlock, M.G., T. Jebe, and R.T. Sleeter, Unpublished.
4. Woestenburg, W.J., and J. Zaalberg, *Fette Seifen Anstrichm. 88:*53 (1986).
5. Mehlenbacher, V.C., *The Analysis of Fats and Oils,* Garrard Press, Champaign, IL, 1960, p. 199.
6. The Oxidative Stability Instrument, Owner's Manual, AIR SUPPLY.

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Table 1
Statistical results of interlaboratory study in which test samples were analyzed at 110°C.

	Test sample[a]								
	A	B	C	D	E	F	I	J	L
Number of labs after removal of outliers	15	14	15	15	13	14	13	12	13
n	58	55	56	57	48	52	49	45	51
Outliers	0	3	1	1	8	4	0	4	0
Mean, hours[b]	10.1	7.7	17.9	23.7	13.9	46.5	68.1	69.7	18.6
Repeatability, S_r	0.6	0.4	0.8	0.9	0.3	1.2	4.2	3.5	1.8
RSD_r	5.96	4.80	4.34	4.00	2.11	2.51	6.22	5.03	9.62
r ($2.8 \times S_r$)	1.68	1.12	2.24	2.52	0.8	3.36	11.76	9.80	5.04
Reproducibility, S_R	1.3	0.9	2.0	1.9	1.14	4.1	13.5	9.0	4.4
RSD_R	12.33	11.23	11.09	8.13	8.18	8.92	19.88	12.92	23.93
R ($2.8 \times S_R$)	3.64	2.52	5.60	5.32	3.19	11.48	37.91	25.23	12.32

[a]Key to test samples: A, sunflower/soybean oil blend; B, nonhydrogenated soybean oil; C, liquid/hydrogenated soybean oil blend; D, hydrogenated soybean oil/cottonseed oil blend; E, liquid/hydrogenated corn oil blend; F, hydrogenated corn oil/cottonseed oil blend; I, jojoba oil; J, high-stability oil; L, crude corn oil.
[b]Induction period in hours.

Table 2
Statistical results of interlaboratory study in which test samples were analyzed at 130°C.

	Test sample[a]						
	A	C	D	E	F	I	J
Number of labs after removal of outliers	14	14	14	14	14	11	14
n	51	51	53	54	50	41	49
Outliers	1	0	2	2	3	4	4
Mean, hours[b]	2.6	4.4	5.8	3.4	10.9	11.5	16.1
Repeatability, S_r	0.13	0.18	0.18	0.13	0.46	0.39	0.60
RSD_r	4.98	4.14	3.13	3.85	4.24	3.44	3.70
r ($2.8 \times S_r$)	0.36	0.50	0.50	0.36	1.29	1.09	1.01
Reproducibility, S_R	0.32	0.49	0.54	0.39	1.09	1.74	1.70
RSD_R	12.26	11.04	9.27	11.37	10.01	15.17	10.51
R ($2.8 \times S_R$)	0.90	1.37	1.51	1.09	3.05	4.87	4.76

[a]Key to test samples: A, sunflower/soybean oil blend; C, liquid/hydrogenated soybean oil blend; D, hydrogenated soybean oil/cottonseed oil blend; E, liquid/hydrogenated corn oil blend; F, hydrogenated corn oil/cottonseed oil blend; I, jojoba oil; J, high-stability oil.
[b]Induction period in hours.

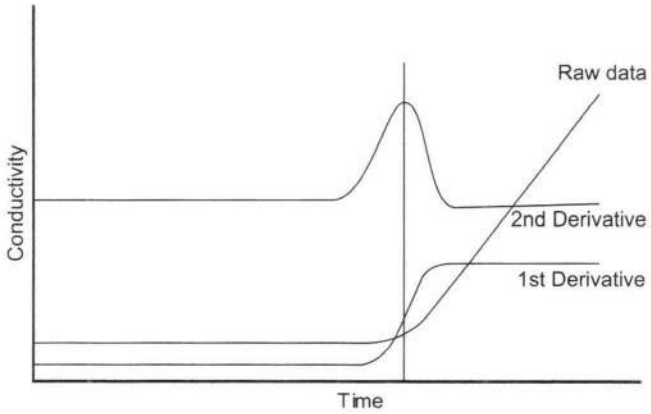

Figure 1. OSI determined by first and second derivatives.

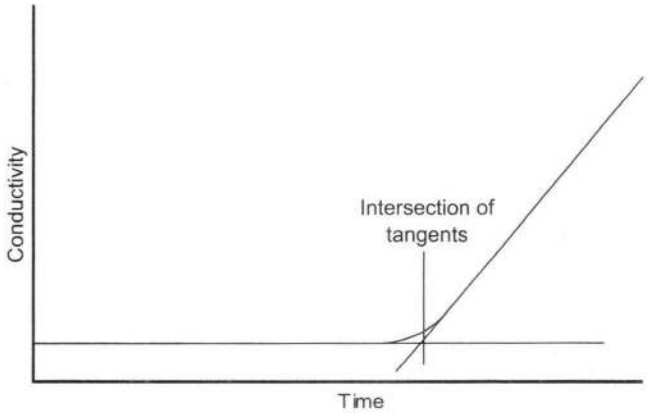

Figure 2. OSI determined by tangential method.

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AOCS Official Method Cd 13-60

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Hydroxyl Value

DEFINITION

The hydroxyl value is defined as the number of milligrams of potassium hydroxide equivalent to the hydroxyl content of one gram of test sample.

SCOPE

Applicable to fatty oils and their derivatives, such as fatty alcohols, mono- and diglycerides, and hydroxystearic acid.

APPARATUS

1. Erlenmeyer flasks—250 mL T ground-glass joints.
2. Pipet—5 mL, class A, volumetric.
3. Burette—50 mL, class A, graduated in 0.1 mL divisions.
4. Reflux condensers—ground-glass joints.
5. Steam bath—standard openings.

REAGENTS

1. Pyridine—reagent grade, distilling at 114–115°C (see Notes, *Caution*).
2. Acetic anhydride—reagent grade, fresh (see Notes, *Caution*).
3. Pyridine-acetic anhydride reagent—freshly prepared before using by mixing 3 volumes of pyridine with 1 volume of acetic anhydride.
4. Normal butyl alcohol—reagent grade, neutralized with 0.5 M ethanolic KOH to a faint pink phenolphthalein end point.
5. Ethanolic potassium hydroxide (KOH) solution—0.5 M, accurately standardized according to AOCS Specification H 15-52 (see Notes, 1).
6. Phenolphthalein indicator solution—1% in ethanol.

PROCEDURE

1. Weigh to the nearest 0.1 mg into an Erlenmeyer flask the indicated amount of test sample for acetylation:

Hydroxyl value	Mass of test portion, g
0–20	10 ± 0.1 g
20–50	5 g
50–100	3 g
100–200	2 g

2. Weigh 9.0–11.0 g of the test portion into another flask for determination of the acidity (see Notes, 2). For fatty acids, such as hydroxystearic acid, the weight should be 0.9–1.1 g.
3. Pipet 5.0 mL of the pyridine-acetic anhydride reagent into the flask containing the test portion for acetylation. (For test portions having a 0–20 hydroxyl value, add an additional 5 mL of pyridine to the flask.) Thoroughly mix the contents by gentle swirling.
4. Pipet 5.0 mL of pyridine-acetic anhydride reagent into an empty flask for the reagent blank. (If 10.0 mL was used for the acetylation, use 10.0 mL for the blank.)
5. Add 10 mL of pyridine (neutralized to phenolphthalein) to the test portion for which the acidity is to be determined (Procedure, 2). Thoroughly mix the contents by gentle swirling, add 1 mL of phenolphthalein indicator solution and titrate to a faint pink end point with 0.5 M alcoholic KOH.
6. Place the flask on a steam bath, under a reflux condenser and heat for 1 hr (see Notes, 3).
7. Add 10 mL of water through the condenser to the flask and heat on the steam bath for an additional 10 min with the reflux condenser attached (see Notes, 3).
8. Allow the flask to cool to room temperature with condenser attached.
9. Using a total of about 25 mL of butyl alcohol (neutralized to phenolphthalein), wash the condenser with about 12 mL, remove the condenser and use the remaining butyl alcohol to wash the sides of the flask.
10. Add 1 mL of phenolphthalein indicator solution. Titrate to a faint pink end point with 0.5 M alcoholic KOH solution.

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CALCULATIONS

1. The hydroxyl value is calculated as follows:

$$\text{Hydroxyl value} = \frac{\left[B + \left(\frac{WA}{C}\right) - S\right] \times M \times 56.1}{W}$$

Where—

A = volume, mL KOH solution required for the acidity titration (Procedure, 5)
B = volume, mL KOH solution required for the reagent blank
C = mass, g of test portion used for the acidity titration (Procedure, 2)
S = volume, mL KOH solution required for titration of the acetylated sample (Procedure, 10)
W = mass, g of test portion used for acetylation (Procedure, 1)

Report the results to the first decimal place.

PRECISION

For castor oil and dehydrated castor oil—

1. Two single determinations performed in one laboratory should not differ by more than 2.4.
2. Single determinations performed in two different laboratories should not differ by more than 3.1.

NOTES

Caution

Pyridine is flammable and a dangerous fire risk. The explosive limits in air are 1.8–12.4%. It is toxic by ingestion and inhalation. The TLV is 5 ppm in air. The danger from crude pyridine is greater than from pure pyridine, the associated homologs and impurities being even more toxic than pyridine itself.

Acetic anhydride is a strong irritant and corrosive. It can cause burns and eye damage. Eyes and hands should be protected when working with this compound. Work should be carried out in a properly operating fume hood.

NUMBERED NOTES

1. The caustic strength should not be less than 0.5 M, so that less than 50 mL is used for the blank titration. This will avoid refilling the burette.
2. For many routine analyses, the acid value of the test samples may be used in place of the acidity titrations, and the calculation altered accordingly.
3. A steam bath must be used. A heating mantle, hot plate, or other higher temperature source of heat will cause erroneous results.

REFERENCES

1. AOAC International, 16th ed. Vol. 11, Gaithersburg, MD, 1995, Chapt. 41, p. 6, Method 965.32.
2. *J. Biol. Chem. 104:*627 (1934)
3. *Ind. Eng. Chem. Anal. Ed. 17:*394 (1945)
4. *JAOAC 48:*131 and 175 (1965)

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AOCS Official Method Cd 14-95

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Isolated *trans* Isomers Infrared Spectrometric Method

DEFINITION

Isolated *trans* bonds in long-chain fatty acids, esters, and triglycerides may be measured by infrared spectrometry. An absorption band with a maximum at about 966 cm^{-1} (10.3 μm), arising from a C-H deformation about a *trans* double bond, is exhibited in the spectra of all compounds containing an isolated *trans* group, and not observed in the spectra of the corresponding *cis* and saturated compounds. Measurement of the intensity of this absorption band under analytically controlled conditions is the basis for a quantitative method for the determination of isolated *trans* content. For high accuracy, common interfering absorptions associated with the glycerol backbone of triglycerides and the carboxyl group of fatty acids must be eliminated by conversion of these test samples to their methyl esters prior to analysis.

SCOPE

This method is applicable to the accurate determination of isolated *trans* bonds in natural or processed long-chain acids, esters, and triglycerides with *trans* levels ≥5%. It is not applicable or is applicable only with specific precautions for fats and oils containing large quantities (over 5%) of conjugated unsaturation, such as tung oil; to materials containing functional groups which modify the intensity of the C-H deformation about the *trans* double bond, such as castor oil containing ricinoleic acid or its geometrical isomer, ricinelaidic acid (12-hydroxy-9-octadecenoic acid); to mixed glycerides having long- and short-chain moieties, such as diacetostearin; or in general, to any materials containing constituents which have functional groups which give rise to specific absorption bands at or sufficiently close to interfere with the 966 cm^{-1} band of the C-H deformation of the isolated *trans* double bond. For accurate determinations on materials with *trans* levels below 5%, AOCS method Ce 1f-96 is recommended. For the direct analysis of triglycerides, Association of Official Analytical Chemists (AOAC) method 965.35 is recommended. Replaces Cd 14-61.

APPARATUS

1. Infrared spectrometer (FT-IR or dispersive) capable of making measurements at 8 cm^{-1} resolution in the spectral range covering 1050–900 cm^{-1}. The instrument's data handling capabilities should allow conversion of spectra to absorbance, scale expansion of the x and y axes, and readout of wavenumbers to the nearest 1 cm^{-1} and absorbance to the nearest 0.001 A.U. FT-IR spectrometers should employ TGS or DTGS detectors to ensure linearity.
2. Infrared liquid sampling cells with NaCl or KBr windows and a fixed path-length of 1 mm. For use in nulltype instruments, pairs of cells matched to within 0.01 absorbance units are required. In split-beam type instruments with both cells filled with the solvent, electronic balance of the two beams to within these limits should be attained.
3. Volumetric flasks—Class A, 10 mL, 25 mL, and 50 mL.
4. Pipets—Class A, 1 mL, 3 mL, 4 mL, 5 mL, 7 mL, 9 mL.
5. Disposable Pasteur pipets for filling of infrared cells.
6. Analytical balance with a 60 g capacity, capable of weighing 0.2 g to an accuracy of ± 0.0001 g.

REAGENTS

1. Carbon disulfide (CS_2)—dry, reagent grade (see Notes, *Caution*).
2. Primary standards—Methyl elaidate and methyl oleate with purity of 99+% (see Notes, 1).

PREPARATION OF STANDARDS

1. *Stock solutions.* Weigh accurately to the nearest 0.0001 g, 0.5 g ± 0.01 g of methyl elaidate into a 25 mL volumetric flask. Dilute to volume with CS_2, stopper, and mix well. Prepare a second solution of methyl oleate as above. Prepare a 0.0020 g/mL stock solution of methyl elaidate by pipetting 5.00 mL of the 0.020 g/mL methyl elaidate stock solution into a 50 mL volumetric flask. Dilute to volume with CS_2, stopper, and mix well.
2. *1, 4, and 7%* trans *standards.* Into 10 mL volumetric flasks, weigh to the nearest 0.0001 g, the weight of methyl oleate specified below to ± 0.005 g. Pipet the specified volume of the 0.0020 g/mL methyl elaidate stock solution into each, then dilute each to volume with CS_2, stopper, and mix well.

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Nominal % *trans* mL	Elaidate Soln. g	Methyl Oleate
1	1.00	0.198
4	4.00	0.192
7	7.00	0.186

3. *10, 30, 50, and 70%* trans *standards.* From the 0.020 g/mL stock solutions prepared above, prepare a set of calibration solutions by pipetting the following specified volumes of solutions into 10 mL volumetric flasks. Bring to volume, stopper, and mix well.

Nominal % *trans* mL	Elaidate Soln. mL	Oleate Soln.
10	1.00	9.00
30	3.00	7.00
50	5.00	5.00
70	7.00	3.00

CALIBRATION (See Notes, 2)

1. For each standard solution, calculate the exact weight of methyl elaidate diluted in 10 mL of solvent. Analyze each solution and determine the baseline corrected infrared absorbance at 966 cm^{-1} as described in the Procedure and Calculations sections below.
2. *Trans* <10%. Using a first-order regression analysis, determine the slope and intercept of the line which best fits the plot of the baseline corrected absorbance at 966 cm^{-1} for the 1, 4, 7, and 10% *trans* standards (y-axis) as a function of the grams of methyl elaidate/10 mL of solution (x-axis).
3. *Trans* >10%. Repeat Step 2 for the 10, 30, 50, and 70% *trans* standards.

PREPARATION OF TEST SAMPLES

1. Solid fats must be melted on a steam bath and mixed before sampling. Test samples that appear cloudy due to presence of water should be treated with anhydrous sodium sulfate and filtered before removing the test sample for analysis.
2. Convert 0.5–1 g of the test sample to methyl esters using AOCS method Ce 2-66, AOAC method 969.33, or other procedure which is appropriate for the test sample type.
3. Weigh accurately to the nearest 0.0001 g, 0.20 g ± 0.01 g of the prepared undiluted methyl esters into a 10 mL volumetric flask. Dilute to volume with CS_2, stopper, and mix well.

PROCEDURE

1. Set up the infrared spectrometer operating parameters according to the manufacturer's recommendations to collect infrared spectra at 4 cm^{-1} resolution covering the range from 1050–900 cm^{-1}. Conditions employed should be identical for test samples and standards. (See Notes, 3)
2. *For single-beam instruments.* Fill a clean infrared liquid cell with CS_2, remove any air bubbles, stopper, and place in the spectrometer's cell holder. Collect the single-beam spectrum to be used as a reference (background). Refill the clean cell with the test solution and collect the single-beam spectrum of the test sample. Ratio the test sample spectrum against the background and convert to absorbance.
   For split or dual-beam instruments. Fill a clean infrared liquid cell with CS_2, remove any air bubbles, stopper, and place in the reference beam cell holder. Fill a second matched cell with the test solution, remove any air bubbles, stopper, and place in the sample beam cell holder. Collect the spectrum and convert to absorbance.

CALCULATIONS

With the spectrum of the test sample scale expanded in the region from 1050–900 cm^{-1}, draw a baseline (XY) tangent to the "feet" of the infrared absorbance band at 966 cm^{-1} as demonstrated in Figure 1. The points in a spectrum between which line XY is drawn will vary with peak absorbance. For accurate results, baselines must be drawn the same for both test samples and standards. For high *trans* containing test samples, the baseline should be drawn to the minimum located at 985 cm^{-1}. Occasionally, this minimum may be absent and the point to which the baseline is drawn will need to be estimated.

Determine the baseline corrected absorbance of the absorbance band (A_C) by subtracting the absorbance of the baseline at the peak maximum (A_B) from the absorbance of the peak at 966 cm^{-1} (A_P). (The measured position of the peak maximum will vary with instrument and *trans* level of the test sample analyzed. The position of the maximum should be established separately for each instrument with the 70% *trans* standard. This same position should then be used at all concentrations.)

From Figure 1,

$$A_C = (A_P - A_B)$$

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Using the calibration data for the appropriate trans range (≤10% or >10%), calculate the weight equivalent of methyl elaidate in the test portion from the following equation:

$$\text{Methyl elaidate mass equivalent, g} = (A_C - \text{intercept}) / \text{slope}$$

$$\%\ \textit{trans}\ \text{as methyl elaidate} = \frac{\text{Methyl elaidate mass equivalents, grams}}{\text{Test portion mass, grams/10 mL of } CS_2} \times 100$$

Report results to the nearest 0.1%.

REPRODUCIBILITY

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit R as shown in Table 1.

REPEATABILITY

Absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than repeatability limit r as shown in Table 1.

NOTES

Caution

Prolonged breathing of carbon disulfide (CS_2) vapors is dangerous. This solvent should be handled only under conditions which provide adequate ventilation, preferably under a chemical hood.

NUMBERED NOTES

1. Primary standards are available from Nu-Check-Prep, Inc., P.O. Box 295, Elysian, MN 56028, USA. Telephone (507) 267-4689.
2. Once calibration data have been established, they should be checked periodically to ensure their validity. For highest quantitative accuracy and precision, a primary or secondary reference should be methylated and analyzed with each group of test portions, then checked to ensure that its measured *trans* level is in agreement with the known or established mean value.
3. Infrared spectrometers should be checked to ensure that they are operating within the manufacturer's established specifications. Checks should include signal-to-noise as well as photometric and wavelength accuracy.

Table 1

An international interlaboratory study completed by the AOCS Chromatography Committee in 1995, coordinated by David Henry, in which 16 laboratories participated, each obtaining two test results for each test sample analyzed by IR, gave statistical results determined by ISO 5725–1986, which are summarized in the following table.

	Me-1	Tg-1	Tg-2	Tg-3	Tg-4	Tg-5	Tg-6
Labs after outliers	15	16	16	15	16	16	14
Labs removed	1	001	0 02				
Values, *n*	30	32	32	30	32	32	27
Mean, %	20.23	6.09	1.33	16.14	16.02	30.91	47.25
Known value, %	21.15	6.14	—	—	—	—	—
S_r	0.49	0.22	0.18	0.20	0.38	0.46	0.81
RSD_r	2.40	3.65	13.86	1.21	2.38	1.49	1.71
$r = 2.8 \times S_r$	1.37	0.62	0.50	0.56	1.09	1.29	2.27
S_R	1.30	0.60	0.44	1.19	1.07	1.86	1.22
RSD_R	6.42	9.82	32.78	7.38	6.69	6.03	2.59
$R = 2.8 \times S_R$	3.64	1.68	1.23	3.33	3.00	5.21	3.42

Test sample key for Table 1:
Me-1 = Methyl elaidate/Methyl stearate with 21.15% *trans*
Tg-1 = Trielaidin in zero-*trans* peanut oil with 6.14% *trans*
Tg-2 = Canola oil
Tg-3 = }Shortening—
Tg-4 = }Blind duplicates
Tg-5 = Margarine
Tg-6 = Pan fry oil

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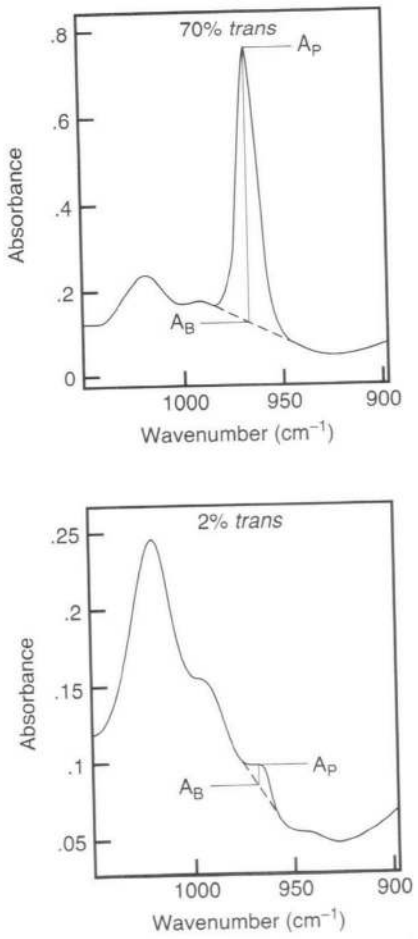

Figure 1. Infrared spectra of methyl esters containing 70 and 2% *trans*.

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AOCS Official Method Cd 14d-99

Revised 2013

Rapid Determination of Isolated *trans* Geometric Isomers in Fats and Oils by Attenuated Total Reflection Infrared Spectroscopy

DEFINITION

In most naturally occurring vegetable fats and oils, unsaturated constituents contain only isolated, i.e., non-conjugated, double bonds in the *cis* configuration. These *cis* bonds may be isomerized to the *trans* configuration during extraction and processing procedures, due to oxidation, conversion during heating, and/or partial hydrogenation. Animal and marine fats may contain measurable amounts of naturally occurring *trans* isomers. Isolated *trans* bonds in long-chain fatty acids, fatty acid methyl esters (FAME), soaps, and triacylglycerols (TAG) may be measured by infrared spectroscopy. An absorption band with a maximum of about 966 cm^{-1} (10.3 μ), arising from a C-H deformation about a *trans* double bond, is exhibited in the spectra of all compounds containing an isolated *trans* group. This band is not observed in the spectra of the corresponding *cis* and saturated compounds. Measurement of the intensity of this absorption band under analytically controlled conditions is the basis for a quantitative method for the determination of isolated *trans* content.

A Fourier transform infrared spectrometer capable of operating at 4 cm^{-1} resolution, equipped with a TGS or DTGS detector, and an attenuated total reflection (ATR) infrared cell are required.

SCOPE

This method is applicable to the rapid (about 5 min) and accurate determination of isolated *trans* double bonds in natural or processed oils and fats with *trans* levels equal to or greater than about 1.0%.

It is not applicable to fats and oils containing over about 1% of conjugated unsaturation, such as tung oil; to materials containing functional groups which modify the intensity of the C-H deformation about the *trans* double bond, such as castor oil, which contains hydroxy groups; or in general, to any materials containing constituents with functional groups that give rise to specific absorption bands at or sufficiently close to interfere with the 966 cm^{-1} band of the C-H deformation of the isolated *trans* double bond. For accurate determinations of materials with *trans* levels below about 1.0%, gas chromatography of FAME (e.g., AOCS Method Ce 1f-96, JAOCS 73:275–282, 1996) is recommended; GC has also been the industry standard for the routine determination of fatty acid composition.

This method requires no weighing and no quantitative dilution of TAG test samples in any solvent.

APPARATUS

1. Fourier transform infrared (FTIR) spectrometer—capable of making measurements at 4 cm^{-1} resolution in the spectral range covering 1050–900 cm^{-1}. The instrument data handling capabilities should allow conversion of the spectra to absorbance, scale expansion of the x and y axes, readout of wavenumbers to the nearest 1 cm^{-1} and absorbance to the nearest 0.001 A.U., and integration of the area under the absorption band at 966 cm^{-1}. FTIR spectrometer should be equipped with TGS and DTGS detectors for greatest linearity. With no test sample on the attenuated total reflection (ATR) crystal (see number 2 below), a 1 min data collection at 4 cm^{-1} resolution should yield an absorption spectrum between 1050 and 900 cm^{-1}, with a peak-to-peak noise level of 0.0005 A.U. or less.
2. ATR infrared cell—with a zinc selenide (or equivalent) crystal. The capacity of the ATR cell should be about 50 μL. For the 966 cm^{-1} band, the signal-to-noise ratio (SNR) in the 1050–900 cm^{-1} range must equal or exceed 10:1 for a 1% trielaidin test sample.
3. Analytical balance—capable of weighing 0.3 g to an accuracy of ± 0.0001 g.
4. ATR accessory—capable of maintaining a constant temperature of about 65 (± 2) °C.

REAGENTS

1. Primary standards: trielaidin (TE) and triolein (TO) with purity of 99% (see Notes, 1).

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PREPARATION OF STANDARDS

1. *Trans* calibration standards: weigh accurately to the nearest 0.0001 g, (0.3-x) g of TO, and x g of TE, into a 10 mL beaker, where x equals 0.0030, 0.0150, 0.0300, 0.0600, 0.0900, 0.1200, and 0.1500 g in order to prepare 1, 5, 10, 20, 30, 40, and 50% *trans* calibration standards, respectively.

CALIBRATION (SEE NOTES, 2)

1. For each neat TE/TO standard mixture, calculate the exact percent *trans* as TE per total TAG. Analyze each mixture and determine the integrated area under the absorption band at 966 cm^{-1} as described in the Procedures and Calculations sections below.
2. Using a first-order regression analysis, determine the slope and intercept of the line which best fits the plot of the area of the *trans* band for all the *trans* standard mixtures (y axis) as a function of % *trans* expressed as percent TE in TO (x axis).

PREPARATION OF TEST SAMPLES

Solid fats must be gently melted and mixed before sampling. Test samples that appear cloudy due to the presence of water should be treated with anhydrous sodium sulfate and filtered before removing the test sample for analysis.

PROCEDURES

1. Set up the FTIR spectrometer operating parameters according to the manufacturer's recommendations for using an ATR cell with the following parameters: 1050–900 cm^{-1} range, 64 scans (or appropriate number of scans needed to give a peak-to-peak noise level of 0.0005 A.U. or less in this range), 4 cm^{-1} resolution, and triangular apodization (if available). Conditions should be identical for test samples and calibration standards (see Notes, 3). For solid TAG test samples maintain ATR cell at 65°C (± 2°C).
2. Using a disposable pipet, transfer about 50 µL without weighing (use enough material to cover the entire surface of the ATR crystal) of the neat reference background material; for TE/TO calibration standard mixtures, use TO as reference background material. For spiked (accuracy standards) test samples, use the unspiked material for measuring the reference background single-beam spectrum. For unknown test samples, use an appropriate reference as background, for example, the refined bleached source oil for the material to be analyzed (see Notes, 4). Place the reference background material on the horizontal (face-up) ZnSe sampling surface of the ATR cell (see Notes, 5). Collect and save the single-beam spectrum to be used as background. Clean the ZnSe crystal by wiping off the TAG analyte with a disposable soft lint-free or low-lint tissue paper (see Notes, 6). Place the neat test sample (without weighing) on the horizontal ZnSe crystal. Collect and save the single-beam spectrum of the test sample. Ratio the test sample single-beam spectrum against that of the background, and convert to absorbance. Save the absorption spectrum.

CALCULATIONS

With the absorbance spectrum wavenumber scale expanded in the region from 1050 to 900 cm^{-1}, integrate (electronically) the area under the 966 cm^{-1} band between the limits 990 cm^{-1} and 945 cm^{-1}. Calculate the linear regression equation for area vs. % *trans* plot of the *trans* calibration standard mixtures.

Using the slope and intercept generated for *trans* standard mixtures, calculate the % *trans* for test samples by plugging the value of the integrated area of the *trans* band in the following equation:

$$\%\ trans\ \text{as ME} = \frac{(\text{area} - \text{intercept})}{\text{slope}}$$

Report results to the nearest 0.1%.

NOTES

1. Primary standards are available from Nu-Check-Prep, Inc., P.O. Box 295, Elysian, MN 56028.
2. Once a calibration curve has been established, it should be checked periodically to ensure that it has not shifted.
3. The performance of FTIR spectrometers should be evaluated for wavenumber accuracy and noise level to ensure that they are operating within the manufacturer's established specifications.
4. TAG reference material ultra degummed bleached expeller soybean oil is recommended (available commercially from Owensboro Grain Co, Owensboro, KY).
5. The analyte must *completely* cover the horizontal surface of the ZnSe crystal.
6. In order to minimize contamination, wipe the ZnSe crystal with the next test sample to be analyzed, and then clean the crystal.

REFERENCES

1. Mossoba, M.M., M.P. Yurawecz, and R.E. McDonald, Rapid Determination of the Total *trans* Content of Neat Hydrogenated Oils by Attenuated Total Reflection Spectroscopy, *J. Am. Oil Chem. Soc. 73*:1003 (1996).

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2. Ali, L.H., G. Angyal, C.M. Weaver, J.I. Rader, and M.M. Mossoba, Determination of Total *trans* Fatty Acids in Foods: Comparison of Capillary Column GC and SB-HATR Infrared Spectroscopy, *J. Am. Oil Chem. Soc. 73:*1699 (1996).
3. Adam, M., M. Chew, S. Wasserman, A. McCollum, R.E. McDonald, and M.M. Mossoba, Determination of *trans* Fatty Acids in Hydrogenated Vegetable Oils by Attenuated Total Reflection Infrared Spectroscopy: Two Limited Collaborative Studies, *J. Am. Oil Chem. Soc.* 75:353 (1998).
4. Adam, M., M.M. Mossoba, M. Chew, and S. Wasserman, Comparison of Attenuated Total Reflection Infrared Spectroscopy to Capillary Gas Chromatography for *trans* Fatty Acid Determination, *J. Am. Oil Chem. Soc. 76:*375 (1999).

APPENDIX 1

Summary of statistical results, determined by the AOAC AOACBUBR computer program, for an international collaborative ATR FTIR study among 12 laboratories that was coordinated by Michael Adam (Lipton, Baltimore, MD) and Magdi M. Mossoba (FDA, Washington, DC).

The study reports data for seven pairs of blind duplicate accuracy standards that were spiked with known amounts of *trans* fat, and 10 pairs of blind duplicate test samples.

For test samples, the reference background material was ultra-degummed bleached expeller soybean oil (Owensboro Grain Co.). Test samples consisted of a commercial vegetable oil (namely, soybean oil, rapeseed oil, palm kernel oil, cottonseed oil, or sunflower oil), partially hydrogenated soybean oil, or the same oil (or a blend of two oils) mixed with partially hydrogenated soybean oil (Lipton, Baltimore, MD).

Accuracy standards consisted of cold-pressed bleached degummed soybean oil spiked with trielaidin; the reference gackground material was cold-pressed bleached degummed soybean oil (Lipton).

The statistical parameters:

- XBAR, the overall mean of the laboratory values
- s(r), repeatability standard deviation
- s(R), reproducibility standard deviation
- RSD(r), repeatability relative standard deviation
- RSD(R), reproducibility relative standard deviation
- r, repeatability value = 2.8 s(r)
- R, reproducibility value = 2.8 s(R)

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Table 1
Pairs of blind duplicate accuracy standards

Lab	Acc1/a	Acc2/b	Acc3/c	Acc4/d	Acc5/e	Acc6/f	Acc7/g
one	0.93	1.08	5.12	10.13	15.40	20.16	40.36
	0.80	1.14	5.27	10.17	15.42	19.97	43.30
two	0.61	0.89	5.10	9.95	15.20	20.40	40.44
	0.64	0.85	5.14	10.24	15.07	20.50	40.37
three	0.81	0.81	4.90	10.13	15.37	20.61	40.41
	0.81	0.81	4.90	10.13	15.37	20.03	40.41
four	1.13	1.40	5.10	10.86	16.53	15.69[b]	38.07
	1.24	1.33	4.87	11.46	15.99	17.87[b]	43.63
five	0.54	0.78	5.37	10.44	16.11	20.99	39.38
	0.52	0.69	5.40	10.83	15.86	21.01	39.37
six	0.74	0.85	5.15	9.53	15.61	20.59	40.31
	0.95	1.23	5.29	10.21	15.18	20.45	40.29
seven	0.93	1.15	5.03	9.75	16.06	20.82	37.48
	0.97	1.15	5.11	10.01	17.14	23.70	38.02
eight	0.84	0.99	5.02	10.12	15.43	20.22	39.96
	0.80	1.26	5.34	10.16	15.33	20.06	40.53
nine	0.81	1.08	5.01	9.88	15.51	19.79	38.87
	0.75	0.99	5.00	10.54	15.03	19.56	39.22
ten	0.07[a]	0.31	5.17	10.73	16.12	20.36	41.34
	0.10[a]	0.28	5.09	9.50	15.27	20.63	39.11
eleven	0.84	0.90	5.03	11.33	15.44	23.54	40.69
	0.78	0.91	5.38	11.15	15.71	20.32	40.35
twelve	0.93	1.15	5.26	10.04	15.30	20.09	40.63
	0.94	1.14	5.26	10.37	15.23	20.09	39.47
TRUE[c]	0.81	1.00	5.04	10.00	15.03	20.00	39.98
XBAR	0.83	0.97	5.14	10.32	15.61	20.63	40.08
sr	0.06	0.10	0.12	0.37	0.34	0.93	1.40
SR	0.18	0.28	0.16	0.52	0.51	1.04	1.41
RSD_r	7.46	10.46	2.30	3.55	2.17	4.53	3.48
RSD_R	21.07	29.27	3.06	5.06	3.26	5.03	3.52
r	0.17	0.28	0.33	1.03	0.95	2.62	3.91
R	0.49	0.79	0.44	1.46	1.42	2.91	3.95

[a] Single Grubbs' outlier
[b] Cochran's outlier
[c] Determined gravimetrically

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Table 2
Pairs of blind duplicate test samples

Lab	TS1/j	TS2/i	TS3/h	TS4/g	TS5/f	TS6/e	TS7/d	TS8/c	TS9/b	TS10/a
one	0.75	0.06	39.93	0.32	4.99	14.79	1.39	28.72	19.77	1.73
	0.71	0.21	40.12	0.35	4.83	14.94	1.49	28.64	20.19	1.84
two	0.12	0.11	39.95	0.00	4.30	14.58	1.20	28.99	20.22	1.05
	0.12	0.13	39.92	0.00	4.27	14.66	1.23	28.86	20.20	0.96
three	0.23	0.23	39.83	0.23	4.30	14.79	1.39	28.76	20.61	1.39
	0.23	0.23	40.41	0.23	4.30	14.79	1.39	28.76	20.61	1.39
four	0.81	0.00	40.32	0.32	4.94	15.00	1.82	29.86	21.67	2.12
	0.80	0.00	40.46	0.37	4.47	15.16	1.76	29.13	21.83	2.13
five	0.07	0.00	40.23	0.00	4.83	15.78	1.26	30.24	21.50	1.75
	0.00	0.00	40.87	0.00	4.68	15.74	1.30	30.37	21.25	1.77
six	0.28	0.00	39.30	0.00	4.64	14.59	1.36	28.94	20.26	2.21
	0.41	0.00	39.24	0.00	4.69	14.45	1.61	28.85	20.47	1.81
seven	0.64	0.66[a]	37.34	0.44	4.45	13.97	1.56	27.10	19.23	2.03
	0.64	0.66[a]	37.37	0.37	4.49	13.90	1.56	27.06	19.30	2.05
eight	0.53	0.00	39.85	0.00	4.66	14.49	1.38	28.74	20.00	1.89
	0.45	0.00	39.75	0.00	4.50	14.40	1.41	28.97	20.09	1.85
nine	0.44	0.00	39.00	0.00	4.43	14.19	1.38	28.13	19.84	1.88
	0.40	0.00	39.82	0.00	4.37	14.33	1.37	28.15	19.83	1.85
ten	0.00	0.00	41.42	0.00	4.32	15.38	0.66[a]	30.41	21.60	1.00
	0.00	0.00	41.66	0.00	4.22	15.50	0.66[a]	30.35	21.43	0.95
eleven	0.48	0.37	39.14	0.00	4.17	13.75	1.48	28.50	19.81	1.84
	0.59	0.27	39.35	0.00	4.35	13.72	1.61	27.83	19.64	2.19
twelve	0.91	0.13	39.40	1.00[b]	4.65	14.25	1.57	28.97	18.92	1.93
	0.80	0.29	35.48	0.63[b]	4.78	14.52	1.83	25.15	18.05	2.23
XBAR	0.43	0.09	39.59	0.12	4.53	14.65	1.47	28.73	20.26	1.74
sr	0.05	0.05	0.84	0.02	0.12	0.09	0.09	0.81	0.22	0.13
SR	0.30	0.12	1.34	0.17	0.24	0.59	0.18	1.19	0.96	0.41
RSD_r	11.11	55.88	2.12	16.25	2.72	0.62	5.87	2.81	1.07	7.39
RSD_R	68.35	133.46	3.38	143.15	5.32	4.00	12.35	4.15	4.74	23.69
r	0.13	0.14	2.35	0.05	0.34	0.25	0.24	2.26	0.61	0.36
R	0.83	0.34	3.75	0.48	0.67	1.64	0.51	3.34	2.69	1.16

[a] Single Grubbs' outlier
[b] Cochran's outlier

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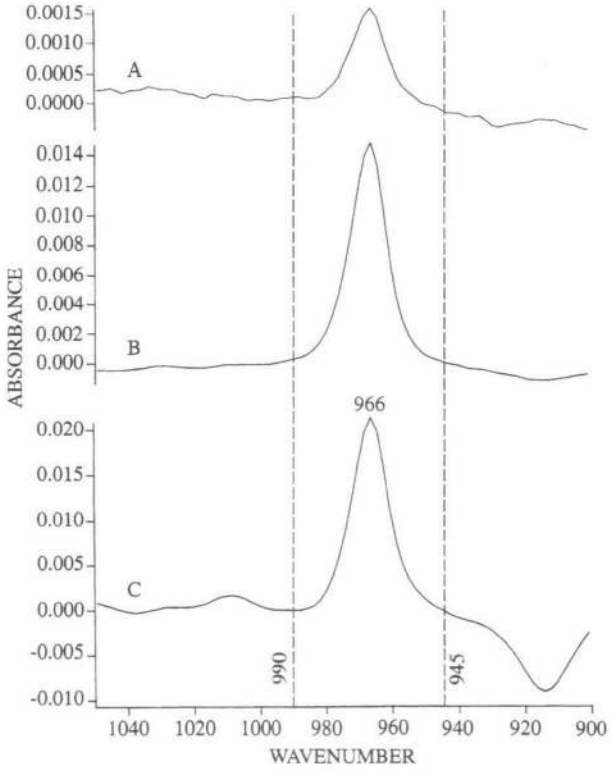

Figure 1. ATR-FTIR spectra in the range 1050-900 cm^{-1} showing the 966 cm^{-1} band attributed to isolated *trans* double bonds for: (A) a 1% trielaidin standard. The signal-to-noise ratio in this range was equal to 16:1 under our experimental conditions of 64 scans and 4 cm^{-1} resolution; (B) a 10% trielaidin standard; and (C) a test sample consisting of a blend of commercial vegetable oils and partially hydrogenated soybean oil found to contain about 14% *trans* fat, as percent of total fat.

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AOCS Official Method Cd 14e-09
Approved 2009

Negative Second Derivative Infrared Spectroscopic Method for the Rapid (5 min) Determination of Total Isolated *trans* Fat

DEFINITION

This method provides a Fourier transform infrared (FTIR) spectroscopic procedure for the rapid (5 min) determination of the total isolated *trans* fat content (as percent of total fat) of vegetable, animal, dairy, ruminant, and marine fats and oils. The fats and oils are measured by FTIR in the attenuated total reflection (ATR) mode (Note 1, References 1 and 2).

SCOPE

This procedure is designed to determine in a single FTIR measurement the level of total isolated (non-conjugated) *trans* fats in *trans*-monounsaturated or mono-*trans*-polyunsaturated molecules with an isolated, methylene-interrupted, or non-methylene-interrupted double bond(s). *Trans* fats and oils exhibit a unique infrared absorption band at 966 cm^{-1} arising from a C-H deformation about an isolated *trans* double bond. This method entails the measurement of the height of the negative second derivative of the band at 966 cm^{-1}.

The method is applicable to neat (undiluted in any solvent) fat and oil test samples of crude, refined, partially or fully hydrogenated products derived from vegetable, animal, dairy, ruminant, or marine sources.

This method is suitable for the analysis of isolated *trans* fat and oil products containing or supplemented with *trans* conjugated linoleic acid (CLA) isomers that exhibit absorption bands near 990, 984 and 950 cm^{-1}.

This method does not require any derivatization of the oil or fat test materials to their corresponding FAMEs. It also does not require any weighing or quantitative dilution of unknown test samples in any solvent.

APPARATUS

1. FTIR spectrometer—It should be equipped with a deuterium triglycine sulfate (DTGS) or mercury cadmium telluride (MCT) detector, capable of making measurements at 4 cm^{-1} resolution in the spectral range covering 1050-900 cm^{-1}. Its performance must meet the following criteria. In the absence of a test sample, a 3 min data collection at 4 cm^{-1} resolution must yield between 1050-900 cm^{-1} a peak-to-peak noise level < 0.0005 absorbance unit (AU) for absorption spectra (Note 2).
2. Heated ATR infrared cell—It should be equipped with an internal reflection element made of zinc selenide (ZnSe), diamond, or equivalent with capacity of approximately 1–10μL. It must be capable of maintaining a constant temperature of the sample of (65 ± 1)°C.
3. Steam water bath.
4. Vials with screw caps.
5. Low-lint tissue paper.
6. Disposable plastic pipets.
7. 10 mL Beakers or vials.

REAGENTS

Unless otherwise stated, use only reagents as specified in ISO 6353 (parts 2 and 3), if listed in Reference 3.

1. Reference trielaidin (TE) with purity of >99% from commercial sources (e.g., Nu-Check-Prep, Elysian, MN, Matreya, Inc., Pleasant Gap, PA, and Sigma Chemical Co., St. Louis, MO) for preparing calibration standards.
2. A high purity saturated tripalmitin (TP) *trans*-free reference oil with purity of >99% from same commercial sources for preparing TE calibration standards. It is noted that the impurities in TP are usually other saturated *trans*-free oils.

PROCEDURE

1. Prepare neat (without solvent) *trans* reference TE calibration standards by weighing accurately to the nearest 0.0001 g, (0.3–x) g of a saturated TP *trans*-free reference oil, and x g of TE, into a 10 mL beaker or vial, where x equals 0.0015, 0.0030,

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0.0060, 0.0150, 0.0300, 0.0450, and 0.0600 g in order to prepare 0.5, 1, 2, 5, 10, 15, and 20% (as percent of total fat) *trans* calibration standards, respectively.

2. Allow the ATR cell to warm up to about 65 (±1)°C for 30 min.
3. Place no sample on the clean ATR horizontal surface, collect a 256-scan single-beam FTIR spectrum at 4 cm^{-1} resolution, and save it. This is the open beam (air) *reference* single beam background spectrum.
4. Place each of the TE calibration standards on the ATR horizontal surface making sure that the surface of the ZnSe or diamond crystal is *completely* covered (Note 3), collect a 256-scan single-beam FTIR spectrum at 4 cm^{-1} resolution, and save it.
5. After each measurement, clean the ATR crystal by thoroughly wiping it with low-lint tissue papers as many times as needed. To insure absence of cross contamination, apply the next test sample to be analyzed, and then clean the crystal again with low-lint tissue papers as many times as needed (Note 4).
6. (a) Place each of the *trans* fat test samples on the ATR horizontal surface making sure that the surface of the ZnSe or diamond crystal is *completely* covered (Note 2), collect a 256-scan single-beam FTIR spectrum at 4 cm^{-1} resolution, and save it. (b) Repeat step 5. (c) Measure each test sample in duplicate (repeat steps 6a and 6b).
7. Electronically generate the absorbance spectra for all the TE reference standards. This is carried out electronically by "ratioing" each of the saved TE single-beam spectra against that of the *reference* single beam spectrum. Electronically convert each absorbance spectrum to its negative second derivative spectrum (-2nd der) (References 4–7). Save each -2nd der spectrum.
8. Electronically generate the absorbance spectra for all the *trans* fat test samples. This is carried out electronically by "ratioing" each of the saved *trans* fat single-beam spectra against that of the *reference* single beam spectrum. Electronically convert each absorbance spectrum to its -2nd der spectrum (References 4–7). Save each -2nd der spectrum.
9. For each of the TE reference standards, display the -2nd der spectrum (see Figs. 1–4) in the expanded wavenumber range 1030-880 cm^{-1}, and record the height of the -2nd der *trans* band at 966 cm^{-1} (between zero and maximum band height, see Fig. 2). Tabulate the data.
10. For each of the *trans* fat test samples, display the -2nd der spectrum (see Figs. 1–4) in the expanded wavenumber range 1030-880 cm^{-1}, and record the height of the -2nd der *trans* band at 966 cm^{-1} (between zero and maximum band height, see Fig. 2). Tabulate the data (Note 5, Figure 4).

CALCULATIONS

1. Create a calibration curve by performing a linear regression analysis of the height of the -2nd der *trans* band at 966 cm^{-1} versus the amount of TE (as percent of total fat) in the *trans* calibration standards. Calibration curves should be checked periodically to insure that they had not shifted.
2. Using the slope and intercept of the linear regression equation generated for TE calibration standards, calculate the *trans* level (as percent of total fat) for each fat or oil test sample by substituting the height of the -2nd der *trans* band into the equation: %*Trans* = [height—Intercept] / Slope. Report results to the nearest 0.01%. This equation assumes that *trans* test samples consist of TE.

METHOD PRECISION

Table 1. IR collaborative study precision data

Test sample[a,b]	1	2	3	4	5	6	7	8	9	10
No. of Labs	10	10	10	10	10	10	10	8	9	10
No. of Replicates	18	20	20	20	19	19	20	16	18	20
MEAN	2.15	4.27	5.13	7.26	9.14	12.55	12.27	1.29	1.34	12.38
s(r)	0.05	0.21	0.11	0.24	0.14	0.22	0.32	0.14	0.15	0.40
s(R)	0.16	0.21	0.22	0.34	0.19	0.29	0.37	0.14	0.16	0.46
RSD(r)	2.17	4.87	2.14	3.26	1.55	1.76	2.57	10.68	11.19	3.21
RSD(R)	7.41	4.87	4.38	4.71	2.03	2.35	2.99	10.68	11.58	3.68
HORRAT	2.08	1.52	1.40	1.59	0.71	0.86	1.09	2.77	3.03	1.34

[a]Test samples 1-6 consist of a mixture of canola oil and partially hydrogenated canola oil
[b]Test samples 9 (lard) and 10 (margarine oil) are blind duplicates of 8 (lard) and 7 (margarine oil), respectively

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Table 2. Comparison of IR collaborative study precision data to those collaboratively obtained by GC (Ce 1h-05) for the two common test samples

	Margarine oil			Lard		
	IR	IR	GC[b] Ce 1h-05	IR	IR	GC[b] Ce 1h-05
Test sample[a]	7	10		8	9	
MEAN	12.27	12.38	**11.62**	1.29	1.34	**0.90**
s(r)	0.32	0.40	**0.160**	0.14	0.15	**0.067**
s(R)	0.37	0.46	**0.253**	0.14	0.16	**0.195**
RSD(r)	2.57	3.21	**1.380**	10.68	11.19	**7.440**
RSD(R)	2.99	3.68	**2.180**	10.68	11.58	**21.700**
HORRAT	1.09	1.34	**0.708**	2.77	3.03	**5.340**

[a]Test samples 9 (lard) and 10 (margarine oil) are blind duplicates of 8 and 7, respectively
[b]GC official method Ce 1h-05 (Reference 8)

QUALITY ASSURANCE AND CONTROL

Blank test sample: The first test sample in an analysis batch is always a blank, namely air. This is the blank test sample that should give no detectable bands relative to the *reference* single beam spectrum (see Procedure, step 3). Repeat this test after every 10 test samples.

NUMBERED NOTES

1. The present negative second derivative method was developed to overcome the high variability in precision measurements obtained for *trans* fats and oils levels below 5% (as percent of total fat) with infrared *Official Methods* AOCS Cd14d-99 (Reference 1) and AOAC 2000.10 (Reference 2).
2. To achieve a minimum signal-to-noise ratio (SNR) of 10:1, a high sensitivity linearized MCT detector operating at liquid nitrogen temperature would be recommended when a single-reflection ATR crystal is used. For room temperature DTGS detectors, a 3-reflection (or higher) ATR crystal would be recommended.
3. ATR sampling—It is essential to ensure that the test portion of the fat being analyzed *completely* covers the horizontal surface of the ATR crystal for the quantitative determination to succeed. In rare cases, after placing a fat test portion on the ATR crystal, the melted or liquid fat beads up and partially rolls off the surface of the ATR crystal. The only recourse the analyst would have is to try again.
4. Cleaning the ATR crystal—Thoroughly wiping the horizontal surface of the ATR crystal with low-lint paper has been the method of choice for cleaning it without the use of any solvent. However, to insure the complete removal of a test sample the analyst should also apply the subsequent test portion to be analyzed, and clean the crystal once again. Additionally, the analyst could make an infrared measurement after cleaning the crystal; the absence of a fat spectrum would confirm that the crystal is clean; this last step is particularly useful when a test sample having a relatively low level of *trans* fat is measured *after* another unknown test sample that is *high* in *trans* fat. However, for accurate background measurement, more precautions are required: Use a lint- and glue-free cotton swab with a very small amount of ethanol, then a dry cotton swab. A blank sample should be run to confirm the removal of all residues.
5. If a spectral feature is observed at slightly lower wavenumbers, near or below 960 cm^{-1} (Figure 4), it should *not* be attributed to a band for isolated *trans* double bonds (reference 5).

REFERENCES

1. AOCS Official Method Cd14d-99, Rapid determination of isolated *trans* geometric isomers in fats and oils by attenuated total reflection infrared spectroscopy.
2. AOAC Official Method 2000.10, Determination of total isolated *trans* unsaturated fatty acids in fats and oils. ATR-FTIR spectroscopy.
3. ISO 6353, Reagents for Chemical Analysis, Part 2 (1983) and 3 (1987); Specifications.
4. M. Milosevic, V. Milosevic, J.K.G. Kramer, H. Azizian, and M.M. Mossoba, *Lipid Technology*, 16:252–264 (2004).
5. M.M. Mossoba, J.K.G. Kramer, M. Milosevic, V. Milosevic, and H. Azizian, (2007), *J Am Oil Chem Soc,* 84:339-342.
6. M.M. Mossoba, V. Milosevic, M. Milosevic, J.K.G. Kramer, H. Azizian, (2007), *Analytical Bioanalytical Chemistry* (special issue on Food and Dietary supplements), 389, 87-92.
7. M.M. Mossoba, A. Seiler, J.K.G. Kramer, V. Milosevic, M. Milosevic, H. Azazian, H. Steinhart, (2009), *J Am Oil Chem Soc,* 86:1037-1045.
8. AOCS Official Method Ce1h-05, Determination of *cis-, trans-*, saturated, monounsaturated and polyunsaturated fatty acids in vegetable or non-ruminant animal oils and fats by capillary GLC.

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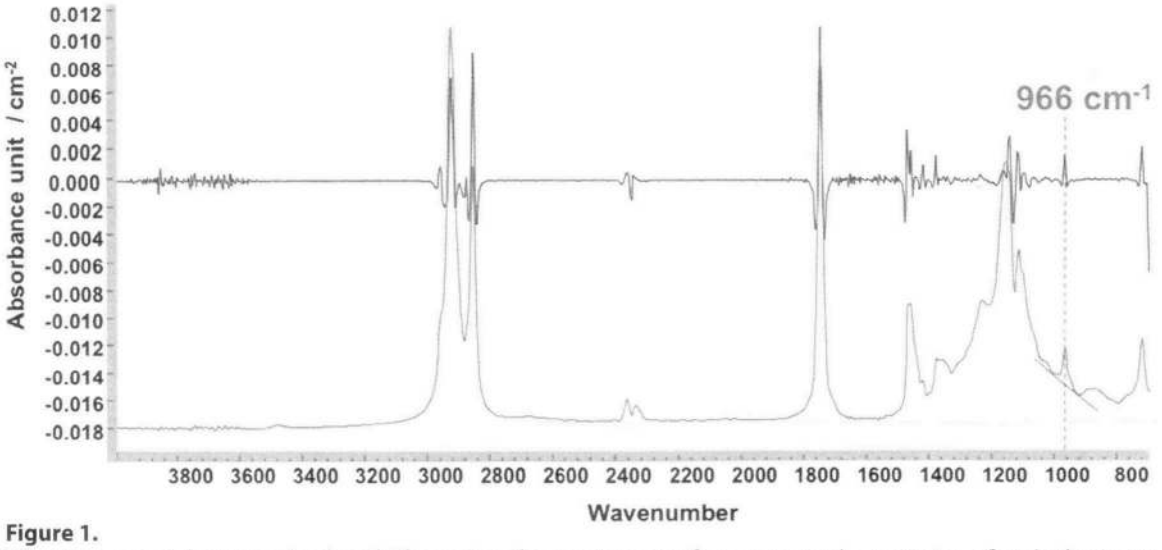

Figure 1.
Negative second derivative (top) and Absorption (bottom) spectra for a test sample consisting of trielaidin in tripalmitin with a *trans* level of 12.58% (as percent of total fat). A vertical line indicates the position of the unique band due to isolated *trans* double bonds at 966 cm^{-1}. The second derivative spectrum was multiplied by –1 only to have the bands point upwards for convenience.

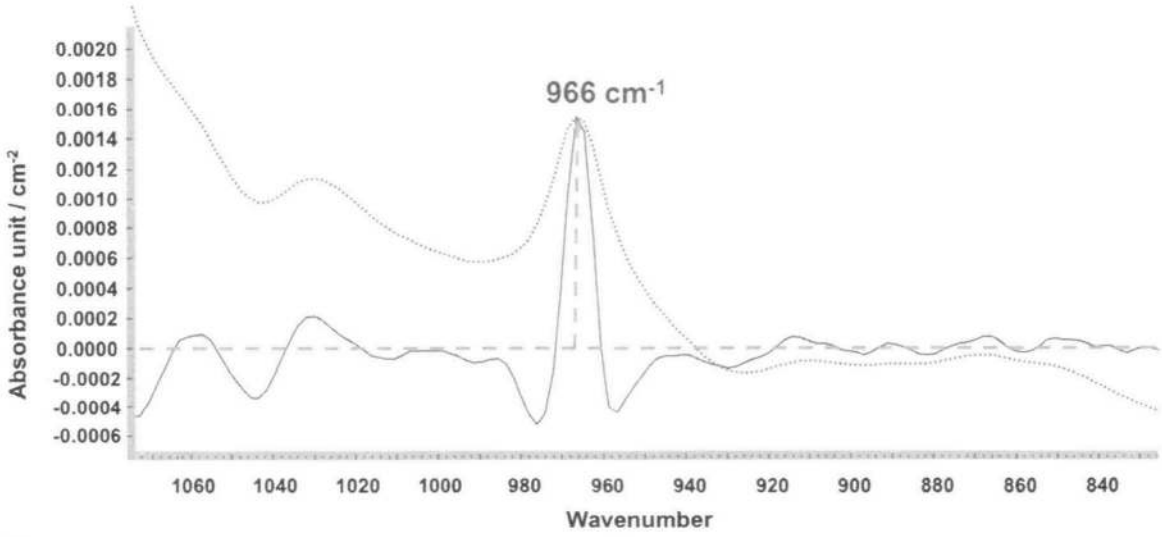

Figure 2.
The region of the spectra that exhibit the deformation band for isolated *trans* double bonds at 966 cm^{-1} is expanded for the Negative second derivative (solid line) and Absorption (dotted line) spectra for a test sample consisting of trielaidin in tripalmitin with a *trans* level of 12.58% (as percent of total fat). The height of the negative second derivative band, as indicated by the vertical arrow, can be accurately measured from a horizontal baseline. It is noted that several weak bands observed in the same spectral region are also more pronounced in the –2nd derivative spectrum (narrower bandwidths) than in the absorption spectrum.

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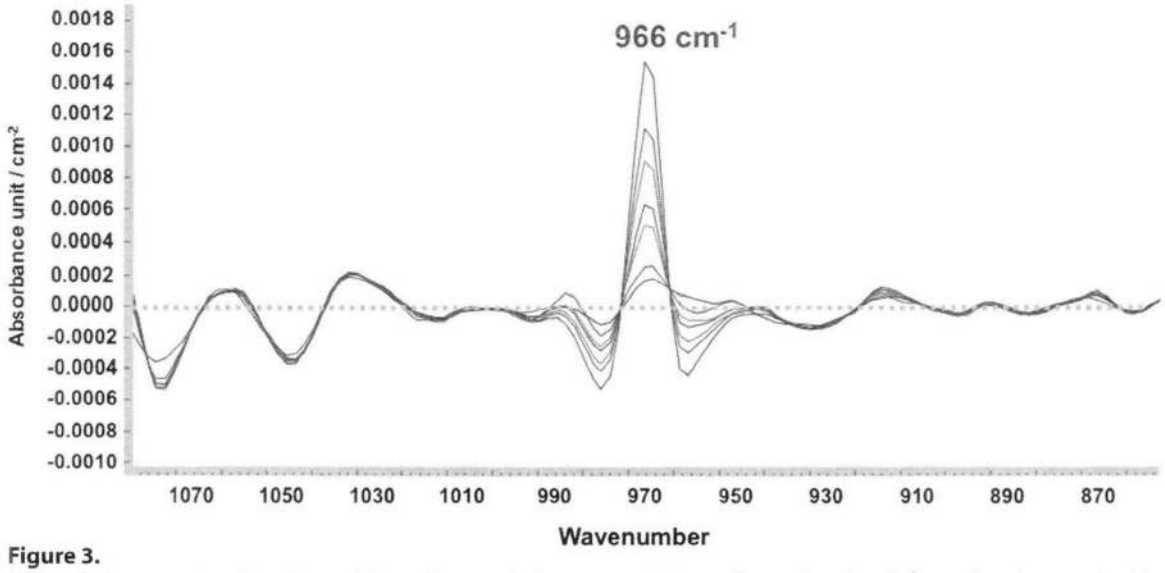

Figure 3.
Expanded spectral region that exhibits the –2nd derivative of the deformation band for isolated *trans* double bonds at 966 cm^{-1} for representative test samples covering approximately the 1–12% range and containing *trans* fat levels determined by IR to be for Lard 1.21%, and Canola oil mixtures 2.21%; 4.20%; 4.72%; 7.35%; 9.11%; and 12.62%, as percent of total fat. The height of the –2nd derivative band can be easily measured from the horizontal baseline (dotted line).

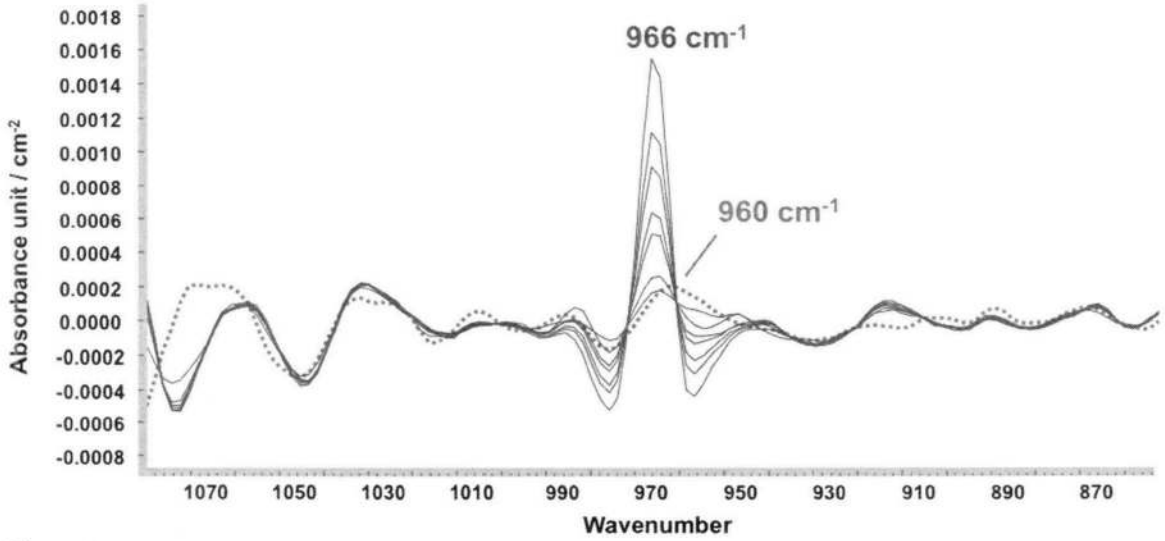

Figure 4.
Expanded spectral region that exhibits the –2nd derivative of the deformation band for isolated *trans* double bonds at 966 cm^{-1} for several test samples containing *trans* fat (solid lines), as well as for coconut oil (dotted line) that is high in saturated fat and contains only a trace (approximately 0.1%, reference 8) of *trans* fat. Coconut oil exhibited a spectral feature at slightly lower wavenumbers, near 960 cm^{-1}, which is easy to misidentify as a band for isolated *trans* double bonds (reference 5).

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Appendix **Collaborative IR study results: Total isolated trans fat, as percent of total fat (provided in duplicate)**

Lab No.		1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9	10	10
Test sample[a,b]																					
Canola mixture	1	2.18	2.15	2.78[c]	2.33	1.78	1.90	1.94	2.06	2.16	2.84[c]	2.14	2.21	2.16	2.18	2.33	2.34	2.31	2.30	2.09	2.09
Canola mixture	2	4.20	3.78	4.13	4.58	4.59	4.07	4.44	4.19	4.27	4.15	4.31	4.20	4.15	4.35	4.30	4.23	4.49	4.49	4.21	4.21
Canola mixture	3	5.09	4.95	5.48	5.48	5.06	5.37	5.06	5.06	4.98	4.82	4.84	4.72	4.96	5.25	5.22	5.17	5.40	5.39	5.19	5.19
Canola mixture	4	7.21	6.62	7.73	7.28	6.93	6.24	7.25	7.38	7.46	7.32	7.51	7.35	7.31	7.43	7.30	7.18	7.58	7.58	7.23	7.24
Canola mixture	5	8.94	9.21	9.08	9.08	9.74[c]	8.84	9.44	9.13	8.82	9.20	9.29	9.11	9.19	9.23	9.10	8.99	9.45	9.44	9.04	9.04
Canola mixture	6	12.62	12.13	10.87[c]	12.22	12.54	11.88	12.56	12.69	12.63	12.45	12.85	12.62	12.84	12.72	12.46	12.17	12.99	12.99	12.56	12.56
margarine oil	7	11.98	11.71	11.77	12.67	12.54	11.88	12.56	12.38	12.33	12.76	12.58	12.35	12.60	12.18	12.03	11.56	12.66	12.66	12.10	12.10
lard	8	1.40	1.21	1.88[d]	1.88[d]	1.31	1.47	1.31	1.19	1.43	1.03	1.28	1.21	1.44	1.19	1.58[d]	1.62[d]	1.34	1.33	1.25	1.25
lard	9	1.26	1.20	1.88[d]	1.88[d]	1.31	1.47	1.31	1.19	1.33	1.81	1.25	1.24	1.32	1.20	1.58	1.24	1.41	1.40	1.29	1.29
margarine oil	10	12.25	11.70	12.22	13.12	13.01	11.88	12.56	12.25	13.12	12.87	12.57	12.36	12.60	12.13	12.07	11.49	12.60	12.60	12.05	12.05

[a]Test samples 1-6 consist of mixtures of canola oil and partially hydrogenated canola oil
[b]Test samples 9 (lard) and 10 (margarine oil) are blind duplicates of 8 and 7, respectively
[c]Cochran's outlier
[d]Single Grubbs' outlier

AOCS

AOCS Official Method Cd 15-78

Reapproved 2009

cis, cis Polyunsaturated Fatty Acids, Essential Fatty Acids (EFA)

DEFINITION

This method determines the *cis, cis* 1, 4 methylene interrupted diene structure (–CH=CH–CH2–CH=CH–) of polyunsaturated fatty acids, using the enzyme lipoxidase.

SCOPE

Applicable to esters of fatty acids and to triglycerides having two or more double bonds. The enzyme is specific for the double bond structure listed under Definition, and will not react with conjugated double bonds, monoenoic acids, or *trans* double bonds.

APPARATUS

1. Spectrophotometer—with matched pair of silica cuvettes.
2. Analytical balance—accurate to ± 0.1 mg.
3. Volumetric flasks—100 mL, 10 mL, and 1 L with ground glass stoppers.
4. Pipets—0.5 mL, graduated in 0.05 mL; and 1, 2, 3, 5, 10, 20, and 100 mL pipets.
5. Test tubes—13 × 100 mm (disposable test tubes may be used).

REAGENTS

1. Potassium borate buffer, 1.0 M, pH 9.0—Prepare by dissolving 61.9 g boric acid (H_3BO_3) and 25.0 g potassium hydroxide (KOH) in 800 mL distilled water with stirring and gentle heating. Cool to room temperature and adjust pH to 9.0 by adding 1.0 M hydrochloric acid (HCl) or 1.0 M KOH as required. Dilute to exactly 1000 mL with distilled water and mix.
2. Potassium borate buffer, 0.2 M, pH 9.0—Prepare by diluting 200 mL 1.0 M potassium borate buffer (Reagents, 1) to 1000 mL with distilled water and mixing.
3. Lipoxidase solutions—Prepare a stock solution by dissolving 10 mg of lipoxidase from soybeans with an activity of 50,000 units/mg (ICN Nutritional Biochemicals, Cleveland, OH 44128, USA) in 10 mL of 0°C 0.2 M potassium borate buffer (Reagents, 2).
   (a) Dilute lipoxidase solution—Mix 2 mL of stock solution with 8 mL of 0°C 0.2 M buffer.
   Note—If a large number of analyses are to be performed, 5 mL of stock solution mixed with 20 mL 0°C 0.2 M buffer, giving 25 mL of dilute lipoxidase solution, is more desirable.
   (b) Boiled dilute lipoxidase solution—Transfer 4 mL of dilute lipoxidase solution to a 10 mL volumetric flask and hold in boiling water for 5 min.
4. Alcoholic KOH, 0.5 M—Prepare by dissolving 1.40 g reagent grade KOH in 95% ethanol and diluting it to 50 mL with 95% ethanol. Prepare fresh each day.

PROCEDURE

1. Preparation of vegetable oil test sample—Accurately weigh 100 ± 0.1 mg of test sample and transfer with n-hexane or acetone into a 100 mL volumetric flask which has just been flushed with nitrogen. Dilute to volume with the solvent. Transfer 1.00 mL of this diluted solution to a 100 mL volumetric flask and completely evaporate the solvent under a stream of nitrogen.
2. Saponification of test samples—Add 1 mL of 0.5 M alcoholic KOH solution to the solvent-free test sample in the 100 mL volumetric flask from Procedure, 1. Heat gently on steam plate to dissolve the saturated fatty acids. Allow saponification to proceed in an atmosphere of nitrogen, in the dark, for a minimum of 5 hr or overnight. After saponification is complete, add 20 mL of 1.0 M potassium borate buffer solution and 50 mL of distilled water and mix. Add 1 mL of 0.5 M HCl and dilute to volume with distilled water and mix completely.
3. Determination of the PUFA content—Pipet 3.0 mL of the saponified fatty acid solution into each of four test tubes (13 × 100 mm). To the first two tubes (blanks) add 0.10 mL of the boiled (inactive) dilute enzyme solution and shake vigorously to mix, or use a vortex mixer. To the third and fourth test tubes (duplicate aliquots) add 0.10 mL of the (active) dilute enzyme solution. Mix all the test tubes vigorously for about 30 sec immediately after adding enzyme. Let stand exposed to air at room temperature for 30 min. Make certain to treat all tubes identically, including the reaction time. Transfer the contents of the tubes to matched 1 cm quartz cuvettes and place in the spectrophotometer (Beckmann DU or equivalent). Adjust the instrument with the blank samples and measure the absorbance of the reacted test samples at 234 nm. Space test samples to permit cleaning and checking absorbance of cuvettes after each test sample.

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CALCULATIONS

1. Standard curve—
   Weigh a 100 mg quantity of trilinolein (99% pure, Nu-Chek-Prep, P. O. Box 172, Elysian, MN, USA) and quantitatively transfer to a 100 mL volumetric flask and dilute to volume with n-hexane or acetone. Quantitatively transfer a 10 mL aliquot of the solution to a 100 mL volumetric flask and dilute to volume with n-hexane or acetone. Quantitatively transfer aliquots of 2, 4, 6, 8, 10, and 12 mL to separate 100 mL volumetric flasks. Reduce the aliquot in each volumetric flask to dryness with a stream of nitrogen. Pipet 1 mL of 0.5 M alcoholic KOH into each triglyceride-containing volumetric flask and store in the dark for 4 hr or overnight. To each saponified test sample add 20 mL of 1.0 M potassium borate solution, 50 mL of distilled water, and 1 mL of 0.5 M HCl. Dilute to final volume with distilled water and mix. Pipet 3.00 mL of the prepared fatty acid solution from each of the standard samples into four separate test tubes. To the first and second tubes add 0.1 mL of the boiled (inactive) enzyme; to the third and fourth tubes add 0.1 mL of the unboiled (active) enzyme; shake vigorously for about 30 sec and allow to stand at room temperature for 30 min. Repeat this procedure for each of the six standard sample levels. Transfer contents of each tube to matched 1 cm quartz cuvettes. Read and record the absorbance at each level.
   Draw a standard curve (Beer-Lambert plot) using absorbance vs. µg/mL for each level of standard.
2. Formula for calculating the grams of polyunsaturated fatty acid as triglyceride/100 g of product—Trilinolein, g/100 g product = $W \times DF \times 10^{-4}$
   Where—
   W = µg *cis, cis* PUFA/mL test portion (obtained from the calibration plot)
   DF = dilution factor

PRECISION

1. Two determinations made by this procedure in one laboratory should not differ by more than 1.6%.
2. Two determinations made by this procedure between two laboratories should not differ by more than 2.2%.

REFERENCES

1. *J. Assoc. Off. Anal. Chem. 60:*895 (1977).
2. *Ibid. 61:*1419 (1978).
3. *Ibid. 66:*81 (1983).

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AOCS

AOCS Official Method Cd 16-81

Revised 2013

Solid Fat Content (SFC) by Low-Resolution Nuclear Magnetic Resonance—The Indirect Method

DEFINITION

This method describes procedures for the determination of the solid fat content (SFC) by low resolution nuclear magnetic resonance (NMR), using the indirect method. An alternative NMR method to determine the SFC, the direct method, is described in the AOCS Official Method Cd 16b-93.

The SFC-NMR method determines what percentage of all hydrogen nuclei (protons) in the test sample, composed of hydrogen nuclei in both liquid and solid phases, is due to hydrogen nuclei in the solid phase. This percentage is called the solid fat content.

TERMINOLOGY

Indirect method—In the indirect method the NMR signal from the liquid phase of the test sample is compared with the signal from the same test sample when fully melted. In this method the SFC is defined as one minus the ratio between the NMR response obtained from the hydrogen nuclei in the liquid phase of the test sample and the response from the hydrogen nuclei in the fully melted test sample, corrected for NMR temperature sensitivity differences. The result is generally converted to a percentage.

The indirect method is based on measurements of the liquid NMR signal only of the fat test sample. To eliminate temperature effects on the NMR signal, a liquid reference oil is also measured at all measuring temperatures.

Solid fat content as determined by NMR does not determine the percentage of solids by weight as in the case of dilatation, since in general the hydrogen nuclei weight ratios for the solid and the liquid phase in the mixture are not known. No correction is made for variations of the density of hydrogen nuclei in the test sample between solid and liquid phases. Therefore, the SFC as determined by NMR is not equal to the solid fat index (SFI) as determined by dilatometry.

APPARATUS

1. NMR instrument—indirect SFC-NMR measurements can be performed by any low resolution NMR instrument that is capable of measuring the signal from the liquid part of the test sample.

   The following requirements must also be observed: magnet with a field that is sufficiently homogeneous to ensure that the measurements are reproducible and fulfill the requirements for precision.

   The half-life time of the magnetization of a reference sample of oil must be longer than 1000 ms.

   The magnet and sample probe temperature must be stable and consistent.

   A microswitch must be fitted to start the measurement automatically on insertion of the sample tube.

   The probe head in the magnet gap should be suitable for sample tubes of 10 mm o.d.

   A calculating device should be provided which gives a direct readout of percentage of solids.

   For more details, see Notes 1,2.

2. Sample tubes:

Outer diameter:	10.0 ± 0.13 mm
Wall thickness:	0.6 mm
Length:	at least 150 mm
Material:	glass

3. Oven—should be maintained at 100 ± 2°C.
4. Constant temperature water baths or heating blocks—capable of keeping the temperature of the liquid at any set temperature between 10 and 60°C ± 0.1°C.

   Constant temperature baths, one for each temperature, equipped with thermostatic controls, an efficient stirring device, and a calibrated thermometer, accurate to 0.05°C.

   Alternatively, heating blocks may be used, one for each temperature.
5. Metal blocks with holes for the sample tubes—Dimensions of the holes:

Internal diameter	10.35 ± 0.05 mm
Depth	70 mm
Distance between the holes	17 mm (center to center)
Thickness of metal under the holes	10 mm
Immersion depth in water bath	60 mm

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6. 0°C bath—a cooling unit maintained at 0°C, filled with a suitable cooling liquid (e.g., 20% aqueous ethylene glycol) is essential. The 0°C bath can also be an electronically controlled aluminum block capable of maintaining 0 ± 0.1°C. An ice bath at 0°C must not be used.
7. Calibration samples—a sample of triolein which remains fully liquid at all measurement temperatures is needed to compensate for the NMR sensitivity at different measurement temperatures. In case of a non-linear detector, mixtures of tristearin in triolein are used as reference samples for the calibration of the instrument. These mixtures are melted and liquid readings taken at 70°C before being tempered and measured by this method. The SFC values measured agree well with the percentage of solids by weight. A single fifth order polynomial regression equation is recommended for solids contents of 95% or less. Polynomial coefficients, correlation coefficients, and interpolation tables for conversion of calculated solids to calibration-corrected solids can be obtained from the manufacturer for each instrument. The calibration procedure is also provided by the manufacturer.

ANALYSIS METHODS

The indirect NMR method can be performed in parallel and serial mode; of the two, the parallel mode is recommended (see Notes, 8). The indirect method using parallel measurements applied to non-stabilizing and stabilizing fats is described in detail below.

METHOD I—NON-STABILIZING FATS AND OILS

SCOPE

This method is applicable to margarine or conventional oils and fats (also termed non-stabilizing oils), to frying and baking oils, and to non-stabilizing specialty fats like cocoa butter substitutes and replacers. These oils and fats do not need the additional polymorphic stabilization described in Method II. The method is applicable only to oils and fats for which crystallization is complete within a crystallization period of 1 h at 0°C. Slower crystallizing oils and fats will require a longer crystallization period (see Notes, 6).

INDIRECT METHOD—PARALLEL MEASUREMENTS

1. Checking the instrument calibration. This check is to be run at the beginning of each working period to detect possible instrument failures, according to the methods described in the manual of the instrument.
2. Stabilizing and tempering procedure:

   The stabilization and tempering procedure for all fats that do not need additional polymorphic stabilization is as follows:

   Melt the test sample at 100°C and store for 15 min at 100°C and then at least 5 min at 60°C, followed by 60 ± 2 min at 0°C, and finally 30–35 min at each chosen measuring temperature.

   Suitable measuring temperatures are 10, 15, 20, 25, 30, 35, 40, and every 5°C up to 60°C as required. The requirement to measure the SFC after stabilization for 30–35 min determines the number of sample tubes that can be handled in one run, and this is about 20 tubes. If more tubes have to be measured, a second run of no more than 20 tubes can be started from the 60°C bath 10 min after the first.
3. Measuring and handling procedures:

   In order to calculate the SFC of a test sample from the NMR signal using the indirect method it is necessary to measure the test sample in its fully melted state at 60°C as well as to measure the temperature compensation sample at the appropriate temperature. Before tempering begins, the two test samples stored at 60°C should be measured. By following this procedure all the necessary information can be available to calculate SFC for each test sample as it is measured.

   (a) Heat the fat to at least 100°C and store for 15 min at this temperature. Filter through paper filter if the test sample is not clear, and reheat to 100°C.
   (b) Homogenize the test sample thoroughly and fill a sample tube to a depth of 4 ± 1 cm. Do not weigh and deaerate. The outside of the sample tubes must be clean and dry (see Notes, 9).
   (c) Fill one sample tube for each measuring temperature, and reheat to 100°C.
   (d) Place the sample tubes containing the completely melted fat after reheating to 100°C and the reference oil in a rack in the 60°C water bath and equilibrate for 30 min.
   (e) Remove the test sample from the 60°C water bath and transfer it as quickly as possible into the sample holder of the NMR instrument, ensuring that the tube is in the homogeneous region of the magnet.
   (f) Use a pulse repetition time [recycle delay (RD) or trigger time] of 2 s and 4 pulses (used for signal averaging).
   (g) Read the liquid signal at 70 μs according to the instructions in the manual.
   (h) Record the liquid signal at 70 μs; this value is used for further calculations (see Calculations).
   (i) Remove the tube from the instrument.
   (j) Repeat steps (e)–(i) until all the test samples have been measured.
   (k) Repeat steps (e)–(i) for the reference oil.
   (l) Reheat the test samples to 100°C.

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(m) Place the sample tubes containing the completely melted fat in a rack in the 60°C water bath for at least 5 min but not more than 15 min.
(n) After tempering at 60°C, place the tubes in a rack in the 0°C bath for 60 ± 2 min.
(o) Remove the sample tubes from the 0°C bath, ensure that they are dry, and place them in the blocks (the holes must be clean and dry) of the water baths at the desired measuring temperatures. Use one tube for each temperature.
(p) Remove the tubes from the block after 30–35 min and transfer each one as quickly as possible into the sample holder of the NMR instrument, ensuring that the test sample is in the homogeneous region of the magnet.
(q) Use a pulse repetition time [recycle delay (RD) or trigger time] of 2 sec and 4 pulses for signal averaging.
(r) Read the liquid signal at 70 μs according to the instructions in the manual.
(s) Record the liquid signal at 70 μs; this value is used for further calculations (see Calculations).
(t) Remove the tube from the instrument,
(u) Repeat steps (p)–(t) until all the test samples have been measured.
(v) Measure the reference oil sample according to steps (p)–(t) at each desired temperature after equilibration at this temperature for 30–35 min.

METHOD II—STABILIZING CONFECTIONERY FATS

SCOPE

This method is suitable for stabilizing confectionery fats such as cocoa butter and specialty fats, which contain appreciable quantities of 2-oleo-disaturated glycerides.

With these fats the polymorphic form must be stabilized before reproducible SFC results can be obtained, and for this reason a period of 40 h of tempering at 26°C is used. The stabilization procedure is adopted from AOCS Method Cd 10-57 (SFI by dilatometry). See also Notes, 7.

INDIRECT METHOD—PARALLEL MEASUREMENTS

1. Checking the instrument calibration—same as in Method I.
2. Stabilization and tempering procedures:

   The stabilization and tempering procedure for cocoa butter and all cocoa butter like fats which need additional polymorphic stabilization is as follows:

   Melt and store for 15 min at 100°C, then at least 5 min at 60°C, followed by 90 ± 5 min at 0°C, 40 ± 0.5 h at 26°C, 90 ± 5 min at 0°C and finally 60–65 min at each chosen measuring temperature.

   Suitable measuring temperatures are 20, 25, 30, 35, 40, and every 5°C up to 60°C as required. The requirement to measure the SFC after stabilization for 60–65 min determines the number of sample tubes which can be handled in one run, and this is about 20 tubes. If more tubes have to be measured, a second run of no more than 20 tubes can be started from the 26°C bath 10 min after the first.

   Alternatively, tubes may be started from the 26°C bath at 15 sec intervals and progressively transferred through the 0°C bath to blocks in water baths at the measuring temperatures at 15 sec intervals. This refinement of the procedure is useful when it can be introduced conveniently, and is not subject to the 20 tubes per batch limitation. In both cases, however, the total number of tubes that can be handled is limited by the capacity of the racks in the 0°C and 26°C baths.
3. Measuring and handling procedures:

   In order to calculate the SFC of a test sample from the NMR signal using the indirect method it is necessary to measure the test sample in its fully melted state at 60°C as well as to measure the temperature compensation sample at the appropriate temperature. Before tempering begins, the two test samples stored at 60°C should be measured. By following this procedure all the necessary information can be available to calculate SFC for each test sample as it is measured.

   (a) Heat the fat to at least 100°C and store for 15 min at this temperature. Filter through paper filter if the test sample is not clear, and reheat to 100°C.
   (b) Homogenize the test sample thoroughly and fill a sample tube to a depth of 4 ± 1 cm. Do not weigh and deaerate. The outside of the sample tubes must be clean and dry (see Notes, 9).
   (c) Fill one sample tube for each measuring temperature, and reheat to 100°C.
   (d) Place the sample tubes containing the completely melted fat after reheating to 100°C and the reference oil in a rack in the 60°C water bath and equilibrate for 30 min.
   (e) Remove the test sample from the 60°C water bath and transfer it as quickly as possible into the sample holder of the NMR instrument ensuring that the tube is in the homogeneous region of the magnet.
   (f) Use a pulse repetition time [recycle delay (RD) or trigger time] of 2 sec and 4 pulses (used for signal averaging).
   (g) Read the liquid signal at 70 μs according to the instructions in the manual.
   (h) Record the liquid signal at 70 μs; this value is used for further calculations (see Calculations).
   (i) Remove the tube from the instrument.
   (j) Repeat steps (e)–(i) until all the test samples have been measured.
   (k) Repeat steps (e)–(i) for the reference oil.

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(l) Reheat the test samples to 100°C.
(m) Place the sample tubes containing the completely melted fat in a rack in the 60°C water bath for at least 5 min but not more than 15 min.
(n) After tempering at 60°C, place the tubes in a rack in the 0°C bath for 90 ± 5 min.
(o) Transfer the tubes (optionally together with the rack) to the 26°C (± 0.2°C) bath for 40 ± 0.5 h (see Notes, 2).
(p) Transfer the tubes (optionally together with the rack) back to the 0°C bath and keep them there for 90 ± 5 min.
(q) Remove the sample tubes from the 0°C bath, dry them quickly to avoid warming, and place them in the blocks (the holes must be clean and dry) of the water baths at the desired measuring temperatures. Use one tube for each temperature.
(r) Remove the tube from the block after 60–65 min and transfer each one as quickly as possible into the sample holder of the NMR instrument, ensuring that the tube is in the homogeneous region of the magnet.
(s) Use a pulse repetition time [recycle delay (RD) or trigger time] of 2 sec and 4 pulses for signal averaging.
(t) Read the liquid signal at 70 μs according to the instructions in the manual.
(u) Record the liquid signal at 70 μs; this value is used for further calculations (see Calculations).
(v) Remove the tube from the instrument.
(w) Repeat steps (q)–(v) until all the test portions have been measured.
(x) Measure the reference oil according to steps (q)–(v) at each desired temperature after equilibration at this temperature for 30–35 min.

2. Presentation of data—the solids content of a fat sample (SFC) should be reported in % with one decimal place. The temperature at which this solids fraction is measured should also be given, e.g.:
   Solid fat content of 75.3% of test portion at 10°C is designated N10 = 75.3%
   Solid fat content of 44.1% of test portion at 20°C is designated N20 = 44.1%
   Solid fat content of 20.7% of test portion at 30°C is designated N30 = 20.7%
   Together with the data the exact mode of operation and tempering procedure should always be reported.

CALCULATIONS

1. Indirect method—solid fat content (SFC) at T°C:

$$NT = 100 - \left(\frac{R60 \times ST \times 100}{S60 \times RT} \right)$$

Where—
R60 = Signal intensity of the reference oil at 60°C
S60 = Signal intensity of the test portion at 60°C
RT = Signal intensity of the reference oil at T°C
ST = Signal intensity of the test portion at T°C

The ratio R60/RT expresses the correction factor for the temperature dependence of the NMR signal at T°C.

PRECISION

For the precision of the indirect method the results of the international collaborative study carried out in 1992/1993 by AOCS and Unilever (in which 24 companies participated) were used. (This study also included the direct NMR and the dilatometry methods; general conclusions and a summary of statistical results are given in the AOCS Method Cd 16b-93.)

The test was set up using a double split level design using four different test sample types: a margarine, palm oil, bean oil, and kaomel. Every type consisted of two slightly different batches which were offered in (blind) duplicates (16 test samples in total). Every test sample was analyzed at six different temperatures.

According to the statistical procedure, tests to detect outliers and suspected values were carried out on the data set. Outliers were defined as data which lie outside the expected data distribution with a confidence level between 95 and 99%.

After deletion of possible outliers the repeatability, r, and the between laboratory reproducibility, R, were calculated.

The repeatability, r, represents the reliability of the method when duplicate analyses are carried out within one company/laboratory and using the same (batch of) reagents, the same instrument and within a short period of time. The between-laboratory reproducibility, R, represents the reliability of the method when an analysis is carried out by two different companies or laboratories.

In daily practice the difference between two measurements on the same test sample should not exceed the value of r or R when performed by one laboratory or by two laboratories, respectively, in a 95% confidence interval. In principle pooling of the standard deviations in order to calculate one value for r and one for R is not allowed. Nevertheless this has been done from a practical point of view.

From the collaborative study the following values were found:

repeatability	2.0%
between-laboratory reproducibility:	4.0%

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NOTES

Caution

Persons with a pacemaker should not come within a distance of 30 cm of the NMR equipment. Heavy metal objects should not be in the vicinity of the NMR equipment.

NUMBERED NOTES

1. Instrument siting. The room temperature where the low resolution NMR equipment is placed should be stable—never below 15°C or above 30°C. The instrument should be placed on a robust table to prevent influence of vibrations. Large moving metal objects should be kept away from the instrument because they may interfere with the magnetic field. The magnet should be thermostated (usually 40°C) to reduce drift.
2. Instrument temperature control. Because NMR instruments have heated magnets, test portions warm up as soon as they are placed inside an instrument. This can affect the accuracy of the SFC measurements if the sample heating rate is variable. Therefore, the magnet and sample probe temperature must be stabilized by the thermostat. Variable temperature-controlled probe heads are not practical and are not applicable to the present method.
3. Drying of tubes and blocks. After being tempered in racks, the tubes should be dried quickly and carefully before being placed in the metal block in the water bath at the measuring temperature. Tubes should not be wiped with tissues because this may interfere with the results. The holes in the metal blocks in the 10 or 15°C bath must be dried frequently because moisture may condense in the holes.
4. Tube enclosures. A method of closing off the tube (e.g., a cap or a rubber stopper) can limit contamination by dust, solvent vapors or moisture during the tempering steps especially during the long stabilization period of 40 h at 26°C for stabilizing fats.
5. Test sample temperature control. SFC values depend on the thermal history of the crystallized fat. Therefore it is important to adhere to the prescribed times given in the method. Temperatures of tempering and measurements are also extremely important, and thermostat settings together with both dimensions of the tubes and the holes in the metal blocks should be strictly observed, the latter in order to ensure the appropriate thermal contact. The effect of temperature and time are especially important at temperatures where the confectionery fats have a steep melting profile (e.g., at 30°C for cocoa butter).
6. Slow crystallizing oils and fats. Slow crystallizing oils and fats will not have completely crystallized during the 1 h at 0°C period, leading to a lower SFC value than would be obtained after complete crystallization. The applicability of the method may be verified by determining the SFC after a range of range of crystallization periods at 0°C. Slower crystallizing oils and fats will require a longer crystallization period.
7. Stabilizing fats. A more accurate description would be "fats which need polymorphic stabilization," as cocoa butter and specialty fats are found in fully stable, partially stable, and unstable forms. However, the term "stabilizing fats" has been used for many years in connection with dilatometry and has now become the accepted description.
8. This method describes the parallel tempering method (separate test samples for every temperature). Two distinct advantages of the parallel method are as follows:
   Measurements are independent of the number of temperatures used and the speed of the entire procedure. However, the serial method (same test sample at each temperature) is suited to situations when only small amounts of test sample are available, and less preparation time and greater throughput are required. The serial method may be used if the SFC results using the particular type of test sample have been shown to be not significantly different from the SFC using the standard parallel method. The time between the temperature readings should be 30–35 min.
9. In some instances a measuring depth of 1 cm has been used. The AOCS NMR committee is considering the results of collaborative studies addressing this issue.

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AOCS Official Method Cd 16b-93

Reapproved 2009

Solid Fat Content (SFC) by Low-Resolution Nuclear Magnetic Resonance—The Direct Method

DEFINITION

This method describes procedures for the determination of the solid fat content (SFC) by low resolution nuclear magnetic resonance (NMR), using the direct method. An alternative NMR method to determine the SFC, the indirect method, is described in the AOCS Official Method Cd 16-81.

The SFC-NMR method determines what percentage of all hydrogen nuclei (protons) in the test sample, composed of hydrogen nuclei in both liquid and solid phases, is due to hydrogen nuclei in the solid phase. This percentage is called the solid fat content.

TERMINOLOGY

Direct method—In the direct method, NMR signals from both the solid and liquid phase are measured and compared. In this method the SFC is defined as the ratio, expressed as a percentage, between the NMR response obtained from the hydrogen nuclei in the solid phase of the test sample and the NMR response obtained from the hydrogen nuclei in both the solid and the liquid phases of the test sample.

Solid fat content as determined by NMR does not determine the percentage of solids by weight as in the case of dilatation, since in general the hydrogen nuclei weight ratios for the solid and the liquid phase in the mixture are not known. No correction is made for variations of the density of hydrogen nuclei in the test sample between solid and liquid phases. Therefore, the SFC as determined by NMR is not equal to the solid fat index (SFI) as determined by dilatometry.

APPARATUS

1. NMR instrument—
   (a) Direct SFC-NMR measurements can be performed by any low resolution pulsed NMR instrument which is capable of measuring the signal from the solid part of the test sample. In practice, this means any pulsed instrument of 20 MHz or higher operating frequency that fulfills specifications regarding accuracy and reproducibility stated in Precision section. The instrument must have a dead time plus pulse width of 10 μs or less. The instrument should allow a factor (known as the "F" factor) to be set, with a value between l and 2, to correct measurements for the instrument's dead time. The instrument should also have a variable repetition delay between pulses (also known as trigger time).
   (b) The following requirements must also be observed:

   A magnet with a field that is sufficiently homogeneous to ensure that the measurements are reproducible and fulfill the requirements for precision

   The half-life time of the magnetization of a reference sample of oil must be longer than 1000 ms. The magnet and sample probe temperature must be stable and consistent.

   A microswitch must be fitted to start the measurement automatically on insertion of the sample tube.

   The probe head in the magnet gap should be suitable for sample tubes of 10 mm o.d.

   A calculating device should be provided which gives a direct readout of percentage of solids.

   For more details see General Notes 1,2, and 10.
2. Sample tubes:

Outer diameter:	10.0 ± 0.13 mm
Wall thickness:	0.6 mm
Length:	at least 150 mm
Material:	glass

3. Sample filling height—Sample height is not critical for direct measurements. A filling height of 4 ± 1 cm is recommended, but the analyst should be sure that the level of the test sample is at least 5 mm below the outside surfaces of the aluminum tempering blocks.
4. Oven—should be maintained at 100 ± 2°C.
5. Constant temperature water baths or heating blocks—capable of keeping the temperature of the liquid at any set temperature between 10 and 60°C ± 0.1°C.

   Constant temperature baths, one for each temperature, fitted with thermostatic controls and equipped with an efficient stirring device and a calibrated thermometer, accurate to 0.05°C.

   Alternatively, heating blocks may be used, one for each temperature.

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6. Metal blocks with holes for the sample tubes—dimensions of the holes:

Internal diameter	10.35 ± 0.05 mm
Depth	70 mm
Distance between the holes	17 mm (center to center)
Thickness of metal under the holes	10 mm
Immersion depth in water bath	60 mm

7. 0°C bath—a cooling unit maintained at 0°C, filled with a suitable cooling liquid (e.g., 20% aqueous ethylene glycol) is essential. The 0°C bath can also be an electronically controlled aluminum block capable of maintaining 0 ± 0.1°C. An ice bath at 0°C must not be used.
8. Calibration samples—calibration in the direct SFC NMR method is done with plastic-in-oil standards whose calibration will be referenced to Triolein/Tristearin mixtures. Plastic-in-oil standards will be made available from a source approved by the AOCS. If calibration standards of other sources are used, this must be mentioned in (reported with) the SFC results.

   Records should be kept of the F-factors derived from plastic-in-oil standards and the standards should be replaced if significant variations are noted.

ANALYSIS METHODS

The direct NMR method can be performed in parallel and serial mode, of which the parallel mode is recommended (General Notes, 9). The direct method using parallel measurements applied to non-stabilizing and stabilizing fats is described in full detail below.

METHOD I-NON-STABILIZING FATS AND OILS

SCOPE

This method is applicable to margarine or conventional oils and fats (also termed non-stabilizing oils), to frying and baking oils, and to non-stabilizing specialty fats like cocoa butter substitutes and replacers. These oils and fats do not need additional polymorphic stabilization as described in Method II. The method is applicable only to oils and fats for which crystallization is complete within a crystallization period of 1 h at 0°C. Slower crystallizing oils and fats will require a longer crystallization period (General Notes, 6).

DIRECT METHOD—PARALLEL MEASUREMENTS

1 Checking the instrument calibration—This check is to be run at the beginning of each working period to detect possible instrument failures, according to the methods described in the manual of the instrument.
2. Stabilizing and tempering procedures:
   (a) The stabilization and tempering procedure for all fats that do not need additional polymorphic stabilization is as follows:

   Melt the test sample at 100°C and store for 15 min at 100°C, at least 5 min at 60°C, then 60 ± 2 min at 0°C, and then 30–35 min at each chosen measuring temperature.

   Suitable measuring temperatures are 10, 15, 20, 25, 30, 35, 40, and every 5°C up to 60°C as required. The requirement to measure the SFC after stabilization for 30–35 min determines the number of sample tubes that can be handled in one run, and this is about 20 tubes. If more tubes have to be measured, a second run of no more than 20 tubes can be started from the 60°C bath 10 min after the first.
3. Measuring and handling procedure:
   (a) Heat the fat to at least 100°C and store for 15 min at this temperature. Filter through paper filter if the test sample is not clear, and reheat to 100°C.
   (b) Homogenize the test sample thoroughly and fill a sample tube to a depth of 4 ± 1 cm. Do not weigh and deaerate. The outside of the sample tubes must be clean and dry.
   (c) Fill one sample tube for each measuring temperature.
   (d) Place the sample tubes containing the completely melted fat after reheating to 100°C in a rack in the 60°C water bath for at least 5 min but not more than 15 min.
   (e) After tempering at 60°C place the tubes in a rack in the 0°C bath for 60 ± 2 min.
   (f) Remove the sample tubes from the 0°C bath, ensure that they are dry, and place them in the blocks (the holes must be clean and dry) of the water baths at the desired measuring temperatures. Use one tube for each temperature.
   (g) Remove the tubes from the block after 30–35 min and transfer each one as quickly as possible into the sample holder of the NMR instrument, ensuring that the tube touches the bottom of the holder.
   (h) Use a pulse repetition time [recycle delay (RD) or trigger time] of 2 sec, 3 pulses (for signal averaging) and the F-factor found during the calibration routine.
   (i) Read the solids content according to the instructions in the manual.
   (j) Record the SFC value observed at T°C.
   (k) Remove the tube from the instrument.
   (1) Repeat the whole procedure until all the test samples have been measured.

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METHOD II—STABILIZING CONFECTIONERY FATS

SCOPE

This method is suitable for stabilizing confectionery fats such as cocoa butter and specialty fats, which contain appreciable quantities of 2-oleo-disaturated glycerides. With these fats the polymorphic form must be stabilized before reproducible SFC results can be obtained, and for this reason a period of 40 h of tempering at 26°C is used. The stabilization procedure is adopted from AOCS Method Cd 10-57 (SFI by dilatometry). See also General Notes, 7.

However, a further distinction between stabilizing and unstabilizing fats occurs when the solids content is measured by pulsed NMR. This is because the protons in the stable beta crystal form recover relatively slowly from the radio frequency pulse. Therefore, different pulse repetition time of 6 seconds combined with a single pulse must be used to compensate for this distinction (see General Notes, 8).

DIRECT METHOD-PARALLEL MEASUREMENTS

1. Checking the instrument calibration—same as in Method I.
2. Stabilization and tempering procedures:
   (a) The stabilization and tempering procedure for cocoa butter and all cocoa butter-like fats which need additional polymorphic stabilization is as follows:

   Melt and store for 15 min at 100°C, at least 5 min at 60°C, then 90 ± 5 min at 0°C, followed by 40 ± 0.5 h at 26°C, 90 ± 5 min at 0°C, and finally 60–65 min at each chosen measuring temperature.

   Suitable measuring temperatures are 20, 25, 30, 35, 40, and every 5°C up to 60°C as required. The requirement to measure the SFC after stabilization for 60–65 min determines the number of sample tubes which can be handled in one run, and this is about 20 tubes. If more tubes have to be measured, a second run of no more than 20 tubes can be started from the 26°C bath 10 min after the first.

   Alternatively, tubes may be started from the 26°C bath at 15 sec intervals and progressively transferred through the 0°C bath to blocks in water baths at the measuring temperatures at 15 sec intervals. This refinement of the procedure is useful when it can be introduced conveniently, and is not subject to the 20 tubes per batch limitation. In both cases, however, the total number of tubes that can be handled is limited by the capacity of the racks in the 0°C and 26°C baths.
3. Measuring and handling procedures:
   (a) Heat the fat to at least 100°C in a water bath or oven and store for 15 min at this temperature. If the test sample is not clear, filter through paper filter and reheat to 100°C.
   (b) Homogenize the test sample thoroughly and fill a sample tube to a depth of 4 ± 1 cm. Do not weigh and deaerate. The outside of the sample tubes must be clean and dry.
   (c) Fill one sample tube for each measuring temperature, and reheat to 100°C.
   (d) Place the sample tubes containing the completely melted fat after reheating to 100°C in a rack in the 60°C water bath for at least 5 min but not more than 15 min.
   (e) After tempering at 60°C, place the tubes in a rack in the 0°C bath for 90 ± 5 min.
   (f) Transfer the tubes (optionally together with the rack) to the 26°C ± 0.2°C bath for 40 ± 0.5 h (see General Notes, 2).
   (g) Transfer the tubes (optionally together with the rack) back to the 0°C bath and keep them there for 90 ± 5 min.
   (h) Remove the sample tubes from the 0°C bath, dry them quickly to avoid warming, and place them in the blocks (the holes must be clean and dry) of the water baths at the desired measuring temperatures. Use one tube for each temperature.
   (i) Remove the tube from the block after 60–65 min and transfer each one as quickly as possible into the sample holder of the NMR instrument, ensuring that the tube touches the bottom of the holder.
   (j) Use a pulse repetition time [recycle delay (RD) or trigger time] of 6 sec, 1 pulse and the F factor found during the calibration routine.
   (k) Read the solids content according to the instructions in the manual.
   (l) Record the SFC value observed at T°C.
   (m) Remove the tube from the instrument.
   (n) Repeat the whole procedure until all the test portions have been measured
4. Presentation of data—the solids content of a fat sample (SFC) should be reported in % with one decimal place. The temperature at which this solids fraction is measured should also be given, e.g.:
   Solid fat content of 75.3% of test portions at 10°C is designated N10 = 75.3%
   Solid fat content of 44.1% of test portion at 20°C is designated N20 = 44.1%
   Solid fat content of 20.7% of test portion at 30°C is designated N30 = 20.7%
   Together with the data the exact mode of operation and the tempering procedure should always be reported.

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CALCULATIONS

1. For the Direct method the SFC is calculated by the instrument. For further explanations on modes of calculation, see manufacturers' manuals.

Direct method—solid fat content (SFC) at T°C:

$$NT = \frac{(E11–E70) \times F \times 100}{E70 + [(E11–E70) \times F] + D}$$

Where

E11 = NMR signal measured at 11 μs after the pulse
E70 = NMR signal measured at 70 μs after the pulse
F = An empirical factor which corrects for the dead time of the receiver (calculated from the calibration standards)
D = Digital offset factor, to correct for the non-linearity or offset of the detector (calculated from the calibration standards).

For instruments without diode detectors, omit D in the formula above for SFC calculations. (E11–E70) represents the solid signal after 11 μs and E70 represents the liquid signal after 70 μs.

PRECISION

For the precision of the direct method the results of the international collaborative study, carried out in 1992/1993 by AOCS and Unilever (in which 24 companies participated) were used. (This study also included the indirect NMR and the dilatometry methods; general conclusions and a summary of statistical results are given in General Notes, 11 and Tables 1–10, respectively).

The test was set up using a double split level design using four different test sample types: a margarine, palm oil, bean oil, and kaomel. Every type consisted of two slightly different batches which were offered in (blind) duplicates (16 test samples in total). Every test sample was analyzed at 10, 21.1, 26.7, 33.3, and 37.8°C (by the AOCS serial and parallel procedures) and at 10, 20, 25, 30, 35, and 40°C (by the IUPAC serial and parallel procedures).

Tests to detect outliers and suspected values were carried out on the data set according to statistical procedures. Outliers were defined as data which lie outside the expected data distribution with a confidence level between 95 and 99%.

After deletion of possible outliers the repeatability, r, and the between-laboratory reproducibility, R, were calculated.

The repeatability, r, represents the reliability of the method when duplicate analyses are carried out within one company/laboratory and using the same (batch of) reagents, the same instrument, and within a short period of time.

The between-laboratory reproducibility, R, represents the reliability of the method when an analysis is carried out by two different companies or laboratories.

In daily practice the difference between two measurements on the same test sample should not exceed the value of r or R when performed by one laboratory or by two laboratories, respectively, in a 95% confidence interval.

In principle pooling of standard deviations in order to calculate one value for r and one value for R is not allowed. Nevertheless this has been done from a practical point of view.

From the collaborative study the following values were found:

repeatability	1.3%
between-laboratory reproducibility:	3.3%

NOTES

Caution

Persons with a pacemaker should not come within a distance of 30 cm of the NMR equipment. Heavy metal objects should not be in the vicinity of the NMR equipment.

GENERAL NOTES

1. Instrument siting. The room temperature where the low resolution NMR equipment is placed should be stable—never below 15°C or above 30°C. The instrument should be placed on a robust table to prevent influence of vibrations. Large moving metal objects should be kept away from the instrument because they may interfere with the magnetic field. The magnet should be thermostated (usually 40°C) to reduce drift.
2. Instrument temperature control. Because NMR instruments have heated magnets, test portions warm up as soon as they are placed inside an instrument. This can affect the accuracy of the SFC measurements if the sample heating rate is variable. Therefore, the magnet and sample probe temperature must be stabilized by the thermostat. Variable temperature controlled probe heads are not practical and are not applicable to the present method.
3. Drying of tubes and blocks. After being tempered in racks, the tubes should be dried carefully and quickly before being placed in the metal block in the water bath at the measuring temperature. Tubes should not be wiped with tissues, because this may interfere with the results. The holes in the metal blocks in the 10 or 15°C bath must be dried frequently, because moisture may condense in them.

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4. Tube enclosures. A method of closing off the tube (e.g., a cap or a rubber stopper) can limit contamination by dust, solvent vapors, or moisture during the tempering steps, especially during the long stabilization period of 40 hours at 26°C for stabilizing fats.
5. Sample temperature control. SFC values depend on the thermal history of the crystallized fat. Therefore, it is important to adhere to the prescribed times given in the method. Temperatures of tempering and measurements are also extremely important, and thermostat settings together with both dimensions of the tubes and the holes in the metal blocks should be strictly observed, the latter in order to ensure the appropriate thermal contact. The effect of temperature and time are especially important at temperatures where the confectionery fats have a steep melting profile (e.g , at 30°C for cocoa butter).
6. Slow crystallizing oils and fats. Slow crystallizing oils and fats will not have completely crystallized during the 1 h at 0°C period, leading to a lower SFC value than would be obtained after complete crystallization. The applicability of the method may be verified by determining the SFC after a range of crystallization periods at 0°C. Slower crystallizing oils and fats will require a longer crystallization period.
7. Stabilizing fats. A more accurate description would be "fats which need polymorphic stabilization," as cocoa butter and specialty fats are found in fully stable, partially stable, and unstable forms. However, the term "stabilizing fats" has been used for many years in connection with dilatometry and has now become the accepted description.
8. Repetition delay. The usual repetition delay time for commercial margarine and unstabilized confectionery fats is two sec. For those fats which crystallize in a beta form after extended stabilization (such as cocoa butter and cocoa butter-like fats), a two-second trigger time leads to saturation effects, and for these fats a trigger time of six seconds is used, combined with a single pulse. If there are doubts about the occurrence of saturation effects, one can check this by repeating the entire tempering procedure, but measuring with a single pulse and a trigger time of six seconds. When the solids content thus obtained is not higher than the one obtained with a trigger time of two seconds, one can safely assume that the latter method gives the correct solid fat content value. Although a single pulse has a poorer signal-to-noise ratio than a four-pulse sequence, increasing the number of pulses using a trigger time of 6 seconds for the purpose of increasing accuracy is not recommended because the temperature and the SFC value will start to vary.
9. This method describes the parallel tempering method (separate test samples for every temperature). There are two distinct advantages of the parallel method: measurements are independent of the number of temperatures used and the speed of the entire procedure. However, the serial method (same test sample at each temperature) is suited to situations when only small amounts of test sample are available, less preparation time, and greater throughput are required. The serial method may be used if the SFC results using the particular type of test sample has been shown to be not significantly different from the SFC using the standard parallel method. The time between the temperature readings should be 30–35 min.
10. Comments on the F-factor: Direct versus Indirect method (AOCS Official Method Cd 16-81). The SFC can only be measured by the Direct method if the F-factor is accurately determined. The calibration method defined in this Official Method establishes a mean F-factor for fat blends which can be approximated by tristearin/triolein mixtures. In other cases the F-factor will not be accurate and the results will not be the true solid fat content of the test samples, but rather an empirical "NMR value" related to the solids content. However, the NMR value (or "N-value") will always be the same under identical conditions, so the method provides a reliable quality control measure. This is similar to the dilatometry case, where SFI is an "index" of the solids content and not the precise solids content. Accurate F-factors can be predetermined for particular groups of fats exposed to the same temperature treatment by carrying out an Indirect measurement and then calculating the F-factor necessary to achieve the same answer by the Direct method. For commercial comparisons, however, it is essential that F-factors calculated from the defined calibration procedure are used; otherwise, the comparisons cannot be made. Some variation of F-factors between instruments at various sites is unavoidable because the F-factor depends partly on the instrument. Therefore, during the establishment of commercial contracts one should exchange reference samples whose solids content is accurately known. This will ensure better reproducibility between different sites.

    The Direct method works by ratioing the signals for the solid and liquid phases within the same test sample. It is therefore relatively immune to external influences such as sample filling, tube dimension variations, and small temperature variations, because all these factors affect the solid and liquid signal equally.

    The Indirect method does not rely on F-factors and is calibrated by direct reference to each test sample in its fully melted state. The Indirect method therefore determines the solids content accurately under all circumstances. However, the Indirect method involves extra steps and complexity, and it is subject to inaccuracies caused by variations in sample filling and minor temperature changes.
11. General conclusions on the precision and accuracy of the different NMR methods and the dilatometry method from the collaborative AOCS study, performed in 1992/1993:
    (a) Direct NMR methods give better repeatability than indirect NMR methods, but the latter ones are more accurate.
    (b) Repeatability of both direct and indirect NMR methods is in general as good as the AOCS SFI dilatometric method (Official Method Cd 10-57).
    (c) Reproducibility is acceptable and about a factor of 2–3 times greater than the repeatability. This is the usual observation in a collaborative study of this type.
    (d) Serial and parallel tempering procedures are not different. The choice between these procedures will not affect results.
    (e) The AOCS and IUPAC tempering procedures are not different with respect to precision.

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Table 1
Summary of mean statistical results for determinations at 10°C.

		AOCS Serial Procedure			AOCS Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.55	0.59	0.88	0.69	0.77	0.29
K	2.13	0.36	0.95	0.29	0.44	0.39
PO	2.06	0.61	0.67	0.44	0.70	0.57
MO	0.38	0.70	1.24	0.35	0.97	0.29
S_R—						
HSBO	0.73	2.45	2.25	2.00	2.10	2.23
K	2.55	1.56	2.58	1.32	1.13	1.44
PO	3.26	2.50	2.56	2.04	2.91	2.21
MO	0.55	1.74	2.65	0.96	1.72	1.20
		IUPAC Serial Procedure			IUPAC Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.55	0.58	1.24	0.63	0.48	0.52
K	2.13	0.28	0.44	0.17	0.24	0.19
PO	2.06	0.53	0.72	0.36	0.58	0.38
MO	0.38	0.49	1.44	0.29	0.86	0.38
S_R—						
HSBO	0.73	2.40	2.72	2.13	1.41	1.16
K	2.55	1.71	4.08	1.25	1.46	1.12
PO	3.26	1.96	2.44	1.49	2.05	1.52
MO	0.55	1.77	3.23	0.98	1.64	0.98

Key:
HSBO = Hydrogenated soybean oil 1 = SFI's by AOCS Cd 10-57
K = Kaomel 2 = Bruker instrument
PO = Palm oil 3 = Praxis instrument
MO = Margarine oil

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Table 2
Summary of mean statistical results for determinations at 21.1°C (AOCS) or 20°C (IUPAC).

		AOCS Serial Procedure			AOCS Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.49	0.60	0.39	0.53	0.60	0.58
K	1.17	0.50	0.34	0.29	0.37	0.51
PO	0.31	0.61	0.58	0.26	0.55	0.30
MO	0.28	0.64	0.59	0.36	0.76	0.39
S_R—						
HSBO	0.70	1.49	1.48	1.59	2.06	1.67
K	1.17	1.07	2.42	1.40	1.59	1.42
PO	0.59	1.66	1.10	0.94	1.67	0.85
MO	0.37	1.51	1.63	0.96	1.19	0.59
		IUPAC Serial Procedure			IUPAC Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.49	0.48	0.44	0.44	0.63	0.45
K	1.17	0.18	0.21	0.20	0.49	0.21
PO	0.31	0.47	0.45	0.45	0.83	0.37
MO	0.28	0.53	0.49	0.33	0.71	0.38
S_R—						
HSBO	0.70	0.68	1.65	1.25	1.33	1.15
K	1.17	0.44	3.13	1.11	1.17	0.97
PO	0.59	1.44	1.88	1.28	1.45	1.03
MO	0.37	1.64	2.35	0.83	1.33	0.96

Key:
HSBO = Hydrogenated soybean oil 1 = SFI's by AOCS Cd 10-57
K = Kaomel 2 = Bruker instrument
PO = Palm oil 3 = Praxis instrument
MO = Margarine oil

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Table 3
Summary of mean statistical results for determinations at 26.7°C (AOCS) or 25°C (IUPAC).

		AOCS Serial Procedure			AOCS Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.41	0.71	0.60	0.58	0.75	0.80
K	1.69	0.57	0.54	0.40	0.59	0.56
PO	0.54	0.58	0.35	0.39	0.83	0.38
MO	0.24	0.65	0.73	0.28	0.51	0.36
S_R—						
HSBO	0.71	1.42	0.74	1.72	1.75	1.75
K	1.86	1.87	2.00	1.99	1.66	1.29
PO	0.59	1.57	0.97	0.97	1.97	1.03
MO	0.30	1.38	1.07	0.63	0.94	0.96
		IUPAC Serial Procedure			IUPAC Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.41	0.49	0.33	0.40	0.82	0.36
K	1.69	0.44	0.49	0.31	0.62	0.27
PO	0.54	0.56	0.51	0.29	0.66	0.27
MO	0.24	0.50	0.78	0.24	0.58	0.36
S_R—						
HSBO	0.71	1.04	2.58	1.43	1.37	1.51
K	1.86	1.10	2.57	1.25	1.79	1.29
PO	0.59	1.31	2.10	0.55	1.35	0.67
MO	0.30	1.33	2.37	0.39	1.21	0.74

Key:
HSBO = Hydrogenated soybean oil 1 = SFI's by AOCS Cd 10-57
K = Kaomel 2 = Bruker instrument
PO = Palm oil 3 = Praxis instrument
MO = Margarine oil

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Table 4
Summary of mean statistical results for determinations at 33.3°C (AOCS) or 30°C (IUPAC).

		AOCS Serial Procedure			AOCS Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.42	0.55	0.63	0.44	0.83	0.51
K	1.86	0.59	0.57	0.34	0.63	0.25
PO	0.40	0.56	0.48	0.34	0.78	0.34
MO	0.21	0.62	0.25	0.30	0.59	0.37
S_R—						
HSBO	0.76	1.70	0.94	1.26	1.59	1.30
K	2.56	1.50	0.86	1.67	1.03	1.65
PO	0.53	1.58	1.29	0.90	1.34	0.65
MO	0.28	1.36	1.25	0.68	0.90	0.79
		IUPAC Serial Procedure			IUPAC Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.42	0.34	0.63	0.50	0.45	0.43
K	1.86	0.31	0.35	0.42	0.45	0.40
PO	0.40	0.32	0.60	0.36	0.45	0.40
MO	0.40	0.32	0.60	0.36	0.74	0.29
S_R—						
HSBO	0.76	1.38	2.25	1.55	1.29	1.36
K	2.56	0.75	1.97	2.16	1.42	1.76
PO	0.53	1.18	1.01	0.69	1.14	0.42
MO	0.28	1.09	1.58	0.51	0.94	0.48

Key:
HSBO = Hydrogenated soybean oil 1 = SFI's by AOCS Cd 10-57
K = Kaomel 2 = Bruker instrument
PO = Palm oil 3 = Praxis instrument
MO = Margarine oil

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Table 5
Summary of mean statistical results for determinations at 37.8°C (AOCS) or 35°C (IUPAC).

		AOCS Serial Procedure			AOCS Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.19	0.58	0.74	0.35	0.45	0.41
K	0.95	0.43	0.63	0.27	0.29	0.41
PO	0.45	0.68	0.81	0.35	0.55	0.30
MO	0.25	0.58	0.73	0.34	0.41	0.45
S_R—						
HSBO	0.51	1.11	1.06	0.83	0.97	0.62
K	1.30	1.27	0.95	0.90	0.71	0.72
PO	0.55	1.23	1.42	1.03	0.76	0.86
MO	0.33	1.05	0.94	0.45	0.74	0.53
		IUPAC Serial Procedure			IUPAC Parallel Procedure	
	1	2 Indirect	3 Indirect	2 Direct	2 Indirect	2 Direct
S_r—						
HSBO	0.19	0.37	0.67	0.27	0.41	0.41
K	0.95	0.31	0.63	0.26	0.45	0.46
PO	0.45	0.32	0.50	0.27	0.68	0.33
MO	0.25	0.41	0.67	0.33	0.68	0.31
S_R—						
HSBO	0.51	1.10	3.62	0.88	0.65	0.78
K	1.30	1.01	5.35	1.38	1.16	1.17
PO	0.55	1.07	2.12	0.54	1.22	0.51
MO	0.33	1.03	2.18	0.56	0.88	0.57

Key:
HSBO = Hydrogenated soybean oil 1 = SFI's by AOCS Cd 10-57
K = Kaomel 2 = Bruker instrument
PO = Palm oil 3 = Praxis instrument
MO = Margarine oil

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Table 6
Summary of all mean repeatability (S_r) and reproducibility (S_R) from all methods at all temperatures, including results determined at 40°C, using IUPAC serial and parallel procedures.

	AOCS Serial Procedures								AOCS Parallel Procedures			
	1		2 Indirect		3 Indirect		2 Direct		2 Indirect		2 Direct	
Repeatability, S_r	ISO	Unilever	ISO	Unilever	ISO	Unilever	ISO	Unilever	ISO	Unilever	ISO	Unilever
HSBO	0.41	0.42	0.61	0.57	0.65	NA	0.52	0.5	0.68	0.64	0.52	0.55
K	1.56	1.61	0.49	0.44	0.51	NA	0.32	0.32	0.47	0.52	0.43	0.4
PO	0.78	0.69	0.61	0.55	0.58	NA	0.36	0.35	0.68	1.0	0.38	0.38
MO	0.28	0.27	0.64	0.72	0.71	NA	0.33	0.32	0.65	0.82	0.37	0.38
Reproducibility, S_R												
HSBO	0.68	0.82	1.63	1.58	1.3	NA	1.5	1.63	1.7	1.56	1.52	1.5
K	1.86	2.35	1.46	1.38	1.76	NA	1.46	1.42	1.23	1.24	1.31	1.26
PO	1.11	0.99	1.71	1.77	1.47	NA	1.18	1.08	1.73	1.79	1.12	1.23
MO	0.37	0.36	1.41	1.47	1.51	NA	0.71	0.59	1.1	1.21	0.82	0.82

	IUPAC Serial Procedures								IUPAC Parallel Procedures			
	1		2 Indirect		3 Indirect		2 Direct		2 Indirect		2 Direct	
Repeatability, S_r	ISO	Unilever	ISO	Unilever	ISO	Unilever	ISO	Unilever	ISO	Unilever	ISO	Unilever
HSBO	0.41	0.42	0.44	0.47	0.61	NA	0.44	0.42	0.52	0.73	0.44	0.42
K	1.56	1.61	0.31	0.31	0.64	NA	0.28	0.25	0.5	0.4	0.26	0.29
PO	0.78	0.69	0.44	0.47	0.6	NA	0.35	0.32	0.66	0.7	0.33	0.34
MO	0.28	0.27	0.47	0.52	0.8	NA	0.32	0.32	0.69	0.7	0.36	0.36
Reproducibility, S_R												
HSBO	0.68	0.82	1.13	1.29	2.51	NA	1.32	1.36	1.41	1.47	1.08	1.34
K	1.89	2.35	0.92	0.97	3.01	NA	1.43	1.31	1.34	1.26	1.05	1.07
PO	1.11	0.99	1.3	1.28	1.77	NA	0.88	0.91	1.41	1.42	0.8	0.93
MO	0.37	0.36	1.26	1.39	2.15	NA	0.64	0.64	1.21	1.12	0.72	0.76

Key:
HSBO = Hydrogenated soybean oil
K = Kaomel
PO = Palm oil
MO = Margarine oil
1 = SFI's by AOCS Cd 10-57
2 = Bruker instrument
3 = Praxis instrument
ISO = statistics determined by ISO 5725–1986 using program developed at United States Food and Drug Administration provided by Dr. David Firestone
Unilever = statistics determined by Unilever in accordance with ISO 5275–1986

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Table 7
Comparison of SFC values obtained for hydrogenated soybean oil by AOCS method (A) and NMR methods (B, C).

	10°C			21.1°C			26.7°C			33.3°C			37.8°C		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
No. labs	15	24	19	15	24	19	15	24	19	15	24	19	15	24	19
No. results	29	48	38	29	48	38	29	48	38	29	48	38	30	48	38
Labs retained	12	22	19	12	22	17	12	21	17	13	23	17	8	22	17
Labs after outliers	12	22	19	12	22	17	12	21	17	12	23	17	8	22	17
No. accepted results	24	44	38	24	44	34	24	42	34	24	46	34	16	44	34
Mean, SFC	51.71	62.92	63.24	36.85	39.32	40.86	30.64	30.89	32.04	14.73	12.58	15.04	4.86	3.81	5.16
S_L	0.79	1.97	2.41	0.65	1.44	1.47	0.68	1.69	1.36	0.77	1.26	1.64	0.67	0.77	1.18
S_r	0.55	0.64	0.69	0.44	0.62	0.59	0.42	0.70	0.72	0.39	0.53	0.64	0.25	0.36	0.67
S_R	0.88	2.02	2.45	0.72	1.50	1.53	0.74	1.76	1.45	0.82	1.31	1.70	0.69	0.81	1.27
$2.8 \times S_r$	1.54	1.79	1.65	1.23	1.74	1.65	1.18	1.96	2.02	1.09	1.48	1.79	0.70	1.01	1.88
$2.8 \times S_R$	2.46	5.66	6.86	2.02	4.20	4.28	2.07	4.93	4.06	2.30	3.67	4.76	1.93	2.27	3.56

Key:
A = AOCS Official Method, Cd 10-57, AOCS serial procedure
B = NMR direct, AOCS serial procedure (Bruker)
C = NMR indirect, AOCS serial procedure (Bruker)

Table 8
Comparison of SFC values obtained for kaomel by AOCS method (A) and NMR methods (B, C).

	10°C			21.1°C			26.7°C			33.3°C			37.8°C		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
No. labs	15	24	19	15	24	19	15	24	19	15	24	19	15	24	19
No. results	28	48	38	28	48	38	28	48	38	29	48	38	30	48	38
Labs retained	12	22	19	11	22	17	12	22	17	12	23	17	9	22	17
Labs after outliers	10	22	19	10	22	17	10	22	17	12	22	17	9	22	17
No. accepted results	20	44	38	20	44	34	20	44	34	24	44	34	18	44	34
Mean, SFC	70.14	87.76	87.76	60.87	70.04	69.94	53.71	59.54	58.81	26.57	24.29	26.56	3.99	3.28	4.39
S_L	0.87	1.27	1.52	1.08	1.33	1.10	1.08	1.93	1.75	2.22	1.69	1.56	1.08	0.88	1.24
S_r	1.99	0.25	0.29	1.68	0.28	0.39	1.63	0.38	0.48	2.147	0.22	0.54	0.85	0.25	0.50
S_R	1.99	1.28	1.54	1.68	1.35	1.14	1.63	1.95	1.79	2.68	1.69	1.61	1.23	0.90	1.29
$2.8 \times S_r$	5.57	0.70	0.81	4.70	0.78	1.09	4.56	1.06	1.34	5.99	0.62	1.51	2.38	0.70	1.40
$2.8 \times S_R$	5.57	3.58	4.31	4.70	3.78	3.19	4.56	5.46	5.01	7.50	4.73	4.51	3.44	2.52	3.61

Key:
A = AOCS Official Method, Cd 10-57, AOCS serial procedure
B = NMR direct, AOCS serial procedure (Bruker)
C = NMR indirect, AOCS serial procedure (Bruker)

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Table 9
Comparison of SFC values obtained for palm oil by AOCS method (A) and NMR methods (B, C).

	10°C			21.1°C			26.7°C			33.3°C			37.8°C		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
No. labs	15	24	19	15	24	19	15	24	19	15	24	19	15	24	19
No. results	30	48	38	30	48	38	30	48	38	30	48	38	30	48	38
Labs retained	14	22	16	13	22	17	13	22	17	13	23	17	11	22	17
Labs after outliers	14	22	16	11	22	17	11	22	17	10	23	17	10	22	17
No. accepted results	28	44	32	22	44	34	22	44	34	20	46	34	20	44	34
Mean, SFC	33.01	42.89	41.90	14.30	15.92	15.87	11.66	12.37	12.27	7.81	6.72	7.78	6.38	4.23	5.88
S_L	1.94	1.72	3.11	0.37	0.88	0.72	0.25	0.80	1.57	0.26	0.76	1.63	0.36	0.89	1.20
S_r	2.33	0.42	0.67	0.31	0.21	0.71	0.27	0.46	0.74	0.30	0.31	0.75	0.43	0.41	0.79
S_R	2.55	1.75	3.14	0.41	0.89	1.79	0.32	0.87	0.65	0.33	0.79	1.71	0.48	0.94	1.32
$2.8 \times S_r$	6.52	1.18	1.88	0.87	0.59	1.99	0.76	1.29	2.07	0.84	0.87	2.10	1.20	1.15	2.21
$2.8 \times S_R$	7.14	4.90	8.79	1.15	2.49	5.01	0.90	2.44	4.62	0.92	2.21	4.79	1.34	2.63	3.70

Key:
A = AOCS Official Method, Cd 10-57, AOCS serial procedure
B = NMR direct, AOCS serial procedure (Bruker)
C = NMR indirect, AOCS serial procedure (Bruker)

Table 10
Comparison of SFC values obtained for margarine oil by AOCS method (A) and NMR methods (B, C).

	10°C			21.1°C			26.7°C			33.3°C			37.8°C		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
No. labs	15	24	19	15	24	19	15	24	19	15	24	19	15	24	19
No. results	30	48	38	30	48	38	30	48	38	30	48	38	30	48	38
Labs retained	14	22	16	14	22	17	14	23	17	14	23	17	11	22	17
Labs after outliers	14	22	16	14	22	17	14	22	17	17	23	17	11	21	17
No. accepted results	28	44	32	28	44	34	28	44	34	28	46	34	22	42	34
Mean, SFC	8.43	9.60	8.39	6.40	6.03	4.98	6.19	5.35	4.55	5.31	4.07	3.65	4.95	2.90	3.18
S_L	0.38	0.94	1.67	0.26	0.75	1.45	0.22	0.42	1.31	0.27	0.82	1.41	0.30	0.36	1.05
S_r	0.47	0.34	0.42	0.32	0.24	0.95	0.28	0.30	0.93	0.29	0.34	0.91	0.26	0.37	0.87
S_R	0.51	0.97	1.70	0.35	0.77	1.60	0.29	0.47	1.47	0.34	0.86	1.55	0.36	0.45	1.22
$2.8 \times S_r$	1.32	0.95	1.18	0.90	0.67	2.66	0.78	0.84	2.60	0.81	0.95	2.55	0.73	1.04	2.44
$2.8 \times S_R$	1.43	2.72	4.76	0.98	2.16	4.48	0.81	1.32	4.12	0.95	2.41	4.34	1.01	1.26	3.42

Key:
A = AOCS Official Method, Cd 10-57, AOCS serial procedure
B = NMR direct, AOCS serial procedure (Bruker)
C = NMR indirect, AOCS serial procedure (Bruker)

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AOCS Official Method Cd 18-90

Revised 2011

p-Anisidine Value

DEFINITION

The p–anisidine value is defined by convention as 100 times the optical density measured at 350 nm in a 1 cm cuvette of a solution containing 1.00 g of the oil in 100 mL of a mixture of solvent and reagent according to the method described.

SCOPE

This method determines the amount of aldehydes (principally 2–alkenals and 2,4–dienals) in animal and vegetable fats and oils, by reaction in an acetic acid solution of the aldehydic compounds in an oil and the p-anisidine (see Notes, 1), and then measuring the absorbance at 350 nm.

APPARATUS

1. Test tubes—10 mL min. with either ground-glass stoppers or Teflon™–lined screw caps.
2. Volumetric flasks—25 mL.
3. Automatic pipet or automatic burette.

   Note—Any pipette and/or burette capable of delivering exactly 1 mL and 5 mL is satisfactory.
4. Spectrophotometer suitable for observation at 350 nm.
5. Glass cuvettes—1.00 ± 0.01 cm, the two cuvettes of each pair must be identical.

REAGENTS

1. Isooctane (2,2,4–trimethylpentane)—optically clear (see Notes, *Caution* and 2).
2. Glacial acetic acid—analytical reagent quality (see Notes, 3).
3. p–Anisidine—analytical reagent quality (see Notes, *Caution* and 4) 0.25 g/100 mL solution in glacial acetic acid (Reagents, 2) (see Notes, 5).

PROCEDURE

Note—The test sample should be perfectly clear and dry (see Notes, 3).

1. Weigh 0.5–4.0 ± 0.001 g of the test sample into a 25 mL volumetric flask. Dissolve and dilute to volume with isooctane.
2. Measure the absorbance (Ab) of the solution at 350 nm in a cuvette with the spectrophotometer, using the reference cuvette filled with solvent as a blank.
3. Pipet exactly 5 mL of the fat solution into one test tube (Apparatus, 1) and exactly 5 mL of the solvent into a second test tube. By means of an automatic pipet (Apparatus, 3) add exactly 1 mL of the p-anisidine reagent (Reagents, 3) to each tube, and shake (see Notes, 6).
4. After exactly 10 min measure the absorbance (As) of the solvent in the first test tube in a cuvette (Apparatus, 5) at 350 nm, using the solution from the second test tube as a blank in the reference cuvette.

CALCULATIONS

The p–anisidine value (p–A.V.) is given by the formula

$$\text{p–A.V.} = \frac{25 \times (1.2\text{As} - \text{Ab})}{\text{m}}$$

Where—

As = absorbance of the fat solution after reaction with the p-anisidine reagent (Reagents, 3)

Ab = absorbance of the fat solution

m = mass of the test portion, g

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PRECISION (see References, 2)

	Crude Rapeseed Oil		Refined Palm Oil	
	Test Sample 1	Test Sample 2	Test Sample 1	Test Sample 2
No. of labs	20	20	20	20
Mean value	2.0	2.0	2.3	2.3
Repeatability, CV, %	4.0	5.8	4.8	4.6
Reproducibility, CV, %	35	37	30	31

NOTES

Caution

Isooctane is flammable and a fire risk. Explosive limits in air are 1.1–6.0%. It is toxic by ingestion and inhalation. A properly operating fume hood should be used when working with this solvent.

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

p–Anisidine is an irritant and should be handled with care, preferably in a fume hood. p–Anisidine is an aromatic amine, a class of toxic and possibly carcinogenic chemicals. o–Anisidine is a carcinogen in rats and mice, causing urinary carcinomas or papillomas. [Fourth Annual Report on Carcinogens, NTP 85–002, 1985, p. 2; *Chem. Res. Toxicol. 4:*474 (1991)]. The TLV is 0.1 ppm.

NUMBERED NOTES

1. In the presence of acetic acid, p–anisidine reacts with aldehydic compounds in oils or fats. The intensity of color of the yellowish reaction products formed depends not only on the amount of aldehydic compounds present but also on their structure. It has been found that a double bond in the carbon chain conjugated with the carbonyl double bond increases the molar absorbance four to five times. This means that 2–alkenals and dienals, especially, will contribute substantially to the value found.
2. In most cases *n*–hexane can be substituted for isooctane as a solvent. However, oils containing high amounts of oxidized fatty acids will not dissolve completely in *n*-hexane. For such oils isooctane should be used as the solvent. The absorbance of the solvent used (isooctane or *n*-hexane), measured in a 1.00 cm cuvette between 300 and 380 nm, must be nil or nearly nil. The commercial product can be freed from absorptive material by percolating it through a glass column (3–5 cm i.d., and 100 cm long) filled with silica gel.
3. The reaction between p-anisidine and aldehydes involves the formation of water. Hence, the presence of moisture in any of the reagents or in the test sample leads to incomplete reaction and, consequently, low values. Since glacial acetic acid is highly hygroscopic, it is essential to check its moisture content by a Karl Fischer determination. If the content exceeds 0.1 percent, the acetic acid must be discarded.
4. In storage, p-anisidine tends to darken as a result of oxidation. The p–anisidine crystals, which should be cream colored, should be stored at 0–4°C in a dark bottle. The crystals should not be exposed to strong light and should be used before any color change is observed. A discolored reagent can be reduced and decolorized in the following way. Dissolve 4.0 g of p–anisidine in 100 mL of water at 75°C. Add 0.2 g of sodium sulphite and 2.0 g of active carbon and stir for 5 min. Then filter through a double filter paper. If carbon passes through, repeat filtration. Cool the filtered solution to about 0°C, allow to stand at this temperature for at least 4 hr, or, preferably, overnight. Filter off the crystallized p-anisidine and wash with a small amount of water at a temperature of about 0°C. After drying in a vacuum desiccator, transfer the crystals into a brown glass bottle. If stored in the dark and at low temperature, the crystals obtained should not darken appreciably for 1 yr.
5. Reagent solutions having an absorbance greater than 0.200 when measured in a 1.00 cm cuvette at 350 nm against isooctane or *n*-hexane as a blank should be discarded.
6. The mixture should be completely homogenized with minimum shaking and then allowed to react for 10 min before proceeding with the absorbance measurement (References, 4).

REFERENCES

1. IUPAC, *Standard Methods for the Analysis of Oils, Fats and Derivatives,* 7th ed., Method Number 2.504 Determination of the p-anisidine value (p-A.V.), Blackwell Scientific Publications, Boston, MA and Oxford, UK (1987).
2. FOSFA International Collaborative Study #P15, May 1986, Document No. 384, ISO/TC 34/SC 11, February 12, 1987.
3. *JAOCS 51:*17 (1974).
4. Hamilton, R.J., and S. Hamilton, *Lipid Analysis,* Oxford University Press, New York, 1992, pp. 45–47.

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AOCS Official Method Cd 19-90
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2-Thiobarbituric Acid Value Direct Method

DEFINITION

The TBA value is defined as the increase of absorbance measured at 530 nm due to the reaction of the equivalent of 1 mg of test sample per 1 mL volume with 2-thiobarbituric acid. Secondary oxidation products of oils and fats react with 2-thiobarbituric acid forming condensation products the absorbance of which is measured at 530 nm, the wavelength of one of their absorption maxima.

SCOPE

This method permits the direct determination of the 2-thiobarbituric acid value (TBA value) in oils and fats without preliminary isolation of secondary oxidation products. This standard is applicable to animal and vegetable fats and oils, fatty acids and their esters, partial glycol esters, and similar materials (see Notes, 1).

APPARATUS

1. Volumetric flask—25 mL.
2. Volumetric flask—100 mL.
3. Pipet—5 mL.
4. Test tubes—10–15 mm i.d., fitted with ground-glass stoppers.
5. Glass cuvettes—10 mm, suitable for spectrometric measurements.
6. Thermostated bath—maintained at 95 ± 0.5°C.
7. Spectrometer—allowing the reading at 530 nm to the nearest 0.001 nm.

REAGENTS

1. Pure 1-butanol—containing less than 0.5% water (see Notes, 2).
2. 2-Thiobarbituric acid—chemically pure (see Notes, 3).
3. The TBA reagent is prepared by dissolving 200 mg 2-thiobarbituric in 100 mL 1-butanol. Leave the weighed amount with butanol overnight or use an ultrasonic apparatus, filter, or centrifuge the suspension to remove the undissolved residue; make up the filtrate to 100 mL with 1-butanol. The reagent should not be stored for more than 1 wk in the refrigerator.

PROCEDURE

1. Accurately weigh 50–200 mg of the test sample (see Notes, 3) into a 25 mL volumetric flask (Apparatus, 1). Dissolve the test sample in a small volume of 1-butanol and make up to volume with 1-butanol.
2. Transfer, using a pipet (Apparatus, 3), 5.0 mL of the test solution to a dry test tube (Apparatus, 4); add by pipet (Apparatus, 3) 5.0 mL of the reagent solution (Reagents, 3). Close the test tube with a ground-glass stopper and mix thoroughly.
3. Place the prepared test tube into a thermostated bath (Apparatus, 6) at 95°C.
4. After 120 min, remove the test tube from the thermostated bath and cool it under running tap water for about 10 min until it reaches room temperature.
5. Measure the absorbance of the reaction solution in a 10 mm cuvette (Apparatus, 5) at 530 nm (Apparatus, 7) using distilled water in the reference cuvette (see Notes, 4).
6. Prepare a reagent blank at the same time as the test portion. The reading of the blank determination should not exceed 0.1 in a 10 mm cuvette (see Notes, 5).

CALCULATIONS

Results are calculated as follows—

$$\text{TBA value} = \frac{50 \times (A - B)}{m}$$

Where—

A = absorbance of the test solution
B = absorbance of the reagent blank
m = mass of the test portion, mg

50 is a valid factor if the volume of the volumetric flask is 25 mL (Procedure, 1) and the cuvette width is 10 mm (Procedure, 4) (see Notes, 5).

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PRECISION (see References, 6)

Test Sample	A	B	C	D	E
No. of labs	20	20	20	20	20
mean	0.099	0.041	0.098	0.165	0.018
Repeatability, CV, %	6.1	9.7	5.5	6.9	16.8
Reproducibility, CV, %	50	50	45	65	66

A = refined zero erucic rapeseed oil
B = partially hydrogenated A
C = blind duplicate of A
D = soybean oil stored at room temperature for 2 yr
E = butterfat stored at room temperature for 14 d

NOTES

1. This method is not applicable to phospholipid concentrates and to test samples containing carbohydrates or proteins that could react either with the reagent or with TBA-active substances. For the analysis of such test samples, the lipid fraction should be isolated by extraction before the analysis, or the volatile TBA-active substances should be isolated by steam distillation.

   Under the conditions of the method hydroperoxides and dioxolanes may be partially decomposed with formation of TBA-active compounds, and the decomposition is catalyzed by a trace of heavy metals. On the other hand, antioxidants may react with some TBA-active substances thus decreasing the TBA value. Some substances present in the test sample might react with TBA to form other colored complexes or reaction products for which the absorbance is not at 530 nm. This can be recognized by another color tone.
2. The absorbance of the reagent blank measured according to the procedure, but without the test portion, should not exceed 0.1. If so, a new reagent solution should be prepared using TBA of better purity. In the case of reagent blanks higher than 0.1, the high blank value may be due to impurities in 1-butanol. They are removed by refluxing the solvent with 0.1% TBA for 2 hr and distilling. Water may be removed by distillation, and the rejection of the first, opalescent fraction.
3. Solid test samples are melted at not more than 10°C above the melting point and, if not entirely clear, filtered. Butter is melted at 40°C and the water removed on filtering with a hydrophilic filter.
4. If the absorbance falls outside the range of 0.2–0.8, the determination is repeated with more suitable cuvettes or with a more appropriate amount of test portion.
5. In the case of low blank values (0.05), the absorbance of the test solution can be measured directly against distilled water. The value is then calculated as follows (see Calculations):

$$\text{TBA value} = \frac{50 \times A}{m}$$

6. An interference by formaldehyde has been reported by M. Careche and M. Tejada (see Other References, 7).

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats, and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1st Supplement 1992, IUPAC method 2.531.

OTHER REFERENCES

1. Sinnhuber, R.O., and T.C. Yu, *Food Technology, 12:*9 (1958).
2. *Ibid. 16:*115 (1962)
3. Yukagaku, *Ibid. 26:*259 (1977).
4. Marcuse, R., and L. Johansson, *J. Am. Oil Chem. Soc. 50:*387 (1973).
5. ISO 5725: Precision of test methods—Determination of repeatability and reproducibility by interlaboratory tests (1986).
6. Pokorny, J., and A. Dieffenbacher, *Pure Appl. Chem. 61:*1165 (1989).
7. Careche, M., and M. Tejada, *J. Sci. Food Agric. 43:*49 (1988).

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AOCS Official Method Cd 20-91

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Determination of Polar Compounds in Frying Fats

DEFINITION

Polar compounds are those compounds in oils and fats which are determined by column chromatography under the conditions specified (see Notes, 1). Frying oils and fats are separated by column chromatography into nonpolar and polar compounds, followed by the elution of the nonpolar compounds. The polar compounds are determined by calculating the difference between the weight of the test sample added to the column and that of the nonpolar fraction eluted.

SCOPE

This method is for the determination of polar compounds in frying fats. Polar compounds are formed during the heating of fats. This method is applicable to animal and vegetable oils and fats. The method serves to assess the deterioration of used frying fats.

APPARATUS

1. Round-bottomed flasks—250 and 500 mL with ground necks.
2. Beakers—100 mL.
3. Ground-glass stoppers—to fit the 500 mL flasks.
4. Chromatographic glass column—21 mm i.d., 450 mm in length, with stopcock (preferably in Teflon™) and ground-glass joint.
5. Dropping funnel—250 mL with ground-glass joint to fit the column (Apparatus, 4).
6. Glass funnel—about 8 cm diameter.
7. Glass rod—about 60 cm in length.
8. Volumetric flask—50 mL.
9. Volumetric pipet—20 mL.
10. Capillary pipets—2 µL for thin-layer chromatography.
11. Glass plates for thin-layer chromatography—20 × 20 cm, coated with silica gel (without fluorescence indicator), 0.25 mm layer thickness.
12. Glass developing tank for thin-layer chromatography—with ground-glass lid.
13. Spray—for thin-layer chromatography.
14. Porcelain dish—about 20 cm dia.
15. Oven—regulated at 103 ± 2°C.
16. Drying oven—controllable between 120 and 160°C.
17. Water bath.
18. Desiccator—containing a suitable desiccant such as silica gel with moisture indicator (blue gel); see AOCS Specification H 9-87.
19. Apparatus for removing solvent under vacuum—e.g., rotary evaporator.
20. Shaking machine.

REAGENTS

1. Light petroleum ether (bp 40–60°C)—chromatographic quality, redistilled (see Notes, *Caution*).
2. Ethanol—95% (v/v).
3. Chloroform—pure (see Notes, *Caution* and 2).
4. Diethyl ether—free from peroxides and residue (see Notes, *Caution*).
5. Acetic acid—100% analytical reagent quality (see Notes, *Caution*).
6. Elution solvent—mixture of light petroleum and diethyl ether 87/13, (v/v) (see Notes, 3).
7. Developing solvent—mixture of light petroleum, diethyl ether, and acetic acid, 70/30/2, (v/v/v).
8. Silica gel—particle size 0.063–0.200 mm (70–230 mesh), Merck N 7734 or equivalent, adjusted to a water content of 5% (m/m, see Notes, 2).
9. Phosphomolybdic acid—analytical reagent quality, 100 g/L solution in ethanol.
10. Sea sand—purified by acid and calcined.
11. Cotton wool—surgical quality.
12. Nitrogen—99.0–99.8%.

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PROCEDURE

Test Sample preparation—

1. Remove visible impurities by filtration after homogenization. If water is present, use a hydrophobic filter paper.
2. For semiliquid and solid test samples, warm to a temperature slightly above the melting point and homogenize carefully; avoid overheating.

Column preparation—

1. Fill the column (Apparatus, 4) with about 30 mL of the elution solvent (Reagents, 6). Introduce a wad of cotton wool into the lower part of the column with the aid of a glass rod and remove air by pressing the wool.
2. Prepare in a 100 mL beaker a slurry of 25 g of silica gel (Reagents, 8) in about 80 mL of the elution solvent and pour this slurry into the column with the aid of the funnel (Apparatus, 6). To ensure complete transfer of the silica gel into the column, rinse with the elution solvent.
3. Open the stopcock and drain off the elution solvent into a second 100 mL beaker until the level of the elution solvent is 10 cm above the silica gel. Level the silica gel by tapping against the column.
4. Add about 4 g of sea sand with the aid of the funnel. Drain off the supernatant elution solvent as far as the sand layer (see Notes, 3).

Column chromatography—

Note—For the determination of polar compounds, only the nonpolar fraction is used. However, if the efficiency of the fractionation is assessed by thin-layer chromatography or by recovery of the test sample, both the polar and nonpolar fractions are required.

1. Weigh 2.4–2.6 ± 0.001 g of the test sample prepared (Test Sample preparation, 1) into a 50 mL volumetric flask. Dissolve in about 20 mL of the elution solvent while warming slightly. Allow to cool to room temperature and fill up to the mark with the elution solvent.
2. Introduce with a 20 mL volumetric pipet 20 mL of this solution into the column prepared as noted in Column preparation, Avoid disturbing the surface (see Notes, 4).
3. Dry two 250 mL flasks in the oven at a temperature of 103 ± 2°C. Allow to cool to room temperature and weigh accurately to within 0.001 g. Place one of them under the outlet of the column.
4. Open the stopcock and let the test solution drain off to the level of the sand layer (flow rate of 2.1–2.5 mL/min). Collect the eluate in the flask.
5. Elute nonpolar compounds with 150 mL of the elution solvent using the 250 mL dropping funnel. Adjust the flow rate so that 150 mL passes through the column within 60–70 min. Collect the eluate.
6. After completion of the elution, wash any substance adhering to the outlet of the column into the flask with 20 mL of the elution solvent.
7. If the polar compounds are required, elute them into a second 250 mL dry flask with 150 mL diethyl ether as described in Procedure, 5.
8. After completing the elution, discard the silica gel.
9. Remove the solvent from the flask(s) with the aid of a rotary evaporator using a water bath at a temperature no higher than 60°C. Avoid losses due to foaming (see Notes, 5).
10. Shortly before the end of the distillation, introduce nitrogen into the system.
11. Dry the flask at 103 ± 2°C in an oven and allow to cool. Weigh the flask.

CALCULATIONS

The content of polar compounds, in percent (m/m), is given by the formula:

$$\frac{m - m_1}{m} \times 100$$

Where—

m_1 = mass of the nonpolar fraction, g
m = mass of the test portion contained in 20 mL of the solution added to the column, g

THIN-LAYER CHROMATOGRAPHY (TLC) ASSESSMENT OF COLUMN EFFICIENCY

The efficiency of the fractionation can be assessed by thin-layer chromatography (see Notes, 6).

1. For the thin-layer chromatographic investigation prepare 10% solutions of the substance in chloroform and apply 2 µL spots onto a TLC plate (Apparatus, 11) using a capillary pipet (Apparatus, 10).
2. Line the developing tank with filter paper to achieve saturation. Place the plate in the developing tank and carry out the development with the developing solvent (Reagents, 7). Normally, after 36 min, the solvent front ascends to a height of about 17 cm. Remove the plate and allow the solvent to evaporate.
3. Spray the plate with the phosphomolybdic acid solution (Reagents, 9). After evaporation of ethanol, heat the plate in the drying oven at 120–130°C. As an example see Figure 1, showing a chromatogram obtained after fractionating a frying fat into individual fractions.

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NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light or upon prolonged standing. It should not be stored for longer than the recommended period. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using ethyl ether.

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

NUMBERED NOTES

1. The polar compounds include polar substances such as monoglycerides, diglycerides, free fatty acids which occur in unused fats, as well as polar transformation products formed during frying of foodstuffs and/or during heating. Nonpolar compounds are mostly unaltered triglycerides.
2. An alternate solvent has been recommended to replace chloroform. A mobile phase of petroleum ether/ethyl ether/acetic acid, 80:20:1, v/v/v, has been shown to work well. See Figure 2.
3. Adding 1% acetic acid to the 87/13 mixture may increase separation. See Figure 3.
4. Place the silica gel in the porcelain dish, dry in an oven at 160°C for at least 4 hr and cool in a desiccator to room temperature. Adjust the silica gel to a water content of 5%, e.g., weigh 152 g of silica gel and 8 g of water into a 500 mL flask. Close the flask with a stopper and shake mechanically with the aid of the shaking machine.
5. The excess solvent mixture drained off should not be used for elution.
6. For fats containing low amounts of polar components the amount of test sample added to the column can be raised from 1 to 2 g.
7. If the rotary evaporator is not available, the elution solvent can be evaporated on a steam plate under a stream of nitrogen.
8. The efficiency of the fractionation can also be assessed by checking the recovery of the test portion. For test samples containing greater amounts of polar material, recovery of the test portion may be incomplete. This is due to small amounts of highly polar material, generally not more than 1–2%, which is not eluted under the conditions specified.
9. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.507.
2. *Pure Appl. Chem. 54:*242 (1982).
3. *Ibid. 55:*1381 (1983).

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PRECISION

Table 1
Results of an IUPAC collaborative study on the determination of polar compounds.

Test Sample	1	2	3	4
Number of laboratories accepted	9	9	8	8
Number of results accepted	18	18	16	16
Mean of the laboratory values (%, m/m)	8.00	7.30	11.50	25.90
Repeatability				
Standard deviation, s_r	0.36	0.33	0.23	0.52
Relative standard deviation, RSD_r%	4.5	4.5	2.0	2.0
Repeatability value, $2.8 \times s_r$	1.02	0.94	0.66	1.47
Reproducibility				
Standard deviation, s_R	0.37	0.39	0.48	0.77
Relative standard deviation, RSD_R%	4.6	5.3	4.2	3.0
Reproducibility value, $2.8 \times s_R$	1.04	1.09	1.35	2.17

Table 2
Results of a 2000 collaborative study on the determination of polar compounds.

Test Sample	1	2	3	4	5
Number of laboratories accepted	13	13	11	14	10
Number of results accepted	26	26	22	28	20
Mean of the laboratory values (%, m/m)	16.01	22.28	11.74	32.06	20.80
Repeatability					
Standard deviation, s_r	0.27	0.29	0.34	0.33	0.14
Relative standard deviation, RSD_r%	1.7	1.3	2.9	1.0	0.7
Repeatability value, $2.8 \times s_r$	0.74	0.81	0.95	0.92	0.38
Reproducibility					
Standard deviation, s_R	1.04	1.29	0.56	1.66	0.61
Relative standard deviation, RSD_R%	6.5	5.8	4.8	5.2	3.0
Reproducibility value, $2.8 \times s_R$	2.92	3.61	1.58	4.64	1.72

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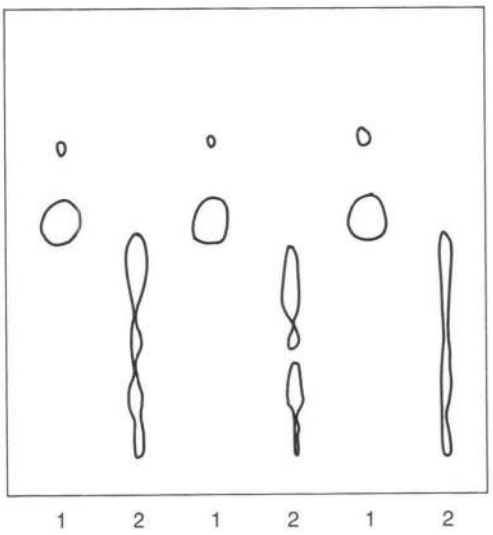

Figure 1. Fractionation of a frying fat into individual fractions: fraction 1 = nonpolar compounds, fraction 2 = polar compounds.

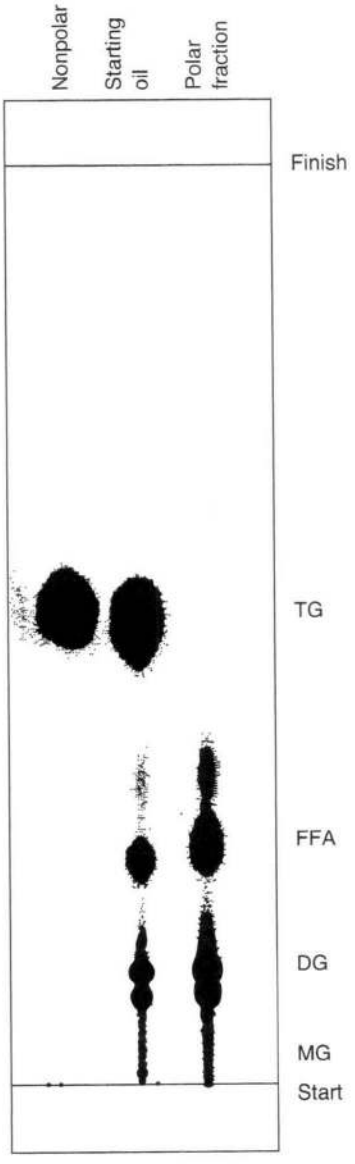

Figure 2. Mobile phase of petroleum ether/diethyl ether/acetic acid, 80:20:1, v/v/v, on Whatman K6F silica gel 60A 250 µm, 5 × 20 cm plates. TG = triglycerides; FFA = free fatty acids; DG = diglycerides; MG = monoglycerides.

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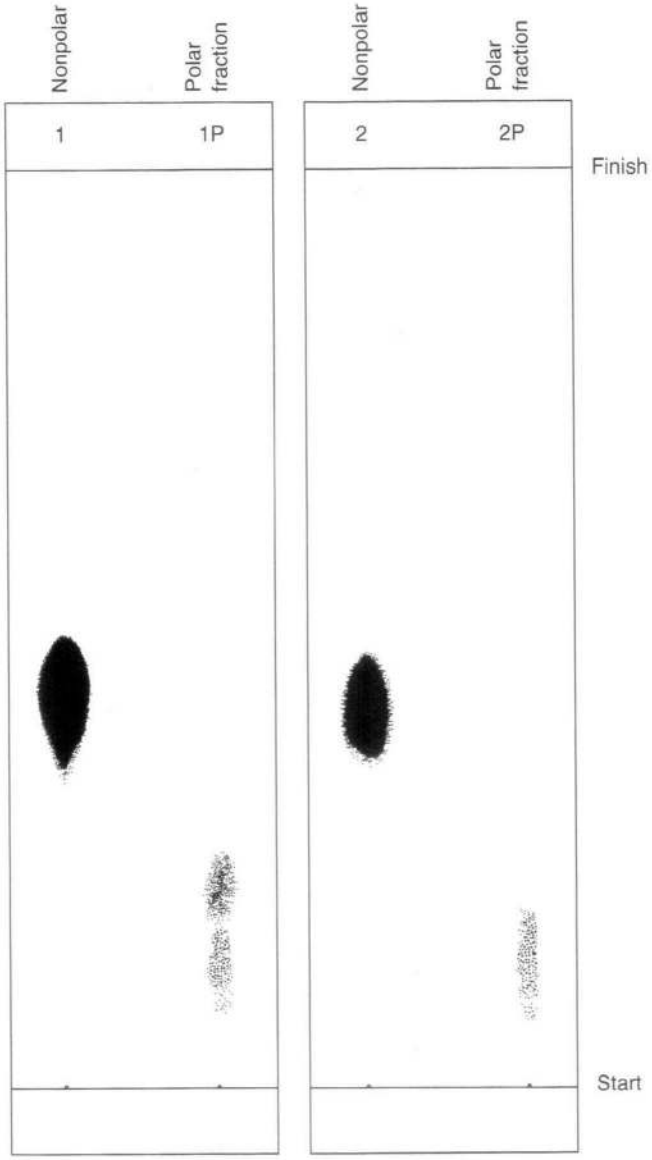

Figure 3. Mobile phase of petroleum ether/diethyl ether/acetic acid, 87:13:1, v/v/v, on Whatman K6F silica gel 60A 250 µm, 5 × 20 cm plates.

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Determination of Benzo(a)pyrene in Edible Oils and Fats

DEFINITION

A suitable amount of oil or fat in light petroleum is absorbed on an alumina column; subsequently any benzo(a)pyrene (BaP) present is eluted with light petroleum. Final analysis is carried out by reversed-phase, high-performance, liquid chromatography using a fluorimetric detector (References, 1).

SCOPE

This method can be used for determining benzo(a)pyrene in crude and refined edible oils and fats with a lowest limit of detection of about 0.1 µg/kg. Interference by other polynuclear aromatic hydrocarbons should not occur if the specified excitation and emission wavelengths are used with the fluorimetric detector.

APPARATUS

1. Glass chromatography column—30 cm × 15 mm i.d., fitted with sintered-glass disks and (Teflon™) plug taps.
2. Water bath—60–70°C.
3. Minivials—about 1 mL, with Teflon™-layered septa and aluminum caps, e.g., Chrompack catalog no. 10201 and 10210, respectively, or equivalent.
4. Handcrimper—for crimping the aluminum cap onto the vial, Chrompack catalog number 10231, or equivalent.
5. HPLC equipment—consisting of liquid pump (e.g., Waters model 510, or equivalent); injector (e.g., Waters model UK 6, or equivalent); reversed-phase column [see Procedure, 2, (d)]; fluorimetric detector (e.g., Perkin-Elmer model LS 4, or equivalent); integrator (e.g., Hewlett-Packard model 3390A, or equivalent).
6. HPLC syringes—10 and 20 µL.
7. Silanized glass wool.

REAGENTS

1. Light petroleum ether—bp 40–60°C, or Hexane—analytical grade, redistilled over potassium hydroxide pellets (4 g/L).
2. Acetonitrile—HPLC grade.
3. Tetrahydrofuran (THF)—HPLC grade.
4. Toluene—HPLC grade.
5. Sodium sulfate—analytical grade, granular, anhydrous.
6. Water—double distilled, filtered over a 0.45 µm membrane filter (e.g., Norganic Water Purification System from Waters, catalog no. 83097, or equivalent).
7. Alumina—activity 4 grade, prepared as follows: In suitable vessel, neutral aluminum oxide, activity super I (supplied by Woelm, Eschwege, Germany) is deactivated by the addition of 10 mL distilled water to 90 g alumina. The vessel is shaken for about 15 min and equilibrated for 24 hr before use. The alumina is stored in a closed vessel at ambient temperature.
8. Benzo(a)pyrene (BaP)—see Notes, *Caution*, purity 99.0%, supplied by the Commission of the European Community, Community Bureau of References, BCR, Rue de la Loi 200, B-1049 Brussels, Belgium.

PROCEDURE

1. Preparation of standard BaP solutions—
   (a) Weigh accurately about 12.5 mg BaP in a 25 mL volumetric flask. Dissolve the BaP in toluene and dilute with toluene to the mark. The BaP concentration of this standard stock solution is about 0.5 mg/mL. When stored in the dark at 4°C, this standard stock solution is stable for at least 6 months.
   (b) Prepare BaP standard solutions of about 0.2 µg/mL and 0.01 µg/mL by diluting aliquots of the standard stock solution of BaP with acetonitrile.
2. HPLC conditions—
   (a) Eluent—acetonitrile/deionized water, 88/12, v/v.
   (b) Flow—about 1 mL/min.
   (c) Precolumn—7.5 cm × 4.6 mm i.d. (one only), with Lichrosorb RP-18, 5 µm (e.g., Merck catalog no. 9333, or equivalent).

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(d) Column—reversed-phase HPLC column, C18 polymeric phase, 25 cm × 4.6 mm i.d. (e.g., spheresorb PAH, Supelcosil LC-PAH, Vydac PAH). The LC column should be capable of achieving baseline resolution and resolving benzo(k)fluoranthene, benzo(a)pyrene, benzo(e)pyrene, and perylene.
(e) Detector—excitation wavelength at 384 nm, slit 10 nm; emission wavelength at 406 nm, slit 10 nm.

3. HPLC calibration—
   (a) Prepare a 4-point calibration curve by injecting 0.04 ng (4 µL, 0.01 ng/µL), 0.2 ng (20 µL, 0.01 ng/µL), 1 ng (5 µL, 0.2 ng/µL) and 2 ng (10 µL, 0.2 ng/µL) of the BaP standard solutions. Use peak height or peak area, whichever is convenient. If an autosampler (constant volume injected) is used, prepare four standard BaP solutions of concentrations such that the amounts injected correspond to 0.04, 0.2, 1, and 2 ng, respectively. Use the same injection procedure for test portions and standards. Flush the sample loop with acetonitrile after each injection of standard or test portion.
4. Sample cleanup—
   (a) Weigh to the nearest mg about 2 g of the oil sample in a 10 mL volumetric flask. Dissolve the oil in light petroleum ether and fill to the mark with light petroleum ether.
   (b) Fill the chromatography column (Apparatus, 1) half-full with light petroleum ether.
   (c) Rapidly weigh 22 g alumina (activity 4 grade) into a small beaker and transfer the alumina immediately to the column. Promote settling of the alumina by gently tapping the column.
   (d) Add anhydrous sodium sulfate on the top of the column in a layer of about 3 cm.
   (e) Open the plug and allow the light petroleum ether to become level with the top of the sodium sulfate layer.
   (f) Place a 20 mL measuring flask under the column.
   (g) Pipet 2.00 mL of the oil solution onto the column and rinse the column with minimal amounts of light petroleum, allowing the solvent layer to run into the sodium sulfate layer between rinsings.
   (h) Elute the column with light petroleum ether at a flow rate of 1 mL/min.
   (i) Discard the first 20 mL of eluate.
   (j) Collect the next 60 mL of eluate in a 100 mL round-bottomed flask.
   (k) Concentrate the eluate in a water bath at 65°C to about 0.5–1 mL and transfer the concentrated solution into a preweighed (to the nearest 0.1 mg) crimp-top minivial of about 1 mL volume. Continue the evaporation at 35°C under a gentle stream of nitrogen to dryness.
   (l) Weigh the minivial to the nearest 0.1 mg and calculate the weight (R, mg) of residue.
   (m) Stopper the minivial with Teflon™ -layered septum and an aluminum cap and store at 4°C.
5. Sample analysis—
   (a) With a syringe, inject 20.0 µL THF into the vial from Procedure, 4, (m).
   (b) Dissolve the residue in the vial in THF by careful swirling, avoiding contact of the solvent with the septum.
   (c) Inject an accurately known volume of about 10 µL of the solution onto the column and start the HPLC run.

CALCULATIONS

1. Calculate from the calibration curve the amount of BaP in the injected volume of the test solution (see Notes, 1).

$$P = \frac{5 \times A \times (20.0 + 1.25\ R)}{V \times G}$$

Where—

A = amount of benzo(a)pyrene, in ng, obtained from calibration curve
G = the amount of oil in g weighed in the measuring flask
V = the volume in µL of the THF solution injected onto the HPLC column
R = the mass of the residue in mg, assuming that the density of the residue is 0.8 and, therefore, the volume is 1.25 R µL
P = BaP level in µg/kg in the oil sample

PRECISION (see References, 2, 3)

1. Repeatability—This value, indicated with r in Tables 1–3, is a measure for the difference obser ved when repeating the analysis on the same test sample in the same laboratory with the same analyst. At level 1 (actual concentration 0.75 ppb), results of two analyses on the same test sample are expected to differ not more than 0.57 ppb with a probability of 95%. At level 2 (actual concentration 2.55 ppb), the corresponding value is 0.66 ppb (References, 4).
2. Reproducibility—This value, indicated with R in Tables 1–3, is a measure for the difference in analytical results observed when the same test sample is analyzed in different laboratories with different analysts. At level 1 (actual concentration 0.75 ppb), results of single analyses in two different laboratories are expected to differ not more than 0.97 ppb with a probability of 95%. At level 2, the corresponding figure is 2.12 ppb (References, 4).

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NOTES

Caution

BaP is a known carcinogen. All work using BaP should be carried out in a fume hood. Gloves should be worn at all times to minimize exposure.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

NUMBERED NOTES

1. Using the calibration curve determined in Procedure, 3, BaP levels of 0.1–10 µg/kg (ppb) in the oil can be determined. If the BaP concentration in the oil sample is above 10 ppb, then an aliquot of the test portion should be injected. This can be achieved by diluting the residue from Procedure, 5, (a) with THF, or by injecting a smaller volume of the residue solution from Procedure, 5, (c) onto the HPLC column.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats, and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.608.
2. International Union of Pure and Applied Chemistry working group report, St. Andrews, Scotland, August 1988.
3. International Union of Pure and Applied Chemistry working group report, July 1990, pp. A1, A2, M1, M2.
4. Results of the collaborative study and the standardized method published in *Pure Appl. Chem. 63:*1659 (1991).

Table 1
Repeatability (r) and reproducibility (R) of results for BaP in fish oil from first collaborative study.

			Mean (ppb)		Repeatability			Reproducibility		
Level	Batch	Number of labs	Batch	Level	SD	r	RSD_r, %	SD	R	RSD_R, %
1	1	10	0.59	0.76	0.20	0.57	26.8	0.35	0.97	45.8
	2	10	0.93							
2	1	10	2.13	2.25	0.24	0.66	10.5	0.76	2.12	33.8
	2	10	2.37							

Table 2
Repeatability and reproducibility for BaP in fish oil from second collaborative study.

			Mean (µg/kg)		Repeatability			Reproducibility		
Level	Batch	Number of lab	Batch	Level	SD	r	RSD_r, %	S_R	R	RSD_R, %
1	1	16	4.54	4.76	0.85	2.38	17.9	1.74	4.88	36.6
	2	16	4.98							
2	1	16	2.84	3.26	0.69	1.92	21.1	0.99	2.76	30.3
	2	16	3.64							
3	1	16	0.82	0.95	0.24	0.66	25.0	0.28	0.80	30.1
	2	16	1.08							

Table 3
Repeatability and reproducibility for BaP in rapeseed oil.

			Mean (µg/kg)		Repeatability			Reproducibility		
Level	Batch	Number of labs	Batch	Level	SD	r	RSD_r, %	S_R	R	RSD_R, %
High	1	17	4.02	4.16	0.63	1.77	15.2	0.82	2.28	19.6
	2	17	4.31							
Med	1	17	2.38	2.54	0.44	1.22	17.2	0.93	2.60	36.5
	2	17	2.71							
Low	1	17	0.67	0.82	0.10	0.29	12.5	0.43	1.21	53.0
	2	17	0.97							

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Determination of Polymerized Triglycerides by Gel-Permeation HPLC

DEFINITION

This method is for the determination of polymerized triglyceride content in oils and fats. The separation is based on the relative retention of solubilized polymer molecules in terms of their molecular size by gel-permeation chromatography.

SCOPE

This method is applicable to vegetable and animal fats and oils, heated or not. It has been applied to the determination of polymers in animal feed fats and used frying fats or oils at levels >3%. Not suitable for test samples containing Olean.

APPARATUS

Note—HPLC equipment suitable for gel-permeation chromatography is required, including the following:

1. Solvent reservoir—with a mobile-phase line filter (pore size 1 µm).
2. HPLC pump—pulseless, with a flow of 0.7–1.5 mL/min.
3. Injection port—with low dead volume, applicable with high pressure, provided with a 20 µL sample loop.
4. Syringe—with a volume of 50–100 µL, compatible with injector.
5. Stainless steel column—length 300 mm, 7.8-mm i.d.
6. Stationary phase—consisting of a high-performance spherical gel made of polystyrene-divinylbenzene microporous particles with 5 µm particle size and 10 nm pore size (see Notes, 1).
   Note—The column must be stored as recommended by the manufacturer or in toluene (solvent B) (Reagents, 2).
7. Detector—refractive index detector with minimum sensitivity at full-scale of at least 1.10×10^{-4} refraction index units, capable of being maintained at a temperature a few degrees above ambient temperature (e.g., 40°C). If the detector is not provided with built-in heating, a recirculating water bath with constant temperature may be used.
8. Recorder—suitable to display, and to quantify with accuracy, the peak areas. Suggested specifications:
   —response time 1.5 sec (time to give a response of 90% with a signal at 100%)
   —paper at least 20 cm wide
   —chart speed 0.1–10 mm/min
   —sensitivity 1–100 mV, adjustable to signal output from the refractive index detector
9. Calculator and/or integrator and/or suitable chromatography data system.
10. Round-bottom flask—250 mL, with suitable heating mantle capable of heating the contents of the flask to 180 ± 2°C.
11. Thermometer—to measure a temperature of 180 ± 1°C.
12. Conical flask—25–50 mL capacity, for test sample preparation.

REAGENTS

1. Tetrahydrofuran (solvent A)—analytical reagent grade (see Notes, *Caution*).
2. Toluene (solvent B)—analytical reagent grade (see Notes, *Caution*).
3. Soybean oil—refined.
4. Sodium sulfate—anhydrous.

PROCEDURE

1. Starting up HPLC equipment—
   (a) It is advisable to read and to follow carefully the manufacturer's recommendations. In choosing the optimal working conditions, take into account the following variables:
   —choice of column for optimal separation
   —concentration of the test sample
   —sensitivity of the refractive index detector
   —constant temperature of the refractive index detector
   —use of column heater
   —pump speed
   —optimal pump performance
   —straight baseline
   —time of analysis

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(b) To condition and stabilize the column before test sample analyses are carried out, the mobile phase must be changed stepwise from the storage solvent or toluene (solvent B), to tetrahydrofuran (solvent A). Prepare mixtures of solvent B with increasing amounts of solvent A (e.g., step 1, 25% A in B; step 2, 50% A in B; step 3, 75% A in B; step 4, 100% A). To stabilize the column, pump solvent A for at least 12 hr. The mobile phase must also be changed stepwise when changing back to solvent B for storage of the column.
(c) Pump tetrahydrofuran at a rate of 1 mL/min to purge the whole system up to the injection valve.
(d) Connect the column to the injection valve and wash it with about 30 mL of tetrahydrofuran.
(e) Connect the column to the sample cell of the detector.
(f) Fill the reference cell with tetrahydrofuran.
(g) Adjust the mobile-phase flow to 0.5-1.0 mL/min.
(h) Wait until the system becomes stabilized (no appreciable deviation of the baseline).

2. Preparation of standard oil—With the aid of a heating mantle, heat a 250 mL round-bottom flask containing approximately 185 mL of refined soybean oil to 180 ± 2°C until the amount of polymerized triglycerides is between 10 and 15%, analyzed and calculated as noted in Procedure, step 5, below.
3. Preparation of test sample—If the test sample is not completely melted at ambient temperature, heat the test sample to a temperature of not more than 10°C above the temperature at which it is completely melted. Mix the test sample thoroughly.
4. Determination of theoretical plates—Analyze the standard oil (Procedure, 2) according to step 5 in Procedure, in such a way that the peak of the triglycerides is more than half the chart width (refer to Fig. 1). Calculate the theoretical plate number with the formula:

$$n = 16 \frac{dR}{(w)^2}$$

Calculate the resolution with the formula:

$$R = \frac{\Delta}{w}$$

Where—

n = theoretical plate number

R = resolution

dR = elution volume from the start of the chromatogram to the peak maximum for triglycerides, in mm

w = width of the triglycerides peak at the baseline, measured between points of intersection between tangents and baseline, in mm

Δ = the distance between peak maxima of the triglycerides peak and the neighbor peak of the polymerized triglycerides (peaks numbered 3 and 2, respectively, in Fig. 1), in mm

Conditions for the analysis shall be chosen in such a way that the theoretical plate number (n) for the triglycerides is at least 6000 and the resolution (R) as calculated above is at least 1.

5. Analysis—
(a) Accurately weigh 0.2 ± 0.01 g prepared test sample into a conical flask. Add 15 mL tetrahydrofuran (see Notes, 2), swirl, and leave until the fat is completely dissolved. The test samples must be free from water. Add about 50 mg anhydrous sodium sulfate, shake and wait 2 min. Filter through medium-porosity filter paper.
(b) Using the syringe, take 50-100 µL of the test solution. Fill the injection loop with 20 µL of filtered (see Notes, 3) test solution.
(c) Inject the test sample onto the HPLC column and simultaneously switch on the integrator, or note injection point on chart recorder.
(d) The processes above may be automated using a heated autosampler.
(e) Elute the test sample with a mobile-phase flow rate of 0.5-1 mL/min. At a flow rate of 1 mL/min, the analysis takes about 10 min.

RESULTS

1. The basic chromatographic pattern (e.g., Fig. 1) resulting from this determination shows a main peak, representing monomeric triglycerides (MW about 900), and one or several smaller peaks with a shorter retention time than the triglycerides peak, representing polymerized triglycerides (dimers and oligomers).
2. Three typical chromatograms are presented (Fig. 2). Under suitable conditions, triglycerides and polymerized triglycerides can be separated with good resolution (Fig. 2, A and B), even at low levels of polymerized triglycerides (Fig. 2, A). However, with those fats and oils in which complex degradation phenomena (possibly hydrolysis) may have occurred, the peak pattern preceding the triglyceride peak is less well defined (Fig. 2, C), with corresponding difficulties in calculation.

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3. Figure 1 is the chromatograph of the standard oil sample (heated soybean oil, prepared as noted in Procedure, 2), used to check the efficiency and the resolution power of the column, which should be at least as good as shown in the reference chromatogram.

CALCULATIONS

1. The area normalization method is used to calculate the percentage of polymerized triglycerides, under the assumption that all components of the test sample are eluted.
2. In case there is a valley between the triglycerides peak and the highest peak of the polymerized triglycerides, and the lowest point of that valley is lower than 75% of the height of that highest peak of the polymerized triglycerides, then the amount of polymerized triglycerides has to be calculated using the formula:

$$PT = \frac{A_{PT}}{\Sigma A} \times 100$$

Where—

PT = content of polymerized triglycerides, in % (A/A)

A_{PT} = sum areas of the polymerized triglycerides peaks (these are the peaks with a smaller elution volume than the triglycerides peak)

ΣA = sum of areas of all peaks.

Give the result to one decimal place.

3. In case there is no valley between the triglycerides peak and the highest peak of the polymerized triglycerides, or the lowest point of that valley is higher than 75% of the height of that highest peak of the polymerized triglycerides, then the amount of the polymerized triglycerides has to be calculated using the formula (see Notes, 4):

$$PT = \frac{A'_{PT}}{\Sigma A} \times 100$$

Where—

PT = content of polymerized triglycerides, in % (A/A)

A'_{PT} = area above the baseline measured from the start until the elution volume (dR – w/2) (see Procedure, 4)

ΣA = sum of areas of all peaks

Give the result to one decimal place.

4. For calculating A_{PT} two cases are possible:
   (a) Good resolution between peaks (R > 1) (Fig. 2, A and B). The general methods of integration (manual and electronic) can be used to calculate individual and total areas.
   (b) Poor resolution between peaks (R < 1) (Fig. 2, C). It is assumed that all components before (dR – w/2) are polymerized triglycerides (see Fig. 1), where w is the width of the triglycerides peak at the baseline, measured between points of intersection between tangents and baseline, and dR is the retention distance from the start of the chromatogram to peak maximum for triglycerides.

   With a manual technique, it is necessary to determine the triglyceride peak area by triangulation.

   With electronic integration, the integrator has to be carefully adjusted to integrate all the surfaces included between the curve and the baseline.

PRECISION

1. The statistical analysis of collaborative study results is shown in Table 1.

NOTES

Caution

Tetrahydrofuran is flammable and a dangerous fire risk. Flammable limits in air are 2–11%. It is toxic by ingestion and inhalation. The TLV in air is 200 ppm. A fume hood should be used at all times when using tetrahydrofuran.

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

NUMBERED NOTES

1. Suppliers: Phenomenex, Torrance, CA, USA, or Polymer Laboratories, Stretton, Shropshire, United Kingdom.
2. To avoid possible interferences from tetrahydrofuran breakdown products, collect tetrahydrofuran at the end of a chromatographic run, after the tetrahydrofuran breakdown products have eluted from the column, and use this tetrahydrofuran to dissolve the test sample.

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3. To avoid blockage of the porous fritted filter at the top of the column, when the test sample contains suspended particles, filter the test sample through 1 µm pore size polytetrafluoroethylene (Teflon™ TFE) or cellulose esters filter (commercial LC disposable syringe filter units are suitable).
4. With an increasing amount of products of hydrolysis in a feed fat or in used frying oils, the peak pattern preceding the triglycerides peak becomes less clear or even disappears. It is, however, important that for these fats (especially feed fats) the amount of polymerized triglycerides can be determined. In Calculations, 3, it is assumed that all components eluting before (dR – w/2) are polymerized triglycerides.

REFERENCES

1. *Nederlandse Norm,* Draft NEN 6348, November, 1986.
2. *Pure Appl. Chem. 63:*1163 (1991).
3. *Standard Methods for the Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.508.

Table 1
Statistical analysis of collaborative study results.

Test Sample no.	1	2	3	4
No. of labs	17	17	17	16
No. of assays	34	34	34	32
No. of labs retained[a]	16	15	16	15
No. of assays retained[a]	32	30	32	30
Average value, PT, %	9.7	1.8	5.2	10.0
Repeatability standard deviation (S_r)	0.3	0.1	0.1	0.2
RSD_r, %	3.2	5.5	1.9	2.2
Repeatability value, r ($2.83 \times S_r$)	0.9	0.3	0.3	0.6
Reproducibility standard deviation (S_R)	0.4	0.4	0.3	1.8
RSD_R, %	4.6	23.7	6.71	12.2
Reproducibility value, R ($2.83 \times S_R$)	1.3	1.2	1.0	3.5

[a] Outliers detected and eliminated by the Dixon and Cochran test methods.

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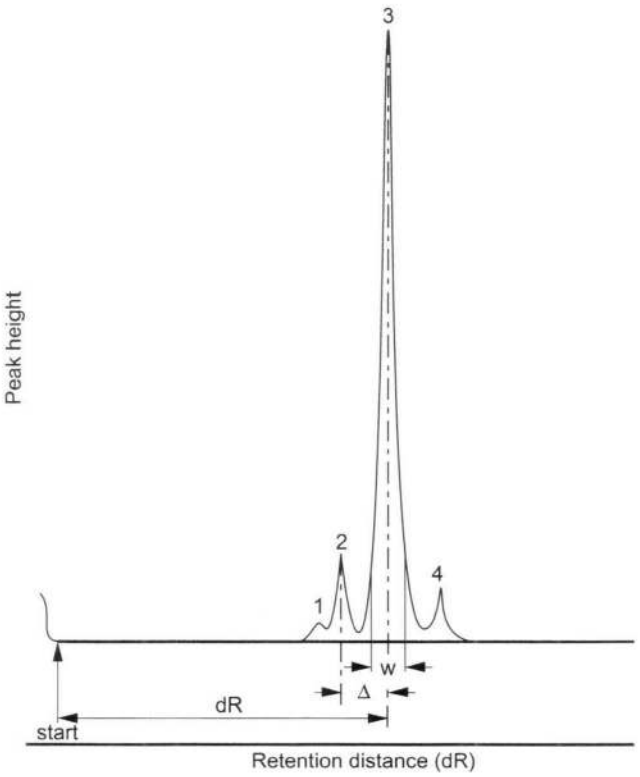

Figure 1. Determination of resolution and theoretical plate number for standard oil (heated soybean oil). 1 and 2, polymerized triglycerides; 3, triglycerides; 4, free fatty acids.

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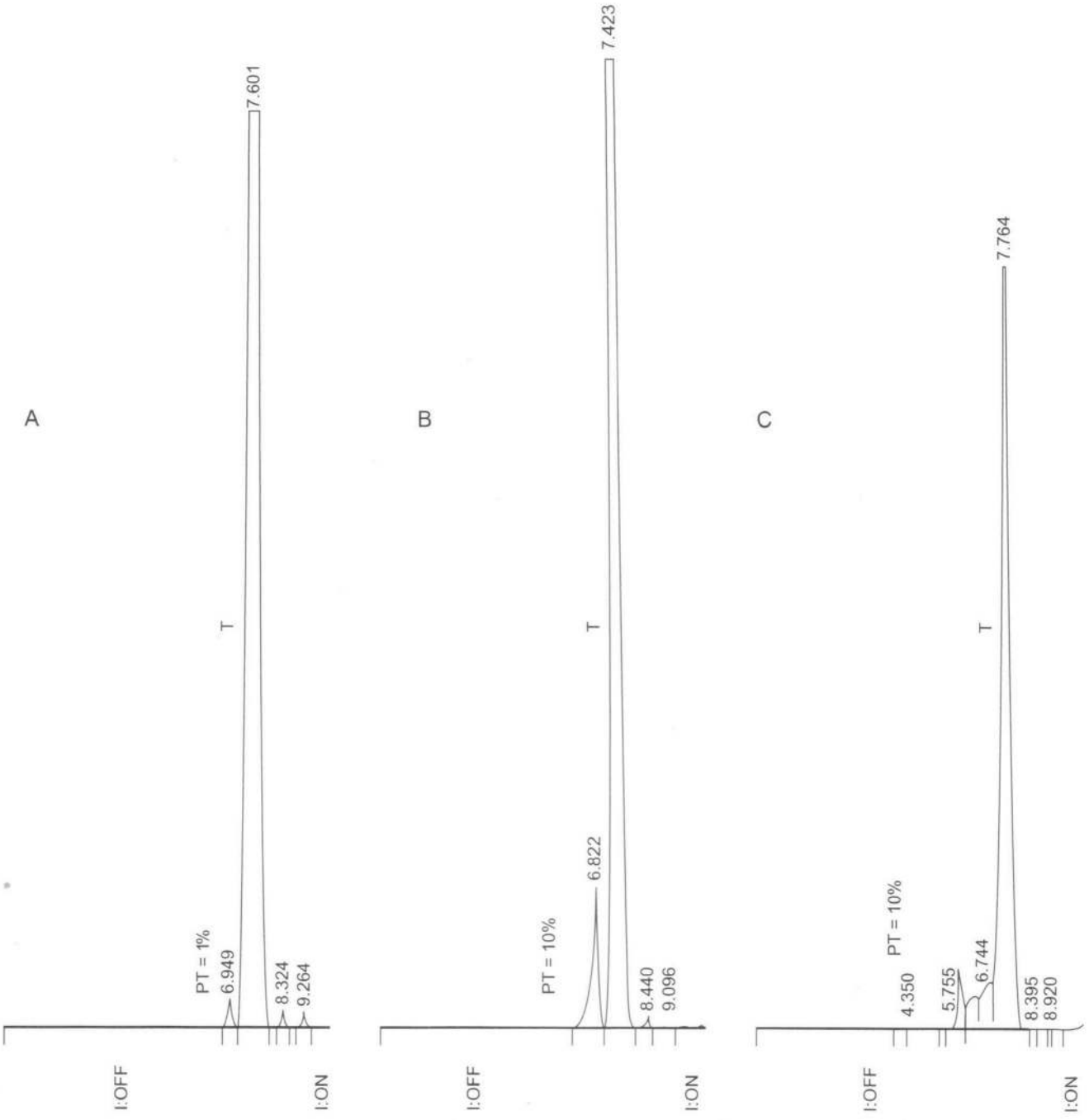

Figure 2. Typical chromatograms. T, triglycerides; PT, polymerized triglyceride; I, injector. Conditions: chart speed, 0.5 cm/min; attenuation, 16; zero, 10%; 5 min/tick.

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Separation and Analysis of Organophosphorus and Organochlorine Pesticide Residues from Edible Oils Using Multiple Solid Phase Extraction Cartridges

DEFINITION

This method is a practical and effective procedure for the separation of trace levels of pesticides from edible oils in preparation for analysis by gas chromatography. The method combines a reverse-phase system and a normal-phase solid-phase extraction (SPE) cleanup system for the determination of organophosphate (OP) and organochlorine (OC) pesticide residues in olive oil and sunflower oil. Partitioning of the OP and OC pesticides is effected using disposable mini-columns of a macroporous Kieselguhr-type material (Extrelut QE) (References, 1,2).

SCOPE

This procedure is applicable to olive oil, sunflower oil, and other vegetable oils, and to butterfat and similar fats.

APPARATUS

1. Solid-phase extraction cartridges—Sep-Pak C-18, 5 g and Sep-Pak Alumina N, 5 g may be obtained from Waters Associates (Milford, MA, USA).
2. Extrelut QE disposable columns—3 mL capacity, obtained from EM Science (Gibbstown, NJ, USA).
3. Gas chromatographs—
   (a) OP quantitation—GC equipped with an FPD (P mode). Column, 2–3 mm × 0.53 mm i.d. intermediate polar deactivated retention gap and a 15 m × 0.53 mm i.d., 1.0 µm film thickness, 50% phenyl methylpolysiloxane fused silica open tubular column (J & W Scientific, Fullerton, CA, or equivalent). Injector liner, direct flash vaporization type. Nitrogen carrier gas with ca. 20 mL/min flow rate. Column temperature, 170°C; detector and injector temperature, 220°C. Adjust GC and recorder sensitivities so that ca. 1.0 ng chlorpyrifos injected gives ca. 50% Full Scale Detection (FSD).
   (b) OC quantitation—GC equipped with an ECD. Column, 30 m × 0.53 mm i.d., 1.5 µm film thickness with a 1 m × 0.53 mm i.d. retention gap of uncoated, deactivated fused silica. Injection liner, direct flash vaporization type containing inlet sleeve and glass wool (References, 5). Detector makeup gas, nitrogen at ca. 55 mL/min flow rate. Carrier gas, hydrogen at 15 mL/min flow rate. Column temperature, 200°C. Injector temperature, 220°C; detector temperature, 350°C. Adjust GC and recorder sensitivities so that 0.1 ng heptachlor epoxide injected gives about 50% FSD.
4. Vacuum rotary evaporator.

REAGENTS

1. Solvents (see Notes, *Caution*)—distilled in glass, or equivalent, and residue grade: acetone, petroleum ether, acetonitrile, dichloromethane, hexane, isooctane, and methyl alcohol.
2. Standards—diazinon, methamidophos, chlopyrifos, acephate, methyl parathion, azodrin, aldrin, heptachlor epoxide, dieldrin, endrin, pp´DDT, and pp´methoxychlor reference standards obtained from the U.S. Environmental Protection Agency, Pesticide and Industrial Chemicals Repository, Research Triangle Park, NC 27711.
   (a) An OP working standard solution is prepared in acetone to contain ca. 0.2 µg/mL diazinon, 0.17 µg/mL methamidophos, 0.7 µg/mL chlorpyrifos, and 0.2 µg/mL acephate.
   (b) An OC working standard solution is prepared in 1:1 isooctane-hexane to contain ca. 0.03 µg/mL aldrin, 0.04 µg/mL heptachlor epoxide, 0.04 µg/mL dieldrin, 0.04 µg/mL endrin, 0.07 µg/mL pp'DDT, and 0.21 µg/mL methoxychlor.

PREPARATION OF TEST SAMPLE

1. Mix the edible oil well and weigh out a 4 g test sample into a glass tube. Liquefy solid fats by warming to ca. 50°C. Separate fat from butter or margarine by removing a portion of fat for analysis. Mix the oil or liquid fat and weigh out a 4 g test portion into a glass tube. Add hexane to obtain a total volume of 7 mL for a concentration of approximately 0.6 g lipid/mL and mix well.

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PROCEDURE

1. Test of alumina N cartridge suitability—
   (a) Test each new lot of alumina N cartridges for proper elution of OC pesticides. Prepare a solution of mixed pesticide standard in hexane to contain per mL ca. 0.15 µg heptachlor epoxide, 0.12 µg aldrin, 0.16 µg dieldrin, 0.16 µg endrin, 0.38 µg pp′DDT, and 0.90 µg pp′methoxychlor. Test each new lot of alumina N cartridges as follows: weigh out 4 g pesticide-free butterfat and dilute to 7 mL with hexane and mix well. Refer to Fig. 1 for column-cartridge configuration. Pipette 3.0 mL of the butterfat solution onto the Extralut QE column and take the test portion through the extraction and SPE cleanup procedure through "split the solution in half and transfer each half to a 100 mL evaporating flask." Evaporate both butterfat blanks to dryness using a vacuum rotary evaporator with water bath at 50–55°C. Treat both butterfat blanks to the following procedure: Add 2.0 mL of the mixed pesticide standards to the butterfat residue and dissolve in 8 mL of 20% methylene chloride in hexane. Deactivate the 5 g alumina N cartridge by passing 10 mL of acetonitrile containing 0.2 mL water through the cartridge prior to washing it with 50 mL of 20% methylene chloride in hexane. Discard this prewash and elute the standard solution through the alumina N cartridge with an additional 25 mL of 20% methylene chloride in hexane. Discard this prewash and elute the standard solution through the alumina N cartridge with an additional 25 mL of 20% methylene chloride in hexane (total 33 mL) following the steps in the procedure for determination of OC pesticides. Concentrate the eluents to 8.0 mL hexane in a glass tube and determine the recovery of each pesticide by injecting into a GC equipped with an ECD using a standard quantitation. Transfer the contents of the glass tube to a weighed fat flask and evaporate to dryness on a water bath at 50–55°C under nitrogen. Dry the flask and weigh to determine lipid carryover. Satisfactory alumina N will allow a 90–105% recovery of the pesticides and a lipid carryover of 1.5 mg or less.
   (b) If the alumina N does not meet the specifications in (a), a second unused cartridge should be tested as in (a) but omitting the deactivation of the alumina with the 10 mL of acetonitrile containing 0.2 mL of water.
   (c) If the alumina N lot does not meet specifications after testing as described in (a) or (b), it should not be used.
2. Pipet 3.0 mL (ca. 1.8 g) of prepared test portion in hexane onto the Extrelut QE column (Apparatus, 2) and allow 10 min for even distribution of the lipid solution. Meanwhile, place the C-18 cartridge (Apparatus, 1) on a vacuum system, prewash with about 50 mL methyl alcohol (Reagents, 1) at a flow rate of about 5 mL/min, and discard the washings.
3. Place the Extrelut QE column over the C-18 cartridge and elute both with 20 mL of methyl alcohol/acetonitrile (1:1, v/v), saturated with hexane, using light air pressure on the Extrelut QE column and light vacuum on the C-18 cartridge to maintain a 3 mL/min flow. (Obtain air pressure by connecting an air jet to a vacuum bottle half filled with cotton to catch particulate matter. Attach a hollow rubber tube connected to a hollow glass tube and a #1 rubber stopper to the vacuum bottle. Fit the stopper onto the top of the Extrelut column.) Allow the solution to pass through the C-18 cartridge using gravity flow only and then elute *only* the C-18 cartridge with 20 mL methyl alcohol using gravity flow. Collect eluate in a suitable receiver.
4. Transfer the eluate to a 50 mL volumetric flask using acetone (Reagents, 1). Dilute to volume and mix well. Split the solution in half and transfer each half to a 100 mL evaporating flask. If left in methyl alcohol-acetonitrile overnight, low recovery will result. Evaporate the OP solution to dryness and the OC solution to ca. 5 mL using a vacuum rotary evaporator with a water bath at 50–55°C.
5. (a) For the OC analysis, add 20 mL isooctane and evaporate to ca. 2–3 mL using the vacuum rotary evaporator with the water bath set at 60–65°C.
   (b) For the OP analysis, take one of the flasks and reconstitute the residue in acetone and transfer to a suitable glass tube.

OC Analysis

1. Assume the alumina N cartridge (Apparatus, 1) must be deactivated with 10 mL of acetonitrile containing 0.2 mL of water. If this is not the case, omit the deactivation step.
2. Dissolve the residue, from half of the Extrelut/C-18 eluate [Procedure, 5(a)] in 8 mL of 20% dichloromethane in hexane. Place a 5 g alumina N cartridge or equivalent on a vacuum manifold. Deactivate by adding 10 mL acetonitrile containing 0.2 mL of water. Prewash with 50 mL of 20% dichloromethane in hexane at ca. one drop per second and discard the washings. Transfer the test solution onto the alumina N cartridge and elute with an additional 25 mL of 20% dichloromethane in hexane using gravity flow. Collect eluate in a suitable receiving flask.
3. Transfer the eluate to a 100 mL evaporating flask using ca. 10 mL of acetone and evaporate to less than 3 mL but not to dryness using the vacuum rotary evaporator with a water temperature of 50–55°C. Transfer to a suitable glass tube with hexane and dilute or evaporate to 4.0 mL and proceed with quantitation of 0°C.
4. Inject an appropriate volume of test solution and quantitate by area or peak height. Correct for small amounts of GC interference that might be present in the Extrelut QE or other reagents by carrying a pesticide-free fat or oil (of the same type as that being analyzed) through the same analytical steps and subtracting any interfering GC peaks from any corresponding pesticide peaks found in the oil or fat being analyzed.

OP Analysis

1. Dilute or evaporate the test solution [Procedure, 5(b)] to 5.0 mL and inject an appropriate volume of test solution into the GC and screen for OP. Make additional dilutions or evaporations as needed. Quantitation is carried out with both a matrix standard (Reference, 4) and a neat standard. The matrix protects the OP compounds from adsorption and/or decomposition in hot vaporizing injectors, ensuring a more complete transfer from injector to column as compared to results obtained

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when standards in matrix-free solvent are used. In the absence of matrix, recoveries in excess of 100% are observed when neat standards are used for calibration (1,10).

Note—Acetone solutions tend to concentrate rapidly due to solvent evaporation. The volume of both test solution and standard solution must be checked closely and adjusted to avoid serious quantitation errors.

GC ANALYSIS OF ORGANOPHOSPHORUS RESIDUES (References, 4)

1. Adjust the GC detector and recorder sensitivities so that 0.2 ng of chlorpyifos injected produces about 50% fullscale deflection. Inject 5 µL (for OP determination) of test solution into the GC system and screen for organophosphorus pesticides. Dilute test solutions further with acetone as necessary.
2. For quantification of the residues detected, prepare a "matrix standard solution" as follows: Select a test solution of a fat matrix (see Notes, 3) of the same type as the test solution to be analyzed, but which has been carried through the method and found to contain no detectable residues of the pesticides in question. Add to this solution an appropriate volume of standard solution, then dilute with acetone, or concentrate (with a stream of nitrogen and a 50°C water bath) to the same final volume as the test solution to be analyzed.
3. Determine the pesticide residues in the test solution by comparison of peak heights or areas with the matrix standard so prepared.

RESULTS AND DISCUSSION

1. The procedure consists of an on-column partitioning on a macroporous Kieselguhr material (Extrelut QE) in place of the usual liquid-liquid partitioning step. Removal of highly polar and nonpolar components of olive oil and sunflower oil is effected by a combination of SPE cartridges containing reverse-phase (C-18) and normal-phase packings. The C-18 column strongly retains lipids, which typically consist of acids and esters with long hydrocarbon chains, while the pesticides with relatively little alkyl content are readily eluted. Sep-Pak cartridges containing 5 g of C-18 packing were tested and found to provide adequate cleanup of lipids for OP pesticide quantitation using the FPD. The combined Extrelut QE column and C-18 cartridge retained 98% of the lipids. A matrix standard solution was used for the quantitation of the OP residues by FPD. This technique and the rationale for using it is described in References, 4 and will therefore not be discussed here. Recent studies using a widebore capillary column combined with test sample injection on-column have eliminated the need for a matrix standard. The results of these studies are being finalized and will be published.
2. Additional cleanup of the lipid matrix by normal phase SPE is required to quantitate the OC pesticides by GC-ECD. Adsorption SPE is typically used to extract moderately polar compounds from nonpolar solutions. The active surface of the normal-phase adsorbent retains polar coextractives, whereas the relatively nonpolar OC pesticides are unretained. Adsorption chromatography using silica, Florisil, and alumina N cartridges was evaluated. Alumina N cartridges produced cleaner GC-ECD chromatograms than either silica or Florisil cartridges. Sep-Pak cartridges containing 5 g alumina N packing provided optimal retention of the lipid material remaining in the split eluate after C-18 cartridge cleanup.
3. The ODS bonded silica and alumina N cartridges were repeatedly regenerated and reused throughout this investigation. Following elution of pesticides, the cartridges were washed with at least 50 mL of dichloromethane to remove lipids and traces of pesticides, whereas the Extrelut QE columns were used only once and discarded, as recommended by the manufacturer.
4. Recoveries of OC and OP pesticides from fortified olive oil and sunflower oil are shown in Table 1 and Table 2, respectively. The average recoveries for OC pesticides ranged from 79.2 to 93.5% and for OP pesticides from 78.9 to 100.9% in both matrices. For OP pesticides, the limit of quantitation defined as the pesticide level producing a peak height of about 10% FSD for 0.9 mg test sample equivalent (5 µL from a 5 mL dilution) ranges from about 0.03 ppm diazinon to about 0.23 ppm azodrin. For OC pesticides, the limits of quantitation for 0.9 mg test sample equivalent (4 µL injected from a 4 mL dilution) range from about 0.04 ppm aldrin to 0.25 ppm pp′methoxychlor. Chromatograms of fortified and unfortified olive oil for OC and OP quantitation are shown in Figure 2 and Figure 3, respectively.

CONCLUSION

1. The multiple cartridge technique is fast and simple, requiring a minimum of reagents and glassware. Quantitative separation of the analytes from the two lipid matrices was achieved with minimal extraneous interferences in the GC chromatograms. Butterfat and additional edible oils are being tested through the procedure and results will be published in the near future.

NOTES

Caution

Acetone is highly flammable. Forms explosive peroxides with oxidizing agents. Use effective fume-removal device. Do not mix with chloroform.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Dichloromethane (methylene chloride) is toxic and a carcinogen. The TLV is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

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Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Isooctane is flammable and a fire risk. Explosive limits in air are 1.1–6.0%. It is toxic by ingestion and inhalation. A properly operating fume hood should be used when working with this solvent.

Methyl alcohol (methanol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform, and perchloric acid.

NUMBERED NOTES

1. ODS bonded silica and alumina N cartridges can be repeatedly regenerated and reused. Following elution of pesticides, the cartridges were washed with at least 50 mL dichloromethane to remove lipids and traces of pesticides, whereas the Extrelut QE columns were used only once and discarded, as recommended by the manufacturer.
2. For butterfat and similar fats, dichloromethane/hexane (20:80, v/v) should be used for elution of the organochlorine pesticides from the alumina N cartridge to prevent excessive elution of butterfat and similar fats with pesticides.
3. No significant GC-FDP interferences from the test sample matrix were noted with 5 µL injections of the 5 mL acetone dilutions of the test solutions tested (sunflower, corn, safflower, soybean, olive oils; milk fat). However, more concentrated solutions containing lipid residue in excess of about 5 mg/mL produced background peaks in some instances. Olive oil was "dirtier" than the other oils and minor peaks were seen in control sample chromatograms. The practical limit of determination, defined as the pesticide level producing a peak height of about 10% full-scale deflection for 3 mg test sample equivalent (5 µL from a 5 mL dilution) ranges from 0.01 µg/g (diazinon, methamidophos) to 0.08 µg/g (azodrin).

REFERENCES

1. Gillespie, A.M., S.M. Walters, and D.M. Gilvydis, United States Food & Drug Administration, Laboratory Information Bulletin, Number 3633 (1992).
2. Walters, S.M., *Anal. Chim. Acta 236:*77 (1990).
3. Gillespie, A.M., and S.M. Walters, *J. Liq. Chromatogr. 12:*1687 (1989).
4. Gillespie, A.M., and S.M. Walters, *Anal. Chim. Acta 245:*259 (1991).

OTHER REFERENCES

1. This method was submitted by AOCS Uniform Methods Committee Chairperson David Firestone in 1992.
2. Ansari, G.A.S., and P.Y. Hendrix, *J. Chromatogr. 346:*435 (1985).
3. Chiang, T.C.H., and W. Liao, *J. Assoc. Off. Anal. Chem. 70:*100 (1987).
4. DiMuccio, A., A.M. Cicero, I. Camoni, D. Pontecorvo, and R. Dommarco, *Ibid. 70:*106 (1987).
5. DiMuccio, A., A. Ausili, L. Vergori, I. Camoni, R. Dommarco, and L. Gambetti, *Analyst 115:*1167 (1990).

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Table 1
Recovery of organochlorine pesticides from fortified olive oil and sunflower oil test samples[a].

	Olive Oil			Sunflower Oil		
	Added, ppm	Recovered, % avg	Range, n = 2	Added, ppm	Recovered, % avg	Range, n = 3
Aldrin	0.18	79.2	77.0–81.5	—	—	—
Heptachlor Epoxide	0.22	83.8	79.3–88.3	0.22	82.6	76.1–92.8
Dieldrin	0.24	90.9	88.5–93.4	0.24	86.1	82.3–93.8
Endrin	0.24	89.7	86.8–92.6	0.24	83.4	80.2–89.7
pp'DDT	0.42	88.1	84.6–91.7	0.42	82.8	78.7–88.6
pp'Methoxychlor	1.23	90.6	83.7–97.6	1.23	93.5	89.4–95.9

[a]References, 1.

Table 2
Recovery of organophosphorus pesticides from fortified olive oil and sunflower oil test samples[a].

	Olive Oil			Sunflower Oil		
	Added, ppm	Recovered, % avg	Range, n = 2	Added, ppm	Recovered, % avg	Range, n = 3
Diazinon	0.245	90.00	92.7–87.3	0.245	100.9	90.2–106.5
Methdamidophos	0.600	78.90	77.5–80.3	0.600	84.7	78.7–91.5
Chlorpyrifos	0.483	94.80	97.3–92.3	0.483	98.8	93.6–102.1
Acephate	2.960	96.40	98.0–94.9	2.960	93.3	88.9–96.6
Methyl parathion	1.230	82.10	79.5–84.6	1.230	97.0	91.1–100.0
Azodrin	2.170	97.00	98.6–95.4	2.170	97.7	96.3-100.0

[a]References, 1.

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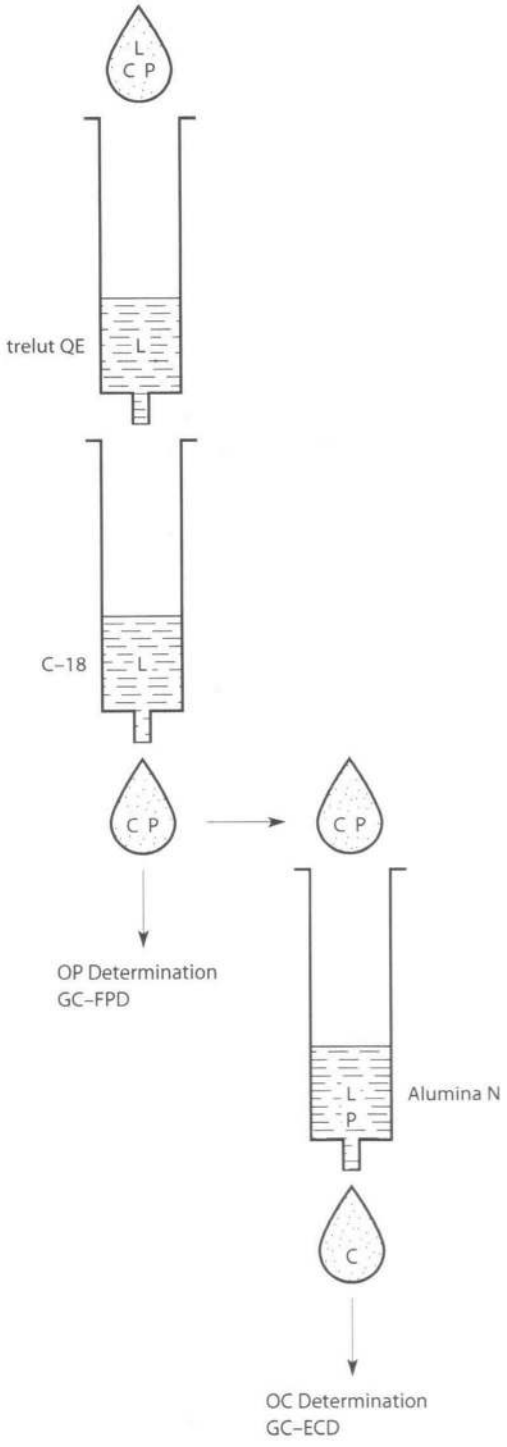

Figure 1. SPE cartridge set-up. Key: L, Lipids; C, OC pesticides; P, OP pesticides. From References, 1.

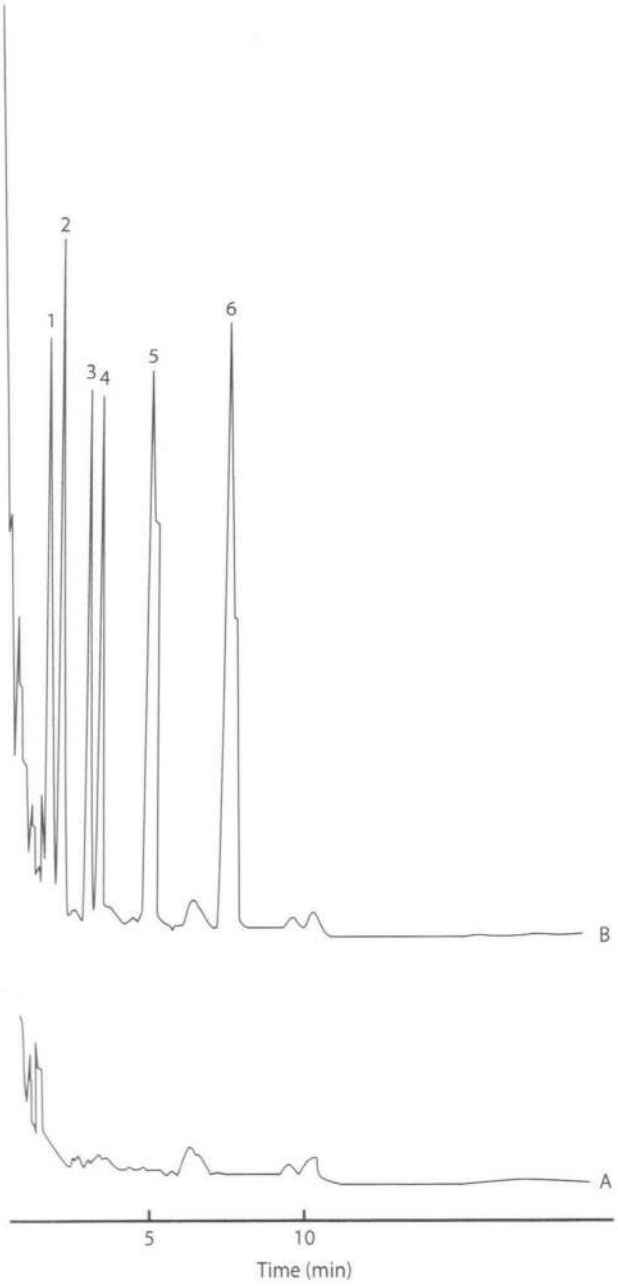

Figure 2. GC-ECD olive oil extract. A: Control. B: Test Sample fortified with 1, 0.18 ppm aldrin; 2, 0.22 ppm heptachlor epoxide; 3, 0.24 ppm dieldrin; 4, 0.24 ppm endrin; 5, 0.42 ppm p, p′ DDT; 6, 1.23 ppm p, p′ methoxychlor. From References, 1.

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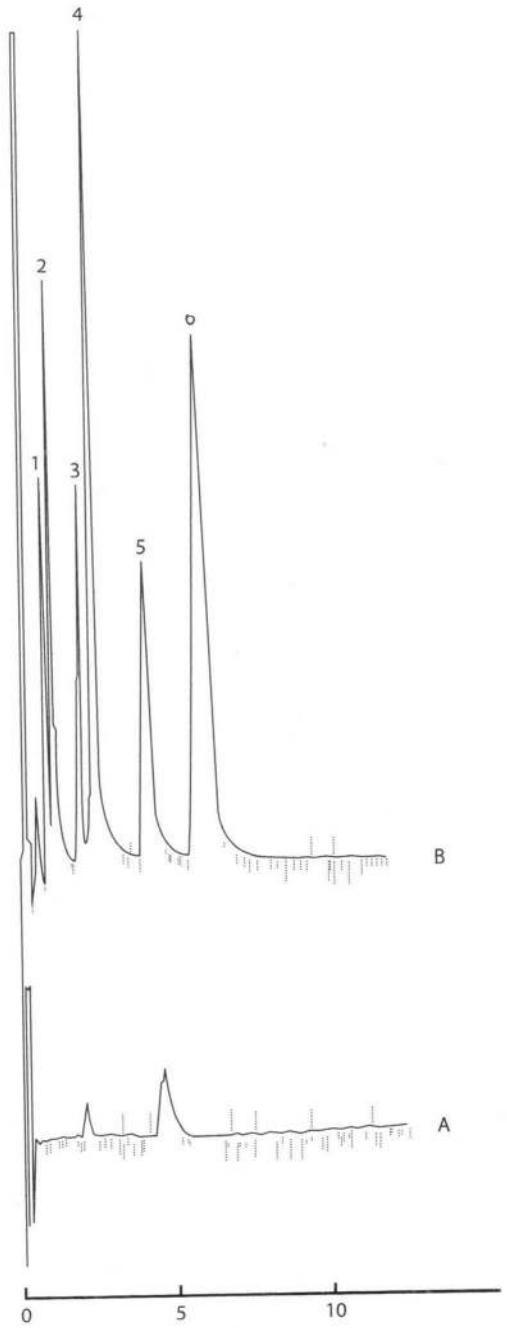

Figure 3. GC-FPD olive oil extract. A: Control. B: Test Sample forified with 1, 0.25 ppm diazinon; 2, 0.60 ppm methamidophos; 3, 0.48 ppm chlorpyrifos; 4, 2.96 ppm acephate; 5, 1.23 ppm methyl parathion; 6, 2.17 ppm azodrin. From References, 1.

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AOCS Recommended Practice Cd 24-95
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Determination of Dimethylpolysiloxanes in Fats and Oils

DEFINITION

A direct aspiration, flame atomic absorption method has been developed for the determination of dimethylpolysiloxanes (DMPS) in fats and oils. The detection limit of the method is approximately 1 µg/g with a standard deviation for test samples containing 1–10 µg/g DMPS of 0.3.

SCOPE

The technique is reasonably rapid, requires a minimum of test sample preparation, and is applicable to the analysis of both hydrogenated and nonhydrogenated products. Dimethylpolysiloxanes or methyl silicones are commonly added to fats and oils to prevent oxidative foaming in products used in frying applications. In spite of their widespread use, methodology for their determination in hydrogenated products remains relatively limited. In general, the methods employed for their determination in fats and oils require an isolation of the DMPS from the fat followed by an atomic absorption (AA) determination of silicon in the isolates. One of the more frequently applied techniques was developed by Neal and co-workers (References, 1), who reported on the low temperature extraction of DMPS from fats and oils with petroleum ether followed by AA analysis of the extract. This technique, while successfully applied in some laboratories, is a reasonably tedious and technique-dependent method for the determination of DMPS. More recently, Kundu (References, 2) reported on a method employing flameless AA. In this work, the fat is converted to a soap, the siloxanes extracted into an organic solvent, and the extract analyzed for silicon. This technique is also relatively time-consuming and technique-dependent. Freeman (References, 3) eliminated the need for an isolation step by employing a direct aspiration flame AA technique for estimating the DMPS content of sunflower seed oil. While applicable to the analysis of lightly hydrogenated oils, this method is not directly applicable to the analysis of more heavily hydrogenated products. In contrast, the procedure detailed in this method is relatively rapid and is applicable to the analysis of both hydrogenated and nonhydrogenated fats and oils. The detection limit is ca. 1 µg/g with a standard deviation of 0.3 for test samples containing 1–10 µg/g DMPS.

APPARATUS

1. Perkin-Elmer model 403 atomic absorption spectrophotometer—with a silicon hollow-cathode lamp. Instrument parameters were wavelength 251.6 nm, recorder response 3 and slit 4 (0.7 nm). The instrument was used in the concentration mode at an expansion setting of 900 (ca. 0.01 absorbance units full scale).

   The selection of a wider slit setting than that recommended by the manufacturer, combined with proper alignment of the cork gasket in the nebulizer head so that it did not even partially block the gas inlets, resulted in a reasonably stable baseline at a sensitivity of 0.01 absorbance units full scale.
2. A nitrous oxide-acetylene flame—flow rates of 13 L/min for nitrous oxide and 7 L/min for acetylene.
3. Data were collected on a 10 MV recorder.
4. Water bath—70°C to heat test solution and standard solutions prior to analysis.

   Heating both test solution and standard solutions to 70°C prior to analysis reduced solution viscosity, increased the aspiration rate, and consequently improved the sensitivity of the method.
5. Volumetric flasks—50 mL.
6. Volumetric flasks—10, 8, 6, 4 and 2 mL.

REAGENTS

1. Dow Corning 200 or equivalent. Dimethylpolysiloxane standard.
2. DMPS-free hydrogenated soybean oil—to match the viscosities of the standards to those of the test samples.
3. Industrial grade mineral spirits—boiling range of 157–197°C as supplied by Central Solvents and Chemical Co., Chicago, Illinois, to be used as a solvent for both standards and test samples.

STANDARDS

Working standards were prepared by pipetting 10, 8, 6, 4 and 2 mL of a 20 µ/mL DMPS standard in mineral spirits solution into separate 50 mL volumetric flasks each containing 20 g of DMPS-free fat and diluting to volume with mineral spirits. These solutions contained 10, 8, 6, 4 and 2 µg DMPS per g of fat, respectively. A solution containing 40% fat and 30 µg DMPS per g of fat was also prepared for use in the optimization of the instrument.

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PROCEDURE

1. Test solutions were prepared by dissolving 20 g of melted fat in mineral spirits and diluting to 50 mL.
2. Both standard and test solutions were then placed in a water bath at 70°C and allowed to equilibrate for ca. 1 hr.
3. Aspiration rate, flame conditions, and burner position were optimized while aspirating the unheated 20 µg/mL DMPS stock solution. An absorbance of ca. 0.04 was generally considered desirable.
4. The heated 30 µg DMPS per g of fat solution was then aspirated and the nebulizer adjusted to yield the maximum response.
5. The standard and test solutions were then alternately removed from the 70°C bath and immediately aspirated into the flame.
6. Mineral spirits at ambient temperature were continually aspirated between test and standard solutions.

INSTRUMENT CALIBRATION

The recorder tracings obtained for standards containing 2, 4, 6, 8 and 10 µg DMPS per g of fat are presented in Figure 1. A calibration curve was prepared by plotting average peak height vs. concentration in µg DMPS per g of fat for these standards. The curve is presented in Figure 2. The DMPS content of the fat or oil is read directly from the calibration curve.

RECOVERY STUDIES AND TEST SAMPLE ANALYSES

A test sample of DMPS-free, hydrogenated fat was fortified with known levels of the DMPS standard and analyzed in triplicate. The results obtained are presented in Table 1. Single determinations were also made on a second set of test samples containing known amounts of DMPS. The results are presented in Table 2. In both instances the precision and recovery data are satisfactory. Three frying fats of unknown DMPS concentration were also analyzed in duplicate on two different days. These results are presented in Table 3.

Based on the data obtained in these and other studies, the standard deviation of the method was found to be ca. 0.3 over the range of 1–10 µg/g or ± 0.6 µg/g at 95% confidence. The detection limit of the method, defined as a response equal to double the noise, was found to be below 1 µg DMPS per g of fat. In those instances when optimum instrument performance was obtained, it was possible to achieve a detection limit as low as 0.5 µg/g DMPS.

REFERENCES

1. Neal, P., A.D. Campbell, and D. Firestone, *J. Am. Oil Chem. Soc. 46*:561 (1969).
2. Kundu, M.K., *Fette Seifen Anstrichm. 79*:170 (1977).
3. Freeman, I.P., F.B. Padley, and W.L. Sheppard, *J. Am. Oil Chem. Soc. 50*:101 (1973).

Table 1
Replicate Determination of Dimethylpolysiloxane in Hydrogenated Vegetable Oil[a]

	Dimethylpolysiloxane (µg/g)		
	Found		
Added	1	2	3
—	ND[b]	ND	ND
2.0	1.9	2.0	2.1
4.0	3.8	4.0	3.9
7.0	6.7	6.7	7.0
10.0	9.0	9.6	9.6

[a] IV-65.
[b] ND = None detected.

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Table 2
Recovery of Dimethylpolysiloxane from Hydrogenated Vegetable Oil[a]

Dimethylpolysiloxane (µg/g)			
Added	Found	Added	Found
0.5	0.8	7.0	6.7
1.0	1.1	7.0	6.6
3.0	3.0	9.0	8.7
5.0	4.6	11.0	10.4
5.0	5.1	13.0	12.6
5.5	5.4		

[a] IV-65.

Table 3
Dimethylpolysiloxane in Commercial Frying Fats

	Dimethylpolysiloxane (µg/g)			
	Day I		Day II	
Test Sample	I	II	I	II
1	5.3	5.1	5.0	5.1
2	3.9	3.8	3.4	3.6
3	2.5	2.4	2.2	2.1

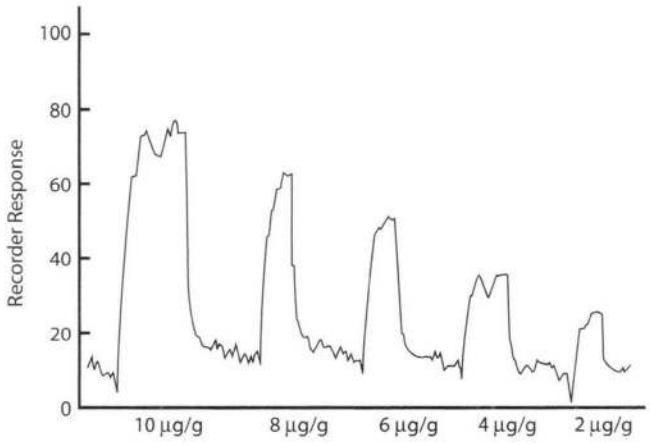

Figure 1. Recorder response obtained for DMPS working standards. Concentrations are in µg DMPS/g of fat.

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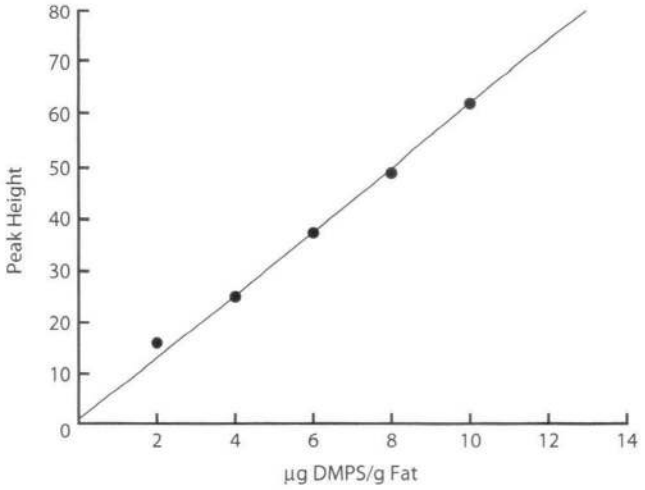

Figure 2. Calibration curve obtained from DMPS working standards. Concentrations are in µg DMPS/g of fat.

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Heat Transfer Fluids in Oils—Dowtherm™ by GC

DEFINITION

This method determines the concentration of Dowtherm™ (see Numbered Notes, 1) in the test sample by gas chromatography (see References, 1).

SCOPE

Applicable to deodorizer distillate and to finished oils.

APPARATUS

1. Volumetric flasks—100 mL.
2. Jars—236.6 mL (8 oz).
3. Separatory funnels—250 mL and 1000 mL.
4. Beakers—100 mL.
5. Small specimen bottles.
6. Graduated cylinders—10 mL, 50 mL, and 500 mL.
7. Syringe—10 µL.
8. Pipets—various sizes.
9. GLC column—stainless steel, 6′ × 1/8″, packed with 10% SE-30 on 80/100 mesh Chromosorb W.
10. Gas chromatograph, equipped with flame ionization detector and capable of meeting the following conditions—
    (a) Injection port temperature: 200°C.
    (b) Detector temperature: 250°C.
    (c) Column temperature: 180°C.
    (d) Helium flow rate: 30 mL/min.
    (e) Range: 10.
    (f) Attenuation: 4.

REAGENTS (see Notes, *Caution*)

1. Dowtherm™.
2. Hexane, reagent grade.
3. Corn oil, deodorized.
4. Petroleum ether, reagent grade.
5. Acetonitrile, reagent grade.
6. Sodium chloride, saturated solution.
7. Sodium sulfate, reagent grade.

PROCEDURE

1. Deodorizer distillate standards preparation—Weigh 0.100 g of Dowtherm™ (Reagents, 1) into a 100 mL volumetric flask (Apparatus, 1). Dilute to volume with hexane (Reagents, 2) and mix. Pipet 1.0, 2.0, 5.0, and 10.0 mL aliquots of this solution into respective 100 mL volumetric flasks. Dilute each aliquot to volume with hexane and mix. The solutions in the four flasks will then contain, respectively, 10, 20, 50, and 100 ppm of Dowtherm™.
2. Oil standards preparation—Weigh 0.100 g of Dowtherm™ into a tared 8 oz. jar (Apparatus, 2) and add deodorized corn oil (Reagents, 3) until the total weight of the jar contents is 100.0 g. Mix thoroughly. Weigh 0.100 g, 0.200 g, 0.500 g, and 1.000 g portions of this solution into respective tared 8 oz. jars. Add deodorized corn oil to each until the total weight of the contents is 100.0 g and mix thoroughly. The oil solutions in the four jars will now contain, respectively, 1, 2, 5, and 10 ppm of Dowtherm™.
3. Extraction of deodorizer distillate sample—
    (a) Pipet a 5.0 mL aliquot of deodorizer distillate into a 250 mL separatory funnel (Apparatus, 3). Add 50 mL of petroleum ether (Reagents, 4) and swirl to effect solution. Add 30 mL of CH_3CN (Reagents, 5) and shake for 1 min. When the layers have separated, drain the lower CH_3CN layer into a second 250 mL separatory funnel.
    (b) Repeat the extraction of the petroleum ether with a second 30 mL portion of CH_3CN, again draining the CH_3CN layer into the second separatory funnel.
    (c) Add 30 mL petroleum ether to the combined extracts and shake for 1 min. When the layers have separated, drain the CH_3CN layer into a 1000 mL separatory funnel (Apparatus, 3).

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(d) Add 300 mL water, 20 mL saturated NaCl solution (Reagents, 6), 50 mL petroleum ether, and shake for 1 min. When the layers have completely separated, drain and discard the bottom layer.
(e) Add 30 mL water and wash by shaking gently. Discard the lower water layer and wash for a second time with another 30 mL portion of water, again discarding the water layer.
(f) Drain the remaining petroleum ether solution into a 100 mL beaker (Apparatus, 4) and add 2-3 g Na_2SO_4 (Reagents, 7). Swirl and then allow to settle for several minutes. Decant the dried solution and store in a closed container.

4. Extraction of oil test sample and standards—
   (a) Weigh 5 g of test sample into test tube. Do the same for 1 µg/g, 2 µg/g, and 5 µg/g Dowtherm™ standards of oils (Procedure, 2).
   (b) Add 5 mL of CH_3CN into each tube, shake for 1 min, and centrifuge for 5 min at 2000 rpm.
   (c) Transfer top layer from each tube to a corresponding 250 mL volumetric flask.
   (d) Repeat the extraction [steps (b) and (c)] two times more.
   (e) Add 20 mL saturated NaCl solution and 3 mL petroleum ether to each volumetric flask and dilute to 250 mL with water.
   (f) Shake well.
   (g) Transfer top layer from each volumetric flask to a corresponding small specimen bottle containing 2-3 g anhydrous Na_2SO_4 and shake well.
5. Analysis—
   (a) To generate standard curves, inject deodorizer distillate standard prepared as in Procedure, 1, or oil standard prepared as in Procedure, 2 and extracted as in Procedure, 4. Inject 5 µL of each standard and measure the height of the Dowtherm™ peak obtained for each standard.
   (b) To analyze unknown test samples, inject 5 µL of an oil test sample extracted as in Procedure, 4, or 5 µL of deodorizer distillate extract prepared as in Procedure, 3. For oil test samples, measure the height of the peak with the same retention time as obtained with the oil standards; for deodorizer distillate, measure the peak with the same retention time as for the deodorizer distillate standards. For deodorizer distillate test samples it may be necessary to draw in a baseline to correct for interference from a previous peak.
   (c) Determine Dowtherm™ concentration using the applicable standard curve. Report results as µg/g directly from the oil standard curve; multiply results obtained on the hexane standard curve by 10.

CALCULATIONS

1. The amount of Dowtherm™ present may be determined directly from a standard curve by plotting the intersection of Dowtherm™concentration with GC peak height response. An alternate means is to calculate least squares correlation by linear regression and fit peak height to get Dowtherm™concentration:

$$Y = MX + B$$

Where—
Y = Dowtherm™concentration, mg/kg (ppm)
M= Slope of response to concentration
X = Peak height of response
B = Y intercept

METHOD PERFORMANCE

The relative standard deviation (RSD) for 4 replicates at 10 ppm level = 5.10%.

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1-6%. A fume hood should be used at all times when using petroleum ether.

Hexane is a flammable solvent and a dangerous fire risk. All work should be performed in a fume hood, with no open flames. Always ensure that your hood is functioning before introducing volatile solvents. The TLV for hexane is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

NUMBERED NOTES

1. Dowtherm™ is a registered trademark of Dow Chemical Co.

REFERENCES

1. This method is a paraphrase of a method submitted by A. Waltking of Best Foods, Inc.

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AOCS Recommended Practice Cd 25a-00

Repproved 2009

Thermal Heating Fluids in Edible Oils and Oleochemicals—Dowtherm A™ by HPLC Coupled with Fluorescence Detector

DEFINITION

This method describes a procedure for the determination of Dowtherm A™ in edible oils and oleochemicals by HPLC. The procedure does not require a pre-treatment step.

SCOPE

Applicable to edible oils and oleochemicals which have no interfering components that fluoresce in the same region as Dowtherm A™. The method is also applicable to other thermal heating fluids based on mixture of biphenyl and diphenyl oxide, e.g., Therminol VP-1™ (see References, 1). The method may be used either semi-quantitatively or quantitatively.

APPARATUS

1. HPLC system—with minimum requirements of a high pressure pump, sample injection device, detector, and chart recorder. A fluorescence detector should be used, with the excitation and emission wavelengths set at 247 nm and 310 nm, respectively.
2. HPLC analytical column—250 × 4.6 mm i.d., with 5 µm reversed-phase LiChrospher C18 (GL Sciences Inc., Tokyo, Japan) or equivalent.
3. Column temperature—maintained at 30°C with a column oven.
4. Injection syringe—capable of accurately injecting 20 µL.
5. Volumetric flasks—10 mL and 100 mL.
6. Beaker—100 mL.
7. Graduated cylinders—100 mL and 500 mL.
8. Micropipets—100–1000 µL capacity.

REAGENTS

1. Dowtherm A™ (Dow Chemical Company, Midland, Michigan, USA).
2. Biphenyl and diphenyl oxide standards (98% purity, Fluka Chemika, AG, Buchs, Switzerland) or equivalent.
3. HPLC grade solvents.
   (a) Methanol.
   (b) Tetrahydrofuran.
4. Deionized water.
5. HPLC mobile phase—water in methanol (10:90, v/v).
6. Blank oil—may be any refined oil or oleochemical (palmolein, corn oil, sunflower oil, fatty acid, fatty alcohol, etc.) free of possible contact with thermal heating fluid.

PREPARATION OF REFERENCE SOLUTIONS

1. Biphenyl and diphenyl oxide—
   (a) Weigh separately 10 mg reference standards (Reagents, 2) into each of two 100 mL volumetric flasks (Apparatus, 5), and dilute with tetrahydrofuran [Reagents, 3(b)] to the mark.
   (b) Pipet (Apparatus, 8) 1 mL of each reference standard solution [Procedure 1(a)] into a 10 mL volumetric flask (Apparatus, 5) and bring to 10 mL with tetrahydrofuran [Reagents, 3(b)]. Use this solution to determine the retention time and resolution of biphenyl and diphenyl oxide by HPLC. Make serial dilutions of this mixture to check the linearity of response and limits of detection of both of the components.
2. Dowtherm A™—
   (a) Stock solution (100 µg/mL)—Accurately weigh 10 mg Dowtherm A™ into a 100 mL volumetric flask [Apparatus, 5], and dissolve in tetrahydrofuran. Make up to the mark.
   (b) Further dilute aliquots in tetrahydrofuran to give working standards with 10.0, 5.0, 1.0, 0.5, 0.01, and 0.005 µg/mL. The linearity of fluorescence detector response may be checked by injecting 20 µL of these solutions in duplicates. Record the area of biphenyl peak and plot against concentration. Dowtherm A™ is resolved into biphenyl and diphenyl oxide peaks (Fig. 1). Biphenyl fluoresces more intensely than diphenyl oxide.

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CALIBRATION TEST SOLUTIONS FOR THE SEMI-QUANTITATIVE ANALYSIS OF DOWTHERM A™ CONTAMINATION

1. Weigh accurately 1 g of blank oil, to within 0.1 mg, into each of five 10 mL volumetric flasks (Apparatus, 5).
2. Using a micropipet (Apparatus, 8), add 1.0, 0.5, 0.2, 0.1, and 0 mL working standard solution of 1 µg/mL [Reference Solutions, 2(a)] to all five flasks and dilute to 10 mL with tetrahydrofuran [Reagent, 3(b)]. The final solution contains 1.0, 0.5, 0.2, 0.1, and 0 µg Dowtherm A™. Figure 2 shows a calibration graph of a palm olein test sample spiked with Dowtherm A™ (0.1–2.0 µg/g) plotted against the area under the biphenyl peak.

PROCEDURE I—SEMI-QUANTITATIVE

1. Determine the presence of interfering compounds. Figure 3 shows stacked chromatograms of control blank sample solutions of palm olein, fatty alcohol, fatty acid, corn, and sunflower oils. No fluorescent components co-elute with Dowtherm A™.
   (a) Weigh approximately 1 g of blank oil, to within 0.1 mg, into a 10 mL volumetric flask (Apparatus, 5).
   (b) Dissolve the test sample in tetradrofuran solvent [Reagent, 3(b)] and bring to 10 mL.
   (c) Inject 20 µL onto the HPLC column to determine any interfering components that co-elute in the Dowtherm A™ region.
2. Determine Dowtherm A™ in test samples.
   (a) Weigh accurately 1 g of test sample into a 10 mL volumetric flask, and dilute to the mark with tetrahydrofuran.
   (b) Inject 20 µL of the test sample solution into the column. Record the areas of biphenyl peak.
   (c) Repeat at least three injections to obtain an average.
3. Calculate.
   The amount of Dowtherm A™ present may be determined directly by interpolation from the calibration graph established using Reference Solutions, 2:

$$y = mx + b$$

   Where—
   y = Dowtherm A™ peak area of biphenyl
   m = Slope of area to concentration
   x = Dowtherm A™ concentration, µg/g
   b = y intercept

PROCEDURE II—QUANTITATIVE

1. Determine the presence of interfering compounds as described in Procedure I, 1.
2. Determine biphenyl and diphenyl oxide in test samples.
   (a) Weigh accurately 1 g of test sample into a 10 mL volumetric flask and dilute to the mark with tetrahydrofuran.
   (b) Inject 20 µL of the test sample solution into the column. Record the areas of biphenyl peak.
   (c) Repeat at least three injections to obtain an average.
3. Calculate.
   (a) The individual amounts of biphenyl and diphenyl oxide present may be determined directly by interpolation from the calibration graphs established using Reference Solutions, 1. Use the following relationship to determine the individual concentrations of each compound:

$$y = mx + b$$

   Where—
   y = Peak area of biphenyl *or* diphenyl oxide
   m = Slope of area to concentration
   x = Dowtherm A™ concentration, µg/g
   b = y intercept
   (b) Sum the individual amounts of each isomer to determine the amount of heat exchanger present. Compare the ratio of each isomer with the ratio seen in the reference sample of Dowtherm A™. If the test sample contains other thermal heating fluids containing biphenyl and diphenyl oxide, compare the test results with an authentic reference sample or with a sample of fluid drawn from the heat exchanger system.
   Note—The presence of breakdown products may prevent the accurate estimation of the extent of contamination, and a semi-quantitative estimate may be sufficient.

RESULTS OF INTERLABORATORY STUDIES

The mean recoveries of Dowtherm A™ from spiked test samples sent to three different laboratories are given in Table 1. Overall, the results were satisfactory. The highest average within-laboratory recovery was 116% for corn oil at a concentration of 0.2 µg/g, and the lowest recovery was 93% for sunflower oil at a concentration of 0.1 µg/g.

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NOTES

1. Dowtherm A™ is a registered trademark of Dow Chemical Co., USA.
2. Therminol VP-1™ is a registered trademark of Solutia Inc., USA.

REFERENCES

1. Moh, M.H., and T.S. Tang, Liquid Chromatographic Detection of Dowtherm A in Oleochemicals and Edible Oils, *J. Assn. Off. Anal. Chem. 82*:893–896 (1999).
2. Moh, M.H., T.S. Tang, and G.H. Tan, Optimization and Validation of High-Performance Liquid Chromatographic Method for the Determination of Dowtherm A™ in Edible Oils and Oleochemicals, *J. Am. Oil Chem. Soc. 77*:1077–1082 (2000).

Table 1
Statistical analysis of results from interlaboratory study (3 laboratories) on the recovery of Dowtherm A™ from spiked test samples ($n = 6$).

	Fatty alcohol	Fatty acid	Corn oil	Sunflower oil
Laboratory 1	0.956 (0.42)[a]	0.515 (1.86)	0.246 (2.85)	0.098 (0.82)
Laboratory 2	0.975 (1.12)	0.505 (1.25)	0.238 (0.97)	0.081 (4.58)
Laboratory 3	0.982 (0.23)	0.483 (4.33)	0.212 (8.21)	0.100 (4.10)
Amount added, µg/g	1.0	0.5	0.2	0.1
Amount recovered, µg/g	0.971	0.501	0.232	0.093
Recovery, %	97.1	100.2	116.0	93.0
Within-lab CV, %	0.589	2.479	4.006	3.165
Between-lab CV, %	1.287	2.435	3.836	3.118

[a]Laboratory values in parentheses are coefficients of variation for six replicate analyses. For experimental details see References, 2.

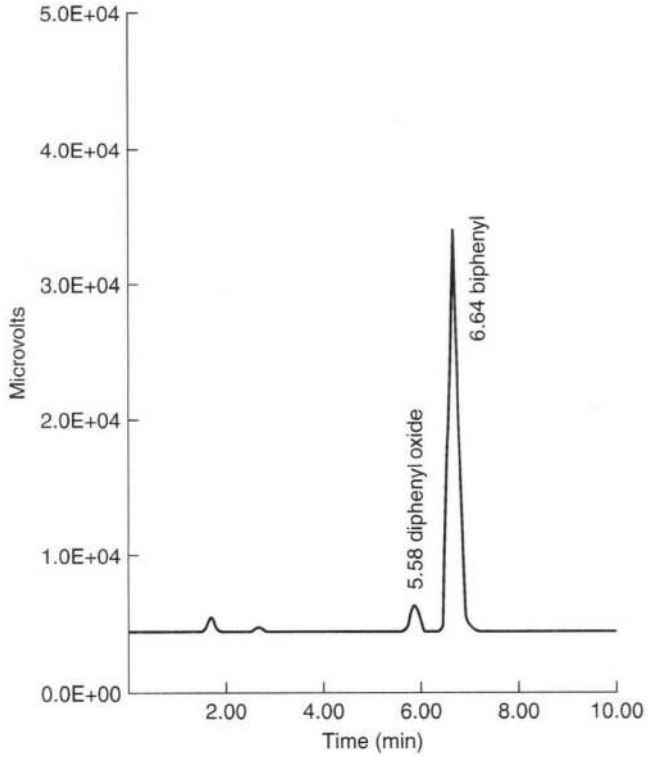

Figure 1. Chromatograms of Dowtherm A™ resolved into diphenyl oxide and biphenyl. Biphenyl fluoresces more intensely than diphenyl oxide.

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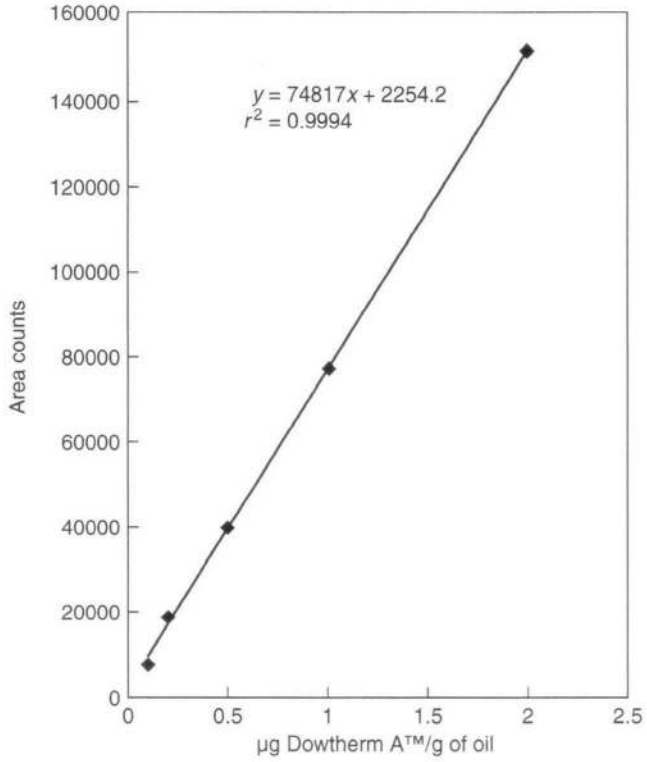

Figure 2. Calibration graph of palm olein test samples spiked with Dowtherm A™ plotted against areas of biphenyl peak.

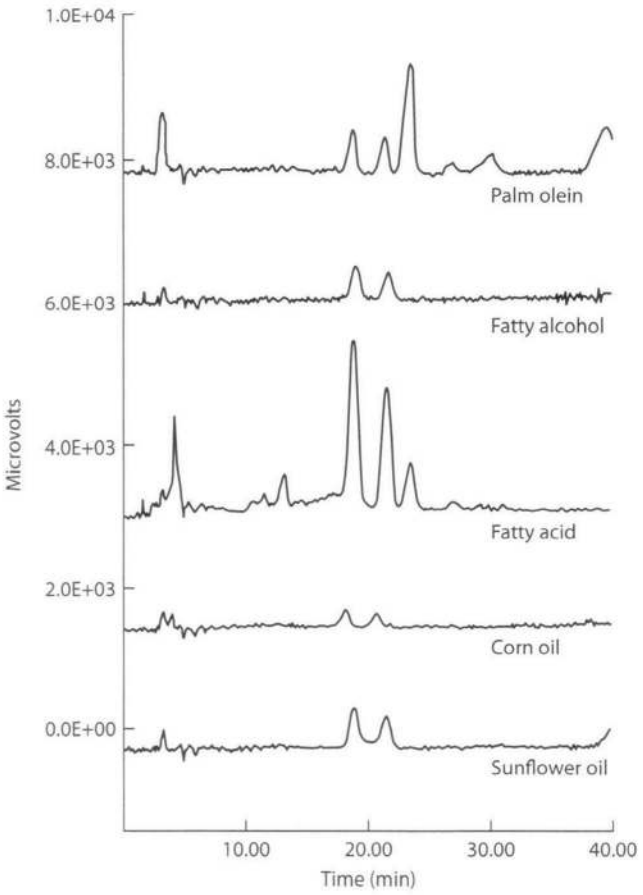

Figure 3. Stacked chromatograms of control blank samples.

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AOCS Recommended Practice Cd 25b-07
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Thermal Heating Fluids in Vegetable Oils

DEFINITION

This method quantitatively determines the Dowtherm J™, Dowtherm A™, and Syltherm™ content in vegetable oils, using capillary gas chromatography (GC) with a flame ionization detector (FID). Oil test samples are dissolved in heptane. The diluted test samples are subsequently injected onto a capillary column and detected with flame ionization.

SCOPE

This procedure is applicable to vegetable oils.

APPARATUS

1. Gas chromatograph, HP6890 with flame ionization detector (FID) and a split/splitless injection port—
   (a) Column—
   Initial carrier flow—2.3 mL/min
   Initial pressure—12.00 psi
   Average velocity—39 cm/sec
   (b) Oven—
   Initial temperature—80°C
   Initial time—1.0 minute
   Rate—15.0°C/min
   Final temperature—330°C
   Final time—10.0 minutes
   Total run time—27.67 minutes
   (c) Injector—
   1.0 µL injection volume
   Splitless mode
   Injector temperature—325°C
   Pressure—12.00 psi
   Purge flow—54.0 mL/min
   Purge time—1.0 minute
   Total flow—58.9 mL/min
   Helium carrier gas
   (d) Detector—
   Flame Ionization Detector (FID)
   Temperature—350°C
   Hydrogen flow—40.0 mL/min
   Air flow—450.0 mL/min
   Nitrogen make-up gas—50.0 mL/min
2. Data system capable of measuring and recording peak area and/or height (e.g. HP Chemstation).
3. DB-17MS capillary column, 30 m × 0.32 mm ID, 0.25 mm film thickness (P/N 123–4732, J&W Scientific, Inc.).
4. 4 mm splitless injection port liner, single taper (e.g. 5062–3587, Agilent).
5. 11 mm high temperature, low bleed, injection port septa (e.g. 5182–0739, Agilent).
6. 4 mL autosampler wash vials (e.g. 9301–0723, Agilent).
7. Diffusion caps for autosampler 4 mL wash vials (e.g. 07673–40180, Agilent).
8. 2 mL autosampler vials (e.g. 5182–0714, Agilent).
9. Aluminum crimp caps for 2 mL autosampler vials (e.g. 5181–1210, Agilent).
10. Autoinjector vial crimper (e.g. 8710–0979, Agilent).
11. Analytical balance, electronic top-loading, accuracy to 0.0001 g.
12. Borosilicate glass transfer pipettes.
13. 20 mL volumetric flasks.
14. 20 mL borosilicate glass scintillation vials (e.g. 03340–129, Fisher).
15. Caps for 20 mL scintillation vials, PTFE-lined (e.g. 3340131, Fisher).
16. 60 mL Qorpak bottles, PTFE caps (33204A, Fisher).

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17. 10 mL pipettor (e.g. P-10ML, Rainin).
18. 1000 µL pipettor (e.g. P-1000, Rainin).
19. 100 µL pipettor (e.g. P-100, Rainin).
20. 20 µL pipettor (e.g. P-20, Rainin).

REAGENTS

1. Heptane—HPLC grade.
2. Toluene—HPLC grade.
3. Dowtherm—J™.
4. Dowtherm—A™.
5. Syltherm™.
6. Helium—compressed, Ultra High Purity/Zero Grade for GC carrier.
7. Hydrogen—compressed, Zero Grade for GC FID detector.
8. Air—compressed, Zero Grade for GC FID detector.
9. Nitrogen—compressed, Zero Grade for GC FID detector make-up gas.

PROCEDURE

1. Calibration Standard Stock Solutions—
   (a) To prepare stock solutions for the calibration standards, weigh 0.6 g (to 0.0001 g) of each analyte separately into a 20 mL volumetric flask using an analytical balance (see Numbered Notes, 1).
   (b) Dilute to 20 mL with heptane, cap, and mix well.
   (c) This will produce three individual stocks of Dowtherm J™, Dowtherm A™, and Syltherm™ at a concentration of 30000 mg/L.
   (d) To a clean 60 mL bottle, pipette 10 mL of each of the 30000 mg/L stock solutions to combine the analytes into one Calibration Stock Solution with a concentration of 10000 mg/L for each analyte (Table 1).
   (e) The stock solutions should be refrigerated if they are to be stored for extended periods.
2. Working Standard Solutions—
   (a) Prepare the Working Standard Solutions from the Calibration Stock Solution by serial dilution into heptane to obtain the desired concentration levels (Table 2).
3. Vegetable oils test sample preparation—
   (a) Using an analytical balance, weigh 0.1000 g test sample into a 5 m Lvolumetric flask.
   (b) Dilute to volume with heptane, cap, and mix.
   (c) This will yield a test solution concentration of 20000 mg/L.
   (d) Using a glass pipette, transfer the test solution extract into a 2 mL GC autosampler vial and crimp seal.

CALCULATIONS

1. A data system or excel spreadsheet should be used to calculate the calibration curve and test sample analyte amounts.
2. A relative response factor (RRF) should be calculated for each analyte and be linear with an R^2 value of 0.995 or better.
3. Since Syltherm™ and Dowtherm A™ give multiple peaks, the areas of these compounds were summed in the calculations.
4. Table 3 illustrates the retention times of the analytes under the current method conditions (see Fig. 1).

METHOD PRECISION

This method is for reference only. No collaborative study has been performed.

NOTES

Caution

Always follow the Chemical Hygiene Plan and established laboratory safety procedures for handling materials, cleaning up spills, and disposing of wastes.

Read and observe all precautionary measures and hazards noted in the Material Safety Data Sheets for all chemicals used in this procedure.

Heptane and toluene are highly flammable solvents. Caution must be taken at all times to keep them away from sparks, heat, and open flames. Know the location of the fire extinguishers in the work area before proceeding with the test. Also, all work should be performed under a hood when handling organic solvents.

The hydrogen gas used as a fuel for the GC/FID is explosive in a confined space and under certain conditions. Caution must be taken to ensure that there are no leaks at any of the fittings or in the plumbing lines leading away from the primary source.

NUMBERED NOTES

1. A solvent blank should be analyzed prior to performing a calibration or analyzing test samples to ensure a contaminant free chromatographic system.

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2. A matrix spike should be analyzed and used as a reference to facilitate identification of analytes of interest. For example, a spike level of 1000 mg/kg. In a 10 mL volumetric flask, spike 0.10 g of blank sample with 10 µL of the 10000 mg/L Stock Standard and dilute to volume.
3. The calibration standard curve should be linear with an R^2 value of 0.995 or better.
4. A mid-level calibration standard should be analyzed as a continuing calibration verification at the end of a sequence of test samples or every twenty test samples, whichever comes first. If response varies greatly (>15–20 %) the method and chromatographic system should be investigated to determine the cause of the variation.

Table 1
Calibration Standard Stock Preparation

Standard Name	Weight of Standard (g)	Final Volume (mL)
30000 mg/L Dowtherm J™	0.6000	20
30000 mg/L Therminol—Dowtherm A™	0.6000	20
30000 mg/L Syltherm™	0.6000	20
	Volume of Standard Solution (mL)	
10000 mg/L Calibration Stock Solution	10 mL of 30000 mg/L Dowtherm J™ 10 mL of 30000 mg/L Therminol™ 10 mL of 30000 mg/L Syltherm™	30

Table 2
Working Standard Solutions

Standard Name	Standard Volume	Final Volume (mL)
5000 mg/L Cal Std	5.0 mL of 10000 mg/L Calibration Stock Solution	10
1000 mg/L Cal Std	1.0 mL of 10000 mg/L Calibration Stock Solution	10
500 mg/L Cal Std	0.5 mL of 10000 mg/L Calibration Stock Solution	10
100 mg/L Cal Std	1.0 mL of 1000 mg/L Calibration Stock Solution	10
50 mg/L Cal Std	0.5 mL of 1000 mg/L Calibration Stock Solution	10
10 mg/L Cal Std	100 uL of 1000 mg/L Calibration Stock Solution	10
5 mg/L Cal Std	50 uL of 1000 mg/L Calibration Stock Solution	10
1 mg/L Cal Std	10 uL of 1000 mg/L Calibration Stock Solution	10

Table 3
(Retention times of analytes)

Compound	Retention time (min)
Dowtherm J™	3.566
Syltherm-1™	5.783
Syltherm-2™	6.921
Dowtherm A-1™	7.370
Dowtherm A-2™	7.565
Syltherm-3[a]™	7.964
Syltherm-4[a]™	8.926

[a] Syltherm™ peaks (1) and (2) are used for quantitation. Peaks (3) and (4) are used for identification only.

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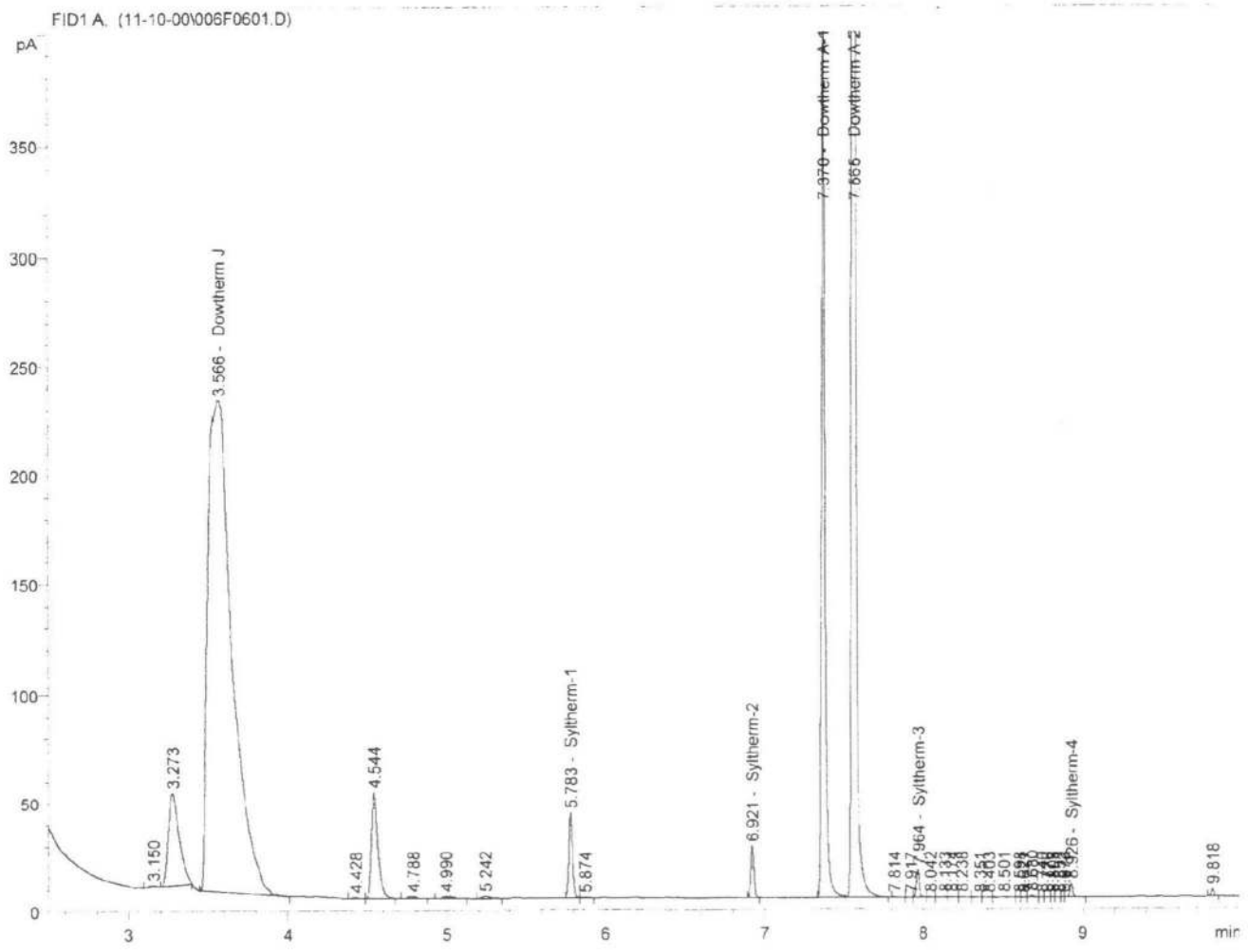

Figure 1. Chromatogram of the calibration standard (100 mg/kg).

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AOCS Official Method Cd 26-96

Reapproved 2009

Stigmastadienes in Vegetable Oils

DEFINITION

This is a rigorous method for the determination of stigmasta-3,5-diene in vegetable oils containing low concentrations of these hydrocarbons, particularly virgin olive oils and crude olive pomace oil. The method is particularly suited to detecting the presence of refined vegetable oils (olive, olive pomace, sunflower, palm, etc.) in virgin olive oil, since refined oils contain stigmastadienes and virgin oils do not. The method consists of isolation of unsaponifiable matter, separation of steroidal hydrocarbon fraction by column chromatography on silica gel, and analysis by capillary gas chromatography (References, 1).

SCOPE

Applicable to all vegetable oils, although measurements are reliable only where the content of these hydrocarbons lies between 0.01 and 4.0 mg/kg. This method is ideal for detecting the presence of refined veg oils (olive, olive-pomace, sunflower, soybean, palm, etc.) in virgin olive oil since refined oils contain stigmastadienes and virgin oils do not.

APPARATUS

1. 250 mL flat-bottom flasks with reflux condenser.
2. Separating funnels—500 mL.
3. Round-bottom flasks—100 mL.
4. Rotary evaporator.
5. Glass chromatography column—1.5 cm i.d. × 50 cm length with Teflon stopcock and a plug of glass wool fiber at the bottom.
6. Gas chromatograph—with flame ionization detector, split or on-column injector, and oven programmable to within ±1°C.
7. Fused silica capillary columns for gas chromatography, 0.25 or 0.32 mm i.d. × 25 m length, coated with 5% phenylmethylsilicone phase, 0.25 µm film thickness (see Notes, 1).
8. Integrator-recorder—with possibility of valley-valley integration mode.
9. Microsyringe for gas chromatography—5–10 µL, with cemented needle.

REAGENTS (see Notes, *Caution*)

All reagents should be analytical grade unless otherwise specified. The water used should be distilled water, or water of at least equivalent purity.

1. Hexane, or mixture of alkanes—b.p. interval 65–70°C, distilled with rectifying column (see Notes, 2).
2. Ethanol—96%, v/v.
3. Sodium sulfate—anhydrous.
4. Alcoholic potassium hydroxide solution at 10% w/v, prepared by adding 10 mL of water to 50 g KOH, stirring, and then dissolving the mixture in ethanol to 500 mL (see Notes, 3).
5. Silica gel 60 for column chromatography—70–230 mesh (Merck ref. 7734, or equivalent) (see Notes, 4).
6. Stock solution—(200 ppm) of cholesta-3,5-diene (Sigma, or equivalent, 99% purity) in hexane (10 mg in 50 mL) (see Notes, 5).
7. Standard solution of cholesta-3,5-diene in hexane at concentration 20 µg/g obtained by dilution of stock solution (Reagents, 6) (see Notes, 5).
8. Carrier gas for chromatography—N-50 helium or hydrogen.
9. Auxiliary gases for flame ionization detector, N-50 hydrogen, and purified air.
10. Solution of n-nonacosane in hexane—approximately 100 µg/g.

GC OPERATING CONDITIONS

1. Injector temperature—300°C.
2. Detector temperature—320°C.
3. Integrator-recorder—The parameters for integration should be fixed so as to give a correct assessment of the areas. Valley-valley integration mode is recommended.
4. Sensitivity—about 16 times the minimum attenuation.
5. Amount of solution injected: 1 µL.
6. Oven programming temperatures: Initial 235°C for 6 min and then programming at 2°C/min up to 285°C.
7. Injector—with 1:15 split flow divider.
8. Carrier—helium or hydrogen at about 120 kPa pressure.

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9. These conditions may be adjusted in accordance with the characteristics of the chromatograph and the column to give chromatograms meeting the following requirements:
   (a) Internal standard peak should be within approximately 5 min of the time given in Procedures, 5(a).
   (b) Internal standard peak should be at least 80% of the full scale.
10. The gas chromatographic system must be checked by injecting a mixture of the stock solution of cholestadiene (Reagents, 6) and n-nonacosane solution (Reagents, 10). The cholesta-3,5-diene peak should appear before the n-nonacosane (Figure 1c); if it does not, the initial oven temperature should be reduced or the GC column should be replaced by a less polar or wider-i.d. one.

PROCEDURE

1. Treatment of silica gel (if required; see Notes, 4)—
   (a) Deactivate the silica gel by heating for a minimum of 4 hr at 550°C.
   (b) After heating, place the silica gel in a desiccator while the gel is cooling.
   (c) Transfer the silica gel to a stoppered flask.
   (d) Add 2% of water. Shake until no lumps can be seen and the powder flows freely.
2. Preparation of silica gel column—
   (a) Pour hexane (Reagents, 1) into the chromatography column (Apparatus, 5) to a depth of approximately 5 cm and then fill with a slurry of silica gel (Reagents, 5) in hexane (15 g in 40 mL) with the help of hexane portions.
   (b) Allow to settle and finish settling by applying slight vibration.
   (c) Add anhydrous Na_2SO_4 (Reagents, 3) to a height of 0.5 cm.
   (d) Finally, elute the excess hexane.
3. Preparation of unsaponifiable matter—
   (a) Weigh 20 ± 0.1 g of oil into a 250 mL flask, add 1 mL of the standard solution of cholesta-3,5-diene (20 mg) and 75 mL of alcoholic potash at 10%, attach reflux condenser, and heat to slight boiling for 30 min. Remove the flask containing the test sample from the heat and allow the solution to cool slightly (do not allow to cool completely, as the test sample will set). Add 100 mL of water and transfer the solution to a decanting funnel with the aid of 100 mL of hexane. Shake the mixture vigorously for 30 s and leave to stratify. (If an emulsion is produced, wait, it disappears rapidly, or add small quantities of ethanol.)
   (b) Transfer the aqueous phase beneath to a second separating funnel and extract again with 100 mL of hexane. Once more, run off the lower phase and wash the hexane extracts (combined in another separating funnel) three times, with 100 mL each time of ethanol/water (1:1), until neutral pH is reached.
   (c) Pass the hexane solution through anhydrous Na_2SO_4 (50 g), wash with 20 mL hexane, and evaporate in a rotary evaporator at 30°C and low pressure until dryness. (See Notes, 6.)
4. Separation of steroidal hydrocarbon fraction—
   (a) Take the residue from Procedure, 3 to the fractioning column with the aid of two 1 mL portions of hexane, run the test sample onto the column (by allowing the solution level to drop to the top of the Na_2SO_4), and start chromatographic elution with hexane at a flow rate of 1 mL/min, approximately. Discard the first 25-30 mL of the elution, and then collect the following 40 mL fraction (see Notes, 7).
   (b) Evaporate the second fraction from Procedure, 4(a) in an evaporator at 30°C and low pressure until dryness, and immediately dissolve the residue in 0.2 mL of hexane. Keep the solution in the refrigerator until analysis (see Notes, 6).
5. Gas chromatography (see GC Operating Conditions)—
   (a) Peak identification—The internal standard peak appears at approximately 19 min and the stigmasta-3,5-diene at a relative retention time of 1.29 (see Figure 1b). The stigmasta-3,5-diene goes with small quantities of an isomer, and usually, both originate a single chromatographic peak. Nevertheless, if the column is too polar or shows high resolving power, the isomer can appear as a small peak before and close to that of stigmasta-3,5-diene (Figure 2); in that case the areas of the two peaks must be summed. It is advisable to eliminate the stigmastadiene split by replacing the column with a less polar or a wider-i.d. one. (See Notes, 8 and 9.)
   (b) Quantitative analysis—The stigmastadiene content is determined according to the formula:

$$\text{mg stigmastadienes/kg} = \frac{(A_s)\,(M_c)}{(A_c)\,(M_o)}$$

Where—
A_s = area of stigmastadiene peak (if the peak is split, sum of areas of the two isomers)
A_c = area of internal standard (cholestadiene) peak
M_c = mass of standard added (μg)
M_o = mass of oil taken (g)

METHOD PERFORMANCE

Detection limit—about 0.01 mg/kg (see Notes, 10). The approximate percentages of refined vegetable oils that can be detected in virgin olive oil by this method are shown in Table 1.

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PRECISION

1. Repeatability—When the mean of the values obtained from two single determinations, carried out in rapid succession by the conditions for the analysis of the same operator, using the same apparatus under the same conditions for the analysis of the same laboratory sample, lies within the range of the mean values cited in the tables below, the difference between the two values obtained should not be greater than the repeatability limit (r), which can generally be deduced by linear interpolation from the values in Tables 2 and 3.
2. Reproducibility—When the values for the final result, obtained by operators in different laboratories using different apparatus under the same conditions for the analysis of the same laboratory sample, lie within the range of mean values cited in the tables, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility limit (R), which can generally be deduced by linear inter-polation from the values in Tables 2 and 3.
3. Analysis of the collaborative test results—The precision values of the method are given in Table 4. Nineteen laboratories holding International Olive Council(IOC) recognition at the time took part in the collaborative test arranged by the Executive Secretariat in 1999. The laboratories were from eight countries. The test was performed on five test samples: 1) extra virgin olive oil, 2) virgin olive oil + refined sunflower oil, 3) virgin olive oil + refined olive-pomace oil, 4) virgin olive oil + refined soybean oil + refined sunflower oil, 5) refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil. The results of the collaborative test organized by the IOC Executive Secretariat have been statistically processed according to the rules laid down in the international standards ISO 5725 Accuracy (trueness and precision) of measurement methods and results. Outliers were examined by applying Cochran's and Grubbs's test to the laboratory results for each determination (replicates a and b) and each test sample.

NOTES

Caution

Hexane is a flammable solvent and a dangerous fire risk. All work should be performed in a fume hood, with no open flames. Always ensure that your hood is functioning before introducing volatile solvents. The TLV for hexane is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/M_3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

Potassium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials, such as potassium hydroxide, always add pellets to the water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event that the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. Other columns of similar or lower polarity can be used.
2. The hexane must be distilled to remove impurities.
3. Alcoholic potash turns brown on standing. It should be prepared fresh each day and kept in well-stoppered dark glass bottles.
4. Usually, silica gel can be used directly from the container without any treatment. However, some batches of silica may show low activity, resulting in bad chromatographic separations. If this occurs, or if batches of silica gel result in chromatograms with interfering peaks, the silica gel should be treated as described in Procedure, 1. An alternative could be the use of extra pure silica gel 60 (Merck, ref. 7754).
5. If kept at a temperature below 4°C, cholesta-3,5-diene solutions (Reagents, 6 and 7) will not deteriorate over a period of at least 4 months.
6. Residues from Procedure, 3(c) and Procedure, 4(b) are unstable at room temperature. As soon as the residues are obtained, the solvent should be added and the solutions should be kept in the refrigerator.
7. The first fraction from Procedure, 4(a) contains saturated hydrocarbons (Figure 1a), and the second fraction contains the steroidal ones. Further elution provides squalene and related compounds. To achieve a good separation between saturated and steroidal hydrocarbons, the fraction volumes must be optimized. For this, the volume of the first fraction should be adjusted so that, when the second fraction is analyzed, the peaks representing the saturated hydrocarbons are low (see Figure 1c); if they do not appear, but the intensity of the standard peak is low, the volume should be reduced. A complete separation between the components of the first and second fractions is unnecessary, because there is no overlapping of peaks during GC analysis. The optimization of the volume of the second fraction is generally not needed, since a good separation exists with the further components. Nevertheless, the presence of a large peak at approximately 1.5 min lower retention time than the standard is due to squalene and indicates a bad separation.
8. Stigmastadienes for reference can be obtained from the analysis of a refined vegetable oil by applying Method Cd 27-96, Steroidal Hydrocarbons in Vegetable Oils. Stigmastadienes produce a significant and easily identifiable peak.
9. Care must be taken that there is no overlap between the peaks due to the internal standard or the stigmastadienes and any of the peaks that appear in the first fraction, eluted from the silica gel column [Procedure, 4(a) and Notes, 7].

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10. When stigmastadienes appear in concentrations of more than 4 mg/kg, if quantifying is required, the method for determination of steroidal hydrocarbons (Cd 27-96) must be applied.

REFERENCES

1. This method paraphrases *International Olive Council* (IOC) Method COI/T-20/Doc. no. 11-Rev. 2-2001.
2. *IOC* Doc. IOC/T.20/no. 28–4, October 22, 1993, Annex 2, pp. 2–3.
3. *International Union of Pure and Applied Chemistry* (IUPAC) *Commission on Oils, Fats and Derivatives* (1992). Minutes of Meeting, Budapest, Hungary, September 9–11, 1992, pp. A9–A11.
4. *IUPAC Commission on Oils, Fats and Derivatives* (1993). Working Report of Meeting, Lisbon, Portugal, July 1993, pp. A2–A4.
5. *Pure Appl. Chem. 71*:349 (1999).

Table 1
Percent refined vegetable oil that can be detected in virgin olive oil using the stigmasta-3,5-diene method.[a]

Oil	% Refined vegetable oil in virgin olive oil
Olive, physically refined	1.0
Olive, alkali refined	15
Olive pomace	2
Cottonseed, grapeseed	0.3
Palm, corn	1.5
Rapeseed	1.2
Soybean	2
Sunflower	4
Sunflower, high-oleic	4
Desterolized sunflower	0.15

[a] *Source*: References, 2.

Table 2
Interlaboratory study of the standard method for stigmasta-3,5-diene in vegetable oils.[a,b]

Laboratory	1	2	3	4
x (mg/kg stigmasta-3,5-diene)	0.29	0.006	23.2	36.2
S_r (mg/kg)$_c$	0.01	0.009	2.46	1
S_R (mg/kg)$_c$	0.03	12.94		
RSD_r %[c]	4.88	120	3.94	4.02
RSD_R %[c]	9.22	163	6.94	8.10

[a] Test Samples: 1, virgin olive oil with 1.5% refined olive oil; 2, virgin olive oil; 3, refined olive oil; 4, mixture of refined olive oil, virgin olive oil, and desterolized sunflower oil (25:70:5).

[b] *Source*: References, 3 and 4.

c Sr = intralaboratory standard deviation; S_R = interlaboratory standard deviation; RSD_r = intralaboratory relative standard deviation; RSD_R = interlaboratory relative standard deviation.

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Table 3
Interlaboratory study (10 laboratories) of the revised and amplified standard method for stigmasta-3,5-diene in vegetable oils.[a,b]

	Test Sample		
	1	2	3
No. of laboratories	9	9	9
Mean (mg/kg)	0.014	0.177	1.017
Repeatability			
Sr[c]	0.0032	0.0078	0.0747
RSD_r	23.00	4.40	7.30
r_c	0.0089	0.0218	0.2092
Reproducibility			
S_R[c]	0.0055	0.0249	0.1500
RSD_R	39.60	14.10	14.70
Rc	0.0153	0.0697	0.4200

[a] *Source*: References, 5.
[b] For statistical calculations, results of ND (none detected) as well as <0.02 or <0.01 were considered as 0. Test samples were as follows: 1, virgin olive oil; 2, blend of virgin olive oil with 0.7% refined olive oil containing 23 mg/kg stigmasta-3,5-diene; 3, blend of virgin olive oil with 1.2% refined sunflower oil containing 80 mg/kg stigmasta-3,5-diene.
[c] mg/kg.

Table 4
Stigmastadiene content (ppm).

	Extra virgin olive oil	Virgin olive oil + refined sunflower oil	Virgin olive oil + refined olive-pomace oil	Virgin olive oil + refined soybean oil + refined sunflower oil	Refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil
n = number of participating laboratories	19	19	19	19	19
outliers = number of laboratories with outlying values	3	5	7	2	5
Mean = mean of the accepted results	0.01	0.80	9.49	0.22	7.55
r	0.01	0.08	0.39	0.05	0.48
S_r = Repeatability standard deviation	0.004	0.03	0.14	0.01	0.17
RDS_r (%) = Repeatability coefficient of variation ($S_r \times 100$/mean)	32.43 (not sig.)	3.70	1.5	8.41	2.29
R	0.033	0.15	1.66	0.06	1.59
S_R = Reproducibility standard deviation	0.012	0.05	0.59	0.025	0.57
RDS_R (%) = Reproducibility coefficient of variation ($S_R \times 100$/mean)	98.59 (not sig.)	6.73	6.26	11.45	7.55

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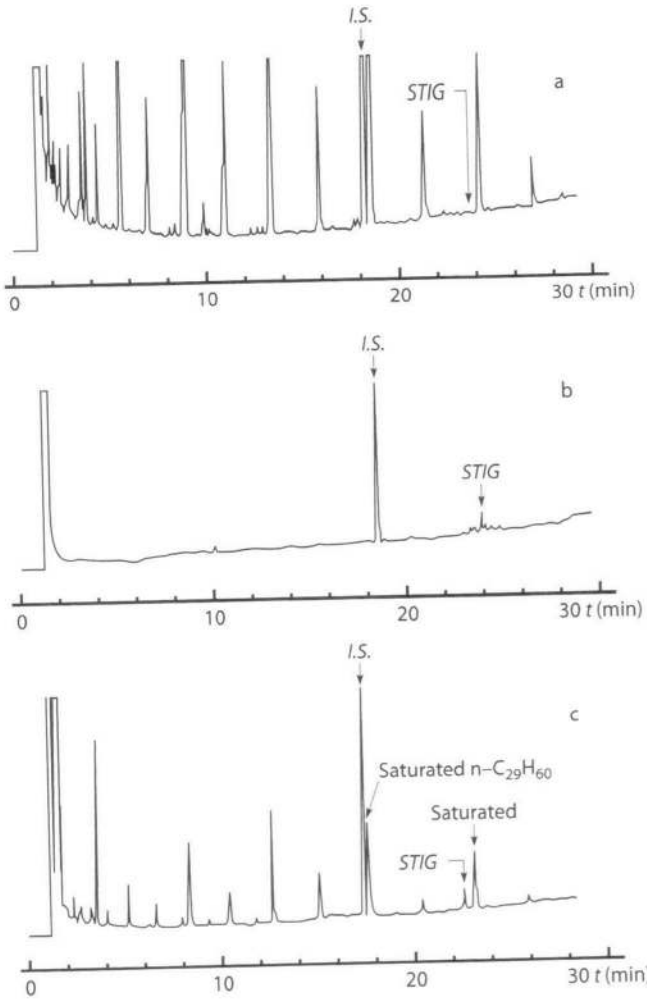

Figure 1. Gas chromatograms obtained from olive oil test samples analyzed on a fused silica capillary column (0.25 µm i.d. × 25 m) coated with 5% phenylmethylsilicone, 0.25 µm film thickness. (a) First fraction (30 mL) from a virgin oil, spiked with standard. (b) Second fraction (40 mL) from an olive oil containing 0.10 mg/kg of stigmastadienes. (c) Second fraction (40 mL) containing a small proportion of the first fraction.

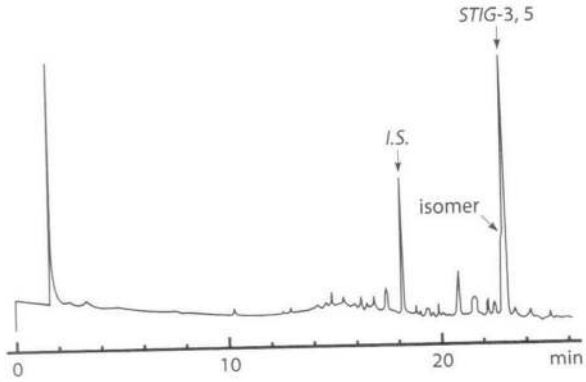

Figure 2. Gas chromatogram obtained from a refined olive oil test sample analyzed on 5% phenylmethylsilicone column, showing the isomer of stigmasta-3,5-diene.

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AOCS Official Method Cd 27-96

Reapproved 2009

Steroidal Hydrocarbons in Vegetable Oils

DEFINITION

This method should be used for the determination of campesta-3,5-diene, stigmasta-3,5,22-triene, and stigmasta-3,5-diene (hydrocarbons originating from sterols during refining or desterolizing treatments applied to vegetable oils). The standard may be used to detect desterolized oils—particularly desterolized seed oils—in refined olive oil and refined olive residue oil. The procedure may also be used to quantify stigmasta-3,5-diene in oils where it occurs at a concentration greater than 4.0 mg/kg; this method is less rigorous for this purpose than AOCS method Cd 26-96 for the determination of stigmasta-3,5-diene in vegetable oils. The method consists of isolation of the steroidal hydrocarbon fraction by column chromatography on silica gel and analysis by capillary gas chromatography (References, 1).

SCOPE

Vegetable oils.

APPARATUS

1. 100 mL round-bottom flasks.
2. Rotary evaporator.
3. Glass chromatography column—1.5 cm i.d. × 50 cm length with Teflon stopcock and a plug of glass wool fiber at the bottom.
4. Gas chromatograph—with flame ionization detector, split injector, and oven programmable to within ± 1°C.
5. Fused silica capillary columns for gas chromatography—0.25 or 0.32 mm i.d. × 25 m length, coated with 5% phenylmethylsilicone phase, 0.25 µm film thickness.
6. Integrator-recorder—with possibility of valley-valley integration mode.
7. Microsyringe for gas chromatography—5 to 10 µL, with cemented needle.

REAGENTS (see Notes, *Caution*)

All reagents should be analytical grade unless otherwise specified.

1. Hexane—or mixture of alkanes of b.p. interval 65-70°C, distilled with rectifying column (see Numbered Notes, 1).
2. Sodium sulfate—anhydrous.
3. Silica gel 60 for column chromatography—70–230 mesh (Merck ref. 7734), or equivalent.
4. Stock solution—(200 µg/g) of cholesta-3,5-diene (Sigma, 99% purity), or equivalent, in hexane (10 mg in 50 mL) (see Numbered Notes, 3).
5. Standard solution of cholesta-3,5-diene in hexane at concentration 20 µg/g—obtained by dilution of stock solution (Reagents, 4) (see Numbered Notes, 3).
6. Carrier gas for chromatography—N-50 helium or hydrogen.
7. Auxiliary gases for flame ionization detector—N-50 hydrogen and purified air.

GC OPERATING CONDITIONS

1. Injector temperature—300°C.
2. Detector temperature—320°C.
3. Integrator-recorder—The parameters for integration of the peaks should be fixed so as to give a correct assessment of the areas.
4. Sensitivity—about 16 × the minimum attenuation.
5. Amount of solution injected: 1 µL.
6. Oven programming temperatures: initial 235°C for 6 min and then programming at 2°C/min up to 285°C.
7. Injector—with 1:15 split flow divider.
8. Carrier—helium or hydrogen at about 120 kPa pressure.
9. These conditions may be adjusted in accordance with the characteristics of the chromatograph and the column to give chromatograms meeting the following requirements—
   (a) Internal standard peak within approximately 5 min of the time given in Procedure, 4(a).
   (b) Internal standard peak should be at least 80% of the full scale.
   (c) Column efficiency should be 7000 or more theoretical plates/m, calculated on the cholesta-3,5-diene peak.

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PROCEDURE

1. Treatment of silica gel (if required; see Numbered Notes, 2)—
   (a) Deactivate the silica gel by heating for a minimum of 2 hr at 110°C.
   (b) After heating, place the silica gel in a desiccator while the gel is cooling.
   (c) Transfer the silica gel to a stoppered flask.
   (d) Add 30 g of activated silica gel to a round-bottomed flask covered in black paper. Add dropwise a solution of silver nitrate (3.0 g silver nitrate dissolved in 7 mL distilled water) shaking from time to time. Stopper the flask and shake vigorously for 20 s, then place on a rotary evaporator and rotate under atmospheric pressure at room temperature for 30 min. The silica gel turns slightly grey and can be kept in a stoppered flask in the dark.
2. Preparation of silica gel column—
   (a) Pour hexane (Reagents, 1) into the chromatography column (Apparatus, 3) to a depth of approximately 5 cm and then fill with a slurry of silver-impregnated silica gel [Procedure, 1 (d)] in hexane (15 g in 40 mL) with the help of hexane portions.
   (b) Allow to settle and finish settling by applying slight vibration.
   (c) Add anhydrous Na_2SO_4 (Reagents, 2) to a height of 0.5 cm.
   (d) Finally, elute the excess hexane. Wrap the column in black paper.
3. Separation of steroidal hydrocarbon fraction—
   (a) Weigh 1 ± 0.01 g of oil in a 25 mL beaker.
   (b) Add 1 mL of standard solution of cholesta-3,5-diene (20 µg) (Reagents, 5).
   (c) Take the mixture from Procedure, 3(b) to the fractioning column with the aid of two 1 mL portions of hexane, run the test sample onto the column (by allowing the solution level to drop to the top of the Na_2SO_4), and start chromatographic elution with hexane at a flow rate of approximately 1 mL/min. Discard the first 25–30 mL of the elution, and then collect the following 40 mL fraction (see Numbered Notes, 4).
   (d) Evaporate the fraction from Procedure, 3(c) in the rotary evaporator at 30°C and reduce pressure. Dissolve the residue in 0.2 mL of hexane. Keep the solution in the refrigerator until analysis (see Numbered Notes, 5).
4. Gas chromatography (see GC Operating Conditions)—
   (a) Peak identification—The internal standard peak appears at a retention time of approximately 19 min and the stigmasta-3,5-diene at a relative retention time of 1.29 (see Figures 1b and 1c). The campesta-3,5-diene appears at approximately 22 min (retention time relative to standard 1.16; relative to stigmasta-3,5-diene 0.90) (see Figure 2a). The stigmasta-3,5,22-triene appears at approximately 23 min (retention time relative to standard 1.21; relative to stigmasta-3,5-diene 0.93) (see Figure 2a). See Numbered Notes, 6–8.
   (b) Quantitative analysis—The stigmasta-3,5-diene content is determined according to the formula:

$$\text{mg stigmasta-3,5-diene/kg} = \frac{(A_s)(M_c)}{(A_c)(M_o)}$$

Where—

A_s = area of stigmasta-3,5-diene peak
A_c = area of internal standard (cholestadiene) peak
M_c = mass of standard added (µg)
M_o = mass of oil taken (g)

The ratios between the hydrocarbons are calculated as follows:

$$R1 = \frac{\text{area of stigmasta-3,5-diene}}{\text{area of campesta-3,5-diene}}$$

$$R2 = \frac{\text{area of stigmasta-3,5-diene}}{\text{area of stigmasta-3,5,22-triene}}$$

These ratios are valid only if the stigmasta-3,5-diene peak reaches at least 50% of its full scale. See Numbered Notes, 9.

PRECISION

1. Interlaboratory test—Details of an interlaboratory test on the precision of the method are summarized in Table 1. An international collaborative test involving 19 laboratories in 8 countries was carried out on five test samples: extra virgin olive oil, virgin olive oil + refined sunflower oil, virgin olive oil + refined olive-pomace oil, virgin olive oil + refined soybean oil + refined sunflower oil, refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil. The test was organized by the Executive Secretariat in 1999 and the results obtained were subjected to statistical analysis in accordance with ISO 5725-1 [3] and ISO 5725-2 [4] to give the precision data shown in Table 1. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

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2. Repeatability (r)— value below which the absolute difference between two single independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time may be expected to lie with a probability of 95%.
3. Reproducibility (R)—value below which the absolute difference between two single test results obtained with the same method on identical test material in different laboratories with different operators using different equipment may be expected to lie with a probability of 95%.

NOTES

Caution

Hexane is a flammable solvent and a dangerous fire risk. All work should be performed in a fume hood, with no open flames. Always ensure that your hood is functioning before introducing volatile solvents. The TLV for hexane is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/M_3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

NUMBERED NOTES

1. The hexane must be distilled to remove impurities.
2. Usually, silica gel can be used directly from the container without any treatment. However, some batches of silica may show low activity, resulting in bad chromatographic separations. If this occurs, or if batches of silica gel result in chromatograms with interfering peaks, the silica gel should be treated as described in Procedure, 1. An alternative could be the use of extra pure silica gel 60 (Merck, ref. 7754).
3. If kept at a temperature below 4°C, cholesta-3,5-diene solutions (Reagents, 4 and 5) will not deteriorate over a period of at least 4 months.
4. The first fraction from Procedure, 3(c) contains saturated hydrocarbons (Figure 1a), and the second fraction contains steroidal hydrocarbons (see Figure 1b). Further elution provides squalene and related compounds. To achieve a good separation between saturated and steroidal hydrocarbons, the fraction volumes must be optimized. For this, the volume of the first fraction should be adjusted so that, when the second fraction is analyzed, the peaks representing the saturated hydrocarbons are low (see Figure 1c); if they do not appear but the intensity of the standard peak is low, the volume should be reduced. The presence of important peaks coming from the first fraction in the second fraction may interfere with the determination of campesta-3,5-diene. The volume of the second fraction generally does not need to be optimized, because a good separation of the components can be achieved. Nevertheless, the presence of a large peak at approximately 1.5 min lower retention time than the standard is due to squalene and indicates a bad separation.
5. The residue from Procedure, 3(d) is unstable at room temperature. As soon as the residues are obtained, the solvent should be added and the solutions should be kept in the refrigerator.
6. Gas chromatographic references for these steroidal hydrocarbons can be obtained by analyzing a test sample of sunflower oil, decolored with 5% of an acid bleaching earth (Tonsyl or similar) at 100°C during 30 min (Figure 2b), or which has been dehydrated by heating the sunflower oil with 10% v/v of sulfuric acid/water (1:1) at 150°C for 5 min.
7. Special care must be taken to identify the stigmasta-3,5,22-triene, since some oils produce other peaks very close in retention time (Figure 2c).
8. The presence of high proportions of saturated hydrocarbons may interfere with the determination of campesta-3,5-diene (see Numbered Notes, 4).
9. Eventually, the second fraction may show a group of intense peaks next to standard, making its identification and integration difficult. Such peaks may come from:
   (a) Presence of squalene and related compounds as a result of a bad separation in the silica gel column (see Numbered Notes, 4).
   (b) Presence of high proportions of other steroidal hydrocarbons derived from triterpenic alcohols.

   In the first case the fractioning should be improved; in the second case, the analysis should be repeated using a smaller amount of oil, thus increasing the standard/interferences ratio.

REFERENCES

1. This method paraphrases International Olive Council (IOC) Method COI/T.20/Doc. no. 1b/Rev. 1.
2. IUPAC Commission on Oils, Fats, and Derivatives (1994). Working Report of Meeting, Bruges, Belgium, July 1994, pp. M7-M11.
3. ISO 5725-1, Accuracy (trueness and precision) of measurement methods and results - Part 1: General principles and definitions, 1994.
4. ISO 5725-2, Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method, 1994.
5. ISO 5725-5, Accuracy (trueness and precision) of measurement methods and results - Part 5: Alternative methods for the determination of the precision of a standard measurement method, 1994.
6. ISO 5725-6, Accuracy (trueness and precision) of measurement methods and results - Part 6: Use in practice of accuracy, 1994.

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Table 1
Precision data.

	Virgin olive oil + refined olive-pomace oil	Refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil
Number of laboratories	18	19
Outliers	5	7
Mean	19.96	15.86
Repeatability limit, r	1.67	1.25
Repeatability standard deviation, S_r	0.59	0.45
Repeatability coefficient of variation ($S_r \times 100$/mean)	3.00	2.84
Reproducibility limit (R)	4.38	3.04
Reproducibility standard deviation, S_R	1.56	1.08
Reproducibility coefficient of variation ($S_R \times 100$/mean)	7.85	6.86

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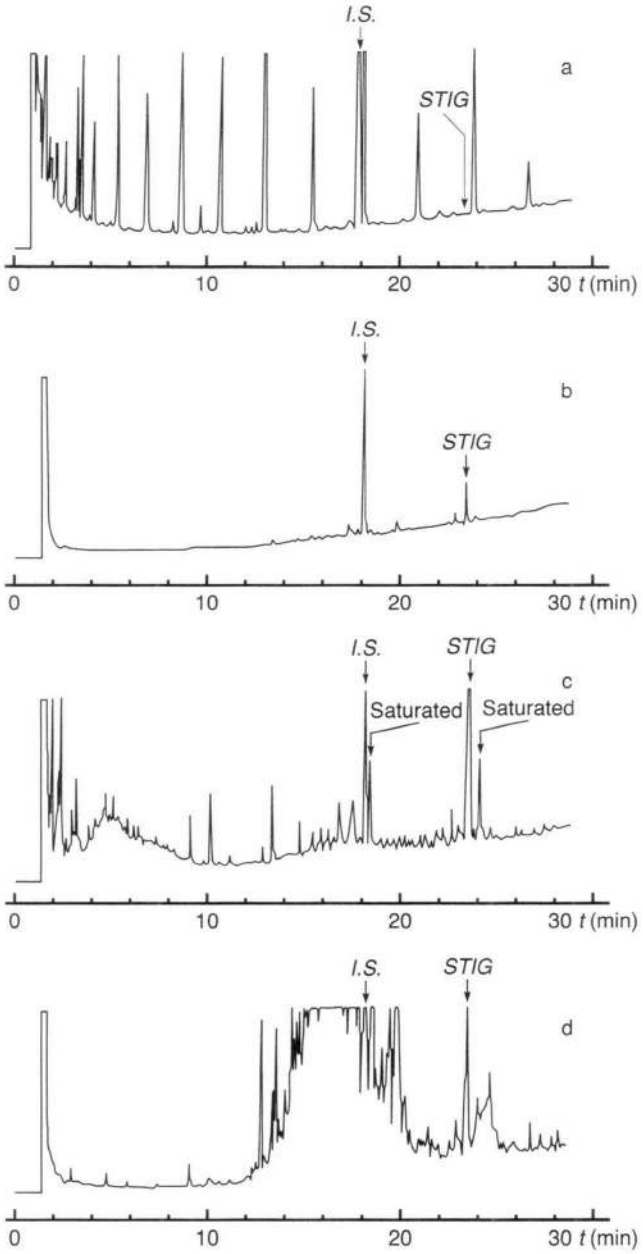

Figure 1. Gas chromatograms obtained from olive oil test samples analyzed on a fused silica capillary column (0.25 mm i.d. × 25 m) coated with 5% phenylmethylsilicone, 0.25 µm film thickness. (a) First fraction (30 mL) from a virgin oil, spiked with standard (cholesta-3,5-diene). (b) Second fraction (40 mL) from a refined oil containing 2 mg/kg of stigmasta-3,5-diene. (c) Second fraction (40 mL) from a refined oil containing 45 mg/kg of stigmasta-3,5-diene showing small amounts of saturated hydrocarbons (first fraction). (d) Second fraction (40 mL) from a refined oil containing 11 mg/kg of stigmasta-3,5-diene and showing the presence of squalene and isomers.

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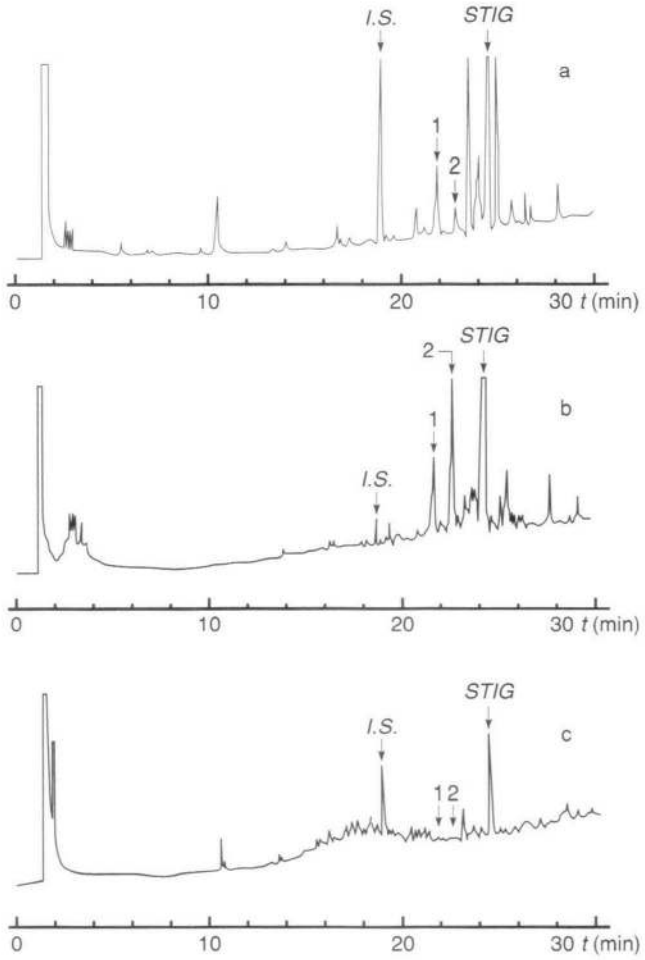

Figure 2. Gas chromatograms of the steroidal hydrocarbon fraction from vegetable oils analyzed on a capillary column (0.25 mm i.d. × 25 m) coated with 5% phenylmethylsilicone, 0.25 µm film thickness. (a) Refined olive oil containing 60 mg/kg of stigmasta-3,5-diene. (b) Sunflower oil decolored with 5% acid bleaching earth (Tonsyl) at 100°C for 30 min. (c) Olive oil containing 35 mg/kg of stigmasta-3,5-diene. 1: Campesta-3,5-diene; 2: stigmasta-3,5,22-triene.

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AOCS

Joint AOCS/JOCS Official Method Cd 28-10

Approved 2012

JOCS

Glycidyl Fatty Acid Esters in Edible Oils

DEFINITION

This method is used for the determination of glycidyl (glycidol) fatty acid esters (GEs) in edible oils using double solid-phase extraction (SPE) and liquid chromatography—mass spectrometry (LC-MS).

SCOPE

Applicable for the determination of glycidyl fatty acid esters (GEs) in commercial vegetable oils and fats.

APPARATUS

1. Vortex mixer.
2. Solid phase extraction cartridges—
   (a) C18, 500 mg (e.g. Waters Sep-Pak Vac RC, WAT036945).
   (b) Silica 500 mg (e.g. Waters Sep-Pak Vac RC, WAT036920).
3. Evaporation unit (nitrogen).
4. Glass Pasteur pipettes.
5. HPLC-MS equipment consisting of—
   (a) High performance liquid chromatography system (HPLC), comprising eluent reservoir, two pumps for gradient, a data processing work station, coupled with a single quadrupole mass spectrometer using an atmospheric pressure chemical ionization source (e.g., Agilent 1100 series LC-MSD SL® single quadrupole system). The mass spectrometer used must have a signal-to-noise ratio greater than 10 when a 20 µL injection of a 5 ng/mL GE standard solution is analyzed.
   (b) Analytical reversed-phase ODS HPLC column (4.6 mm I.D. × 150 mm), e.g., L-column ODS® 4.6 mm I.D. × 150 mm with 5 µm packing material.

REAGENTS

1. *tert*-Butyl methyl ether—HPLC grade (see Notes, *Caution*).
2. Ethyl acetate—reagent grade (see Notes, *Caution*).
3. *n*-Hexane—HPLC grade (see Notes, *Caution*).
4. Methanol—HPLC grade (see Notes, *Caution*).
5. Isopropyl alcohol—HPLC grade.
6. Water—ultrapure (e.g. water prepared using a Millipore Milli-Q purification system).

STANDARDS (SEE NOTES, 1)

1. Glycidol palmitic acid ester—C16:0-GE, > 90% purity.
2. Glycidol stearic acid ester—C18:0-GE, > 90% purity.
3. Glycidol oleic acid ester—C18:1-GE, > 90% purity.
4. Glycidol linolic acid ester—C18:2-GE, > 90% purity.
5. Glycidol linolenic acid ester—C18:3-GE, > 80% purity.

PREPARATION OF STANDARD SOLUTIONS (SEE NOTES, 2)

1. All solutions are prepared with methanol/isopropyl alcohol 1:1 (v/v).
2. Individual Stock Solutions, 500 µg/mL—
   Weigh accurately a sufficient amount of each Working Standard into a 100 mL volumetric flask to ensure that 50 mg of the standard is present (e.g. if the purity is 95%, weigh 52.6 mg). Fill up to the mark with methanol/isopropyl alcohol 1:1 (v/v). Standard solutions are stable at 4°C with a nitrogen headspace for at least six months.
3. Working Solutions—
   (a) Mixed Solution I, 10 µg/mL—Using a glass pipette add 2 mL of each 500 µg/mL Individual Stock Solution (2) into a 100 mL volumetric flask and dilute to the mark with methanol/isopropyl alcohol solution 1:1 (v/v) just prior to use.
   (b) Mixed Working Solution II, 1000 ng/mL—Using a glass pipette add 10 mL of the 10 µg/mL Mixed Solution I (3a) into a 100 mL volumetric flask and dilute to the mark with methanol/isopropyl alcohol solution 1:1 (v/v).
   (c) Prepare Mixed Working Solutions of 200 ng/mL, 50 ng/mL, 10ng/mL and 5 ng/mL by diluting appropriately the Mixed Working Solution II, 1000 ng/mL (3b). The solutions are stable for two weeks at 4°C under nitrogen.

TEST SAMPLE PREPARATION (SEE NOTES 2, 3)

1. Weigh 1 g edible oil/fat into a 20 mL beaker. Add 6 mL *tert*-butyl methyl ether/ethyl acetate 4:1 (v/v) and dissolve the test sample completely.

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2. Transfer the solution to a 10 mL volumetric flask quantitatively with a Pasteur pipette. Carefully wash the beaker with several small volumes of *tert*-butyl methyl ether/ethyl acetate 4:1 (v/v), add to the flask.
3. Add *tert*-butyl methyl ether/ethyl acetate 4:1 (v/v) up to the mark and mix the solution well.

PROCEDURE (SEE NOTES, 2)

1. Reversed-phase SPE—Condition a Sep-Pak C18 cartridge with 4 mL of methanol (1 mL, four times). Take care to wash the inner wall of the cartridge just prior to use (do not allow the cartridge to dry out after conditioning). Rinse thoroughly a micro-syringe with methanol/isopropyl alcohol 1:1 (v/v) and transfer 100 µL of the test sample solution to the cartridge. Add 2 mL of methanol three times to the cartridge. Take care to wash the inner wall of the cartridge. Collect the eluted methanol (ca. 6 mL) in a clean glass tube under gravity. Do not use vacuum or other equipment, such as joints and manifolds, during the SPE step. After the elution is complete under gravity, hold the cartridge in one hand and press the palm of the other hand on the open top of the cartridge to drive out as much solvent as possible from the cartridge (See Notes, 4). Evaporate the solvent at room temperature using a nitrogen stream to obtain a dried residue.
2. Transfer 2 mL of *n*-hexane/ethyl acetate 95:5 (v/v) into the glass tube containing the residue (1). Rinse the solvent around the tube wall to collect the residue. Agitate the tube using a vortex mixer for more than 30 s to completely dissolve the dried residue.
3. Normal-phase SPE—Condition a Sep-Pak Silica cartridge with 4 ml of *n*-hexane/ethyl acetate 95:5 (v/v) (1 mL, four times). Take care to wash the inner wall of the cartridge just prior to use (do not allow the cartridge to dry out after conditioning). Transfer the solution (2) to the cartridge and then collect the eluted *n*-hexane/ethyl acetate under gravity in a clean glass tube. Wash the inner wall of the empty glass tube (2) using 2 mL of *n*-hexane/ethyl acetate and apply to the cartridge. Take care to wash the inner wall of the cartridge. Collected the eluted solvent. Repeat the above procedures (from washing the empty glass tube to collecting the solvent) three times (totally ca. 8 mL). Do not use vacuum or other equipment, such as joints and manifolds, during the SPE step. After the elution is complete under gravity, hold the cartridge in one hand and press the palm of the other hand on the open top of the cartridge to drive out as much solvent as possible from within the cartridge (See Notes, 4). Evaporate the solvent at room temperature using a nitrogen stream to obtain a dried residue.
4. Accurately transfer 1 mL of methanol/isopropyl alcohol 1:1 (v/v) into the glass tube (3) containing the residue using a Class A glass volumetric pipette. Rinse the solvent around the tube wall to collect the residue. Agitate the glass tube using a vortex mixer for more than 30 s to completely dissolve the dried residue. If the residue is not dissolved completely, prepare a supernatant solution (e.g. by centrifugation). Do not use membrane filters or other filtration systems. Transfer the solution to a glass vial for LC-MS analysis.
5. Analyze 20 µL aliquots of the test sample solution and Mixed Working Solutions by LC-MS.
6. LC-MS analysis—
   (a) LC conditions—
   Mobile phase A—methanol/water 92:8 (v/v)
   Mobile phase B—isopropyl alcohol.
   Gradient—Isocratic elution Mobile phase A 100% (B 0%) from 0.0 min to 18.0 min.
   Linear gradient of A 100% (B 0%) to A 0% (B 100%) from 18.0 min. to 18.1 min.
   Isocratic elution A 0% (B 100%) from 18.1 min to 25.0 min for column washing
   Linear gradient of A 0% (B 100%) to A 100% (B 0%) from 25.0 min. to 25.1 min.
   Isocratic elution A 100% (B 0%) from 25.1 min to 35.0 min.
   (see Note 5)
   Flow rate—1 mL/min.
   Injection volume—20 µL.
   Set column temperature at 40°C.
   Acquisition time window—0 to 18 min (the mobile phase is not introduced in the mass spectrometer from 18 to 35 min to avoid contamination).
   (b) MS conditions—
   Polarity—positive.
   Vaporizer temperature—500°C.
   Heater temperature of nitrogen gas—350°C.
   Flow of heated dry nitrogen gas—5.0 L/min.
   Nebulizer gas pressure—0.241 MPa (35 psi).
   Corona current—8.0 µA.
   Fragmenter voltage—150 V.
   Selected ion monitoring (SIM) for protonated molecular ions $[M + H]^+$– *m/z* 313.5 for C16:0-GE, *m/z* 341.5 for C18:0-GE, *m/z* 339.4 for C18:1-GE, *m/z* 337.4 for C18:2-GE and *m/z* 335.4 for C18:3-GE.
   Dwell time—200 ms.
   For references guidelines on optimal conditions using different LC-MS equipment—see Notes 6.
7. Figures 1 & 2 show LC-MS selected ion monitoring chromatograms of five species of standard GEs and two samples of edible oil.

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VERIFICATION

1. Blank test—Perform double SPE treatment and LC-MS measurement on solvents (without oil samples) according to Procedure 1–6 above. If there are no contaminant peaks that affect the quantitative results of GEs, the experimental procedures are acceptable. If there are significant peaks that affect the quantitative results, the procedure requires improve-ment. Investigate the procedure in a step-wise fashion until the contamination problem is resolved (see Note, 2). Do not proceed with sample analysis until all concerns with the blank have been resolved.
2. Recovery test—Accurately add 1 mL of 10 µg/mL Mixed Working Solution I (10 µg of each GE) using a Class A glass volumetric pipette into a 20 mL glass beaker. Evaporate the solvent using a nitrogen stream. Weigh 1 g edible oil/fat into a 20 mL beaker. Add 6 mL *tert*-butyl methyl ether/ethyl acetate 4:1 (v/v) and dissolve the test sample completely. Transfer the solution to a 10 mL glass volumetric flask quantitatively with a glass Pasteur pipette. Carefully wash the beaker with several small volumes of *tert*-butyl methyl ether/ethyl acetate 4:1 (v/v), add to the flask. Add *tert*-butyl methyl ether/ethyl acetate 4:1 (v/v) up to the mark and mix the solution well. The amounts spiked correspond to 10 µg/g GE in the oil. Perform the double SPE and LC-MS measurement according to Procedure 1-6 above. Recovery values (%) are calculated using both the quantified levels of the GEs in the spiked oil and those in the non-spiked oil. Unless the recovery values range from 80 to 120%, quantitative results cannot be reported.

CALCULATIONS

1. Prepare a calibration curve by plotting the concentration (x) of the standard GEs (range 5ng/mL to 200 ng/mL Mixed Working Solution) against their SIM peak areas (y), as follows:

$$y = Ax + B$$

Where—
A = slope
B = y-intercept

Confirm that the linearity is better than 0.99 (see Notes, 7).

2. Determine the concentration of individual GEs in the test sample (mg/kg) (Q) using the SIM peak areas (S), as follows:

$$Q = V / (10AW)$$

Where—
Q = GE in the test sample (mg/kg)
S = SIM peak area determined in the sample
B = y-intercept of calibration curve
V = corrected SIM peak area for each GE, (S–B)
A = slope of calibration curve
W = sample weight (g) See Test Sample Preparation

PRECISION

Intermediate precision values are given in Tables 1 and 2. Precision data determined from a collaborative study of this method by 17 laboratories analyzing 18 samples are given in Table 3. Each of 9 samples of commercial vegetable fats and oils was determined as a blind duplicate. Reproducibility and repeatability values are calculated according to the AOAC/IUPAC Harmonized Protocol for Collaborative Study. Data submitted as below LOQ were not considered in the evaluation of the Collaborative Study results, hence some data sets were from fewer than 17 laboratories before statistical analysis. Extra virgin olive oil previously determined to contain no detectable level of GE was used as the blank sample and also to prepare a spiked sample containing approximately 50 ng/ml total GE (See Notes, 8 and Table 4). Determination of LOQ is strongly influenced by the configuration of the chosen mass spectrometer and individual measurement uncertainty. Performance of the test at the lowest concentration of the calibration curve may be used to guide each analyst.

NOTES

Caution

tert-Butyl methyl ether is extremely flammable and toxic. Avoid inhalation, ingestion, and eye or skin contact. The TLV is 50 ppm in air. OSHA recommends that exposure not exceed 100 mg/M^3 for a time-weighted average. Respiratory irritation, dizziness, and disorientation have been reported. A fume hood should be used at all times when using tert-Butyl methyl ether.

Ethyl acetate is flammable and toxic. Avoid inhalation, ingestion, and eye or skin contact. The TLV is 3.9 ppm in air. OSHA recommends that exposure not exceed 1,400 mg/ M^3 for a time-weighted average. Contact between ethyl acetate and nitrates, strong oxidizers, strong alkalies, or strong acids may cause fires and explosions. The use of a fume hood is recommended when using ethyl acetate.

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n-Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Methanol (methyl alcohol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Methanol can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform, and perchloric acid.

NUMBERED NOTES

1. Standards may be purchased from Wako Pure Chemical Industry Ltd., Tokyo, Japan; and Tokyo Chemical Industry Co. Ltd., Tokyo, Japan. GE standards are available from other manufacturers, however, their purity must be clearly indicated because some standards may contain other alkyl GEs as impurities. In which case, their levels must be taken into consideration when the standard solutions are prepared. GE standards are recommended to be stored at around –20°C with a nitrogen headspace to avoid any changes.
2. Use glass equipment during all procedures, e.g. glass Pasteur pipettes, glass run-out pipettes, glass volumetric flasks, glass beakers, glass tubes and glass micro-syringes. Metal equipment like metal spatulas can be used. With the exception of the essential SPE cartridges, do not use any plastic equipment, e.g. plastic pipette tips, plastic stop valves for SPE, plastic liners for SPE and membrane filters made of plastic resins, for any step in the preparation of standard solutions and samples. Plastic resins may be the cause of contaminants that interfere with the resolution of the analytes.
3. Solid oil (fats) at room temperature may be non-homogeneous. Warm the test sample to not more than 5°C above the melting point and thoroughly homogenize. Long exposure to high temperature may cause possible decomposition or conversion of GEs in the oil.
4. Pressure may also be applied by gently inserting an appropriately-sized syringe plunger into the mouth of the SPE cartridge.
5. If the pressure goes over the upper limit during column when washing (T 18.1 to 25.0 min) with 100% mobile phase B, the flow rate can be reduced. In which case, the wash period must be prolonged to maintain the total volume, e.g., 0.5 mL/min for 13.8 min can be used instead of 1.0 mL/min for 6.9 min.
6. MS conditions described in this method are optimized for Agilent 1100 series LC-MS SL® single quadruple system. Consult the user's manual for optimal MS conditions if other LC-MS equipment is used.
7. If the high sensitivity of the mass spectrometer does not allow a linear response to GE standards at concentrations of 5, 10, 50, 100 and 200 ng/mL, dilute both the standards and the sample solutions to the same degree. For example, if 0.5, 1, 5, 10 and 20 ng/mL standard solutions are used, the sample solutions must be diluted with 10 mL methanol/isopropyl alcohol 1:1 (v/v) at Procedure 4 above, instead of 1 mL.
8. A spiked sample was prepared for the collaborative study to contain 10 µg/ml of each GE (C16:0, C18:0, C18:1, C18:2, C18:3) with a nominal total concentration of 50 µg/ml in extra virgin olive oil. All samples were stored under identical conditions in the dark, at room temperature, in individual nitrogen-flushed vials during the study period. Results returned in the first precollaborative study carried out one month after preparation (Table 4) show mean values slightly less than the original value. At 6 months (2nd precollaborative study) returned values showed a further decrease in GE content. The same sample, at month 10 during the full collaborative trial, was again determined by participants to have a further reduced value. These results indicate that GE may not be stable in certain oil matrices. Analysts should be aware of this potential instability when designing analytical protocols and reporting results.

REFERENCES

1. Masukawa Y., Shiro H., Nakamura S., Kondo, N., Jin N., Suzuki N., Ooi N., Kudo N. (2010) *J. Oleo Sci.* 59:81–88.
2. Masukawa Y., Shiro H., Kondo N., Kudo, N. (2010) *J. Am. Oil Chem. Soc.* 88:15–21.
3. Shiro, H., Kondo, N., Kibune, N., Masukawa, Y. (2011). *Eur. J. Lipid Sci. Technol.* 113:356–360.

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Table 1
Percentages of relative standard (RSD%) during consecutive runs, limit of detection (LOD), limit of quantification (LOQ) and calibration line obtained from standard glycidol fatty acid esters (GEs).

	RSD% [a]		LOD [b]	LOQ [b]	Calibration line [c]	
GE	Retention time	Area	(ng/mL)	(ng/mL)	Equation	R2
C16:0-GE	0.17	4.0	0.16	0.52	y = 2238x - 1714	1.0000
C18:0-GE	0.17	4.4	0.23	0.78	y = 2223x - 1719	1.0000
C18:1-GE	0.19	4.6	0.12	0.42	y = 2230x + 3156	1.0000
C18:2-GE	0.16	3.9	0.19	0.65	y = 1923x + 2446	1.0000
C18:3-GE	0.16	4.6	0.21	0.69	y = 1344x - 2540	1.0000

[a] Obtained by six consecutive runs of the standard GE solution at a concentration of 200 ng/mL.
[b] Defined as signal to noise = 3 for LOD and 10 for LOQ based on 20 µL injection of the standard GE solution at a concentration of 5 ng/mL.
[c] Calculated from the equation y = Ax + B, where x is the injected concentration (ng/mL), y is the peak area, A is the slope, and B is the intercept in the range between 5 and 1000 ng/mL (5, 10, 50, 200 and 1000 ng/mL).

Table 2
Levels of glycidol fatty acid esters (GEs) in a commercial edible oil sample (palm) and recovery values obtained by a newly developed method.

	Level [mg/g] [a]	Recovery% [a, b]	
GE	Mean ± S.D.	Mean ± S.D.	RSD%
C16:0-GE	8.4 ± 0.23	109.4 ± 1.4	1.3
C18:0-GE	1.2 ± 0.015	108.0 ± 0.9	0.9
C18:1-GE	12.4 ± 0.059	111.9 ± 3.5	3.2
C18:2-GE	3.6 ± 0.025	110.4 ± 1.6	1.4
C18:3-GE	< 0.5 [c]	107.7 ± 3.7	3.4

[a] Analyzed in triplicate.
[b] Obtained from the ratio of the difference between quantified levels of the GEs in the spiked oil and those in the non-spiked oil to the known levels spiked (10 µg/g-oil of each GE).
[c] Determined by the lowest concentration of the standard solution for calibration and dilution degrees (100-fold dilution) in the oil sample preparation.

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Table 3
Results of glycidyl fatty acid ester (mg/kg, GEs) determination by 17 laboratories in a collaborative study[a].

Sample ID	C16:0	C18:0	C18:1	C18:2	C18:3	Total
Extra Virgin Olive Oil	no results reported above 0.5 mg/kg					
Spiked Extra Virgin Olive Oil[b]	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Total**
n	17	17	17	17	17	17
Mean	3.94	3.91	4.70	4.52	4.31	21.29
repeatability						
s(r)	0.35	0.34	0.38	0.37	0.49	1.86
RSD(r)	8.88	8.62	7.99	8.09	11.28	8.72
r	0.98	0.94	1.05	1.02	1.36	5.20
Reproducibility						
s(R)	0.81	0.69	0.78	0.91	1.05	3.96
RSD(R)	20.47	17.62	16.58	20.06	24.34	18.61
R	2.26	1.93	2.18	2.54	2.94	11.09
Palm Oil (1)	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Total**
n	17	12	17	17		17
Mean	2.34	0.50	5.11	1.33	N.Q.	9.26
s(r)	0.46	0.08	0.96	0.26		1.92
RSD(r)	19.88	16.95	18.83	19.17		20.77
r	1.30	0.24	2.69	0.72		5.38
s(R)	0.78	0.15	1.50	0.44		2.74
RSD(R)	33.16	29.31	29.45	33.32		29.58
R	2.17	0.41	4.21	1.24		7.67
Palm Oil (2)	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Total**
n	17	13	17	16		17
Mean	2.37	0.48	5.12	1.35	N.Q.	9.40
s(r)	0.34	0.10	0.68	0.21		1.42
RSD(r)	14.49	19.91	13.27	15.90		15.14
r	0.96	0.27	1.90	0.60		3.99
s(R)	0.63	0.14	1.13	0.35		2.47
RSD(R)	26.64	29.76	21.99	26.18		26.31
R	1.77	0.40	3.15	0.99		6.93
Palm Oil + GS	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Total**
n	17	17	15	17		17
Mean	2.68	7.26	5.66	1.39	N.Q.	16.44
s(r)	0.36	0.74	0.60	0.18		1.61
RSD(r)	13.50	10.24	10.63	13.20		9.79
r	1.01	2.08	1.68	0.51		4.50
s(R)	0.91	2.40	1.09	0.49		5.25
RSD(R)	33.97	33.08	19.33	35.52		31.93
R	2.55	6.72	3.06	1.38		14.70
Rapeseed Oil	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Total**
n			15			14
Mean	N.Q.	N.Q.	0.45	N.Q.	N.Q.	0.52
s(r)			0.17			0.20
RSD(r)			36.44			37.67
r			0.46			0.55
s(R)			0.20			0.22
RSD(R)			43.77			42.42
R			0.56			0.62

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Table 3, cont.

Soybean Oil	C16:0	C18:0	C18:1	C18:2	C18:3	Total
n				15		14
Mean	N.Q.	N.Q.	N.Q.	0.48	N.Q.	0.56
s(r)				0.08		0.09
RSD(r)				17.45		16.08
r				0.23		0.25
s(R)				0.21		0.23
RSD(R)				43.51		41.39
R				0.58		0.64
Corn Oil	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Total**
n	9		15	16		15
Mean	0.20	N.Q.	0.82	1.49	N.Q.	2.51
s(r)	0.03		0.06	0.10		0.15
RSD(r)	13.71		7.57	6.85		5.95
r	0.08		0.15	0.29		0.42
s(R)	0.11		0.15	0.36		0.45
RSD(R)	53.61		18.35	23.97		17.95
R	0.31		0.42	1.00		1.26
Palm Oil (1) dupilcate	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Total**
n	17	14	17	17		17
Mean	2.39	0.49	5.37	1.39	N.Q.	9.44
s(r)	0.23	0.09	0.51	0.15		0.94
RSD(r)	9.84	17.42	9.45	10.51		10.00
r	0.66	0.24	1.42	0.41		2.64
s(R)	0.54	0.15	0.98	0.32		1.85
RSD(R)	22.79	30.10	18.30	22.88		19.58
R	1.52	0.42	2.75	0.89		5.17

[a]Results obtained in a collaborative study organized by AOCS in 2011. Precision data calculated according to the AOAC/IUPAC Harmonized Protocol. Each laboratory was supplied with blind duplicates of 9 samples of commercial vegetable fats and oils.
[b]Refer to Notes, 8.
N.Q. No laboratory returned results above their individual quantification limit for this analyte.

Table 4
Values of individual and total glycidyl ester content determined by study participants in the "spiked" sample at different times after preparation.

	C16:0	C18:0	C18:1	C18:2	C18:3	Total
Spiked level (mg/kg)	10	10	10	10	10	50
Precollab 1 (month 3)						
Mean	9.07	8.84	8.85	8.53	9.50	43.20
SD	1.83	1.90	2.34	1.87	2.97	10.09
Precollab 2 (month 10)						
Laboratories	15	15	15	15	15	15
Mean	5.32	5.04	5.92	5.77	5.37	27.96
s(R)	0.57	0.63	0.71	0.75	0.86	3.26
Collaborative Study (month 13)						
n	17	17	17	17	17	17
Mean	3.94	3.91	4.70	4.52	4.31	21.29
s(R)	0.81	0.69	0.78	0.91	1.05	3.96

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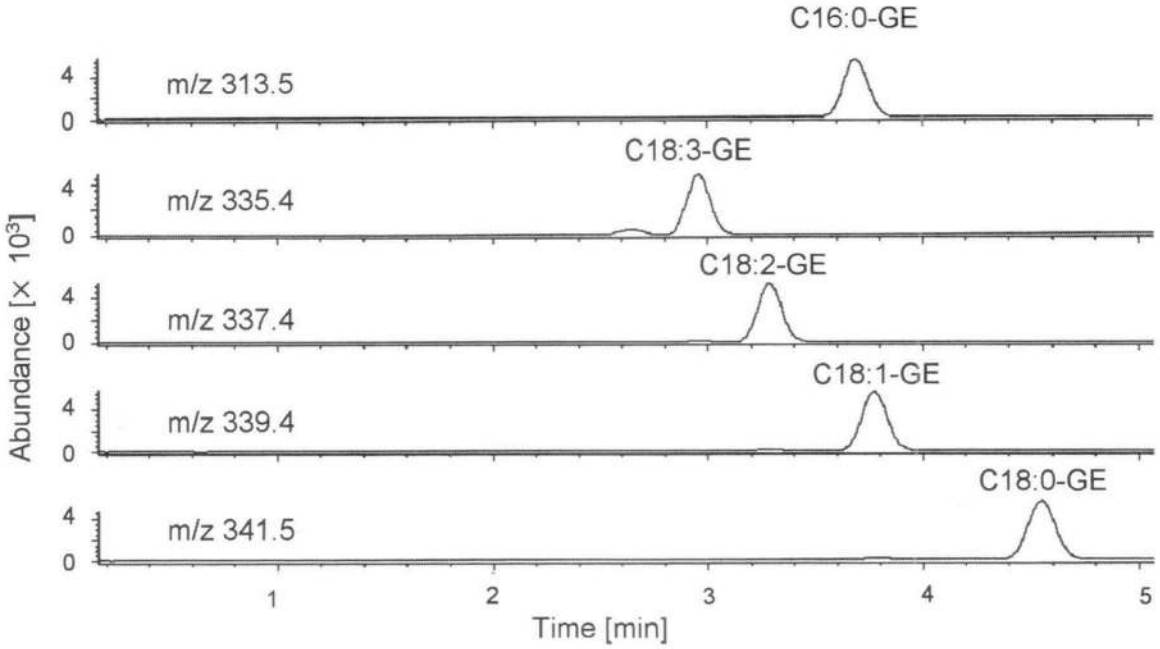

Figure 1.
LC-MS selected ion monitoring chromatograms of five species of standard GEs (200 ng/mL each).

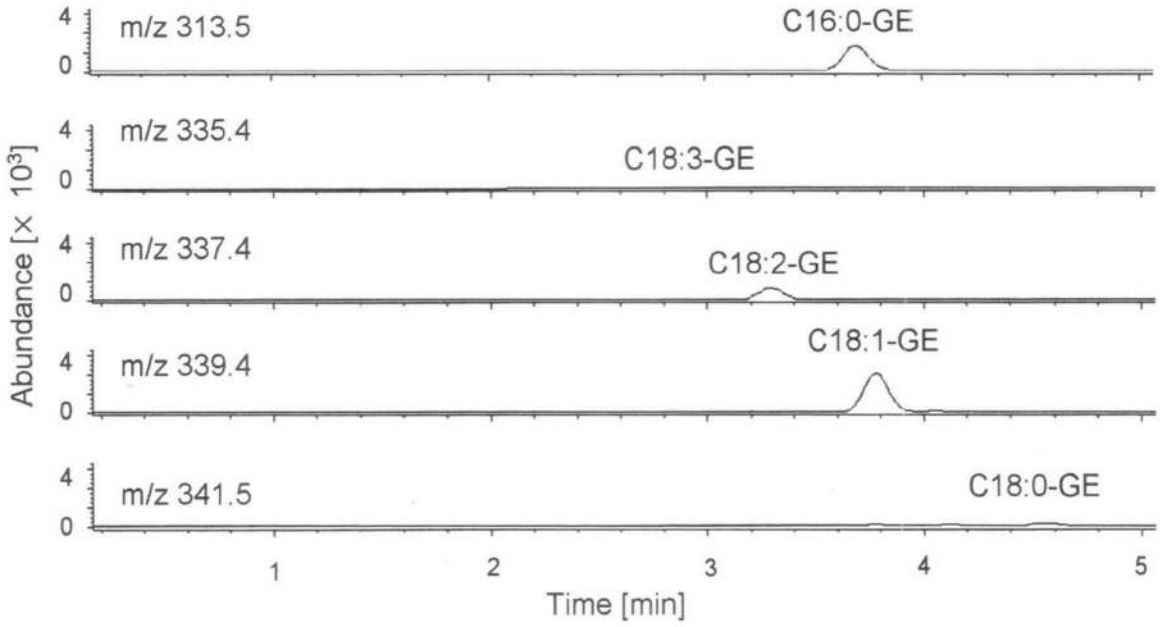

Figure 2.
LC-MS selected ion monitoring chromatograms of GEs in a commercial edible oil (palm).

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AOCS Official Method Cd 29a-13
Approved 2013

2- and 3-MCPD Fatty Acid Esters and Glycidol Fatty Acid Esters in Edible Oils and Fats by Acid Transesterification

DEFINITION

This method is used for the determination of fatty acid esters of 2-chloropropane-1,3-diol (2-MCPD), 3-chloropropane-1,2-diol (3-MCPD) and glycidol in edible oils and fats; see also AOCS Official Methods Cd 29b-13 or Cd 29c-13 (see Notes, 1).

Glycidyl esters are converted to 3-monobromopropanediol (3-MBPD) monoesters in an acid solution containing a bromide salt. 3-MBPD esters, together with 2- and 3-MCPD esters, are then converted into the free (non-esterified) form in acid methanolic solution. The fatty acid methyl esters generated during the reaction are extracted from the sample; and 2- and 3-MCPD as well as 3-MBPD, are then derivatized with phenylboronic acid prior to GC-MS analysis.

SCOPE

Applicable for the determination of 2- and 3-MCPD fatty acid esters and glycidyl fatty acid esters (as a sum of all the respective esters) in edible oils/fats.

APPARATUS

1. Vortex mixer.
2. Oven capable of 45°C ± 5°C.
3. Ultrasonic bath.
4. Evaporation unit (nitrogen).
5. Centrifuge.
6. GC-MS equipment consisting of:
   (a) Capillary gas chromatograph coupled with a quadrupole mass selective detector (e.g., Agilent Technologies 7890A gas chromatograph equipped with Agilent 5975C MSD) and data processing system.
   (b) Bonded, poly(dimethylsiloxane) capillary column (e.g., Supelco Equity-1, 30 m length × 0.25 mm i.d. × 1.0 µm film thickness).

REAGENTS

1. Tetrahydrofuran, anhydrous
2. Methanol, analytical grade
3. *n*-Heptane, analytical grade
4. Acetone, analytical grade
5. Toluene, analytical grade
6. Water, ultra-pure (e.g., obtained by using a Millipore Milli-Q purification system)
7. Sulfuric acid (purity ≥ 95%)
8. Sodium hydrogen carbonate (purity ≥ 99%)
9. Sodium sulfate (purity ≥ 99%)
10. Phenylboronic acid (purity ≥ 97%)
11. Sodium bromide (purity ≥ 99.5%)

SOLUTIONS: REAGENTS

1. Acid aqueous solution of sodium bromide (sodium bromide 3 mg/mL, sulfuric acid 5% v/v). Prepare a concentrated aqueous solution of sodium bromide by dissolving 1 g of sodium bromide (Reagents, 11) in 10 mL of ultra-pure water (Reagents, 6). Transfer 180 µL of the concentrated solution into an empty conical flask. Add 0.3 mL of sulfuric acid (Reagents, 7) and 5.5 mL of ultra-pure water (Reagents, 6). Shake vigorously (see Notes, 2).
2. Sodium hydrogen carbonate solution (0.6%, w/v). Weigh 0.6 g of sodium hydrogen carbonate (Reagents, 8) in a 100 mL volumetric flask and fill up to the mark with ultra-pure water (Reagents, 6). Use ultrasonic bath to ensure the complete dissolution of the reagent (see Notes, 3).
3. Sulfuric acid/methanol solution (1.8% v/v). Pipette 1.8 mL of sulfuric acid (Reagents, 7) in a 100 mL volumetric flask and fill up to the mark with methanol (Reagents, 2; see Notes, 2).

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4. Sodium hydrogen carbonate solution (saturated). Weigh 9.6 g of sodium hydrogen carbonate (Reagents, 8) in a 100 mL volumetric flask and fill up to the mark with ultra-pure water (Reagents, 6). Use ultrasonic bath to ensure the dissolution of the reagent (see Notes, 4).
5. Sodium sulfate solution (20%, w/v). Weigh 20 g of sodium sulfate (Reagents, 9) in a 100 mL volumetric flask and fill up to the mark with ultra-pure water (Reagents, 6). Use ultrasonic bath to ensure the complete dissolution of the reagent.
6. Phenylboronic acid solution (saturated). Weigh 3 g of phenylboronic acid (Reagents, 10) and add 12 mL of an acetone (Reagents, 4)/ultra-pure water (Reagents, 6) mixture (19/1 v/v). Shake vigorously (see Notes, 5).

STANDARDS

1. 1,2-Dipalmitoyl-3-chloropropanediol (PP-3-MCPD), purity ≥ 95% (e.g., supplied by Santa Cruz Biotechnology Inc., Santa Cruz, CA or synthesized from 3-MCPD and palmitoyl chloride as described by Kraft et al. [1]; see Notes, 6).
2. 1,3-Dipalmitoyl-2-chloropropanediol (PP-2-MCPD), purity ≥ 95% (e.g., synthesised from 2-MCPD and palmitoyl chloride as described by Kraft et al. [1]; see Notes, 7).
3. Pentadeuterated 1,2-dipalmitoyl-3-chloropropanediol (PP-3-MCPD-d5), purity ≥ 95% (the same consideration applied to 1,2-dipalmitoyl-3-chloropropanediol is valid also for this standard, see Notes, 6).
4. Glycidyl palmitate (Gly-P), purity ≥ 98% (e.g., supplied by Wako Chemicals GmbH, Neuss, Germany; see Notes, 8).
5. Pentadeuterated glycidyl palmitate (Gly-P-d5), purity ≥ 98% (e.g., supplied by Toronto Research Chemicals Inc., North York, ON, Canada; the same consideration applied to glycidyl palmitate is valid also for this standard, see Notes, 8).

SOLUTIONS: STANDARDS

1. All standard solutions can be prepared with either toluene (Reagents, 5) or tetrahydrofuran (Reagents, 1). Toluene is preferred for standard solutions containing glycidyl esters.
2. Stock solutions (1 mg/mL).
   (a) Weigh 10 mg of PP-3-MCPD (Standards, 1) in a 10 mL volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.
   (b) Weigh 10 mg of PP-2-MCPD (Standards, 2) in a 10 mL volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.
   (c) Weigh 10 mg of PP-3-MCPD-d5 (Standards, 3) in a 10 mL volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.
   (d) Weigh 10 mg of Gly-P (Standards, 4) in a 10 mL volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.
   (e) Weigh 10 mg of Gly-P-d5 (Standards, 5) in a 10 mL volumetric flask. Fill up to the mark, making sure that the standard is completely dissolved in the solvent.

   Stock solutions are stable for at least three months when stored at –18°C.
3. Working solutions.
   (a) Calibration I (PP-3-MCPD, 55 µg/mL). Pipette 550 µL of the stock solution (Solutions: Standard, 2a) into a 10 mL volumetric flask and fill up to the mark with the solvent.
   (b) Calibration II (PP-3-MCPD, 5.5 µg/mL). Pipette 1 mL of the Calibration I solution (Solutions: Standard, 3a) into a 10 mL volumetric flask and fill up to the mark with the solvent.
   (c) Calibration III (PP-2-MCPD, 55 µg/mL). Pipette 550 µL of the stock solution (Solutions: Standard, 2b) into a 10 mL volumetric flask and fill up to the mark with the solvent.
   (d) Calibration IV (PP-2-MCPD, 5.5 µg/mL). Pipette 1 mL of the Calibration III solution (Solutions: Standard, 3c) into a 10 mL volumetric flask and fill up to the mark with the solvent.
   (e) Calibration V (Gly-P, 100 µg/mL). Pipette 1 mL of the stock solution (Solutions: Standard, 2d) into a 10 mL volumetric flask and fill up to the mark with the solvent.
   (f) Calibration VI (Gly-P, 10 µg/mL). Pipette 1 mL of the Calibration V solution (Solutions: Standard, 3e) into a 10 mL volumetric flask and fill up to the mark with the solvent.
   (g) Internal standard I (PP-3-MCPD-d5, 40 µg/mL). Pipette 400 µL of the stock solution (Solutions: Standard, 2c) into a 10 mL volumetric flask and fill up to the mark with the solvent.
   (h) Internal standard II (Gly-P-d5, 50 µg/mL). Pipette 500 µL of the stock solution (Solutions: Standard, 2e) into a 10 mL volumetric flask and fill up to the mark with the solvent. (see Notes, 9 and 10).

PROCEDURE: TEST SAMPLE PREPARATION

1. Weigh 100–110 mg of oil/fat (± 0.01 mg) in a screw cap glass tube. Add 50 µL of Internal Standard Working Solution I (Solutions: Standard, 3g) and II (Solutions: Standard, 3h) and 2 mL of tetrahydrofuran (Reagent, 1). Mix for 15 s (or until complete dissolution) by means of a vortex mixer (see Notes, 11, 12, 13 and 14).
2. Add 30 µL of acid aqueous solution of sodium bromide (Solutions: Reagent, 1) to the sample, shake vigorously (vortex) and incubate the mixture at 50°C for 15 min. Stop the reaction by the addition of 3 mL of 0.6% aqueous solution of sodium hydrogen carbonate (Solutions: Reagent, 2). In order to separate the oil/fat from the water phase, add 2 mL of *n*-heptane

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(Reagent, 3) and shake vigorously (vortex, 15 s). After separation of the two phases, transfer the upper layer to an empty glass tube and evaporate to dryness under a nitrogen stream (max 15-20 min at 35–40°C). Dissolve the residue in 1 mL of tetrahydrofuran (Reagent, 1; see Notes, 15, 16 and 17).

3. Add 1.8 mL of sulfuric acid/methanol solution (Solutions: Reagent, 3) to the sample and shake vigorously (vortex) for 10 s. Close the cap of the glass tube tightly and incubate the mixture at 40°C for 16 h.
4. After the incubation period, stop the reaction by adding 0.5 mL of sodium hydrogen carbonate saturated solution (Solutions: Reagent, 4) to the sample. Shake (vortex) for 10 s. Evaporate the organic solvents of the mixture under a nitrogen stream (see Notes, 18).
5. Add 2 mL of sodium sulfate solution (Solution: Reagent, 5) and 2 mL of *n*-heptane (Reagent, 3). Shake (vortex) for 10 s. The two phases will spontaneously separate in few seconds. Discard the upper phase (that contains fatty acid methyl esters dissolved in *n*-heptane) and repeat the extraction with *n*-heptane (see Notes, 19).
6. Add 250 µL of phenylboronic acid solution (Solution: Reagent, 6), shake for 10 s (vortex) and incubate the mixture for 5 min in an ultrasonic bath (room temperature).
7. Extract the phenylboronic derivatives of 2- and 3-MCPD as well as 3-MBPD by adding 1 mL of *n*-heptane (Reagent, 3), shake for 10 s (vortex) and transfer the upper phase to an empty glass test tube. Repeat the extraction with 1 mL of *n*-heptane and combine the two extracts. Evaporate the extracts to dryness under a nitrogen stream. Dissolve the residue in 400 µL of *n*-heptane by shaking the mixture for 10 s (vortex) and transfer the supernatant to an empty GC vial (a glass insert of about 250 µL of volume is typically used) (see Notes, 20, 21 and 22).

PREPARATION OF THE CALIBRATION CURVE

1. Prepare 9 calibration samples by pipetting 50 µL of both internal standard working solutions (Solutions: Standard, 3g and 3h) and the volume of calibration solutions (Solutions: Standard, 3a–f) indicated in Table 1. Add 2 mL of tetrahydrofuran (Reagent , 1) and shake vigorously for 10 s (vortex)(see Notes, 23).
2. Treat the calibration samples according to the procedure used for the test samples (Procedure: Test Sample Preparation, 2–7).

EQUIPMENT

1. GC conditions
   (a) Injection volume: 1.0 µL
   (b) Injection mode: pulsed splitless
   (c) Injection temperature: 250°C
   (d) Carrier gas: helium
   (e) Flow rate: 0.8 mL/min
   (f) Temperature program: 80°C (1 min), from 80°C to 170°C at 10°C/min, from 170°C to 200°C at 3°C/min, from 200°C to 300°C at 15°C/min, 15 min at 300°C
2. MS conditions
   (a) Transfer line temperature: 300°C
   (b) Ion source temperature: 230°C
   (c) Quadrupole temperature: 150°C
   (d) Ionization mode: EI, SIM mode
   (e) Parameters for SIM mode:
      (i) phenylboronic derivative of 3-MCPD (*m/z*) 147 (quantifier ion); 196, 198 (qualifier ions);
      (ii) phenylboronic derivative of 2-MCPD (*m/z*) 196 (quantifier ion); 198 (qualifier ion);
      (iii) phenylboronic derivative of 3-MCPD-d5 (*m/z*) 150 (quantifier ion for 3-MCPD); 201 (quantifier ion for -MCPD); 203 (qualifier ion);
      (iv) phenylboronic derivative of 3-MBPD (*m/z*) 147 (quantifier ion); 240 (qualifier ion);
      (v) phenylboronic derivative of 3-MBPD-d5 (*m/z*) 150 (quantifier ion); 245 (qualifier ion).
   (f) Acquisition time window: 5-20 min (see Notes, 24).

CALCULATIONS

Quantification of 3-MCPD esters

1. Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as free 3-MCPD equivalent) to the amount of internal standard (expressed as free 3-MCPD-d5 equivalent) on the x-axis, against the ratio of the corresponding peak areas on the y-axis (Figure 1). Ions at *m/z* 147 (3-MCPD) and 150 (3-MCPD-d5) are used for quantification. Calculate the regression line as follows:

$$y = ax + b$$

where a is the slope and b is the y-intercept. Confirm that the linearity is good ($R^2 > 0.99$) and the y-intercept preferably $<|0.02|$ in order to achieve a good accuracy for samples at very low concentration of 3-MCPD esters.

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2. Determine the concentration of 3-MCPD esters in the test sample (mg/kg) by applying the following formula:

$$c = [(A_{147}/A_{150}) - b] \times IS \times 1/a \times 1/W$$

Where—

c = concentration of 3-MCPD esters in the test sample (expressed as free 3-MCPD equivalent, mg/kg oil)
A_{147} = area of the peak corresponding to the 3-MCPD derivative (*m/z* 147)
IS = absolute amount (in µg, adjusted for the purity) of internal standard added to the test sample
A_{150} = area of the peak corresponding to the 3-MCPD-d5 derivative (*m/z* 150)
a = slope of the calibration curve
W = weight of the sample (in g)
b = y-intercept of the calibration curve

Quantification of 2-MCPD esters

1. Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as free 2-MCPD equivalent) to the amount of internal standard (expressed as free 3-MCPD-d5 equivalent) on the x-axis, against the ratio of the corresponding peak areas on the y-axis. Ions at *m/z* 196 (2-MCPD) and 201 (3-MCPD-d5) are used for quantification. Calculate the regression line as follows:

$$y = ax + b$$

where a is the slope and b is the y-intercept. Confirm that the linearity is good ($R^2 > 0.99$) and the y-intercept preferably <|0.05| in order to achieve a good accuracy for samples at very low concentration of 2-MCPD esters.

2. Determine the concentration of 2-MCPD esters in the test sample (mg/kg) by applying the following formula:

$$c = [(A_{196}/A_{201}) - b] \times IS \times 1/a \times 1/W$$

Where—

c = concentration of 2-MCPD esters in the test sample (expressed as free 2-MCPD equivalent, mg/kg oil)
A_{196} = area of the peak corresponding to the 2-MCPD derivative (*m/z* 196)
IS = absolute amount (in µg, adjusted for the purity) of internal standard added to the test sample
A_{201} = area of the peak corresponding to the 3-MCPD-d5 derivative (*m/z* 201)
a = slope of the calibration curve
W = weight of the sample (in g)
b = y-intercept of the calibration curve

Quantification of glycidyl esters

1. Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as glycidol equivalent) to the amount of internal standard (expressed as deuterated glycidol equivalent) on the x-axis, against the ratio of the corresponding peak areas on the y-axis (Figure 2). Ions at *m/z* 147 (3-MBPD) and 150 (3-MBPD-d5) are used for the quantification. Calculate the regression line as follows:

$$y = ax + b$$

where a is the slope and b is the y-intercept. Confirm that the linearity is good ($R^2 > 0.99$) and the y-intercept preferably <|0.02| in order to achieve a good accuracy for samples at very low concentration of glycidyl esters.

2. Determine the concentration of glycidyl esters in the test sample (mg/kg) by applying the following formula:

$$c = [(A_{147}/A_{150}) - b] \times IS \times 1/a \times 1/W$$

Where—

c = concentration of glycidyl esters in the test sample (expressed as glycidol equivalent, mg/kg oil)
A_{147} = area of the peak corresponding to the 3-MBPD derivative (*m/z* 147)
IS = absolute amount (in µg, adjusted for the purity) of internal standard added to the test sample
A_{150} = area of the peak corresponding to the 3-MBPD-d5 derivative (*m/z* 150)
a = slope of the calibration curve
W = weight of the sample (in g)
b = y-intercept of the calibration curve

3. Typical chromatograms of a real sample are given in Figures 3 and 4.

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METHOD PRECISION

Precision data determined from a 2012 AOCS collaborative study for comparison of this method with Cd 29b-13 and Cd 29c-13 by 12 laboratories analyzing 7 samples are given in Table 2-4. The same 7 samples were used in each of these methods. Reproducibility and repeatability values are calculated according to the AOAC/IUPAC Harmonized Protocol for Collaborative Study. The known composition of the reference samples is given in Table 5.

NUMBERED NOTES

1. AOCS Official Methods Cd 29a-13, Cd 29b-13 and Cd 29c-13 are three different methods that have been found through international collaborative studies to produce statistically the same results, and therefore are considered equivalent for use in the determination of 2- and 3-MCPD fatty acid esters and glycidyl fatty acid esters in edible oils and fats (Tables 2–4).
2. It is advisable to freshly prepare the solution on daily basis.
3. As an alternative, the solution can be prepared by dilution of the sodium hydrogen carbonate saturated solution (Solutions: Reagents, 4).
4. The saturated solution of sodium hydrogen carbonate can be substituted with a solution of the exact concentration (9%, w/v) to improve the robustness of the method.
5. The phenylboronic acid does not dissolve completely in the solvent mixture. Only the supernatant is used for the derivatization reaction (Procedure: Test Sample Preparation, 6).
6. 1,2-Dipalmitoyl-3-chloropropanediol can be substituted by 1,2-dioleyl-3-chloropropanediol or other fatty acid diesters of 3-MCPD with similar chain length (C16-C18 should be preferred as they are the most abundant in the majority of oils/fats).
7. As an analogy with the recommendations given for PP-3-MCPD, 1,3-dipalmitoyl-2-chloropropanediol can be substituted by other fatty acid diesters of 2-MCPD with similar chain length (C16-C18 should be preferred as they are the most abundant in the majority of oils/fats).
8. Glycidyl palmitate can be substituted by glycidyl oleate or other fatty acid esters of glycidol with similar chain length (C16-C18 should be preferred as they are the most abundant in the majority of oils/fats).
9. In alternative to the preparation of separate standard solutions for each analyte, Solutions: Standard 3a, 3c, and 3e can be combined in one single standard solution at high concentration of all three analytes (mixed Calibration I). To prepare the mixed solution, pipette 550 µL of PP-3-MCPD stock solution (Solutions: Standard, 2a), 550 µL of PP-2-MCPD stock solution (Solutions: Standard, 2b), and 1 mL of Gly-P stock solution (Solutions: Standard, 2d) into a 10 mL volumetric flask and fill up to the mark with the solvent. Also Solutions: Standard 3b, 3d, and 3f can be combined in one single standard solution at low concentration of all three analytes (mixed Calibration II). To prepare the mixed solution, pipette 1 mL of mixed Calibration I into a 10 mL volumetric flask and fill up to the mark with the solvent.
10. In analogy to Note 9, the internal standard solutions (Solutions: Standard, 3g and 3h) can be combined in a single solution (mixed internal standard). To prepare the mixed solution, pipette 400 µL of PP-3-MCPD-d5 (Solutions: Standard, 2c) and 500 µL of Gly-P-d5 (Solutions: Standard, 2e) into a 10 mL volumetric flask and fill up to the mark with solvent.
11. The presence of water interferes with the conversion of glycidyl esters into MBPD monoesters (Procedure: Test Sample Preparation, 2); therefore, it is recommended to use anhydrous tetrahydrofuran (Reagent, 1) as described by Ermacora & Hrnčiřík [2].
12. If the oil/fat is solid at room temperature, the sample must be warmed to its melting point before weighing.
13. If the mixed internal standard solution is used (Solutions: Standard, 3h; see Notes, 8), add 50 µL of this solution to the sample.
14. When a sample is suspected to contain high levels of chloride salts, an additional step of liquid/liquid extraction with water is required as described by Ermacora & Hrnčiřík [3]. Particular attention must be paid to discarding the water layer completely after the extraction.
15. Both time and temperature of reaction must be carefully controlled to avoid either sub-optimal conversion of the glycidyl esters or *ex-novo* formation of 3-MBPD esters as described by Ermacora & Hrnčiřík [2].
16. The clear separation of the organic phase from the water phase can be difficult. Therefore, it is often necessary to centrifuge the sample (approx. 1–2 min at 250 × g).
17. The evaporation of the organic phase under nitrogen stream must be carefully monitored and stopped immediately after the evaporation of the solvent. A small amount of residual solvent (< 50 µL) dissolved in the sample does not affect the quality of the results and should be preferred over a prolonged exposure of the sample to a nitrogen stream.
18. The removal of organic solvents enhances the sensitivity of the method. The evaporation can be stopped when, upon visual check, the volume in the test tube is about 1 mL.
19. During the second extraction, it is important to remove the upper organic phase completely.
20. The evaporation of the extracts under nitrogen stream can be carried out at approximately 35–40°C to facilitate the evaporation of the n-heptane, but it must be stopped as soon as the solvent is evaporated in order to avoid loss of the highly volatile phenylboronic derivatives.

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21. If the evaporation of the extracts is carried out at elevated temperature (max 40°C), the test tubes must be allowed to cool to room temperature before dissolving the residue.
22. During the evaporation of the extracts, excess of phenylboronic acid that did not react with 2- and 3-MCPD, as well as 3-MBPD, forms a white precipitate on the walls of the test tube. The addition of 400 µL of n-heptane (final step of the sample preparation) does not lead to its complete dissolution that would otherwise interfere with the analysis.
23. The addition of 100-110 mg of blank oil (containing no 2-MCPD/3-MCPD esters and glycidyl esters) to the calibration samples can improve the robustness of the method, since the oil matrix helps retain the analytes during the evaporation step.
24. Molecular ions (*m/z* 196 and 201 for 3-MCPD and 3-MCPD-d5, respectively and *m/z* 240 and 245 for 3-MBPD and 3-MBPD-d5, respectively) can also be used for quantification purposes; but they are not preferred because of the lower intensity of their signal, which results in a significant decrease of sensitivity.

REFERENCES

1. Kraft, R., Brachwitz, H., Etzold, G., Langen, P., Zöpel, H-J (1979). *J Prakt Chem* 321: 756–768.
2. Ermacora, A., Hrnčiřík, K. (2013). *J Am Oil Chem Soc*. 90: 1–8.
3. Ermacora, A., Hrnčiřík, K. (2012). *J Am Oil Chem Soc* 89: 211–217.

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Table 1
Scheme for the construction of the calibration curve for PP-3-MCPD, PP-2-MCPD and Gly-P.

	Calibration sol. (5.3a-f)	2-MCPD* [µg]	3-MCPD* [µg]	Gly* [µg]	Internal standard sol. (5.3g-h)	3-MCPD-d5* [µg]	Gly-d5* [µg]
Cal 0	—	0.00	0.00	0.00	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62
Cal 1	25 µL (5.3b) + 25 µL (5.3d) + 25 µL (5.3f) or 25 µL mixed cal II	0.03	0.03	0.06	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62
Cal 2	50 µL (5.3b) + 50 µL (5.3d) + 50 µL (5.3f) or 50 µL mixed cal II	0.05	0.05	0.12	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62
Cal 3	100 µL (5.3b) + 100 µL (5.3d) + 100 µL (5.3f) or 100 µL mixed cal II	0.10	0.10	0.24	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62
Cal 4	20 µL (5.3a) + 20 µL (5.3c) + 20 µL (5.3e) or 20 µL mixed cal I	0.21	0.21	0.47	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62
Cal 5	30 µL (5.3a) + 30 µL (5.3c) + 30 µL (5.3e) or 30 µL mixed cal I	0.31	0.31	0.71	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62
Cal 6	50 µL (5.3a) + 50 µL (5.3c) + 50 µL (5.3e) or 50 µL mixed cal I	0.52	0.52	1.19	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62
Cal 7	70 µL (5.3a) + 70 µL (5.3c) + 70 µL (5.3e) or 70 µL mixed cal I	0.72	0.72	1.66	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62
Cal 8	90 µL (5.3a) + 90 µL (5.3c) + 90 µL (5.3e) or 90 µL mixed cal I	0.93	0.93	2.13	50 µL (5.3g) + 50 µL (5.3h) or 50 µL mixed IS sol	0.39	0.62

*These values must be corrected for the purity of the standard used.

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Table 2
Results of glycidol, 3-MCPD, and 2-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method Cd 29a-13

Sample 1	Glycidol	3-MCPD	2-MCPD
N	11	10	10
Mean	8.76	47.28	0.32
Repeatability (Sr)	0.31	0.46	0.03
%RSDr	3.51	0.97	9.62
r (2.8×Sr)	0.86	1.28	0.09
Reproducibility (SR)	0.82	3.97	0.09
%RSDR	9.33	8.40	27.79
R (2.8×Sr)	2.29	11.12	0.25
Sample 2	Glycidol	3-MCPD	2-MCPD
n	10	10	11
Mean	1.46	0.49	0.21
Repeatability (Sr)	0.03	0.03	0.03
%RSDr	2.19	5.45	14.24
r (2.8×Sr)	0.09	0.08	0.08
Reproducibility (SR)	0.05	0.08	0.05
%RSDR	3.15	16.12	25.28
R (2.8×Sr)	0.13	0.22	0.15
Sample 3	Glycidol	3-MCPD	2-MCPD
n	8	12	11
Mean	0.28	5.82	0.22
Repeatability (Sr)	0.03	0.35	0.04
%RSDr	12.28	5.99	16.20
r (2.8×Sr)	0.10	0.98	0.10
Reproducibility (SR)	0.12	0.79	0.06
%RSDR	44.38	13.54	26.56
R (2.8×Sr)	0.35	2.20	0.16
Sample 4	Glycidol	3-MCPD	2-MCPD
n	12	11	11
Mean	4.69	18.92	0.26
Repeatability (Sr)	0.16	0.35	0.03
%RSDr	3.46	1.85	13.49
r (2.8×Sr)	0.45	0.98	0.10
Reproducibility (SR)	0.41	1.87	0.07
%RSDR	8.84	9.88	25.62
R (2.8×Sr)	1.16	5.23	0.19
Sample 5	Glycidol	3-MCPD	2-MCPD
n	8	9	10
Mean	0.32	0.50	0.22
Repeatability (Sr)	0.04	0.05	0.03
%RSDr	12.13	9.76	12.57
r (2.8×Sr)	0.11	0.14	0.08
Reproducibility (SR)	0.13	0.05	0.03
%RSDR	40.12	9.76	15.20
R (2.8×Sr)	0.36	0.14	0.09

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Table 2
(Continued)

Sample 6	Glycidol	3-MCPD	2-MCPD
n	12	11	11
Mean	10.91	1.95	0.93
Repeatability (Sr)	0.42	0.09	0.06
%RSDr	3.87	4.65	6.31
r (2.8×Sr)	1.18	0.25	0.16
Reproducibility (SR)	1.37	0.13	0.13
%RSDR	12.56	6.42	13.56
R (2.8×Sr)	3.84	0.35	0.35
Sample 7	Glycidol	3-MCPD	2-MCPD
n	12	12	12
Mean	4.92	3.41	1.61
Repeatability (Sr)	0.13	0.08	0.09
%RSDr	2.70	2.43	5.58
r (2.8×Sr)	0.37	0.23	0.25
Reproducibility (SR)	0.49	0.41	0.22
%RSDR	9.93	11.96	13.76
R (2.8×Sr)	1.37	1.14	0.62

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Table 3
Results of glycidol, 3-MCPD, and 2-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method Cd 29b-13

Sample 1	Glycidol	3-MCPD	2-MCPD
n	9	10	9
Mean	9.37	49.90	0.32
Repeatability (Sr)	0.23	2.85	0.03
%RSDr	2.46	5.72	9.14
r (2.8×Sr)	0.65	7.99	0.08
Reproducibility (SR)	2.11	8.73	0.11
%RSDR	22.51	17.50	35.68
R (2.8×Sr)	5.91	24.46	0.32
Sample 2	Glycidol	3-MCPD	2-MCPD
n	9	8	10
Mean	1.34	0.51	0.24
Repeatability (Sr)	0.04	0.02	0.01
%RSDr	2.85	3.08	5.64
r (2.8×Sr)	0.11	0.04	0.04
Reproducibility (SR)	0.18	0.11	0.08
%RSDR	13.50	22.15	32.87
R (2.8×Sr)	0.51	0.31	0.22
Sample 3	Glycidol	3-MCPD	2-MCPD
n	8	10	9
Mean	0.18	5.93	0.24
Repeatability (Sr)	0.02	0.12	0.01
%RSDr	10.67	2.05	3.79
r (2.8×Sr)	0.05	0.34	0.03
Reproducibility (SR)	0.14	0.72	0.06
%RSDR	78.10	12.18	23.97
R (2.8×Sr)	0.39	2.02	0.16
Sample 4	Glycidol	3-MCPD	2-MCPD
n	9	8	9
Mean	5.34	18.89	0.27
Repeatability (Sr)	0.21	0.31	0.02
%RSDr	3.86	1.63	6.38
r (2.8×Sr)	0.58	0.86	0.05
Reproducibility (SR)	2.12	1.43	0.09
%RSDR	39.61	7.56	31.13
R (2.8×Sr)	5.93	4.00	0.24
Sample 5	Glycidol	3-MCPD	2-MCPD
n	8	8	10
Mean	0.17	0.50	0.24
Repeatability (Sr)	0.01	0.05	0.01
%RSDr	5.57	10.83	3.85
r (2.8×Sr)	0.03	0.15	0.03
Reproducibility (SR)	0.12	0.13	0.07
%RSDR	71.36	25.93	30.81
R (2.8×Sr)	0.33	0.36	0.21

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Table 3
(Continued)

Sample 6	Glycidol	3-MCPD	2-MCPD
n	10	9	10
Mean	11.34	1.93	0.95
Repeatability (Sr)	0.40	0.09	0.09
%RSDr	3.54	4.86	9.94
r (2.8×Sr)	1.12	0.26	0.26
Reproducibility (SR)	2.32	0.19	0.27
%RSDR	20.49	9.94	28.83
R (2.8×Sr)	6.51	0.54	0.76
Sample 7	Glycidol	3-MCPD	2-MCPD
n	9	10	9
Mean	4.75	3.46	1.73
Repeatability (Sr)	0.11	0.09	0.04
%RSDr	2.31	2.71	2.21
r (2.8×Sr)	0.31	0.26	0.11
Reproducibility (SR)	0.53	0.42	0.49
%RSDR	11.14	12.26	28.53
R (2.8×Sr)	1.48	1.19	1.38

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Table 4
Results of glycidol and 3-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method Cd 29c-13[a]

Sample 1	Glycidol	3-MCPD
n	9	8
Mean	11.88	47.09
Repeatability (Sr)	3.10	0.90
%RSDr	26.12	1.90
r (2.8×Sr)	8.69	2.51
Reproducibility (SR)	3.67	4.53
%RSDR	30.91	9.61
R (2.8×Sr)	10.28	12.68
Sample 2	Glycidol	3-MCPD
n	9	8
Mean	1.42	0.47
Repeatability (Sr)	0.10	0.01
%RSDr	6.77	1.68
r (2.8×Sr)	0.27	0.02
Reproducibility (SR)	0.41	0.08
%RSDR	28.54	16.64
R (2.8×Sr)	1.13	0.22
Sample 3	Glycidol	3-MCPD
n	6	8
Mean	0.44	5.86
Repeatability (Sr)	0.23	0.12
%RSDr	52.41	2.10
r (2.8×Sr)	0.65	0.34
Reproducibility (SR)	0.29	0.64
%RSDR	66.05	10.90
R (2.8×Sr)	0.82	1.79
Sample 4	Glycidol	3-MCPD
n	9	9
Mean	5.27	19.02
Repeatability (Sr)	0.45	0.36
%RSDr	8.45	1.91
r (2.8×Sr)	1.25	1.02
Reproducibility (SR)	1.95	1.72
%RSDR	36.92	9.07
R (2.8×Sr)	5.45	4.83
Sample 5	Glycidol	3-MCPD
n	6	8
Mean	0.14	0.51
Repeatability (Sr)	0.03	0.01
%RSDr	21.63	2.62
r (2.8×Sr)	0.08	0.04
Reproducibility (SR)	0.05	0.04
%RSDR	35.34	8.62
R (2.8×Sr)	0.14	0.12

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Table 4
(Continued)

Sample 6	Glycidol	3-MCPD
n	9	8
Mean	10.15	2.09
Repeatability (Sr)	0.93	0.08
%RSDr	9.12	3.84
r (2.8×Sr)	2.59	0.22
Reproducibility (SR)	3.15	0.35
%RSDR	31.08	16.91
R (2.8×Sr)	8.83	0.99
Sample 7	**Glycidol**	**3-MCPD**
n	9	9
Mean	4.99	3.39
Repeatability (Sr)	0.14	0.06
%RSDr	2.72	1.86
r (2.8×Sr)	0.38	0.18
Reproducibility (SR)	1.50	0.29
%RSDR	30.15	8.42
R (2.8×Sr)	4.21	0.80

[a]No more than 3 labs reported data for 2-MCPDs using AOCS Official Method Cd 29c-13. According to the AOAC/IUPAC Harmonized Protocol there were too few labs to run statistical analysis.

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Table 5
Known composition of process contaminant reference samples used in the 2012 AOCS collaborative study[a]

Reference Sample #1

Analyte	Conc. mg/kg
Glycidyl Laurate	5.75
Glycidyl Myristate	8.14
Glycidyl Palmitate	4.68
Glycidyl Stearate	4.46
Glycidyl Oleate	4.62
Glycidyl Linoleate	5.01
Glycidyl Linolenate	3.95
Lauric Acid - Monochloropropanediol monoester	4.09
Myristic Acid - Monochloropropanediol monoester	4.57
Palmitic Acid - Monochloropropanediol monoester	4.84
Stearic Acid - Monochloropropanediol monoester	6.07
Oleic Acid - Monochloropropanediol monoester	3.53
Linoleic Acid - Monochloropropanediol monoester	4.72
Linolenic Acid - Monochloropropanediol monoester	3.94
Di-Lauric Acid - Monochloropropanediol diester	3.95
Lauric Acid - Myristic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Palmitic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Oleic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Myristic Acid - Monochloropropanediol diester	3.95
Myristic Acid - Palmitic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Oleic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Palmitic Acid - Monochloropropanediol diester	3.95
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Oleic Acid - Monochloropropanediol diester	3.95
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Oleic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Linoleic Acid - Monochloropropanediol diester	3.95
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Linolenic Acid - Monochloropropanediol diester	3.95
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Stearic Acid - Monochloropropanediol diester	3.95

Reference Sample #2

Analyte	Conc. mg/kg
Glycidyl Palmitate	1.49
Glycidyl Stearate	0.88
Glycidyl Oleate	1.11
Glycidyl Linoleate	1.22
Glycidyl Linolenate	1.32

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Table 5
(Continued)

Reference Sample #3

Analyte	Conc. mg/kg
Di-Palmitic Acid - Monochloropropanediol diester	1.28
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Oleic Acid - Monochloropropanediol diester	1.28
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	2.56
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Oleic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Linoleic Acid - Monochloropropanediol diester	1.28
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Linolenic Acid - Monochloropropanediol diester	1.28
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Stearic Acid - Monochloropropanediol diester	1.28

Reference Sample #4

Analyte	Conc. mg/kg
Glycidyl Palmitate	5.26
Glycidyl Stearate	3.11
Glycidyl Oleate	3.93
Glycidyl Linoleate	4.33
Glycidyl Linolenate	4.68
Di-Palmitic Acid - Monochloropropanediol diester	4.48
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Oleic Acid - Monochloropropanediol diester	4.48
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	8.96
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Oleic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Linoleic Acid - Monochloropropanediol diester	4.48
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Linolenic Acid - Monochloropropanediol diester	4.48
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Stearic Acid - Monochloropropanediol diester	4.48

Reference Sample #5

Analyte	Conc. mg/kg
Glycidyl Oleate	0.20

[a]Reference Sample #6 was an unknown trade sample of palm oil. Reference Sample #7 was B5 Palm Oil B & GS from the 2011 GE Study. All reference samples were made up in blank canola oil, and all reference samples contain up to 1500 mg/kg dichloromethane. The above information was used in determining the statistical equivalence of the AOCS Official Methods Cd 29a-13, Cd 29b-13 and Cd 29c-13.

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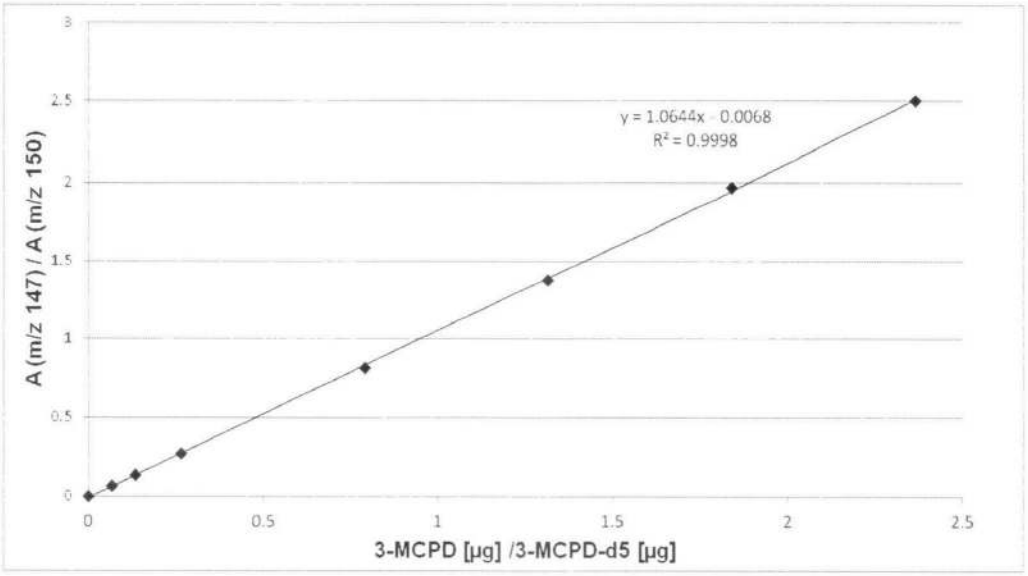

Figure 1. Calibration curve of PP-3-MCPD.

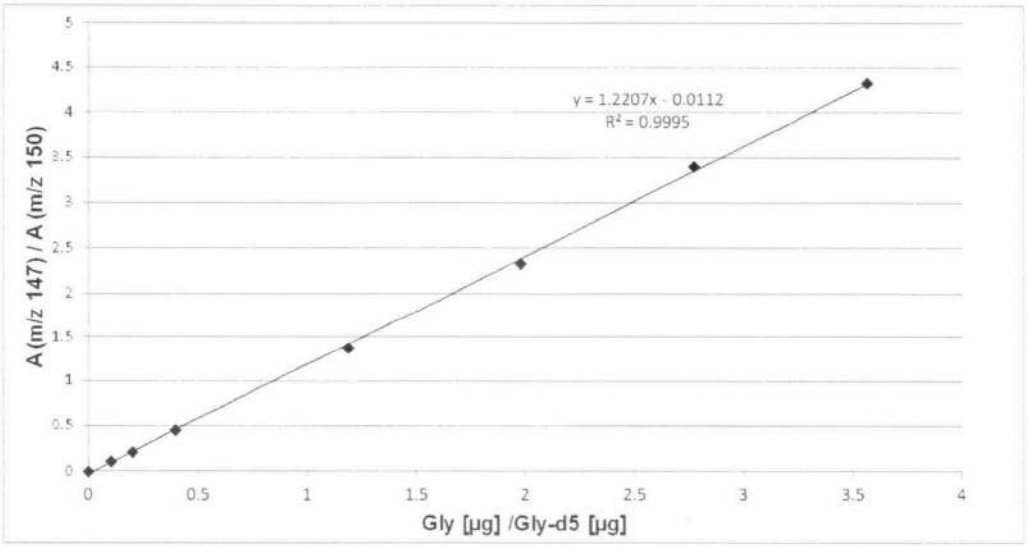

Figure 2. Calibration curve of Gly-P.

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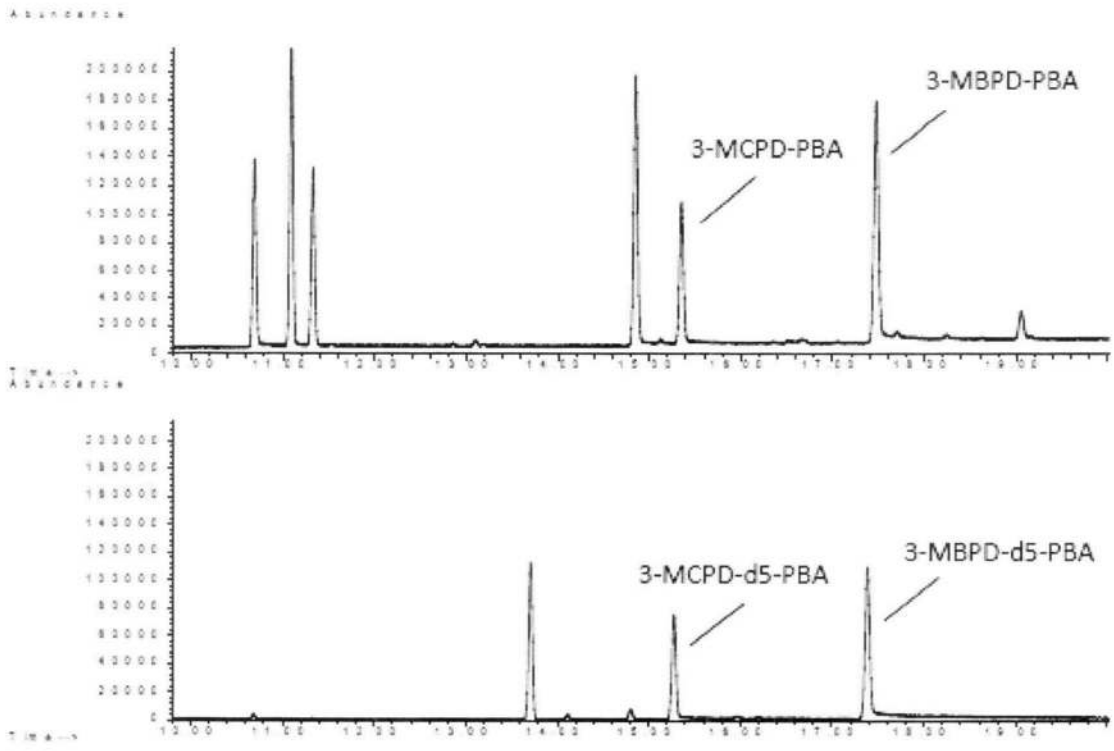

Figure 3. SIM chromatogram of a sample of fully refined palm oil (extracted ions *m/z* 147 and 150).

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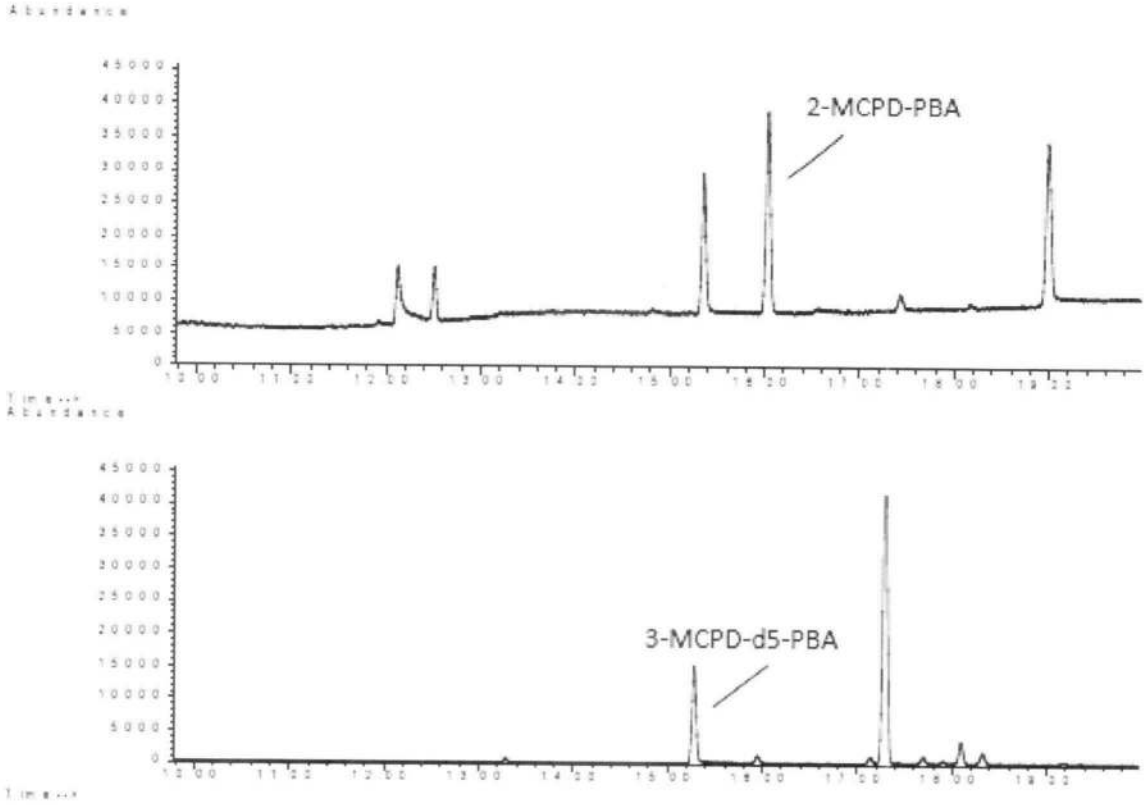

Figure 4. SIM chromatogram of a sample of fully refined palm oil (extracted ions *m/z* 196 and 201).

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AOCS Official Method Cd 29b-13

Approved 2013

Determination of Bound Monochloropropanediol- (MCPD-) and Bound 2,3-epoxy-1-propanol (glycidol-) by Gas Chromatography/Mass Spectrometry (GC/MS)

DEFINITION

This method is used for the determination of fatty acid esters of 2-chloropropane-1,3-diol (2-MCPD), 3-chloropropane-1,2-diol (3-MCPD) and glycidol in edible oils and fats; see also AOCS Official Methods Cd 29a-13 or Cd 29c-13 (see Notes, 1).

Bound glycidol is the sum of all glycidyl derivatives that are cleaved by alkaline-catalyzed alcoholysis. The content of bound glycidol is reported in milligrams per kilogram (mg/kg). Bound 2-MCPD is the sum of all 2-MCPD-derivatives that are cleaved by alkaline-catalyzed alcoholysis. The content of bound 2-MCPD is reported in milligrams per kilogram (mg/kg). Bound 3-MCPD is the sum of all 3-MCPD-derivatives that are cleaved by alkaline-catalyzed alcoholysis. The content of bound 3-MCPD is reported in milligrams per kilogram (mg/kg).

SCOPE

This method describes a procedure for the parallel determination of glycidol together with 2-MCPD and 3-MCPD present in bound or free form in oils and fats. The method is based on alkaline-catalyzed ester cleavage, transformation of the released glycidol into monobromopropanediol (MBPD) and derived free diols (MCPD and MBPD) with phenylboronic acid (PBA). Though free MCPD and glycidol are only present in fats and oils in low to negligible quantities, significant content would increase proportionately the determination of bound analytes. This method is applicable to solid and liquid fats and oils.

APPARATUS

1. Analytical balance; capable of measuring ± 0.0001g.
2. Screw cap vials -ca. 2 mL capacity - and screw caps with Teflon®-coated septa.
3. Micro inserts -ca. 200 µL capacity - for screw cap vials - ca. 2 mL capacity.
4. Universal pH-indicator strips or pH meter.
5. GC/MS-system with temperature programmable injector.

REAGENTS

1. Toluene.
2. *tert*-Butyl methyl ether.
3. Methanol.
4. *iso*-Hexane.
5. Ethyl acetate.
6. Diethyl ether.
7. *iso*-Octane.
8. Sodium sulfate anhydrous, granular.

STANDARDS

1. 2-MCPD.
2. 2-MCPD-d_5.
3. 2-MCPD-1,2-*bis*-stearoylester *.
4. 2-MCPD-d_5-1,2-*bis*-stearoylester *.
5. 3-MCPD.
6. 3-MCPD-d_5.
7. 3-MCPD-1,3-*bis*-palmitoylester *.
8. 3-MCPD-d_5-1,3-*bis*-palmitoylester *.

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9. Glycidyl oleate*.
10. Glycidyl-d_5 oleate*.

*Other commercially available fatty acid esters of the analytes may be substituted.

SOLUTIONS

1. 2-MCPD: 10.0 µg/mL in methanol.
2. 2-MCPD-d_5: 10.0 µg/mL in methanol.
3. 3-MCPD: 10.0 µg/mL in methanol.
4. 3-MCPD-d_5: 10.0 µg/mL in methanol.
5. 2-MCPD-1,2-*bis*-stearoylester: 26.6 µg/mL; equivalent to 5.0 µg/mL free 3-MCPD in toluene.
6. 2-MCPD-d_5-1,2- *bis*-stearoylester: 26.9 µg/mL in toluene; equivalent to 5.0 µg/mL free 3-MCPD.
7. 3-MCPD-1,3- *bis*-palmitoylester: 29.2 µg/mL in toluene; equivalent to 5.0 µg/mL free 2-MCPD.
8. 3-MCPD-d_5-1,3- *bis*-palmitoylester: 29.4 µg/mL in toluene; equivalent to 5.0 µg/mL free 2-MCPD.
9. Glycidyl oleate: 22.8 µg/mL in toluene; equivalent to 5.0 µg/mL free glycidol.
10. Glycidyl-d_5 oleate: 23.2 µg/mL in toluene; equivalent to 5.0 µg/mL free glycidol.
11. Sodium hydroxide solution: Weigh 0.25 g freshly ground sodium hydroxide in a 100 mL plastic bottle. Add 100 mL methanol and tightly seal the bottle. Shake vigorously (vortex) until the sodium hydroxide has been dissolved completely. Store in a freezer at –22°C to –25°C. (see Notes, 1).
12. Acidified sodium bromide solution: Weigh 600 g sodium bromide in a 1 L screw cap glass volumetric flask, add deionised water up to the 1 L mark. Acidify the mixture with 3 mL of *ortho*-phosphoric acid (85%), seal tightly and shake (vortex) until the solution is clear. 600 µL of this solution must neutralize 350 µL of sodium hydroxide solution (Solutions, 1) and adjust the pH-value to the acidic range (pH 3-1). Store the solution in a freezer at –22°C to –25°C. (see Notes, 2).
13. Saturated solution of phenylboronic acid (PBA) in diethyl ether: Add approximately 200 mg PBA to 10 mL diethyl ether in a screw cap vial. Shake well, allow non-dissolved PBA to settle and remain as precipitate. For derivatization purposes, use only the clear supernatant.

PROCEDURE (SEE NOTES, 3)

1. Aliquot liquid samples directly. Melt solid or semi-solid fats at approximately 80°C in a drying oven or water bath. For high-melting point fats, the temperature can be increased in 10°C steps until the melting process starts. Aliquot solid samples that contain higher amounts of water without melting to avoid phase separation.
2. Weigh two 100 ± 0.5 mg aliquots of the sample into two screw cap vials, ca. 2 mL capacity. To assay A, add 50 µL each 2- and 3-MCPD-d5 standard working solutions (Solutions, 2 and 4). To assay A, pipette 100 µL glycidyl-d5 ester standard working solution (Solutions, 10). To assay B, transfer 100 µL 2-MCPD-d5-diester standard working solution (Solutions, 6.) and 100 µL 3-MCPD-d5-diester standard working solution (Solutions, 8). To each sample, add 600 µL diethyl ether and shake (vortex) the mixtures until completely dissolved. In the case of high melting point samples, the reaction vessels may require gentle warming.
3. Place both assays for a minimum of 15 min in a freezer to cool down to –22°C to –25°C (precipitation of sample material at this stage of sample preparation is not a problem). To each assay, add 350 µL methanolic sodium hydroxide solution (Solutions, 11). To complete the ester cleavage, seal the vials, briefly shake (vortex) and keep for at least 16 h at –22°C to –25°C. Stop the reactions with 600 µL acidified sodium bromide solution (Solutions, 12) kept at –22°C to –25°C. Briefly shake (vortex) the mixtures and place under a gentle stream of nitrogen to reduce the volume of the organic phase to approximately 100 µL. To both assays, add 600 µL *iso*-hexane, seal the vials and shake vigorously (vortex). Leave the mixtures at RT for approximately 5–10 min to complete the transformation of glycidol into MBPD. (see Notes, 4).
4. Remove the organic phase by Pasteur pipette. Wash the aqueous phase in each assay with a second 600 µL *iso*-hexane.
5. Using new Pasteur pipettes, extract each assay three times with 600 uL of 3:2 (v/v) mixture of diethyl ether/ethyl acetate. Take care that the pipette does not touch the aqueous phase. For each assay A & B, combine the organic extracts in a new screw cap vial containing a small amount of anhydrous sodium sulfate (Reagents, 8). If the drying agent gets sticky, transfer the solution into a new screw cap vial with fresh sodium sulfate. Add 20 µL of the derivatization reagent (Solutions, 13) to both organic extracts to achieve derivatization. If the derivatization reaction results in a small analyte signal response, increase the amount of derivatization agent until the signal to noise ratio in the corresponding chromatogram does not increase. The maximum amount of derivatization reagent that can be used is limited by the individual capacity of the gas chromatographic –mass spectrometric system to exclude excess PBA. To complete the derivatization reaction and to remove excess reagent, evaporate to dryness both assays using a gentle stream of nitrogen. Re-dissolve soluble fractions in approximately 300 µL to 500 µL *iso*-octane and vigorously shake (vortex) the mixture for a few seconds. For GC/MS measurement, transfer a portion of each solution into a 200 µL micro-insert.

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ANALYSIS

1. Gas chromatography
   (a) Injection volume: 1 µL to 2 µL
   (b) Carrier gas: Helium 5.0 (99.999 %), const. flow 1 mL/min to 1.5 mL/min
   (c) PTV temperature program: e.g. 85°C, increase temperature at 300°C/min to 165°C, hold 10 min isothermal, increase at 300°C/min to 320°C, hold 8 min isothermal
   (d) Injector: e.g. split-less, purge flow 50 mL/min 0.5 min to 1 min, septum purge 3 mL/min
   (e) GC oven temperature program: e.g. 85°C, isothermal 0.5 min, rise at 6°C/min to 150°C, then 12°C/min to 180°C, then 25°C/min to 280°C, hold isothermal 8 min
2. Mass spectrometry
   (a) Electron-impact (EI), Selected Ion Monitoring (SIM)
   (b) Detected ion traces related to the mass to charge ratios (*m/z*) from the phenylboronic derivatives of the analytes:
      (i) 3-MCPD-d_5: *m/z* = 149/150/201/203
      (ii) 3-MCPD: *m/z* = 146/147/196/198
      (iii) 2-MCPD-d_5: *m/z* = 201/203
      (iv) 2-MCPD: *m/z* = 196/198
      (v) 3-MBPD-d_5 (glycidol-d_5 transformation product): *m/z* = 245/247
      (vi) 3-MBPD (glycidol transformation product): *m/z* = 240/242, for target ions of labeled and unlabeled analytes should correspond for quantification purposes; e.g. *m/z* = 150 *vs.* 147 (3-MCPD-d_5 *vs.* 3-MCPD), *m/z* = 201 *vs.* 196 (2-MCPD-d_5 *vs.* 2-MCPD), *m/z* = 245 *vs.* 240 (3-MBPD-d_5 *vs.* 3-MBPD)
3. Suitable software with automatic data analysis is recommended for quantification

CALCULATIONS (SEE NOTES, 5)

1. Determination of bound glycidol
   (a) Bound glycidol in assay A is quantified in Calculations, 1.i without correction, when 3-MCPD is low in proportion to the glycidol content. In the presence of larger amounts of 3-MCPD, 3-MCPD conversion into glycidol may be incomplete leading to overestimation of glycidol. To compensate for this, the conversion ratio of 3-MCPD-d_5-diester into 3-MBPD-d_5 in assay B is determined and a factor is applied to quantify the potential conversion of 3-MCPD into 3-MBPD in assay A. This procedure is described in Calculations, 1.ii to 1.v.
      (i) For the quantitative determination of bound glycidol, the signal area of the phenylboronic derivative of 3-MBPD in assay A is multiplied by the spiking level of the internal standard glycidol-d_5 (introduced as glycidyl-d_5 ester) related to its concentration in the sample and divided by the signal area of the phenylboronic derivative of 3-MBPD-d_5:

$$W_{\text{glycidol uncorrected (A)}} = \frac{SA_{3\text{-MBPD(A)}} \times W_{\text{glycidol-d5(A)}}}{SA_{3\text{-MBPD-d5(A)}}}$$

Where—

$W_{\text{glycidol uncorrected(A)}}$ is the uncorrected mass fraction of glycidol in assay A in mg/kg
$W_{\text{glycidol-d5(A)}}$ is the mass fraction, in mg/kg, of glycidol-d_5 in assay A
$SA_{3\text{-MBPD(A)}}$ is the signal area of the phenylboronic derivative of 3-MBPD in assay A
$SA_{3\text{-MBPD-d5(A)}}$ is the signal area of the phenylboronic derivative of 3-MBPD-d_5 in assay A

If the sample contains significant amounts of 3-MCPD, the uncorrected glycidol value can be adjusted by a factor as follows.

(ii) To determine the undesired transformation of 3-MCPD via glycidol into 3-MBPD during sample preparation, the corresponding transformation ratio 3-MCPD-d_5 → 3-MBPD-d_5 in assay B is calculated by dividing the signal area of the phenylboronic derivative of 3-MCPD-d_5 by the signal area of the phenylboronic derivative of 3-MBPD-d_5 in assay B:

$$\tau_{3\text{MCPD}} = \frac{SA_{3\text{-MBPD-d5(B)}}}{SA_{3\text{-MCPD-d5(B)}}}$$

Where—

$\tau_{3\text{-MCPD}}$ is the transformation ratio 3-MCPD-d5 → 3-MBPD-d5
$SA_{3\text{-MBPD-d5(B)}}$ is the signal area of the phenylboronic derivative of 3-MBPD-d5 in assay B
$SA_{3\text{-MCPD-d5(B)}}$ is the signal area of the phenylboronic derivative of 3-MCPD-d5 in assay B

(iii) After sample preparation and GC/MS analysis according to Procedure and Equipment sections, the ratio of detected 3-MBPD and 3-MBPD-d_5 correlates to the isotopic factor (see Notes, 6):

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$$I = \frac{SA_{3\text{-MBPD}}}{SA_{3\text{-MBPD-d5}}}$$

Where—

I is the isotopic factor
$SA_{3\text{-MBPD}}$ is the signal area of the phenylboronic derivative of 3-MBPD
$SA_{3\text{-MBPD-d5}}$ is the signal area of the phenylboronic derivative of 3-MBPD-d_5

(iv) Subsequently the corrected amount of glycidol can be determined as follows:

$$W_{\text{glycidol corrected(A)}} = \frac{\left(SA_{3\text{-MBPD(A)}} - \left(\tau_{3\text{-MCPD}} \times SA_{3\text{-MCPD(A)}} \times I\right)\right) W_{\text{glycidol-d5(A)}}}{SA_{3\text{-MBPD-d5(A)}}}$$

Where—

$W_{\text{glycidol corrected(A)}}$ is the corrected mass fraction of glycidol in assay A, in mg/kg
$\tau_{3\text{-MCPD}}$ is the transformation ratio 3-MCPD → 3-MBPD (Calculations, 1.iii)
I is the isotopic factor (Calculations, 1.iv)
$W_{\text{glycidol-d5(A)}}$ is the mass fraction of glycidol-d5 in assay A, in mg/kg
$SA_{3\text{-MBPD(A)}}$ is the signal area of the phenylboronic derivative of 3-MBPD in assay A
$SA_{3\text{-MCPD(A)}}$ is the signal area of the phenylboronic derivative of 3-MCPD in assay A
$SA_{3\text{-MBPD-d5(A)}}$ is the signal area of the phenylboronic derivative of 3-MBPD-d_5 in assay A

2. Determination of bound 2-MCPD

(a) For the quantitative determination of bound 2-MCPD, the signal area of the phenylboronic derivative of 2-MCPD in assay B is multiplied by the spiking level of the internal standard 2-MCPD-d_5 (introduced as 2-MCPD-d_5 ester) related to its concentration in the sample and divided by the signal area of the phenylboronic derivative of 2-MCPD-d_5:

$$W_{2\text{-MCPD(B)}} = \frac{SA_{2\text{-MCPD(B)}} \times W_{2\text{-MCPD-d5(B)}}}{SA_{2\text{-MCPD-d5(B)}}}$$

Where—

$W_{2\text{-MCPD(B)}}$ is the mass fraction of 2-MCPD in assay B, in mg/kg
$W_{2\text{-MCPD-d5(B)}}$ is the mass fraction of 2-MCPD-d5 in assay B, in mg/kg
$SA_{2\text{-MCPD(B)}}$ is the signal area of the phenylboronic derivative of 2-MCPD in assay B
$S_{A2\text{-MCPD-d5(B)}}$ is the signal area of the phenylboronic derivative of 2-MCPD-d_5 in assay B

3. Determination of bound 3-MCPD

(a) For the quantitative determination of bound 3-MCPD, the signal area of the phenylboronic derivative of 3-MCPD in assay B is multiplied by the spiking level of the internal standard 3-MCPD-d_5 (introduced as 3-MCPD-d_5 ester) related to its concentration in the sample and divided by the signal area of the phenylboronic derivative of 3-MCPD-d_5:

$$W_{3\text{-MCPD(B)}} = \frac{SA_{3\text{-MCPD(B)}} \times W_{3\text{-MCPD-d5(B)}}}{SA_{3\text{-MCPD-d5(B)}}}$$

Where—

$W_{3\text{-MCPD(B)}}$ is the mass fraction of 3-MCPD in assay B, in mg/kg
$W_{3\text{-MCPD-d5(B)}}$ is the mass fraction of 3-MCPD-d_5 in assay B, in mg/kg
$SA_{3\text{-MCPD(B)}}$ is the signal area of the phenylboronic derivative of 3-MCPD in assay B
$SA_{3\text{-MCPD-d5(B)}}$ is the signal area of the phenylboronic derivative of 3-MCPD-d_5 in assay B

4. Determination of the degree of diester cleavage (see Notes, 7).

(a) For the quantitative determination of the ester cleavage, bound 3-MCPD is quantified in assay A via free 3-MCPD-d_5. Therefore, the signal area of the phenylboronic derivative of 3-MCPD is multiplied by the spiking level of 3-MCPD- d_5 and divided by the signal area of the phenylboronic derivative of 3-MCPD-d_5:

$$W_{3\text{-MCPD(A)}} = \frac{SA_{3\text{-MCPD(A)}} \times W_{3\text{-MCPD-d5(A)}}}{SA_{3\text{-MCPD-d5(A)}}}$$

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Where—

$W_{3\text{-MCPD(A)}}$ is the mass fraction of 3-MCPD in assay A, in mg/kg
$W_{3\text{-MCPD-d5(A)}}$ is the mass fraction of 3-MCPD-d_5 in assay A, in mg/kg
$SA_{3\text{-MCPD(A)}}$ is the signal area of the phenylboronic derivative of 3-MCPD in assay A
$SA_{3\text{-MCPD-d5(A)}}$ is the signal area of the phenylboronic derivative of 3-MCPD-d_5 in assay A

The degree of 3-MCPD-d_5-diester cleavage is subsequently calculated as follows (see Notes, 8):

$$ec_{3\text{-MCPD-diester}} = \frac{100 \times W_{3\text{-MCPD(A)}}}{W_{3\text{-MCPD(B)}}}$$

Where—

$W_{3\text{-MCPD(A)}}$ is the mass fraction of 3-MCPD in assay A, in mg/kg
$W_{3\text{-MCPD(B)}}$ is the mass fraction of 3-MCPD in assay B, in mg/kg
$ec_{3\text{-MCPD-diester}}$ is the degree of 3-MCPD-diester cleavage, in %

5. Quality control
   Method validation should be carried out according to international analytical method validation guidelines.
   (a) To control specificity and trueness of the method, analyze a reference material daily. If no reference material is available, a spiked oil sample should be used.
   (b) For quality contro l purposes, it is recommended to analyze a blank oil sample (e.g. virgin vegetable oils) daily. Virgin vegetable oils contain bound glycidol or 2- and 3-MCPD only in trace amounts (<< 25 µg/kg).

METHOD PRECISION

Precision data determined from a 2012 AOCS collaborative study for comparison of this method with Cd 29a-13 and Cd 29c-13 by 12 laboratories analyzing 7 samples are given in Table 1–3. The same 7 samples were used in each of these methods. Reproducibility and repeatability values are calculated according to the AOAC/IUPAC Harmonized Protocol for Collaborative Study. The known composition of the reference samples is given in Table 4.

NOTES

Caution

Some consumables like membrane filters or glassware may cause contamination. If there is some indication of contamination by consumables, glassware should be heated at 400°C to 500°C. Replace non thermo-resistant material or clean by rinsing with sufficient amounts of organic solvents (e.g. acetone and hexane).

NUMBERED NOTES

1. AOCS Official Methods Cd 29a-13, Cd 29b-13 and Cd 29c-13 are three different methods that have been found through international collaborative studies to produce statistically the same results, and therefore are considered equivalent for use in the determination of 2- and 3-MCPD fatty acid esters and glycidyl fatty acid esters in edible oils and fats (Tables 1–3).
2. For method establishment purposes, it can be useful to work with double- or triple- concentrated solutions of sodium hydroxide in methanol (0.5 g/100 mL or 0.75 g/100 mL) may be used. Adjust the acidity of the sodium bromide solution (Solutions, 12) as described.
3. Divide every sample into two aliquots (A and B). Spike Aliquot A with an isotopically labeled glycidyl ester and isotopic labeled free 2- and 3-MCPD as standards. Spike Aliquot B with isotopic labeled 2- and 3-MCPD esters as standards. Add diethyl ether to both assays followed by a dilute solution of sodium hydroxide in methanol in the cold to release free MCPD and free glycidol. Under these controlled conditions the undesired conversion of free 3-MCPD to induced glycidol, a well-known side reacti on by alkaline treatment of 3-MCPD at room temperature (RT), will be minimized. The cleaving reaction is stopped in the cold by the addition of an excess amount of a cold solution of sodium bromide in diluted phosphoric acid. Under acidic conditions the unstable free glycidol reacts rapidly with inorganic bromide to stable MBPD (3-MBPD >> 2-MBPD). The analytes MCPD and MBPD are water soluble so can be separated from the fractions of the sample aliquots by double extraction of the aqueous phase with *iso*-hexane. The analytes and the standards are recovered into an organic solvent by triple extraction of the aqueous phase with diethyl ether, ethyl acetate or a mixture of both solvents. The diol-selective derivatization is performed in the extraction solvent by reaction with PBA at room temperature. The absence of the aqueous phase and the avoidance of heat during the derivatization reaction minimizes the risk of undesired side reactions. In order to remove excess amounts of PBA, concentrate the analytes and transfer them into a more inert organic solvent. The reaction mixture is then placed over a small amount of anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen before being re-dissolved in *iso*-octane for the measurement by GC/MS. Differences in the results for 2- and

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3-MCPD between the assays A and B serve for the quantitative control of ester cleavage. To determine the undesired conversion of 3-MCPD to glycidol during alkaline treatment, the transformation rate of isotopic labeled 3-MCPD into isotopic labeled 3-MBPD in assay B is monitored and adapted to the "naturally" occurring analytes in assay A. 2-MCPD is more resistant to alkaline-catalyzed transformation into glycidol and is not a significant glycidol precursor under the conditions used within this method.

4. It is important that the samples are not allowed to warm up at any stage of alkaline treatment. Furthermore, it is recommended to occasionally test the acidity of the aqueous phase of the mixture after the addition of sodium bromide solution (Solutions, 12).
5. Assay A determines the bound glycidol while assay B determines the bound MCPD. In parallel, the transformation of isotopic labeled 3-MCPD ester into 3-MBPD-d_5 can be determined via assay B. It is assumed that the undesired transformation of bound 3-MCPD to glycidol (determined as 3-MBPD) in assay A during sample preparation follows similar kinetics and can be determined quantitatively when the ratio itself is known. Finally, the combination of both assays A and B evaluates the completeness of the ester cleavage. All quantifications refer to the sample spiked with isotopic labeled internal standards (Procedure, 2 - internal one point calibration). External calibrations are not intended to serve for analyte quantification but for the determination of the linearity of the method.
6. Slight differences in the physical and chemical properties of d_5-labeled and unlabeled 3-MCPD have indicated a little faster conversion of the unlabeled analyte in comparison to the labeled 3-MCPD-d_5. This assumed "isotopic effect" has been experimentally determined for the alkaline treatment that is described in Procedure 3. Under these conditions, 3-MCPD is converted approximately 1.2-fold faster via glycidol into 3-MBPD than 3-MCPD-d_5 via glycidol-d_5 into 3-MBPD-d_5. Consequently, the isotopic factor I = 1.2 has to be considered for the quantitative determination of the amount of glycidol that has been generated accidentally from the non-labeled 3-MCPD by alkaline treatment in assay A. If the conditions of alkaline-catalyzed ester cleavage are modified, a new isotopic factor has to be determined, e.g. by spiking a blank oil (only one assay necessary in this case) with the same amount of isotopic labeled and non-labeled 3-MCPD-1,2-*bis*-palmitoylester (not less than 100 mg/kg each). It is not yet known if impurities in the reference compounds (3-MCPD-diester and 3-MCPD-d5-diester) may have an influence on the observed effect. In most cases the corrected value for bound glycidol (Calculations, 1.v) does not differ significantly from the uncorrected value (Calculations, 1.i) so that the impact of the isotopic factor is negligible.
7. This method uses slow and mild alkaline-catalyzed ester cleavage to minimize the undesired transformation of 3-MCPD into glycidol. Under these conditions, the ester cleavage might not be complete. As quantification of the analytes is based on the use of esterified isotopic labeled internal standards, an incomplete ester cleavage should not have a major impact on the results as long as the cleavability of the selected internal standards is representative of one of the analytes. In particular matrices, this might not be the case as the cleavability, especially of 3-MCPD monoesters and diesters, probably is different. 3-MCPD diesters are supposed to be more resistant to alkaline catalyzed ester cleavage than 3-MCPD mono-esters, 2-MCPD mono- and diesters, as well as glycidyl esters. Therefore, the determination of 3-MCPD diester cleavage can be a useful tool to overcome this potential source of error. To determine the degree of 3-MCPD diester cleavage, the released 3-MCPD in both assays A and B serves as a reference. The spiked free 3-MCPD-d_5 in assay A represents complete hydrolysis; whereas, the spiked 3-MCPD-d_5-diester in assay B serves as a model substance that will be cleaved the least. If ester cleavage of the 3-MCPD-d_5-diester in assay B is absolute, both assays are assumed to give the same value for 3-MCPD within acceptable measurement uncertainty. If the 3-MCPD-d_5-diester cleavage is incomplete, the 3-MCPD value will rise proportionally.
8. If *ec* is < 90%, it is recommended to repeat analysis with a higher concentration of sodium hydroxide or under extended reaction time for alkaline hydrolysis. Therefore the use of double or triple concentrated solutions of sodium hydroxide (0.5% or 0.75%) in methanol combined with equivalently higher acidified solutions of sodium bromide (5.4 mL/L or 8.1 mL/L *ortho*-phosphoric acid) has shown to be suitable.

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Table 1
Results of glycidol, 3-MCPD, and 2-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method Cd 29a-13

Sample 1	Glycidol	3-MCPD	2-MCPD
N	11	10	10
Mean	8.76	47.28	0.32
Repeatability (Sr)	0.31	0.46	0.03
%RSDr	3.51	0.97	9.62
r (2.8×Sr)	0.86	1.28	0.09
Reproducibility (SR)	0.82	3.97	0.09
%RSDR	9.33	8.40	27.79
R (2.8×Sr)	2.29	11.12	0.25
Sample 2	Glycidol	3-MCPD	2-MCPD
n	10	10	11
Mean	1.46	0.49	0.21
Repeatability (Sr)	0.03	0.03	0.03
%RSDr	2.19	5.45	14.24
r (2.8×Sr)	0.09	0.08	0.08
Reproducibility (SR)	0.05	0.08	0.05
%RSDR	3.15	16.12	25.28
R (2.8×Sr)	0.13	0.22	0.15
Sample 3	Glycidol	3-MCPD	2-MCPD
n	8	12	11
Mean	0.28	5.82	0.22
Repeatability (Sr)	0.03	0.35	0.04
%RSDr	12.28	5.99	16.20
r (2.8×Sr)	0.10	0.98	0.10
Reproducibility (SR)	0.12	0.79	0.06
%RSDR	44.38	13.54	26.56
R (2.8×Sr)	0.35	2.20	0.16
Sample 4	Glycidol	3-MCPD	2-MCPD
n	12	11	11
Mean	4.69	18.92	0.26
Repeatability (Sr)	0.16	0.35	0.03
%RSDr	3.46	1.85	13.49
r (2.8×Sr)	0.45	0.98	0.10
Reproducibility (SR)	0.41	1.87	0.07
%RSDR	8.84	9.88	25.62
R (2.8×Sr)	1.16	5.23	0.19
Sample 5	Glycidol	3-MCPD	2-MCPD
n	8	9	10
Mean	0.32	0.50	0.22
Repeatability (Sr)	0.04	0.05	0.03
%RSDr	12.13	9.76	12.57
r (2.8×Sr)	0.11	0.14	0.08
Reproducibility (SR)	0.13	0.05	0.03
%RSDR	40.12	9.76	15.20
R (2.8×Sr)	0.36	0.14	0.09

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Table 1
(Continued)

Sample 6	Glycidol	3-MCPD	2-MCPD
n	12	11	11
Mean	10.91	1.95	0.93
Repeatability (Sr)	0.42	0.09	0.06
%RSDr	3.87	4.65	6.31
r (2.8×Sr)	1.18	0.25	0.16
Reproducibility (SR)	1.37	0.13	0.13
%RSDR	12.56	6.42	13.56
R (2.8×Sr)	3.84	0.35	0.35
Sample 7	Glycidol	3-MCPD	2-MCPD
n	12	12	12
Mean	4.92	3.41	1.61
Repeatability (Sr)	0.13	0.08	0.09
%RSDr	2.70	2.43	5.58
r (2.8×Sr)	0.37	0.23	0.25
Reproducibility (SR)	0.49	0.41	0.22
%RSDR	9.93	11.96	13.76
R (2.8×Sr)	1.37	1.14	0.62

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Table 2
Results of glycidol, 3-MCPD, and 2-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method Cd 29b-13

Sample 1	Glycidol	3-MCPD	2-MCPD
n	9	10	9
Mean	9.37	49.90	0.32
Repeatability (Sr)	0.23	2.85	0.03
%RSDr	2.46	5.72	9.14
r (2.8×Sr)	0.65	7.99	0.08
Reproducibility (SR)	2.11	8.73	0.11
%RSDR	22.51	17.50	35.68
R (2.8×Sr)	5.91	24.46	0.32
Sample 2	Glycidol	3-MCPD	2-MCPD
n	9	8	10
Mean	1.34	0.51	0.24
Repeatability (Sr)	0.04	0.02	0.01
%RSDr	2.85	3.08	5.64
r (2.8×Sr)	0.11	0.04	0.04
Reproducibility (SR)	0.18	0.11	0.08
%RSDR	13.50	22.15	32.87
R (2.8×Sr)	0.51	0.31	0.22
Sample 3	Glycidol	3-MCPD	2-MCPD
n	8	10	9
Mean	0.18	5.93	0.24
Repeatability (Sr)	0.02	0.12	0.01
%RSDr	10.67	2.05	3.79
r (2.8×Sr)	0.05	0.34	0.03
Reproducibility (SR)	0.14	0.72	0.06
%RSDR	78.10	12.18	23.97
R (2.8×Sr)	0.39	2.02	0.16
Sample 4	Glycidol	3-MCPD	2-MCPD
n	9	8	9
Mean	5.34	18.89	0.27
Repeatability (Sr)	0.21	0.31	0.02
%RSDr	3.86	1.63	6.38
r (2.8×Sr)	0.58	0.86	0.05
Reproducibility (SR)	2.12	1.43	0.09
%RSDR	39.61	7.56	31.13
R (2.8×Sr)	5.93	4.00	0.24
Sample 5	Glycidol	3-MCPD	2-MCPD
n	8	8	10
Mean	0.17	0.50	0.24
Repeatability (Sr)	0.01	0.05	0.01
%RSDr	5.57	10.83	3.85
r (2.8×Sr)	0.03	0.15	0.03
Reproducibility (SR)	0.12	0.13	0.07
%RSDR	71.36	25.93	30.81
R (2.8×Sr)	0.33	0.36	0.21

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Table 2
(Continued)

Sample 6	Glycidol	3-MCPD	2-MCPD
n	10	9	10
Mean	11.34	1.93	0.95
Repeatability (Sr)	0.40	0.09	0.09
%RSDr	3.54	4.86	9.94
r (2.8×Sr)	1.12	0.26	0.26
Reproducibility (SR)	2.32	0.19	0.27
%RSDR	20.49	9.94	28.83
R (2.8×Sr)	6.51	0.54	0.76
Sample 7	Glycidol	3-MCPD	2-MCPD
n	9	10	9
Mean	4.75	3.46	1.73
Repeatability (Sr)	0.11	0.09	0.04
%RSDr	2.31	2.71	2.21
r (2.8×Sr)	0.31	0.26	0.11
Reproducibility (SR)	0.53	0.42	0.49
%RSDR	11.14	12.26	28.53
R (2.8×Sr)	1.48	1.19	1.38

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Table 3
Results of glycidol and 3-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method Cd 29c-13[a]

Sample 1	Glycidol	3-MCPD
n	9	8
Mean	11.88	47.09
Repeatability (Sr)	3.10	0.90
%RSDr	26.12	1.90
r (2.8×Sr)	8.69	2.51
Reproducibility (SR)	3.67	4.53
%RSDR	30.91	9.61
R (2.8×Sr)	10.28	12.68
Sample 2	Glycidol	3-MCPD
n	9	8
Mean	1.42	0.47
Repeatability (Sr)	0.10	0.01
%RSDr	6.77	1.68
r (2.8×Sr)	0.27	0.02
Reproducibility (SR)	0.41	0.08
%RSDR	28.54	16.64
R (2.8×Sr)	1.13	0.22
Sample 3	Glycidol	3-MCPD
n	6	8
Mean	0.44	5.86
Repeatability (Sr)	0.23	0.12
%RSDr	52.41	2.10
r (2.8×Sr)	0.65	0.34
Reproducibility (SR)	0.29	0.64
%RSDR	66.05	10.90
R (2.8×Sr)	0.82	1.79
Sample 4	Glycidol	3-MCPD
n	9	9
Mean	5.27	19.02
Repeatability (Sr)	0.45	0.36
%RSDr	8.45	1.91
r (2.8×Sr)	1.25	1.02
Reproducibility (SR)	1.95	1.72
%RSDR	36.92	9.07
R (2.8×Sr)	5.45	4.83
Sample 5	Glycidol	3-MCPD
n	6	8
Mean	0.14	0.51
Repeatability (Sr)	0.03	0.01
%RSDr	21.63	2.62
r (2.8×Sr)	0.08	0.04
Reproducibility (SR)	0.05	0.04
%RSDR	35.34	8.62
R (2.8×Sr)	0.14	0.12

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Table 3
(Continued)

Sample 6	Glycidol	3-MCPD
n	9	8
Mean	10.15	2.09
Repeatability (Sr)	0.93	0.08
%RSDr	9.12	3.84
r (2.8×Sr)	2.59	0.22
Reproducibility (SR)	3.15	0.35
%RSDR	31.08	16.91
R (2.8×Sr)	8.83	0.99
Sample 7	Glycidol	3-MCPD
n	9	9
Mean	4.99	3.39
Repeatability (Sr)	0.14	0.06
%RSDr	2.72	1.86
r (2.8×Sr)	0.38	0.18
Reproducibility (SR)	1.50	0.29
%RSDR	30.15	8.42
R (2.8×Sr)	4.21	0.80

[a]No more than 3 labs reported data for 2-MCPDs using AOCS Official Method Cd 29c-13. According to the AOAC/IUPAC Harmonized Protocol there were too few labs to run statistical analysis.

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Table 4
Known composition of process contaminant reference samples used in the 2012 AOCS collaborative study[a]

Reference Sample #1

Analyte	Conc. mg/kg
Glycidyl Laurate	5.75
Glycidyl Myristate	8.14
Glycidyl Palmitate	4.68
Glycidyl Stearate	4.46
Glycidyl Oleate	4.62
Glycidyl Linoleate	5.01
Glycidyl Linolenate	3.95
Lauric Acid - Monochloropropanediol monoester	4.09
Myristic Acid - Monochloropropanediol monoester	4.57
Palmitic Acid - Monochloropropanediol monoester	4.84
Stearic Acid - Monochloropropanediol monoester	6.07
Oleic Acid - Monochloropropanediol monoester	3.53
Linoleic Acid - Monochloropropanediol monoester	4.72
Linolenic Acid - Monochloropropanediol monoester	3.94
Di-Lauric Acid - Monochloropropanediol diester	3.95
Lauric Acid - Myristic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Palmitic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Oleic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Myristic Acid - Monochloropropanediol diester	3.95
Myristic Acid - Palmitic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Oleic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Palmitic Acid - Monochloropropanediol diester	3.95
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Oleic Acid - Monochloropropanediol diester	3.95
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Oleic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Linoleic Acid - Monochloropropanediol diester	3.95
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Linolenic Acid - Monochloropropanediol diester	3.95
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Stearic Acid - Monochloropropanediol diester	3.95

Reference Sample #2

Analyte	Conc. mg/kg
Glycidyl Palmitate	1.49
Glycidyl Stearate	0.88
Glycidyl Oleate	1.11
Glycidyl Linoleate	1.22
Glycidyl Linolenate	1.32

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Table 4
(Continued)

Reference Sample #3

Analyte	Conc. mg/kg
Di-Palmitic Acid - Monochloropropanediol diester	1.28
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Oleic Acid - Monochloropropanediol diester	1.28
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	2.56
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Oleic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Linoleic Acid - Monochloropropanediol diester	1.28
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Linolenic Acid - Monochloropropanediol diester	1.28
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Stearic Acid - Monochloropropanediol diester	1.28

Reference Sample #4

Analyte	Conc. mg/kg
Glycidyl Palmitate	5.26
Glycidyl Stearate	3.11
Glycidyl Oleate	3.93
Glycidyl Linoleate	4.33
Glycidyl Linolenate	4.68
Di-Palmitic Acid - Monochloropropanediol diester	4.48
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Oleic Acid - Monochloropropanediol diester	4.48
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	8.96
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Oleic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Linoleic Acid - Monochloropropanediol diester	4.48
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Linolenic Acid - Monochloropropanediol diester	4.48
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Stearic Acid - Monochloropropanediol diester	4.48

Reference Sample #5

Analyte	Conc. mg/kg
Glycidyl Oleate	0.20

[a]Reference Sample #6 was an unknown trade sample of palm oil. Reference Sample #7 was B5 Palm Oil B & GS from the 2011 GE Study. All reference samples were made up in blank canola oil, and all reference samples contain up to 1500 mg/kg dichloromethane. The above information was used in determining the statistical equivalence of the AOCS Official Methods Cd 29a-13, Cd 29b-13 and Cd 29c-13.

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AOCS Official Method Cd 29c-13
Approved 2013

Fatty-acid-bound 3-chloropropane-1, 2-diol (3-MCPD) and 2,3-epoxi-propane-1-ol (glycidol), Determination in Oils and Fats by GC/MS (Differential Measurement)

DEFINITION

This method is used for the determination of fatty acid esters of 2-chloropropane-1,3-diol (2-MCPD), 3-chloropropane-1,2-diol (3-MCPD) and glycidol in edible oils and fats; see also AOCS Official Methods Cd 29a-13 or Cd 29b-13 (see Notes, 1).

The sum of bound 3-MCPD and bound glycidol is determined as free 3-MCPD (Assay A). The addition of a dilute solution of sodium hydroxide or sodium methoxide in methanol releases free 3-MCPD and free glycidol by base catalysis. This reaction is stopped by the addition of an excess amount of an acidic chloride-containing salt solution. Under acidic conditions, free glycidol reacts with inorganic chloride to generate additional 3-MCPD and a small amount of 2-MCPD. Derivatization is completed by phenylboronic acid for the measurement by GC/MS.

Bound 3-MCPD (Assay B) is released by a dilute solution of sodium hydroxide or sodium methoxide in methanol. This reaction is stopped by the addition of an excess amount of an acidic chloride-free salt solution (e.g. sodium sulphate, ammonium sulphate, sodium bromide). Under chloride-free acidic conditions, free glycidol does not generate additional 3-MCPD.

SCOPE

This method describes a procedure for the direct determination of 3-MCPD esters (bound 3-MCPD) and free 3-MCPD after alkaline-catalyzed ester cleavage and derivatization with phenylboronic acid. Furthermore, this method enables the indirect determination of glycidyl esters (bound glycidol) under the assumption that no other substances are present that react at room temperature with inorganic chloride to generate 3-MCPD.

This method is applicable to solid and liquid fats and oils present as such or obtained by cautious extraction from processed fat- and oil-containing foodstuffs (see Notes, 2).

APPARATUS

1. Analytical balance, capable of measuring ± 0.0001g.
2. Screw cap vials (ca. 1.5 mL capacity) and screw caps with Teflon-coated septa.
3. Micro inserts (ca. 200 µL capacity) for screw cap vials (ca. 1.5 mL capacity).
4. GC/MS-system with temperature programmable injector.
5. Fused-silica-GC-column (e.g. Restek Rxi® 17 or HP DB 17-MS, length 30 m, ID 0.25 mm, stationary phase 0.25 µm, if necessary with pre-column).
6. Drying oven or water bath.

REAGENTS

1. Toluene.
2. *tert*-Butyl methyl ether.
3. Methanol.
4. *iso*-Hexane.
5. Ethyl acetate.
6. Diethyl ether.
7. *iso*-Octane.
8. Aqueous sodium chloride solution.
9. Sulfuric acid.
10. Aqueous sodium bromide solution.
11. Sodium sulphate anhydrous, granular.

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SOLUTIONS

1. Sodium hydroxide solution, 20 g/L in methanol (Reagents, 3) or sodium methoxide solution, 25 g/L in methanol (Reagents, 3).
2. Acidified sodium chloride solution (200 g/L): Add to 1 L of aqueous sodium chloride solution (Reagents, 8) 35 mL of sulfuric acid (Reagents, 9) (25%). 600 µL of this solution shall neutralize 200 µL of sodium hydroxide or sodium methoxide solution (Solutions, 1) and adjust the pH-value to the acidic range. The use of other acids (e.g. acetic acid) is possible. Test suitability in spiking experiments or analysis of ring test material.
3. Acidified chloride-free salt solution, e.g. sodium bromide solution (600 g/L): Add to 1 L of aqueous sodium bromide solution (Reagents, 10) 35 mL of sulfuric acid (Reagents, 9) (25%). 600 µL of this solution shall neutralize 200 µL of sodium hydroxide or sodium methoxide solution (Solutions, 1) and adjust the pH-value to the acidic range. The use of other acids (e.g. acetic acid) is possible. Test suitability in spiking experiments or analysis of ring test material.
4. Phenylboronic acid, saturated in diethyl ether.

STANDARDS

1. Working solution standard: d_5-3-MCPD-1,2-*bis*-palmitoylester (e.g. 26.87 µg/mL in toluene (Reagents, 1); equivalent 5.0 µg/mL free 3-MCPD) (Supplier: e.g. Toronto Research Chemicals, Canada)
2. 3-MCPD-1,2-*bis*-palmitoylester (26.6 µg/mL in toluene (Reagents, 1); equivalent 5.0 µg/mL free 3-MCPD) (Supplier: e.g. Toronto Research Chemicals, Canada)
3. 3-MCPD, 99.0% (Supplier: e.g. Dr. Ehrenstorfer GmbH, Germany)
4. Glycidyl stearate, 98.0% (Supplier: e.g. Toronto Research Chemicals, Canada)

PROCEDURE

1. Use liquid samples without further treatment. Melt solid or turbid fats at approximately 80°C in a drying oven or water bath. For high-melting fats, increase the temperature 10°C steps until the melting process starts. (See Notes, 3).
2. Solid samples containing higher amounts of water, that result in a phase separation after melting, shall be used as is.
3. Weigh, to the nearest 0.5 mg, two 100 mg portions of the sample into two individual 1.5 mL screw cap vials. Add to each vial 100 µL of working solution standard (Standards, 1) and 100 µL of *tert*-butyl methyl ether (Assays A and B). Shake both assays until the sample is completely dissolved. In the case of high melting point samples, warm the reaction vessel. (see Notes, 4).
4. Add to each assay 200 µL of sodium hydroxide or sodium methoxide solution (Solutions, 1), seal the vials and shake briefly to mix.
5. The reaction time for ester cleavage is 3.5 min to 5.5 min. Shake occasionally turbid solutions or solutions with precipitates to obtain a clear solution. (see Notes, 5).
6. Stop the ester cleavage by the addition of:
   (a) 600 µL of acidified sodium chloride solution (Solutions, 2) to one vial (Assay A) and
   (b) 600 µL of acidified chloride-free salt solution (Solutions, 3) to the second vial (Assay B).
7. Add to both assays 600 µL of *iso*-hexane (Reagents, 4), seal the vials and shake vigorously. Allow both vials to stand at room temperature for approximately 5 min. (see Notes, 6).
8. Shake both vials vigorously and then separate and discard the organic phase. Repeat this step with another 600 µL of *iso*-hexane for both assays.
9. For each assay, extract the aqueous phase (Procedure, 5) three times with each 600 µL of a mixture of diethyl ether/ethyl acetate (e.g. 600 µL/mL diethyl ether; 400 µL/mL ethyl acetate) (Reagents, 5 and 6). Combine the organic extracts of each assay avoiding the aqueous phase in new screw cap vials containing a small amount of anhydrous sodium sulphate (Reagents, 11), Add 20 µL of the derivatization reagent (Reagents, 11) to both organic extracts. If the derivatization reaction results in a small signal response of the analytes, the amount of derivatization agent can be increased until the signal-to-noise ratio in the corresponding chromatogram does not increase. The maximum amount of derivatization reagent that can be used is limited by the individual capacity of a gas chromatographic–mass spectrometric system to exclude excess PBA. If the drying agent gets sticky, the solution shall be transferred into a new screw cap vial with fresh sodium sulphate (Reagents, 11).
10. Evaporate both assays to dryness using a gentle stream of nitrogen. Redissolve the soluble fractions in approximately 500 µL of *iso*-octane.

ANALYSIS

For GC/MS measurement, one part of each redissolved assay is transferred in a 200 µL micro insert.

1. GC conditions
   (a) Injection volume: 1 µL to 2 µL
   (b) Injector: e.g. splitless
   (c) Carrier gas: Helium 99.999%
   (d) Const. flow: 1 mL/min to 1.2 mL/min
   (e) GC oven temperature program: e.g. 85°C, isothermal 0.5 min, with 6°C/min to 150°C, with 12°C/min to 180°C, with 25°C to 280°C, isothermal 7 min

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(f) PTV temperature program: e.g. 85°C, with 300°C/min to 165°C, 10 min isothermal, with 300°C/min to 320°C, 8 min isothermal
(g) Purge flow: 50 mL/min 0.5 min to 1 min, septum purge 3 mL/min

2. MS Conditions
   (a) Mass spectrometric detector: Electron-Impact (EI), Selected Ion Monitoring (SIM), detected ion traces, 149/150/201/203 for standard, 146/147/196/198 for analyte. Generally the corresponding ion traces 150 and 147 or 201 and 196 are targeted for quantification whereas the other ion traces serve as qualifiers.
   (b) For quantification, suitable software with automatic analysis is recommended. (see Notes, 7).

CALCULATIONS

1. For sum of 3-MCPD from bound 3-MCPD and glycidyl esters, multiply the ratio of signal areas of the analyte and the isotopic labeled standard based on corresponding ion traces with the spiking level of the isotopic labeled standard in Assay A:

$$W_{3\text{-MCPD(A)}} = \frac{SF_a \times W_{d_5\text{-3-MCPD(A)}}}{SF_{iA}}$$

Where—

$W_{3\text{-MCPD(A)}}$ is the mass fraction, in mg/kg, of 3-MCPD in Assay A
$W_{d5\text{-3-MCPD(A)}}$ is the mass fraction, in mg/kg, of d5-3-MCPD Assay A
SF_A is the area of 3-MCPD in Assay A
SF_{iA} is the area of d5-3-MCPD in Assay A

2. For bound 3-MCPD, multiply the ratio of signal areas of the analyte and the isotopic labeled standard based on corresponding ion traces with the spiking level of the isotopic labeled standard in Assay B:

$$W_{3\text{-MCPD(B)}} = \frac{SF_B \times W_{d_5\text{-3-MCPD(B)}}}{SF_{iB}}$$

Where—

$W_{3\text{-MCPD(B)}}$ is the mass fraction, in mg/kg, of 3-MCPD in Assay B
$W_{d5\text{-3-MCPD(B)}}$ is the mass fraction, in mg/kg, of d5-3-MCPD in Assay B
SFB is the area of 3-MCPD in Assay B
SFiB is the area of d5-3-MCPD in Assay B
Report the results to one decimal place.

3. The transformation factor that quantifies the 3-MCPD generated from glycidol can be determined by a matrix calibration in the presence of chloride (Assay A). Therefore, a non-contaminated oil or fat sample is processed (Assay A) after spiking with different amounts of glycidol (added as glycidyl ester; e.g. glycidyl stearate) in a way that regular concentration levels will result. The reciprocal value of the resulting calibration curve y = mx + n equates to the transformation factor *t*:

$$t = \frac{1}{m}$$

Where—

y is the mass fraction of $W_{3\text{-MCPD}}$ related to the calibration curve
x is the mass fraction of $W_{glycidol}$ related to the calibration curve
n is the intercerpt of the calibration curve
m is the gradient of the calibration curve

4. The content of glycidol is the difference of the result obtained in Calculations, 1 and Calculations, 2 multiplied with the transformation factor t (from Calculations, 3; see Notes, 8):

$$W_{glycidol} = t \times (W_{3-MCPD(A)} - W_{3-MCPD(B)}$$

Where—

$W_{Glycidol}$ is the mass fraction, in mg/kg, of glycidol in the sample

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METHOD PRECISION

The precision of the method is the result of an international, interlaboratory study organized by the *Joint Committee for the Analysis of Fats, Oils, Fat Products, Related Products and Raw Materials (GA Fett)*. The study was carried out in 2010 on six samples with the results given in Tables 1 and 2 (see Notes, 9).

Additional precision data determined from a 2012 AOCS collaborative study for comparison of this method with Cd 29a-13 and Cd 29b-13 by 12 laboratories analyzing 7 samples are given in Table 3–5. The same 7 samples were used in each of these methods. Reproducibility and repeatability values are calculated according to the AOAC/IUPAC Harmonized Protocol for Collaborative Study. The known composition of the reference samples is given in Table 6.

REPORTING OF RESULTS

Report results, method number and Assay A or B for each analysis. Additionally, report all sample identification details and operations not specified within the method (see Notes, 10).

NUMBERED NOTES

1. AOCS Official Methods Cd 29a-13, Cd 29b-13 and Cd 29c-13 are three different methods that have been found through international collaborative studies to produce statistically the same results, and therefore are considered equivalent for use in the determination of 2- and 3-MCPD fatty acid esters and glycidyl fatty acid esters in edible oils and fats (Tables 3–5).
2. To extract fat from fat-containing foodstuffs, neither acidic nor alkaline cleavage procedures shall be used. Digestion procedures are not suitable because significant amounts of 3-MCPD esters are formed. Suitable solvents for fat extraction are *tert*-butyl methyl ether or mixtures of *tert*-butyl methyl ether with hexane or petroleum ether. Under certain circumstances, it is possible that 3-MCPD monoesters cannot be extracted completely with hexane or petroleum ether.
3. Solid samples containing higher amounts of water should be used without pretreatment.
4. Quantification of the analytes is carried out using d_5-3-MCPD-1,2-*bis*-palmitoyl ester as standard. Therefore, no control of the completeness of the ester cleavage is necessary. A matrix calibration over the complete method is carried out periodically and serves for the determination of method linearity, relative recovery and, in the case of bound glycidol, for the determination of the transformation factor. As long as the method is not changed, calibration does not have to be done every day. It is recommended that a blank sample virgin vegetable oil is analyzed every day. Contamination may also occur from consumables, e.g. screw lid vials or filter. Baking the glassware at 400°C to 500°C can reduce this problem. A better solution is the use of non-contaminated materials. Samples which do contain 3-MCPD only and no glycidol result in lower values (ca. 2% to 10%) when using Procedure B instead of Procedure A. The difference is due to an isotope effect in Procedure B. This effect can be determined by dosing a non-contaminated oil sample with equal amounts of 3-MCPD-1,2-*bis*-dipalmitoylester and d_5-3-MCPD-1,2-*bis*-palmitoylester before being divided into two aliquots. The aliquots are then analyzed three times according to Procedure A and Procedure B. From the determinations a correction factor can be determined which is the ratio of the results from Procedures A and B. The isotope effect also appears in the presence of bound glycidol.
5. It is very important to follow the given reaction time because in alkaline solution and at higher temperature it is possible to liberate free 3-MCPD and d_5-3-MCPD which reacts to form glycidol and d_5-glycidol.
6. Glycidol reacts under acidic catalysis to form 3-MCPD and partly to 2-MCPD in Assay A. The completeness of this reaction depends on the acidity of the reaction solution and so does the ratio of the formed MCPD isomers. If the acidity is not strong enough due to the use of a weak acid or the use of too basic reaction components, the reaction time and temperature shall be increased. Otherwise the reaction will not be complete and reproducible.
7. Following the described sample preparation, the use of chromatographic back-flush-technique is not necessary.
8. For the calculation of bound glycidol, the difference of both determinations (Assays A and B) is multiplied by a nonstoichiometric factor reflecting the transformation of glycidol to 3-MCPD. This procedure is based on the assumption that the difference between the determinations is caused only by the exclusive occurrence of glycidyl esters.
9. Evaluated according to DGF Standard Method A-II 1: *Execution and evaluation of ring tests* and ISO 5725:1994: *Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.*
10. For the determination of the true amount of 3-MCPD only Assay B shall be used; whereas Assay A shall be used to determine the combined amount of 3-MCPD and glycidol.

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Table 1
Summary of statistical results for Assay A (results in mg/kg).

Sum of 3-MCPD and glycidol	A	B	C	D	E	F
Number of participating laboratories (N)	13	13	13	13	13	13
Number of laboratories retained after eliminating outliers (n)	12	12	13	13	13	9
Number of individual test results of all laboratories on each sample (z)	24	24	26	26	26	18
Mean value (m), %	**0.08**	**1.21**	**5.35**	**10.33**	**1.07**	**2.66**
Repeatability standard deviation (s_r)	0.02	0.06	0.23	0.23	0.09	0.10
Repeatability coefficient of variation (RCV_r), %	25.6	4.6	4.4	2.3	8.1	3.9
Repeatability limit r ($s_r \times 2.8$)	**0.06**	**0.15**	**0.66**	**0.65**	**0.24**	**0.29**
Reproducibility standard deviation (s_R)	0.10	0.27	0.97	1.51	0.21	0.45
Reproducibility coefficient of variation (RCV_R), %	131.7	22.6	18.2	14.6	20.0	16.9
Reproducibility limit R ($s_R \times 2.8$)	**0.29**	**0.77**	**2.72**	**4.23**	**0.60**	**1.26**

*A rapeseed oil extra virgin
B maize germ oil, refined
C deep-frying fat (hydrogenated fat)
D walnut oil/extra virgin grapeseed oil
E soybean oil/sunflowerseed oil
F sample A with addition of 3 mg/kg free 3-MCPD

Table 2
Summary of statistical results for Assay B (results in mg/kg).

3-MCPD	A	B	C	D	E	F
Number of participating laboratories (N)	13	13	13	13	13	13
Number of laboratories retained after eliminating outliers (n)	12	13	13	13	12	12
Number of individual test results of all laboratories on each sample (z)	24	26	26	26	24	24
Mean value (m), %	**0.09**	**0.46**	**2.31**	**8.12**	**0.62**	**2.37**
Repeatability standard deviation (s_r)	0.04	0.07	0.11	0.22	0.04	0.17
Repeatability coefficient of variation (RCVr), %	44.5	14.7	4.7	2.7	5.7	7.3
Repeatability limit r (s_r x 2.8)	**0.11**	**0.19**	**0.30**	**0.62**	**0.10**	**0.48**
Reproducibility standard deviation (s_R)	0.12	0.17	0.54	1.41	0.18	0.57
Reproducibility coefficient of variation (RCV_R), %	130.9	37.7	23.6	17.4	28.5	24.1
Reproducibility limit R (s_R x 2.8)	**0.34**	**0.48**	**1.52**	**3.95**	**0.50**	**1.60**

*A rapeseed oil extra virgin
B maize germ oil, refined
C deep-frying fat (hydrogenated fat)
D walnut oil/extra virgin grapeseed oil
E soybean oil/sunflowerseed oil
F sample A with addition of 3 mg/kg free 3-MCPD

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Table 3
Results of glycidol, 3-MCPD, and 2-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method 29a-13

Sample 1	Glycidol	3-MCPD	2-MCPD
N	11	10	10
Mean	8.76	47.28	0.32
Repeatability (Sr)	0.31	0.46	0.03
%RSDr	3.51	0.97	9.62
r (2.8×Sr)	0.86	1.28	0.09
Reproducibility (SR)	0.82	3.97	0.09
%RSDR	9.33	8.40	27.79
R (2.8×Sr)	2.29	11.12	0.25
Sample 2	Glycidol	3-MCPD	2-MCPD
n	10	10	11
Mean	1.46	0.49	0.21
Repeatability (Sr)	0.03	0.03	0.03
%RSDr	2.19	5.45	14.24
r (2.8×Sr)	0.09	0.08	0.08
Reproducibility (SR)	0.05	0.08	0.05
%RSDR	3.15	16.12	25.28
R (2.8×Sr)	0.13	0.22	0.15
Sample 3	Glycidol	3-MCPD	2-MCPD
n	8	12	11
Mean	0.28	5.82	0.22
Repeatability (Sr)	0.03	0.35	0.04
%RSDr	12.28	5.99	16.20
r (2.8×Sr)	0.10	0.98	0.10
Reproducibility (SR)	0.12	0.79	0.06
%RSDR	44.38	13.54	26.56
R (2.8×Sr)	0.35	2.20	0.16
Sample 4	Glycidol	3-MCPD	2-MCPD
n	12	11	11
Mean	4.69	18.92	0.26
Repeatability (Sr)	0.16	0.35	0.03
%RSDr	3.46	1.85	13.49
r (2.8×Sr)	0.45	0.98	0.10
Reproducibility (SR)	0.41	1.87	0.07
%RSDR	8.84	9.88	25.62
R (2.8×Sr)	1.16	5.23	0.19
Sample 5	Glycidol	3-MCPD	2-MCPD
n	8	9	10
Mean	0.32	0.50	0.22
Repeatability (Sr)	0.04	0.05	0.03
%RSDr	12.13	9.76	12.57
r (2.8×Sr)	0.11	0.14	0.08
Reproducibility (SR)	0.13	0.05	0.03
%RSDR	40.12	9.76	15.20
R (2.8×Sr)	0.36	0.14	0.09

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Table 3
(Continued)

Sample 6	Glycidol	3-MCPD	2-MCPD
n	12	11	11
Mean	10.91	1.95	0.93
Repeatability (Sr)	0.42	0.09	0.06
%RSDr	3.87	4.65	6.31
r (2.8×Sr)	1.18	0.25	0.16
Reproducibility (SR)	1.37	0.13	0.13
%RSDR	12.56	6.42	13.56
R (2.8×Sr)	3.84	0.35	0.35
Sample 7	**Glycidol**	**3-MCPD**	**2-MCPD**
n	12	12	12
Mean	4.92	3.41	1.61
Repeatability (Sr)	0.13	0.08	0.09
%RSDr	2.70	2.43	5.58
r (2.8×Sr)	0.37	0.23	0.25
Reproducibility (SR)	0.49	0.41	0.22
%RSDR	9.93	11.96	13.76
R (2.8×Sr)	1.37	1.14	0.62

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Table 4
Results of glycidol, 3-MCPD, and 2-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method 29b-13

Sample 1	Glycidol	3-MCPD	2-MCPD
n	9	10	9
Mean	9.37	49.90	0.32
Repeatability (Sr)	0.23	2.85	0.03
%RSDr	2.46	5.72	9.14
r (2.8×Sr)	0.65	7.99	0.08
Reproducibility (SR)	2.11	8.73	0.11
%RSDR	22.51	17.50	35.68
R (2.8×Sr)	5.91	24.46	0.32
Sample 2	Glycidol	3-MCPD	2-MCPD
n	9	8	10
Mean	1.34	0.51	0.24
Repeatability (Sr)	0.04	0.02	0.01
%RSDr	2.85	3.08	5.64
r (2.8×Sr)	0.11	0.04	0.04
Reproducibility (SR)	0.18	0.11	0.08
%RSDR	13.50	22.15	32.87
R (2.8×Sr)	0.51	0.31	0.22
Sample 3	Glycidol	3-MCPD	2-MCPD
n	8	10	9
Mean	0.18	5.93	0.24
Repeatability (Sr)	0.02	0.12	0.01
%RSDr	10.67	2.05	3.79
r (2.8×Sr)	0.05	0.34	0.03
Reproducibility (SR)	0.14	0.72	0.06
%RSDR	78.10	12.18	23.97
R (2.8×Sr)	0.39	2.02	0.16
Sample 4	Glycidol	3-MCPD	2-MCPD
n	9	8	9
Mean	5.34	18.89	0.27
Repeatability (Sr)	0.21	0.31	0.02
%RSDr	3.86	1.63	6.38
r (2.8×Sr)	0.58	0.86	0.05
Reproducibility (SR)	2.12	1.43	0.09
%RSDR	39.61	7.56	31.13
R (2.8×Sr)	5.93	4.00	0.24
Sample 5	Glycidol	3-MCPD	2-MCPD
n	8	8	10
Mean	0.17	0.50	0.24
Repeatability (Sr)	0.01	0.05	0.01
%RSDr	5.57	10.83	3.85
r (2.8×Sr)	0.03	0.15	0.03
Reproducibility (SR)	0.12	0.13	0.07
%RSDR	71.36	25.93	30.81
R (2.8×Sr)	0.33	0.36	0.21

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Table 4
(Continued)

Sample 6	Glycidol	3-MCPD	2-MCPD
n	10	9	10
Mean	11.34	1.93	0.95
Repeatability (Sr)	0.40	0.09	0.09
%RSDr	3.54	4.86	9.94
r (2.8×Sr)	1.12	0.26	0.26
Reproducibility (SR)	2.32	0.19	0.27
%RSDR	20.49	9.94	28.83
R (2.8×Sr)	6.51	0.54	0.76
Sample 7	Glycidol	3-MCPD	2-MCPD
n	9	10	9
Mean	4.75	3.46	1.73
Repeatability (Sr)	0.11	0.09	0.04
%RSDr	2.31	2.71	2.21
r (2.8×Sr)	0.31	0.26	0.11
Reproducibility (SR)	0.53	0.42	0.49
%RSDR	11.14	12.26	28.53
R (2.8×Sr)	1.48	1.19	1.38

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Table 5
Results of glycidol and 3-MCPD determination by 12 laboratories in a collaborative study organized by AOCS in 2012.

AOCS Official Method 29c-13[a]

Sample 1	Glycidol	3-MCPD
n	9	8
Mean	11.88	47.09
Repeatability (Sr)	3.10	0.90
%RSDr	26.12	1.90
r (2.8×Sr)	8.69	2.51
Reproducibility (SR)	3.67	4.53
%RSDR	30.91	9.61
R (2.8×Sr)	10.28	12.68
Sample 2	Glycidol	3-MCPD
n	9	8
Mean	1.42	0.47
Repeatability (Sr)	0.10	0.01
%RSDr	6.77	1.68
r (2.8×Sr)	0.27	0.02
Reproducibility (SR)	0.41	0.08
%RSDR	28.54	16.64
R (2.8×Sr)	1.13	0.22
Sample 3	Glycidol	3-MCPD
n	6	8
Mean	0.44	5.86
Repeatability (Sr)	0.23	0.12
%RSDr	52.41	2.10
r (2.8×Sr)	0.65	0.34
Reproducibility (SR)	0.29	0.64
%RSDR	66.05	10.90
R (2.8×Sr)	0.82	1.79
Sample 4	Glycidol	3-MCPD
n	9	9
Mean	5.27	19.02
Repeatability (Sr)	0.45	0.36
%RSDr	8.45	1.91
r (2.8×Sr)	1.25	1.02
Reproducibility (SR)	1.95	1.72
%RSDR	36.92	9.07
R (2.8×Sr)	5.45	4.83
Sample 5	Glycidol	3-MCPD
n	6	8
Mean	0.14	0.51
Repeatability (Sr)	0.03	0.01
%RSDr	21.63	2.62
r (2.8×Sr)	0.08	0.04
Reproducibility (SR)	0.05	0.04
%RSDR	35.34	8.62
R (2.8×Sr)	0.14	0.12

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Table 5
(Continued)

Sample 6	Glycidol	3-MCPD
n	9	8
Mean	10.15	2.09
Repeatability (Sr)	0.93	0.08
%RSDr	9.12	3.84
r (2.8×Sr)	2.59	0.22
Reproducibility (SR)	3.15	0.35
%RSDR	31.08	16.91
R (2.8×Sr)	8.83	0.99
Sample 7	Glycidol	3-MCPD
n	9	9
Mean	4.99	3.39
Repeatability (Sr)	0.14	0.06
%RSDr	2.72	1.86
r (2.8×Sr)	0.38	0.18
Reproducibility (SR)	1.50	0.29
%RSDR	30.15	8.42
R (2.8×Sr)	4.21	0.80

[a]No more than 3 labs reported data for 2-MCPDs using AOCS Official Method Cd 29c-13. According to the AOAC/IUPAC Harmonized Protocol there were too few labs to run statistical analysis.

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Table 6
Known composition of process contaminant reference samples used in the 2012 AOCS collaborative study[a]

Reference Sample #1

Analyte	Conc. mg/kg
Glycidyl Laurate	5.75
Glycidyl Myristate	8.14
Glycidyl Palmitate	4.68
Glycidyl Stearate	4.46
Glycidyl Oleate	4.62
Glycidyl Linoleate	5.01
Glycidyl Linolenate	3.95
Lauric Acid - Monochloropropanediol monoester	4.09
Myristic Acid - Monochloropropanediol monoester	4.57
Palmitic Acid - Monochloropropanediol monoester	4.84
Stearic Acid - Monochloropropanediol monoester	6.07
Oleic Acid - Monochloropropanediol monoester	3.53
Linoleic Acid - Monochloropropanediol monoester	4.72
Linolenic Acid - Monochloropropanediol monoester	3.94
Di-Lauric Acid - Monochloropropanediol diester	3.95
Lauric Acid - Myristic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Palmitic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Oleic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Lauric Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Myristic Acid - Monochloropropanediol diester	3.95
Myristic Acid - Palmitic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Oleic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Myristic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Palmitic Acid - Monochloropropanediol diester	3.95
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Oleic Acid - Monochloropropanediol diester	3.95
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	7.89
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Oleic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Linoleic Acid - Monochloropropanediol diester	3.95
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	7.89
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Linolenic Acid - Monochloropropanediol diester	3.95
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	7.89
Di-Stearic Acid - Monochloropropanediol diester	3.95

Reference Sample #2

Analyte	Conc. mg/kg
Glycidyl Palmitate	1.49
Glycidyl Stearate	0.88
Glycidyl Oleate	1.11
Glycidyl Linoleate	1.22
Glycidyl Linolenate	1.32

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Table 6
(Continued)

Reference Sample #3

Analyte	Conc. mg/kg
Di-Palmitic Acid - Monochloropropanediol diester	1.28
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Oleic Acid - Monochloropropanediol diester	1.28
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	2.56
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Oleic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Linoleic Acid - Monochloropropanediol diester	1.28
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	2.56
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Linolenic Acid - Monochloropropanediol diester	1.28
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	2.56
Di-Stearic Acid - Monochloropropanediol diester	1.28

Reference Sample #4

Analyte	Conc. mg/kg
Glycidyl Palmitate	5.26
Glycidyl Stearate	3.11
Glycidyl Oleate	3.93
Glycidyl Linoleate	4.33
Glycidyl Linolenate	4.68
Di-Palmitic Acid - Monochloropropanediol diester	4.48
Palmitic Acid - Oleic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Linoleic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Palmitic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Oleic Acid - Monochloropropanediol diester	4.48
Oleic Acid - Linoleic Acid - Monochloropropanediol diester	8.96
Oleic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Oleic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Linoleic Acid - Monochloropropanediol diester	4.48
Linoleic Acid - Linolenic Acid - Monochloropropanediol diester	8.96
Linoleic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Linolenic Acid - Monochloropropanediol diester	4.48
Linolenic Acid - Stearic Acid - Monochloropropanediol diester	8.96
Di-Stearic Acid - Monochloropropanediol diester	4.48

Reference Sample #5

Analyte	Conc. mg/kg
Glycidyl Oleate	0.20

[a]Reference Sample #6 was an unknown trade sample of palm oil. Reference Sample #7 was B5 Palm Oil B & GS from the 2011 GE Study. All reference samples were made up in blank canola oil, and all reference samples contain up to 1500 mg/kg dichloromethane. The above information was used in determining the statistical equivalence of the AOCS Official Methods Cd 29a-13, Cd 29b-13 and Cd 29c-13.

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Fatty Acid Composition by Packed Column Gas Chromatography

DEFINITION

Methyl esters of fatty acids are separated and determined quantitatively by GLC using a packed column.

SCOPE

The method is applicable to methyl esters of fatty acids having 8–24 carbon atoms and to animal fats, vegetable oils, marine oils, and fatty acids after their conversion to methyl esters. The method permits quantitative separation of mixtures containing saturated and unsaturated methyl esters. The conditions specified in this method are not suitable for determining epoxy or oxidized fatty acids or fatty acids that have been polymerized.

APPARATUS

1. The gas chromatograph, which is commercially available, should have as a minimum the following characteristics—
   (a) Column oven—capable of heating the column to at least 220°C and of maintaining the desired temperature to within ± 1°C.
   (b) Sample inlet port—with minimum dead space which is independently heated to a temperature 20–50°C higher than column temperature.
   (c) Detectors—thermal conductivity (TC) or flameionization (FID), separately thermostated, which can be maintained at or above column temperature.
2. Recorder—If the recorder curve is to be used to calculate the composition of the mixture analyzed, an electronic recorder of high precision is required. The characteristics of the recorder should be:
   (a) Rate of response—below 1.0 sec (the rate of response is the time taken for the recording pen to pass from 0 to 90% following the momentary introduction of a 100% signal).
   (b) Chart paper—width 25 cm (10 in.) min.
   (c) Chart paper speed—25–100 cm/hr (10–40 in./hr).
3. Integrator or calculator (optional)—rapid and accurate calculation can be performed with the help of an electronic integrator or calculator. This must give a linear response with adequate sensitivity and baseline correction should be consistent with good chromatographic practice. Horizontal, non-horizontal, and tangential baseline correction must be controlled by selectable electronic peak logic.
4. Syringe—maximum capacity 10 µL, graduated in 0.1 µL.
5. Chromatographic column—
   (a) The column must be constructed of a material inert to the substances to be analyzed—glass, or failing that, stainless steel (see Notes, 1), with a length of 1–3 m and a 2–4 mm i.d.
   (b) Packing support—acid-washed and silanized diatomaceous earth, or other suitable inert support with a narrow range (25 µm) of grain size between the limits of 60–120 mesh (125–250 µm).
   (c) Stationary phase—polyester type of polar liquid (diethylene glycol polysuccinate, butanedial polysuccinate, ethylene glycol polyadipate), or any liquid (e.g., cyanosilicones), meeting the requirements below. The stationary phase should amount to 5–20% of the packing. A non-polar stationary phase, such as methyl silicone, fluid or gum, can be used for separations of fully saturated materials.

REAGENTS

1. Gases—
   (a) Carrier gas for TC detector—helium, min purity 99.95 mol%; for FID, helium, nitrogen or argon, min purity 99.95 mol%.
   (b) FID—hydrogen, min purity 99.95 mol%; air, dry [dew point –59°C (–75°F) maximum] and hydrocarbon free (less than 2 µg/g hydrocarbons equivalent CH_4).
2. Reference standards—a mixture of methyl esters, or the methyl esters of an oil of known composition, preferably similar to that of the fatty matter to be analyzed. Reference mixtures simulating most fats and oils may be obtained from:
   Applied Science Laboratories, Inc., P. O. Box 440, State College, PA, USA. Supelco, Inc., Supelco Park, Bellefonte, PA, USA. Nu-Chek-Prep, Inc., P. O. Box 172, Elysian, MN, USA. Analabs, Inc., 80 Republic Drive, North Haven, CT, USA. Alltech Associates, Inc., 2501 Waukegan Rd., Deerfield, IL, USA.

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PREPARATION OF METHYL ESTERS

AOCS Official Method Ce 2-66 is recommended.

PROCEDURE

1. Condition a new column while it is disconnected from detector by holding it about 10°C above its operating temperature with flow of inert gas at 20–60 mL/min for approximately 16 hr and then an additional 2 hr at 20°C above its operating temperature. In no case exceed the manufacturer's recommended maximum temperature.
2. Determining optimal operating conditions—
   (a) In selecting the test conditions, the following variables must be taken into account: length and diameter of the column, temperature of the column, carrier gas flow, resolution required, size of the sample for analysis, and time of analysis. The size of the sample should be chosen so that the assembly of detector and electrometer gives a linear response. As a rule, the following figures will lead to the desired results, viz., at least 2,000 theoretical plates for methyl stearate and its elution within about 15 min.

Column i.d., mm	Carrier Gas Supply, mL/min
2	15–20
3	20–40
4	40–60

Concentration of Stationary Phase, %	Temperature,°C
5	175
10	180
15	185
20	185

   (b) Where the apparatus allows, the injection port should be at a temperature of about 250–275°C and the detector at a temperature equal to, or higher than, that of the column.
   (c) The flow of hydrogen to the flame ionization detector is, as a rule, about 0.5–1 times that of the carrier gas, and the flow of air about 5–10 times that of the hydrogen.
3. Determining the efficiency and the resolution—
   (a) Carry out the analysis of a mixture of methyl stearate and oleate in about equivalent proportions (e.g., methyl esters from cocoa butter). Choose the size of the test portion, the temperature of the column, and the carrier gas flow so that the maximum of the methyl stearate peak is recorded about 15 min after the solvent peak and rises to three-quarters of the full scale. Calculate the number of theoretical plates n (efficiency) by the formula:

$$n = 16(dR_1/w_1)^2$$

   and the resolution, R, by the formula—

$$R = 2\Delta/(w_1 + w_2)$$

   Where—

   dR_1 = the retention distance, measured in mm, from the start to the maximum peak of methyl stearate.

   w_1 = width in mm of the peak for methyl stearate, measured between the points of intersection of the tangents of the inflection points of the curve with the baseline.

   w_2 = width in mm of the peak for methyl oleate, measured between the points of intersection of the tangents of the inflection points of the curve with the baseline.

   Δ = distance between the respective peak maxima for methyl stearate and oleate.

   (b) Operating conditions to be selected are those which will afford at least 2,000 theoretical plates for methyl stearate and a resolution of at least 1.25. Additionally, linolenic acid ($C_{18:3}$) ester should be separable from arachidic acid ($C_{20:0}$) and gadoleic acid ($C_{20:1}$) esters.
   (c) As a rule, the operating conditions will be those defined above. Nevertheless, it is possible to work with a lower column temperature where the determination of acids below C_{12} is required, or at a higher temperature when determining fatty acids above C_{20}.
   (d) On occasion, it is possible to employ temperature programming in both the previous cases. For example, if the test portion contains the methyl esters of fatty acids below C_{12}, inject the test portion at a column temperature of 100°C and immediately raise the temperature at a rate of 4–8°C/min to the optimum temperature. In some cases, the two procedures can be combined. After the programmed heating, continue the elution at a constant temperature until all the components have been eluted. If the instrument does not employ programmed heating, work at two fixed temperatures

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between 100°C and 195°C. Liquid phase characteristics will determine the starting temperature or the upper temperature if the analysis is performed isothermally.

4. Analysis—
   (a) The test portion for examination should be 0.1–2 µL of the solution of methyl esters obtained according to AOCS Official Method Ce 2-66. In the case of esters not in solution, prepare an approximate 1–10% solution and inject 0.1–1 µL of this solution.
   (b) If the object is to determine constituents present only in trace amounts, the test portion size may be increased (up to tenfold).

CALCULATIONS

1. Identification of peaks—
   (a) Analyze the reference standard mixture of known composition under the same operating conditions as those employed for the test portion, and measure the retention distances (or retention times) for the constituent esters.
2. Quantitative analysis—
   (a) Apart from exceptional cases, assume that the whole of the components of the test portion are represented on the chromatogram, so that the total of the areas under the peaks represents 100% of the constituents (total elution).
   (b) If the equipment includes an integrator, use the figures obtained therefrom. If not, determine the area under each peak by multiplying the height by the breadth at mid-height and, where necessary, taking into account the various attenuations used during the recording.
   (c) For the general case, in which significant amounts of components below C_{12} are absent, calculate the content of a particular constituent (expressed as percent of methyl esters) by determining the percentage represented by the area of the corresponding peak relative to the sum of the areas of all the peaks.

$$\text{Area percent of the component i expressed as methyl esters} = \frac{A_i}{\sum A_i} \times 100$$

Where—

Ai = area of the peak corresponding to component i

$\sum A_i$ = sum of the areas under all the peaks

   (d) Correction factors, particularly in the presence of acids below C_{12}, of acids with secondary groups, or when using a TC detector, must be used to convert the percentages of peak areas into mass percentages of the components. Determine the correction factors with the help of a chromatogram derived from the analysis of a reference mixture of methyl esters of known composition under operating conditions identical to those used for the test portion.

For this reference mixture:

$$\text{mass \% (m/m) of component i} = \frac{B_i}{\sum B_i} \times 100$$

Where—

Bi = mass of component i in the reference mixture

$\sum B_i$ = total of the masses of the various components of the reference mixture

From the chromatogram of the reference mixture, one can calculate:

$$\text{Area percent of component i} = \frac{C_i}{\sum C_i} \times 100$$

Where—

C_i = area under the peak corresponding to component i

$\sum C_i$ = sum of the area under all the peaks

From which:

$$\text{Correction factor } K_i = \frac{B_i \times \sum C_i}{C_i \times \sum B_i}$$

Commonly, the correlation factors are made relative to Kc_{16} so the relative factors become:

$$K'_i = \frac{K_i}{Kc_{16}}$$

Then the content of each component in the sample is given by:

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$$\text{mass \% (m/m) of component i, expressed as methyl esters} = \frac{(K'_i \times A_i)}{\Sigma(K'_i \times A_i)} \times 100$$

(e) Use an internal standard, notably in determinations when all of the fatty acids are not eluted. The internal standard may be the methyl ester of the C_{13} fatty acid. The correction factor for the internal standard should be determined:

$$\text{mass \% (m/m) of component i, expressed as methyl esters} = \frac{mc_{13} \times K'_i \times A_i}{m \times K'c_{13} \times Ac_{13}} \times 100$$

Where—

mc_{13} = mass of the internal standard added to sample, mg

m = mass of the sample, mg

*$K'c_{13}$ = correction factor for the internal standard relative to Kc

Ac_{13} = area of the peak corresponding to the internal $_{16}$ standard

A_i= area of the peak corresponding to component i

K'_i = correction factor of component i relative to Kc_{16}

$$*K'c_{13} = \frac{Kc_{13}}{Kc_{16}}$$

*Determined by adding a known amount of C_{13} methyl ester to the reference mixture and then following the above procedure for determining K'_i.

(f) Expression of the results—Give the results to three significant figures for contents over 10%, two significant figures for contents from 1–10%, one significant figure for contents below 1%, i.e., with one figure beyond the decimal point in every case.

PRECISION

1. Repeatability—The difference between the results of two determinations carried out on the same day by the same operator using the same apparatus for the same esters and for constituents present in excess of 5% should not exceed a relative figure of 3% of the determined value, with an absolute maximum of 1%. For components present in amounts of less than 5%, the repeatability in relative terms diminishes progressively as the content is reduced.
2. Reproducibility—The difference between the results obtained in two different laboratories for constituents present in excess of 5% should not exceed a relative figure of 10% of the determined value, with an absolute maximum of 3%. For constituents present in amounts less than 5%, the reproducibility in relative terms diminishes progressively as the content is reduced.

NOTES

1. If polyunsaturated components with more than three double bonds are present, they may decompose in a stainless steel column.
2. It is recommended that chromatographers read *Standard Recommended Practice for General Gas Chromatography Procedures,* ASTM Designation E260-73; *Standard Recommended Practice for Gas Chromatography Terms and Relationships,* ASTM Designation E355-77; and *Standard Recommended Practice for Testing Flame Ionization Detectors Used in Gas Chromatography,* ASTM Designation E594-77.
3. In 1990, several laboratories reported the following:
   (a) Using a medium polarity column packing in a column 2.44 m long and 6.3 mm i.d. produces better separation, especially for the $C_{18:3}$ and $C_{20:1}$ fatty acids.
   (b) The initial column temperature appears to affect the final result more than any other temperature variable.
   (c) The GC operating conditions should be adjusted to give satisfactory separation and recovery of a suitable fatty acid reference material, e.g., AOCS Laboratory Proficiency Program GC reference sample.

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AOCS Official Method Ce 1a-13

Approved 2013

Determination of Fatty Acids in Edible Oils and Fats by Capillary GLC

DEFINITION

This method provides a general gas–liquid chromatography (GLC) procedure for the determination of the fatty acid composition of vegetable or non-ruminant animal oils and fats.

SCOPE

This method is applicable to the determination of fatty acid methyl esters obtained from vegetable oils and fats from a standard procedure, for example, see also ISO 12966-2:2011, AOCS Official Method Ce 2-66 or ISO 5509-(References 1 and 2). This method is not applicable to polymerized and oxidized fats or fatty acids (see Notes, 1).

The fatty acid methyl esters (FAME) of the sample are separated on a capillary gas–liquid chromatography column having a highly polar stationary phase, according to chain length (CL), degree of unsaturation, and geometry and position of the double bonds.

APPARATUS

The instructions given apply to normal equipment used for gas–liquid chromatography employing capillary columns and flame-ionization detection.

1. Gas chromatograph—suitable for use with a capillary column equipped with:
   (a) Oven for the capillary column, capable of heating the column to at least 250°C maintaining the desired temperature to ± 0.1°C.
   (b) Temperature-controlled injection unit (see Notes, 2).
   (c) Flame-ionization detector (FID), amplifier and computer based data system.
   (d) Capillary column—fused silica capillary 25-100 m, 0.20-0.35 mm i.d., polar stationary phase, mainly of the type polyglycol (polyethylene glycol 20,000), polyester (butanediol polysuccinate) or highly polar polysiloxane (cyanosilicones), with a film thickness of 0.20-0.25 µm (see Notes, 3).
2. Installation and conditioning of the column:
   (a) Follow instrument manufacturer's instructions to install capillary columns—note the arrangement of the column in the oven (support), the correct position of the ends of the column in the injector and the detector to reduce dead volume.
   (b) Condition the column according to the manufacturer's instructions or use the following temperature program:

   Initial Temperature: 35°C, hold time: 30 mins., 16°C/min. to 220°C, hold until the baseline stablizes. Return to 180°C to work under isothermal conditions.
3. Microsyringe for gas chromatography, 10 µL delivery, with a hardened needle.

REAGENTS

1. Carrier gas—hydrogen or helium, 99.995% pure or better, gas chromatography quality, dried, and oxygen removed by suitable filters (see Notes, 4).
2. Auxiliary (make-up) gas—nitrogen 99.995% min., chromatography grade.
3. Flame gases—hydrogen and air, gas chromatography quality.
4. Reference standards—a mixture of methyl esters of fatty acids (see Notes, 5).

PROCEDURE

System Suitability Test

Reference standards of known fatty acid composition (containing, e.g., C16:0, C18:0, C18:1, C18:2, C18:3 and C20:0 methyl esters) or certified reference material (e.g., CRM 162) are used to check the performance of the system (see Notes, 5). If the quantitative results obtained correspond to the theoretical values or the certified values, the system can be regarded as suitable.

Sample Analysis

1. Inject 1 µL of a solution containing 20–25 mg/ml methyl esters, equivalent to 15–20 ug of fatty acids, prepared according to AOCS Official Method Ce 2-66 or ISO 5509-(References 1 and 2).
2. Suitable gas chromatography operating conditions are as follows:
   Injection port temperature: 250°C
   Detector temperature: 250°C
   Oven temperature: 180°C

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Carrier gas—hydrogen; column head pressure, 170 kPa (25 psi); flow rate, 1.0 mL/min; linear velocity, 40-45 cm/sec; split ratio, 100:1. Determine linear gas linear velocity by injecting an unretained compound like methane. Optimal hydrogen carrier gas linear velocity is typically around 40 cm/sec. Linear velocity is determined by dividing the column length in cm by 60 and then by the experimentally determined retention time for the unretained peak.

Carrier gas—helium; column head pressure, 286 kPa (41 psi); flow rate, 1.0 mL/min; linear velocity, 19-25 cm/sec; split ratio, 100:1. Determine linear gas linear velocity by injecting an unretained compound like methane. Optimal hydrogen carrier gas linear velocity is typically around 20 cm/sec. Linear velocity is determined by dividing the column length in cm by 60 and then by the experimentally determined retention time for the unretained peak.

CALCULATION AND EXPRESSION OF RESULTS

Performance check

Column performance is checked using a suitable mixture of fatty acid methyl esters covering the range of fatty acids under investigation. Use a mixture containing C12:0; 9c-18:1; 11c-18:1; 9c,12c,15c-18:3; 11c-20:1; and the Internal Standard C21:0 for each carrier gas and column combination to determine critical pair separation. Since commercial GC designs are different, and the critical pair separation obtained is not identical, small changes in the sample size, sample concentration or oven temperature may be required. If so, adjust the sample size, sample concentration or oven temperature until the best separation results are obtained. If column oven temperature needs to be adjusted, it should be adjusted in small increments, preferably by steps of ± 1°C (see Notes, 6).

Peak identification

The individual FAMEs are identified by their retention times, by comparison with the FAME reference standards. When unknown peaks are observed, attempt to identify such peaks using appropriate procedures such as GC-MS, FTIR, silver-ion chromatography and classical chemical methods (Reference 4). Peaks of unknown identity should not be included in the summation of peak areas when quantifying the concentrations of total fat, saturated, cis-monounsaturated, cis-polyunsaturated and trans fatty acids unless they have been confirmed to be fatty acids.

Quantitative Analysis

(a) Apart from exceptional cases, assume that all of the components of the sample are represented on the chromatogram, so that the total of areas under the peaks represents 100% of the constituents (excluding the solvent peak).
(b) For samples in which significant amounts of components below C12 are absent (recommended > 1%), calculate the content of a particular constituent (expressed as percent of methyl ester) by determining the percentage represented by the area of the corresponding peak relative to the sum of the area of all the peaks.

Area percent of the component I expressed as methyl ester =

$$(A_i/\Sigma A_i)100$$

Where—

ΣA_i = sum of the areas under all the peaks

In the presence of significant amounts of components below C12, the areas obtained from the gas chromatogram have to be multiplied by correction factors in order to convert area percentages into mass percentages.

Correct the area of each peak to compensate for the flame ionization detector (FID) response for each component. The FID correction factors are calculated relative to C16 from the molecular weight of the FAME as follows:

$$FID_x = \frac{MW_x}{(n_x - 1)(AW_C)(FID_{16:0})}$$

Where—

FID_x = the FID factor for component x
MW_x = molecular weight of component x
n_x = the number of carbon atoms in the FAME of component x
AW_C = the atomic weight of carbon (12.01)
$FID_{16:0}$ = the FID correction factor for 16:0 (1.407)

All the FID correction factors used in the calculation are relative to $FID_{16:0}$. For example, the correction factor for 10:0 becomes 1.10. FID correction factors are listed in Table 1 (see Notes, 7).

Calculate the (relative) percentage x of each component by determining the corrected area of the corresponding peak relative to the sum of the corrected areas of all the peaks, as follows:

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$$x = A_x / A_t$$

Where—

A_x = the corrected area of the peak corresponding to component x
A_t = sum of the corrected areas under all the peaks, excluding the solvent peak

QUALITY ASSURANCE AND CONTROL

1. Blank sample—The first sample in an analysis batch is always a blank. No peaks should be detected in the blank run. Repeat this test after every ten samples.
2. Proficiency test—For external reference, the method is tested each year by participation in the AOCS Laboratory Proficiency Program or any other suitable ring test.
3. Reference materials, with chromatograms, and samples from the collaborative study are available from the AOCS (www.aocs.org/tech/samples.asp).

PRECISION

The results of interlaboratory studies, organized at the international level, evaluated in accordance with International Organization for Standardization (ISO) 5725-1986 reference the following parameters:

Repeatability—When the mean of the values obtained from two single determinations, carried out in rapid succession by the same operator using the same apparatus under the same conditions for the analysis of the same test sample, the difference between the two values obtained should not be greater than the repeatability value (r) for the comparable level of fatty acids reported from interlaboratory studies.

Reproducibility—When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions from the analysis of the same laboratory sample, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility (R) for the comparable level of fatty acids reported from interlaboratory studies (see Notes, 8).

NUMBERED NOTES

1. If the analyzed oils and fats do contain polymerized and/or oxidized fatty acids, these are not all converted to FAMEs. Polymerized FAMEs are not volatile at recommended analysis conditions and will decompose in the injector. This may lead to unexpected losses of FAMEs.
2. The injection system should be specially designed for use with capillary columns. It should be of the split type or on-column. On-column injection is preferred because it generally gives less discrimination than split injection mode. Dilute the solution to 0.05% before injection. An automatic injection system is recommended because it improves the precision of results. If a split injector is used, adjust the split ratio of approximately 100:1.
3. As column diameter increases, sample capacity increases though columns with smaller diameters give greater efficiency and better resolution. Internal diameters range from 0.1 mm for fast GC columns to 0.53 mm wide bore columns, with a film thickness between 0.1 μm and 0.3 μm. The column diameter is typically chosen according to the type of sample injection system. Examples include 0.25- or 0.32-mm-I.D. columns for split and splitless injection systems, 0.32-mm-I.D. for splitless and on column injections, and 0.53-mm-I.D. for direct injection systems. As film thickness increases, retention and sample capacity increases and the column efficiency may decrease, especially with films greater than 1.0 μm thickness. Film thickness is directly proportional to the time of analysis. Thicker phase films result in increased retention times in isothermal conditions and a higher final elution temperature under temperature programmed conditions. The suitable column length is 10 m to 100 m depending on the required resolution and complexity of the sample mixture. In the separation of FAMEs, column length is a significant factor especially if the separation of isomers is required. With doubling the column length (e.g. 30 m to 60 m) the resolution is increased by a factor of $\sqrt{2} = 1.41$, which is an increase of only 40%. At the same time, increased column length increases both the time of analysis and the column head pressure. Shorter columns are used as a compromise between speed of analysis and resolution (See Table, 2 and Figure, 1) However the separation of the geometrical and positional isomers of FAMEs is best performed on a 100 m column. It is recommended to use 100 m, 0.25-mm-i.d., 0.20 μm film thickness columns with SP-2560 or CP-Sil 88 as the stationary phase to separate most C18:1 *trans*- and *cis*-isomers. If this degree of separation is not required, a 60 m column can be used. Commercially prepared columns are available from: SP-2560 (part # 24056, Supelco Inc. Bellefonte, PA, USA) and CP-Sil 88 (part # CP7489, Agilent Technologies Inc. Santa Clara, CA, USA). Commercially prepared Polyethylene glycol (PEG) coatings are available as Carbowax 20M, Durabond 225, FFAP, Silar 5 CP or Supelcowax 10 from various manufacturers. The recommended capillary column is a 100% cyanopropylsilicone such as SP-2560, SP-2340 or CP-Sil 88.
4. Nitrogen gas is not recommended as a carrier gas for this method due to the long run time.
5. Standards of most known fatty acids are available from suppliers such as: Nu-Check-Prep, Inc., P.O. Box 172, Elysian, MN 56028, USA; Bast of Copenhagen V, Denmark; Lanodon Fine Chemicals AB, Limhamnsgarden alle 9, S21616, Malmo,

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Sweden; Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, USA. Otherwise oils with specific fatty acids should be used as standards, such as black currant seed oil containing C18:3 (n-6) (γ-linoleic acid)* and C18:4 (n-3) (stearidonic acid)**.

* systematic name, all *cis* 6,9,12-octadecatrienoic acid
** systematic name, all *cis* 6,9,12,15-octadecatrienoic acid

Alternatively, certified reference material (e.g., CRM 162) from BCR, Joint Research Center, Institute for Reference Materials and Measurement, Relieseweg, B-2440, Belgium, can be used.

6. On SP-2560, CP-Sil 88 or any other cyanopropylsilicone capillary columns, the analysis temperature has profound effect on the elution pattern of 13t+14t-C18:1, 16t-C18:1, 14c-C18:1, 9c,12c,15t-C18:3, 11c-C20:1 and 9c,12c,15c-C18:3 (Reference 3). The best resolution of these and other fatty acids may be obtained when the column is operated in isothermal mode.
7. It is important to understand the difference (and hence the relationship) between TCF and TRF.
   (a) Theoretical flame ionization response factor (TRF) is actually the proportion (with respect to the total weight, which is the molecular weight) of the total amount of carbon atoms that are responding to the FID. For fatty acids, the carbons that are responding to FID are CH, CH_2 and CH_3 units. Carbonyl carbon and ester carbonyls carbon do not respond to FID. So that in FAME, all the carbon atoms are active, except the ester carbon. Thus, there are N_{x-1} active carbon atoms in a FAME so the TRF for a fatty acid methyl ester would be:

   TRF = (N_{x-1}) (Atomic Weight of Carbon)/ MW of FAME
   TRF of C21:0 FAME = 21 x 12.011/340.5776 = 0.7406

   (b) Since the FID response depends on the proportion of active carbon atoms, to determine the actual weight of a FAME, the experimental measured area (or the weight) needs to be corrected. This factor is termed as TCF and it is equal to 1/ TRF. In other words the formula for TCF is as follows:

   TCF = MW/(N_{x-1}) (Atomic Weight of Carbon).

   The TCF of C21:0 FAME is 1.3503.

   For example, the TRF of C18:0 FAME is 0.7243. Therefore, its TCF is 1/0.7243 = 1.3806. Thus TCF of C18:0 FAME with respect to C21:0 FAME = 1.3806/1.3503 = 1.0225.
8. Representative values may be found in the tables contained in AOCS Ce 1h-05, Ce 1i-07 and Ce 1j-07.

REFERENCES

1. *AOCS Official Method Ce 2-66,* Preparation of Methyl Esters of Long-Chain Fatty Acids.
2. ISO Method 5509, Animal and vegetable fats and oils—Preparation of methyl esters of fatty acids, 12-23-2004.
3. Ratnayake, W.M.N. Analysis of trans fatty acids. In Trans Fatty Acids in Human Nutrition. Eds. J.L. Sebedio and W.W. Christie. The Oily Press, Dundee, pp. 115-161 (1998).
4. Ratnayake, W.M.N., J. AOAC Int. 87:523-539 (2004).
5. The Role of Edible Oils and Fats in Human Nutrition, WHO/FAO, Rome (1977).
6. IUPAC, Standard Methods for Analysis of Oils, Fats and Derivatives, Blackwell Scientific Publications, 7th edn. (1987); IUPAC Methods 2.303 and 2.304.
7. *Animal and vegetable fats and oils—Gas chromatography of fatty acid methyl esters. Part 1: Guidelines on modern gas chromatography of fatty acid methyl esters,* International Standards Organization, 2013, ISO/DIS 12966-1.

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Table 1
List of FID response factors

FAME	MW	N-1	FID Response Factor	FID Correction Factor Relative to C16
4:0	102.13	4	2.126	1.51
6:0	130.19	6	1.807	1.28
8:0	158.24	8	1.647	1.17
9:0	172.27	9	1.594	1.13
10:0	186.30	10	1.551	1.10
11:0	200.32	11	1.516	1.08
12:0	214.35	12	1.487	1.06
13:0	228.37	13	1.463	1.04
14:0	242.40	14	1.442	1.02
15:0	156.42	15	1.423	1.01
16:0	270.46	16	1.407	1.00[a]
17:0	284.49	17	1.393	0.99
18:0	298.52	19	1.370	0.97
19:0	312.52	19	1.370	0.97
20:0	326.57	20	1.360	0.97
21:0	340.57	21	1.350	0.96
22:0	354.62	22	1.342	0.95
23:0	368.62	23	1.334	0.95
24:0	382.68	24	1.328	0.94

[a]Reference

Table 2
Effect of Column Length on Resolution and Speed of Analysis

Column Length	Resolution	Speed of Analysis
Long (> 60 m)	High	Slow
Medium (25 m to 50 m)	Good (compromise between resolution and speed)	
Short (< 15 m)	Moderate	Fast

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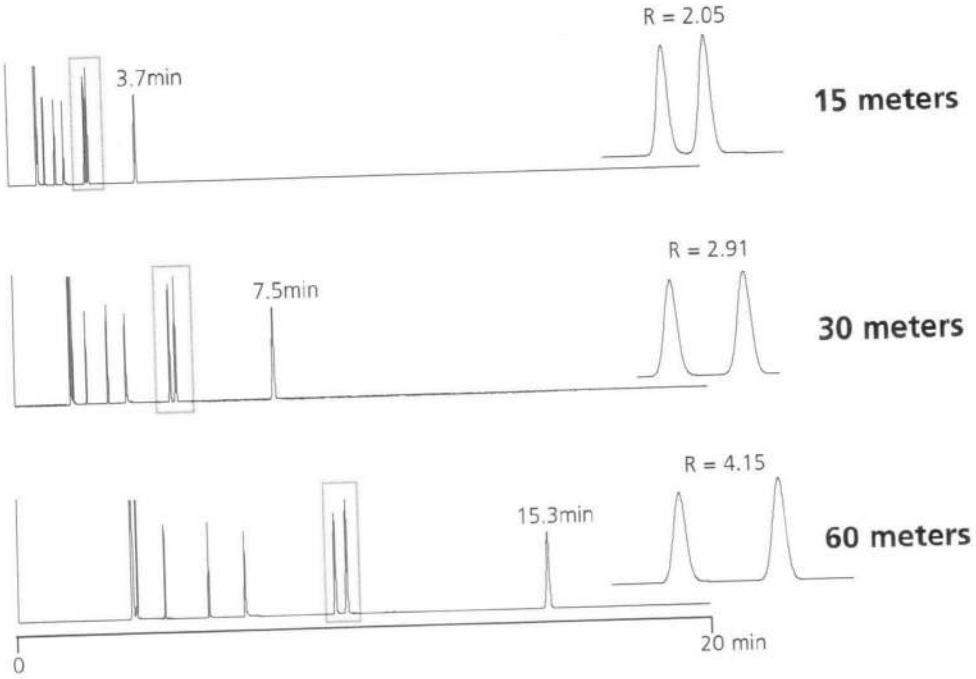

Figure 1. Effect of Column Length on Resolution
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AOCS

AOCS Official Method Ce 1b-89

Reapproved 2009

Fatty Acid Composition of Marine Oils by GLC

DEFINITION

This method determines the fatty acid composition of marine oils and marine oil esters by capillary column gas-liquid chromatography.

SCOPE

This method is designed to determine the fatty acid composition of marine oils and marine oil esters in relative (area %) values, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in absolute (mg/g) values using a bonded polyglycol liquid phase in a flexible fused silica capillary column. $C_{23:0}$ fatty acid is used as the internal standard. The method is applicable to the analysis of marine oil or marine oil ethyl or methyl esters, capsules of EPA and DHA, and minor naturally occurring polyunsaturated fatty acids (see Notes, 1).

APPARATUS

1. Gas chromatograph—with capillary injection system (split mode preferred, operated at a split ratio of 1:50; see Notes, 2) and flame-ionization detector (FID; see Notes, 3), capable of meeting the following minimum requirements (see Notes, 4):
   Injection port, 250°C
   Detector, 270°C
   Oven temperature profile:
   Initial temperature, 170°C
   Initial hold time, 0 min
   Program rate, 1.0°C/min
   Final temperature, 225°C
   Final hold time, 0 min
2. The capillary GLC column should be of flexible fused silica, 25 m or more in length and 0.20–0.35 mm i.d. The liquid phase must be bonded Carbowax-20M or an equivalent polyglycol. Examples of the latter are Supelcowax-10, 30 m × 0.25 mm (or 0.32 mm) with 0.25 µm coating (Supelco, Inc., Bellefonte, PA, USA; catalog no. 2-4079 or no. 2-4080) and Omegawax 320, 30 m × 0.32 mm i.d. with 0.25 µm coating (Supelco, Inc., catalog no. 2-4152). No constraints are placed on the column supplier; however, the manufacturer's claim for the upper temperature limit of the column should be noted and verified.
3. Carrier gas—hydrogen or helium, 99.99% pure or better, is required.
   Note—An oxygen scrubber is mandatory with Carbowax-20M and similar columns.
4. Any suitable amplifier, recorder, integrator, or data processor may be used.
5. Constant-temperature water bath or dry heating block—maintained at 100°C.
6. Screw-cap tubes—16 × 125 mm, with leak-tight Teflon™-lined caps.
7. Vial—screw- or crimp-cap, 12 mL.
8. Volumetric pipets—1 and 2 mL.
9. Pasteur-type pipets.
10. Volumetric flasks—25 and 100 mL.
11. Analytical balance—± 0.0001 g.
12. Source of dry nitrogen.

REAGENTS

1. Sodium hydroxide—reagent grade.
2. Methyl alcohol—reagent grade (see Notes, *Caution*).
3. BF_3—12% in methanol, 2 mL ampules (Supelco, Inc., catalog no. 3-3020, or equivalent).
4. Isooctane—reagent grade (see Notes, *Caution*).
5. Sodium chloride—reagent grade.
6. $C_{23:0}$ methyl ester—see Notes, 5.
7. $C_{23:0}$ ethyl ester—see Notes, 5.
8. Solutions
   (a) Alcoholic sodium hydroxide (NaOH), 0.5 M-dissolve 2.0 g NaOH in methanol and make to 100 mL with methanol.
   (b) Sodium chloride (NaCl)—saturated solution, dissolve 36 g NaCl in 100 mL distilled water.

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PROCEDURE

1. Oils
   (a) Accurately weigh (±0.1 mg) approximately 25 mg of $C_{23:0}$ methyl ester internal standard (IS) into a 25 mL volumetric flask and make to volume with isooctane.
   (b) Pipet 1.0 mL aliquots of IS (see Notes, 6) into culture tubes and evaporate the solvent. Store the tubes in a freezer if they will not be used immediately.
   (c) Accurately weigh (± 0.1 mg) approximately 25 mg of marine oil (may be from fish oil capsule) into the culture tube containing the IS.
   (d) Add 1.5 mL of 0.5 M NaOH. Blanket with nitrogen, cap tightly, mix, and heat at 100°C for 5 min.
   (e) Cool, add 2 mL BF_3/methanol reagent, blanket with nitrogen, cap tightly, mix, and heat at 100°C for 30 min.
   (f) Cool to 30–40°C, add 1 mL of isooctane, blanket with nitrogen, cap, and vortex or shake vigorously for 30 sec while still warm.
   (g) Immediately add 5 mL of saturated NaCl solution, blanket with nitrogen, cap, and agitate thoroughly.
   (h) Cool to room temperature. When isooctane layer separates from the aqueous layer, transfer the isooctane layer to a clean glass tube, blanket with nitrogen, and cap.
   (i) Extract the methanol/water phase again with an additional 1 mL of isooctane.
   (j) Combine isooctane extracts (see Notes, 7) and concentrate to approximately 1 mL with a stream of dry nitrogen.
   (k) Inject (see Notes, 8) 1–2 µL under appropriate gas chromatographic conditions (see Apparatus, 1).
2. Methyl or ethyl esters
   (a) Accurately weigh (± 0.1 mg) approximately 25 mg of $C_{23:0}$ methyl or ethyl ester (appropriate to the substrate to be analyzed) IS into a 25 mL volumetric flask and make to volume with isooctane.
   (b) Pipet a 1.0 mL aliquot (see Notes, 6) of the IS into a culture tube.
   (c) Evaporate the solvent.
   (d) Accurately weigh (± 0.1 mg) no more than 15 mg of esters into the tube containing the IS, add 1 mL of isooctane, blanket with nitrogen, cap, and mix thoroughly.
   (e) Inject 1–2 µL under appropriate gas chromatographic conditions (see Apparatus, 1).

   See Figures 1, 2, and 3 for chromatograms of methyl and ethyl esters of various marine oils; see Tables 1 and 2 for retention times of marine oil methyl esters.

CALCULATIONS

1. Area percentages—Calculate the area percent for fatty acids by the formula:

$$\text{Area \% fatty acids} = \frac{100\,(A_x)}{A_t - A_{IS}}$$

Where—

A_x = area counts for fatty acid x
A_t = total area counts for the chromatogram
A_{IS} = area counts of the internal standard

2. Calculation of mg of EPA and DHA per g of test portion
   (a) For marine oils and fish oil capsules (methyl esters)—weight of EPA and DHA (see Notes, 9), expressed as mg of EPA/DHA fatty acid per g of oil:

$$\text{EPA or DHA, mg/g} = \frac{(A_x)(W_{IS})(CF_x)}{(A_{IS})(W_S)(1.04)} \times 1000$$

   (b) For ethyl esters—weight of EPA and DHA (see Notes, 9), expressed as mg of EPA/DHA fatty acid per g of sample:

$$\text{EPA or DHA, mg/g} = \frac{(A_x)(W_{IS})(CF_x)}{(A_{IS})(W_S)(1.08)} \times 1000$$

Where—

A_x = area counts for EPA (20:5n-3) or DHA (22:6n–3) A_x
A_{IS} = area counts for internal standard A_{IS}
CF_x = theoretical correction factor for EPA or DHA CF_x
W_{IS} = mass of internal standard added to the sample, in mg W_{IS}
W_S = test portion mass, in mg W_S

3. Detector correction factors—Theoretical detector correction factors relative to $C_{23:0}$ (the internal standard, IS) for $C_{20:5n-3}$ (0.99) and $C_{22:6n-3}$ (0.97) should be applied to the analytical data for optimal accuracy.

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PRECISION

1. Collaborative study statistics, based on results from 19 laboratories that participated in the study, appear in Table 3.

NOTES

Caution

Methanol (methyl alcohol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide.

Isooctane is highly flammable. An effective fume hood should be used at all times when working with isooctane.

NUMBERED NOTES

1. It has been shown that the alkali-catalyzed methanolysis step for the determination of long-chain marine oil fatty acids produces dimethyl and mixed-alcohol esters from the plasticizer dioctyl [actually di(2-ethylhexyl)] phthalate. An independent boron trifluoride-catalyzed methanolysis step produced a lower level of artifacts, but the official two-step process produced a higher conversion than either catalyst did independently. The retention times for the dimethyl, mixed-alcohol, and dioctyl phthalates are discussed (References, 1) in relation to methyl esters of common fatty acids on polyglycol wall-coated open-tubular GLC columns.
2. There is usually sufficient material in marine oils analysis to permit operation in the split mode.
3. The column end may be inserted directly in the detector jet, or there may be a connection at the base of the jet, with or without makeup gas. If the FID requires makeup gas, nitrogen is satisfactory.
4. These are suggested operating conditions for a 30 m column.
5. Authentic standards of saturated fatty acid esters, if needed for peak identification, are widely available. $C_{21:5n-3}$ should be baseline separated from $C_{23:0}$ (IS), and $C_{24:0}$ should be baseline separated from $C_{22:6n-3}$ (see Fig. 1).
6. If the peak height of the IS is ≤one-half that of the EPA or DHA peaks, repeat the analysis using 2.0 mL of IS.
7. Optional—Wash isooctane with 2 mL of water and dry over anhydrous sodium sulfate.
8. Manual injection with a microsyringe is preferred; however, an autoinjection may be used.
9. Please note that this calculation gives results for fatty acids and not for esters of fatty acids. The analyst can confirm detector correction factors with pure reference standards on individual instruments and capillary columns. The weight % calculation should always be based on theoretical FID correction factors. In case of strongly deviating experimental factors (>5% deviation from the theoretical factor), the analyst should locate the cause of the deviation, rather than applying the experimental factor.

REFERENCES

1. *J. Chromatogr. 587:*263 (1991).

OTHER REFERENCES

*INFORM 1:*987 (1990).
*J. Am. Oil Chem. Soc. 64:*499 (1987).
*Ibid. 66:*1822 (1989).
General referee report on oils and fats, *J. Assoc. Off. Anal. Chem. 73:*105 (1990).
*J. Chromatogr. Biomed. Appl. 533:*1 (1990).
*J. AOAC Int. 75:*488 (1992).
AOAC International, 16th ed., Vol. II, Method 991.39, Gaithersburg, MD, 1995, Chap. 41, p. 20.

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Table 1
Retention times and area percent of fatty acid methyl esters of steam-deodorized menhaden oil (collaborative study sample 1). See Figure 1.

Fatty acid	Retention time (min)	Relative retention time	Area percent
14:0	4.14	0.335	8.3
16:0	7.13	0.578	18.7
16:1n-7	7.66	0.621	12.1
18:0	12.34	1.00	3.1
18:1n-9	12.97	1.05	11.9
18:1n-7	13.22	1.07	
18:2n-6	14.57	1.18	1.2
18:3n-3	17.08	1.38	0.8
18:4n-3	18.38	1.40	2.9
20:0	20.32	1.65	0.2
20:1n-11	20.91	1.69	1.8
20:1n-9	21.12	1.71	
20:2n-6	23.37	1.89	0.1
20:3n-6	24.66	2.00	0.2
20:3n-3	26.66	2.16	0.2
20:4n-6	25.74	2.09	0.8
20:4n-3	28.07	2.27	1.3
20:5n-3	29.29	2.37	13.8
22:0	30.32	2.46	0.2
22:1n-11+13	31.27	2.53	1.4
22:1n-9	31.63	2.56	
22:4n-6	37.17	3.03	0.2
22:5n-6	38.83	3.14	0.2
22:5n-3	41.11	3.33	2.1
22:6n-3	42.86	3.47	7.9
24:0	42.45	3.44	0.1
24:1n-9	43.54	3.52	0.3

Table 2
Retention times and area percent of cod liver oil methyl esters (collaborative study sample 3). See Figure 3.

Fatty acid	Retention time (min)	Relative retention time	Area percent
14:0	4.17	0.335	5.7
16:0	7.17	0.576	13.3
16:1n-7	7.71	0.620	8.8
18:0	12.44	1.00	2.3
18:1n-9	13.08	1.05	18.0
18:1n-7	13.32	1.07	
18:2n-6	14.68	1.18	2.0
18:3n-3	17.20	1.38	1.0
18:4n-3	18.50	1.49	2.0
20:0	20.47	1.65	0.2
20:1n-11	21.08	1.69	10.0
20:1n-9	21.28	1.71	
20:2n-6	23.52	1.89	0.3
20:3n-6	24.81	1.99	0.1
20:3n-3	26.86	2.16	0.1
20:4n-6	25.91	2.08	0.4
20:4n-3	28.25	2.27	0.6
20:5n-3	29.43	2.37	7.8
22:0	30.50	2.45	0.3
22:1n-11+13	31.50	2.53	9.0
22:1n-9	31.83	2.56	
22:4n-6	37.70	3.03	0.2
22:5n-6	39.04	3.14	0.1
22:5n-3	41.32	3.32	1.0
22:6n-3	43.77	3.52	7.0
24:0	42.67	3.43	0.2
24:1	43.77	3.52	0.9

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Table 3
Statistical analysis of results for 6 fish oils, based on results received from 21 laboratories.

	Samples[a]	1	2	3	4	5	6
$\bar{x}$, %	14:0	8.3	0.4	5.7	7.0	5.4	8.3
	16:0	18.7	0.9	13.3	15.0	7.8	19.0
	18:0	3.1	2.1	2.3	2.9	0.8	3.1
	18:1	11.9	8.7	18.0	14.1	10.2	11.9
	18:4n-3	2.9	1.9	2.0	2.1	4.4	2.9
	20:1	1.8	14.2	10.0	3.2	1.3	1.8
	20:5n-3	13.8	26.4	7.8	17.0	27.5	13.4
	22:1	1.4	10.6	9.0	2.4	1.0	1.3
	22:5n-3	2.0	4.3	1.0	2.1	2.2	2.0
	22:6n-3	7.9	18.7	7.0	12.3	12.5	7.8
RSD_R	14:0	11.7	17.1	13.1	8.3	12.0	11.6
	16:0	7.0	12.2	7.5	6.9	5.7	10.0
	18:0	3.6	7.0	3.1	14.4	5.6	5.7
	18:1	1.9	5.7	2.8	3.0	6.7	3.6
	18:4n-3	2.4	7.6	4.2	6.3	5.5	4.5
	20:1	8.0	3.2	4.5	9.7	18.1	16.2
	20:5n-3	5.8	5.6	5.5	7.4	7.5	9.8
	22:1	17.2	4.7	8.9	10.0	13.4	16.1
	22:5n-3	14.6	8.5	9.7	12.2	8.8	16.2
	22:6n-3	16.1	7.5	9.9	11.7	7.5	15.5
$\bar{x}$, mg/g	20:5n-3	115.3	223.1	64.3	157.5	241.4	117.7
	22:6n-3	66.0	157.9	56.4	105.3	105.4	67.2
RSD_R	20:5n-3	6.0	9.2	7.7	19.8	5.4	7.9
	22:6n-3	4.2	9.0	8.3	12.6	5.2	8.6
RSD_R—Blind duplicate pair (Samples 1 and 6)		$\bar{x}$, %	RSD_r				
	14:0	8.3	5.8				
	16:0	18.7	2.9				
	18:0	3.1	1.8				
	18:1	11.9	1.6				
	18:4n-3	2.9	1.5				
	20:1	1.8	7.3				
	20:5n-3	13.8	1.9				
	22:1	1.4	7.9				
	22:5n-3	2.0	6.7				
	22:6n-3	7.9	3.7				
$\bar{x}$, mg/g		$\bar{x}$, mg/g					
	20:5n-3	116.5	5.9				
	22:6n-3	66.6	5.3				

[a] All samples were soft-gelatin encapsulated. 1. Steam-deodorized menhaden oil; 2. commercial ethyl ester preparation; 3. cod liver oil; 4. commercial fish oil; 5. commercial fish oil enriched in EPA and DHA; 6. steam-deodorized menhaden oil (blind duplicate of sample 1).

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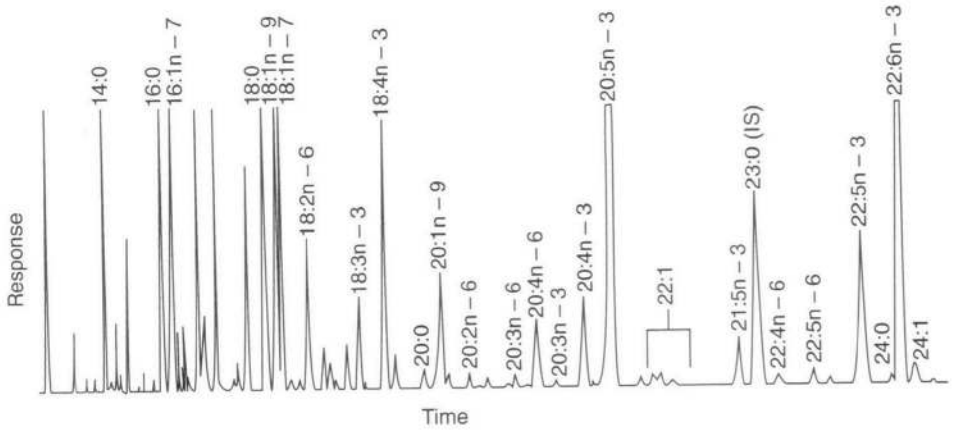

Figure 1. Fatty acid methyl esters of soft-gelatin-encapsulated, steam-deodorized menhaden oil (collaborative study sample 1) analyzed on a flexible fused silica capillary column (30 m × 0.25 mm), coated with Supelcowax-10, under the conditions noted in the text.

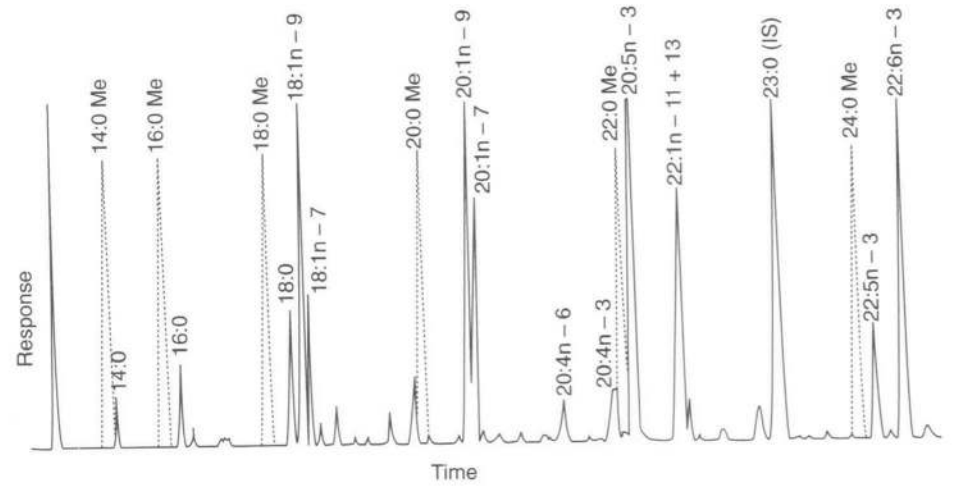

Figure 2. Fatty acid ethyl esters of a soft-gelatin-encapsulated commercial product, SuperEPA 500 (collaborative study sample 2) analyzed on a flexible fused silica capillary column (30 m × 0.25 mm), coated with Supelcowax-10, under the conditions noted in the text. The location of added saturated methyl esters is indicated for reference.

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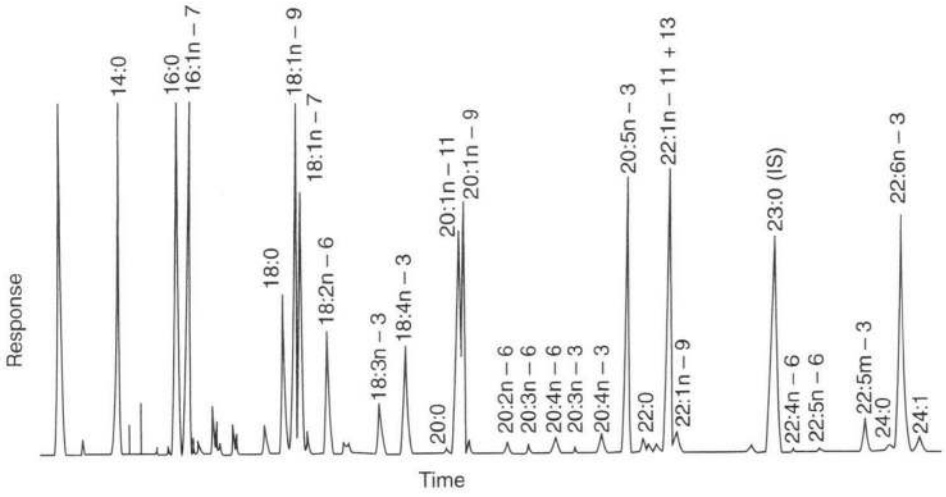

Figure 3. Chromatogram of cod liver oil (collaborative study sample 3) methyl esters analyzed on a flexible fused silica capillary column (30 m × 0.25 mm), coated with Supelcowax-10, under the conditions noted in the text.

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AOCS Recommended Practice Ce 1g-96

Revised 2013

trans Fatty Acids by Silver-Ion Exchange HPLC

DEFINITION

This Recommended Practice (see References, 1) is for isolating fatty acids (FA) as their methyl esters (FAME) using special HPLC columns packed with a silver-loaded ion exchange resin (see References, 2–4).

SCOPE

The FA, as their FAME, are separated according to the number and geometry of double bonds (DB) according to the so-called E-class distribution, wherein a *trans* DB counts for one E-class unit, or a *cis* DB for two E-class units. The E-class value of some example FA are shown in Table 1. (The effects of DB position are believed to be less pronounced on Ag-ion exchange columns than in Ag-SiO_2 systems.) Once isolated, the fractions are further analyzed for identification and quantification by a suitable GLC method (e.g., on highly polar stationary phases).

APPARATUS

1. HPLC column—CP Lipids, 25 cm × 4.6 mm i.d. (Chrompack, Middelburg, The Netherlands) or equivalent.
2. HPLC system—capable of producing ternary gradients.
3. Autosampler—
   (a) Autosampler vials.
   (b) Crimp caps for autosampler vials—PTFE-faced seal.
4. Evaporative light scattering detector—Varex ELSD Mark II or equivalent.
5. Fraction collector—equipped with solvent switch valve, operated on timed events, connected between the column and the detector.
6. HPLC organic eluent particle filters—0.5 μm pore size.
7. Syringe—disposable, 2 mL.
8. Automatic pipets—
   (a) Volume 100 μL, with disposable tips.
   (b) Volume range 0–1000 μL, with disposable tips.

REAGENTS (See Notes, *Caution*)

1. Dichloromethane (DCM).
2. Acetone—analytical grade.
3. Acetonitrile—analytical grade.
4. Heptane.
5. Nitrogen gas—free from water and organic matter by carbon filter and moisture trap.
6. HPLC mobile phases—
   (a) DCM (Reagents, 1) (mobile phase A)
   (b) Acetone (Reagents, 2) (mobile phase B)
   (c) Acetone/acetonitrile (80:20, v/v) (mobile phase C)
7. Fatty acid mixture—suitable mixture of *cis* and *trans* FA [e.g., Nucheck GLC 68B and 87].
8. Solvent for use in GLC analysis, such as hexane.

PROCEDURE

1. Sampling and preparation—
   (a) From the oils or fats to be analyzed, prepare the methyl esters from the triglycerides using the BF_3 method (as described in AOCS Official Method Ce 2-66).
   (b) Before taking test portions from test samples, mix the test samples thoroughly. Melt solid test samples to ensure proper mixing.
   (c) Dissolve the methyl esters prepared in step (a) in heptane (Reagents, 4). Evaporate this solvent using modest temperature conditions under nitrogen (Reagents, 5).
2. High-performance liquid chromatography—
   (a) Set up the liquid chromatograph with the gradient system described in Table 2.
   (b) Inject 10 μL (dissolve 1 drop in 1.5 mL DCM; concentration approximately 10 mg/mL) of the methyl esters from a test sample [Procedure, 1(a)] into the system. Compare the result with the example chromatogram (Figure 1).
   (c) If the separation obtained is not identical to the example chromatogram, small changes in gradient profile may be required. Increase in $(CH_3)_2CO/CH_3CN$ (mobile phase C) content at the mobile phase will decrease the elution time

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of unsaturated FA; decrease of acetone (mobile phase B) content at the start of the gradient will improve the separation between *trans* and *cis* monoenoic FAME.

3. Performance check—
   (a) If the HPLC system is set up properly, the separation obtained should result in a single, sharp peak for the saturated FA (see Figure 1).
   (b) The *trans* and *cis* monoenoic FA should elute baseline-separated. Within each E-class, small details may be visible, caused by the positional isomers and/or chain-length differences within each group.
   (c) Repeat the separation as obtained with a test sample several times. The start/end retention time for each of the different FA groups should remain constant.
4. Time correction—Determine (e.g., with air bubbles) the time difference between the detector signal and the fraction collector split valve.
5. Fraction collection—
   (a) Using suitable FA standards (Reagents, 7), determine the retention windows for each E-class. Small deviations in retention time may exist due to chain-length differences. In general, retention windows for a certain E-class start with the front of the peak of the standard used and continue until the next E-class. This will compensate for possible late-eluting short-chain FA, present in real samples.
   (b) If necessary, the collected fractions of the standard FA mixtures can be checked with GC analysis. In general, three fractions are collected: (1) all saturated FA and *trans* monoenoic FA (E-class <2); (2)all *cis* monoenoic and *trans*, *trans* dienoic FA (E-class = 2); (3) the remainder (E-class >2).
   (c) Specific FA may be fractionated based on standards. Fractionation in a larger number of fractions as indicated above will result in "overlap" of the different FA isomers. At higher levels of unsaturation, the E-class separation is distorted.
   (d) Check the retention windows as obtained with the FA standards using a real, well-known sample to confirm the time settings. Collected fractions may be analyzed on GC to assess the purity of each fraction.
   (e) Once the correct time windows for fraction collection are set, check the repeatability of the retention times with multiple injection/collections from several known and unknown samples (see Method Performance, 2).
   (f) If the system is stable, fractions of test portions can be collected. After the analysis/fractionation, always recheck the retention time windows with known samples or standards.
6. GLC analysis—
   (a) Transfer of the FAME to a more GLC-friendly solvent may be required. Evaporate the HPLC solvent from the collected fraction under nitrogen and at modest temperatures. Redissolve the residue in the appropriate GLC solvent, such as hexane (Reagents, 8).
   (b) For quantitative purposes, suitable internal standards (IS) are added to the samples before fractionation. Suitable IS are (1) for fraction 1 (E-class <2), the natural amount of $C_{16:0}$; (2) for fraction 2 (E-class = 2), $C_{17:1}$ cis; (3) for fraction 3 (E-class >2), to be determined.
7. Calculation and expression of result—
   (a) FA are quantified with a suitable GLC technique. The content within each fraction is related to the IS, which depends on sample type (see Procedure). Check the test portion with the final GC procedure for "empty" spots in the chromatogram. For example, the amount of fraction 1 as obtained with Ag-ion exchange HPLC is related to the total test portion by the following formula:

$$FA_x = \frac{A_{x,\text{fraction}}}{A_{\text{IS, fraction}}} \times \frac{A_{\text{IS, total}}}{A_{\text{fraction, total}}} \times \frac{A_{\text{fraction}}}{A_{\text{total}} - A_{\text{IS}}} \times 100\%$$

Where—

FA_x (%) = percentage of FA type x in total sample
$A_{x,\text{fraction}}$ = area of FA type x in fraction
$A_{\text{IS,total}}$ = area of IS in fraction
$A_{\text{fraction,total}}$ = total area of all peaks as fractionated in total test portion injection
A_{fraction} = total area of fraction
A_{total} = total sample area
A_{IS} = area of IS in total injection

   (b) Raw data is defined as a hard copy of the sample sequence list and the chromatogram, printed during or directly after finishing each analysis. On the hard copy of the chromatogram, the timed vents for each fraction collection should be visible (mark).

METHOD PERFORMANCE

1. Precision of the method has not been established.
2. Quality assurance and control—System Suitability Test (SST): Perform replicate injections (minimum 6) of a suitable test sample containing FA isomers in the three relevant fractions from the same test sample solution. Record the retention time (Rt), area, and peak width at half height with the data system. Complete Table 3.

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NOTES

Caution

Refer to most recent NIOSH or OSHA manual for TLC and TLV ratings. Dichloromethane (methylene chloride) is toxic and a carcinogen. A fume hood should be used at all times when using dichloromethane.

Acetone is highly flammable and forms explosive peroxides with oxidizing agents. It should not be used near an open flame. The use of a fume hood is recommended when using acetone. Do not mix with chloroform.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. A fume hood should be used at all times when using acetonitrile.

Hexane is a flammable solvent and a dangerous fire risk. All work should be performed in a fume hood, with no open flames. Always ensure that your hood is functioning before introducing volatile solvents. OSHA recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

REFERENCES

1. This method is a paraphrase of one submitted by Dr. Guus S.M.J.E. Duchateau of Unilever Research Laboratorium, Vlaardingen, The Netherlands.
2. *J. Chromatogr. 469:*261 (1989).
3. *J. Chromatogr. 659A:*95 (1994).
4. *J. Am. Oil Chem. Soc. 72:*571 (1995).
5. IUPAC, *Standard Methods for Analysis of Oils, Fats and Derivatives.* Blackwell Scientific Publications; IUPAC Method 2.301.
6. AOCS Method Ce 2-66.

Table 1
E-class values of some representative fatty acids.

Class	Value	Fatty acid
S	0	All saturated FA
E	1	All monoenoic *trans* FA, such as elaidic acid
O	2	All monoenoic *cis* FA, such as oleic acid
L	4	All dienoic *cis, cis* FA, such as linoleic acid
Ln	6	All trienoic *cis, cis, cis* FA, such as linolenic acid
$C_{18:2}$ *cis, trans*	3	

Table 2
Solvent composition of mobile phase.

Time	% bottle B [Reagents, 6(b)]	% bottle C [Reagents, 6(c)]
0.0	2	0
1.0	2	0
6.2	14	0
8.0	50	50
15.0	50	50
16.0	99	0
30.0	99	0

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Table 3
SST parameters and requirements for Ag-ion exchange FAME analysis. Table to be completed by user of method.

Replicate	Rt (min)	Area counts	Area %	Peak width(s)	N for FA...	Resolution between... and ...
1						
2						
3						
4						
5						
6						
Mean RSD%	TBD[a]	TBD	TBD	TBD	TBD	TBD
Requirement	<2%	<2%	<2%		>...	>...

[a]TBD = to be determined.

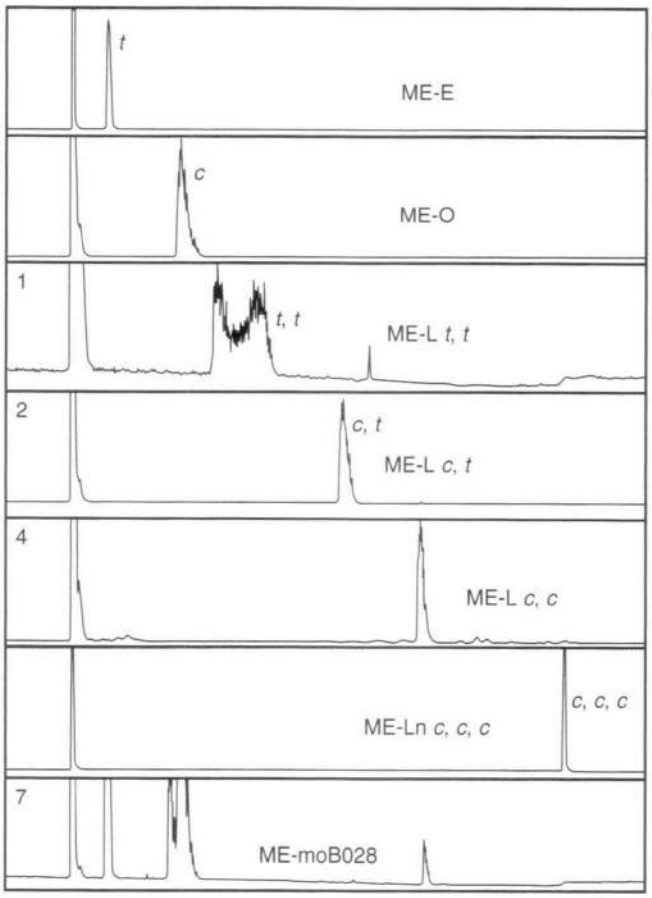

Figure 1. Chromatograms of FAME standards used for correct window setting for fractionation and one chromatogram of a real sample (moBO28) (Chrompack Ag-ion exchange column). Please note the unusual shape of the *trans, trans* standard peak.

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AOCS Official Method Ce 1h-05

Reapproved 2009

Determination of *cis-*, *trans-*, Saturated, Monounsaturated and Polyunsaturated Fatty Acids in Vegetable or Non-Ruminant Animal Oils and Fats by Capillary GLC

DEFINITION

This method provides a gas-liquid chromatography (GLC) procedure for the determination of the fatty acid composition, including the *trans* fatty acid isomers of vegetable or non-ruminant animal oils and fats. The fatty acid methyl esters (FAME) of the sample are separated on a capillary gas chromatography column having a highly polar stationary phase, according to their chain length (CL), degree of unsaturation, and geometry and position of the double bonds.

SCOPE

This method is specially designed to determine, by a single capillary GLC procedure, the levels of *trans* isomers, saturated fatty acid (SAFA), *cis-* and *trans*-monounsaturated fatty acid (MUFA), and *cis-* and *trans*-polyunsaturated fatty acid (PUFA) levels in the same sample and same analysis. For nutritional labeling purposes the total fat, saturated, *cis*-monounsaturated, *cis*-polyunsaturated, and *trans* fatty acid contents may need to be determined.

The method is applicable to crude, refined, partially hydrogenated or fully hydrogenated oils and fats derived from vegetable or non-ruminant animal sources. The method is not suitable for the analysis of dairy, ruminant, marine, long chain polyunsaturated (PUFA) fats and oils, or products supplemented with conjugated linoleic acid (CLA). There is minor co-elution of *cis-* and *trans*-fatty acid isomers, particularly in the C18:1 (oleic acid) region, using this technique.

APPARATUS

1. Gas chromatograph—suitable for use with a capillary column equipped with:
   a. Thermostatic chamber for the capillary column, capable of maintaining the desired temperature to ± 0.1°C.
   b. Temperature-controlled split mode injection unit.
   c. Flame-ionization detector (FID), amplifier and electronic recorder-integrator.
2. Capillary column—fused silica capillary 100 m, and 0.25 mm i.d., coated with SP-2560 or CP-Sil 88, 100% cyanopropyl-silicone stationary phase, to a thickness of 0.20 µm. Commercially prepared columns are available from: SP-2560 (part # 24056, Supelco Inc. Bellefonte, PA, USA) and CP-Sil 88 (part # CP7489, Varian, Walnut Creek, CA, USA; Chrompack, Middelburg, the Netherlands).
3. Microsyringe for gas chromatography—10 µL delivery, with a hardened needle.
4. Carrier gas—hydrogen or helium, 99.99% pure or better, gas chromatography quality, dried, and oxygen removed by suitable filters (Note 1).
5. Flame gases—hydrogen and air, gas chromatography quality.
6. Makeup gas—nitrogen or helium, gas chromatography quality.
7. Injection port split liner—4 mm i.d., 6.3 mm o.d. × 78.5 mm split liner with glass wool (e.g., Agilent part #5183-4647 or equivalent) (Note 2).

REAGENTS

Unless otherwise stated, use only reagents as specified in ISO 6353 (parts 2 and 3) (Reference 1) if listed there; if not, then use reagents of recognized analytical grade and water of at least grade 3 as defined in ISO 3696 (Reference 2).

1. Reference fatty acid methyl esters (FAME)—Methyl esters of pure fatty acids, in particular *cis-* and *trans* isomers of octadecenoic (oleic), *trans* isomers of octadecadienoic (linoleic) and octadecatrienoic (α- linolenic) acids. Wide ranges of *cis-* and *trans* octadecenoic methyl ester isomers are available on the market (e.g., from Alltech Associates, Inc., Deerfield, IL, Nu-Chek-Prep, Elysian, MN, Supelco Inc., Bellefonte, PA, and Sigma Chemical Co., St. Louis, MO). *trans-* Geometrical isomers of linoleic and α-linolenic acids can be prepared in the laboratory with the aid of *p-* toluenesulfonic acid (Reference 3).
2. Triacylglycerol (TAG) internal standard (IS) solution (for calculating fatty acid data as mg per 100 g oil or 100 g of food sample)—C21:0 TAG—triheneicosanoin (purity >99%), 5.0 mg/mL in chloroform. The TAG internal standard solution is

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stable indefinitely if precautions are taken to eliminate the loss of chloroform and therefore a change in the concentration of the IS. For example, store the solution in a refrigerator in well-sealed amber bottle when not in use. Pure triheneicosanoin is available on the market (e.g., from Nu-Chek-Prep). Purity of the IS should be confirmed by thin-layer chromatography, high-performance liquid chromatography, and gas chromatography analysis or by any other appropriate technique.

PROCEDURE

1. Preparation of fatty acid methyl esters (FAMEs) —
   a. From oils and fats: Use a standard procedure for example, AOCS Official Method Ce 2-66 (Reference 4) or ISO 5509 (Reference 12). Prior to methylation, add enough internal standard solution to the reaction flask, so that after the oil or fat is added, the concentration of the final solution is between 0.05 and 0.10 mg IS/mg oil or fat. Since chloroform is used in the IS solution, it should be evaporated from the flask prior to the methylation procedure.
   b. Dissolve the prepared FAME in n-hexanes or n-heptane, concentration should be approximately 15–20 mg/mL *n*-hexanes or heptane.
2. Chromatography—
   a. Operating conditions are as follows:

Injection port temperature:	250°C
Detector temperature:	250°C
Oven temperature:	180°C

      Carrier gas: hydrogen; column head pressure, 170 kPa (25 psi); flow rate, 1.0 mL/min; linear velocity; 26 cm/s; split ratio, 100:1.

      Carrier gas: helium; column head pressure, 286 kPa (41 psi); flow rate, 1.0 mL/min; linear velocity; 19 cm/s; split ratio, 100:1.
   b. Inject 1 µL, which is equivalent to 15–20 µg fatty acid.
3. Performance check: Column performance is checked using a suitable mixture of fatty acid methyl esters covering the range of fatty acids under investigation. Figure 1 shows the separation of a mixture containing C12:0; 9*c*-18:1; 11*c*-18:1, 9*c*,12*c*,15*c*-18:3; 11*c*- 20:1; and the Internal Standard C21:0 using each carrier gas and column combination. Since commercial GC designs are different, and the separation obtained is not identical to the example chromatograms, small changes in the test portion size, test portion concentration or oven temperature may be required. If so, adjust the test portion size, test portion concentration, or oven temperature until the best separation results are obtained. If column oven temperature needs to be adjusted, it should be adjusted with small increments, preferably by steps of 1°C. Note: on SP-2560, CP-Sil 88 or any other cyanopropylsilicone capillary columns, the column temperature has a profound effect on the elution pattern of 13*t*+14*t*-C18:1, 16*t*-C18:1, 14*c*-C18:1, 9*c*,12*c*,15*t*-C18:3, 11*c*-C20:1 and 9*c*,12*c*,15*c*-C18:3 (Reference 5). The best isothermal resolution of these and other fatty acids is obtained when the column is operated at 180°C.
4. Peak identification: The individual FAMEs are identified by their retention times, and by comparison with the FAME reference standards and the reference hydrogenated oil samples. See Figures 2–5 for examples of chromatograms of the reference samples. When unknown peaks are observed, attempt to identify such peaks using appropriate procedures such as GC-MS, FTIR, silver-ion chromatography and classical chemical methods (Reference 6). Peaks of unknown identity should *not* be included in the summation of peak areas when quantifying the concentrations of total fat, saturated, *cis*-monoun- saturated, *cis*-polyunsaturated, and *trans* fatty acids unless they have been confirmed to be fatty acids.

   Food labeling regulations in North America (References 7,8) define total fat as the sum of fatty acids originated from all the lipid classes present in the food matrix and express it as TAG equivalents. This requires the conversion of GC measured FAMEs into TAG equivalents. The factors for conversion of the individual FAMEs into TAG equivalents are given in Table 1. The fatty acids listed in Table 1 cover all the chain lengths (from C4 to C24) and double bonds (from 0 to 6) that are encountered in most foods and food supplements. CLA is not normally considered.

   The food labeling regulations in Canada (Reference 7) and the USA (Reference 8) require the mandatory declaration of the amounts of *cis*-monoun-saturated and *cis*-polyunsaturated fatty acids, total saturated fatty acids and *trans* fattty acids. In addition, the amounts of total n-6 and n-3 polyunsaturated fatty acids may be declared on the food label on a voluntary basis (Note 3). In contrast to total fat, fatty acids should be expressed as free fatty acids. The factors for conversion of the individual FAMEs into free fatty acids are given in Table 1.

CALCULATIONS

Calculate individual fatty acids (g) and total fat per 100 g of test sample:

1. Calculate the amount (in g) of individual fatty acids, expressed as FAMEs (W_{FAMEx}) or as TAG (W_{TAGx}) in test sample as follows (Note 5):

$$W_{FAMEx} = \frac{A_x \times W_{is} \times 1.0040 \times R_x}{A_{is}}$$

$$W_{TAGx} = W_{FAMEx} \times F_{TAGx}$$

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Where—

A_x = area counts for fatty acid x;

W_{is} = weight of C21:0 internal standard (in g) added to the test portion;

A_{is} = peak area counts of the IS;

1.0040 = conversion of IS in test (see Note 4);

R_x = theoretical flame ionization detector correction factor (TCF) for FAMEs relative to C21:0 TAG IS.

Note: TCF should be applied to the analytical data for optimum accuracy (References 10 and 11) and to minimize variation between laboratories because of differences in calculating response factors. The TCF of a number of FAME that are commonly encountered in dietary fats are listed in Table 2. They were calculated using the following formula (Note 6):

$$TCF_x = MW_x/(N_x - 1)\ (AWC)\ (1.3503)$$

Where—

TCF = theoretical flame ionization detector response factor for fatty acid x (as methyl ester) with respect to C21:0 FAME (internal standard);

MW_x = molecular weight of component x;

N_x = number of carbon atoms in the FAME of component x;

AWC = atomic weight of carbon (12.011);

1.3503 = TCF for C21:0 FAME.

2. Calculate the amount of total fat in test sample (sum of all fatty acids; expressed as TAGs) as follows (Note 7):

$$\text{Total fat (g/100 g portion test sample)} = (\Sigma W_{TAGi}/W_{TS}) \times 100$$

Where—

W_{TS} = weight of test sample, g

3. Calculate weight (in g) of each individual fatty acid (W_x) as follows:

$$W_x \text{ (g per test portion)} = W_{FAMEx} \times F_{FAx}$$

Where—

F_{FAx} = conversion factors for conversion of FAME to their corresponding fatty acids (see Table 1).

4. Calculate weight of saturated fats (sum of all saturated fatty acids) as follows:

$$\text{Saturated fat (g per 100 g test sample)} = (\Sigma \text{Saturated } W_x/W_{TS}) \times 100$$

Where—

ΣSaturated W_x = sum of all saturated fatty acids

5. Calculate weight of trans fat as follows:

$$\textit{trans} \text{ fat (g per 100 g test sample)} = (\Sigma \textit{trans}\ W_x/W_{TS}) \times 100$$

Where—

Σ*trans* W_x = sum of all *trans* fatty acids excluding *trans* isomers with a conjugated double bond

6. Calculate weight of *cis*-monounsaturated fat, fatty acids containing one double bond in the *cis* configuration, as follows:

$$\textit{cis}\text{-monounsaturated fat (g per 100 g test sample)} = (\Sigma \textit{cis}\text{-monounsaturated } W_x/W_{TS}) \times 100$$

Where—

Σ*cis*-monounsaturated W_x = sum of all *cis*-monounsaturated fatty acids.

7. Calculate weight of *cis*-polyunsaturated fat, fatty acids containing two or more double bonds in the cis configuration, as follows:

$$\textit{cis}\text{-polyunsaturated fat (g per 100 g test sample)} = (\Sigma \textit{cis}\text{-polyunsaturated } W_x/W_{TS}) \times 100$$

Where—

Σ*cis*-polyunsaturated W_x = sum of all *cis*-polyunsaturated fatty acids

METHOD PRECISION

See Tables 3–5.

QUALITY ASSURANCE AND CONTROL

1. Blank sample: The first sample in an analysis batch is always a blank (*n*-heptanes or *n*-hexane). No peaks should be detected in the blank run. Repeat this test after every ten samples.

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2. Proficiency test: For external reference, the method is tested each year by participation in the AOCS Laboratory Proficiency Program or any other suitable ring test.
3. Reference materials, with chromatograms, and samples from the collaborative study are available from the AOCS (www.aocs.org/tech).

NUMBERED NOTES

1. Nitrogen gas is not acceptable as a carrier gas for this method.
2. No other liner types (e.g., straight through, etc.) should be used with this method.
3. Polyunsaturated fatty acids can be classified into n-3 and n-6 polyunsaturated fatty acids as follows:
   a. Calculate weight of n-6 polyunsaturated fat as follows:

$$\text{n-6 Polyunsaturated fat (g per 100 g test sample)} = (\sum \text{n-6 polyunsaturated } W_x / W_{TS}) \times 100$$

   Where—

   n-6 polyunsaturated W_x = Sum of all *cis* n-6 polyunsaturated fatty acids which include:
   C18:2n-6 (9*c*,12*c*-C18:2),
   C18:3n-6 (6*c*,9*c*,12*c*-C18:3),
   C20:3n-6 (8*c*,11*c*,14*c*-C20:3),
   C20:4n-6 (5*c*,8*c*,11*c*,14*c*-C20:4),
   C22:4n-6 (7*c*,10*c*,13*c*,16*c*-C22:4) and
   C22:5n-6 (4*c*,7*c*,10*c*,13*c*,16*c*-C22:5)

   b. Calculate weight of n-3 polyunsaturated fat as follows:

$$\text{n-3 Polyunsaturated fat (g per 100 g test sample)} = (\sum \text{n-3 polyunsaturated } W_x / W_{TS}) \times 100$$

   Where—

   n-3 polyunsaturated Wx = Sum of all *cis* n-3 polyunsaturated fatty acids which include:
   C18:3n-3 (9*c*,12*c*,15*c*-C18:3),
   C18:4n-3 (6*c*,9*c*,12*c*,15*c*-C18:4),
   C20:4n-3 (8*c*,11*c*,14*c*,17*c*-C20:3),
   C20:5n-3 (5*c*,8*c*,11*c*,14*c*,17*c*-C20:4),
   C22:4n-3 (7*c*,10*c*,13*c*,16*c*,19*c*-C22:5) and
   C22:6n-3 (4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-C22:6)
4. 1.0040 is the factor for conversion of the weight of the internal standard (which is C21:0 TAG) from the TAG form to its corresponding weight of the FAME form. In calculating the factor, we have to take into consideration that each mole of TAG yields three mole of FAME. So that the general formula for the conversion factor is as follows:

$$\text{MW of C21:0 FAME} \times 3/\text{MW of C21:0 TAG} = 340.5771 \times 3/1017.6993 = 1.0040$$

5. 1.3503 is the theoretical flame ionization detector response correction factor (TCF) for 21:0 FAME. It is calculated as follows:

$$\text{TCF of C21:0 FAME} = \text{MW of C21:0}/ (N_{x-1}) \text{ (Atomic weight of carbon)} = 340.5776/(22 \times 1) \times 12.011 = 1.3503$$

   Where—

   N_x is the number of carbon atoms in C21:0 FAME, which is 22 and 12.011 is the atomic weight of carbon.
6. It is important to understand the difference (and hence the relationship) between TCF and TRF.
   a. Theoretical flame ionization response factor (TRF) is actually the proportion (with respect to the total weight, which is the molecular weight) of the total amount of carbon atoms that are responding to the FID. For fatty acids, the carbons that are responding to FID are CH, CH_2 and CH_3 units. Carbonyl carbon and ester carbonyls carbon do not respond to FID. So that in FAME, all the carbon atoms are active, except the ester carbon. Thus, there are N_{x-1} active carbon atoms in a FAME so the TRF for a fatty acid methyl ester would be:

$$\text{TRF} = (N_{x-1}) \text{ (Atomic Weight of Carbon)/MW of FAME TRF of C21:0 FAME} = 21 \times 12.011/340.5776 = 0.7406$$

   b. Since the FID response depends on the proportion of active carbon atoms, to determine the actual weight of a FAME, the experimental measured area (or the weight) needs to be corrected. This factor is termed as TCF and it is equal to 1/TRF. In other words the formula for TCF is as follows:

$$\text{TCF} = \text{MW}/ (N_{x-1}) \text{ (Atomic Weight of Carbon)}.$$

   As shown in 5, the TCF of C21:0 FAME is 1.3503.

   For example, the TRF of C18:0 FAME is 0.7243. Therefore, its TCF is 1/0.7243 = 1.3806. Thus TCF of C18:0 FAME with respect to C21:0 FAME = 1.3806/1.3503 = 1.0225.
7. When reporting results, the labeling regulations of the country where the product will be sold need to be consulted to determine how this is done.

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REFERENCES

1. ISO 6353, Reagents for Chemical Analysis, Part 2 (1983) and 3 (1987); Specifications.
2. ISO 3696, Water for Analytical Laboratory Use— Specifications and Test Methods (1987).
3. J.M. Snyder and C.R. Scholfield, *J. Am. Oil Chem. Soc. 59:*469–470 (1982).
4. *AOCS Official Method* Ce 2-66, Preparation of Methyl Esters of Long-Chain Fatty Acids.
5. Ratnayake, W.M.N., *J. AOAC Int. 87:*523–539 (2004).
6. Ratnayake, W.M.N. Analysis of *trans* Fatty Acids, in *Trans Fatty Acids in Human Nutrition.* Eds. J.L. Sebedio and W.W. Christie. The Oily Press, Dundee, pp. 115–161 (1998).
7. Regulations Amending the Food and Drug Regulations (Nutrition Labelling, Nutrient Content Claims and Health Claims). Department of Health, *Canada Gazette,* Part 11. January 1, 2003.
8. Department of Health and Human Services. Food and Drug Administration. 21 CFR Part 101 [Docket No. 94P-0036]. Food Labelling: *Trans* Fatty Acids in Nutrition Labelling, Nutrient Content Claims, and Health Claims. Washington, DC. July 2003, p. 254.
9. R.G. Ackman and J.C. Sipos, *J. Am. Oil Chem. Soc. 41:*377 (1964).
10. J.D. Craske and C.D. Bannon, *J. Am. Oil Chem. Soc. 64:*1413–1417 (1987).
11. J.D. Craske and C.D. Bannon, *J. Am. Oil Chem. Soc. 65:*1190 (1988).
12. ISO Method 5509, Animal and vegetable fats and oils—Preparation of methyl esters of fatty acids, 12-23-2004.

Table 1
Factors for conversion of FAME to fatty acids and TAG equivalents

Fatty acid	F_{FAx} [a]	F_{TAGx} [b]	Fatty acid	F_{FAX} [a]	F_{TAGX} [b]
4:0	0.8627	0.9868	18:4	0.9517	0.9954
6:0	0.8923	0.9897	20:0	0.9570	0.9959
8:0	0.9114	0.9915	20:1	0.9568	0.9959
10:0	0.9247	0.9928	20:2	0.9565	0.9958
11:0	0.9300	0.9933	20:3	0.9562	0.9958
12:0	0.9346	0.9937	20:4	0.9560	0.9958
13:0	0.9386	0.9941	20:5	0.9557	0.9958
14:0	0.9421	0.9945	21:0	0.9588	0.9961
14:1	0.9417	0.9944	22:0	0.9604	0.9962
15:0	0.9453	0.9948	22:1	0.9602	0.9962
15:1	0.9449	0.9947	22:2	0.9600	0.9962
16:0	0.9481	0.9950	22:3	0.9598	0.9961
16:1	0.9477	0.9950	22:4	0.9595	0.9961
17:0	0.9507	0.9953	22:5	0.9593	0.9961
17:1	0.9503	0.9952	22:6	0.9590	0.9961
18:0	0.9530	0.9955	23:0	0.9620	0.9964
18:1	0.9527	0.9955	24:0	0.9633	0.9965
18:2	0.9524	0.9954	24:1	0.9632	0.9965
18:3	0.9520	0.9954			

[a] F_{FAx} is the conversion factor for conversion of FAMEs to corresponding fatty acids
[b] F_{TAGx} is the conversion factor for conversion of FAMEs to corresponding TAG equivalents

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Table 2
Theoretical flame ionization detector correction factor (TCF) for fatty acid methyl esters (FAME) [a]

FAME	TCF	FAME	TCF	FAME	TCF	FAME	TCF
4:0	1.5742	14:1	1.0587	18:2	1.0087	22:1	0.9881
5:0	1.4324	15:0	1.0540	18:3	1.0017	22:2	0.9825
6:0	1.3378	15:1	1.0457	18:4	0.9949	22:3	0.9769
7:0	1.2702	16:0	1.0422	19:0	1.0142	22:4	0.9713
8:0	1.2195	16:1	1.0345	20:0	1.0067	22:5	0.9655
9:0	1.1802	16:2	1.0267	20:1	1.0005	22:6	0.9599
10:0	1.1486	16:3	1.0189	20:2	0.9943	23:0	0.9882
11:0	1.1228	16:4	1.0111	20:3	0.9880	24:0	0.9830
12:0	1.1013	17:0	1.0318	20:4	0.9819	24:1	0.9779
12:1	1.0910	17:1	1.0244	20:5	0.9665		
13:0	1.0831	18:0	1.0225	21:0	1.0000		
14:0	1.0675	18:1	1.0155	22:0	0.9939		

[a]Atomic weights used: carbon 12.011; hydrogen, 1.0079; oxygen, 15.994. Factors are relative to 21:0, which has a factor of 1.0000 by definition. Only one factor is given for all positional and geometric isomers and for branched-chain FAME, as the factors are dependent only on the content of carbon to which hydrogen is bonded. Calculations were adopted from Reference 12.

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Table 3
AOCS Ce 1h-05 Collaborative Study Data

Sample No.	Total Fat	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
hydrogenated lard	Total Fat	97.39	0.541	0.560	1.516	3.256	3.340	9.116	1.660	6.013
margarine oil	Total Fat	96.74	1.241	1.290	3.474	2.984	3.090	8.354	1.540	2.405
canola oil	Total Fat	97.20	1.976	2.030	5.532	2.472	2.540	6.921	1.270	1.251
lard	Total Fat	97.11	1.169	1.200	3.274	3.436	3.540	9.620	1.760	2.938
sunflower oil	Total Fat	98.61	1.206	1.220	3.376	4.573	6.640	12.804	2.310	3.793
coconut oil	Total Fat	96.46	1.619	1.680	4.533	6.456	6.690	18.078	3.330	3.988
canola oil	Total Fat	96.20	1.855	1.930	5.194	3.069	3.190	8.594	1.590	1.655
vegetable shortening	Total Fat	95.46	2.718	2.850	7.610	5.286	5.540	14.801	2.750	1.945
cocoa butter	Total Fat	98.61	1.383	1.400	3.871	4.076	4.130	11.413	2.060	2.948
coconut oil	Total Fat	96.73	2.433	2.520	6.813	3.216	3.330	9.005	1.650	1.322
	MONO	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
hydrogenated lard	MONO	38.75	0.359	0.930	1.004	1.416	3.650	3.964	1.580	3.948
margarine oil	MONO	21.29	0.312	1.470	0.874	0.553	2.600	1.547	1.030	1.770
canola oil	MONO	39.43	0.913	2.320	2.557	1.188	3.010	3.327	1.310	1.301
lard	MONO	40.44	0.549	1.360	1.536	0.760	1.880	2.128	0.820	1.385
sunflower oil	MONO	81.00	1.100	1.360	3.080	4.042	4.990	11.319	2.420	3.675
coconut oil	MONO	5.32	0.060	1.130	0.168	0.138	2.590	0.385	0.830	2.292
canola oil	MONO	39.23	0.817	2.080	2.286	1.379	3.510	3.861	1.530	1.689
vegetable shortening	MONO	22.57	0.494	2.190	1.384	1.068	4.730	2.990	1.890	2.160
cocoa butter	MONO	32.28	0.930	2.880	2.603	2.027	6.280	5.676	2.650	2.181
coconut oil	MONO	5.25	0.090	1.710	0.251	0.136	2.590	0.381	0.830	1.518
	PUFA	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
hydrogenated lard	PUFA	12.85	0.172	1.340	0.482	0.499	3.890	1.398	1.430	2.900
margarine oil	PUFA	41.70	1.317	3.160	3.689	1.716	4.110	4.805	1.800	1.303
canola oil	PUFA	10.70	0.240	2.240	0.672	0.338	3.160	0.945	1.130	1.406
lard	PUFA	13.29	0.302	2.270	0.846	0.602	4.530	1.687	1.670	1.994
sunflower oil	PUFA	5.41	0.075	1.380	0.209	0.142	2.630	0.398	0.850	1.904
coconut oil	PUFA	1.30	0.012	0.930	0.034	0.027	2.050	0.074	0.530	2.176
canola oil	PUFA	10.70	0.188	1.760	0.526	0.323	3.020	0.904	1.080	1.719
vegetable shortening	PUFA	0.49	0.067	13.720	0.187	0.112	23.110	0.315	5.180	1.684
cocoa butter	PUFA	3.12	0.112	3.580	0.313	0.115	3.700	0.323	1.100	1.032
coconut oil	PUFA	1.29	0.055	4.300	0.155	0.056	4.350	0.157	1.130	1.013
	SAFA	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
hydrogenated lard	SAFA	40.27	0.165	0.410	0.463	1.684	4.180	4.714	1.820	10.181
margarine oil	SAFA	17.75	0.191	1.080	0.534	0.783	4.410	2.191	1.700	4.103
canola oil	SAFA	16.28	0.373	2.290	1.045	0.546	3.350	1.528	1.280	1.462
lard	SAFA	37.45	1.250	3.340	3.500	1.668	4.450	4.669	1.920	1.334
sunflower oil	SAFA	7.51	0.128	1.700	0.358	0.246	3.280	0.690	1.110	1.927
coconut oil	SAFA	84.19	1.469	1.740	4.112	6.058	7.200	16.963	3.510	4.125
canola oil	SAFA	16.06	0.250	1.560	0.700	0.826	5.140	2.312	1.950	3.303
vegetable shortening	SAFA	23.30	0.141	0.610	0.395	1.170	5.020	3.275	2.020	8.291
cocoa butter	SAFA	58.71	0.449	0.760	1.257	2.197	3.740	6.151	1.730	4.893
coconut oil	SAFA	84.42	2.321	2.750	6.500	3.164	3.750	8.859	1.830	1.363
	trans	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
hydrogenated lard	*trans*	1.00	0.100	10.060	0.281	0.216	21.640	0.605	5.410	2.153
margarine oil	*trans*	11.62	0.160	1.380	0.448	0.253	2.180	0.708	0.790	1.580
canola oil	*trans*	26.55	0.637	2.400	1.783	0.650	2.450	1.821	1.000	1.021
lard	*trans*	0.90	0.067	7.440	0.187	0.195	21.700	0.546	5.340	2.920
sunflower oil	*trans*	0.17	0.026	15.940	0.074	0.099	60.340	0.278	11.500	3.757
coconut oil	*trans*	0.10	0.036	35.350	0.101	0.037	35.880	0.103	6.370	1.020
canola oil	*trans*	26.27	0.510	1.940	1.428	0.780	2.970	2.185	1.210	1.530
vegetable shortening	*trans*	45.01	0.656	1.460	1.838	2.049	4.550	5.738	2.020	3.122
cocoa butter	*trans*	0.06	0.032	53.710	0.090	0.042	69.660	0.117	11.400	1.300
coconut oil	*trans*	0.11	0.013	12.280	0.037	0.016	14.800	0.045	2.650	1.216

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Table 4
Compilation of precision data from a collaborative study carried out among 13 laboratories. Data are calculated as triacylglycerols (W_{TAGx}) and organized according to the mean value.

Calculation method	FA	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
TAGx	Total Fat	97.05	1.614	1.668	4.519	3.882	4.203	10.871	1.992	2.826
TAGx	C 18:1*c*	84.32	1.155	1.370	3.233	4.177	4.950	11.695	2.410	3.617
TAGx										
TAGx	C 18:1*t*	45.39	0.837	1.840	2.345	2.041	4.500	5.714	2.000	2.437
TAGx	C 12:0	45.50	1.115	2.440	3.122	4.444	9.750	12.441	4.335	4.424
TAGx	C 18:2*c*	38.92	0.788	2.020	2.205	1.370	3.520	3.837	1.530	1.740
TAGx	C 18:1*c*	38.30	0.752	1.978	2.106	1.367	3.656	3.827	1.574	1.959
TAGx	C 18:0	34.20	0.704	2.060	1.972	1.563	4.570	4.375	1.940	2.219
TAGx	C 16:0	24.58	0.562	2.280	1.572	1.322	5.353	3.702	2.173	3.909
TAGx	C 18:1*c*	22.66	0.411	1.805	1.149	0.768	3.355	2.150	1.345	1.821
TAGx	C 18:1*t*	22.35	0.425	1.900	1.189	0.605	2.710	1.694	1.080	1.520
TAGx	C 14:0	17.76	0.304	1.715	0.851	0.907	5.110	2.540	1.970	3.205
TAGx	C 18:0	14.15	0.235	1.730	0.658	0.575	4.117	1.611	1.530	3.664
TAGx	C 18:2*c*	11.74	0.186	1.610	0.520	0.361	3.133	1.010	1.130	1.993
TAGx	C 18:1*t*	10.56	0.167	1.580	0.468	0.175	1.660	0.490	0.590	1.047
TAGx	C 16:0	9.95	0.202	2.043	0.565	0.447	4.487	1.252	1.585	2.446
TAGx	C 08:0	7.50	0.436	5.800	1.219	0.630	8.395	1.763	2.840	1.733
TAGx	C 18:0	6.05	0.142	2.420	0.397	0.282	4.513	0.791	1.487	2.331
TAGx	C 10:0	5.72	0.290	5.065	0.811	0.507	8.860	1.420	2.880	1.699
TAGx	C 18:1*c*	5.49	0.087	1.580	0.242	0.135	2.455	0.377	0.795	1.694
TAGx	C 18:2*t*	5.01	0.192	3.825	0.537	0.352	7.030	0.985	2.240	1.987
TAGx	C 18:3*c*	4.89	0.092	1.880	0.257	0.154	3.140	0.430	1.000	1.673
TAGx	C 18:2*c*	4.35	0.092	2.430	0.258	0.167	4.435	0.465	1.345	1.789
TAGx	C 18:0	3.62	0.061	1.690	0.170	0.088	2.437	0.245	0.737	1.476
TAGx	C 16:0	3.25	0.059	1.800	0.164	0.135	4.150	0.378	1.240	2.305
TAGx	C 16:1*c*	2.19	0.113	5.175	0.318	0.190	8.665	0.531	2.435	1.680
TAGx	C 18:2*t*	1.40	0.211	15.020	0.590	0.457	32.580	1.279	8.570	2.168
TAGx	C 14:0	1.40	0.017	1.250	0.048	0.072	5.140	0.202	1.355	5.701
TAGx	C 18:2*c*	1.35	0.036	2.645	0.100	0.042	3.130	0.118	0.815	1.490
TAGx	C 20:0	1.09	0.023	2.110	0.065	0.032	2.880	0.088	0.730	1.354
TAGx	C 22:0	0.87	0.017	1.930	0.047	0.023	2.630	0.064	0.640	1.362
TAGx	C 18:2*t*	0.84	0.132	15.790	0.371	0.169	20.140	0.473	4.900	1.275
TAGx	C 20:1	0.69	0.015	2.235	0.042	0.022	3.155	0.061	0.745	1.534
TAGx	C 18:3*t*	0.69	0.048	6.900	0.133	0.225	32.600	0.630	7.710	4.737
TAGx	C 18:1*t*	0.67	0.077	11.645	0.216	0.088	13.210	0.246	3.105	1.165
TAGx	C 18:3*c*	0.62	0.031	4.990	0.088	0.038	5.990	0.105	1.400	1.175
TAGx	C 06:0	0.53	0.092	17.245	0.256	0.168	31.685	0.470	7.195	1.849
TAGx	C 18:2*c*	0.49	0.073	15.030	0.206	0.110	22.440	0.307	5.040	1.490
TAGx	C 17:0	0.38	0.013	3.230	0.035	0.030	7.925	0.084	1.715	2.419
TAGx	C 22:0	0.34	0.012	3.430	0.033	0.019	5.495	0.052	1.165	1.595
TAGx	C 20:0	0.31	0.007	2.194	0.019	0.018	5.600	0.049	1.177	2.699
TAGx	C 16:1*c*	0.28	0.012	4.260	0.034	0.025	8.990	0.071	1.860	2.088
TAGx	C 20:2	0.27	0.088	30.670	0.247	0.268	101.190	0.750	20.705	61.953
TAGx	C 17:1*c*	0.26	0.014	5.285	0.038	0.021	7.965	0.058	1.625	1.494
TAGx	C 24:0	0.25	0.094	37.450	0.263	0.124	49.400	0.346	10.030	1.316
TAGx	C 17:0	0.23	0.005	2.110	0.013	0.010	4.490	0.028	0.900	2.154
TAGx	C 22:0	0.20	0.008	4.070	0.023	0.012	6.070	0.034	1.190	1.478
TAGx	C 18:3*c*	0.20	0.013	6.443	0.035	0.027	13.533	0.075	2.650	2.474
TAGx	C 20:1	0.17	0.009	5.213	0.025	0.014	8.223	0.039	1.565	1.540
TAGx	C 16:1*t*	0.16	0.011	7.085	0.032	0.169	107.960	0.471	20.400	16.583
TAGx	C 18:3*t*	0.15	0.021	13.765	0.057	0.054	36.895	0.150	6.895	2.648

(Continued)

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Table 4
(Continued)

Calculation method	FA	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
TAGx	C 18:2*t*	0.15	0.038	26.135	0.106	0.061	43.825	0.170	8.125	1.652
TAGx	C 17:0	0.11	0.005	3.998	0.012	0.008	6.840	0.021	1.223	1.834
TAGx	C 20:0	0.10	0.005	5.135	0.015	0.011	10.660	0.030	1.880	2.054
TAGx	C 18:1*t*	0.087	0.024	30.943	0.067	0.038	49.433	0.106	8.345	1.622
TAGx	C 16:1*c*	0.087	0.006	6.885	0.017	0.010	11.363	0.028	1.965	1.808
TAGx	C 12:0	0.084	0.005	5.265	0.013	0.007	8.245	0.020	1.420	2.400
TAGx	C 10:0	0.085	0.004	4.470	0.011	0.010	11.750	0.028	2.025	2.750
TAGx	C 14:0	0.074	0.004	5.178	0.011	0.006	8.325	0.017	1.397	1.722
TAGx	C 24:0	0.072	0.037	51.722	0.105	0.047	65.872	0.133	11.084	1.280
TAGx	C 20:4	0.072	0.003	3.750	0.008	0.108	150.140	0.301	25.250	37.625
TAGx	C 15:0	0.070	0.004	4.690	0.009	0.010	13.635	0.027	2.285	3.013
TAGx	C 20:1	0.054	0.008	13.415	0.021	0.008	14.320	0.022	2.310	1.042
TAGx	C 17:1*c*	0.054	0.004	6.340	0.010	0.010	17.805	0.027	2.870	3.500
TAGx	C 18:2*t*	0.049	0.006	12.980	0.018	0.038	77.130	0.105	12.240	5.833
TAGx	C 18:3*c*	0.046	0.006	19.935	0.017	0.012	47.900	0.034	6.770	1.971
TAGx	C 18:3*t*	0.040	0.015	41.023	0.041	0.043	108.513	0.119	16.600	3.142
TAGx	C 17:0	0.036	0.005	14.940	0.015	0.007	18.090	0.018	2.750	1.200
TAGx	C 22:1	0.036	0.001	3.750	0.004	0.078	213.160	0.217	32.360	54.250
TAGx	C 12:0	0.035	0.002	6.190	0.006	0.007	18.750	0.018	2.830	3.000
TAGx	C 20:3	0.032	0.002	4.760	0.004	0.062	193.500	0.173	28.800	43.250
TAGx	C 16:1*t*	0.031	0.016	53.370	0.046	0.033	106.040	0.091	15.700	1.978
TAGx	C 17:1*c*	0.022	0.002	12.370	0.007	0.017	77.925	0.046	10.763	8.092
TAGx	C 16:1*c*	0.022	0.003	15.890	0.010	0.016	73.990	0.046	10.410	4.600
TAGx	C 20:1	0.022	0.008	33.570	0.021	0.019	83.590	0.052	11.755	4.048
TAGx	C 22:0	0.020	0.017	71.445	0.047	0.028	136.535	0.078	18.950	5.313
TAGx	C 15:0	0.018	0.002	9.448	0.004	0.009	60.680	0.025	8.004	6.440
TAGx	C 13:0	0.016	0.002	9.875	0.004	0.019	115.675	0.051	15.480	16.833
TAGx	C 24:0	0.010	0.012	122.420	0.033	0.017	173.750	0.047	21.600	1.424

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Table 5
Compilation of precision data from a collaborative study carried out among 13 laboratories. Data are calculated as fatty acid (WFAMEx; g/100g) and organized according to the mean value.

Calculation method	FA	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
FAMEx	SAFA	84.31	1.895	2.245	5.306	4.611	5.475	12.911	2.670	2.744
FAMEx	MONO	81.00	1.100	1.360	3.080	4.042	4.990	11.319	2.420	3.675
FAMEx	C 18:1*c*	80.66	1.104	1.370	3.090	4.032	5.000	11.289	2.420	3.653
FAMEx	SAFA	58.71	0.449	0.760	1.257	2.197	3.740	6.151	1.730	4.893
FAMEx	*trans*	45.01	0.656	1.460	1.838	2.049	4.550	5.738	2.020	3.122
FAMEx	C 18:1*t*	43.45	0.774	1.780	2.168	1.995	4.590	5.587	2.030	2.577
FAMEx	C 12:0	42.77	1.047	2.435	2.932	4.190	9.780	11.732	4.305	4.447
FAMEx	PUFA	41.70	1.317	3.160	3.689	1.716	4.110	4.805	1.800	1.303
FAMEx	SAFA	38.86	0.708	1.875	1.982	1.676	4.315	4.692	1.870	5.758
FAMEx	MONO	38.03	0.714	1.914	1.997	1.354	3.666	3.791	1.578	2.101
FAMEx	C 18:2*c*	37.22	0.753	2.020	2.109	1.329	3.570	3.720	1.540	1.764
FAMEx	C 18:1*c*	36.64	0.712	1.960	1.995	1.331	3.718	3.727	1.590	2.006
FAMEx	C 18:0	32.73	0.674	2.060	1.886	1.511	4.620	4.231	1.950	2.243
FAMEx	*trans*	26.41	0.574	2.170	1.606	0.715	2.710	2.003	1.105	1.276
FAMEx	C 16:0	23.42	0.535	2.280	1.497	1.261	5.360	3.530	2.157	3.921
FAMEx	MONO	21.93	0.403	1.830	1.129	0.811	3.665	2.269	1.460	1.965
FAMEx	C 18:1*c*	21.74	0.399	1.820	1.116	0.778	3.540	2.178	1.410	1.892
FAMEx	C 18:1*t*	21.37	0.417	1.950	1.166	0.590	2.760	1.650	1.095	1.522
FAMEx	SAFA	18.35	0.239	1.385	0.669	0.831	4.480	2.327	1.738	4.290
FAMEx	C 14:0	16.81	0.288	1.715	0.807	0.862	5.125	2.414	1.960	3.215
FAMEx	C 18:0	13.55	0.230	1.767	0.644	0.559	4.187	1.565	1.543	3.662
FAMEx	PUFA	11.88	0.226	1.903	0.632	0.441	3.650	1.234	1.328	2.005
FAMEx	*trans*	11.62	0.160	1.380	0.448	0.253	2.180	0.708	0.790	1.580
FAMEx	C 18:2*c*	11.22	0.178	1.610	0.498	0.352	3.185	0.984	1.143	2.036
FAMEx	C 18:1*t*	10.11	0.191	1.890	0.535	0.191	1.890	0.535	0.670	1.000
FAMEx	C 16:0	9.48	0.192	2.042	0.537	0.426	4.488	1.193	1.573	2.453
FAMEx	SAFA	7.51	0.128	1.700	0.358	0.246	3.280	0.690	1.110	1.927
FAMEx	C 08:0	6.89	0.401	5.805	1.122	0.575	8.340	1.609	2.790	1.711
FAMEx	C 18:0	5.79	0.136	2.420	0.380	0.271	4.527	0.758	1.477	2.339
FAMEx	C 10:0	5.32	0.270	5.065	0.755	0.472	8.855	1.321	2.850	1.699
FAMEx	MONO	5.28	0.075	1.420	0.210	0.137	2.590	0.383	0.830	1.905
FAMEx	C 18:1*c*	5.25	0.082	1.560	0.229	0.133	2.525	0.372	0.815	1.788
FAMEx	C 18:2*t*	4.79	0.184	3.825	0.514	0.338	7.050	0.945	2.230	1.992
FAMEx	C 18:3*c*	4.68	0.088	1.880	0.246	0.147	3.150	0.412	0.990	1.675
FAMEx	PUFA	4.27	0.094	2.480	0.261	0.129	3.165	0.361	0.975	1.468
FAMEx	C 18:2*c*	4.16	0.088	2.435	0.247	0.161	4.480	0.451	1.350	1.816
FAMEx	C 18:0	3.13	0.058	1.683	0.163	0.085	2.477	0.239	0.747	1.508
FAMEx	C 16:0	3.10	0.056	1.800	0.156	0.128	4.120	0.358	1.220	2.295
FAMEx	C 16:1*c*	2.08	0.108	5.175	0.303	0.179	8.600	0.502	2.400	1.667
FAMEx	C 18:2*t*	1.34	0.201	15.020	0.564	0.436	32.540	1.221	8.500	2.165
FAMEx	C 14:0	1.33	0.016	1.245	0.045	0.068	5.105	0.190	1.335	5.816
FAMEx	C 18:2*c*	1.29	0.034	2.615	0.095	0.042	3.200	0.116	0.830	1.595
FAMEx	PUFA	1.29	0.034	2.615	0.095	0.042	3.200	0.116	0.830	1.595
FAMEx	C 20:0	1.05	0.022	2.100	0.062	0.030	2.900	0.085	0.730	1.371
FAMEx	*trans*	0.95	0.084	8.750	0.234	0.206	21.670	0.576	5.375	2.536
FAMEx	C 22:0	0.84	0.016	1.870	0.044	0.018	2.160	0.051	0.530	1.159
FAMEx	C 18:2*t*	0.79	0.141	17.840	0.394	0.190	24.140	0.533	5.820	1.353
FAMEx	C 20:1	0.66	0.014	2.210	0.041	0.022	3.200	0.059	0.750	1.546
FAMEx	C 18:3*t*	0.64	0.108	16.870	0.302	0.245	38.350	0.686	8.960	2.272
FAMEx	C 18:1*t*	0.64	0.074	11.635	0.206	0.084	13.170	0.235	3.075	1.161
FAMEx	C 18:3*c*	0.58	0.023	3.860	0.064	0.024	4.075	0.067	0.940	1.048

(Continued)

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Table 5
(Continued)

Calculation method	FA	MEAN	S_r	RSD_r	r	S_R	RSD_R	R	horrat	R/r
FAMEx	PUFA	0.49	0.067	13.720	0.187	0.112	23.110	0.315	5.180	1.684
FAMEx	C 06:0	0.48	0.082	17.215	0.230	0.151	31.565	0.422	7.060	1.853
FAMEx	C 18:2*c*	0.47	0.070	15.020	0.197	0.105	22.440	0.294	5.000	1.492
FAMEx	C 17:0	0.37	0.012	3.220	0.033	0.029	7.900	0.080	1.695	2.431
FAMEx	C 22:0	0.33	0.011	3.343	0.030	0.017	5.165	0.047	1.088	1.532
FAMEx	C 20:0	0.30	0.007	2.283	0.019	0.016	5.271	0.044	1.099	2.405
FAMEx	C 16:1c	0.27	0.012	4.270	0.032	0.024	8.990	0.038	1.850	1.188
FAMEx	C 20:2	0.26	0.085	30.655	0.237	0.258	101.190	0.721	20.580	71.286
FAMEx	C 17:1*c*	0.25	0.013	5.315	0.037	0.020	7.960	0.055	1.610	1.487
FAMEx	C 24:0	0.24	0.091	37.380	0.254	0.120	49.460	0.336	9.990	1.323
FAMEx	C 17:0	0.22	0.005	2.120	0.013	0.010	4.470	0.027	0.890	2.077
FAMEx	C 22:0	0.20	0.008	4.120	0.023	0.012	6.230	0.034	1.220	1.478
FAMEx	C 18:3*c*	0.19	0.012	6.367	0.033	0.026	13.577	0.072	2.640	2.578
FAMEx	C 20:1	0.17	0.009	5.235	0.024	0.013	8.123	0.037	1.538	1.516
FAMEx	C 16:1*t*	0.15	0.011	7.025	0.030	0.161	108.380	0.450	20.325	17.035
FAMEx	C 18:3*t*	0.14	0.021	14.440	0.057	0.052	37.335	0.146	6.930	2.538
FAMEx	C 18:2*t*	0.14	0.037	26.365	0.102	0.058	44.115	0.163	8.120	1.652
FAMEx	*trans*	0.11	0.027	29.320	0.076	0.049	45.170	0.136	7.980	1.823
FAMEx	C 17:0	0.10	0.004	4.025	0.012	0.007	6.808	0.020	1.210	1.798
FAMEx	C 20:0	0.095	0.004	3.690	0.010	0.011	11.400	0.031	1.995	3.126
FAMEx	C 18:1*t*	0.084	0.023	31.125	0.065	0.036	49.338	0.102	8.278	1.596
FAMEx	C 16:1*c*	0.083	0.006	6.903	0.016	0.010	11.383	0.026	1.955	1.792
FAMEx	C 12:0	0.080	0.006	7.310	0.016	0.008	8.880	0.020	1.515	1.278
FAMEx	C 10:0	0.080	0.004	4.555	0.010	0.010	11.880	0.027	2.025	2.896
FAMEx	C 14:0	0.070	0.003	4.653	0.009	0.007	9.407	0.019	1.570	2.507
FAMEx	C 24:0	0.070	0.036	50.818	0.100	0.046	65.844	0.129	11.026	1.303
FAMEx	C 20:4	0.069	0.003	3.810	0.007	0.103	150.140	0.289	25.090	41.286
FAMEx	C 15:0	0.066	0.004	5.145	0.010	0.010	13.700	0.026	2.275	2.683
FAMEx	C 20:1	0.052	0.007	13.240	0.020	0.008	14.310	0.021	2.295	1.043
FAMEx	C 17:1*c*	0.052	0.004	6.340	0.009	0.010	17.685	0.026	2.830	3.438
FAMEx	C 18:3*c*	0.044	0.006	19.795	0.016	0.011	47.570	0.032	6.675	1.924
FAMEx	C 18:3*t*	0.038	0.014	41.903	0.040	0.041	109.310	0.114	16.607	3.086
FAMEx	C 17:0	0.035	0.005	14.740	0.014	0.006	17.860	0.017	2.700	1.214
FAMEx	C 12:0	0.033	0.002	5.650	0.005	0.006	18.440	0.017	2.760	3.400
FAMEx	C 20:3	0.031	0.002	4.960	0.004	0.059	193.590	0.166	28.630	41.500
FAMEx	C 16:1*t*	0.029	0.014	49.890	0.041	0.031	106.320	0.086	15.600	2.098
FAMEx	C 17:1*c*	0.021	0.003	14.140	0.007	0.016	78.388	0.044	10.735	7.861
FAMEx	C 16:1*c*	0.021	0.003	15.550	0.009	0.015	73.950	0.043	10.330	4.778
FAMEx	C 20:1	0.021	0.008	34.105	0.020	0.018	83.665	0.050	11.695	3.938
FAMEx	C 22:0	0.019	0.016	71.620	0.046	0.027	136.530	0.075	18.835	5.065
FAMEx	C 15:0	0.017	0.002	9.170	0.004	0.008	60.802	0.023	7.958	7.223
FAMEx	C 13:0	0.015	0.002	9.230	0.004	0.018	115.560	0.048	15.345	15.833
FAMEx	C 14:1	0.003	0.006	197.130	0.018	0.007	213.700	0.019	22.510	1.056

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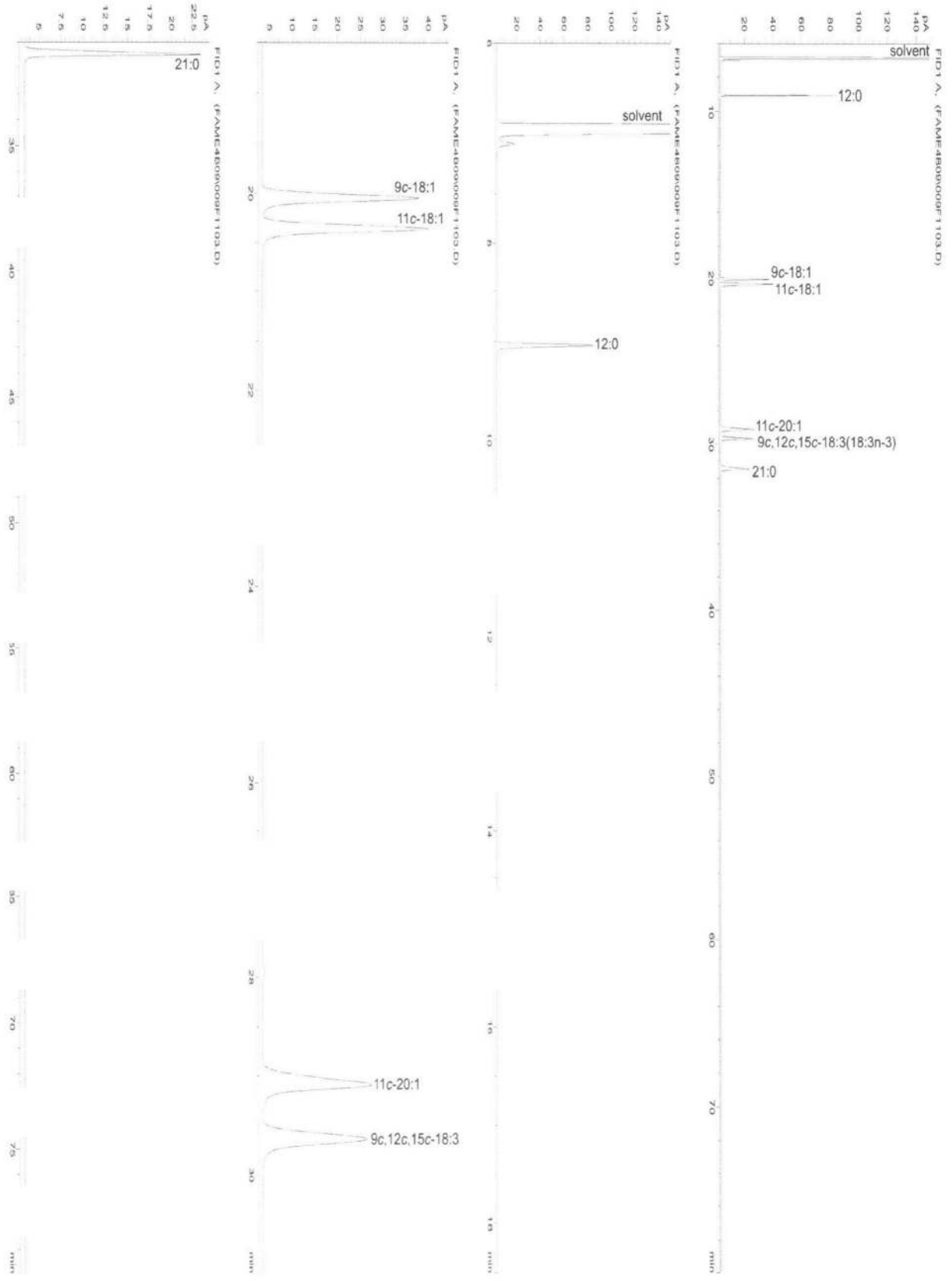

Figure 1. System Suitabillity Mix (CP-Sil 88; Iso @ 180°C @ 1.0 mL/min (26 cm/s); H_2; 1:100)

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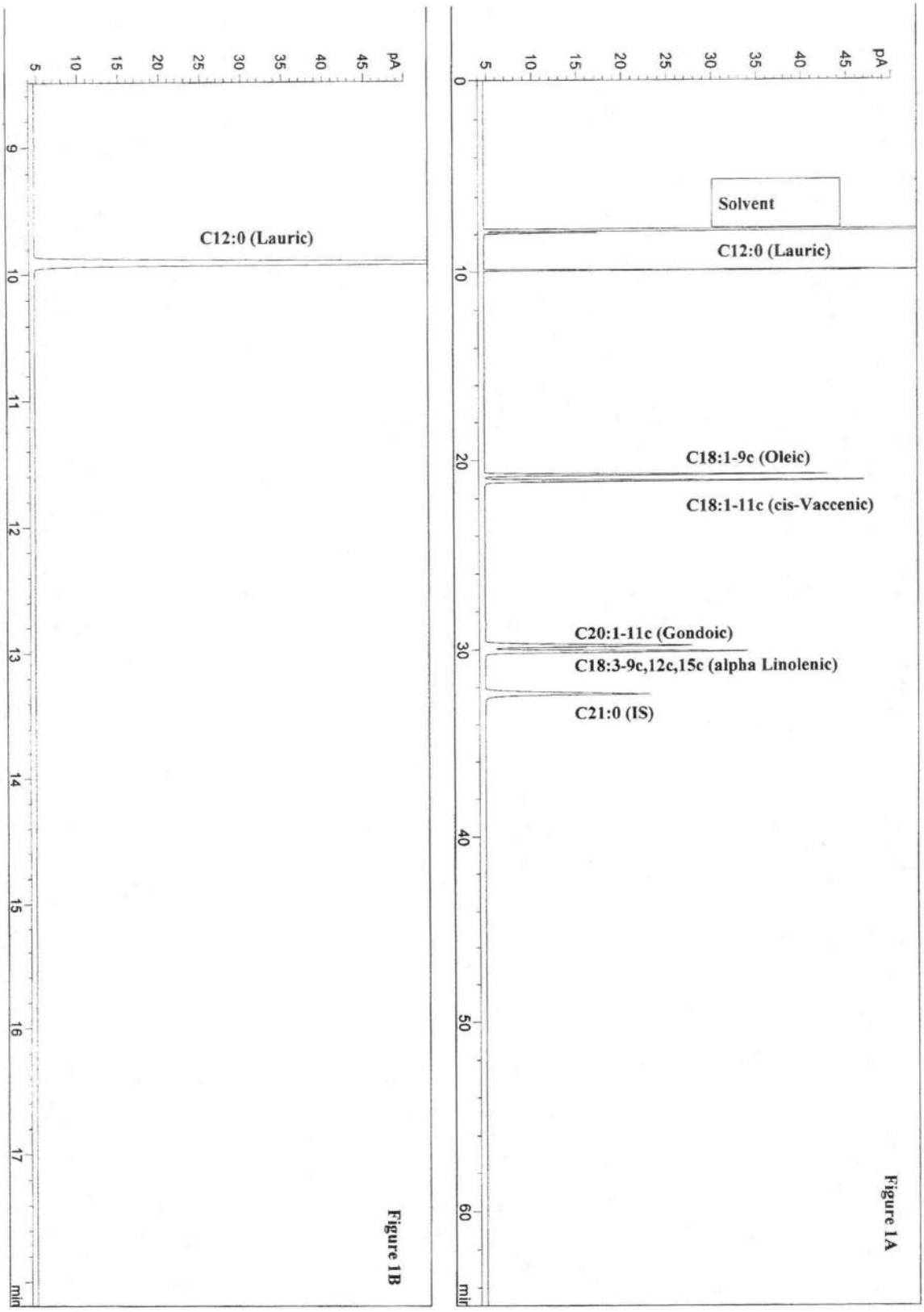

Figure 1. System Suitability Mix, AOCS Method Ce 1h-05, (SP-2560; Isothermal @ 180°C @ 1.0 mL/min (26 cm/s); H_2; Split ratio-1:100)

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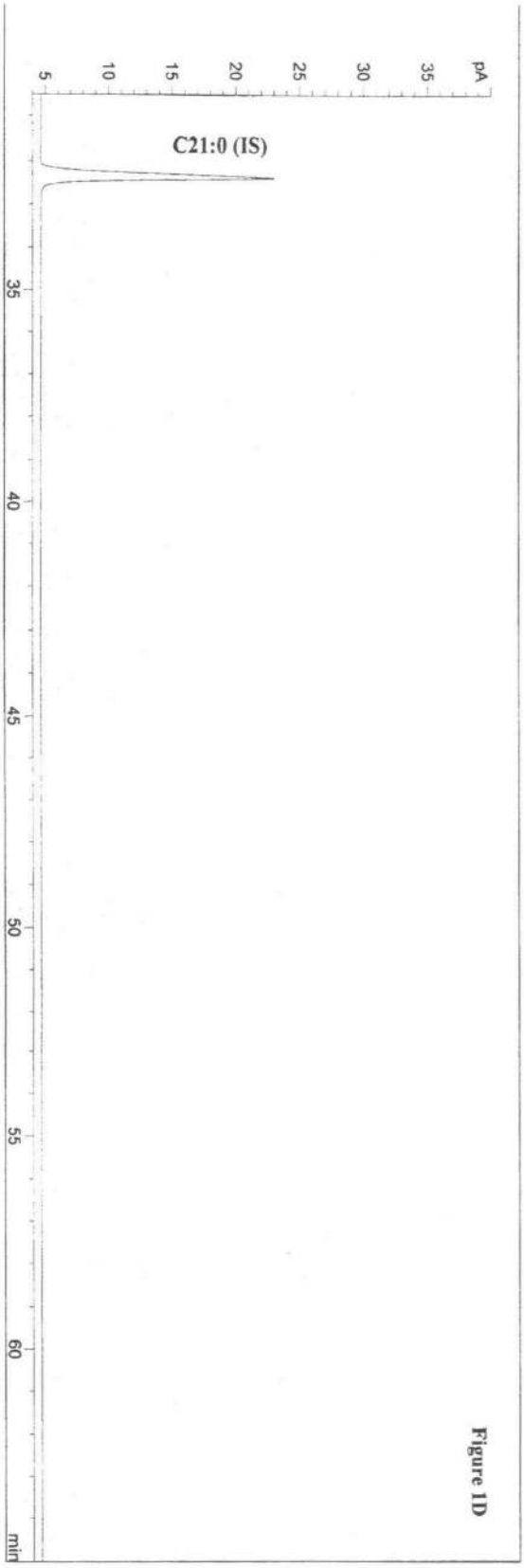

Figure 1D

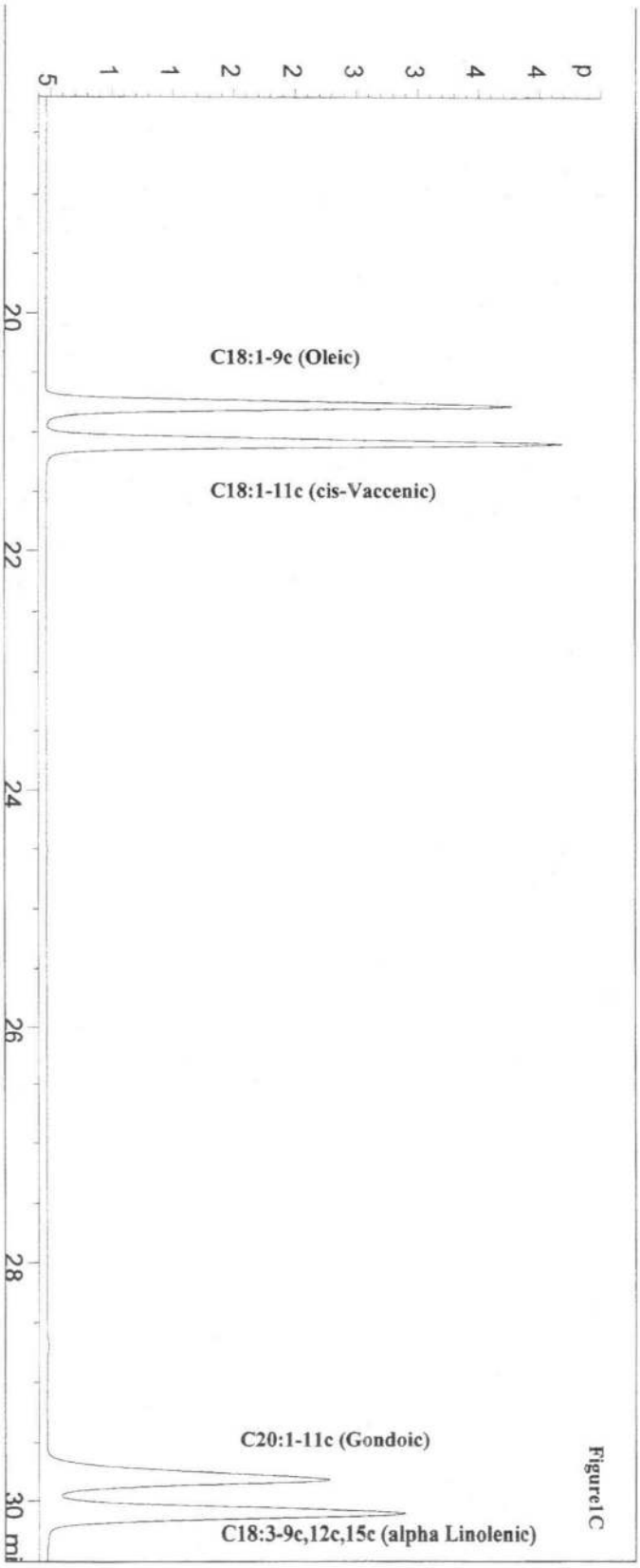

Figure 1C

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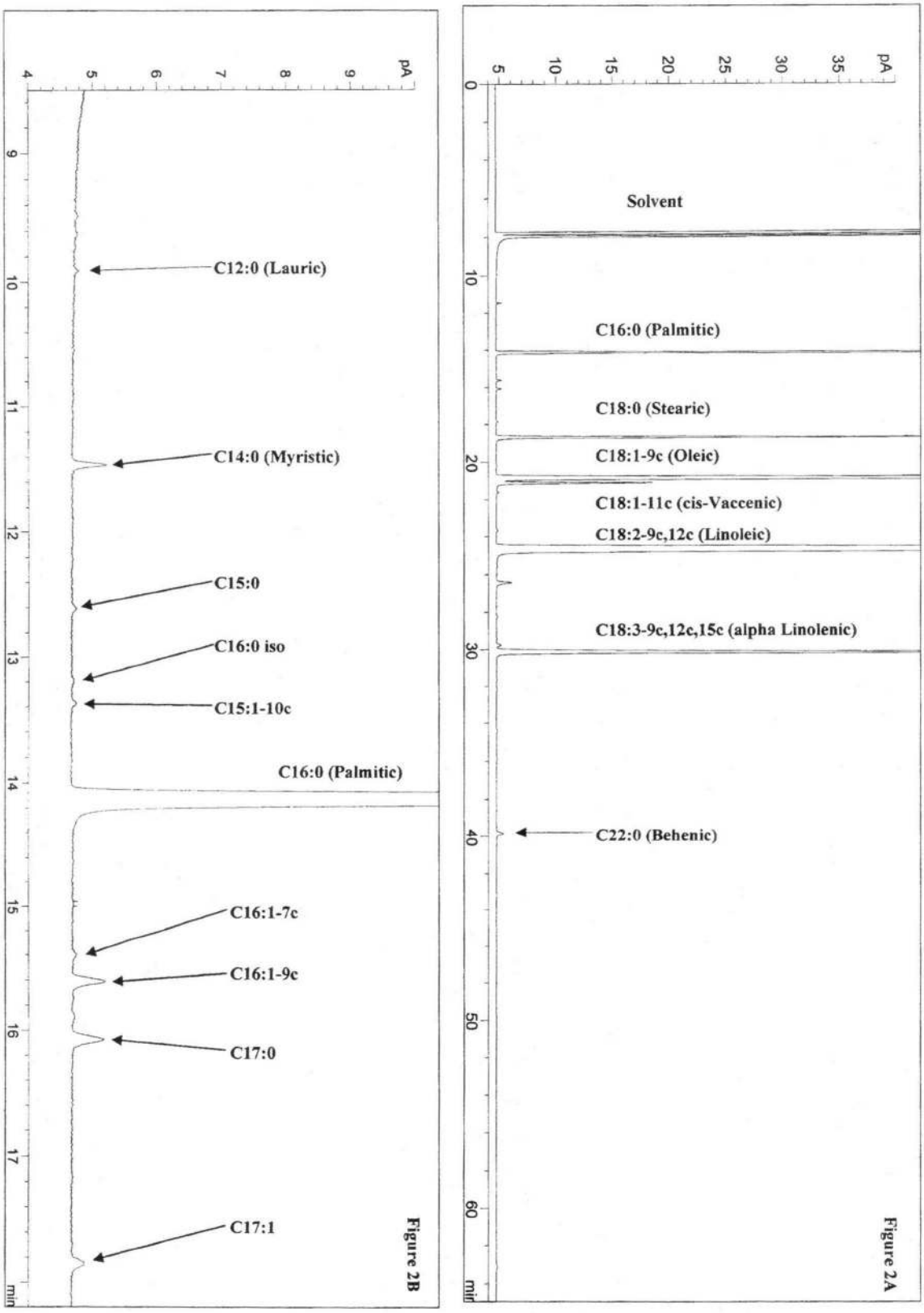

Figure 2. Salad Oil (Soybean), AOCS Method Ce 1h-05, (SP-2560; Isothermal @ 180°C @ 1.0 mL/min (26 cm/s); H_2; Split ratio-1:100)

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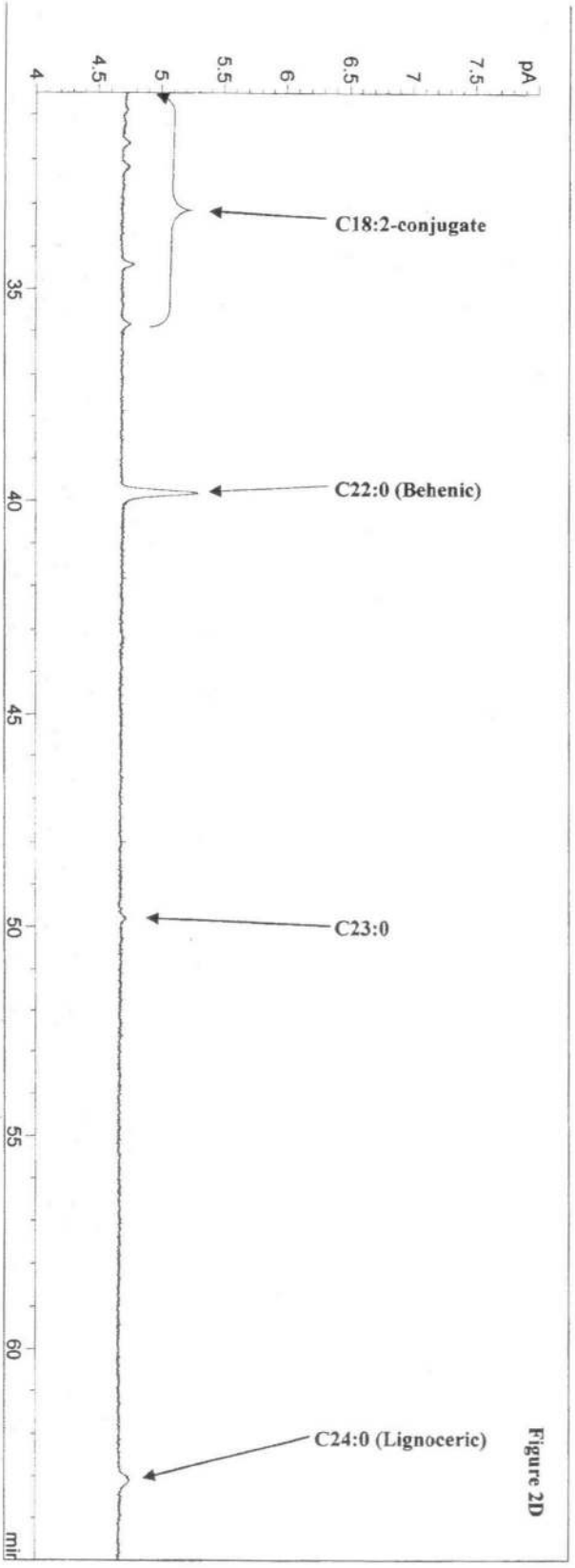

Figure 2D

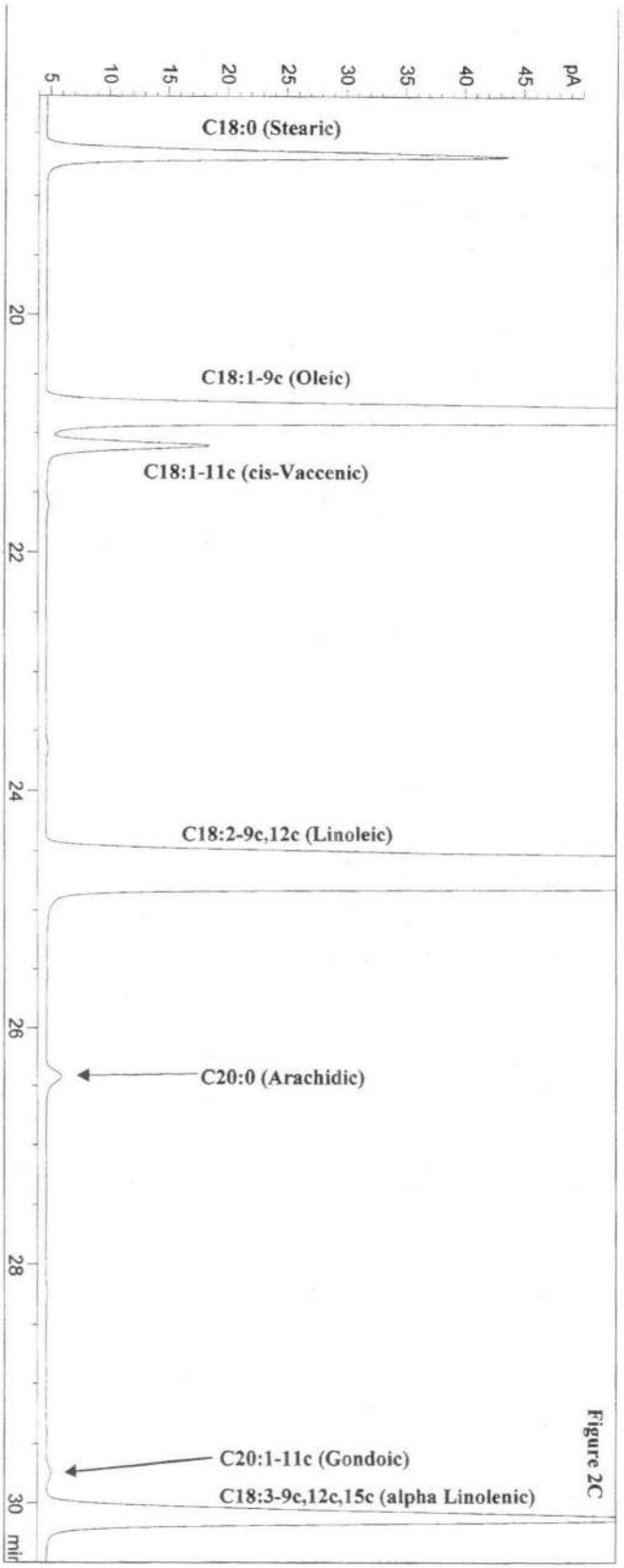

Figure 2C

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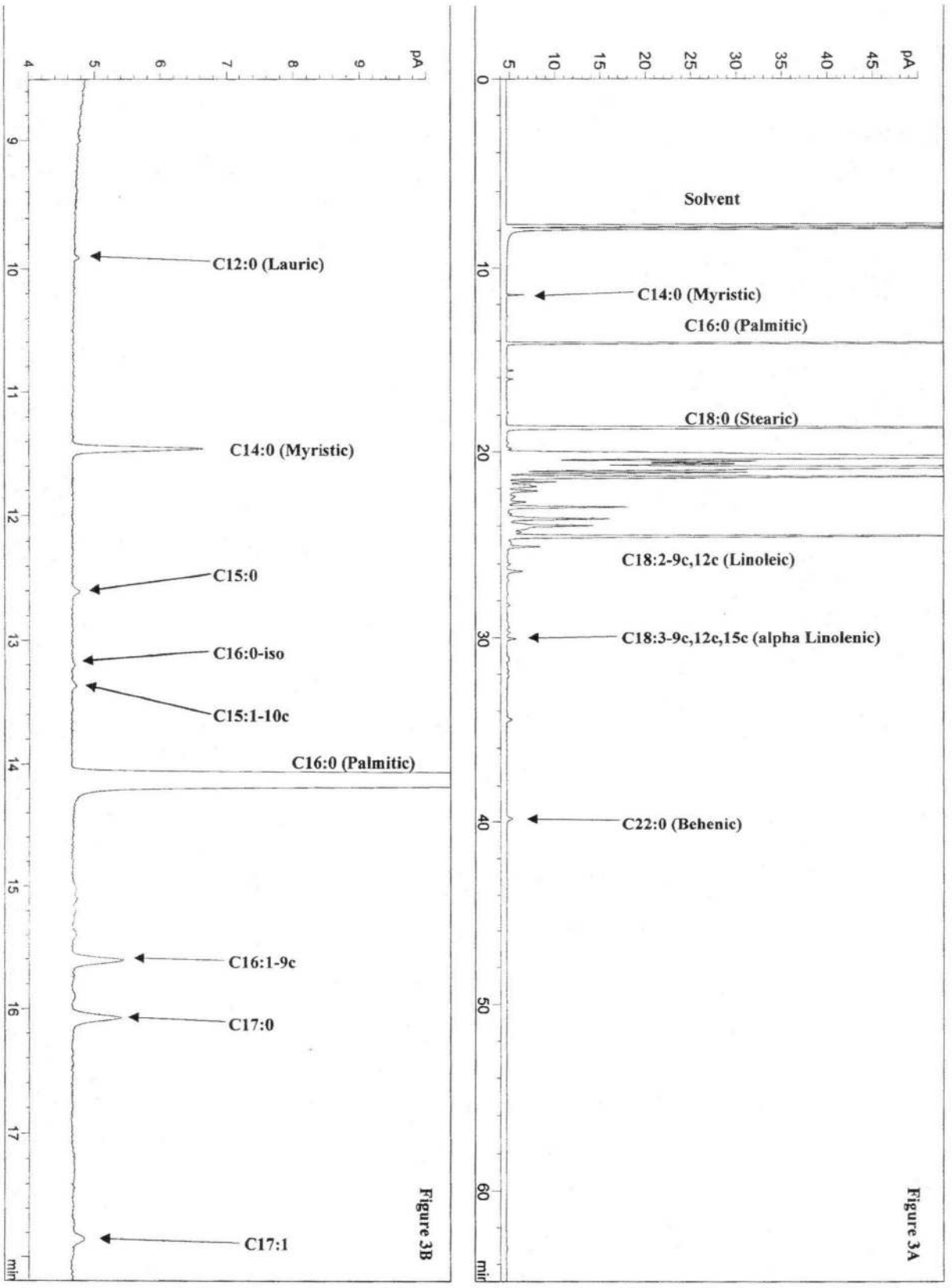

Figure 3. Margarine, AOCS Method Ce 1h-05, (SP-2560; Isothermal @ 180°C @ 1.0 mL/min (26 cm/s); H_2; Split ratio-1:100)

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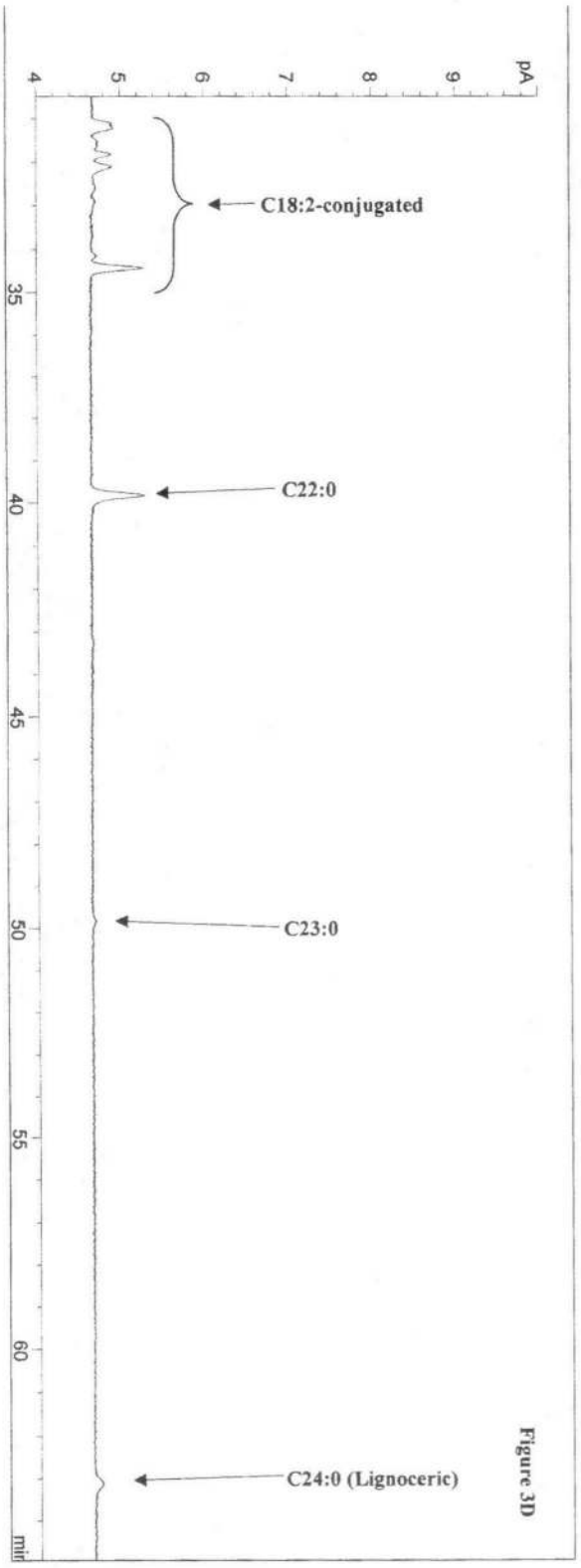

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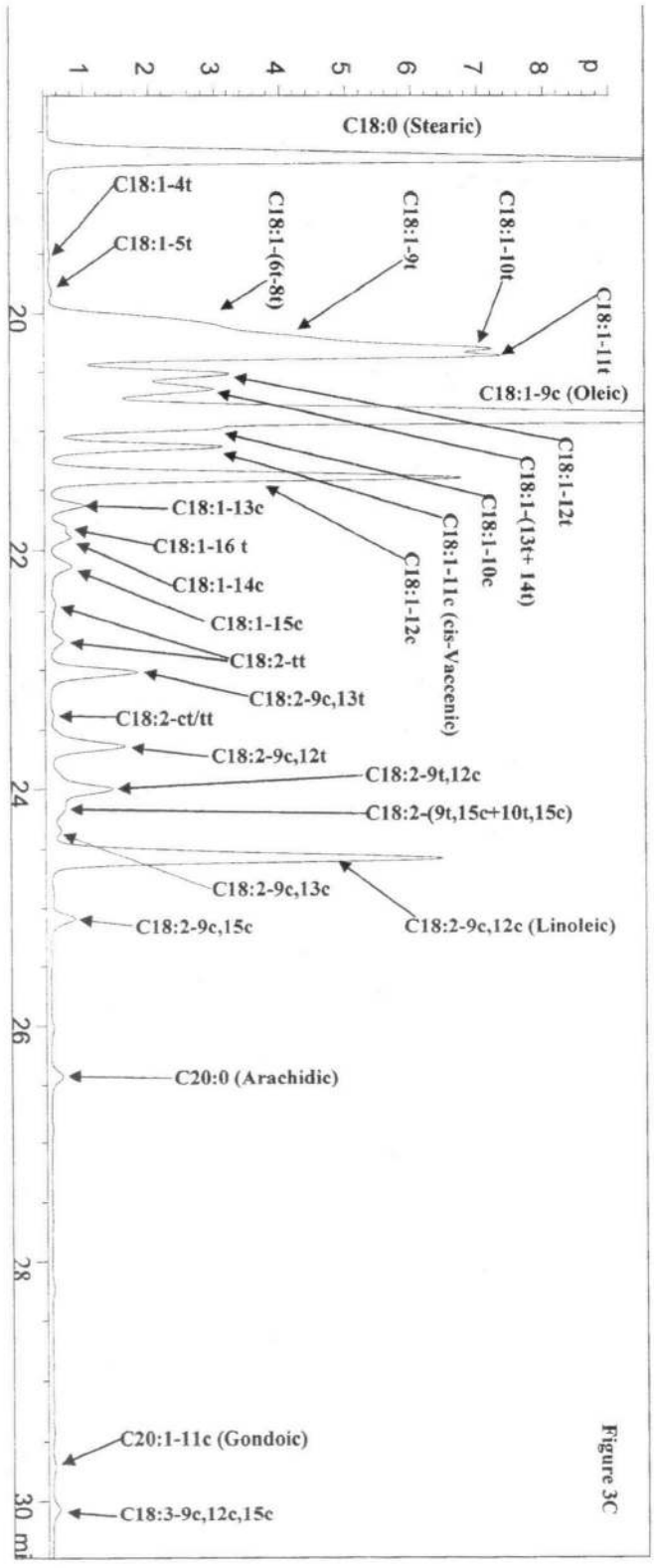

Figure 3C

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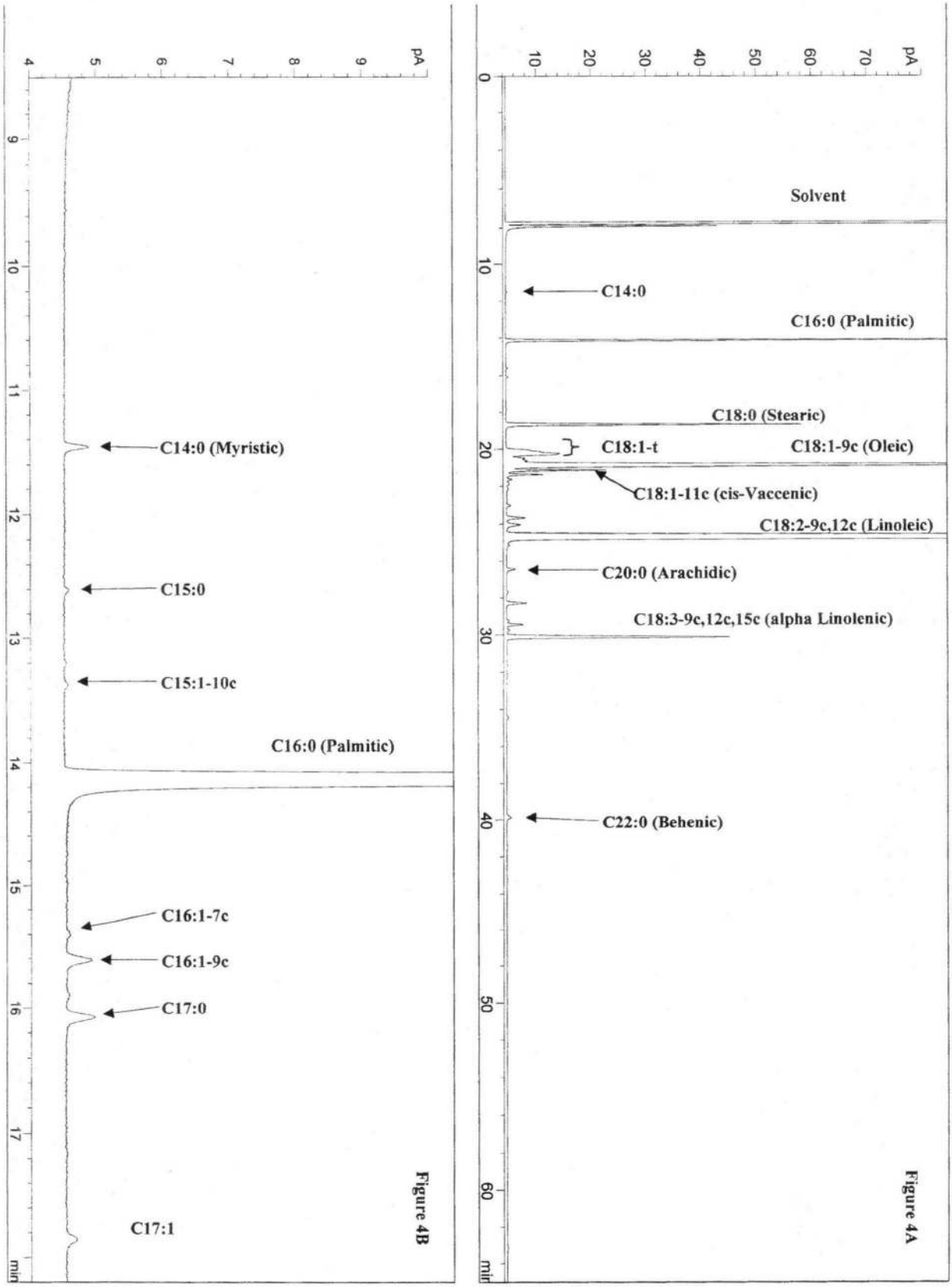

Figure 4. Low Trans Oil. AOCS Method Ce 1h-05, (SP-2560; Isothermal @ 180°C @ 1.0 mL/min (26 cm/s); H_2; Split ratio-1:100)

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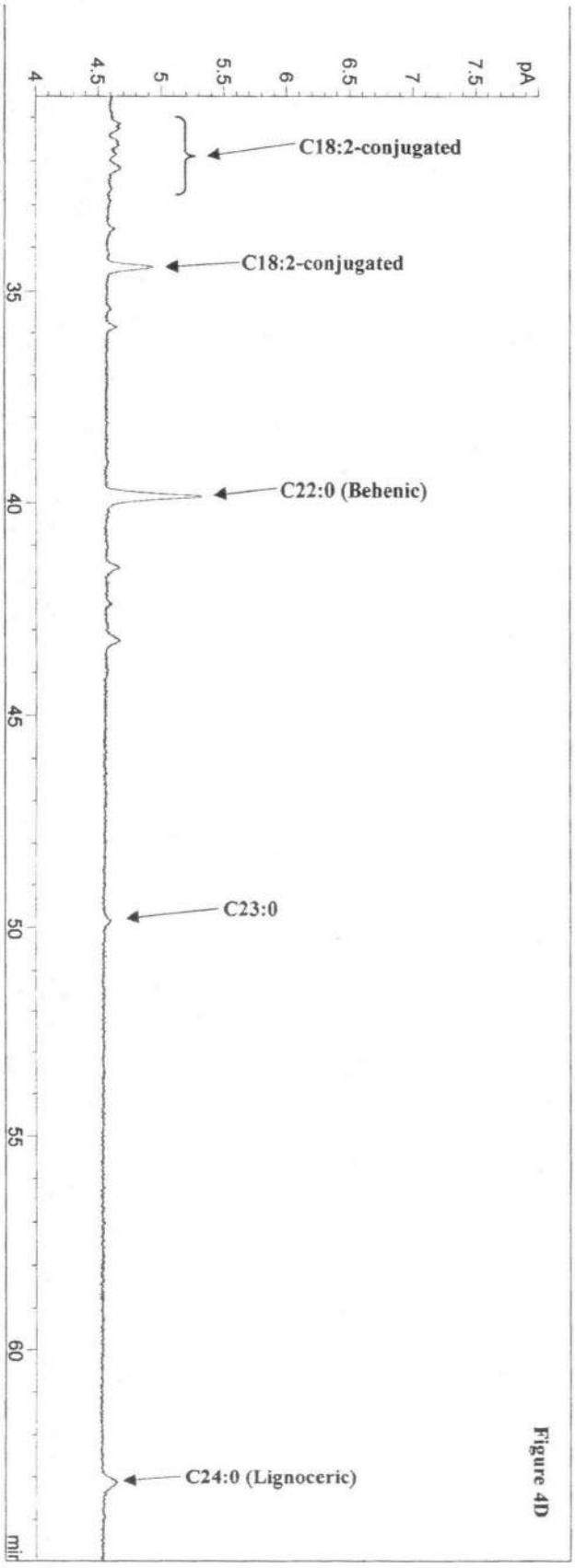

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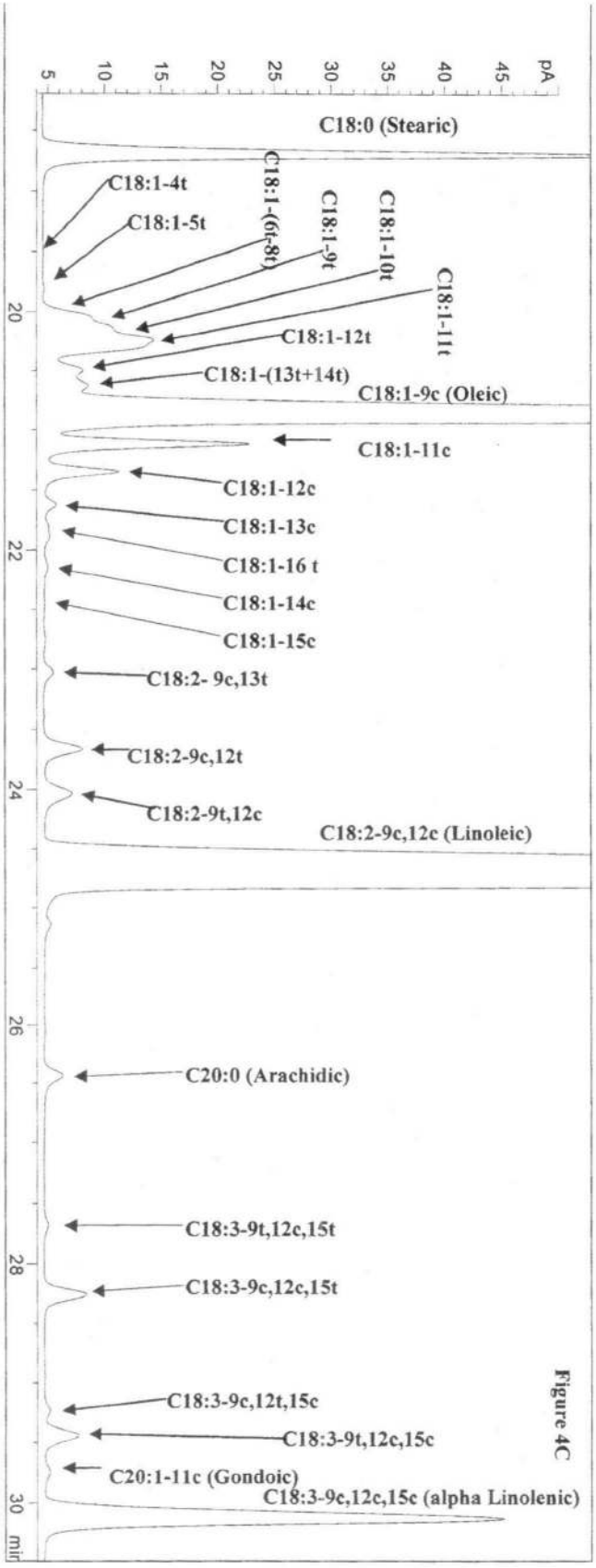

Figure 4C

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Figure 5. Compound Coating, AOCS Method Ce 1h-05, (SP-2560; Isothermal @ 180°C @ 1.0 mL/min (26 cm/s); H_2; Split ratio-1:100)

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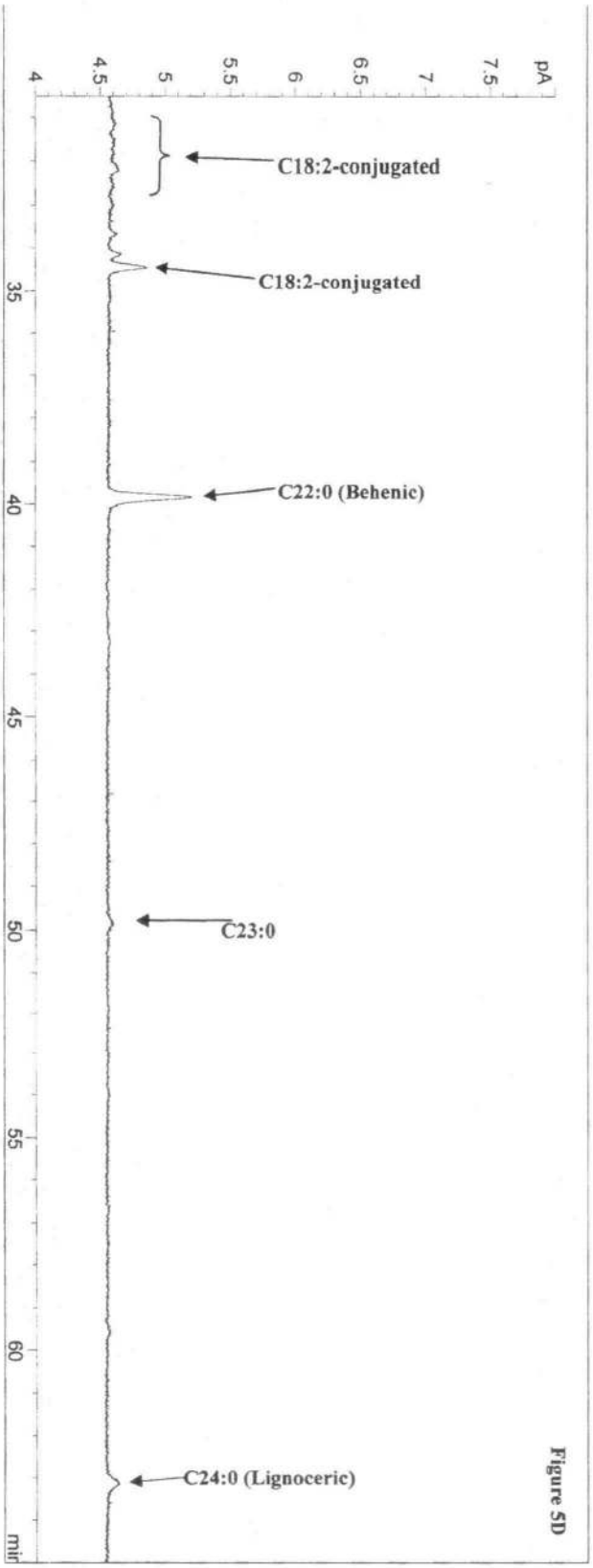

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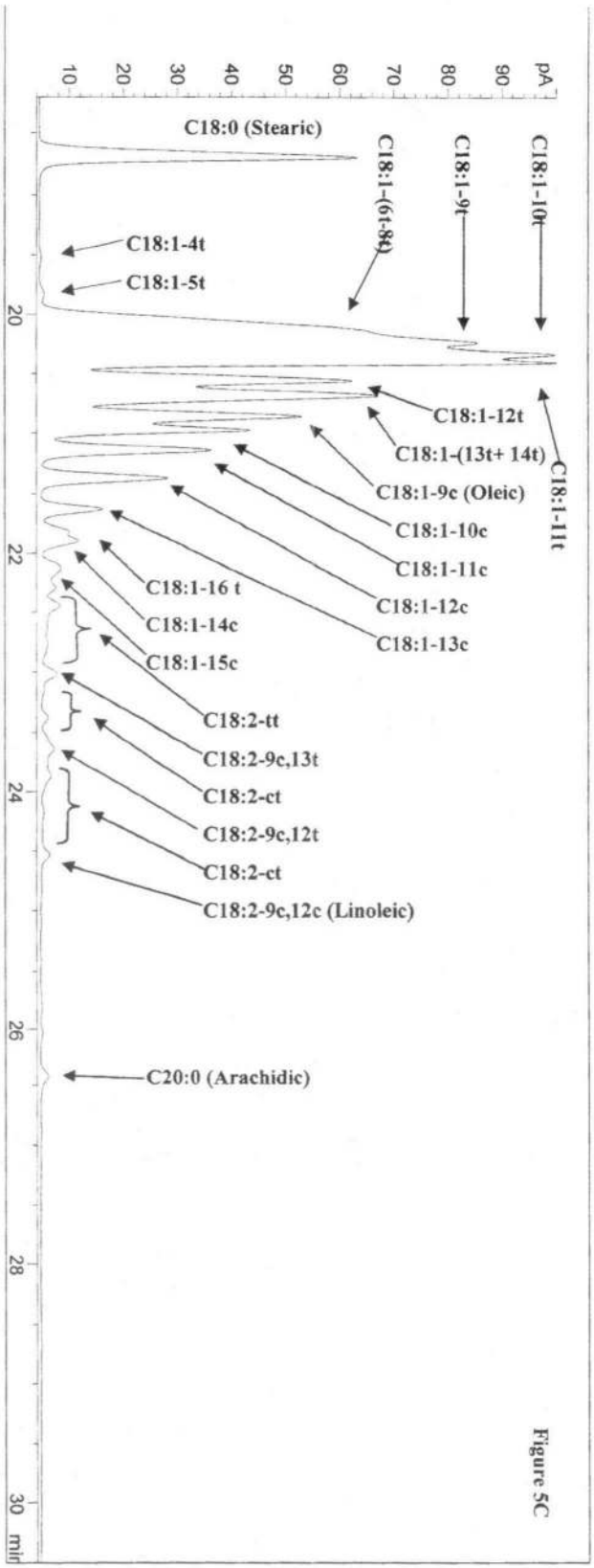

Figure 5C

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AOCS Official Method Ce 1i-07

Reapproved 2009

Determination of Saturated, *cis*-Monounsaturated, and *cis*-Polyunsaturated Fatty Acids in Marine and Other Oils Containing Long Chain Polyunsaturated Fatty Acids (PUFAs) by Capillary GLC

DEFINITION

This method provides a gas–liquid chromatography (GLC) procedure for the determination of the fatty acid composition of marine oils and other oils containing long chain polyunsaturated fatty acids (PUFAs). The fatty acid methyl esters (FAMEs) of the sample are separated on a capillary gas chromatography column having a polar stationary phase, according to their chain length (CL), degree of unsaturation, and double bond position (i.e., positional isomers). This method, however, is not suitable for separation of *cis* and *trans* isomers of unsaturated FAMEs.

SCOPE

This method determines the content and concentrations of saturated fatty acids (SFA), *cis*-monounsaturated fatty acids (MUFA), and *cis*-polyunsaturated fatty acids (PUFA), including arachidonic acid (ArA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), in marine oils and other oils such as single cell oils (SCOs) using capillary GLC. For nutritional labeling purposes the total fat, saturated, *cis*-monounsaturated, and *cis*-polyunsaturated fatty acid contents are to be determined with this method. This method is not suitable for determining the *trans* fatty acid composition of hydrogenated marine oils or other oils containing long chain polyunsaturated fatty acids. AOCS Ce 1h-05 or Ce 1j-07 are the methods of choice for quantitating and separating oils with *trans* fatty acids, and short chain fatty acids (≤ 10 carbons) should be quantitated with AOCS Ce 1j-07.

This method utilizes a triacylglycerol (23:0 TAG) internal standard (IS) for determining the concentration of the individual FAMEs in the oil samples after methylation. This method may also be used with marine oil fatty acid ethyl esters (FAEEs). FAEEs are methylated to their corresponding FAMEs. This conversion allows for ease of interpretation and quantitation for determining the fat and fatty acid contents plus the conversion of any residual acylglycerols, such as triacylglycerols (TAGs) to FAME. FAEEs should not be analyzed without derivatization since nutritional labeling requires all fatty acids to be quantitated as FAME.

Theoretical Correction Factors (TCFs) are used to quantitate *all* saturated and monounsaturated fatty acids greater than or equal to 12:0 and polyunsaturated fatty acids (PUFA) of 18 carbons. TCFs are also used for fatty acids, which lack standards, such as 6*c*, 9*c*, 12*c*, 15*c*, 18*c*-21:5 (21:5n-3). Empirical Correction Factors (ECFs) are used for long chain PUFA of 20 carbons or more and three or more double bonds for which standards are readily available.

Very long chain fatty acids (VLC FAs) may be present in SCOs and certain marine fats and oils. VLC FAs (25:0 and greater) require a longer run time with a pressure flow ramp for elution.

Phthalate esters are also known to be present in marine oils and therefore their retention times are noted in Tables 1 and 2, however, their identification is not required for fatty acid or nutritional labeling purposes.

APPARATUS

1. Gas chromatograph: suitable for use with a capillary column equipped with:
   (a) Thermostatic chamber for the capillary column, capable of maintaining the desired temperature to ± 0.1°C.
   (b) Temperature-controlled split mode injection unit.
   (c) Flame-ionization detector (FID), amplifier and electronic recorder-integrator.
2. Capillary columns: The capillary GC column should be of fused silica capillary 30 m in length, and 0.25 mm i.d., 0.25 um coating of polyethylene glycol (PEG). For example: SUPELCOWAX®-10 (Part Number 24079, Supelco, Inc., Bellefonte, PA, USA), FAMEWAX™ (Part Number 12497, Restek, Bellefonte, PA, USA), HP-INNOwax™ (Part Number 19091N-133, Agilent Technologies, Palo Alto, California, USA), CP-WAX™ 52 CB (Part Number CP8713, Varian, Inc., Walnut Creek, CA, USA), Carbowax™-20M (Part Number 200-0020, Agilent Technologies, Palo Alto, California, USA), and Omegawax™ 320 (Part Number 24152, Supelco Inc. Bellefonte, PA, USA) are columns that may be used.

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3. Microsyringe for gas chromatography—10 µL delivery, with a hardened needle.
4. Carrier gas—hydrogen or helium, 99.99% pure or better, gas chromatography quality, dried, and oxygen removed by suitable filters (Note 1).
5. Flame gases—hydrogen and air, gas chromatography quality.
6. Make-up gas—nitrogen or helium, gas chromatography quality.
7. Injection port split line—4 mm i.d., 6.3 mm o.d. × 78.5 mm base deactivated precision (Focus) split liner with glass wool (e.g., Part Number 21022-211.5, Restek; Part Number 092002, SGE or equivalent) (Note 2).

REAGENTS

Unless otherwise stated, use only reagents as specified in ISO 6353 (parts 2 and 3) (Reference 1) if not listed there, then use reagents of recognized analytical grade and water of at least grade 3 as defined in ISO 3696 (Reference 2).

1. Reference fatty acid methyl esters (FAME)—High purity methyl esters of fatty acids are available on the market (e.g., from Indofine Chemical Company, Inc., Somerville, NJ; Nu-Chek-Prep, Inc., Elysian, MN; Supelco Inc., Bellefonte, PA).
2. Triacylglycerol (TAG) internal standard (IS) solution (for calculating fatty acid data as mg per 100 g oil)—23:0 TAG, Tritricosanoin (purity >99%) (Note 3)—2.0 mg/mL in chloroform (Note 4). This TAG IS solution is stable indefinitely if precautions are taken to eliminate the loss of chloroform and therefore a change in the concentration of the IS. For example, store the solution in a refrigerator in a well-sealed amber bottle when not in use. Pure tritricosanoin is available on the market (e.g., from Nu-Chek-Prep, Inc.). Purity of the IS should be confirmed by thin-layer chromatography, high-performance liquid chromatography, gas chromatography, or by any other appropriate technique.
3. GLC-714, 20 mg/mL in *n*-heptane or *n*-hexanes, 100 mg ampoule (Nu-Chek-Prep, Inc.). This mixture is used to calculate the Empirical Correction Factors (ECFs). A Certificate of Analysis (COA) must be requested from Nu-Chek-Prep for every lot of the standard mixture used in determining ECFs. The actual amounts weighed into the ampoule and purities are listed on the COA and are used to calculate the actual amount of each FAME standard present in the mixture. The actual amount is necessary for determining the response factor (area/amount) for each FAME in the mixture. This mixture (100 mg) is diluted in 5 mL of *n*-heptane or *n*-hexanes with a 5 mL Class A volumetric pipette. The ampoule is rinsed with *n*-heptane or *n*-hexanes to ensure complete and homogenous transfer of the mixture. The final concentration should be approximately 20 mg/mL. The FAME standards in GLC-714 were chosen to represent the majority of fatty acids present in edible vegetable, marine, and single cell oils which were commercially available along with the internal standards to be used with each method.
4. System Suitability Mixture—Menhaden Oil from a commercial source (e.g., Arista Industries) to use for identification of fatty acids peaks and to ensure that 6*c*, 9*c*, 12*c*, 15*c*, 18*c*-21:5 (21:5n-3) is baseline separated from 23:0 (IS) and 24:0 is baseline separated from 4*c*, 7*c*, 10*c*, 13*c*, 16*c*, 19*c*-22:6 (DHA or 22:6n-3) which should be almost baseline resolved from the 24:1 isomers.

PROCEDURE

1. Methylation of Oils—Preparation of fatty acid methyl esters (FAMEs) from oils and fatty acid ethyl esters (FAEEs)
   (a) GLC-714—Analyze the 20 mg/mL preparation to determine ECFs (Figures 1 and 2).
   (b) For oils and FAEEs—Use a standard procedure for example, AOCS Official Method Ce 2-66 (References 3 and 4) or ISO 5509 (Reference 5). Prior to methylation, add enough TAG IS solution to the reaction flask with a Class A volumetric pipette, so that after the oil is added, the concentration of the final solution is between 0.05 and 0.10 mg TAG IS/1 mg oil (e.g., 5 mL of a 2 mg/mL TAG IS solution per 100 mg of oil). Since chloroform is used in the IS, it should be evaporated from the flask prior to addition of the oil and methylation procedure. Addition of antioxidants (e.g., pyrogallol, BHT, etc.) (1 mg/1 mg sample) may help protect the highly unsaturated fatty acids from oxidation during methylation (Note 5).
   (c) Dissolve the prepared FAME in *n*-hexanes or *n*-heptane. The concentration should be approximately 15–20 mg/mL in *n*-hexanes or *n*-heptane.
2. Chromatography (FAME)
   (a) Operating conditions are as follows:
   Injection port temperature: 235°C
   Detector temperature: 325°C
   Oven temperature program: Initial temperature 170°C; ramp 1°C/min; final temperature 225°C (References 6 and 7)
   Carrier gas: *hydrogen;* column head pressure, 77.9 kPa (11.3 psi); constant flow rate, 1.2 mL/min; linear velocity; 43 cm/s; split ratio, 100:1.
   Carrier gas: *helium;* column head pressure, 226 kPa (32.77 psi); constant flow rate, 2.4 mL/min; linear velocity; 53 cm/s; split ratio, 100:1.
   Oven temperature program for VLCFAs (> 25:0): Initial temperature 170°C; ramp 1°C/min; final temperature 225°C (hold 55 min)
   Carrier gas for VLCFAs (> 25:0): *hydrogen;* column head pressure, 77.9 kPa (11.3 psi); ramp flow, 1.2 mL/min (55 min) to 2.4 mL/min @ 1.2 mL/min^2 (hold 54 min); linear velocity; 43 cm/s; split ratio, 100:1.
   Carrier gas for VLCFAs (> 25:0): *helium;* column head pressure, 226 kPa (32.77 psi); ramp flow, 2.4 mL/min (55 min) to 4.8 mL/min @ 2.4 mL/min^2 (hold 54 min); linear velocity; 53 cm/s; split ratio, 100:1.
3. Inject 1 µL, which is equivalent to 15 to 20 µg of total FAMEs.

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PERFORMANCE CHECK: (SYSTEM SUITABILITY MIX)

1. Column performance is checked using fatty acid methyl esters (FAMEs) of Menhaden Oil (e.g., Arista Industries) and single cell oils (SCOs) covering the range of fatty acids under investigation (Figures 3-22). Since commercial GC designs and columns are different, and the separation obtained may not identical to the example chromatograms, small changes in the carrier gas flow rate, sample size, sample concentration, or oven temperature may be required. If so, adjust the carrier gas flow rate, sample size, sample concentration, or oven temperature until the best separation results are obtained. If column oven temperature needs to be adjusted, it should be adjusted with small increments, preferably by steps of 1°C. Authentic standards of fatty acid esters, if needed for peak identification, are available. 6*c*, 9*c*, 12*c*, 15*c*, 18*c*-21:5 (21:5n-3) should be baseline separated from 23:0 (IS) and 24:0 should be baseline separated from 4*c*, 7*c*, 10*c*, 13*c*, 16*c*, 19*c*-22:6 (DHA or 22:6n-3) which should be almost baseline resolved from the 24:1 isomers (Reference 6 and Figures 1, 2, 7, and 17).
2. The Empirical Correction Factors (ECFs) are determined by analysis of GLC-714 at approximately 20 mg/mL and plotted as the percent difference between the ECF and Theoretical Correction Factors (TCFs) divided by the TCFs (Figures 23 and 24). Figure 24 shows the plot from collaborative study laboratories. Different laboratories will most likely have different plots and therefore should be determined for each laboratory. An example calculation is as follows:

$$\% \text{ Difference} = ((ECF_{FAMEx} - TCF_{FAMEx})/TCF_{FAMEx}) \times 100$$

   GLC-714 can also be used to help determine if a chromatographic system is operating properly. Operation under the GC parameters listed in the method should result in a similar trend line with the long chain PUFA ECFs deviating furthest from TCFs, while the saturated and monounsaturated FAME greater than 12 carbons in length deviate from the TCFs the least. If the % Difference is greater than three for the saturated and monounsaturated FAME, it is suggested the GC parameters (e.g., detector gases, carrier gas flows, and column choice, etc.) be reoptimized to achieve a difference of 3% or less before continuing with analysis. ECFs will need to be calculated and used for the long chain PUFA (≥ 20 carbons and three double bonds). Figure 24 shows the plot of three different methods AOCS Ce 1h-05, Ce 1i-07, and Ce 1j-08 from one laboratory.
3. Peak identification—The individual FAMEs are identified by their retention times, which are compared with the FAME reference standards and reference oils, see tables 1 and 2. The marine FAME reference oil is LCP menhaden oil (Arista Industries, Inc.). See Figures 3–7 and 13–17 for example sample chromatograms of LCP menhaden oil. The SCO reference oil is produced by fermentation of *Mortierella alpina ALK-1* fungus. See Figures 8–12 and 18–22 for example sample chromatograms of SCO. When peaks of unknown identity are observed, attempt to identify such peaks using appropriate procedures that usually involve GC-MS, FTIR, silver-ion chromatography, and classical chemical methods (References 8 and 9). Peaks of unknown identity should *not* be included in the summation of peak areas when quantifying the concentrations of total fat, saturated, monounsaturated, polyunsaturated, EPA, DHA, other LC and VLC PUFA unless they have been confirmed to be fatty acids.

CALCULATIONS

1. Calculation of grams (g) of individual fatty acids and total fat per 100 g of test sample:

The food labeling regulations in Canada (Reference 10) and the USA (Reference 11) define the total fat as the sum of fatty acids originating from all the lipid classes present in the food matrix and are expressed as TAG equivalents. This requires converting the GC measured FAMEs into TAG equivalents. The fatty acids listed in Table 3 cover most of the fatty acid chain lengths (from 4:0 to 24:0) and double bonds (from 0 to 6) that are encountered in marine and single cell oils (SCO). The factors for conversion of the individual FAMEs into TAG equivalents are also given in Table 3.

The food labeling regulations in Canada (Reference 10) and the USA (Reference 11) require mandatory declaration of total fat and the amounts of saturated fatty acids (SAFAs) and *trans* fatty acids. Declaration of the amounts of *cis*-monounsaturated fatty acids (MUFAs), n-6 and n-3 *cis*-polyunsaturated fatty acids (PUFAs) is not mandatory, however, they could be declared in the nutrition facts table on a voluntary basis. Furthermore, the amounts of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) could also be declared on the label, but their values should be displayed outside the nutrition fact table. For nutritional labeling purposes the total fat, saturated, *cis*-monounsaturated, and *cis*-polyunsaturated fatty acid contents are to be determined with this method. For the determination of *trans* fatty acid concentration AOCS Ce 1h-05 or Ce 1j-08 methods are recommended. In contrast to total fat, fatty acids should be expressed as free fatty acids (FFAs). The factors for conversion of the individual FAMEs into FFAs are also given in Table 3.

(a) Calculate, the amount (in g) of individual fatty acids, expressed as FAME (W_{FAMEx}) and TAG (W_{TAGx}) equivalents as follows. Please note that this calculation is applicable to both oil and fatty acid ethyl ester (FAEE) samples, because both oil and FAEE samples use the same TAG (i.e., 23:0 TAG) as the internal standard (IS) and that all samples are converted to FAME for GC analysis.

$$W_{FAMEX} = \frac{A_X \times W_{TAG\ is} \times 1.0037 \times R_x}{A_{is}}$$

$$W_{TAGx} = W_{FAMEx} \times F_{TAGx}$$

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Where A_x = area counts for fatty acid x; $W_{TAG\ is}$ = weight of 23:0 TAG IS (in g) added to the oils; A_{is} = peak area counts of the IS; 1.0037, conversion of IS in test portion (Note 6); F_{TAGx} = conversion factor for FAMEs to TAGs for individual fatty acids (Table 3) and R_x = theoretical flame ionization detector correction factor (TCF) for FAMEs relative to 23:0 ME IS, respectively (Table 4; Note 7) or the Empirical Correction Factor (ECF) as determined via GLC-714 (Table 4). TCF should be applied to the analytical data for optimum accuracy (References 12–14) and to minimize variation between laboratories because of differences in calculating response factors. TCFs are also used for fatty acids where standards are not available. ECFs are needed due to the large deviation from TCFs for long chain PUFA of 20 carbons or more and three or more double bonds. The TCFs and ECFs of a number of FAMEs that are commonly encountered in marine and SCOs are listed in Table 4. The TCF were calculated using the following formula:

$$TCF_x = MW_x/(N_x - 1) \times (AWC) \times (1.3344)$$

Where TCF = theoretical flame ionization detector response factor for fatty acid x (as methyl ester) with respect to 23:0 FAME IS; MW_x = molecular weight of component x; N_x = number of carbon atoms in the FAME of component x; AWC = atomic weight of carbon (12.011); 1.3344 = TCF for 23:0 FAME (Notes 7–9).

ECFs are required for long chain PUFA FAME of 20 carbons or more and three or more double bonds of which standards are readily available. The ECFs are calculated using the Certificate of Analysis (COA) supplied with each lot of GLC-714 from Nu-Chek-Prep, Inc. The COA will list both the purity (P) and amount (Amt_{FAMEx}) of each FAME used to make up the standard. The actual amount ($AAmt_{FAMEx}$) is calculated by the following equation:

$$AAmt_{FAMEx} = P \times Amt_{FAMEx}$$

The response factor (RF) for each peak are determined by the following equation:

$$RF_{FAMEx} = Area_{FAMEx}/AAmt_{FAMEx}$$

Each RF is then made relative to the 23:0 RF by the flowing equation:

$$RRF_{FAMEx} = RF_{FAMEx}/RF_{23:0}$$

The Empirical Correction Factor (ECF) for each FAME is then calculated by the taking the inverse of the RRF:

$$ECF_{FAMEx} = 1/RRF_{FAMEx}$$

(b) Calculate the amount of *total fat* in test sample (sum of all fatty acids; expressed as TAGs) as follows:

$$\text{Total fat (g/100 g portion test sample)} = (\Sigma W_{TAG}/W_{TS}) \times 100$$

where W_{TS} = weight of test sample, g

(c) Calculate weight (in g) of each *individual fatty acid* (W_x) as follows:

$$W_x \text{ (g per test portion)} = W_{FAMEx} \times F_{FAx}$$

where F_{FAx} = conversion factors for conversion of FAME to their corresponding fatty acids (Table 3).

(d) Calculate weight of *saturated fats* (sum of all saturated fatty acids) as follows:

$$\text{Saturated fat (g per 100 g test sample)} = (\Sigma\text{Saturated } W_x/W_{TS}) \times 100$$

Where ΣSaturated W_x = sum of all saturated fatty acids (4:0; 5:0; 6:0; 7:0; 8:0; 9:0; 10:0; *iso* 10:0; 11:0; 12:0; *anteiso* and *iso* 12:0; 13:0; *anteiso* and *iso* 13:0; 14:0; *anteiso* and *iso* 14:0; 15:0; *anteiso* and *iso* 15:0; 2,6,10,14-tetramethyl 15:0; 16:0; *anteiso* and *iso* 16:0; 3,7,11,15-tetramethyl 16:0; 17:0; *anteiso* and *iso* 17:0; 18:0; *anteiso* and *iso* 18:0; 19:0; *anteiso* 19:0; 20:0; *iso* 20:0; 21:0; *iso* 21:0; 22:0; 24:0; 25:0; 26:0; 27:0; 28:0; 29:0; and 31:0).

(f) Calculate weight of *cis-monounsaturated fat,* fatty acids containing one double bond, as follows:

$$\textit{cis}\text{-monounsaturated fat (g per 100 g test sample)} = (\Sigma\textit{cis}\text{-monounsaturated } W_x/W_{TS}) \times 100$$

Where Σ*cis*-monounsaturated W_x = sum of all *cis*-monounsaturated fatty acids (10*c*-11:1; 9*c*-14:1; 10*c*-15:1; 7*c*-, 9*c*-, 11*c*-16:1; 10*c*-17:1; 9*c*-, 11*c*-, 13*c*-18:1; 10*c*-19:1; 9*c*-, 11*c*-, 13*c*-20:1; 9*c*-, 11*c*-, 13*c*-22:1; and 24:1 isomers).

(g) Calculate weight of *cis-polyunsaturated fat,* fatty acids containing two or more double bonds, as follows:

$$\textit{cis}\text{-polyunsaturated fat (g per 100 g test sample)} = (\Sigma\textit{cis}\text{-polyunsaturated } W_x/W_{TS}) \times 100$$

Where Σ*cis*-polyunsaturated W_x = sum of all *cis*-polyunsaturated fatty acids (7*c*,10*c*-16:2, 9*c*,12*c*-16:2; 6*c*,9*c*,12*c*-16:3; 7*c*,10*c*,13*c*-16:3; 4*c*,7*c*,10*c*,13*c*-16:4; 6*c*,9*c*,12*c*,15*c*-16:4; 5*c*,11*c*-18:2; 8*c*,11*c*-18:2; 9*c*,12*c*-18:2; 11*c*,14*c*-18:2; 6*c*,9*c*,12*c*-18:3; 8*c*,11*c*,14*c*-18:3;9*c*,12*c*,15*c*-18:3; 6*c*,9*c*,12*c*,15*c*-18:4; 8*c*,11*c*,14*c*,17*c*-18:4;

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3*c*,6*c*,9*c*,12*c*,15*c*-18:5; 11*c*,14*c*-20:2; 8*c*,11*c*,14*c*-20:3; 11*c*,14*c*,17*c*-20:3; 5*c*,8*c*,11*c*,14*c*-20:4; 8*c*,11*c*,14*c*,17*c*-20:4; 5*c*,8*c*,11*c*,14*c*,17*c*-20:5; 6*c*,9*c*,12*c*,15*c*,18*c*-21:5; 13*c*,16*c*-22:2; 13*c*,16*c*,19*c*-22:3; 7*c*,10*c*,13*c*,16*c*-22:4; 4*c*,7*c*,10*c*,13*c*,16*c*-22:5; 7*c*,10*c*,13*c*,16*c*,19*c*-22:5, and 4*c*,7*c*,10*c*,13*c*,16*c*,19*c* 22:6).

(h) Calculate weight of *EPA* or *DHA* as follows:

EPA or DHA fat (g per 100 g test sample) = (ΣEPA or DHA W_x/W_{TS}) × 100

(i) Calculate weight of *n-3 PUFAs* as follows:

n-3 PUFA (g per 100 g test sample) = (Σn-3 PUFA W_x/W_{TS}) × 100

Where Σn-3 PUFAs W_x = sum of all n-3 PUFAs (9*c*,12*c*,15*c*-18:3; 6*c*,9*c*,12*c*,15*c*-18:4; 8*c*,11*c*,14*c*,17*c*-20:4; 5*c*,8*c*,11*c*,14*c*,17*c*-20:5; 7*c*,10*c*,13*c*,16*c*,19*c*-22:5, and 4*c*,7*c*,10*c*,13*c*,16*c*,19*c* 22:6) (References, 10).

(j) Calculate weight of *n-6 PUFAs* as follows:

n-6 PUFAs (g per 100 g test sample) = (Σn-6 PUFAs W_x/W_{TS}) × 100

where Σn-6 PUFAs W_x = sum of all n-6 PUFAs (9*c*,12*c*-18:2; 6*c*,9c,12*c*-18:3; 8*c*,11*c*,14*c*-20:3; 5*c*, 8*c*,11*c*,14*c*-20:4; 7*c*,10*c*,13*c*,16*c*-22:4; and 4*c*,7*c*, 10*c*, 13*c*,16*c*-22:5) (References, 10).

METHOD PRECISION

1. Pre-Collaborative Data from four labs using TCFs and ECFs for six samples for the major fatty acids, PUFAs, and the nutritional labeling requirements for total fat, saturated, monounsaturated, and polyunsaturated fatty acids are shown in table 5.
2. Collaborative Data from 13 labs using TCFs and ECFs for seven samples for the major fatty acids, PUFAs, and the nutritional labeling requirements for total fat, saturated, monounsaturated, and polyunsaturated fatty acids are shown in Table 6. All data are expressed as FA Wt.% except for NLEA Total Fat, which is expressed as FA Wt.%.

QUALITY ASSURANCE AND CONTROL

1. Blank sample—The first sample in an analysis batch is always a blank (*n*-hexanes and/or *n*-heptane). No peaks other than the solvent should be detected in the blank run. Repeat this test after every ten samples.
2. Proficiency test—For external reference, the method is tested annually by participation in the AOCS Laboratory Proficiency Program or any other suitable test.

NOTES

1. Nitrogen gas is *not* acceptable as a carrier gas for this method.
2. No other liner types (e.g., straight through, etc.) should be used with this method.
3. Tritricosanoin (23:0 TAG) was decided upon as the only IS for this method, as opposed to the Ethyl Ester (EE), because a) the exact composition of the oil (e.g., FAEE, FFA, or TAG) may not be known and b) harmonization with AOCS Ce 1h-05 and AOCS Ce 1j-08 methods.
4. Tritricosanoin (23:0 TAG) is not readily soluble in any solvents at 5 mg/mL. However, it is soluble in chloroform at 2 mg/mL or less. Care must also be taken to prevent the loss of chloroform during use and storage.
5. Preliminary identification of peaks associated with antioxidants should first be determined before analyzing oil samples to determine if co-elution will occur with peaks of interest.
6. 1.0037 is the factor for conversion of the weight of the IS (which is 23:0 TAG) from the TAG form to its corresponding weight of the FAME form. In calculating the factor, we have to take into consideration that each mole of TAG yields three moles of FAME. So that the general formula for the conversion factor is as follows:

$$\text{MW of 23:0 FAME} \times 3/\text{MW of 23:0 TAG} = (368.6312 \times 3)/1101.8620 = 1.0037$$

7. 1.3344 is the theoretical flame ionization detector response correction factor (TCF) for 23:0 FAME. It is calculated as follows.

$$\text{TCF of 23:0 FAME} = \text{MW of 23:0}/(N_{x-1})\ (\text{Atomic weight of carbon}) = 368.6312/((24-1) \times 12.011) = 1.3344$$

Here N_x is the number of carbon atoms in 23:0 FAME, i.e., 24. 12.011 is the atomic weight of carbon.

8. It is important to understand the difference (and hence the relationship) between TCF and TRF.
   (a) Theoretical flame ionization response factor (TRF) is actually the proportion (with respect to the total weight, which is the molecular weight) of the total amount of carbon atoms that are responding to the FID. For fatty acids, the carbons that are responding to FID are CH, CH_2, and CH_3 units. Carbonyl carbon and ester carbonyls carbon do not respond to FID. So that in FAME, all the carbon atoms are active, except the ester carbon. Thus, there are N_{x-1} active carbon atoms in a FAME. So that the TRF for a FAME would be:

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$$TRF = (N_{x-1}) \text{ (Atomic Weight of Carbon)/MW of FAME}$$

$$TRF \text{ of 23:0 FAME} = 23 \times 12.011/368.6312 = 0.7494$$

(b) Since the FID response depends on the proportion of active carbon atoms, to determine the actual weight of a FAME, the experimental measured area (or the weight) needs to be corrected. This factor is termed as TCF and it is equal to 1/TRF. In other words formula for TCF is as follows:

$$TCF = MW/(N_{x-1}) \text{ (Atomic Weight of Carbon).}$$

The TCF of 23:0 FAME is 1.3343

9. The TRF of 18:0 FAME is 0.7243. Therefore, its TCF is 1/0.7243 = 1.3806. Thus TCF of 18:0 FAME with respect to 23:0 FAME = 1.3806/1.3343 = 1.0347 (Table 4).

REFERENCES

1. ISO 6353, Reagents for Chemical Analysis, Part 2 (1983) and 3 (1987); Specifications.
2. ISO 3696, Water for Analytical Laboratory Use—Specifications and Test Methods (1987).
3. *AOCS Official Method* Ce 2-66, Preparation of Methyl Esters of Long-Chain Fatty Acids.
4. N.C. Shantha and R.G. Ackman, *J. Chromatography, 533,* 1–10, 1990.
5. ISO Method 5509, Animal and vegetable fats and oils—Preparation of methyl esters of fatty acids (2004).
6. *AOCS Official Method* Ce 1b-89, Fatty Acid Composition by GLC, Marine Oils.
7. *AOAC International* 991.39, Fatty Acids in Encapsulated Fish Oils and Fish Oil Methyl and Ethyl Esters.
8. R.G. Ackman, Application of gas-liquid chromatography to lipid separation and analysis: Qualitative and quantitative analysis. *Analyses of Fats, Oils and Lipoproteins,* E.G. Perkins, Ed.; AOCS Press, Champaign, IL, 1991, pp. 270–300.
9. J.L. Sebedio, Classical chemical techniques for fatty acid analysis. *New Trends in Lipid and Lipoprotein Analyses.* J.L. Sebedio and E.G. Perkins, Ed.; AOCS Press, Champaign, IL, 1995, pp. 277–289.
10. Regulations Amending the Food and Drug Regulations (Nutrition Labelling, Nutrient Content Claims and Health Claims). Department of Health, Canada Gazette, Part 11. January 1, 2003.
11. Department of Health and Human Services. Food and Drug Administration. 21 CFR Part 101 [Docket No. 94P-00036]. Food Labelling: *Trans* Fatty Acids in Nutrition Labelling, Nutrient Content Claims, and Health Claims. Washington, DC. July 2003, pp. 254.
12. R.G. Ackman and J.C. Sipos, *J. Am. Oil Chem. Soc. 41:*377–378, 1964.
13. J.D. Craske and C.D. Bannon, *J. Am. Oil Chem. Soc. 64:*1413–1417, 1987.
14. J.D. Craske and C.D. Bannon, *J. Am. Oil Chem. Soc. 65:*1190–1991, 1988.
15. R.G. Ackman, J.C. Sipos, and C.S. Tocher, *J. Fish. Res. Bd. Canada, 24:*635–650, 1967.
16. R.G. Ackman and S.N. Hooper, *Comp. Biochem. Physiol., 24:*549–565, 1968.
17. P. Mayzaud, C.A. Eaton, and R.G. Ackman, *Lipids, 11:*858–862, 1976.
18. G.E. Napolitano, W.M.N. Ratnayake, and R.G. Ackman, *Phytochemistry, 27:*1751–1755, 1988.
19. A. Timmins, E.J. Macpherson, and R.G. Ackman, *Food Chemistry, 70:*425–426, 2000.
20. E. Durnford, F, Shahidi, and R.G. Ackman, *J. Am. Oil Chem. Soc., 80:*405, 2003.
21. N.C. Shantha and R.G. Ackman, *J. Chromatography, 587:*263–267, 1991.
22. R.G. Ackman, *Analytica Chimica Acta, 465,* 175–192, 2002.
23. Van Pelt, C.K., M.-C. Huang, C.L. Tschanz, and J.T. Brenna, *J. Lipid Res., 40:*1501–1555, 1999.
24. Käkelä, R., R.G. Ackman, and H. Hyuärinen. *Lipids, 30:*725–731, 1995.

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Table 3
Factors for conversion of FAME to FA and TAG equivalents

Fatty Acid[a]	F_{FAx}[b]	F_{TAGx}[c]	Fatty Acid[a]	F_{FAx}[b]	F_{TAGx}[c]
4:0	0.8626	0.9868	20:0	0.9570	0.9959
5:0	0.8792	0.9884	20:1	0.9568	0.9959
6:0	0.8922	0.9897	20:2	0.9565	0.9958
7:0	0.9027	0.9907	20:3	0.9562	0.9958
8:0	0.9114	0.9915	20:4	0.9560	0.9958
9:0	0.9186	0.9922	20:5	0.9557	0.9958
10:0	0.9247	0.9928	21:0	0.9588	0.9961
10:1	0.9239	0.9927	21:5	0.9576	0.9959
11:0	0.9300	0.9933	22:0	0.9604	0.9962
11:1	0.9293	0.9932	22:1	0.9602	0.9962
12:0	0.9346	0.9937	22:2	0.9600	0.9962
12:1	0.9339	0.9937	22:3	0.9598	0.9961
13:0	0.9386	0.9941	22:4	0.9595	0.9961
13:1	0.9380	0.9941	22:5	0.9593	0.9961
14:0	0.9421	0.9945	22:6	0.9590	0.9961
14:1	0.9416	0.9944	23:0 (IS)	0.9619	0.9964
15:0[d]	0.9453	0.9948	23:5	0.9609	0.9963
Tetra Methyl 15:0	0.9551	0.9957	24:0	0.9633	0.9965
15:1	0.9449	0.9947	24:1	0.9631	0.9965
16:0[e]	0.9481	0.9950	24:3	0.9628	0.9964
Tetra Methyl 16:0	0.9570	0.9959	24:4	0.9626	0.9964
16:1	0.9477	0.9950	24:5	0.9624	0.9964
16:2	0.9473	0.9950	24:6	0.9621	0.9964
16:3	0.9469	0.9949	25:0	0.9646	0.9966
16:4	0.9465	0.9949	26:0	0.9658	0.9967
17:0	0.9507	0.9953	26:5	0.9650	0.9966
17:1	0.9503	0.9952	26:6	0.9648	0.9966
18:0	0.9530	0.9955	27:0	0.9670	0.9968
18:1	0.9527	0.9955	28:0	0.9680	0.9969
18:2	0.9524	0.9954	28:7	0.9670	0.9968
18:3	0.9520	0.9954	28:8	0.9668	0.9968
18:4	0.9517	0.9954	29:0	0.9690	0.9970
18:5	0.9514	0.9953	30:0	0.9700	0.9971
19:0	0.9551	0.9957	31:0	0.9708	0.9972
19:1	0.9548	0.9957			

[a]Only one factor is given for all positional and geometric isomers and for branched-chain FAME, as the factors are dependent only on the content of carbon to which hydrogen is bonded.
[b]F_{FAx} is the conversion factor for conversion of FAMEs to corresponding fatty acids.
[c]F_{TAGx} is the conversion factor for conversion of FAMEs to corresponding TAG equivalents.
[d]3,7,11-trimethyldodecanoic acid (TMDD) has equivalent F_{Fax} and F_{TAGx} to 15:0.
[e]4,8,12-trimethyltridecanoic acid (TMTD) has equivalent F_{Fax} and F_{TAGx} to 16:0.

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Table 4
Theoretical flame ionization detector correction factor (TCF) for fatty acid methyl esters (FAME)[a].

FAME	TCF	FAME	TCF	FAME	TCF/ECF	FAME	TCF/ECF
4:0	1.5929	15:1	1.0582	20:1	1.0124	24:3	0.9791
5:0	1.4494	16:0[d]	1.0546	20:2	1.0061	24:4	0.9738
6:0	1.3537	Tetra methyl 16.0	1.0187	20:3	ECF[b]	24:5	0.9686
7:0	1.2853	16:1	1.0468	20:4	ECF[b]	24:6	0.9634
8:0	1.2340	16:2	1.0389	20:5	ECF[b]	25:0	0.9900
9:0	1.1942	16:3	1.0310	21:0	1.0119	26:0	0.9856
10:0	1.1623	16:4	1.0232	21:5	0.9819	26:5	0.9614
10:1	1.1497	17:0	1.0441	22:0	1.0057	26:6	0.9566
11:0	1.1362	17:1	1.0367	22:1	1.0000	27:0	0.9815
11:1	1.1247	18:0	1.0347	22:2	0.9942	28:0	0.9777
12:0	1.1144	18:1	1.0277	22:3	0.9885	28:7	0.9463
12:1	1.1039	18:2	1.0207	22:4	0.9828	28:8	0.9418
13:0	1.0960	18:3	1.0137	22:5	ECF[b]	29:0	0.9742
13:1	1.0863	18:4	1.0067	22:6	ECF[b]	30:0	0.9709
14:0	1.0802	18:5	0.9997	23:0 (IS)	1.0000	31:0	0.9678
14:1	1.0713	19:0	1.0263	23:5	0.9727		
15:0[c]	1.0666	19:1	1.0197	24:0	0.9948		
Tetra methyl 15:0	1.0263	20:0	1.0187	24:1	0.9896		

[a]Atomic weights used: carbon 12.011; hydrogen, 1.0079; oxygen, 15.994. Factors are relative to 23:0, which has a factor of 1.0000 by definition. Only one factor is given for all positional and geometric isomers and for branched-chain FAME, as the factors are dependent only on the content of carbon to which hydrogen is bonded. Calculations were adopted from Reference 4.
[b]ECFs are to be calculated from GLC-714. Any isomers (i.e., ω-3 and ω-6) will have the same TCFs and ECFs.
[c]3,7,11-trimethyldodecanoic acid (TMDD) has a TCF equivalent to 15:0.
[d]4,8,12-trimethyltridecanoic acid (TMTD) has a TCF equivalent to 16:0.

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Table 5
Precollaborative study data.

	DHA SCO FA Wt. %			DHA-S SCO FA Wt. %			ArA SCO FA Wt. %		
Labs = 4	Mean	sd	% rsd	Mean	sd	% rsd	Mean	sd	% rsd
12:0 (Lauric)	4.21	0.15	3.60	0.17	0.01	3.98	0.02	0.00	6.09
14:0 (Myristic)	12.13	0.42	3.49	5.74	0.15	2.70	0.67	0.02	3.43
16:0 (Palmitic)	10.61	0.26	2.45	18.14	0.27	1.50	11.17	0.25	2.25
9*c*-18:1 (Oleic)	16.89	0.22	1.31	1.67	0.06	3.42	12.71	0.12	0.94
5*c*,8*c*,11*c*,14*c*-20:4 (ArA)	NA	NA	NA	1.53	0.06	3.90	39.45	1.45	3.66
5*c*,8*c*,11*c*,14*c*,17*c*-20:5 (EPA)	0.02	NA	NA	1.08	0.03	2.40	0.09	0.01	5.96
4*c*,7*c*,10*c*,13*c*,16*c*-22:5 (n-6 DPA)	NA	NA	NA	16.02	0.49	3.04	NA	NA	NA
7*c*,10*c*,13*c*,16*c*,19*c*-22:5 (n-3 DPA)	0.35	0.01	2.93	0.42	0.02	3.98	0.06	NA	NA
4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-22:6 (DHA)	39.82	1.28	3.21	39.74	1.01	2.54	1.61	0.08	5.04
NLEA Total Fat (TAG Wt. %)	93.42	0.51	0.55	92.68	2.07	2.23	95.15	1.06	1.11
Total Saturated	29.14	0.46	1.57	25.64	0.60	2.32	23.65	0.23	0.95
Total Monounsaturated (MUFA)	18.97	0.50	2.62	2.08	0.20	9.37	13.93	0.61	4.35
Total Polyunsaturated (PUFA)	41.25	1.22	2.96	61.18	1.81	2.96	53.59	1.15	2.15
Total n-3 Polyunsaturated (PUFA)	40.25	1.23	3.06	42.63	1.09	2.55	2.04	0.15	7.56
Total n-6 Polyunsaturated (PUFA)	0.99	0.02	2.11	18.48	0.69	3.75	51.96	1.48	2.85

	60/03 Fish Oil FA Wt. %			40/20 EE FA Wt. %			18/12 Fish Oil FA Wt. %		
Labs = 4	Mean	sd	% rsd	Mean	sd	% rsd	Mean	sd	% rsd
12:0 (Lauric)							0.14	0.00	2.70
14:0 (Myristic)	0.06	0.00	2.98	0.07	0.00	1.57	7.19	0.37	5.18
16:0 (Palmitic)	0.35	0.01	3.76	0.37	0.01	2.67	14.76	0.60	4.05
9*c*-18:1 (Oleic)	7.82	0.12	1.49	3.83	0.06	1.59	6.66	0.22	3.35
5*c*,8*c*,11*c*,14*c*-20:4 (ArA)	2.24	0.08	3.76	2.04	0.10	4.76	1.07	0.07	6.22
5*c*,8*c*,11*c*,14*c*,17*c*-20:5 (EPA)	47.02	2.20	4.68	38.34	2.16	5.64	16.62	1.00	6.04
4*c*,7*c*,10*c*,13*c*,16*c*-22:5 (n-6 DPA)	0.20	0.01	5.73	0.62	0.03	4.41	0.35	0.03	8.69
7*c*,10*c*,13*c*,16*c*,19*c*-22:5 (n-3 DPA)	1.22	0.05	4.17	2.57	0.14	5.39	2.01	0.11	5.45
4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-22:6 (DHA)	5.66	0.22	3.84	21.30	1.16	5.45	10.11	0.71	7.07
NLEA Total Fat (TAG Wt. %)	87.32	2.54	2.91	91.69	4.12	4.49	92.14	1.26	1.37
Total Saturated	3.99	0.23	5.69	3.99	0.38	9.62	26.94	0.59	2.19
Total Monounsaturated (MUFA)	14.84	0.35	2.36	11.89	1.36	11.45	19.98	0.69	3.44
Total Polyunsaturated (PUFA)	64.95	2.33	3.59	72.17	4.15	5.75	41.19	1.21	2.95
Total n-3 Polyunsaturated (PUFA)	59.41	2.42	4.08	66.95	3.45	5.16	33.36	1.88	5.64
Total n-6 Polyunsaturated (PUFA)	4.43	0.16	3.62	4.63	1.24	26.75	4.22	0.65	15.50

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Table 6
Collaborative study data from 13 laboratories.

	18/12 Fish Oil FA Wt%								
Labs = 13	Mean	Sr	RSD r	SR	RSD R	r	R	horrat	R/r
12:0 (Lauric)	0.10	0.00	3.52	0.01	13.54	0.01	0.04	2.40	3.86
14:0 (Myristic)	6.69	0.26	3.82	0.31	4.64	0.72	0.87	1.54	1.21
16:0 (Palmitic)	14.91	0.73	4.89	0.83	5.58	2.04	2.33	2.10	1.14
9c-18:1 (Oleic)	9.03	0.06	0.63	0.76	8.42	0.16	2.13	2.93	13.27
5*c*,8*c*,11*c*,14c-20:4 (ArA)	0.77	0.02	1.99	0.09	11.81	0.04	0.26	2.84	5.94
5*c*,8*c*,11*c*,14*c*,17c-20:5 (EPA)	15.18	0.24	1.57	0.76	4.97	0.67	2.11	1.87	3.17
7*c*,10*c*,13*c*,16*c*,19c-22:5 (n-3 DPA)	1.78	0.02	0.99	0.10	5.52	0.05	0.28	1.50	5.56
4*c*,7*c*,10*c*,13*c*,16*c*,19c-22:6 (DHA)	11.56	0.14	1.21	0.39	3.40	0.39	1.10	1.23	2.81
NLEA Total Fat (TAG wt. %)	93.61	0.60	0.64	2.54	2.71	1.68	7.11	1.34	4.24
NLEA Fatty Acid	89.52	0.57	0.64	2.43	2.72	1.60	6.81	1.34	4.25
Total Saturated	28.15	0.31	1.09	2.41	8.57	0.86	6.76	3.54	7.88
Total Monounsaturated (MUFA)	20.70	0.23	1.12	0.51	2.46	0.65	1.42	0.97	2.19
Total Polyunsaturated (PUFA)	41.41	0.33	0.80	1.08	2.60	0.92	3.01	1.14	3.26
Total n-3 Polyunsaturated (PUFA)	33.33	0.48	1.45	2.79	8.36	1.35	7.80	3.54	5.78
Total n-6 Polyunsaturated (PUFA)	2.89	0.05	1.66	0.25	8.61	0.13	0.70	2.52	5.17

	30/20 EE FA Wt%								
Labs = 13	Mean	Sr	RSD r	SR	RSD R	r	R	horrat	R/r
12:0 (Lauric)	0.00	0.00	43.56	0.00	48.38	0.00	0.00	0.25	0.00
14:0 (Myristic)	0.02	0.00	4.43	0.02	80.00	0.00	0.05	11.42	18.07
16:0 (Palmitic)	1.07	0.02	2.06	0.08	7.04	0.06	0.21	1.78	3.41
9c-18:1 (Oleic)	9.77	0.06	0.65	0.21	2.19	0.18	0.60	0.77	3.38
5*c*,8*c*,11*c*,14c-20:4 (ArA)	1.37	0.04	3.18	0.09	6.62	0.12	0.25	1.74	2.08
5*c*,8*c*,11*c*,14*c*,17c-20:5 (EPA)	27.99	0.17	0.60	0.47	1.69	0.47	1.33	0.70	2.80
7*c*,10*c*,13*c*,16*c*,19c-22:5 (n-3 DPA)	2.32	0.05	1.94	0.11	4.69	0.13	0.30	1.33	2.42
4*c*,7*c*,10*c*,13*c*,16*c*,19c-22:6 (DHA)	18.77	0.25	1.36	1.21	6.46	0.71	3.40	2.51	4.76
NLEA Total Fat (TAG Wt. %)	84.87	6.43	7.58	6.43	7.58	18.01	18.01	3.70	1.00
NLEA Fatty Acid	81.45	6.17	7.58	6.17	7.58	17.29	17.29	3.67	1.00
Total Saturated	6.46	0.08	1.28	0.26	3.97	0.23	0.72	1.31	3.11
Total Monounsaturated (MUFA)	17.91	0.13	0.74	1.01	5.66	0.37	2.84	2.19	7.66
Total Polyunsaturated (PUFA)	59.85	1.02	1.71	1.23	2.05	2.87	3.43	0.95	1.20
Total n-3 Polyunsaturated (PUFA)	54.43	0.37	0.68	1.43	2.62	1.03	4.00	1.20	3.87
Total n-6 Polyunsaturated (PUFA)	3.70	0.17	4.63	0.31	8.46	0.48	0.88	2.58	1.83

	Tuna Oil FA Wt%								
Labs = 13	Mean	Sr	RSD r	SR	RSD R	r	R	horrat	R/r
12:0 (Lauric)	0.02	0.00	3.72	0.02	74.65	0.00	0.05	10.65	20.04
14:0 (Myristic)	2.93	0.07	2.40	0.14	4.91	0.20	0.40	1.44	2.05
16:0 (Palmitic)	18.37	0.40	2.15	1.51	8.21	1.11	4.22	3.18	3.81
9c-18:1 (Oleic)	12.41	0.27	2.16	0.85	6.89	0.75	2.39	2.52	3.19
5*c*,8*c*,11*c*,14c-20:4 (ArA)	1.27	0.01	1.01	0.09	7.23	0.04	0.26	1.87	7.17
5*c*,8*c*,11*c*,14*c*,17c-20:5 (EPA)	4.81	0.11	2.36	0.28	5.73	0.32	0.77	1.82	2.43
7*c*,10*c*,13*c*,16*c*,19c-22:5 (n-3 DPA)	0.90	0.02	2.30	0.05	6.10	0.06	0.15	1.50	2.65
4*c*,7*c*,10*c*,13*c*,16*c*,19c-22:6 (DHA)	20.87	0.42	2.02	1.04	4.99	1.18	2.92	1.97	2.47
NLEA Total Fat (TAG Wt. %)	87.89	1.96	2.23	3.79	4.31	5.48	10.60	2.11	1.93
NLEA Fatty Acid	84.15	1.87	2.23	3.62	4.31	5.24	10.15	2.10	1.93
Total Saturated	30.64	0.67	2.18	2.23	7.28	1.87	6.25	3.05	3.34
Total Monounsaturated (MUFA)	20.88	0.48	2.29	1.26	6.03	1.34	3.53	2.38	2.64
Total Polyunsaturated (PUFA)	32.75	0.45	1.36	2.72	8.30	1.25	7.61	3.51	6.10
Total n-3 Polyunsaturated (PUFA)	28.73	0.57	1.98	1.61	5.60	1.59	4.50	2.32	2.83
Total n-6 Polyunsaturated (PUFA)	4.34	0.13	2.94	0.32	7.42	0.36	0.90	2.31	2.52

(Continued)

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Table 6
(Continued)

	ArA SCO FA Wt%								
Labs = 13	Mean	Sr	RSD r	SR	RSD R	r	R	horrat	R/r
12:0 (Lauric)	0.01	0.00	4.65	0.01	117.71	0.00	0.03	14.69	25.00
14:0 (Myristic)	0.48	0.01	2.20	0.05	10.19	0.03	0.14	2.28	4.64
16:0 (Palmitic)	10.35	0.14	1.32	0.80	7.70	0.38	2.23	2.74	5.84
9c-18:1 (Oleic)	16.60	0.18	1.09	1.00	6.03	0.51	2.80	2.30	5.53
5*c*,8*c*,11*c*,14c-20:4 (ArA)	39.62	1.16	2.92	1.29	3.24	3.24	3.60	1.41	1.11
5*c*,8*c*,11*c*,14*c*,17c-20:5 (EPA)	0.07	0.01	6.93	0.05	73.44	0.01	0.15	12.36	10.57
7*c*,10*c*,13*c*,16*c*,19c-22:5 (n-3 DPA)	0.00	0.00	49.82	0.00	63.28	0.00	0.00	0.31	0.00
4*c*,7*c*,10*c*,13*c*,16*c*,19c-22:6 (DHA)	0.00	0.00	38.92	0.00	44.93	0.00	0.00	0.23	0.00
NLEA Total Fat (TAG Wt. %)	97.18	1.41	1.45	4.47	4.60	3.95	12.53	2.29	3.17
NLEA Fatty Acid	93.10	1.35	1.45	4.26	4.57	3.78	11.92	2.26	3.16
Total Saturated	22.25	0.18	0.82	1.15	5.16	0.51	3.22	2.06	6.29
Total Monounsaturated (MUFA)	17.69	0.21	1.16	1.19	6.73	0.57	3.33	2.59	5.80
Total Polyunsaturated (PUFA)	51.95	0.71	1.36	2.05	3.94	1.98	5.74	1.79	2.90
Total n-3 Polyunsaturated (PUFA)	0.17	0.03	18.50	0.14	84.02	0.09	0.40	16.06	4.54
Total n-6 Polyunsaturated (PUFA)	50.91	0.69	1.36	2.02	3.98	1.94	5.67	1.80	2.92

	Tuna Oil FA Wt%								
Labs = 13	Mean	Sr	RSD r	SR	RSD R	r	R	horrat	R/r
12:0 (Lauric)	0.03	0.01	26.46	0.03	83.63	0.02	0.07	12.43	3.16
14:0 (Myristic)	2.97	0.03	1.10	0.17	5.81	0.09	0.48	1.71	5.29
16:0 (Palmitic)	18.40	0.22	1.20	1.65	8.98	0.62	4.62	3.48	7.50
9c-18:1 (Oleic)	12.45	0.19	1.50	1.01	8.14	0.52	2.84	2.97	5.41
5*c*,8*c*,11*c*,14c-20:4 (ArA)	1.32	0.04	2.81	0.06	4.41	0.10	0.16	1.15	1.57
5*c*,8*c*,11*c*,14*c*,17c-20:5 (EPA)	4.72	0.08	1.62	0.59	12.53	0.21	1.66	3.96	7.73
7*c*,10*c*,13*c*,16*c*,19c-22:5 (n-3 DPA)	0.90	0.02	2.51	0.14	15.20	0.06	0.38	3.74	6.04
4*c*,7*c*,10*c*,13*c*,16*c*,19c-22:6 (DHA)	20.58	0.21	1.01	0.80	3.88	0.58	2.24	1.53	3.84
NLEA Total Fat (TAG Wt. %)	90.05	0.85	0.94	4.61	5.12	2.38	12.91	2.52	5.42
NLEA Fatty Acid	86.20	0.82	0.95	4.41	5.11	2.28	12.34	2.50	5.41
Total Saturated	30.12	0.33	1.10	1.58	5.26	0.93	4.44	2.20	4.78
Total Monounsaturated (MUFA)	21.67	0.49	2.27	2.56	11.80	1.38	7.16	4.69	5.19
Total Polyunsaturated (PUFA)	32.76	1.04	3.18	3.53	10.76	2.92	9.87	4.55	3.39
Total n-3 Polyunsaturated (PUFA)	27.83	0.80	2.88	1.71	6.14	2.24	4.79	2.53	2.13
Total n-6 Polyunsaturated (PUFA)	4.35	0.14	3.32	0.31	7.25	0.40	0.88	2.26	2.18

	DHA SCO FA Wt%								
Labs = 13	Mean	Sr	RSD r	SR	RSD R	r	R	horrat	R/r
12:0 (Lauric)	3.19	0.08	2.57	0.20	6.21	0.23	0.55	1.85	2.41
14:0 (Myristic)	10.88	0.24	2.23	0.43	3.93	0.68	1.20	1.41	1.76
16:0 (Palmitic)	10.46	0.19	1.77	0.92	8.77	0.52	2.57	3.12	4.95
9c-18:1 (Oleic)	21.10	0.34	1.63	1.46	6.91	0.96	4.08	2.73	4.25
5*c*,8*c*,11*c*,14c-20:4 (ArA)	0.00	0.00	69.13	0.00	69.13	0.00	0.00	0.34	0.00
5*c*,8*c*,11*c*,14*c*,17c-20:5 (EPA)	0.00	0.00	78.18	0.00	78.18	0.00	0.00	0.38	0.00
7*c*,10*c*,13*c*,16*c*,19c-22:5 (n-3 DPA)	0.35	0.01	3.10	0.02	6.31	0.03	0.06	1.35	2.03
4*c*,7*c*,10*c*,13*c*,16*c*,19c-22:6 (DHA)	39.39	0.71	1.81	2.07	5.26	1.99	5.80	2.29	2.91
NLEA Total Fat (TAG Wt. %)	95.11	1.37	1.44	4.83	5.07	3.83	13.51	2.52	3.52
NLEA Fatty Acid	91.00	1.31	1.44	4.59	5.04	3.67	12.85	2.49	3.51
Total Saturated	26.49	0.66	2.49	1.57	5.92	1.85	4.39	2.42	2.38
Total Monounsaturated (MUFA)	23.49	0.41	1.74	1.68	7.17	1.14	4.72	2.88	4.13
Total Polyunsaturated (PUFA)	40.96	0.74	1.80	1.97	4.81	2.06	5.52	2.10	2.68
Total n-3 Polyunsaturated (PUFA)	39.77	0.71	1.79	2.02	5.08	2.00	5.65	2.21	2.83
Total n-6 Polyunsaturated (PUFA)	1.16	0.04	3.33	0.13	11.03	0.11	0.36	2.82	3.32

(Continued)

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Table 6
(Continued)

	48/25 Fish Oil FA Wt%								
Labs = 13	Mean	Sr	RSD r	SR	RSD R	r	R	horrat	R/r
12:0 (Lauric)	0.00	0.00	53.68	0.00	60.91	0.00	0.00	0.30	0.00
14:0 (Myristic)	0.28	0.01	3.05	0.03	10.42	0.02	0.08	2.15	3.41
16:0 (Palmitic)	0.68	0.01	1.75	0.06	8.53	0.03	0.16	2.01	4.86
9c-18:1 (Oleic)	4.92	0.15	3.10	0.28	5.69	0.43	0.78	1.81	1.84
5*c*,8*c*,11*c*,14c-20:4 (ArA)	1.80	0.06	3.16	0.08	4.68	0.16	0.24	1.28	1.48
5*c*,8*c*,11*c*,14*c*,17c-20:5 (EPA)	31.92	1.15	3.60	1.55	4.87	3.22	4.35	2.05	1.35
7*c*,10*c*,13*c*,16*c*,19c-22:5 (n-3 DPA)	2.37	0.09	3.93	0.11	4.61	0.26	0.31	1.31	1.17
4*c*,7*c*,10*c*,13*c*,16*c*,19c-22:6 (DHA)	17.81	0.55	3.11	0.86	4.84	1.55	2.41	1.87	1.56
NLEA Total Fat (TAG Wt. %)	82.68	1.82	2.20	4.50	5.45	5.09	12.61	2.65	2.48
NLEA Fatty Acid	79.38	1.75	2.20	4.32	5.45	4.89	12.11	2.63	2.48
Total Saturated	5.61	0.18	3.14	0.72	12.76	0.49	2.01	4.14	4.07
Total Monounsaturated (MUFA)	11.60	0.36	3.15	1.94	16.69	1.02	5.42	6.03	5.30
Total Polyunsaturated (PUFA)	62.23	1.46	2.34	4.52	7.26	4.08	12.65	3.38	3.10
Total n-3 Polyunsaturated (PUFA)	56.70	1.49	2.62	2.09	3.69	4.17	5.86	1.70	1.41
Total n-6 Polyunsaturated (PUFA)	3.40	0.11	3.23	0.85	25.01	0.31	2.38	7.52	7.73

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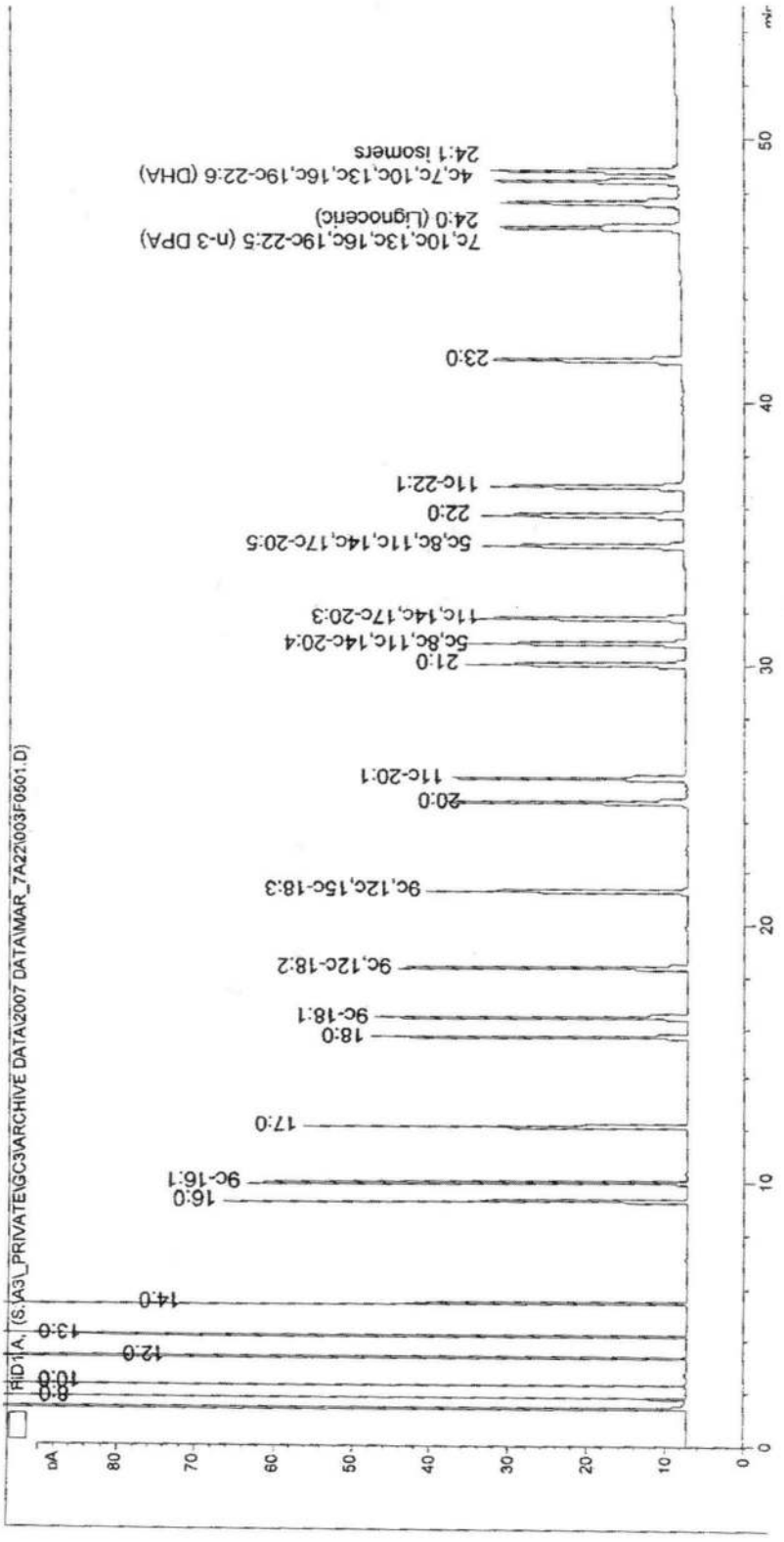

Figure 1. GC/FID profile of GLC-714 (25 peaks). Separations were on a SUPELCOWAX®-10 column (30 m × 0.25 mm × 0.25 µm). Column temperature was 170°C to 225°C @ 1°C/min. Carrier gas was hydrogen at 1.2 mL/min (constant flow) with a linear vleocity of 43 cm/s. Pressure was 11.34 psi @ 170°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100.

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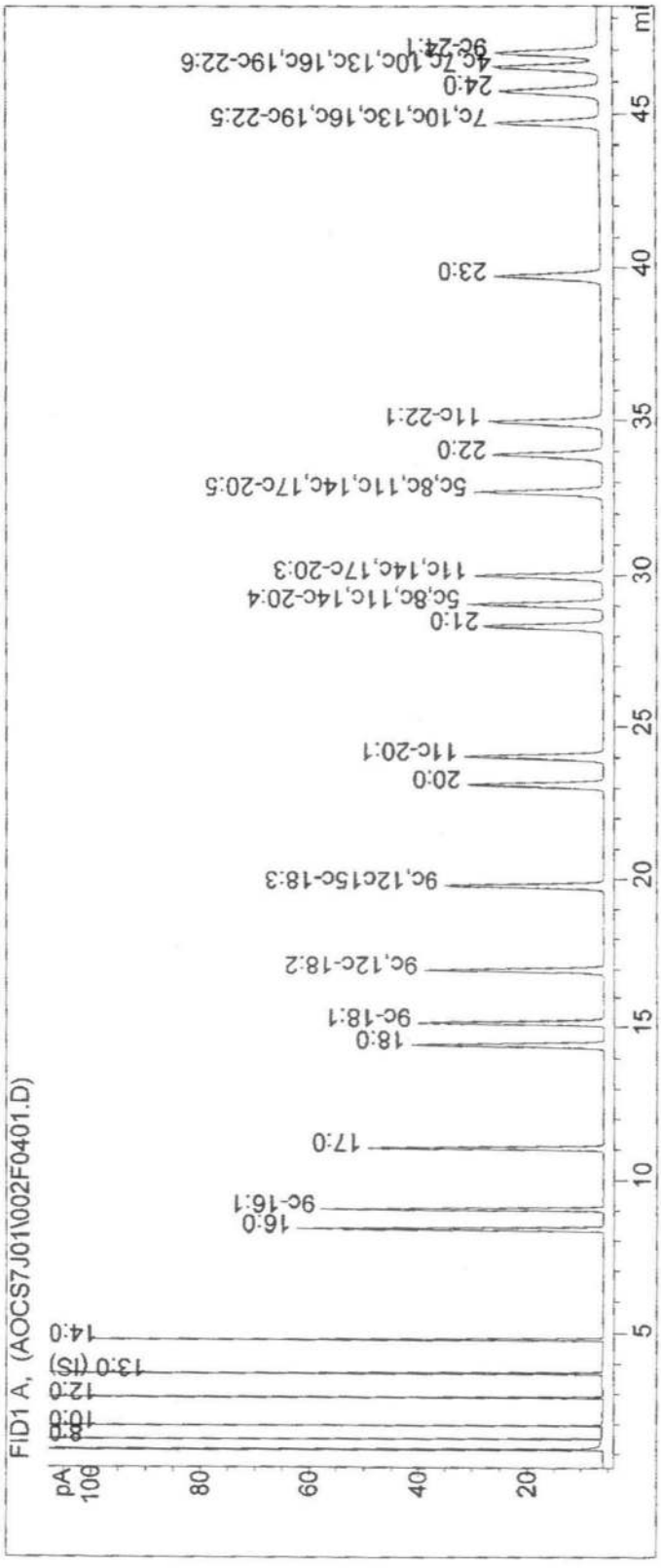

Figure 2. GC/FID profile of GLC-714 (25 peaks) at 20 mg/mL. Separations were on a SUPELCOWAX®-10 column (30 m × 0.25 mm × 0.25 µm). Column temperature was 170°C to 225°C @ 1°C/min. Carrier gas was Helium at 2.4 mL/min (constant flow) with a linear vleocity of 53 cm/s. Pressure was 32.77 psi @ 170°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100.

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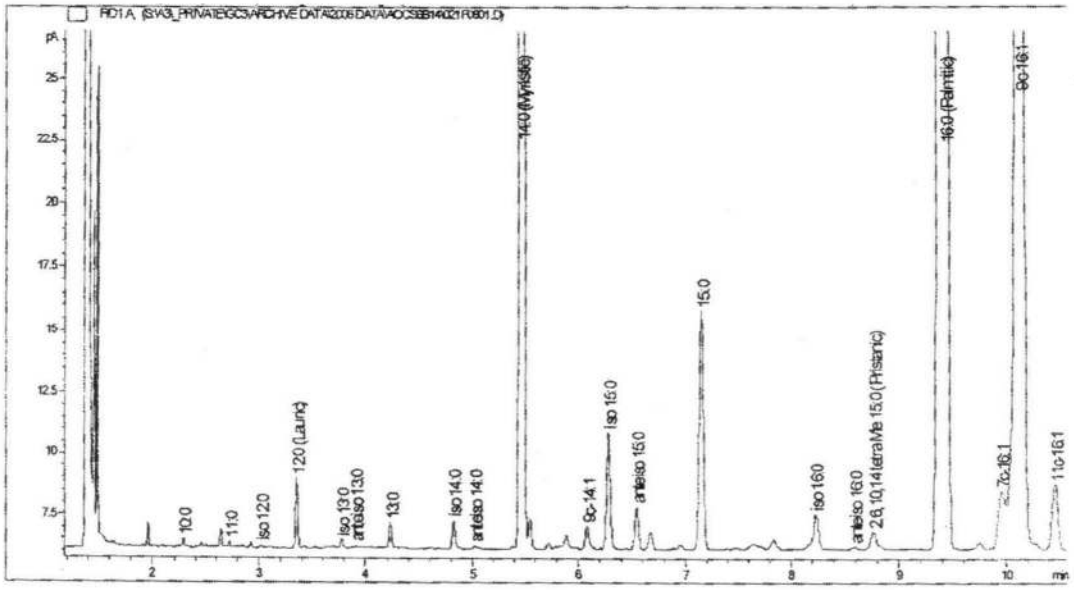

Figure 3. GC/FID profile of LCP Menhaden Oil (Arista Industries, Inc.) from start to 11*c*-16:1. Separations were on a SUPELCOWAX®-10 column (30 m × 0.25 mm × 0.25 µm). Column temperature was 170°C to 225°C @ 1°C/min. Carrier gas was *hydrogen* at 1.2 mL/min (constant flow) with a linear vleocity of 43 cm/s. Pressure was 11.34 psi @ 170°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100. Pristanic acid is a doublet peak composed of diastereomers 2L, 6D, 10D and 2D, 6D, 10D, respectively (References 15 and 16).

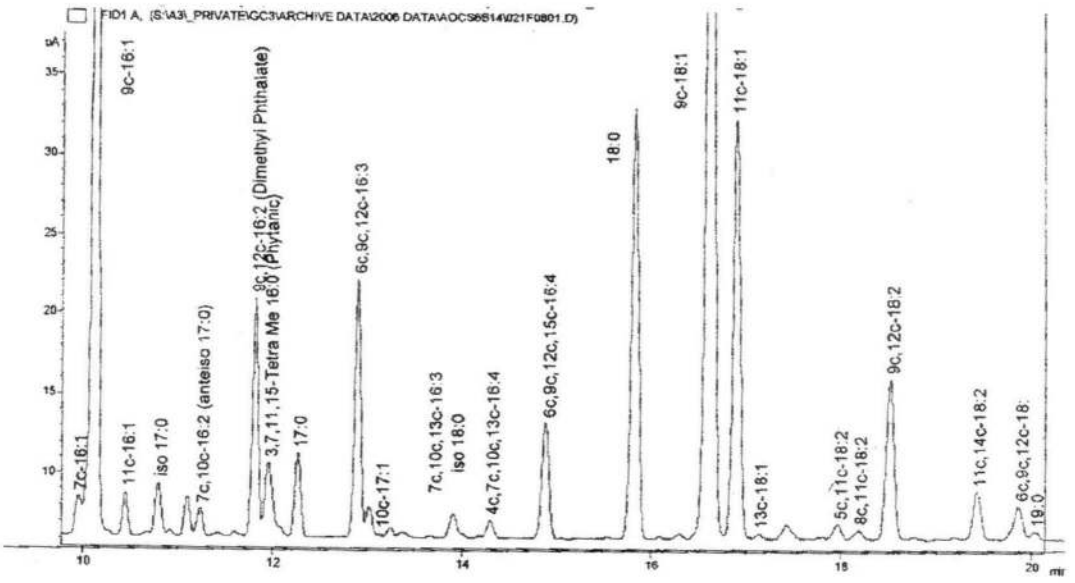

Figure 4. GC/FID profile of LCP Menhaden Oil (Arista Industries, Inc.) from 7*c*-16:1 to 19:0. Phytanic acid is a doublet peak composed of diastereomers 3L, 7D, 11D and 3D, 7D, 11D, respectively (References 15 and 16). See Figure 3 for GC parameters.

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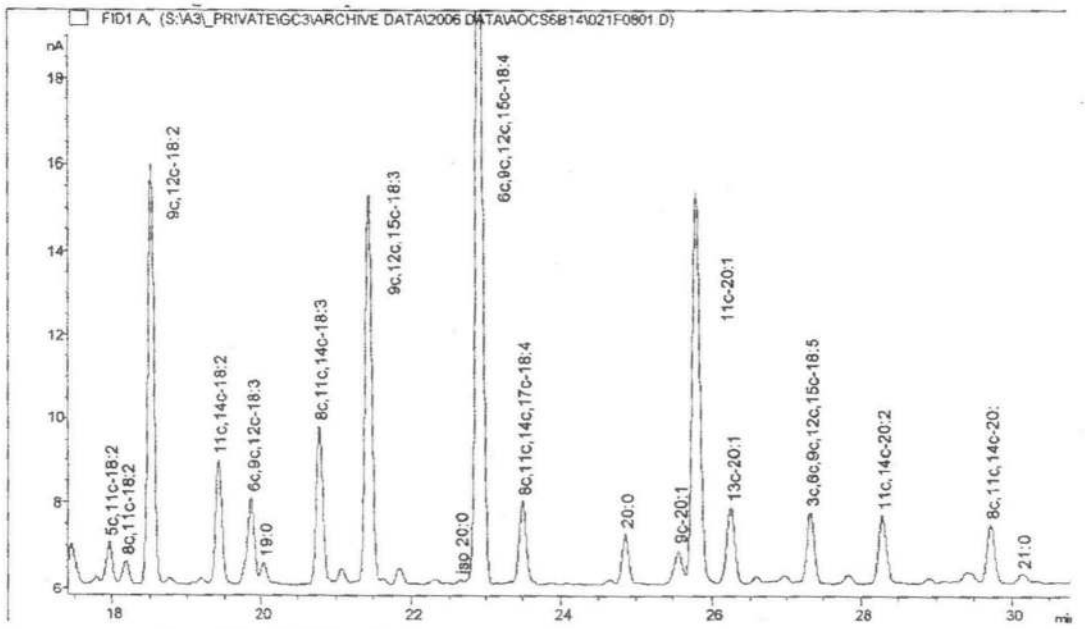

Figure 5. GC/FID profile of LCP Menhaden Oil (Arista Industries, Inc.) from 5*c*,11*c*-18:2 to 21:0. See Figure 3 for GC parameters.

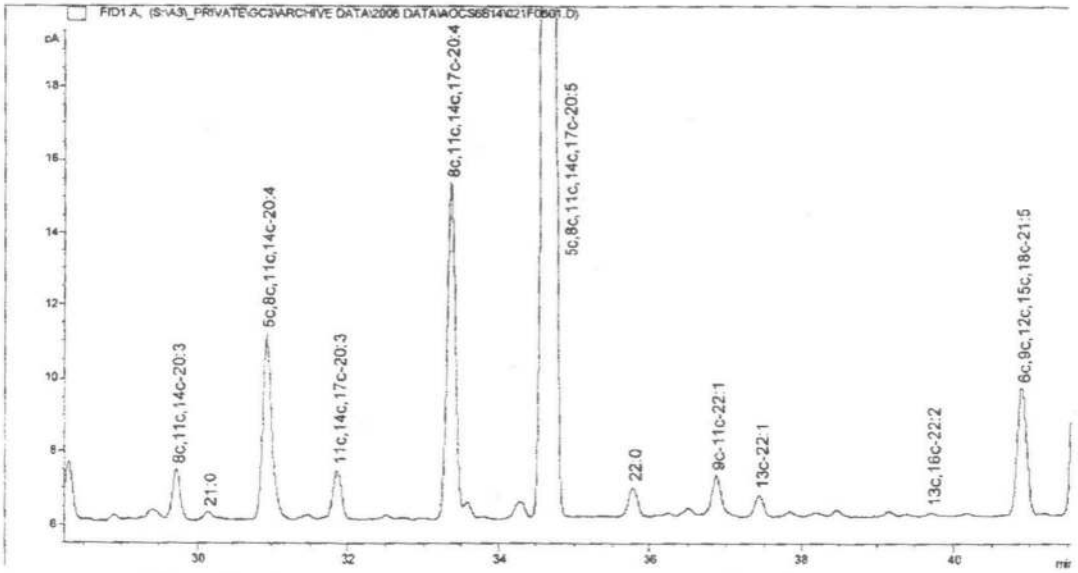

Figure 6. GC/FID profile of LCP Menhaden Oil (Arista Industries, Inc.) from homogamma 8*c*,11*c*,14*c*-20:3 to 6*c*,9*c*,12*c*,15*c*,18*c*-21:5. See Figure 3 for GC parameters.

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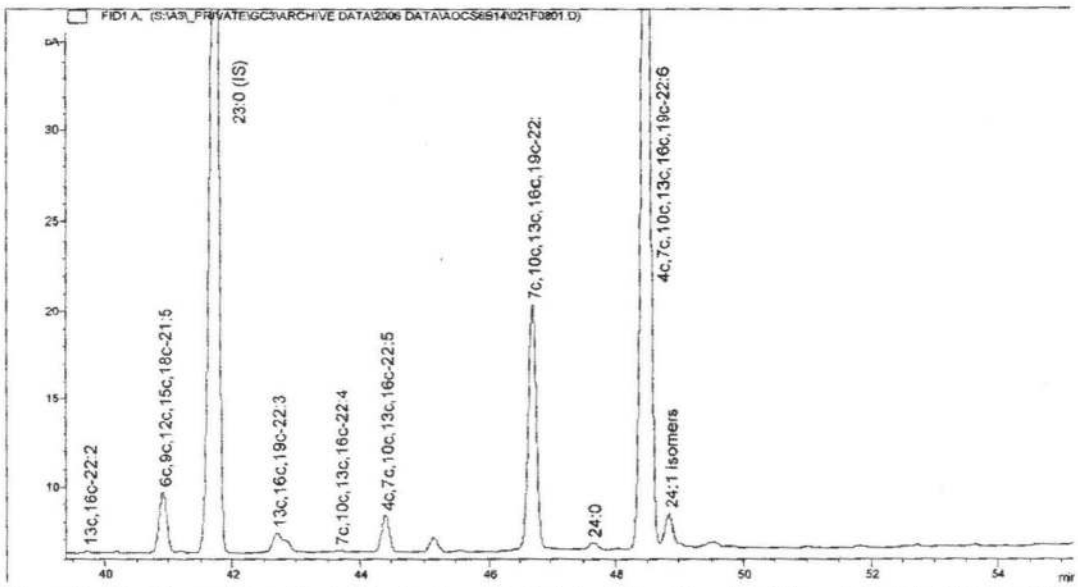

Figure 7. GC/FID profile of LCP Menhaden Oil (Arista Industries, Inc.) from 13*c*,16*c*-22:2 to 24:1 isomers. See Figure 3 for GC parameters.

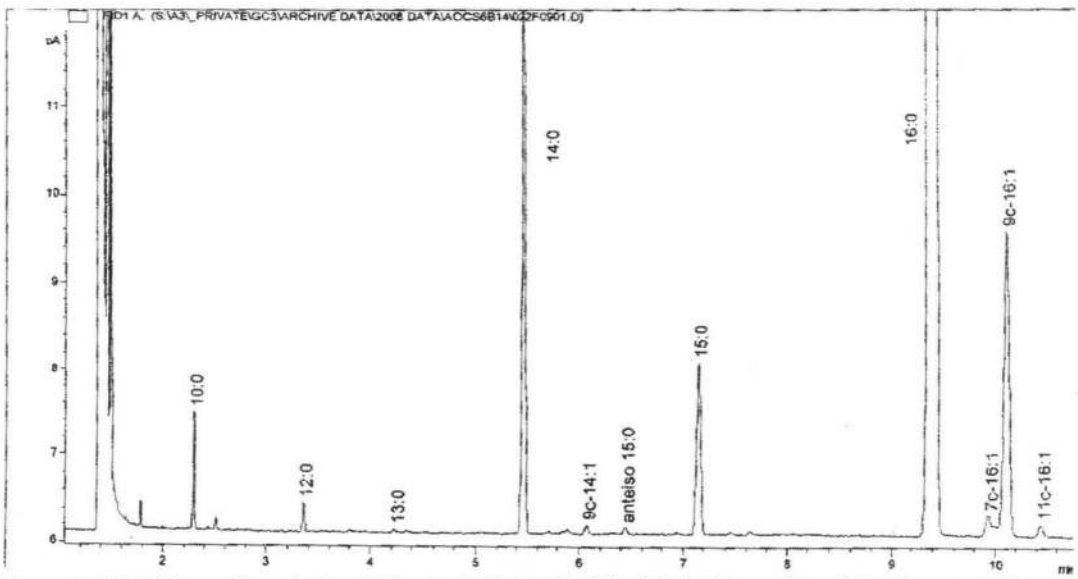

Figure 8. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from start to 11*c*-16:1. Separations were on a SUPELCOWAX®-10 column (30 m × 0.25 mm × 0.25 µm). Column temperature was 170°C to 225°C @ 1°C/min. Carrier gas was *hydrogen* at 1.2 mL/min (constant flow) with a linear vleocity of 43 cm/s. Pressure was 11.34 psi @ 170°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100.

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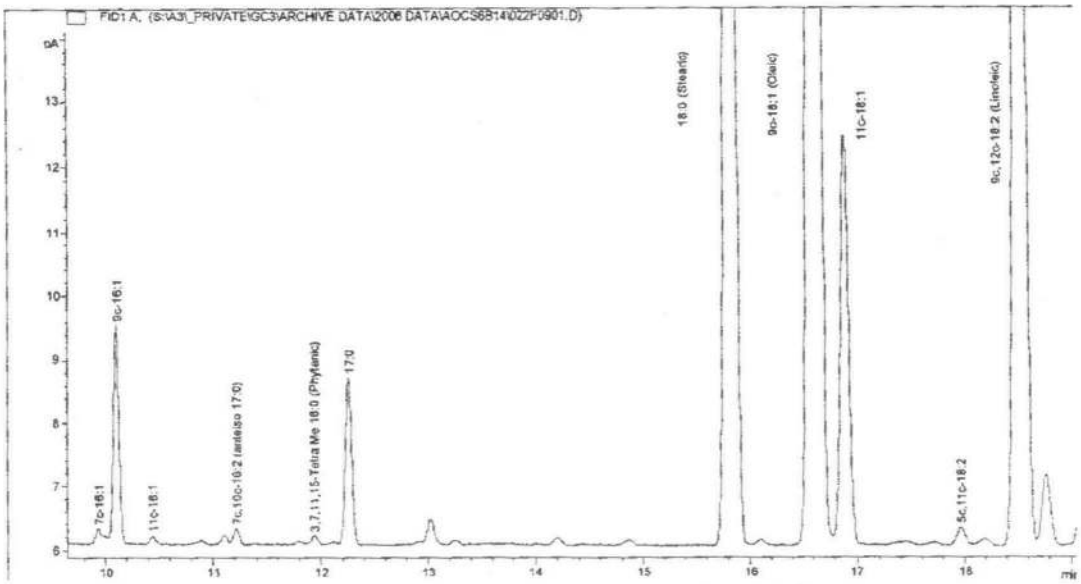

Figure 9. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from 7*c*-16:1 to 9*c*,12*c*-18:2. See Figure 8 for GC parameters.

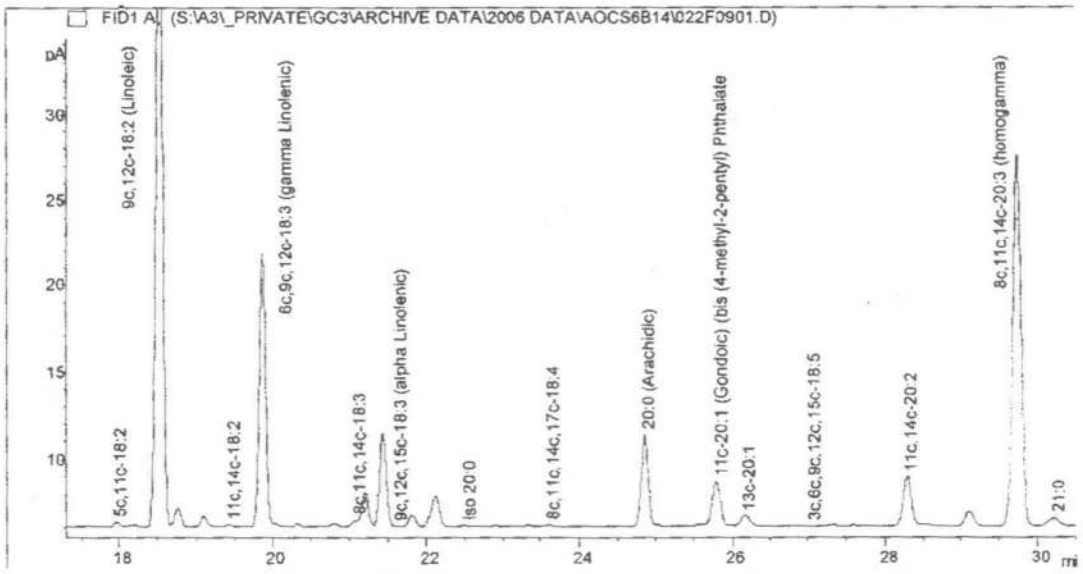

Figure 10. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from 5*c*,11*c*-18:2 to 21:0. See Figure 8 for GC parameters.

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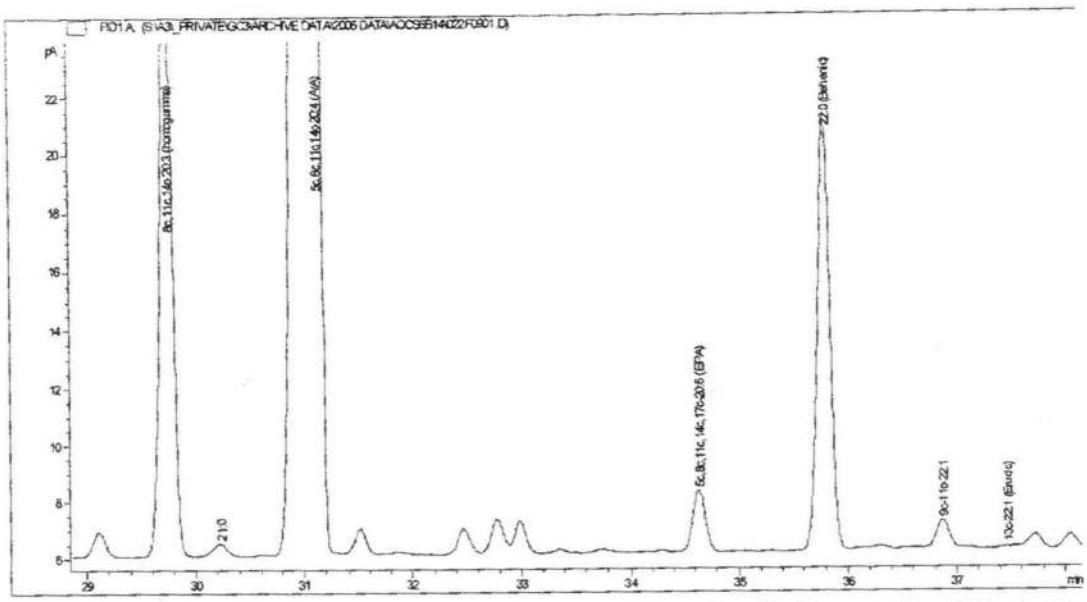

Figure 11. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from homogamma 8c,11c,14c-20:3 to 13c-22:1. See Figure 8 for GC parameters.

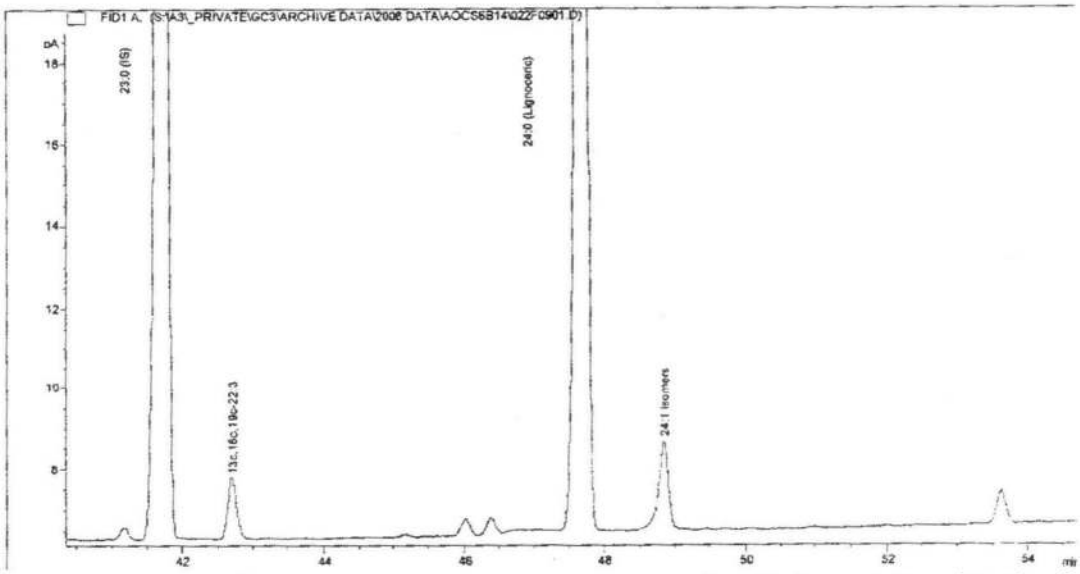

Figure 12. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from 23:0 to 24:1 isomers. See Figure 8 for GC parameters.

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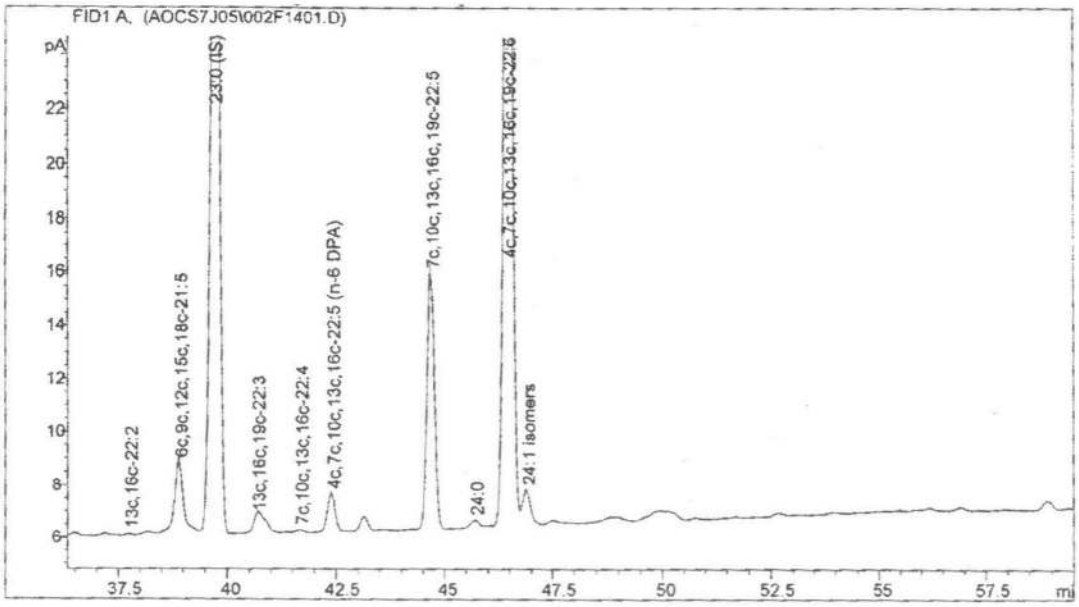

Figure 17. GC/FID profile of LCP Menhaden Oil (Arista Industries, Inc.) from 13c,16c-22:2 to 24:1 isomers. See Figure 13 for GC parameters.

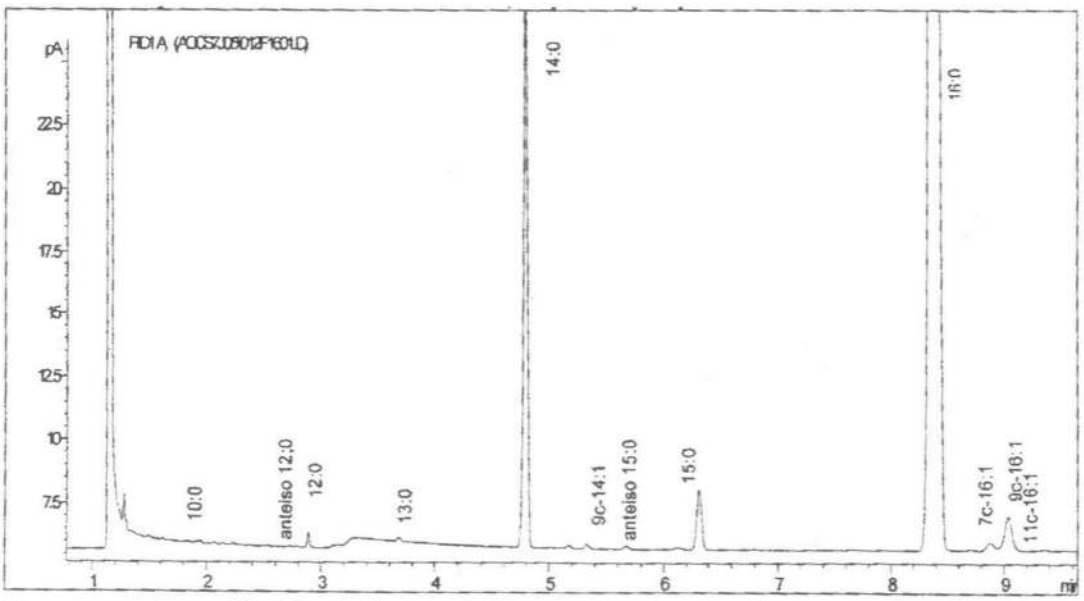

Figure 18. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from start to 11c-16:1. Separations were on a SUPELCOWAX®-10 column (30 m × 0.25 mm × 0.25 µm). Column temperature was 170°C to 225°C @ 1°C/min. Carrier gas was *helium* at 2.4 mL/min (constant flow) with a linear vleocity of 53 cm/s. Pressure was 32.77 psi @ 170°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100.

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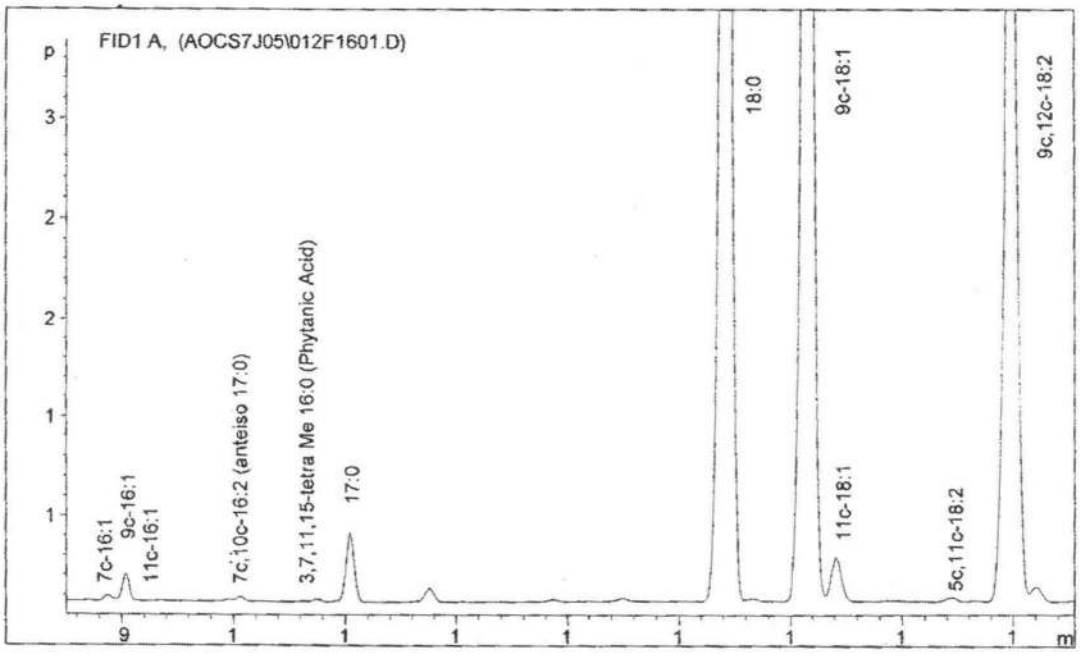

Figure 19. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from 7*c*-16:1 to 9c,12*c*-18:2. See Figure 18 for GC parameters.

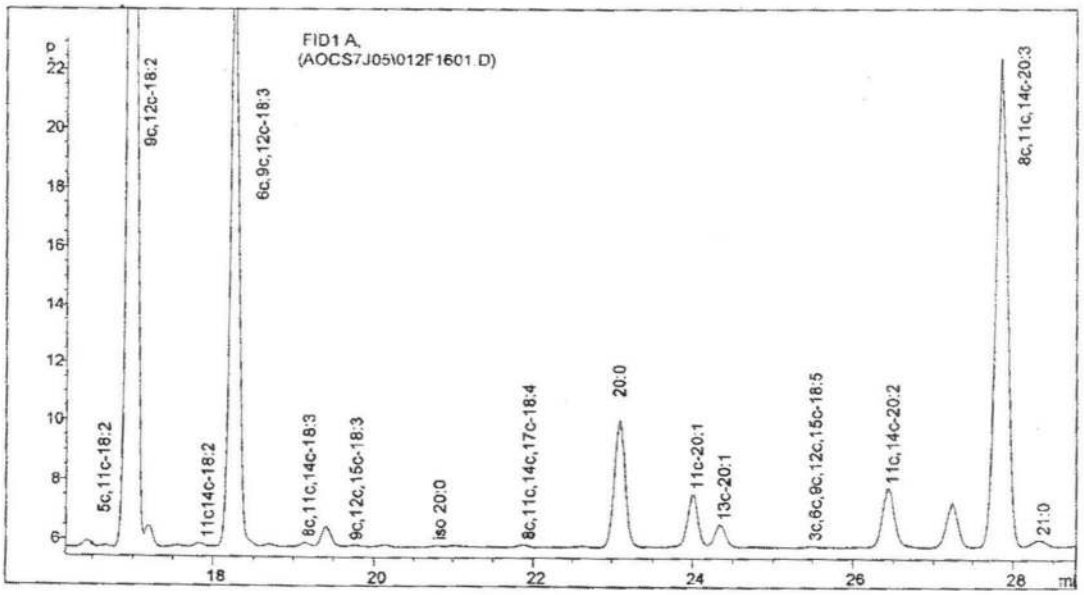

Figure 20. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from 5*c*,11*c*-18:2 to 21:0. See Figure 18 for GC parameters.

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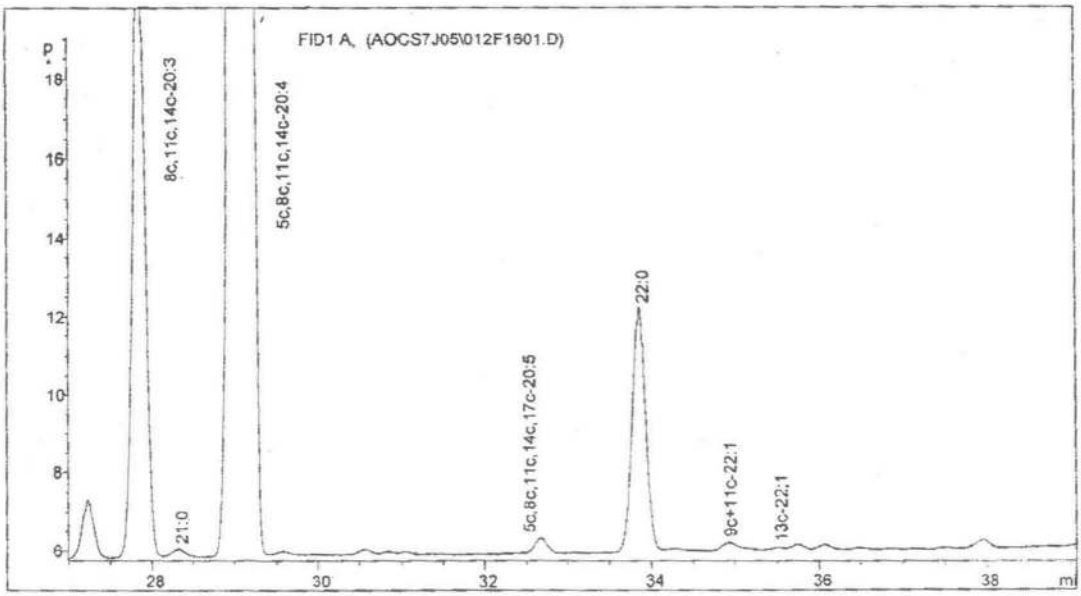

Figure 21. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from homogamma 8*c*,11*c*,14*c*-20:3 to 13*c*-22:1. See Figure 18 for GC parameters.

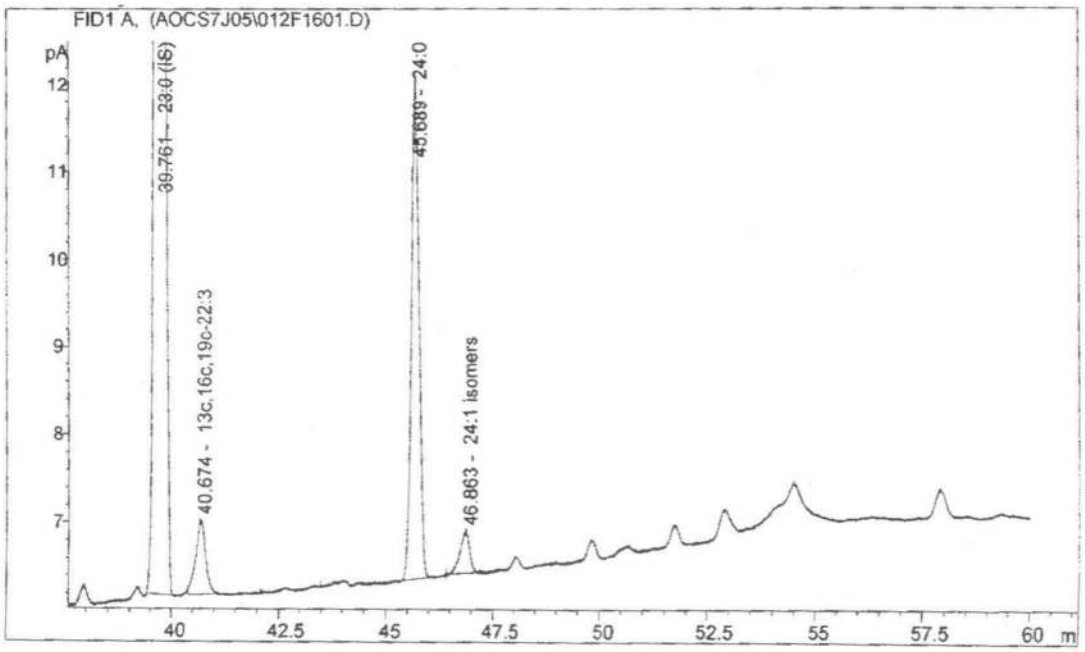

Figure 22. GC/FID profile of Arachidonic Acid (ArA) Oils (CABIO) produced by fermentation of *Mortierella alpina ALK-1* Fungus from 23:0 to 24:1 isomers. See Figure 18 for GC parameters.

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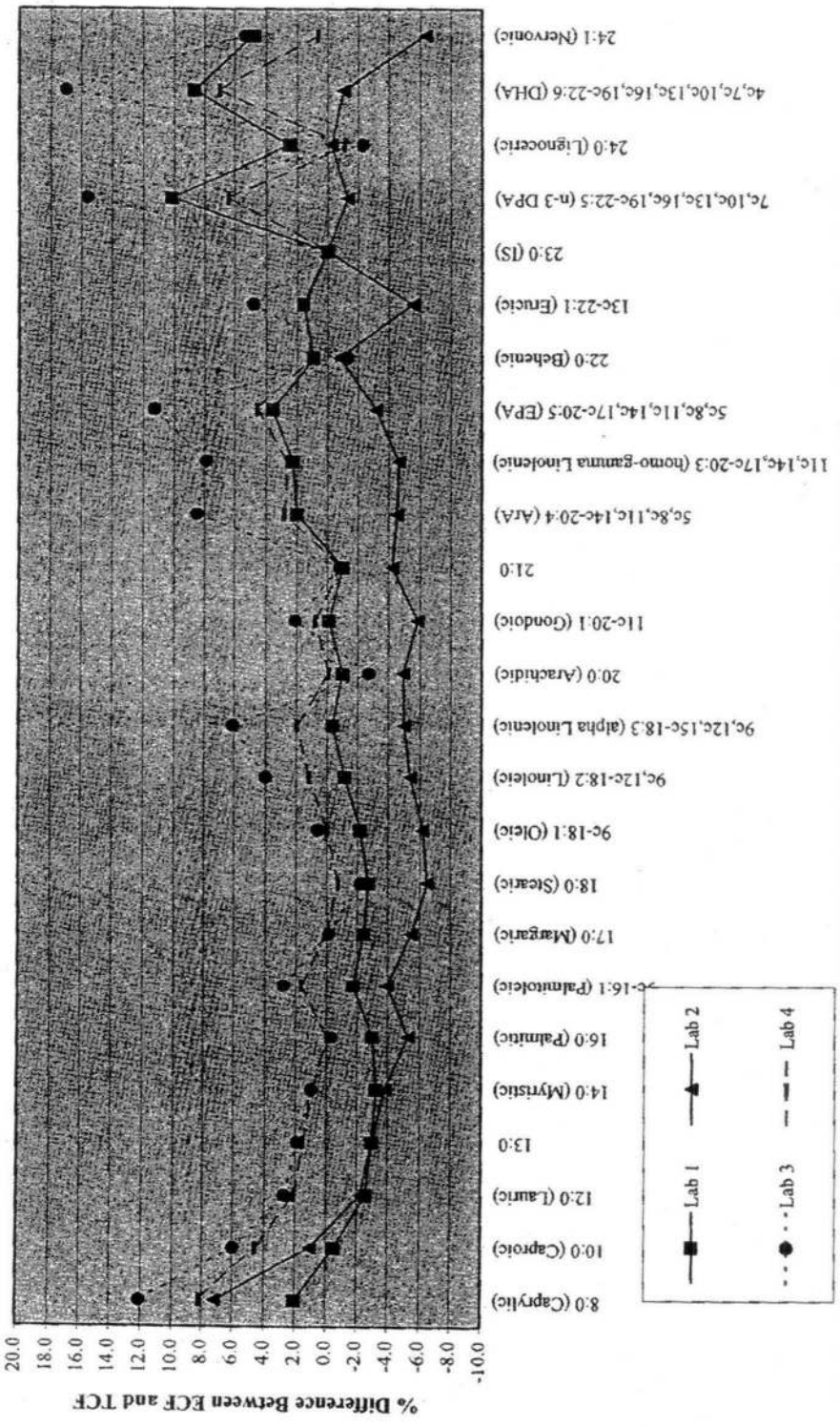

Figure 23. Plot of % Difference between ECF and TCF from four different labs for the Pre-Collaborative. If the % Difference is greater than three for the saturated and monounsaturated FAME, it is suggested the GC parameters (e.g., detector gases, carrier gas flows, and column choice, etc.) be reoptimized to achieve a difference of 3% or less before continuing with analysis.

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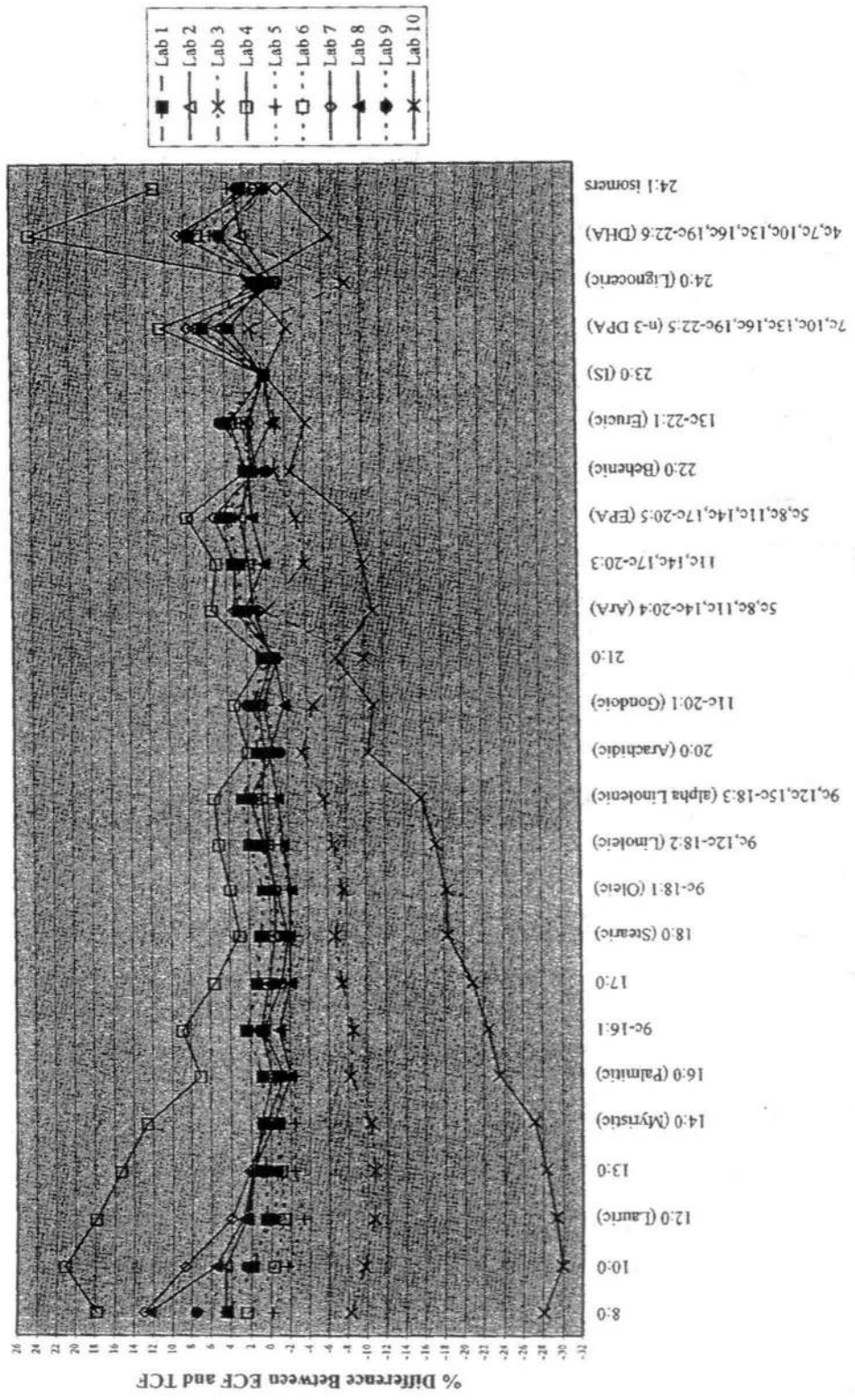

Figure 24. Plot of % Difference between ECF and TCF from ten collaborative labs (outliers have been removed). If the % Difference is greater than three for the saturated and monounsaturated FAME, it is suggested the GC parameters (e.g., detector gases, carrier gas flows, and column choice, etc.) be reoptimized to achieve a difference of 3% or less before continuing with analysis.

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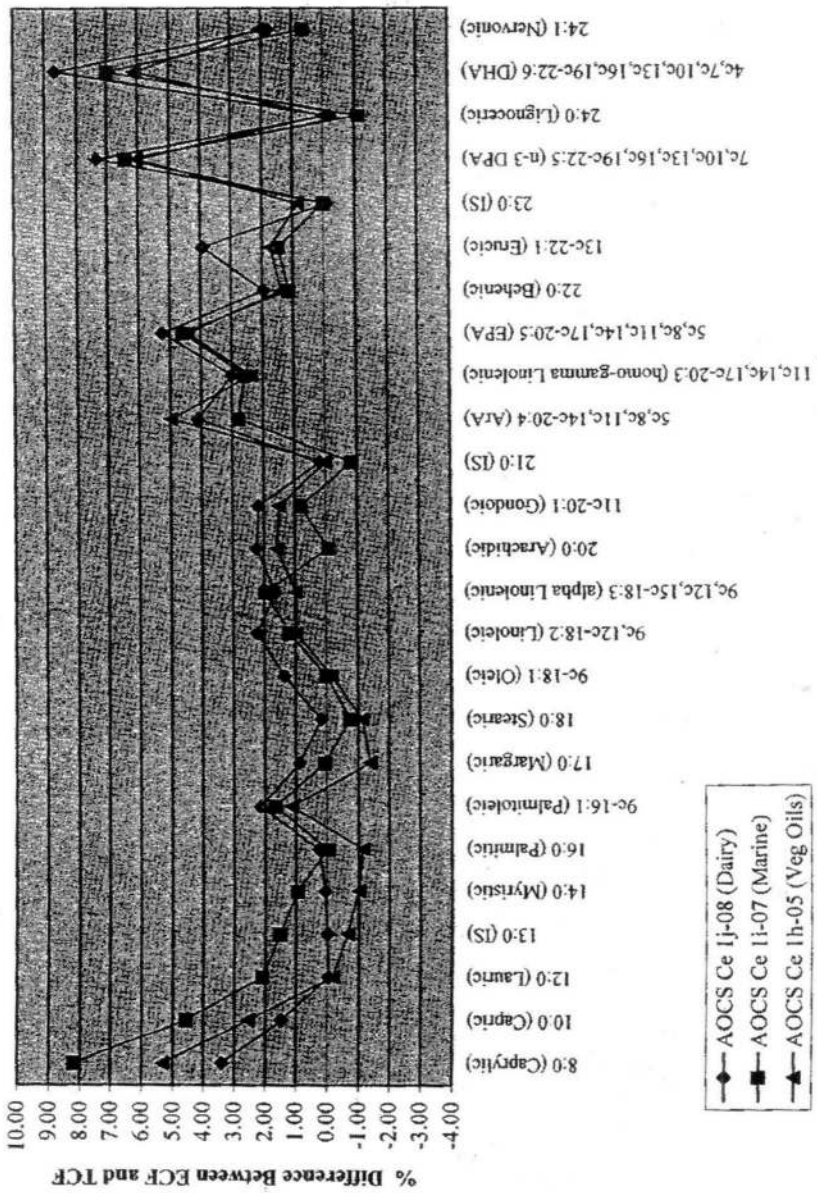

Figure 25. Plot of % Difference between ECF and TCF from one lab for the different AOCS methods. If the % Difference is greater than three for the saturated and monounsaturated FAME, it is suggested the GC parameters (e.g., detector gases, carrier gas flows, and column choice, etc.) be reoptimized to achieve a difference of 3% or less before continuing with analysis.

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AOCS

AOCS Official Method Ce 1j-07

Revised 2013

Determination of *cis-*, *trans-*, Saturated, Monounsaturated, and Polyunsaturated Fatty Acids in Extracted Fats by Capillary GLC

DEFINITION

This method provides a gas–liquid chromatography (GLC) procedure for the determination of the fatty acid composition, including the *trans* fatty acid isomers of extracted fats. The fatty acid methyl esters (FAME) are separated on a capillary gas chromatography column having a highly polar stationary phase, according to their chain length (CL), degree of unsaturation, and geometry and position of the double bonds [DB(s)].

SCOPE

This method evaluates, by a single capillary GLC procedure, the levels of *trans* isomers, saturated fatty acid (SFA), *cis-* and *trans-*monounsaturated fatty acid (MUFA), and *cis-* and *trans-*polyunsaturated fatty acid (PUFA) in fat samples where the source of the fat is unknown or is of dairy or ruminant origins. The method is not designed to provide detailed definitive isomer composition that may be desired for nutritional and/or biochemical purposes. To obtain more detailed isomeric composition information prior argentation chromatographic separations and/or additional GC analyses are required. For nutritional labeling purposes the total fat, saturated, *cis-*monounsaturated, *cis-*polyunsaturated and *trans* fatty acid contents are to be determined. This method may also determine *cis-*polyunsaturated fatty acids (PUFA), including arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).

This method utilizes a triacylglycerol (13:0 TAG) internal standard (IS) for determining the concentration of the individual fatty acids in the oil samples after methylation. The method is applicable to fats derived from dairy and ruminant products. This method is not applicable to products containing mixtures of both dairy and vegetable fats as the *trans* linolenic acid (18:3) isomers will coelute with the gondoic acid (20:1) isomers. In that case both this method coupled with AOCS Ce 1h-05 would be required for analysis. Conjugated linoleic acids (CLAs) will be present in dairy and ruminant fats and may be quantitated with this method, however for nutritional labeling purposes CLA are not included either as *cis-* or *trans-*PUFA (References 1, 2). There is minor co-elution of *cis-* and *trans-* fatty acid isomers, particularly in the 16:1, 17:1, and 18:1 regions, using this technique.

Theoretical Correction Factors (TCFs) are used to quantitate all saturated, monounsaturated, and polyunsaturated fatty acids (PUFA) of 18 carbons. TCFs are also used for fatty acids, which lack pure standards. Empirical Correction Factors (ECFs) are used for very long chain PUFA of 20 carbons or more and three or more double bonds for which standards are readily available.

APPARATUS

1. Gas chromatograph: suitable for use with a capillary column equipped with:
   (a) Thermostatic chamber for the capillary column, capable of maintaining the desired temperature to ± 0.1°C.
   (b) Temperature-controlled split mode injection unit.
   (c) Flame-ionization detector (FID), amplifier, and electronic recorder-integrator.
2. Capillary column: Fused silica capillary 100 m, and 0.25 mm i.d., coated with SP™-2560 or CP-Sil™ 88, 100% cyanopropylsilicone stationary phase, to a thickness of 0.20 μm. Commercially prepared columns are available from: SP™-2560 (Part Number 24056, Supelco, Inc. Bellefonte, PA, USA) and CP-Sil™ 88 (Part Number CP7489, Varian, Walnut Creek, CA, USA; Chrompack, Middleburg, The Netherlands).
3. Microsyringe for gas chromatography—10 μL delivery, with a hardened needle.
4. Carrier gas—hydrogen or helium, 99.99% pure or better, gas chromatography quality, dried, and oxygen removed by suitable filters (see Notes, 1).
5. Flame gases—hydrogen and air, gas chromatography quality.
6. Make-up gas—nitrogen or helium, gas chromatography quality.
7. Injection port split liner—4 mm i.d., 6.3 mm o.d. × 78.5 mm base deactivated precision (Focus) split liner with glass wool (e.g., Catalog #21022-211.5, Restek; P/N 092002, SGE or equivalent) (see Notes, 2).

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REAGENTS

Unless otherwise stated, use only reagents as specified in ISO 6353 (Parts 2 and 3) (Reference, 3) if listed there; if not, then use reagents of recognized analytical grade and water of at least grade 3 as defined in ISO 3696 (Reference, 4).

1. Reference FAMEs—Methyl esters of pure fatty acids, in particular *cis-* and *trans* isomers of octadecenoic (oleic), *trans* isomers of octadecadienoic (linoleic) and octadecatrienoic (α-linolenic) acids. Wide ranges of *cis-* and *trans* octadecenoic methyl ester isomers are available on the market (e.g., from Alltech Associates, Inc., Deerfield, IL, Nu-Chek-Prep, Elysian, MN, Supelco Inc., Bellefonte, PA, and Sigma Chemical Co., St. Louis, MO). *trans* Geometrical isomers of linoleic and α-linolenic acids can be prepared in the laboratory with the aid of p-toluenesulfonic acid (Reference 5) and are commercially available from Supelco, Inc.
2. Triacylglycerol (TAG) IS solution (for calculating fatty acid data as mg per 100 g oil or 100 g of food sample)—13:0 TAG—tritridecanoin (purity >99%) 5.0 mg/mL in *n*-hexanes or *n*-heptane. This TAG IS solution is stable indefinitely if precautions are taken to eliminate the loss of *n*-hexanes or *n*-heptane and therefore a change in the concentration of the internal standard. For example, store the solution in refrigerator in well-sealed amber bottle when not in use. Pure tritridecanoin is available on the market (e.g., from Nu-Chek-Prep). Purity of the IS should be confirmed by thin-layer chromatography, high-performance liquid chromatography, and gas chromatography analysis or by any other appropriate technique.
3. GLC-714, 20 mg/ mL in *n*-heptane or *n*-hexanes, 100 mg ampoule (Nu-Chek-Prep, Inc.). This mixture is used to calculate the Empirical Correction Factors (ECFs). A Certificate of Analysis (COA) must be requested from Nu-Chek-Prep for every lot of the standard mixture used in determining ECFs. The actual amounts weighed into the ampoule and purities are listed on the COA and are used to calculate the actual amount of each FAME standard present in the mixture. The actual amount is necessary for determining the response factor (area/amount) for each FAME in the mixture. This mixture (100 mg) is diluted in 5 mL of *n*-heptane or *n*-hexanes with a 5 mL Class A volumetric pipette. The ampoule is rinsed with *n*-heptane or *n*-hexanes to ensure complete and homogenous transfer of the mixture. The final concentration should be approximately 20 mg/mL. The fatty acid methyl esters (FAMEs) standards in GLC-714 were those commercially available chosen to represent the majority of fatty acids present in edible vegetable, dairy, and ruminant fats, marine and single cell oils. Included were the internal standards used in AOCS Ce 1h-05, AOCS Ce 1i-07, and AOCS Ce 1j-07.

PROCEDURE

1. Preparation of FAMEs:
   (a) GLC-714—Analyze the 20 mg/mL preparation to determine ECFs.
   (b) From oils and fats—Use a standard procedure for example, Alcoholysis (Reference 6), ISO 5509 (Reference, 7), or transmethylation (References, 8, 9). Prior to methylation, add enough IS solution to the reaction flask, so that after the oil or fat is added, the concentration of the final solution is between 0.05 and 0.10 mg IS/mg oil or fat (e.g., 5 mL of a 2 mg/mL TAG IS solution per 100 mg of oil).
   (c) Dissolve the prepared FAME in *n*-hexanes or *n*-heptane, concentration should be approximately 15–20 mg/mL *n*-hexanes or *n*-hetpane.
2. Chromatography
   (a) Operating conditions are as follows:
   Injection port temperature: 235°C
   Detector temperature: 325°C
   Oven temperature program: Isothermal at 180°C (32 min) ramped at 20°C/min to 215°C (hold 31.25 min)
   Carrier gas: *hydrogen*, constant flow rate 1.0 mL/min; 26 cm/s; 24.63 psi; split ratio, 100:1.
   Carrier gas: *helium*; constant flow rate; flow rate 2.0 mL/min; 28 cm/s; 63.29 psi; split ratio, 100:1
   (b) Inject 1 µL, which is equivalent to 15 to 20 µg of total FAMEs (Table 1 and Figures 1–32).

Performance check: (System Suitability Mix)

1. Column performance is checked using fatty acid methyl esters (FAMEs) of tallow, anhydrous butterfat, and Certified Reference Material (BCR) 163, respectively, covering the range of fatty acids under investigation (Figures 2–16 and 18–32). Since commercial GC designs are different, and the separation obtained may not be identical to the example chromatograms, small changes in the sample size, sample concentration, or oven temperature may be required. If so, adjust the sample size, sample concentration, or oven temperature until the best separation results are obtained. If the column oven temperature needs to be adjusted, it should be adjusted with small increments, preferably by steps of 1°C. Authentic standards of fatty acid esters, if needed for peak identification, are available. On SP™-2560, CP-Sil™ 88, or any other cyanopropylsilicone capillary columns, the column temperature has profound effect on the elution pattern of 13*t*+14*t*-18:1, 16*t*-18:1, 14*c*-18:1, 9*c*,12*c*,15*t*-18:3, 11*c*-20:1, and 9*c*,12*c*,15*c*-18:3 (Reference 10). From the chromatograms of the butterfat:
   (a) Baseline separation between 9*c*-18:1 and 11*c*-18:1 should be obtained.
   (b) Resolution greater than 1.0 but less than 1.5 between 11*c*-20:1 and 9*c*,12*c*,15*c*-18:3 should be obtained (Reference 11).

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2. The Empirical Correction Factors (ECFs) are determined by analysis of GLC-714 at approximately 20 mg/mL and plotted as the percent difference between the ECF and Theoretical Correction Factors (TCFs) divided by the TCFs (Figure 34). Figure 34 shows the plots from four different laboratories. Different laboratories will most likely have different plots and therefore should be determined for each laboratory. An example calculation follows:

$$\% \text{ Difference} = 100 \times \frac{ECF_{FAMEx} - TCF_{FAMEx}}{TCF_{FAMEx}}$$

GLC-714 can also be used to help determine if a chromatographic system is operating properly. Operation under the GC parameters listed in the method should result in a similar trend line with the long chain PUFA ECFs deviating furthest from TCFs, while all saturated and monounsaturated FAME deviate from the TCFs the least. Ideally if the % Difference is three or less for the saturated and monounsaturated FAME, then the GC system is considered to be operating properly and TCFs can be used for those FAME. ECFs will need to be calculated and used for the long chain PUFA (≥ 20 carbons and three double bonds). Figure 33 shows the plot of three different methods AOCS Ce 1h-05, Ce 1i-07, and Ce 1j-07 within one laboratory.

3. Peak identification—the individual FAMEs are identified by their retention times, which are compared with the FAME reference standards and reference oils. The FAME reference oils are tallow and anhydrous butterfat. See Figures 2–11 and 18–27 for sample chromatograms of both. When peaks of unknown identity are observed, attempt to identify such peaks using appropriate procedures that usually involve GC-MS, FTIR, silver-ion chromatography, and classical chemical methods (References 12 and 13). Peaks of unknown identity should not be included in the summation of peak areas when quantifying the concentrations of total fat, saturated, *cis-* or *trans*-monounsaturated, *cis-* or *trans*-polyunsaturated, AA, EPA, or DHA, unless they have been confirmed to be fatty acids.

CALCULATIONS

1. Calculation of grams (g) of individual fatty acids and total fat per 100 g of sample:

The food labeling regulations in Canada (Reference 1) and the USA (Reference 2) define the total fat as the sum of fatty acids originating from all the lipid classes present in the food matrix and is expressed as TAG equivalents. This requires converting the GC measured FAMEs into TAG equivalents. The fatty acids listed in Tables 1 and 2 cover most of the fatty acid chain lengths (from 4:0 to 26:0) and double bonds (from 0 to 6) that are encountered in dairy and ruminant fats. The factors for conversion of the individual FAMEs into TAG equivalents are given in Table 3.

The food labeling regulations in Canada (Reference 1) and the USA (Reference 2) require mandatory declaration of total fat and the amounts of saturated fatty acids (SFAs) and *trans* fatty acids. Declaration of the amounts of *cis*-monounsaturated fatty acids (MUFAs), n-6, and n-3 *cis*-polyunsaturated fatty acids (PUFAs) is not mandatory, however, they could be declared in the nutrition facts table on a voluntary basis. Furthermore, the amounts of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) could also be declared on the label, but their values should be displayed outside the nutrition fact table. For nutritional labeling purposes the total fat, saturated, *cis*-monounsaturated, and *cis*-polyunsaturated fatty acid contents are to be determined with this method. For the determination of vegetable oil fatty acid concentrations the AOCS Ce 1h-05 method is recommended. For the determination of marine oil or single cell oils (SCOs) fatty acid concentrations the AOCS Ce 1i-07 method is recommended. In contrast to total fat, fatty acids should be expressed as free fatty acids (FFAs). The factors for conversion of the individual FAMEs into FFAs are given in Table 2.

(a) Calculate, the amount (in g) of individual fatty acids, expressed as FAME (W_{FAMEx}) and TAG (W_{TAGx}) equivalents as follows.

$$W_{FAMEx} = \frac{A_x \times W_{TAG\ is} \times 1.0059 \times R_x}{A_{is}}$$

$$W_{TAGx} = W_{FAMEx} \times F_{TAGx}$$

Where A_x = area counts for fatty acid x; $W_{TAG\ is}$ = weight of 13:0 TAG IS (in g) added to the oils; A_{is} = peak area counts of the IS; 1.0059, conversion of IS in sample (Note 3); F_{TAGx} = conversion factor for FAMEs to TAGs for individual fatty acids (Table 3) and R_x = theoretical flame ionization detector correction factor (TCF) for FAMEs relative to 13:0 FAME IS, respectively (Table 4; Note 4) or the Empirical Correction Factor (ECF) as determined via GLC-714 (Table 4). TCF should be applied to the analytical data for optimum accuracy (References 14–16) and to minimize variation between laboratories because of differences in calculating response factors. TCFs are also used for fatty acids where standards are not available. ECFs are needed due to the large deviation from TCFs for long chain PUFA of 20 carbons or more and three or more double

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bonds. The TCFs and ECFs of a number of FAMEs that are commonly encountered in dairy and ruminant fats are listed in Table 4. The TCF were calculated using the following formula:

$$TCF_x = \frac{MW_x}{(N_x - 1) \times (AWC) \times (1.4625)}$$

Where TCF_x = theoretical flame ionization detector response factor for fatty acid x (as methyl ester) with respect to 13:0 FAME IS; MW_x = molecular weight of component x; N_x = number of carbon atoms in the FAME of component x; AWC = atomic weight of carbon (12.011); 1.4625 = TCF for 13:0 FAME (Notes 5 and 6).

ECFs are required for long chain PUFA FAME of 20 carbons or more and three or more double bonds of which standards are readily available. The ECFs are calculated using the Certificate of Analysis (COA) supplied with each lot of GLC-714 from Nu-Chek-Prep, Inc. The COA will list both the purity (P) and amount (Amt_{FAMEx}) of each FAME used to make up the standard. The actual amount ($AAmt_{FAMEx}$) is calculated by the following equation:

$$AAmt_{FAMEx} = P \times Amt_{FAMEx}$$

The response factor (RF) for each peak is determined by the following equation:

$$RF_{FAMEx} = Area_{FAMEx}/AAmt_{FAMEx}$$

Each RF is then made relative to the 13:0 RF by the following equation:

$$RRF_{FAMEx} = RF_{FAMEx}/RF_{13:0}$$

Where RRF = Relative Response Factor

The Empirical Correction Factor (ECF) for each FAME is then calculated by the taking the inverse of the RRF:

$$ECF_{FAMEx} = 1/RRF_{FAMEx}$$

(b) Calculate the amount of *total fat* in sample (sum of all fatty acids; expressed as TAGs) as follows:

$$\text{Total fat (g/100 g portion sample)} = (\Sigma W_{TAG}/W_{TS}) \times 100$$

Where W_{TAG} = weight of the sum of all TAG, g and W_{TS} = weight of test sample, g

(c) Calculate weight (in g) of each *individual fatty acid* (W_x) as follows:

$$W_x \text{ (g per sample)} = W_{FAMEx} \times F_{FAx}$$

Where W_{FAMEx} = individual fatty acids expressed as FAME and F_{FAx} = conversion factors for conversion of FAME to their corresponding fatty acids (Table 3).

(d) Calculate weight of *saturated fats* (sum of all saturated fatty acids) as follows:

$$\text{Saturated fat (g per 100 g sample)} = (\Sigma \text{Saturated } W_x/W_{TS}) \times 100$$

Where ΣSaturated W_x = sum of all saturated fatty acids (4:0; 5:0; 6:0; 7:0; 8:0; 9:0; 10:0; *iso* 10:0; 11:0; 12:0; *iso* 12:0; 13:0; *anteiso* and *iso* 13:0; 14:0; *iso* 14:0; 15:0; *anteiso* and *iso* 15:0; 2,6,10,14-tetramethyl 15:0; 16:0; *iso* 16:0; 3,7,11,15-tetramethyl 16:0; 17:0; *anteiso* and *iso* 17:0; 18:0; *iso* 18:0; 19:0; 20:0; *iso* 20:0; 21:0; *iso* 21:0; 22:0; 24:0; 25:0 and 26:0) and W_{TS} = weight of sample, g

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(f) Calculate weight of *cis-monounsaturated fat,* fatty acids containing one double bond, as follows:

$$\textit{cis}\text{-monounsaturated fat (g per 100 g sample)} = (\Sigma \textit{cis}\text{-monounsaturated } W_x/W_{TS}) \times 100$$

Where Σ*cis*-monounsaturated W_x = sum of all *cis*-monounsaturated fatty acids (11:1; 14:1; 15:1; 16:1; 17:1; 18:1; 19:1; 20:1; 22:1; and 24:1) and W_{TS} = weight of sample, g

(g) Calculate weight of *trans* fat as follows:

$$\textit{trans}\text{ fat (g per 100 g sample)} = (\text{Total } \textit{trans}\ W_x/W_{TS}) \times 100$$

Where total *trans* W_x = sum of all *trans* fatty acids excluding *trans* isomers with a conjugated double bond and W_{TS} = weight of sample, g

(h) Calculate weight of *cis-polyunsaturated fat,* fatty acids containing two or more double bonds, as follows:

$$\textit{cis}\text{-polyunsaturated fat (g per 100 g sample)} = (\Sigma \textit{cis}\text{-polyunsaturated } W_x/W_{TS}) \times 100$$

Where Σ*cis*-polyunsaturated W_x = sum of all *cis*-polyunsaturated fatty acids (18:2; 18:3; 18:4; 20:2; 20:3; 20:4; 20:5; 22:2; 22:3; 22:4; 22:5; and 22:6).

(i) Calculate weight of *EPA* or *DHA* as follows:

$$\text{EPA or DHA fat (g per 100 g sample)} = (\Sigma \text{EPA or DHA}/W_{TS}) \times 100$$

Where W_{TS} = weight of sample, g

(j) Calculate weight of *n-3 PUFAs* as follows:

$$\text{n-3 PUFA (g per 100 g sample)} = (\Sigma \text{n-3 PUFA } W_x/W_{TS}) \times 100$$

Where Σn-3 PUFAs W_x = sum of all n-3 PUFAs (9*c*,12*c*,15*c*-18:3; 6*c*,9*c*,12*c*,15*c*-18:4; 8*c*,11*c*,14*c*,17*c*-20:4; 5*c*,8*c*,11*c*,14*c*,17*c*-20:5; 7*c*,10*c*,13*c*,16*c*,19*c*-22:5 and 4*c*,7*c*,10*c*,13*c*,16*c*,19*c* 22:6) and W_{TS} = weight of sample, g.

(k) Calculate weight of *n-6 PUFAs* as follows:

$$\text{n-6 PUFAs (g per 100 g sample)} = (\Sigma \text{n-6 PUFAs } W_x/W_{TS}) \times 100$$

Where Σn-6 PUFAs W_x = sum of all n-6 PUFAs (9*c*,12*c*-18:2; 6*c*,9*c*,12*c*-18:3; 8*c*,11*c*,14*c*-20:3; 5*c*,8*c*,11*c*,14*c*-20:4; 7*c*,10*c*,13*c*,16*c*-22:4; and 4*c*,7*c*,10*c*,13*c*,16*c*-22:5) and W_{TS} = weight of sample, g.

Peak Identification

Identification of the all the FAME components in complex matrices such as dairy and ruminant fats can be quite challenging. Identification of the peaks in this method has been done by running numerous standards such as; GLC-463 and GLC-714 (Nu-Check Prep, Elysian, MN), Pamolyn 380 (Hercules, DE), as well as many other individual pure standards available from a variety of different vendors. In addition, there have been a number of papers published that use more detailed techniques like argentation chromatographic separations and/or additional GC analyses to separate out and identify many of the different isomers (References 17–24). These papers all have excellent chromatograms that were used to identify some of the more difficult peaks in the fats that have been analyzed in this method.

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PRECISION

Precision data determined from a 2011 AOCS collaborative study by 9 laboratories analyzing 24 samples are given in Table 5. Samples were prepared according to Ce 2b-11. Reproducibility and repeatability values are calculated according to the AOAC/IUPAC Harmonized Protocol for Collaborative Study.

QUALITY ASSURANCE AND CONTROL

1. Blank sample—The first sample in an analysis batch is always a blank (*n*-hexanes and/or *n*-heptane). No peaks should be detected in the blank run except for the internal standard. Analyze a blank sample with every run.
2. Control sample: TBD.
3. Proficiency test—For external reference the method is tested each year by participation in the AOCS Laboratory Proficiency Program or any other suitable ring test.

NOTES

1. Nitrogen gas is not acceptable as a carrier gas for this method.
2. No other liner types (e.g., straight through, etc.) should be used with this method.
3. 1.0059 is the factor for conversion of the weight of the IS (which is 13:0) from the TAG form to its corresponding weight of the FAME form. In calculating the factor, hydrolysis (and transmethylation), TAG yields three moles of FAME. So that the general formula for the conversion factor is as follows:

$$\text{MW of 13:0 FAME} \times 3/\text{MW of 13:0 TAG} = 228.363 \times 3/681.058 = 1.0059$$

4. 1.4625 is the theoretical flame ionization detector response correction factor (TCF) for 13:0 FAME. It is calculated as follows.

$$\text{TCF of 13:0 FAME} = \text{MW of 13:0}/(N_{x-1})\ \text{(Atomic weight of carbon)}$$

$$= 228.363/(14-1) \times 12.011 = 1.4625$$

Here N_x is the number of carbon atoms in 13:0 FAME, which is 14. 12.011 is the atomic weight of carbon.

5. It is important to understand the difference (and hence the relationship) between TCF and TRF.
   (a) Theoretical flame ionization response factor (TRF) is actually the proportion (with respect to the total weight, which is the molecular weight) of the total amount of carbon atoms that are responding to the FID. For fatty acids, the carbons that are responding to FID are CH, CH_2, and CH_3 units. Carbonyl carbon and ester carbonyls carbon do not respond to FID. So that in FAME, all the carbon atoms are active, except the ester carbon. Thus, there are N_{x-1} active carbon atoms in a FAME. So that the TRF for a FAME would be:

$$\text{TRF} = (N_{x-1})\ \text{(Atomic Weight of Carbon)}/\text{MW of FAME}$$

$$\text{TRF of 13:0 FAME} = 13 \times 12.011/228.363 = 0.6837$$

   (b) Since the FID response depends on the proportion of active carbon atoms, to determine the actual weight of a FAME, the experimental measured area (or the weight) needs to be corrected. This factor is termed as TCF and it is equal to 1/TRF. In other words formula for TCF is as follows:

$$\text{TCF} = \text{MW}/(N_{x-1})\ \text{(Atomic Weight of Carbon)}$$

$$\text{The TCF of 13:0 FAME is } 1.4625$$

6. The TRF of 18:0 FAME is 0.7243. Therefore, its TCF is 1/0.7243 = 1.3806. Thus TCF of 18:0 FAME with respect to 13:0 FAME = 1.3806/1.4625 = 0.9440 (Table 3).

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REFERENCES

1. Regulations Amending the Food and Drug Regulations (Nutrition Labelling, Nutrient Content Claims and Health Claims). Department of Health, Canada Gazette, Part 11. January 1, 2003.
2. Department of Health and Human Services. Food and Drug Administration. 21 CFR Part 101 [Docket No. 94P-0036]. Food Labelling: *Trans* Fatty Acids in Nutrition Labelling, Nutrient Content Claims, and Health Claims. Washington, DC. July 2003, pp. 254.
3. ISO 6353, Reagents for Chemical Analysis, Part 2 (1983) and 3 (1987); Specifications.
4. ISO 3696, Water for Analytical Laboratory Use—Specifications and Test Methods (1987).
5. J.M. Snyder and C.R. Scholfield, *J. Am. Oil Chem. Soc. 59*:469–470 (1982).
6. S.W. Christopherson and R.L. Glass, *J. Dairy Sci.* 52:1289–1290 (1969).
7. ISO Method 5509, *Animal and vegetable fats and oils – Preparation of methyl esters of fatty acids*, 12-23-2004
8. W.W. Christie, *J. Lipid Res. 23:*1072–1075 (1982).
9. M.P. Yurawecz, J.K. Hood, J.A.G. Roach, M.M. Mossoba, D.H. Daniels, Y. Ku, M.W. Pariza, S.F. Chin, *J. Am. Oil Chem. Soc. 71:*1149–1155 (1994).
10. W.M.N. Ratnayake, L.L. Plouffe, E. Pasqier and C. Gagnon, *J. AOAC Int. 85:*1112–1118 (2002)
11. W.M.N. Ratnayake, S.L. Hansen, and M.P. Kennedy, *J. Am. Oil Chem. Soc. 83:*475–488 (2006)
12. R.G. Ackman, Application of gas-liquid chromatography to lipid separation and analysis: Qualitative and quantitative analysis. *Analyses of Fats, Oils and Lipoproteins*, Edited by E.G. Perkins, AOCS Press, Champaign, IL, 1991, pp. 270–300.
13. J.L. Sebedio, Classical chemical techniques for fatty acid analysis. *New Trends in Lipid and Lipoprotein Analyses.* Eds. J.L. Sebedio and E.G. Perkins, AOCS Press, Champaign, IL, 1995, pp. 277–289.
14. J.D. Craske and C.D. Bannon, *J. Am. Oil Chem. Soc. 64:*1413–1417, 1987.
15. J.D. Craske and C.D. Bannon, *J. Am. Oil Chem. Soc. 65:*1190–1991, 1988.
16. R.G. Ackman, J.C. Sipos, and C.S. Tocher, *J. Fish. Res. Bd. Canada, 24:*635–650, 1967.
17. C. Cruz-Hernandez, Z. Deng, J. Zhou, A.R. Hill, M.P. Yurawecz, P. Delmonte, M.G. Mossoba, M.E.R. Dugan, and J.K.G. Kramer, *J. AOAC International 87:*545–562 (2004).
18. D. Precht and J. Molkentin, Fett./*Lipid, 9(5)*, 319–326, 1997.
19. F. Destaillats, P. A. Golay, F. Joffre, M. de Wispelaere, B. Hug, F. Giuffrida, L. Fauconnot, and F. Dionisi, *J. Chromat. A, 1145*, 222–228, 2007.
20. V. Santercole, R. Mazzette, E. P. L. De Santis, S. Banni, L. Goonewardene, and J. K. G. Kramer, *Lipids, 42:*361–382, 2007.
21. F. Dionisi, P.A. Golay, and L.B. Fay, *Analytica Chimica Acta, 465*, 395–407, 2002.
22. D. Precht and J. Molkentin, *Eur. J. Lipid Sci. Technol.* 102–113 (2000).
23. J.K.G. Kramer, M. Hernandez, C. Cruz-Hernandez, J. Kraft, M.E.R. Dugan, *Lipids 43:*259–273 (2008).
24. J.A.G. Roach, M.P Yurawecz, J.K.G. Kramer, M.M. Mossoba, K. Eulitz, and Y. Ku, *Lipids, 35:*797–802 (2000).

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Table 1
Example elution order of fatty acid methyl esters (FAME). Separations were on a SP™ -2560 (100 m x 0.25 mm x 0.20 µm). Column temperature was 180°C (32 min) to 215°C @ 20°C/min (hold 31.25 min). Carrier gas was *hydrogen* at 1.0 mL/min (constant flow) with a linear velocity of 26 cm/s. Pressure was 24.63 psi @ 180°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100. Please note that different columns will have different retention times and sometimes the order of elution of the fatty acids can change for later eluting compounds. Fatty acids in parenthesis coleute with the peaks listed in the same cell for this column.

Fatty Acids	Tr (min)	Fatty Acids	Tr (min)	Fatty Acids	Tr (min)
2:0	7.750	4*t*-18:1	19.640	9*t*,12*c*,15*c*-18:3	29.950
4:0	7.909	5*t*-18:1	19.934	11*c*-20:1	29.979
5:0	8.000	6*t*-8*t*-18:1	20.158	9*c*,12*c*,15*c*-18:3	30.377
6:0	8.108	9*t*-18:1	20.256	*ct*-6,8-18:2	30.010
7:0	8.249	10*t*-18:1	20.387	7*c*,9*c*-18:2	31.388
8:0	8.433	11*t*-18:1	20.446	9*c*,11*t*-18:2	31.466
9:0	8.674	12*t*-18:1	20.615	8*t*,10*c*-18:2	31.600
iso 10:0	8.800	13*t*-14*t*-18:1 (6*c*-8*c*-18:1)	20.751	10*c*,12*t*-18:2	31.700
10:0	8.984	9*c*-18:1 (18:1n-9) (15*t*-10*c*-18:1)	21.037	9*t*,11*c*-18:2	31.962
11:0	9.442	11*c*-18:1	21.268	11*c*,13*t*-18:2	32.100
iso 12:0	9.618	12*c*-18:1	21.508	10*t*,12*c*-18:2	32.355
12:0	9.917	13*c*-18:1	21.794	21:0	32.371
iso 13:0	10.215	16*t*-18:1	21.952	8*c*,10*c*-18:2	32.500
anteiso 13:0	10.390	14*c*-18:1	22.148	9*c*,11*c*-18:2	32.687
13:0 (IS)	10.607	15*c*-18:1 (19:0)	22.309	10*c*,12*c*-18:2	32.750
iso 14:0	10.993	*t*,*t*-NMID_1	22.500	11*c*,13*c*-18:2	32.800
14:0	11.505	*t*,*t*-NMID_2	22.622	*t*,*t*-conj.-18:2_1	33.000
12*c*-13:1	11.705	*t*,*t*-NMID_3	22.800	*t*,*t*-conj.-18:2_2	33.281
iso 15:0	12.011	9*t*,12*t*-18:2	22.941	12*t*,14*t*-18:2	33.300
anteiso 15:0	12.212	9*t*,13*c*-18:2/8*t*,12*c*-18:2	23.227	11*t*,13*t*-18:2	33.489
9*t*-14:1	12.289	17-cyclo	23.487	*t*,*t*-8,10;9,11;10,12-18:2	33.599
9*c*-14:1 (14:1n-5)	12.616	9*c*,12*t*-18:2/8*t*,13*c*-18:2	23.743	7*t*,9*t*-18:2	33.738
15:0	12.666	16*c*-18:1	23.847	6*c*,9*c*,12*c*,15*c*-18:4	33.904
iso 16:0	13.340	9*t*,12*c*-18:2	24.302	11*c*,14*c*-20:2	34.133
10*c*-15:1 (15:1n-5)	13.983	11*t*,15*c*-18:2	24.403	22:0	35.425
16:0	14.237	9*c*,12*c*-18:2	24.794	8*c*,11*c*,14*c*-20:3 (20:3n-6)	36.134
iso 17:0	15.079	9*c*,15*c*-18:2	25.190	13*c*-22:1 (22:1n-9)	37.164
9*t*-16:1	15.206	9*t*,12*t*,15*t*-18:3	26.578	11*c*,14*c*,17*c*-20:3 (20:3n-3)	37.337
11*t*-12*t*-16:1	15.384	20:0	26.604	5*c*,8*c*,11*c*,14*c*-20:4 (20:4n-6)	37.732
7*c*-13*t*-16:1/					
anteiso 17:0	15.530	9*t*,12*t*,15*c*-18:3	27.885	23:0	38.000
9*c*-16:1 (16:1n-7)	15.730	9*t*,12*c*,15*t*-18:3	27.971	13*c*,16*c*-22:2	39.980
10*c*-16:1	15.868	6*c*,9*c*,12*c*-18:3	28.249	5*c*,8*c*,11*c*,14*c*,17*c*-20:5 (20:5n-3)	41.430
11*c*-16:1	16.026	9*c*,12*t*,15*t*-18:3 + 9*c*,12*c*,15*t*-18:3	28.578	24:0	41.655
17:0	16.177	6*t*-10*t*-20:1	28.885	15*c*-24:1	43.196
13*c*-16:1	16.308	11*t*-20:1	28.949	13*c*,16*c*,19*c*-22:3	43.667
iso 18:0	17.347	12*t*-13*t*-20:1	29.000	7*c*,10*c*,13*c*,16*c*-22:4	44.760
9*t*-17:1	17.602	14*t*-20:1	29.400	25:0	45.232
9*c*-17:1 (17:1n-7)	18.000	9*c*-20:1	29.549	7*c*,10*c*,13*c*,16*c*,19*c*-22:5	49.443
18:0	18.845	9*c*,12*t*,15*c*-18:3	29.549	4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-22:6	51.719

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Table 2
Example elution order of fatty acid methyl esters (FAME). Separations were on a SP™ -2560 (100 m × 0.25 mm × 0.20 µm). Column temperature was 180°C (32 min) to 215°C @ 20°C/min (hold 31.25 min). Carrier gas was *helium* at 2.0 mL/min (constant flow) with a linear velocity of 28 cm/s. Pressure was 63.29 psi @ 180°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100. Please note that different columns will have different retention times and sometimes the order of elution of the fatty acids can change for later eluting compounds. Fatty acids in parenthesis coleute with the peaks listed in the same cell for this column.

Fatty Acids	Tr (min)	Fatty Acids	Tr (min)	Fatty Acids	Tr (min)
2:0	6.400	4*t*-18:1	16.446	9*t*,12*c*,15*c*-18:3	25.000
4:0	6.542	5*t*-18:1	16.684	11*c*-20:1	25.230
5:0	6.615	6*t*-8*t*-18:1	16.869	9*c*,12*c*,15*c*-18:3	25.479
6:0	6.709	9*t*-18:1	16.990	*ct*-6,8-18:2	25.976
7:0	6.800	10*t*-18:1	17.043	7*c*,9*c*-18:2	26.284
8:0	6.982	11*t*-18:1	17.098	9*c*,11*t*-18:2	26.342
9:0	7.100	12*t*-18:1	17.246	8*t*,10*c*-18:2	26.400
iso 10:0	7.300	13*t*-14*t*-18:1 (6*c*-8*c*-18:1)	17.372	10*c*,12*t*-18:2	26.500
10:0	7.446	9*c*-18:1 (18:1n-9) (15*t*-10*c*-18:1)	17.642	9*t*,11*c*-18:2	26.714
11:0	7.788	11*c*-18:1	17.810	11*c*,13*t*-18:2	27.163
iso 12:0	7.830	12*c*-18:1	17.998	10*t*,12*c*-18:2	27.250
12:0	8.232	13*c*-18:1	18.230	21:0	27.367
iso 13:0	8.479	16*t*-18:1	18.377	8*c*,10*c*-18:2	27.750
anteiso 13:0	8.621	14*c*-18:1	18.563	9*c*,11*c*-18:2	27.886
13:0 (IS)	8.813	15*c*-18:1 (19:0)	18.667	10*c*,12*c*-18:2	28.100
iso 14:0	9.136	*t*,*t*-NMID_1	18.800	11*c*,13*c*-18:2	28.200
14:0	9.569	*t*,*t*-NMID_2	18.947	*t*,*t*-conj.-18:2_1	28.400
12*c*-13:1	9.820	*t*,*t*-NMID_3	19.000	*t*,*t*-conj.-18:2_2	28.455
iso 15:0	9.995	9*t*,12*t*-18:2	19.204	12*t*,14*t*-18:2	28.700
anteiso 15:0	10.227	9*t*,13*c*-18:2/8*t*,12*c*-18:2	19.435	11*t*,13*t*-18:2	28.898
9*t*-14:1	10.350	17-cyclo	19.670	*t*,*t*-8,10;9,11;10,12-18:2	29.159
9*c*-14:1 (14:1n-5)	10.496	9*c*,12*t*-18:2/8*t*,13*c*-18:2	19.871	7*t*,9*t*-18:2	29.300
15:0	10.545	16*c*-18:1	19.929	6*c*,9*c*,12*c*,15*c*-18:4	29.500
iso 16:0	11.115	9*t*,12*c*-18:2	20.272	11*c*,14*c*-20:2	30.383
10*c*-15:1 (15:1n-5)	11.648	11*t*,15*c*-18:2	20.380	22:0	33.176
16:0	11.846	9*c*,12*c*-18:2	20.785	8*c*,11*c*,14*c*-20:3 (20:3n-6)	33.624
iso 17:0	12.582	9*c*,15*c*-18:2	21.082	13*c*-22:1 (22:1n-9)	34.681
9*t*-16:1	12.622	9*t*,12*t*,15*t*-18:3	22.200	11*c*,14*c*,17*c*-20:3 (20:3n-3)	34.945
11*t*-12*t*-16:1	12.655	20:0	22.309	5*c*,8*c*,11*c*,14*c*-20:4 (20:4n-6)	35.880
7*c*-13*t*-16:1/ *anteiso* 17:0	12.962	9*t*,12*t*,15*c*-18:3	23.200	23:0	36.010
9*c*-16:1 (16:1n-7)	13.140	9*t*,12*c*,15*t*-18:3	23.300	13*c*,16*c*-22:2	36.756
10*c*-16:1	13.250	6*c*,9*c*,12*c*-18:3	23.616	5*c*,8*c*,11*c*,14*c*,17*c*-20:5 (20:5n-3)	38.441
11*c*-16:1	13.368	9*c*,12*t*,15*t*-18:3 + 9*c*,12*c*,15*t*-18:3	23.800	24:0	38.954
17:0	13.532	6*t*-10*t*-20:1	24.216	15*c*-24:1	40.687
13*c*-16:1	13.606	11*t*-20:1	24.412	13*c*,16*c*,19*c*-22:3	41.375
iso 18:0	14.498	12*t*-13*t*-20:1	24.500	7*c*,10*c*,13*c*,16*c*-22:4	41.921
9*t*-17:1	14.697	14*t*-20:1	24.700	25:0	45.232
9*c*-17:1 (17:1n-7)	15.038	9*c*-20:1	24.834	7*c*,10*c*,13*c*,16*c*,19*c*-22:5	45.431
18:0	15.732	9*c*,12*t*,15*c*-18:3	24.900	4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-22:6	47.345

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Table 3
Factors for conversion of FAME to fatty acids and TAG equivalents

Fatty Acid[a]	F_{FAx}[b]	F_{TAGx}[c]	Fatty Acid	F_{FAx}[b]	F_{TAGx}[c]
4:0	0.8627	0.9868	18:1	0.9527	0.9955
5:0	0.8792	0.9884	18:2	0.9524	0.9954
6:0	0.8923	0.9897	18:3	0.9520	0.9954
7:0	0.9027	0.9907	18:4	0.9517	0.9954
8:0	0.9114	0.9915	19:0	0.9551	0.9957
9:0	0.9186	0.9922	20:0	0.9570	0.9959
10:0	0.9247	0.9928	20:1	0.9568	0.9959
10:1	0.9239	0.9927	20:2	0.9565	0.9958
11:0	0.9300	0.9933	20:3	0.9562	0.9958
11:1	0.9293	0.9932	20:4	0.9560	0.9958
12:0	0.9346	0.9937	20:5	0.9557	0.9958
12:1	0.9339	0.9937	21:0	0.9588	0.9961
13:0	0.9386	0.9941	22:0	0.9604	0.9962
13:1	0.9380	0.9941	22:1	0.9602	0.9962
14:0	0.9421	0.9945	22:2	0.9600	0.9962
13:1	0.9380	0.9941	22:3	0.9598	0.9961
14:1	0.9417	0.9944	22:4	0.9595	0.9961
15:0	0.9453	0.9948	22:5	0.9593	0.9961
15:1	0.9449	0.9947	22:6	0.9590	0.9961
16:0	0.9481	0.9950	23:0	0.9620	0.9964
16:1	0.9477	0.9950	24:0	0.9633	0.9965
17:0	0.9507	0.9953	24:1	0.9632	0.9965
17:1	0.9503	0.9952	25:0	0.9646	0.9966
18:0	0.9530	0.9955	26:0	0.9658	0.9967

[a] Only one factor is given for all positional and geometric isomers and for branched-chain FAME, as the factors are dependent only on the content of carbon to which hydrogen is bonded.
[b] F_{FAx} is the conversion factor for conversion of FAMEs to corresponding fatty acids.
[c] F_{TAGx} is the conversion factor for conversion of FAMEs to corresponding TAG equivalents.

Table 4
Theoretical flame ionization detector theoretical and empirical correction factors (TCFs/ECFs) for fatty acid methyl esters (FAME)[a]

FAME	TCF	FAME	TCF	FAME	TCF/ECF	FAME	TCF/ECF
4:0	1.4534	14:0	0.9856	18:3	0.9249	22:1	0.9124
5:0	1.3224	14:1	0.9774	18:4	0.9185	22:2	0.9071
6:0	1.2351	15:0	0.9731	18:5	0.9122	22:3	0.9019
7:0	1.1727	15:1	0.9655	19:0	0.9364	22:4	0.8967
8:0	1.1259	16:0	0.9622	19:1	0.9303	22:5	ECF[b]
9:0	1.0896	16:1	0.9550	20:0	0.9295	22:6	ECF[b]
10:0	1.0604	16:2	0.9479	20:1	0.9237	23:0	0.9124
10:1	1.0490	16:3	0.9407	20:2	0.9180	24:0	0.9076
11:0	1.0366	16:4	0.9335	20:3	ECF[b]	24:1	0.9029
11:1	1.0262	17:0	0.9526	20:4	ECF[b]	25:0	0.9033
12:0	1.0168	17:1	0.9458	20:5	ECF[b]	26:0	0.8993
12:1	1.0072	18:0	0.9440	21:0	0.9232		
13:0 (IS)	1.0000	18:1	0.9377	21:5	0.8959		
13:1	0.9912	18:2	0.9313	22:0	0.9176		

[a] Atomic weights used: carbon 12.011; hydrogen, 1.0079; oxygen, 15.994. Factors are relative to 13:0, which has a factor of 1.0000 by definition. Only one factor is given for all positional and geometric isomers and for branched-chain FAME, as the factors are dependent only on the content of carbon to which hydrogen is bonded. Calculations were adopted from Reference 8.
[b] ECFs are to be calculated from GLC-714. Any positional or geometrical isomers will have the same TCFs and ECFs.

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Table 5
Results of *cis*-, *trans*-, Saturated, Monounsaturated, and Polyunsaturated fatty acid determination by 9 laboratories in a collaborative study organized by AOCS in 2011.

Anhydrous milk fat	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	93.59	88.92	58.27	5.11	26.39	21.59	3.78	3.25	0.53	2.64
Repeatability (Sr)	2.48	2.39	1.32	0.41	1.21	0.27	0.39	0.20	0.06	0.04
%RSDr	2.65	2.69	2.26	8.06	4.60	1.27	10.23	6.08	11.21	1.60
r (2.8xSr)	6.94	6.69	3.69	1.15	3.40	0.77	1.08	0.55	0.17	0.12
Reproducibility (SR)	2.48	2.39	2.39	0.67	1.53	0.73	0.54	0.20	0.14	0.24
%RSDR	2.65	2.69	4.10	13.14	5.81	3.38	14.28	6.08	26.83	8.94
R (2.8xSR)	6.94	6.69	6.69	1.88	4.29	2.05	1.51	0.55	0.40	0.66

Tallow	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	99.68	95.21	45.44	7.14	46.53	39.31	2.65	1.99	0.33	1.66
Repeatability (Sr)	1.01	0.96	1.03	0.19	0.78	0.33	0.12	0.05	0.01	0.04
%RSDr	1.01	1.01	2.26	2.73	1.67	0.84	4.43	2.41	3.80	2.33
r (2.8xSr)	2.82	2.69	2.88	0.55	2.18	0.92	0.33	0.13	0.04	0.11
Reproducibility (SR)	1.66	1.58	1.36	0.30	1.71	0.80	0.34	0.24	0.07	0.18
%RSDR	1.66	1.66	2.99	4.20	3.68	2.03	12.74	12.24	22.64	11.13
R (2.8xSR)	4.64	4.42	3.81	0.84	4.80	2.24	0.94	0.68	0.21	0.52

Chocolate cake mix	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	10.81	10.34	4.44	0.90	3.21	2.47	2.65	2.49	0.23	2.27
Repeatability (Sr)	0.23	0.22	0.11	0.03	0.06	0.04	0.07	0.06	0.01	0.05
%RSDr	2.09	2.09	2.37	3.29	1.79	1.72	2.77	2.60	2.46	2.20
r (2.8xSr)	0.63	0.61	0.30	0.08	0.16	0.12	0.21	0.18	0.02	0.14
Reproducibility (SR)	0.42	0.40	0.25	0.07	0.10	0.09	0.12	0.10	0.01	0.08
%RSDR	3.92	3.91	5.68	7.43	3.25	3.45	4.67	4.20	4.63	3.62
R (2.8xSR)	1.19	1.13	0.71	0.19	0.29	0.24	0.35	0.29	0.03	0.23

Cheese Powder	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	29.78	28.38	13.49	7.27	14.08	6.95	1.24	0.97	0.14	0.83
Repeatability (Sr)	0.43	0.41	0.22	0.12	0.22	0.12	0.04	0.02	0.00	0.01
%RSDr	1.43	1.44	1.62	1.68	1.55	1.66	3.10	2.22	3.22	1.60
r (2.8xSr)	1.19	1.14	0.61	0.34	0.61	0.32	0.11	0.06	0.01	0.04
Reproducibility (SR)	2.39	2.28	0.71	0.37	0.75	0.57	0.12	0.02	0.03	0.05
%RSDR	8.02	8.02	5.24	5.04	5.31	8.18	9.54	2.46	19.48	6.58
R (2.8xSR)	6.69	6.38	1.98	1.03	2.09	1.59	0.33	0.07	0.08	0.15

DHA/EPA fortified infant formula	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	28.94	27.58	12.07	0.15	10.15	10.07	5.21	5.14	0.53	4.62
Repeatability (Sr)	0.66	0.63	0.07	0.02	0.20	0.20	0.13	0.12	0.01	0.09
%RSDr	2.29	2.29	0.59	15.01	1.96	2.02	2.43	2.41	2.16	2.01
r (2.8xSr)	1.86	1.77	0.20	0.06	0.56	0.57	0.35	0.35	0.03	0.26
Reproducibility (SR)	1.48	1.42	0.45	0.12	0.65	0.65	0.39	0.38	0.02	0.33
%RSDR	5.11	5.14	3.77	78.47	6.39	6.46	7.52	7.48	3.49	7.08
R (2.8xSR)	4.14	3.97	1.27	0.33	1.82	1.82	1.10	1.08	0.05	0.92

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Table 5
(Continued)

Extruded dog food (high protein and high fat)	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	22.04	21.06	6.72	0.31	9.75	9.31	4.76	4.72	0.33	4.36
Repeatability (Sr)	1.49	1.42	0.43	0.11	0.64	0.61	0.31	0.30	0.03	0.28
%RSDr	6.75	6.75	6.38	34.60	6.53	6.60	6.44	6.41	9.24	6.41
r (2.8xSr)	4.17	3.98	1.20	0.30	1.78	1.72	0.86	0.85	0.09	0.78
Reproducibility (SR)	1.49	1.42	0.43	0.11	0.64	0.61	0.31	0.30	0.04	0.28
%RSDR	6.75	6.75	6.38	34.97	6.53	6.60	6.44	6.41	11.77	6.41
R (2.8xSR)	4.17	3.98	1.20	0.31	1.78	1.72	0.86	0.85	0.11	0.78

Oatmeal Cookie	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	19.19	18.33	8.95	0.05	7.12	7.08	2.25	2.20	0.05	2.16
Repeatability (Sr)	0.27	0.26	0.20	0.02	0.08	0.08	0.02	0.02	0.00	0.02
%RSDr	1.42	1.42	2.23	28.55	1.13	1.11	1.03	1.02	2.41	0.99
r (2.8xSr)	0.76	0.73	0.56	0.04	0.22	0.22	0.06	0.06	0.00	0.06
Reproducibility (SR)	0.72	0.68	0.39	0.02	0.31	0.31	0.09	0.10	0.00	0.09
%RSDR	3.74	3.72	4.30	44.84	4.38	4.40	4.00	4.33	4.89	4.36
R (2.8xSR)	2.01	1.91	1.08	0.07	0.87	0.87	0.25	0.27	0.01	0.26

Evaporated milk	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	6.28	5.97	3.93	0.33	1.78	1.47	0.23	0.19	0.03	0.16
Repeatability (Sr)	0.29	0.27	0.17	0.03	0.09	0.08	0.02	0.01	0.00	0.01
%RSDr	4.61	4.57	4.44	8.45	5.11	5.17	7.06	4.73	8.53	4.38
r (2.8xSr)	0.81	0.76	0.49	0.08	0.25	0.21	0.05	0.03	0.01	0.02
Reproducibility (SR)	0.29	0.27	0.22	0.05	0.09	0.08	0.03	0.02	0.01	0.02
%RSDR	4.61	4.57	5.54	15.89	5.11	5.17	14.13	10.75	32.47	10.66
R (2.8xSR)	0.81	0.76	0.61	0.15	0.25	0.21	0.09	0.06	0.03	0.05

Peanut butter	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	54.01	51.69	10.11	0.06	30.28	30.13	11.23	11.17	0.08	10.79
Repeatability (Sr)	1.17	1.12	0.33	0.01	0.70	0.74	0.29	0.40	0.02	0.26
%RSDr	2.16	2.16	3.29	21.71	2.31	2.47	2.61	3.62	21.36	2.37
r (2.8xSr)	3.26	3.12	0.93	0.03	1.96	2.08	0.82	1.13	0.05	0.72
Reproducibility (SR)	3.10	2.96	0.79	0.04	1.57	1.60	1.46	1.48	0.08	1.14
%RSDR	5.73	5.73	7.83	75.73	5.17	5.32	13.02	13.21	98.99	10.58
R (2.8xSR)	8.67	8.30	2.22	0.12	4.38	4.49	4.10	4.13	0.22	3.19

Yogurt (plain)	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	5.80	5.51	3.60	0.32	1.65	1.37	0.23	0.19	0.03	0.15
Repeatability (Sr)	0.55	0.53	0.39	0.03	0.13	0.11	0.03	0.02	0.01	0.01
%RSDr	9.54	9.55	10.90	7.94	8.09	8.26	12.67	13.02	42.60	9.08
r (2.8xSr)	1.55	1.47	1.10	0.07	0.37	0.32	0.08	0.07	0.04	0.04
Reproducibility (SR)	0.55	0.53	0.39	0.03	0.15	0.14	0.03	0.02	0.02	0.01
%RSDR	9.54	9.55	10.90	7.94	9.23	9.92	12.83	13.02	59.74	9.08
R (2.8xSR)	1.55	1.47	1.10	0.07	0.43	0.38	0.08	0.07	0.05	0.04

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Table 5
(Continued)

Canned cat food	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	5.70	5.44	1.71	0.05	2.40	2.01	1.25	1.24	0.42	0.76
Repeatability (Sr)	0.66	0.64	0.15	0.01	0.25	0.23	0.12	0.11	0.05	0.07
%RSDr	11.59	11.67	8.83	13.55	10.58	11.68	9.22	9.19	11.58	9.53
r (2.8xSr)	1.85	1.78	0.42	0.02	0.71	0.66	0.32	0.32	0.14	0.20
Reproducibility (SR)	0.80	0.77	0.26	0.03	0.46	0.93	0.23	0.22	0.24	0.14
%RSDR	14.09	14.11	15.08	49.55	19.14	46.30	18.07	17.88	56.88	18.16
R (2.8xSR)	2.25	2.15	0.72	0.08	1.29	2.60	0.63	0.62	0.68	0.39
DHA/EPA fortified orange juice	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	0.12	0.11	0.03	0.00	0.03	0.03	0.03	0.03	0.02	0.01
Repeatability (Sr)	0.02	0.02	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.01
%RSDr	15.70	17.14	13.26	0.00	8.92	16.09	29.64	29.64	25.75	46.97
r (2.8xSr)	0.05	0.05	0.01	0.00	0.01	0.01	0.03	0.03	0.01	0.02
Reproducibility (SR)	0.02	0.02	0.02	0.00	0.00	0.01	0.02	0.02	0.01	0.01
%RSDR	19.39	20.56	51.60	0.00	12.41	19.13	58.12	58.12	67.03	58.62
R (2.8xSR)	0.06	0.06	0.05	0.00	0.01	0.01	0.05	0.05	0.03	0.02
Butter	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	71.12	67.76	36.52	2.49	22.31	20.23	8.77	8.36	1.73	6.91
Repeatability (Sr)	1.62	1.54	1.00	0.13	0.63	0.54	0.44	0.39	0.14	0.14
%RSDr	2.28	2.28	2.73	5.34	2.81	2.68	4.99	4.72	8.25	2.05
r (2.8xSr)	4.54	4.32	2.80	0.37	1.75	1.52	1.23	1.10	0.40	0.40
Reproducibility (SR)	4.74	4.52	2.32	0.43	1.94	1.74	0.89	0.88	0.15	0.50
%RSDR	6.66	6.68	6.35	17.29	8.69	8.62	10.15	10.50	8.89	7.17
R (2.8xSR)	13.26	12.66	6.49	1.21	5.43	4.88	2.49	2.46	0.43	1.39
Whole egg powder	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	40.24	38.47	14.34	0.43	16.73	16.35	7.36	7.31	0.37	6.84
Repeatability (Sr)	1.20	1.15	0.41	0.02	0.56	0.56	0.22	0.21	0.02	0.20
%RSDr	2.98	2.98	2.85	4.79	3.33	3.41	2.95	2.92	5.99	2.88
r (2.8xSr)	3.36	3.21	1.14	0.06	1.56	1.56	0.61	0.60	0.06	0.55
Reproducibility (SR)	1.20	1.15	0.50	0.06	0.56	0.56	0.35	0.33	0.03	0.38
%RSDR	2.98	2.98	3.52	12.99	3.33	3.41	4.79	4.50	9.04	5.56
R (2.8xSR)	3.36	3.21	1.41	0.16	1.56	1.56	0.99	0.92	0.09	1.06
Full-fat soy flour flakes	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	23.05	22.05	3.70	0.02	4.73	4.74	13.49	13.48	1.61	11.69
Repeatability (Sr)	0.23	0.22	0.11	0.00	0.12	0.05	0.39	0.39	0.03	0.12
%RSDr	0.98	0.98	3.02	22.12	2.55	1.10	2.91	2.91	1.61	1.07
r (2.8xSr)	0.63	0.61	0.31	0.01	0.34	0.15	1.10	1.10	0.07	0.35
Reproducibility (SR)	0.66	0.62	0.39	0.01	0.14	0.12	0.39	0.39	0.15	0.25
%RSDR	2.85	2.83	10.42	73.10	2.93	2.57	2.91	2.91	9.34	2.13
R (2.8xSR)	1.84	1.75	1.08	0.04	0.39	0.34	1.10	1.10	0.42	0.70
Encapsulated	Total Fat	Total Fat	Total	Total	Total	Total *cis*	Total	Total *cis*	Total n-3	Total n-6

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Table 5
(Continued)

DHA/EPA	(TAG%)*	(FA%)*	Saturated	*trans*	MUFA	MUFA	PUFA**	PUFA**	PUFA**	PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	56.05	53.66	18.18	0.68	14.09	13.33	21.02	20.63	18.25	2.19
Repeatability (Sr)	1.31	1.26	0.43	0.08	0.37	0.35	0.47	0.46	0.41	0.09
%RSDr	2.34	2.34	2.36	12.13	2.65	2.64	2.23	2.21	2.25	4.24
r (2.8xSr)	3.68	3.52	1.20	0.23	1.05	0.98	1.31	1.28	1.15	0.26
Reproducibility (SR)	3.32	3.15	0.95	0.23	0.81	0.67	1.83	1.75	1.46	0.93
%RSDR	5.92	5.87	5.22	33.82	5.72	5.01	8.71	8.50	8.01	42.40
R (2.8xSR)	9.29	8.83	2.66	0.64	2.26	1.87	5.13	4.91	4.09	2.60
Creamy ranch dressing	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	46.18	44.16	7.12	0.24	9.66	9.63	25.68	25.51	3.40	23.18
Repeatability (Sr)	1.91	1.82	0.32	0.01	0.52	0.52	0.98	0.98	0.11	0.92
%RSDr	4.13	4.13	4.51	3.71	5.39	5.40	3.83	3.84	3.18	3.98
r (2.8xSr)	5.34	5.11	0.90	0.02	1.46	1.46	2.75	2.74	0.30	2.58
Reproducibility (SR)	1.91	1.82	0.32	0.16	0.52	0.52	2.36	2.37	0.11	1.16
%RSDR	4.13	4.13	4.51	65.50	5.39	5.40	9.17	9.30	3.18	5.02
R (2.8xSR)	5.34	5.11	0.90	0.44	1.46	1.46	6.60	6.64	0.30	3.26
Potato chips	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	36.01	34.44	6.35	0.22	14.16	14.02	13.70	13.59	0.12	13.45
Repeatability (Sr)	1.75	1.67	0.31	0.06	0.74	0.72	0.63	0.61	0.02	0.60
%RSDr	4.85	4.85	4.95	26.22	5.21	5.14	4.57	4.50	13.30	4.50
r (2.8xSr)	4.89	4.68	0.88	0.16	2.07	2.02	1.75	1.71	0.04	1.69
Reproducibility (SR)	1.75	1.67	0.31	0.14	0.74	0.72	0.63	0.61	0.04	0.60
%RSDR	4.85	4.85	4.95	62.69	5.21	5.14	4.57	4.50	37.66	4.50
R (2.8xSR)	4.89	4.68	0.88	0.39	2.07	2.02	1.75	1.71	0.12	1.69
Cheese powder duplicate	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	30.10	28.69	13.35	7.20	13.85	6.96	1.31	0.98	0.13	0.85
Repeatability (Sr)	0.45	0.43	0.27	0.10	0.22	0.11	0.05	0.02	0.01	0.02
%RSDr	1.49	1.48	2.05	1.36	1.59	1.56	4.14	2.12	5.11	2.18
r (2.8xSr)	1.25	1.19	0.77	0.27	0.62	0.30	0.15	0.06	0.02	0.05
Reproducibility (SR)	1.18	1.11	0.30	0.31	0.37	0.21	0.05	0.10	0.04	0.08
%RSDR	3.91	3.88	2.22	4.27	2.70	3.04	4.14	10.08	29.05	9.25
R (2.8xSR)	3.30	3.12	0.83	0.86	1.05	0.59	0.15	0.28	0.11	0.22
Frozen cheese pizza	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	8.06	7.66	3.56	0.37	2.25	1.68	1.33	1.29	0.05	1.23
Repeatability (Sr)	0.67	0.64	0.30	0.04	0.20	0.16	0.13	0.12	0.01	0.12
%RSDr	8.32	8.37	8.54	10.04	9.10	9.76	9.45	9.46	20.01	9.39
r (2.8xSr)	1.88	1.80	0.85	0.10	0.57	0.46	0.35	0.34	0.03	0.32
Reproducibility (SR)	1.59	1.51	1.63	0.07	0.45	0.77	0.24	0.24	0.03	0.23
%RSDR	19.72	19.76	45.76	18.70	19.92	45.85	18.19	18.38	56.05	18.41
R (2.8xSR)	4.45	4.24	4.56	0.19	1.25	2.15	0.68	0.66	0.07	0.64

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Table 5
(Continued)

Peanut butter duplicate	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	51.50	49.29	9.66	0.05	30.84	30.55	8.78	8.77	0.08	8.55
Repeatability (Sr)	3.97	3.79	0.88	0.03	2.40	2.26	0.59	0.59	0.01	0.59
%RSDr	7.70	7.70	9.13	53.93	7.79	7.41	6.76	6.77	6.55	6.91
r (2.8xSr)	11.10	10.62	2.47	0.08	6.73	6.34	1.66	1.66	0.02	1.65
Reproducibility (SR)	4.43	4.24	0.92	0.05	2.60	2.89	1.51	1.50	0.09	1.22
%RSDR	8.61	8.61	9.51	85.63	8.42	9.46	17.22	17.15	105.13	14.32
R (2.8xSR)	12.41	11.88	2.57	0.13	7.27	8.09	4.23	4.21	0.24	3.43

Dry cereal fortified with flax	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	1.91	1.82	0.28	0.00	0.36	0.36	1.20	1.20	0.52	0.64
Repeatability (Sr)	0.03	0.02	0.02	0.00	0.01	0.01	0.03	0.03	0.01	0.02
%RSDr	1.34	1.35	6.75	0.00	2.04	2.04	2.53	2.53	2.48	3.42
r (2.8xSr)	0.07	0.07	0.05	0.00	0.02	0.02	0.08	0.08	0.04	0.06
Reproducibility (SR)	0.27	0.26	0.04	0.00	0.06	0.06	0.09	0.09	0.03	0.09
%RSDR	14.17	14.19	14.49	0.00	16.81	16.81	7.26	7.26	6.12	13.26
R (2.8xSR)	0.76	0.72	0.12	0.00	0.17	0.17	0.24	0.24	0.09	0.24

Horse feed	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	3.22	3.07	0.72	0.00	0.76	0.75	1.58	1.58	0.21	1.38
Repeatability (Sr)	0.17	0.16	0.04	0.00	0.05	0.04	0.07	0.07	0.01	0.06
%RSDr	5.31	5.26	6.22	0.00	6.27	5.89	4.52	4.52	3.57	4.68
r (2.8xSr)	0.48	0.45	0.13	0.00	0.13	0.12	0.20	0.20	0.02	0.18
Reproducibility (SR)	0.19	0.18	0.07	0.00	0.06	0.04	0.09	0.09	0.02	0.07
%RSDR	5.86	5.82	9.52	0.00	7.85	5.89	5.54	5.54	8.39	5.36
R (2.8xSR)	0.53	0.50	0.19	0.00	0.17	0.12	0.25	0.25	0.05	0.21

Gamebird feed	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	3.00	2.87	0.57	0.00	0.64	0.63	1.65	1.65	0.13	1.52
Repeatability (Sr)	0.07	0.07	0.02	0.00	0.02	0.02	0.02	0.02	0.00	0.02
%RSDr	2.25	2.29	4.27	0.00	3.40	3.47	1.49	1.44	1.68	1.50
r (2.8xSr)	0.19	0.18	0.07	0.00	0.06	0.06	0.07	0.07	0.01	0.06
Reproducibility (SR)	0.40	0.38	0.07	0.00	0.08	0.07	0.26	0.26	0.03	0.23
%RSDR	13.39	13.41	12.91	0.00	12.14	10.97	15.48	15.46	19.07	15.19
R (2.8xSR)	1.12	1.08	0.21	0.00	0.22	0.19	0.72	0.71	0.07	0.64

*NLEA (Nutritional Labeling and Education Act) according to the Office of Nutritional Products, Labeling, and Dietary Supplements in the Center for Food Safety and Applied Nutrition at the U.S. Food and Drug Administration
**Does not include CLA

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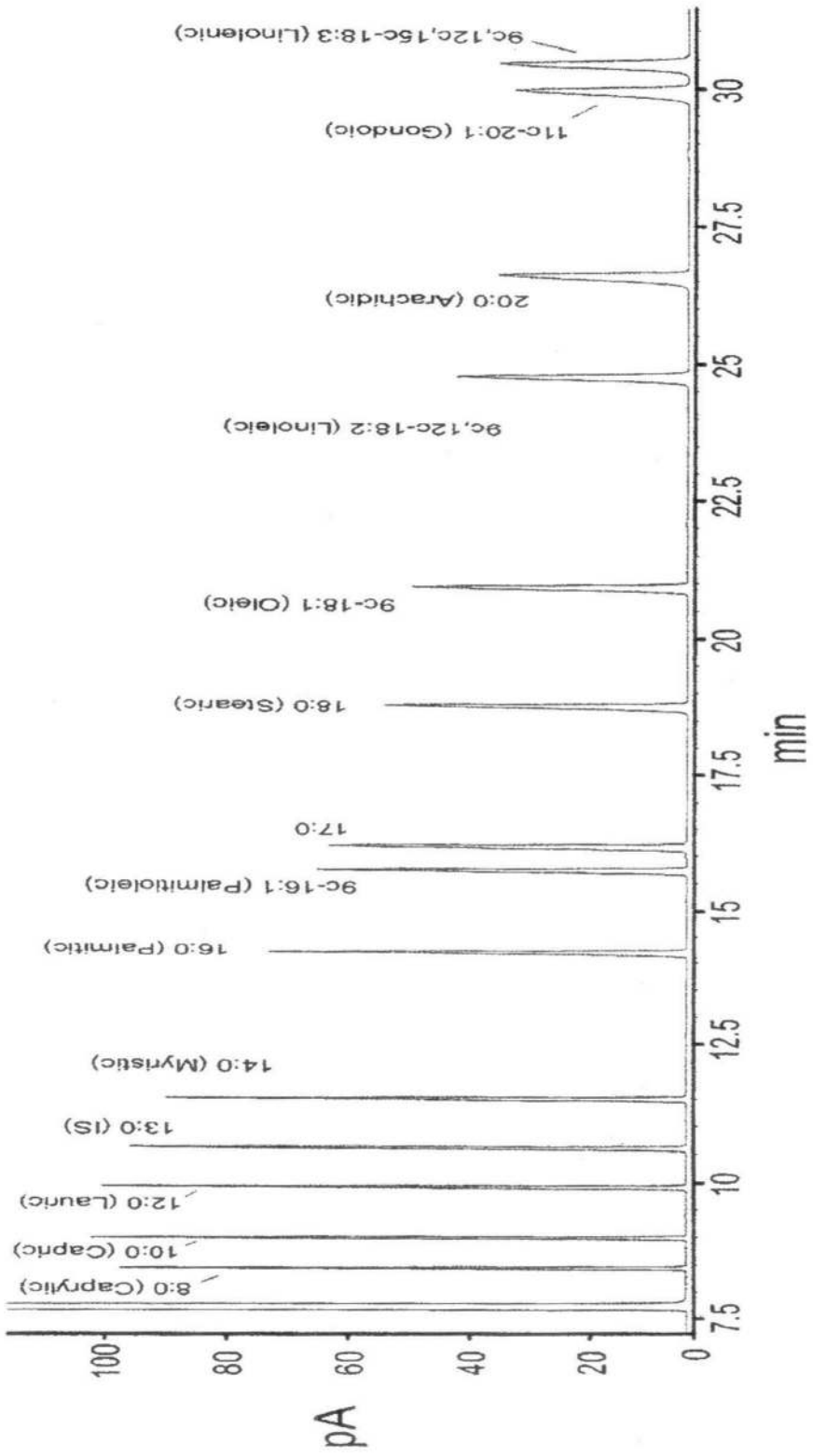

Figure 1a. GC/FID profile of GLC-714. Separations were on a SP™-2560 (100 m × 0.25 mm × 0.20 µm). Column temperature was 180°C (32 min) to 215°C @ 20°C/min (hold 31.25 min). Carrier gas was *hydrogen* at 1.0 mL/min (constant flow) with a linear velocity of 26 cm/s. Pressure was 24.63 psi @ 180°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100.

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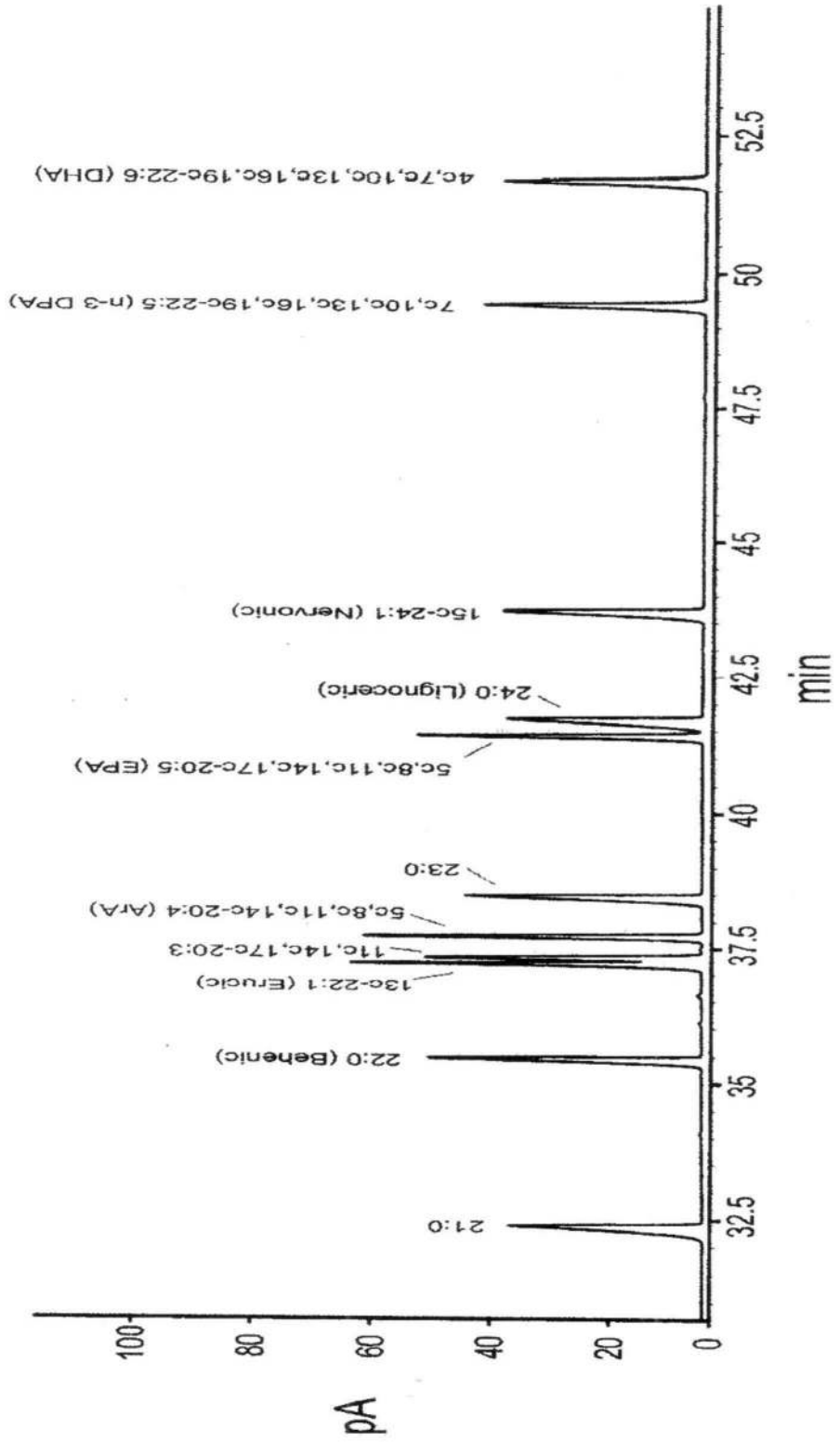

Figure 1b. GC/FID profile of GLC-714. See Figure 1a for chromatographic parameters.

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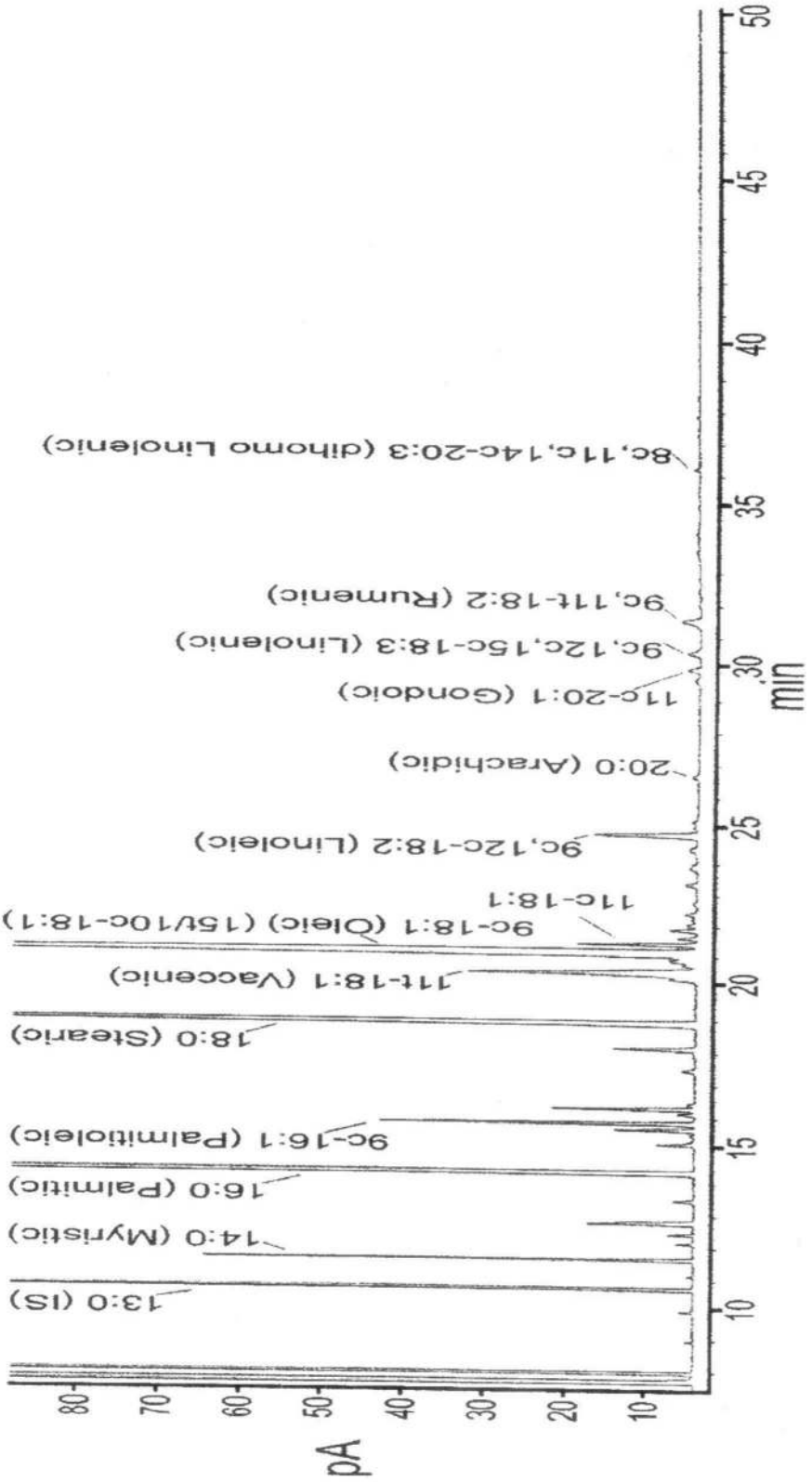

Figure 2. GC/FID profile of major fatty acid methyl esters (FAME) in Tallow (References 17–24). See Figure 1a for chromatographic parameters.

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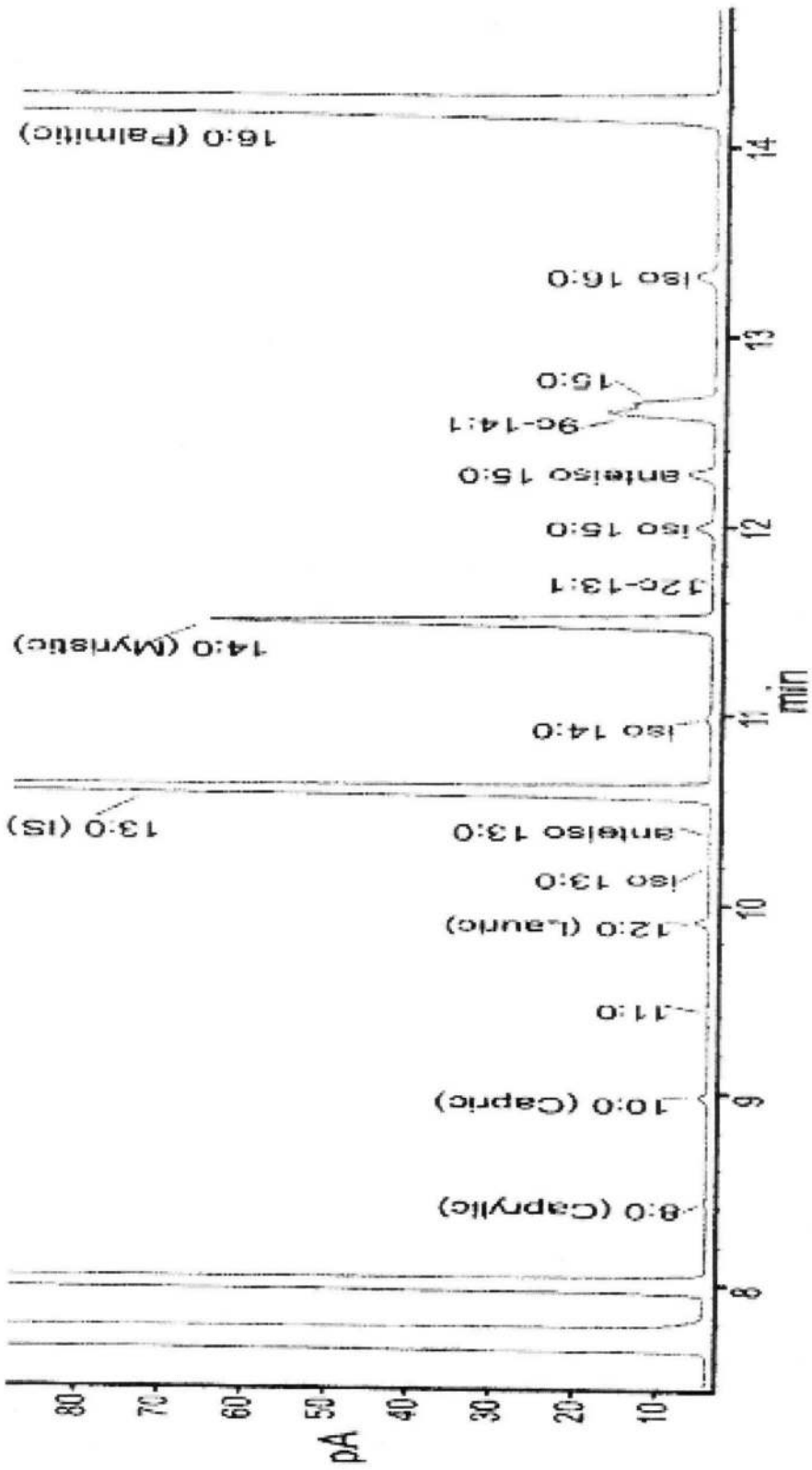

Figure 3. GC/FID profile of fatty acid methyl esters (FAME) in Tallow from start to 16:0 (References 17–24) . See Figure 1a for chromatographic parameters.

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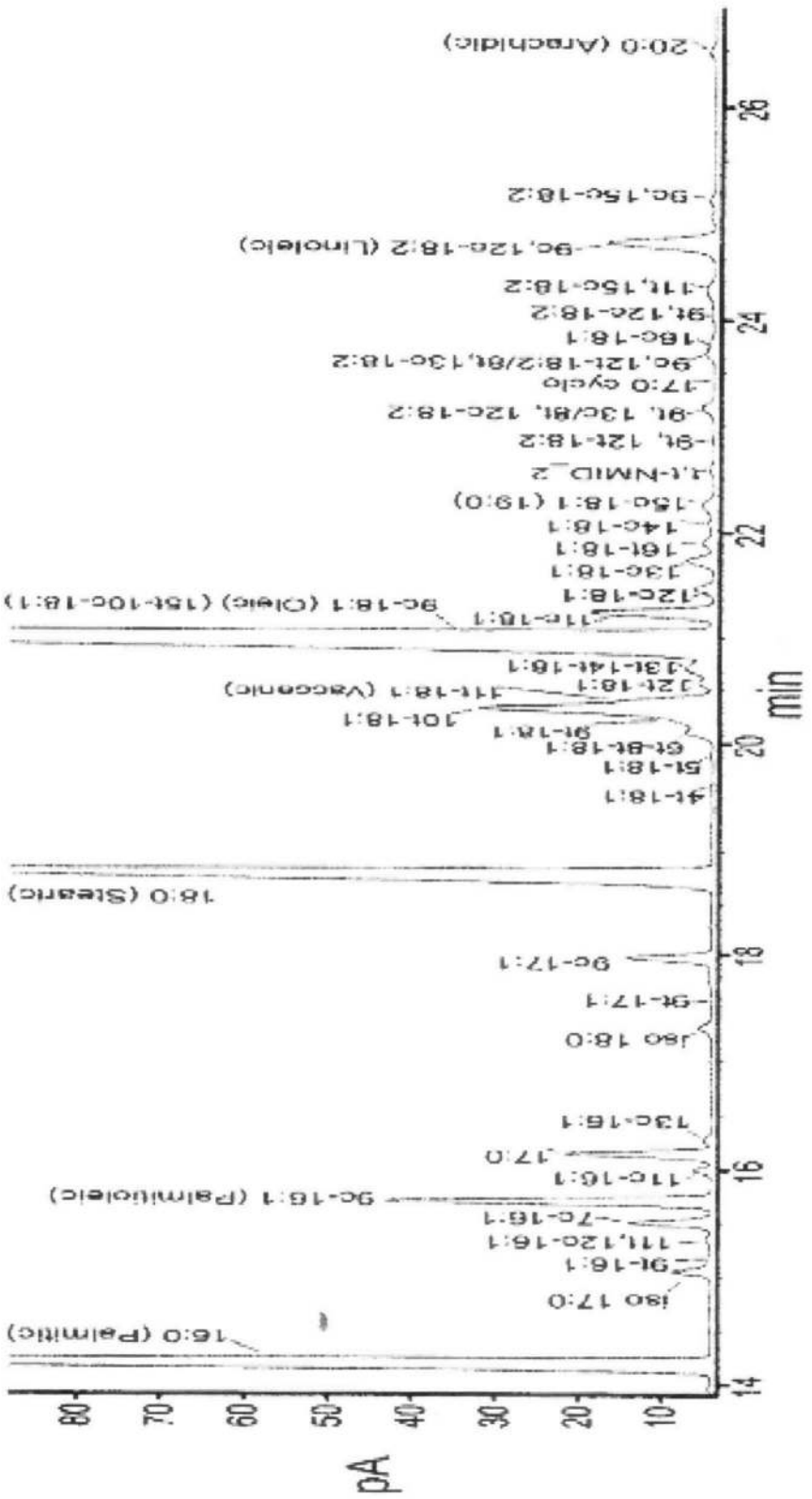

Figure 4a. GC/FID profile of fatty acid methyl esters (FAME) in Tallow from 16:0 to 20:0 (References 17–24). See Figure 1a for chromatographic parameters.

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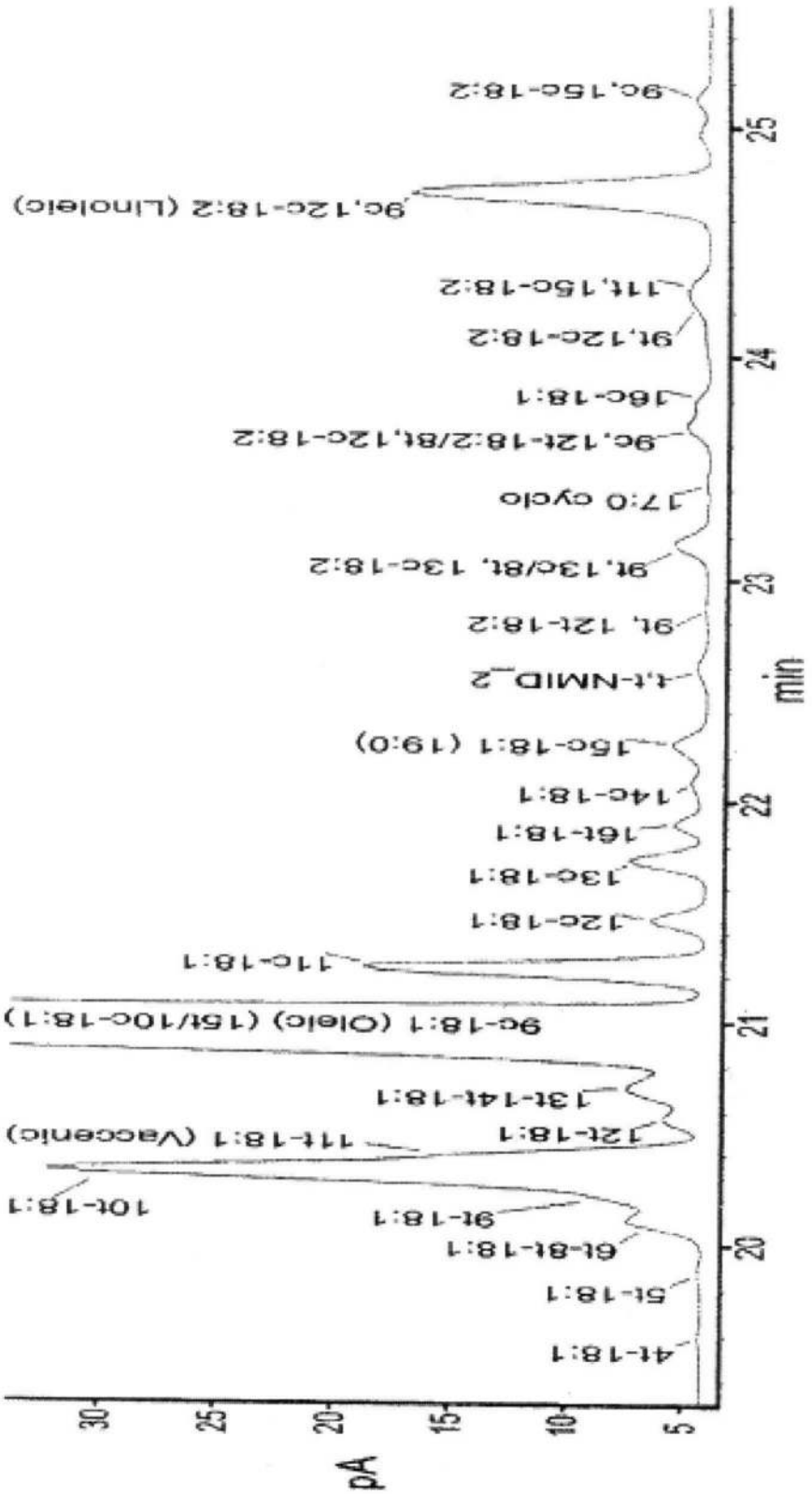

Figure 4b. GC/FID profile of fatty acid methyl esters (FAME) in Tallow zoomed in to Oleic and Linoleic regions (References 17–24). See Figure 1a for chromatographic parameters.

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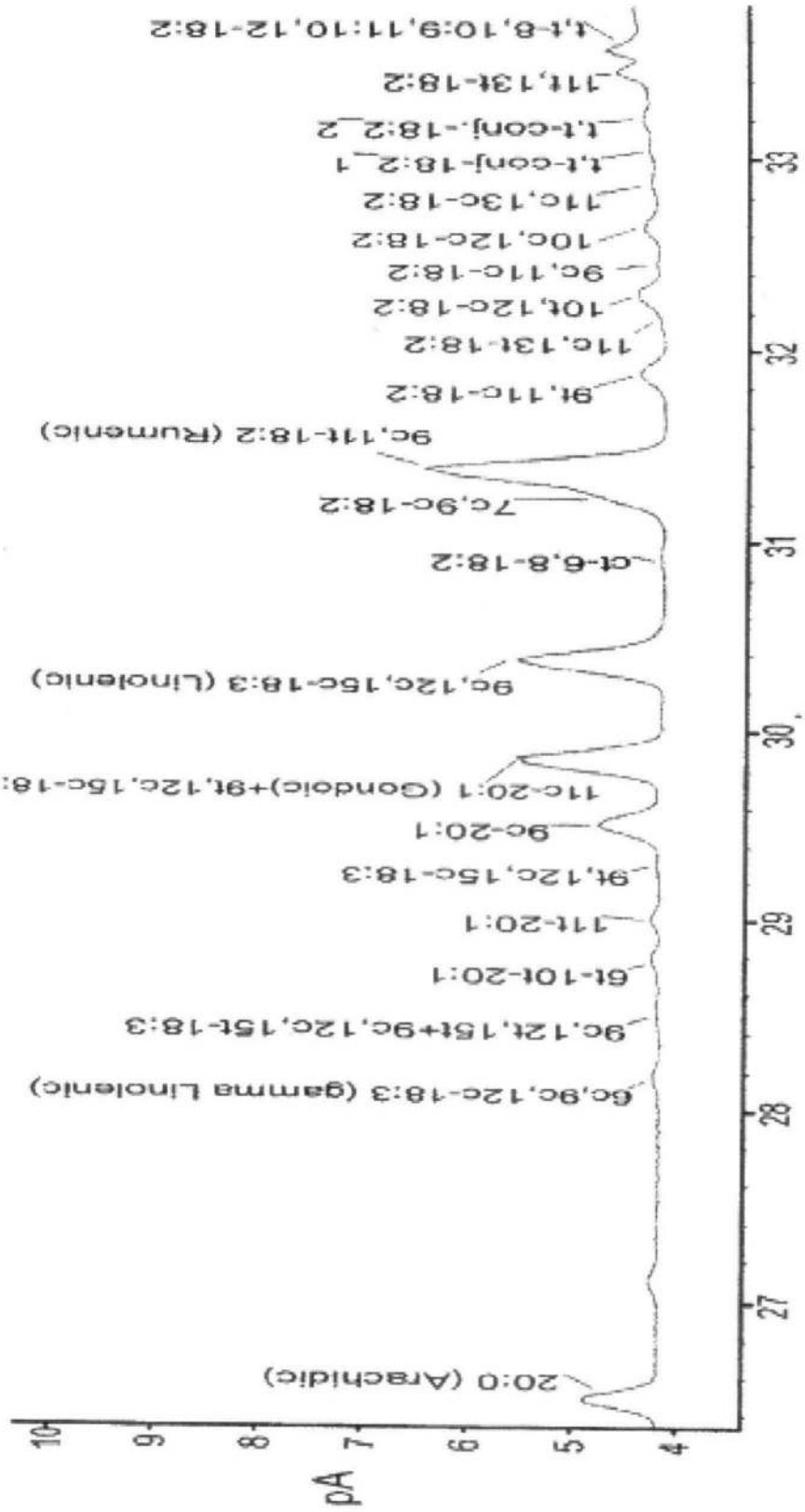

Figure 5. GC/FID profile of fatty acid methyl esters (FAME) in Tallow from 20:0 to the CLA region (References 17–24). See Figure 1a for chromatographic parameters. 9c,12t,15c-18:3 will coelute with 9c-20:1 (21).

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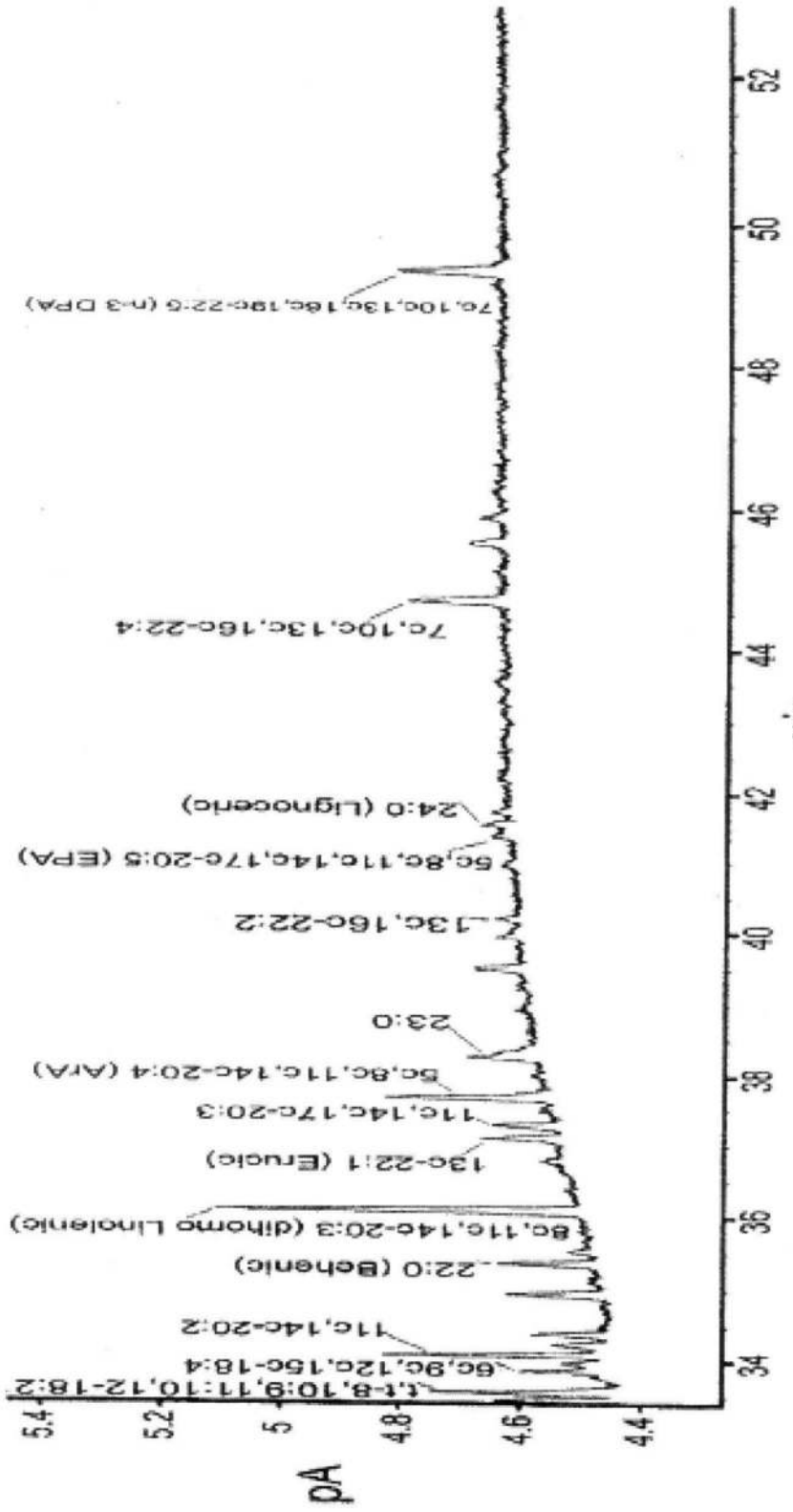

Figure 6. GC/FID profile of fatty acid methyl esters (FAME) in Tallow from the CLA region to end of the chromatogram (References 17–24). See Figure 1a for chromatographic parameters.

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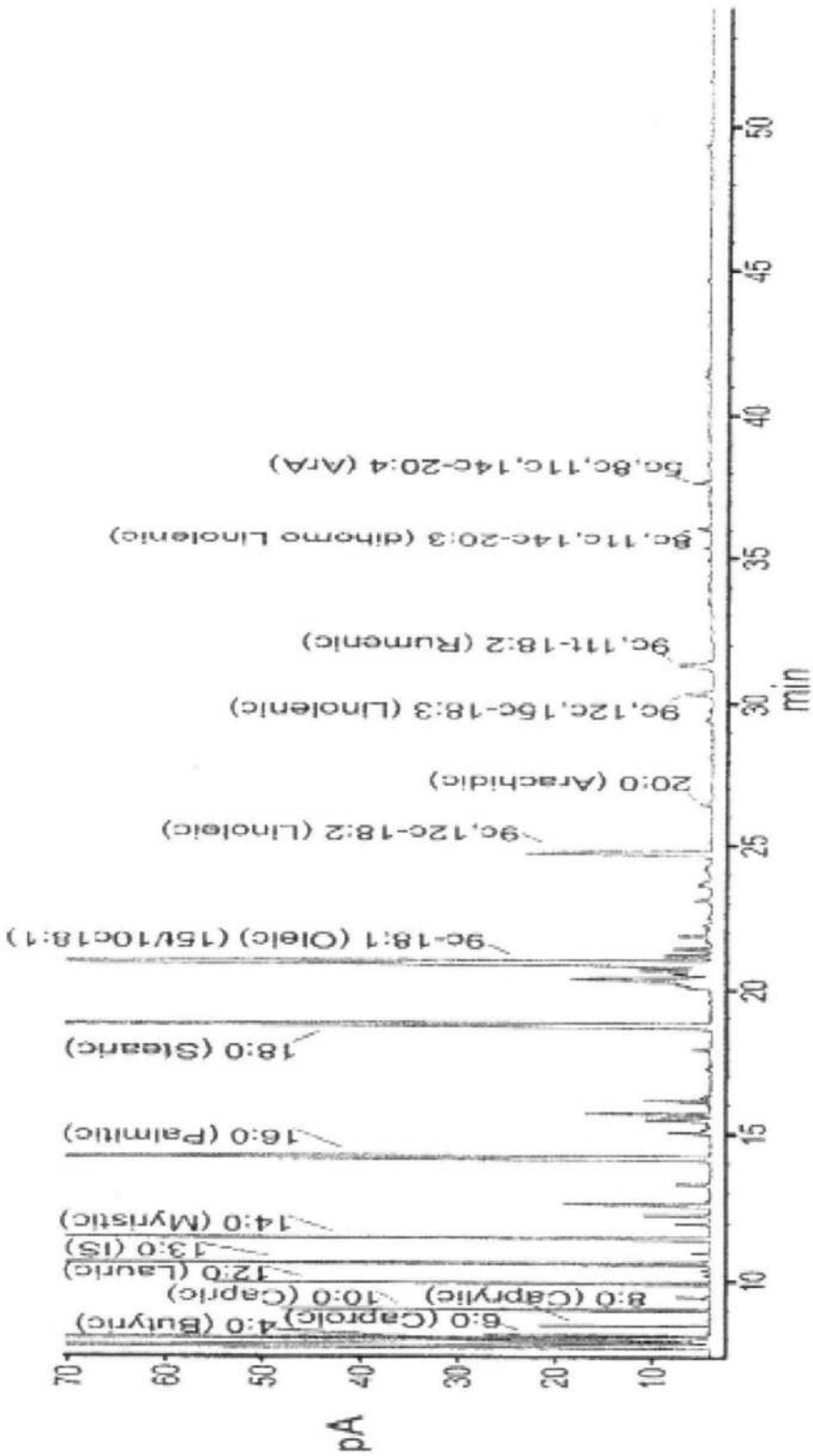

Figure 7. GC/FID profile of major fatty acid methyl esters (FAME) in Anhydrous Butterfat (References 17–24). See Figure 1a for chromatographic parameters.

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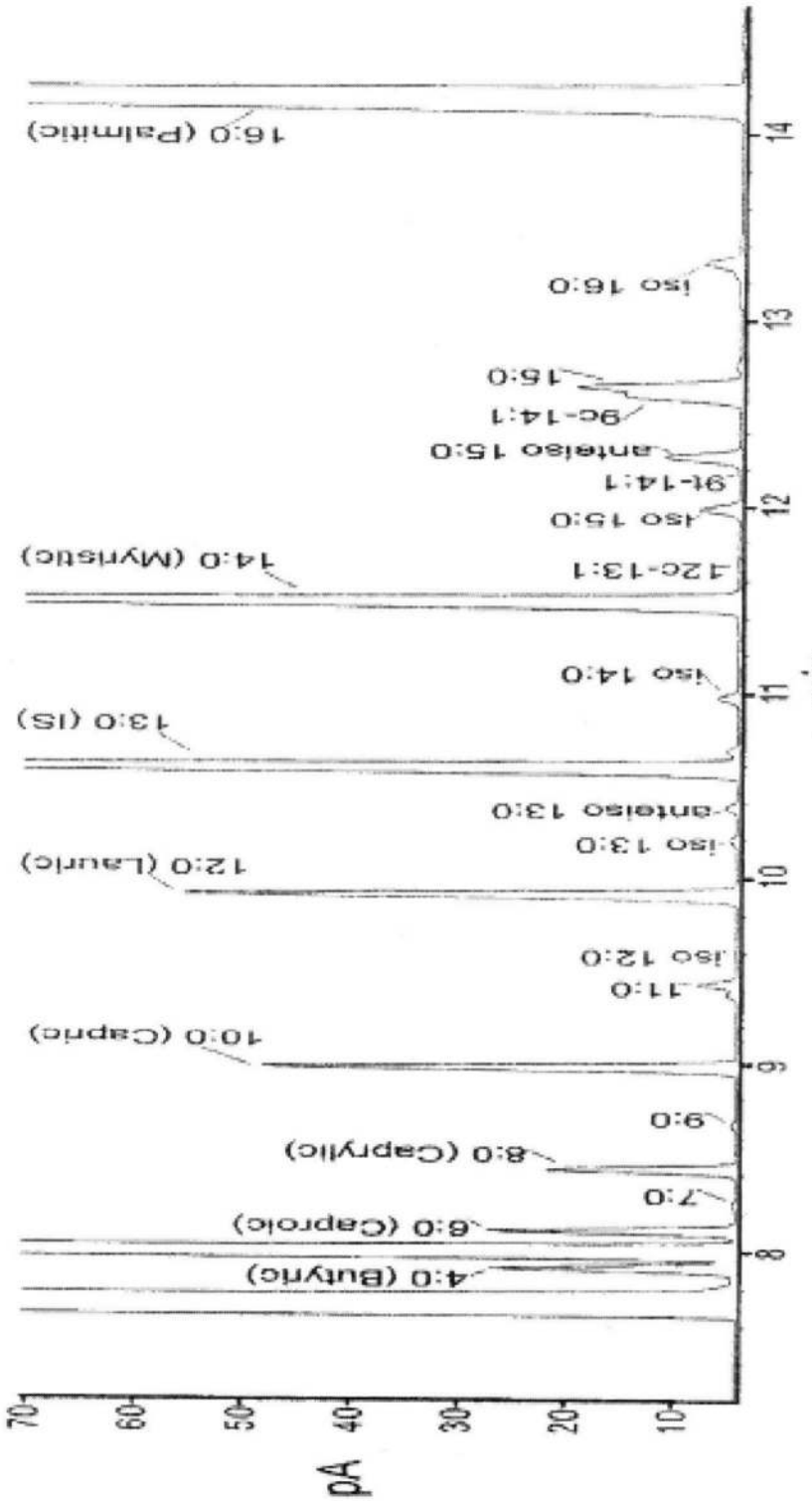

Figure 8. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat from start to 16:0 (References 17–24) . See Figure 1a for chromatographic parameters.

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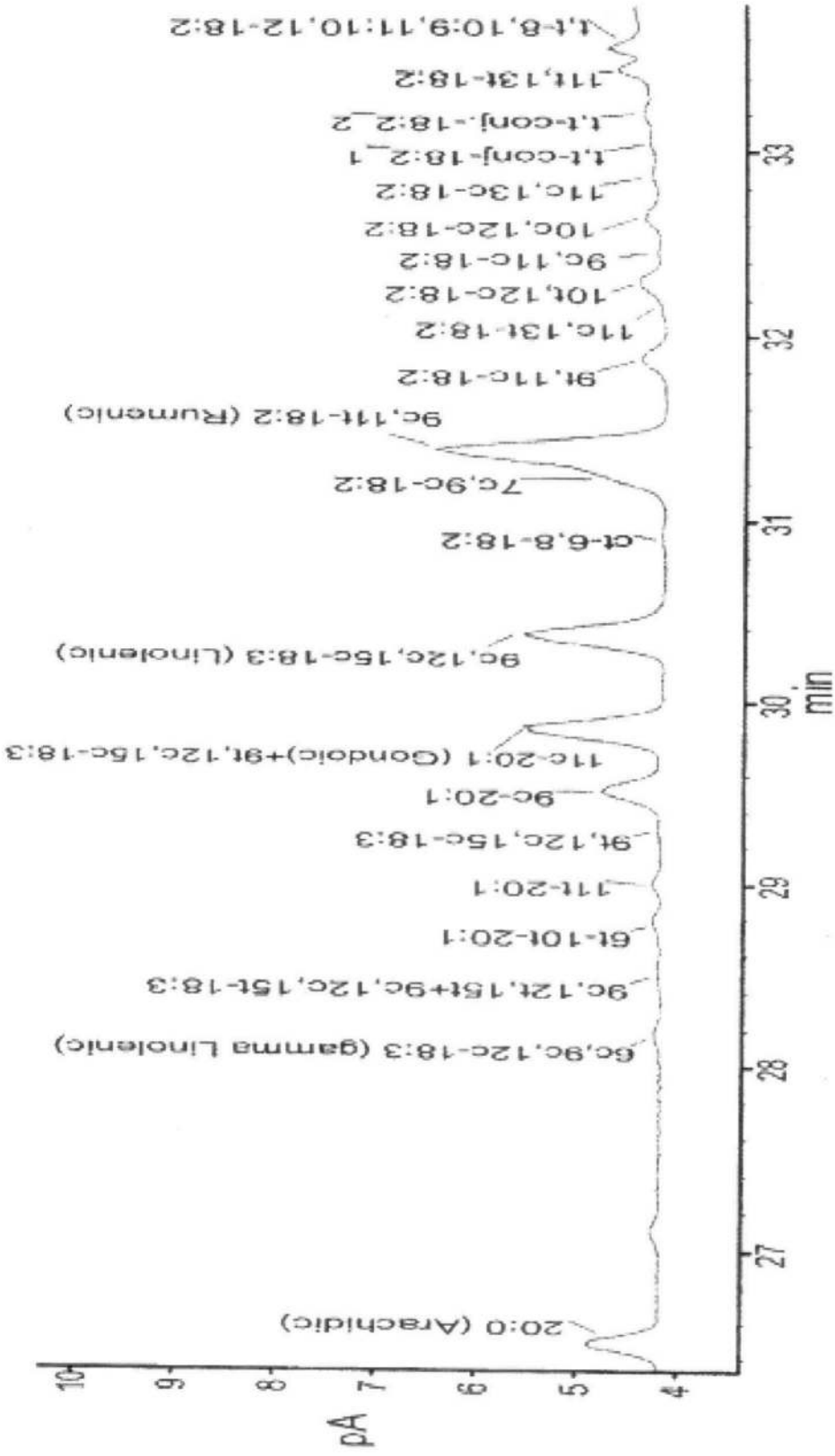

Figure 9a. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat from 16:0 to 20:0 (References 17–24). See Figure 1a for chromatographic parameters.

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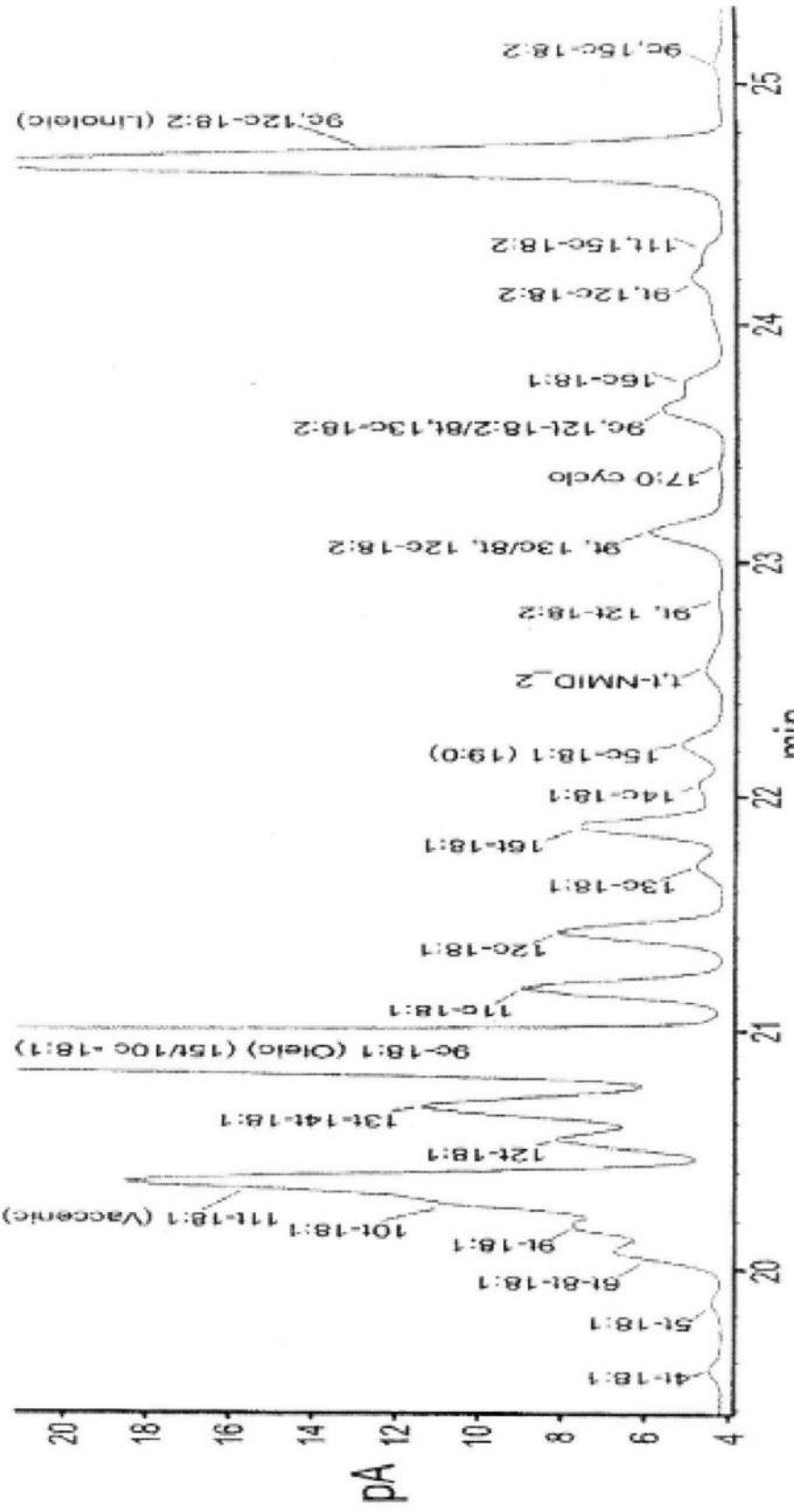

Figure 9b. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat zoomed in to Oleic and Linoleic regions (References 17–24). See Figure 1a for chromatographic parameters.

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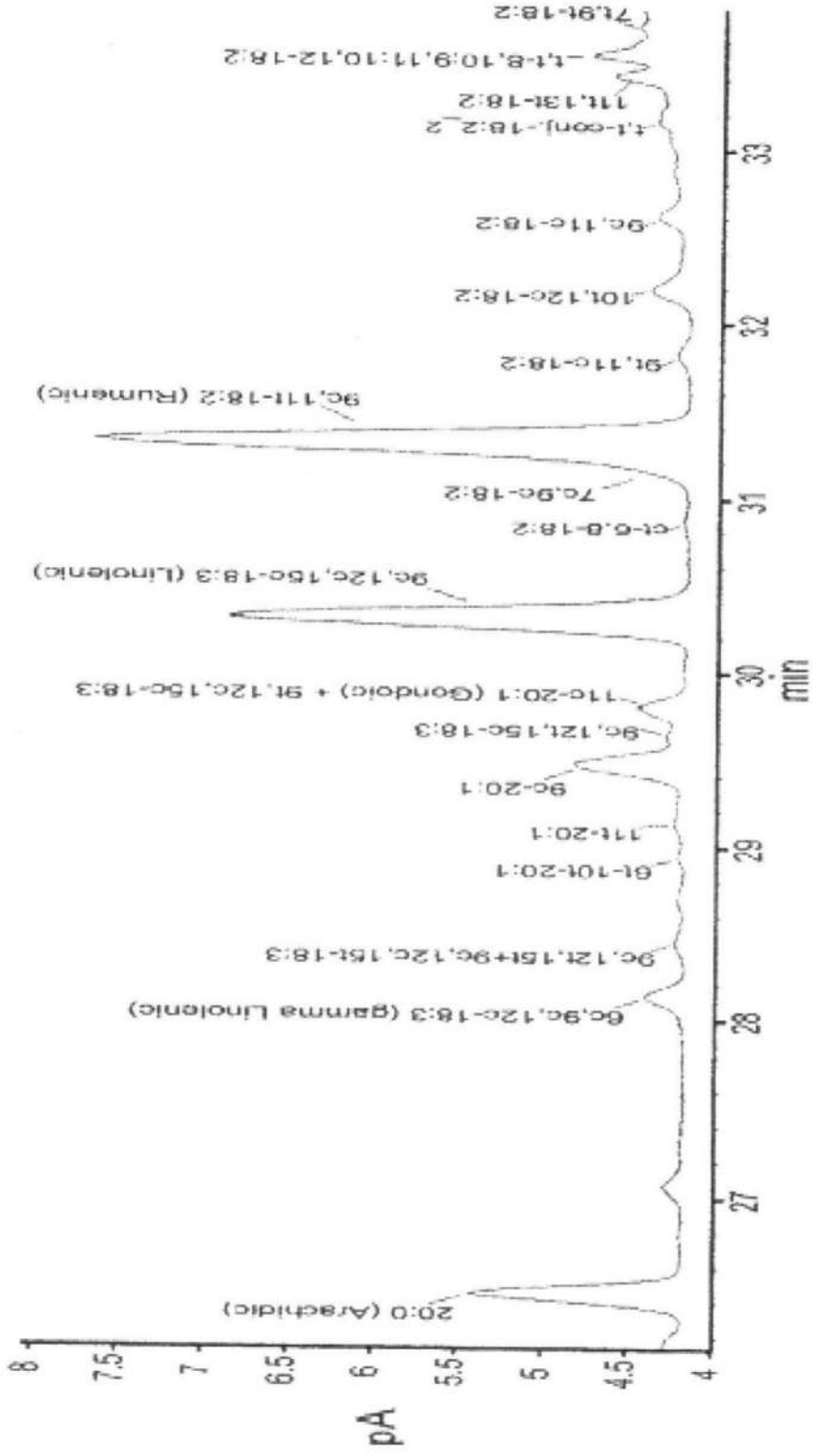

Figure 10. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat from 20:0 to the CLA region (References 17–24). See Figure 1a for chromatographic parameters. 9c,12t,15c-18:3 will coelute with 9c-20:1 (21).

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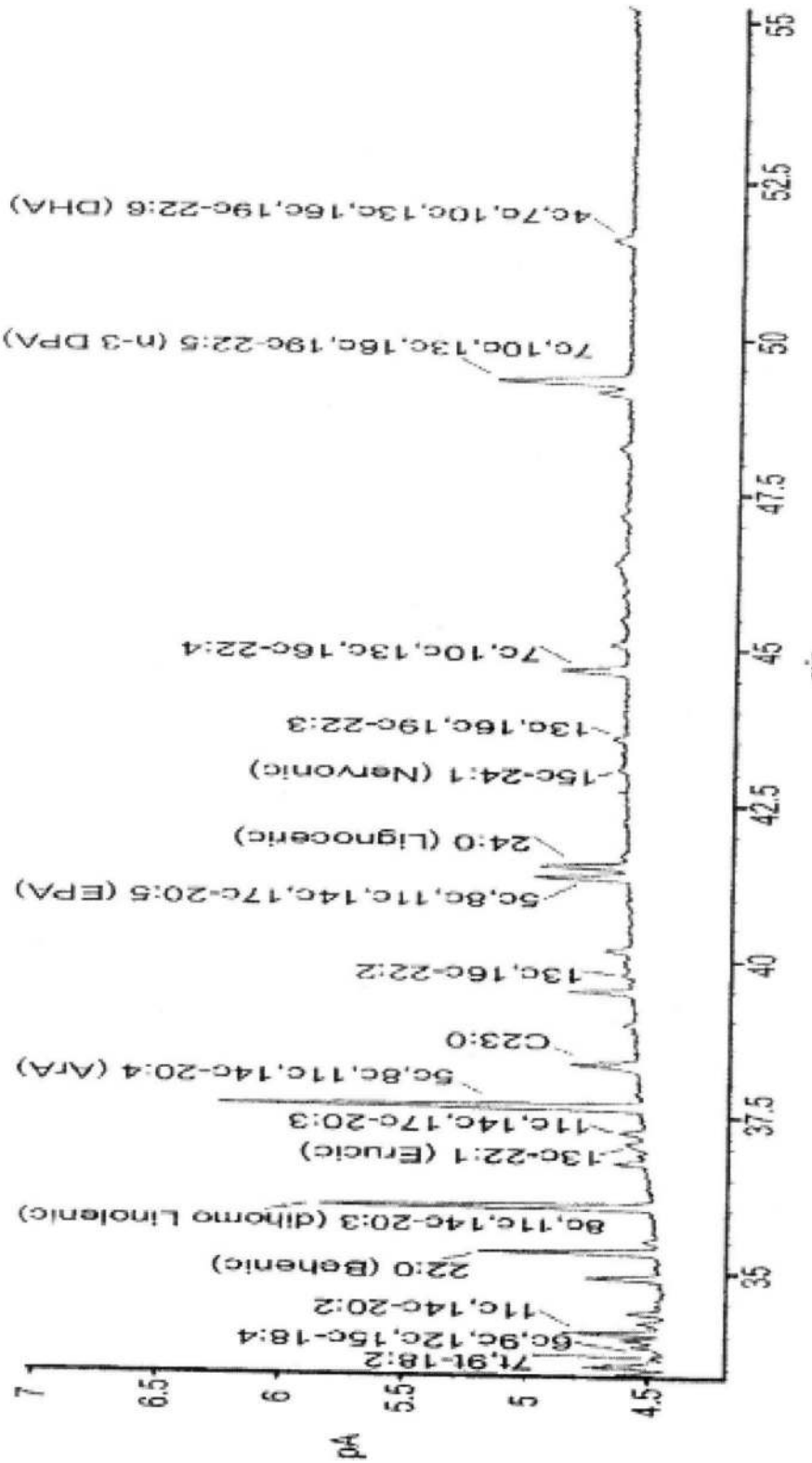

Figure 11. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat from the CLA region to end of the chromatogram (References 17–24). See Figure 1a for chromatographic parameters.

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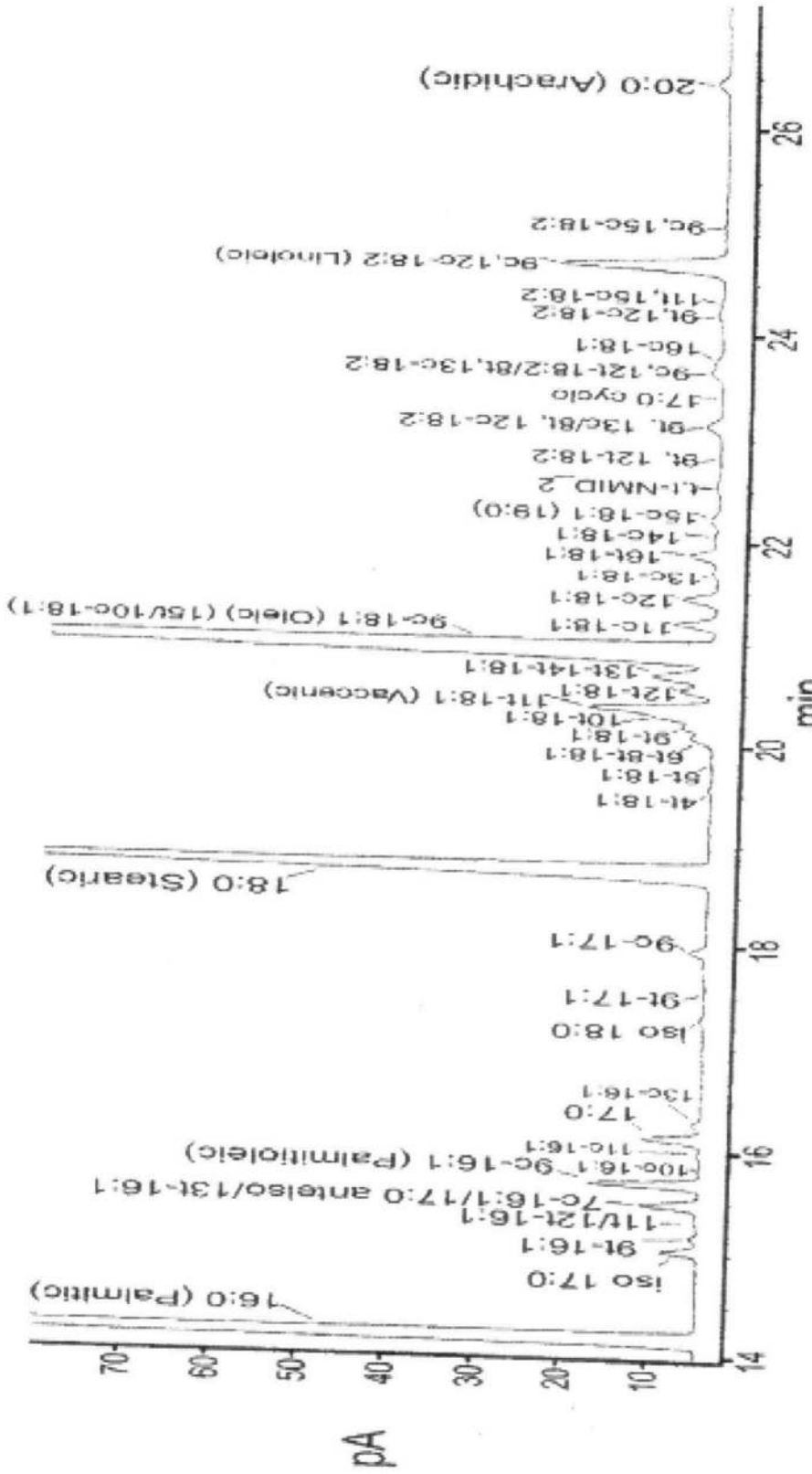

Figure 12. GC/FID profile of the major fatty acids in BCR-163 fatty acid methyl esters (FAME) (References 17–24). See Figure 1a for chromatographic parameters.

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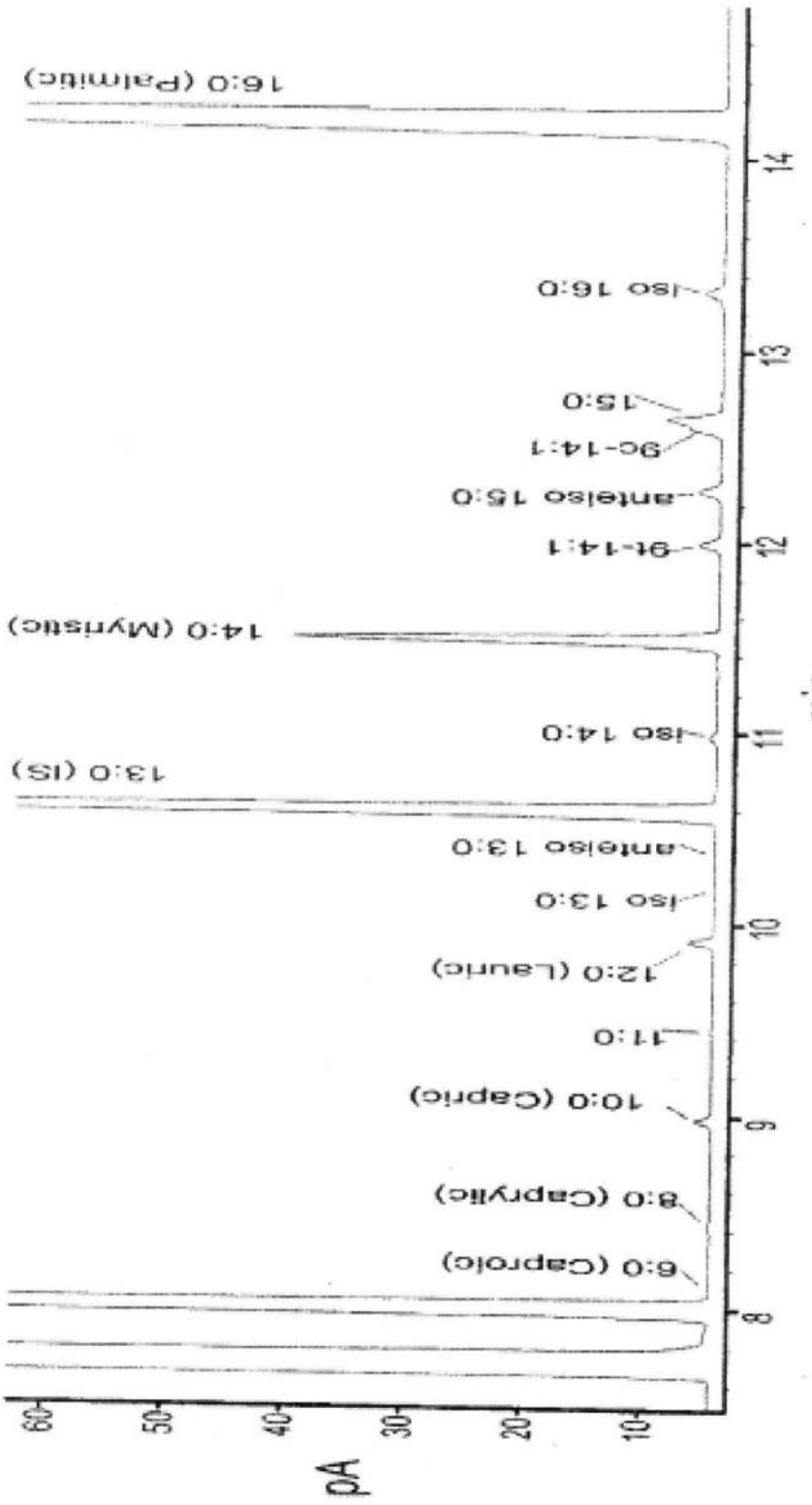

Figure 13. GC/FID profile of fatty acid methyl esters (FAME) in BCR-163 from start to 16:0 (References 17–24). See Figure 1a for chromatographic parameters.

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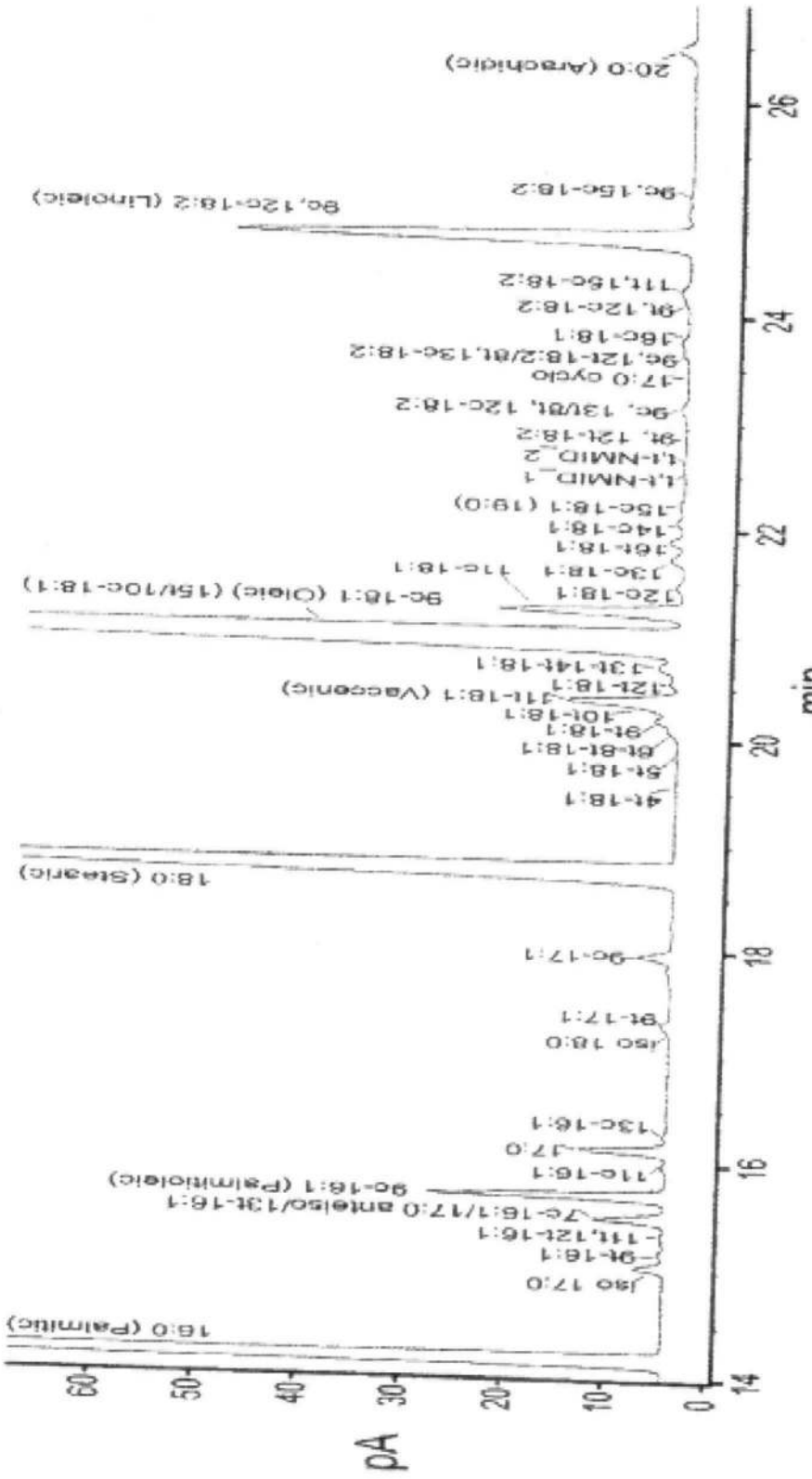

Figure 14a. GC/FID profile of major fatty acid methyl esters (FAME) in BCR-163 from 16:0 to 20:0 (References 17–24). See Figure 1a for chromatographic parameters.

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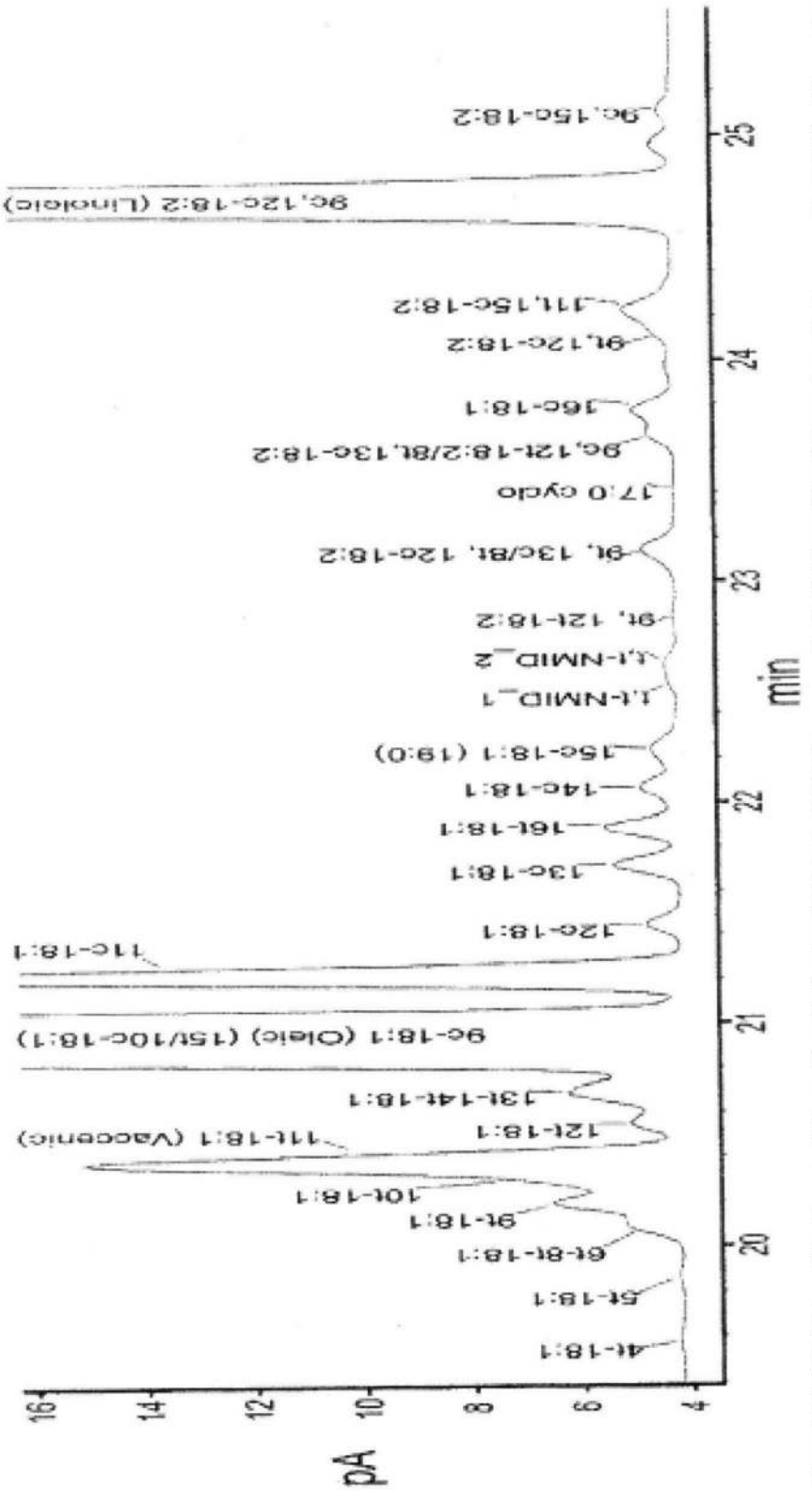

Figure 14b. GC/FID profile of major fatty acid methyl esters (FAME) in BCR-163 zoomed in to Oleic and Linoleic regions (References 17–24). See Figure 1a for chromatographic parameters.

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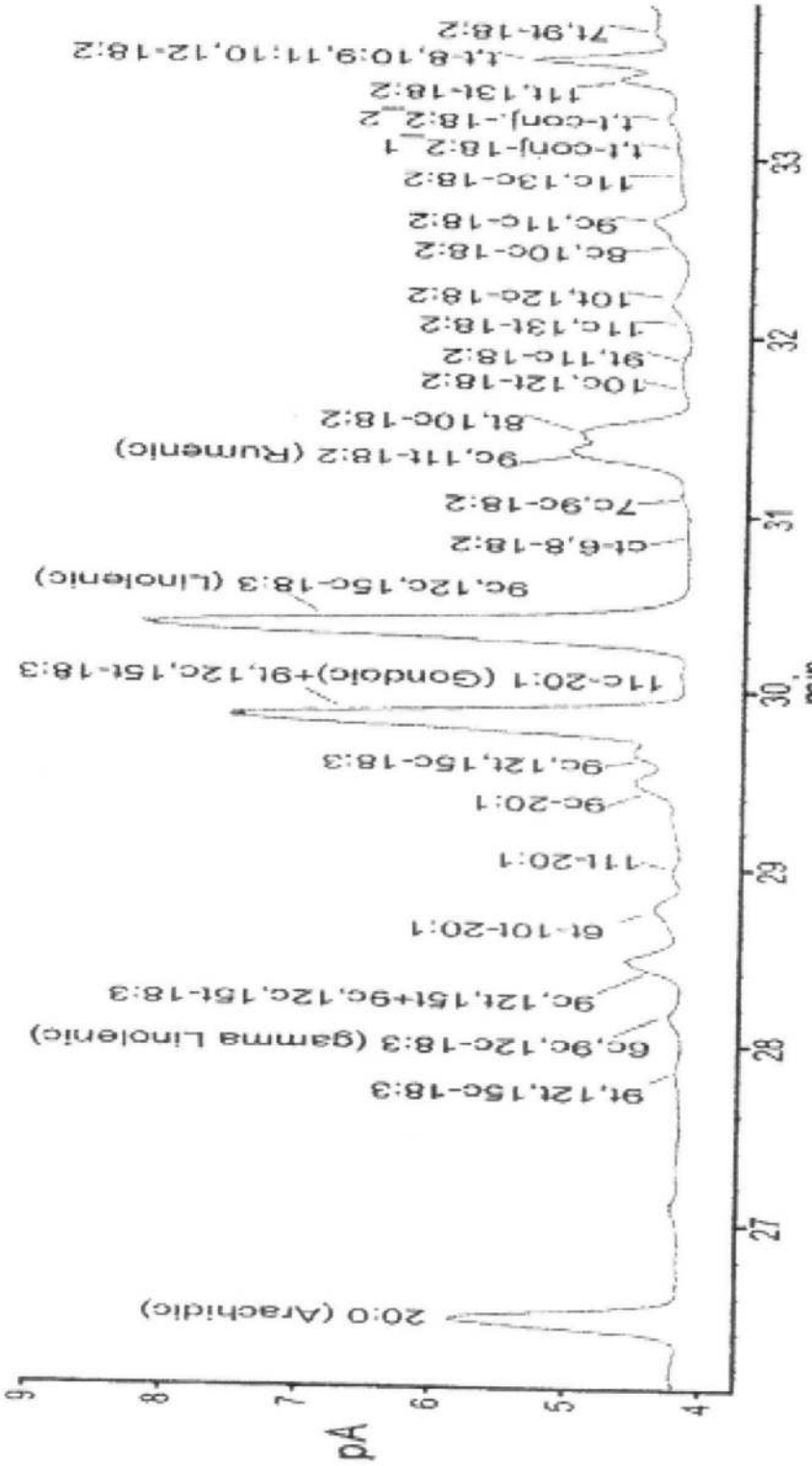

Figure 15. GC/FID profile of fatty acid methyl esters (FAME) in BCR-163 from 20:0 to the CLA region (References 17–24). See Figure 1a for chromatographic parameters. 9c,12t,15c-18:3 will coelute with 9c-20:1 (21).

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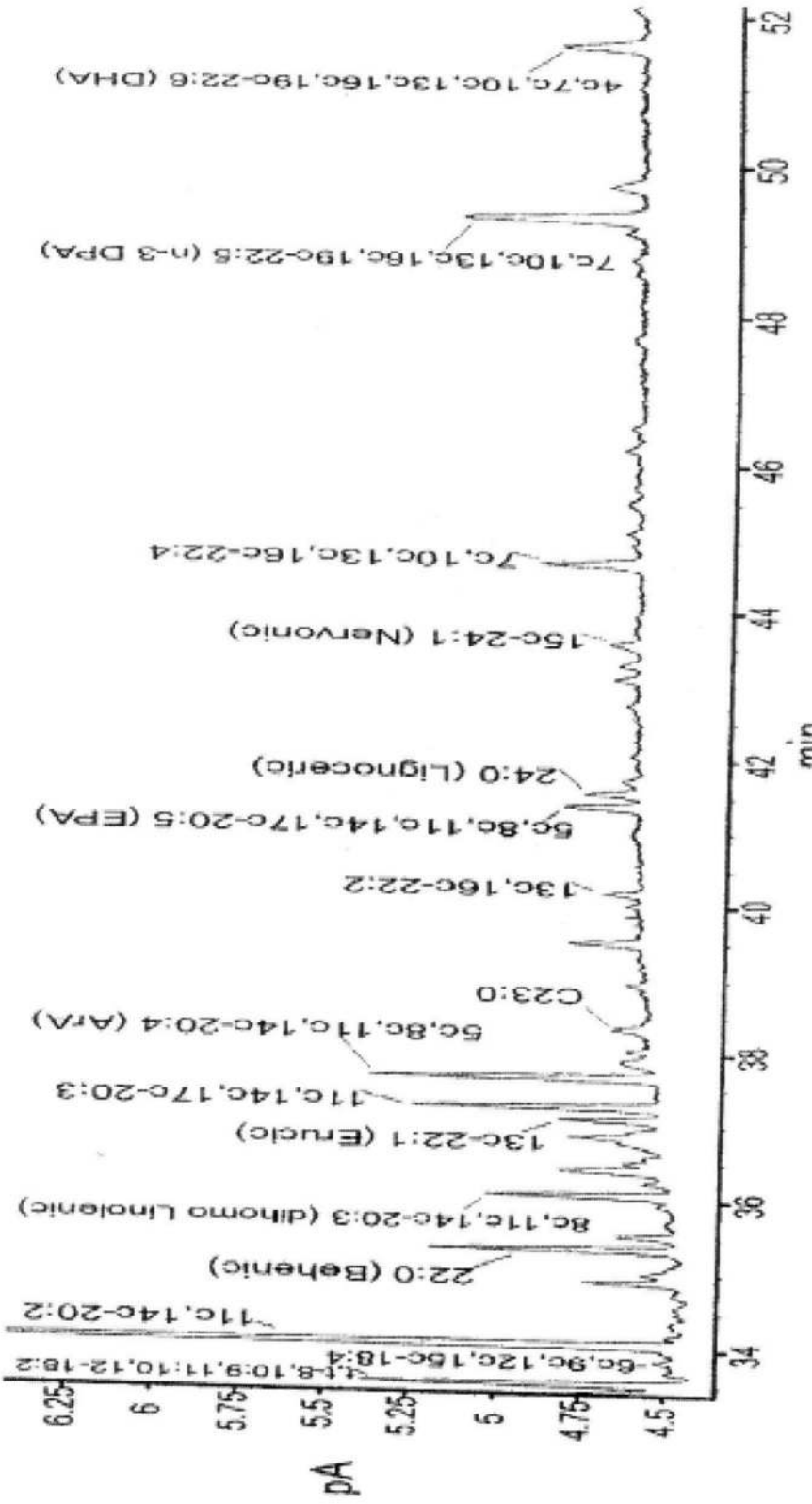

Figure 16. GC/FID profile of fatty acid methyl esters (FAME) in BCR-163 from the CLA region to end of the chromatogram (References 17–24). See Figure 1a for chromatographic parameters.

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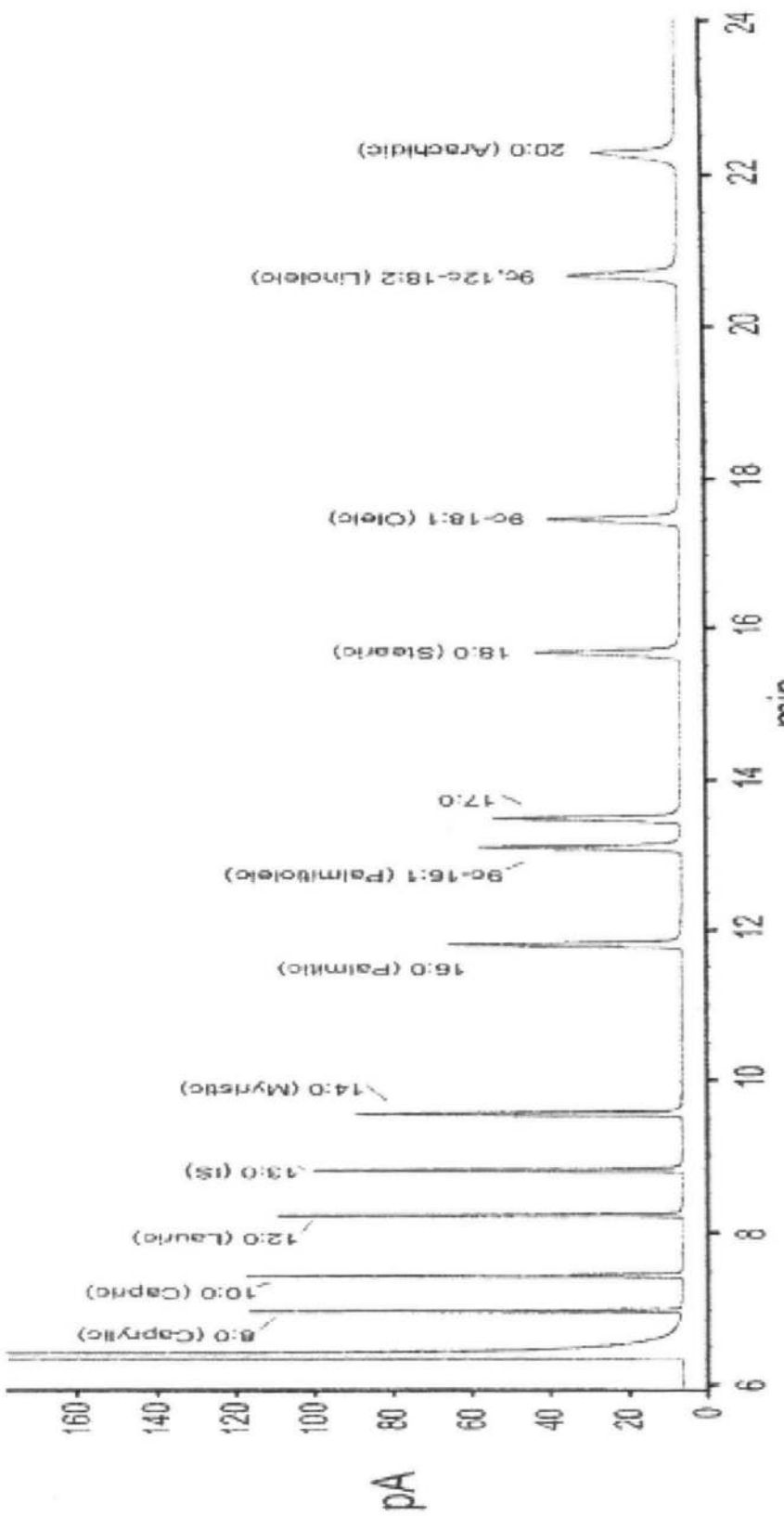

Figure 17a. GC/FID profile of GLC-714. Separations were on a CP-SIL-88 (100 m × 0.25 mm × 0.20 µm). Column temperature was 180°C (32 min) to 215°C @ 20°C/min (hold 31.25 min). Carrier gas was *helium* at 2.0 mL/min (constant flow) with a linear velocity of 28 cm/s. Pressure was 63.29 psi @ 180°C. Injection and FID temperatures were 235°C and 325°C, respectively. Split ratio was 1:100.

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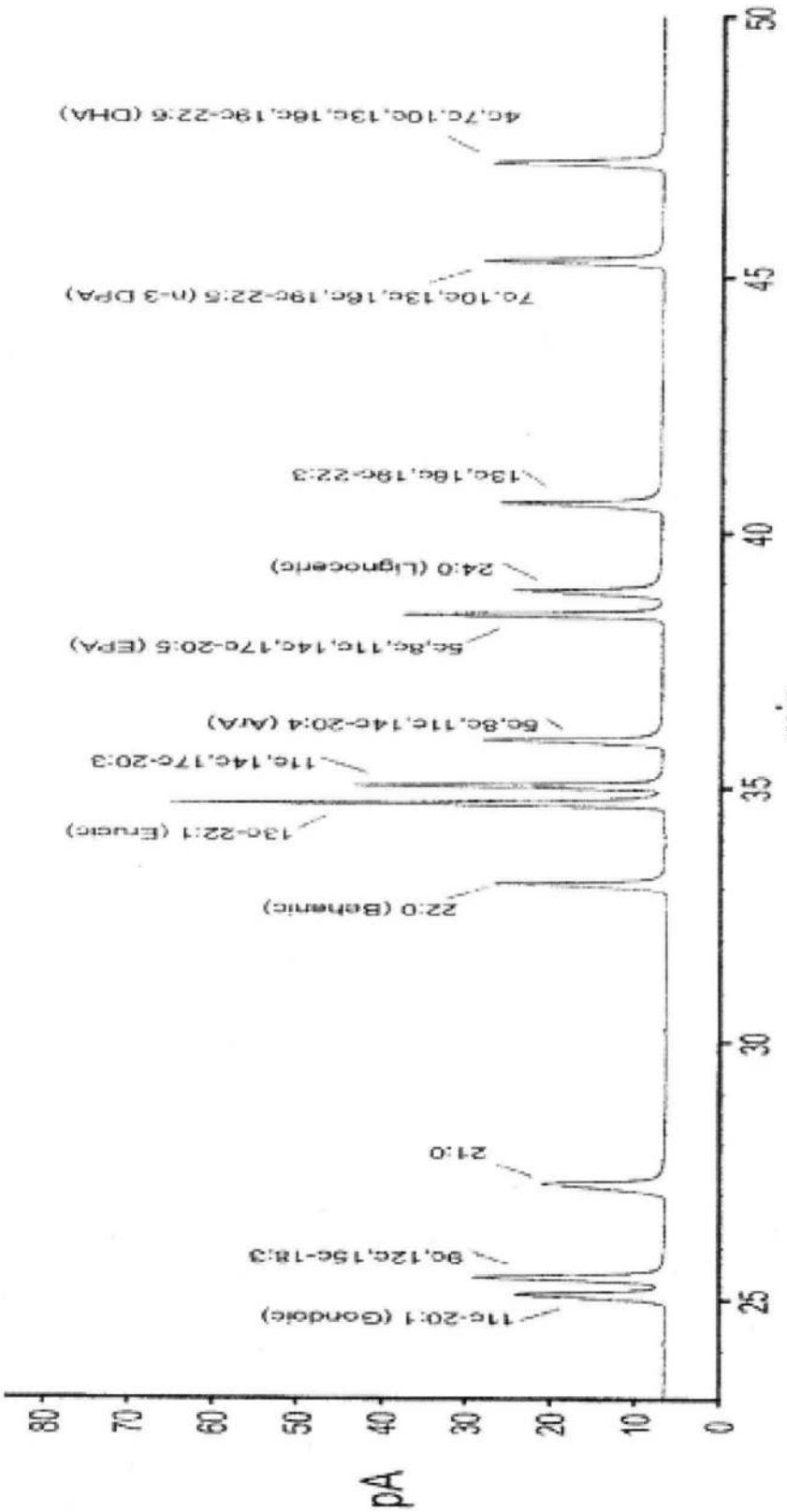

Figure 17b. GC/FID profile of GLC-714. See Figure 17a for chromatographic parameters.

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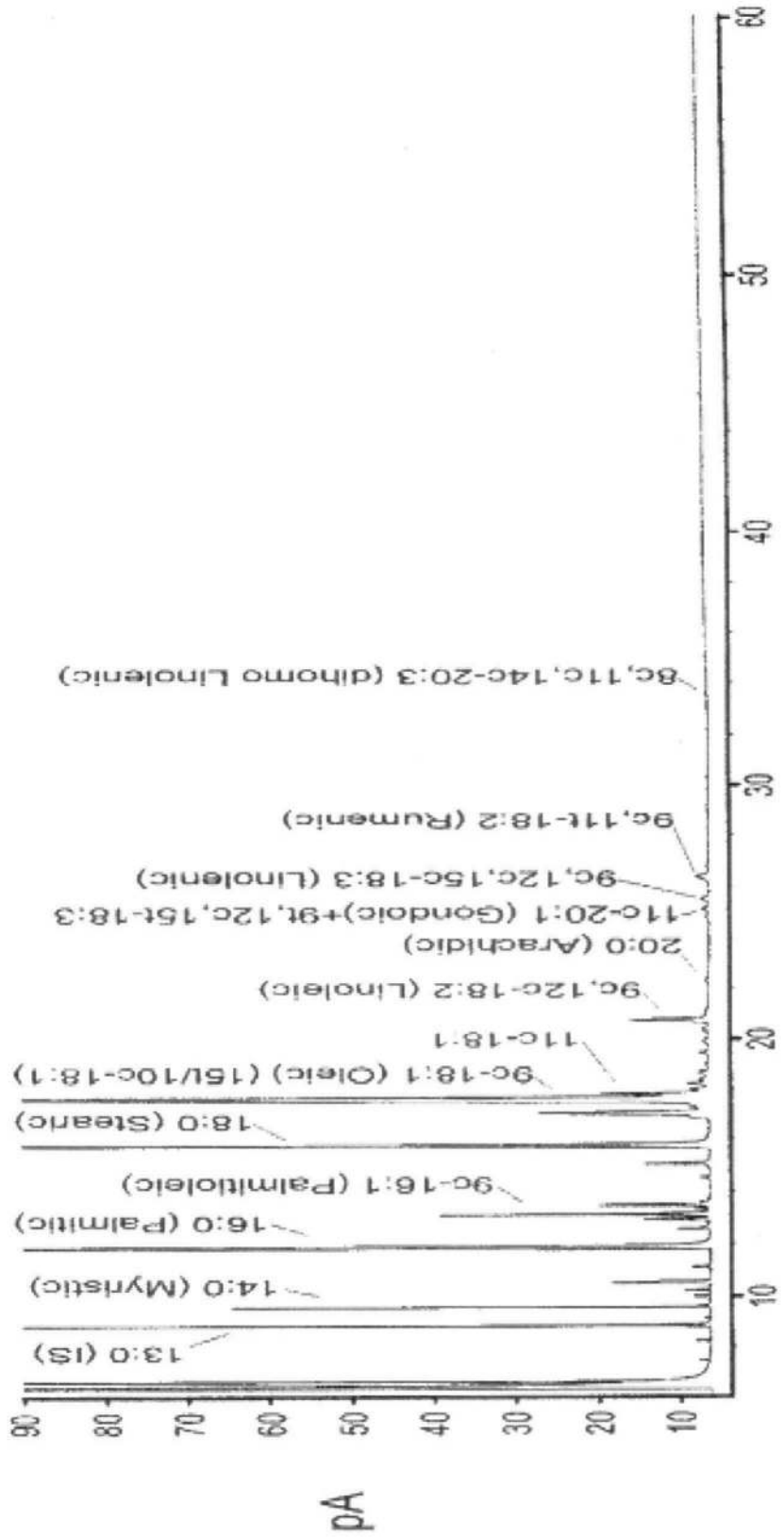

Figure 18. GC/FID profile of major fatty acid methyl esters (FAME) in Tallow (References 17–24). See Figure 17a for chromatographic parameters.

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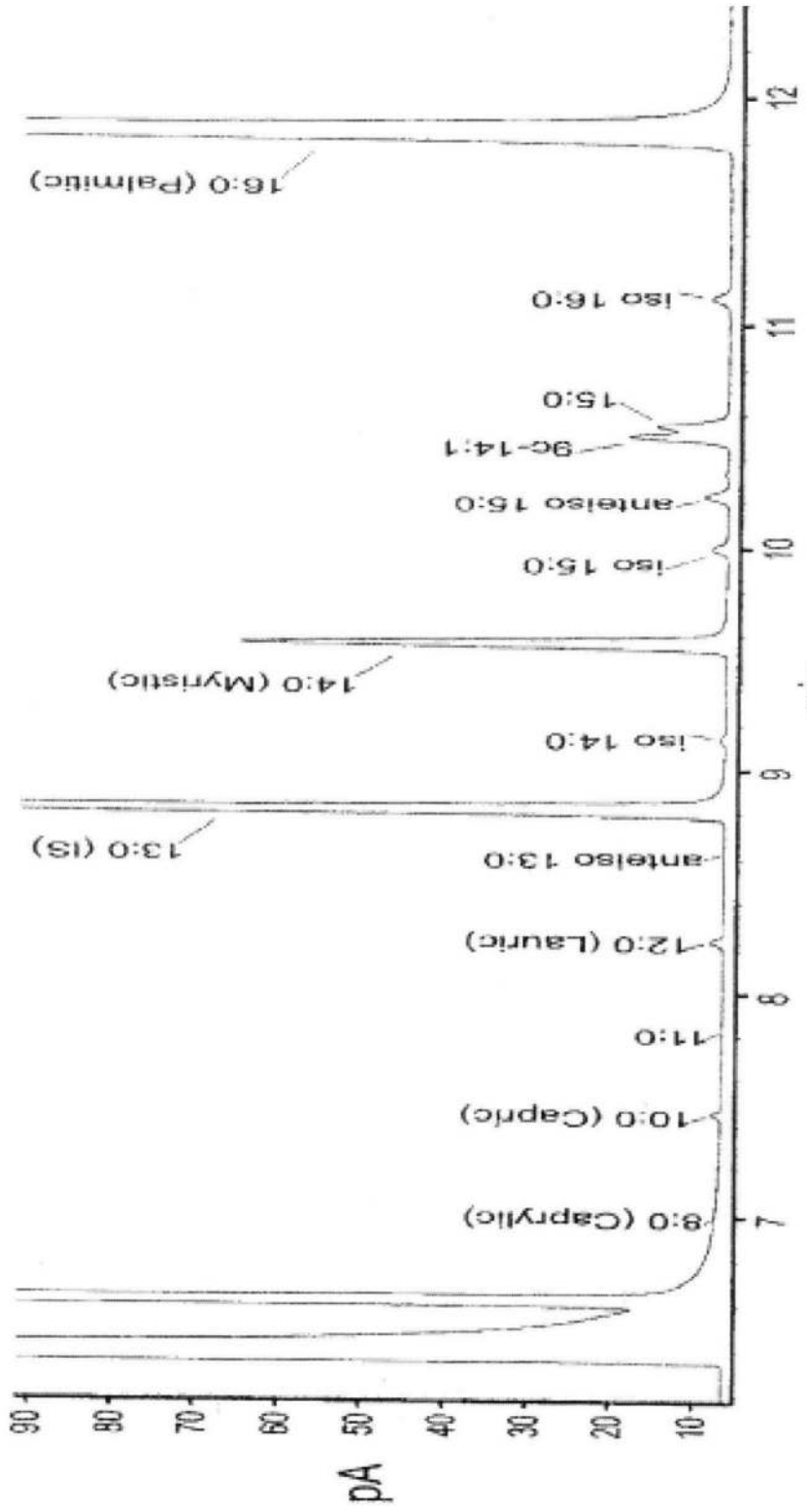

Figure 19. GC/FID profile of fatty acid methyl esters (FAME) in Tallow from start to 16:0 (References 17–24) . See Figure 17a for chromatographic parameters.

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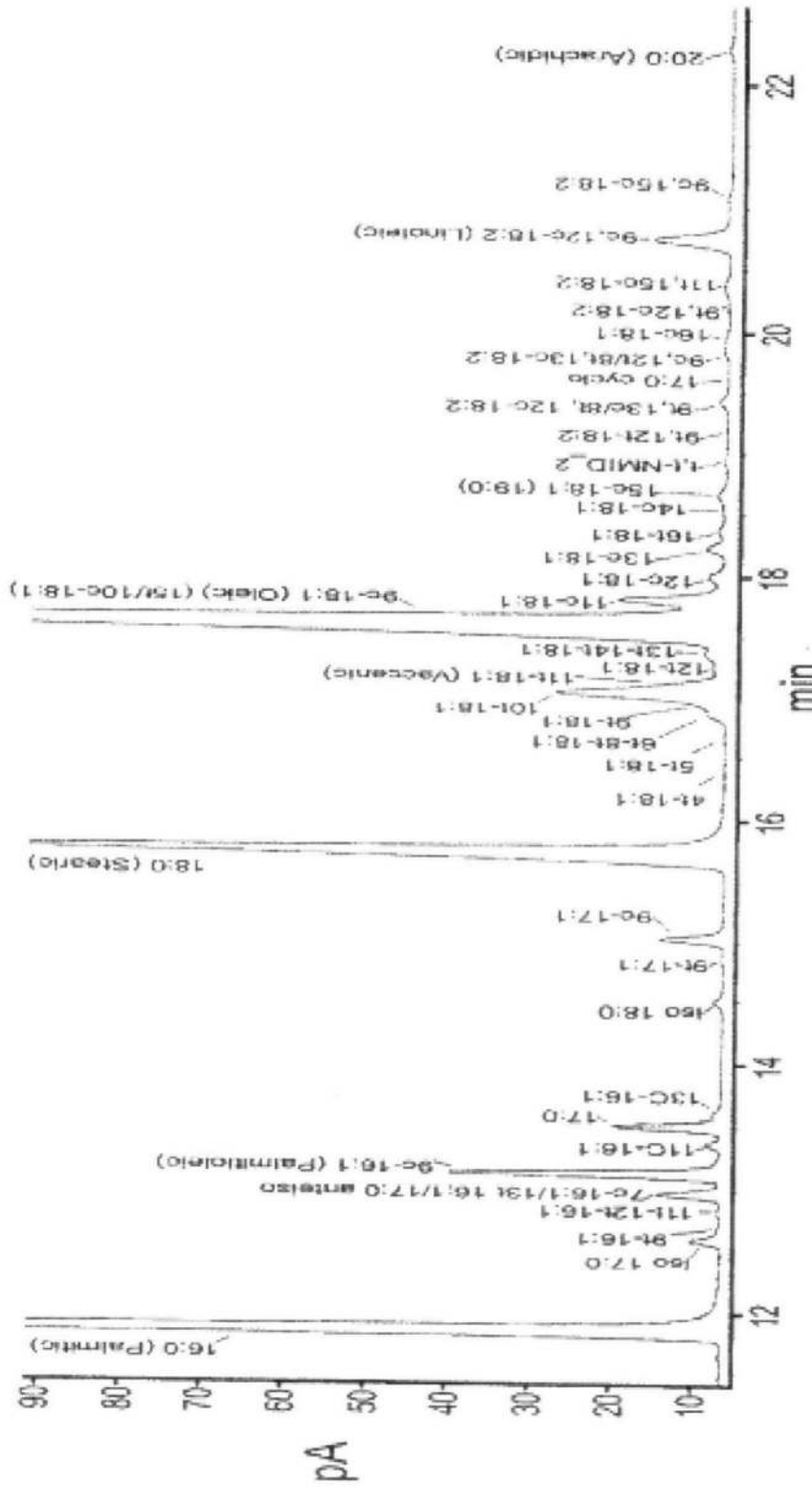

Figure 20a. GC/FID profile of fatty acid methyl esters (FAME) in Tallow from 16:0 to 20:0 (References 17–24). See Figure 17a for chromatographic parameters.

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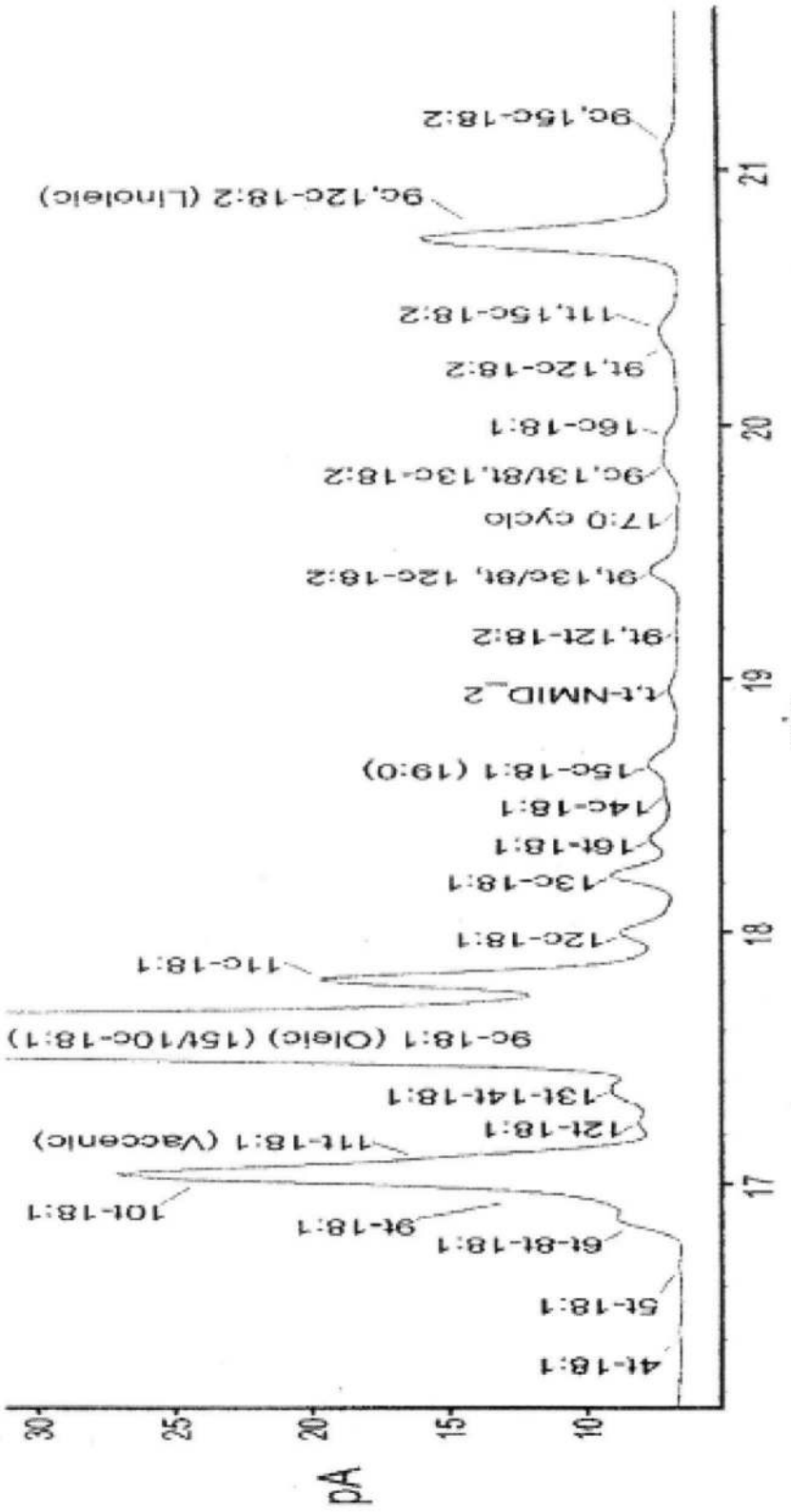

Figure 20b. Blow up GC/FID profile of fatty acid methyl esters (FAME) in Tallow for the Oleic and Linoleic regions. See Figure 17a for chromatographic parameters.

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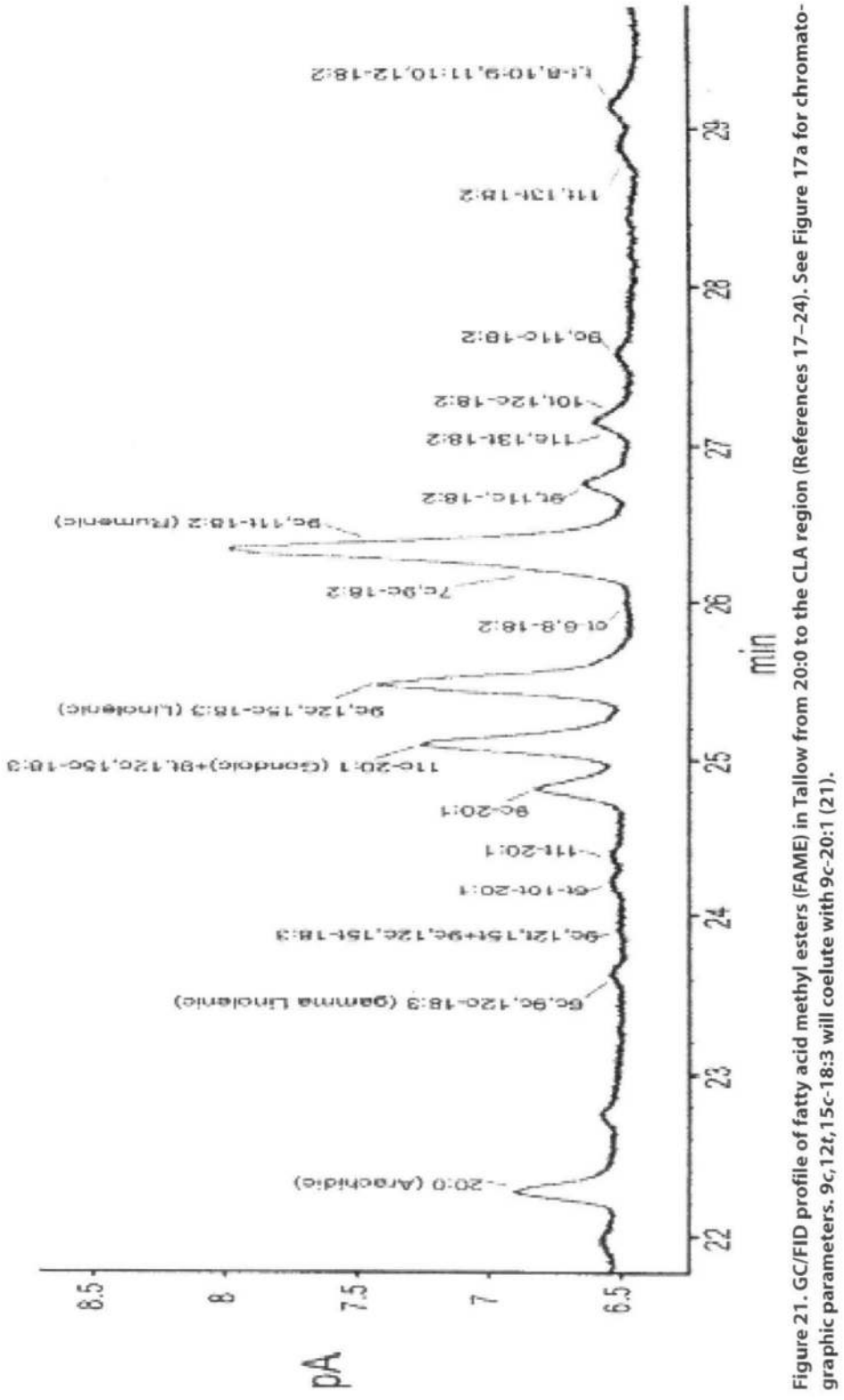

Figure 21. GC/FID profile of fatty acid methyl esters (FAME) in Tallow from 20:0 to the CLA region (References 17–24). See Figure 17a for chromatographic parameters. 9*c*,12*t*,15*c*-18:3 will coelute with 9*c*-20:1 (21).

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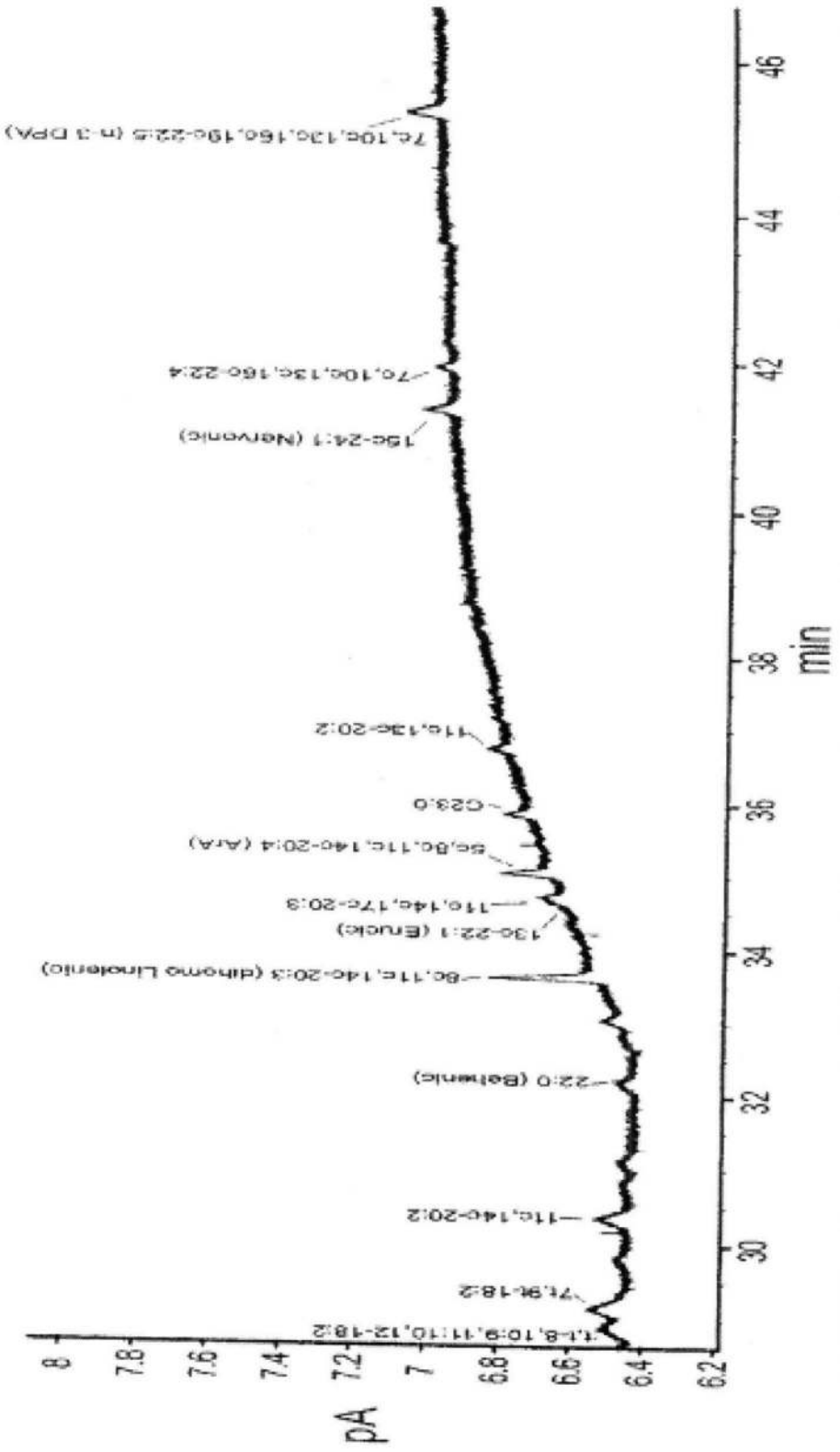

Figure 22. GC/FID profile of fatty acid methyl esters (FAME) in Tallow from the CLA region to end of the chromatogram (References 17–24). See Figure 17a for chromatographic parameters.

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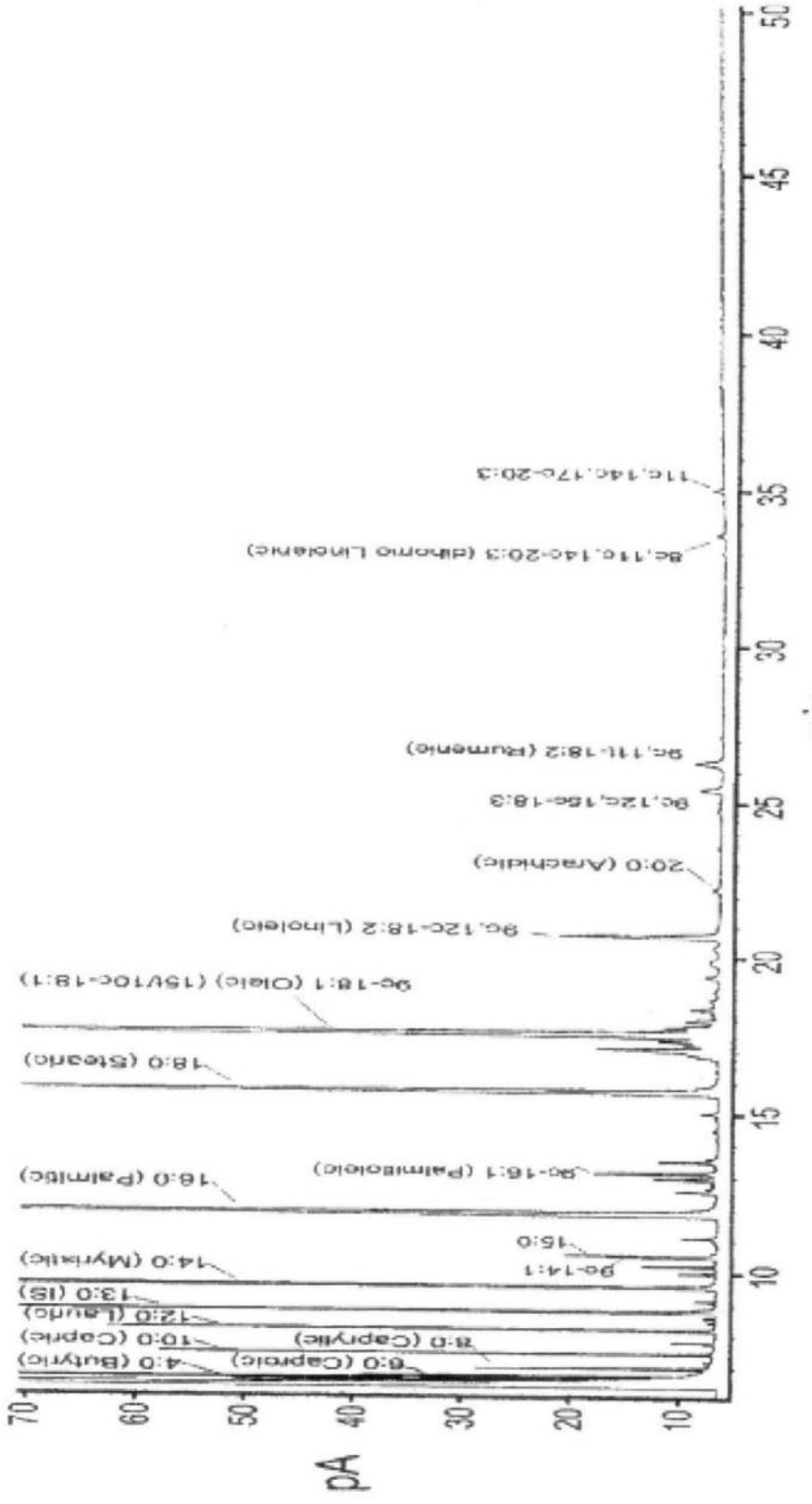

Figure 23. GC/FID profile of major fatty acid methyl esters (FAME) in Anhydrous Butterfat (References 17–24). See Figure 17a for chromatographic parameters.

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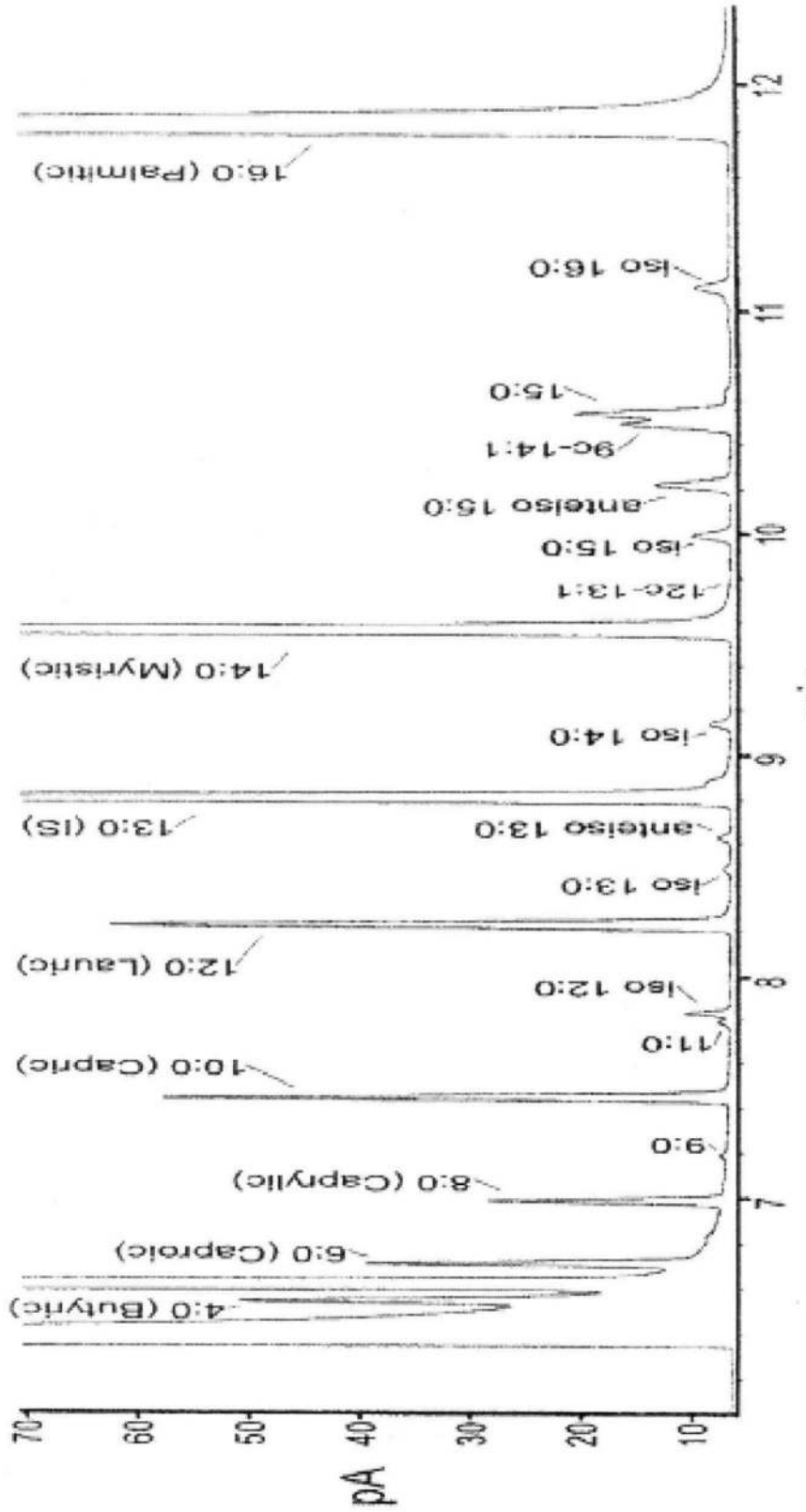

Figure 24. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat from start to 16:0 (References 17–24). See Figure 17a for chromatographic parameters.

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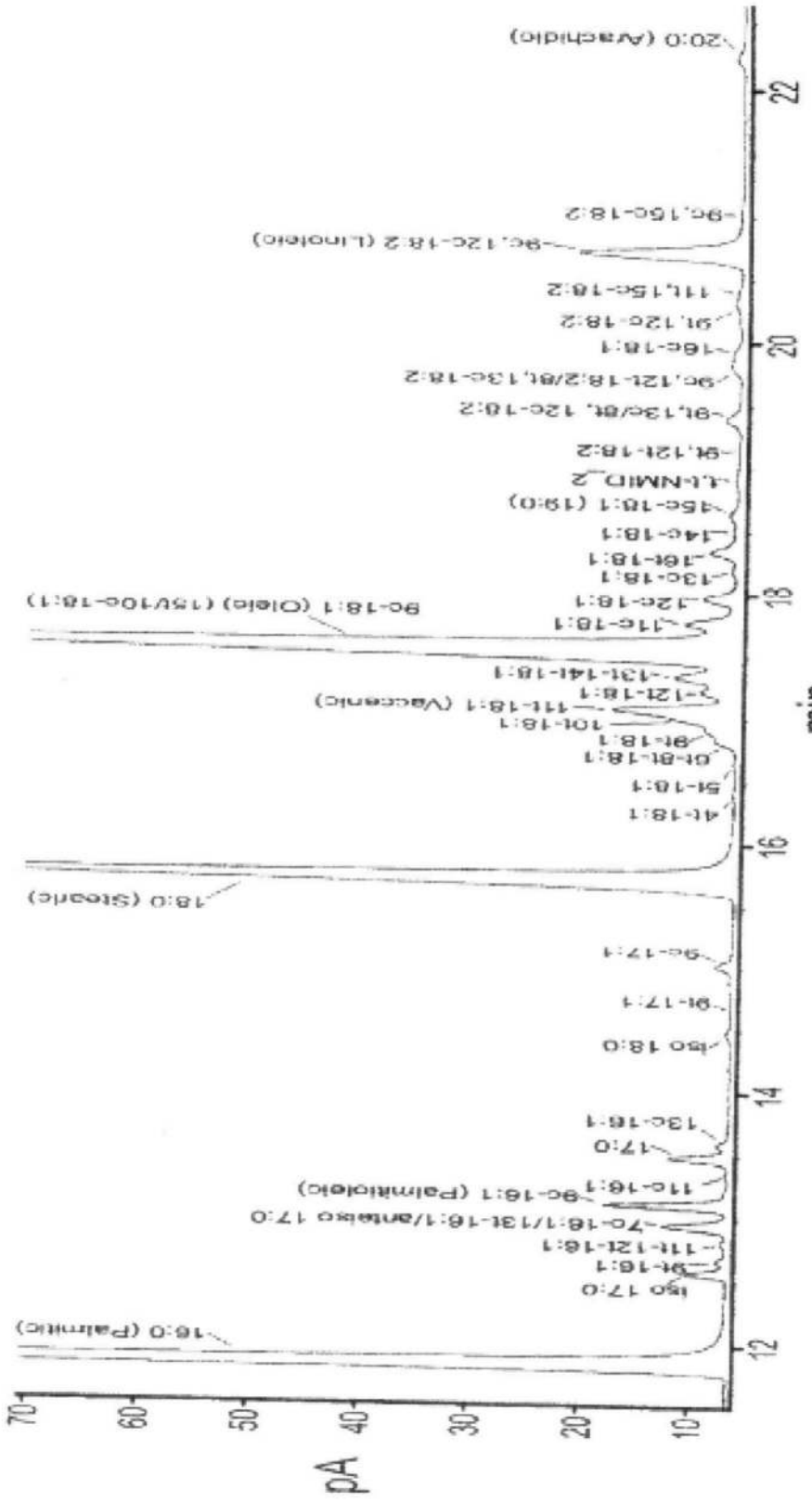

Figure 25a. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat from 16:0 to 20:0 (References 17–24). See Figure 17a for chromatographic parameters.

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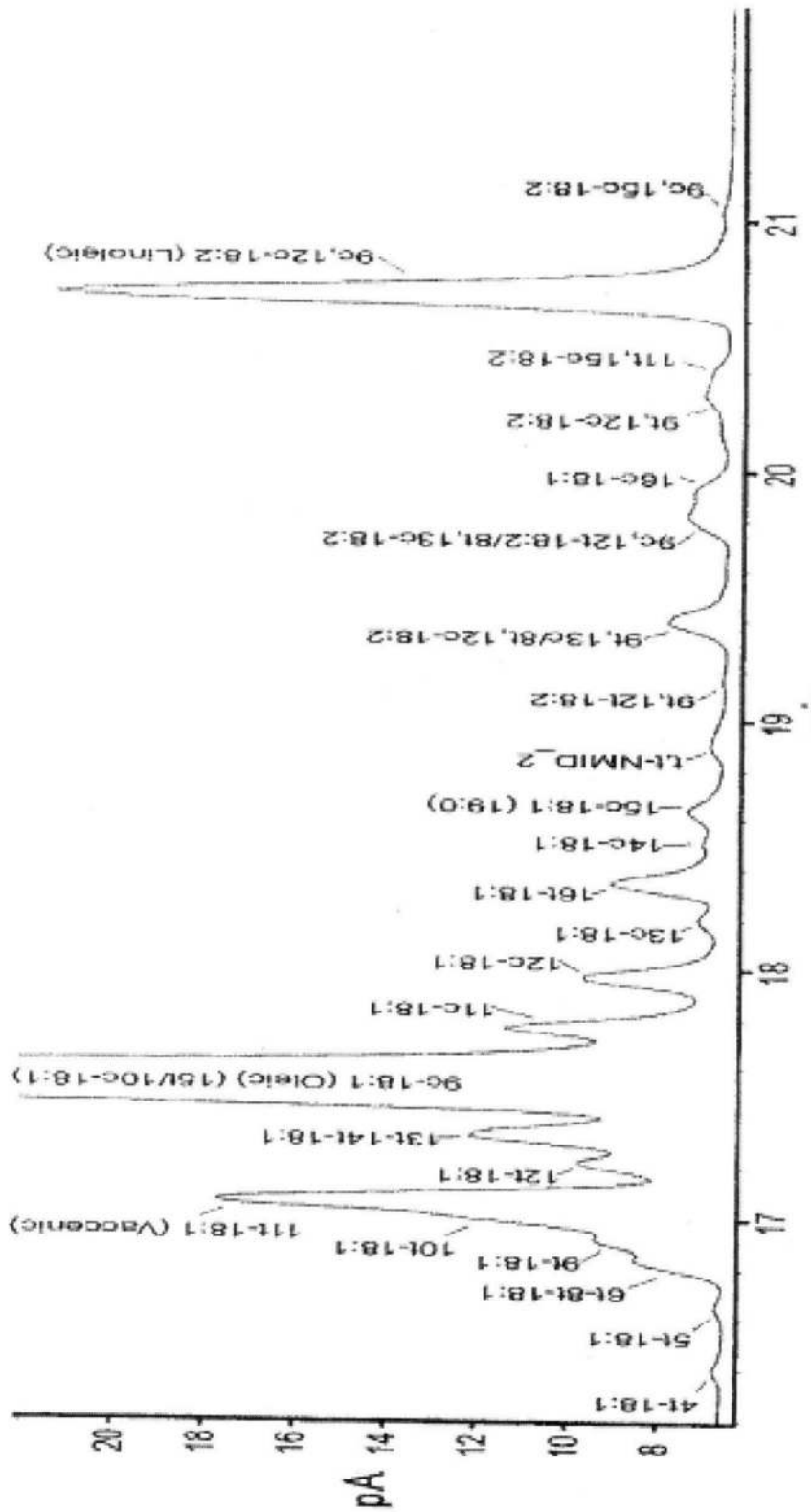

Figure 25b. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat zoomed in to Oleic and Linoleic regions (References 17–24). See Figure 17a for chromatographic parameters.

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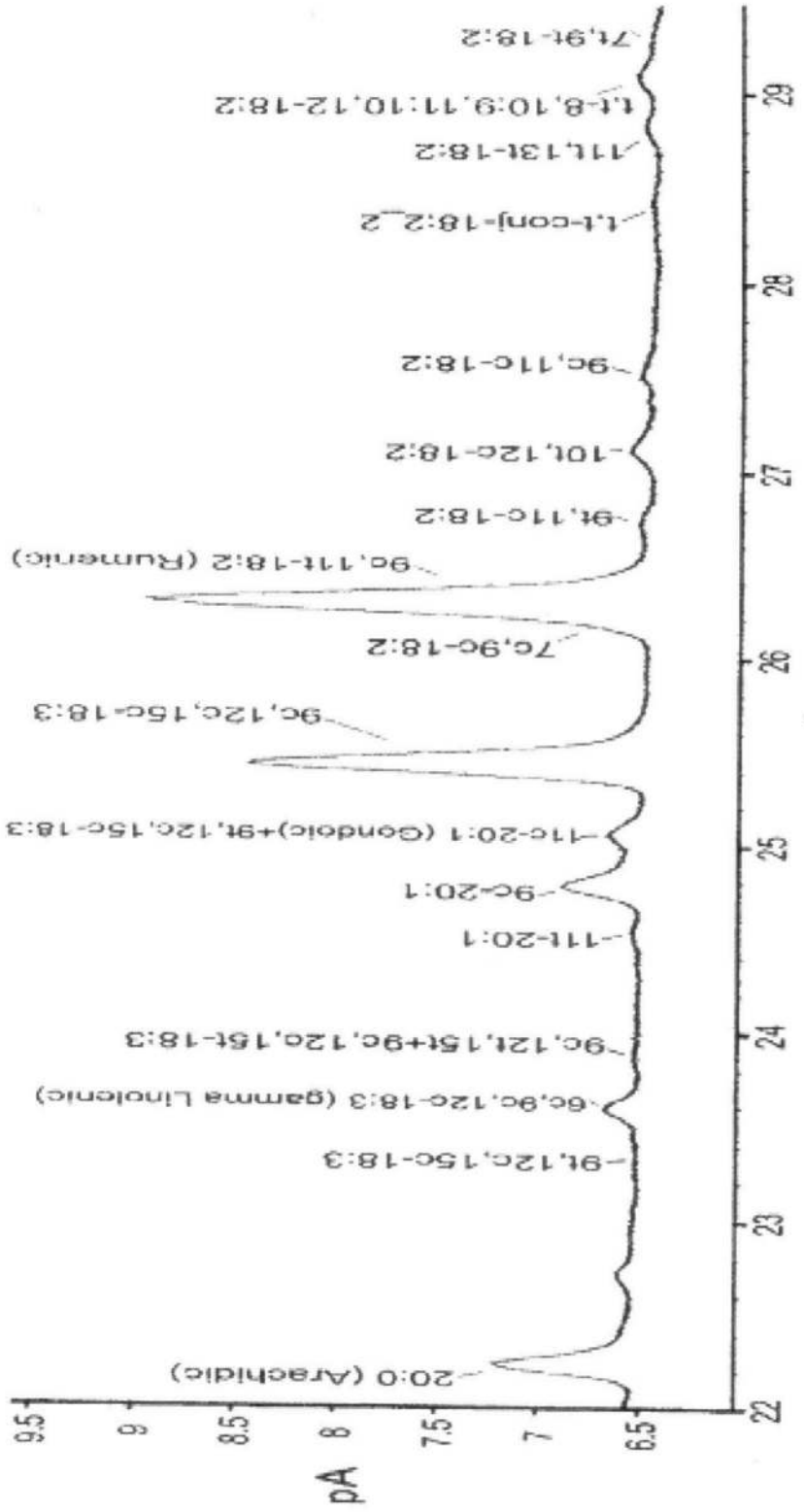

Figure 26. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat from 20:0 to the CLA region (References 17–24). 9c,12t,15c-18:3 will coelute with 9c-20:1 (21). See Figure 17a for chromatographic parameters.

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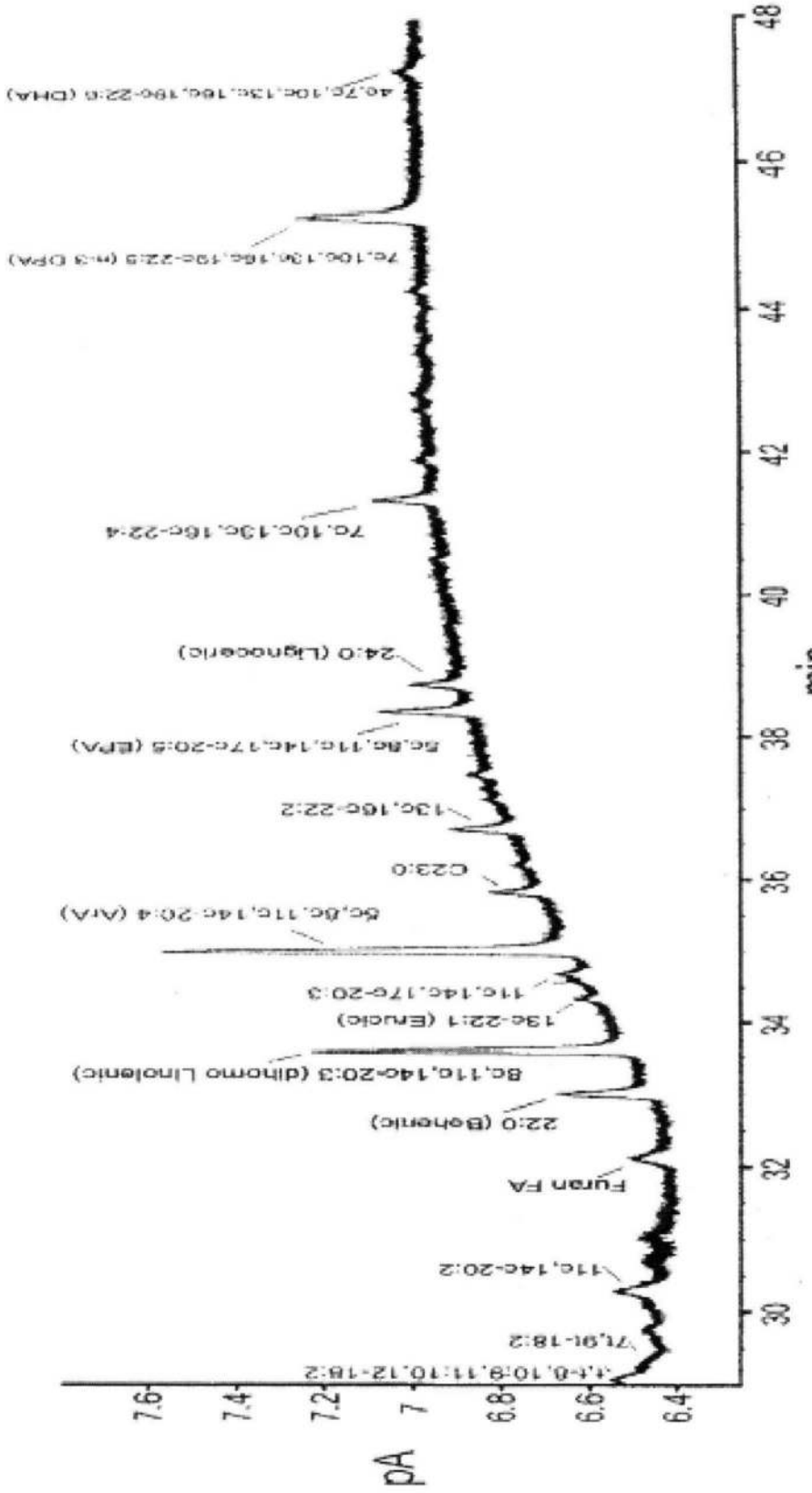

Figure 27. GC/FID profile of fatty acid methyl esters (FAME) in Anhydrous Butterfat from the CLA region to end of the chromatogram (References 17–24). See Figure 17a for chromatographic parameters.

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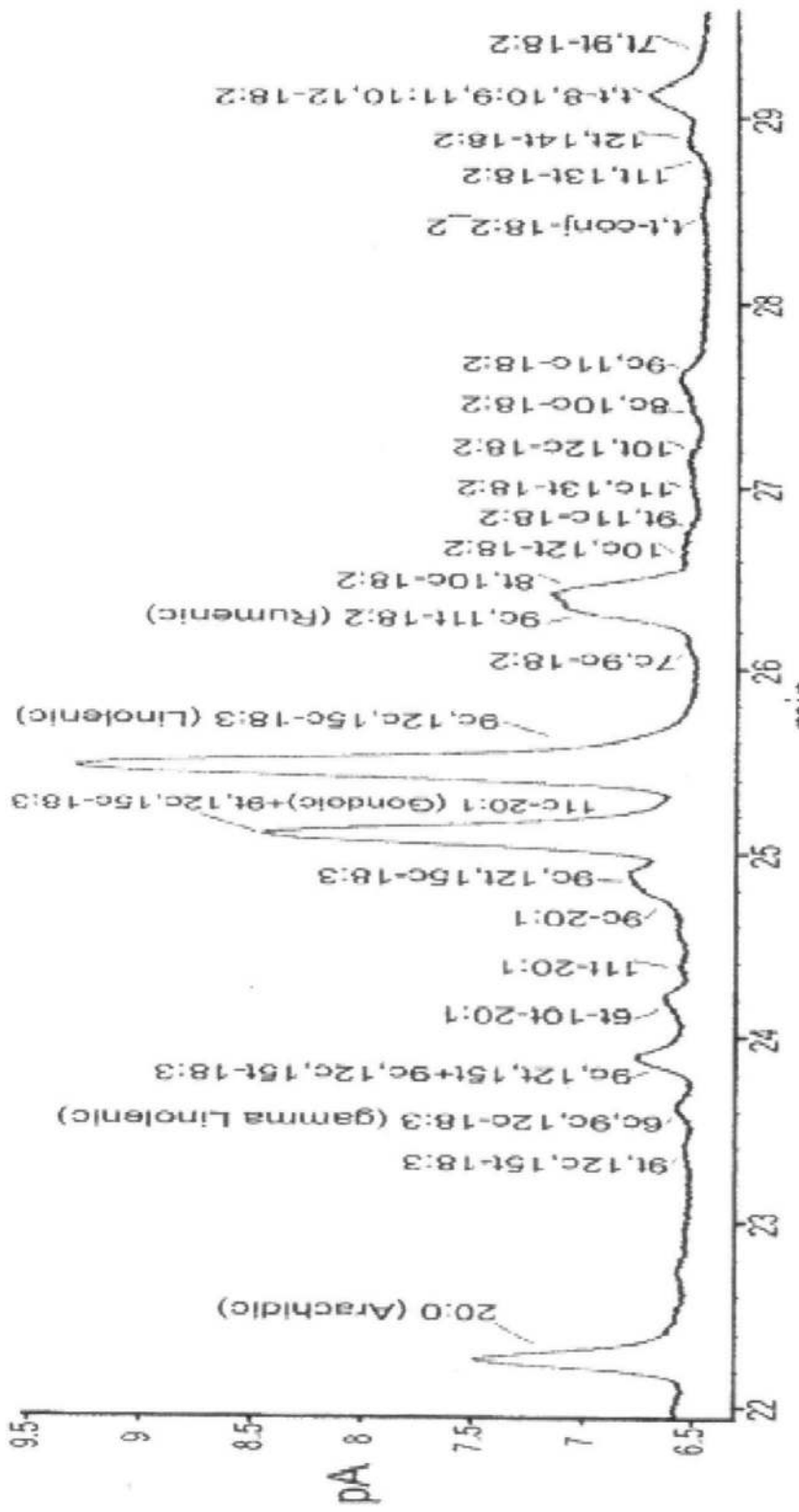

Figure 31. GC/FID profile of fatty acid methyl esters (FAME) in BCR-163 from 20:0 to the CLA region (References 17–24). 9c,12t,15c-18:3 will coelute with 9c-20:1 (21). See Figure 17a for chromatographic parameters.

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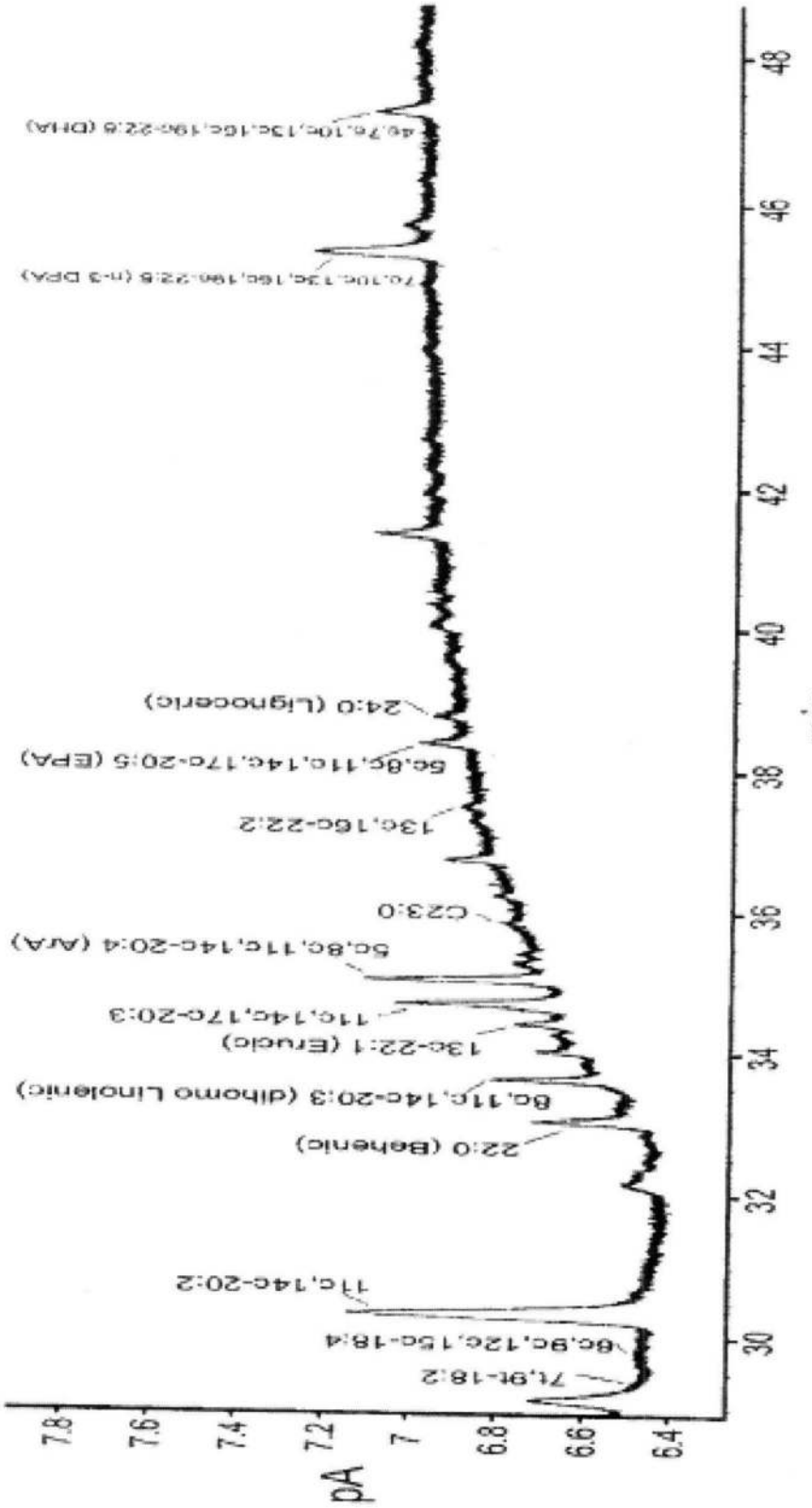

Figure 32. GC/FID profile of fatty acid methyl esters (FAME) in BCR-163 from the CLA region to end of the chromatogram (References 17–24). See Figure 17a for chromatographic parameters.

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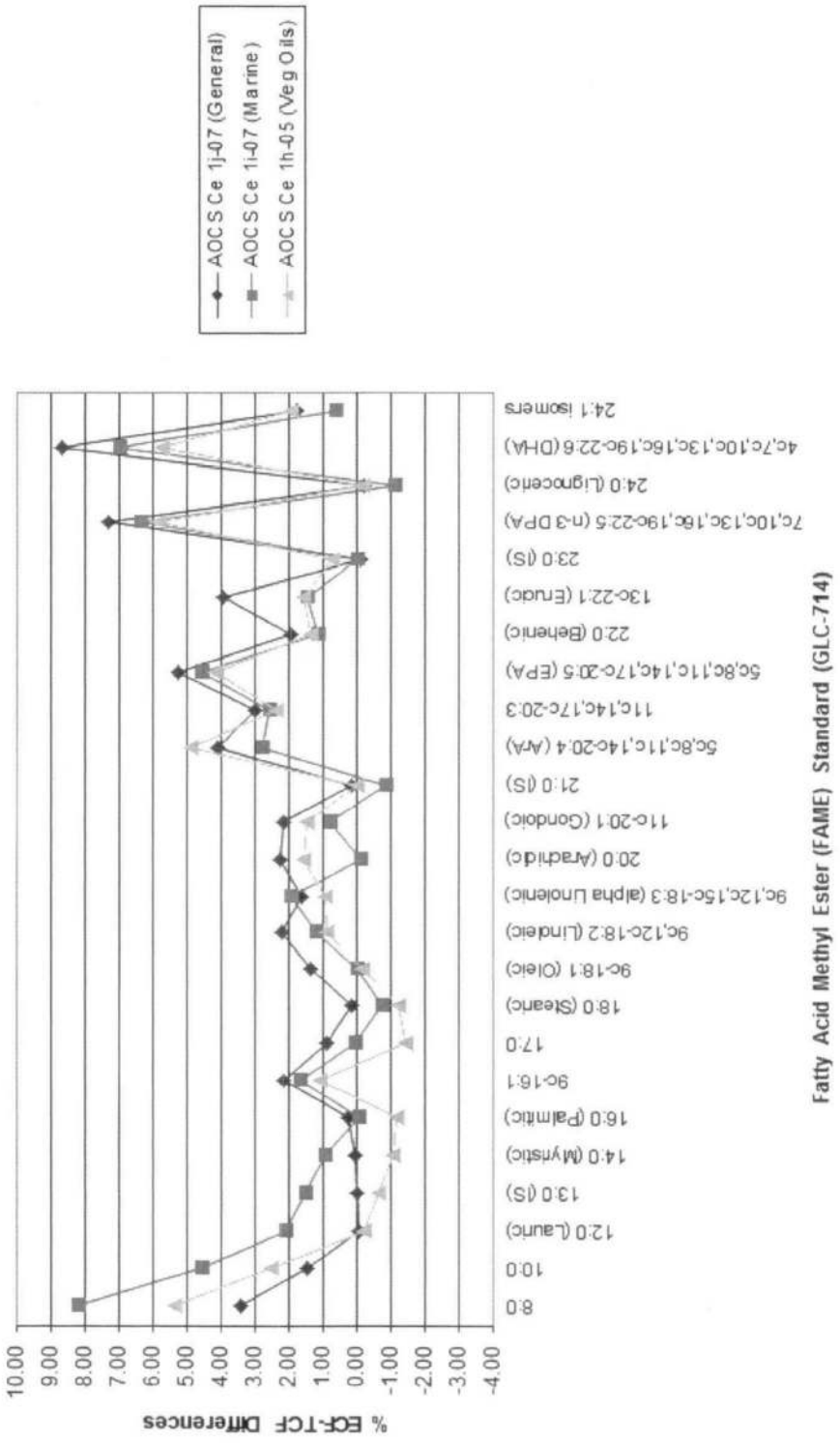

Figure 33. Plot of % ECF – TCF Differences from one lab for the different AOCS methods. Values should fall between ± 3% for saturated, monounsaturated, and long chain fatty acids with 18 or less carbons and three or less double bonds.

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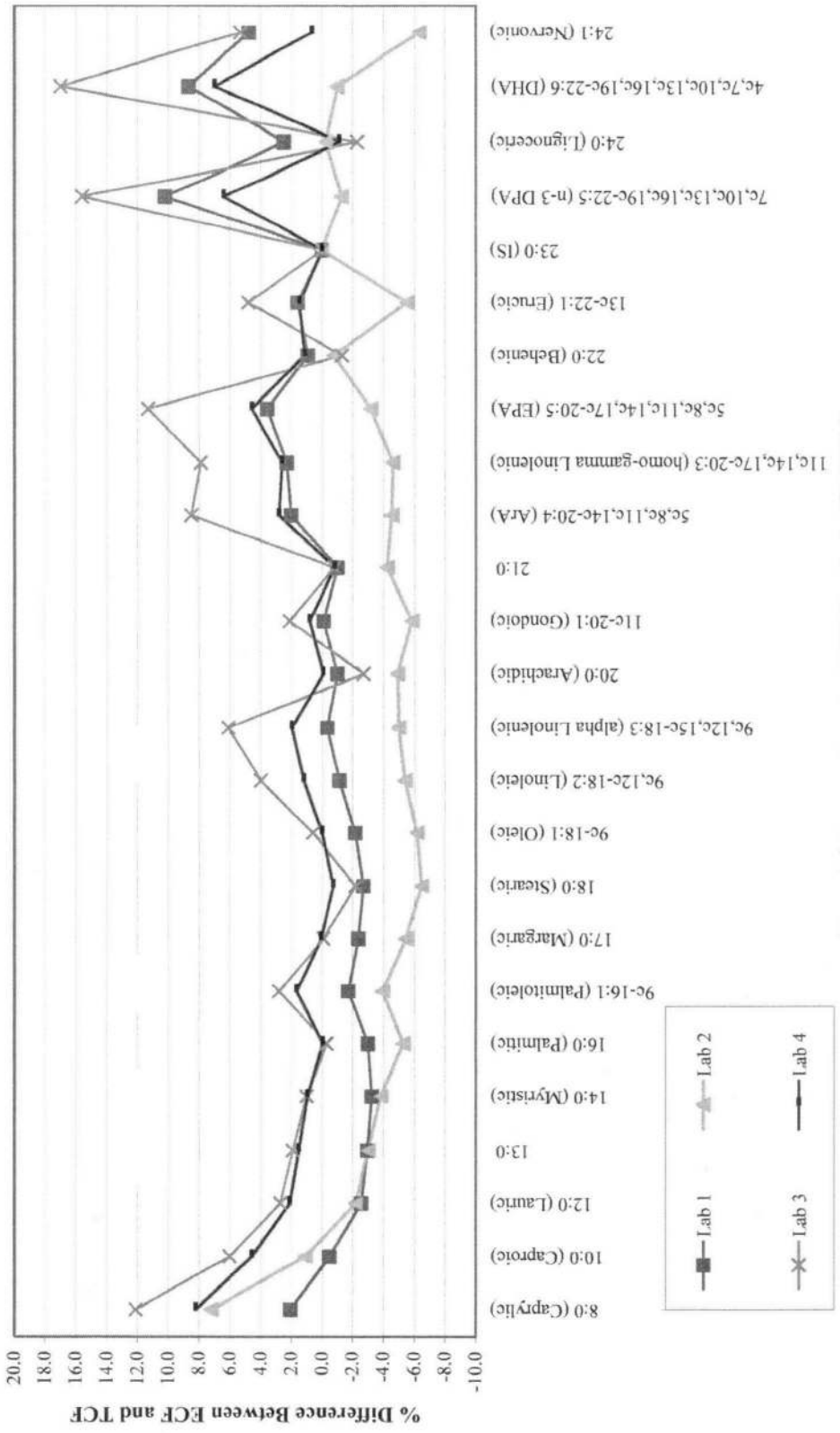

Figure 34. Plot of % ECF – TCF Differences from four different labs. Values should fall between ± 3% for saturated, monounsaturated, and long chain fatty acids with 18 or less carbons and three or less double bonds.

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AOCS

AOCS Official Method Ce 2-66

Reapproved 2009

Preparation of Methyl Esters of Fatty Acids

DEFINITION

This method provides a means for preparing methyl esters of long-chain fatty acids for further analysis by GLC.

SCOPE

The method is applicable to common fats, oils, and fatty acids with the exception of milk fats (see Notes, 1). Unsaponifiables are not removed but, if present in large amounts, they may interfere with subsequent analyses. The procedure will result in partial or complete destruction of the following groups: epoxy, hydroperoxy, cyclopropenyl, cyclopropyl, and possibly hydroxyl, and acetylenic fatty acids, and is not suitable for the preparation of methyl esters of fatty acids containing these groups.

APPARATUS

1. Flasks—50 and 125 mL flat-bottom boiling flasks, or s 24/40 outer necks.
2. Water-cooled condensers—Liebig or West design, s 24/40 inner joint.
3. Separatory funnels—250 mL.
4. Boiling flask—200 mL, for solvent removal.
5. Boiling chips—free of fat.

REAGENTS

1. BF_3-methanol reagent, 12% to 15%, available commercially as 14% and 50% solution (see Notes, *Caution,* Notes, 5) (125 g BF_3 per liter of methanol)— available commercially, or may be prepared using BF_3 gas and methanol [see Section 3 (d) of American Society for Testing and Materials (ASTM) Method D-1983-64T, or References, 1].
2. Sodium hydroxide (NaOH)—0.5 M in methanol.
3. Sodium chloride (NaCl)—saturated solution in water.
4. Petroleum ether—redistilled, bp 30–60°C (see Notes, *Caution*).
5. Heptane—gas chromatographically clean (see Notes, *Caution*).
6. Sodium sulfate (Na_2SO_4)—anhydrous, reagent grade.
7. Methyl red indicator—0.1% in 60% ethanol.
8. Nitrogen gas—high purity.

PROCEDURE

1. Accurate weighing is not required. Test portion size need be known only to determine the size of flask and amounts of reagents that should be used according to the following table:

Test portion, mg	Flask, mL	NaOH 0.5 M, mL	BF_3-methanol reagent, mL
100–250	50	4	5
250–500	50	6	7
500–750	125	8	9
750–1000	125	10	12

2. For fatty acids—
   (a) Introduce the fatty acids into the 50 or 125 mL reaction flask. Add the specified amount of BF_3-methanol reagent, attach a condenser and boil for 2 min (see Notes, 1). Add 2–5 mL of heptane through the condenser and boil for 1 min longer. Remove from heat, remove condenser and add about 15 mL of saturated sodium chloride solution (Reagents, 3). Stopper the flask and shake vigorously for 15 sec while the solution is still tepid. Add sufficient saturated sodium chloride solution to float the heptane solution of the methyl esters into the neck of the flask (References, 2). Transfer about 1 mL of the heptane solution into a test tube and add a small amount of anhydrous sodium sulfate. The dry heptane solution may then be injected directly into a gas chromatograph. (See Notes, 2.)
   (b) To recover dry esters, transfer the salt solution and heptane phase to a 250 mL separatory funnel. Extract twice with 50 mL portions of redistilled petroleum ether (bp 30–60°C). Wash the combined extracts with 20 mL portions of water

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until free of acids (test water with methyl red indicator), dry with sodium sulfate and evaporate the solvent under a stream of nitrogen on a steam bath (see Notes, 3 and 4).

3. For fats and oils—
   (a) Introduce the fat into the 50 or 125 mL reaction flask. Add the specified amount of 0.5 M methanolic sodium hydroxide and add a boiling chip. Attach a condenser and heat the mixture on a steam bath until the fat globules go into solution. This step should take 5–10 min. Add the specified amount of BF_3-methanol reagent through the condenser, and proceed as directed in the fatty acid section (Procedure, 2).
4. Alternate method for fats and oils (acid value <2) (References, 3)—An alternate method for the preparation of fatty acid methyl esters (FAME) is as follows: Accurately weigh a test portion (approximately 200 mg) into a stoppered-glass centrifuge vial. Add 2.0 mL of hexane (see Notes, *Caution*) or heptane, followed by 0.1 mL of 2 M methanolic KOH. Close the vial and shake well for 30 sec, centrifuge, remove two drops of the upper layer and dilute with 2.0 mL of hexane or heptane. The concentration of the FAME in hexane is approximately 0.5%. Inject 0.2 µL for capillary column GLC analysis using split injection; in case of on-column injection, dilute to 0.05% before injection.

NOTES

Caution

Petroleum ether, hexane and heptane are extremely flammable. Use effective fume-removal device. Avoid static electricity.

The BF_3-methanol reagent has a limited shelf life, even when refrigerated, and the use of old or too-concentrated solutions can result in production of artifacts and losses of appreciable amounts of polyunsaturated fatty acids (References, 4). BF_3 is poisonous. For this reason it is not recommended that the analyst prepare the methanolic solution. Precautions should be taken for eye protection and for protection from the dangers of corrosive chemical burns.

NUMBERED NOTES

1. The required reaction time appears to increase as both the carbon number and degree of unsaturation increase. For longer chain and more unsaturated fatty acids, a general observation is that the reaction should be allowed to proceed just until there is no visual evidence of oil globules in the reaction mixture.
2. It is recommended that all methyl esters be washed with water (to the disappearance of a methyl red end point) prior to injection. An alternative is to partition the hexane-methyl ester phase against water containing a small amount of mixed-bed (indicating resin).
3. There is danger of losing some of the more volatile esters if the solvent-removal step is prolonged, or if too vigorous a stream of nitrogen is used. For infrared spectroscopy, this step should be terminated as soon as all solvent is removed. For gas-liquid chromatography the method may be extended to fatty acids with eight carbon atoms, if the solvent is not completely removed.
4. The methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen in a screw-cap vial at 2°C for 24 hr. For longer storage they should be sealed in a glass ampule, subjected first to a vacuum and then backfilled with nitrogen and stored at –20°C (freezer).
5. Prepare the BF_3 solution only if it is absolutely necessary using BF_3 g gas and methanol (125 g BF_3 per liter of methanol). Warning: Operate under a ventilated hood [see Section 3(d) of American Society for Testing and Materials (ASTM) Method D-1948-64T, or Reference 1].

REFERENCES

1. *Anal. Chem. 38:*514 (1966).
2. *J. Chromatogr. 247:*63 (1982).
3. *Standard Methods for Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.301.
4. *INFORM 3:* 1031 (1992)

OTHER REFERENCES

*J. Am. Oil Chem. Soc. 70:*325 (1993).

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AOCS Official Method Ce 2b-11

Revised 2013

Direct Methylation of Lipids in Foods by Alkali Hydrolysis

DEFINITION

This method describes a simultaneous alkali hydrolysis and methylation procedure without prior digestion option for the preparation of fatty acid methyl esters (FAMEs) directly from food matrices. The incorporation of triacylglycerol (TAG) standards allows the quantification of total fat and fatty acids using Theoretical Correction Factors (TCFs) and Empirical Correction Factors (ECFs).

SCOPE

This method may be applicable for fat-containing matrices (e.g., food stuffs, beverages, tissues, and oils). The results of the collaborative study indicate that most matrices do not require acid pretreatment. Extruded cat, dog foods and oat based foods gave low results, thus, analysts are advised to test the chosen method against appropriate reference methods. Materials with high moisture contents do not require drying before analysis. However, if drying is required (except when freeze drying) antioxidants should be added to protect from oxidation. An appropriate grinding technique for homogenization of the test sample should be used, when necessary.

This method is not suitable for steryl ester concentrates and steryl ester-fortified food products.

APPARATUS

1. 50-mL round flat-bottom flasks with 24/40 joints or equivalent
2. Teflon™ flask-neck sleeves (size 24/40)
3. Hollow, full-length stopper (size 24/40), barrel-shaped head
4. Explosion-proof hot plates or equivalent (see Notes, 1)
5. Water-cooled condensers or equivalent (see Notes, 1)
6. 2-mL amber write-on autosampler vials with Teflon™-lined caps
7. Glass powder funnel
8. Analytical balance capable of reading to ± 0.0001 g or ± 0.1 mg
9. Weighing funnels
10. 1-L transparent polymethylpentene screw top flask
11. 500-mL Nalgene® bottle
12. Porous boiling chips

REAGENTS

1. Sodium Hydroxide, reagent grade
2. Methanol (MeOH), high purity gas chromatographic grade
3. NaOH/MeOH (0.5 M NaOH in MeOH). Add 20 g of NaOH to a 1-L polymethylpentene flask. Add MeOH and mix well to dissolve before diluting to volume.
4. Sodium Chloride, reagent grade, saturated. Dissolve 36 g NaCl in 100 mL distilled water.
5. Hexanes or *n*-heptane, high purity gas chromatographic grade
6. Chloroform, high purity gas chromatographic grade
7. 14% Boron Trifluoride in MeOH (see Notes, 2)
8. Sodium Sulfate, anhydrous, reagent grade
9. Pyrogallol, ACS reagent
10. Internal standard: 13:0, Tritridecanoin (P/N T-135, Nu-Chek-Prep, Inc. or equivalent) for unknowns, dairy or ruminant fats. Accurately weigh out 2.5 g of 13:0 triacylglycerol (TAG) into a weighing funnel. Empty the weighing funnel into a 500-mL class A volumetric flask and rinse the funnel with hexanes or *n*-heptane to ensure complete quantitative transfer into the flask. Add hexanes or *n*-heptane to the flask, mix to dissolve, and dilute to volume (see Calculations, 1). The 13:0 TAG internal standard solution is stable indefinitely if precautions are taken to eliminate the loss of *n*-heptane and therefore a change in the concentration of the internal standard.
11. Internal standard: 21:0, Triheneicosanoin (P/N T-175, Nu-Chek-Prep, Inc. or equivalent) for all samples other than dairy or ruminant fats and marine or long chain polyunsaturated oils. Accurately weigh out 2.5 g of 21:0 TAG into a weighing funnel. Empty the weighing funnel into a 500-mL class A volumetric flask and rinse the funnel with chloroform to ensure complete quantitative transfer into the flask. Add chloroform to the flask, mix to dissolve, and dilute to volume (see Calculations,

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1). The 21:0 TAG internal standard solution is stable indefinitely if precautions are taken to eliminate the loss of chloroform and therefore a change in the concentration of the internal standard.

12. Internal standard: 23:0, Tritricosanoin (P/N T-185, Nu-Chek-Prep, Inc. or equivalent) for marine oils or other oils containing long chain polyunsaturated fatty acids (PUFAs). Accurately weigh out 1.0 g of 23:0 TAG into a weighing funnel (see Notes, 3). Empty the weighing funnel into a 500-mL class A volumetric flask and rinse the funnel with chloroform to ensure complete quantitative transfer into the flask. Add chloroform to the flask, mix to dissolve, and dilute to volume (see Calculations, 1). The 23:0 TAG internal standard solution is stable indefinitely if precautions are taken to eliminate the loss of chloroform and therefore a change in the concentration of the internal standard.

SAMPLE PREPARATION

1. Samples should be homogeneous before extraction, which may be done by drying, grinding or melting. High moisture matrices (e.g., food stuffs, tissues, etc.) may need to have the moisture removed to allow for grinding. Use the appropriate grinding apparatus for the matrix. Solid and semi-solid fats should be melted and mixed thoroughly at the lowest temperature that ensures homogeneity.
2. Prior to methylation, pipette (with a 0.5, 1, 2 or 5-mL class A volumetric pipette) enough internal standard (IS) into a tared 50-mL round flat-bottom reaction flask, so that after the test portion is added, the concentration of the final solution is between 0.05 and 0.1 mg IS per 1 mg oil or fat from the test portion. See Table 1 to determine the appropriate amount (mL) of IS to add based upon the expected fat or oil concentration of the test portion (see Calculations, 2). Evaporate the solvent (hexanes, *n*-heptane or chloroform) to dryness. This is the value for W_{is} in AOCS Ce 1h-05, Ce 1i-07 or Ce 1j-07.
3. Add the appropriate amount (mg) of sample; see Table 1, into the tared 50-mL flat bottom flask containing the dried IS. (This will yield a final concentration of 15–20 mg fat or oil per mL of final solution. Where samples contain less than 20% fat or oil, the range will be <10 to 20 mg/mL). For samples containing less than 10% fat, use 1000 mg or 1 g (maximum). Record the exact weight to 0.1 mg as this amount will be used in the total fat, saturated, *cis*-monounsaturated, *cis*-polyunsaturated and *trans* fatty acid content determinations. This value is W_{TS} in AOCS Ce 1h-05, Ce 1i-07 and Ce 1j-07.
4. Add 2 or 3 porous boiling chips.
5. Add 1 mg of antioxidant (e.g., pyrogallol)/1 mg oil (see Notes, 4).

ALKALI CATALYZED FAME PREPARATION

1. Add 5 mL of NaOH/methanol to flask (see Reagents, 3).
2. Attach coolant (≤30°C), water or air-cooled condensers to the flasks (see Notes, 1).
3. Reflux for 15 min after boiling begins (see Notes, 5). *CAUTION:* If moisture is still present the mixture may bump during reflux/boiling.
4. Add 5 mL 14% BF_3/MeOH to the boiling flask. Reflux for an additional two min (References 6-9).
5. Add 5 mL of hexanes or *n*-heptane.
6. Remove from heat source; cool flask to room temperature then add saturated sodium chloride until the organic layer is in the neck of the 50-mL round flat-bottom flask. Stopper the flask and shake. Allow the layers to separate and transfer a clear portion of the top layer (hexanes or *n*-heptane) with a transfer pipette into a 2-mL autosampler vial containing 50 mg of sodium sulfate. Crimp on the aluminum cap and vortex mix. The test solution is ready for chromatographic separation and quantification.

CHROMATOGRAPHY

1. For unknowns, dairy or ruminant fats use AOCS Official Method Ce 1j-07 for the chromatographic separation and calculations (see Calculations, 3).
2. For all samples containing vegetable oil, use AOCS Official Method Ce 1h-05 for the chromatographic separation and calculations (see Calculations, 3).
3. For samples containing marine oils or other oils containing long chain PUFAs, use AOCS Official Method Ce 1i-07 for the chromatographic separation and calculations (see Calculations, 3).

CALCULATIONS

1. The internal standard solution concentrations are calculated as follows:

If using AOCS Official Method Ce 1j-07:

2.5 g 13:0 TAG/500 mL hexanes or *n*-heptane = 0.0050 g/mL
0.0050 g/mL × 1000 mg/g = 5.0 mg/mL

If using AOCS Official Method Ce 1h-05:

2.5 g 21:0 TAG/500 mL chloroform = 0.0050 g/mL
0.0050 g/mL × 1000 mg/g = 5.0 mg/mL

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If using AOCS Official Method Ce 1i-07:

1.0 g 23:0 TAG/500 mL chloroform = 0.0020 g/mL
0.0020 g/mL × 1000 mg/g = 2.0 mg/mL

2. For example, for a product formulated with 40% vegetable fat or oil, the sample amount would be 200 mg (This value is W_{TS} in Ce 1h-05, Ce 1i-07 or Ce 1j-07). Since the oil added is other than dairy or ruminant fats and marine or long chain polyunsaturated oils, 21:0 TAG internal standard (IS) would be used.

   The fat or oil amount (mg) in the sample is calculated as follows:

   200 mg sample × 0.40 (40%) fat = 80 mg of fat or oil in the sample

   The amount of internal standard added to the 50-mL round flat-bottom reaction flask should be between 0.05 and 0.1 mg IS per 1 mg of fat or oil (Ce 1h-05, Ce 1i-07 or Ce 1j-07). The amount (mg) of IS that may be added is calculated as follows:

   80 mg of fat or oil × 0.05 mg IS/1 mg fat or oil = 4 mg IS
   80 mg of fat or oil × 0.1 mg IS/1 mg fat or oil = 8 mg IS

   Therefore, between 4–8 mg of IS should be added to the 50-mL flat-bottom reaction flask and evaporated to dryness before sample addition. Since the concentration of the 21:0 TAG IS is 5.0 mg/mL, the amount (mL) of IS added is calculated as follows:

   If 1 mL of IS is added, then 5.0 mg/mL × 1 mL = 5.0 mg IS
   If 2 mL of IS is added, then 5.0 mg/mL × 2 mL = 10.0 mg IS

   This is the value for W_{is} in Ce 1h-05, Ce 1i-07 or Ce 1j-07.

   The 80 mg of fat or oil will be diluted in 5 mL hexanes or *n*-heptane to give a concentration of:

   80 mg fat or oil/5 mL hexanes or *n*-heptane = 16 mg fat or oil/mL hexanes or *n*-heptane

   This falls within the 15–20 mg/mL stipulated in Ce 1h-05, Ce 1i-07 or Ce 1j-07. However, for products with fat or oil contents less than 20%, the fat or oil solution concentration may be <10 to 20 mg/mL due to sample amount limitations.

3. For samples analyzed as received (without further drying) report the total fat, saturated, *cis*-monounsaturated, *cis*-polyunsaturated, and *trans* fatty acids as directed in Ce 1h-05, Ce 1i-07 or Ce 1j-07 methods.

PRECISION

Precision data determined from a 2011 AOCS collaborative study by 9 laboratories analyzing 24 samples are given in Table 2. Reproducibility and repeatability values are calculated according to AOAC/IUPAC Harmonized Protocol for Collaborative Study.

NOTES

1. One example of an apparatus that may be specifically manufactured for sample preparation is the 10-position 50 mL flat bottom flask S-Evap derivatization unit with West-style condensers, intrinsically safe bath purge system with electrical interlock, and a pressure reducing regulator and gauge for z-purge. The condensers can be manufactured to allow reagent addition through holes in the top (see Figure 1).
2. BF_3/MeOH should be refrigerated when not in use.
3. Tritricosanoin (23:0 TAG) internal standard is not soluble in chloroform at 5 mg/mL; therefore 2 mg/mL is used.
4. If the sample has been dried, calculate the fat content on a dry basis and use the appropriate sample amount to achieve a fat concentration of between 15–20 mg oil per mL of *n*-hexane or *n*-heptane.
5. Preliminary identification of peaks associated with antioxidants should first be determined before analyzing oil samples to determine if co-elution will occur with peaks of interest. BHA co-elutes with 14:0 in AOCS Ce 1h-05 and Ce 1j-07, BHT and TBHQ elute. There is no co-elution of BGA, BHT, or TBHQ with fatty acids peaks in AOCS Ce 1i-07. Many times antioxidants are not needed. The use of antioxidants is not routinely required.
6. The appropriate time for some matrices is more than 15 min.

SAFETY

1. Always follow a Chemical Hygiene Plan and established laboratory safety procedures for handling materials, cleaning up spills and disposing of wastes.
2. Read and observe all precautionary measures and hazards noted in the Material Safety Data Sheets for all chemicals used in this procedure.

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3. Hexanes, *n*-heptane and MeOH are highly flammable. Caution must be taken at all times to keep them away from sparks, heat and open flames. Know the location of the fire extinguishers in the area before proceeding with the test. All solvent work (usage) should be done in a hood.
4. Chloroform should be disposed of in a halogenated waste container. Internal standard (IS) solutions prepared in chloroform should be used as long as the concentration does not change.
5. BF_3/MeOH should be stored in a flammable liquid refrigerator when not in use.

REFERENCES

1. *AOCS Official Method* Ce 2-66, Preparation of Methyl Esters of Long-Chain Fatty Acids.
2. *AOCS Official Method* Ce 1h-05, Determination of *cis*-, *trans*-, Saturated, Monounsaturated and Polyunsaturated Fatty Acids in Vegetable or Non-Ruminant Animal Oils and Fats by Capillary GLC.
3. *AOCS Official Method* Ce 1i-07, Determination of Saturated, *cis*-Monounsaturated and *cis*-Polyunsaturated Fatty Acids in Marine and Other Oils Containing Long Chain Polyunsaturated Fatty Acids (PUFAs) by Capillary GLC.
4. *AOCS Official Method* Ce 1j-07, Determination of *cis*-, *trans*-, Saturated, Monounsaturated and Polyunsaturated Fatty Acids in Dairy and Ruminant Fats by Capillary GLC.
5. Barnes, Jr., P.C. and Holaday, C.E., Rapid Preparation of Fatty Acid Esters Directly From Ground Peanuts, *J. Chromat. Sci.* 10, 181–183, 1972.
6. Hougen, F.W., and Bodo, V., Extraction and Methanolysis of Oil from Whole or Crushed Rapeseed for Fatty Acid Analysis, *J. Am. Oil Chem.* 50, 230–234, 1973.
7. Dahmer, M.L., Fleming, P.D., Collins, G.B., and Hildebrand, D.F., A Rapid Screening Technique for Determining the Lipid Composition of Soybean Seeds, *J. Am. Oil Chem.* 66 (4), 543–548, 1989.
8. Ulberth, F. and Henninger, M., One-Step Extraction/Methylation for Determining the Fatty Acid Composition of Processed Foods, *J. Am. Oil Chem.* 69 (2), 174–177, 1992.

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Table 1
Guide to test portion size and internal standard volume.

Expected Fat or Oil Concentration	Test Portion Amount (mg)	Fat or Oil Concentration (mg/mL)	Fat or Oil Amount (mg)	Amount of TAG IS Added (mg)	13:0 TAG IS (mL)	21:0 TAG IS (mL)	23:0 TAG IS (mL)
90–100%	100	18–20	90–100	4.5–10	1–2	1–2	2–5
80–89%	100	16–18	80–90	4–9	1–2	1–2	2–5
70–79%	125	17.5–20	87.5–100	4.4–10	1–2	1–2	2–5
60–69%	125	15–17.5	75–87.5	3.8–8.8	1–2	1–2	2–5
50–59%	150	15–18	75–90	3.8–9	1–2	1–2	2–5
40–49%	200	16–20	80–100	4–10	1–2	1–2	2–5
30–39%	250	15–20	75–100	3.8–10	1–2	1–2	2–5
20–29%	325	13–19.5	65–97.5	3.3–9.8	1–2	1–2	2–5
10–19%	500	10–20	50–100	2.5–10	0.5–2	0.5–2	1–5
ND–9%	1000	<20	<100	<10	0.5–2	0.5–2	1–5

Table 2
Results of *cis*-, *trans*-, Saturated, Monounsaturated, and Polyunsaturated fatty acid determination by 9 laboratories in a collaborative study organized by AOCS in 2011. FAMES were analyzed using AOCS Ce 1j-07.

Anhydrous milk fat	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	93.59	88.92	58.27	5.11	26.39	21.59	3.78	3.25	0.53	2.64
Repeatability (Sr)	2.48	2.39	1.32	0.41	1.21	0.27	0.39	0.20	0.06	0.04
%RSDr	2.65	2.69	2.26	8.06	4.60	1.27	10.23	6.08	11.21	1.60
r (2.8xSr)	6.94	6.69	3.69	1.15	3.40	0.77	1.08	0.55	0.17	0.12
Reproducibility (SR)	2.48	2.39	2.39	0.67	1.53	0.73	0.54	0.20	0.14	0.24
%RSDR	2.65	2.69	4.10	13.14	5.81	3.38	14.28	6.08	26.83	8.94
R (2.8xSR)	6.94	6.69	6.69	1.88	4.29	2.05	1.51	0.55	0.40	0.66
Tallow	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	99.68	95.21	45.44	7.14	46.53	39.31	2.65	1.99	0.33	1.66
Repeatability (Sr)	1.01	0.96	1.03	0.19	0.78	0.33	0.12	0.05	0.01	0.04
%RSDr	1.01	1.01	2.26	2.73	1.67	0.84	4.43	2.41	3.80	2.33
r (2.8xSr)	2.82	2.69	2.88	0.55	2.18	0.92	0.33	0.13	0.04	0.11
Reproducibility (SR)	1.66	1.58	1.36	0.30	1.71	0.80	0.34	0.24	0.07	0.18
%RSDR	1.66	1.66	2.99	4.20	3.68	2.03	12.74	12.24	22.64	11.13
R (2.8xSR)	4.64	4.42	3.81	0.84	4.80	2.24	0.94	0.68	0.21	0.52
Chocolate cake mix	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	10.81	10.34	4.44	0.90	3.21	2.47	2.65	2.49	0.23	2.27
Repeatability (Sr)	0.23	0.22	0.11	0.03	0.06	0.04	0.07	0.06	0.01	0.05
%RSDr	2.09	2.09	2.37	3.29	1.79	1.72	2.77	2.60	2.46	2.20
r (2.8xSr)	0.63	0.61	0.30	0.08	0.16	0.12	0.21	0.18	0.02	0.14
Reproducibility (SR)	0.42	0.40	0.25	0.07	0.10	0.09	0.12	0.10	0.01	0.08
%RSDR	3.92	3.91	5.68	7.43	3.25	3.45	4.67	4.20	4.63	3.62
R (2.8xSR)	1.19	1.13	0.71	0.19	0.29	0.24	0.35	0.29	0.03	0.23

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Table 2
(Continued)

Cheese Powder	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	29.78	28.38	13.49	7.27	14.08	6.95	1.24	0.97	0.14	0.83
Repeatability (Sr)	0.43	0.41	0.22	0.12	0.22	0.12	0.04	0.02	0.00	0.01
%RSDr	1.43	1.44	1.62	1.68	1.55	1.66	3.10	2.22	3.22	1.60
r (2.8xSr)	1.19	1.14	0.61	0.34	0.61	0.32	0.11	0.06	0.01	0.04
Reproducibility (SR)	2.39	2.28	0.71	0.37	0.75	0.57	0.12	0.02	0.03	0.05
%RSDR	8.02	8.02	5.24	5.04	5.31	8.18	9.54	2.46	19.48	6.58
R (2.8xSR)	6.69	6.38	1.98	1.03	2.09	1.59	0.33	0.07	0.08	0.15
DHA/EPA fortified infant formula	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	28.94	27.58	12.07	0.15	10.15	10.07	5.21	5.14	0.53	4.62
Repeatability (Sr)	0.66	0.63	0.07	0.02	0.20	0.20	0.13	0.12	0.01	0.09
%RSDr	2.29	2.29	0.59	15.01	1.96	2.02	2.43	2.41	2.16	2.01
r (2.8xSr)	1.86	1.77	0.20	0.06	0.56	0.57	0.35	0.35	0.03	0.26
Reproducibility (SR)	1.48	1.42	0.45	0.12	0.65	0.65	0.39	0.38	0.02	0.33
%RSDR	5.11	5.14	3.77	78.47	6.39	6.46	7.52	7.48	3.49	7.08
R (2.8xSR)	4.14	3.97	1.27	0.33	1.82	1.82	1.10	1.08	0.05	0.92
Extruded dog food (high protein and high fat)	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	22.04	21.06	6.72	0.31	9.75	9.31	4.76	4.72	0.33	4.36
Repeatability (Sr)	1.49	1.42	0.43	0.11	0.64	0.61	0.31	0.30	0.03	0.28
%RSDr	6.75	6.75	6.38	34.60	6.53	6.60	6.44	6.41	9.24	6.41
r (2.8xSr)	4.17	3.98	1.20	0.30	1.78	1.72	0.86	0.85	0.09	0.78
Reproducibility (SR)	1.49	1.42	0.43	0.11	0.64	0.61	0.31	0.30	0.04	0.28
%RSDR	6.75	6.75	6.38	34.97	6.53	6.60	6.44	6.41	11.77	6.41
R (2.8xSR)	4.17	3.98	1.20	0.31	1.78	1.72	0.86	0.85	0.11	0.78
Oatmeal Cookie	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	19.19	18.33	8.95	0.05	7.12	7.08	2.25	2.20	0.05	2.16
Repeatability (Sr)	0.27	0.26	0.20	0.02	0.08	0.08	0.02	0.02	0.00	0.02
%RSDr	1.42	1.42	2.23	28.55	1.13	1.11	1.03	1.02	2.41	0.99
r (2.8xSr)	0.76	0.73	0.56	0.04	0.22	0.22	0.06	0.06	0.00	0.06
Reproducibility (SR)	0.72	0.68	0.39	0.02	0.31	0.31	0.09	0.10	0.00	0.09
%RSDR	3.74	3.72	4.30	44.84	4.38	4.40	4.00	4.33	4.89	4.36
R (2.8xSR)	2.01	1.91	1.08	0.07	0.87	0.87	0.25	0.27	0.01	0.26
Evaporated milk	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	6.28	5.97	3.93	0.33	1.78	1.47	0.23	0.19	0.03	0.16
Repeatability (Sr)	0.29	0.27	0.17	0.03	0.09	0.08	0.02	0.01	0.00	0.01
%RSDr	4.61	4.57	4.44	8.45	5.11	5.17	7.06	4.73	8.53	4.38
r (2.8xSr)	0.81	0.76	0.49	0.08	0.25	0.21	0.05	0.03	0.01	0.02
Reproducibility (SR)	0.29	0.27	0.22	0.05	0.09	0.08	0.03	0.02	0.01	0.02
%RSDR	4.61	4.57	5.54	15.89	5.11	5.17	14.13	10.75	32.47	10.66
R (2.8xSR)	0.81	0.76	0.61	0.15	0.25	0.21	0.09	0.06	0.03	0.05

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Table 2
(Continued)

Peanut butter	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	54.01	51.69	10.11	0.06	30.28	30.13	11.23	11.17	0.08	10.79
Repeatability (Sr)	1.17	1.12	0.33	0.01	0.70	0.74	0.29	0.40	0.02	0.26
%RSDr	2.16	2.16	3.29	21.71	2.31	2.47	2.61	3.62	21.36	2.37
r (2.8xSr)	3.26	3.12	0.93	0.03	1.96	2.08	0.82	1.13	0.05	0.72
Reproducibility (SR)	3.10	2.96	0.79	0.04	1.57	1.60	1.46	1.48	0.08	1.14
%RSDR	5.73	5.73	7.83	75.73	5.17	5.32	13.02	13.21	98.99	10.58
R (2.8xSR)	8.67	8.30	2.22	0.12	4.38	4.49	4.10	4.13	0.22	3.19

Yogurt (plain)	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	5.80	5.51	3.60	0.32	1.65	1.37	0.23	0.19	0.03	0.15
Repeatability (Sr)	0.55	0.53	0.39	0.03	0.13	0.11	0.03	0.02	0.01	0.01
%RSDr	9.54	9.55	10.90	7.94	8.09	8.26	12.67	13.02	42.60	9.08
r (2.8xSr)	1.55	1.47	1.10	0.07	0.37	0.32	0.08	0.07	0.04	0.04
Reproducibility (SR)	0.55	0.53	0.39	0.03	0.15	0.14	0.03	0.02	0.02	0.01
%RSDR	9.54	9.55	10.90	7.94	9.23	9.92	12.83	13.02	59.74	9.08
R (2.8xSR)	1.55	1.47	1.10	0.07	0.43	0.38	0.08	0.07	0.05	0.04

Canned cat food	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	5.70	5.44	1.71	0.05	2.40	2.01	1.25	1.24	0.42	0.76
Repeatability (Sr)	0.66	0.64	0.15	0.01	0.25	0.23	0.12	0.11	0.05	0.07
%RSDr	11.59	11.67	8.83	13.55	10.58	11.68	9.22	9.19	11.58	9.53
r (2.8xSr)	1.85	1.78	0.42	0.02	0.71	0.66	0.32	0.32	0.14	0.20
Reproducibility (SR)	0.80	0.77	0.26	0.03	0.46	0.93	0.23	0.22	0.24	0.14
%RSDR	14.09	14.11	15.08	49.55	19.14	46.30	18.07	17.88	56.88	18.16
R (2.8xSR)	2.25	2.15	0.72	0.08	1.29	2.60	0.63	0.62	0.68	0.39

DHA/EPA fortified orange juice	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	0.12	0.11	0.03	0.00	0.03	0.03	0.03	0.03	0.02	0.01
Repeatability (Sr)	0.02	0.02	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.01
%RSDr	15.70	17.14	13.26	0.00	8.92	16.09	29.64	29.64	25.75	46.97
r (2.8xSr)	0.05	0.05	0.01	0.00	0.01	0.01	0.03	0.03	0.01	0.02
Reproducibility (SR)	0.02	0.02	0.02	0.00	0.00	0.01	0.02	0.02	0.01	0.01
%RSDR	19.39	20.56	51.60	0.00	12.41	19.13	58.12	58.12	67.03	58.62
R (2.8xSR)	0.06	0.06	0.05	0.00	0.01	0.01	0.05	0.05	0.03	0.02

Butter	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	71.12	67.76	36.52	2.49	22.31	20.23	8.77	8.36	1.73	6.91
Repeatability (Sr)	1.62	1.54	1.00	0.13	0.63	0.54	0.44	0.39	0.14	0.14
%RSDr	2.28	2.28	2.73	5.34	2.81	2.68	4.99	4.72	8.25	2.05
r (2.8xSr)	4.54	4.32	2.80	0.37	1.75	1.52	1.23	1.10	0.40	0.40
Reproducibility (SR)	4.74	4.52	2.32	0.43	1.94	1.74	0.89	0.88	0.15	0.50
%RSDR	6.66	6.68	6.35	17.29	8.69	8.62	10.15	10.50	8.89	7.17
R (2.8xSR)	13.26	12.66	6.49	1.21	5.43	4.88	2.49	2.46	0.43	1.39

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Table 2
(Continued)

Whole egg powder	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	40.24	38.47	14.34	0.43	16.73	16.35	7.36	7.31	0.37	6.84
Repeatability (Sr)	1.20	1.15	0.41	0.02	0.56	0.56	0.22	0.21	0.02	0.20
%RSDr	2.98	2.98	2.85	4.79	3.33	3.41	2.95	2.92	5.99	2.88
r (2.8xSr)	3.36	3.21	1.14	0.06	1.56	1.56	0.61	0.60	0.06	0.55
Reproducibility (SR)	1.20	1.15	0.50	0.06	0.56	0.56	0.35	0.33	0.03	0.38
%RSDR	2.98	2.98	3.52	12.99	3.33	3.41	4.79	4.50	9.04	5.56
R (2.8xSR)	3.36	3.21	1.41	0.16	1.56	1.56	0.99	0.92	0.09	1.06

Full-fat soy flour flakes	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	23.05	22.05	3.70	0.02	4.73	4.74	13.49	13.48	1.61	11.69
Repeatability (Sr)	0.23	0.22	0.11	0.00	0.12	0.05	0.39	0.39	0.03	0.12
%RSDr	0.98	0.98	3.02	22.12	2.55	1.10	2.91	2.91	1.61	1.07
r (2.8xSr)	0.63	0.61	0.31	0.01	0.34	0.15	1.10	1.10	0.07	0.35
Reproducibility (SR)	0.66	0.62	0.39	0.01	0.14	0.12	0.39	0.39	0.15	0.25
%RSDR	2.85	2.83	10.42	73.10	2.93	2.57	2.91	2.91	9.34	2.13
R (2.8xSR)	1.84	1.75	1.08	0.04	0.39	0.34	1.10	1.10	0.42	0.70

Encapsulated DHA/EPA	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	56.05	53.66	18.18	0.68	14.09	13.33	21.02	20.63	18.25	2.19
Repeatability (Sr)	1.31	1.26	0.43	0.08	0.37	0.35	0.47	0.46	0.41	0.09
%RSDr	2.34	2.34	2.36	12.13	2.65	2.64	2.23	2.21	2.25	4.24
r (2.8xSr)	3.68	3.52	1.20	0.23	1.05	0.98	1.31	1.28	1.15	0.26
Reproducibility (SR)	3.32	3.15	0.95	0.23	0.81	0.67	1.83	1.75	1.46	0.93
%RSDR	5.92	5.87	5.22	33.82	5.72	5.01	8.71	8.50	8.01	42.40
R (2.8xSR)	9.29	8.83	2.66	0.64	2.26	1.87	5.13	4.91	4.09	2.60

Creamy ranch dressing	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	46.18	44.16	7.12	0.24	9.66	9.63	25.68	25.51	3.40	23.18
Repeatability (Sr)	1.91	1.82	0.32	0.01	0.52	0.52	0.98	0.98	0.11	0.92
%RSDr	4.13	4.13	4.51	3.71	5.39	5.40	3.83	3.84	3.18	3.98
r (2.8xSr)	5.34	5.11	0.90	0.02	1.46	1.46	2.75	2.74	0.30	2.58
Reproducibility (SR)	1.91	1.82	0.32	0.16	0.52	0.52	2.36	2.37	0.11	1.16
%RSDR	4.13	4.13	4.51	65.50	5.39	5.40	9.17	9.30	3.18	5.02
R (2.8xSR)	5.34	5.11	0.90	0.44	1.46	1.46	6.60	6.64	0.30	3.26

Potato chips	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	36.01	34.44	6.35	0.22	14.16	14.02	13.70	13.59	0.12	13.45
Repeatability (Sr)	1.75	1.67	0.31	0.06	0.74	0.72	0.63	0.61	0.02	0.60
%RSDr	4.85	4.85	4.95	26.22	5.21	5.14	4.57	4.50	13.30	4.50
r (2.8xSr)	4.89	4.68	0.88	0.16	2.07	2.02	1.75	1.71	0.04	1.69
Reproducibility (SR)	1.75	1.67	0.31	0.14	0.74	0.72	0.63	0.61	0.04	0.60
%RSDR	4.85	4.85	4.95	62.69	5.21	5.14	4.57	4.50	37.66	4.50
R (2.8xSR)	4.89	4.68	0.88	0.39	2.07	2.02	1.75	1.71	0.12	1.69

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Table 2
(Continued)

Cheese powder duplicate	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	30.10	28.69	13.35	7.20	13.85	6.96	1.31	0.98	0.13	0.85
Repeatability (Sr)	0.45	0.43	0.27	0.10	0.22	0.11	0.05	0.02	0.01	0.02
%RSDr	1.49	1.48	2.05	1.36	1.59	1.56	4.14	2.12	5.11	2.18
r (2.8xSr)	1.25	1.19	0.77	0.27	0.62	0.30	0.15	0.06	0.02	0.05
Reproducibility (SR)	1.18	1.11	0.30	0.31	0.37	0.21	0.05	0.10	0.04	0.08
%RSDR	3.91	3.88	2.22	4.27	2.70	3.04	4.14	10.08	29.05	9.25
R (2.8xSR)	3.30	3.12	0.83	0.86	1.05	0.59	0.15	0.28	0.11	0.22
Frozen cheese pizza	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	8.06	7.66	3.56	0.37	2.25	1.68	1.33	1.29	0.05	1.23
Repeatability (Sr)	0.67	0.64	0.30	0.04	0.20	0.16	0.13	0.12	0.01	0.12
%RSDr	8.32	8.37	8.54	10.04	9.10	9.76	9.45	9.46	20.01	9.39
r (2.8xSr)	1.88	1.80	0.85	0.10	0.57	0.46	0.35	0.34	0.03	0.32
Reproducibility (SR)	1.59	1.51	1.63	0.07	0.45	0.77	0.24	0.24	0.03	0.23
%RSDR	19.72	19.76	45.76	18.70	19.92	45.85	18.19	18.38	56.05	18.41
R (2.8xSR)	4.45	4.24	4.56	0.19	1.25	2.15	0.68	0.66	0.07	0.64
Peanut butter duplicate	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	51.50	49.29	9.66	0.05	30.84	30.55	8.78	8.77	0.08	8.55
Repeatability (Sr)	3.97	3.79	0.88	0.03	2.40	2.26	0.59	0.59	0.01	0.59
%RSDr	7.70	7.70	9.13	53.93	7.79	7.41	6.76	6.77	6.55	6.91
r (2.8xSr)	11.10	10.62	2.47	0.08	6.73	6.34	1.66	1.66	0.02	1.65
Reproducibility (SR)	4.43	4.24	0.92	0.05	2.60	2.89	1.51	1.50	0.09	1.22
%RSDR	8.61	8.61	9.51	85.63	8.42	9.46	17.22	17.15	105.13	14.32
R (2.8xSR)	12.41	11.88	2.57	0.13	7.27	8.09	4.23	4.21	0.24	3.43
Dry cereal fortified with flax	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	1.91	1.82	0.28	0.00	0.36	0.36	1.20	1.20	0.52	0.64
Repeatability (Sr)	0.03	0.02	0.02	0.00	0.01	0.01	0.03	0.03	0.01	0.02
%RSDr	1.34	1.35	6.75	0.00	2.04	2.04	2.53	2.53	2.48	3.42
r (2.8xSr)	0.07	0.07	0.05	0.00	0.02	0.02	0.08	0.08	0.04	0.06
Reproducibility (SR)	0.27	0.26	0.04	0.00	0.06	0.06	0.09	0.09	0.03	0.09
%RSDR	14.17	14.19	14.49	0.00	16.81	16.81	7.26	7.26	6.12	13.26
R (2.8xSR)	0.76	0.72	0.12	0.00	0.17	0.17	0.24	0.24	0.09	0.24
Horse feed	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	3.22	3.07	0.72	0.00	0.76	0.75	1.58	1.58	0.21	1.38
Repeatability (Sr)	0.17	0.16	0.04	0.00	0.05	0.04	0.07	0.07	0.01	0.06
%RSDr	5.31	5.26	6.22	0.00	6.27	5.89	4.52	4.52	3.57	4.68
r (2.8xSr)	0.48	0.45	0.13	0.00	0.13	0.12	0.20	0.20	0.02	0.18
Reproducibility (SR)	0.19	0.18	0.07	0.00	0.06	0.04	0.09	0.09	0.02	0.07
%RSDR	5.86	5.82	9.52	0.00	7.85	5.89	5.54	5.54	8.39	5.36
R (2.8xSR)	0.53	0.50	0.19	0.00	0.17	0.12	0.25	0.25	0.05	0.21

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Table 2
(Continued)

Gamebird feed	Total Fat (TAG%)*	Total Fat (FA%)*	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total *cis* PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	9	9	9	9	9	9	9
Mean	3.00	2.87	0.57	0.00	0.64	0.63	1.65	1.65	0.13	1.52
Repeatability (Sr)	0.07	0.07	0.02	0.00	0.02	0.02	0.02	0.02	0.00	0.02
%RSDr	2.25	2.29	4.27	0.00	3.40	3.47	1.49	1.44	1.68	1.50
r (2.8xSr)	0.19	0.18	0.07	0.00	0.06	0.06	0.07	0.07	0.01	0.06
Reproducibility (SR)	0.40	0.38	0.07	0.00	0.08	0.07	0.26	0.26	0.03	0.23
%RSDR	13.39	13.41	12.91	0.00	12.14	10.97	15.48	15.46	19.07	15.19
R (2.8xSR)	1.12	1.08	0.21	0.00	0.22	0.19	0.72	0.71	0.07	0.64

*NLEA (Nutritional Labeling and Education Act) according to the Office of Nutritional Products, Labeling, and Dietary Supplements in the Center for Food Safety and Applied Nutrition at the U.S. Food and Drug Administration
**Does not include CLA

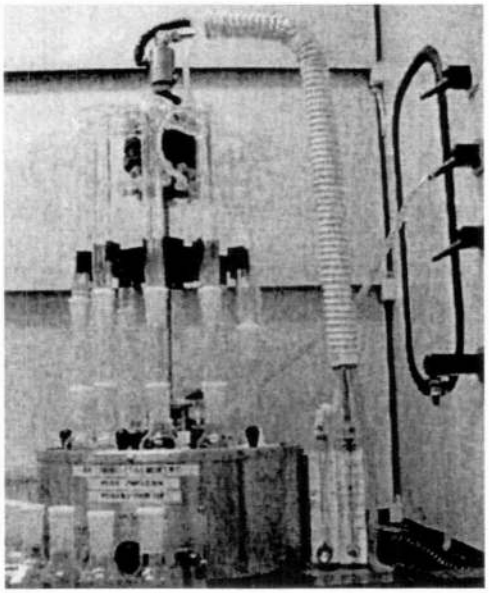

Figure 1. Example of suitable extraction apparatus (see Notes, 1)

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AOCS

AOCS Official Method Ce 2c-11

Revised 2013

Direct Methylation of Lipids in Foods by Acid-Alkali Hydrolysis

DEFINITION

This method describes an acid-alkaline procedure for the preparation of fatty acid methyl esters (FAMEs) directly from food matrices. The fats or oils are released from the matrix by *in situ* acid digestion (hydrochloric acid in methanol) followed by alkali hydrolysis (sodium hydroxide in methanol) and methylation (with boron trifluoride in methanol as the catalyst).

SCOPE

This method is only required when AOCS Official Method Ce 2b-11 does not release all fatty acids quantitatively. Some food matrices such as extruded cat and dog foods, oat based foods, and some encapsulated oils require acid pretreatment. Some long chain polyunsaturated fortified beverages show lower recoveries using this method. Since not all matrices could be evaluated, analysts are advised to test the chosen method against appropriate reference methods. Many materials with high moisture contents do not require drying before analysis. However, antioxidants should be added if drying is required (except when freeze drying) to protect from oxidation. An appropriate grinding technique for homogenization of the test sample should be used, when necessary.

Analysis of steryl ester concentrates and steryl ester-fortified food products are outside the scope of this method since greatly increased saponification times are required.

APPARATUS

1. 50-mL round flat-bottom flasks with 24/40 joints or equivalent
2. Teflon™ flask-neck sleeves (size 24/40)
3. Hollow, full-length stopper (size 24/40), barrel-shaped head
4. Explosion-proof hot plates or equivalent (see Notes, 1)
5. Water-cooled condensers or equivalent (see Notes, 1)
6. 2-mL amber write-on autosampler vials with Teflon™-lined caps
7. Glass powder funnel
8. Analytical balance capable of reading to ± 0.0001 g or ± 0.1 mg
9. Weighing funnels
10. 1-L transparent polymethylpentene screw top flask
11. 500-mL Nalgene® bottle
12. Porous boiling chips

REAGENTS

1. Hydrochloric Acid, 1.25 M in methanol (MeOH). Do not use aqueous HCl.
2. Sodium Hydroxide in MeOH, 2.3 M analytical reagent grade. Add 92 g of NaOH to a 1-L polymethylpentene flask. Add MeOH and mix well to dissolve before diluting to volume.
3. Methanol (MeOH), high purity gas chromatographic grade
4. Sodium Chloride, analytical reagent grade, saturated. Dissolve 36 g NaCl in 100 mL distilled water.
5. Hexanes or *n*-heptane, high purity gas chromatographic grade
6. Chloroform, high purity gas chromatographic grade
7. 14% Boron Trifluoride in MeOH (see Notes, 2)
8. Sodium Sulfate, anhydrous, reagent grade
9. Pyrogallol, ACS reagent
10. Internal standard: 13:0, Tritridecanoin (P/N T-135, Nu-Chek-Prep, Inc. or equivalent) for unknowns, dairy or ruminant fats. Accurately weigh out 2.5 g of 13:0 triacylglycerol (TAG) into a weighing funnel. Empty the weighing funnel into a 500-mL class A volumetric flask and rinse the funnel with hexanes or *n*-heptane to ensure complete quantitative transfer into the flask. Add hexanes or *n*-heptane to the flask, mix to dissolve, and dilute to volume (see Calculations, 1). The 13:0 TAG internal standard solution is stable indefinitely if precautions are taken to eliminate the loss of *n*-heptane and therefore a change in the concentration of the internal standard.

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11. Internal standard: 21:0, Triheneicosanoin (P/N T-175, Nu-Chek-Prep, Inc. or equivalent) for all samples other than dairy or ruminant fats and marine or long chain polyunsaturated oils. Accurately weigh out 2.5 g of 21:0 TAG into a weighing funnel. Empty the weighing funnel into a 500-mL class A volumetric flask and rinse the funnel with chloroform to ensure complete quantitative transfer into the flask. Add chloroform to the flask, mix to dissolve, and dilute to volume (see Calculations, 1). The 21:0 TAG internal standard solution is stable indefinitely if precautions are taken to eliminate the loss of chloroform and therefore a change in the concentration of the internal standard.
12. Internal standard: 23:0, Tritricosanoin (P/N T-185, Nu-Chek-Prep, Inc. or equivalent) for marine oils or other oils containing long chain polyunsaturated fatty acids (PUFAs). Accurately weigh out 1.0 g of 23:0 TAG into a weighing funnel (see Notes, 3). Empty the weighing funnel into a 500-mL class A volumetric flask and rinse the funnel with chloroform to ensure complete quantitative transfer into the flask. Add chloroform to the flask, mix to dissolve, and dilute to volume (see Calculations, 1). The 23:0 TAG internal standard solution is stable indefinitely if precautions are taken to eliminate the loss of chloroform and therefore a change in the concentration of the internal standard.

SAMPLE PREPARATION

1. Samples should be homogeneous before extraction, which may be done by drying, grinding or melting. High moisture matrices (e.g., food stuffs, tissues, etc.) may need to have the moisture removed to allow for grinding. Use the appropriate grinding apparatus for the matrix. Solid and semi-solid fats should be melted and mixed thoroughly at the lowest temperature that ensures homogeneity.
2. Prior to methylation, pipette (with a 0.5, 1, 2 or 5-mL class A volumetric pipette) enough internal standard (IS) into a tared 50-mL round flat-bottom reaction flask, so that after the test portion is added, the concentration of the final solution is between 0.05 and 0.1 mg IS per 1 mg oil or fat from the test portion. See Table 1 to determine the appropriate amount (mL) of IS to add based upon the expected fat or oil concentration of the test portion (see Calculations, 2). Evaporate the solvent (hexanes, *n*-heptane or chloroform) to dryness. This is the value for W_{is} in AOCS Ce 1h-05, Ce 1i-07 or Ce 1j-07.
3. Add the appropriate amount (mg) of sample, see Table 1, into the tared 50-mL flat bottom flask containing the dried IS. (This will yield a final concentration of 15–20 mg fat or oil per mL of final solution. Where samples contain less than 20% fat or oil, the range will be <10 to 20 mg/mL). For samples containing less than 10% fat, use 1000 mg or 1 g (maximum). Record the exact weight to 0.1 mg as this amount will be used in the total fat, saturated, *cis*-monounsaturated, *cis*-polyunsaturated and *trans* fatty acid content determinations. This value is W_{TS} in AOCS Ce 1h-05, Ce 1i-07 and Ce 1j-07.
4. Add a couple of porous boiling chips.
5. Add 1 mg of antioxidant (e.g., pyrogallol) 1 mg oil (see Notes, 4)

ACID/ALKALI CATALYZED FAME PREPARATION

1. Add 5 mL 1.25 M HCl/MeOH to flask.
2. Attach coolant (≤30°C), water or air-cooled condensers to the flasks (see Notes, 1).
3. Reflux for 15 min after boiling begins (see Notes, 5). *CAUTION:* If moisture is still present the mixture may bump during reflux/boiling.
4. Add 5 mL of 2.3 M NaOH/MeOH to flask.
5. Attach coolant (≤30°C), water or air-cooled condensers to the flasks (see Notes, 1).
6. Reflux for 15 min after boiling begins (see Notes, 5). *CAUTION:* If moisture is still present the mixture may bump during reflux/boiling.
7. Add 10 mL 14% BF_3/MeOH to the boiling flask. Reflux for additional 5 min.
8. Add 5 mL of hexanes or *n*-heptane.
9. Remove from heat source; once the flask is cooled to room temperature add enough saturated sodium chloride so the organic layer is in the neck of the 50-mL round flat-bottom flask. Stopper the flask and shake. Allow the layers to separate and transfer a clear portion of the top layer (hexanes or *n*-heptane) with a transfer pipette into a 2-mL autosampler vial containing 50 mg of sodium sulfate. Crimp on the aluminum cap and shake well. The test solution is ready for chromatographic separation and quantification.

CHROMATOGRAPHY

1. For unknowns, dairy or ruminant fats use AOCS Official Method Ce 1j-07 for the chromatographic separation and calculations (see Calculations, 3).
2. For all samples containing vegetable oil, use AOCS Official Method Ce 1h-05 for the chromatographic separation and calculations (see Calculations, 3).
3. For samples containing marine oils or other oils containing long chain PUFAs, use AOCS Official Method Ce 1i-07 for the chromatographic separation and calculations (see Calculations, 3).

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CALCULATIONS

1. The internal standard solution concentrations are calculated as follows:

   If using AOCS Official Method Ce 1j-07:

   2.5 g 13:0 TAG/500 mL hexanes or n-heptane = 0.0050 g/mL
   0.0050 g/mL × 1000 mg/g = 5.0 mg/mL

   If using AOCS Official Method Ce 1h-05:

   2.5 g 21:0 TAG/500 mL chloroform = 0.0050 g/mL
   0.0050 g/mL × 1000 mg/g = 5.0 mg/mL

   If using AOCS Official Method Ce 1i-07:

   1.0 g 23:0 TAG/500 mL chloroform = 0.0020 g/mL
   0.0020 g/mL × 1000 mg/g = 2.0 mg/mL

2. For example, for a product formulated with 40% vegetable fat or oil, the sample amount would be 200 mg (This value is W_{TS} in Ce 1h-05, Ce 1i-07 or Ce 1j-07). Since the oil added is other than dairy or ruminant fats and marine or long chain polyunsaturated oils, 21:0 TAG internal standard (IS) would be used.

   The fat or oil amount (mg) in the sample is calculated as follows:

   200 mg sample × 0.40 (40%) fat = 80 mg of fat or oil in the sample

   The amount of internal standard added to the 50-mL round flat-bottom reaction flask should be between 0.05 and 0.1 mg IS per 1 mg of fat or oil (Ce 1h-05, Ce 1i-07 or Ce 1j-07). The amount (mg) of IS that may be added is calculated as follows:

   80 mg of fat or oil × 0.05 mg IS/1 mg fat or oil = 4 mg IS
   80 mg of fat or oil × 0.1 mg IS/1 mg fat or oil = 8 mg IS

   Therefore, between 4-8 mg of IS should be added to the 50-mL flat-bottom reaction flask and evaporated to dryness before sample addition. Since the concentration of the 21:0 TAG IS is 5.0 mg/mL, the amount (mL) of IS added is calculated as follows:

   If 1 mL of IS is added, then 5.0 mg/mL × 1 mL = 5.0 mg IS
   If 2 mL of IS is added, then 5.0 mg/mL × 2 mL = 10.0 mg IS

   So if 1 mL is added, then 5 mg of IS are added and if 2 mL is added, then 10 mg IS is added. This is the value for W_{is} in Ce 1h-05, Ce 1i-07 or Ce 1j-07.

   The 80 mg of fat or oil will be diluted in 5 mL hexanes or n-heptane to give a concentration of:

   80 mg fat or oil/5 mL hexanes or *n*-heptane = 16 mg fat or oil/mL hexanes or *n*-heptane

   This falls within the 15–20 mg/mL stipulated in Ce 1h-05, Ce 1i-07 or Ce 1j-07. However, for products with fat or oil contents less than 20%, the fat or oil solution concentration may be <10 to 20 mg/mL due to sample amount limitations.

3. For samples analyzed as received (without further drying) report the total fat, saturated, *cis*-monounsaturated, *cis*-polyunsaturated, and *trans* fatty acids basis Ce 1h-05, Ce 1i-07 or Ce 1j-07 methods.
4. Store in liquid refrigerator when not in use.

PRECISION

Precision data determined from a 2011 AOCS collaborative study by 9 laboratories analyzing 24 samples are given in Table 2. Reproducibility and repeatability values are calculated according to AOAC/IUPAC Harmonized Protocol for Collaborative Study.

NOTES

1. One example of an apparatus that may be specifically manufactured for sample preparation is the 10-position 50 mL flat bottom flask S-Evap derivatization unit with West-style condensers, intrinsically safe bath purge system with electrical interlock, and a pressure reducing regulator and gauge for z-purge. The condensers can be manufactured to allow for reagent addition through holes in the top. A picture is provided in Figure 1.
2. BF_3/MeOH should be refrigerated when not in use.
3. Tritricosanoin (23:0 TAG) internal standard is not soluble in chloroform at 5 mg/mL; therefore 2 mg/mL is used.

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4. If the sample has been dried, calculate the fat content on a dry basis and use the appropriate sample amount to achieve a fat concentration of between 15–20 mg oil per mL of *n*-hexane or *n*-heptane.
5. Preliminary identification of peaks associated with antioxidants should first be determined before analyzing oil samples to determine if co-elution will occur with peaks of interest.
6. The appropriate time for some matrices may require more than 15 min.

SAFETY

1. Always follow a Chemical Hygiene Plan and established laboratory safety procedures for handling materials, cleaning up spills and disposing of wastes.
2. Read and observe all precautionary measures and hazards noted in the Material Safety Data Sheets for all chemicals used in this procedure.
3. Hexanes, *n*-heptane and MeOH are highly flammable. Caution must be taken at all times to keep them away from sparks, heat and open flames. Know the location of the fire extinguishers in the area before proceeding with the test. All solvent work (usage) should be done in a hood.
4. Chloroform should be disposed of in a halogenated waste container. Internal standard (IS) solutions prepared in chloroform should be used as long as the concentration does not change so as to not generate unnecessary chlorinated solvent waste.
5. BF_3/MeOH should be stored in a flammable liquid refrigerator when not in use

REFERENCES

1. *AOCS Official Method* Ce 2-66, Preparation of Methyl Esters of Long-Chain Fatty Acids.
2. IUPAC, *Standard Methods for Analysis of Oils, Fats and Derivatives,* Blackwell Scientific Publications IUPAC Method 2.301.
3. *AOCS Official Method* Ce 1h-05, Determination of *cis-, trans-,* Saturated, Monounsaturated and Polyunsaturated Fatty Acids in Vegetable or Non-Ruminant Animal Oils and Fats by Capillary GLC.
4. *AOCS Official Method* Ce 1i-07, Determination of Saturated, *cis*-Monounsaturated and *cis*-Polyunsaturated Fatty Acids in Marine and Other Oils Containing Long Chain Polyunsaturated Fatty Acids (PUFAs) by Capillary GLC.
5. *AOCS Official Method* Ce 1j-07, Determination of *cis-, trans-,* Saturated, Monounsaturated and Polyunsaturated Fatty Acids in Dairy and Ruminant Fats by Capillary GLC.
6. Barnes, Jr., P.C. and Holaday, C.E., Rapid Preparation of Fatty Acid Esters Directly From Ground Peanuts, *J. Chromat. Sci.* 10, 181-183, 1972.
7. Hougen, F.W., and Bodo, V., Extraction and Methanolysis of Oil from Whole or Crushed Rapeseed for Fatty Acid Analysis, *J. Am. Oil Chem.* 50, 230–234, 1973.
8. Dahmer, M.L., Fleming, P.D., Collins, G.B., and Hildebrand, D.F., A Rapid Screening Technique for Determining the Lipid Composition of Soybean Seeds, *J. Am. Oil Chem.* 66 (4), 543–548, 1989.
9. Ulberth, F. and Henninger, M., One-Step Extraction/Methylation for Determining the Fatty Acid Composition of Processed Foods, *J. Am. Oil Chem,* 69 (2), 174–177, 1992.

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Table 1
Guide to test portion size and internal standard volume.

Expected Fat or Oil Concentration	Test Portion Amount (mg)	Fat or Oil Concentration (mg/mL)	Fat or Oil Amount (mg)	Amount of TAG IS Added (mg)	13:0 TAG IS (mL)	21:0 TAG IS (mL)	23:0 TAG IS (mL)
90–100%	100	18–20	90–100	4.5–10	1–2	1–2	2–5
80–89%	100	16–18	80–90	4–9	1–2	1–2	2–5
70–79%	125	17.5–20	87.5–100	4.4–10	1–2	1–2	2–5
60–69%	125	15–17.5	75–87.5	3.8–8.8	1–2	1–2	2–5
50–59%	150	15–18	75–90	3.8–9	1–2	1–2	2–5
40–49%	200	16–20	80–100	4–10	1–2	1–2	2–5
30–39%	250	15–20	75–100	3.8–10	1–2	1–2	2–5
20–29%	325	13–19.5	65–97.5	3.3–9.8	1–2	1–2	2–5
10–19%	500	10–20	50–100	2.5–10	0.5–2	0.5–2	1–5
ND–9%	1000	<20	<100	<10	0.5–2	0.5–2	1–5

Table 2
Results of *cis-, trans-*, saturated, monounsaturated, and polyunsaturated fatty acid determination by 9 laboratories in a collaborative study organized by AOCS in 2011. FAMES were analyzed using AOCS Ce1j-07.

Anhydrous Milk Fat	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	8	9	7	7	8	7	9	8
Mean	91.90	87.36	56.26	5.08	26.39	22.03	3.78	3.14	0.52	2.60
Repeatability (Sr)	1.67	1.58	1.38	0.65	0.85	0.69	0.17	0.08	0.07	0.11
%RSDr	1.82	1.81	2.45	12.84	3.21	3.15	4.59	2.55	13.72	4.33
r (2.8xSr)	4.68	4.43	3.86	1.83	2.37	1.94	0.49	0.22	0.20	0.32
Reproducibility (SR)	2.63	2.52	2.14	1.16	1.57	1.36	0.33	0.21	0.11	0.22
%RSDR	2.86	2.88	3.81	22.92	5.96	6.15	8.75	6.78	21.35	8.44
R (2.8xSR)	7.37	7.05	6.00	3.26	4.41	3.80	0.93	0.60	0.31	0.61
Tallow	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	7	7	7	7	9	9	9	9
Mean	97.65	93.28	44.53	6.86	45.57	39.30	2.64	1.97	0.34	1.64
Repeatability (Sr)	1.50	1.44	0.78	0.15	0.63	0.51	0.08	0.06	0.01	0.06
%RSDr	1.54	1.54	1.75	2.21	1.39	1.31	3.09	3.18	3.35	3.61
r (2.8xSr)	4.21	4.02	2.18	0.42	1.77	1.44	0.23	0.18	0.03	0.17
Reproducibility (SR)	2.93	2.81	1.35	0.56	1.29	1.11	0.33	0.25	0.10	0.19
%RSDR	3.00	3.01	3.03	8.12	2.84	2.83	12.61	12.45	29.90	11.78
R (2.8xSR)	8.21	7.86	3.78	1.56	3.62	3.11	0.93	0.69	0.28	0.54
Chocolate cake mix	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	7	7	8	7	8	8	8	8
Mean	10.73	10.26	4.44	0.88	3.13	2.44	2.63	2.46	0.22	2.26
Repeatability (Sr)	0.23	0.22	0.10	0.03	0.09	0.07	0.05	0.04	0.03	0.04
%RSDr	2.16	2.17	2.14	3.20	2.73	2.75	1.74	1.52	14.72	1.60
r (2.8xSr)	0.65	0.62	0.27	0.08	0.24	0.19	0.13	0.10	0.09	0.10
Reproducibility (SR)	0.69	0.66	0.32	0.10	0.20	0.13	0.11	0.09	0.03	0.10
%RSDR	6.39	6.38	7.16	11.93	6.35	5.42	4.33	3.53	15.90	4.26
R (2.8xSR)	1.92	1.83	0.89	0.29	0.56	0.37	0.32	0.24	0.10	0.27

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Table 2
(Continued)

Cheese Powder	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	8	7	7	8	9	9	7	8
Mean	28.70	27.35	13.13	6.99	13.68	6.80	1.18	0.93	0.14	0.79
Repeatability (Sr)	0.80	0.77	0.24	0.16	0.31	0.15	0.08	0.04	0.00	0.02
%RSDr	2.79	2.80	1.83	2.29	2.29	2.24	7.19	4.63	1.94	2.46
r (2.8xSr)	2.24	2.15	0.67	0.45	0.88	0.43	0.24	0.12	0.01	0.05
Reproducibility (SR)	2.25	2.15	0.48	0.52	0.82	0.56	0.17	0.08	0.02	0.07
%RSDR	7.83	7.86	3.65	7.39	5.98	8.19	14.63	8.10	12.96	8.26
R (2.8xSR)	6.29	6.02	1.34	1.45	2.29	1.56	0.48	0.21	0.05	0.18
Extruded dog food (high protein and high fat)	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	8	8	8	7	8	8	8	8	8	8
Mean	22.23	21.24	6.67	0.31	9.75	9.40	4.80	4.75	0.38	4.37
Repeatability (Sr)	0.71	0.68	0.25	0.04	0.35	0.33	0.11	0.09	0.01	0.09
%RSDr	3.21	3.22	3.79	12.10	3.56	3.47	2.21	1.96	2.03	2.09
r (2.8xSr)	2.00	1.92	0.71	0.11	0.97	0.91	0.30	0.26	0.02	0.26
Reproducibility (SR)	1.21	1.16	0.49	0.06	0.53	0.59	0.25	0.24	0.06	0.25
%RSDR	5.45	5.45	7.28	18.79	5.45	6.26	5.28	5.03	16.23	5.71
R (2.8xSR)	3.39	3.24	1.36	0.16	1.49	1.65	0.71	0.67	0.17	0.70
Oatmeal cookie	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	7	7	7	7	7	7	7	7
Mean	19.25	18.39	9.31	0.06	7.47	7.45	2.29	2.25	0.06	2.20
Repeatability (Sr)	0.39	0.37	0.20	0.01	0.18	0.17	0.05	0.04	0.01	0.05
%RSDr	2.04	2.04	2.15	20.27	2.35	2.31	1.98	1.87	10.67	2.34
r (2.8xSr)	1.10	1.05	0.56	0.04	0.49	0.48	0.13	0.12	0.02	0.14
Reproducibility (SR)	0.65	0.62	1.05	0.03	0.87	0.86	0.10	0.09	0.01	0.08
%RSDR	3.38	3.38	11.27	47.13	11.62	11.56	4.16	3.83	18.61	3.81
R (2.8xSR)	1.82	1.74	2.94	0.08	2.43	2.41	0.27	0.24	0.03	0.23
Evaporated milk	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	8	8	9	8	8	8	9	9	9	9
Mean	5.96	5.67	3.69	0.32	1.69	1.41	0.23	0.19	0.03	0.16
Repeatability (Sr)	0.29	0.28	0.18	0.02	0.08	0.06	0.02	0.01	0.00	0.01
%RSDr	4.82	4.86	4.85	7.40	4.93	4.60	6.57	5.93	11.62	5.55
r (2.8xSr)	0.80	0.77	0.50	0.07	0.23	0.18	0.04	0.03	0.01	0.02
Reproducibility (SR)	0.70	0.67	0.44	0.05	0.19	0.15	0.03	0.02	0.01	0.02
%RSDR	11.79	11.86	12.00	17.04	11.45	10.67	14.77	12.28	31.54	12.15
R (2.8xSR)	1.97	1.88	1.24	0.15	0.54	0.42	0.10	0.06	0.02	0.05
Peanut butter	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	6	6	6	6	8	8	7	7	7	7
Mean	54.66	52.32	10.47	0.08	29.88	29.70	11.02	10.95	0.28	10.59
Repeatability (Sr)	0.84	0.80	0.22	0.01	5.53	5.83	0.25	0.13	0.02	0.12
%RSDr	1.54	1.54	2.09	9.37	18.51	19.64	2.27	1.16	5.30	1.09
r (2.8xSr)	2.35	2.25	0.61	0.02	15.49	16.33	0.70	0.36	0.04	0.32
Reproducibility (SR)	5.68	5.44	1.25	0.06	7.12	7.47	1.71	1.71	0.35	1.50
%RSDR	10.39	10.39	11.90	71.88	23.82	25.16	15.56	15.64	122.95	14.14
R (2.8xSR)	15.90	15.22	3.49	0.16	19.93	20.92	4.80	4.80	0.98	4.19

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Table 2
(Continued)

Yogurt (plain)	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	8	8	7	7	8	8	8	8	7	7
Mean	5.54	5.26	3.42	0.30	1.54	1.29	0.22	0.18	0.04	0.14
Repeatability (Sr)	0.50	0.47	0.17	0.02	0.14	0.10	0.02	0.02	0.00	0.01
%RSDr	8.97	8.97	4.86	7.34	9.19	8.05	7.58	9.90	10.64	4.30
r (2.8xSr)	1.39	1.32	0.47	0.06	0.40	0.29	0.05	0.05	0.01	0.02
Reproducibility (SR)	0.73	0.69	0.46	0.04	0.18	0.14	0.03	0.03	0.01	0.02
%RSDR	13.19	13.20	13.39	13.86	11.53	10.62	16.05	15.12	25.57	12.25
R (2.8xSR)	2.04	1.94	1.28	0.12	0.50	0.38	0.10	0.08	0.03	0.05

Canned cat food	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	8	7	7	7	7	7	8	8
Mean	7.12	6.81	2.03	0.08	3.13	3.07	1.54	1.52	0.60	0.95
Repeatability (Sr)	0.53	0.51	0.21	0.01	0.27	0.26	0.08	0.08	0.11	0.07
%RSDr	7.46	7.44	10.30	7.97	8.48	8.55	5.08	5.03	17.81	7.47
r (2.8xSr)	1.49	1.42	0.59	0.02	0.74	0.73	0.22	0.21	0.30	0.20
Reproducibility (SR)	0.57	0.54	0.22	0.02	0.37	0.37	0.20	0.20	0.19	0.10
%RSDR	7.97	7.97	10.63	23.16	11.88	12.16	12.80	13.27	31.35	10.24
R (2.8xSR)	1.59	1.52	0.60	0.05	1.04	1.04	0.55	0.57	0.53	0.27

DHA/EPA fortified orange juice	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	7	9	7	7	7	7	7	7
Mean	0.10	0.10	0.03	0.00	0.03	0.03	0.04	0.04	0.02	0.02
Repeatability (Sr)	0.05	0.05	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
%RSDr	45.70	45.70	49.36	0.00	10.61	9.02	11.07	11.07	19.67	3.46
r (2.8xSr)	0.13	0.13	0.04	0.00	0.01	0.01	0.01	0.01	0.01	0.00
Reproducibility (SR)	0.05	0.05	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.00
%RSDR	45.70	45.70	49.36	0.00	20.58	27.87	30.09	30.09	53.67	12.30
R (2.8xSR)	0.13	0.13	0.04	0.00	0.02	0.02	0.03	0.03	0.03	0.01

Butter	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	8	8	9	8	9	9	7	7	6	8
Mean	68.38	65.16	34.68	2.29	21.17	19.29	8.75	8.32	1.62	6.75
Repeatability (Sr)	3.26	3.11	2.51	0.17	2.26	1.98	0.32	0.31	0.06	0.24
%RSDr	4.76	4.77	7.23	7.32	10.69	10.24	3.65	3.75	3.89	3.57
r (2.8xSr)	9.11	8.70	7.02	0.47	6.34	5.53	0.89	0.87	0.18	0.67
Reproducibility (SR)	4.76	4.56	2.52	0.39	2.73	2.38	0.36	0.32	0.08	0.45
%RSDR	6.96	7.00	7.26	17.02	12.90	12.36	4.13	3.80	5.21	6.67
R (2.8xSR)	13.32	12.77	7.04	1.09	7.65	6.67	1.01	0.88	0.24	1.26

Whole egg powder	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	9	9	7	7	8	8	8	8
Mean	40.66	38.87	13.95	0.40	16.94	16.56	7.26	7.21	0.36	6.77
Repeatability (Sr)	0.97	0.93	0.69	0.02	0.49	0.48	0.13	0.13	0.03	0.13
%RSDr	2.39	2.39	4.94	5.60	2.90	2.93	1.77	1.83	7.51	1.96
r (2.8xSr)	2.72	2.61	1.93	0.06	1.38	1.36	0.36	0.37	0.08	0.37
Reproducibility (SR)	1.19	1.14	1.32	0.08	0.64	0.62	0.51	0.50	0.04	0.66
%RSDR	2.92	2.92	9.43	19.66	3.78	3.72	7.04	6.97	10.99	9.80
R (2.8xSR)	3.32	3.18	3.68	0.22	1.79	1.73	1.43	1.41	0.11	1.86

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Table 2
(Continued)

Full-fat soy flour flakes	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	9	8	7	7	9	9	8	9
Mean	22.72	21.73	3.74	0.02	5.00	4.99	13.20	13.18	1.61	11.60
Repeatability (Sr)	0.76	0.73	0.17	0.01	0.27	0.27	0.33	0.33	0.03	0.32
%RSDr	3.34	3.34	4.52	26.37	5.48	5.48	2.52	2.52	1.79	2.76
r (2.8xSr)	2.12	2.03	0.47	0.02	0.77	0.77	0.93	0.93	0.08	0.90
Reproducibility (SR)	2.19	2.10	0.40	0.02	0.27	0.27	1.23	1.24	0.14	1.10
%RSDR	9.65	9.65	10.69	88.64	5.48	5.48	9.33	9.37	8.99	9.52
R (2.8xSR)	6.14	5.87	1.12	0.06	0.77	0.77	3.45	3.46	0.40	3.09

Encapsulated DHA/EPA	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	8	8	7	8	7	7	8	8	7	7
Mean	55.46	53.08	17.79	0.80	13.92	13.38	20.51	20.09	17.72	2.55
Repeatability (Sr)	2.05	1.95	0.23	0.24	0.49	0.32	0.45	0.40	0.14	0.04
%RSDr	3.70	3.68	1.27	30.34	3.55	2.40	2.19	1.97	0.81	1.51
r (2.8xSr)	5.75	5.47	0.63	0.68	1.38	0.90	1.26	1.11	0.40	0.11
Reproducibility (SR)	2.72	2.60	0.74	0.24	0.75	0.67	1.88	2.01	1.60	0.76
%RSDR	4.90	4.90	4.18	30.34	5.37	5.03	9.15	10.02	9.05	29.89
R (2.8xSR)	7.61	7.29	2.08	0.68	2.09	1.88	5.26	5.63	4.49	2.14

Creamy ranch dressing	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	9	9	7	7	9	9	8	7
Mean	46.67	44.63	7.00	0.21	9.47	9.43	26.35	26.18	3.29	23.94
Repeatability (Sr)	0.87	0.84	0.15	0.02	0.20	0.20	0.63	0.63	0.07	0.45
%RSDr	1.87	1.87	2.17	10.74	2.10	2.11	2.38	2.39	2.10	1.88
r (2.8xSr)	2.44	2.34	0.43	0.06	0.56	0.56	1.76	1.75	0.19	1.26
Reproducibility (SR)	1.87	1.78	0.73	0.14	1.10	1.07	2.45	2.47	0.33	0.84
%RSDR	4.00	3.99	10.42	65.73	11.57	11.35	9.29	9.44	10.07	3.52
R (2.8xSR)	5.23	4.99	2.04	0.39	3.07	3.00	6.85	6.92	0.93	2.36

Potato chips	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	8	8	8	8	9	9	9	9
Mean	34.12	32.63	6.11	0.21	13.62	13.52	13.10	12.98	0.13	12.85
Repeatability (Sr)	1.11	1.06	0.19	0.03	0.45	0.45	0.46	0.45	0.01	0.45
%RSDr	3.24	3.25	3.05	14.12	3.31	3.30	3.50	3.50	6.11	3.50
r (2.8xSr)	3.10	2.96	0.52	0.08	1.26	1.25	1.28	1.27	0.02	1.26
Reproducibility (SR)	2.66	2.54	0.50	0.06	1.06	1.04	0.95	0.92	0.03	0.90
%RSDR	7.79	7.79	8.16	29.33	7.79	7.69	7.23	7.13	24.25	7.02
R (2.8xSR)	7.44	7.12	1.40	0.18	2.97	2.91	2.65	2.59	0.09	2.53

Cheese powder duplicate	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	8	8	8	8	7	9	9	9	9	9
Mean	29.29	27.92	13.28	6.67	13.37	6.78	1.19	0.94	0.13	0.81
Repeatability (Sr)	0.61	0.58	0.21	0.17	0.17	0.31	0.07	0.02	0.01	0.02
%RSDr	2.08	2.07	1.57	2.55	1.24	4.61	5.72	2.51	9.08	3.08
r (2.8xSr)	1.70	1.62	0.58	0.48	0.46	0.87	0.19	0.07	0.03	0.07
Reproducibility (SR)	2.73	2.61	1.48	0.87	1.31	0.87	0.19	0.14	0.03	0.11
%RSDR	9.33	9.35	11.12	13.12	9.84	12.82	16.15	14.65	25.82	13.98
R (2.8xSR)	7.65	7.31	4.14	2.45	3.68	2.43	0.54	0.39	0.10	0.32

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Table 2
(Continued)

Frozen cheese pizza	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	8	7	7	7	8	8	8	8
Mean	9.77	9.30	4.90	0.43	2.76	2.38	1.65	1.59	0.06	1.53
Repeatability (Sr)	0.58	0.55	0.29	0.03	0.18	0.15	0.10	0.10	0.00	0.10
%RSDr	5.91	5.91	5.83	7.85	6.57	6.38	6.35	6.24	6.42	6.26
r (2.8xSr)	1.62	1.54	0.80	0.09	0.51	0.43	0.29	0.28	0.01	0.27
Reproducibility (SR)	0.67	0.64	0.43	0.04	0.18	0.16	0.10	0.10	0.02	0.10
%RSDR	6.90	6.86	8.72	9.32	6.57	6.77	6.35	6.24	25.81	6.26
R (2.8xSR)	1.89	1.79	1.20	0.11	0.51	0.45	0.29	0.28	0.05	0.27
Peanut butter duplicate	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	7	7	6	7	6	6	6	6	6	6
Mean	54.08	51.76	10.20	0.06	32.33	32.29	8.66	8.64	0.10	8.46
Repeatability (Sr)	0.79	0.75	0.15	0.01	0.46	0.46	0.07	0.07	0.01	0.07
%RSDr	1.46	1.46	1.46	21.32	1.43	1.42	0.87	0.82	13.12	0.87
r (2.8xSr)	2.21	2.11	0.42	0.04	1.30	1.29	0.21	0.20	0.04	0.20
Reproducibility (SR)	3.14	3.01	0.83	0.04	1.73	1.72	0.63	0.63	0.10	0.51
%RSDR	5.81	5.81	8.12	63.63	5.36	5.33	7.32	7.28	99.62	6.04
R (2.8xSR)	8.80	8.42	2.32	0.11	4.85	4.82	1.77	1.76	0.29	1.43
Dry cereal fortified with flax	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	9	9	7	9	7	7	9	9	9	7
Mean	2.66	2.54	0.55	0.00	0.58	0.58	1.54	1.54	0.46	1.25
Repeatability (Sr)	0.08	0.08	0.01	0.00	0.02	0.01	0.04	0.04	0.01	0.04
%RSDr	3.03	3.04	2.65	0.00	2.70	2.44	2.40	2.40	2.60	2.86
r (2.8xSr)	0.23	0.22	0.04	0.00	0.04	0.04	0.10	0.10	0.03	0.10
Reproducibility (SR)	0.80	0.77	0.05	0.00	0.05	0.05	0.52	0.52	0.19	0.14
%RSDR	30.14	30.15	9.21	0.00	8.80	9.35	33.61	33.69	41.10	10.90
R (2.8xSR)	2.24	2.14	0.14	0.00	0.14	0.15	1.45	1.45	0.53	0.38
Horse feed	Total Fat (TAGS%)*	Total Fat (FA%)	Total Saturated	Total *trans*	Total MUFA	Total *cis* MUFA	Total PUFA**	Total PUFA**	Total n-3 PUFA**	Total n-6 PUFA**
n	8	8	8	9	7	7	8	8	8	8
Mean	3.14	3.00	0.72	0.00	0.75	0.74	1.51	1.51	0.20	1.31
Repeatability (Sr)	0.08	0.08	0.02	0.00	0.02	0.02	0.04	0.04	0.01	0.03
%RSDr	2.67	2.78	2.32	0.00	2.10	2.48	2.47	2.53	3.57	2.40
r (2.8xSr)	0.24	0.23	0.05	0.00	0.04	0.05	0.10	0.11	0.02	0.09
Reproducibility (SR)	0.26	0.25	0.06	0.00	0.05	0.05	0.16	0.16	0.03	0.13
%RSDR	8.27	8.31	7.78	0.00	7.32	7.10	10.67	10.69	15.69	10.06
R (2.8xSR)	0.73	0.70	0.16	0.00	0.15	0.15	0.45	0.45	0.09	0.37

*NLEA (Nutritional Labeling and Education Act) according to the Office of Nutritional Products, Labeling, and Dietary Supplements in the Center for Food Safety and Applied Nutrition at the U.S. Food and Drug Administration
**Does not include CLA

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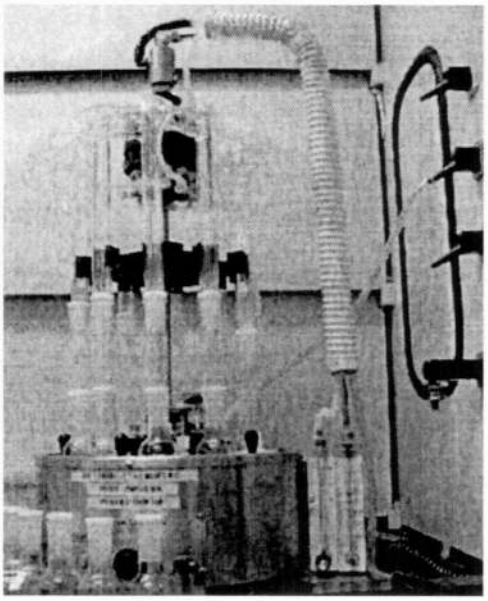

Figure 1. Example of suitable extraction apparatus (see Notes, 1)

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Determination of Tocopherols and Sterols in Soy Sludges and Residues by Gas-Liquid Chromatography

DEFINITION

Samples are saponified and the extracted unsaponifiables are reacted with butyric anhydride. The butyrate esters of tocopherols and sterols in the unsaponifiables are separated and determined quantitatively by gas-liquid chromatography (GLC), using a packed glass column.

SCOPE

The method is applicable to soybean oil deodorizer distillate and distillation residues from hydrolyzed soybean acid soapstock. The method is also applicable to the by-products of other vegetable oils provided they do not contain components eluting in the same place on the gas chromatogram as the internal standard. It is not directly applicable to by-products from rapeseed oil or animal fat.

APPARATUS

1. The gas chromatographic apparatus should have as a minimum the following characteristics—
   (a) Column oven—capable of constant temperature operation to ± 1.0°C at a temperature from 220–250°C. The oven should have a configuration capable of accepting coiled or preferably U-shaped glass columns.
   (b) Sample inlet port—independently heated to a temperature about 50°C higher than the column oven. One end of the column is an integral part of the sample inlet port, permitting sample injection directly on the column.
   (c) Column packing—prepared by first dissolving 0.1 g SE-30 silicone gum rubber in 100 mL hot toluene. This solution is poured into a 500-mL flask containing 10 g of 100–120 mesh Gas Chrom Q, or equivalent. The solvent is stripped with a water aspirator while the mixture is stirred in a 70°C water bath. After the packing becomes free flowing, it is transferred to a Petri dish and heated at 70°C on a hot plate until solvent odor is not detected.
   (d) Columns—183 cm (6 ft) long, 3.5 mm i.d., and made of glass. Prior to packing, the column is rinsed with potassium dichromate sulfuric acid cleaning solution for 5 min. Then 500 mL of distilled water is flushed through the column followed by 200 mL of absolute methyl alcohol. Next, the column is rinsed with 1% dimethyl dichlorosilane in chloroform (see Notes, *Caution*), washed with 100 mL absolute methyl alcohol and dried by aspirating with air. The column is filled with packing aided by a vibrator and the ends are plugged with glass wool previously rinsed with 1% dimethyldichlorosilane in chloroform or washed with absolute methyl alcohol and air dried.
   (e) Detectors—flame-ionization (FID) and others with equal or greater sensitivity. They should be maintained at a temperature 25°C higher than the column temperature.
   (f) Recorder—0 to 1.0, 2.5, or 5.0 mv range, 1 sec full scale deflection with a chart speed of 1.25–2.5 cm per minute.
   (g) Gases—carrier gas, helium of 99.95 mol% min purity is preferred; FID, hydrogen, min purity 99.95 mol%; air, dry, dew point –59°C (–75°F) maximum.
2. Syringe—for injecting sample, 5 µL or 10 µL capacity with known and reproducible volume.

SAMPLE PREPARATION

1. Apparatus—
   (a) Volumetric flasks—500 mL.
   (b) Water-cooled condensers—Liebig or West design, Ʈ24/40 inner joint.
   (c) Separatory funnels—125 mL.
   (d) Volumetric pipets—25 mL.
   (e) Round bottom boiling flasks—50 mL.
   (f) Boiling chips.
2. Reagents—
   (a) Potassium hydroxide (KOH)—160 g dissolved in 100 mL water.
   (b) Pyrogallol—5 g dissolved in 95 mL absolute ethyl alcohol. This solution must be prepared fresh each day.
   (c) Cholesteryl isovalerate—prepared as in *JAOCS 47*:259 (1970).
   (d) Butyric anhydride—distilled using a 20 cm long unpacked Vigreux column. After discarding the first 10%, a 70–80% cut of the remaining material is collected.
   (e) Benzene—analytical reagent grade (see Notes, *Caution*).
   (f) α-Tocopherol—minimum purity 99.5%.

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(g) Stigmasterol—minimum purity 99.5%.
(h) Cholesteryl isovalerate-butyric anhydride reagent—Weigh about 0.30 ± 0.0001 g cholesteryl isovalerate into a 50-mL volumetric flask. Dissolve material in a solvent consisting of 2 parts pyridine (see Notes, *Caution*) and 1 part butyric anhydride and dilute to the mark with this solvent. This solution cannot be used more than 3 d.
(i) Pyridine—analytical reagent grade (see Notes, *Caution*).
(j) Ethyl alcohol—95%, USP (see Notes, *Caution*).
(k) Diethyl ether—reagent grade (see Notes, *Caution*).

3. Extraction and derivatization of unsaponifiables—
   (a) Accurately weigh about 5 ± 0.0001 g of molten sample into a 500-mL volumetric flask. Dissolve and dilute to the mark with benzene. Pipet a 25-mL aliquot into a 50-mL round bottom flask and evaporate the solvent on a steam bath under a stream of nitrogen (requires about ½ hr). Then add 4 mL of pyrogallol solution to the residue and attach a water-cooled condenser to the flask. After heating the solution to reflux, add 1 mL of potassium hydroxide solution and continue refluxing an additional 3 min. Remove the flask and cool under tap water.
   (b) Add 50 mL distilled water to the flask and transfer the solution quantitatively to a 125-mL separatory funnel. Use two 10-mL portions of diethyl ether to rinse the flask for quantitative transfer. Extract the solution in the separatory funnel with the diethyl ether, taking care not to shake too vigorously since emulsions tend to form. Emulsions can be broken by adding 5–10 mL saturated aqueous sodium chloride solution. Extract the solution five more times with 25-mL portions of diethyl ether and combine the extracts. Transfer the combined extracts to a separatory funnel and wash with 20-mL portions of distilled water until the washings are neutral to phenolphthalein indicator (usually requires four to eight washings).
   (c) Concentrate the diethyl ether solution to 25 mL under nitrogen on a steam bath, then transfer quantitatively to a 50-mL round bottom flask. Evaporate the diethyl ether under nitrogen on a steam bath. Then add 10 mL of 5:1, v/v, benzene-ethyl alcohol solution and also evaporate this solvent under nitrogen on a steam bath. If traces of water are still visible in the flask, repeat the addition and evaporation of benzene-ethyl alcohol solution.
   (d) Pipet 4 mL of a solution consisting of 2 parts butyric anhydride and 1 part pyridine into the residue in the flask. Reflux the solution 10 min and then cool under tap water. Inject this sample as in Procedure, 3 before preparing the solution in (e), below. Also, refer to Calculation, 1.
   (e) Pipet 4 mL cholesteryl isovalerate-butyric anhydride reagent into the solution prepared in Sample Preparation, 3 (d) after it has been injected.

PROCEDURE

1. With carrier gas flowing through the apparatus, adjust to operating temperature and record a baseline to check for stability of the instrument. With FID, it is recommended that the apparatus be operated at medium sensitivity rather than near its maximum sensitivity. A new column should be conditioned by holding it about 10°C above operating temperature with helium flowing for at least 24 hr. The column should be disconnected from the detector during the conditioning period.
2. Proper carrier gas flow rate and column temperature should permit elution of all sample components in 25–30 min. Follow the manufacturer's recommendations for FID air and hydrogen flow rate.
3. Analyze the solutions prepared in Sample Preparation, 3, (b) and (c). Measure the sample solution in a syringe. For an instrument equipped with FID, approximately 2–4 µL are injected. Pierce the septum of the sample inlet port and quickly discharge the sample. Withdraw the needle immediately after sample injection and note on the recorder chart where solvent begins eluting, which marks the sample introduction reference point. Sample size must be adjusted so the major peak, other than solvent, is not attenuated more than two times.
4. Watch the recorder pen to see that peaks do not go off scale. Change the setting of the attenuator as necessary to keep the peaks on the chart paper. Mark the attenuator setting on the chart.
5. After all peaks have been traced and the pen has returned to baseline, remove the chart for calculation.

CALCULATIONS

1. Observe the chromatogram of the sample prepared in Sample Preparation, 3, (d). If a peak with a peak height >1% of the chart span elutes in that part of the chromatogram where cholesteryl isovalerate elutes, this method is not directly applicable to the sample.
2. Identify the peaks by determining their relative retention times. Do this first measuring the retention time from the sample introduction reference point to the apex of each peak. Then divide this time by the retention time of the cholesteryl isovalerate internal standard peak. Compounds should elute in the order listed in the table below and within the limit of ± 0.02 relative retention time units, shown in Table 1.
3. Determine the area of each peak. If the instrument is equipped with an electromechanical or electronic integrator, the area is best measured by following the manufacturer's instructions. When an integrator is employed, the baseline must remain constant or the integrator must be equipped with a baseline corrector. Otherwise, the area is obtained by drawing lines tangent to the sides of the peak and intersecting the baseline. Determine the area of the resulting triangle by multiplying the height by one half the base. For an attenuated peak the outer sides of the peak must be full chart span, and the tangents drawn intersecting the baseline should utilize the upper two-thirds to obtain the peak width. Determine the area by multiplying the

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height by one half the base. The height must be corrected for attenuation including the correction required if the baseline is not the recorder zero. The percentage of each component is calculated as follows:

$$\% \text{ tocopherol or sterol} = \frac{A \times W \times 20}{RR \times As \times S} \times 100$$

Where—

A = area of tocopheryl or steryl butyrate peak
As = area of cholesteryl isovalerate peak
W = mass cholesteryl isovalerate in butyric anhydride pyridine reagent, mg
S = sample mass, mg
RR = relative response of tocopheryl or steryl butyrate obtained from calibration curve

4. The instrument is calibrated with weighted mixtures of stigmasterol, α-tocopherol and cholesteryl isovalerate. At least two mixtures should be weighed covering the expected sample composition range. Typical mixtures for a FID equipped instrument are—
   (a) 50 mg α-tocopherol, 70 mg stigmasterol and 50 mL cholesteryl isovalerate-butyric anhydride, or
   (b) 130 mg α-tocopherol, 80 mg stigmasterol and 10 mL cholesteryl isovalerate, butyric anhydride. These solutions are refluxed 10 min and allowed to cool. The solutions are prepared and chromatographed in duplicates each of 3 d. Relative response values (relative to cholesteryl isovalerate) for α-tocopheryl butyrate and stigmasteryl butyrate are calculated as follows:

$$\text{relative response (RR)} = \frac{A \times Ws}{W \times As}$$

Where—

A = area of stigmasteryl or α-tocopheryl butyrate
As = area of cholesteryl isovalerate
W = mass of stigmasterol or α-tocopherol, mg
Ws = mass of cholesteryl isovalerate, mg

A calibration curve is prepared by plotting peak area of α-tocopheryl butyrate and stigmasteryl butyrate vs. relative response. The relative response of β-tocopheryl butyrate and β + γ-tocopheryl butyrate are assumed to be the same as α-tocopheryl butyrate. Similarly, campesteryl butyrate and sitosteryl butyrate are assumed to have the same relative response as stigmasteryl butyrate.

5. Instrument and column performance are monitored by—
   (a) Calculating the theoretical plate number for the stigmasteryl butyrate peak as follows:

$$N = 16\left(\frac{x}{y}\right)^2$$

Where—

N = theoretical plate number
x = peak retention time
y = peak width measured between the baseline intercepts of lines drawn tangent to the peak sides

A theoretical plate number >2000 indicates an acceptable column.

   (b) Calculating the peak separation of the campesteryl butyrate and stigmasteryl butyrate peaks which is expressed as peak resolution.

$$\text{peak resolution} = \frac{2y}{S + O}$$

Where—

y = retention time difference between peak maxima for campesteryl butyrate and stigmasteryl butyrate
S = base width of the campesteryl butyrate peak
O = base width of the stigmasteryl butyrate peak

These values should be determined on a sample containing approximately equal quantities of stigmasteryl butyrate and campesteryl butyrate. The sample size used should produce peak heights about 25–50% of the chart width. A peak resolution of 1.07 or greater indicates an acceptable column and instrument performance. All columns when used will show a gradual loss in peak resolution. When the value becomes less than 1.07, a new column should be installed.

PRECISION

1. Two single determinations performed in one laboratory should not differ by a coefficient of variation more than—
   Total tocopherols, 9.7%
   Total sterols, 14.7%

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2. Two single determinations performed in different laboratories should not differ by a coefficient of variation more than—
   Total tocopherols, 11.0%
   Total sterols, 26.0%

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Benzene is a known carcinogen and is highly toxic. Benzene vapor can be absorbed not only through the lungs by inhalation, but also directly through the skin. It may accumulate in the system as a result of regular exposure to even low concentrations. Avoid contact with the skin. It is flammable. The explosive limits in air are 1.5–8% by volume. The TLV is 10 ppm in air. A fume hood should be used at all times when using benzene.

Pyridine is flammable and a dangerous fire risk. The explosive limits in air are 1.8–12.4%. It is toxic by ingestion and inhalation. The TLV is 5 ppm in air. The danger from crude pyridine is greater than from pure pyridine, the associated homologs and impurities being even more toxic than pyridine itself.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating this solvent.

Diethyl ether is extremely flammable and a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. Store protected from the light. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. Can react explosively when in contact with chlorine, ozone, lithium aluminum hydride or strong oxidizing agents. A fume hood should be used at all times when using ethyl ether. Avoid static electricity.

NUMBERED NOTES

1. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

REFERENCES

J. Am. Oil Chem. Soc. 47:259 (1970).

Table 1

Compound	Relative retention time
δ-Tocopheryl butyrate	0.49
β + γ-Tocopheryl butyrate	0.63
α-Tocopheryl butyrate	7.05
Cholesteryl isovalerate	1.00
Campesteryl butyrate	1.11
Stigmasteryl butyrate	1.20
Sitosteryl butyrate	1.39

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AOCS Official Method Ce 5-86

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Triglycerides by Gas Chromatography

DEFINITION

Triglyceride groups having the same carbon number are separated by direct gas-liquid chromatography of the oil or fat, using a packed column, under temperature-programmed conditions. Based on peak area measurements, the triglyceride content, by carbon number, is expressed as a percentage relative to the total triglyceride content of the sample.

SCOPE

The method is applicable to animal and vegetable oils and fats. Triglycerides having the same carbon number cannot be separated and determined individually, nor can triglycerides having different degrees of unsaturation but an identical carbon number.

APPARATUS

1. The gas chromatograph should have as a minimum the following characteristics—
   (a) Column oven—capable of temperature programming up to at least 350°C.
   (b) Sample inlet system—capable of on-column injection. An all-glass system should be used whenever possible.
   (c) Flame-ionization detector—separately thermostated, which can be maintained at least 25°C above maximum column temperature.
2. Recorder—if the recorder trace is to be used to calculate the composition of the samples analyzed, an electronic recorder of high precision is required. The characteristics of the recorder should be—
   (a) Rate of response—below 1.0 seconds. The rate of response is the time taken for the recording pen to pass from 0 to 90% following the momentary introduction of a 100% signal.
   (b) Width of paper—25 cm (10 in.) minimum.
   (c) Paper speed—25–100 cm/hr (10–40 in./hr).
   (d) Attenuator switch—for range change.
3. Electronic integrator (preferred)—Rapid and accurate calculation can be performed using an electronic integrator or computer. This must give a linear response with adequate sensitivity and baseline correction consistent with good chromatographic practice. Horizontal, non-horizontal, and tangential baseline correction must be controlled by selectable electronic peak logic.
4. Microsyringe—maximum volume 10 µL graduated to 0.1 µL.
5. Chromatographic column—
   (a) Column—should be constructed of glass, 0.5–0.6 m long and 2–4 mm i.d. An equivalent result may be obtained by use of a short capillary column, i.e., 6 m or less.
   (b) Packing—support, acid-washed, and silanized diatomaceous earth of appropriate mesh size for column i.d. Stationary phase, methyl polysiloxane (OV-1) consisting of 3% of the packaging.

REAGENTS

1. Gases—
   (a) Carrier gas—helium (preferred for better resolution) or nitrogen, min purity 99.95 mol%, dried and containing a max of 10 mg O_2/kg.
   (b) Flame-ionization detector (FID)—hydrogen, min purity 99.95 mol% and air, dry [dew point -59°C (-75°F) max], hydrocarbon-free (less than 2 ppm hydrocarbon equivalents to CH_4).
2. Chloroform—analytical reagent grade (see Notes, *Caution* and 2). Di-isopropyl ether is a satisfactory substitute.
3. Triglyceride reference standards—tricaprin, tricaprylin, trilaurin, trimyristin, tripalmitin, tristearin, 99% purity.

PREPARATION OF TRIGLYCERIDE STANDARD SOLUTION

Prepare a solution containing approximately 10 mg/mL each of tricaprin, tricaprylin, trilaurin, trimyristin, tripalmitin, and tristearin according to the type of oil or fat to be analyzed.

CHROMATOGRAPHIC SPECIFICATIONS

1. Condition a new column prior to use by heating it at about 350°C for at least 36 hr with a carrier gas flow rate of about 5 mL/min.
2. GC operating conditions—
   (a) Carrier gas flow depends on carrier gas and packing density. Helium is recommended, but nitrogen may be used. Some loss of resolution between peaks may be experienced with nitrogen. The carrier gas flow through the column should be about 50 mL/min.

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(b) The flow of hydrogen to the flame-ionization detector should be one-half that of the carrier gas, and the flow of air should be about 5–10 times that of the hydrogen.
(c) The injector and detector temperatures should be set at about 350°C.
(d) The oven should be programmed to increase the temperature at a rate of about 4–5°C/min (not to exceed 5°C) from an initial temperature of about 220°C to a max temperature of 350°C. The maximum temperature should be maintained until all triglycerides have eluted from the column.

PROCEDURE

1. Determination of triglyceride correction factors—By means of a microsyringe, inject about 1 µL of the triglyceride standard solution onto the column and program the oven temperature as prescribed in Chromatographic Specifications, 2.
2. Assume that trilaurin is completely recovered from the column and calculate the correction factor, f_i, for each of the remaining triglycerides from:

$$f_i = \frac{C_{si}}{C_L} \times \frac{A_L}{A_{si}}$$

Where—
A_L = peak area for trilaurin
A_{si} = peak area for standard triglyceride, i
C_L = concentration of trilaurin, mg/mL
C_{si} = concentration of standard triglyceride, i, mg/mL

3. Determine f from at least two injections of the standard solution.
Note—Correction factors greater than 1.1 should be considered unsatisfactory.
4. A reduction in the stationary phase loading or an increase in the carrier gas flow rate may achieve acceptable correction factors.
5. Plot a graph of the average values for f of each triglyceride against the corresponding carbon number.
6. Identification graph—Plot the values of the retention time for each standard triglyceride peak against the corresponding carbon number. Normally a straight line will be obtained from which the expected retention times for other triglycerides can be determined.
7. Preparation of test sample solution—Melt the test sample if necessary by warming just above the melting point for short periods. To avoid rearrangement of partial glycerides, use extreme caution in applying heat to test samples. Homogenize the liquid test sample by gently shaking or swirling the container.
8. Prepare a 50 mg/mL solution of the test sample in chloroform or di-isopropyl ether. If the test sample is known to contain significant amounts of mono- or diglycerides or free fatty acids, these should be removed by column chromatography according to IUPAC Method 2.321 before proceeding with the analysis.

EXPRESSION OF RESULTS

1. Determination of the triglyceride group composition is carried out by identifying each peak using the identification graph (Procedure, 6).
2. Calculation of the triglyceride group content—Determine the peak areas of each group of triglycerides. Calculate the corrected peak areas using the correction factors determined either by calculation (Procedure, 1) or by interpolation from the graph of correction factors obtained for the standard triglycerides (Procedure, 1). The quantity of each group of triglycerides having the same carbon number expressed as a percentage relative to the total triglyceride content is given by the formula:

$$\frac{A_{TGi}}{A_T} \times 100$$

Where—
A_{TGi} = corrected peak area of triglyceride group, i
A_T = total corrected peak area of triglyceride group contained in the sample ($A_T = \sum A_{TGi}$).

PRECISION

1. Repeatability—The difference between the results of two determinations carried out on the same day by the same analyst using the same apparatus for the same test material and for triglycerides present in excess of 10% should not exceed a figure of 1% absolute. For triglycerides present at a level of 10% or less, the difference should not exceed 0.5% absolute. Triglycerides present at a level of 5% or less are determined less accurately.

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NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

NUMBERED NOTES

1. This method is identical with the official IUPAC Method 2.323.
2. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350) is less than those of chloroform, carbon tetrachloride and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

REFERENCE

*Pure & Appl. Chem. 57:*1515 (1985).

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AOCS Official Method Ce 5b-89

Revised 2011

Triglycerides in Vegetable Oils by HPLC

DEFINITION

This method is for the separation and quantitative determination of the triglycerides in liquid vegetable oils, in terms of their molecular weight and degree of unsaturation as a function of their equivalent carbon number (ECN), which is sometimes referred to as the partition number, using high-performance liquid chromatography (see Notes, 1, and References, 1).

SCOPE

This method is applicable to all vegetable oils containing triglycerides of long-chain fatty acids. The method is especially applicable to the detection of the presence of small quantities of semidrying oils (rich in linoleic acid) in vegetable oils containing oleic acid as the predominant unsaturated fatty acid, such as olive oil, palm oil, and peanut oil. The triglycerides are separated according to their equivalent carbon number by high-performance liquid chromatography (reversed-phase polarity) and quantitated by interpretation of the chromatograms. Three detector options are given: (a) differential refractometer, (b) UV detector, and (c) mass detector.

APPARATUS

1. High-performance liquid chromatograph—allowing thermostatic control of column temperature.
2. Injection unit—for 10 µL delivery.
3. Detector—differential refractometer, with full-scale sensitivity, which should be at least 10^{-4} unit of refractive index.
4. Column—stainless steel tube 250 mm in length and 4.5 mm i.d., packed with 5 µm diameter particles of silica with 22–23% carbon in the form of octadecyl-dimethylsilyl (see Notes, 2).
5. Recorder and/or integrator.

REAGENTS

All reagents should be of spectral grade quality. Elution solvents should be degassed and may be recycled several times without effect on the separations.

1. Chloroform (see Notes, *Caution,* and 3).
2. Acetone (see Notes, *Caution*).
3. Acetonitrile (see Notes, *Caution*).
4. HPLC mobile phase—acetonitrile + acetone, with proportions to be adjusted to obtain the desired separation. Begin with 50:50 (v/v) mixture (see Procedure, 1). The mobile phase should be degassed before use. It may be recycled several times without effect on the separation of the triglycerides.
5. Solubilization solvent—acetone or acetone-chloroform mixture (1:1, v/v).
6. Reference triglycerides—Use either commercial triglycerides (tripalmitin, triolein, etc.), plotting retention times in accordance with the equivalent carbon number; or, alternatively, use a reference chromatogram obtained from soybean oil (see Notes, 4, 5 and Figs. 1 and 2).
7. Reference triglyceride solution—a 5% solution (w/v) of the samples to be analyzed is prepared by weighing 0.5 ± 0.001 g of the sample into a 10 mL graduated flask and making up to 10 mL with the solubilization solvent (Reagents, 5).

PROCEDURE

1. Optimization of working parameters—Pump the mobile phase (Reagents, 4) through the column at a flow rate of about 1.5 mL/min for at least 30 min or until a stable baseline is obtained. Adjustment of the relative proportions of the solvents in the mobile phase and the flow rate should be made as required in order to achieve the desired separation of the triglycerides according to their ECN (see Fig. 1).
2. Preparation of test sample solution—Prepare a 5% (w/v) solution by transferring a 0.5 ± 0.01 g test sample to a 10 mL graduated flask and making up to volume with the solubilization solvent (Reagents, 5). Inject 10 µL of this solution. Identify the triglyceride peaks (see Procedure, 3). Record the retention times of the solvent and triglyceride peaks; also record the peak area of each triglyceride.
3. Identification of triglyceride peaks—Analyze the reference triglyceride solution (Reagents, 7) by injecting 10 µL of the solution under the same conditions as the test sample solution (Procedure, 2), recording retention times and peak areas as in the case of the chromatographed test sample (Procedure, 2). Identify, where possible, the triglyceride peaks by comparison of retention times of the triglyceride peaks obtained from the chromatography of vegetable oils of known triglyceride composition or reference triglycerides (see Notes, 5 and References, 2). To identify the remaining triglycerides:

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(a) Calculate the relative retention time (α_i) of the triglyceride i with respect to the one of triolein as given by the formula:

$$\alpha_i = \frac{RT'_i}{RT'_{triolein}}$$

Where—
RT'_i and $RT'_{triolein}$ are, respectively, the reduced retention times of the triglyceride i and triolein according to

$$RT'_{i \text{ or triolein}} = RT_{i \text{ or triolein}} - RT_{solvent}$$

(b) Plot log α of the reference triglycerides against their number of double bonds (n). The graph thus obtained allows the relative retention times to be determined for all triglycerides whose component fatty acids are present in the reference triglyceride standards (see Fig. 3).

Following identification of all the triglyceride peaks, calculate the partition number (ECN) (see Notes, 5) of each triglyceride. On the chromatogram, divide the triglycerides into groups according to their partition numbers as shown in the example in Figure 1.

CALCULATIONS

1. Using the internal normalization method, the total sum of the peak areas corresponding to all the chromatographed triglycerides is assumed to be 100%. The relative percentage (Ti) of the triglycerides with the same partition number is then given by the formula:

$$Ti = \frac{\text{area of peaks } i_{ECN}}{\text{sum of peak areas}} \times 100$$

Where—
i_{ECN} are the triglycerides with the same partition number
Express the results to one decimal place.

PROCEDURE USING A UV DETECTOR (see Notes, 6)

1. Preparation of solutions for chromatography— Accurately weigh 40–60 mg (2–3 drops) of melted sample into a small glass container. The glass container is warmed slightly if necessary to melt the test sample. Tetrahydrofuran (see Notes, *Caution*) is added with a pipet to form a solution approximately 5% (w/v) of test portion in tetrahydrofuran. In order to protect the HPLC columns from particulate contamination, the test portion should be passed through a 0.5 µm syringe filter into a glass container (or vial if an autosampler is used).
2. HPLC column—The column system consists of two 150 mm × 4.5 mm i.d. Spherisorb S3ODS-2 (Phase Separations Ltd.) arranged in series. These are packed with 3 µm C_{18} bonded phase particles. Columns must be maintained at a constant temperature of 20 ± 0.05°C (thermostated column oven, direct water cooling, etc.). Phase Separations Ltd. can supply columns tailored for triglyceride analysis. Contact The Director, Phase Separations Ltd., Queensbury, UK.
3. Injection—10 µL filtered sample solution through a 10 µL loop injector, either manual or automatic.
4. Mobile phase—Acetonitrile and tetrahydrofuran, 73/27 volume percent, respectively, prepared by adding appropriate volumes of the HPLC-grade solvents to a flask and degassing by immersing the solvent container in an ultrasonic bath (10 watts). The mobile phase should be continuously degassed during the analysis.
5. Flow rate—1.0 mL/min.
6. Detector—a UV detector capable of measuring at 220 nm (see Fig. 2 and References, 3).

CALCULATIONS

1. Use the internal standardization method, i.e., assume that the sum of the areas of the peaks corresponding to the various triglycerides is equal to 100%. Calculate the relative percentage of each triglyceride to one decimal place, using the formula:

$$\% \text{ triglyceride} = \frac{\text{area of peak}}{\text{sum of all peak areas}} \times 100$$

PROCEDURE USING A MASS DETECTOR (see Notes, 5)

1. Preparation of solutions for chromatography—Solutions of 1% m/v of the oils or fat in 50:50 dichloromethane/acetonitrile are prepared by filtering through a 0.45 µm filter (see Notes, *Caution*).

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2. HPLC column—The column is 150 mm × 4.6 mm i.d., stainless steel, packed with Spherisorb ODS-2 (3 µm particle size, 12% carbon loading), available from Phase Separations Ltd.
3. Injection—20 µL, using a loop injector.
4. Mobile phase—stepped gradient as follows: 70% dichloromethane in acetonitrile over 60 min, with 45% dichloromethane in acetonitrile after 20 min, and 70% dichloromethane in acetonitrile after 60 min.
5. Flow rate—0.6 mL/min.
6. Detector—mass detector.

PRECISION

1. Repeatability—When the mean of the values obtained from two single determinations, carried out in rapid succession by the conditions for the analysis of the same operator, using the same apparatus under the same conditions for the analysis of the same laboratory sample, lies within the range of the mean values cited in the tables below, the difference between the two values obtained should not be greater than the repeatability limit (r), which generally can be deduced by linear interpolation from the values in Tables 1 and 2.
2. Reproducibility—When the values for the final result, obtained by operators in different laboratories using different apparatus under the same conditions for the analysis of the same laboratory sample, lie within the range of mean values cited in the tables below, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility limit (R), which generally can be deduced by linear interpolation from the values in Tables 1 and 2.
3. Results of the interlaboratory tests (see References, 4)—Two interlaboratory tests (in which 18 and 16 laboratories participated, each obtaining two test results for each sample), carried out at an international level in 1986–1987 by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Oils, Fats and Derivatives, gave the statistical results (evaluated in accordance with International Organization for Standardization method ISO-5725) summarized in Tables 1 and 2. Additional RSD_r and RSD_R values are given in Table 3.

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Acetone is a flammable solvent. It should not be used near an open flame. The use of a fume hood is recommended when using this solvent.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Tetrahydrofuran is flammable and a dangerous fire risk. The flammable limits in air are 2–11%. It is toxic by ingestion and inhalation. The TLV in air is 200 ppm.

Dichloromethane (methylene chloride) is toxic and a carcinogen. The TLC is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

NUMBERED NOTES

1. The elution order can be determined by calculating the equivalent carbon numbers, often defined by the relation ECN = CN – $2n$, where CN is the carbon number and n is the number of double bonds. It can be calculated much more precisely by taking into account the origin of the double bond. If n_O, n_L, and n_{Ln} are the numbers of double bonds attributable to oleic, linoleic, and linolenic acids, respectively, the equivalent carbon number can be calculated by means of the formula:

$$ECN = CN - d_O n_O - d_L n_L - d_{Ln} n_{Ln}$$

Where—

the coefficients d_O, d_L, and d_{Ln} can be calculated by means of standard reference triglycerides. Under the conditions specified in this method, the relation obtained will be close to:

$$ECN = CN - [2.60\ n_O] - [2.35\ n_L] - [2.17\ n_{Ln}]$$

2. Examples: Lichrosorb (Merck) RP18 Art 50333; Lichrosphere (Merck) 100 CH18 Art 50377.
3. Usually oils and fats are readily soluble in acetone, with the exception of those containing appreciable amounts of saturated triglycerides, such as high melting fats and hydrogenated oils and blends thereof. In such cases the proportion of chloroform in the solubilization solvent should be increased.

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4. With several reference triglycerides (i) it is also possible to calculate the resolution (α) with respect to triolein:

$$\alpha_i = RT'_i / RT'_{triolein}$$

by use of the reduced retention time

$$RT'_i = RT_i - RT_{solvent}$$

5. The graph of log α against n (number of double bonds) enables the retention values to be determined for all the triglycerides of fatty acids contained in the reference triglycerides (see Fig. 3).
6. The procedures using the UV detector and the mass detector are not part of IUPAC method 2.324.

REFERENCES

1. AOCS Official Method Ce 5b-89 was adopted from *Standard Methods for the Analysis of Oils, Fats and Derivatives*, International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.324.
2. Perrin and Naudet, *Rev. Fr. Corps Gras 30*:279 (1983).
3. Inclusion of UV detector option based on method developed by Dr. V. K. S. Shukla.
4. Collaborative study results published in *Pure Appl. Chem. 63*:1173 (1991).

Table 1
Statistical analysis of results for test sample C (sunflower oil) from the second interlaboratory study.

Triglyceride groups, ECN	40	42	44	46	48	50
Number of participating laboratories	18	18	18	18	18	18
Number of values	36	36	36	36	36	34
Number of accepted laboratories[a]	16	17	16	14	17	16
Number of values	32	34	32	28	34	32
Mean value, % m/m	0.3	29.4	38.4	22.5	8.2	1.3
Repeatability						
Standard deviation (S_r)	0.1	0.5	0.4	0.4	0.9	0.5
Coefficient of variation (RSD_r), %	21.4	1.8	1.2	1.6	10.7	37.0
Repeatability value (r)	0.2	1.5	1.3	1.0	2.5	1.4
Reproducibility						
Standard deviation (S_R)	0.2	0.95	1.3	0.55	1.0	1.2
Coefficient of variation (RSD_R), %	70.4	3.2	3.4	2.4	11.7	92.6
Reproducibility value (R)	0.6	2.7	3.65	1.55	2.7	3.4

[a]Elimination on basis of the Cochran and Dixon tests.

Table 2
Statistical analysis of results for test sample B (olive oil) from the third interlaboratory study.

Triglyceride groups, ECN	42	44	46	48	50
Number of participating laboratories	16	16	16	16	16
Number of values	31	31	31	31	31
Number of accepted laboratories[a]	12	13	14	14	14
Number of values	24	26	28	28	28
Mean value, % m/m	0.6	5.85	21.2	64.7	7.15
Repeatability					
Standard deviation (S_r)	0.09	0.09	0.28	0.52	0.26
Coefficient of variation (RSD_r), %	16.2	1.5	1.3	0.8	3.6
Repeatability value (r)	0.26	0.25	0.78	1.49	0.74
Reproducibility					
Standard deviation (S_R)	0.12	0.18	0.34	1.01	0.61
Coefficient of variation (RSD_R), %	21.1	3.0	1.6	1.6	8.5
Reproducibility value (R)	0.34	0.50	0.95	2.85	1.73

[a]Elimination on basis of the Cochran and Dixon tests.

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Table 3
RSD_r (%) and RSDR values for other oils.

		ECN					
Test sample		40	42	44	46	48	50
Soybean oil	$\bar{x}$, %	8.2	25.5	31.0	21.6	10.7	1.5
	RSD_r, %	3.4	1.8	1.3	3.5	7.3	14.5
	RSD_R, %	6.7	3.5	3.9	4.3	9.5	71.7
Almond oil	$\bar{x}$,%	0.1	2.2	13.0	30.7	50.4	4.4
	RSD_r, %	—	18.8	4.4	1.7	1.6	5.4
	RSD_R, %	—	27.8	6.9	2.3	2.7	9.1
Rapeseed oil	$\bar{x}$,%	3.1	9.8	22.9	28.7	31.2	3.0
	RSD_r, %	5.3	1.8	0.7	1.7	1.7	13.2
	RSD_R, %	8.5	2.3	2.8	2.4	2.5	40.8
Palm oil	$\bar{x}$,%	—	—	3.3	22.1	64.7	9.4
	RSD_r, %	—	—	8.1	2.3	1.2	9.6
	RSD_R, %	—	—	16.2	2.8	2.3	9.6

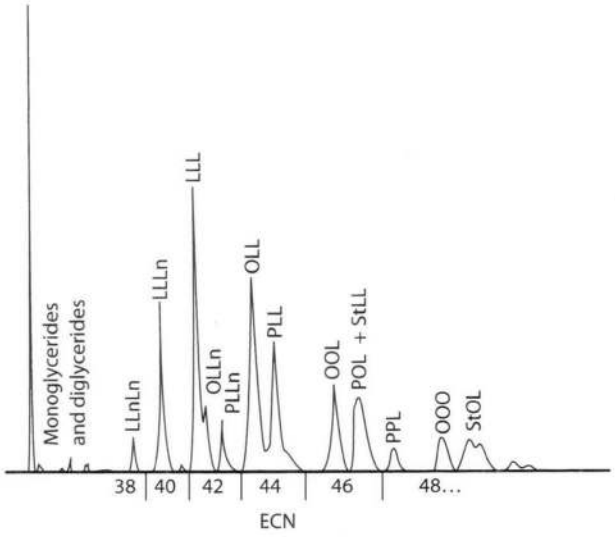

Figure 1. Chromatogram of a soy oil sample. Components of the triglycerides: O, oleic acid; P, palmitic acid; L, linoleic acid; S, stearic acid; Ln, linolenic acid; thus, OOO represents triolein and LLL represents trilinolein.

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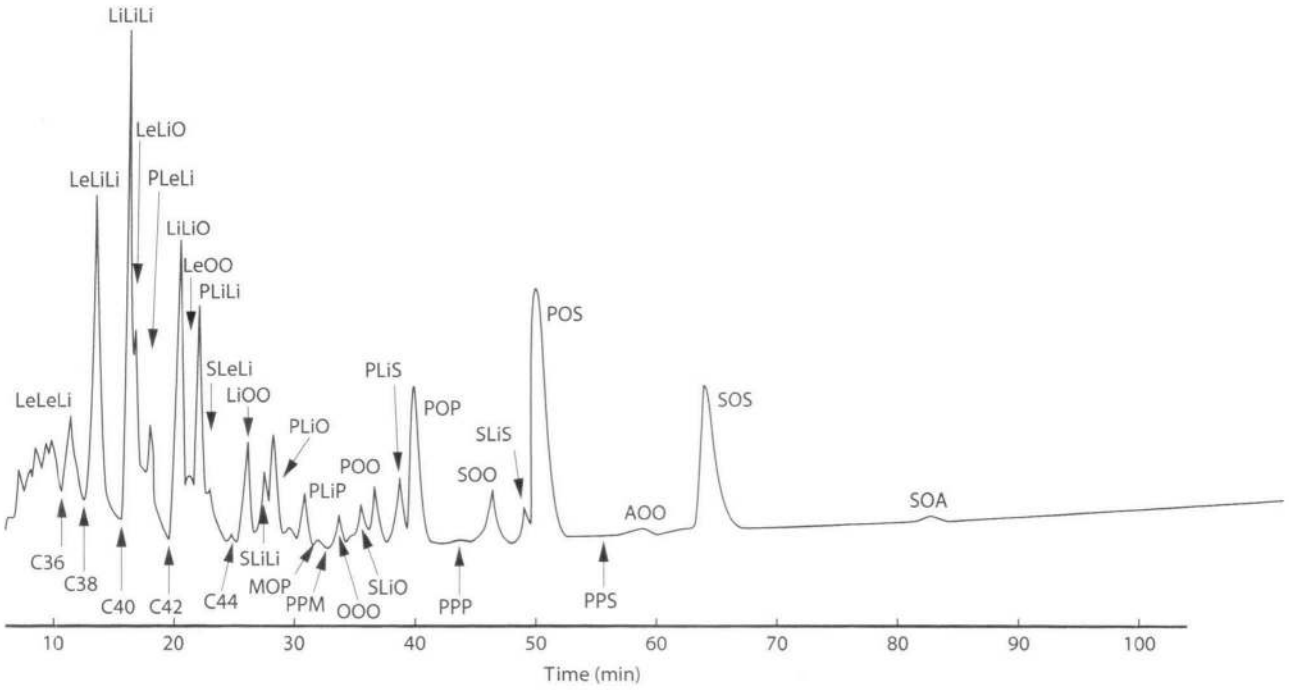

Figure 2. Elution pattern using UV detector method, using two Spherisorb S30DS-2 columns, 150 4.5 mm i.d. coupled in series, with mobile phase consisting of acetonitrile/tetrahydrofuran 68/32; flow rate 0.8 mL/min; injection volume 10 µL of a 5% solution in THF; chart speed 0.25 cm/min. Notation: M, myristic acid C_{14}; P, palmitic acid C_{16}; S, stearic acid C_{18}; O, oleic acid $C_{18:1}$; L, linoleic acid $C^{18:2}$; Ln, linolenic $C_{18:3}$; A, arachidic acid C_{20}.

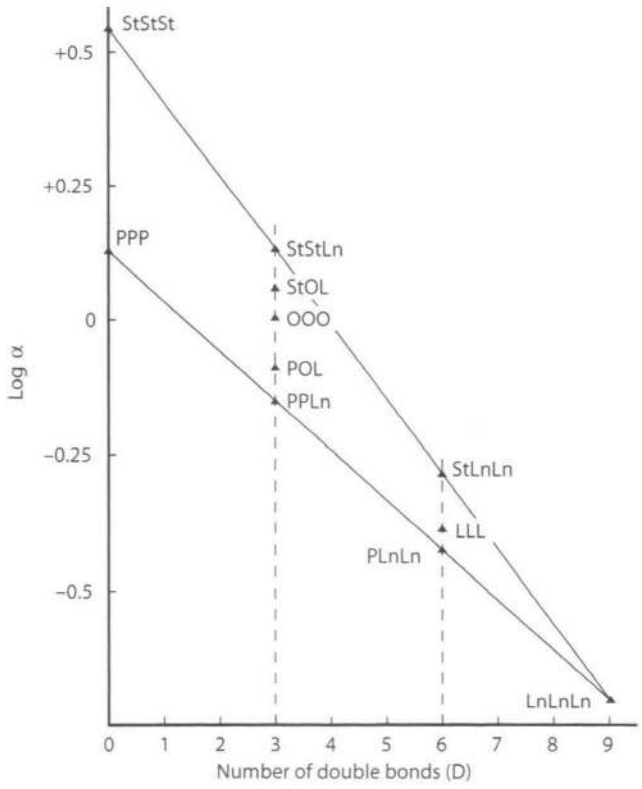

Figure 3. Graph log α = *f* (*n*). For abbreviations see caption of Figure 1.

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AOCS

AOCS Official Method Ce 5c-93

Revised 2011

Individual Triglycerides in Oils and Fats by HPLC

DEFINITION

This method is for the separation, identification, and quantitative determination of individual triglycerides in edible fats and oils using HPLC. The method is complementary to AOCS Official Method Ce 5b-89 (Triglycerides in Vegetable Oils by HPLC). Identification and quantitation are carried out by reference to external standards and the determined fatty acid composition of the test sample.

SCOPE

This method is applicable to vegetable and animal oils and fats which contain only triglycerides of long-chain fatty acids (in particular, with 16–24 carbon atoms; see Notes, 1).

APPARATUS

1. High-performance liquid chromatograph—with thermostatic control of column temperature.
2. Injection unit—for 10 µL delivery.
3. Detector—either a differential refractometer detector, with full-scale sensitivity of at least 10^{-4} unit of refractive index; or mass detector; or UV detector capable of measuring at 220 nm (see Procedure using a UV detector in AOCS Official Method, Ce 5b-89).
4. Column—stainless steel, preferably 250 mm in length and 4.5 mm i.d.; or two columns, each 150 mm in length and 4.5 mm i.d., packed with 5 µm or less diameter particles of silica with 22–23% octadecylsilane coating (see Notes, 2 in AOCS Official Method Ce 5b-89).
5. Recorder and/or integrator.

REAGENTS

Note—All reagents should be of spectral grade quality. Elution solvents should be degassed, and may be recycled several times without effect on the separations.

1. Chloroform (see Notes, *Caution*, and 2).
2. Acetone (see Notes, *Caution*).
3. Acetonitrile (see Notes, *Caution*).
4. Elution solvent—acetonitrile + acetone, with proportions to be adjusted to obtain the desired separation. Begin with 25:75, v/v, acetonitrile/acetone (see Procedure, 1).
5. Solubilization solvent—either acetone alone or acetone/chloroform, 1:1, v/v (see Notes, 2).
6. Reference triglycerides—either commercial triglycerides (tripalmitin, triolein, tristearin, etc.), or alternatively, a reference chromatogram obtained from a vegetable oil of known triglyceride composition, e.g., soybean oil (see Figure 1 in AOCS Official Method Ce 5b-89).

TEST PORTION PREPARATION

1. Prepare a 5% solution of the test samples to be analyzed by weighing 0.5 ± 0.001 g into a 10 mL graduated flask and make up to 10 mL with solubilization solvent (Reagents, 5).

PROCEDURE

1. Set up the chromatographic system. Pump elution solvent (Reagents, 4) at a rate of about 1.5 mL/min for at least 30 min and until a stable baseline is obtained. Adjust the relative proportions of the mobile phase solvents and flow rate in order to achieve satisfactory separation of individual triglycerides (see Figure 1).
2. Inject 10 µL of the test portion solution prepared as in Test Portion Preparation.
3. Analyze the reference triglyceride solution (Reagents, 6) by injecting 10 µL of the solution under the same conditions as the test sample solution, recording retention times and peak areas as in the case of the chromatographed test portion solution.
4. Identify, where possible, the triglyceride peaks by comparison of retention times of the triglyceride peaks obtained from chromatography of vegetable oils of known triglyceride composition or reference triglycerides (see Notes, 3).

CALCULATIONS

1. Use the internal normalization method, i.e., assume that the sum of the areas of the peaks corresponding to the various triglycerides is equal to 100%. Calculate the relative percentage of each triglyceride to one decimal place, using the formula:

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$$\% \text{ Triglyceride} = \frac{\text{area of peak}}{\text{sum of all peak areas}} \times 100$$

2. Verification of triglyceride identification—Determine the fatty acid composition according to either AOCS Official Methods Ce 2-66 and Ce 1-62, IUPAC standard methods 2.301 and 2.302, or IUPAC standard methods 2.303 and 2.304.

   Use a "balance sheet" to verify that the correct identification of the triglycerides has been made. Thus, for a specific fatty acid, compare the sum of the amounts of this fatty acid occurring in the individual test sample triglycerides (expressed as a percentage [m/m]) with the total amount of this fatty acid in the test sample as determined by either AOCS Official Methods Ce 1-62 or Ce 1h-05 or IUPAC Standard Method 2.304. For example, the "balance sheet" applicable to oleic acid is given by the formula:

$$To = T_{0\text{–}tri}$$

   Where—

   To = amount, expressed as a percentage (m/m) of the oleic acid in the sample's fatty acid composition

   $T_{0\text{–}tri}$ = sum of the amounts, expressed as a percentage (m/m), of the oleic acid occurring in the test portion's triglycerides

   Confirmation that all the individual triglycerides have been identified correctly is indicated when the sum of the amounts (expressed as a percentage (m/m)) of each fatty acid present in the test portion triglycerides corresponds, respectively, to the amount (expressed as a percentage (m/m)) of each fatty acid represented in the fatty acid composition of the test portion.

PRECISION

1. Statistics resulting from the collaborative study of the method are shown in Table 1.

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Acetone is highly flammable. It forms explosive peroxides with oxidizing agents. Use an effective fume-removal device. Do not mix with chloroform.

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

NUMBERED NOTES

1. Triglycerides of hydrogenated fats are generally not very well resolved under the working conditions described, resulting very often in broad triglyceride peaks. The presence of hydrogenated fats in blends may thus be indicated in the chromatogram by a more or less pronounced shoulder on the side of a triglyceride peak, indicating the presence of a partially superimposed peak of another triglyceride. The method does not allow separation of all the individual triglycerides in lauric oils (e.g., coconut and palm kernel oils), because of the complex nature of their glyceride composition.
2. Usually oils and fats are readily soluble in acetone, with the exception of those containing appreciable amounts of saturated triglycerides, such as high melting fats and hydrogenated oils and blends thereof. Chloroform should not be used for oils which are acetone soluable. In such cases the proportion of chloroform in the solubilization solvent should be increased.
3. The elution order of the triglycerides can be determined by calculating either their partition numbers or their equivalent carbon numbers (ECN) as described in either AOCS Official Method, Ce 5b-89, Notes, 1; or IUPAC standard method, 2.324, Note 4, paragraph 9 (the procedures are identical).
4. For ease of reference, the abbreviations below are used for those fatty acids that occur in the triglycerides most commonly encountered:

   La, lauric acid
   My, myristic acid
   P, palmitic acid
   S, stearic acid
   Ar, arachidic acid
   Po, palmitoleic acid
   O, oleic acid
   L, linoleic acid
   Ln, linolenic acid

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The triglycerides can then be designated as shown in the following examples:

PPP, tripalmitin
OOO, triolein
POP, palmito-oleopalmitin
POS, palmito-oleostearin
PPO, palmitopalmito-olein
PSO, palmitostearo-olein

REFERENCES

1. This method is identical to IUPAC Standard Method 2.325. *IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives*, 1st Supplement to the 7th Edition, Blackwell Scientific Publications, 1992.
2. Litchfield, C. *Analysis of Triglycerides*, Academic Press, New York, 1972.
3. Pei, P.T.S., R.S. Henly, and S. Ramachandran. *Lipids 10:*152 (1975).
4. Perrin, J.L., and M. Naudet. *Rev. Franc. Corps. Gras 30:*279 (1983).
5. Podlaha, O., and B. Toregard. *J. High Res. Chrom. 5:*553 (1982).

Table 1
Statistical analysis of results for individual triglycerides, based on results received from 13 laboratories[a]

Test Sample	Individual triglycerides			
Soybean Oil	LLnLn	OLL	OOL	OOO
x, %	1.1	17.3	8.7	3.4
RSD_r%	7.9	3.2	3.2	7.1
RSD_R,%	14.1	4.3	3.2	12.8
r	0.25	1.57	0.79	0.69
R	0.45	2.13	0.79	1.26
Palm Oil	PPL	POO	SOO	
x, %	10.8	21.7	2.4	
RSD_r%	2.4	1.0	5.0	
RSD_R%	3.4	4.2	8.6	
r	0.74	0.61	0.33	
R	1.05	2.58	0.58	
Beef Tallow	POO	PPP	SOO	
x, %	20.2	4.1	1.2	
RSD_r%	2.3	8.2	9.1	
RSD_R%	9.2	17.7	17.4	
r	1.33	0.94	0.32	
R	5.24	2.03	0.61	

[a] See IUPAC Commission on Oils, Fats, and Derivatives, Minutes of the Meeting in Montreux, Switzerland, August 13–17, 1990, pp. A6–A12 (L, linoleic acid; Ln, linolenic acid; O, oleic acid; P, palmitic acid; S, stearic acid).

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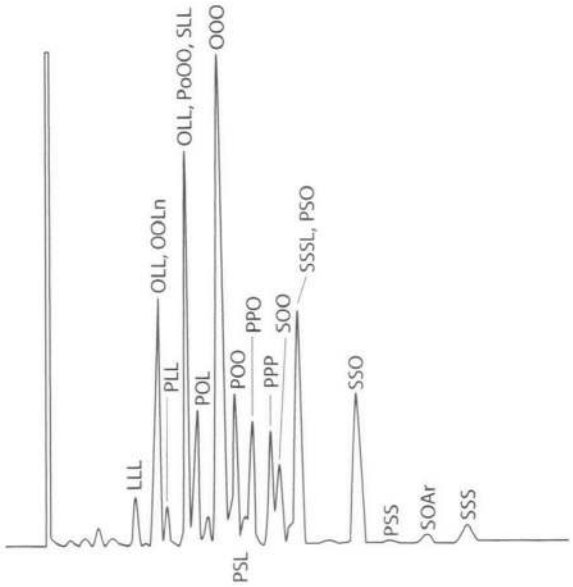

Figure 1. Chromatogram of a blend of vegetable oils spiked with tripalmitin and tristearin. For the abbreviations used for the designation of triglycerides see Notes, 4.

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AOCS

AOCS Official Method Ce 6-86

Reapproved 2009

Antioxidants—Liquid Chromatographic Method

DEFINITION

This method determines propyl gallate (PG), 2,4,5-trihydroxybutyrophenone (THBP), *tert*-butylhydroquinone (TBHQ), nordihydroguaiaraetic acid (NDGA), 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT) in oils, under the conditions of the test (see References, 1).

SCOPE

Applicable to animal and vegetable fats and oils and shortenings. The antioxidants are dissolved in hexane, partitioned into acetonitrile (which is concentrated and diluted with isopropanol to give a 1:1, v/v, isopropanol-acetonitrile solution) and then separated by reverse-phase gradient elution by HPLC and detected at 280 nm.

APPARATUS

1. Gradient liquid chromatograph—equipped with 10 mv strip chart recorder, 20 µL sample loop injection valve, and detector to measure absorbance at 280 nm.
2. Typical operating conditions—detector sensitivity, 0.05 AUFS; time constant, 0; temperature, ambient; flow rate, 2 mL/min.
3. HPLC column—stainless steel, 250 mm length, 4.6 mm i.d., packed with 10 µm LiChrosorb RP-18 (E. Merck, Darmstadt, Germany), or equivalent. Use guard column if desired. Baseline separation of all seven antioxidants should be obtained as shown in Figure 1.
4. Pyrex™ beakers—50 and 150 mL (see Notes, 1).
5. Separators—125 and 250 mL (see Notes, 1).
6. Volumetric flasks—50 and 100 mL (see Notes, 1).
7. Round-bottomed flasks—250 mL (see Notes, 1).
8. Graduated glass cylinders—with ground-glass stoppers, 10 mL.

REAGENTS

1. Solvents—distilled in glass, HPLC grade: acetonitrile, 2-propanol, and hexane (see Notes, *Caution*).
2. HPLC mobile phase, HPLC-grade solvents, or equivalent—
   (a) Distilled H_2O, add 5% acetic acid.
   (b) Acetonitrile, add 5% acetic acid.
   (c) Use linear gradient, from 30% (b) in (a) to 100% (b) over 10 min, followed by 4 min hold at 100% (b) at 2 mL/min.
   (d) For test portion only, increase flow rate to 6 mL/min at 100% (b) for 5 min, or until nonpolar lipids are eluted.
   (e) For test portions and standards, return to 30% (b) over 1 min at 2 mL/min, and let baseline, pressure, and mobile-phase composition stabilize, requiring about 10 min.
   (f) Run blank gradient (no injection).
   (g) Peaks interfering with detection of any antioxidant should not be present. If small peaks that cannot be eliminated are present, all relevant peak heights are to be corrected for interferences.
3. Antioxidants for standards—BHA (mixture of 2- and 3-isomers), BHT, TBHQ, Ionox-100, THBP, and PG (available from Polyscience Corp., Niles, IL, USA); NDGA (Food Chemicals Codex Reference Standard), or equivalent.
4. Standard solutions—Refrigerate all antioxidant solutions out of direct light. Prepare all solutions with 2-propanol + acetonitrile (1:1).
   (a) Stock solution (1 mg/mL)—Accurately weigh and transfer 50 mg of each antioxidant into one 50 mL volumetric flask, dissolve, dilute to volume and mix.
   (b) Standard solution (0.01 mg/mL; 10 µg/mL)—Pipet 1 mL stock solution into a 100 mL volumetric flask, dilute to volume and mix.
5. Extracting solvents—Saturate hexane and acetonitrile by shaking together for 2 min and separate. Unless otherwise specified, use these saturated solvents for the extraction below.

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PROCEDURE

1. Extraction of liquid oils—
   (a) Accurately weigh about 20 g oil into a 50 mL beaker and quantitatively transfer to a 100 mL volumetric flask, rinsing the beakers with hexane. Dilute to volume with hexane and mix.
   (b) Pipet 25 mL aliquot into a 125 mL separator funnel and extract with three 50 mL portions of acetonitrile. If emulsions form, break by holding separator funnel under hot tap water 5–10 sec. Collect extracts in 250 mL separator, and let combined extracts drain slowly into a 250 mL round-bottomed flask to aid removal of hexane-oil droplets.

      Note—At this point, the 150 mL acetonitrile extract may be stored overnight under refrigeration.
   (c) Evaporate acetonitrile extract to 3–4 mL using a flash evaporation with water bath at a temperature of no more than 40°C. Evaporation should be completed in less than 10 min.

      Note—Losses of TBHQ may occur if evaporation time is prolonged. Use an efficient vacuum source and ice-water cooling to decrease evaporation time.
   (d) Using a disposable pipet, transfer acetonitrile-oil droplet mixture to a 10 mL graduate. Rinse flask with small portions of nonsaturated acetonitrile, and transfer rinsings to the graduate with a disposable pipet until 5 mL is collected. Rinse disposable pipet, and continue to rinse flask with small portions of 2-propanol, transferring all rinsings to the graduate until exactly 10 mL is collected. Mix contents of the graduate.

      Note—Avoid delays in analysis after preparing the test sample solution because loss of TBHQ may occur.
2. Extraction of lards or shortenings—
   (a) Accurately weigh 10 g lard or shortening into a 150 mL beaker. Add approximately 30 mL hexane and dissolve sample, heating gently if necessary. Dilute to volume and mix. Pipet 25 mL aliquot into a 125 mL separator.
   (b) Continue extractions as in Procedure, 1, (b).
3. Chromatography—
   (a) Using a sample loop injection valve, inject, in duplicate, 20 µL of the prepared sample solutions onto the column, and solvent program as described in Reagents, 2. Inject 20 µL antioxidant standard solution, Reagents, 4, (b), and solvent program as described, before and after each test portion. For test portion peaks off-scale, quantitatively dilute the sample solution with 2-propanol + acetonitrile (1:1).
   (b) Identify peaks by comparison with retention times of the standard.

      Note—Octyl gallate (available from Pfaltz and Bauer, Inc., Stamford, CT, USA), if present, may coelute with Ionox-100, but can be separated with a H_2O-methanol gradient as follows: 30% (c) (methanol with 5% acetic acid) in (a) (H_2O with 5% acetic acid) to 100% (c) over 10 min. If both Ionox-100 and octyl gallate are present, accurate quantitation may not be possible.
   (c) Carry out reagent blank determination, substituting 25 mL hexane for hexane-oil. Continue extraction as in Procedure, 1, (b). Inject 20 µL reagent blank solution, and solvent program as described. Peaks interfering with determination of any antioxidant should not be present. Using blank gradient chromatogram as a guide to follow baseline, determine average peak height of antioxidant test samples from duplicate injections (corrected for reagent and gradient blanks) and average peak height of antioxidant standard from duplicate injections before and after sample (corrected for gradient blank).

CALCULATIONS

1. Calculate concentrations of antioxidant as follows:

$$\text{Antionxidant, mg/kg} = \frac{R \times C}{R' \times W}$$

Where—

R and R′ = peak heights from samples and standard, respectively
C = concentration of standard in µg/mL
W = mass of sample in g/mL in 10 mL final extract
D = dilution factor if solution injection is diluted

METHOD PERFORMANCE

Average recovery (av rec); S_r, repeatability (within-laboratory) standard deviation; S_R, reproducibility (including repeatability) standard deviation are as follows (as noted in References, 3):

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1. Oils—

PG	av rec at 19–201 mg/kg = 95% (S_R = 3.3–7.6, Sr = 2.2–7.6)
THBP	av rec at 20–201 mg/kg = 97% (S_R = 1.3–6.5, Sr = 1.0–5.2)
TBHQ	av rec at 19–205 mg/kg = 98% (S_R = 2.5–26.0, Sr = 1.5–9.5)
NDGA	av rec at 18–98 mg/kg = 97% (S_R = 0.5–4.9, Sr = 0.3–4.7)
BHA	av rec at 19–207 mg/kg = 99% (S_R = 0.7–7.8, Sr = 0.4–6.5)
Ionox-100	av rec at 20–217 mg/kg = 96% (S_R = 0.7–14.7, Sr = 0.7–12.2)
BHT	av rec at 20–210 mg/kg = 84% (S_R = 0.9–5.2, Sr = 0.9–3.5)

2. Lard—

PG	av rec at 37–101 mg/kg = 91% (S_R = 1.6–4.0, Sr = 1.6–3.2)
THBP	av rec at 39–105 mg/kg = 92% (S_R = 1.4–13.8, Sr = 1.4–7.2)
TBHQ	av rec at 38–102 mg/kg = 92% (S_R = 11.6–25.0, Sr = 6.0–7.7)
NDGA	av rec at 36–98 mg/kg = 93% (S_R = 1.0–5.4, Sr = 1.0–2.6)
BHA	av rec at 38–103 mg/kg = 97% (S_R = 1.9–3.9, Sr = 1.9–2.5)
Ionox-100	av rec at 40–108 mg/kg = 95% (S_R = 1.8–5.9, Sr = 1.2–4.8)
BHT	av rec at 39–105 mg/kg = 86% (S_R = 1.0–5.6, Sr = 1.1–3.4)

NOTES

Caution

Acetonitrile is flammable. There is toxic action by skin absorption and inhalation. The TLV is 40 ppm in air. A fume hood should be used at all times when using acetonitrile.

Acetone, 2-propyl alcohol, and methyl alcohol are flammable solvents. Use a fume hood when heating, evaporating, and working with these solvents.

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

NUMBERED NOTES

1. Rinse all glassware with $CHCl_3$ (see Notes, *Caution*), acetone, and methyl alcohol, in that order, and blow dry with nitrogen.

REFERENCES

1. This method was adopted from *Official Methods of Analysis*, AOAC International, 16th ed., Vol. II, Gaithersburg, MD, 1995, Chapt. 47, p. 2, Method 983.15 and *Standard Methods for the Analysis of Oils, Fats and Derivatives*, International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.642.
2. From Page, B. D., *J. Assoc. Off. Anal. Chem. 66*:729 (1983).
3. Horwitz, W., *Ibid. 67*:432 (1984).

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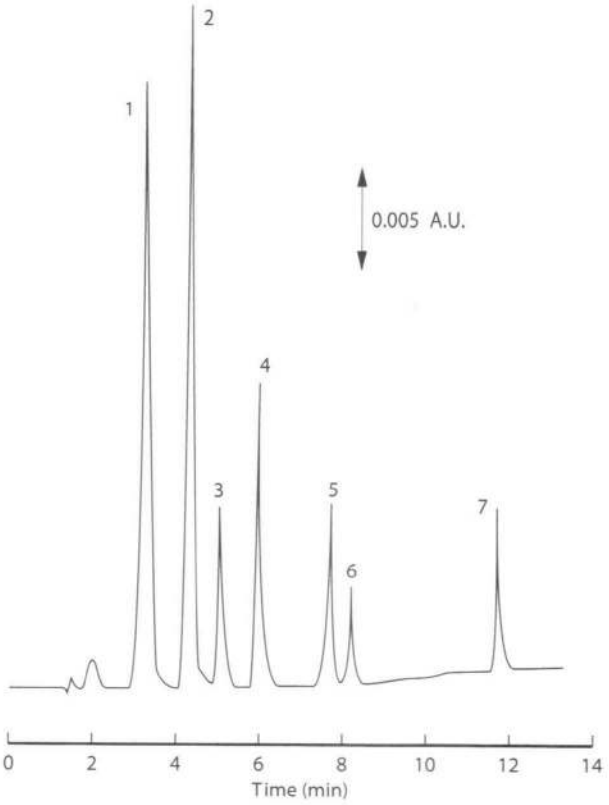

Figure 1. Chromatographic separation of antioxidant standards, about 0.2 µg of each antioxidant. 1, PG; 2, THBP; 3, TBHQ; 4, NDGA; 5, BHA; 6, Ionox-100; 7, BHT. See References, 2.

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AOCS

AOCS Recommended Practice Ce 7-87

Reapproved 2009

Total Tocopherols in Deodorizer Distillate

DEFINITION

This method determines total tocopherols in deodorizer distillate by capillary gas chromatography (see References, 1).

SCOPE

Applicable to deodorizer distillates. This method is based on the ability of silylated tocopherols to be chromatographed by capillary gas chromatography. Materials are silylated using Sylon BFT as the silylating reagent and pyridine as the silylation solvent. Heptadecanyl stearate is the internal standard and α-tocopherol is the primary standard. Response factors for β-, γ-, and δ-tocopherols are taken to be equivalent to that of α-tocopherol (see Notes, 1).

APPARATUS

1. Capillary gas chromatograph—capable of meeting the operating conditions noted in Procedure, 1 (Hewlett-Packard 5880A, or equivalent), equipped with a flame-ionization detector and a 30 m DB-5 capillary column.
2. Electronic balance—sensitive to 0.0001 g; checked daily with standard weights, 20.0 and 50.0 mg.
3. Regular laboratory glassware—consisting of transfer pipets and Erlenmeyer flasks with ground-glass stoppers.

REAGENTS (See Notes, *Caution*)

1. Pyridine—Mallinckrodt Crom AR grade (available from American Scientific Products), or equivalent.
2. Sylon BFT—available from Supelco, or equivalent.
3. Heptadecanyl stearate, internal standard solution—laboratory prepared as follows: Accurately weigh appropriate amount of heptadecanyl stearate and dissolve in chloroform (see Notes, *Caution*) to give a final concentration of approximately 4.0 mg/mL. See Notes, 2 for preparation of heptadecanyl stearate.
4. α-Tocopherol reference standard.
5. Chloroform—reagent grade.

PROCEDURE

1. Capillary gas chromatographic conditions—
   (a) Capillary column, 30 m DB-5, 0.25 μm film thickness, 0.25 mm i.d. (see Notes, 3).
   (b) Oven temperature program, from 140 to 300°C at 10°C/min, hold 6 min; from 300 to 320°C at 5°C/min, hold 10 min.
   (c) Helium carrier gas flow rate, approximately 2 mL/min.
   (d) Split flow rate, 150 mL/min.
   (e) Injector temperature, 240°C.
   (f) Detector temperature, 345°C.
   (g) Plotter chart speed, 1 cm/min.
   (h) Attenuation, 2.
   (i) Threshold, 1.
   (j) Peak width, 0.08.
2. Determination of response factor for α-tocopherol—
   (a) Accurately weigh 20–30 mg of α-tocopherol into a 50 mL Erlenmeyer flask.
   (b) Add 1 mL of pyridine and 2 mL of Sylon BFT. Heat contents to 50°C, with occasional swirling, for approximately 10 min, or until contents are completely dissolved.
   (c) Allow flask to cool slightly. Add 4.00 mL of the internal standard solution (Reagents, 3) and 15 mL of chloroform. Mix well.
   (d) Adjust α-tocopherol standard for purity per AOCS Method Ce 8-89, procedure 1, (b).
   (e) Inject 1 μL aliquot in the gas chromatograph set at previously described conditions (see Procedure, 1). A typical capillary chromatogram is shown in Figure 1.
   (f) Calculate the response factor (FC):

$$FC = \frac{A_{IS} \times C_{Standard}}{A_{Standard} \times C_{IS}}$$

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Where—

A_{IS} = area of heptadecanyl stearate internal standard
$A_{Standard}$ = area of α-tocopherol
$C_{Standard}$ = mg of α-tocopherol
C_{IS} = mg heptadecanyl stearate internal standard (g) Response factors for β-, γ-, and δ-tocopherols are taken to be equivalent to that of α-tocopherol.

Note—These factors are determined each day. New solutions should be prepared each week and should be refrigerated when not in use.

3. Preparation of test sample—
   (a) Warm the material and shake well. Accurately weigh 180–250 mg of test portion into a 50 mL Erlenmeyer flask.
   (b) Add 1.0 mL of pyridine followed by approximately 2 mL of Sylon BFT. Heat the contents to 50°C for 5–10 min, or until the test portion has completely dissolved.
   (c) Add 4.00 mL of the internal standard solution (Reagents, 3) and 15 mL of chloroform. Mix well.
   (d) Inject 1 µL aliquot in the gas chromatograph set at previously described conditions (see Procedure, 1).

CALCULATIONS

1. Total tocopherol content of a sample is expressed as the total weight percent of α-, β-, γ-, and δ-tocopherols:

$$\% X = \frac{A_X \times FC \times C_{IS}}{A_{IS} \times S} \times 100$$

Where—

% X = weight percent α-, β-, γ-, or δ-tocopherol
A_X = area of α-, β-, γ- or δ-tocopherol
FC = response factor from Procedure, 2, (e)
C_{IS} = mg of heptadecanyl stearate
S = sample weight (mg)

2. Total tocopherol % = % α- + % β- + % γ- + % δ-tocopherol

CALIBRATION

1. Preparation and calculation of response factors [Procedure, 2, (e)] is sufficient for calibration.
2. A secondary standard containing a known concentration of total tocopherols is desirable for additional calibration.

PRECISION

1. Accuracy—The accuracy of this assay has been measured by spiking known amounts of α-tocopherol, ranging from 12 to 72 mg/g, into six deodorizer distillate test samples. Average recovery was 99.2% of the spiked amount. Accuracy of β-, γ-, and δ-tocopherol was not determined.
2. Precision—The precision of this assay has been measured by dividing one test sample into ten vials; test one vial was assayed per day for a total of ten assays. The assays were performed by one operator using one instrument. The calculated total tocopherols had a coefficient of variation of ± 1.1%.
3. See Notes, 1 for potential sources of error.
4. Collaborative study statistics are summarized in Table 1.

NOTES

Caution

Sylon BFT is a powerful silylating reagent, composed of mixing 1 part trimethyl chlorosilane with 99 parts of bistrimethylsilyltrifluoroacetamide, and should be used only in a properly operating fume hood. This reagent is highly flammable.

Pyridine is flammable and a dangerous fire risk. The explosive limits in air are 1.8–12.4%. It is toxic by ingestion and inhalation. The TLV is 5 ppm in air. The danger from crude pyridine is greater than from pure pyridine, the associated homologs and impurities being even more toxic than pyridine itself.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Gloves and proper ventilation should be used at all times when working with pyridine, Sylon BFT, and chloroform.

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NUMBERED NOTES

1. Sources of error—Failure to warm and thoroughly mix test portions will give erratic results. Failure to completely silylate tocopherols will give low results. Improper storage of test samples may result in oxidation of the tocopherols, which will yield low results.
2. Preparation of heptadecanyl stearate (HDS)—
   (a) Weigh 50 g heptadecanol (Eastman Chemicals no.10411, or equivalent) into a 1 L beaker and allow to solidify. Add 30 g stearoyl chloride (98+% pure, Eastman Chemicals no. P2227, or equivalent). Heat in a hood on a steam bath for 2 hr.

   Caution—As the reactants melt and reaction proceeds, hydrogen chloride is liberated vigorously.

   (b) After bubbling subsides, stir reaction mixture occasionally with a glass rod. Dissolve crude product in 800 mL alcohol (heat will be necessary). Allow to crystallize at room temperature for at least 2 hr. Separate crystals on a Buchner funnel. Repeat the dissolution in alcohol, performing crystallization and filtration four times. On the final filtration, allow product to dry on the Buchner funnel for 1–2 hr. Transfer product to a beaker and melt on a 150°C hot plate until a constant weight is obtained. The HDS is ready for use.
3. Laboratories having only packed-column gas chromatographs may want to try using a wide-bore capillary column; however, a wide-bore column may not resolve β-, δ-, and γ-tocopherols. The resolution of these tocopherol isomers on a wide-bore column should be checked using β-, δ-, and γ-tocopherol standards.

REFERENCES

1. This method was developed at the Distillation Products Division of Eastman Kodak, Rochester, NY. The method was studied collaboratively during 1985 and 1986, and is published in *J. Am. Oil Chem. Soc. 65:*1936 (1988).
2. Collaborative study results appear in *Ibid. 65:*881 (1988).

Table 1
Collaborative study statistics[a].

Concn., mg/g	α	7.3	6.8	21.3	19.3	6.8	7.0	6.9	7.0
	β	0.5	0.5	2.5	2.3	1.1	1.3	0.8	0.5
	γ	31.1	30.5	72.5	65.3	54.6	56.4	52.1	52.6
	δ	14.5	14.5	35.8	34.5	25.6	25.9	20.9	20.8
	Total	53.4	52.3	132.0	121.3	88.4	90.5	80.8	81.0
RSD_r	α	0	7.4	2.4	2.6	0	0	5.1	7.1
	β	0	0	20.0	0	70.3	69.3	116	100
	γ	1.1	2.5	1.5	1.1	0.9	2.6	1.1	1.2
	δ	3.4	3.4	2.4	1.4	1.4	2.4	1.7	0
	Total	1.1	2.5	1.5	1.1	0.9	2.6	1.1	1.2
RSD_R	α	6.9	16.3	14.5	27.9	18.6	0	12.9	7.7
	β	116	116	316.6	22.2	70.3	71.2	116	108
	γ	4.3	12.1	15.9	30.4	18.2	8.4	10.6	8.9
	δ	3.7	3.7	6.1	5.3	6.3	6.8	6.4	9.1
	Total	3.0	8.6	12.3	22.1	14.3	6.2	8.8	7.9

[a]See References, 2.

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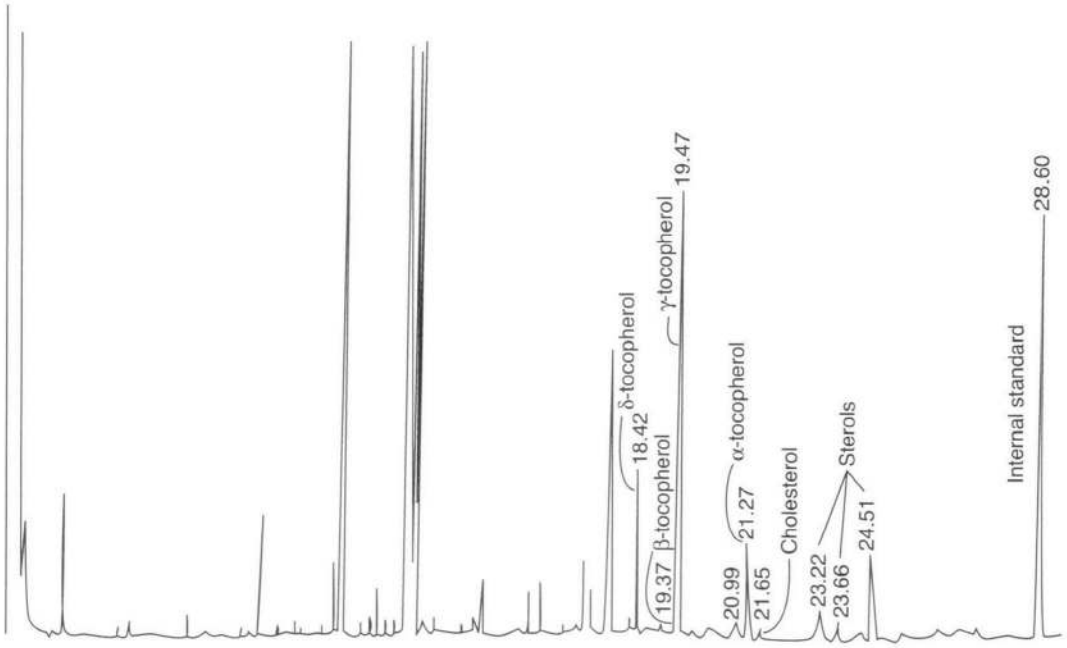

Figure 1. Typical capillary chromatogram of a deodorizer distillate containing cholesterol and internal standard.

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AOCS

AOCS Official Method Ce 8-89

Revised 2013

Determination of Tocopherols and Tocotrienols in Vegetable Oils and Fats by HPLC

DEFINITION

This method describes a procedure for the determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC. The procedure is not directly applicable to processed products, such as margarines containing tocopherol esters, but it may be used to determine tocopherols in the unsaponifiable matter obtained from such products (see Notes, 1, 2 and References, 1).

SCOPE

The oil or fat (or the unsaponifiable matter obtained from a processed product containing tocopherol esters) is dissolved in an organic solvent and subjected to direct high-performance liquid chromatographic (HPLC) separation of the individual tocopherols and tocotrienols. Calibration factors are determined for each tocopherol from the chromatography of solutions of standard tocopherols; calibration factors for tocotrienols are taken to be equivalent to that of the corresponding tocopherols. The tocopherol and tocotrienol content of an oil or fat is the quantity of tocopherols and tocotrienols, determined in the sample by the described procedure and expressed in micrograms per gram (μg/g).

APPARATUS

Note—All glassware should be of low actinic activity, or procedure should be performed under yellow lights.

1. HPLC system—consisting of a high-pressure pump, sample injection device, detector, and chart recorder, or recording integrator. A fluorescence detector should be used, with the excitation wavelength set at 290 nm and emission wavelength at 330 nm. A UV detector may be used if a fluorescence detector is not available. The wavelength of the UV detector should be set at 292 nm.
2. HPLC analytical column—250 × 4 mm, packed with microparticulate silica having a mean particle size of about 5 μm (see Notes, 3).
3. UV spectrometer—capable of absolute measurement of absorbance at precisely defined wavelengths.
4. Rotary film evaporator.

REAGENTS

Note—All solvents should be of HPLC grade, or equivalent. For additional reagents required when prepared fats containing tocopherol esters are being analyzed, see Reagents section in Notes, 2.

1. α-, β-, γ-, and δ-Tocopherol standards (see Notes, 4).
2. Methanol (see Notes, *Caution*).
3. Dichloromethane.
4. Hexane (see Notes, *Caution*).
5. Isopropanol.
6. HPLC mobile phase—isopropanol in hexane (0.5:99.5, v/v).

SAMPLING

1. It is essential that the laboratory sample used for preparing the test sample is taken according to a recognized sampling procedure [such as that described in International Organization for Standardization (ISO) 5555], and that the laboratory sample was subsequently protected from the effects of heat and light.

PROCEDURE

1. Preparation of solutions of tocopherol standards—
   (a) α-Tocopherol standard stock solution—Prepare a stock solution of α-tocopherol by accurately weighing about 10 mg of the standard (Reagents, 1) into a 100 mL volumetric flask, and making up to volume with hexane (Reagents, 4). Pipet 10 mL of this solution into an amber glass round-bottom flask, and remove all hexane on a rotary evaporator (Apparatus, 4) at a temperature not higher than 40°C. Restore atmospheric pressure with nitrogen, and remove the flask from the evaporator as soon as all the solvent has been removed. Pipet into the flask 10 mL of methanol (Reagents, 2),

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and swirl to dissolve the tocopherol. Measure the absorbance of this solution at 292 nm, and calculate the concentration (as μg/mL α-tocopherol) by dividing the absorbance value by 0.0076 (see Notes, 5).

(b) β-, γ-, and δ-Tocopherol standards stock solutions—Prepare similar stock solutions and aliquots for UV spectrometry of β-, γ-, and δ-tocopherol standards (Reagents, 1) as described for the α-tocopherol standard stock solution. Measure the absorbance of each of these solutions at the following wavelengths, and use the corresponding divisor factors (see Notes, 5) for calculation of concentration:

296 nm β-tocopherol = 0.0089
298 nm γ-tocopherol = 0.0091
298 nm δ-tocopherol = 0.0087

(c) Mixed tocopherol standards working solution—Mix appropriate volumes of the stock solutions of the tocopherol standards to obtain a mixed tocopherol standards working solution, and dilute with hexane to give a solution containing between 1 and 5 μg/mL of each tocopherol.

Note—A more concentrated solution may have to be prepared if a UV detector is used. It is important that all standards are protected from light and are stored refrigerated (see Notes, 6).

2. Optimization of working parameters—Condition the column (Apparatus, 2), if necessary (see Notes, 7). Pump the isopropanol/hexane mobile phase (Reagents, 6) through the column at a flow-rate of 1 mL/min for at least 30 min. Inject about 20 μL of the mixed tocopherol standards working solution onto the column and, if necessary, adjust the isopropanol content of the mobile phase and the flow-rate (see Notes, 8) to achieve the following conditions:
   (a) α-tocopherol retention time not less than 5 mins;
   (b) resolution factor (R) for the separation of β- and γ- tocopherol not less than 1.0, i.e., an almost baseline separation (see Notes, 9).

Select the optimum settings for detector and integrator sensitivity and chart speed. Inject about 20 μL of the mixed tocopherol standards working solution. Repeat the injection, and check that reproducible chromatograms are obtained.

3. Preparation of the test sample—The test sample, in the case of liquid laboratory samples, should be prepared by homogenization, except that filtration should be avoided. In the case of solid samples, transfer a representative portion (i.e. not less than 10% by weight of the laboratory sample) to a glass beaker and carefully homogenize by melting, with gentle mixing, in a water bath at a temperature not exceeding 40 C. The preparation of the test sample should be carried out, as far as is practicable, in subdued light and, in any case, out of direct sunlight.
4. Preparation of the test solution—Weigh accurately about 2 g of the prepared test sample (see Notes, 10) into a 25 mL volumetric flask. Add a quantity of hexane (Reagents, 4), swirling to dissolve the sample, and make up to volume with the same solvent. If a fluorescent detector is used it may be necessary to make further dilution of this solution prior to chromatography. It is important that the test solutions are protected from light prior to analysis and analyzed on the day of preparation.
5. HPLC determination of tocopherols in the test solution—Inject 20 μL of the mixed tocopherol standards working solution onto the column and record the areas of the tocopherol peaks. If an integrator is not available, record peak heights (measured in mm). Inject 20 μL of the test solution onto the column and identify the tocopherols (and tocotrienols) present by reference to the chromatograms obtained from standards (see Notes, 11). Record the areas of the tocopherol peaks (or peak heights). Record the areas of any tocotrienol peaks, if these are present and are to be quantified. Duplicate injections should be made. Inject a further 20 μL of the mixed tocopherol standards working solution and record the areas of the tocopherol peaks.
6. Number of determinations—Carry out two determinations (each consisting of duplicate injections of the prepared test solutions) in rapid succession, using a fresh test portion for each determination.

CALCULATIONS

1. The α-tocopherol content of the sample in μg/g is given by:

$$\frac{C \times a \times D \times 25}{A \times m} \times 100$$

Where—

C = concentration of the α-tocopherol standard (μg/mL)
A = mean of the peak areas obtained for the α-tocopherol standard
a = mean of the peak areas obtained for the α-tocopherol in test portion
m = mass of test portion taken
D = dilution factor, e.g., for a test solution prepared from a 1:10 dilution of a 25 mL solution of the test portion this factor would be 10

2. The β-, γ-, and δ-tocopherol contents of the test portion are calculated in the same way, using the data from chromatography of the corresponding tocopherol standard (see Notes, 12).

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3. The tocotrienol content of a test portion can be estimated using the C and A values for the corresponding tocopherol (see Notes, 13).
4. Report as the final result the mean of the values obtained from the two determinations, provided the requirements noted in Precision, 2 are met. If the requirements for repeatability are not met, carry out a further two determinations on the test portion. If the range ($x_{max} - x_{min}$) of the four values obtained is <1.3× r (where r = the repeatability value; see Table 1), report as the final result the mean of the four values; otherwise report as the final result the median of the four values, i.e. the mean of the two intermediate values. Report the results for each tocopherol and tocotrienol to the nearest µg.

PRECISION

1. The results of an interlaboratory study organized at the international level gave the statistical results which are summarized in Table 1.
2. Repeatability—When the mean of the values obtained from two single determinations, carried out in rapid succession by the same operator using the same apparatus under the same conditions for the analysis of the same test portion, lies within the range of the mean values cited in Table 1, the difference between the two values obtained should not be greater than the repeatability value (r), which can generally be deduced by linear interpolation from Table 1.
3. Reproducibility—When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions from the analysis of the same laboratory sample, lie within the range of mean values cited in Table 1, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility value (R) (see Notes, 14), which can generally be deduced by linear interpolation from Table 1.

NOTES

Caution

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Ethanol and methanol are flammable. Use effective fume-removal device when heating or evaporating. Methanol is toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform, and perchloric acid.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

NUMBERED NOTES

1. It is recommended that the unsaponifiable matter is obtained by a method involving a cold saponification procedure such as that described in Notes, 2. Particular attention must be paid to saponification temperature and time, otherwise low recoveries of tocopherols from tocopherol esters may be obtained.
2. Cold saponification procedure for samples containing added tocopherol esters—

   Reagents

   (a) Ethanol—approximately 96% pure (see Notes, *Caution*).
   (b) Ethanol—absolute, 99% pure (see Notes, *Caution*).
   (c) Pyrogallol.
   (d) Potassium hydroxide solution—aqueous, 60% (m/m).
   (e) Diethyl ether—peroxide-free, containing 0.1% (m/m) pyrogallol (see Notes, *Caution*).
   (f) Hydrochloric acid—0.01 mol/L.
   (g) Sodium sulphate—anhydrous.

   Procedure

   (a) Weigh accurately about 2 g of the prepared laboratory sample (see Sampling section) into a 100 mL flat-bottom flask, and thoroughly disperse the molten test portion in approximately 8 mL of ethanol [Notes, 2, Reagents, (a)] by gentle swirling. Add 100 mg of pyrogallol [Notes, 2, Reagents, (c)] and swirl to dissolve. Purge the flask with nitrogen, add 4 mL of potassium hydroxide solution [Notes, 2, Reagents (d)], repurge the flask with nitrogen, and close with a glass stopper. Place the flask in a 26°C water bath and shake vigorously for 10 min. All operations must be performed in the absence of direct sunlight; use amber glassware or shield with aluminum foil.
   (b) Add 50 mL of deionized water to the flask and transfer contents quantitatively to a 250 mL separating funnel. Wash the flask with 50 mL of diethyl ether [Notes, 2, Reagents, (e)] and transfer the washings to the funnel. Shake the separator vigorously for 1 min, releasing the pressure occasionally. Allow the layers to separate, and draw off the lower aqueous layer. Extract the aqueous layer an additional four times with 30 mL aliquots of diethyl ether and combine the ether extracts.
   (c) Wash the combined diethyl ether extracts with 50 mL of water (shaking carefully to avoid emulsion formation) and then with 30 mL of dilute hydrochloric acid [Notes, 2, Reagents, (f)]. Add about 3 g of anhydrous sodium sulfate [Notes, 2,

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Reagents, (g)] with gentle mixing to absorb water. Filter the ether extracts through a phase-separating paper, and collect the filtrate in a round-bottom amber rotary evaporator flask.

(d) Remove the ether under reduced pressure, using a rotary film evaporator, at a temperature of not more than 40°C. If a liquid residue remains in the flask, add ethanol [see Notes, 2, Reagents, (b)] and reevaporate to dryness. Wash the sides of the flask with hexane (Reagents, 5), transfer the contents quantitatively to a 50 mL volumetric flask and make up to volume. Make a suitable dilution of the prepared test solution (as described in Procedure, 4 in the main method), and proceed to Procedure, 5 (main method).

3. Suitable silica column packing materials are 5 µm LiChrosorb SI 60 or Spherisorb S5W.
4. β-, γ-, and δ-Tocopherol standards can be obtained from Merck; α-tocopherol can be obtained from various suppliers. It has been reported that the purity of some commercially available tocopherol standards may vary between 85 and 100% (see References, 2). This confirms the importance of determining the concentration of prepared standard solutions by UV spectrometry.
5. The divisor factors quoted for the tocopherols are derived from their E values (1%/1 cm) quoted in References, 3. For example the E value (1%/1 cm) of α-tocopherol is 76 at 292 nm (in methanol); therefore a 1 µg/mL solution of α-tocopherol will have an absorbance of 0.0076 at 292 nm.
6. Stock standard solutions can be satisfactorily stored in amber low-actinic glassware for up to a week if refrigerated. Working standard solutions must be prepared each working day. Protection from light is of the utmost importance.
7. If the HPLC column is new or of unknown history, wash and condition for about 10 min with methanol, then dichloromethane, followed by hexane at a flow rate of about 1 mL/min.
8. Mobile-phase flow rates in the range 0.7–1.5 mL/min have been found to be satisfactory. Higher flow-rates can result in poor chromatography, which must be avoided when UV detection is used.
9. The resolution factor (R) is calculated from:

$$R = \frac{(Rd_1) - (Rd_2)}{0.5\,(W_1 + W_2)} \times 100$$

Where—

Rd_1 = retention distance of γ-tocopherol
Rd_2 = retention distance of β-tocopherol
W_1 = width at base of γ-tocopherol peak
W_2 = width at base of β-tocopherol peak

It should be possible to achieve an efficiency of 10,000 plates/meter calculated on the δ-tocopherol peak. The efficiency (n), in plates/meter, may be calculated from:

$$n = 5.54 \left(\frac{Rd_3}{W_h}\right)^2$$

Where—

Rd_3 = retention distance of δ-tocopherol
W_h = peak width at half height

10. When analyzing processed products such as margarines, and materials containing added tocopherol esters, a cold saponification procedure must be performed prior to chromatography.

Note—When materials contain tocopherol esters, parallel samples spiked with known amounts of α-tocopherol acetate should be analyzed to enable a check to be made on the recovery of tocopherols from tocopherol esters. The saponification procedure is described in Notes, 2.

11. If any tocopherol standards are not available, a blend of wheat germ and soybean oil can be used to obtain chromatograms that contain α-, γ-, and δ-tocopherols. These can be used to assist peak identification in test sample chromatograms. Palm oil can be used to identify α- and γ-tocotrienols, if required. The following relative retention times have been found to be typical:

α-tocopherol = 1.0
β-tocopherol = 1.6
δ-tocopherol = 3.0
γ-tocopherol = 1.7

See Figure 1, showing the elution order of tocopherols and tocotrienols.

12. If fluorescence detection is used and the only standard available is α-tocopherol, relate all tocopherols to the α-tocopherol standard, but make this clear when reporting results. If UV detection is used, again relate all tocopherols to the α-tocopherol standard, but normalize the peak areas to α-tocopherol using the divisor values noted in the procedure for preparing standard stock solutions.
13. According to the literature, the fluorescence intensity of tocotrienols is the same as the corresponding tocopherol (References, 4), and the UV absorbencies are similar (References, 3).

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14. It should be noted that the reproducibility values (R) cited in Table 1 apply in the particular case when the results of single determinations obtained by two laboratories are being compared. If, when following the method described, it is desired to compare the final results (which have been derived from the means of duplicate determinations) obtained by two laboratories, the values for (R) should be converted to 95% probability critical difference values (CrD_{95}), applicable to the means of two determinations, using the formula:

$$CrD_{95} = V \times \frac{R^2 - r^2}{2}$$

REFERENCES

1. Adopted by the AOCS in 1989 from the International Union of Pure and Applied Chemistry (IUPAC) collaborative study and IUPAC Standard Method 2.432 as published in *Pure Appl. Chem. 60:*877 (1988).
2. Ramnell, C. G., and J. J. L. Hoogenboom, *J. Liq. Chromatogr. 8:*707 (1985).
3. Horwitz, W., *Anal. Chem. 54:*67A (1982).
4. Schudel, P., H. Mayer, and O. Isler, in *The Vitamins*, Vol. V, 2nd edn., edited by W. H. Sebrell and R. H. Harris, Academic Press, New York, 1972, p. 1658.

OTHER REFERENCES

Arens, M., S. Kroll, and H. Muller, *Fette Seifen Anstrichm. 86:*148 (1984).
Thompson, J. N., and G. Hatina, *J. Liq. Chromatogr. 2:*327 (1979).

Table 1
Statistical analysis of results for tocopherols and tocotrienols[a].

		Tocopherols			
		α–	β–	γ–	δ–
Soybean oil	no. of labs	17	16	17	16
	x, μg/g	69	17	508	263
	RSD_r, %	5	5	3	2
	RSD_R, %	25	317	7	1
Soybean oil-wheat germ oil (1 + 1)	no. of labs	15	16	16	14
	x, μg/g	69	217	225	130
	RSD_r, %	6	3	5	5
	RSD_R, %	19	26	24	20
Soybean oil-corn oil (1 + 1)	no. of labs	17	17	17	16
	x, μg/g	124	12	603	134
	RSD_r, %	6	7	3	3
	RSD_R, %	24	19	10	14
Margarine	no. of labs	10	—	10	10
	x, μg/g	9	—	17	67
	RSD_r, %	19	—	8	3
	RSD_R, %	23	—	20	10
		Tocotrienols			
		α–	β–	γ–	δ–
Refined palm oil	no. of labs	16	—	16	15
	x, μg/g	203	—	213	45
	RSD_r, %	7	—	6	8
	RSD_R, %	23	—	14	39

[a]IUPAC collaborative studies, 1985–1986 and 1986–1987 (References, 1). Statistical analysis according to ISO 5725–1986.

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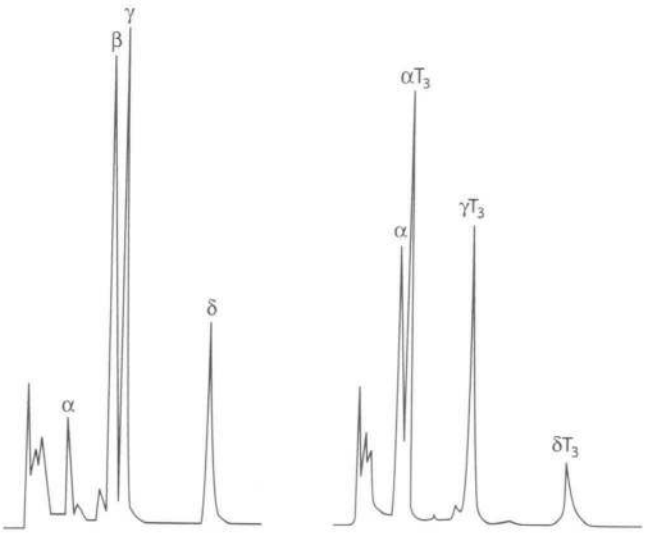

Figure 1. Chromatogram showing the elution order of tocopherols and tocotrienols (References, 6).

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AOCS Recommended Practice Ce 9-01
Reapproved 2009

Determination of β-Carotene in Margarine

DEFINITION

This Recommended Practice describes a procedure for the determination of β-carotene in margarine by high-performance liquid chromatography (HPLC).

SCOPE

The margarine is saponified and then extracted with *tert*-butyl methyl ether. The extract is washed, concentrated, and then dissolved into an adequate solvent for HPLC quantification. Calibration factors are determined for β-carotene, α-carotene, and lycopene from the chromatography of standard solutions. The β-carotene content of margarine is expressed in mg/100 g.

APPARATUS

1. HPLC system—consisting of a high-pressure pump, sample injection device, detector, and chart recorder or recording integrator. The wavelength of the UV detector should be set at 450 nm.
2. HPLC analytical column—250 × 4 mm, packed with a reverse phase microparticulate silica having a mean particle size of about 5 µm. A precolumn of the same material may also be used.
3. Rotary evaporator.

REAGENTS

Note—All solvents should be HPLC grade, or equivalent.

1. Lycopene, α- and β-carotene standards.
2. Butylated hydroxytoluene (BHT)
3. Dichloromethane
4. Ethanol
5. *n*-Hexane
6. Methanol
7. Potassium hydroxide
8. 2-Propanol
9. *tert*-Butyl methyl ether
10. Tetrahydrofuran
11. Acetonitrile

PROCEDURE

1. Preparation of standard solutions
   (a) β-Carotene stock solution—Prepare a stock solution of β-carotene by accurately weighing 0.3 mg of the standard (Reagents, 1) in 20 mL dichloromethane (Reagents, 3) followed by 30 s treatment in an ultrasonic bath. Transfer solution to a 100 mL volumetric flask and dilute to 100 mL with *n*-hexane (Reagents, 5). Pipet 10 mL of this solution to a 100 mL volumetric flask and bring to volume with *n*-hexane. This yields a standard solution of 3 µg β-carotene/mL in *n*-hexane/dichloromethane (4:1, v/v). Measure the absorbance of this solution at 450 nm and calculate the concentration by using E (1%/1cm) value 2592 (References, 1). Pipet a 20 mL portion of the standard solution into a round-bottomed flask. Remove solvent with a rotary evaporator under partial vacuum at 50°C. Dissolve residue in 20 mL of solvent compatible with the reverse phase HPLC system used.
   (b) α-Carotene and lycopene standard solutions—Prepare stock solutions of α-carotene and lycopene by accurately weighing 0.3 mg of the appropriate standard (Reagents, 1) in 10 mL tetrahydrofuran. Mix until dissolved. Dilute to 100 mL with ethanol (Reagents, 4) or another solvent compatible with the HPLC system.
   (c) Thermal isomerization of β-carotene—Mix a solution of almost pure (all-E) β-carotene in ethanol-tetrahydrofuran (9:1, v/v) containing 1000 ppm BHT (Reagents, 2). Transfer solution to HPLC vials. Close the vials and place in an 80°C waterbath. Heat for 2 h.
2. Optimization of working parameters—Condition the column (Apparatus, 2) if necessary (see Notes, 1). Pump the chosen solvent system (see Notes, 2 for list of possible solvents) through the column for at least 30 min at a flow rate of 1mL/min. Inject 20 µL of the individual standards and adjust the HPLC to obtain baseline separation of the carotenes and lycopene.
3. Preparation of test portion—Weigh 5 g of margarine into a 250 mL round-bottom flask. Add 10 mL water and mix to disperse. Add 60 mL of ethanol (Reagents, 4), 1 g BHT (Reagents, 2), and 10 mL *tert*-butylmethyl ether (Reagents, 9). Mix vigorously. Add 50 mL of 50% potassium hydroxide, mix, heat to 80°C, and then reflux for 15 min. Cool the saponified mixture. Extract the cooled mixture with two 100 mL portions of *tert*-butyl methyl ether (Reagents, 9), combine the two extracts, and wash the extracts four times with 50 mL portions of water. Concentrate a portion of the extract in a

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round-bottom flask using a rotary evaporator under partial vacuum at 50°C. Dissolve the residue in a solvent compatible with the reverse phase HPLC system used to a defined volume (5–50 mL) to obtain test solution with a concentration of 5 µg/mL.

4. HPLC determination of β-carotene in the test solution—Inject 20 µL of the mixed β-carotene standard working solution onto the column and record the areas of the β-carotene peaks. If an integrator is not available, record peak heights (measured in mm). Inject 20 µL of the test solution onto the column and identify the carotenes present by reference to the chromatograms obtained from standards. Record the areas of the β-carotene peaks (or peak heights). Record the areas of any lycopene and α-carotene peaks, if these are present and are to be quantified. Duplicate injections should be made. Inject an additional 20 µL of the mixed β-carotene standard working solution and record the area of the β-carotene peak.
5. Number of determinations—Carry out two determinations (each consisting of duplicate injections of the prepared test solutions) in rapid succession, using a fresh test portion for each determination.

CALCULATIONS

1. The β-carotene content of the test portion in µg/g is given by:

$$(C \times a \times D \times 25)/(A \times m)$$

Where—

C = concentration of the β-carotene standard (µg/mL)

A = mean of the peak areas obtained for the β-carotene standard

a = mean of the peak areas obtained for the β-carotene in test sample

m = mass of test sample taken

D = dilution factor

2. The lycopene and α-carotene contents of the test portion are calculated in the same manner using the data from chromatography of the corresponding standard.
3. Report as the final result the mean of the values obtained from the two determinations, provided the requirements noted in Precision, 2 are met. If the requirements for repeatability are not met, carry out two further determinations on the test portion. If the range (x_{max}-x_{min}) of the four values obtained is $<1.3 \times r$ (where r = the repeatability value; see Table 1), report as the final result the mean of the four values; otherwise, report as the final result the median of the four values, i.e., the mean of the two intermediate values. Report the results for each β-carotene, lycopene, and α-carotene to the nearest mg/100g.

PRECISION

1. The results of an interlaboratory study organized at the international level gave the statistical results summarized in Table 1.
2. Repeatability—When the mean of the values obtained from two single determinations, carried out in rapid succession by the same operator using the same apparatus under the same conditions for the analysis of the same test portion, lies within the range of the mean values cited in Table 1, the difference between the two values obtained should not be greater than the repeatability value (r), which can generally be deduced by linear interpolation from Table 1.
3. Reproducibility—When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions from the analysis of the same laboratory sample, lie within the range of mean values cited in Table 1, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility value (R), which can generally be deduced by linear interpolation from Table 1.

NOTES

Caution

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/m^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Ethanol and methanol are flammable. Use an effective fume-removal device when heating or evaporating. Methanol is toxic. Avoid contact with eyes. Avoid breathing vapors. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform and perchloric acid.

NUMBERED NOTES

1. If the HPLC column is new or of unknown history, wash and condition for about 10 min with methanol, then dichloromethane, followed by hexane at a flow rate of about 1 mL/min.
2. The following solvent systems may be used: a) 0.05 M ammonium acetate/acetonitrile in methanol/dichloromethane (75:20:5, v/v/v); b) acetonitrile/methanol/dichloromethane (75:20:5, v/v/v); c) methanol/acetonitrile/2-propanol/0.2%

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ammonium acetate in water N-ethyldiisopropylamine (500:455:20:25:0.2, by vol.) and 50 µg/g butylated hydroxytoluene; d) acetonitrile/methanol/tetrahydrofuran (55:35:15, v/v/v) or (40:55:5, v/v/v); e) methanol/acetonitrile/2-propanol/0.2% ammonium acetate in water (500:455:20:25, by vol.) and 50 µg/g butylated hydroxytoluene; f) methanol/methyl tert-butyl ether (gradient 30–80%) and 0.1% butylated hydroxytoluene; g) methanol/tetrahydrofuran (99:1, v/v) or (95:5, v/v); h) methanol/tetrahydrofuran (95:5, v/v) and 0.1% butylated hydroxytoluene.

REFERENCES

1. Schierle, J., Hardi, W., Faccin, N., Buhler, I., and Schuep, W. (1995) *Carotenoids, Vol. 1A, Isolation and Analysis,* G. Britton, S. Liaaen-Jensen, and H. Pfander (eds.), Birkhauser Verlag, Basel, Switzerland, pp. 265–272.
2. Schuep, W., and Schierle, J. (1997) Determination of β-Carotene in Commercial Foods: Interlaboratory Study, *J. AOAC Int. 80,* 1057–1063.

Table 1
Statistical results and precision data for determinaion of (all-E) and total β-carotene.

	Margarine	
Parameter	Total	All-E
Total no. of laboratories	13	13
Number of outliers	1	1
No. of laboratories after elimination of outliers	12	12
No. of accepted results	55	53
Mean content, mg/100g	0.253	0.223
Repeatability s_r, mg/100g	0.011	0.009
Relative repeatability RSDr, %	4.5	3.9
Repeatability value r, mg/100g	0.032	0.025
Reproducibility S_R, mg/100g	0.024	0.026
Relative reproducibility RSD_R, %	9.7	12
Reproducibility value R, mg/100g	0.069	0.073

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AOCS Recommended Practice Ce 10-02

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Detection of Non-Cocoa Butter Vegetable Fats and Oils in Chocolate by the Analysis of Hydrocarbon Sterol Degradation Products

DEFINITION

This Recommended Practice is for the detection of hydrocarbon sterol degradation recommended practice products (sterenes) adapted for the analysis of non-cocoa butter vegetable fats in chocolate. This involves solvent extraction of the fat, separation of the sterene fraction, and analysis of individual sterenes with mass spectrometric detection.

SCOPE

Applicable to detection of all known sterenes in refined vegetable fats and cocoa butter.

APPARATUS

1. Beaker.
2. Lipid fractionation chromatographic column (1.5 cm i.d. by 50 cm) fitted with a polytetrafluoroethylene stopcock.
3. Gas chromatograph.
   (a) e.g., Carlo Erba 4160 gas chromatograph or similar equipment capable of reproducing the following conditions: oven temperature of 200 to 280°C at a rate of 7°C/min.
   (b) Column, e.g., 50 m × 0.32 mm column with a 0.2 μm polar stationary phase (e.g., J&W DBWax, CP Wax 52CB).
   (c) VG 12-250 Quadrupole mass spectrometer (Thermo Separations) or comparable model.
4. Spatula, warmed.
5. Soxhlet thimble.
6. Cellulose filter paper.

REAGENTS

1. Silica gel 60, 70-230 mesh (Merck Co.).
2. Anhydrous sodium sulfate.
3. Hexane.
4. Stigmasta-3,5,22-triene (92%) (Chiron AS).
5. Cholesta-3,5-diene (Chiron AS).
6. Campesta-3,5-diene plus stigmasta 3,5-diene (97%) (Chiron AS).
7. Isooctane.
8. Celite.
9. Dichloromethane.

TEST SAMPLE PREPARATION

Chocolate should be chilled in a refrigerator, grated to a powder and mixed.

PROCEDURE

1. Extraction (cocoa butter and vegetable fat test samples receive no treatment prior to column chromatography)—
   (a) Place a 2.5 g test portion in a beaker (Apparatus, 1).
   (b) Add internal standard (cholestadiene (Reagents,5), 5 μg, in 0.05 mL isooctane (Reagents, 7)).
   (c) Melt chocolate at 55°C for 5 minutes.
   (d) Add Celite (Reagents, 8), 3 g at 55°C and mix with a warm spatula.
   (e) Place mixture in a Soxhlet thimble (Apparatus, 7) and extract overnight with 150 mL dichloromethane (Reagents, 9)
   (f) Filter extract through a cellulose filter paper (Apparatus, 8) and evaporate solvent.
   (g) Transfer residue to a fractionating column (Apparatus, 2).
2. Column chromatography—
   (a) Top column (Apparatus, 2) with 0.5 cm anhydrous sodium sulfate (Reagents, 2). Transfer a 1 g sample to the fractionating column with three 1 mL volumes of hexane.
   (b) Elute column with hexane at a flow rate of approximately 2 mL/min.
   (c) Discard the first 25 mL fraction (alkanes).
   (d) Collect the next 40 mL fraction (sterenes).

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(e) Evaporate the hexane and transfer the residue to a 1 mL vial with three 0.3 mL volumes of hexane.
(f) Evaporate the solution to dryness and redissolve in 0.1 mL isooctane prior to GC-MS.

3. Calibration curve preparation—Curves should be prepared such that they are linear up to 100 µg/mL sterene with correlation coefficients (r) of typically 0.996.
4. GC-MS—
   a. Scan spectra in electron ionization mode at 200°C with an electron current of 70 electron volts and a trap current of 200 microamps. Acquire spectra over the mass range of 35 to 600 daltons at a rate of 0.7 s/scan with an interscan delay of 0.5 ms.
5. Quantitative analysis—
   (a) Analysis is done by selected ion monitoring. Molecular ions of cholestadiene (m/z 368), campestadiene (m/z 382), stigmastatriene (m/z 394), and stigmastadiene (m/z 396) were selected. Ions corresponding to [M-alkyl]+ and the common ring fragment ions at m/z 255 and m/z 275 should be monitored to confirm peak identity. Campestatriene (m/z 380) and ions thought to be derived from nonpolar degradation products of triterpene alcohols (m/z 2 = 189, 218, 365, 393, and 408) may also be monitored.
   (b) Measurement and validation—
      1. Calibration graphs should be constructed and their slopes used to calculate the sterene content of the test portion. Calibrations should be linear up to 100 µg/mL sterene with correlation coefficients r, typically 0.996 for each analyte.
      2. Sterenes should be quantified on the basis of peak area measurements relative to the internal standard using the response in the molecular ion channel of each sterene.

PRECISION

1. Validation—limited validation experiments performed during previous work on olive oil containing 0.4 mg/kg stigmasta-3,5-diene showed the standard deviation to be typically 0.04. Analysis of six replicate test portion of a chocolate labeled as containing vegetable fat gave coefficients of variance for the sterene to internal standard peak area ratio within the range of 8 to 9% for each analyte.
2. Repeatability—replicate analyses (6) of a chocolate test sample containing about 3 mg/kg each of campesta-3,5-diene and stigmasta-3,5-diene. Coefficient of variance for the sterene-to-internal standard peak area ratios of three ions each of campestadiene and stigmastadiene ranged from 8 to 9%.

NOTES

Caution

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

REFERENCES

1. *JAOCS 74*(10):1273 (1997).

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Table 1
The levels of the quantified sterenes (campesta-3,5-diene, stigmasta-3,5,22-triene, and stigmasta-3,5-diene) measured in the chocolate samples (see Reference, 1).

Code	Camp	Triene	Diene	Total
Without fat				
1—	—	0.02	0.02	
2	—	—	0.010.01	
3	—	—	—	
4	0.03	0.010.08 2	0.1	
5	0.02	0.010.06	0.09	
6	—	—	0.010.01	
7	—	—	0.010.01	
8	—	0.010.04	0.05	
9	0.03	0.02	0.07	0.12
10	—	—	—	
11	—	—	—	
With fat				
12[b]	—	—	—	—
13	2.55	0.04	3.53	6.12
14	0.42	0.05	0.75	1.22
15	0.23	0.02	0.35	0.60
16	0.11	0.08	0.39	0.58
17	0.26	0.13	1.08	1.47
18	0.20	0.09	0.46	0.75
19	0.35	0.05	0.82	1.22

[a]—, less than 0.01 mg/kg.
[b]Contained unhydrogenated fat.
[c]Abbreviations: CAMP, campesta-3,5-diene; TRIENE, stigmasta- 3,5,22-triene; DIENE, stigmasta-3,5-diene.

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Table 2
Sterene levels (mg/kg) measured in the vegetable fat samples.[a]

Code	Sample	Camp	Triene	Diene	Total
Fat mixtures					
CB60	Palm	6.10	1.81	19.25	27.16
CB57	Fat mixture	5.81	1.78	18.39	25.98
CB62	PMF	3.07	0.99	10.47	14.53
CB61	CBE	.9101	.78	6.919	.60
CB67	CBR	1.68	1.25	3.79	6.72
CB53	Fat mixture	0.95	0.312	.26	3.52
CB49	CBE	0.94	0.25	1.51	2.70
CB51	Fat mixture	0.51	0.18	1.76	2.45
CB56	Choclin	0.42	0.19	1.77	2.38
CB47	CBE	0.69	0.17	1.50	2.36
CB64	Fat mixture	0.46	0.13	1.48	2.07
CB50	CBE	0.60	0.16	0.75	1.51
CB48	CBE	0.43	0.12	0.73	1.28
CB54	Fat mixture	0.33	0.13	0.81	1.27
CB55	Coberine	0.23	0.11	0.87	1.21
CB52	Fat mixture	0.28	0.12	0.79	1.19
CB66	CBE	0.09	0.04	0.27	0.40
CB65	CBE	0.06	0.03	0.20	0.29
Illipe, shea, and miscellaneous test samples					
B42	Illipe	4.54	1.17	20.10	25.81
CB58	Illipe	0.54	0.11	2.71	3.36
CB59	Illipe	0.01	0.1	2.64	3.25
CB63	Illipe	0.43	0.14	1.79	2.36
CB40	Illipe	—	—	0.11	0.11
CB41	Illipe	—	—	0.08	0.08
CB44	Shea	0.33	—	0.44	0.77
CB43	Shea	—	—	—	—
CB39	Shea	—	—	—	—
CB45	Chocolate crumb	—	—	0.02	0.02
CB46	Lecithin	1.20	0.09	3.11	4.40

[a]Abbreviations: CAMP, campesta-3,5-diene; TRIENE, stigmasta-3,5,22-triene; DIENE, stigmasta-3,5-diene. —, less than 0.01 mg/kg. For other abbreviations see Table 1. SAMPLING AND ANALYSIS OF COMMERCIAL FATS AND OILS Ce 10-02 • Detection of Non-Cocoa Butter Vegetable Fats and Oils in Chocolate

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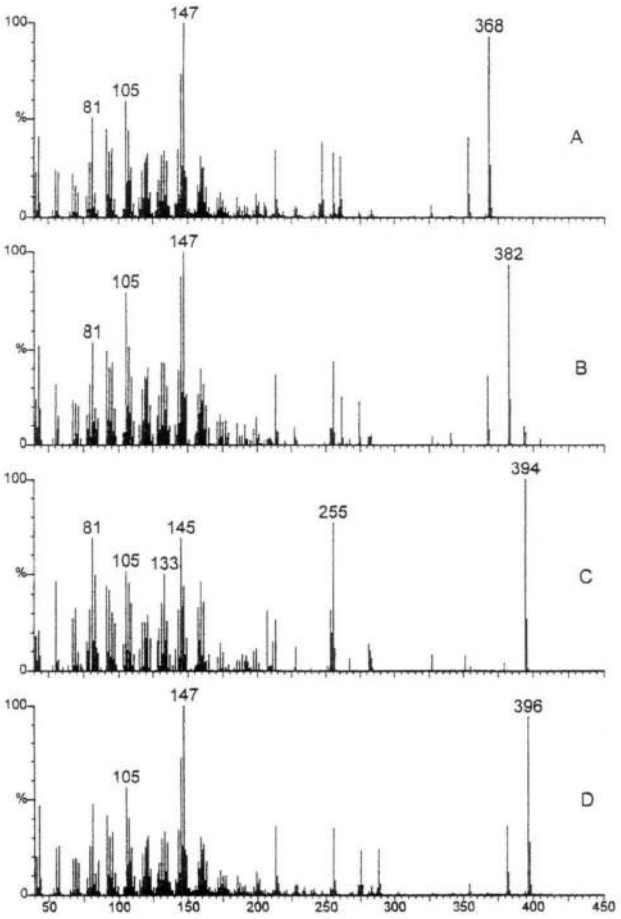

Figure 1. Mass spectra of the major sterene analytes in vegetable fats. (A) Cholestra-3,5-diene; (B) campesta-3,5-diene; (C) stigmasta-3,5,22-triene; (D) stigmasta-3,5-diene.

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AOCS Official Method Ce 11-05

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Determination of Cocoa Butter Equivalents in Cocoa Butter and Plain Chocolate

DEFINITION

This method describes a procedure for the detection and quantification of cocoa butter equivalents (CBEs) (see Notes, 1) in cocoa butter (CB) and plain chocolate by high-resolution capillary gas chromatography (HR-GC) of triacylglycerols and subsequent data evaluation by either linear regression analysis or partial least squares regression analysis.

SCOPE

This method is suitable for the detection and quantification of CBEs in cocoa butter or plain chocolate. When the presence of CBEs is estimated by linear regression analysis applied to the relative proportions of the three main triacylglycerol fractions of the fat analyzed, the presence of CBEs in CB and plain chocolate can be determined down to a level of 2% (this is equivalent to 0.4% if the fat content of chocolate is assumed to be 20%). When the amount of the CBE admixture is estimated by partial least-squares regression analysis applied to the relative proportions of the five main triacylglycerols, CBEs may be reliably quantified at a level of 5%.

APPARATUS

1. Analytical balance—accurate to ± 0.1 mg.
2. Drying oven—maintained at 55°C or dry heater block may be used.
3. Food grater—a kitchen blender with the motor above the receiving container to avoid melting the test samples.
4. Pipets—1 mL.
5. Volumetric flasks—20 mL.
6. Micro syringe, maximum volume 10 µL, graduated to 0.1 µL. (An automatic sampler may be used.)
7. Gas chromatograph (GC) fitted with a cold on-column injection system and a flame ionization detector (FID). [An alternative injection system, e.g., a split injector, a programmed-temperature vaporizer (PTV), or a moving-needle injector, may be used, provided the same results are obtained as indicated in Notes, 2—System suitability.]
8. The following experimental conditions give suitable results:
   (a) GC column: 25–30 m × 0.25 i.d. fused silica coated with thermo-stable 50% phenylmethylpoly-siloxane to a film thickness of 0.1–0.15 µm. (Suitable columns are listed in Notes, 2 and Table 3).
   (b) Temperature program: 100°C (initial temperature), program rate 30°C/min to 340°C (final temperature). Operating conditions may be changed to obtain optimal separation of cocoa butter triacyl-glycerols.
   (c) Carrier gas: helium or hydrogen (purity ≥ 99.999%).

   Alternative experimental conditions, used in an international collaborative study, are listed in Notes, 2 and Table 3.
9. Chromatographic data system.

REAGENTS

1. All reagents shall be of recognized analytical grade, unless otherwise stated.
2. Cocoa butter Certified Reference Material (CRM) IRMM-801 (References, 1), for calibration purposes and system suitability check.
3. Fat solvent (non-chlorinated solvents e.g., diethyl ether, *n*-heptane, isooctane).

PROCEDURE

1. Preparation of the cocoa butter CRM—Before opening and using the cocoa butter CRM, the ampoule has to be warmed in a drying oven until the contents have melted. When a clear solution is obtained, mix the contents by repeated inversion for not less than 20 s, open and transfer the contents to a clean vial, which can be tightly sealed and preserved in a cool place for future usage (see Notes, 3).
2. Preparation of test sample—Chill 200 g chocolate until hard, and mechanically prepare a fine granular powder. Mix thoroughly and preserve in tightly stoppered bottle in a cool place.
3. Fat extraction—Obtain the fat and determine the fat content from the chocolate test sample by Soxhlet extraction (References, 2). Other extraction procedures may be used, e.g., by accelerated solvent extraction, by supercritical carbon dioxide, or by using microwaves, provided that the same results are obtained.

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4. Separation of individual triacylglycerols by HR-GC—Warm the test samples (cocoa butter, fat extracted from chocolate, cocoa butter CRM) in a drying oven until completely melted. If the liquid sample contains sediment, filter the test sample inside the oven to obtain a clear filtrate. Pipets (or similar equipment) used for transferring the test sample during weighing operations should be brought to a temperature of approx. 55°C in a drying oven in order to avoid partial fat fractionation.

   Weigh 0.2 g test portion in a 20 mL volumetric flask and bring to volume with a suitable fat solvent. Pipet 1 mL of the resulting solution in another 20 mL volumetric flask and bring to volume with the same solvent.

   Inject 0.5–1.0 µL of the final test solution (0.5 mg fat/mL) into the HR-GC system using the cold on-column injection system. Alternative test portion amounts and injectors may be used provided that the detection system employed gives a linear response and the system suitability criteria are met.
5. Identification—Compare the retention times of the five major triacylglycerol fractions 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 1-palmitoyl-2,3-dioleoyl-glycerol (POO), 1,3-distearoyl-2-oleoylglycerol (SOS), and 1-stearoyl-2,3-dioleoyl-glycerol (SOO) of the test portion with those of the cocoa butter CRM. In general, triacylglycerols appear in order of increasing number of C atoms and of increasing unsaturation for the same number of C atoms. The elution order of the triacylglycerols of the cocoa butter CRM is given in the example chromatogram (Figure 1).

CALCULATIONS

1. Determination of response factors

   Determine the response factors of the triacylglycerols POP, POS, POO, SOS, and SOO by injection of the cocoa butter CRM solution using experimental conditions identical to those used for the test portion. Calculate the area percentage for each of the five triacylglycerol fractions by

$$AR_i(\%) = \frac{AR_i}{\sum AR_i} \times 100 \qquad [1]$$

$$RF_i = \frac{Mr_i(\%)}{Ar_i(\%)} \qquad [2]$$

   where—

   AR_i (%) = the area percentage of triacylglycerol i in the cocoa butter CRM
   AR_i = the peak area of the triacylglycerol i in the cocoa butter CRM
   ΣAR_i = the sum of the peak areas attributed to POP, POS, POO, SOS, SOO in the cocoa butter CRM
   RF_i = the detector response factor of triacylglycerol i in the cocoa butter CRM.
   MR_i (%) = the mass percentage of triacylglycerol i in the cocoa butter CRM.

   Report the results to two decimal places.
2. Calculation of mass percentages of triacylglycerols

   Calculate the mass percentage of the triacylglycerols (a) POP, POS, and SOS, and (b) POP, POS, POO, SOS, and SOO in the test sample by

$$MS_i(\%) = \frac{RF_i \times AS_i}{\sum (RF_i \times AS_i)} \times 100 \qquad [3]$$

   where—

   MS_i (%) = the mass percentage of triacylglycerol i in the test portions
   AS_i = the peak area corresponding to the triacylglycerol i in the test portion
   RF_i = the response factors as determined in 1

   Report the results to two decimal places in grams per 100 grams.
3. Detection of %CBE in cocoa butter by linear regression analysis

   The variability of the triacylglycerol composition of cocoa butter is expressed by Equation 4 using the normalised triacylglycerols, i.e., %POP + %POS + %SOS = 100% as determined in 2 (a) Equation 3:

   POP =
   43.734 – 0.733 × SOS (residual standard deviation = 0.125) [4]
   (see Notes, 4)

   For 99% of all analyses, pure cocoa butter complies with:

   $$POP < 44.025 - 0.733 \times SOS \qquad [5]$$

   A greater value of POP, as given by Equation 5, means that the sample is not pure cocoa butter. Using the cocoa butter CRM for calibration purposes, allows the laboratory to verifying the purity of cocoa butter, without establishing a "CB-line" by linking the laboratory results to the cocoa butter triacylglycerol database and the decision rule given in Equation 5.

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4. Calculation of %CBE in cocoa butter by partial least square regression analysis
   The CBE content of the cocoa butter, expressed in grams CBE per 100 grams of cocoa butter, is calculated by using a partial least squares regression analysis (Equation 4) of the relative proportions of the five main triacylglycerols, i.e., %POP + %POS + %POO + %SOS + %SOO = 100% as determined in 2 (b) Equation 3.
   %CBE in cocoa butter =

$$37.439 + 1.175 \times POP - 1.939 \times POS - 0.121 \times POO + 0.982 \times SOS - 0.097 \times SOO \quad [6]$$

   Give the results to two decimal places. (See Notes, 5)
   In 99% of cases where commercially available fats are used for blend formulation the prediction error does not exceed ± 2.60% related to cocoa butter.
5. Calculation of %CBE in chocolate
   The CBE content of the final product chocolate, expressed in grams CBE per 100 grams of chocolate, is calculated by applying Equation 7.

$$CBE_{Choc} = \frac{fat \cdot CBE_{CB}}{100} \quad [7]$$

   where—
   CBE_{Choc} = the content of CBEs in chocolate in grams per 100 grams
   fat = the fat content of the chocolate in grams per 100 grams as determined in Procedure, 3—Fat extraction
   CBE_{CB} = the cocoa butter content in grams per 100 grams as determined in Procedure, 3
   Report the results to two decimal places. In 99% of all cases where commercially available fats are used for blend formulation the prediction error does not exceed ± 0.52% related to chocolate (assumed fat content of chocolate 20%).

PRECISION

1. Linear regression analysis
   (a) Interlaboratory test
   The analytical approach was validated in a European interlaboratory test with 13 participants (References, 4, 6). The results derived from this interlaboratory test demonstrate that the procedure performs well with a detection limit of at least 2% CBE admixture to CB corresponding to 0.4% CBE in chocolate (assumed fat content of chocolate 20%) without giving false-positive or false-negative results. Precision data for the three triacylglycerols POP, POS, and SOS (normalized to 100%) are given below. Details of an interlaboratory test on precision of the method are summarized in Tables 1a–d.
   (b) Repeatability
   The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases be greater than: r = 0.514 g/100 g for POP values ranging from 18.99 to 25.37 g/100 g r = 0.293 g/100 g for POS values ranging from 43.76 to 47.73 g/100 g r = 0.621 g/100 g for SOS values ranging from 30.87 to 33.80 g/100 g
   (c) Reproducibility
   The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment (References, 6), will in not more than 5% of cases be greater than:
   R = 0.741 g/100 g for POP values ranging from 18.99 to 25.37 g/100 g
   R = 0.588 g/100 g for POS values ranging from 43.76 to 47.73 g/100 g
   R = 0.782 g/100 g for SOS values ranging from 30.87 to 33.80 g/100 g
2. Partial least squares regression analysis
   (a) Interlaboratory test—The analytical approach was validated in an interlaboratory test with 13 participants (References 4, 5). The method was validated for CBE levels around the statutory addition limit of 5% CBE in chocolate, i.e., admixture of 15, 25, and 30 g CBE/100 g cocoa butter corresponding to 3, 5, and 6 g of CBE in chocolate (fat content of the chocolate assumed to be 20%). Details of the collaborative study on the precision of the method are listed in Table 2 a–c.
   (b) Repeatability—The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases be greater than 0.721 g/100 g related to cocoa butter and 0.144 g/100 g related to the final product chocolate with the assumption that the fat content of the chocolate is 20%. Figures for r as found in the validation study are summarized in Table 2a–c.
   (c) Reproducibility—The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5% of cases be greater than 1.719 g/100 g related to cocoa butter and 0.344 g/100 g related to the final product chocolate

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with the assumption that the fat content of the chocolate is 20%. Figures for R as found in the validation study are summarised in Table 2a–c.

NUMBERED NOTES

1. Cocoa butter equivalents is the general term for fats used to replace cocoa butter in chocolate. They resemble the chemical composition and physical properties of cocoa butter very closely, making them therefore extremely difficult to quantify and even in some cases to detect. Cocoa butter equivalents are fats low in lauric acid, rich in symmetrical monounsaturated triacylglycerols of the type 1,3-dipalmitoyl-2-oleoyl-glycerol, 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol and 1,3-dis-tearoyl-2-oleoyl-glycerol, miscible with cocoa butter, and obtained only by refining and fractionation. The following vegetable fats, obtained from the plants listed below, are used singly or in blends: Illipé, Borneo tallow or Tengkawang (*Shorea* spp.), Palm oil (*Elaeis guineensis, Elaeis olifera*), Sal (*Shorea robusta*), Shea (*Butyrospermum parkii*), Kokum gurgi (*Garcinia indica*), and Mango kernel (*Mangifera indica*).
2. System suitability—The details of the chromatographic procedure depend, among other factors, on the equipment, the type, age, and supplier of the column, the means of introduction of the test solution, the sample size, and the detector. Different column lengths and brands may be used, and injection volumes may be varied (Table 3), if the requirements of the system suitability tests are met. The cocoa butter CRM has to be used to check the suitability of the separation system.
   a. Resolution—The HR-GC separation system must be capable of separating the critical pairs POS/POO and SOS/SOO with a chromatographic resolution of at least 1.0. In case of failure, the chromatographic conditions (e.g., sample size, column temperature, carrier gas flow, etc.) have to be optimized.
   b. Determination of detector response factors—To check that the assumption that flame ionization detector response factors of triacylglycerols do not differ significantly from unity, analyze the cocoa butter CRM applying standard GC conditions. Experience has shown that for a properly functioning chromatographic system the response factors for the five main triacylglycerols (POP, POS, POO, SOS, SOO) vary within a range of 0.80–1.20.
3. The use of the cocoa butter CRM for calibration purposes and as a system suitability check ensures high comparability of the results between individual testing laboratories and commutability of the elaborated procedure (References, 3).
4. The principle of the detection method is that for cocoa butter test samples POS is practically constant for wide variations of POP and SOS, resulting in a linear relationship (so-called "CB-line", Equation 4) between POP and SOS. All CB/CBE mixtures will cause the triacylglycerol analysis to deviate from the "CB-line" to the extent that their POS value differs from the POS value of cocoa butter. Equation 4 was established by using a standardized database of the triacylglycerol profile of 74 individual genuine cocoa butters evaluated and in-house validated by the authors (References, 3). The cocoa butter CRM was used to standardise the applied analytical methodology for the determination of the triacylglycerol profile of the cocoa butters.
5. Equation 6 was established by using a calibration set of 798 CB/CBE blends containing 10, 15, and 20% CBEs.

REFERENCES

1. Koeber, R., M. Buchgraber, F. Ulberth, R. Bacarolo, A. Bernreuther, H. Schimmel, E. Anklam, and J. Pauwels, *EUR 20781 EN*, ISBN 92-894-6036-9 (2003).
2. Official Methods of Analysis of AOAC International, AOAC Official Method 963.15, Fat in Cacao Products, Gaithersburg, MD, (1995).
3. Buchgraber, M., Ch. Senaldi, F. Ulberth, and E. Anklam, *J. AOAC Internat. 87:*1153–1163 (2004).
4. Buchgraber, M., F. Ulberth, and E. Anklam, *J. AOAC Internat. 87:*1164–1172 (2004).
5. Buchgraber, M., and E. Anklam, *EUR 20777 EN*, ISBN 92–894–6035–0 (2003).
6. Buchgraber, M., and E. Anklam, *EUR 20685 EN*, ISBN 92-894-5510-1 (2003).

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Table 1a
Precision data—pure cocoa butter samples.

Year of collaborative study	Pure CB[a]			Pure CB[b]			Pure C[B]		CB[c]
				2002					
	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS
Number of laboratories	13	13	13	13	13	13	13	13	13
Number of outliers	1	1	1	1	1	1	1	1	1
Number of accepted results	12	12	12	12	12	12	12	12	12
Mean value (g/100 g)	20.396	47.731	31.873	19.491	47.421	33.089	18.991	47.210	33.799
Repeatability standard deviation s_r, g/100 g	0.057	0.056	0.093	0.069	0.095	0.097	0.092	0.084	0.154
Repeatability relative standard deviation RSD_r, %	0.28	0.12	0.29	0.36	0.20	0.29	0.49	0.18	0.45
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.160	0.157	0.261	0.194	0.265	0.271	0.258	0.236	0.430
Reproducibility standard deviation s_R, g/100 g	0.142	0.108	0.169	0.081	0.148	0.166	0.120	0.090	0.168
Reproducibility relative standard deviation RSD_R, %	0.70	0.23	0.53	0.42	0.31	0.50	0.63	0.19	0.50
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	0.397	0.302	0.473	0.227	0.413	0.464	0.337	0.253	0.471

[a] Country origin: Grenada
[b] Country origin: Ghana
[c] Country origin: Ivory Coast

Table 1b
Precision data—real chocolate samples.

Year of collaborative study	Chocolate (CBE added)			Chocolate (no CBEs added)		
			2002			
	POP	POS	SOS	POP	POS	SOS
Number of laboratories	13	13	13	13	13	13
Number of outliers	2	2	2	1	1	1
Number of accepted results	11	11	11	12	12	12
Mean value (g/100 g)	25.375	43.757	30.868	19.740	47.401	32.859
Repeatability standard deviation s_r, g/100 g	0.128	0.101	0.180	0.089	0.070	0.074
Repeatability relative standard deviation RSD_r, %	0.50	0.23	0.58	0.45	0.15	0.23
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.358	0.282	0.503	0.250	0.195	0.208
Reproducibility standard deviation s_R, g/100 g	0.265	0.210	0.198	0.120	0.106	0.111
Reproducibility relative standard deviation RSD_R, %	1.04	0.48	0.64	0.61	0.22	0.34
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	0.741	0.588	0.553	0.335	0.298	0.310

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Table 1c
Precision data—cocoa butter with ca. 2% CBE admixture

Year of collaborative study	2.06 % CBE/CB[a]			1.98 % CBE/CB[b]			2.04 % CBE/CB[c]			2.09 % CBE/CB[d]		
	2002											
	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS
Number of laboratories	13	13	13	13	13	13	13	13	13	13	13	13
Number of outliers	0	0	0	1	1	1	2	2	2	1	1	1
Number of accepted results	13	13	13	12	12	12	11	11	11	12	12	12
Mean value (g/100 g)	20.611	47.082	32.307	19.938	46.795	33.267	19.698	46.619	33.682	19.859	46.646	33.495
Repeatability standard deviation s_r, g/100 g	0.184	0.095	0.222	0.050	0.070	0.082	0.093	0.063	0.137	0.029	0.083	0.077
Repeatability relative standard deviation RSD_r, %	0.89	0.20	0.69	0.25	0.15	0.25	0.47	0.14	0.41	0.14	0.18	0.23
Repeatability limit r $[r = 2.8 \times s_r]$, g/100 g	0.514	0.267	0.621	0.140	0.196	0.230	0.261	0.177	0.383	0.080	0.233	0.217
Reproducibility standard deviation s_R, g/100 g	0.193	0.132	0.250	0.118	0.091	0.181	0.113	0.142	0.149	0.094	0.118	0.185
Reproducibility relative standard deviation RSD_R, %	0.94	0.28	0.77	0.59	0.19	0.54	0.57	0.31	0.44	0.47	0.25	0.55
Reproducibility limit R $[R = 2.8 \times s_R]$, g/100 g	0.540	0.368	0.701	0.331	0.254	0.506	0.317	0.399	0.417	0.262	0.330	0.517

[a]CB from Grenada mixed with CBE type PMF/(Shea + Illipé) (35/65)
[b]CB from Ghana mixed with CBE type PMF/Shea (50/50)
[c]CB from Ivory coast mixed with CBE type PMF/Sal/Mango (50/25/25)
[d]CB from Ivory coast mixed with CBE type PMF/Shea/Illipé (50/25/25)

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Table 1d

Precision data—cocoa butter with ca. 4% CBE admixture

Year of collaborative study	4.0 % CBE/CB[a]			3.96 % CBE/CB[b]			4.04 % CBE/CB[c]			4.00 % CBE/CB[d]		
	2002											
	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS
Number of laboratories	13	13	13	13	13	13	13	13	13	13	13	13
Number of outliers	0	0	0	1	1	1	1	1	1	1	1	1
Number of accepted results	13	13	13	12	12	12	12	12	12	12	12	12
Mean value (g/100 g)	20.747	46.341	32.912	20.348	46.176	33.475	20.421	46.075	33.504	20.765	46.100	33.135
Repeatability standard deviation s_r, g/100 g	0.141	0.077	0.171	0.075	0.053	0.075	0.115	0.105	0.134	0.109	0.079	0.126
Repeatability relative standard deviation RSD_r, %	0.68	0.17	0.52	0.37	0.11	0.22	0.56	0.23	0.40	0.53	0.17	0.38
Repeatability limit r $[r = 2.8 \times s_r]$, g/100 g	0.395	0.215	0.478	0.209	0.148	0.209	0.322	0.293	0.377	0.306	0.221	0.354
Reproducibility standard deviation s_R, g/100 g	0.156	0.182	0.279	0.214	0.066	0.213	0.157	0.148	0.196	0.164	0.125	0.226
Reproducibility relative standard deviation RSD_R, %	0.75	0.39	0.85	1.05	0.14	0.64	0.77	0.32	0.59	0.79	0.27	0.68
Reproducibility limit R $[R = 2.8 \times s_R]$, g/100 g	0.437	0.510	0.782	0.601	0.183	0.596	0.441	0.414	0.549	0.459	0.351	0.633

[a]CB from Grenada mixed with CBE type PMF/(Shea + Illipé) (35/65)
[b]CB from Ghana mixed with CBE type PMF/Shea (50/50)
[c]CB from Ivory Coast mixed with CBE type PMF/Sal/Mango (50/25/25)
[d]CB from Ivory Coast mixed with CBE type PMF/Shea/Illipé (50/25/25)

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Table 2a
Precision data—Chocolate sample and CB/CBE blends prepared with a CB from Grenada and a CBE of the type PMF/(Shea + Illipé) (35/65)

Sample	Chocolate sample	Low level	Medium level	High level
Year of collaborative study		2002		
Number of laboratories	13	13	13	13
Number of outliers	1	1	0	0
Number of accepted results	12	12	13	13
Mean value, g CBE/100 g CB	13.992	15.142	24.798	29.563
True value, g CBE/100 g CB	—	14.790	24.920	30.010
Bias, g CBE/100 g CB	—	-0.352	0.122	0.447
Repeatability standard deviation s_r, g/100 g	0.258	0.167	0.181	0.222
Repeatability relative standard deviation RSD_r, %	1.84	1.11	0.73	0.75
Repeatability limit r ($r = 2.8 \times s_r$), g/100 g	0.721	0.469	0.506	0.623
Reproducibility standard deviation s_R, g/100 g	0.614	0.357	0.410	0.541
Reproducibility relative standard deviation RSD_R, %	4.39	2.36	1.65	1.83
Reproducibility limit R ($R = 2.8 \times s_R$), g/100 g	1.719	1.000	1.148	1.514

Table 2b
Precision data - CB/CBE blends prepared with a CB from Ghana and a CBE of the type PMF/Shea (50/50)

Sample	Low level	Medium level	High level
Year of collaborative study		2002	
Number of laboratories	13	13	13
Number of outliers	2	1	0
Number of accepted results	11	12	13
Mean value, g CBE/100 g CB	16.627	25.871	30.686
True value, g CBE/100 g CB	15.070	24.900	30.010
Bias, g CBE/100 g CB	-1.557	-0.971	-0.676
Repeatability standard deviation s_r, g/100 g	0.214	0.258	0.158
Repeatability relative standard deviation RSD_r, %	1.29	1.00	0.51
Repeatability limit r ($r = 2.8 \times s_r$), g/100 g	0.599	0.722	0.442
Reproducibility standard deviation s_R, g/100 g	0.229	0.427	0.452
Reproducibility relative standard deviation RSD_R, %	1.37	1.65	1.47
Reproducibility limit R ($R = 2.8 \times s_R$), g/100 g	0.640	1.196	1.265

Table 2c
Precision data - CBE/CB blends prepared with a CB from Ivory Coast and a CBE of the type PMF/Sal/Mango (50/25/25)

Sample	Low level	Medium level	High level
Year of collaborative study		2002	
Number of laboratories	13	13	13
Number of outliers	2	1	0
Number of laboratories	13	13	13
Number of outliers	0	1	0
Number of accepted results	13	12	13
Mean value, g CBE/100 g CB	15.655	24.170	28.535
True value, g CBE/100 g CB	14.970	24.970	29.960
Bias, g CBE/100 g CB	-0.685	0.800	1.425
Repeatability standard deviation s_r, g/100 g	0.223	0.189	0.222
Repeatability relative standard deviation RSD_r, %	1.43	0.78	0.78
Repeatability limit r ($r = 2.8 \times s_r$), g/100 g	0.625	0.530	0.621
Reproducibility standard deviation s_R, g/100 g	0.309	0.356	0.406
Reproducibility relative standard deviation RSD_R, %	1.98	1.47	1.42
Reproducibility limit R ($R = 2.8 \times s_R$), g/100 g	0.866	0.997	1.138

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Table 3

Suitable HR-GC conditions to be used for triacylglycerol analyses of cocoa butter, CBEs, CB/CBE blends, and chocolate.

Method Possibility	1	2	3	4	5	6	7	8	9	10	11
Column characteristics[a]											
stationary phase	DB-17HT	RTx-65TG	CB-TAP	RTx-65TG	CB-TAP	RTx-65TG	CB-TAP	DB-17HT	CB-TAP	CB-TAP	CB-TAP
Length (m)	30	30	25	30	25	30	25	30	25	25	25
i.d. (mm)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Film thickness (μm)	0.15	0.1	0.1	0.1	0.1	0.1	0.1	0.15	0.1	0.1	0.1
Temperature mode											
Oven											
Injection temperature (°C)/hold time (min)	80/2	340/1	280/0	100/0.5	340/0	200/0	100/0.1	50/2	200/2	100/1	200/2
Program rate 1 (°C/min)	50	1	10	50	1	15	70	50	20	30	12
Temperature 1 (°C)/hold time (min)	300/0	—	320/0	330/2	—	360/0	—	300/1	320/0	300/2	—
Program rate 2 (°C/min)	30	—	2	1	—	1	—	10	1	30	—
Temperature 2 (°C)/hold time (min)	—	—	—	—	—	—	—	340/2	—	—	—
Program rate 3 (°C/min)	—	—	—	—	—	—	—	0.5	—	—	—
Final temperature (°C)/hold time (min)	350/30	360/3	360/6	350/5	360/10	370	350/21	345/26	360/10	340/35	350/10
—Injector temperature (°C)	oven track	390	370	oven track	360	390	oven track	50	65-220-370	100	
—Detector temperature (°C)	360	370	370	355	360	390	360	360	370	360	360
Injection mode	OCI	split	split	OCI	split	split	OCI	OCI	OCI	OCI	hot OCI
Carrier gas											
Type	H_2	H_2	H_2	He	He	H_2	H_2	H_2	He	H_2	H_2
Pressure (kPa)	—	120	100	—	150	150	—	120	90	150	—
Flow (mL/min)	0.8	—	—	0.8	—	—	1	—	—	—	2.4
Sample											
Concentration (mg/mL)	0.3	50	12.5	0.3	10	15	0.5	1 - 2	0.5	0.65	
Volume injected (μL)	0.5	0.1	0.6	0.5	1	0.5	0.5	0.5	0.1	0.4	0.3

[a]These types of columns are examples of suitable products which are available commercially.

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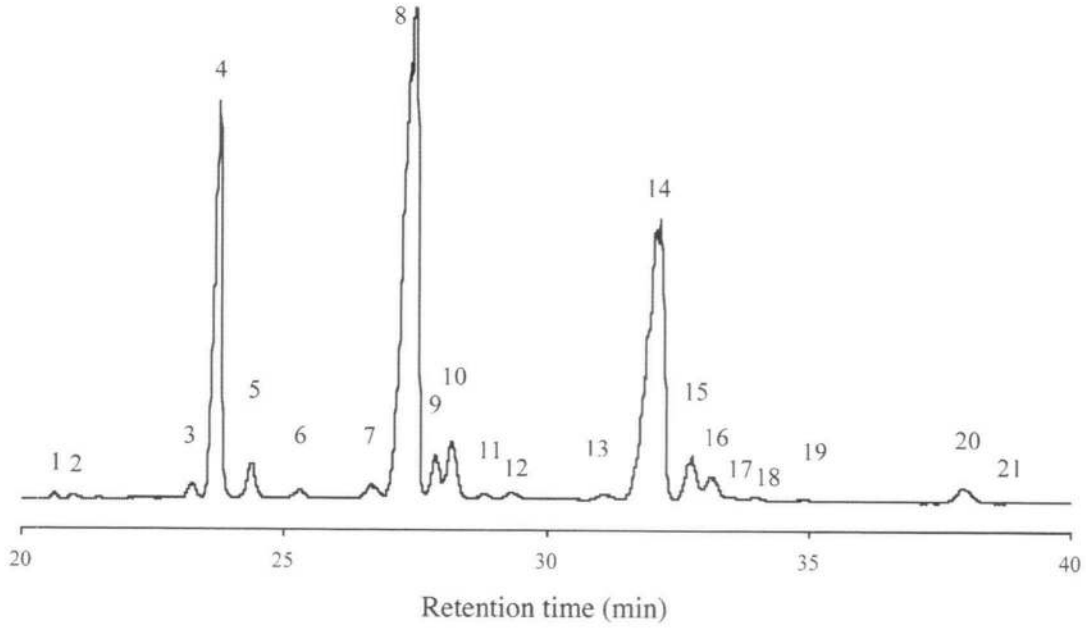

Peak identification: 1. PPP; 2. MOP; 3. PPS; 4. POP; 5. PLP; 6. unidentified; 7. PSS; 8. POS; 9. POO; 10. PLS; 11. PLO; 12. unidentified; 13. SSS; 14. SOS; 15. SOO; 16. SLS + OOO; 17. SLO; 18. unidentified; 19. unidentified; 20. SOA; 21. AOO

Experimental conditions:

GC column:	25 m × 0.25 mm fused silica capillary column coated with 0.1 μm Chrompack TAP
Oven temperature	100°C held for min, 30°C/min to 340°C held for 35 min
Injector	Cold on-column
Detector (FID)	360°C
Carrier gas	H_2 at 1.6 bar head pressure
Amount injected	0.5 μL of a 0.5 mg/mL solution

Abbreviations:

PPP	Tripalmitin	SSS	Tristearin
MOP	1-Margaroyl-2-oleoyl-3-palmitoylglycerol	SOS	1,3-Distearoyl-2-oleoylglycerol
PPS	1,2-Dipalmitoyl-3-stearoylglycerol	SOO	1-Stearoyl-2,3-dioleoylglycerol
POP	1,3-Dipalmitoyl-2-oleoylglycerol	SLS	1,3-Distearoyl-2-linoleoyl glycerol
PLP	1,3-Dipalmitoyl-2-linoleoylglycerol	OOO	Triolein
PSS	1-Palmitoyl-2,3-distearoylglycerol	SLO	1-Stearoyl -2-linoleoyl-3-oleoylglycerol
POS	1-Palmitoyl-2-oleoyl-3-stearoylglycerol	SOA	1-Stearoyl-2-oleoyl-arachidoylglycerol
POO	1-Palmitoyl-2,3-dioleoylglycerol	AOO	1-Arachidoyl-2,3- dioleoylglycerol
PLS	1-Palmitoyl-2-linoleoyl-3-stearoylglycerol		

Figure 1. Triacylglycerol profile of the cocoa butter CRM

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Detection and Quantification of Cocoa Butter Equivalents in Milk Chocolate

DEFINITION

This method determines cocoa butter equivalents (CBEs) and milk fat (MF) in milk chocolate by triacylglycerol (TAG) profiling using high-resolution capillary gas-liquid chromatography (HR-GLC), and subsequent data evaluation by simple and partial least squares regression analysis.

SCOPE

This method specifies a procedure for the detection and quantification of cocoa butter equivalents (CBEs) and milk fat (MF) in milk chocolate by triacylglycerol (TAG) profiling using high-resolution capillary gas liquid chromatography (HR-GLC), and subsequent data evaluation by simple and partial least squares regression analysis. Chocolate fats obtained from milk chocolate using a rapid fat extraction procedure, are separated by HR-GLC into TAG fractions according to their molecular weight and degree of unsaturation. Individual TAG fractions, i.e., 1-palmitoyl-2-stearoyl-3-butyroylglycerol (PSB), 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 1,2-dioleoyl-3-palmitoyl-glycerol (POO), 1,3-distearoyl-2-oleoyl-glycerol (SOS) and 1,2- dioleoyl-3-stearoyl-glycerol (SOO) are used to calculate the MF content in the chocolate fat and to determine the presence/absence of CBEs to quantify the amount of the CBE admixture in chocolate fat. CBE admixtures can be detected down to a level of 0.5 g CBE/100 g milk chocolate and quantified well around levels of 5% CBE addition to milk chocolate with a prediction error of 0.7 g CBE/100 g milk chocolate (see Notes, 1).

APPARATUS

1. Analytical balance—capable of accurately measuring 0.1 mg.
2. Drying oven. (A dry heater block may be used.)
3. Filter paper—15 cm.
4. Food grater—a kitchen blender with the motor above the receiving container to avoid melting the samples
5. Rotary evaporator or alternative evaporation procedures
6. Evaporation block—with nitrogen supply.
7. Desiccator.
8. Soxhlet extractor, with standard taper joints, siphon capacity 100 mL (33 mm × 88 mm extraction thimble), 250 mL Erlenmeyer flask, and regulated heating mantle (or equivalent).
9. Volumetric flasks—capacity of 10, 25, 50, and 100 mL (or other capacities if needed).
10. Pipettes—capacities ranging from 1 to 10 mL (or other capacities if needed).
11. Micro syringe—with maximum volume 10 µL, graduated to 0.1 µL, or automatic sample injector.
12. Gas chromatograph (GC) fitted with a cold on-column and a flame ionization detector (FID). [Alternative injection systems (e.g., a split injector, see Notes, 2, a programmed-temperature vaporizer (PTV) or a moving-needle injector) may be used (see Notes, 3 System suitability.)]
13. The separation and quantification have proven to be satisfactory if the following experimental conditions are followed:
    (a) GC column: CB-TAP 25 m × 0.25 mm i.e., fused silica coated with a medium polar thermo stable phenylmethylpolysiloxane stationary phase with a film thickness of 0.10 µm.
    (b) Oven program: 100°C hold for 2 min; 30°C/min to 270°C hold for 1 min; 2.5°C/min to 340°C hold for 7 min (see Notes, 2 a).
    (c) Detector (FID): 360°C.
    (d) Carrier gas: H_2 (purity ≥ 99.999%) with a constant flow rate of 3.5 mL/min. (Another suitable carrier gas is helium) (see Notes, 2 b).

    Suitable columns and alternative experimental conditions, used in an international collaborative study (References, 3), are listed in Table 1. Operating conditions may be changed to obtain optimal separation.
14. Chromatographic data system.

REAGENTS

1. Use only reagents of recognized analytical grade, unless otherwise stated.
2. Cocoa butter Certified Reference Material IRMM-801 (References, 2)—for calibration purposes and system suitability tests.

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3. Pure milk fat—for system suitability tests.
4. 1-Palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB)—for calibration purposes.
5. alpha-Cholestane—dissolve 50 mg alpha-cholestane in 50 mL of *iso*-octane and then dilute to 0.004 mg/mL (1:250) (see Notes, 2 c).
6. Fat solvent—non-chlorinated solvents (e.g. petroleum ether, *n*-hexane, *n*-heptane, *iso*-octane).
7. Hydrochloric acid—4 mol/L.

PROCEDURE

1. Preparation of IRMM-801 for calibration purposes and system suitability tests—Before opening and using the IRMM-801, the ampoule shall be warmed in an oven until the contents have melted. When a clear solution is obtained, mix the contents by repeated inversion for not less than 20 s. Then open and transfer the contents to a clean vial, which can be tightly sealed and preserved in a cool place for future usage.
2. Preparation of calibration standards—Dissolve about 40 mg of PSB in a 50 mL volumetric flask with *iso*-octane resulting in a stock solution of about 0.8 mg/mL. Mix thoroughly until completely dissolved. From this PSB stock solution prepare a series of five calibration solutions ranging from 0.016 to 0.128 mg/mL in the matrix (IRMM-801) by weighing 250 mg IRMM-801 into 25 mL volumetric flasks and adding the respective volumes of the PSB stock solution. Fill up to the mark with *iso*- octane.

   Prior to cold-on column injection, dilute each calibration solution 1:5 with *iso*-octane to obtain a final IRMM-PSB concentration ($C_{IRMM\text{-}PSB}$) of 2 mg/mL in each solution and PSB concentrations (C_{PSB}) ranging from 0.0256 to 0.0032 mg/mL (see Notes, 2 d).

   Calculate the final PSB concentrations using the actual weighed mass in the stock standard solution.
3. Preparation of pure milk fat for system suitability tests—If no pure milk fat is available it can be obtained from a butter test sample by melting and passing the fat layer through a folded filter at 50°C in an oven.
4. Preparation of test sample—Chill approx. 200 g of chocolate until hard, and grate to fine granular condition using a mechanical device. Mix thoroughly and preserve in a tightly sealed bottle in a cool place.
5. Fat extraction—
   (a) Determine the fat content from the chocolate test sample by Soxhlet extraction (References, 1).
   (b) Rapid fat extraction—The chocolate fat is separated from 5 g grated chocolate test portion by extracting with two to three 10 mL portions of a suitable solvent. Centrifuge and decant. Combine the extracts and evaporate most of the solvent and finally dry it under a stream of nitrogen (see Notes, 3, 4).
6. Calibration curve for determination of PSB content— Prepare five calibration solutions containing different concentrations of PSB ranging from 0.0016 to 0.0128 with the same concentration of alpha-cholestane (0.002 mg/mL). Inject 0.5 µL of each calibration solution into the HR-GLC system (see Notes, 2 e).
7. Separation of individual TAGs by HR-GLC—Warm the test portions (cocoa butter, fat extracted from chocolate, cocoa butter CRM) in a drying oven until completely melted. If the liquid contains sediment, filter it inside the oven to obtain a clear filtrate. Pipettes (or similar equipment) used for transferring should be brought to a temperature of 55°C in a drying oven in order to avoid partial fat fractionation.
   (a) Separation of individual TAGs of IRMM-801—Weigh 0.1 g of IRMM-801 in a 10 mL volumetric flask and dilute to the mark with *iso*-octane. Pipette 1 mL of the resulting solution in another 50 mL volumetric flask and dilute to the mark with the same solvent (0.2 mg/mL). Inject 0.5 µL of the final test solution into the HR-GLC system (see Notes, 2 f).
   (b) Separation of individual TAGs of pure MF—Weigh 0.05 g of pure milk fat in a 50 mL volumetric flask and dilute to the mark with *iso*-octane (1 mg/mL). Transfer 1 mL of this solution in a test tube and add 1 mL of alpha-cholestane solution (resulting test sample solution 0.5 mg/mL). Inject 0.5 µL of the final test solution into the HR-GLC system (see Notes, 2 g).
   (c) Separation of individual TAGs of chocolate fat—Weigh 0.1 g of chocolate fat on an analytical balance in a 100 mL volumetric flask and dilute to the mark with *iso*-octane (1 mg/mL). Transfer 1 mL of this solution in a test tube and add 1 mL of alpha-cholestane solution (resulting test sample solution 0.5 mg/mL). Inject 0.5 µL of the final test solution into the HR-GLC system (see Notes, 2 h).
8. Identification—Compare the retention times of 1-palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB) and alpha-cholestane of the test portion with those of the reference standards. Identify the five major TAG fractions 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS), 1-palmitoyl-2,3-dioleoyl-glycerol (POO), 1,3-distearoyl-2-oleoylglycerol (SOS) and 1-stearoyl-2,3-dioleoyl-glycerol (SOO) by comparison of the retention times of the test portion with those of the IRMM-801. In general, TAGs appear in order of increasing number of carbon atoms and of increasing unsaturation for the same number of carbon atoms. The elution order of the TAGs of IRMM-801 is given in Figure 1. The elution order of the TAGs of an average pure MF is given in (References, 5).

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CALCULATIONS

1. CBE detection in chocolate fat—
Determine the response factors of the TAGs i, (a) POP, POS, and SOS and (b) POP, POS, POO, SOS and SOO by injection of the IRMM-801 solution using experimental conditions identical to those used for the test portion. Calculate the percentage of each of the three TAGs with respect to all TAGs present in IRMM-801 by the following equations:

$$P_{i;\,ref} = \frac{A_{i;\,ref}}{\Sigma A_{all\ TAGs;\,ref}} \times 100 \quad [1]$$

$$P_i = \frac{M_{i;\,ref}}{P_{i;ref}} \quad [2]$$

Where—

$A_{i;\,ref}$ = the peak area of TAG i, i.e., POP, POS, and SOS, in IRMM-801
$\Sigma A_{all\ TAGs;\,ref}$ = the sum of the peak areas attributed to all TAGs in IRMM-801
$P_{i;\,ref}$ = the percentage of TAG i, i.e., POP, POS, in IRMM-801 (see Equation 1)
$M_{i;\,ref}$ = the mass fraction in percent of TAG i in IRMM-801 as given in the certificate (POP = 16.00%, POS = 39.40%, SOS = 27.90%) (References, 2)
P_i = the detector response factor of TAG i, i.e., POP, POS, and SOS, in IRMM-801

Report the results to two decimal places.

2. Calculation of mass percentages of POP, POS, and SOS in chocolate fat—
Calculate the mass percentage of the TAGs i, POP, POS, and SOS in the test sample by

$$M_{i;\,total} = \frac{F_i \times A_i}{\Sigma A_{all\ TAGs}} \times 100 \quad [3]$$

Where—

A_i = the peak area corresponding to the TAG i, i.e., POP, POS, and SOS, in the test sample
$\Sigma A_{all\ TAGs}$ = the sum of the peak areas attributed to all TAGs in the test portion
F_i = the response factor for the TAG i, i.e., POP, POS, and SOS (see Equation 2)
$M_{i;\,total}$ = the mass fraction in percent of TAG i, i.e., POP, POS, and SOS, in the test portion

Report the results to two decimal places.

3. PSB and MF quantification in chocolate fat and chocolate—
Determine the response factor of PSB by injection of the five calibration solutions using the same experimental conditions. For each calibration solution calculate a response factor for PSB, F_{PSB}

$$F_{PSB;\,i} = \frac{C_{PSB;\,i} \times A_{Cholestane;\,i}}{C_{Cholestane;\,i} \times A_{PSB;\,i}} \quad [4]$$

Where—

$A_{PSB;\,i}$ = the peak area of PSB in calibration solution i
$A_{Cholestane;\,i}$ = the peak area of the internal standard alpha-cholestane in calibration solution i
$C_{PSB;\,i}$ = the concentration (mg/mL) of PSB used in calibration solution i
$C_{Cholestane;\,i}$ = the concentration (mg/mL) of the internal standard alpha-cholestane used in calibration solution i
$F_{PSB;\,i}$ = the detector response factor of PSB in the calibration solution i

An average response factor for PSB, $F_{PSB;\,mean}$, obtained from the five calibration solutions has to be calculated and used for further calculations.

4. PSB quantification in chocolate fat—
The mass fraction in percent of PSB in the test portion (chocolate fat), $M_{PSB;\,choc\ fat}$, is calculated as follows:

$$M_{PSB;\,choc\ fat} = \frac{A_{PSB} \times C_{Cholestane} \times F_{PSB,\,mean}}{A_{Cholestane} \times C_{Sample}} \times 100 \quad [5]$$

Where—

A_{PSB} = the peak area of PSB in the test portion
$A_{Cholestane}$ = the peak area of the internal standard alpha-cholestane in the test portion
$F_{PSB;\,mean}$ = the average response factor for PSB

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$C_{Cholestane}$ = the concentration (mg/mL) of the internal standard alpha-cholestane in the test portion
C_{Sample} = the concentration (mg/mL) of the test portion
$M_{PSB;\ choc\ fat}$ = the mass fraction in percent of PSB in the test portion

Report the results to two decimal places.

5. MF quantification in chocolate fat—
The mass fraction in percent of milk fat in the chocolate fat, $M_{MF;\ choc\ fat}$, is calculated as follows. The calibration function was established from a database of 900 mixtures (References, 4).

$$M_{MF;\ choc\ fat} = 0.19 + (44.04 \times M_{PSB;\ choc\ fat}) \quad [6]$$

Where—
$M_{PSB;\ choc\ fat}$ = the mass fraction in percent of PSB in chocolate fat (see Equation 5)
$M_{MF;\ choc\ fat}$ = the mass fraction in percent of MF in chocolate fat

Report the results to two decimal places.

6. MF quantification in chocolate—
The mass fraction in percent of MF in chocolate, $M_{MF;\ choc}$, is calculated as follows:

$$M_{MF;\ choc} = \frac{M_{fat;\ choc} \times M_{MF;\ choc\ fat}}{100} \quad [7]$$

Where—
$M_{fat;\ choc}$ = the mass fraction in percent of total fat in chocolate (see Procedure 5 a)
$M_{MF;\ choc\ fat}$ = the mass fraction in percent of MF in chocolate fat (see Equation 6)
$M_{MF;\ choc}$ = the mass fraction in percent of MF in chocolate

7. Correction for MF contribution—
Calculate the contribution of the mass percentages of the TAGs POP, POS, and SOS originating from MF by

$$M_{i;\ mf} = \frac{M_{MF;\ choc\ fat} \times M_{i;\ ref\ MF}}{100} \quad [8]$$

Where—
$M_{i;\ ref\ MF}$ = the average mass fraction in percent of TAG i in a MF, i.e., POP = 3.99%, POS = 2.19%, SOS = 0.45% [values obtained from database, (References, 5)]
$M_{MF;\ choc\ fat}$ = the mass fraction in percent of MF in the test portion (see Equation 6)
$M_{i;\ mf}$ = the mass fraction in percent of TAG i, i.e., POP, POS, and SOS, originating from MF in the test portion

Subtract the obtained mass percentages of the three TAGs originating from MF (Equation 8) from the mass percentages of the three TAGs obtained for the test sample (Equation 3).

$$M_{i;\ corr.} = M_{i;\ total} - M_{i;\ mf} \quad [9]$$

Normalize the obtained mass percentages of the three TAGs (Equation 9) to 100% (Equation 11):

$$POP_{corr.} + POS_{corr.} + SOS_{corr.} = 100\% \quad [10]$$

Report the results to two decimal places.

8. Decision whether chocolate fat is pure cocoa butter—
The variability of the TAG composition of CB is expressed by Equation 11 (References, 6, 7).

$$POP - \% = 43.73 - 0.73 \times SOS - \% \quad [11]$$
$$(\text{residual SD} = 0.125)$$

The principle of the method is that for pure CB POS is practically constant for wide variations of POP and SOS, resulting in a linear relationship (so-called "CB-line," Equation 11) between POP and SOS. CBE and other fat admixtures will cause the TAG analysis to deviate from the "CB-line" to the extent that their POS value differs from the POS value of cocoa butter (see Notes, 5). For 99% of all analyses, pure cocoa butter complies with:

$$POP_{corr.} < 44.03 - 0.73 \times SOS_{corr.} \quad [12]$$

A greater value of POPcorr., as given by Equation 12, means that the sample is not pure CB.

9. CBE quantification in chocolate fat and chocolate—
Determine the response factors of the TAGs j, i.e., POP, POS, POO, SOS, and SOO, by injection of IRMM-801 solution using experimental conditions identical to those used for the test portions. Calculate the percentage of each of the five TAG fractions by the following equations:

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$$P_{j;\,ref} = \frac{A_{j;\,ref}}{\Sigma A_{j;\,ref}} \times 100 \qquad [13]$$

$$F_j = \frac{M_{j;\,ref}}{P_{j;\,ref}} \qquad [14]$$

Where—

$A_{j;\,ref}$ = the peak area of the TAG j, i.e., POP, POS, POO, SOS, and SOO, in IRMM-801
$S_{Aj;\,ref}$ = the sum of the peak areas attributed to POP, POS, POO, SOS, and SOO, in IRMM-801
$P_{j;\,ref}$ = the percentage of TAG j, i.e., POP, POS, POO, SOS, and SOO, in IRMM-801
$M_{j;\,ref}$ = the mass fraction in percent of TAG j, i.e., POP, POS, POO, SOS, and SOO, in IRMM-801 as given in the certificate POP = 18.14%, POS = 44.68%, POO = 2.26%, SOS = 31.63%, and SOO = 3.29%, i.e., normalized to 100%) (References, 2)
F_j = the detector response factor of TAG j, i.e., POP, POS, POO, SOS, and SOO, in IRMM-801

Report the results to two decimal places.

10. Calculation of the mass percentages of TAGs—
Calculate the mass percentages of TAGs j, i.e., POP, POS, POO, SOS, and SOO, in the test sample by

$$M_{j;\,choc\ fat} = \frac{F_j \times A_j}{\Sigma(F_j \times A_j)} \times 100 \qquad [15]$$

Where—

F_j = the response factor of the TAG j, i.e., POP, POS, POO, SOS, and SOO, (see Equation 14)
A_j = the peak area corresponding to the TAG j, i.e., POP, POS, POO, SOS, and SOO, in the test portion
$M_{j;\,choc\ fat}$ = the mass fraction in percent of TAG j, i.e., POP, POS, POO, SOS, and SOO, in the test portion

Report the results to two decimal places.

11. CBE quantification in chocolate fat—
Calculate the mass fraction in percent of CBE in the chocolate fat, $M_{CBE;\,choc\ fat}$, using Equation 16, which was derived by PLS regression analysis of the relative proportions of the five main TAGs; i.e., $POP_{choc\ fat}$, $POS_{choc\ fat}$, $POO_{choc\ fat}$, $SOS_{choc\ fat}$, and $SOO_{choc\ fat}$ as determined in Equation 15 and the MF content of the chocolate fat, i.e., $M_{MF;\,choc\ fat}$ as determined in Equation 6.

$$\begin{aligned} M_{CBE;\,choc\ fat} = {} & -4.24 - (0.23 \times M_{MF;\,choc\ fat}) \\ & + (1.52 \times POP_{choc\ fat}) \\ & - (1.47 \times POS_{choc\ fat}) \\ & + (1.09 \times POO_{choc\ fat}) \\ & + (1.29 \times SOS_{choc\ fat}) \\ & + (0.26 \times SOO_{choc\ fat}) \end{aligned} \qquad [16]$$

Report the result to one decimal place.

12. CBE quantification in chocolate—
The mass fraction in percent of CBE in chocolate, $M_{CBE;\,choc}$, is calculated by applying Equation 14:

$$M_{CBE;\,choc} = \frac{M_{fat;\,choc} \times M_{CBE;\,choc\ fat}}{100} \qquad [17]$$

Where—

$M_{fat;\,choc}$ = the mass fraction in percent of total fat in chocolate (see Procedure 5 a)
$M_{CBE;\,choc\ fat}$ = the mass fraction in percent of CBE in chocolate fat (see Equation 16)
$M_{CBE;\,choc}$ = the mass fraction in percent of CBE in chocolate

Report the result to one decimal place.

Peak identification: 1, PPP; 2, MOP; 3, PPS; 4, POP; 5, PLP; 6, unidentified; 7, PSS; 8, POS; 9, POO; 10, PLS; 11, PLO; 12, unidentified; 13, SSS; 14, SOS; 15, SOO; 16, SLS + OOO; 17, SLO; 18, unidentified; 19, unidentified; 20, SOA; 21, AOO

Experimental conditions

GLC column: 25 m × 0.25 mm fused silica capillary column coated with 0.1 μm Chrompack TAP
Temperature program: 100°C held for at least 2 min; 30°C/min to 270°C held for 1 min; 2.5°C/min to 340°C held for 7 min
Injector: Cold on-column

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Detector (FID): 360°C
Carrier gas: H_2 with a constant flow rate of 3.5 mL/min

Abbreviations:

PPP	Tripalmitin
MOP	1-Margaroyl-2-oleoyl-3-palmitoylglycerol
PPS	1,2-Dipalmitoyl-3-stearoylglycerol
POP	1,3-Dipalmitoyl-2-oleoylglycerol
PLP	1,3-Dipalmitoyl-2-linoleoylglycerol
PSS	1-Palmitoyl-2,3-distearoylglycerol
POS	1-Palmitoyl-2-oleoyl-3-stearoylglycerol
POO	1-Palmitoyl-2,3-dioleoylglycerol
PLS	1-Palmitoyl-2-linoleoyl-3-stearoylglycerol
SSS	Tristearin
SOS	1,3-Distearoyl-2-oleoylglycerol
SOO	1-Stearoyl-2,3-dioleoylglycerol
SLS	1,3-Distearoyl-2-linoleoylglycerol
OOO	Triolein
SLO	1-Stearoyl-2-linoleoyl-3-oleoylglycerol
SOA	1-Stearoyl-2-oleoyl-arachidoylglycerol
AOO	1-Arachidoyl-2,3-dioleoylglycerol

PRECISION

1. Interlaboratory test
   Details of the collaborative trial of the method are listed in Table 1. The values derived from this interlaboratory study test may not be applicable to concentration ranges and matrices other than those given.
2. Repeatability
   The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases be greater than 0.3 g/100 g milk chocolate. Values for the repeatability limit, *r*, as found in the validation study are summarized in Table 2.
3. Reproducibility
   The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5% of cases be greater than 0.6 g/100 g milk chocolate. Values for the reproducibility limit, R, as found in the validation study are summarized in Table 2.

NUMBERED NOTES

1. Chocolate and cocoa based products may contain vegetable fats in addition to cocoa butter. Six vegetable fats can be used singly or in blends, i.e., Illipé (*Shorea spp.*); palm oil (*Elaeis guineensis, Elaeis olifera*); sal (*Shorea robusta*); shea (*Butyrospermum parkii*); kokum gurgi (*Garcinia indica*); and mango kernel (*Mangifera indica*). For correct labelling of milk chocolate, the results related to chocolate fat are converted into g MF/100 g chocolate and g CBE/100 g chocolate, requiring the accurate determination of the total fat content of the chocolate using a Soxhlet extraction procedure (References, 1). If the detection approach proves the absence of CBEs in chocolate fat, the quantification of CBEs and the determination of the total fat content of chocolate are not required.
2. For split injection, the following should be taken into consideration:
   (a) Oven program: 200°C hold for at least 1 min; 14°C/min to 270°C hold for 1 min; 2.5°C/min to 340°C hold for 10 min.
   (b) Carrier gas for split: H_2 (purity ≥ 99.999%) with a constant flow rate of 2.5 mL/min. (Another suitable carrier gas is helium.)
   (c) Dilute 1:100 (0.01 mg/mL).
   (d) Dilute each calibration solution 1:2 with *iso*-octane to obtain a final IRMM-PSB concentration ($C_{IRMM\text{-}PSB}$) of 5 mg/mL in each solution and PSB concentrations (C_{PSB}) ranging from 0.064 to 0.008 mg/mL.
   (e) Prepare five calibration solutions containing different concentrations of PSB ranging from 0.004 to 0.032 with the same concentration of alpha-cholestane of 0.005 mg/mL. Inject 1 µL of the final test solution into the HR-GLC system.
   (f) Weigh 0.1 g of IRMM-801 in a 10-mL volumetric flask and dilute to the mark with *iso*-octane. Pipette 1 mL of the resulting solution in another 10-mL volumetric flask and dilute to the mark with the same solvent (1 mg/mL). Inject 1 µL of the final test solution into the HR-GLC system.
   (g) Weigh 0.25 g of pure milk fat in a 50-mL volumet-ric flask and dilute to the mark with *iso*-octane (5 mg/mL). Transfer 1 mL of this solution in a test tube and add 1 mL of alpha-cholestane solution (resulting test sample solution 2.5 mg/mL). Inject 1 µL of the final test solution into the HR-GLC system.

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(h) Weigh 0.5 g of chocolate fat on an analytical balance in a 100 mL volumetric flask and dilute to he mark with *iso*-octane (5 mg/mL). Transfer 1 mL of this solution in a test tube and add 1 mL of alpha-cholestane solution (resulting test sample solution 2.5 mg/mL). Inject 1 μL of the final test solution into the HR-GLC system.

3. System suitability—The details of the chromatographic procedure depend, among other factors, on equipment, type, age, and supplier of the column, means of injection of the test solution, sample size, and detector. Different column lengths and brands may be used, and injection volumes may be varied, if the requirements of the system suitability tests are met.
   (a) Resolution—The HR-GLC separation system shall be capable of separating, 1. the critical pairs POS/POO and SOS/SOO with a chromatographic resolution of at least 1.0, 2. PSB from neighboring peaks within CN 38 group (References, 5) and 3. showing no co-elution for the internal standard alpha-cholestane. Adjust portion size, column temperature, carrier gas flow, etc. to optimize separation and peak resolution.
   (b) Determination of detector response factors—To check that flame-ionization detector response factors of TAGs do not differ by more than 20% from unity, analyze IRMM-801 under standard HR-GLC conditions. In a properly functioning chromatographic system the response factors for the five TAGs (POP, POS, POO, SOS, SOO) vary within a range of 0.80 to 1.20. Verify the system by repeat analysis (at least in triplicate). The relative standard deviations of the detector response factors should be less than 5%. To check the stability of the separation system, establish a calibration curve for PSB with alpha-cholestane as internal standard (inject each calibration solution at least in duplicate). Calculate the average detector response factor for PSB. Individual calibration solutions should not deviate by more than 5% from the average value.
4. The chocolate fat obtained by rapid fat extraction is used for the final TAG analysis by HR-GLC. For the detection of CBEs in chocolate the accurate amount of total fat in chocolate is not needed. When no CBEs are detected the second part of the standard, i.e., quantification of CBEs around the statutory limit, is not necessary. If CBEs are detected, the quantification part has to be performed using the same TAG profile as used for the CBE detection.
5. The use of IRMM-801 allows individual testing laboratories to verify the purity of CB without establishing a "CB-line." Calibration using IRMM-801 links the results to the cocoa butter TAG database and the decision rule (Equation 12).

REFERENCES

1. Official Methods of Analysis of AOAC International, AOAC Official Method 963.15: Fat in Cacao Products, Gaithersburg, MD, (1995).
2. Koeber, R., M. Buchgraber, F. Ulberth, R. Bacarolo, A. Bernreuther, H. Schimmel, E. Anklam, and J. Pauwels, *EUR 20781 EN,* ISBN 92-894-6036-9 (2003).
3. Buchgraber, M., and S. Androni, *EUR 22553 EN,* ISBN 92-79-03731-5 (2006).
4. Buchgraber, M., S. Androni, and E. Anklam, *J. Agric. Food Chem. 55*(9):3275–3283 (2007).
5. Buchgraber, M., S. Androni, and E. Anklam, *J. Agric. Food Chem. 55*(9):3284–3291 (2007).
6. Buchgraber, M., C. Senaldi, F. Ulberth, and E. Anklam, *J. AOAC Internat. 87*:1153–1163 (2004).
7. Buchgraber, M., F. Ulberth, and E. Anklam, *J. AOAC Internat. 87*:1164–1172 (2004).

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Table 1
Suitable HR-GLC conditions to be used for TAG analysis of chocolate fats.

Laboratory code	1	2	3	4	5	6	7	8	9	10	11	12
						Carrier gas						
Type	He	He	He	H_2	H_2	H_2	H_2	He	H_2	H_2	H_2	H_2
If constant pressure (kPa)	100	180	—	—	—	—	150	135	130	140	—	—
If constant flow (mL/min)	—	—	2.2	2	2	1.5	—	—	—	—	3.5	2
						Column characteristics						
Stationary phase	Ultimetal	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	RTx-65TG	CB-TAP	CB-TAP	CB-TAP
Length	25	25	25	25	25	25	25	25	30	25	25	25
i.d. (mm)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Film thickness (µm)	0.05	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
						Temperature mode						
Oven												
Injection temperature (°C), hold time (min)	200/2	200/1	100/0.5	200/1	200/0	200/1	100/2	200/1	200/1	200/1	100/2	200/1
Program rate 1 (°C/min)	20	14	40	14	20	24	30	14	15	30	30	14
Temperature (°C) hold time (min)	320/0	270/0	280/0	270/0	270/0	270/0	270/1	270	360/0	270/0	270/1	270/0
Program rate 2 (°C/min)	1	2.5	2.5	2.5	5	2.5	3	2	1	2.5	3.5	2.5
Temperature, hold time	360/10	340/30	340/17	340/10	340/15	340/13	340/10	340/30	370	355/2	340/7	340/10
Program rate 3 (°C/min)	—	10	—	—	25	—	—	—	—	—	—	—
Final temperature (°C), hold time (min)	—	350/9	—	—	200	—	—	—	—	—	—	—
Injector temperature (°C)	65-370	360	oven track	365	370	350		340	380	140-340	oven track	360
Detector temperature (°C)	370	360	360	365	360	370	350	360	380	350	360	360
						Injection mode						
Manuel (M)/automatic (A)	M	A	—	A	A	A	M	A	A	A	A	A
Split/on-column/PTV	PTV	split	OCI	split	split	split	OCI	split	split	PTV	OCI	split
If split (split ratio)	—	1:20	—	1:10	1:10	1:10	—	1:7	—	—	—	1:10

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Table 2
Precision data for chocolate samples (samples 5–6) and for chocolate fat samples (samples 10–13).

Sample	5	6	10	11	12	13
Number of laboratories	12	12	12	12	12	12
Number of outliers	0	1	0	0	0	0
Number of accepted laboratories	12	11	12	12	12	12
Mean value, g CBE/100 g chocolate	5.20	4.08	4.62	5.81	5.35	8.23
True value, g CBE/100 g chocolate	5.11	3.95	4.81	5.99	6.02	8.41
Bias, g CBE/100 g chocolate	–0.09	–0.13	0.19	0.18	0.67	0.18
Repeatability limit r ($r=2.8 \times s_r$), g/100 g	0.28	0.15	0.26	0.19	0.19	0.23
Repeatability standard deviation s_r, g/100 g	0.10	0.05	0.09	0.07	0.07	0.08
Repeatability relative standard deviation RSD_r, %	1.9	1.3	2.0	1.2	1.2	1.0
Reproducibility limit R ($r=2.8 \times s_R$), g/100 g	0.59	0.53	0.50	0.46	0.53	0.52
Reproducibility standard deviation s_R, g/100 g	0.21	0.19	0.18	0.17	0.19	0.19
Reproducibility relative standard deviation RSD_R, %	4.1	4.7	3.8	2.8	3.5	2.2
HorRAT value = RSD_R/predicted RSDR[a]	1.30	1.45	1.21	0.93	1.13	0.77

[a]Predicted $RSD_R = 2C^{-0.15}$ C = estimated mean concentration

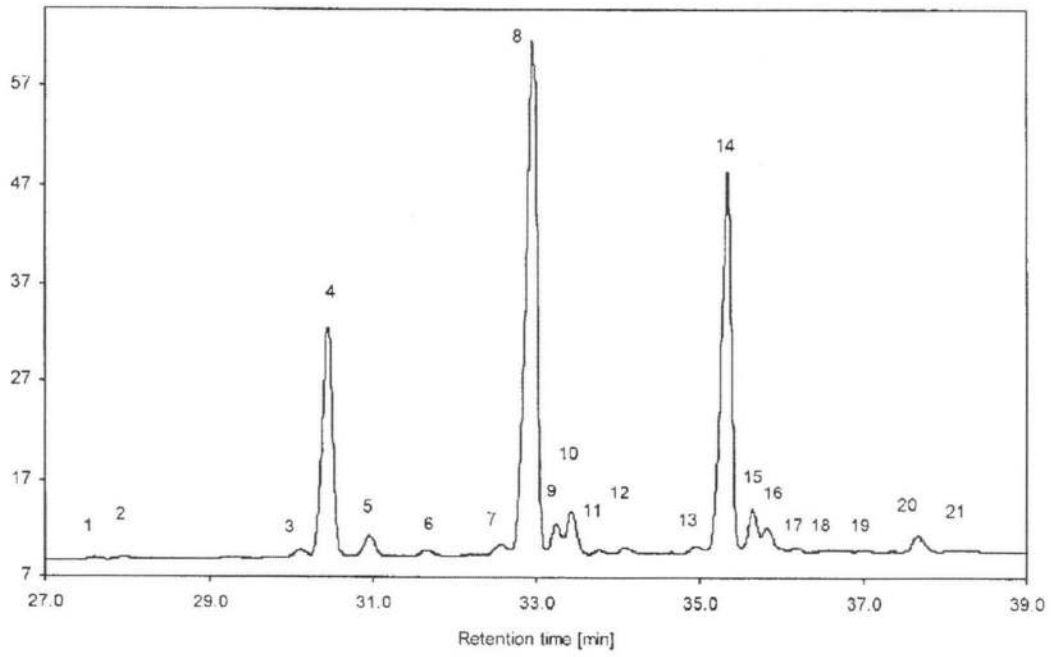

Figure 1. Triacylglycerol profile of IRMM-801.

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AOCS Analytical Guidelines Cf 1-68
Reapproved 2009

Analytical Guidelines for Testing Feed Grade Fat Products

SCOPE

(a) These Analytical Guidelines are applicable to the selection and application of procedures for testing fat products designated for use in animal feed formulations. Feed grade fat products include animal fat, vegetable fat or oil, hydrolyzed fat or oil, fatty ester, or any mixture of these components. The test methods are listed with the appropriate method number in Table 1.

(b) The use of the term "feed grade" requires that the specific type of product will have been adequately tested to prove its safety for feeding purposes.

SAMPLING

1. Fat products are frequently solids or semisolids at room temperature and should be completely liquified and blended prior to testing. Temperature during melting should not exceed the melting point of the sample by more than 10°C.
2. Sample material in accordance with AOCS Method C 1-47.

MOISTURE

1. The Karl Fischer Moisture Method, AOCS Method Ca 2e-84, is used to determine the water content of the test portion.

MOISTURE AND VOLATILES

1. These methods determine moisture and any other substances volatile under the conditions of the test. They are useful for solvent-extracted fats and oils to indicate possible residual volatile solvent. The methods do not differentiate between moisture and other volatile hydrocarbons. If such a differentiation is required, a static headspace method (e.g., AOCS Official Method Ca 3b-87, Hexane in Fats and Oils) may be used.
2. Technique is important for accuracy and precision. The methods cited in this section are highly empirical, therefore attention to detail is necessary.
3. Determine moisture and volatiles by AOCS Official Method Ca 2b-38 (Hot Plate Moisture), or by AOCS Official Method Ca 2c-25 (Air Oven).

ACID VALUE OR FREE FATTY ACID

1. The acid value is a measure of the quantity of free fatty acids and mineral acids present in the test portion. It is defined as the mg of potassium hydroxide (KOH) required to neutralize 1 g of test portion.
2. The free fatty acid content can be calculated from the acid value if the average molecular weight of the fatty acids is known, or if an assumed species (usually oleic acid) is used for the calculation. The basis for the calculation must be given with every reported value for free fatty acid, *e.g.*, "___% free fatty acids, calculated as oleic acid."
3. Determine the acid value by AOCS Official Method Cd 3d-63. To calculate as free fatty acids in terms of lauric, oleic, or palmitic acids, divide the acid value by 1.99, 2.81, or 2.56, respectively.

SAPONIFICATION VALUE

1. The saponification value is essentially a measure of the molecular weight of the fatty acid portion of the test portion, varying inversely with the molecular weight. It is not a measure of the quality of the fat or oil and it is not related to the identity of the fat or oil.
2. The ester value, which is a measure of the glyceride and alkyl ester content of the test portion, is determined by subtracting the acid value from the saponification value.
3. Determine the saponification value by AOCS Official Method Cd 3-25.

UNSAPONIFIABLE MATTER

1. The term unsaponifiable matter includes all of those substances that are not converted to water-soluble soaps by alkali and that are soluble in ordinary fat solvents. Included in this category are the longer chain aliphatic alcohols, sterols, pigments, and hydrocarbons.
2. A small amount of unsaponifiable matter is characteristic of all natural fats and oils, varying with the extraction and processing conditions. An excessive amount of unsaponifiable matter indicates contamination with nonglyceride matter, such as mineral oil, hydrocarbon resins, sterols, or other foreign substances.

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3. Because the exact amount of unsaponifiable matter obtained is governed by the partition coefficient of the matter between the soap solution and the solvent, different results may be expected if some other solvent is used. The recommended method employs hexane. Extraction techniques can affect the results, especially when severe emulsions are encountered.
4. Determine the hexane-soluble unsaponifiable matter by AOCS Official Method Ca 5b-71.

INSOLUBLE IMPURITIES

1. The insoluble impurities test is a measure of those materials such as mineral soap, dirt, meal, bone, and foreign substances that are insoluble in hot kerosene and petroleum ether.
2. Determine the insoluble impurities by AOCS Official Method Ca 3-46.

CRUDE FATTY ACIDS

1. Crude fatty acid is the hexane-soluble fraction after saponification of the test portion and acidification to split the soaps. It consists of the free fatty acids, combined fatty acids, and unsaponifiable matter.
2. Determine the crude fatty acids by AOCS Official Method Ca 5b-71.
3. Total fatty acid consists of free fatty acids and combined fatty acids. It may be calculated by subtracting the unsaponifiable from the crude fatty acid value, using AOCS Official Method Ca 5b-71.

IODINE VALUE

1. Iodine value is a convenient test for expressing the degree of unsaturation of a fatty acid. By definition, iodine value is the number of g of iodine absorbed per 100 g of test portion.
2. Determine the iodine value in accordance with AOCS Official Method Cd 1b-87.

   Note—The analyst is cautioned to follow the method exactly as written. Small deviations in test portion size, reagents, and time of reaction will definitely affect the accuracy and precision of the method. Generally, as the fatty acid chain length increases and the degree of unsaturation increases, longer reaction times may be required. This is especially true when using solvents other than carbon tetrachloride.
3. The iodine value may be calculated from the fatty acid profile obtained by GLC analysis; see AOCS Recommended Practice Cd 1d-92. The GLC method tends to give low results for both low iodine value products and for oils with an unsaponifiable content greater than 0.5% (e.g., fish oils).

TITER

1. Titer is the solidification point of fatty acids under specified conditions. The titer value can be correlated with the degree of unsaturation of a fatty acid and is occasionally useful in determining the identity of a fat, or if the fat has been blended with other fats or oils.
2. Determine the titer by AOCS Official Method Cc 12-59.

HYDROCARBONS

1. Non-volatile hydrocarbons (*e.g.,* mineral oil) are included in the unsaponifiable matter. The test for these hydrocarbons is useful in cases of suspected gross contamination during shipment or other situations. The lower limit of detection is approximately 0.1% hydrocarbons for mineral oil-like materials.
2. The test portion in solvent is passed through an aluminum oxide chromatographic column and hydrocarbons recovered from the eluate by removal of solvent. The recovery of hydrocarbons depends on their volatility; volatile hydrocarbons will be lost during evaporation of the solvent.
3. Determine the hydrocarbon content by AOCS Official Method Ca 6c-65.
4. Trace levels [mg/kg (ppm) and µg/kg (ppb)] of hydrocarbon contamination may be determined by static headspace techniques (*e.g.*, AOCS Official Method Ca 3b-87), purge and trap techniques coupled with GC/MS, or HPLC/MS techniques.

FATTY ACID COMPOSITION

1. The use of gas chromatography (GLC) for the separation of fatty acids by chain length and unsaturation is a common practice. AOCS Method Ce 1-62 is applicable to fatty acids. Work done to date shows the method to be simple and accurate with good precision.
2. All test portions must be converted to methyl esters of the fatty acids prior to analysis. Prepare the methyl esters by AOCS Official Method Ce 2-66.

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Table 1
Recommended methods for analyses.

Test	AOCS Method
Sampling	C 1-47
Moisture,	
Modified Karl Fischer Method	Ca 2e-84
Moisture and Volatiles,	
Hot Plate Method	Ca 2b-38
Air Oven Method	Ca 2c-25
Acid Value or Free Fatty Acids	Cd 3d-63
Saponification Value	Cd 3-25
Unsaponifiable Matter	Ca 5b-71
Insoluble Impurities	Ca 3-46
Crude Fatty Acids	Ca 5b-71
Iodine Value	Cd 16-87 or Cd 1d-92
Titer	Cc 12-59
Hydrocarbons	Ca 6c-65
Fatty Acid Composition,	
(Gas Chromatography)	Ce 1-62/Ce 2-66

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AOCS Recommended Practice Cg 1-83

Reapproved 2009

Correlation of Oil Volatiles with Flavor Scores of Edible Oils

DEFINITION

This Recommended Practice provides a representative condition for test portion preparation and procedures for the analysis of gas chromatographic volatiles, such as direct injection, static headspace, and dynamic headspace (purge and trap), in order to permit the generation of correlation factors for vegetable oil flavor, utilizing a trained flavor panel.

SCOPE

Applicable to deodorized edible vegetable oils with potential application to other untested edible oils.

PRINCIPLE

1. Volatiles isolated and measured from edible oils have been shown to be related to oil quality (see References, 1-7).
2. The isolated volatiles represent those formed by the breakdown of hydroperoxides and other flavor precursors as well as other free volatiles present in the samples.
3. The volatile procedure offers an objective means of evaluating oil quality and stability, if used under standardized conditions.
4. Correlation studies between volatiles and taste panel results for each particular oil, or processing treatment, such as hydrogenation, are necessary.

PROCEDURE

1. Specific test portions should be stored under standardized conditions of light or absence of light, temperature, and time prior to analysis by a formal taste panel and one of the volatile measurement techniques.
2. A typical procedure, given only as a reference to show the conditions of control of storage history, might be as follows—
   (a) The test portion of oil is stored in a 240 mL clear glass bottle with 2.25 cm of headspace. A screw cap is tightened to a consistent torque.
   (b) The test portion is then maintained at room temperature (22–25°C) or under Schaal oven conditions (60°C). Refer to AOCS Recommended Practice Cg 5-97. Storage time would normally be 2–6 months at room temperature (22–25°C) and 2–8 days at 60°C.
   (c) A separate test portion bottle (for each volatile and taste analysis) is taken at time zero and then repeated once a week for a minimum of three analyses during the storage period.
   (d) See Method Cg 4-94 on Volatile Organic Compounds (VOC) in fats and oils by gas chromatography for descriptions of three gas chromatographic methods: direct injection, static headspace, and purge and trap.
   (e) See Method Cg 2-83 on Flavor Panel Evaluation of Vegetable Oils for descriptions of sensory methods.
   (f) Volatiles should be measured against a known internal standard, or if not feasible, an external standard for final quantitation. Specific components, such as hexanal, might be used for correlation; however, total volatiles are recommended for most applications. Results are usually expressed as total parts per million relative to a standard, although integrator counts may be used.
   (g) Correlation of volatiles with taste panel scores can be established by using linear multiple regression analysis on a series of test portions taken over the storage period. Total volatiles converted to their logarithms have been preferred for correlation in some procedures vs. taste panel scores (References, 1 and 3). In either respect, the larger the database is, the more reliable will be the correlations.
   (h) The precision of the analyses should also be calculated at different levels of volatiles to establish the level of expected error. Such reproducibility studies should be established prior to attempts to establish taste panel correlations.

REFERENCES

For a list of references for flavor volatile methods successfully utilized in collaborative studies of the Flavor Nomenclature Committee see References, 6.

1. DuPuy, H.P., E.T. Rayner, J.F. Wadsworth, and M.G. Legendre, *JAOCS 54:*445 (1977).
2. Waltking, A.E., and H. Zmachinski, *JAOCS 54:*454 (1977).
3. Jackson, H.W., and D. Giacherio, *JAOCS 54:*458 (1977).

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4. Warner, K., C.D. Evans, G.R. List, H.P. DuPuy, J.I. Wadsworth, and G. E. Goheen, *JAOCS 55:*252 (1978).
5. Min, D.B., *J. Food Sci. 46:*1453 (1981).
6. Waltking, A.E., *JAOCS 59:*116A (1982).
7. Snyder, J.M., E.N. Frankel, E. Selke, and K. Warner, *JAOCS 65:*1617 (1988).

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AOCS Recommended Practice Cg 2-83
Reapproved 2009

Flavor Panel Evaluation of Vegetable Oils

DEFINITION

This Recommended Practice provides a standardized technique for the sensory evaluation of edible vegetable oils. It encompasses standard preparation and presentation of test portions, as well as the procedures for tasting and scoring the oils.

SCOPE

Applicable to all deodorized edible vegetable oils, with potential application to other untested edible oils (see Notes, 1). The scales for both intensity and quality are applicable to most testing situations from quality control to research studies.

APPARATUS

1. Isolated taste panel room—
   (a) Temperature range, 24 ± 1°C.
   (b) Lighting, white and red fluorescent light, with white light to provide 80 foot-candles of light on table area of individual booth and red light and white light to be operated separately or together.
   (c) Filtered air, under positive pressure to create an airflow out of panel room to adjacent areas.
   (d) Individual booths for panelists or tables with 45 cm (18 in.) high dividers to separate panelists.
2. Test portion presentation utensils—
   (a) 50 mL covered glass beakers.
   (b) Aluminum blocks, drilled to ½ the height of the glass beakers, to comfortably hold 4 beakers.
   (c) Hot plate or oven capable of heating the aluminum blocks to a constant temperature such that the oil attains a temperature of 50 ± 1°C.

REAGENTS

1. Carbon-filtered tap water—heated to 38°C.
2. Reference standards for oil quality and intensity—consisting of one or a group of the following, which, immediately after preparation, were constantly flushed with nitrogen until stored frozen (≤10°C) in glass jars completely filled with no headspace for up to 1 year. The jar size should be adequate for the needs of the panel for a single day's evaluation. Any excess reference oil from an opened jar should be discarded.
   (a) Control, lightly hydrogenated—100–110 iodine value (IV) soybean oil double deodorized, with 2 µg/g citric acid added after deodorization as a metal scavenger. Flavor score should be 8–9 (1–10 scale).
   (b) Buttery odor/flavor—Store control (a) 3 days at 60°C (140°F), air exposed.
   (c) Hydrogenated odor/flavor—Add freshly hydrogenated, undeodorized soybean oil (100–110 IV) to a control (a) at a level of 25%.
   (d) Bitter taste—0.1% caffeine in a good control.
   (e) Raw, grassy odor/flavor—1% undeodorized, crude soybean oil in a good control.
   (f) Corny odor/flavor—5% undeodorized corn oil in control (a).
   (g) Rancid odor/flavor—cottonseed oil aged ≥4 days at 60°C (140°F) to a peroxide value of between 5 and 10. See AOCS Recommended Practice Cg 5-97.
   (h) Painty odor/flavor—low erucic acid rapeseed oil aged ≥8 days at 60°C (140°F) (Schaal oven test) to a peroxide value of between 15 and 20.

PREPARATION AND PRESENTATION OF OIL TEST PORTION

1. Up to four 50 mL glass beakers containing 10 mL of oil each are capped with a tight-fitting ground-glass cover and are placed in hollowed-out areas of an aluminum block.
2. The aluminum blocks containing the beakers are heated in a circulating air oven or on an electrically controlled hot plate.
3. Hot plate or oven temperature is adjusted such that the oil is maintained at 50 ± 1°C.
4. Oils are heated for 30 min in the dark prior to serving and should be evaluated within 30 additional minutes.
5. If the panel size is large enough to permit using a statistical design (*i.e.*, Cochran and Cox; see References, 1), then permutations of the order of test portion presentation are balanced.
6. Test portions should be coded using random numbers of at least 3 digits.

ODOR AND FLAVOR TESTING PROCEDURES

1. Odor is used in the following manner to set the order of oil evaluation if the panel size is too small for a valid balanced statistical design to have been used to establish presentation order.

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(a) Oils are evaluated for odor in the order in which test portion are presented from left to right.
(b) Covered beaker is swirled and lifted to nose.
(c) Cover is removed as tester sniffs the volatiles.
(d) Beaker is swirled again if necessary.
(e) Test portion with the weakest odor is evaluated first for flavor; the remaining oils are evaluated in the order of increasing odor intensity.

2. Flavor—
   (a) Unless odor has been used to establish an evaluation order, evaluate oils as presented from left to right.
   (b) 10 mL of the oil is taken into the mouth and thoroughly swished throughout the mouth, or cupped on the tongue while air is drawn over or through it.
   (c) Oil is expectorated into paper waste cups.
   (d) Carbon-filtered tap water heated to 38°C is used to rinse the mouth before testing and after testing each oil.

EVALUATIONS

1. Overall scores for quality or intensity—
   Note—Quality and intensity levels on the scale must be defined for each specific oil type prior to using the scale. Listing specific flavor characteristics, such as buttery, rancid, etc., directly next to specific scores on each scale is not recommended. However, taste testers should be trained to recognize various quality or intensity levels based on specific flavor characteristics of each oil type.
   (a) If the objective of the test is to evaluate overall oil quality, the scoring scale in Table 1 is recommended. This scale is appropriate for oils with distinctive flavors, such as corn, peanut, or olive oil (see Notes, 1). Standards for the various quality levels should be determined by the flavor panel for each type of oil.
   (b) If the objective of the test is to evaluate overall oil blandness, the scoring scale in Table 2 is recommended. This scale is appropriate only for oils that have no distinct flavors immediately after deodorization, such as soybean, sunflower, or low erucic acid rapeseed (canola) oils.
2. Description of intensity scores—These scores are calculated for each odor or flavor identified in the oils. After rating for either overall quality or intensity, the panelists should identify each odor or flavor detected and rate the intensity of the odor or flavor as "weak," "moderate," or "strong."
3. Data calculation—
   (a) Calculate overall mean scores for intensity or quality by totalling all panelists' scores and dividing by the number of panelists.
   (b) Calculate description intensity scores by the following equation:
   $1 \times$ (number weak responses) + $2 \times$ (number moderate responses) + $3 \times$ (number strong responses)/number of panelists
   (c) Use statistical trial analysis, such as a two-way analysis of variance, to determine significant differences between the overall mean scores for the oils.

NOTES

1. The International Olive Council (IOC) has developed a special sensory method for olive oil. Because of the length of the method, and because of the limited application, the method is not reproduced here. It may be obtained by contacting IOC, Principe de Vergara 154, 28002 Madrid, Spain. (www.internationaloliveoil.org)

REFERENCES

1. Cochran and Cox, *Experimental Designs,* 2nd edn., J. Wiley and Sons, 1957.

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Table 1
Scoresheet for flavor quality evaluation.

Name_____ Date_____

Directions: Take 5 to 10 mL of warm oil into the mouth, pull air through the oil and evaluate the flavor by exhaling through the nose. Mark on the intensity scale below the overall flavor intensity for the sample.

Quality	Overall quality scores Sample numbers				Descriptions	Description intensity Sample numbers			
	____	____	____	____		____	____	____	____
10 Excellent	____	____	____	____	Nutty	____	____	____	____
9 Good	____	____	____	____	Buttery	____	____	____	____
8	____	____	____	____	Corny	____	____	____	____
7 Fair	____	____	____	____	Beany	____	____	____	____
6	____	____	____	____	Hydrogenated	____	____	____	____
5 Poor	____	____	____	____	Burned	____	____	____	____
4	____	____	____	____	Weedy	____	____	____	____
3 Very poor	____	____	____	____	Grassy	____	____	____	____
2	____	____	____	____	Rubbery	____	____	____	____
1____ ____	____	____			Melon	____	____	____	____
					Painty	____	____	____	____
					Fishy	____	____	____	____
					Other_____	____	____	____	____
					Other_____	____	____	____	____

Table 2
Scoresheet for flavor intensity evaluation.

Name_____ Date_____

Directions: Take 5 to 10 mL of warm oil into the mouth, pull air through the oil and evaluate the flavor by exhaling through the nose. From the master list of descriptions, list the one(s) appropriate for this sample and indicate the amount of flavor.

Intensity	Overall intensity scores Sample numbers				Descriptions	Description intensity Sample numbers			
____	____	____	____	____		____	____	____	
10 Bland	____	____	____	____	Nutty	____	____	____	____
9 Trace	____	____	____	____	Buttery	____	____	____	____
8 Faint	____	____	____	____	Corny	____	____	____	____
7 Slight	____	____	____	____	Beany	____	____	____	____
6 Mild	____	____	____	____	Hydrogenated	____	____	____	____
5 Moderate	____	____	____	____	Burned	____	____	____	____
4 Definite	____	____	____	____	Weedy	____	____	____	____
3 Strong	____	____	____	____	Grassy	____	____	____	____
2 Very strong	____	____	____	____	Rubbery	____	____	____	____
1 Extreme	____	____	____	____	Melon	____	____	____	____
					Painty	____	____	____	____
					Fishy	____	____	____	____
					Other_____	____	____	____	____
					Other_____	____	____	____	____

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AOCS Analytical Guidelines Cg 3-91
Reapproved 2009

Analytical Guidelines for Assessing Oil Quality and Stability

DEFINITION

These Analytical Guidelines list those methods which may be used to assess the quality and stability of oils and fats.

SCOPE

These Analytical Guidelines list the application of procedures for testing and measuring the quality and stability of edible oils and fats. Only AOCS Official Methods or Recommended Practices are listed in Tables 1 and 2; however, other procedures are reported in the scientific literature.

VOLATILE ORGANIC COMPOUNDS (VOC) IN FATS AND OILS BY GAS CHROMATOGRAPHY

The three methods for volatile compound analyses—direct injection, static headspace, and purge and trap—measure secondary oxidation products that can be primarily responsible for flavors in oxidized vegetable oils. The types of compounds in the oil will vary based on the type of oil being tested. For example, linoleate-containing oils will produce volatile compounds including pentane, pentanal, hexanal, and 2,4-decadienal. Other factors that affect the type of volatile compounds include oxidation conditions such as temperature and light exposure. Also, each of the three methods may show selectivity toward specific types of compounds. For example, low molecular weight compounds are detected more easily by static headspace than are higher molecular weight compounds.

PEROXIDE VALUE

This titration analysis is a measure of all substances—peroxides and similar oil or fat oxidation products—that oxidize potassium iodide under the prescribed test conditions. This test will not detect secondary oxidation products of off-quality caused by problems other than oxidation. A freshly deodorized oil or fat should have a peroxide value of zero to be considered good quality. For soy oil, peroxide levels range from 1.0–5.0 for low oxidation; 5.0–10.0 for moderate oxidation; and 10.0 and above for high levels of oxidation. A peroxide value above 20 is usually only of academic interest since the oil flavor quality is very poor.

OIL STABILITY INDEX (OSI)

The OSI is an accelerated method to determine the oil or fat stability by oxidizing the sample at 100–140°C and measuring the time required for the oil or fat to reach induction. Extrapolating the results of this test to ambient storage conditions is difficult because of the extreme heat stress on the oil or fat, especially if testing a polyunsaturated oil.

p-ANISIDINE

This spectrophotometric analysis of oil or fat oxidation products primarily measures the amounts of α- and β- unsaturated aldehydes (secondary oxidation products) present in the sample. The presence of anisidine-reactive substances in a deodorized oil may indicate prior oil oxidation or damage to the seeds before extraction. Anisidine values for an unaged soybean oil should be less than 2.0 to indicate good stability. This test will not detect off-quality from non-oxidative sources. The use of peroxide analysis and anisidine analysis together gives a more complete picture of total oxidation than each test separately. The equation is TOTOX = anisidine value + 2(peroxide value). The TOTOX value for an unaged soybean oil should be less than 4.0 to indicate good stability.

FLAVOR PANEL EVALUATION

Analysis of oil or fat flavor can only be done by human subjects. Some instrumental and chemical analyses may correlate highly with sensory evaluations but they are not capable of measuring flavor. The main disadvantage of using human testers—person-to-person variability—is diminished with training and experience. The AOCS Intensity Scale (AOCS Official Method Cg 2-83) is used for rating oils for the intensity of total flavor ranging from bland to extreme. Oils such as soy, sunflower, low erucic acid rapeseed, or cottonseed, which are processed for bland flavor, can be rated with this scale. Oils with distinct flavors such as olive, peanut, or corn should be evaluated with the AOCS Quality Scale (AOCS Official Method Cg 2-83), which has quality levels ranging from excellent to very poor. All oils may be rated on the AOCS Quality Scale; however, the Intensity Scale should not be used for oils with distinct flavors. In addition to rating overall flavor quality or flavor intensity, panel members should also indicate the individual flavor they detect and its intensity. Overall flavor should be rated first, followed by individual flavor description intensity.

General guidelines for interpreting flavor panel data are:

—Score of 8.0 for a fresh, unaged soy oil is indicative of an oil with good quality and faint overall flavor intensity.

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— Score of 5.0 for a soy oil aged 4 d at 60°C in the AOCS Recommended Practice Cg 5-97 is indicative of a poor quality oil and moderate overall flavor intensity.

CORRELATION OF OIL VOLATILES WITH FLAVOR SCORES

Numerous publications have shown that volatiles analysis can correlate highly with flavor panel evaluations. These data have identified total volatiles and/or individual volatile compounds such as pentane, pentanal, hexanal, and 2,4-decadienal that may serve as markers for oil stability. These volatiles are not individually responsible for the total flavor of the oil as in a cause-effect relationship; however, they do increase rapidly with increasing oil storage time and are indicative of oxidation levels. Flavor problems from non-oxidative sources are not detected by volatiles analysis. Data on volatiles found in the literature are difficult to extrapolate to other chromatographic conditions to determine oil stability, because the data are usually expressed as integrator counts for peaks rather than in parts per million (ppm). Integrator counts for the same oil can vary widely depending on the chromatographic conditions used.

Table 1
Methods to detect markers of oil oxidation.

Test	Method
Volatile organic compounds	AOCS Cg 4-94
Peroxide value	AOCS Cd 8b-90
Oil stability index	AOCS Cd 12b-92
p-Anisidine	AOCS Cd 18-90
Flavor panel evaluation	AOCS Cg 2-83
Correlation of oil volatiles with flavor scores	AOCS Cg 1-83

Table 2
Methods to oxidize oils.

Oxidation type	Method
Oven storage/accelerated temperature	AOCS Cg 5-97
Accelerated light storage	AOCS Cg 6-01

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AOCS Recommended Practice Cg 4-94
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Volatile Organic Compounds (VOC) in Fats and Oils by Gas Chromatography

DEFINITION

This Recommended Practice describes three gas chromatographic methods for measuring the volatile compounds related to the flavor, quality, and oxidative stability of oils and fats.

SCOPE

Applicable to edible fats and oils.

PRINCIPLES

1. The three basic gas chromatographic procedures for volatile compound analysis are direct injection (References, 1-7), static headspace (References, 8-11), and dynamic (purge & trap) headspace (References, 12-14).
2. Volatile compounds measured by gas chromatography have shown a high correlation with sensory analysis of oil quality and stability (References, 1, 3, 6, 8); AOCS Recommended Practices Cg 1-83, Cg 2-83.
3. The choice of procedure will depend on available equipment and/or on the type of volatile compounds of interest. The two headspace methods described in this method require an additional piece of instrumentation attached to the gas chromatograph to collect volatile compounds. Contact manufacturers of gas chromatographic equipment for information on headspace instruments.
4. The static headspace method is selective toward the low molecular weight compounds, such as pentane, whereas dynamic headspace is selective toward medium and higher molecular weight compounds, such as 2,4-decadienal. The entire range of low to high molecular weight compounds is detected by direct injection (14).
5. Gas chromatographic procedures such as temperature and thermostating times are shown in Table 1 with ranges to allow for individual variation that will affect the type and amount of volatile compounds detected.
6. In current methods, capillary columns have replaced packed columns in older methods and collection procedures have been modified (References, 7, 9, 18).
7. More than one volatile is usually responsible for the flavors in oils. Not all flavors present in oils can be detected by gas chromatography, e.g., metallic, bitter. Volatile compounds responsible for complex flavors, such as fishy and hydrogenated, are usually present at low levels and are not easily detected.

PROCEDURES

1. General procedures for all methods—
   (a) Times and temperatures given were used in an AOCS collaborative study. Ranges for purge temperatures have been from 50–190°C with purging times of 5–60 min. Generally shorter purging times are used with higher temperatures. Static headspace and direct injection both require higher purge (injector) temperatures than dynamic headspace. Consulting literature references and conducting methods development will help determine exact times and temperatures.
   (b) Fused silica capillary columns with either polar or nonpolar liquid phases may be used. Nonpolar phases separate based on molecular weight and boiling point, whereas polar phases separate by polarity which improves isomer separation. Nonpolar phases include polysiloxane equivalents such as OV-1, OV-5, OV-17, DB-1, DB-5, SPB-1, and Rtx-5. Polar phases include polyesters, Carbowax 20M, Stabilwax, DB-WAX, and Supelcowax. Column length depends on the type of volatiles. Longer columns with smaller diameters are generally used, such as 30 m × 0.32 mm i.d. or 60 m × 0.32 mm i.d. with 1 µm film thickness.
   (c) Temperature for detectors, usually flame-ionization detectors (FID), is typically set the same as or 10°C higher than the highest column temperature.
   (d) Compounds can be identified by matching retention times with authentic compounds or by mass spectrometry. Results in a previous collaborative study showed that all three gas chromatographic methods were able to correctly rank the four test samples in the order of no, low, moderate, and high oxidation levels.
2. Direct injection—
   (a) Condition clean, silanized glass wool for 15 hr at 250°C to remove any volatile compounds.
   (b) Place a plug of conditioned, silanized glass wool into a clean glass liner of the injector of a gas chromatograph.
   (c) Inject 2 µL of oil onto the glass wool in the liner through the injector septum, or pipet oil onto the glass wool in the liner before inserting the liner into the injector.
   (d) Depending on the material, either hold the capillary column temperature at –50°C for 1 min with the injector at 180°C in the splitless mode, or for 10 min with the injector at 120°C in the splitless mode (Table 1). Lower temperatures enhance recovery of volatile compounds.

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(e) Program the temperature to 250°C at a ramp rate specific to the column.
(f) Remove liner with glass wool from injector port.

3. Static headspace—
   (a) Place 0.5 g oil into a clean 6 or 10 mL glass vial and seal by crimping the septum cap.
   (b) Place vial in headspace magazine of headspace sampler and heat at temperature and time depending on the sample, e.g., 180°C for 5 min, 110°C for 20 min, (Table 1). A specific combination of time and temperature can be determined during methods development.
   (c) Set injector temperature at 200°C.
   (d) Move the magazine into injection position and pressurize the system for 1 min.
   (e) Hold the capillary column temperature at –50°C for 5–20 min (Table 1).
   (f) Program the capillary column temperature to 250°C at ramp rate specific for the column.
4. Dynamic headspace (purge & trap)—
   (a) Place 5 g oil in headspace tube, then heat and purge with helium at various times and temperatures depending on the sample, e.g., 60°C for 25 min; 90°C for 15 min; 180°C for 5 min, etc. (Table 1).
   (b) Condition adsorbent, such as Tenax GC, with gas flowing and at a temperature 20°C higher than desorption temperature.
   (c) Collect volatile compounds on a porous polymer trap, such as Tenax GC adsorbent, at 25°C.
   (d) Thermally desorb the volatile compounds from the polymer adsorbent at 150–220°C for up to 5 min onto the capillary column with the chromatograph in splitless mode.
   (e) Hold the capillary column temperature at –50°C during the desorption process.
   (f) Program the capillary column temperature to 250°C at 5°C/minute.
   (g) Heat trap at 270°C and backflush with helium to remove compounds left in the trap.
   (h) Run a blank with a clean sample tube between oils to check for residual contaminants.

REFERENCES

Direct Injection—

1. Evans, C.D., G.R. List, R.L. Hoffman, and H. Moser, *JAOCS 46:*501 (1969).
2. Dupuy, H.P., E.T. Rayner, and J.I. Wadsworth, *JAOCS 53:*628 (1976).
3. Warner, K., C.D. Evans, G.R. List, H.P. Dupuy, J.I. Wadsworth, and G.E. Goheen, *JAOCS 55:*252 (1978).
4. Warner, K. and E.N. Frankel, *JAOCS 62:*100 (1985).
5. Jackson, H.W. and D.J. Giacherio, *JAOCS 54:*458 (1977).
6. Jackson, H.W., G.K. Mirmira, and W.M. Wagner, *JAOCS 63:*117 (1986).
7. Dupuy, H.P., G.J. Flick, M.E. Bailey, A.J. St. Angelo, M.G. Legendre, and G. Sumrell, *JAOCS 62:*1690 (1985).
8. Warner, K., E.N. Frankel, and K.J. Moulton, *JAOCS 65:*386 (1988).
9. Przybylski, R., *Proceedings of the Eighth International Rapeseed Congress,* D.I. McGregor, ed., Organizing Committee of the Eighth International Rapeseed Congress and Canola Council of Canada, Vol. 3, pp. 861–866.

Static Headspace—

10. Warner, K., E.N. Frankel, and T.L. Mounts, *JAOCS 66:*558 (1989).
11. Marsali, R.T., *J. Chromatog. Sci. 22:*100 (1984).
12. Frankel, E.N., *JAOCS 70:*767 (1993).
13. Snyder, J. and T.L. Mounts, *JAOCS 67:*800 (1990).

Dynamic Headspace—

14. Snyder, J., E.N. Frankel, E. Selke, and K. Warner, *JAOCS 65:*1617 (1988).
15. Selke, E. and E.N. Frankel, *JAOCS 64:*749 (1987).
16. Hinshaw, J.V., *LC-GC 7:*904 (1989).
17. Nunez, A.J., L.F. Gonzalez, and J. Janak, *J. Chromatogr. Sci. 300:*127 (1984).

General Information on Volatile Compounds in Oils—

18. Waltking, A.E. and A.G. Goetz, *CRC Crit. Rev. Food Sci. Nutr. 19:*99 (1983).
19. Przybylski, R., L. Malcolmson, N.A.M. Eskin, S. Durance-Tod, J. Mickle, and R. Carr, *Lebensm. Wiss. u. Technol. 26:*205 (1993).

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Table 1
Range of gas chromatographic conditions for volatile compound analysis[a].

Conditions	Direct Injection	Static Headspace	Dynamic Headspace
Purge temp/time			60°C/25 min 90°C/15 min 180°C/5 min
Trap temp/time	120°C/10 min 180°C/1 min	110°C/20 min 180°C/5 min	25°C/5–15 min
Desorption temp			150–220°C/5 min
Injector	200°C	200°C	200°C
Initial column temp/hold time[b]	–50°C/1–10 min	–50°C/5–20 min	–50°C/5–15
Temp program rate	5°C/min	5°C/min	5°C/min
Final column temp[c]	250°C	250°C	250°C
Detector temp	250°C	250°C	250°C

[a]Times and temperatures may have to be modified depending on the type of oil.
[b]Initial column temperatures of –50°C focus the volatile compounds cryogenically at the head of the column and enhance the separation of the low molecular weight compounds. Using higher initial column temperatures may be applicable if separation of low molecular weight compounds is not an objective.
[c]Hold at 250°C until column is clear.

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Oven Storage Test for Accelerated Aging of Oils

DEFINITION

This Recommended Practice describes the procedures for an accelerated test to measure the stability of oil by aging in an oven at 60°C. Methods such as peroxide value, sensory, gas chromatographic volatile compounds are then employed to measure oxidation levels and endpoints in the oils.

SCOPE

Applicable to edible fats and oils. This practice is based on optimum conditions that would be met by controlling the various parameters; any modifications will affect results.

PRINCIPLES

1. To create accelerated conditions for aging oils, storage temperature should be greater than ambient (approximately 25°C) but less than 80°C. High temperatures (generally above 80°C) are not representative of the same oxidation mechanisms that occur at 25°C (References, 1,2). In addition, ambient temperatures cause less reproducibility than higher temperatures because increased storage time permits more time for other environmental factors to affect the samples. Thus, temperatures within the 25–80°C range provide for accelerations in oxidation while retaining the same oxidation mechanism at lower temperature conditions but without the problems of long storage periods. A 60°C storage temperature is recommended based on the use of this temperature in an AOCS collaborative study of oil oxidation and the use of this temperature in published studies of oil oxidation (see References, 3–6).
2. Oven storage tests should be conducted in the dark to avoid more than one oxidation mechanism such as both heat and light (References, 7–10).
3. Starting oils should have as little oxidation as possible to avoid having any oxidation products present in the oil acting as catalysts for further oxidation (see References, 7–10). For example, an initial peroxide value of zero is recommended.
4. The surface to volume ratio[surface area (cm)/oil volume (mL)] is a critical factor affecting rate of oxidation (see References 5,11). The ratio should remain constant from one trial to another.

GENERAL PRECAUTIONS

1. Glassware should be unetched and scrupulously clean to avoid any contamination such as oxidized material or trace metals. Detergents without surface-active agents should be used. Use deionized water for rinsing.
2. Do not expose oil to conditions that affect oxidation including high temperature, oxygen, metals, or light, prior to storage.

APPARATUS

1. Forced draft (mechanical convection) oven to operate at uniform sample temperature of 60°C ± 1°C. Refer to AOCS Specification H 1-39 for information about forced draft ovens.
2. Thermometer or thermocouple accurately calibrated.
3. Glass storage container; amber (or brown)-colored; clear glass may be used if precautions are taken to minimize exposure of oil to light while handling the samples before and after storage. Container size will be determined by amount of material needed in tests.

PROCEDURES

1. Fill container with proper amount of oil, taking care to minimize exposure of oil to conditions such as heat, light, or metals that promote oxidation. Keep the surface to volume ratio [surface area (cm) /oil volume (mL)] (surface area = $r^2 \times 3.14$) constant from one test portion to another. Examples of oil and storage containers include 250 mL in 8.8 cm beaker, 150 mL in 5.5 cm wide-mouth jar, and 75 mL in 4.5 cm beaker. Since the reaction mechanism is oxygen-dependent, as the ratio of volume to surface is increased, the oxidation rate will decrease. Narrow jars that are filled to a high level could have an altered oxidation reaction because of oxygen deficiency compared to the same container with less oil (see References,12).
2. Oil storage may be either uncovered or covered. If storage containers are covered, keep top loosely closed or stoppered. Caps or tops should be lined with an inert material and it is recommended that corks and stoppers be wrapped in inert material such as cellophane or Teflon™ tape. When using open, uncovered containers, care should be taken to avoid cross-contamination of volatile compounds from the oil in one container to that of another container (see References, 13).
3. If storage containers are covered, the amount of headspace in the containers should be uniform from one sample to another.
4. Separate storage containers are recommended for each sampling time rather than removing aliquots. Aliquots may be taken from the storage container if the amounts removed are very small and do not affect the surface to volume ratio.

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5. After storage period ends, measure oxidation level immediately in the oil or sparge headspace of container with nitrogen, tightly stopper or cap container, and store oil below 0°C until oxidation level can be measured.
6. To measure oxidation level or endpoint, use more than one method to determine extent of oxidation; preferably one each for primary and secondary oxidation products (References, 2). Tests to measure primary oxidation products include peroxide value (AOCS Cd 8-53 or Cd 8b-90) and conjugated dienes (AOCS Ti 1a-64). Tests to measure secondary oxidation products include volatile organic compounds by gas chromatography (AOCS Cg 4-94), sensory evaluation (AOCS Cg 2-83), anisidine value (AOCS Cd 18-90), carbonyl value (JOCS 2.4.22-73). See References, 11,12 for reviews of methods to measure oxidation.
7. Take measurements over a period of storage time to produce an induction curve of the oxidation products. Initial and final rates of oxidation can also be calculated.

OPTIONS

1. The test may be modified to include gentle shaking of containers to increase rate of oxidation.

REFERENCES

1. Ragnarsson, J.O., and T.P. Labuza, *Food. Chem:* 291–308 (1977).
2. Frankel, E.N., *Trends in Food Sci. Tech. 4:*220–225 (1993).
3. Warner, K., and T. Nelsen, *J. Am. Oil Chem. Soc. 73:*157–166 (1996).
4. Warner, K., E.N. Frankel, and T.L. Mounts, *J. Am. Oil Chem. Soc. 4:*558–564 (1989).
5. Malcolmson, L.J., M. Vaisey-Genser, R. Przybylski, and N.A.M. Eskin, *J. Am. Oil Chem. Soc. 71:*435–440 (1994).
6. Warner, K., in *Food Lipids and Health,* (McDonald, M., and Min, D., eds.), Marcel Dekker, Inc., New York, pp. 345–369 (1995).
7. Frankel, E.N., *in Flavor Chemistry of Fats and Oils,* (Min, D., and Smouse, T., eds.), American Oil Chemists' Society, Champaign, IL, pp. 1–36 (1985).
8. Frankel, E.N., *Prog. Lipid Res. 19:*1–22 (1980).
9. Frankel, E.N., *Prog. Lipid Res. 19:*197–222 (1984).
10. Frankel, E.N., *Prog. Lipid Res. 22:*1–33 (1982).
11. Garcia-Mesa, J.A., M.D. Luque de Castro, and M. Valcarcel, *J. Am. Oil Chem. Soc. 70:*245–247 (1993).
12. Labuza, T.P., *Critical Reviews in Food Technology 2:*355 (1971).
13. el-Magoli, S.B., M. Karel, and S. Yong, *J. Food Biochemistry 3:*111–123 (1980).
14. *Methods to Assess Quality and Stability of Oils and FatContaining Foods,* AOCS Press, Champaign, IL (1995).

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AOCS Recommended Practice Cg 6-01

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Accelerated Light Exposure of Edible Vegetable Oils

DEFINITION

This Recommended Practice describes procedures for accelerated tests to photoxidize oils under controlled conditions in order to evaluate the stability of oil to light. No light exposure apparatus is available commercially; therefore, three published procedures are described by which to photoxidize oils. The three procedures represent a range of cost as well as complexity in reproducing each apparatus. To measure oxidation levels and end points in the oil after photoxidation, methods such as peroxide value, odor or flavor by sensory analysis, and gas chromatographic volatile compounds are used.

SCOPE

Applicable to edible vegetable oils. This practice is based on optimal conditions that would be met by controlling the various parameters; any modifications will affect results.

PRINCIPLES

1. These methods are intended to create accelerated conditions for evaluating the stability of oil to light. For accelerated light oxidation, light intensity should be greater than ambient which is generally 700 lux. Light intensity levels range from 4200 to 7500 lux in the following three procedures. Temperature in the storage chamber should be as close to ambient (25°C) as possible to avoid effects of heat on the oil. Temperature of the storage chamber should remain constant during the test to avoid effects of temperature changes. An accelerated light storage condition of 7500 lux was used in an AOCS collaborative study of the effects of light oxidation on oils (1).
2. Light exposure tests should be conducted under constant light and temperature conditions. Care should be taken to avoid more than one oxidation mechanism such as both heat and light. These methods oxidize oil at controlled light and temperature conditions in contrast to using uncontrolled conditions such as placing a container of oil on the laboratory bench or near natural light with fluctuating light intensity and temperature.
3. Starting oils should have as little oxidation as possible to avoid having any oxidation products present in the oil acting as catalysts for further oxidation (2). Instrumental, sensory, and/or chemical methods of analyzing oxidation (as listed in AOCS Cg 3-91, Recommended Practice for Assessing Oil Quality and Stability) should be used, such as peroxide value (AOCS Cd 8b-90), volatile oxidation products (AOCS Method Cg 4-94), or sensory analysis (AOCS Method Cg 2-83). For example, an initial peroxide value of zero is ideal; however, it is recommended that the peroxide value of the starting oil be as low as possible—preferably less than 1.0.
4. The surface-to-volume ratio [surface area (cm)/oil volume (mL)] is a critical factor affecting rate of oxidation (3,4). The ratio should remain constant from one trial to another.
5. By exposure to light, the light stability of vegetable oils can be determined rapidly within hours or a few days. Decreasing the light intensity and/or increasing the length of light oxidation can allow autoxidation to occur also and the test would not be a measure of light oxidation only. The mechanism of photoxidation is different from that of autoxidation (2,5,6). Although the types of volatile degradation products would be similar in the two types of oxidation, the amounts of specific compounds would vary significantly (5,6). Therefore, characteristic flavors produced from light oxidation and from dark oxidation will vary accordingly (7). This test is especially useful for soybean oil and other vegetable oils that are sensitive to light oxidation. This light stability test cannot be used as a substitute to determine effects of autoxidation.
6. Storage container size will be determined by amount of oil needed for analysis.

GENERAL PRECAUTIONS

1. None of the apparatus are commercially available as described and must be assembled for each laboratory.
2. Glassware should be unetched and scrupulously clean to avoid any contamination such as oxidized material or trace metals. Detergents without surface-active agents should be used. Use deionized water for rinsing.
3. Prior to the storage test, oil should not be exposed to conditions that affect oxidation including high temperature, oxygen, metals, or light.
4. Because of the higher light intensities, temperature in the storage chamber may increase from ambient level; however, the temperature should be as close to ambient as possible.
5. Light intensity of bulbs will change over time; therefore, light intensity should be measured regularly with a light meter and bulbs replaced to maintain light intensity level.

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METHOD A

Apparatus (Fig. 1)

1. Metal storage cabinet—(122 cm wide × 147 cm high × 56 cm deep with shelf positioned 18 cm from light source) with temperature control; two 40 watt cool white rapid-start fluorescent bulbs 89 cm in length mounted on ceiling of cabinet.
2. Thermometer or thermocouple—accurately calibrated.
3. Light meter—to set light intensity on oil surface.
4. Pyrex glass crystallizing dish—(125 mm diameter × 65 mm high).

PROCEDURES

1. Place 270 g oil in uncovered 125 mm × 65 mm crystallizing dish (surface/volume, 0:4).
2. Position dish under lights to give light intensity of 4500 lux at surface of oil in modified metal cabinet (Fig. 1). Door should be closed during test.
3. Maintain temperature of storage cabinet at 40°C.
4. Use one dish for each sampling time.

METHOD B

Apparatus (Fig. 2)

1. Round metal cylinder—(42 mm diameter × 42 mm height) (open at top and bottom) with 6–35 mm, 14 watt daylight fluorescent tubes mounted vertically on the walls of the cylinder. Base is elevated 3 mm from bench/table top to allow for air circulation. 22 mm diameter metal platform in middle of cylinder revolves with 4–7.5 mm metal platforms rotating individually to allow for even light exposure.
2. Thermometer or thermocouple, accurately calibrated.
3. Light meter to set light intensity on oil surface.
4. 250 mL narrow-mouthed, clear glass bottles with cap with Teflon™ lined plastic screw-top lids.

PROCEDURES

1. Fill 250 mL narrow-mouthed, clear glass bottles to 2/3 level with oil; cap with Teflon™ lined plastic screwtop lids; air in the headspace.
2. Position bottles on individual revolving platforms so that a light intensity of 7500 lux is maintained on the sides of the storage bottles (Fig. 2).
3. Maintain temperature of storage cabinet at 30°C.
4. Store oil 2 to 16 h depending on light stability of oil; sample every 2 h.
5. Use one bottle for each sampling time.

METHOD C

Apparatus (Fig. 3)

1. Fluorescent light fixture with two 40 watt fluorescent bulbs (300 cm length) suspended on two 56 cm-high weighted ring stands.
2. Aluminum foil to cover light and ring stand; overall enclosure dimensions 366 cm length × 46 cm width × 56 cm height. Assembled unit may be placed in vented box or in large cabinet.
3. Thermometer or thermocouple, accurately calibrated.
4. Light meter to set light intensity on oil surface.
5. 120 mL wide-mouth clear glass jars.
6. Clear plastic wrap.

PROCEDURES

1. Place 100 g oil in 120 mL wide-mouth clear glass jar; cover jar loosely with clear plastic wrap.
2. Position jar under lights to give light intensity of 4200 lux at surface of oil in the aluminum foil enclosure.
3. Maintain temperature of storage unit at 25 ± 1°C.
4. Determine oxidation level every 12 h during 60 h exposure period.
5. Use one jar for each sampling time.

POST-STORAGE HANDLING OF OIL

1. Transfer oil from storage container to narrow-mouth amber glass bottle; blanket headspace with inert gas (nitrogen, argon), and freeze at 0°C to −10°C until oxidation level is measured.

METHODS TO DETERMINE OXIDATION LEVELS (9)

1. Flavor (AOCS Method Cg 2-83 for sensory evaluation procedures)
2. Gas chromatographic volatile compounds (AOCS Method Cg 4-94)

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3. Peroxide value (AOCS Method Cd 8b-90). It is recommended that more than one method be used to determine the level of oxidation in the oil and that at least one method measure primary oxidation products and another method measure secondary oxidation products or effects of the oxidation products (10).

REFERENCES

1. Waltking, A.E., Progress Report of the AOCS Flavor Nomenclature and Standards Committee, *J. Am. Oil Chem. Soc. 59:*116A-120A (1982).
2. Frankel, E.N., *Lipid Oxidation,* The Oily Press, Dundee, Scotland (1998).
3. Malcolmson, L.J., M. Vaisey-Genser, R. Przybylski, and N.A.M. Eskin, *J. Am. Oil Chem. Soc. 71:*435–440 (1994).
4. Przybylski, R., L.J. Malcolmson, N.A.M. Eskin, S. Durance-Todd, J. Mickle, and R.A. Carr, *Lebensmittel Wissenschaft und Technologie 23:*205–209 (1993).
5. Frankel, E.N., W.E. Neff, and E. Selke, *Lipids 16:*279–285 (1981).
6. Frankel, E.N., W.E. Neff, E. Selke, and D. Weisleder, *Lipids 17:*11–18 (1982).
7. Warner, K., and E.N. Frankel, *J. Am. Oil Chem. Soc. 64:*213–218 (1987).
8. Hall III, C., S. Cuppett, D. Wheeler, and X. Fu, *J. Am. Oil Chem. Soc. 71:*533–535 (1994).
9. Methods to Assess Quality and Stability of Oils and Fat-Containing Foods, K. Warner and N.A.M. Eskin (eds.), American Oil Chemists' Society, Champaign, IL (1995).
10. Frankel, E.N., *Trends in Food Sci. and Tech. 4:*220–225 (1993).

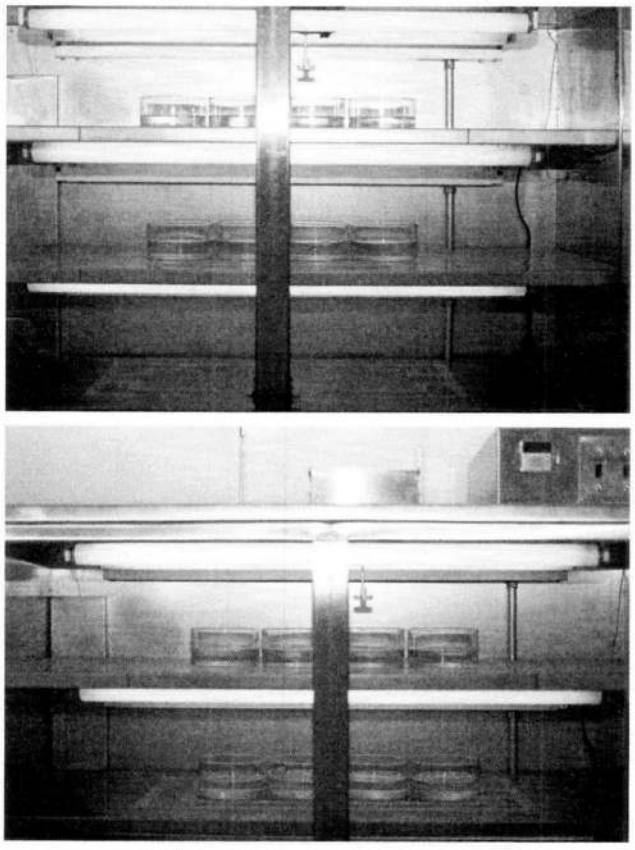

Figure 1. Method A. Light cabinet showing placement of oil samples.

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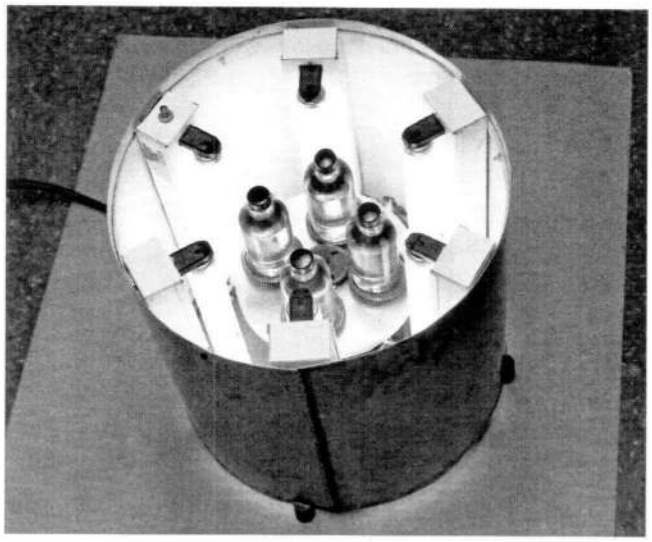

Figure 2. Method B. Metal cylinder containing oil samples in bottles.

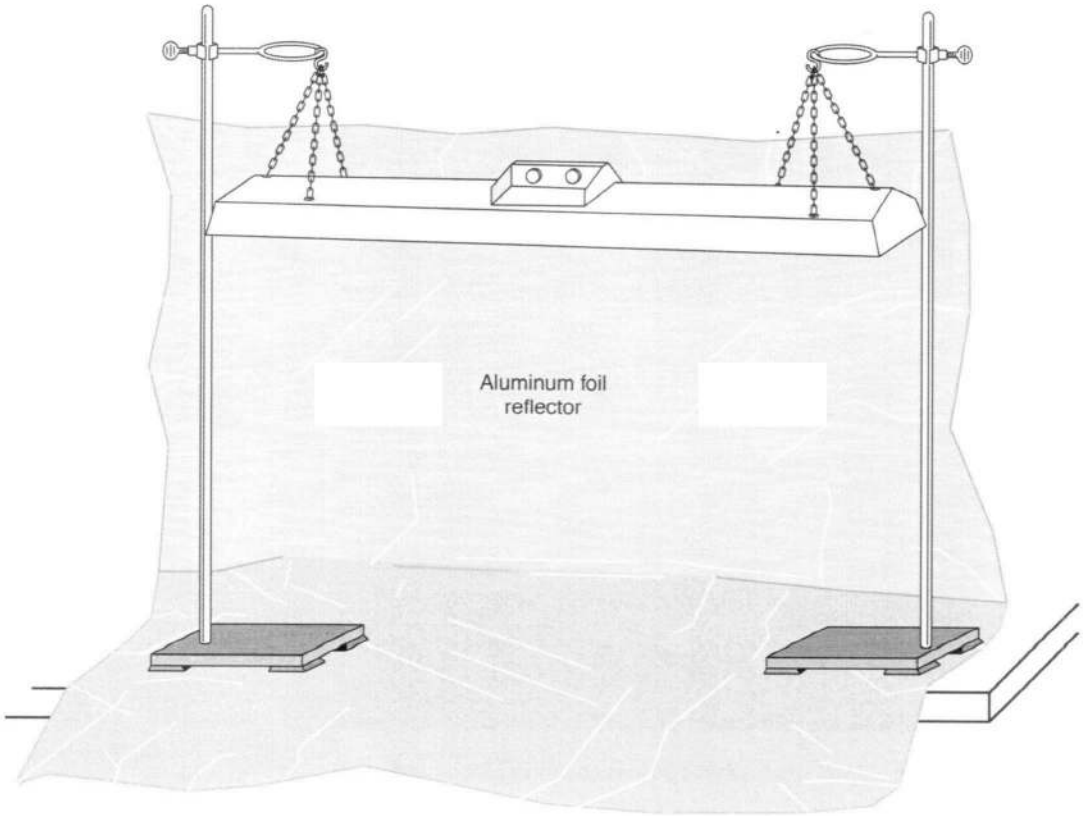

Figure 3. Method C. Line drawing of stand and fluorescent light arrangement. Aluminum foil is used to surround the test portions and focus the light.

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AOCS Analytical Guidelines Cg 7-05
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Techniques for Assessing the Effects of Antioxidants in Oils and Fats

DEFINITION

These Analytical Guidelines describe the procedures for evaluating the effects of antioxidants in vegetable oils and fats such as soybean, canola, sunflower, corn, cottonseed, rice bran, and peanut that are intended for food applications.

SCOPE

These Analytical Guidelines are for measuring the effects of antioxidants in vegetable oils or fats and in purified oils or fats that have been stripped of all minor oil constituents. A procedure is included for stripping minor oil constituents from oils. Protocols are included for oxidizing oils or fats containing either naturally occurring antioxidants or added antioxidants. Protocols are included for measuring the amount of oxidation and the effectiveness of the antioxidants in inhibiting oxidation. These Analytical Guidelines are not intended for emulsions because they behave differently than oils in antioxidant testing and require separate procedures.

PROCEDURES FOR OXIDIZING PURIFIED OR UNPURIFIED OILS BY TEMPERATURE OR LIGHT

1. Autoxidation: Refer to AOCS Recommended Practice "Oven Storage Test for Accelerated Aging of Oils" (Cg 5-97) (References, 1) for procedures. The storage oven should be set no higher than 60°C. Higher temperatures may produce misleading results that may not agree with ambient temperature storage, because the mechanism of oxidation for antioxidants is different (References, 2). Sampling should be done a minimum of every two days in order to determine induction period.
2. Photo-oxidation: Refer to AOCS Recommended Practice "Light Exposure Procedures for Oils" (Cg 6-02) (References, 1) for procedures. Sampling during photooxidation should be carried out at set times such as every 4, 8, 12, or 24 hours depending on the rate of oxidation.
3. End point: Set a defined end point to indicate the maximum level of oxidation that is preferred, relative to the goal of the experiment prior to initiating the project. For example, a typical end point is the number of hours to reach a peroxide value of 10 or 20 meq/kg. A peroxide value of 20 is indicative of poor quality oil.
4. Size of storage container: Refer to AOCS Recommended Practice "Oven Storage Test for Accelerated Aging of Oils" (Cg 5-97) (References, 1) for procedures on shape and size of container and many other details to use for oxidizing the oil test portion. The size will depend on the amount of oil to be tested. For example, a 240 mL bottle with narrow mouth can be filled 2/3 full of oil with 1/3 air in the headspace. To sample the oil, either of the following options may be used.
   a. Option 1: Use smaller individual glass bottles or jars with sufficient oil for one analysis with some left over to assure randomness. This controls the surface area-to-volume ratio that is a factor in oxidation.
   b. Option 2: Use larger glass bottles or jars and take sub-samples over time. This allows an over-time analysis using the statistical procedure of Repeated Measures (References, 3) and allows evaluation of the reliability of the replicates.

METHODS TO MEASURE LEVELS OF OXIDATION (REFERENCES, 1)

Test portions should be analyzed for oxidation level by more than one procedure and should include analyses of primary oxidation products such as peroxide value (AOCS method Cd 8-53) (References, 1) or conjugated dienes (AOCS Method Ti 1a-64 (References, 1) and secondary oxidation products such as volatile compounds (AOCS Method Cg 4-94 (References, 1) or anisidine values (AOCS Method Cd 18-90 (References, 1). Secondary oxidation products usually correlate well with flavor. If only two chemical and instrumental methods are used, peroxide value and gas chromatographic volatile compounds are recommended. The nature of headspace volatile compounds usually depends on the fatty acid composition of the oil/fat unless a contaminant is present. For example, hexanal is derived from linoleic acid and propanal from linolenic acid; other volatile compounds can also be monitored, as well as total volatile if the method is properly standardized (References, 4). Odor and flavor analysis of the oil/fat by trained sensory judges is recommended.

Flavor or odor evaluations of the oil/fat should be conducted according to the AOCS Recommended Practice Cg 2-83 (References, 1).

ANTIOXIDANT SAMPLE

For reference compounds, use pure synthetic antioxidants, such as BHA, BHT, or TBHQ, or natural antioxidants such as pure tocopherols or tocopherol mixtures of known concentrations, or crude extracts of known composition, such as rosemary or tea extracts. When the effects of crude plant extracts are evaluated, it is ideal to measure the amounts of individual phenolic compounds and other antioxidants present in the extract; however, this may be difficult if all the phenolic compounds cannot

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be identified. Alternatively, the total phenol contents can be measured by the Folin-Ciocalteu spectrophotometric method to give the total phenol content expressed as gallic acid equivalents and reported in order to compare samples (References, 5). The use of a crude extract of unknown composition limits the conclusions that can be drawn since no actual cause and effect from specific compounds can be determined. The use of pure compounds allows a better understanding of how the compounds function as antioxidants alone and in combination with other compounds, and of the assessment of their stability and the effect of degradation products on their activity. If appropriate, measure the disappearance of the additive(s) after oxidation. For example, tocopherol contents can be measured with high performance liquid chromatography by AOCS Method Ce 8-89 (References, 1) or antioxidants such as TBHQ can be measured with high performance liquid chromatography by AOCS Method Ce 6-86 (References, 1).

STATISTICAL DESIGN AND ANALYSIS

1. Clearly define the experimental design. Conduct statistical analyses to establish treatment effects such as Analysis of Variance (ANOVA).
2. Indicate the level of significance; report significant differences in tables or figures such as Least Significant Differences (LSD) of the means or other common statistical notations.

PROCEDURES FOR STRIPPING OR PURIFYING VEGETABLE OIL

1. Select refined, bleached, and deodorized oil without added antioxidants. Utilize molecular distillation procedures according to manufacturer's instructions or strip oil by the following two-stage process of bleaching and purification (References, 6).
2. Batch process bleaching: The bleaching material, blend of Actisil:Activated Charcoal:Celite:HyFlo SuperCel (37:33.3:14.8:14.8, w/w, respectively) should be used at a ratio of 1.67:1 of oil) and added slowly to the stripping vessel (e.g., round-bottom flask) containing 1:3 oil:hexane. Stir the oil-solvent mixture during addition of bleaching materials with a stirring bar. Then seal the system under a flow of nitrogen and maintain stirring until bleaching is complete, which usually requires between 2–4 hours depending on how much time is needed to remove all the tocopherols. Beginning at 2 h, open the system and take a small subsample of oil of approximately 5–10 grams. Filter the oil through #1 Whatman filter paper under a stream of nitrogen, then remove hexane under vacuum before analyzing the oil for tocopherol content (AOCS method Ce 8-89 (References, 1). If tocopherols(s) remain, continue the stripping process and take oil subsamples at regular intervals and analyze oil for tocopherol(s). After bleaching is complete, remove bleaching material by filtration through #1 Whatman filter paper under a stream of nitrogen. Maintain a headspace of nitrogen during filtration because of the increased susceptibility of bleached oil to oxidation.
3. Purification: Conduct the second stage of the stripping process using column chromatography. First remove solvent from the oil-hexane mixture from the bleaching stage using a roto-evaporator at 30°C. Then dissolve the oil in hexane (1:2, oil:solvent). Pack dry, a blend of silicic acid:absorptive magnesia:HyFlo SuperCel: Florisil (36.4:27.2:18.2:18.2, w/w, respectively) over Actisil (0.1 parts to the amount of purification material) into a chromatography column (300 mm length × 11 mm i.d.). Condition the chromatographic column prior to filtration of the oil-hexane mixture by passing hexane through the column. Use vacuum to pull the oil-hexane mixture through the column that is kept under nitrogen and covered with aluminum foil to protect the oil from light exposure. Analyze oil for removal of all carotenoids and chlorophyll (AOCS method Cc 13d-55 using spectrophotometric methods (References, 1,6), tocopherols (AOCS method Ce 8-89 (References, 1) and fatty acid composition by gas chromatography (AOCS Official Method Ce 1-62) (References, 1) to check that no significant changes occurred.
4. Store bleached oil in hexane, in amber bottle, under nitrogen at –20°C. When ready to conduct oxidation studies with the oil, remove hexane from the oil with a rotoevaporator at 30°C, breaking the vacuum with nitrogen and purge the oil by bubbling gently with nitrogen. Protect the oil from light during this process. Use the oil immediately (within 24 hr) after hexane is removed. Check the peroxide value of the oil before oil is used for antioxidant testing (AOCS Official Method Cd 8b-90) (References, 1). Peroxide value of oil should be low (<1.0) to avoid problems with oxidized material in starting oil that can act as a catalyst for further oxidation.

OTHER GUIDANCE AND NOTES (SEE ALSO REFERENCES, 2,7)

1. Substrates

   Use triacylglycerols or oils/fats. Oils/fats can be used that contain the naturally occurring range of minor constituents and antioxidants such as tocopherols. If oils without any minor constituents are needed, strip or purify oils by molecular distillation or use oils stripped of all minor constituents that are available commercially. Alternatively, use the bleaching and chromatographic purification procedures described previously in this practice to strip oils/fats of all non-triacylglycerol compounds. In addition, companies specializing in distillation technologies can be contracted to prepare stripped oil. Avoid the use of free fatty acids as a substrate because they form micelles, and of fatty acid methyl esters because the effect of antioxidants is different than in triacylglycerols. Protect the oil/fat from unintended oxidation during handling before and after oxidation conditions by keeping the oil/fat under inert gas such as argon or nitrogen. Protect the oil/fat from light by using amber bottles and covering processing equipment with aluminum foil. The unpurified oil/fat should be analyzed for fatty acid composition and amounts of minor constituents such as metals, pigments, mono- and diacylglycerols, tocopherols, chelators, and phytosterols before adding antioxidants and should be analyzed subsequently for the level of the added

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antioxidant(s). The levels of the added antioxidant(s) should be measured after its addition if possible. The purified oil should be analyzed for its composition before and after purification and should be analyzed for the level of the antioxidant after its addition.

2. Oxidation conditions
   Use various oxidation conditions including different temperatures (between 25 and 60°C) or controlled light exposure. Oxidation levels should not be extreme but should be relevant to real storage conditions. Avoid high temperatures for oxidation because the mechanism for oxidation is different at high temperatures (above 60°C) than at ambient temperature (References 2,7). Avoid high oxidation levels such as peroxide values higher than 20–30, because they are not relevant to actual food samples and exceed normal levels of rancidity. For example, an AOCS collaborative study reported that corn, sunflower, canola, and soybean oils oxidized to peroxide values of 17.2–17.9 had significant levels of rancid and/or painty flavors (References, 8). Care should be taken not to splash oil on sides of storage containers as the results may vary.
3. Analyses
   Measure both initial primary oxidation products (hydroperoxides, peroxides, conjugated dienes) and secondary decomposition products (carbonyls, volatile compounds). Sensory evaluations of odor or flavor should be conducted to confirm results from instrumental and chemical analyses used to measure oxidation products. Use methods that are sufficiently sensitive to measure relatively low levels of oxidation (below 0.1% or peroxide values below 5–10). Methods that measure specific oxidation products such as volatile compounds are preferable to methods that measure non-specific compounds or changes in the oil such as the TBA test, weight gain, decrease of linoleic or linolenic acid, or oxygen absorption. Measure the disappearance of naturally occurring compounds in the oil such as tocopherols or of added antioxidants.
4. Antioxidant concentrations
   Use same molar concentration of active components. If the amounts of individual phenolic compounds in crude plant extracts cannot be measured, measure and report total phenol contents in order to compare data with other investigators.
5. Other additives
   Other types of additives such as metal chelators may be used, but their presence and amount used should be indicated in the report of the data.
6. Presentation of calculations
   Express results as one of the following: induction period, % inhibition of hydroperoxide formation, rates of hydroperoxide formation or decomposition, I_{50} (antioxidant concentration required to achieve 50% inhibition). Investigator should refer to papers in the scientific literature on antioxidants to get details on these calculations.

REFERENCES

1. *AOCS Official Methods and Recommended Practices,* 5th edition, AOCS Press, Champaign, IL (1998).
2. Frankel, E. N. Lipid Oxidation, Oily Press, Dundee, Scotland, 1998, pp. 129–160.
3. Kuehl, R.O. Design of Experiments: Statistical Principles of Research Design and Analysis, Chapter 15, pp. 492–519, Duxbury Thomson Learning, Washington, DC (2000).
4. Frankel, E.N., *Progress in Lipid Research 19:*1–22 (1980).
5. Satue, M.T., S.W. Huang, and E.N. Frankel, *J. Am. Oil Chem. Soc. 72:*1131–1137 (1995).
6. Hall III, C., and S. Cuppett, *J. Am. Oil Chem. Soc. 77:*937–943 (1993).
7. Frankel, E.N., and A.S. Meyer, *J. Sci. Food Agric. 80:*1925–1941 (2000).
8. Warner, K., and T. Nelsen, *J. Am. Oil Chem. Soc. 73:*157–166 (1996).

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AOCS Official Method Ch 1-91

Reapproved 2009

Preparation of Methyl Esters of Long-Chain Fatty Acids

DEFINITION

This method provides a means for preparing methyl esters of long-chain fatty acids for further analysis by GLC, as in AOCS Official Methods Ch 2-91 (olive oil, capillary GC), Ce 1-62 (fats and oils, packed-column GLC), Ce 1b-89 (marine oil, GLC) and Cd 14-95 (*trans* fatty acids by IR).

SCOPE

The method is applicable to common fats, oils, and fatty acids (see Notes, 1). Unsaponifiables are not removed but, if present in large amounts, they may interfere with subsequent analyses. The procedure will result in partial or complete destruction of the following groups: epoxy, hydroperoxy, cyclopropenyl, cyclopropyl, conjugated polyunsatured, acetylenic, and possibly hydroxyl, and is not suitable for the preparation of methyl esters of fatty acids containing these groups.

APPARATUS

1. Flasks—50 and 125 mL flat-bottom boiling flasks, or Erlenmeyer flasks with ground glass $^{24}/_{40}$ outer necks.
2. Water-cooled condensers—Liebig or West design, 20–30 cm jacket, with ground glass $^{24}/_{40}$ inner joint.
3. Separatory funnels—250 mL.
4. Boiling flask—200 mL, for solvent removal.
5. Boiling chips—free of fat.

REAGENTS

1. BF_3-Methanol reagent (125 g BF_3 per liter of methanol)—available commercially, or may be prepared using the gas and methanol [see Section 3 (d) of American Society for Testing and Materials Method D- 1983-64T, or References, 1].
2. Sodium hydroxide (NaOH)—0.5 M in methanol.
3. Sodium chloride (NaCl)—saturated solution in water.
4. Petroleum ether—redistilled, bp 30–60°C (see Notes, *Caution*).
5. Heptane—gas-chromatographically clean (see Notes, *Caution*).
6. Sodium sulfate (Na_2SO_4)—anhydrous, reagent grade.
7. Methyl red indicator—0.1% in 60% ethanol.
8. Nitrogen gas—high purity.

PROCEDURE

1. Accurate weighing is not required. Test portion size need be known only to determine the size of flask and amounts of reagents that should be used according to the following table:

Test portion, mg	Flask, mL	NaOH 0.5 M, mL	BF_3-Methanol reagent, mL
100–250	50	4	5
250–500	50	6	7
500–750	125	8	9
750–1000	125	10	12

2. For fatty acids—
   (a) Introduce the fatty acids into the 50 or 125 mL reaction flask. Add the specified amount of BF_3-methanol reagent, attach a condenser and boil for 2 min (see Notes, 1). Add 2–5 mL of heptane through the condenser and boil for 1 min longer. Remove from heat, remove condenser and add about 15 mL of saturated sodium chloride solution (Reagents, 3). Stopper the flask and shake vigorously for 15 sec while the solution is still tepid. Add sufficient saturated sodium chloride solution to float the heptane solution of the methyl esters into the neck of the flask (References, 2). Transfer about 1 mL of the heptane solution into a test tube and add a small amount of anhydrous sodium sulfate. The dry heptane solution may then be injected directly in a gas chromatograph. (See Notes, 2.)

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(b) To recover dry esters, transfer the salt solution and heptane phase to a 250 mL separatory funnel. Extract twice with 50 mL portions of redistilled petroleum ether (bp 30–60°C). Wash the acids (test water with methyl red indicator), dry with sodium sulfate and evaporate the solvent under a stream of nitrogen on a steam bath (see Notes, 3 and 4).

3. For fats and oils—
   (a) Introduce the fat into the 50 or 125 mL reaction flask. Add the specified amount of 0.5 M methanolic sodium hydroxide and add a boiling chip. Attach a condenser and heat the mixture on a steam bath until the fat globules go into solution. This step should take 5–10 min. Add the specified amount of BF_3-methanol reagent through the condenser, and proceed as directed in the fatty acid section (Procedure, 2).
4. Alternate method for fats and oils (acid value <2) (References, 3)—An alternate method for the preparation of fatty acid methyl esters (FAME) is as follows: Accurately weigh a test portion of analytical sample (approximately 200 mg) into a glass-stoppered centrifuge vial. Add 2.0 mL of hexane (see Notes, *Caution*) or heptane, followed by 0.1 mL of 2 M methanolic KOH. Close the vial and shake well for 30 sec, centrifuge, remove two drops of the upper layer and dilute with 2.0 mL of hexane or heptane. The concentration of the FAME in hexane is approximately 0.5%. Inject 0.2 µL for capillary column GLC analysis.

NOTES

Caution

Petroleum ether, hexane, and heptane are extremely flammable. Use effective fume removal device. Avoid static electricity.

The BF_3-methanol reagent has a limited shelf life, even when refrigerated, and the use of old or too-concentrated solutions can result in the production of artifacts and losses of appreciable amounts of polyunsatured fatty acids (References, 4).

NUMBERED NOTES

1. The required reaction time appears to increase as both the carbon number and degree of unsaturation increase. For longer chain and more unsaturated fatty acids, a general observation is that the reaction should be allowed to proceed just until there is no visual evidence of oil globules in the reaction mixture.
2. It is recommended that all methyl esters be washed with water (to the disappearance of a methyl red end point) prior to injection. An alternative is to partition the hexane-methyl ester phase against water containing a small amount of mixed-bed (indicating resin).
3. There is a danger of losing some of the more volatile esters if the solvent-removal step is prolonged, or if too vigorous a stream of nitrogen is used. For infrared spectroscopy, this step should be terminated as soon as all solvent is removed. For gas-liquid chromatography the method may be extended to fatty acids with eight carbon atoms, if the solvent is not completely removed.
4. The methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen in a screw-cap vial at 2°C for 24 hr. For longer storage they should be sealed in a glass ampule, subjected first to a vacuum and then backfilled with nitrogen and stored at –20°C (freezer).

REFERENCES

1. *Anal. Chem. 38:*514 (1966).
2. *J. Chromatogr. 247:*63 (1982).
3. *Standard Methods for Analysis of Oils, Fats and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 2.301.
4. *INFORM 3:*1031 (1992).

OTHER REFERENCES

*J. Am. Oil Chem. Soc. 70:*325 (1993).
*J. Am. Oil Chem. Soc. 73:*275 (1996).

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AOCS Official Method Ch 2-91

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Determination of Fatty Acids in Olive Oils by Capillary GLC

DEFINITION

This method is for the determination of fatty acid methyl esters in olive oils by capillary gas-liquid chromatography.

SCOPE

This method is applicable to the determination of fatty acid methyl esters obtained from olive oils according to AOCS Official Method Ce 2-66, Ch 1-91 IUPAC Standard 2.301, AOAC 969.33, or other accepted standard methods.

APPARATUS

The instructions given apply to normal equipment used for gas-liquid chromatography employing capillary columns and flame-ionization detection.

1. Gas-liquid chromatograph.
2. Injection system.
   Note—The injection system should be specially designed for use with capillary columns. It should be of the split-type or on-column (see Notes, 1).
3. Oven.
   Note—The oven should be capable of heating the column to at least 220°C and of maintaining the desired temperature to within ± 0.1°C (see Notes, 2).
4. Capillary column, with the following general specifications—constructed of fused silica or glass; length 25–30 m or 50–60 m, the length depending on the desired separation; 0.20–0.35 mm i.d.
5. Stationary phase either moderately polar, e.g., polyglycol (polyethylene glycol 20,000), polyester (butanediol polysuccinate), or polar, e.g., Carbowax 20M, Durabond 225, FFAP, Silar 5 CP, Supelcowax 10, CP Sil 88. The coating should be 0.1–0.2 µm in thickness.
6. Assembly and conditioning of the column—
   (a) Observe the normal precautions for assembling capillary columns—arrangement of the column in the oven (support), choice and assembly of joints (leak tightness), positioning of the ends of the column in the injector and the detector (reduction of dead spaces). Place the column under a carrier gas flow [e.g., 0.3 bar (0.03 MPa) for a 25 m column of 0.3 mm i.d.].
   (b) Condition the column by temperature programming the oven at 3°C/min from ambient temperature to a temperature 10°C below the decomposition limit of the stationary phase. Maintain at this temperature for 1 hr or until the baseline stabilizes. Return to 180°C to work under isothermal conditions.
7. Syringe—max capacity of 10 µL, graduated in 0.1 µL.
8. Integrator or Calculator—The integrator or calculator should be compatible with the apparatus used. Rapid and accurate calculations can be performed with the help of an electronic integrator or calculator, but it must give a linear response with adequate sensitivity and the correction for deviation of the baseline must be satisfactory.

REAGENTS

1. Carrier gas—either helium or hydrogen (see Notes, 3).
2. Auxiliary gas—hydrogen 99.9% min, free from organic impurities, air, or oxygen.
3. Reference standards—a mixture of methyl esters of fatty acids (see Notes, 4).

PROCEDURE

Test Conditions

1. Selection of optimal operating conditions—The properties of efficiency and permeability of capillary columns mean that the separation between constituents and duration of analysis are largely dependent on the flow rate of the carrier gas in the column. It will be necessary to optimize the operating conditions by acting on this parameter (or more simply on the dead-space of the column), according to whether one wishes to improve the separations or to make a rapid analysis.
2. Determination of efficiency and resolution—
   (a) Apply the usual working conditions (isothermal or temperature programming) to give maximum resolution of C_{18} signals.

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(b) Use the procedures and the formulas given in AOCS Official Method Ce 1-62 (Procedure, 3) or IUPAC Standard 2.302 (both procedures are the same) or other accepted standard method to calculate efficiency and resolution. The number of theoretical plates calculated should be at least 2000 per meter.

Test Sample Analysis

1. Use the syringe (Apparatus, 7) to take 0.1–1 µL of the solution of 1–5% of methyl esters prepared according to AOCS Official Method Ce 2-66, IUPAC Standard 2.301, or AOAC 969.33.
2. Operate the oven isothermally at a temperature of 180–210°C or operate by linear heating from 80–220°C (see Notes, 5).
3. Set injector and detector temperatures 30–50°C above the column temperature.
4. Carry out two determinations (each consisting of duplicate injections of the prepared test solution) in rapid succession, using a fresh test portion for each determination.

CALCULATION

1. Obtain the area for each peak by electronic integration and express it as percent of the total fatty acid methyl esters. Use formulas given in AOCS Official Method Ce 1-62 or IUPAC Standard 2.302 (both procedures are the same), or other accepted method. Identify peaks with available standards and material of known composition (see Notes, 4).

   Note—Critical pairs or triplets of fatty acid peaks may be formed depending on the stationary phase used, e.g., C 20:0 and C 18:3 (n-6); C 22:0 and C 20:3 (n-6); C 20:3 (n-3), C 22:1 (n-9) and C 20:4 (n-3) (see Notes, 6).

REPORTING OF RESULTS

Report as the final result the mean of the values obtained from two determinations, provided that the requirements for repeatability are met.

PRECISION

The user of this method is referred to AOCS Official Methods Ce 1b-89 and regarding an approximation of the precision which can be expected with this method.

NUMBERED NOTES

1. On-column injection is preferred because it generally gives better resolution.
2. Apparatus equipped with a temperature programmer is recommended.
3. Either helium or nitrogen may be suitable as a carrier gas, but these may increase helium elution times with respect to hydrogen. Hydrogen, which is used only with capillary columns, can double the speed of analysis. Safety devices are available and should be used.
4. Standards of most known fatty acids are available from suppliers such as, for US: Nu Check Prep, Inc., P. O. Box 172, Elysian, MN 56028, USA; Bast of Copenhagen V, Denmark. Otherwise, oils with specific fatty acids should be used as standards such as blackcurrant seed oil containing C 18:3 (n-6) (γ-linolenic acid)* and C 18:4 (n- 3) (stearidonic acid)**.
   * systematic name, 6,9,12-octadecatrienoic acid.
   ** systematic name, 6,9,12,15-octadecatetraenoic acid.
5. Operating the oven by linear heating increases the speed of fatty acid elution but does not improve or reduce the resolution of the gas chromatograms. A suitable heating program is—
   (a) Start at an oven temperature of 80°C.
   (b) Hold for 2 min, then heat at a rate of 20°C/min until 125°C.
   (c) Hold at 125°C for 1 min, then continue to increase temperature at a rate of 3°C/min until 220°C.
   (d) Hold at 220°C for at least 5 min until all high carbon number fatty acid methyl esters are eluted.
6. In the abbreviated symbols C18:2 and C18:3, C18 stands for the number of carbon atoms (18) whereas :2 and :3 indicate the number of double bond in the fatty acids chain, and :0 means saturated fatty acids.

REFERENCES

1. IUPAC, *Standard Methods for Analysis of Oils, Fats and Derivatives,* Blackwell Scientific Publications, 7th ed. (1987); IUPAC Method 2.303.
2. *The Role of Edible Oils and Fats in Human Nutrition,* WHO/FAO, Rome (1977).
3. *Pure Appl. Chem. 62:*795 (1990).

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AOCS Recommended Practice Ch 2a-94

Revised 2011

trans Unsaturated Fatty Acids by Capillary Column Gas Chromatography

DEFINITION

This Recommended Practice describes a procedure for the determination of the *trans* fatty acid content of vegetable fats and oils by capillary column gas chromatography (GC). Fatty acids are identified by their retention times and quantitated by determination of the ratio of the area under the relevant peak to the sum of the areas under all the peaks for the fatty acids. The fat or oil is converted to its fatty acid methyl ester and then analyzed by capillary column GC. This method is identical to an IOC Method (References, 1). According to this method, the *trans* fatty acid content of a vegetable fat or oil is given by the sum of the following acid contents relative to the total fatty acids: *trans*-octadecenoic (*t* 18:1); *trans,trans*-octadecadienoic (*tt* 18:2); *cis,trans* and *trans,cis* octadecadienoic [(*ct* + *tc*) 18:2] and *trans,cis,trans*; *cis,cis,trans*; *cis,trans,cis*; and *trans,cis,cis* octadecatrienoic [(*tct* + *cct* + *ctc* + *tcc*) 18:3].

SCOPE

Applicable to oils and fats containing fatty acids with 10–24 carbon atoms. Including *trans* unsaturated fatty acids with 18 carbon atoms that may be present at specific concentrations in natural vegetable fats and oils and in such oils that have undergone refining. It is not applicable to vegetable fats that have undergone any hydrogenation process.

APPARATUS

1. Gas chromatograph—suitable for use with a capillary column equipped with a splitting system consisting of:
   (a) Thermostatic chamber for the column—capable of maintaining the desired temperature to ±0.1°C.
   (b) Temperature-controlled injection unit.
   (c) Flame-ionization detector and converter-amplifier.
   (d) Flow meters—for carrier and auxiliary gases.
2. Glass or fused silica capillary column—50 m × 0.25–0.32 mm i.d. coated with cyanopropylsilicone to a thickness of 0.1–0.3 μm. Commercially prepared chromatographic columns are available on the market (e.g., SP 2340, CP Sil 88, Silar 10, etc.).
3. Recorder-integrator—suitable for use with a converter-amplifier, with a response time of not more than 1 sec and a variable paper speed.
4. Microsyringe for gas chromatography—10 μL delivery, with a hardened needle.
5. Rotary evaporator.
6. Apparatus needle—to prepare the methyl esters.

REAGENTS

1. Carrier gas—pure helium or hydrogen, gas chromatography quality (see Notes, *Caution*).
2. Auxiliary gas—air and hydrogen, gas chromatography quality (see Notes, *Caution*).
3. Reference samples—methyl esters of pure fatty acids, in particular *cis* and *trans* isomers of octadecenoic and octadecatrienoic. Methyl esters of octadecenoic and octadecatrienoic acid are available on the market (e.g., from firms like Alltech Associates, Inc., Deerfield, IL; NuChek Prep, Elysian, MN; Supelco Inc., Bellefonte, PA). Isomers of octadecadienoic can be prepared in the laboratory by isomerization of pure linoleic acid with the aid of selenium (References, 1 and 2).
4. *n*-hexane (see Notes, *Caution*).

PROCEDURE

1. Preparation of methyl esters—use a standardized procedure that involves basic catalysis (Na methylate closed vial procedure).
2. Fats and oils with a free acidity >3% should be neutralized prior to start of analysis.
3. Check the gas chromatograph and condition of the column:
   (a) Carry out the preliminary checks of the injection unit and recorder.
   (b) Fit the column in the gas chromatograph without connecting the outlet to the detector.
   (c) Run a light flow of carrier gas (1–2 mL/min) through the column and gradually heat to 210°C in no less than 4 hr. Hold the column at this temperature at least overnight and allow to cool. Connect the outlet to the detector, check the stability of the connection, and bring the injection unit to the conditioning temperature. Connect the detector and recorder and, after 3–4 hr, check that the linearity and drift of the baseline are satisfactory, operating at a sensitivity at least four times greater than that intended for the analysis (a positive drift of more that 5%/hr for the full-scale value indicates that the column has not been properly conditioned).

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4. Operating conditions and checking of column efficiency. General operating conditions are as follows:
   Column temperature, 150–200°C, if possible by temperature programming (e.g., 165°C for 15 min, then raising the temperature by 5°C/min to 200°C).
   Injector temperature: 250°C.
   Detector temperature: 260–280°C.
   Volume of carrier gas (helium or hydrogen): 1.2 mL/min.
   Quantity of substance injected: 1 µL of solution at 2% in hexane.
   Note—These conditions may be varied in light of the composition of the test portion and the characteristics of the column and gas chromatograph in order to ensure separation of all the methyl esters and satisfactory peak resolution (see Procedure, 5, and Fig. 1). Check that the linolenic acid (C18:3) peak appears as a resolved peak just before the eicosenoic acid (C20:1) peak. If it does not, two steps can be taken: change the oven temperature and/or use a column of different polarity.
5. Column efficiency—The efficiency of a column is determined by its separating and resolving power. It must provide chromatograms meeting the following requirements if the analyses are to be performed correctly:
   Separating power—the peaks for the methyl esters of all the fatty acids present must be quite distinct;
   Resolving power—the peaks must be completely resolved (i.e., the peak trace should return to the baseline before leaving for the next peak). For some typical pairs, for instance the *trans* $C_{18:1}$/*cis* $C_{18:1}$ acids, incomplete resolution is tolerated. In such cases, the column is considered satisfactory if the resolution index >2. The resolution index between two peaks is defined by the ratio:

$$\frac{a}{b}$$

   Where—
   a = distance from the baseline to the tip of the smallest peak
   b = distance from the baseline to the lowest point of the trace line between two peaks

   To check that the above requirements are satisfied, inject equal quantities of the test portion of the methyl esters, doing so several times, and adjust the temperature and flow of the carrier gas until the best separation results are obtained. Also, check the resolving power by reducing the test portion, if necessary, and increasing the sensitivity until the best peak resolution is obtained.
   If all the fatty acids present are separated and the resolution index >2, the column is suitable and the operating conditions selected should be maintained in all the successive determinations. If not, the column is not efficient.
6. Analytical procedure—Using the 10 µL microsyringe, take up 1 µL of hexane, draw in 0.5 µL of air and 0.5 µL + 1 µL of the sample solution. Raise the plunger of the syringe farther, so that no sample is in the needle of the syringe. Push the needle through the membrane of the injection unit and after 1–2 sec inject rapidly, then slowly remove the needle after 5 sec. Continue recording until the methyl esters present are completely eluted. The baseline must always satisfy the requirements as specified in Procedure, 5.
7. Peak identification—The order in which the fatty acid methyl esters appear on the chromatogram is a direct function of the number of carbon atoms. The unsaturated esters are eluted after the corresponding saturated esters and their elution is a direct function of the number of double bonds. The *trans* fatty acid esters are eluted before the corresponding *cis* isomers.
8. The individual methyl esters are then identified by their retention times, which are compared with the retention times of the reference samples. A sample chromatogram is given in Figure 1.

QUANTITATIVE ANALYSIS

1. Calculate the areas of each peak using the electronic integrator. To ensure the correct analytical sensitivity, the height of the peak corresponding to the methyl ester of arachidic acid must not be less than 20% of the full-scale value. In the case of oils high in arachidic content, the height must not be less than 50%.
2. The percentage for each fatty acid is calculated from the ratio of the area under the corresponding peak to the sum of the areas under all the peaks using the formula:

$$\frac{Ax}{\sum A} \times 100$$

   Where—
   Ax = peak area for fatty acid x
   $\sum A$ = total of all the peak areas
3. The "total *trans* fatty acids, %" is the sum of the percentage contents of the following acids: *trans*-octadecenoic (C18:1T); *trans,trans* (C18:2T); *cis,trans* and *trans,cis* octadecadienoic (C18:CTTC); *trans,cis,trans,cis,cis,trans; cis,trans,cis* and *trans,cis,cis* octadecatrienoic.
4. Express the result to two decimal points.

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PRECISION

1. The precision of the method was assessed on the basis of an interlaboratory trial involving the participation of 10 laboratories that performed two tests on three samples, one of virgin olive oil + 20% soybean oil, one of virgin olive oil + 10% soybean oil, and one of straight soybean oil. The test results are shown in Table 1.
   (a) Repeatability—When the method is applied in the correct manner, the difference between two single test results obtained on identical test material tested by the same operator using the same equipment within short intervals of time should not, on average, be greater than the repeatability limit given in the table, at a probability of 95%.
   (b) Reproducibility—When the method is applied in the correct manner, the difference between two single test results obtained on identical test material in different laboratories with different operators using different equipment should not, on average, be greater than the reproducibility limit given in Table 1, at a probability of 95%.
2. Analysis of the collaborative test results: The precision values of the method are given in Tables 2 and 3. Nineteen laboratories holding IOC recognition at the time took part in the collaborative test arranged by the Executive Secretariat in 1999. The laboratories were from eight countries. The test was performed on five test samples: a) extra virgin olive oil, b) virgin olive oil + refined sunflower oil, c) virgin olive oil + refined olive-pomace oil, d) virgin olive oil + refined soybean oil + refined sunflower oil, and e) refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil. The results of the collaborative test organized by the IOC Executive Secretariat have been statistically processed according to the rules laid down in the International Standards ISO 5725 (References, 2–5) Accuracy (trueness and precision) of measurement methods and results. Outliers were examined by applying Cochran's and Grubbs's test to the laboratory results for each determination (replicates a and b) and each test portion.

NOTES

Caution

Bottled, compressed air: Compressed gas at high pressure. Use with caution in the presence of combustible substances since the self-ignition temperature of most of the organic compounds in air is considerably lower under high pressure. Make sure the bottle valve is always closed when not in use. Always use a pressure regulator. Release the tension of regulator spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Do not transfer gas from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep them away from sunlight or sources of heat. Store in a corrosion-free environment. Do not use damaged or unlabeled bottles. Air intended for technical purposes must not be used for inhaling or for respiratory apparatus.

Bottled, compressed helium: Compressed gas at high pressure. It reduces the amount of oxygen available for breathing. Keep the bottle shut. Ensure proper ventilation during usage. Do not enter storage areas unless they are properly ventilated. Always use a pressure regulator. Release the tension of regulator spring before opening the bottle valve. Do not transfer gas from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Do not stand in front of the bottle outlet when opening the valve. Keep bottles away from sunlight and sources of heat. Store in a corrosion-free environment. Do not inhale. Use solely for technical purposes.

Bottled, compressed hydrogen: Highly inflammable, under pressure. Keep away from sources of heat, sparks, naked flames or electric apparatus not manufactured from nonflammable material. Make sure the bottle valve is shut when not in use. Always use with a pressure regulator. Release the tension of the regulator spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Ensure proper ventilation during usage. Do not transfer hydrogen from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep bottles away from sunlight and sources of heat. Store in a corrosion-free environment. Do not use damaged or unlabeled bottles.

n-Hexane: Highly flammable, fumes may ignite. Keep away from sources of heat, sparks, or naked flames. Make sure the bottles are always properly closed. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from nonflammable material. It is harmful if inhaled since it can damage the cells of the nervous system. Avoid breathing the fumes. Use a suitable respiratory apparatus if necessary. Avoid contact with eyes and skin.

NUMBERED NOTES

1. Methyl esters of octadecenoic and octadecatrienoic acid are available on the market (e.g., from firms like Alltech, Supelco, etc.)
2. Isomers of octadecadienoic acid can be prepared in the laboratory by isomerization of pure linoleic acid with the aid of selenium [see Strocchi, A. et al., *Rivista Italiana delle Sostanze Grasse, 45* (8) 607, 1968; Schonfield et al., *Journal of the American Oil Chemists' Society 38:*208, 1961].
3. Certain precautions are required to ensure proper analytical evaluation of the results. Small quantities of *trans* oleic acid could be formed when the sample is introduced into the gas chromatographic injector. To prevent this from occurring, it is important to check procedure in the following manner: A sample of methyl esters of an extra virgin olive oil of known origin (or a methyl oleate standard or a mixture with fatty acid methyl esters with a minimum oleic acid content of 60% that are certified free of *trans* fatty acids) is prepared as described in section 7.1 and analyzed under the conditions specified in the method. The percentage of *trans* octadecenoic fatty acids is then measured. Normally, no *trans* fatty acids are present. However, the presence of 0.01% *trans* octadecenoic fatty acids is tolerated. If higher values are recorded, the injector insert

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should be replaced by a perfectly clean, deactivated insert and the analysis should be repeated. A further precaution has to be taken in the case of injector inserts packed with a variety of materials (glass wool, diatomaceous earth, etc.) since the presence of such materials may catalyze the formation of *trans* fatty acids. Certain precautions, therefore, have to be taken. Analyze a sample of extra virgin olive oil of known origin, as in the preceding case. Measure the percentage of *trans* octadecenoic fatty acids. If the values are above 0.01%, proceed as follows:

a. Replace the injector insert with a clean, deactivated insert.
b. Lower the injector temperature by 30–50°C and repeat the test until the values obtained for the *trans* octadecenoic fatty acids are equal to or below 0.01%.
c. Remove the packing material from the injector.
(*Note:* It is necessary to use packing material (glass wool, diatomaceous earth, etc.) if automatic samplers are employed. If injection is manual, the packing material may be removed.)

It is wise to take one further precaution when analyzing extra virgin olive oils. When authentic oils are analyzed, a peak forms whose retention time is almost identical to that of *cis-trans* linoleic. This peak, the value of which varies around 0.01%, should not be taken into consideration in the calculation when it appears on its own in the chromatogram. It should, however, be taken into account if it appears on the chromatogram together with *trans-cis* linoleic.

REFERENCES

1. IOC: COI/T20/DOC 17 Rev. 1 (2001).
2. ISO 5725-1: 1994 Accuracy (trueness and precision) of measurement methods and results - Part 1: General principles and definitions.
3. ISO 5725-2: 1994 Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of the repeatability and reproducibility of a standard measurement method.
4. ISO 5725-5: 1994 Accuracy (trueness and precision) of measurement methods and results - Part 5: Alternative methods for the determination of the precision of a standard measurement method.
5. ISO 5725-6: 1994 Accuracy (trueness and precision) of measurement methods and results - Part 6: Use in practice of accuracy values.

Table 1
Precision parameters for the determination of *trans* fatty acids in oils by capillary column gas chromatography.

trans Fatty Acids[a]			x	s_r	r	s_R	R	RSD_R%
18:1	*t*	3	0.048	0.008	0.022	0.013	0.037	27
		1,2	0.351	0.017	0.047	0.039	0.110	11
18:2	*tt*	1,2,3	0.043-0.059	0.008	0.023	0.016	0.045	38,27
	ct	1,2	0.175-0.253	0.014	0.039	0.023	0.065	13,9
		3	1.127	0.014	0.039	0.062	0.175	6
	tc	1,2	0.159-0.235	0.011	0.030	0.022	0.061	14,24
		3	1.067	0.063	0.150	0.100	0.281	9
18:3	*tct*	1,2	0.046-0.072	0.011	0.030	0.016	0.046	35,22
		3	0.363	0.011	0.030	0.054	0.152	15
	cct	1,2	0.141-0.249	0.009	0.026	0.032	0.092	23,13
		3	1.301	0.009	0.026	0.084	0.237	6
	ctc	1,2	0.024-0.050	0.004	0.010	0.016	0.045	67,32
		3	0.255	0.022	0.061	0.038	0.108	15
	tcc	1,2	0.118-0.204	0.011	0.030	0.026	0.074	22,13
		3	1.131	0.011	0.030	0.075	0.212	7
18:2		1,2	0.373-0.527	0.022	0.063	0.046	0.130	12,9
		3	2.247	0.045	0.126	0.132	0.372	6
18:3		1	0.301	0.018	0.051	0.059	0.168	20
		2	0.534	0.020	0.056	0.096	0.272	18
		3	2.999	0.045	0.127	0.268	0.758	9

[a]Sample description: 1, virgin olive oil + 20% soybean oil; 2, virgin olive oil + 10% soybean oil; and 3, soybean oil.

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Table 2
trans Fatty acid composition (%): C18:1T.[1]

	A	B	C	D	E
n = number of participating laboratories	17	17	17	17	17
Outliers = number of laboratories with outlying values	5	2	4	0	0
Mean = mean of the accepted results	0.012	0.020	0.020	0.021	0.051
r = Repeatability	0.007	0.009	0.010	0.010	0.020
S_r = Repeatability standard deviation	0.003	0.003	0.004	0.004	0.007
RDS_r (%) = Repeatability coefficient of variation ($s_r \times 100$/mean)	22.610	20.160	32.110	22.001	14.080
R = Reproducibility	0.010	0.021	0.021	0.023	0.031
S_R = Reproducibility standard deviation	0.004	0.008	0.008	0.008	0.011
RDS_R (%) = Reproducibility coefficient of variation ($S_R \times 100$/mean)	30.960	46.580	32.110	50.003	21.741

[1]The test was performed on five samples:
A: extra virgin olive oil
B: virgin olive oil + refined sunflower oil
C: virgin olive oil + refined olive-pomace oil
D: virgin olive oil + refined soybean oil + refined sunflower oil
E: refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil

Table 3
***trans* Fatty acid composition (%): C18:2T + C18:3T.[1]**

	A	B	C	D	E
n = number of participating laboratories	16	17	16	17	17
Outliers = number of laboratories with outlying values	5	2	2	4	0
Mean = mean of the accepted results	0.014	0.032	0.011	0.044	0.303
r = Repeatability	0.011	0.010	0.007	0.010	0.043
s_r = Repeatability standard deviation	0.004	0.004	0.003	0.004	0.015
RDS_r (%) = Repeatability coefficient of variation ($s_r \times 100$/mean)	34.920	13.361	18.983	9.852	5.061
R = Reproducibility	0.016	0.020	0.017	0.023	0.131
S_R = Reproducibility standard deviation	0.006	0.007	0.006	0.008	0.047
RDS_R (%) = Reproducibility coefficient of variation ($S_R \times 100$/mean)	47.702	25.643	42.801	22.413	15.392

[1]The test was performed on five samples:
A: extra virgin olive oil
B: virgin olive oil + refined sunflower oil
C: virgin olive oil + refined olive-pomace oil
D: virgin olive oil + refined soybean oil + refined sunflower oil
E: refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil

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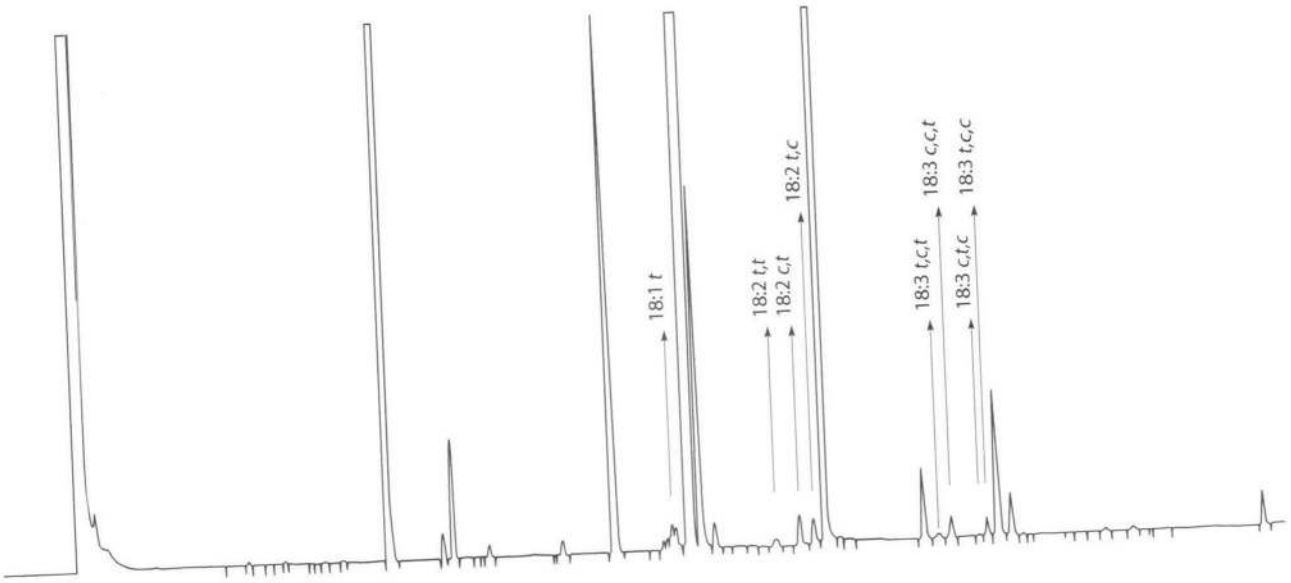

Figure 1. Specimen chromatogram obtained from capillary column analysis of the *trans* fatty acids.

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AOCS

AOCS Official Method Ch 3-91

Revised 2011

Determination of Fatty Acids in the 2-Position in the Triglycerides of Oils and Fats

DEFINITION

This method is for the determination by the use of pancreatic lipase of the composition of that fraction of the fatty acids of an oil or fat which is esterified at the 2-position (β, or internal position) of glycerol. The method consists of neutralization of acid oils and fats in a solvent, purification by passing onto an alumina column, partial hydrolysis of the triglycerides by pancreatic lipase during a determined time, separation of the formed monoglycerides by thin-layer chromatography (TLC), methanolysis of these monoglycerides, and analysis of these methyl esters by gas-liquid chromatography.

SCOPE

This method is applicable to oils and fats having a melting point below 45°C, owing to the peculiarities of the action of pancreatic lipase. It is not applicable unreservedly to oils and fats containing substantial amounts of the following: fatty acids with 12 or fewer carbon atoms (coconut and palm kernel oils, butterfat); highly unsaturated fatty acids (with more than four double bonds) containing 20 or more carbon atoms (fish and marine animal oils); fatty acids containing oxygenated groups, other than the acid group.

APPARATUS

1. Round-bottomed flask—100 mL.
2. Round-bottomed flask—with ground-glass joint.
3. Air condenser—1 m long, to fit the 25 mL round-bottomed flask.
4. Conical flask—250 mL.
5. Beaker—50 mL.
6. Separating funnel—500 mL.
7. Chromatographic glass column—13 mm i.d., 400 mm length, fitted with a fritted glass disc and a tap.
8. Centrifuge tube—with ground-glass stopper.
9. Buret—5 mL graduated, in 0.05 mL.
10. Hypodermic syringe—1 mL fitted, with a thin needle.
11. Microsyringe—to deliver drops in a volume of 3–4 μL.
12. Spreader for thin-layer chromatography (TLC).
13. Glass plates for thin-layer chromatography—20 × 20 cm.
14. Glass developing tank for thin-layer chromatography—with ground-glass lid, suitable for the 20 × 20 cm plates.
15. Spray—for thin-layer chromatography.
16. Oven—regulated at 103 ± 2°C.
17. Thermostated water bath—regulatable between 30–45°C to within 0.5°C.
18. Rotary evaporator.
19. Vibrating electric shaker—allowing vigorous agitation of the centrifuge tube.
20. Ultraviolet lamp—for the examination of the thin-layer plates.

For the control of the lipase activity—

21. pH meter.
22. Spiral stirrer.
23. Buret, 5 mL.
24. Stopwatch.

For the possible preparation of the lipase—

25. Laboratory stirrer—suitable for the dispersion and mixing of heterogeneous materials.

REAGENTS

1. *n*-Hexane or light petroleum ether—(bp 30–50°C), chromatographic quality (see Notes, *Caution*).
2. 2-Propanol or ethanol 95% (v/v)—analytical reagent quality (see Notes, *Caution*).
3. 2-Propanol/water or ethanol/water, 1/1 (v/v)—aqueous solution.
4. Diethyl ether—free from peroxides (see Notes, *Caution*).
5. Acetone (see Notes, *Caution*).
6. Formic acid—98% (m/m) min.

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7. Developing solvent for TLC—mixture of n-hexane, diethyl ether, and formic acid in proportions 70/30/1 (v/v/v).
8. Activated alumina for chromatography—neutral, grade-Brockmann I.
9. Silica powder—with binder, of suitable quality for thin-layer chromatography.
10. Pancreatic lipase—of suitable quality (see Notes, 1 and 2).
11. Sodium hydroxide—120 g/L, aqueous solution.
12. Hydrochloric acid, 6 M—aqueous solution.
13. Calcium chloride—220 g/L, aqueous solution.
14. Sodium cholate (enzymatic quality)—1 g/L, aqueous solution.
15. Buffer solution, 1 M—aqueous solution of tris-hydroxymethylaminomethane brought to pH 8.0 by addition of hydrochloric acid (check with pH meter).
16. Phenolphthalein—10 g/L solution in 95% (v/v) ethanol.
17. 2′,7′-Dichlorofluorescein—2 g/L solution in 95% (v/v) ethanol, rendered slightly alkaline by the addition of 1 drop of 1 M sodium hydroxide per 100 mL.

For the control of the lipase activity—

18. Neutralized oil.
19. Sodium hydroxide, 0.1 M—aqueous solution.
20. Sodium cholate (enzymatic quality)—200 g/L aqueous solution.
21. Gum arabic—100 g/L, aqueous solution.

PREPARATION OF TEST PORTION

If the test sample has an acidity below 3%, determined according to AOCS Official Method Cd 3d-63, purify directly through alumina according to Test Sample Preparation, 2.

If the test sample has an acidity over 3%, determined according to AOCS Official Method Cd 3d-63, neutralize by alkali in the presence of a solvent according to Test Sample Preparation, 1, then pass through alumina according to Test Sample Preparation, 2.

1. Neutralization by alkali in the presence of solvent— Into a 500 mL separating funnel, introduce about 10 g of the crude oil and add 100 mL of hexane and 50 mL of 2-propanol (Reagents, 2), a few drops of phenolphthalein solution, and an amount of the sodium hydroxide solution corresponding to the free acidity of the oil plus 0.3% excess. Shake vigorously for 1 min, add 50 mL of distilled water, shake again, and allow to settle. After separation, remove the bottom soap layer. Also remove any intermediate layers (mucilage, insoluble matter, etc.). Wash the hexane solution of the neutralized oil with successive 25–30 mL portions of the 2-propanol solution (Reagents, 3) until the pink color of the phenolphthalein disappears. Remove most of the hexane by distillation under a vacuum in the rotary evaporator, dry the oil at 30–40°C under vacuum with the help of a current of pure nitrogen until the hexane has been removed completely.
2. Purification through alumina—Prepare a suspension of 15 g of activated alumina (Reagents, 8) in 50 mL of hexane and pour it, while stirring, into the chromatographic column (Apparatus, 7). Let the alumina settle evenly, and allow the solvent level to fall to within 1–2 mm above the alumina absorbent. Carefully pour into the column a solution of 5 g of oil in 25 mL hexane; collect the entire effluent from the column in a 100 mL round-bottomed flask.

PREPARATION OF TLC PLATES

Note—TLC plates are available commercially and may be substituted.

1. Thoroughly clean glass plates with ethanol, light petroleum ether, and acetone to eliminate any trace of fatty matter.
2. In a conical flask, place 30 g of silica powder (Reagents, 9).
3. Add 60 mL of distilled water.
4. Stopper and shake vigorously for 1 min.
5. Transfer the slurry immediately to the spreader and coat the clean plates with a layer 0.25 mm thick.
6. Dry the plates in the air for 15 min and then for 1 hr in an oven at 103 ± 2°C.
7. Cool the plates in desiccator to room temperature before use.

PROCEDURE

1. Hydrolysis with pancreatic lipase—
   (a) Into the centrifuge tube (Apparatus, 8), weigh about 0.1 g of the prepared test sample; if the test sample is a liquid oil proceed directly as below; if it is a solid fat it must be dissolved in 0.2 mL of *n*-hexane with gentle warming (see Notes, 3).
   (b) Add 20 mg of lipase (Reagents, 10) and 2 mL of the buffer solution (Reagents, 15).
   (c) Shake well, but carefully, and then add 0.5 mL of the sodium cholate solution (Reagents, 14) and 0.2 mL of the calcium chloride solution (Reagents, 13).
   (d) Close the tube with the ground-glass stopper, shake cautiously (avoid wetting the stopper), and place the tubes immediately in the thermostated water bath maintained at 40 ± 0.5°C and shake by hand for exactly 1 min (see Notes, 4 and 6).
   (e) Remove the tube from the water bath, and agitate vigorously by means of the electric shaker for exactly 2 min. Add 1 mL of hydrochloric acid and 1 mL of diethyl ether. Stopper and mix vigorously by means of the electric shaker. Allow to stand and remove the organic layer by means of the syringe (Apparatus, 10), after centrifuging, if necessary.

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2. Separation of the monoglycerides by TLC—
   (a) Apply the extract to the chromatographic plate with the microsyringe (Apparatus, 11), about 1.5 cm from the bottom edge, in a thin, uniform line, as narrow as possible.
   (b) Set the plate in a well-saturated TLC-developing tank and develop with the developing solvent (Reagents, 7) at about 20°C, up to about 1 cm from the top edge of the plate.
   (c) Dry the plate in air at the temperature of the tank, and spray it with the 2′,7′-dichlorofluorescein solution (Reagents, 17).
   (d) Identify the monoglyceride band (R_f about 0.035) under ultraviolet light.
3. Analysis of the monoglycerides by GLC—
   (a) Remove the band obtained in Procedure, 2, d with the help of a spatula (avoid removing components remaining on the baseline) and transfer to the methylation flask (Apparatus, 2).
   (b) Treat the collected silica directly as described in AOCS Official Method Ch 1-91, so as to convert the monoglycerides into methyl esters, and then examine the ester by packed column gas chromatography as described in AOCS Official Method Ce 1-62, or by capillary column gas chromatography according to AOCS Official Methods Ce 1e-91 or Ce 1h-05.

CALCULATION

Calculate the fatty acid composition in the 2-position to one decimal place (see Notes, 3).

NOTES

Caution

n-Hexane or light petroleum ether is extremely flammable. Use effective fume removal device. Avoid static electricity.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which diethyl ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Acetone is highly flammable and a dangerous fire risk. It is a narcotic in high concentrations and moderately toxic by ingestion and inhalation. The explosive limits in air are 2.6–12.8%. The TLV in air is 750 ppm.

NUMBERED NOTES

1. Checking the activity of the lipase—
   (a) Prepare an oil emulsion by shaking a mixture of 165 mL gum arabic solution (Reagents, 21), 15 g crushed ice, and 20 mL neutralized oil (Reagents, 18) in a suitable agitator. In a 50 mL beaker, place 10 mL of this emulsion, followed successively by 0.3 mL of the sodium cholate solution (Reagents, 20) and 20 mL of distilled water.
   (b) Put the beaker into a water bath maintained at 37 ± 0.5°C (see Notes, 6); insert the electrodes of a pH meter and a spiral stirrer (Apparatus, 22).
   (c) By means of a buret (Apparatus, 23) add dropwise the sodium hydroxide solution (Reagents, 19) until the pH reaches 8.5.
   (d) Add sufficient aqueous suspension of the lipase (see below).
   (e) As soon as the pH meter indicates a pH of 8.3, start the stopwatch (Apparatus, 24) and drip in the sodium hydroxide solution (Reagents, 19) at such a rate as to maintain the pH at 8.3. Read off the volume of alkali solution consumed every minute.
   (f) Record the observations in the form of a graph, using the time readings as abscissae and the mL of alkali solution required to maintain the pH constant as ordinates. A linear graph should be obtained.
   (g) The lipase suspension mentioned above is a 1 part per thousand (m/m) suspension in water. For each test, a sufficient amount of this suspension should be used so that about 1 mL of the alkali solution is consumed in 4–5 min. Usually about 1–5 mg of the lipase powder is required.
   (h) The lipase unit is defined as the amount of enzyme which will liberate 10 microequivalents of acid per minute. Then the activity A of the powder used, measured in lipase units per mg, is given by the formula:

$$A = \frac{V \times 10}{m}$$

   Where—
   V = mL of sodium hydroxide solution consumed per minute, calculated from the graph
   m = mass of the test portion of the powder, mg
2. Preparation of the lipase—
   Lipases having satisfactory lipase activity are available commercially. It is also possible to prepare the lipase powder in the laboratory as follows:
   (a) Chill 5 kg of fresh pig pancreas to 0°C; remove the surrounding solid fat and connective tissue and triturate in a blender so as to obtain a pasty fluid.
   (b) Stir this paste with the stirrer (Apparatus, 25) for 4–6 hr with 2.5 L of anhydrous acetone and centrifuge.

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(c) Extract the residue three times more with the same volume of acetone, then twice with a 1/1 (v/v) mixture of acetone and diethyl ether, and twice with diethyl ether.
(d) Dry the residue in vacuo for 48 hr to obtain a stable powder, which should be stored in a refrigerator (about 4°C).

3. Based on practical experience, a test portion size of 0.1 g or less gives poor repeatability; 0.5 g of test portion gives better repeatability.
4. Based on practical experience, approximately 20% of 2-monoglyceride should be generated, requiring a reaction time of 15 min.
5. In every case it is advisable to determine the composition of the total fatty acids of the same test portion, since the comparison with that of the acids in the 2-position will help in the interpretation of the figures obtained.
6. The temperature of hydrolysis is set at 37°C, as a liquid oil is being used. However, it is set at 40°C for the test sample, so as to allow the examination of fats with melting points up to 45°C.

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AOCS

AOCS Official Method Ch 4-91

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Chlorophyll Pigments

DEFINITION

This method is used to determine mg/kg of chlorophyll-related pigments (predominantly pheophytin a) in oils from spectrophotometric absorption measurements at 630, 670, and 710 nm (see Notes, 1).

SCOPE

Applicable to expelled, refined, and bleached oils. This method is not applicable to hydrogenated oils, deodorized oils, and finished products, because in these processed oils, the absorption maximum does not occur at 670 nm (see Notes, 2,3).

APPARATUS

1. Spectrophotometer—capable of measurement of the appropriate wavelength.
2. Cuvettes—50 mm long, 20 mm wide, and 40 mm deep. These cuvettes must be matched and differ from each other by not more than 0.5% transmittance when checked with distilled water at 400 nm. Any suitable matched cuvettes with 50 mm light path may be used. Cuvettes with shorter light path, carefully matched, should be available for oils with absorbencies >0.8 at the wavelengths used.

REAGENTS

1. Methylene chloride (CH_2Cl_2) or methylisobutylketone (MIBK)—reagent grade (see Notes, *Caution*). The transmittance should not differ from that of distilled water by more than 0.5% at 400 nm.

ADJUSTMENT OF SPECTROPHOTOMETER

1. Before using, the spectrophotometer should be checked carefully following the instructions given in the operating manual obtained with the instrument. The wavelength setting of the instrument should be checked with the blue sensitive phototube in the instrument. With the instrument turned on and with no cuvettes isuvette compartment, the maximum sensitivity obtainable with the instrument should occur at approximately 540 nm. It is advisable to check the instrument occasionally against a mercury vapor lamp using 579.1, 546.1, 435.8, and 404.7 nm as check points. Any mercury vapor lamp can be focused to pass through the instrument in place of the regular incandescent lamp normally used. Transmission peaks should occur at the specified wavelengths.

PROCEDURE

1. Use the spectrophotometer according to the manufacturer's instructions.
2. "Zero" the instrument using CH_2Cl_2 or MIBK.
3. Adjust the temperature of the oil to 25 ± 2°C. The oil sample must be clear and brilliant. Fill a second 50 mm cuvette with the oil test sample and place in the cuvette compartment of the spectrophotometer.
4. Open the aperture and measure the absorption of the oil test portion. Close the cuvette aperture and change the wavelength setting to the next desired wavelength with either CH_2Cl_2 or MIBK in the light path. Obtain a zero reading. Place the test portion in the light path and measure the absorbance. Make all desired readings following this procedure.
5. All absorbance values should preferably fall between 0.3 and 0.8. Use the maximum cuvette size to give the desired values. On bleached oils, absorbencies of less than 0.3 will be obtained.

CALCULATIONS

$$\text{mg/kg C} = \frac{A_{670} - [(A_{630} + A_{710})/2]}{F \times L}$$

Where—

C = chlorophyll pigments
A = absorbance
L = cuvette length, cm
F = calibration factor (see Notes, 4)

NOTES

Caution

Methylene chloride is toxic and a carcinogen. The TLC is 100 ppm in air. A fume hood should be used at all times when using methylene chloride.

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NUMBERED NOTES

1. A new method, based on the complete conversion of chlorophyll to pheophytin, is currently under study by the NMR and the Seed and Meal Analysis technical committees of the AOCS.
2. This method is not directly applicable to processed and heated oils, because in such oils part or all of the chlorophyll-related pigments may be converted to products that do not exhibit an absorbance maximum at 670 nm.
3. Several variations of this method have been proposed; they include the following:
   (a) Treatment with acetic acid (to convert any remaining chlorophyll to pheophytin), and determining the total pheophytin content, to yield more accurate results. (The pheophytin content is related to the original chlorophyll content.)
   (b) Individually determine chlorophyll and pheophytin by HPLC, using appropriate chlorophyll and pheophytin standards.
   (c) Determine the actual absorbance maximum of the sample and substitute this value in the equation used in the calculation for mg/kg chlorophyll.
   (d) Determine the chlorophyll pigments spectrophotometrically as pheophytin a as follows (References, 1,2):

$$C = \frac{34.53 \times (A_{670} - 0.5\,A_{630} - 0.5\,A_{710})}{L}$$

   Where—
   C = concentration of pigments in mg/kg (ppm) oil
   A = absorbance at respective wavelengths (nm)
   L = light path of the spectrophotometer cell (cm)
4. Factors may be determined for spectrophotometer models currently available, using chlorophyll standards. However, due to the instability of chlorophyll, if this approach is taken, the analyst is advised to check the composition of the chlorophyll standards by an appropriate method, e.g., HPLC. The true composition of the standards as so determined may then be used to calibrate the spectrophotometer.

REFERENCES

1. *La Revista Italiana delle Sostanze Grasse 67:*521 (1990).
2. IUPAC Commission on Oils and Fats, Working Report, International Union of Pure and Applied Chemistry, July 9, 1990, p. M-10.

OTHER REFERENCES

Fette, Seifen, Anstrichm. 86 (8):304 (1984).
J. Am. Oil Chem. Soc. 62 (1):113 (1985).
Rev. Fr. Corps Gras 32 (6-7):257 (1985).

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AOCS

AOCS Official Method Ch 5-91

Reapproved 2009

Determination of Specific Extinction of Oils and Fats, Ultraviolet Absorption

DEFINITION

This method describes the procedure for performing a spectrophotometric examination of olive oil and other oils and fats in the ultraviolet. The oil or fat in question is dissolved in the required solvent and the extinction of the solution is then determined at the specified wavelengths with reference to pure solvent. These absorptions are expressed as specific extinctions E^{1cm} (the extinction of 1% solution of the oil in the specified solvent, in a thickness of 1 cm), conventionally indicated by K.

SCOPE

Spectrophotometric examination in the ultraviolet can provide information on the quality of an oil, its state of preservation and changes brought about in it by processing. It is not specifically a measurement of refining. The degree of oxidation of olive oil is related to its absorption at 232 and approximately 268 nm. In some particular cases, abnormally altered virgin olive oils can show spectral characteristics close to those of refined oils. The absorption at the wavelengths specified in the method is due to the presence of conjugated and triene systems. Virgin olive oil of good quality and with proper storage contains few products of oxidation which absorb at a maximum of approximately 270 nm.

APPARATUS

1. Spectrophotometer—for measuring extinction in the ultraviolet between 220 and 360 nm, with the possibility of reading individual nanometric units. (See Notes, 1).
2. Rectangular quartz cuvettes—with covers, having an optical light path of 1 cm. When filled with water or other suitable solvent, the cuvettes should not show a difference between them of more than 0.01 extinction units.
3. Graduated flasks—25 mL.
4. Chromatographic column—450 mm length and approximately 10 mm i.d.

REAGENTS

1. One of the following solvents (see Notes, *Caution* and Reagents, 3)
   (a) Spectrophotometrically pure isooctane (2,2,4-trimethylpentane)—With reference to distilled water this should have a transmittance of not less than 60% at 220 nm and not less than 95% at 250 nm.
   (b) Spectrophotometrically pure cyclohexane—With reference to distilled water this should have a transmittance of not less than 40% at 220 nm and not less than 95% at 250 nm, or:
   (c) Any other suitable solvent—capable of completely dissolving the oil (e.g., ethyl alcohol for castor oil).
2. Basic alumina—for column chromatography prepared and checked as described in Notes, 2.
3. *n*-Hexane—for chromatography, analytical reagent grade.

PROCEDURE

1. The test sample must be perfectly homogeneous and without suspected impurities. Oils which are liquid at ambient temperature are to be filtered through paper at a temperature of approximately 30°C; hard fats are to be homogenized and filtered at a temperature of not more than 10°C above the melting point.
2. Weigh accurately approximately 0.25 g of the test sample into a 25 mL graduated flask, make up to the mark with the specified solvent, and homogenize. The resulting solution must be perfectly clear. If opalescence or turbidity is present, filter quickly through paper.
3. Fill a cuvette with the solution obtained and measure the extinctions at 232 and 268–270 nm (wavelengths of maximum absorption), using the same solvent as a reference.
4. The extinction values recorded must lie within the range 0.1–0.8. If not, the measurements must be repeated using more concentrated or more diluted solutions as appropriate.
5. When a determination of specific extinction is required after passage over alumina, proceed as follows:
   (a) Place 30 g of basic alumina in suspension in hexane in the chromatography column.
   (b) After the adsorbent has settled, remove the excess hexane down to approximately 1 cm above the top of the alumina.
   (c) Dissolve 10 g of the fat, homogenized and filtered as described in Procedure, 1, in 100 mL of hexane and pour the solution into the column.
   (d) Collect the eluate and evaporate off all the solvent under vacuum at a temperature below 25°C.
   (e) Proceed immediately as specified in Procedure, 2, using the fat so obtained.

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6. The maximum wave length of 268 nm or 270 nm shall be selected depending on whether the solvent used is isooctane or cyclohexane, respectively.

CALCULATION

Record the specific extinctions as the various wavelengths calculated as follows:

$$K_\lambda = \frac{E_\lambda}{C \times S}$$

Where—
K_λ = specific extinction at wavelength λ
E_λ = extinction measured at wavelength λ
C = concentration of the solution, g/100 mL
S = thickness of the cuvette, cm

The results are to be expressed to three decimal figures.

DETERMINATION OF ΔK

Ultraviolet absorption characteristics require the determination of the specific extinction in isooctane solution at wavelengths of 232 and 270 nm and determination of the variation of the specific extinction (ΔK), which is given by:

$$\Delta K = K_m - (K_{m-4} + K_{m+4})/2$$

where—
K_m = the specific extinction at wavelength m, the wavelength for maximum absorption around 270 nm

PRECISION

Repeatability and reproducibility values from an international collaborative study are given in Tables 1–3.

NOTES

Caution

Isooctane is flammable and a dangerous fire risk. It is toxic by ingestion and inhalation. The explosive limits in air are 1.1–6%.

Cyclohexane is flammable and a dangerous fire risk. It is moderately toxic by inhalation and skin contact. The TLV in air is 300 ppm.

NUMBERED NOTES

1. Calibration of the spectrophotometer
   (a) The equipment must be checked at intervals (at least every 6 mo) for both wavelength response and the accuracy of the response.
   (b) The wavelength may be checked using a mercury vapor lamp or by means of suitable filters.
   (c) To check the response of the photocell and the photomultiplier proceed as follows: Weigh 0.2000 g of pure potassium chromate for spectrophotometry and dissolve in 1 m potassium hydroxide solution in a 1000 mL graduated flask and dilute to the mark. Take precisely 25 mL of the solution obtained, transfer to a 500 mL graduated flask and dilute to the mark, using the same potassium hydroxide solution.
   (d) Measure the extinction of the solution so obtained at 275 nm, using the potassium hydroxide solution as a reference. The extinction measured using a 1 cm cuvette should be 0.200 ± 0.005.
2. Preparation of the alumina and testing its activity
   (a) Preparation of the alumina—Place alumina which has been previously desiccated in a furnace at 380–400°C for 3 hr into a hermetically sealed container, add distilled water in the ratio of 5 mL per 100 g of alumina, immediately close the container, shake repeatedly, and then allow to rest for at least 12 hr before use.
   (b) Checking the activity of the alumina—Prepare a chromatographic column with 30 g of alumina. Working as described in Procedure, 5, pass a mixture consisting of 95% virgin olive oil having a specific extinction of less than 0.18 at 268 nm and 5% peanut (groundnut) oil treated with bleaching earth in the refining process, having a specific extinction of not less than 4 at 268 nm through the column. If after passage through the column the mixture has a specific extinction of more than 0.11 at 268 nm the alumina is acceptable; if not, the level of dehydration must be increased.
3. Grades of olive oil are defined by ultraviolet absorbance (Table 4).

REFERENCES

1. This method is based on CAC/RS 33-1970 FAO/WHO, Rome, Italy, Italian Method NGD-C 40-1976, ISO 3656, and COI/T20/Doc. no. 19/Rev 2.

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Table 1
Results of a 2000 collaborative study on the determination of ultraviolet absorbance at 232 nm.

Sample	Sunflower seed oil	Olive oil	Rapeseed oil	Palm kernel oil	Soybean oil
Number of laboratories accepted	13	14	13	13	14
Mean of the laboratory values	2.515	2.220	3.459	2.221	3.665
Repeatability					
Standard deviation, s_r	0.1199	0.0639	0.0582	0.0374	0.1093
Relative standard deviation, RSD_r%	4.8	2.9	1.7	1.7	3.0
Repeatability value, $2.8 \times s_r$	0.3389	0.1808	0.1646	0.1058	0.3091
Reproducibility					
Standard deviation, s_R	0.1866	0.0839	0.1405	0.0983	0.1808
Relative standard deviation, RSD_R%	7.4	3.8	4.1	4.4	4.9
Reproducibility value, $2.8 \times s_R$	0.5277	0.2371	0.3973	0.2780	0.5114

Table 2
Results of a 2000 collaborative study on the determination of ultraviolet absorbance at 268 nm.

Sample	Sunflower seed oil	Olive oil	Rapeseed oil	Palm kernel oil	Soybean oil
Number of laboratories accepted	13	12	11	12	13
Mean of the laboratory values	1.872	0.157	0.627	0.675	1.437
Repeatability					
Standard deviation, s_r	0.0470	0.0215	0.0221	0.0211	0.0329
Relative standard deviation, RSD_r%	2.5	13.7	3.5	3.1	2.3
Repeatability value, $2.8 \times s_r$	0.1329	0.0608	0.0624	0.0597	0.0931
Reproducibility					
Standard deviation, s_R	0.0626	0.0202	0.0379	0.0293	0.0517
Relative standard deviation, RSD_R%	3.3	12.9	6.0	4.3	3.6
Reproducibility value, $2.8 \times s_R$	0.1771	0.0572	0.1072	0.0828	0.1461

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Table 3
Results of a collaborative study conducted in 2001 by the International Olive Council involving 19 laboratories holding IOC recognition. The laboratories were from eight countries.

Absorbency in the Ultraviolet (K)—at 270 nm	A	B	C	D	E
n	17	18	18	18	18
outliers	3	0	1	0	5
mean	0.110	0.253	0.202	0.212	0.561
r	0.006	0.009	0.012	0.015	0.018
S_r	0.002	0.004	0.004	0.006	0.007
RSD_r (%)	1.961	1.431	2.283	2.691	1.191
R	0.016	0.026	0.021	0.031	0.020
S_R	0.006	0.009	0.008	0.011	0.007
RSD_R(%)	5.052	3.770	3.872	5.470	1.302

Delta K	A	B	C	D	E
n	17	18	18	18	18
outliers	1	2	0	1	1
mean	0.000	0.020	0.010	0.003	0.040
r	—	0.000	0.003	0.002	0.007
S_r	—	0.001	0.001	0.001	0.003
RSD_r (%)	—	7.720	17.920	25.660	6.421
R	—	0.004	0.006	0.004	0.017
S_R	—	0.002	0.002	0.002	0.006
RSD_R(%)	—	8.271	37.021	50.201	14.210

A. extra virgin olive oil, B. virgin olive oil + refined sunflower oil, C. virgin olive oil + refined olive-pomace oil, D. virgin olive oil + refined soybean oil + refined sunflower oil, E. refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil.

Table 4
The Codex Alimentarius Commission Recommended International Standard for Olive Oil, Virgin and Refined, and for Refined Olive-Pomace Oil (CAC/RS 33-1970) specifies the following ultraviolet absorption quality characteristics for olive oils (see also codex T20/Doc. 28-2 Annex 3, LL 1993/15-FO, May 1993 and the International Olive Council trade standard for olive oils (COI/T15/NC no. 2/Rev. 6, June 5, 1997).

	$E^{1\%}$ cm Absorbency in ultraviolet at 270 nm	$\Delta E^{1\%}$ cm Delta K at or near 270 nm
Extra virgin olive oil	≤0.25	≤0.01
Fine virgin olive oil	≤0.25	≤0.01
Ordinary virgin olive oil	≤0.30[a]	≤0.01
Lampante virgin olive oil	No limit[a]	
Refined olive oil	≤1.10	≤0.16
Olive oil	≤0.90	≤0.15
Crude olive-residue oil	Not specified	Not specified
Refined olive-residue oil	≤2.00	≤0.20
Olive-residue oil	≤1.70	≤0.18

[a] After passage of the sample through activated alumina, absorbency at 270 nm shall be equal to or less than 0.11.

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AOCS

AOCS Official Method Ch 6-91

Revised 2011

Determination of the Composition of the Sterol Fraction of Animal and Vegetable Oils and Fats by TLC and Capillary GLC

DEFINITION

This method specifies a thin-layer chromatography-capillary gas chromatographic method for the determination of the composition of the sterol fraction of animal and vegetable oils and fats. The method requires the saponification of a test portion of sample, extraction of the unsaponifiable matter, and separation of sterols in the unsaponifiable matter by thin-layer chromatography, followed by the analysis of the separated sterols by gas chromatography using capillary columns (see References, 1).

SCOPE

The results of this method can be used for checking the purity of a fat or for checking conformity with a sales description or advertised composition. The method is applicable to olive oil.

APPARATUS

Preparation of unsaponifiable matter—

1. Usual laboratory apparatus of the type described in AOCS Official Method Ca 6a-40, IUPAC Standard Method 2.401, ISO 3596-1, or ISO 5596-2.

Separation of sterols—

1. Developing tank—made of glass, with a ground-glass lid, suitable for use with plates of dimensions 200 × 200 mm.
2. Usual laboratory apparatus for the preparation of TLC plates—spreader, platform, and 200 × 200 mm glass plates. Alternately, commercially available TLC plates, with or without the appropriate indicator, may be used; see Reagents.
3. Micropipets or microsyringes—capable of delivering droplets of 0.3–0.4 µL. An automatic drop applicator may be used.
4. Usual laboratory apparatus for spraying the indicator solution onto the TLC plates, if the plates do not contain the indicator.
5. Microspatula.
6. Oven—capable of being controlled at 103 ± 2°C.
7. Boiling water bath.
8. Filter paper of 55 mm dia—for retaining fine particles of silica gel; fluted.
9. Conical flasks—25 mL and 250 mL.
10. Reflux condenser—to fit the 25 mL conical flask.
11. Desiccator—containing an efficient desiccant, see AOCS Specification H 9-87.
12. Ultraviolet lamp.

Preparation of sterol derivatives, if required (see Notes, 1)—

1. Reaction tube—internally conical, 5 mL, with a screw cap, or a hemolysis tube, 5 mL.
2. Hemolysis tube—10 mL.
3. Graduated micropipets.

Analysis of sterols—

1. Gas chromatograph—with flame-ionization detector (FID), capillary column and suitable recorder (see AOCS Official Method Ch 2-91 for general guidelines).
2. Glass or fused silica capillary column—20–25 m long of 0.2–0.5 mm i.d., deactivated by silanization, then coated with methylpolysiloxanes (SE 30) or methylphenylpolysiloxanes (OV 17) or other suitable phase (see Notes, 2 and 3), and with a suitable injector of the split type, ratio 1:30 to 1:50.

REAGENTS

Preparation of unsaponifiable matter—

1. Usual laboratory reagents for preparation of unsaponifiable matter, see AOCS Official Method Ca 6a-40, IUPAC Standard Method 2.401, ISO 3596-1, or ISO 3596-2.

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Reagents for the separation of sterols—

1. Ethyl alcohol—95% (v/v).
2. Light petroleum ether—distilling in the range 30–60°C (see Notes, *Caution*).
3. Acetone (see Notes, *Caution*).
4. Developing solvent—such as chloroform (see Notes, *Caution*), hexane/ethyl acetate [80 + 20 (v/v)] mixture, toluene/acetone [95 + 5 (v/v)] mixture (see Notes, *Caution*), or petroleum ether/diethyl ether/formic acid (60:40:1).
5. Ethyl acetate or diethyl ether—free from peroxides and residues (see Notes, *Caution*).
6. Silica powder with binder—thin-layer chromatographic quality. Silica gel containing the indicator (Reagents, 7) is commercially available, e.g., Quanta-Gram no. 4851-830 (Whatman, Inc.) or Uniplate no. 2112 (Analtech, Inc.). Alternately, commercially available plates, with or without indicator may be used.
7. Indicator solution—e.g., rhodamine 6G, 0.5 g/L aqueous solution (see Notes, 4).
8. Cholesterol—100 g/L solution in chloroform.

Preparation of sterol derivatives—

1. Free sterols may be analyzed with a capillary column. If sterol derivatives are required, see Notes, 5, for list of reagents.

Analysis by gas chromatography—

1. Inert gas nitrogen, helium, argon, hydrogen, etc.—thoroughly dried and with an oxygen content of less than 10 mg/kg (ppm) (see Notes, 6).
2. Pure sterols—such as cholesterol, stigmasterol, brassicasterol, β-sitosterol, and campesterol, standard solutions in diethyl ether or any other suitable solvent, corresponding to 20 g of sterol/L (see Notes, 7 and 8).

PROCEDURE

Preparation of unsaponifiable matter—

1. Using 5 ± 0.2 g of the test sample (see Notes, 9), prepare the unsaponifiable matter in accordance with AOCS Official Method Ca 6a-40, IUPAC Standard Method 2.401, ISO 3596-1, or ISO 3596-2. Remove the solvent until about 1 mL of solution remains.

Preparation of TLC plates—

1. Commercially available plates, with or without indicator (see Reagents) may be used. If so, follow the manufacturer's directions for activation.
2. If prepared plates are not available, prepare them as instructed in Notes, 10.

Separation of sterols by TLC—

1. By means of a micropipet or microsyringe transfer 50 µL of the solution obtained from saponification (Procedure, Preparation of unsaponifiable matter) onto an activated TLC plate, 20 mm from one of the edges, in a continuous line of droplets which are as fine as possible, leaving a width of 25 mm on the right- and left-hand sides of the plate unused.
2. Place 0.3–0.4 µL of the cholesterol standard solution 10 mm from the left-hand edge and 10 mm from the righthand edge of the plate.
3. Place sufficient developing solvent in the tank and immediately stand the plate in it. Put on the lid and develop the plate until the solvent front reaches a point about 10 mm from the upper edge.
4. Remove the plate from the tank and allow the solvent to evaporate at laboratory temperature, preferably under a fume hood. If TLC plates do not contain indicator, spray with indicator solution (Reagents, 7).
5. Examine the chromatogram obtained under ultraviolet light.
6. Identify the position of the sterol fraction by means of the two cholesterol reference spots and mark the position with a needle (see Notes, 11).

Recovery of the sterols—

1. Collect the silica containing the sterol spot by means of a microspatula or any other suitable means.
2. Transfer the silica into a 25 mL conical flask, add 5 mL of the developing solvent, ethyl acetate or diethyl ether (see Notes, 12), fit the reflux condenser to the flask, and boil gently in the water bath for 15 min.
3. Allow to cool and filter through the fluted filter paper into a 25 mL conical flask.
4. Remove the silica deposited on the filter and again treat it with 5 mL of boiling developing solvent. Repeat this procedure three times.
5. Combine the filtrates and remove the solvent under a gentle stream of nitrogen.
6. Dissolve the residue in the minimum quantity (normally less than 1 mL) of the ethyl acetate or diethyl ether or the developing solvent (see Notes, 12).
7. Analysis of the sterols by gas chromatography may then be carried out—
   (a) Directly with the free sterols in the solution, or
   (b) With derivatives of the sterols (silylethers or acetates). With certain stationary phases, these derivatives give less tailing than the free sterols. If derivatization is required, prepare the silylethers or, failing this, the acetates as instructed in Notes, 13. Once the derivatives have been prepared, carry out the gas chromatography analysis immediately.

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ANALYSIS OF FREE STEROLS BY GLC

Using a capillary column—

The use of capillary columns is recommended for the analysis of free sterols.

1. Set-up of the apparatus (see Notes, 14)—temperature of the column, 250–260°C. Temperature of the injector and the detector, 30°C above that of the column.
2. Testing the column—Inject onto the column an appropriate volume of each of the standard solutions in order to determine the retention distance or time for each sterol.
3. Inject the appropriate quantity of the mixture of isolated free sterols or their derivatives onto the apparatus.

Examination of chromatograms—

1. Identify the sterols present by determining the relative retention distance or times.
2. Under the operating conditions described and when using methylphenylpolysiloxane (OV 17) as the stationary phases the relative retention times (the origin being the peak for air) of the free sterols, silylethers, and acetates, relative to that for cholesterol, are given in Table 1.

RESULTS

1. The method makes it possible to determine the composition of the sterols for a given stationary phase, with certain restrictions as follows:
   (a) The response of each sterol is a function of the type of detector used.
   (b) The precise quantitative interpretation of the chromatograms is possible only if the individual sterols used for the calibration are pure or, failing this, if the exact composition of the solution of sterols indicated in Notes, 8, has been previously definitely determined by this method by calibration with pure sterols. If not, the results should be expressed as a percentage of the peak areas (see Notes, 15).
   (c) The packed column is less suitable than the capillary column for small quantities, e.g., less than 2% of the sample.
   (d) Use the method of internal normalization, i.e., assume that all the sterols present in the sample are represented on the chromatogram, and that the total area of the peaks represents 100% of the constituents (total elution).
   (e) If pure sterols can be tested, determine the correction factor K_i in relation to cholesterol under the same operating conditions as those used for the test.

CALCULATION

The content of sterol i, expressed as a percentage by mass of the total sterols, is given by the formula:

$$\text{sterol i} = \frac{A_i \times K_i}{\Sigma(A_i \times K_i)} \times 100$$

Where—

A_i = area of the peak corresponding to sterol i

K_i = the correction factor, in relation to cholesterol, corresponding to sterol i. In the absence of pure sterols or of a solution of sterols as indicated in Notes, 8, of exactly known composition, it is reasonable to assume that the correction factors K_i are all equal to 1; the results should then be expressed as a percentage of the peak areas.

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

n-Hexane and petroleum ether are extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using these solvents.

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

Both acetone and ethyl acetate are flammable solvents. They should not be used near an open flame. The use of a fume hood is recommended when using these solvents.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

NUMBERED NOTES

1. As sterols tend to decompose at high temperatures if they come into contact with metals (except silver), it is recommended that an entirely glass or fused silica system be used. However, a stainless steel column may be used, provided that its efficiency

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and resolution are tested with a known mixture of sterols and that it is confirmed that there is no decomposition or absorption of sterols present in small quantities.

2. The separation of the sterols depends to a large extent on the stationary phase used (see Examination of Chromatograms).
3. A recent collaborative study suggested the use of SE-52 or SE-54 (References, 3).
4. Any other coloring product or detection agent, such as a 0.5 g/L solution of 2′,7′-dichlorofluorescein in 95% ethanol (v/v) may be used, provided that it does not react with the sterols.
5. For the preparation of sterol derivatives, the following reagents are required: pyridine (anhydrous), hexamethyldisilazane, trimethylcholorosilane, acetic anhydride, hexane, sodium hydrogen carbonate (10 g/L solution), hydrochloric acid (0.5 mol/L solution) and sodium sulfate (anhydrous).
6. Hydrogen, can double the speed of analysis. Safety devices are available and should be used.
7. Most pure sterols are not commercially available; plant sterol mixture No. 1119 (β-sitosterol, stigmasterol, campesterol, and brassicasterol, 25 mg total sterols in 1 mL solvent) is available from Matreya Inc., Pleasant Gap, PA 16823, USA.
8. If it is impossible to obtain the various pure sterols, and particularly if only the qualitative analysis is carried out, the solutions of pure sterols may be replaced by a 25 g/L solution of sterols of sunflower oil, a 25 g/L solution of sterols of rapeseed oil, and a 20 g/L solution of cholesterol.
9. For fats having low sterol contents (less than 0.1%), such as palm oil, it is advisable to increase the size of the test portion in order to obtain at least 5 mg of sterols; consequently, the quantities of reagents need to be modified.
10. Carefully clean the glass plates first using ethyl alcohol; second, light petroleum ether; and last, acetone, until all fatty matter is totally removed from the plates. Put 30 g of the silica powder into a 250 mL conical flask and add 60 mL of water. Stopper and shake vigorously for 1 min. Immediately introduce the paste into the spreaders. Spread a layer of 0.25 mm thickness onto the clean, dry plates. Allow the plates to dry for 15 min in air and dry in the oven at 103 ± 2°C for 1 hr. Allow the plates to cool to ambient temperature in the desiccator before use.
11. The sterol fraction spot should be completely separate from spots of other components.
12. Use of diethyl ether instead of chloroform has certain advantages; for example, rhodamine 6G is soluble in chloroform and insoluble in diethyl ether.
13. Conversion of sterols—
    (a) Formation of silylethers—Place a few milligrams of the sterols in the reaction tube or 5 mL hemolysis tube and add 0.5 mL of pyridine, 0.1 mL of hexamethyldisilazane and 0.04 mL of the trimethylcholorsilane successively. Allow to stand for 5 min. Use between 0.5–1.0 µL of the upper layer for injection into the GC apparatus.
    (b) Formation of acetate esters—Place a few milligrams of the sterols in the 10 mL hemolysis tube. Add 0.1 mL of the acetic anhydride and 0.1 mL of the pyridine. Maintain at 70°C for 1 hr. Add 2 mL of ice-cold water and 5 mL of hexane. Shake vigorously and then remove the water (using a small separating funnel or pipet). Wash successively with 5 mL of the sodium hydrogen carbonate solution, 5 mL of the hydrochloric acid solution, and then with 5 mL of water. Dry the extract over anhydrous sodium sulfate and filter.
14. A collaborative study, using a glass or fused-silica capillary column, 20–30 m long, 0.25–0.35 mm i.d., with stationary phase consisting of 0.1–0.25 µm SE-52 or SE-54 (References, 3), suggests the following operating conditions: column temperature 265 ± 5°C, inlet temperature 290°C, outlet temperature 300°C, carrier gas (hydrogen) flow rate 30–50 mL/sec, split ratio 1:30 to 1:50, FID hydrogen flow 25–30 mL/min, FID air flow 250–300 mL/min, chart velocity 30–60 cm/hr, time of retention of β-sitosterol 20 ± 5 min. See also the EEC method for determination of sterol composition by Capillary GLC, *Official Journal of the European Communities,* 9-5-91, No. L248/15-22, Annex V.
15. In certain cases, it may be desirable to group the sterols determined individually to facilitate interpretation.

REFERENCES

1 This method is based on ISO Standard 6799 (1983).
2. *JAOCS 62:*1558 (1985).
3. *La Rivista Italiana Sostanza Grasse 66:*531 (1989).
4. IUPAC, *Standard Methods for Analysis of Oils, Fats and Derivatives,* Blackwell Scientific Publications, 7th ed. (1987).

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Table 1

Sterol	OV 17 Free sterols	OV 17 Silylethers	OV 17 Acetates
Cholesterol	1.00	1.00	1.00
Brassicasterol	1.13	1.10	1.13
Campesterol	1.30	1.23	1.30
Stigmasterol	1.42	1.32	1.42
β-Sitosterol	1.61	1.47	1.61
δ5-Avenasterol	1.80	1.60	1.79
δ7-Stigmasterol	1.90	1.69	1.90
δ7-Avenasterol	2.13	1.85	2.09

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AOCS Analytical Guidelines Ch 7-09
Reapproved 2009

International Trade Standard Applying to Olive and Olive-Pomace Oils

DEFINITION

These analytical guidelines note the international trade standards applying to olive and olive-pomace oils.

SCOPE

Applicable to extra virgin olive oil, fine virgin olive oil, semifine (or ordinary) virgin olive oil, lampante virgin olive oil, refined olive oil, blends of refined olive oil and virgin olive oil, crude olivepomace oil, refined olive-pomace oil and blends of refined olive-pomace oil, and virgin olive oil. These oils, which are traded at the international level, are either intended for human consumption as they are, or after refining, or they are intended for technical purposes.

DEFINITIONS

1. Olive oil is the oil obtained solely from the fruit of the olive tree (*Olea europaea sativa Hoffm. et Link*), to the exclusion of oils obtained using solvents or reesterification processes and of any mixture with oils of other kinds.
2. Virgin olive oil is the oil obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which has not undergone any treatment other than washing, decantation, centrifugation, and filtration.
3. Virgin olive oil (oil which may be referred to as "natural") fit for consumption as is includes:
   (a) Extra virgin olive oil—virgin olive oil that has a free acidity, expressed as oleic acid, of not more than 0.8 g per 100 g, and the sensory characteristics of which correspond to those fixed for this category in this standard.
   (b) Virgin olive oil—virgin olive oil that has a free acidity, expressed as oleic acid, of not more than 2.0 g per 100 g, and the sensory characteristics of which correspond to those fixed for this category in this standard.
   (c) Ordinary virgin olive oil—virgin olive oil that has a free acidity, expressed as oleic acid, of more than 3.3 g per 100 g, and the sensory characteristics of which correspond to those fixed for this category in this standard.
4. Virgin olive oil not fit for consumption as is, designated lampante virgin olive oil, is virgin olive oil that has a free acidity, expressed as oleic acid, of more than 3.3 g per 100 g, and the sensory characteristics of which correspond to those fixed for this category in this standard. It is intended for refining or for technical purposes.
5. Refined olive oil is the olive oil obtained from virgin olive oils by refining methods which do not lead to alterations in the initial glyceridic structure. It has a free acidity of not more than 0.3 g per 100 g.
6. Olive oil is the oil consisting of a blend of refined and virgin olive oils fit for consumption as is. It has a free acidity of not more than 1g per 100 g.
7. Olive-pomace oil is the oil obtained by treating olive pomace with solvents, to the exclusion of oils obtained by reesterification processes and of any mixture with oils of other kinds. It is marketed in accordance with the following designations and definitions:
   (a) Crude olive-pomace oil—olive-pomace oil intended for refining with a view to its use in food for human consumption, or intended for technical purposes.
   (b) Refined olive-pomace oil—obtained from crude olive-pomace oil by refining methods which do not lead to alterations in the initial glyceridic structure. It has a free acidity of not more than 0.3 g per 100 g.
   (c) Olive-pomace oil—blend of refined olive-pomace oil and virgin olive oil fit for consumption as is. In no case should this blend be called "olive oil." It has a free acidity of not more than 1g per 100 g

PURITY CRITERIA

The identity characteristics comprising the purity criteria applicable to olive oil and olive-pomace oil are listed in Tables 1-7.

QUALITY CRITERIA

Quality criteria are listed in Table 9.

FOOD ADDITIVES

1. Virgin olive oils and crude olive-pomace oils—None permitted.
2. Refined olive oil, olive oil, refined olive-pomace oil and olive-pomace oil; α-tocopherol permitted to restore natural tocopherol lost in the refining process—Maximum level: 200 mg/kg of total α-tocopherol in the final product.

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CONTAMINANTS

Contaminants are listed in Table 10.

HYGIENE

It is recommended that the products intended for human consumption covered by the provisions of this standard be prepared in accordance with the appropriate sections of the General Principles of Food Hygiene recommended by the Codex Alimentarius Commission (CAC/RCP 1-1969, Rev. 3-1997).

PACKING

Olive oils and olive-pomace oils intended for international trade shall be packed in containers complying with the General Principles of Food Hygiene recommended by the Codex Alimentarius Commission (CAC/RCP 1-1969, Rev. 3-1997).
The containers used may be—

1. tanks, containers, or vats which permit the transportation in bulk of olive and olive-pomace oils;
2. metal drums, in good condition, hermetically sealed, which should be covered internally with a suitable varnish;
3. metal tins and cans, lithographed, new, hermetically sealed, which should be covered internally with a suitable varnish;
4. demi-johns, glass bottles, or bottles made of suitable macromolecular material.

CONTAINER-FILLING TOLERANCE

The volume occupied by the contents shall under no circumstances be less than 90% of the capacity of the container, except in the case of tin containers with a capacity ≤1 L, in which the volume occupied shall under no circumstances be less than 80% of the capacity of the container; this capacity is equal to the volume of distilled water at 20°C which the container can hold when full.

LABELING

In addition to sections 2, 3, 7, and 8 of the Codex General Standard for the Labeling of Prepackaged Foods (CODEX STAN 1-1985, Rev. 1-1991) and the guidelines applying to food not intended for direct sale to consumers, the following specific provisions shall be applied.

1. On containers intended for direct sale to consumers—
   (a) Name of the product—The labeling on each container shall indicate the specific designation of the product contained, complying in every way with the relevant provision of this standard.
   Designations of olive oils
   —extra virgin olive oil[1]
   —virgin olive oil[1]
   —ordinary virgin olive oil[1]
   —refined olive oil
   —olive oil[2]
   Designations of olive-pomace oils
   —refined olive-pomace oil
   —olive-pomace oil
   [1]Oil which may likewise be referred to as "natural."
   [2]The terms "pure" or "100% pure" may figure on the label as a specification of the product.
   (b) Net contents—The net contents shall be declared by weight or volume in the metric system ("Système International" units).
   (c) Name and address—The name and address of the manufacturer, packer, distributor, importer, exporter, or seller shall be declared.
   (d) Country of origin—The name of the country of origin shall be declared. When the product undergoes processing or repacking, including in small containers, in a second country, the country in which the processing was performed shall be considered the country of origin for the purposes of labeling.
   (e) Indications of source and appellations of origin—*Indications of source:* The labels of virgin olive oils may indicate their source (country, region, or locality) when they have been empowered to do so by their country of origin and when such virgin olive oils have been produced, packed, and originate exclusively in the country, region, or locality stated. The labels for blends of refined olive oil and virgin olive oil may indicate only the source of the exporting country.
   Appellations of origin: The labels of extra virgin olive oils may indicate their appellation of origin (country, region, or locality) when they have been awarded such an appellation, in accordance with the terms provided by the regulations of their country of origin and when such extra virgin olive oil has been produced, packed, and originates exclusively in the country, region, or locality mentioned. The labels for blends of refined olive oil and extra virgin olive oil packed and exported by the country providing the extra virgin olive oil may indicate the appellation of origin which would have been given to the extra virgin olive oil in the blend.
   (f) Lot identification—Each container must be embossed or otherwise permanently marked in code or in clear language to identify the producing factory and the lot.

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(g) Date marking and storage conditions—Date of packing: The date of packing shall be declared by the month and year in uncoded numerical sequence. The month may be indicated by letters in those countries where such use will not confuse the consumer; if the month is December, the expression "end (stated year)" may be used as an alternative.

Date of minimum durability: In the case of prepackaged products intended for the end consumer, the date of minimum durability (preceded by the words "best before end") shall be declared by the month and year in uncoded numerical sequence. The month may be indicated by letters in those countries where such use will not confuse the consumer; if the shelf life of the product is valid to December, the expression "end (stated year)" may be used as an alternative. The period of durability shall not exceed 12 months after the date of packing. It may however be extended to 18 months for oils packed in metal containers.

Storage instructions: Any special conditions for storage shall be declared on the label if the validity of the date of minimum durability is dependent on storage conditions.

2. Forwarding packs of oils intended for human consumption. In addition to the details noted under Labeling, 1, the following inscription shall appear: number and type of containers held in pack.
3. Containers allowing the transportation in bulk of olive oils and olive-pomace oils. The labeling on each container shall include—

Name of the product: The name shall indicate the specific designation of the product contained, complying in every way with the provisions of this standard.

Net contents: The net contents shall be declared by weight or volume in the metric system (Système International" units).

Name and address: The name and address of the manufacturer, distributor, or exporter shall be declared.

Country of origin: The name of the exporting country shall be declared.

METHODS OF ANALYSIS AND SAMPLING

The methods of analysis and sampling given in Table 11 are international referee methods. The latest version of these methods should be used.

REFERENCE

Trade standard applying to Olive Oils and Olive-Pomace Oil, International Olive Council, COI/T.15/NC no. 3/Rev. 3, November 2008.

Table 1
Sterol composition

Olive Oils and Olive-Pomace Oils	% of Total Sterols
Cholesterol	≤0.5
Brassicasterol	≤0.1[a]
Campesterol	≤4.0
Stigmasterol	< campesterol in edible olive oils
δ-7-Stigmasterol	≤0.5
β-Sitosterol + δ-5-avenasterol + δ-5-23-stigmastadienol + clerosterol + sitostanol + δ-5-24-stigmastadienol	≥93.0

[a]≤0.2 for olive-pomace oils.

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Table 2
Total sterols content, minimum value

	mg/kg
Virgin olive oils Refined olive oil Olive oil	1000
Crude olive-pomace oil	2500
Refined olive-pomace oil	1800
Olive-pomace oil	1600
Fatty acid composition using gas-liquid chromatography (mol% of methyl esters):	
Myristic acid	≤0.05
Palmitic acid	7.5–20.0
Palmitoleic acid	0.3–3.5
Heptadecanoic acid	≤0.3
Heptadecenoic acid	≤0.3
Stearic acid	0.5–5.0
Oleic acid	55.0–83.0
Linoleic acid	3.5–21.0
Linolenic acid	≤1.0
Arachidic acid	≤0.6
Gadoleic acid (eicosanoic)	≤0.4
Behenic acid	≤0.2[a]
Lignoceric acid	≤0.2
Erucic acid	Not established
Lauric acid	Not established

[a]≤0.3 for olive-pomace oils.

Table 3
Content of 2-glyceryl monopalmitate

Virgin olive oil	≤0.9–1.0%
Refined olive oil	≤0.9–1.1%
Olive oil	≤0.9–1.0%
Crude olive-pomace oil	≤1.4%
Refined olive-pomace oil	≤1.4%
Olive-pomace oil	≤1.2%

Table 4
Unsaponifiable matter (using light petroleum)

Olive oils	≤15 g/kg
Olive-pomace oils	≤30 g/kg

Table 5
Detection of olive-pomace oil

	Lampante Virgin Olive Oils	Edible Virgin Oils	Refined Olive Oil	Olive Oil
Waxes, mg/kg $C_{40} + C_{42}$ $C_{44} + C_{46}$	≤300	≤250	≤350	≤350
Erythrodiol+ uvaol/total sterols, %	≤4.5	≤4.5	≤4.5	≤4.5

Table 6
Detection of seed oils

Maximum difference between the real and theoretical ECN 42 triglyceride content	
Edible virgin olive oils	0.2
Refined olive oil	0.3
Olive oil	0.3
Lampante virgin olive oil	0.3
Refined olive-pomace oil	0.5
Olive-pomace oil	0.5
Crude olive-pomace oil	0.6

Table 7
Detection of refined vegetable oils

	Stigmasta-3, 5-diene ppm	R1[a]
Edible virgin olive oils	≤0.15	
Lampante virgin olive oils	≤0.50	
Refined olive oil	≤50[b]	≥12
Olive oil	≤50[b]	≥12
Crude olive-pomace oil	≤5[b]	no limit
Refined olive-pomace oil	≤120[b]	≥10
Olive-pomace oil	≤120[b]	≥10

[a] The ratio $R1 = \frac{\text{stigmasta-3,5-diene}}{\text{campesta-3,5-diene}}$ is to be applied to oils whose stigmastadiene content is greater than 4 µg/g.
[b] Provisional limits.

Table 8
***trans* fatty acid content**

	C 18:1 trans %	C18:2 trans & C18:3 trans %
Edible virgin olive oil	<0.05	<0.05
Lampante virgin olive oil	≤0.10	≤0.10
Refined olive oil	≤0.20	≤0.30
Olive oil	≤0.20	≤0.30
Crude olive-pomace oil	≤0.20	≤0.10
Refined olive-pomace oil	≤0.40	≤0.35
Olive-pomace oil	≤0.40	≤0.35

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Table 9
Quality criteria

	Extra Virgin Olive Oil	Virgin Olive Oil	Ordinary Virgin Olive Oil	Lampante Virgin Olive Oil[a]	Refined Olive Oil	Olive Oil	Crude Olive-Pomace Oil	Refined Olive-Pomace Oil	Olive-Pomace Oil
Sensory characteristics[a]									
Odor					Acceptable	Good		Acceptable	Good
Taste					Acceptable	Good		Acceptable	Good
Color					Light yellow	Light yellow to green		Light yellow to brownish yellow	Light yellow to green
Median of defect	Me = 0	0<Me≤2.5	2.5<Me<6.0[c]	Me >6.0					
Median of the fruity attribute	Me >0	Me >0							
Aspect at 20°C for 24 hr[a]					Limpid	Limpid		Limpid	Limpid
Free acidity mol% expressed in oleic acid[a]	≤0.8	≤2.0	≤3.3	>3.3	≤0.3	≤1.0	No Limit	≤0.3	≤1.0
Peroxide value in meq. peroxide oxygen per kg/oil[a]	≤20	≤20	≤20	No Limit	≤5	≤15	No Limit	≤5	≤15
Absorbency in Ultraviolet $(K^{1\%})^{1\ cm}$									
270 nm	≤0.22	≤0.25	≤0.30[b]	No Limit[b]	≤1.10	≤0.90		≤2.00	≤1.70
ΔK	≤0.01	≤0.01	≤0.01		≤0.16	≤0.15		≤0.20	≤0.18

[a]It is not obligatory for the criteria to be concurrent; one is sufficient.
[b]After passage of the sample through activated alumina, absorbency at 270 nm shall be equal to or less than 0.11.
[c]Or when the median of the defect is less than or equal to 2.5 and the median of the fruity attribute is equal to 0.

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Table 10
Contaminants

	Extra Virgin Olive Oil	Virgin Olive Oil	Ordinary Virgin Olive Oil	Lampante Virgin Olive Oil	Refined Olive Oil	Olive Oil	Crude Olive-Pomace Oil	Refined Olive-Pomace Oil	Olive-Pomace Oil
Moisture and volatile matter (mol%)	≤0.2	≤0.2	≤0.2	≤0.3	≤0.1	≤0.1	≤1.5	≤0.1	≤0.1
Insoluble impurities (mol%) in									
light petroleum	≤0.1	≤0.1	≤0.1	≤0.2	≤0.05	≤0.05		≤0.05	≤0.05
Flash point	—	—	—	—	—	—	≥120°C	—	—
Metal traces mg/kg									
Iron	≤3.0	≤3.0	≤3.0	≤3.0	≤3.0	≤3.0		≤3.0	≤3.0
Copper	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1		≤0.1	≤0.1
Lead	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1		≤0.1	≤0.1
Arsenic	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1		≤0.1	≤0.1
Each solvent detected mg/kg	≤0.1	≤0.1	≤0.1		≤0.1	≤0.1		≤0.1	≤0.1
Sum of solvents detected mg/kg	≤0.2	≤0.2	≤0.2		≤0.2	≤0.2		≤0.2	≤0.2

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Table 11
Summary of International Trade Standards Applying to Olive Oils and Olive-Pomace Oils

Determination	Method
Identity, purity:	
Sterol composition	Ch 6-91; ISO 6799-1989; COI (IOOC)/T.201/Doc. no. 10-1992
Total sterol content	COI/T20/Doc. no. 10-1992
Fatty acid composition	Ch 2-91; ISO 5508-1990
Saturated fatty acids in the 2-position of the triglycerides	Ch 3-91; IUPAC 2.210
Unsaponifiable matter	Ca 6a-40 or Ca 6b-53; IUPAC 2.401; ISO 3596-1 or 3596-2
Detection of olive-pomace oil:	
Wax content	NGD C 80-1989
Aliphatic alcohols	NGD C 76-1989
Erythrodiol + uvaol	IUPAC 2.431
Detection of refined vegetable oils:	
Stigmasta-3,5-diene	COI/T.20/Doc. no. 11-1992
Campesta-3,5-diene and stigmasta-3,5,22-triene	COI/T.20/Doc no. 12-1992
trans fatty acid content	Cd 14c-94; NGD C 84
α-tocopherol (total tocopherols)	Ce 8-89; IUPAC 2.432
Detection of seed oils	Ce 5b-89; IUPAC 2.324
Quality tests:	
Sensory characteristics (odor, taste, color)	COI/T.20/Doc. no. 3/Rev. 2 1992
Free acidity	Cd 3d-63
Peroxide value	Cd 8-53; Cd 8b-90
Specific extinction in the UV	Ch 5-91
Moisture and volatile matter	IUPAC 2.601; Ca 2f-93
Insoluble impurities	Ca 3a-46; IUPAC 2.604
Soap test	CAC/RM 27-1970
Flash point	AOCS Cc 9b-55 (77)
Metals (iron, copper)	Ca 18b-91
Halogenated solvents	COI/T.20/Doc. no. 8/corr. 1-1990

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AOCS Official Method Ch 8-02

Revised 2011

Determination of Wax Content by Capillary Column Gas-Liquid Chromatography

DEFINITION

Addition of a suitable internal standard to the fat or oil, then fractionation by chromatography on a hydrated silica gel column. Recovery of the fraction eluted first under the test conditions (whose polarity is less than that of the triglycerides), then direct analysis by capillary column gas-liquid chromatography.

SCOPE

This method describes a procedure for the determination of the wax content of certain fats and oils, under the test conditions. It may be used in particular to distinguish between olive oil obtained by pressing and that obtained by extraction (olive-pomace oil). This method is identical to the IOC COI/T.20/ Doc. no.18/ Rev. 2 document.

APPARATUS

1. 25 mL Erlenmeyer flask.
2. Glass column for gas-liquid chromatography, 15 mm internal diameter and 30–40 cm long.
3. Suitable gas-liquid chromatograph for working with a capillary column, equipped with a system for direct introduction into the column comprising the following:
   a. Thermostat-controlled oven for the columns, capable of maintaining the desired temperature to within 1°C.
   b. Cold injector for direct introduction into the column.
   c. Flame-ionization detector and converter-amplifier.
   d. Recorder-integrator capable of working with the converter-amplifier, rate of response below 1 second.
   e. Capillary column, glass or fused silica, 10–15 m long, 0.25–0.32 mm internal diameter, internally covered with SE-52 or SE-54 liquid, or equivalents, to a uniform thickness of 0.10–0.30 µm.
4. Microsyringe with facilities for on-column injection, capacity 10 µL, equipped with a hardened needle.

REAGENTS

1. Silica gel, 60–200 µm mesh, art. 7754 Merck. Place the gel in the oven at 500°C for 4 hours. Allow to cool, then add 2% water. Shake well to homogenize slurry. Keep in darkness for at least 12 hours prior to use.
2. *n*-Hexane—for chromatography.
3. Ethyl ether—for chromatography.
4. *n*-Heptane—for chromatography.
5. Standard solution of lauryl arachidate—at 0.1% (m/v) in hexane (internal standard).
6. Carrier gas—hydrogen, pure, for gas-liquid chromatography.
7. Auxiliary gases—
   -Hydrogen, pure, for gas-liquid chromatography.
   -Air, pure, for gas-liquid chromatography.
8. Sudan I (1-phenylazo-2-naphthol)

PROCEDURE

1. Separation of the wax fraction—
   a. Preparation of the chromatographic column—Suspend 15 g of silica gel hydrated at 2% in anhydrous *n*-hexane and introduce into the column. Allow to settle spontaneously. Complete settling with the aid of an electric shaker to make the chromatographic band more homogeneous. Percolate 30 mL *n*-hexane to remove any impurities.
   b. Column chromatography—Weigh exactly 500 mg of the test sample into a 25 mL flask, and add a suitable amount of internal standard, depending on the assumed wax content, e.g., add 0.1 mg of lauryl arachidate in the case of olive oil, and 0.25–0.50 mg in the case of olive-pomace oil. Transfer the prepared test sample to the chromatographic column, prepared according to Procedure, 1 with the aid of two 2 mL portions of *n*-hexane. Allow the solvent to flow to 1 mm above the upper level of the absorbent. Then start chromatographic elution; collect 140 mL of the *n*-hexane/ethyl ether mixture, at 99:1, at a flow rate of about 15 drops every 10 seconds (2.1 mL/minute). Evaporate the resultant fraction in a rotary evaporator until almost complete dryness. Remove the last 2 or 3 mL of solvent under a weak current of nitrogen, then add 10 mL *n*-heptane.

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2. Gas-liquid chromatographic analysis—
   a. Preliminary procedure, conditioning of column
      (1) Fit the column to the gas-liquid chromatograph, connecting the inlet port to the on-column system and the outlet port to the detector. Check the gas-liquid chromatography apparatus (operation of gas loops, detector and recorder efficiency, etc.).
      (2) If the column is being used for the first time, it is advisable to condition it. Run a light flow of gas through the column, then switch on the gas-liquid chromatography apparatus. Gradually heat to a temperature at least 20°C above the operating temperature (see Notes,1). Maintain this temperature for at least two hours, then regulate the apparatus to the operating conditions (regulate gas flow, light flame, connect to electronic recorder, regulate oven temperature for column, regulate detector, etc.).
      (3) Record the signal at a sensitivity at least twice as high as that required to perform the analysis. The base line should be linear, with no peaks of any kind, and must not have any drift. Negative rectilinear drift indicates that the column connections are not correct; positive drift indicates that the column has not been properly conditioned.
   b. Choice of operating conditions
      (1) The operating conditions are generally as follows:
      -Column temperature: 80°C at first, rising by 30°C/minute to 120°C, then programmed to increase by 5°C/minute up to 340°C.
      -Detector temperature: 350°C.
      -Linear speed of carrier gas: hydrogen, 20–35 cm/sec.
      -Instrument sensitivity: 4–16 times the minimum attenuation.
      -Recorder sensitivity: 1–2 mV of the full scale.
      -Paper speed: 30 cm/hour.
      -Amount injected: 0.5-1 µL solution.
      These conditions may be modified to suit the characteristics of the column and the gas-liquid chromatographic apparatus (in order to obtain chromatograms meeting the following conditions: retention time of C32 internal standard must be 25 ± 2 minutes and the most representative peak of the waxes must lie between 60 and 100% of the full-scale value).
      (2) Determine the peak integration parameters in such a way as to obtain a correct evaluation of the peak areas considered.
   c. Performance of the analysis
      (1) Take up 1 µL of the solution with the aid of the 10 µL micro-syringe; draw back the plunger until the needle is empty. Introduce the needle in the injection system and inject quickly after 1–2 seconds. After about 5 seconds, gently extract the needle.
      (2) Perform the recording until the waxes are completely eluted. The base line must always satisfy the required conditions (see Procedure, 2).
   d. Peak identification—Identify the peaks from the retention times, by comparing them with mixtures of waxes with known retention times, analyzed under the same conditions. Figure 1 gives a wax chromatogram of a virgin olive oil.
   e. Quantitative analysis—
      (1) Determine the areas of the peaks corresponding to the internal standard and the aliphatic esters from C36 to C46 with the aid of the integrator.
      (2) Determine the wax content of each of the esters, in mg/kg of fat, according to the formula:

$$\text{ester mg/kg} = \frac{A_x \times m_s}{A_s \times M} \times 1000$$

Where—

A_x = area corresponding to the peak for each single ester in square millimeters.

A_s = area corresponding to the lauryl arachidate peak, in square millimeters.

m_s = mass of the lauryl arachidate added, in milligrams.

M = mass of the test portion taken for determination, in grams.

   f. Determination of linear gas speed—Inject 1–3 µL of methane (propane) into the gas-liquid chromatographic apparatus, after adjusting it to the normal operating conditions. Measure the time the gas takes to run through the column, from the moment it is injected until the peak emerges (t_M). The linear speed in cm/sec is given by the formula L/t_M where L is the length of the column, in cm, and t_M is the time measured in seconds.

EXPRESSION OF THE RESULTS

Give the different wax contents, and the sum of those contents, in mg/kg of fat.

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NOTES

1. Keep the conditioning temperature at all times at least 20°C below the maximum temperature specified for the eluent employed.

PRECISION

1. Analysis of the collaborative test results—The precision values of the method are given in Table 1. Nineteen laboratories holding IOC recognition at the time took part in the collaborative test arranged by the Executive Secretariat in 1999. The laboratories were from eight countries. The test was performed on five test samples: (a) extra virgin olive oil, (b) virgin olive oil + refined sunflower oil, (c) virgin olive oil + refined olive-pomace oil, (d) virgin olive oil + refined soybean oil + refined sunflower oil, (e) refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil. The results of the collaborative test organized by the IOC Executive Secretariat have been statistically processed according to the rules laid down in the International Standards ISO 5725 Accuracy (trueness and precision) of measurement methods and results. Outliers were examined by applying Cochran's and Grubbs's test to the laboratory results for each determination (replicates a and b) and each sample.
2. Repeatability (r)—When the method is applied in a correct manner, the difference between two single independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time should not on average be greater than the repeatability limit given in the tables at a probability of 95%.
3. Reproducibility (R)—When the method is applied in a correct manner, the difference between two single test results obtained with the same method on identical test material in different laboratories with different operators using different equipment should not on average be greater than the reproducibility limit given in the tables at a probability of 95%.

REFERENCES

1. ISO 5725-1: 1994 Accuracy (trueness and precision) of measurement methods and results -Part 1: General principles and definitions.
2. ISO 5725-2: 1994 Accuracy (trueness and precision) of measurement methods and results -Part 2: Basic method for the determination of the repeatability and reproducibility of a standard measurement method.
3. ISO 5725-5: 1994 Accuracy (trueness and precision) of measurement methods and results -Part 5: Alternative methods for the determination of the precision of a standard measurement method.
4. ISO 5725-6: 1994 Accuracy (trueness and precision) of measurement methods and results.

Table 1
Wax content (mg/kg)

	A	B	C	D	E
n = number of participating laboratories	19	19	19	19	19
Outliers = number of laboratories with outlying values	5	5	4	3	5
Mean = mean of the accepted results	120.32	123.14	222.41	174.1	345.93
r = Repeatability	9.51	12.56	10.51	12.22	14.91
S_r = Repeatability standard deviation	3.39	4.48	3.75	4.72	5.32
RDS_r (%) = Repeatability coefficient of variation ($s_r \times 100$/mean)	2.82	3.64	1.69	2.71	1.54
R = Reproducibility	38.83	48.89	58.93	25.65	44.39
S_R = Reproducibility standard deviation	13.86	17.46	21.04	9.16	15.85
RDS_R(%) = Reproducibility coefficient of variation ($s_R \times 100$/mean)	11.53	14.18	9.46	5.26	4.58

A: extra virgin olive oil
B: virgin olive oil + refined sunflower oil
C: virgin olive oil + refined olive-pomace oil
D: virgin olive oil + refined soybean oil + refined sunflower oil
E: refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil

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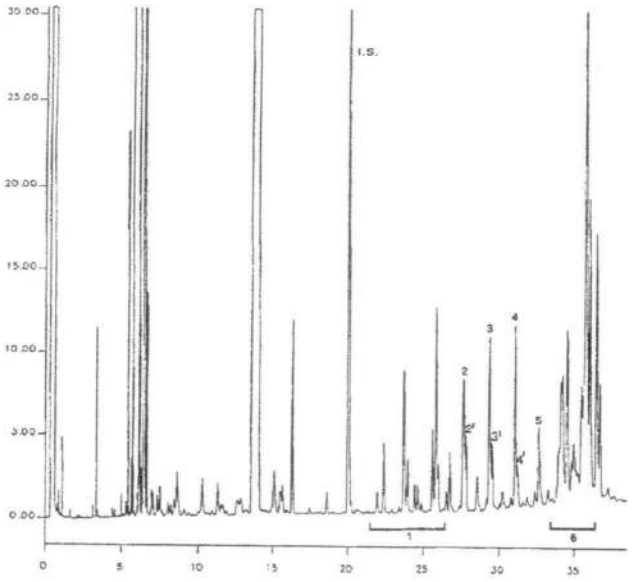

Figure 1. Chromatogram of the wax fraction of an olive oil sample. I.S. = Lauryl arachidate, 1 = Diterpenic esters, 2+2′ = C40 esters, 3+3′ = C42 esters, 4+4′ = C44 esters, 5 = C46 esters, 6 = Sterol esters and triterpene alcohols. Note: After elution of the sterol esters, the gas chromatograph trace should not show any significant peaks (triglycerides).

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AOCS Analytical Guidelines Ci 1-91
Reapproved 2009

Analytical Guidelines for the Determination of Jojoba Oil Quality and Purity

DEFINITION

These Analytical Guidelines are existing AOCS Official Methods and Recommended Practices (Table 1) for the determination of jojoba oil quality and purity (see Notes, 1).

SCOPE

Applicable to jojoba oil quality and purity. The methods listed in this document are not suitable for the identification of jojoba oil.

PEROXIDE VALUE

Although the peroxide value is considered an important property related to the quality of jojoba oil, the committee expressed dissatisfaction with the reliability, accuracy, and precision of the peroxide value method, AOCS Official Method Cd 8b-90, when applied to jojoba oil. While jojoba processors, marketers, and buyers may still use the peroxide value method as a quality specification, the users of the method should be aware of the possible lack of repeatability and reproducibility when the method is applied to jojoba oil.

MOISTURE

The moisture content of jojoba oil is typically <300 μg/g and is sampled only when potential bacteriological contamination is a concern.

RESIDUAL HEXANE

Mechanically extracted jojoba oil contains no hexane or other organic solvents. Residual hexane in solvent-extracted oils is typically near 0 μg/g, although higher levels may be specified for technical grades of oil.

HYDROCARBONS (MINERAL OIL)

Mineral oil content is sampled to detect adulteration of jojoba oil at levels of 5% or more.

PHOSPHORUS

Natural jojoba oil typically contains 50–100 μg/g of phosphorus in the form of phospholipids and phosphatides. The phosphorus content of mechanically extracted and filtered jojoba oil is typically from 20 to 40 μg/g, and refined or solvent-extracted grades contain >1 μg/g. Phospholipids are considered a positive component in some applications, although they may cause cloudiness in jojoba oil.

COLOR

When determining color by the Wesson/Lovibond method, a 13.3 cm column should be used for comparative purposes. The color of natural jojoba oil ranks approximately 4.5 red and >50 yellow on the Lovibond scale. Solvent-extracted oils are typically darker and refined oils lighter; jojoba oil can also be bleached almost water-white.

FAT STABILITY (OSI)

The Jojoba Industry Technical Committee recommends the use of the OSI method at 110°C as the preferred method for evaluating the stability of jojoba oil.

TRIGLYCERIDES

Natural jojoba oil contains no triglycerides or mineral oil. AOCS Recommended Practice Ci 2-91 provides a simple method for detecting the adulteration of jojoba oil with triglycerides at levels of 5% or more.

ACID VALUE

Acid value is a key specification and the most frequently determined property of jojoba oil. Total acid value for natural jojoba oil is typically in the range of 0.2–0.5, technical grades of oil may reach as high as 5 and refined grades may be 0.2 or lower. Acceptable levels are established by the intended application.

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NOTES

1. The committee recommends that the following methods not be used when determining jojoba oil quality: Phosphatides in Vegetable Oils (nephelometry), AOCS Recommended Practice Ca 19-86; Color (FAC method), AOCS Official Method Cc 13a-43; Peroxide Value, AOCS Official Method Cd 8b-90.

Table 1
Recommended methods for jojoba oil quality and purity.

Constituent	AOCS method
Moisture	Ca 2e-84 (quality)
Residual hexane	Ca 3b-87 (quality)
Hydrocarbons (mineral oil)	Ca 6c-65 (purity)
Phosphorus	Ca 12b-92 (quality)
Color	Cc 13b-45 (quality)
Fat stability (OSI)	Cd 12b-92 (quality) (see Notes, 2)
Triglycerides	Ci 2-91 (purity)
Acid value	Ci 4-91 (quality)

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AOCS Recommended Practice Ci 2-91
Reapproved 2009

Detection of Triglyceride Adulteration of Jojoba Oil

DEFINITION

The method uses thin-layer chromatography (TLC) on Silica Gel 60 TLC plates to separate triglycerides present in jojoba wax esters. The triglyceride fraction, having an R_f value of approximately 0.5, is detected by iodine vapor (see Notes, 1). The jojoba wax esters have an R_f value of approximately 0.9.

SCOPE

The method is applicable to the detection of triglyceride adulteration of jojoba oil with a lower detection limit of approximately 0.5% and a standard deviation in the range of 0.07–0.19. The presence of mineral oil in jojoba wax esters is difficult to detect by this method (see Notes, 2).

APPARATUS

1. Silica Gel 60 thin-layer plates—may be purchased premade.
2. Developing tanks—glass, two required.

REAGENTS

1. Hexane, glacial acetic acid, anhydrous diethyl ether—all reagent grade (see Notes, *Caution*).
2. Developing solvent—hexane/diethyl ether/anhydrous acetic acid, in the ratio of 90:10:1, v/v/v.
3. Iodine crystals.

PROCEDURE

1. Add 1 mL hexane to 1 mL jojoba wax esters and mix, using a vortex mixer.
2. Spot 1 µL of test portion on TLC plate with standards (see Notes, 3).
3. Develop in solvent mixture above until the solvent front is approximately 1 cm from top of plate. Remove plate and air dry.
4. Place dry plate in developing tank containing iodine crystals for 0.5 hr for visualization (see Fig. 1).
5. To determine if triglycerides are present, compare the test portion with cottonseed oil standards (see Fig. 2 and Notes, 3–5).

NOTES

Caution

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. The Occupational Safety and Health Administration recommends that exposure not exceed 350 ng/M^3 for a time-weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects. A fume hood should be used at all times when using hexane.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

NUMBERED NOTES

1. Charring with sulfuric acid gives a higher standard deviation than detection with iodine vapor.
2. Mineral oil is hard to detect because it elutes with wax esters. However, mineral oil will give a pink color when treated with iodine vapor.
3. Quantitation is done by comparing the test portions to standards containing known amounts of cottonseed oil (0, 0.5, 1, 2, 5, 7, 10%) in pure jojoba wax esters.
4. Test samples of jojoba wax esters containing different amounts of cottonseed oil were sent to various labs for determining the percent adulteration, using TLC as noted in this method. The test samples contained 0, 0.5, 1, 2, 4, 5, 6, 8, and 10% cottonseed oil.
5. This study shows that this procedure does detect adulteration with consistency. The standard deviation was small, ranging from 0.07 to 0.19 (Table 1). The variance was higher in the test samples for which no exact standard was made. The results were given as a range, with the midpoint of the range used for statistical analysis. One laboratory charring with sulfuric acid because iodine was unavailable produced results with higher test sample variations.

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Table 1
Results from collaborative study among four laboratories (see Notes, 4).

% Adulteration	Average	SD	Variance
0	0.15	0.07	0.17
0.5	0.65	0.07	0.1
1.0	1.24	0.12	0.81
2.0	2.16	0.08	0.59
4.0	3.9	0.18	1.53
6.0	5.2	0.18	1.7
8.0	6.4	0.19	2.36
10.0	9.8	0.1	0.54

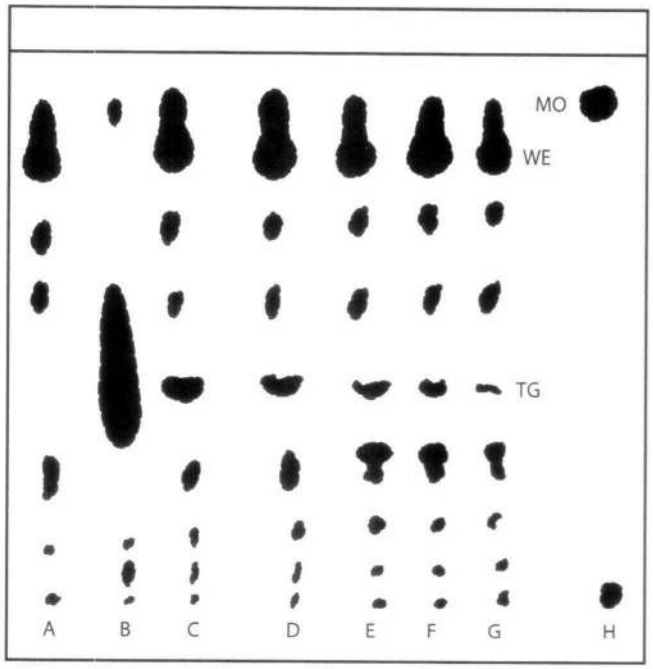
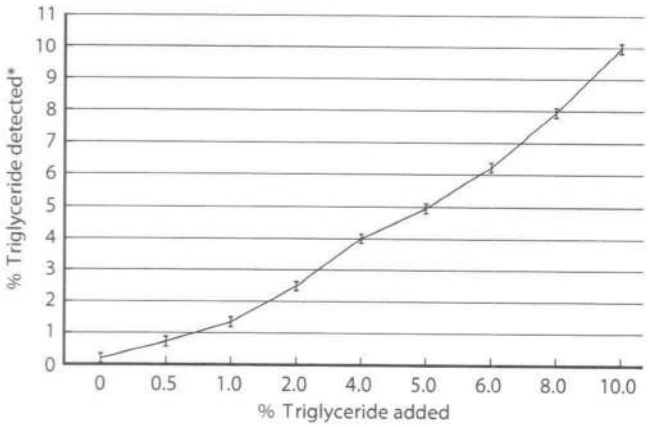


Figure 1. TLC of jojoba lipids. (A) Pure jojoba wax esters; (B) corn oil; (C) jojoba with 5% corn oil; (D) jojoba with 3% corn oil; (E) jojoba with 1% corn oil; (F) jojoba with 0.5% corn oil; (G) jojoba oil with 0.25% corn oil; (H) mineral oil; WE, wax esters; TG, triglycerides; MO, mineral oil. The R_f of the triglycerides is approximately 0.5 and increases in size and density as the amount of triglycerides increases.



*± standard deviation

Figure 2. Results of collaborative study for TLC determination of jojoba oil adulteration (see Notes, 5).

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AOCS Recommended Practice Ci 4-91

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Acid Value, Jojoba Oil

DEFINITION

The acid value is the mg of potassium hydroxide necessary to neutralize the free acids in a 1 g test portion.

SCOPE

This modified version of AOCS Official Method Cd 3d-63 is applicable only to jojoba oil.

APPARATUS

1. Erlenmeyer flasks—250 or 300 mL.
2. Suitable burette—Class A.

REAGENTS

1. Potassium hydroxide (KOH), 0.1 M—accurately standardized and carbonate-free. See AOCS Specification H 15-52 for guidance. Add 6 g reagent grade KOH to 1 L of water in a 2 L Erlenmeyer flask, boil 10 min with stirring, add 2 g reagent grade barium hydroxide [$Ba(OH)_2$], again boil 5–10 min, cool, stopper flask, and let stand several hours. Filter through a sintered-glass funnel, store in an alkali-resistant bottle protected from carbon dioxide (CO_2). Standardize by titration with pure potassium acid phthalate using phenolphthalein indicator (see Notes, 1).
2. Solvent mixture—consisting of equal parts by volume of isopropyl alcohol (AOCS Specification H 18-58) and toluene (AOCS Specification H 19-58) (see Notes, *Caution*). The mixture must give a distinct and sharp end point with phenolphthalein in the titration as noted in Procedure, 4.
3. Phenolphthalein indicator solution—1.0% in isopropyl alcohol.

PROCEDURE

1. Add indicator solution to the required amount of solvent in ratio of 2 mL to 125 mL and neutralize with alkali to a faint but permanent pink color.
2. Weigh 20 grams (± 10%) of jojoba oil into an Erlenmeyer flask.
3. Add 125 mL of the neutralized solvent mixture. Be sure that the test portion is completely dissolved before titrating. Warming may be necessary in some cases.
4. Shake the test portion vigorously while titrating with standard KOH solution to the first permanent pink color of the same intensity as that of the neutralized solvent before the latter was added to the test portion. The color must persist for 30 sec.
5. A blank must be determined in the same manner as the test portion.

CALCULATION

$$\text{The acid value, mg KOH per g of test portion} = \frac{(A - B) \times M \times 56.1}{W}$$

Where—

A = volume, mL of standard KOH used in the titration
B = volume, mL of standard KOH used in the blank titration
M = molarity of standard KOH
W = mass of test portion, g

PRECISION

Single determinations performed in two different laboratories should not differ by more than 0.13.

NOTES

Caution

Isopropyl alcohol is flammable and a dangerous fire risk. The explosive limits in air are 2–12%. It is toxic by ingestion and inhalation. The TLV in air is 400 ppm.

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

NUMBERED NOTES

1. A standard methanolic potassium hydroxide (0.1 M) solution (see AOCS Specification H 15-52) may be used as an alternate titrant in place of the standard aqueous solution. The methanolic potassium hydroxide is reported to provide a complete solvent system, having a distinct, clear end point. See Reference regarding the ruggedness of the acid value method.

REFERENCE

1. *JAOAC 59*:658 (1976).

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Table 1
Jojoba acid value results from four independent laboratories, March, 1991.

	Lab 1		Lab 2		Lab 3		Lab 4		Range					
Sample	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Low	High	Var	Mean	SD	RSD_R %
01	0.31	0.30	0.30	0.31	0.29	0.29	0.25	0.24	0.24	0.31	0.07	0.29	0.027	9.4
02	4.17	4.18	4.21	4.22	4.28	4.28	4.15	4.20	4.15	4.28	0.13	4.21	0.048	1.1
03	0.33	0.32	0.35	0.34	0.31	0.32	0.27	0.28	0.27	0.35	0.08	0.32	0.028	8.8
04	0.60	0.59	0.58	0.57	0.55	0.56	0.54	0.53	0.53	0.60	0.07	0.56	0.024	4.2
05	0.98	0.99	1.04	1.04	1.05	1.07	0.99	1.05	0.98	1.05	0.07	1.03	0.034	3.3
06	0.27	0.27	0.30	0.30	0.28	0.28	0.30	0.29	0.27	0.30	0.03	0.29	0.013	4.5
07	0.28	0.29	0.30	0.29	0.27	0.28	0.27	0.28	0.27	0.30	0.03	0.28	0.010	3.6
08	0.55	0.54	0.54	0.54	0.47	0.48	0.57	0.58	0.47	0.58	0.11	0.53	0.039	7.4
09	0.80	0.79	0.91	0.91	0.73	0.75	0.80	0.81	0.73	0.91	0.18	0.81	0.066	8.1
10	0.33	0.31	0.31	0.32	0.28	0.29	0.35	0.34	0.28	0.35	0.07	0.31	0.024	7.7
Average variance (precision)													0.08	
Standard deviation of precision													0.046	

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AOCS Official Method Cj 1-94

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DSC Melting Properties of Fats and Oils

DEFINITION

The method determines thermal properties of oils and fats by differential scanning calorimetry (DSC).

SCOPE

While the procedure might be used to evaluate five melting characteristics (peak temperature, onset temperature, heat of fusion (ΔH), completion of melt temperature, and identification of polymorphic behavior), this method is limited to the criteria of peak temperature, onset temperature, and completion of melt. This is because only these three criteria provided statistically acceptable values in the collaborative study (see Notes, 1). The method is applicable to animal fats, to liquid and hydrogenated vegetable oils, and to blends of liquid and hydrogenated vegetable oils.

APPARATUS

1. Aluminum or stainless steel capsules.
2. DSC instrument—capable of holding temperature at –60°C and achieving temperature of 80°C.

REAGENTS

1. Indium, powder—60 mesh, 99.999%, Aldrich Chemical Co., Milwaukee, WI 53233, or equivalent.
2. *n*-Decane, 99+%, Aldrich Chemical Co., Milwaukee, WI 53233, or equivalent.
3. Methyl stearate—99%, Aldrich Chemical Co., Milwaukee, WI 53233, or equivalent.

PROCEDURE

1. Standardization of equipment—Proceed with the normal standardization using both indium and *n*-decane as reference standards. Follow instrument manual for adjustment to lock onto these two reference points and flatten the baseline slope as much as possible when empty pans are analyzed. Analyze the secondary standard (methyl stearate). Weigh approximately 5 mg of the standard into the same kind of pan which will be used for the test portion (if hermetically sealed, it may be reused at a later date). Use the method sequence in Procedure, 2–7 to obtain the melting point onset (because of the high purity, only a 2 min hold is necessary for the standard after crystallization). Be certain that the heating rate during the definitive heating pattern is at 5°C/min. The melting point onset should be within ±2.00°C of 36.5°C. If not, recheck calibration.

   Note—Be certain to use identical capsules for the test portion as those used for reference standards and the instrument blank reference.
2. Melt each test portion completely and weigh 7 ± 0.200 mg of each test portion into the same kind of capsule used for the blank and reference samples (aluminum or stainless steel) and seal to minimize oxidation and other changes.
3. Place capsules in DSC at room temperature.
4. Heat rapidly to 80°C and hold for 10 min.
5. Cool to –60°C at 10°C/min and hold for 30 min.
6. Heat to 80°C at 5°C/min.
7. Use the baseline obtained for an empty capsule analysis from the final melt segment of the program to define the position of the baseline under the sample peaks. Overlay the final melting curve of the test portion over the curve for the empty capsule with a flexible ruler or other curve guide to define the baseline of the test portion back to where it intersects the initial deviation of the melting curve from its baseline. The baseline beneath the test portion should be a continuation of the baseline where there are no sample components present. If a shift has occurred in the heat capacity of the test portion after the melt, it will be evident relative to the baseline of the empty capsule. Have the instrument calculate the sigmoid baseline if it can, or connect the end of the peak point with the last point in which the test portion was in conjunction with the baseline of the empty capsule.

RESULTS

1. Determine and report peak temperature, onset temperature, and the point of completion of melt in degrees C. The onset, if not computer generated, is an extrapolation to baseline of the steepest slope of the principal peak. The completion of melt can be estimated as the point of intersection with the baseline of the last slope of the melting curve.

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PRECISION

1. Estimates of precision from collaborative study results, based on 30 min holding time—

	Liquid oils	Solid fats
Repeatability standard deviation S_r:		
Onset	±0.33°C	±1.46°C
Peak	±0.16°C	±0.54°C
Completion of melt	±1.03°C	±0.48°C
Standard deviation between labs, S_L:		
Onset	±1.42°C	±2.79°C
Peak	±0.61°C	±0.85°C
Completion of melt	±3.24°C	±1.42°C
Reproducibility standard deviation S_R:		
Onset	±1.46°C	±3.15°C
Peak	±0.63°C	±1.01°C
Completion of melt	±3.40°C	±1.50°C

NOTES

1. This method was studied collaboratively by the AOCS Physical Methods Committee.
2. Crystallization temperatures above –60°C can be used if the melting point of the fat is sufficiently high. Any deviations from the Official Method should in such cases be noted and commented on.

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AOCS Official Method Cj 2-95

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X-Ray Diffraction Analysis of Fats

DEFINITION

The method determines the short and long spacing of the fat crystal structure and identifies the predominant crystal forms through interpretation of the X-ray diffraction pattern. The method is based on a collaborative study carried out by the AOCS Physical Methods Technical Committee during 1995. The collaborative study results are summarized in Tables 3, 4, and 5.

SCOPE

Applicable to any triglyceride ester that retains a molded shape at the temperature of analysis (temperature up to 25°C).

APPARATUS

1. Any commercial or privately assembled X-ray diffractometer equivalent to those in the labs involved in pretesting the method, e.g., Philips PW 1050, 1710, 1820, 1840, APD 3600, and DPT Rigaku RU 200 and RINT 1100S; Siemens D5000 and Eliott GX-6.
2. Any available sample holder for the instrument that will render immobile a precut lump of hard fat or accept the leveling of a spreadable fat.

PROCEDURE

1. Materials, once received, should not be heated above the temperature of analysis.
2. Test portions are to be deposited into appropriate holders for the instrument.
3. Standard laboratory operating conditions are to be used in accordance with instrument manual for obtaining optimum resolution and intensity of lines for film or peaks for spectrometer. Background scatter should be minimized.
4. Calculate the Bragg spacings of the strongest five short spacings, i.e., between 3.70 and 4.65Å, and up to six of the strongest long spacings, i.e., between 10 and 65Å.
5. Among the long spacings, tabulate the lines in the following categories: 10–20Å, 20–30Å, 30–40Å, 40–50Å, 50–60Å, and 60–65Å. It is then possible to combine data from the same region of diffraction angle (2θ) for a relevant calculation of standard deviation.
6. Define 1st, 2nd, and 3rd order lines/peaks among the long spacings obtained. Multiply spacing in Angstroms by order number.
7. The short spacings are defined as all spacings below 5Å, but for identification of fat crystal form only the following lines or peaks are critical.
   (a) α-form with single line or peak at 4.15Å ± S.D.
   (b) β′-form with two main lines or peaks at 3.8 and 4.2Å ± S.D.
   (c) β-form with a strong 4.6Å ± S.D. line or peak and several other lines or peaks.

   All three groups can be combined for calculation of standard deviation in the short spacings region.

RESULTS

1. Identify crystal forms present from short spacings. Knowledge of the chemical composition of the sample would also benefit interpretation of the acquired data.
2. Average values obtained from first three orders of long spacing to obtain one average value.

PRECISION

Repeatability—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit r, as calculated from 2.8 times the within-laboratory average standard deviation shown in Table 1.

Reproducibility—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit R, as calculated from 2.8 times the within-laboratory average standard deviation shown in Table 2. Collaborative study results are summarized in Tables 3, 4, and 5.

While an explanation of the DCL, TCL terminology follows, typical structures of SCL, DCL, and TCL are shown in Figure 1.

The XRD 1st order long spacings are a measure of longitudinal distances in fat crystals, corresponding to the fatty acid chain length and the packing mode.

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When the fatty acid length of a fat (triglyceride) is known, the packing mode of the triglyceride molecules in the fat crystals can be determined from the long spacings measured by XRD.
There are three typical packing modes for glycerides:

1. *Single chain length (SCL)*—Here the long spacings correspond to the average length of the fatty acids. The SCL packing mode can only be present in glycerides having a single fatty acid esterified to glycerol or other polyols. The SCL structure is often found with surfactants (emulsifiers), where the polar head group is large (i.e., organic esters of monoglycerides, sorbitan esters, etc.), but cannot exist in triglycerides.
2. *Double chain length (DCL)*—This is the most common packing mode in natural triglycerides with C16/C18 chain length fatty acids. The measured long spacings correspond to the thickness of the bilayer structure. Due to a variation in the angle of tilt in different crystal forms (α-β′-β), the long spacings vary accordingly.
3. *Triple chain length (TCL)*—This is found when one fatty acid from a triglyceride molecule "overlaps" an identical fatty acid in another triglyceride molecule. The TCL structure has long spacings corresponding to the length of three fatty acids and is found in triglycerides where the fatty acid in the 2nd position is different from the fatty acids in the 1st or 3rd positions. A typical example is cocoa butter, where a high proportion of the triglycerides has oleic acid in the 2nd position (e.g., POS). Other fats with TCL structure are rarely found among natural vegetable fats, and never in animal based fats. Synthetic fats (e.g., acetoglycerides) with a mixture of long chain fatty acids (C16/C18/C20) and short chain fatty acids (C6/C8/C10) can also form TCL structures [cf. D.R. Kodali et al., *JAOCS 61*:1078 (1984)].

Table 1
Within-Laboratory Average Standard Deviation.

		Within-laboratory S.D.
Long Spacings	40–50Å	0.33Å
	20–30Å	0.22Å
	10–20Å	0.09Å
Calculated Long Spacing Average		0.32Å
Short Spacings		0.025Å

Table 2
Between Laboratory Average Standard Deviation.

		Within-laboratory S.D.
Long Spacings	40–50Å	1.80Å
	20–30Å	0.65Å
	10–20Å	0.28Å
Calculated Long Spacing Average		
With 1st order between	60–65Å	2.04Å
With 1st order between	50–60Å	1.32Å
With 1st order between	40–50Å	1.01Å
Short Spacings		0.023Å

Table 3
Key, Identity, and Crystal Form of Samples Analyzed in AOCS X-Ray Diffraction Collaborative Study.

Sample	Identity	Crystal form	
A	Hydrogenated canola oil, mr 46 C	β-2	(DCL)
B	Margarine fat—soybean oil	β′-2	(DCL)
C	Cocoa butter (West African)	β-3	(TCL)
D	Cocoa butter replacer (CBR)	β′-2	(DCL)
E	Sorbitan tristearate	α-2	(DCL)
I	Duplicate of B	β′-2	(DCL)
M	Blend of C and D (2:1)	β-3, β′-2	(TCL, DCL)
K	Duplicate of A	β-2	(DCL)
O	Duplicate of D	β′-2	(DCL)
T	Glycerol monooleate, 95%	β-2	(DCL)

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Table 4

The interlaboratory study completed by the AOCS Physical Methods Technical Committee in 1995, coordinated by Art Waltking, in which 11 laboratories (using 12 instruments) participated, each obtaining two test results for each of ten samples analyzed, gave results for crystal form summarized in this table.

	Sample Code									
Lab	A	B	C	D	E	I	K	M	O	T
1	β-2 DCL	β'_1-2 + β DCL	β_2-3 TCL	β'_1-2 DCL	α-2 DCL	β'_1-2 + β DCL	β-2 DCL	β-3 + β'_1-2 TCL + DCL	β'_1-2 DCL	β TCL
2	β-2	β′-2	β-3	β′-2	α-2	β-2	β-2	β + β′ DCL + TCL	β′-2	β-2
3	β-2 DCL	β′ DCL	β-3 TCL	β′ DCL	α DCL	β′ DCL	β-2 DCL	β-3 + β′ TCL + DCL	β′ DCL	β-2 TCL
4	β	β′	β-3	β′	α	β′	β	β + β'_1	β′	β
5	β_2-2 DCL	β′-2 DCL	β_1-3 TCL	β′-2 DCL	α-2 DCL	β′-2 DCL	β_2-2 DCL	β′ + β TCL + DCL	β′-2 DCL	β + β′ TCL
6	β-2 DCL	β′-2 DCL	β-3 TCL	β′-2 DCL	α-2 DCL	β′-2 DCL	β-2 DCL	β′-3 TCL	β′-2 DCL	β′-2 TCL
7	β DCL	β′ DCL	β + β′ TCL	β′ DCL	α DCL	β′ DCL	β DCL	β′ + β DCL + TCL	β′ DCL	β TCL
8	β-2 DCL	β'_1-2 DCL	β-3 TCL	β'_1-2 DCL	α-2 DCL	β'_1-2 DCL	β-2 DCL	β + β'_1 TCL + DCL	β'_1-2 DCL	β-2 + β'_1-2 TCL
9	β DCL	β'_1 DCL	β TCL	β'_1 DCL	α DCL	β'_1 DCL	β DCL	β'_1 + β DCL + TCL	β'_1 DCL	β TCL
10	β DCL	β DCL	β TCL	β′ DCL	α DCL	β DCL	β DCL	β DCL + TCL	β′ DCL	β TCL
11	β DCL	β + β′ DCL	β + β′ TCL	β′ DCL	α DCL	β′ + β DCL	β DCL	β + β′ TCL + DCL	β′ DCL	? ?
12	β-2 DCL	β′ DCL	β-3 TCL	β′ DCL	α DCL	β′ DCL	β-2 DCL	β-3 + β′ TCL + DCL	β′ DCL	β-2 DCL

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Table 5

The interlaboratory study completed by the AOCS Physical Methods Technical Committee in 1995, coordinated by Art Waltking, in which 11 laboratories (using 12 instruments) participated, each obtaining two test results for each of ten samples analyzed, gave results for crystal form identification assessment summarized in this table.

Laboratory	Sample code									
	A	B	C	D	E	I	K	M	O	T
1	*	+	+	+	*	+	*	+	+	X
2	*	*	*	*	*	X	*	–	*	*
3	*	*	*	*	*	*	*	*	*	*
4	–	–	*	–	–	–	–	–	–	–
5	+	*	+	*	*	*	+	–	*	+
6	*	*	*	*	*	*	*	X	*	X
7	*	*	+	*	*	*	*	*	*	*
8	*	+	*	+	*	+	*	*	+	+
9	*	+	*	+	*	+	*	+	+	X
10	*	X	*	*	*	X	*	X	*	*
11	*	+	+	*	*	+	*	*	*	X
12	*	*	*	*	*	*	*	*	*	*

Key to Table 5:

* Correct identification as requested (i.e., either β-2 or β(DCL) or both).

+ Correct identification plus added information such as trace of another form or β′ 1-2 types.

– Correct identification but not as complete as requested (Lab 4 not able to do long spacings and other labs not indicating order of concentration when mixed crystals were identified).

X Identification does not match reference or majority.

Summary of Table 5:

Of 120 answers, 9 incorrect; 6 labs—no errors; 1 error labs, #1, 2, 9, and 11; 2 error lab, #6; and 3 error lab #10; 4 of these (#1, 6, 9, and 11) misidentified Sample T, which is a special fat (monoglyceride). Only lab #2 showed inconsistency in identifying a duplicate sample.

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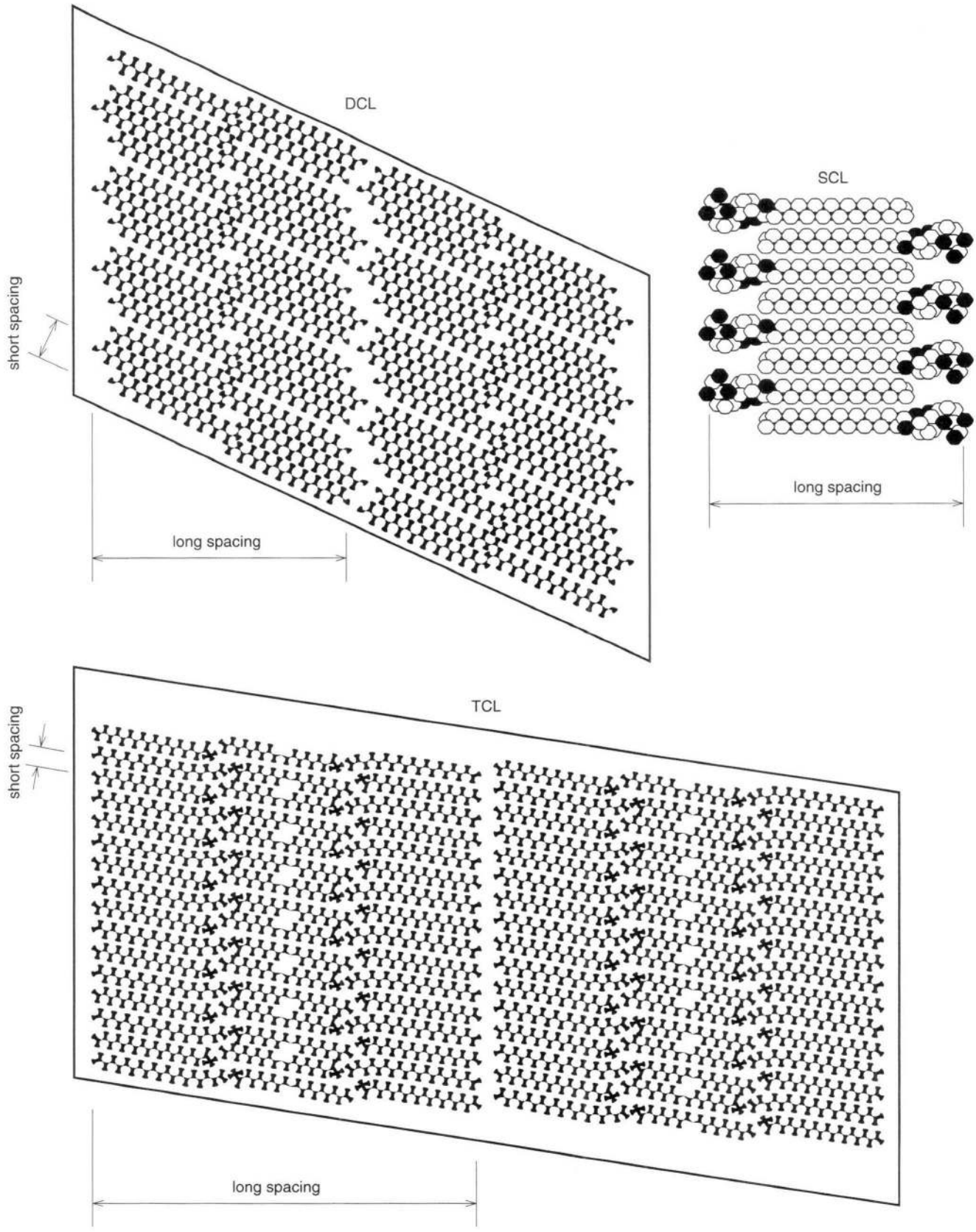

Figure 1. Typical structures of SCL, DCL, and TCL.

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AOCS Recommended Practice Cj 3-99
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Calibration of Rheological Instruments

DEFINITION

The method establishes a criterion for evaluating the accuracy and precision of various viscoelastic and viscosity instruments on an ongoing basis.

SCOPE

Applicable to the evaluation of the validity of measurements from rheological instruments when they are obtained on fluid and semisolid materials such as oils, fats, and products produced from vegetable oilseed materials.

APPARATUS

1. Any commercial rheological instrument equivalent to those in the labs involved in pretesting the method, e.g., Brookfield HADV-III; Carri-med; Haake RV-20; Physica MC100; Rheometrics (RMS800, RFS8400 & DSR); Reologica Stresstech; TA Instruments CSL-500.

REAGENTS

1. Equivalent certified standards as used in the methods collaborative studies.
   (a) Polydimethylsiloxane polymer, obtained from Rheometrics Inc., Stock No. 700-01011.
   (b) Newtonian viscosity oil, obtained from Brookfield Instrument Co., Fluid Standard, 12500. The specific sample in these studies was certified as 12,440 cps.

PROCEDURE

1. Analyze one of the standard reagents in accordance with the capabilities of the instrument to be evaluated.
   (a) Use reagent (A) above or equivalent if the instrument measures the viscoelastic properties, i.e., G′ (elastic or storage modulus), G″ (viscous or loss modulus), and Eta (dynamic viscosity).
   (b) Use reagent (B) above or equivalent if instrument measures only viscosity or if the force range of the instrument does not permit the use of the polymer. (See General Notes, 1).
2. Instrument operating conditions when using the polymer (A) for calibration studies:
   (a) Use a dynamic frequency sweep from 0.1 rad/sec to 100 rad/sec with a set of parallel plates (preferably 25 mm) at a gap of 1.0 mm and a temperature of 25°C (77°F).
   (b) 8000 dynes/cm^2 is used for stress-controlled instruments and 10% strain for strain-controlled instruments.
   (c) Allow for an initial relaxation delay of 60 sec between compressing and shearing of the test portion, during which time the excess of the test portion expressed from the fixtures should be removed.
   (d) Obtain a minimum of 7 data points over the 0.1 to 100 rad/sec range. Record the values of G′ and G″ @ 1.0 rad/sec.
3. Instrument operating conditions for viscosity and yield-force limited instruments using Newtonian standard (B) for calibration studies.
   (a) Use shear rates of 10 and 40 reciprocal sec at a temperature of 25°C (77°F) for a minimum of five min.

RESULTS

1. Determine the viscoelastic properties G′ and G″ or the viscosity in cps in accordance with the calculations in your instrument operating manual.
2. If the same standards are utilized which were used in the collaboratives, compare the results obtained to the data on Table 1. (See General Notes 2, 3).
3. With other standards compare to established values to define the validity of the instrument response.
4. If results are consistently outside the 1 SD error range (S1) of the between-laboratory standard deviations listed in the estimates of precision tabulation in Precision section, an investigation of the cause is warranted.

GENERAL NOTES

1. For most viscoelastic instruments, the use of the polymer standard is preferred since the material is more sensitive to shear rate differences. However, for instruments which will experience an overload error due to the high yield stress of the polymer and all general viscosity instruments, the Newtonian standards should be used.
2. It is not necessary to use precisely the same standards applied in the collaborative study to utilize this procedure. If converted to a percentage basis, the collaborative data still provide reasonable estimates of within- and between-laboratory error if the test conditions spelled out above are used. However, if other standards are used, they should be certified by their supplier or defined independently by two other instruments.

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3. Use of a Newtonian standard in place of the polymer for calibration of instruments measuring viscoelastic properties will not ensure correct measurement of elastic parameters but will still be better than no calibration check.

PRECISION

1. Estimates of precision from collaborative study results. Repeatability standard deviation, S_r:
   G′—polymer evaluation: 2123 dynes/cm^2
   G″—polymer evaluation: 1907 dynes/cm^2
   Newtonian standard @ 10 sec-1: 424 cps
   Newtonian standard @ 40 sec-1: 386 cps
   Standard deviation between labs, S_L:
   G′—polymer evaluation: 4601 dynes/cm^2
   G″—polymer evaluation: 9705 dynes/cm^2
   Newtonian standard @ 10 sec-1: 551 cps
   Newtonian standard @ 40 sec-1: 232 cps
   Reproducibility standard deviation S_R:
   G′—polymer evaluation: 5067 dynes/cm^2
   G″—polymer evaluation: 9890 dynes/cm^2
   Newtonian standard @ 10 sec-1: 698 cps
   Newtonian standard @ 40 sec-1: 450 cps

Table 1
Calibration standards collaborative values.

Polymer standard A for viscoelastic instruments

	1996 Series			1997 Series	
Inst. #	G′ dynes/cm^2	G″ dynes/cm^2	Inst.	G′ dynes/cm^2	G″ dynes/cm^2
1	70900	133830	1	71172	131400
2	74072	137650	2	74378	138550
4	64952	122000	4	64369	119700
5	61690	115575	5	62750	118000
7	70130	114500	7	63540	118800
12	52730	81550	13	51700	99000
Average[a]	68349	124711	Average[a]	67242	125290
Combined 1996–1997			Average[a]	67796	125000

Newtonian standard B for viscosity and other instruments

	1996 Series			1997 Series	
Inst. #	@10 sec-1 CPS	@40 sec-1 CPS	Inst. #	@10 sec-1 CPS	@40 sec-1 CPS
3	14060	13670	3	13360	12300
5	14298	5	12445	12415	
6	11550	11750	6	12300	12450
8	13412	13253	8	12755	12530
9	13590	9	13350	13310	
10	12600	11880			
Average	13252	12638	Average	12842	12601
Combined 1996–1997			Average	13047	12620

[a] Averages adjusted for outliers

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AOCS Recommended Practice Cj 4-00

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Spreadability

DEFINITION

This method determines a measure of spreadability of semi-solid materials.

SCOPE

Applicable to lipid-containing solid suspensions, emulsions, and carbohydrate pastes such as peanut butter, mayonnaise, and starch pastes that will maintain their shape at the temperature of analysis.

APPARATUS

1. Texture Technologies (Scarsdale, NY) TA-XT2 Texture Analyzer or equivalent.
2. 2.54 cm diameter acrylic cylinder probe (if from Texture Technologies, TA-3)
3. 2.1 cm wide, 12.7 cm long stainless steel spatulas (Fisher Scientific, Pittsburg, PA, #14-366C) or equivalent.
4. Lexan base plate.

PROCEDURE

1. Attach the acrylic cylinder probe to the crosshead.
2. Calibrate the instrument for *force*.
3. Place the base plate on top of the sample platform, centered below the probe. *Do not secure in any way.*
4. Set a *loading position* for the crosshead about 10 cm. above the lexan base plate and a *start position* 4 mm above this base plate. The *start position* should be calibrated to precisely 4.0 mm probe height against a flat, smooth base plate in accordance with the instrument manual section on probe height calibration, or as reported in Notes, 1.
5. Incorporate the following instrument parameters.

Mode:	Measure force in compression
Option:	Return to Start
Force units:	Grams
Distance format:	mm
Pre-test speed:	1.0 mm/s
Test speed:	3.0 mm/s
Post-test speed:	10.0 mm/s
Distance:	3.9 mm
Trigger type:	Button
Test output:	Final

6. Test portion loading
   (a) Using the spatula perpendicular to the surface of the material to be tested, penetrate the surface to a depth of about 5 cm near the middle of the surface and force the spatula to one side for about 2.54 cm. This should have created a hole roughly 2.54 cm by 2.54 cm.
   (b) The spatula should be removed, with some of the product forced from the hole, and cleaned with a tissue.
   (c) Re-enter the product with the spatula parallel to the first entry so as to be able to remove a 1.27 cm thick slice of the undisturbed product, which is 2.54 to 3.8 cm long, by again shifting the spatula sideways toward the hole previously created.
   (d) Rest the spatula on the lexan base plate so that this sample is centered under the probe, and with a second spatula butted against the near edge of the sample at a right angle, slip the first spatula from under the sample. This will leave the product sample centered under the probe on the base plate if done properly.
7. Move the crosshead quickly to the start position (4 mm above the base plate) and wait 1 min for relaxation of the sample.
8. Start the run. This will squeeze the sample to 0.1 mm thickness.
9. Record the final force, which is the last force reading before the probe reverses direction and returns to the start position (see Notes, 2).
10. Clean the probe and the base plate by wiping them with tissues.
11. Run the material in triplicate.

RESULTS

1. Use the average of all three values of a given material as the measure of spreadability. Report in grams or kilograms depending on the level and reporting convenience.

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PRECISION

1. Estimates of precision from collaborative study (Notes, 3) show these results:
   Repeatability standard deviation, S_r—116 g
   Standard deviation between labs, S_L—355 g
   Reproducibility standard deviation, S_R—378 g

NOTES

1. To enter the height to be calibrated, turn the key to Machine Configure, press the ENT button until the middle window reads P ht, and then type in 4.0 to establish a return height of 4.0 mm after touching the base. Then turn the key out of the Machine Configure position.

   To calibrate the probe height, position the probe just over the base plate and turn the key to Machine Configure.

   If using the program version up through 5.20—Hold the FAST button down and then also press the CALIBRATE button. The probe will travel downward, touch the base plate, and return precisely to 4.0 mm over the base.

   However, if using program version 6.0 and higher—Press the CALIBRATE button and then press the "+" button on the keyboard to calibrate the probe height—the display will read: "Press the CALIBRATE button." The probe will travel downward, touch the base plate, and return precisely to 4.0 mm over the base.
2. The lexan base plate may rise with the test portion. If the instrument is not equipped with a 50 kg load cell, do not attempt to remove the base plate until the instrument stops, or the instrument may go into overload and require a recalibration from the beginning.
3. This method was studied collaboratively by the AOCS Physical Methods Committee.

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AOCS Analytical Guidelines Ck 1-07
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Analytical Guidelines for Assessing Feedstock to Ensure Biodiesel Quality

DEFINITION

These Analytical Guidelines list those methods which may be used to assess the quality of oils and fats used in the production of biodiesel.

SCOPE

These Analytical Guidelines are restricted to triglyceride feedstocks for biodiesel manufacture, including vegetable oils (soybean oil, rapeseed oil, palm oil, etc.), animal fats (tallow, lard, etc.), and triglyceride greases (yellow grease, etc.). Critical attributes of the triglycerides are considered in light of the requirements of the most common biodiesel process: the alkaline transesterification of the triglyceride feedstock with methanol to produce fatty acid methyl esters. Only AOCS Official Methods or Recommended Practices are listed in Table 1; however, other procedures are reported in the scientific literature.

Table 1
Methods to assess feedstock quality.

Test	Method
Sampling	AOCS C 1-47
Insoluble impurities	AOCS Ca 3a-46
Sediment by centrifugation	AOCS Ca 3d-02
Unsaponifiable matter	AOCS Ca 6b-53
Polar compounds in frying fats	AOCS Cd 20-91
Soap in oil	AOCS Cc 17-95
Polymerized triglycerides by gel-permeation HPLC	AOCS Cd 22-91
Water by modified Karl Fischer method	AOCS Ca 2e-84
Modified moisture and volatiles	AOCS Ca 2f-93
Acid value	AOCS Cd 3d-63
Sulfur, elements in oil by ICP-OES (use general guidance)	AOCS Ca 17-01
Phospholipids in vegetable oils	AOCS Ca 19-86
Phosphorus in oil by ICP-OES	AOCS Ca 20-99
Phosphorus content, colorimetric method;	AOCS Ca 12a-02
Phosphorus	AOCS Ca 12-55
Fat stability, Oil Stability Index (OSI)	AOCS Cd 12b-92
Fat stability, peroxide value	AOCS Cd 8b-90
p-Anisidine value	AOCS Cd 18-90
Polymerized triglycerides by gel-permeation HPLC	AOCS Cd 22-91
Polar compounds in frying fats	AOCS Cd 20-91
Fatty acid composition	AOCS Ce 1-62/Ce 1a-13
Methyl ester preparation	AOCS Ce 2-66
Calculated iodine value	AOCS Cd lc-85

FACTORS TO LOOK FOR IN THE TRIGLYCERIDE FEEDSTOCK TO BE USED IN THE TRANSESTERIFICATION REACTION

Refined vegetable oils destined for food use are an expensive substrate for biofuel development. Although there is interest in developing specialty oil crops, some producers have turned to less desirable or non-edible materials such as animal fats and recycled vegetable oils. A key issue in the production of biodiesel is the amount of intermediates, byproducts, unreacted substrates, and

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a variety of contaminants which may affect engine performance. Regional standards provide some guidance on the methods of analysis to be used, but do not address the analysis of the feedstock. Refined oil quality may be assessed using the Official Methods and Recommended Practices of the AOCS. Rendered animal fats and recycled greases are categorized by grade. Each grade has detailed specifications which can be determined by AOCS methods (for further details and explanation see Notes and Table 2).

SAMPLING

1. Fat products are frequently solids or semisolids at room temperature and should be completely liquified and blended prior to testing. Temperature during melting should not exceed the melting point of the sample by more than 10°C.
2. Sample material in accordance with AOCS Method C 1-47.

CLEANLINESS

1. The feedstock should be free of trash or other foreign material.
2. Possible tests: Insoluble Impurities Ca 3a-46; Sediment in Fats and Oils by Centrifugation Ca 3d-02

PURITY

1. Composition shall be primarily triglyceride in nature. Extensive levels of unsaponifiable material will lower the methyl ester content and possibly cause problems in biodiesel processing. Regional mandates may require the methyl ester content in biodiesel to be a minimum of 96.5%.
2. Possible tests: Unsaponifiable Matter Ca 6b-53; Polar Compounds in Frying Fats Cd 20-91; Soap in Oil Cc 17-95; Polymerized Triglycerides by Gel-Permeation HPLC Cd 22-91.

IMPURITIES

WATER

1. Excessive levels of moisture in the feedstock will reduce catalyst effectiveness, will reduce the total conversion, and will yield undesirable byproducts (soaps).
2. Possible tests: Modified Karl Fischer Method Ca 2e-84; Modified Moisture and Volatiles Ca 2f-93.

ACIDITY

1. High acidity in the feedstock will reduce catalyst effectiveness, will reduce total conversion, and will yield undesirable byproducts (soaps). If the acidity is high enough, alkaline transesterification will not proceed.
2. Possible test: Acid Value of Fats and Oils Cd 3d-63.

SULFUR

1. Limiting the sulfur content of the feedstock will allow the biodiesel manufacturer to more easily meet the stringent specification on sulfur (15 ppm S) in Ultra Low Sulfur Diesel fuels without additional expensive after-treatment.
2. Possible test: Trace Elements in Oil by ICP-OES Ca 17-01.

PHOSPHORUS

1. Phosphorus is an indication of the presence of phospholipids in feedstocks from plant origins. During vegetable oil refining, phospholipids are normally removed. If a significant amount of phospholipid remains, it will cause problems in the biodiesel transesterification and purification steps. If the phosphorus is not removed from the FAME product, it will not meet the phosphorus limit (10 ppm P) allowed in biodiesel. It is easier to start with a feedstock already low in phosphorus than to try to remove it in after-treatment.
2. Possible tests: Phospholipids in Vegetable Oils Ca 19-86; Phosphorus in Oil by ICP-OES Ca 20-99; Phosphorus Content, Colorimetric Method Ca 12a-02; Phosphorus Ca 12-55.

OXIDATIVE STABILITY

1. The stability of the triglyceride feedstock will have great bearing on the oxidative stability of the biodiesel prepared.
2. Possible tests: Fat Stability, Oil Stability Index (OSI) Cd 12b-92; Fat Stability, Peroxide Value Cd 8b-90; p-Anisidine Value Cd 18-90; Polymerized Triglycerides by Gel-Permeation HPLC Cd 22-91; Polar Compounds in Frying Fats Cd 20-91

FATTY ACID COMPOSITION

1. Analysis of the fatty acid composition is useful additional information which may indicate the source of the fat or oil being used as feedstock. The iodine value of the feedstock may be determined by calculation.
2. Possible tests: Fatty Acid Composition Ce 1-62; Preparation of Methyl Esters Ce 2-66; Calculated Iodine Value Cd 1c-85.

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NOTES

Table 2
AOCS Methods for Tallow and Grease Quality Parameters Recognized by the National Renderer's Association.

For an explanation of the tests, refer to 1–15, below.	
1. Boehmer number	Cb 5-40, surplus*
2. a. Color (FAC) standard	Cc 13a-43*
b. R & B Color	Cc 8d-55*
3. Fatty acid profile	Ce 1-62/Ce 1a-13
4. Free fatty acids (FFA as OA)	Ca 5a-40
5. Iodine value (IV)	Cd 1d-92
6. Lead content	Ca 18c-91
7. Moisture, impurities, unsaponifiables (MIU)	
a. Moisture	Ca 2c-25 or Ca 2b-38
b. Unsaponifiable matter	Ca 6a-40
c. Insoluble impurities	Ca 3a-46
8. Peroxide value (PV)	Cd 8b-90
9. Pesticide residue	Use EPA/FDA methods**
10. pH	
11. Polyethylene (PE)	Ca 16-75
12. Rate of filtration	
13. Saponification value (SV)	Cd 3-25
14. Titer	Cc 12-59
15. Total fatty acids (TFA)	

* Methods in normal, and bold text are current or recommended for use in the Tallow and Grease Series of the AOCS Laboratory Proficiency Program (LPP), respectively.

** Only required for food grade or feed grade materials

Rendered fats are defined by the following tests:

1. Boehmer number refers to a test to find out whether tallow is mixed in with lard. If the number is less than 73, contamination has occurred.
2. Color is quantified by comparing a sample of filtered liquid fat to the Fat Analysis Committee (FAC) standard and assigning it a number from 1 (lightest) to 45 (darkest). R & B Color means refined and bleached. For example, the specification for extra fancy tallow is usually one Red, but is sometimes specified at 0.5 Red.
3. Fatty acid profile is the relative amounts of the 16 possible fatty acids as determined by gas chromatography.
4. Free fatty acids (FFA), the amount of fatty acids split from the triglyceride or fat molecule and dissolved in the fat. FFA are a measure of the hydrolysis that has taken place within the fat molecule. Time, temperature, and the presence of moisture, bacteria, and enzymes influence the hydrolysis of fat into free fatty acids and glycerol.
5. Iodine value (IV) is a measure of the chemical unsaturation of the fat. It is expressed as the number of grams of iodine absorbed by 100 g of fat sample.
6. Lead content is determined by automatic analyzer. The tolerance level for lead is 7 ppm (mg/kg), above which it is considered toxic.
7. Moisture, impurities, unsaponifiables (MIU) should not exceed 1 to 2%, depending on how the fat is to be used.
    a. Moisture in fat arises from slight emulsification during processing and is determined by distillation with toluene or by heating; it should be 0.5 to 1%.
    b. Unsaponifiables are any material that will not saponify (form soap) when mixed with an alkali. They are soluble in ordinary fat solvents and include sterols, pigments, and hydrocarbons. These particles are inherent in all fats, both animal and vegetable, and may arise from contamination with petroleum products.
8. Peroxide value (PV) is an indication of stability and rancidity.
9. Pesticide residue must not exceed defined levels for certain chemicals that are toxic to animals; 0.5 ppm for DDT, DDD, and DDE; 0.3 ppm for dieldrin; 2.0 ppm for PCB. The analysis is done by gas chromatography. (Note, the allowable limits frequently change; refer to the Federal Register for current limits.) These tests are not relevant to biodiesel production.
10. pH is determined on a scale of 0 to 14: 7 is neutral, below 7 is acid, above 7 is alkaline.
11. Polyethylene (PE) is a foreign material in tallow, which finds its way into the rendering plant as meat wrappers mixed in with raw material.

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12. Rate of filtration is an analytical method in which a given volume of liquid fat sample will filter in a specified time under standard conditions. Filtration is slowed by the presence of fine particles and glue substances; the rate of filtration indicates whether a batch of fat will give processing difficulties.
13. Saponification value (SV) is an estimate of the mean molecular weight of the constituent fatty acids in a fat sample. It is defined as the number of milligrams of potassium hydroxide required to saponify 1 g of fat. The higher the SV, the lower the mean chain length of the triglycerides.
14. Titer is the solidification point of the fatty acids, an important characteristic in fats used to produce soap or fatty acids. Trade practice is to designate animal fats with titers of 40 °C and up as tallow and those below 40 °C as grease.
15. Total fatty acids (TFA) both the free fatty acids and those combined with glycerol (intact glycerides) should exceed 90% by weight. Fat is composed of approximately 90% fatty acids and 10% glycerol. The calorie content for glycerol is about 4.32 per gram compared with 9.4 for fatty acids. Since fatty acids contain more than twice the energy of glycerol and some other fatty substances, the TFA content of fat indicates energy content. This may be determined from the fatty acid composition when using an internal standard.

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AOCS Standard Procedure Ck 2-09

Revised 2013

Determination of Various Properties of Biodiesel by the QTA® System Method

DEFINITION

This method provides a procedure for using the QTA System for rapid determination of a variety of properties of biodiesel using a QTA-specific infrared spectrometer that uses attenuated total reflectance (ATR). The QTA System maintains a proprietary database of calibration algorithms managed centrally, and accessed by individual method users via a secure website. The spectra of the biodiesel is collected on the local spectrometer, and then transmitted to QTA's central database, where the algorithms are used for prediction. Results are then transmitted back to the user's computer.

SCOPE

This procedure describes the method that QTA Users follow when analyzing a biodiesel sample using the QTA System. A validation procedure is included, as prescribed by QTA and followed by QTA Users. The scope of this procedure does not include the development of the calibration algorithms, which is done in a proprietary process by QTA.

Calibration development by FT-IR requires knowledge of chemometric principles. This method avoids calibration development by the user, by employing a single central calibration that is used by all users. The calibration installed on the QTA Server is the result of a comprehensive calibration development program using the prescribed ASTM D6751 method for each component or property. The QTA calibrations have undergone extensive validation and have been subjected to round robin studies, as described in an AOCS Collaborative Study Report. The spectrometer specifications and operating parameters are set by QTA, and the use of central calibrations, ensure that all users access the calibrations whose performance is documented in the Collaborative Study Report. The QTA participants of the round robin were required only to scan samples and report results; the wet chemistry labs used the prescribed ASTM or EN methods.

The QTA System method is applicable to a variety of biodiesel component analyses and properties. Predictions for moisture, methanol, sulfur, total glycerin, free glycerin and monoglyceride content, as well as acid number, cloud point and oxidative stability are included. The same method may be used for other biodiesel properties when additional calibration algorithms are developed, commercialized and validated by collaborative study. This method is applicable to biodiesel B100 samples that are homogeneous, derived from animal, vegetable and/or waste feedstocks. Non-homogeneous samples cannot be analyzed using this method. The feedstocks evaluated in the 2008 international collaborative study for B100 characterization by the QTA System included two soybean oils (separate facilities), two different canola oils (separate facilities), a blend of tallow and chicken fat, two blends of choice white grease, tallow, and poultry fats (same facility but separate batches), and two blends of soybean oil and poultry fat (same facility but separate batches). For the 2010 collaborative study focusing on monoglycerides, sulfur and oxidative stability, the B100 samples included the following feedstocks: tallow, choice white grease (CWG), waste vegetable oil, canola, a blend of tallow and CWG, and four types of soybean: 1. refined, bleached, distilled; 2. crude, degummed; 3. waste; and 4. distilled after production. Additionally, two samples of unknown feedstock were included.

APPARATUS

1. QTA –specific spectrometer, provided by QTA.
2. Internet-enabled computer.
3. Disposable plastic pipets.
4. Soft white paper towels.

REAGENTS

1. 50% solution of isopropyl alcohol in distilled water.

PROCEDURE (See Notes)

1. Launch the QTA application using the shortcut on the desktop.
2. Enter your secure username and password and allow system to initialize.
3. In the Select Material screen, select B100 for the Material Type.
4. In the Identify Sample section, enter information about your analysis.

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5. Select the properties to be analyzed by checking the appropriate boxes in the Traits section.
6. Clean the ATR surface by wiping with a clean section of a dry white paper towel twice to remove the sample. Place one drop of isopropyl alcohol solution on another clean section of a soft white paper towel. Wipe the ATR with the wetted paper towel twice. Use a dry clean section of a paper towel to wipe the ATR crystal two times. Surface cleanliness will be checked after clicking analyze.
7. Click Analyze.
8. When prompted place one drop of biodiesel on the ATR crystal. Ensure that there are no bubbles in the liquid above the ATR crystal.
9. Click continue to begin the analysis.
10. Results will be returned on the computer screen within a couple of minutes.

CHECK SAMPLES

It is recommended that at least once per week a reference sample having known values be analyzed on QTA. The known values should be generated by a QTA-approved laboratory that utilizes approved methods.

The check sample results returned by QTA should be within 2 standard deviations of the reference value. If it does not, QTA support should be contacted.

VALIDATION

Perform the validation protocol for each new QTA System, when there are any significant changes in feedstock, and annually thereafter. The validation verifies that the sample matrix is included in the QTA central calibration, by comparing the primary method results to the QTA results to ensure that the variability is less than two standard errors. The validation includes:

1. Upon start up, send at least 4 samples of biodiesel from 4 different batches to an approved QTA primary laboratory for primary method analysis. One additional sample will be required annually. Repeat when necessary or when there is a significant feedstock or process change.
2. Analyze an aliquot of each of the above samples on the QTA System.
3. Upon completion of the primary data analysis, the QTA results should fall within two standard errors of the primary result. If true, then the system is validated for use.

If false, then

1. Check the primary data and the QTA spectra for accuracy, and repeat the process defined in step 3.
2. Check the homogeneity of the sample. If non-homogeneous, discard the sample and data and re-validate with homogeneous sample.
3. QTA will adjust the calibration to include the additional sample matrix, provided that: (a) the standard error of the system does not increase; and (b) the error of the prediction results of the other QTA Systems does not exceed two times the standard error.
4. If none of the above enables validation, then the QTA System will not be validated for that facility/process/feedstock.

PRECISION

AOCS Standard Procedure Ck 2-09 was originally published in 2009 based on a collaborative study performed in 2008 that examined the following properties: moisture, methanol, total glycerin, free glycerin, cloud point and acid number. The fuel matrices used in the 2008 study are listed in Table 1. Table 9 is the list of fuel matrices evaluated in 2009 for the validation of this Standard Procedure for the properties of monoglycerides, sulfur and oxidative stability.

REPEATABILITY

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of cases be greater than the values given in Tables 2–7 and 8–12. If the difference is greater than the limit indicated in Tables 2–7 and 8–12, obtain two other test portions. Analyze one as before and keep the other for a fourth determination if necessary. In this case, take as the result the arithmetic mean of the result obtained from the

Table 1. Fuel Matrices

Fuel A*	Soybean Oil Methyl Esters
Fuel B^	Soybean Oil Methyl Esters
Fuel C	Tallow and Chicken Fat Methyl Esters
Fuel D	White Grease, Tallow, and Chicken Fat Methyl Esters
Fuel F	Canola Methyl Esters
Fuel G	Canola Methyl Esters
Fuel H	Soybean Oil and Poultry Fat Methyl Esters
Fuel I	Soybean Oil and Poultry Fat Methyl Esters
Fuel J*	Soybean Oil Methyl Esters
Fuel K^	Soybean Oil Methyl Esters

* Fuel A is a blind duplicate of Fuel J.
^ Fuel B is a blind duplicate of Fuel K.

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third analysis and the nearest result obtained from the previous analyses, provided that the difference does not exceed the allowed limit. Failing this, analyze also the fourth test portion and take as the result the mean of the four determinations.

REPRODUCIBILITY

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5% of cases be greater than the values given in Tables 2–7 and 8–12. Tables 8 and 13 contain the precision data generated by the ASTM D6708 statistical evaluations the precision data generated by the ASTM D6708 statistical evaluation.

NOTES

1. When using the QTA system, the user should always use the manufacturer's instructions as the method revision cycle may not coincide with recent technology or newer technological updates.

Table 2. Precision Data: Karl Fischer Moisture

	A*	B^	C	D	F	G	H	I	J*	K^
Number of Laboratories	8	8	8	8	8	8	8	8	8	8
Number of Replicates	14	16	16	16	16	16	14	14	14	16
Primary Chem. XBAR ± S(R)	*0.038 ± 0.005*	*0.047 ± 0.005*	*0.025 ± 0.005*	*0.041 ± 0.005*	*0.069 ± 0.007*	*0.069 ± 0.005*	*0.044 ± 0.008*	*0.044 ± 0.008*	*0.037 ± 0.004*	*0.046 ± 0.005*
QTA XBAR	0.044	0.046	0.036	0.032	0.055	0.057	0.045	0.042	0.039	0.045
Repeatability										
QTA S(r)	0.003	0.004	0.004	0.007	0.004	0.002	0.003	0.006	0.005	0.003
QTA RSD(r)	6.41	7.60	12.11	21.07	6.55	3.31	6.53	14.75	11.94	5.57
QTA r	0.008	0.010	0.012	0.019	0.010	0.005	0.008	0.018	0.013	0.007
Reproducibility										
QTA S(R)	0.006	0.005	0.006	0.007	0.006	0.004	0.004	0.006	0.005	0.007
QTA RSD(R)	13.83	11.16	17.26	21.07	10.88	7.31	9.39	14.75	12.30	14.53
QTA R	0.017	0.014	0.017	0.019	0.017	0.012	0.012	0.018	0.014	0.018

* Sample A is a blind duplicate of Sample J ^ Sample B is a blind duplicate of Sample K

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Table 3. Precision Data: Acid Value

	A*	B^	C	D	F	G	H	I	J*	K^
Number of Laboratories	8	8	8	8	8	8	8	8	8	8
Number of Replicates	16	14	16	16	16	14	14	16	14	14
Primary Chem. XBAR ± S(R)	*0.313 ± 0.033*	*0.204 ± 0.020*	*0.242 ± 0.029*	*0.530 ± 0.039*	*0.092 ± 0.021*	*0.089 ± 0.019*	*0.423 ± 0.039*	*0.484 ± 0.045*	*0.334 ± 0.050*	*0.211 ± 0.024*
QTA XBAR	0.281	0.207	0.425	0.625	0.063	0.057	0.336	0.419	0.271	0.193
Repeatability										
QTA S(r)	0.043	0.027	0.061	0.050	0.050	0.053	0.046	0.160	0.053	0.060
QTA RSD(r)	15.40	12.90	14.41	8.00	80.00	93.54	13.79	38.23	19.69	30.99
QTA r	0.121	0.075	0.171	0.140	0.140	0.150	0.130	0.448	0.150	0.167
Reproducibility										
QTA S(R)	0.055	0.049	0.069	0.058	0.063	0.053	0.050	0.169	0.085	0.060
QTA RSD(R)	19.59	23.56	16.19	9.32	100.29	93.54	14.89	40.30	31.14	30.99
QTA R	0.154	0.137	0.193	0.163	0.175	0.150	0.140	0.473	0.237	0.167

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Table 4. Precision Data: Cloud Point

	A*	B^	C	D	F	G	H	I	J*	K^
Number of Laboratories	8	8	8	8	8	8	8	8	8	8
Number of Replicates	14	16	16	16	16	16	16	16	16	14
Primary Chem. XBAR ± S(R)	*0.043 ± 0.520*	*0.450 ± 0.788*	*11.679 ± 0.727*	*8.443 ± 1.096*	*-3.007 ± 0.577*	*-2.864 ± 0.656*	*1.850 ± 0.866*	*1.343 ± 0.759*	*0.043 ± 0.569*	*0.236 ± 1.065*
QTA XBAR	-1.071	-0.125	10.125	7.688	-4.938	-4.875	1.500	1.000	-0.875	-0.286
Repeatability										
QTA S(r)	0.267	0.354	0.707	0.433	0.433	0.500	0.935	0.612	0.354	NA
QTA RSD(r)	0.010	0.130	0.250	0.150	0.160	0.190	0.340	0.302	0.130	NA
QTA r	0.748	0.990	1.980	1.212	1.212	1.400	2.619	1.715	0.990	NA
Reproducibility										
QTA S(R)	0.488	0.738	1.044	0.612	0.582	0.906	0.968	0.829	1.184	0.488
QTA RSD(R)	0.180	0.270	0.370	0.220	0.220	0.340	0.350	0.830	0.440	0.180
QTA R	1.366	2.066	2.922	1.715	1.631	2.538	2.711	2.320	3.315	1.366

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Table 5. Precision Data: Total Glycerin

	A*	B^	C	D	F	G	H	I	J*	K^
Number of Laboratories	8	8	8	8	8	8	8	8	8	8
Number of Replicates	16	16	16	16	16	16	16	14	14	16
Primary Chem. XBAR ± S(R)	*0.147 ± 0.020*	*0.100 ± 0.012*	*0.097 ± 0.020*	*0.058 ± 0.013*	*0.200 ± 0.025*	*0.203 ± 0.024*	*0.251 ± 0.0.34*	*0.264 ± 0.027*	*0.148 ± 0.022*	*0.096 ± 0.012*
QTA XBAR	0.144	0.134	0.093	0.093	0.159	0.152	0.258	0.256	0.140	0.128
Repeatability										
QTA S(r)	0.012	0.010	0.008	0.016	0.014	0.009	0.018	0.018	0.009	0.013
QTA RSD(r)	8.30	7.67	8.05	17.60	8.91	5.94	7.00	7.09	6.61	10.00
QTA r	0.034	0.029	0.021	0.046	0.040	0.025	0.050	0.051	0.026	0.036
Reproducibility										
QTA S(R)	0.017	0.017	0.019	0.019	0.017	0.017	0.019	0.042	0.016	0.016
QTA RSD(R)	11.78	12.40	20.47	20.84	10.88	11.35	7.32	16.60	11.13	12.47
QTA R	0.048	0.047	0.053	0.054	0.048	0.048	0.053	0.119	0.044	0.045

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Table 6. Precision Data: Free Glycerin

	A*	B^	C	D	F	G	H	I	J*	K^
Number of Laboratories	8	8	8	8	8	8	8	8	8	8
Number of Replicates	16	16	16	16	16	16	16	16	16	16
Primary Chem. XBAR ± S(R)	*0.013 ± 0.001*	*0.006 ± 0.001*	*0.001 ± 0.002*	*0.002 ± 0.002*	*0.016 ± 0.003*	*0.016 ± 0.004*	*0.002 ± 0.001*	*0.002 ± 0.001*	*0.013 ± 0.001*	*0.006 ± 0.001*
QTA XBAR	0.017	0.010	0.003	0.001	0.015	0.017	0.014	0.013	0.016	0.010
Repeatability										
QTA S(r)	0.002	0.002	0.002	0.001	0.002	0.003	0.003	0.006	0.004	0.001
QTA RSD(r)	11.99	26.05	90.67	124.72	12.65	16.37	18.73	48.89	27.09	10.50
QTA r	0.006	0.007	0.007	0.001	0.005	0.008	0.007	0.018	0.012	0.003
Reproducibility										
QTA S(R)	0.004	0.004	0.003	0.001	0.003	0.003	0.003	0.007	0.007	0.002
QTA RSD(R)	24.95	37.31	102.14	188.98	21.77	20.16	24.72	50.98	42.18	23.01
QTA R	0.012	0.010	0.008	0.003	0.009	0.010	0.010	0.019	0.018	0.006

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Table 7. Precision Data: Methanol Content

	A*	B^	C	D	F	G	H	I	J*	K^
Number of Laboratories	8	8	8	8	8	8	8	8	8	8
Number of Replicates	16	16	16	16	16	16	14	16	16	16
Primary Chem. XBAR ± S(R)	*0.072 ± 0.034*	*0.099 ± 0.032*	*0.011 ± 0.013*	*0.003 ± 0.004*	*0.128 ± 0.044*	*0.135 ± 0.042*	*0.097 ± 0.039*	*0.090 ± 0.042*	*0.076 ± 0.027*	*0.095 ± 0.041*
QTA XBAR	0.076	0.126	0.059	0.032	0.228	0.223	0.155	0.120	0.084	0.149
Repeatability										
QTA S(r)	0.019	0.047	0.006	0.004	0.010	0.026	0.017	0.035	0.019	0.009
QTA RSD(r)	24.54	37.56	10.42	13.58	4.40	11.57	10.77	29.17	22.73	5.82
QTA r	0.052	0.133	0.017	0.012	0.028	0.072	0.047	0.098	0.053	0.024
Reproducibility										
QTA S(R)	0.021	0.047	0.007	0.007	0.028	0.035	0.017	0.050	0.024	0.016
QTA RSD(R)	27.65	37.56	12.35	20.96	12.32	15.95	10.77	41.41	28.92	10.63
QTA R	0.059	0.133	0.020	0.019	0.078	0.099	0.047	0.139	0.068	0.044

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Table 8. ASTM D6708 Precision Summary

	Repeatability	Reproducibility
Cloud Point, deg C (ASTM D2500)	1.2786	2.8918
Water, % (ASTM D2709)	0.2034*(0.1–x)	0.01945
Acid Number, mg KOH/g (ASTM D664)	0.1721	0.2187
Methanol, % (EN 14110)	0.1575*(x+0.25)	0.2275*(x+0.25)
Free glycerin, % (ASTM D6584)	0.1882*(x+0.02)	0.3106*(x+0.02)
Total glycerin, % (ASTM D6584)	0.03526	0.05411

Table 9. Fuel Matrices for monoglycerides, sulfur and oxidative stability.

Fuel AZ	Crude Degummed Soybean Oil Methyl Esters
Fuel CN	Methyl Esters, unknown feedstock
Fuel GA	Methyl Esters, unknown feedstock
Fuel IL	Canola Methyl Esters
Fuel IN	Refined, bleached, distilled Soybean Oil Methyl Esters
Fuel KY	Choice White Grease Methyl Esters
Fuel MI	Waste Vegetable Oil Methyl Esters
Fuel FL	Waste Soybean Oil Methyl Esters
Fuel OH	Soybean Oil Methyl Esters, distilled
Fuel OK	Tallow and Choice White Grease Methyl Esters
Fuel TX	Tallow Methyl Esters

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Table 10. Precision Data: Monoglycerides

Monoglycerides	AZ	CIN	GA	IL	IN	KY	MI	MOR	OH	OK	TX
Number of Laboratories	10	10	10	9	9	10	9	10	10	10	10
Number of Replicates	19	19	19	17	17	19	17	19	19	19	19
Primary Chem. XBAR ± S(R)	*0.516 ± 0.100*	*0.035 ± 0.019*	*0.664 ± 0.118*	*0.586 ± 0.152*	*0.686 ± 0.125*	*0.167 ± 0.063*	*0.731 ± 0.154*	*0.358 ± 0.083*	*0.025 ± 0.016*	*0.359 ± 0.053*	*0.375 ± 0.048*
QTA XBAR	0.588	0.067	0.583	0.425	0.595	0.229	0.708	0.45825	0.041	0.410	0.435
Repeatability											
QTA S(r)	0.037	0.037	0.030	0.021	0.026	0.043	0.021	0.025079	0.029	0.045	0.031
QTA RSD(r)	6.27	55.86	5.18	4.87	4.43	18.91	2.98	5.472726	70.13	10.98	7.18
QTA r	0.103	0.104	0.085	0.058	0.074	0.121	0.059	0.070221	0.081	0.126	0.087
Reproducibility											
QTA S(R)	0.049	0.050	0.051	0.039	0.039	0.043	0.044	0.039607	0.040	0.045	0.038
QTA RSD(R)	8.31	75.63	8.73	9.20	6.63	18.91	6.24	8.643078	95.58	11.05	8.76
QTA R	0.137	0.141	0.143	0.110	0.110	0.121	0.124	0.110899	0.111	0.127	0.107

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Table 11. Precision data: Sulfur

Sulfur	AZ	CIN	GA	IL	IN	KY	MI	MOR	OH	OK	TX
Number of Laboratories	10	10	10	8	10	9	9	9	10	10	10
Number of Replicates	19	19	19	15	19	17	17	17	19	19	19
Primary Chem. XBAR ± S(R)	*1.506 ± 0.533*	*0.942 ± 0.273*	*0.771 ± 0.351*	*1.809 ± 0.447*	*1.506 ± 0.533*	*4.736 ± 1.570*	*3.699 ± 1.052*	*1.166 ± 0.862*	*0.495 ± 0.611*	*11.059 ± 2.032*	*6.053 ± 2.106*
QTA XBAR	1.013	0.216	2.171	3.508	1.866	3.282	4.671	2.126	0.676	8.840	7.215
Repeatability											
QTA S(r)	0.379	0.168	0.578	0.537	0.649	0.681	0.453	0.981	0.502	0.487	0.607
QTA RSD(r)	37.45	77.93	26.62	15.32	34.80	20.75	9.69	46.14	74.21	5.51	8.42
QTA r	1.062	0.471	1.618	1.504	1.818	1.907	1.268	2.746	1.405	1.364	1.701
Reproducibility											
QTA S(R)	0.744	0.275	0.992	0.773	0.788	0.681	1.020	1.080	0.753	1.370	1.006
QTA RSD(R)	73.45	127.38	45.71	22.03	42.24	20.75	21.84	50.80	111.34	15.50	13.95
QTA R	2.083	0.770	2.779	2.164	2.207	1.907	2.857	3.024	2.107	3.836	2.818

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Table 12. Precision Data: Oxidative Stability

Oxidative Stability	AZ	CIN	GA	IL	IN	KY	MI	MOR	OH	OK	TX
Number of Laboratories	10	10	8	9	9	10	10	10	10	9	10
Number of Replicates	19	19	15	17	17	19	19	19	19	17	19
Primary Chem. XBAR ± S(R)	*4.340 ± 0.799*	*4.488 ± 1.452*	*5.203 ± 1.092*	*7.958 ± 1.487*	*5.614 ± 1.463*	*4.443 ± 1.261*	*1.647 ± 0.299*	*3.890 ± 2.103*	*1.263 ± 0.421*	*4.442 ± 1.352*	*11.857 ± 2.775*
QTA XBAR	5.070	2.935	6.613	5.983	6.428	4.710	3.770	3.915	2.335	6.206	9.850
Repeatability											
QTA S(r)	0.837	0.417	0.665	0.508	0.663	0.437	0.823	0.604	0.618	0.508	0.634
QTA RSD(r)	16.50	14.21	10.06	8.49	10.31	9.28	21.84	15.43	26.46	8.19	6.44
QTA r	2.343	1.168	1.863	1.423	1.856	1.224	2.305	1.692	1.730	1.423	1.776
Reproducibility											
QTA S(R)	0.971	0.979	0.665	0.956	0.663	0.974	0.908	0.926	0.903	0.580	1.265
QTA RSD(R)	19.15	33.36	10.06	15.99	10.31	20.68	24.07	23.65	38.67	9.34	12.84
QTA R	2.719	2.742	1.863	2.678	1.856	2.728	2.541	2.593	2.528	1.623	3.543

Table 13. ASTM D6708 Precision Summary

	Repeatability	Reproducibility
Monoglycerides, % (ASTM D6584)	0.0932	0.1266
Sulfur, ppm (ASTM D6453)	0.0697*(X + 20)	0.1083*(X + 20)
Oxidative Stability (EN14112)	1.84	2.26

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AOCS List D 1-99

Comparison of AOCS and ASTM Methods for Soaps and Synthetic Detergent Analysis

D 1-99 is a comparison between AOCS and ASTM methods in use in the analysis of soaps and synthetic detergents. In each case the method immediately to the right of the AOCS method is the most similar or equivalent ASTM method.

1. Soap and Soap Products	AOCS Method	ASTM Method
Sampling	Da 1-45 (09)	D460-91
Moisture and Volatile Matter, Air Oven Method	Da 2a-48 (09)	D820-93
Moisture, Distillation Method	Da 2b-2 (09)	D460-91
Alcohol-Soluble and Alcohol-Insoluble Matter	Da 3-48 (09)	D460-91
Free Acid of Free Alkali (soda soaps)	Da 4a-48 (09)	D500-95
Free Alkali and Potassium Carbonate (potash paste soaps)	Da 5-44 (09)	D460-91
Water-Insoluble Matter	Da 6-48 (09)	D820-93
Total Alkalinity of Alcohol-Insoluble Matter	Da 7-48 (09)	D460-91
Total Anhydrous Soap and Combined Alkali	Da 8-48 (09)	D800-91
Chlorides	Da 9-48 (09)	D820-93
Unsaponified Plus Unsaponifiable Matter		D460-91
Unsaponifiable Matter	Da 11-42 (09)	D460-91
Rosin McNicoll Method	Da 12-48 (09)	D460-91
Titer Test	Da 13-48 (09)	D5565-95
Acid Value of Fatty Acids	Da 14-48 (09)	D460-91
Iodine Value	Da 15-48 (09)	D460-91
Saponification Value	Da 16-48 (09)	D5558-95
Borax	Da 17-52 (09)	D460-91
Alkaline Silicates	Da 18-48 (09)	D460-91
Carbonates, Gravimetric Absorption Method	Da 19a-42 (09)	D460-91
Carbonates, Volumetric Evolution Method	Da 19b-42 (09)	D460-91
Phosphates (gravimetric method)	Da 20a-48 (09)	D460-91
Phosphates (titrimetric method)	Da 20b-57 (09)	D460-91
Tetrasodium Pyrophosphate	Da 21-48 (09)	D460-91
Sulfates	Da 22-48 (09)	D460-91
Free Glycerol	Da 23-56 (09)	D460-91
Sugars	Da 24-48 (09)	D460-91
Starch	Da 25-48 (09)	D460-91
Volatile Hydrocarbons	Da 26-42 (09)	D460-91
Combined Sodium and Potassium Oxides	Da 27-48 (09)	D460-91
Screen Test	Da 28-39 (09)	D502-95
Copper	Da 31-58 (09)	D460-91

2. Soap Containing Synthetic Detergents	AOCS Method	ASTM Method
Moisture and Volatile Matter	Db 1-48 (09)	D460-91
Alcohol-Soluble and Alcohol-Insoluble Matter	Db 2-48 (09)	D460-91
Free Acid or Free Alkali	Db 3-48 (09)	D820-93
Water Insoluble Matter	Db 4-48 (09)	D460-91
Total Alkalinity of Alcohol-Insoluble Matter	Db 5-48 (09)	D460-91
Total Anhydrous Soap	Db 6-48 (09)	D460-91
Chlorides (Indicator method)	Db 7a-48 (09)	D460-91
Chlorides, Potentiometric Determination	Db 7b-55 (09)	D460-91
Saponification Value	Db 8-48 (09)	D5558-95
Alkaline Silicates	Db 9-48 (09)	D501-95
Fatty Matter	Db 10-48 (09)	D500-95
Rosin, McNicoll Method	Db 11-48 (09)	D460-91

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3. Fatty Alkyl Sulfates	AOCS Method	ASTM Method
Sampling	Dc 1-59 (09)	D1568-91
Moisture by Distillation method	Dc 2-59 (09)	D460-91
Alcohol-Soluble Matter	Dc 3a-59 (09)	D460-91
Alcohol-Insoluble Matter	Dc 3b-59 (09)	D460-91
Ester 803	Dc 4-59 (09)	D1570-95
Combined Alcohols	Dc 5-59 (09)	D1570-95
Alkalinity	Dc 6-59 (09)	D500-95
Sodium Sulfate	Dc 7-59 (09)	D1568-91
Unsulfated Material	Dc 8-59 (09)	D1568-91

4. Alkylbenzene Sulfonates	AOCS Method	ASTM Method
Sampling	Dd 1-59 (09)	D1568-91
Moisture by Distillation Method	Dd 2a-59 (09)	D460-91
Modified Karl Fischer Reagent		D1568-91
Sodium Alkylbenzene Sulfonate by Ultraviolet Absorption	Dd 3-60 (09)	D1768-95
Neutral Oil (unsulfonated material)	Dd 4-60 (09)	D1568-91
Color, Linear Alkylbenzene Sulfonates	Dd 5-92 (09)	

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AOCS

AOCS Official Method Da 1-45

Reapproved 2009

Sampling and Preparation of Laboratory Sample

SCOPE

Applicable to cake soaps, soap powders, cleansers, soap flakes; pastes and liquid soaps containing synthetic detergents.

APPARATUS

1. Universal food cutter—with 12-tooth blade, or equivalent.
2. Trier for paste soap—a half-round metal tube 125–250 mm in diameter. The length may be 60–120 cm, depending upon the size of package to be sampled. One end is tapered to a point, the taper to be not more than 2.5 cm long. The other end is attached to a D- or T-shaped handle.
3. Sample tube for liquid product—a glass tube 90–125 mm i.d. The length may be convenient for the size packages to be sampled. One end is constricted by a short taper (not more than 2.5 cm long) to about 60 mm. The other end is constricted sufficiently so that it can be used as a finger valve.
4. Sample containers—airtight and watertight, about 2 kg capacity.
5. Sample containers—airtight and watertight, sufficient capacity to hold samples taken as directed in Procedure, 1-3.
6. Spatula—with 25–30 cm blade.

PROCEDURE

Note—Place all samples in airtight and watertight containers, cover, and seal to prevent any moisture change. Label completely for identification and send to the laboratory for preparation and analysis.

1. Small packages containing cake soaps, powders, flakes, pastes, or liquids—
   (a) Lots of fewer than 1,000 cases—select a minimum of 10 cases at random throughout the lot and take 1 bar or 1 package from each case. In case of lots containing fewer than 10 cases, sample all cases.
   (b) Lots of 1,000 cases or more—select 1% of the total number of cases at random throughout the lot and take 1 bar or 1 package from each case. In case of very large lots where the sample drawn as above will amount to more than 9.1 kg (20 lbs), the percentage of packages sampled shall be reduced so that the amount drawn shall not exceed 9.1 kg (20 lbs).

   Note—In selecting samples from the cases to be sampled, remove as many samples from the layers next to the edges of the package case as from the very middle. Selection of samples should be made so that sampling is thoroughly representative from all positions of the case.
2. Barrels, tierces, or similar large packages containing powder or flakes—
   (a) Lots of fewer than 1,000 packages—select a minimum of 10 packages at random throughout the lot and take about 500 g of solid or 500 mL of liquid product from each package. In case of lots containing fewer than 10 packages, sample all packages.
   (b) Lots of 1,000 packages or more—select 1% of the total number of cases at random throughout the lot and take 500 g of solid or 500 mL of liquid from each package. In case of very large lots where the sample drawn as above will amount to more than 9.1 kg (20 lbs), the percentage of packages sampled shall be reduced so that the amount drawn shall not exceed 9.1 kg (20 lbs).
3. Large packages containing paste or liquid soap—
   (a) Number of packages to be sampled is the same as directed in Procedure, 2, a and b.
   (b) Paste soap products in large packages are conveniently sampled with the trier. Insert the trier diagonally through the package, turn it 2–3 times, and withdraw with the sample. Take enough such portions from each package to make about 500 g and accumulate in clean and dry containers.
   (c) Liquid soap products in large packages are conveniently sampled with the glass tube. The product should be at a temperature of 20–30°C. Mix the package thoroughly and insert the tube slowly, with the upper end open so that the sample gradually fills the tube as it is slowly lowered. When the tube is full, close the upper end with a finger and withdraw the tube. Take enough such portions from each package to make about 500 g (or 500 mL for liquids) and accumulate in a clean and dry container.

   Note—If the consistency is such that neither the metal trier nor the glass tube can be used, take grab samples with a dipper or some similar and convenient tool.

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PREPARATION OF LABORATORY SAMPLE

Note—The samples must be handled as promptly as possible and in such a way as to minimize the possibility of moisture change. Mixing of all samples shall be done on a clean, nonabsorbent, impervious surface. Analysis must be started immediately after preparation of sample.

1. Cake soap—grind all bars through a suitable food chopper. In the case of large samples, it is permissible to quarter the bars and grind one quarter from each bar. However, each ground sample should consist of at least 1500 g. Mix all ground samples thoroughly on a clean, dry, nonabsorbent, impervious surface with a spatula. Divide into four quarters and discard the two opposite quarters. Combine, remix, and requarter the remaining two quarters. Continue in this manner until the sample is reduced to about 1000 g. Place this portion in a clean and dry sample container. Close tightly and label completely for identification. This is the sample for analysis and must be preserved in a cool dry place.
2. Powdered soap and flakes—mix the contents of all packages or all of the 500 g portions on a clean, dry, nonabsorbent, impervious surface with a large spatula. Quarter and proceed as directed for ground cake soap above to obtain a sample of about 1000 g for analysis. Label and preserve as directed above.
3. Liquids—no preparation of a liquid sample, other than a thorough mixing, is necessary unless it is received during very cold weather, in which case it should be allowed to stand at least 1 hr after it has warmed to room temperature (20–30°C) before it is tested, particularly for its lathering qualities. Label and preserve as above.
4. Pastes—store preferably in glass. If crystals separate, melt in water bath. Label and preserve as above. If pastes or liquids are known to be acidic and decomposition of sample can result from heating, the samples should be adequately labeled for precautionary treatment and warmed to room temperature or other maximum temperature as agreed upon for mixing and sampling.

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AOCS Official Method Da 2a-48

Reapproved 2009

Moisture and Volatile Matter, Air Oven Method

DEFINITION

This method determines the moisture and any materials volatile under the conditions of the test.

SCOPE

This method is applicable to soaps and soap products. It is not applicable to products containing appreciable amounts of sodium silicate, more than 1% glycerol, and soaps produced from oils, such as linseed oil, that tend to oxidize readily. The distillation method must be used for these products. Replaces Da 2a-42.

APPARATUS

1. Air oven—maintained at 105 ± 2°C, AOCS Specification H 3-45.
2. Porcelain or glass moisture dishes—60–80 mm dia and 20-40 mm depth.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.

PROCEDURE

1. Weigh 5 ± 0.01 g of test sample into a tared moisture dish which has been previously dried for 1 hr at 105 ± 2°C and cooled to room temperature in a desiccator.
2. Dry the test portion to constant weight in an air oven at 105 ± 2°C, cool to room temperature in a desiccator, and weigh.
3. Constant weight is attained when successive 1 hr heating periods show a loss (or gain) of not more than 0.1%.

CALCULATION

$$\text{Moisture and volatile matter, } \% = \frac{\text{loss in mass, g} \times 100}{\text{mass, g of test portion}}$$

NOTES

If sodium bicarbonate is present, it will undergo partial decomposition under the test conditions in this method and yield water and CO_2.

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AOCS

AOCS Official Method Da 2b-42

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Moisture, Distillation Method

DEFINITION

This method determines the moisture by distillation with an immiscible solvent.

SCOPE

This method is applicable to soaps and soap products except those containing water-miscible volatile substances.

APPARATUS

1. Glass distillation apparatus—with ground-glass connections, constructed and assembled in accordance with the specifications shown in Figure 1.
   (a) Flask—short neck, round-bottom type, 500 mL.
   (b) Condenser—water-cooled, the lower tip of which should be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established.
   (c) Dean and Stark Trap—constructed of well-annealed glass. For greatest accuracy several trap sizes are allowable, depending upon the percentage of moisture expected (see Table 1).

   Traps made of well-annealed glass, constructed essentially as shown in Figure 1, and graduated to contain one of the following specified volumes at 20°C shall be used—

   5 mL trap—subdivided into 0.1 mL divisions with each 1 mL line numbered (5 mL at top). The error in any indicated capacity may not be greater than 0.05 mL.

   10 mL trap—subdivided from 0-2 mL in 0.1 mL divisions and from 2–10 mL in 0.2 mL divisions.

   25 mL trap—subdivided from 0-2 mL in 0.1 mL divisions and from 2–25 mL in 0.2 mL divisions.
   (d) Distillation may be better controlled by wrapping the tube which leads from the flask to the receiver with glass-fiber tape. It is advantageous to wrap the flask also, especially if there are drafts in the vicinity of the distillation apparatus.
   (e) Calibrate the receiver as follows: add an accurately measured portion, 1 ± 0.01 mL, of distilled water to 100 mL of toluene in the distillation flask. Conduct the distillation as described in Procedure, 2-5, and calculate as directed in Calculation. Cool the apparatus, add another mL of water, and repeat the distillation. Continue in this manner up to the capacity of the receiving tube.
2. Pipet—measuring type, capacity 1.0 mL, graduated in 0.01 mL.
3. Heat source—usually an electric heating mantle provided with a suitable means of control.
4. Copper or nichrome wire—long enough to extend through the condenser with one end twisted into a spiral. The diameter of the spiral is such that it will fit snugly within the receiver and yet can be moved up and down.

REAGENTS

1. Xylene or toluene—reagent grade (see Notes, *Caution*). Use toluene for soaps containing 1% or more glycerin. Saturate the xylene or toluene with water by shaking with a small quantity of water and distill. Use the distillate for the determination.
2. Ethyl alcohol (ethanol)—absolute (see Notes, *Caution*).

PROCEDURE

1. The entire apparatus must be completely clean. Clean with potassium dichromate-sulfuric acid cleaning solution to minimize the adherence of water droplets to the sides of the condenser and receiver. Rinse thoroughly and dry completely before using.
2. Transfer to the 1,000 mL flask, equipped with the size of the trap specified in Apparatus, an amount of test sample (see Notes, 1) according to the percentage of moisture expected, as shown in Table 2.

   Add immediately about 100 mL of xylene or toluene. Place a small, thin sheet of long-fiber, chemical-resistant glass wool on the surface of the toluene. The glass wool should be thoroughly dried in the oven and held in the desiccator before use.
3. Assemble the apparatus and fill the receiver with solvent by pouring through the condenser until the solvent begins to overflow into the distillation flask. Insert a loose cotton plug in the top of the condenser to prevent condensation of atmospheric moisture within the tube.
4. Heat the flask so that the distillation rate will be about 100 drops per minute. When the greater part of the water has distilled, increase the distillation rate to about 200 drops per minute and continue until the water level in the receiver remains unchanged for 30 min.
5. Discontinue heating, dislodge any drops of water which may be adhering to the inside of the condenser tube or receiver with a wire spiral and wash down with about 5 mL of toluene or xylene.

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6. Wash down condenser with 1 mL of absolute ethanol.
7. Immerse the receiver in water at 25°C for at least 15 min or until the toluene or xylene layer is clear, then read the volume of water.

CALCULATIONS

$$\text{Moisture, v/w\%} = \frac{\text{volume of water, mL} \times 99.8}{\text{mass of sample, g}}$$

NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27-7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air.

Xylene is flammable and a dangerous fire risk. The TLV is 100 ppm in air.

Ethyl alcohol is flammable. A fume hood should be used at all times when using the solvents specified in this method.

NUMBERED NOTES

1. If sodium bicarbonate is present it will undergo partial decomposition under the conditions of this test and yield water and CO_2.

Table 1

Moisture expected, %	Size of trap, mL
0–5, incl	5
Over 5–17, incl	10
Over 17–30, incl	10
Over 30–50, incl	25
Over 50–70, incl	25
Over 70–85, incl	25

Table 2

Moisture, expected, %	Mass of test portion to be used, g[a]
0–5, incl	50 ± 5
Over 5–17, incl	50 ± 5
Over 17–30, incl	30 ± 5
Over 30–50, incl	30 ± 5
Over 50–70, incl	30 ± 5
Over 70–85, incl	25 ± 5

[a]Weighed to the nearest 0.25 g.

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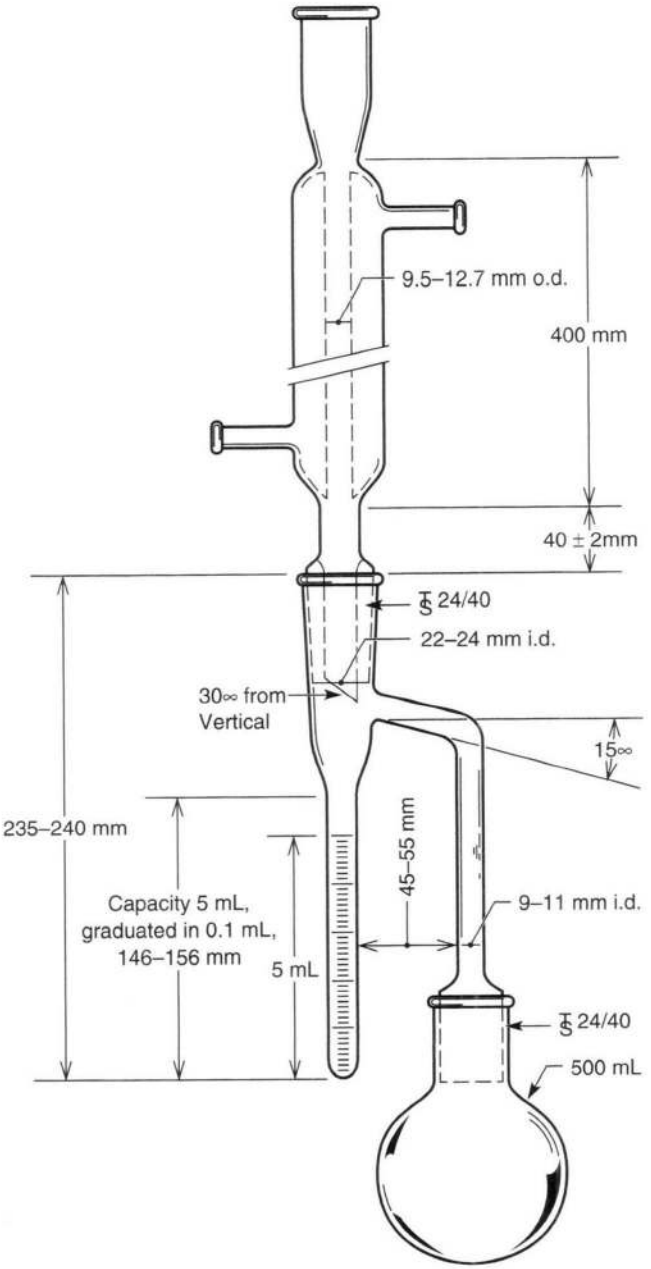

Figure 1. Moisture distillation apparatus.

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AOCS

AOCS Official Method Da 3-48

Revised 2011

Alcohol-Soluble and Alcohol-Insoluble Matter

DEFINITION

This method determines the alcohol-soluble and alcohol-insoluble matter in the sample.

1. The alcohol-soluble matter will contain all of the anhydrous (real) soap and some inorganic salts. Most, if not all, of the sodium chloride will be included. This method is satisfactory for the approximate determination of soap (when buyer and seller agree) with products which contain a high percentage of alcohol-insoluble matter such as cleansers, soap powders, scouring compounds, and pastes.
2. The alcohol-insoluble matter will contain most of the alkaline salts such as carbonates, borates, silicates, and phosphates, as well as sulfates and starch. This method may be used for the approximate determination of these constituents. These salts are not entirely insoluble in alcohol, so for the most accurate results, use separate portions of the sample and follow the specific method for each constituent.

SCOPE

Applicable to soap and soap products. Replaces Da 3-42.

APPARATUS

1. Beakers or Erlenmeyer flasks—250 mL.
2. Filter paper—the filter paper must impart no acidity or alkalinity to neutral alcohol when neutral alcohol is poured through it. Place filter paper in a weighing bottle or metal container (seamless, with tight fitting slip cover) and dry in an oven at 105 ± 2°C with cover removed. Remove from oven, replace cover, cool to room temperature in a desiccator, and weigh. Reweigh after filtration (see Procedure, 5) in the same container.
3. Watch glasses.
4. Air oven—maintained at 105 ± 2°C, AOCS Specification H 3-45.
5. Filter flask—500 mL.
6. Funnel—fitted with rubber stopper to fit the neck of the filter flask.
7. Rotary evaporator.
8. Vacuum pump or aspirator.
9. Round bottom flask—250 mL with 24/40 glass joint.

REAGENTS

1. Ethyl alcohol—95% and absolute ethyl alcohol (see Note, *Caution*). SDA Formulas 30 and 3A are permitted. Add 3–5 drops of 1% phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1–0.2 M solution, to a faint pink color just before using.
2. Phenolphthalein indicator solution—1% in 95% alcohol.

PROCEDURE

1. Weigh a test portion of such size that it will yield 0.1–1.0 g of alcohol-insoluble matter. Place in 250 mL flask or beaker, add 200 mL of alcohol, cover with a watch glass, and heat on a steam bath with frequent stirring until the soap is dissolved.
2. Filter into a flask through the tared filter paper with the aid of vacuum, retaining in the beaker as much of the residue as possible. Repeat this extraction and decantation three times using 25 mL of hot alcohol each time.
3. Wash all of the residue into the filter and wash the filter several times with hot 95% alcohol to remove all of the alcohol-soluble matter.
4. Combine the alcohol filtrate (step 2) and washings (step 3) in a tared 250 mL round bottom flask and evaporate with a rotary evaporator. Dry to constant weight in an oven at 105 ± 2°C. Cool to room temperature in a desiccator and weigh:

$$\text{Alcohol-soluble matter, \%} = \frac{\text{mass of residue in flask, g}}{\text{mass of test portion, g}} \times 100$$

5. Dry the residue on the tared filter paper in an oven at 105 ± 2°C for 3 hr. Cool to room temperature in a desiccator and weigh

$$\text{Alcohol-insoluble matter, \%} = \frac{\text{mass of insoluble matter, g}}{\text{mass of test portion, g}} \times 100$$

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use effective fume-removal device when heating or evaporating.

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AOCS

AOCS Official Method Da 4a-48

Revised 2011

Free Acid or Free Alkali

DEFINITION

This method determines the free (uncombined) acid or alkali in the sample.

SCOPE

Applicable to soda soap products, except those containing borax. For potash soaps see AOCS Official Method Da 5-44. Replaces Da 4a-42.

APPARATUS

1. Erlenmeyer flask—250 mL.
2. Gooch crucible or filter paper—the filter paper must impart no acidity or alkalinity to neutral alcohol when poured through it. A Gooch crucible may be prepared with any suitable glass-fiber filter medium. The filter paper is washed with distilled water, alcohol, and petroleum ether, and then dried at 105 ± 2°C before using.
3. Watch glasses.
4. Steam bath or water bath.

REAGENTS

1. Ethyl alcohol—95%. SDA Formulas 30 and 3A are permitted (see Notes, *Caution*). Add 3–5 drops phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1-0.2 M solution, to a faint pink color just before using.
2. Either hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) solutions, 0.1 M and 0.25 M—accurately standardized. See AOCS Specifications H 13-52 and H 14-52.
3. Sodium hydroxide (NaOH) solution, 0.1 M and 0.25 M—accurately standardized. See AOCS specification H 12-52.
4. Phenolphthalein indicator solution—1% in 95% alcohol.

PROCEDURE

1. Proceed as directed in AOCS Official Method Da 3-48, Procedure, 1 and 2 (see Notes, 1 and 2).
2. Collect the filtrate and washings in a 250 mL Erlenmeyer flask, heat to incipient boiling on either a steam or a water bath, and add 0.5 mL of phenolphthalein indicator solution.
3. Titrate immediately with standard acid if alkaline or with standard alkali if acidic. Use 0.1 M for samples containing small amounts of free acid or free alkali (10% or less) and 0.25 M for all other samples.

CALCULATIONS

1. If free alkali is present and HCl is the titrant:

$$\text{Free alkali as NaOH, \%} = \frac{\text{volume of HCl, mL} \times \text{M of HCl} \times 4}{\text{mass of test portion, g}}$$

2. If free alkali is present, and H_2SO_4 is the titrant:

$$\text{Free alkali as NaOH, \%} = \frac{\text{volume of } H_2SO_4\text{, mL} \times \text{M of } H_2SO_4 \times 8}{\text{mass of test portion, g}}$$

3. If free acid is present:

$$\text{Free acid as oleic, \%} = \frac{\text{volume of alkali, mL} \times \text{M of alkali} \times 28.25}{\text{mass of test portion, g}}$$

where—
M = molarity of standard acid or base

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use effective fume-removal device when heating or evaporating.

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NUMBERED NOTES

1. Soaps containing little or no alkaline salts need not be filtered as directed in AOCS Official Method Da 3-48 as noted in Procedure, 2, before proceeding with the determination of free acid or free alkali. However, when substances such as silicates, phosphates, or other similar salts that influence the titration are present, filtration is essential. Carbonates, up to 0.5% as Na_2CO_3, do not affect the determination appreciably and may be omitted, providing a carbonate determination on a separate sample indicates the quantity not to be in excess of this amount.
2. Avoid exposure to carbonation at any time since this will seriously affect results.

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AOCS

AOCS Official Method Da 5-44

Reapproved 2009

Free Alkali and Potassium Carbonate

DEFINITION

This method determines free potassium hydroxide and potassium carbonate in the sample.

SCOPE

Applicable to potash paste soaps.

APPARATUS

1. Beaker—250 mL.

REAGENTS

1. Sulfuric acid (H_2SO_4), 0.25 M—accurately standardized. See AOCS Specification H 13-52.
2. Sodium hydroxide (NaOH), 0.5 M—accurately standardized. See AOCS Specification H 12-52.
3. Phenolphthalein indicator solution—1.0% in 95% alcohol.
4. Methyl orange indicator solution—0.1% in distilled water.
5. Ethyl alcohol—absolute (see Notes, *Caution*)—SDA Formulas 30 and 3A are permitted. Add 3–5 drops of phenolphthalein indicator solution. Boil and neutralize with dilute NaOH, 0.1-0.2 M solution, to a faint pink color just before using.

PROCEDURE

1. Weigh 10 ± 0.01 g of the test sample into a 250 mL beaker and dissolve in 100 mL of freshly boiled distilled water.
2. Add a measured quantity of 0.25 M H_2SO_4 to make the solution acid to methyl orange. Heat (not to exceed 60°C) until the fatty acids separate in a clear layer.

   Note—Excessive boiling should be avoided to prevent the possibility of volatilization of any low molecular weight fatty acids that may be present.
3. Add 0.5 mL of phenolphthalein indicator and, while stirring, titrate with 0.5 M NaOH until the solution is almost neutral. It should remain slightly on the acid side to phenolphthalein.
4. Evaporate to dryness on a steam bath and dissolve in 200 mL of neutral, absolute ethyl alcohol. Titrate with 0.5 M NaOH to the appearance of a faint pink color.
5. Determine the carbon dioxide (CO_2) on a separate sample by AOCS Official Method Da 19a-42 or Da 19b-42.

CALCULATIONS

1. Potassium carbonate (K_2CO_3), % = % CO_2 (Procedure, 5) × 3.140
2. Potassium hydroxide (KOH), $\% = \dfrac{(A - B) \times 2.805}{\text{mass of test portion}^a} - (\%\ K_2CO_3 \times 0.8119)$

Where—

A = mL 0.25 M H_2SO_4 (Procedure, 2).

B = total mL 0.5 M NaOH (Procedure, 3 and 4).

[a]From Procedure, 1.

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use effective fume-removal device when heating and evaporating.

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AOCS

AOCS Official Method Da 6-48

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Water-Insoluble Matter

DEFINITION

This method determines water-insoluble matter in the sample.

SCOPE

Applicable to soaps and soap products. Replaces Da 6-42.

APPARATUS

1. Beakers or Erlenmeyer flasks—250 mL.
2. Filter paper—the filter paper must impart no acidity or alkalinity to neutral alcohol when neutral alcohol is poured through it.
3. Watch glasses.
4. Air oven—maintained at 105 ± 2°C, AOCS Specification H 3–45.
5. Filter flask—500 mL.

REAGENTS

1. Ethyl alcohol—95% and absolute ethyl alcohol (see Notes, *Caution*). SDA Formulas 30 and 3A are permitted. Add 3–5 drops of 1% phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1–0.2 M solution, to a faint pink color just before using.
2. Phenolphthalein indicator solution-1% in 95% alcohol.

PROCEDURE

1. Proceed as directed in AOCS Official Method Da 3-48, Procedure, 1–5, starting with a fresh test portion. Proceed without drying the filter.
2. After the residue is thoroughly washed with alcohol, change receivers and wash the residue with five 50 mL portions of distilled water at 60°C (see Notes, 1).
3. Reserve the water solution for the determination of total alkalinity as directed in AOCS Official Method Da 7-48.
4. Dry the filter paper or crucible containing the water-insoluble residue in an air oven for 3 hr at 105 ± 2°C. Cool to room temperature in a desiccator and weigh (see Notes, 2).

CALCULATIONS

$$\text{Water-insoluble matter, \%} = \frac{\text{mass of insoluble matter, g}}{\text{mass of test portion, g}} \times 100$$

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use effective fume-removal device when heating or evaporating.

NUMBERED NOTES

1. If protein or starch is present, use ice water (0–10°C).
2. The nature of the water-insoluble material may be determined by further examination if that information is desired.

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AOCS

AOCS Official Method Da 7-48

Reapproved 2009

Total Alkalinity of Alcohol-Insoluble Matter

DEFINITION

This method determines the alkalinity of the alcohol-insoluble matter, expressed as Na_2O or Na_2CO_3 as required.

SCOPE

Applicable to soda soaps and soda soap products. Replaces Da 7-42.

REAGENTS

1. Hydrochloric or sulfuric acid solution, 0.5 N—accurately standardized. See AOCS Specification H 14-52 or H 13-52, respectively.
2. Methyl orange indicator solution—0.1% in distilled water.

PROCEDURE

1. Use the water solution from AOCS Official Method Da 6-48, Procedure, 3, or a sample prepared in an identical manner.
2. Add a few drops of methyl orange indicator solution and titrate at room temperature with 0.5 N acid to a color change from yellow-orange to red.

CALCULATIONS

1. Total alkalinity of alcohol-insoluble matter as Na_2O, $\% = \dfrac{\text{volume of acid, mL} \times M \times 3.1}{\text{mass of test portion for water-insoluble matter, g}}$

2. Total alkalinity of alcohol-insoluble matter as Na_2CO_3, $\% = \dfrac{\text{volume of acid, mL} \times M \times 5.3}{\text{mass of test portion for water-insoluble matter, g}}$

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AOCS

AOCS Official Method Da 8-48

Reapproved 2009

Total Anhydrous Soap and Combined Alkali

DEFINITION

This method determines the total anhydrous soap, fatty anhydride, and combined alkali.

SCOPE

Applicable to all moderately built soaps and soap products. Not applicable to samples containing large amounts of soluble silicates or finely divided matter insoluble in water (see Notes, 1, 2).

APPARATUS

1. Beaker—400 mL or Soxhlet flask, 500 mL.
2. Separatory funnel—500 mL.
3. Extraction cylinder or Stokes flask—
   (a) The extraction cylinder has a 250 mL capacity, is glass-stoppered, about 39 mm dia and 355 mm high.
   (b) The Stokes flask is a 100 mL round-bottom flask sealed onto a 150 mL Erlenmeyer flask (see Figure 1).
4. Siphon—two-hole rubber stopper fitted with small diameter glass tubing constructed to permit removal of the upper layer from the extraction cylinder or Stokes flask.
5. Volumetric pipette—20 mL, to measure NaOH solution for correction factor.
6. Rotary evaporator.
7. Round bottom flask—500 mL with $^{24}/_{40}$ standard taper joint.

REAGENTS

1. Ethyl alcohol—95% SDA Formulas 30 and 3A are permitted (see Notes, *Caution*). Add 3–5 drops phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1–0.2 M solution to a faint pink color just before using.
2. Diethyl ether—reagent grade (see Notes, *Caution*).
3. Sodium chloride solution—a saturated solution of sodium chloride in distilled water.
4. Sodium chloride solution—10% in distilled water.
5. Hydrochloric acid (HCl)—1:1, v/v of concentrated HCl with distilled water (see Notes, *Caution*).
6. Sodium hydroxide solution (NaOH), 1.0 M—accurately standardized. See AOCS Specification H 12-52. This solution should be as free from impurities as possible in order to keep the correction at a minimum. Carbonates are the usual impurity contributing to this factor, but other sodium salts may be present. Determine the correction factor by exactly neutralizing 20 mL of the 1M NaOH solution (containing a little phenolphthalein) with 1 M HCl. Evaporate the neutral solution in a water bath and then dry for 2 hr in an air oven at 105 ± 2°C. Cool in a desiccator to room temperature and weigh. Repeat until constant weight is obtained. Constant weight is obtained when the loss (or gain) in weight does not exceed 0.1% in successive 1 hr drying periods.

   $$\text{The correction factor per mL of NaOH, } K = \frac{\text{mass of residue, g} - (\text{M of NaOH} \times 1.1688)}{20.00}$$

7. Phenolphthalein indicator solution—1% in 95% alcohol.
8. Methyl orange indicator solution—0.1% in distilled water.

PROCEDURE

1. Weigh a test portion of such size that it will yield 1–5 g of fatty acids. The sample may be placed directly in the Stokes flask, extraction cylinder, or into a volumetric flask. If the latter, make up to volume with distilled water, mix thoroughly, and transfer with a pipet a quantity such that it will yield 1–5 g of fatty acids in the extraction cylinder or Stokes flask.
2. Add 75 mL of distilled water or a sufficient quantity to bring to this volume. Warm to promote solution, but do not exceed a temperature of 60°C.
3. Add dilute HCl (1:1, v/v) until the solution is just acid to methyl orange (yellow-orange to red) and then add 10 additional mL. Heat again (do not exceed 60°C) until the fatty acids separate in a clear layer.
4. Cool to room temperature (20–25°C) and add sufficient saturated NaCl solution to make the total volume 160 mL in the cylinder or to bring the upper level within the constricted portion of the Stokes flask. Add 50 mL of diethyl ether (see Notes, *Caution*) without inverting or mixing. Allow to stand until the acids have completely dissolved in the diethyl ether.
5. Remove the upper layer with a siphon, transferring into a 500 mL separatory funnel. Add 50 mL of diethyl ether and shake vigorously. Allow to stand until the layers separate clearly and siphon the diethyl ether layer into the separatory funnel.

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6. Make three more extractions in the separatory funnel with several portions of 10% NaCl solution until the washings are neutral to methyl orange (yellow-orange).
7. If any insoluble matter is present in the diethyl ether extract, filter through a filter paper and wash the paper with diethyl ether to remove all fatty matter. Transfer into a tared round bottom flask and evaporate with a rotary evaporator to a volume of not less than 50 mL, add 1 mL of phenolphthalein indicator, and 100 mL of neutral 95% alcohol. Titrate with 1.0 M NaOH with constant stirring to the first pink color which persists for 30 sec.
8. Evaporate the alcohol and diethyl ether with the rotary evaporator.
9. Dry the soda soap to constant weight in an air oven at 105 ± 2°C. Cool to room temperature in a desiccator and weigh. Constant weight is attained when the loss (or gain) does not exceed 0.1% in successive 1 hr drying periods.

CALCULATIONS

1. Total anhydrous soap—
   (a) If the sample is a soda soap:

$$\text{Total anhydrous potash soap, \%} = \frac{[W - (K \times T)]}{\text{mass of test portion, g}} \times 100 - U$$

Where—
W = mass of soda soap, g from Procedure, 9
K = correction for impurities from Reagents, 7
T = volume of titrant from Procedure, 7, mL
U = % unsaponifiable matter, as determined by AOCS Official Method Da 11-42

   (b) If the sample is a potash soap:

$$\text{Total anhydrous potash soap, \%} = \frac{[W - K \times T) + (T\ 0.0161)]}{\text{mass of test portion, g}} \times 100 - U$$

Note—The anhydrous soap will contain as soap any free fatty acids in the original sample. If present in excess of 0.2% as determined by AOCS Official Method Da 4a-48, a correction should be made. It is usually sufficiently accurate to assume the free fatty acids to be oleic and thus make the correction for sodium oleate. Then the total anhydrous soap corrected for free fatty acids equals:

$$\text{\% total anhydrous soap from Calculations, 1, a or b} - \text{\% free fatty acids} \times 1.08$$

2. Fatty anhydride, $\% = \dfrac{[B - (T \times M \times 0.031)]}{\text{mass of test portion, g}} \times 100 - U$

Where—
B = mass of soda soap (W from Procedure, 9 above) – (K × T)
M = molarity of NaOH solution

3. Combined alkali, % =
   (a) If the alkali is 2NaO: $\% Na_2O = \dfrac{T \times N \times 3.1}{\text{mass of test portion, g}}$
   (b) If the alkali is K_2O: $\% K_2O = \dfrac{T \times N \times 4.71}{\text{mass of test portion, g}}$

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use effective fume-removal device when heating or evaporating.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

NUMBERED NOTES

1. Proceed as follows with soaps containing a large amount of soluble silicates and soap products containing a high percentage of finely divided material insoluble in water.

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(a) Use the neutralized filtrate obtained in the determination of free acid or free alkali, AOCS Official Method Da 4a-48, Procedure, 3, or a sample prepared in an identical manner.
(b) Evaporate the alcohol on a steam bath, dissolve in distilled water, and proceed as directed in Procedure, 1 in this method.

2. Total anhydrous soap (real soap) in soap products containing a high percentage of alcohol insoluble matter and where approximate results will suffice, such as in the case of cleansers, soap powders, scouring compounds, and pastes (this is providing buyer and seller agree) may be analyzed as directed in AOCS Official Method, Da 3-48.

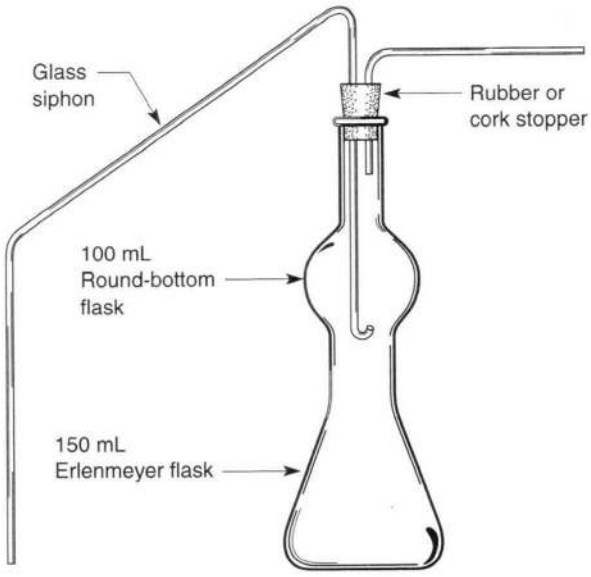

Figure 1. Stokes flask.

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AOCS

AOCS Official Method Da 9-48

Reapproved 2009

Chloride

DEFINITION

This method determines the chlorides in the sample.

SCOPE

Applicable to all soap and soap products. Replaces Da 9-42.

APPARATUS

1. Beaker—500 mL.

REAGENTS

1. Silver nitrate ($AgNO_3$) solution, 0.1 M—accurately standardized.
2. Sulfuric acid solution—about 0.5 M (see Notes, *Caution*).
3. Potassium chromate indicator solution—dissolve 5 g of K_2CrO_4 (see Notes, *Caution*) in distilled water and add $AgNO_3$ solution in distilled water until a slight red precipitate is formed. Filter and dilute to 100 mL with distilled water.
4. Phenolphthalein indicator solution—1% in 95% alcohol.
5. Magnesium nitrate solution—20% $Mg(NO_3)_2 \cdot 6H_2O$ in distilled water. Solution must be neutral and chloride free.
6. Calcium carbonate—reagent grade.

PROCEDURE

1. Accurately weigh 5 g (2–3 g for samples containing more than 5% NaCl) of the test sample into a 500 mL beaker and add 300 mL of chloride-free distilled water. Boil if necessary to effect solution.
2. Add an excess of $Mg(NO_3)_2$ solution (usually about 25 mL of the 20% solution), filter, and wash thoroughly with chloride-free distilled water to remove all chlorides.
3. Cool to room temperature (20–25°C). If the solution is alkaline, add a little phenolphthalein and then 0.5 M H_2SO_4 until just colorless, avoiding an excess of more than 1 drop of acid. Add 1 mL of K_2CrO_4 indicator for every 100 mL of solution and titrate with 0.1 M $AgNO_3$ to the first permanent reddish tinge.
4. Prepare a blank in a 500 mL beaker containing the same volume of distilled water, $Mg(NO_3)_2$ solution and indicator. Add sufficient $CaCO_3$ to the blank to make the turbidity equivalent to that of the test portion when both are swirled.
5. Using the blank for comparison, continue the titration of the sample until a faint, but distinct color change occurs. At the end point, the color of the sample solution should not be dark, but just distinctly different from that of the blank.
6. Add sufficient 0.1 M $AgNO_3$ to the blank so that the color of the blank and sample match.

CALCULATIONS

1. Chlorides, as NaCl, $\% = \dfrac{(S - B) \times M \times 5.85}{\text{mass of test portion, g}}$

   Where—

   S = volume of titrant, mL of test portion
   B = volume of titrant, mL of blank
   M = molarity of $AgNO_3$

2. Chlorides as KCl, $\% = \dfrac{(S - B) \times M \times 7.46}{\text{mass of test portion, g}}$

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

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AOCS

AOCS Official Method Da 11-42

Revised 2013

Unsaponifiable Matter

DEFINITION

The unsaponifiable matter includes those substances frequently found dissolved in fats and oils which cannot be saponified by the caustic alkalies but are soluble in the ordinary fat solvents. Included are the higher aliphatic alcohols, sterols, pigments and hydrocarbons.

SCOPE

Applicable to soaps and soap products.

APPARATUS

1. Beaker—500 mL.
2. Erlenmeyer flasks—250 mL.
3. Beakers or Soxhlet flasks—250 mL.
4. Separatory funnels—500 mL.
5. Extraction cylinder or Stokes flask—
   (a) The extraction cylinder has a 250 mL capacity, is glass-stoppered, about 39 mm dia, and 355 mm high.
   (b) The Stokes flask is a 100 mL round bottom flask sealed onto a 150 mL Erlenmeyer flask (see Figure 1 in AOCS Official Method Da 8-48).
6. Siphon—a two-hole rubber stopper fitted with small diameter glass tubing constructed to permit removal of the upper layer from the extraction cylinder or Stokes flask.
7. Reflux condenser.

REAGENTS

1. Ethyl alcohol—absolute. SDA Formulas 30 and 3A are permitted (see Notes, *Caution*).
2. Aqueous potassium hydroxide (KOH) solution—50% KOH by weight (see Notes, *Caution*).
3. Sodium hydroxide (NaOH) solution, 0.02 M—accurately standardized. See AOCS Specification H 12-52.
4. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
5. Phenolphthalein indicator solution—1.0% in 95% alcohol.

PROCEDURE

1. Weigh accurately 5 g of the test sample into a 200 mL Erlenmeyer flask. Add 30 mL of 95% alcohol and 5 mL of 50% KOH solution. Boil gently but steadily under a reflux condenser for 1 hr or until completely saponified. Complete saponification is essential (see Notes, 1).
2. Transfer to the extraction cylinder and wash to the 40 mL mark with alcohol. Complete the transfer with warm water and then cold distilled water until the total volume is 80 mL. Wash out the flask with a little petroleum ether and add to the cylinder. Cool the cylinder and contents to room temperature (20–25°C) and then add 50 mL of petroleum ether.
3. Shake vigorously for at least 1 min and allow to settle until both layers are clear. Remove the upper layer with a glass siphon.
4. Use a glass siphon to remove the upper layer as completely as possible without including any of the lower portion. The petroleum ether fractions are drawn into and accumulated in a 500 mL separatory funnel.
5. Repeat the extraction using 50 mL portions of petroleum ether each time, for at least six more times, shaking vigorously with each extraction.

   Caution—Vigorous shaking and intimate contact of both phases are essential, otherwise the results may be low. There are some cases, especially if lanolin or similar substances are present, in which seven extractions are not sufficient. This is best judged by making another extraction and evaporating this separately. The residue in this extract should not exceed 0.002 g.
6. Wash the combined extracts with a mixture of 15 mL of 0.1 M NaOH plus 15 mL of 95% alcohol and then three times with 25 mL portions of 10% alcohol, shaking vigorously after each addition. Allow the layers to separate and draw off each portion before adding the next.
7. Transfer the petroleum ether to a round bottom flask and evaporate with a rotary evaporator.
8. Add 50 mL of petroleum ether to the residue at room temperature and filter through a filter paper. Wash the filter paper with petroleum ether to remove the last traces of unsaponified and unsaponifiable matter. Collect the filtrate and washings in a tared flask or beaker.
9. Evaporate the petroleum ether with a rotary evaporator until the odor of petroleum ether is no longer perceptible. Dry in a vacuum oven at 75–80°C, with an internal pressure of not more than 200 mm of mercury for 30 min. Cool to room temperature in a desiccator and weigh. Redry for 15 min under the same conditions and continue in this manner to constant weight.
10. Add 50 mL of 95% alcohol which has previously been neutralized to phenolphthalein with NaOH solution. Warm and titrate to a faint pink color with 0.02 M NaOH.

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CALCULATIONS

$$\text{Unsaponifiable matter, \%} = \frac{(\text{mass of residue, g} - \text{mass of fatty acids, g})}{\text{mass of test portion, g}} \times 100$$

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. Use an effective fume-removal device when heating or evaporating.

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. If the Stokes flask is used, the sample may be weighed directly into and saponification carried out in this flask after attaching an air condenser. This eliminates necessity of transfer of saponified material.

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AOCS

AOCS Official Method Da 12-48

Reapproved 2009

Rosin, McNicoll Method

DEFINITION

This method determines the rosin in fatty acids.

SCOPE

Applicable to all soaps and soap products containing rosin. Replaces Da 12-42.

APPARATUS

1. Esterification flask—150 mL round-bottom or Erlenmeyer type.
2. Reflux condenser—water-cooled. Esterification flask and condenser with ground-glass joints Ʇ 24/40 are preferred.
3. Extraction cylinder or Stokes flask—
   (a) Extraction cylinder, 250 mL capacity, with glass stopper, about 39 mm dia and 355 mm high.
   (b) The Stokes flask is a 100 mL round-bottom flask sealed onto a 150 mL Erlenmeyer flask (see Fig. 1 in AOCS Official Method Da 8-48).
4. Siphon—a two-hole rubber stopper fitted with small diameter glass tubing constructed to permit removal of the upper layer from the extraction cylinder or Stokes flask.
5. Burettes—50 mL.
6. Separatory funnel—500 mL.
7. Graduated cylinders—50 and 100 mL.
8. Glass beads.
9. Beakers—2000 mL, 250 mL.

REAGENTS

1. Ethyl alcohol—95% (see Notes, *Caution*).
2. Sulfuric acid (H_2SO_4), 0.25 M.
3. Sulfuric acid (H_2SO_4)—1:1, v/v with distilled water (see Notes, *Caution*).
4. Sodium chloride (NaCl) solutions—saturated solution in water and 10% solution in water, using reagent grade NaCl.
5. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
6. Methyl orange indicator solution—0.01%, prepared by dissolving 0.1 g of methyl orange indicator in 100 mL of distilled water.
7. Filter paper—qualitative grade, rapid filtering.
8. Alcoholic potassium hydroxide (KOH) solution, 0.2 M—accurately standardized. See AOCS Specification H 15-52. SDA Formulas 30 and 3A are permitted. Due to the volatility of the alcohol, this solution should be standardized frequently.
9. Naphthalene-β-sulfonic acid solution—prepared by dissolving 40 g of naphthalene-β-sulfonic acid in 1 L of absolute methyl alcohol.
10. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol.

PREPARATION OF FATTY AND ROSIN ACIDS

1. Dissolve 50 g of original test sample in 500 mL of hot water contained in a 1000 mL beaker or other convenient container.

   Note—If the sample contains alcohol, this must be completely evaporated from the soap solution before proceeding.
2. Add 100 mL of 1:1 sulfuric acid and a few glass beads to minimize bumping and heat gently until the fatty matter collects in a clear layer on the top.
3. Remove the aqueous acid (lower) layer with a siphon, add 300 mL of hot water, boil gently for a few minutes, and again remove the aqueous acid layer with a siphon.
4. Wash the acids three times in this manner. Complete the acidification and washing in as short a period as possible, keeping the beaker covered to prevent oxidation of the acids.
5. After washing, allow to settle for a few minutes and then decant the fatty acids carefully so as not to include any water. Filter through one or two thicknesses of filter paper into a 250 mL beaker or Soxhlet flask and dry at 105 ± 2°C for 45–60 min.

   Note—An alternate method of drying is to heat the fatty acids rapidly on a hot plate to 130°C. Do not hold at this temperature nor reheat more than once. If water is still present after the first heating, allow to settle, decant the fatty acids, and then refilter and reheat momentarily to 130°C. The acids must be thoroughly dry.

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PROCEDURE

1. Weigh accurately 2 g of fatty acids into the esterification flask and add 25 mL of naphthalene-β-sulfonic acid solution. Add a few glass beads to ensure smooth boiling, attach the reflux condenser, and boil for 30 min.
2. Prepare and conduct a blank determination simultaneously and identical in all respects.
3. At the end of the boiling period, cool the contents of the flask, add 0.5 mL of indicator, and titrate immediately with 0.2 M KOH to the first pink color which persists for 30 sec.

CALCULATIONS

1. Rosin in fatty acids, uncorrected, $\% = \dfrac{(S-B) \times M \times 34.6}{\text{mass of test portion, g}}$
2. Rosin in fatty acids, corrected, % = % rosin, uncorrected (from Calculation, 1) – 1.0 (See Numbered Notes 1)
3. Rosin basis original sample, % = % rosin in fatty acids, corrected (Calculation, 2) $\times \dfrac{C}{100}$
4. Rosin soda (soap basis) in original test sample, % = % rosin basis original test sample × 1.064.
   Where—
   S = volume of titrant, mL of sample
   B = volume of titrant, mL of blank
   N = normality of alcoholic KOH solution
   C = % fatty anhydride + % unsaponified and unsaponifiable + (D × 0.2905)
   D = % combined alkali from AOCS Official Method Da 8a-42, calculated as Na_2O.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Ethyl alcohol (ethanol) is flammable. Use an effective fume-removal device when heating or evaporating.

Methyl alcohol is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device.

NUMBERED NOTES

1. Cooperative studies have shown that the McNicoll method gives results about 1% higher than the amount of actual rosin present. The Technical Committee recommends, therefore, that 1% be deducted from the percentage rosin found in the fatty acids.
2. Whenever less than 5% rosin is found, the qualitative Liebermann-Storch (following) test should be applied to confirm the actual presence or absence of rosin.

   Liebermann Storch Test—Place 1–2 mL of the fatty acids into a test tube, add 5–10 mL of acetic anhydride, and warm on a steam bath. Cool, pour 1-2 mL into a white porcelain dish, and allow 1 or 2 drops of H_2SO_4 (sp. gr. 1.53) to run down the inside of the dish. If rosin is present, a fleeting violet color is produced immediately at the point of contact. The color changes to a brownish tinge. Check the test using a sample of fatty acids to which has been added a small amount of rosin. Cholesterol and some other substances give a similar test.

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AOCS Official Method Da 13-48

Reapproved 2009

Titer Test

DEFINITION

This method determines the solidification point of the fatty acids.

SCOPE

Applicable to the fatty acids of all soaps and soap products. Replaces Da 13-42.

APPARATUS

1. Beaker—Griffin low-form, 2 L.
2. Bottle—wide mouth, 450 mL, height 190 mm and 38 mm i.d. neck.
3. Test tubes—100 mm length, 25 mm dia with or without rim. These tubes may have an etched mark extending around the tube at a distance of 57 mm from the bottom to show the height to which the tube is to be filled.
   Note—Titer test tubes may be obtained from Fisher Scientific, catalog number 14–963A.
4. Stirrer—2–3 mm o.d., with one end bent in the form of a loop 19 mm o.d. Glass, nichrome, stainless steel, or monel wire may be used. The upper end can be formed to accommodate hand stirring or attached to a mechanical stirrer.
5. Thermometers—
   (a) Laboratory thermometer—range 0-150°C.
   (b) Titer test thermometer—range –2–68°C, AOCS Specification H 6-40, calibrated against an NIST traceable thermometer.

PROCEDURE

1. Preparation of the fatty acids—
   (a) The fatty acids are prepared as directed in AOCS Official Method Da 12-48, Preparation, 1-5.
2. Solidification of the fatty acids—
   (a) Fill the water bath to the designated level (see Fig. 1) and adjust the temperature to 20 ± 2°C for all samples having titers of 35°C or higher, and 15-20°C below the titer point for all samples with titers below 35°C.
   (b) Place the tube containing the fatty acids in the assembly, as shown in Figure 1. Insert the titer thermometer to the immersion mark so that it will be equidistant from the sides of the tube.
   (c) Stir with the stirring rod in a vertical manner at the rate of 100 complete up and down motions per minute. The stirrer should move through a vertical distance of 38 mm. The agitation is started while the temperature is at least 10°C above the titer point (see Note, 1).
   (d) Stir at the directed rate until the temperature remains constant for 30 sec, or begins to rise in less than a 30 sec interval. Discontinue stirring immediately, remove the stirrer or raise it out of the sample and observe the increase in temperature. The titer point is the highest temperature indicated by the thermometer during this rise. Duplicate determinations are usually expected to agree within ± 0.2°C.

NOTES

1. Stirring may be performed mechanically by attaching to the stirring rod a small motor with suitable speed-reducing mechanism.

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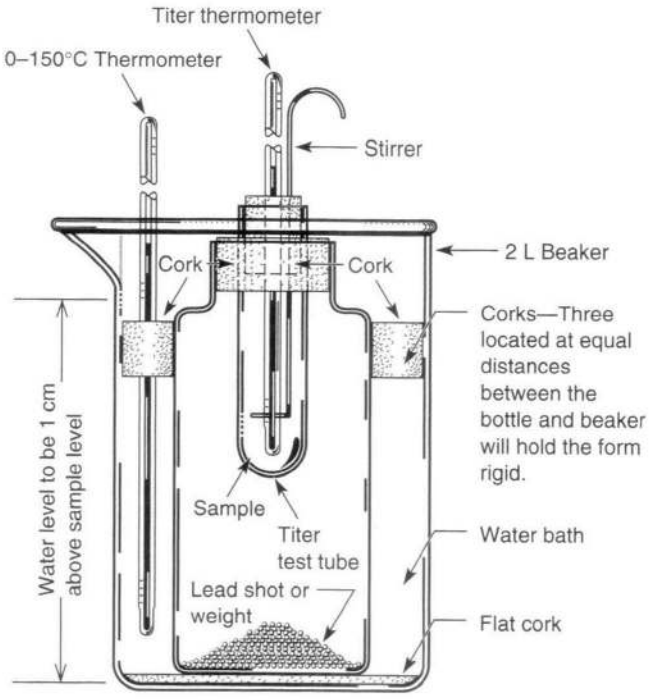

Figure 1. Titer stirring assembly.

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AOCS Official Method Da 14-48

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Acid Value of Fatty Acids

DEFINITION

The acid value is the mg of potassium hydroxide (KOH) necessary to neutralize fatty or rosin acids in 1 g of sample (see Notes, 1).

SCOPE

Applicable to fatty or rosin acids after separation from soap. Replaces Da 14-42.

APPARATUS

1. Erlenmeyer flask—250 mL.

REAGENTS

1. Sodium hydroxide (NaOH) solution, 0.5 M—accurately standardized. See AOCS Specification H 12-52 (see Notes, 1).
2. Ethyl alcohol—95%, SDA Formulas 30 and 3A are permitted (see Notes, *Caution*). Add 3–5 drops phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1-0.2 M solution, to a faint pink color just before using.
3. Phenolphthalein indicator solution—1.0% in 95% alcohol.

PROCEDURE

1. The fatty acids are prepared as in Official Method, Da 12-48, Preparation, 1–5.
2. Weigh accurately about 2 g of fatty acids into 250 mL Erlenmeyer flask and add 20–30 mL of hot, neutralized alcohol.
3. Add 05 mL of indicator and titrate immediately while shaking with 0.5 N NaOH to the first pink color which will persist for 30 sec.

CALCULATIONS

$$\text{Acid value, mg KOH/g of fatty acids} = \frac{\text{mL NaOH} \times \text{M} \times 56.1}{\text{mass of test portion, g}}$$

Where—

M = molarity of NaOH

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. A properly operating fume hood should be used when heating or evaporating

NUMBERED NOTES

1. Although standardized sodium hydroxide is used in the titration, results are expressed in terms of mg KOH/g of fatty acids. This is taken into consideration in the calculation, noting that 1 mL of 1.0 M NaOH is equivalent to 56.1 mg of 1.0 M KOH.

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AOCS

AOCS Official Method Da 15-48

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Iodine Value, Wijs Method

DEFINITION

The iodine value is a measure of the unsaturation of the fatty acids and is expressed in terms of the cg of iodine absorbed per g of sample (% iodine absorbed).

SCOPE

Applicable to all soaps and soap products except those made of fats containing conjugated systems. Replaces Da 15-42. See Recommendations before applying this method.

APPARATUS

1. Bottles, glass-stoppered, or wide mouth Erlenmeyer flasks—500 mL. Wide mouth bottles or flasks are essential if stirring is done by mechanical means.
2. Volumetric flask—glass-stoppered, conforming to National Institute of Testing (NIST) tolerances, and accurately calibrated to contain 1,000 mL.
3. Pipet—20 mL.
4. Two pipets—25 mL.

   Note—One 25 mL pipet is reserved for use with the standard potassium dichromate solution. This pipetmust conform to NIST tolerances, and be accurately calibrated to deliver 25 mL.

REAGENTS

1. Accurately standardized Wijs solution—Because the preparation of the Wijs solution is time-consuming and involves the use of hazardous materials, (see Notes, *Caution*) it is strongly recommended that commercial Wijs solution be used. Commercial Wijs solutions are available which contain no carbon tetrachloride. (The preparation of Wijs solution is noted in AOCS Official Method, Tg 1-64.)
2. Glacial acetic acid—reagent grade. The potassium permanganate ($KMnO_4$) test should be applied to be sure that this specification is met.

   Permanganate test—Dilute 2 mL of the acid with 10 mL of distilled water and add 0.1 mL of 0.1 N $KMnO_4$. The pink color must not be entirely discharged within 2 hr.
3. Potassium iodide (KI)—reagent grade.
4. Soluble starch—tested for sensitivity. Make a paste with 1 g of starch and a small amount of cold distilled water. Add, while stirring, to 200 mL of boiling water. Stir rapidly for a few seconds and immediately remove from heat. Allow to cool. Place 5 mL of this solution in 100 mL of water and add 0.05 mL of 0.1 N iodine solution. The deep blue color produced must be discharged by 0.05 mL of 0.1 N sodium thiosulfate. Test daily. Fresh indicator must be prepared when the end point of the test titration fails to be sharp from blue to colorless. The starch solution should be refrigerated when not being used. Salicylic acid (1.25 g/L) may be added as a preservative.
5. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)—primary standard grade.
6. Potassium dichromate ($K_2Cr_2O_7$)—reagent grade. The potassium dichromate is dried to constant weight at ca. 110°C before using.
7. Carbon tetrachloride (CCl_4)—reagent grade (see Notes, *Caution* and Recommendations).
8. Hydrochloric acid—reagent grade, sp. gr. 1.19 (see Notes, *Caution*).

SOLUTIONS

1. Potassium iodide (KI) solution—dissolve 150 g KI in distilled water and make up to 1 L.
2. Starch indicator solution—prepared and tested as noted in Reagents, 4.
3. Standard potassium dichromate solution, 0.1 N—accurately measure about 4.9 g of finely ground and dried potassium dichromate. Dissolve in distilled water in the 1000 mL volumetric flask and make to volume at 25°C. Calculate the normality of this solution with the following equation:

$$N(K_2Cr_2O_7) = \text{mass of } K_2Cr_2O_7, \text{ g} \times 0.0204$$

4. Sodium thiosulfate solution, 0.1 N—dissolve 24.8 g of sodium thiosulfate in distilled water and dilute to 1 L.

   Standardization—Pipette 25 mL of the standard dichromate solution into Erlenmeyer flask or bottle. Add 5 mL of hydrochloric acid, 10 mL of potassium iodide solution, and rotate to mix. Allow to stand for 5 min and add 100 mL of distilled water. Titrate with sodium thiosulfate solution, shaking continuously, until the yellow color has almost disappeared.

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Add 0.5 mL of indicator and continue titration, adding the thiosulfate solution slowly, until the blue color has just disappeared. The strength of the sodium thiosulfate solution is expressed in terms of its normality:

$$\text{Normality of } Na_2S_2O_3 \text{ solution} = \frac{N(K_2Cr_2O_7) \times 25}{\text{volume } Na_2S_2O_3, \text{ mL}^1}$$

[1]Required in standardization titration in step 4.

5. Wijs solution—purchased commercially. Determine the sodium thiosulfate titer of the Wijs solution by titrating with the standardized $Na_2S_2O_3$ solution as directed in the Procedure, 2-7, with no sample added.

PROCEDURE

1. Fatty acids are prepared as directed in the Procedure of AOCS Official Method, Da 12-48. The temperature of the fatty acids during filtration is not to exceed 15°C above the titer point of the fatty acids. It is essential that the acids be clear and dry; if not, refilter.
2. Weigh the sample accurately into 500 mL flask or bottle to which has been added 20 mL of CCl_4. The weight of sample must be such that there will be an excess of Wijs solution of 50-60% of the amount added, i.e., 100-150% of the amount absorbed. Table 1 is a convenient guide to the size of sample to be used.
3. Make certain that the sample is completely dissolved and then pipet 25 mL of the Wijs solution into the flask containing the sample and swirl to ensure an intimate mixture.
4. Prepare and conduct at least two blank determinations with each group of samples simultaneously and similar in all respects.
5. Store the flask in a dark place for exactly 30 min (see Notes, 1) at a temperature of 25 ± 5°C.
6. Remove the flask from storage and immediately add 20 mL of KI solution, followed by 100 mL of distilled water.
7. Immediately titrate with 0.1 N $Na_2S_2O_3$ solution, adding it gradually and with constant and vigorous shaking (see Notes, 2). Continue the titration until the yellow color has *almost* disappeared. Add 0.5 mL of starch indicator solution and continue the titration until the blue color has just disappeared.

CALCULATIONS

$$\text{Iodine Value} = \frac{(B-S) \times N \times 12.69}{\text{mass of sample, g}}$$

Where—

B = volume of titrant, mL of blank
S = volume of titrant, mL of sample
M = molarity of $Na_2S_2O_3$ solution

NOTES

Caution

Carbon tetrachloride is a known carcinogen. It is toxic by ingestion, inhalation, and skin absorption. It is a narcotic. It should not be used to extinguish fires. It decomposes to phosgene gas at high temperature. The TLV is 5 ppm in air. A fume hood should be used at all times when using carbon tetrachloride.

Wijs solution causes severe burns and vapors can cause lung and eye damage. Use of a fume hood is recommended.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

NUMBERED NOTES

1. Store samples with an iodine value above 150 for 1 hr in a dark place at 25 ± 5°C.
2. Mechanical stirring is satisfactory for agitating during the addition of thiosulfate.

RECOMMENDATIONS

At the time of the revision of this method in 1989, studies were under way to find a replacement for carbon tetrachloride in this method. The most satisfactory replacement found to date has been cyclohexane [see AOCS Official Method, Cd 1b-87 and JAOCS 65:745 (1988)], although erratic results may be obtained for oils with iodine values >100-120 and especially marine oils. An IUPAC collaborative study in progress indicates that the oils with high iodine values may require a longer reaction time, possibly in the range of 2-3 hr. Because of environmental concerns, 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113) is not recommended. Acetic acid alone and a mixture of acetic + cyclohexane, 1:4, respectively, have now been shown to be unsatisfactory.

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1,1,1-Trichloroethane (TCE) has been suggested as a replacement for carbon tetrachloride in this method. Although TCE is a halogenated hydrocarbon, the TLV (350ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternative solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

Table 1
Sample weight

Iodine Value	100% Excess g	150% Excess g	Weighing Accuracy g
<3	10	10	± 0.001
3	10.576	8.4613	0.005
5	6.346	5.0770	0.0005
10	3.1730	2.5384	0.0002
20	1.5865	0.8461	0.0002
40	0.7935	0.6346	0.0002
60	0.5283	0.4231	0.0002
80	0.3966	0.3173	0.0001
100	0.3173	0.2538	0.0001
120	0.2644	0.2115	0.0001
140	0.2266	0.1813	0.0001
160	0.1983	0.1587	0.0001
180	0.1762	0.1410	0.0001
200	0.1586	0.1269	0.0001

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AOCS Official Method Da 16-48

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Saponification Value

DEFINITION

The saponification value is the mg of potassium hydroxide required to saponify 1 g of the sample.

SCOPE

Applicable to the fatty acids of all soaps and soap products. Replaces Da 16-42.

APPARATUS

1. Erlenmeyer flasks—Corning Alkali Resistant, Kimble Resistant, or equivalent, 250 or 300 mL.
2. Air condensers—min 650 mm long.
3. Water bath or a hot plate—with variable heat control.
4. Volumetric pipette—50 mL.

REAGENTS

1. Hydrochloric acid (HCl), 0.5 M—accurately standardized. See AOCS Specification H 14-52.
2. Alcoholic potassium hydroxide (KOH)—place 5–10 g of KOH in a 2 L flask and from 1–1.5 L 95% ethyl alcohol (SDA Formulas 30 and 3A are permitted) and boil in a water bath under a reflux condenser for 30–60 min. Distill and collect the alcohol. Dissolve 27 g of potassium hydroxide, low in carbonate, in 1 L of the distilled alcohol, keeping the temperature below 15.5°C (60°F) while the alkali is being dissolved (see Notes, *Caution*). This solution should remain clear.
3. Phenolphthalein indicator solution—1% in 95% alcohol.

PROCEDURE

1. Fatty acids are prepared as indicated in the Procedure of AOCS Official Method Da 12-48. The temperature of the fatty acids during filtration is not to exceed 15°C above the titer point of the fatty acids. It is essential that the acids be clear and dry; if not, refilter.
2. Weigh a sample of such size that the back titration is 45–55% of the blank. This will usually require a 3–4 g sample. Add 50 mL of the alcoholic KOH with a pipet and allow the pipet to drain for a definite period of time.
3. Prepare and conduct blank determinations simultaneously with the sample.
4. Connect an air condenser and boil gently but steadily until the sample is completely saponified. This usually requires about 30 min for normal samples. Be careful that the vapor ring in the condenser does not rise to the top of the condenser or there may be some loss of sample.
5. After the flask and contents have cooled somewhat, but not sufficiently to gel, wash the inside of the condenser with a few mL of 95% ethyl alcohol, add about 1 mL of indicator and titrate with 0.5 N HCl until the pink color has just disappeared.

CALCULATIONS

$$\text{Saponification Value} = \frac{\text{titrant volume sample, mL} - \text{titrant volume blank, mL}}{\text{wt of test portion, g}} \times \text{M(HCl)} \times 56.1$$

NOTES

Caution

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

Ethyl alcohol (ethanol) is flammable. Use a fume hood when heating or evaporating this solvent.

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Borax

DEFINITION

This method determines borax in the sample.

SCOPE

Applicable to all soaps and soap products.

APPARATUS

1. Beakers—250 and 400 mL.
2. Burette—50 mL.
3. Volumetric flask—250 mL.
4. Watch glasses.
5. Filter paper—a quantitative grade that will retain gelatinous precipitates, such as Whatman no. 1, S & S no. 589 Black Ribbon, or equivalent.
6. pH meter—standardized according to manufacturer's instructions.

REAGENTS

1. Hydrochloric acid (HCl)—1:1, v/v concentrated HCl (see Notes, *Caution*) with distilled water (see Notes, 1).
2. Mannitol—CP grade.
3. Methyl red indicator solution—prepared by dissolving 0.10 g of methyl red in 100 mL of a 1:1, v/v solution of 95% ethyl alcohol and distilled water.
4. α-Naphtholphthalein indicator solution—prepared by dissolving 0.10 g of α-Naphtholphthalein in 100 mL of a 1:1, v/v solution of 95% ethyl alcohol and distilled water.
5. Sodium hydroxide (NaOH) solution—25% by weight in distilled water, carbonate free (see Notes, *Caution*).
6. Sodium hydroxide (NaOH) solution—either 0.05 M or 0.10 M, accurately standardized. See AOCS Specification H 12–52.
7. Strontium chloride ($SrCl_2 \cdot 6H_2O$) solution—33.3% by weight in distilled water, or strontium nitrate [$Sr(NO_3)_2$] solution, 26.5% by weight in distilled water.

PROCEDURE

1. Accurately weigh a test portion, conforming to the estimated borax weight noted in Table 1, into a 250 mL beaker.
2. Add 100 mL of distilled water (see Notes, 1) and warm on the steam bath until the sample has dissolved.
3. Add 2 mL of 25% sodium hydroxide solution, heat the solution almost to boiling, and add, with stirring, 10 mL of either the $SrCl_2 \cdot 6H_2O$ or the $Sr(NO_3)_2$ solution to precipitate the soap and any phosphate or silicate which may be present. Heat the solution for an additional 5 min, stirring frequently and avoiding vigorous boiling.
4. Filter through the quantitative filter paper, collecting the filtrate in a 400 mL beaker. Wash the precipitate thoroughly with hot, distilled water (about 100 mL).
5. Add sufficient 1:1 HCl to the filtrate and washings to change methyl red indicator yellow, then add further 0.5 mL acid.
6. Cool the solution in a cold water bath to room temperature. Rinse the watch glass with water and add the washings to the solution. Using the pH meter, bring the solution to a pH of approximately 6.0–6.2 by the dropwise addition of 25% sodium hydroxide solution, then bring the pH to exactly 6.3 by the dropwise addition of 0.05 N NaOH solution.
7. Add 5 g of mannitol, stir until dissolved and titrate to a pH of 8.0 with 0.05 M or 0.10 M NaOH, depending on the amount of borax present. The titration should be carried out in a cold solution, room temperature or lower. Record the burette reading.
8. Conduct a blank as follows: Take a volume of boiled distilled water corresponding to the volume of solution at the end of the titration (Procedure, 7). Add 0.5 mL of 1:1 HCl. Using the pH meter, bring the pH to approximately 6.0–6.2 by the dropwise addition of 25% sodium hydroxide solution, then bring the pH to exactly 6.3 by the dropwise addition of 0.05 M NaOH solution.
9. Add 5 g of mannitol and stir until dissolved and titrate to a pH of 8.0 with the same strength sodium hydroxide as used in Procedure, 7. Record the burette reading. The blank should not exceed 0.1 mL and is usually less (see Notes, 2).

CALCULATIONS

$$\text{Borax, as } Na_2B_4O_7 \cdot 10H_2O, \% = \frac{(A - B) \times M \times 9.536}{\text{wt of test portion, g}}$$

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Where—

A = mL NaOH to titrate sample (Procedure, 7)
B = mL NaOH to titrate blank (Procedure, 9)
M = molarity of NaOH solution

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. Carbon-dioxide-free distilled water should be used throughout this procedure. Prepare carbon-dioxide-free water by vigorous boiling for several minutes before use and cool in a tightly stoppered bottle or under an atmosphere of nitrogen or argon.
2. An indicator end point may be employed when a pH meter is not available. The procedure is as follows:
   (a) Starting with Procedure, 6, cool the solution in a cold water bath to room temperature.
   (b) Rinse the watch glass with water and add the washings to the solution.
   (c) Add several drops of methyl red indicator solution and then add 1:1 HCl dropwise until the solution is distinctly acid to the indicator. Titrate with 0.05 M NaOH to the methyl red end point (yellow). Record the burette reading.
   (d) Add 5 g of mannitol and stir until dissolved.
   (e) Add 3 mL of α-naphtholphthalein indicator solution, and titrate the boric acid complex with 0.05 M or 0.10 M NaOH to a green-yellow end point. The titration should be carried out in cold solution (room temperature or lower). A blank should be run on the amount of mannitol used in the titration. The blank should not exceed 0.1 mL and is usually less. Methyl purple can be used in place of methyl red.

REFERENCES

1. Bernstein and Hatfel, *JAOCS 27:45* (1950).

Table 1

Estimated borax, %	Sample weight, g
0–10	2.0
10–25	1.0
25–50	0.75
50–90	0.50
90–100	0.40

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Alkaline Silicates

DEFINITION

This method determines alkaline silicates in the sample.

SCOPE

Applicable to all soaps and soap products. Replaces Da 18-42.

APPARATUS

1. Platinum evaporating dish—100 mL.
2. Platinum crucible—30 mL.
3. Watch glasses.
4. Filter paper—ashless, 90 mm dia.
5. pH meter (or see Reagents, 4)

REAGENTS

1. Hydrochloric acid (HCl)—reagent grade, concentrated (sp. gr. 1.19) (see Notes, *Caution*).
2. Hydrofluoric acid (HF)—technical grade, 47% solution (see Notes, *Caution*).
3. Sulfuric acid (H_2SO_4)—reagent grade, concentrated (sp. gr. 1.48) (see Notes, *Caution*).
4. Indicator solution.

PROCEDURE

1. Sample contains no mineral matter insoluble in water—
   (a) Weigh a size of test portion such that it will contain not more than 0.2 g of silicate (SiO_2) into a platinum evaporating dish and ignite over a burner or in a muffle furnace below a dull red heat (350–400°C) until the mass is charred. Add distilled water and filter through ashless filter paper. Wash the dish and residue on the filter paper several times with distilled water so as to extract all water-soluble material.
   (b) Return the filter paper containing the charred residue to the platinum dish and continue the ignition at a bright red heat (850–950°C) until all carbon is removed. Combine the residue and all of the water extract, neutralize carefully with HCl, and then add from 5–10 mL HCl in excess. Keep the dish covered with a watch glass during this addition to prevent loss from spattering.
   (c) Evaporate to dryness on steam bath or hot plate at a temperature not exceeding 120°C. Cool the residue and moisten with HCl, allow to stand for 5–10 min, and then break up all lumps with a stirring rod.
   (d) Add about 25 mL of hot distilled water to the residue, heat for a few minutes, and filter through ashless filter paper. Wash the paper and contents thoroughly with hot water. Repeat Procedure, 1, c and d. Filter through a second ashless filter paper.
   (e) Place the two filter papers in a tared platinum crucible and ignite carefully, first at a low temperature until the paper is burned off and then over a burner or in a muffle furnace at a bright red heat (850–950°C). Cool to room temperature in a desiccator and weigh. Repeat the weighing until constant weight is obtained.
   (f) For the most accurate results, moisten the weighed contents of the crucible with water, add 10 mL of HF, 4 drops of H_2SO_4, and evaporate to dryness over a low flame. Ignite as directed in Procedure, 1, e, cool to room temperature in a desiccator, and weigh. Repeat the weighing until constant weight is obtained.
2. If sample contains mineral matter insoluble in water, or determination of the highest accuracy is not required—
   (a) Use a portion of the solution after titrating the matter insoluble in alcohol, but soluble in water (AOCS Official Method Da 7-48, Procedure, 2), such that it will contain not more than 0.2 g of silicate (SiO_2).
   (b) Add from 5–10 mL HCl and proceed as directed in Procedure 1, c.

CALCULATIONS

1. When SiO_2 is weighed:

$$\text{Sodium Silicate (1 } Na_2O\text{:3.25 } SiO_2\text{), \%} = \frac{\text{mass of residue, g (Procedure 1, e)}}{\text{mass of test portion, g}} \times 131.8$$

2. When SiO_2 is volatilized by HF:

$$\text{Sodium Silicate (1 } Na_2O\text{:3.25 } SiO_2\text{), \%} = \frac{(A - B) \times 131.8}{\text{mass of test portion, g}}$$

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Where—

A = mass, g of crucible and contents from Procedure 1, e.
B = mass, g of crucible and contents from Procedure 1, f.

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Hydrofluoric acid is toxic by ingestion and inhalation. It is highly corrosive to skin and mucous membranes. Protective clothing should be worn at all times when working with this acid. Always add acid to water, never the reverse.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

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Carbonates, Gravimetric Absorption Method

DEFINITION

This method determines all of the carbonates in a sample as carbon dioxide.

SCOPE

Applicable to all soaps and soap products.

APPARATUS

1. Knorr Alkalimeter—see (c) in Figure 1.
2. Turner adsorption bulb—see (d) in Figure 1.
3. Stetser-Norton-Midvale absorption bulb with glass stopper—see (e) in Figure 1.
4. Calcium chloride drying tube—see (a) in Figure 1.
5. Rubber tubing—for connections at (f) in Figure 1, the tubing must be of good grade rubber with inside diameter about 2 mm less than the outside diameter of the connections on which it is used.
6. Cotton and glass wool.
7. Vacuum source.

REAGENTS

1. Ascarite—or equivalent carbon dioxide (CO_2) absorbent.
2. Sulfuric acid (H_2SO_4)—reagent grade, concentrated (sp. gr. 1.48), (see Notes, *Caution*).
3. Calcium chloride—anhydrous, reagent grade.
4. Hydrochloric acid (HCl)—1:1, v/v concentrated HCl with distilled water (see Notes, *Caution*).

Note—All absorbing reagents must be maintained fresh in order to absorb all of the CO_2 and water.

PROCEDURE

1. The apparatus is assembled as shown in Figure 1. All connections are glass to glass except at b and f. The Turner bulb should contain sufficient concentrated H_2SO_4 so that the gas will pass through about a 3 cm column of liquid.
2. Introduce a sample and conduct a preliminary test (or tests), as directed in Procedure, 3–6, to adjust the aspirating rate so that none of the CO_2 will pass through the purifying train without being absorbed. Check this by adding an extra tube containing CO_2 absorbent. Weigh the CO_2-absorbing tubes separately. There should be no gain in weight of the extra tube. The rate of gas passage may be determined by using the H_2SO_4 tube as a bubble counter.
3. Weigh the tube [(e) in Fig. 1] containing Ascarite (or equivalent CO_2 absorbent). Weigh a sample into the flask of the Knorr Alkalimeter (C in Fig. 1) of sufficient size to yield about 0.2 g of CO_2. If the test in Procedure, 2 reveals the sample to be excessively foamy, add a drop of a typical silicone-based defoaming agent. Add 20 mL of freshly boiled distilled water. Place 20 mL of HCl (1:1) in the designated bulb of the Alkalimeter. Close the train with all apparatus in place.
4. Open the stopcock and allow the 20 mL of HCl to drop slowly and gradually into the flask so that the CO_2 formed does not pass through the train too rapidly.
5. As soon as the acid has been added, apply a slight vacuum to the outlet end of the CO_2-absorbing tube. When the reaction begins to subside, start heating the bottom of the sample flask gently and continue heating for 15–20 min or until the reaction appears to be complete.
6. Discontinue heating, but continue aspirating until the flask has cooled to room temperature. Remove the CO_2-absorbing tube and weigh.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to water, never the reverse.

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CALCULATIONS

1. Carbon dioxide (CO_2), % = $\frac{(A - B)}{\text{mass of test portion, g}} \times 100$

   Where—

   A = mass, g of CO_2-absorbing tube (E, Fig. 1) after determination
   B = mass, g of CO_2-absorbing tube before determination

2. To convert results to either sodium carbonate or potassium carbonate:
   Sodium carbonate (Na_2CO_3), % = 2.41% CO_2
   Potassium carbonate (K_2CO_3), % = 3.14% CO_2

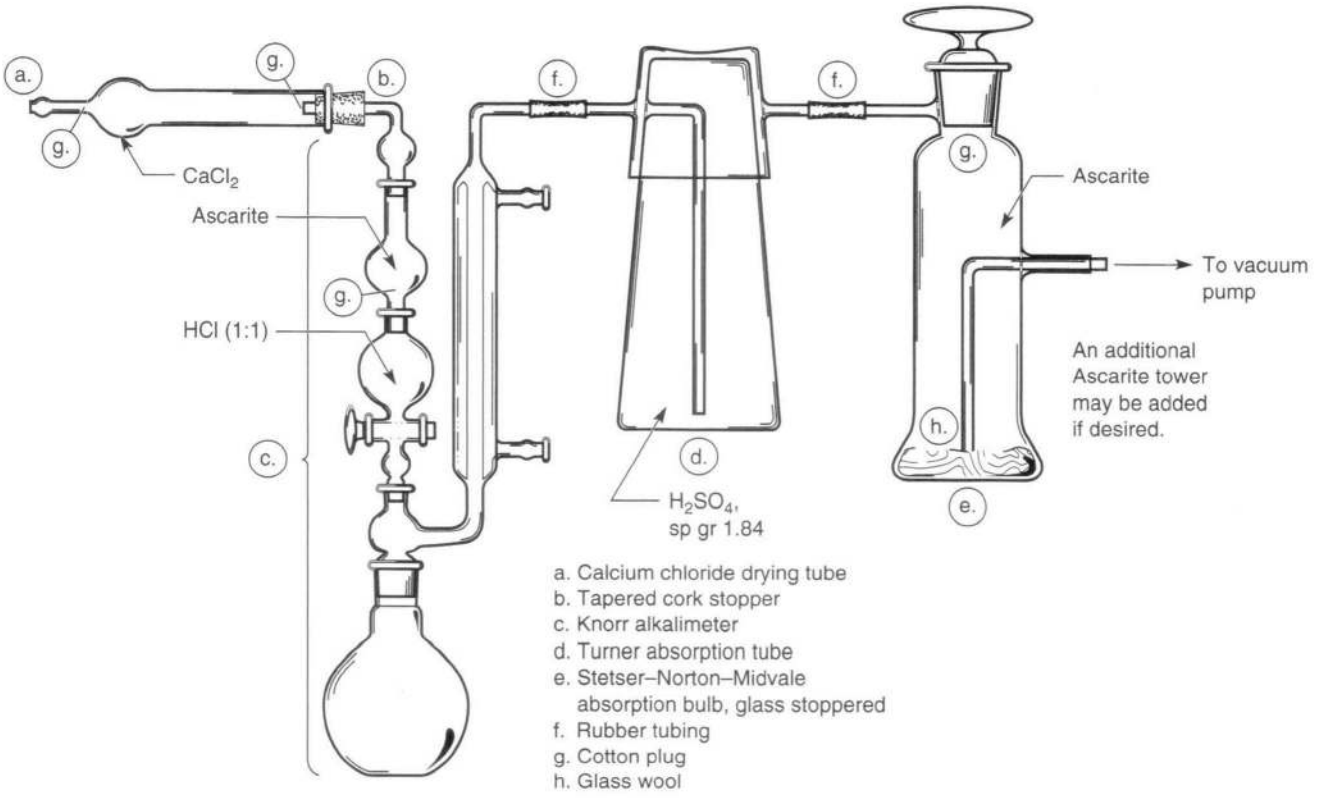

Figure 1. Gravimetric carbonate apparatus.

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AOCS

AOCS Official Method Da 19b-42

Reapproved 2009

Carbonates, Volumetric Evolution Method

DEFINITION

This method determines all of the carbonates in the sample as carbon dioxide.

SCOPE

Applicable to all soaps and soap products.

APPARATUS

1. Glass apparatus constructed and assembled as shown in Figure 1, consisting of the following—
   (a) Evolution or sample flask—1 L round-bottom, ring-necked flask of heat resistant glass provided with a two-hole rubber stopper.
   (b) Dropping funnel—provided with a stopcock and having a stem long enough to reach into the lower bulb of the condenser.
   (c) Erlenmeyer flask—heat resistant glass, 300 mL, provided with a one-hole rubber stopper.
   (d) Condenser—water-cooled, three or four bulb Allihn type with a jacket about 200 mm in length. A side arm is attached for connecting to the Erlenmeyer flask.
   (e) Manometer—for use in maintaining the prescribed pressure within the apparatus.
2. Glass beads.
3. Pipet—25 mL.

REAGENTS

1. Hydrochloric acid (HCl), 0.5 M—accurately standardized. See AOCS Specification H 14-52.
2. Hydrochloric acid (HCl)—1:2, v/v concentrated HCl with distilled water (see Notes, *Caution*).
3. Alkaline absorbent solution—prepared by mixing equal volumes of 1.0 M carbonate-free sodium hydroxide (NaOH) and 1.0 M barium chloride ($BaCl_2$). Allow to settle overnight, filter and preserve in well-stoppered bottles.
4. Magnesium chloride ($MgCl_2$) solution—20% in distilled water.
5. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol.
6. Methyl orange indicator solution—0.1% in distilled water.
7. Trichlorobenzene—1:2:4 isomer, reagent grade quality, bp 213°C, sp. gr. 1.47 (*see* Notes, *Caution*).

PROCEDURE

1. Weigh a sufficient quantity of the test sample into the sample flask (Apparatus, 1, a) to yield about 0.2 g of CO_2. Add about 400 mL of unboiled distilled water to which has been added 2 mL of the alkaline absorbent solution to prevent the loss of CO_2.
2. Heat the flask on a steam bath until the soap is dissolved and then cool until the flask is only slightly warm to the hand. Add 30 mL of the $MgCl_2$ solution and a few glass beads to prevent bumping.
3. Pipet 25 mL of the alkaline absorbent solution into the Erlenmeyer flask (Apparatus, 1, c) and assemble the apparatus as shown in Figure 1. Start the water through the condenser (Apparatus 1, d).
4. Apply a vacuum to the dropping funnel (Apparatus, 1, b) to evacuate the system and reduce the pressure to about 65-85 mm as indicated by the manometer.

   Precaution—Maintain a properly reduced pressure and do not allow air to enter the system at any time during the test.
5. Add dilute HCl (Reagents, 2), containing a few drops of methyl orange indicator, to the mixture in the sample flask until the mixture is acid (yellow to red). Avoid a large excess of acid. Add trichlorobenzene through the dropping funnel in the proportion of about 1 mL to every 2 g of sample.

   Note—Shake the Erlenmeyer flask at frequent intervals from the time that the HCl is added until the sample flask and condenser have been filled with water (see Procedure, 6).
6. Place the small flame of a gas burner immediately in contact with the bottom of the sample flask and heat continuously for 30 min. After 30 min, discontinue heating and pour boiled and cooled CO_2-free water at 50°C through the condenser tube to fill the flask and condenser to just below the side arm of the condenser.
7. Disconnect the Erlenmeyer flask, add 1 mL of phenolphthalein indicator, and titrate immediately with 0.5 M HCl with vigorous agitation until the pink color has just disappeared. Add the acid drop by drop. If the titration cannot be performed immediately, stopper the flask to guard against the entrance of air.
8. Prepare and conduct a blank determination identical in all respects in order to establish the equivalent of the alkaline absorbent solution in terms of 0.5 M HCl and also to correct for any CO_2 in the reagents.

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CALCULATIONS

$$\text{Carbon dioxide, } (CO_2), \% = \frac{(S - B) \times M \times 2.2}{\text{mass of test portion, g}}$$

Where—

B = volume of titrant, mL of blank
S = volume of titrant, mL of sample
M = molarity of HCl

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

1,2,4-Trichlorobenzene is toxic by ingestion and inhalation. The TLV ceiling in air is 5 ppm.

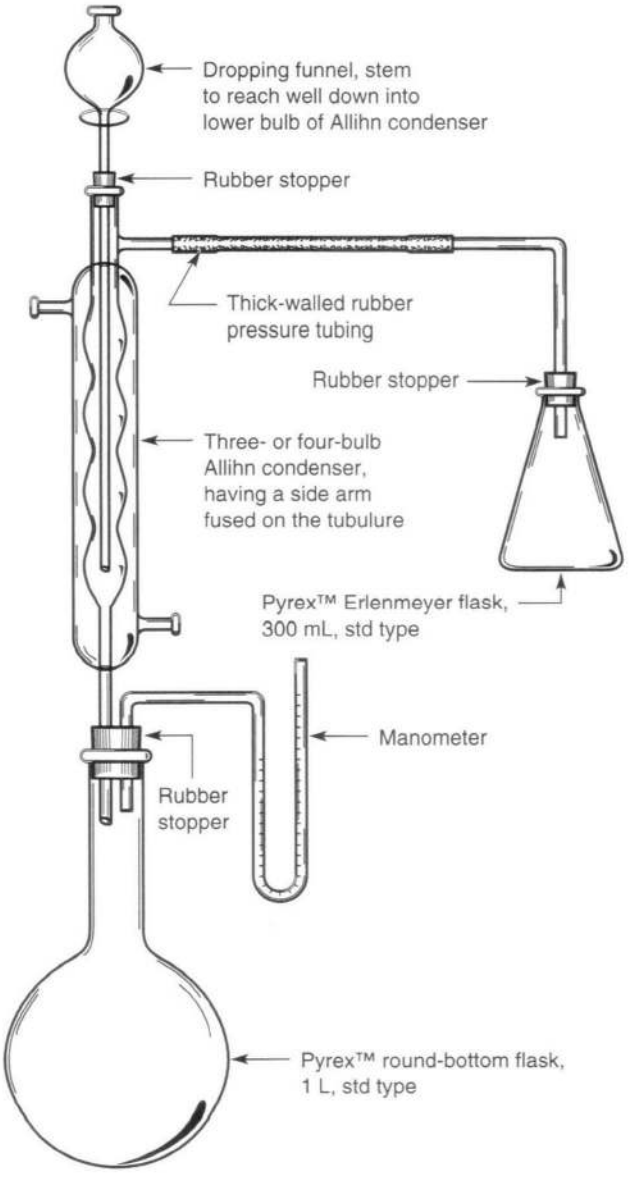

Figure 1. Volumetric carbonate apparatus.

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AOCS

AOCS Official Method Da 20a-48

Reapproved 2009

Phosphates

DEFINITION

This method determines all of the phosphates as phosphorus pentoxide (P_2O_5).

SCOPE

Applicable to all soaps and soap products. Replaces Da 20-42.

APPARATUS

1. Volumetric flasks—250 and 1000 mL.
2. Bottles—with glass stoppers, 1 or 2 L.
3. Beakers—250 mL.
4. Burette—50 mL.
5. Filter paper—ashless, quantitative grade that will retain fine precipitates.

REAGENTS

1. Molybdic acid—reagent grade, 85%.
2. Ammonium nitrate (NH_4NO_3)—reagent grade.
3. Magnesium chloride ($MgCl_2 \cdot 6H_2O$)—reagent grade.
4. Ammonium chloride (NH_4Cl)—reagent grade.
5. Silver nitrate ($AgNO_3$)—reagent grade.
6. Ammonium hydroxide (NH_4OH)—reagent grade, sp. gr. 0.90 (see Notes, *Caution*).
7. Hydrochloric acid—reagent grade, sp. gr. 1.19 (see Notes, *Caution*).
8. Nitric acid—reagent grade, sp. gr. 1.42 (see Notes, *Caution*).
9. pH test paper—or brom thymol blue indicator solution (0.04% in 95% ethyl alcohol).

SOLUTIONS

1. Mix 144 mL of NH_4OH (sp. gr. 0.90) with 271 mL of distilled water and add 100 g of molybdic acid. When molybdic acid is completely dissolved, pour with continuous stirring into a solution of 489 mL of HNO_3 (sp. gr. 1.42) and 1148 mL of distilled water. Keep the final solution in a warm place (30-35°C) for several days and until a portion heated to 40°C deposits no yellow precipitate of ammonium phosphomolybdate. Decant from any sediment and preserve in glass-stoppered bottles.
2. Ammonium nitrate solution—prepared by dissolving 100 g of NH_4NO_3 in distilled water and diluting to 1 L.
3. Magnesium solution—prepared by dissolving 55 g of $MgCl_2 \cdot 6H_2O$ in distilled water and adding 140 g of NH_4Cl, 130.5 mL of NH_4OH (sp. gr. 0.90), and diluting to 1 L with distilled water.
4. Dilute NH_4OH—prepared by diluting 100 mL of NH_4OH (sp. gr. 0.90) to 1 L with distilled water.
5. Ammonium hydroxide solution—1:1, v/v, concentrated ammonium hydroxide solution with distilled water.
6. Hydrochloric acid—1:1, v/v, concentrated hydrochloric acid with distilled water.
7. Silver nitrate solution—10% in distilled water.

PROCEDURE

1. Weigh 2 ± 0.01 g of test sample and proceed as directed in AOCS Official Method Da 18-48, Procedure 1, a-d, to remove silicates and collect the filtrate from Procedure, 1, d in Method Da 18-48 in a 250 mL volumetric flask. Dilute to volume with distilled water and mix well.

   Note—The dry residue after the removal of alcohol-soluble matter (AOCS Official Method Da 3-48, Procedure, 3) may be used, in which case weigh 2 g and remove the silicates as directed in Method Da 18-48, Procedure, 1.
2. Pipet an aliquot from the flask containing 0.05–0.10 g of P_2O_5 into a 250 mL beaker. Neutralize with HNO_3 (sp. gr. 1.42) and then add 5 mL in excess for each 100 mL of solution. Boil vigorously for 15 min. Add NH_4OH (sp. gr. 0.90) until the solution is slightly alkaline to pH test paper (or litmus paper). Stir vigorously and add enough HNO_3 (sp. gr. 1.42) to just dissolve the precipitate.
3. Add about 15 g of dry NH_4NO_3 or a solution containing that quantity. Heat to about 60°C and add 70 mL of the molybdate solution for every 0.1 g of P_2O_5 present.
4. Heat to about 65°C for 1 hr and test for complete precipitation by the addition of 1–2 mL of molybdate solution. The sample solution must remain clear.
5. Filter through filter paper and wash with cold distilled water or preferably with the NH_4NO_3 solution. Discard the filtrate. Dissolve the precipitate on the filter paper with NH_4OH (1:1) and then wash with hot distilled water. Collect this filtrate in a beaker. The total volume of liquid should not exceed 100 mL.

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6. Neutralize with HCl (1:1), using pH paper, litmus paper, or brom thymol blue indicator. Cool and add slowly from a burette (at a rate of about 1 drop per sec) with vigorous stirring, 15 mL of the magnesium solution (Solutions, 3) for each 0.1 g of P_2O_5 present. Allow to stand for 15 min and then add 12 mL of NH_4OH (sp. gr. 0.90).
7. Allow to stand until the supernatant liquid is clear. Two hours is usually sufficient. Filter through a filter paper and wash the precipitate with dilute NH_4OH solution until the washings are free from chlorides (test with dilute $AgNO_3$ solution).
8. Ignite the filter paper and precipitate over a low flame to burn off the filter paper and then ignite in a muffle furnace at 900–1,000°C. Partially cool and place in a desiccator while still warm. Cool to room temperature in the desiccator and weigh the $Mg_2P_2O_7$. Repeat the ignition until constant weight is obtained.

CALCULATIONS

$$\text{Phosphorus pentoxide } (P_2O_5),\ \% = \frac{\text{mass of } Mg_2P_2O_7,\ g \times 63.774}{\text{mass of test portion, g}}$$

NOTES

Caution

Concentrated ammonium hydroxide vapors are extremely irritating to eyes and skin. The liquid can cause burns. Always wear protective clothing when handling ammonium hydroxide. A properly operating fume hood should be used.

Concentrated hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Concentrated nitric acid is a strong acid and will cause severe burns. Use an effective acid-resistant fume-removal device whenever heating the acid or performing reactions which liberate acid fumes. When diluting the acid, always add acid to water unless otherwise directed in a method. Keep the acid off skin and protect eyes when handling. If the acid comes in contact with skin or eyes, wash immediately with large amounts of water. Do not store this acid near organic materials. Mixing organic materials with nitric acid could result in an explosion.

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AOCS

AOCS Official Method Da 20b-57

Revised 2011

Phosphates

DEFINITION

This method determines all the phosphate in a sample as phosphorus pentoxide (P_2O_5) by conversion to the *ortho* form by acid hydrolysis and titration between pH 4.3 and pH 8.8. Two moles of NaOH are equivalent to 1 mole of P_2O_5.

SCOPE

Applicable to any species of alkali metal phosphates free from interfering ions (see Notes, 1). The method may be used for analysis of soap and synthetic detergent builders if the sample is properly prepared.

APPARATUS

1. pH meter—capable of performing titrations accurate to ± 0.1 pH units. The pH meter should be accurately standardized according to the manufacturer's instructions at pH 4.0 and pH 7.0.
2. Gas burners—preferably of the chimney or Argand type.
3. Muffle furnace—with suitable pyrometer and controls for maintaining a temperature up to 550°C.
4. Evaporation dish or large crucible—porcelain or silica.
5. Stirring motor.
6. Beaker—400 mL.

REAGENTS

1. Sodium hydroxide (NaOH) solution (see Notes, 2), 0.5 M or 1.0 M—carbonate-free and accurately standardized. See AOCS Specification H 12-52.
2. Concentrated sodium hydroxide (NaOH) solution—50% by weight, carbonate-free (see Notes, *Caution* and 2).
3. Hydrochloric acid (HCl)—reagent grade, concentrated, sp. gr. 1.19 (see Notes, *Caution*).
4. Mixed indicator solution, optional, (see Notes, 3)—
   (a) 32 mL methyl orange—0.05% in water.
   (b) 32 mL phenolphthalein—0.5% in 50% alcohol (see Reagents, 4, e).
   (c) 8 mL thymol blue—0.04% in water.
   (d) 4 mL methylene blue—0.10% in water.
   (e) 24 mL alcohol—95%, SDA Formula 30 or 3A. Mix the indicated volumes of Reagents 4, a-e. The individual components are stable indefinitely. The mixed indicator should be prepared fresh weekly. In practice, 3 mL of this mixed indicator are used in the final volume of approximately 250 mL of the solution to be titrated. The lower end point (approximately pH 4.3) is taken as the first change from gray to a definite green; the upper end point (approximately pH 8.8) is the change from pink to bright purple.

PREPARATION OF TEST PORTION

1. Commercial sodium or potassium phosphates need no special preparation except solution in distilled water. Weigh a portion of the well-mixed test sample to the nearest 0.001 g, transfer directly to a 400 mL beaker and dissolve in about 100 mL of distilled water. Neutralize to litmus paper with concentrated HCl and add 10 mL of HCl in excess. The optimum size of the test portion is given by the formula:

$$\text{Test portion size, g} = \frac{280 \times M}{\%\ P_2O_5 \text{ expected}}$$

   Where—
   M= molarity of NaOH to be used in the titration (see Procedure, 2)
2. Soap products may be analyzed by using the filtrate from the SiO_2 determination. Use care not to exceed the sample weight prescribed above. Alternatively the sample may be prepared as described in Sample Preparation, 3.
3. Built synthetic products may be analyzed by using the alcohol-insoluble portion, but the following procedure is more rapid and just as accurate. Weigh a sample, of size chosen by the formula in Sample Preparation, 1 (but do not exceed 10 g), to the nearest 0.001 g. Place the sample in a porcelain or silica evaporating dish or large crucible and ignite gently over a low gas burner until most of the volatile combustible matter is burned off. Transfer to a muffle furnace, operated at not over 550°C, for 10–15 min. The ignited residue need not be free from carbon and usually is of a grayish color. Cool and add cautiously 10 mL of concentrated HCl. Evaporate to dryness, take up in 50 mL distilled water and 10 mL concentrated HCl, and transfer to a 400 mL beaker.

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PROCEDURE

1. Each solution in a 400 mL beaker, prepared as described above, should have a volume of about 100 mL and contain an excess of at least 10 mL concentrated HCl. Cover with a watch glass and boil for a minimum of 30 min, and up to 60 min in the presence of phosphates of the glassy type. Cool to room temperature (20–30°C).
2. Dilute to 200 mL volume, place on the titration stand, and neutralize to pH 4.3. Most of the neutralization may be made with 50% NaOH solution, but the final pH adjustment should be made with the standard NaOH to be used in the titration (either 0.5 M or 1.0 M). Cool again, if necessary to maintain the temperature in the range of 20–25°C. Now titrate carefully (using either a pH meter or the mixed indicator), to the upper end point pH of 8.8, recording the volume of titrant used between the end points as "T."

CALCULATIONS

$$\% \text{ Total } P_2O_5 = \frac{T \times M \times 7.098}{\text{mass of test portion, g}}$$

Where—

T = volume of titrant, mL between pH 4.3 and pH 8.8 end points
M = molarity of NaOH solution

NOTES

Caution

Sodium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. Interferences—heavy metals, such as Fe, Al, Ca, Mg, etc., which will precipitate, either as insoluble phosphates or hydroxides, before the upper end point is reached, of course will interfere. Interference also occurs if borates, sulfites, carbonates, or other buffering materials are present. Carbonates and sulfites and much of the borate will be expelled during the boiling in the acid hydrolysis step. NH_4OH or other weak bases will interfere. The most common interference is from silicic acid. Experimentation and experience in analysis of spray-dried synthetics have shown that unless the ratio of SiO_2/P_2O_5 approaches or exceeds 0.2, the interference by silicates will be so slight that it may be neglected. Larger amounts must be dehydrated and removed by filtration during the preparation of the sample.
2. Sodium hydroxide solutions must be protected from carbon dioxide contamination.
3. The mixed indicator may be used for this titration, but with some small sacrifice of accuracy. If the samples have been prepared by the ignition method, they must be filtered and the paper washed thoroughly after the acid hydrolysis because particles of carbon will obscure the visual end point. The color changes can be checked by comparison with pH meter readings to acquire familiarity with the exact shade required. For greatest accuracy, titration with pH meter is recommended.

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AOCS Official Method Da 21-48

Reapproved 2009

Tetrasodium Pyrophosphate

DEFINITION

This method determines tetrasodium pyrophosphate.

SCOPE

Applicable to all soaps and soap products except those containing polyphosphates. Replaces Da 21-42.

APPARATUS

1. Beakers—400 mL.
2. Titration assembly—using a pH meter. The pH meter should be accurately standardized in the range in which it will be used, according to the manufacturer's instructions.
3. Filter paper—thoroughly washed with distilled water and then with 95% ethyl alcohol.

REAGENTS

1. Ethyl alcohol—95% SDA Formulas 30 and 3A are permitted. Add a few drops of phenolphthalein indicator (Reagents, 7). Boil to remove any dissolved carbon dioxide and neutralize with sodium hydroxide (NaOH) (Reagents, 6) to a faint pink color just before using (see Notes, *Caution*).
2. Hydrochloric acid (HCl), 0.5 M—accurately standardized. See AOCS Specification H 14-52 (see Notes, *Caution*).
3. Zinc sulfate solution—7%, prepared by dissolving 125 g of reagent grade $ZnSO_4 \cdot 7H_2O$ in distilled water and diluting to 1 L. Filter and adjust the pH to exactly 3.8 with 0.25 M H_2SO_4, using the pH meter.
4. Sodium pyrophosphate—purified as follows: place 50 g of sodium pyrophosphate ($Na_4P_2O_7$) in 100 mL of distilled water and heat until all is dissolved. Cool to room temperature, allow to settle, and remove all of the liquid by decantation or filtration. Repeat two more times. Finally, filter through filter paper, transfer the salt to a beaker, and dry to constant weight at about 400°C. Preserve the recrystallized salt in a desiccator for the standardization of 0.5 M NaOH solution (Reagents, 6).
5. Sodium hydroxide (NaOH), 0.5 M—standardized as follows: weigh accurately about 1 g of purified tetrasodium pyrophosphate into a 400 mL beaker. Add sufficient distilled water to cover the electrodes. When the salt is dissolved, proceed as directed in Procedure, 3 and 4. Calculate the g tetrasodium pyrophosphate equivalent to 1 mL of the standard 0.5 M NaOH solution.
6. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol.

PROCEDURE

1. Weigh accurately a test portion containing about 1 g of tetrasodium pyrophosphate into a 400 mL beaker. Add 200 mL of hot neutral alcohol and heat on a steam bath until all of the soap is dissolved.
2. Filter through the washed filter paper with the aid of a vacuum filtration flask and wash with hot 95% neutral alcohol until free from soap. Place a clean 400 mL beaker under the funnel and wash the alcohol-insoluble portion with hot distilled water until the washings are neutral to phenolphthalein indicator.
3. Cool and add sufficient distilled water so that the solution will just cover the electrodes of the pH meter. Add 0.5 M HCl very carefully with vigorous stirring until the pH is exactly 3.8.
4. Add 50 mL of the zinc sulfate solution and allow at least 5 min for the reaction to go to completion. This is indicated by the pH becoming constant. Titrate the liberated acid with 0.5 M NaOH, stirring vigorously, until a pH of 3.8 is reached again. This titration is a measure of the pyrophosphate content.

CALCULATIONS

$$\text{Tetrasodium pyrophosphate } (Na_4P_2O_7),\ \% = \frac{A \times B}{\text{mass of test portion, g}} \times 100$$

Where—

A = mL of titrant from Procedure, 4

B = g of $Na_4P_2O_7$ equivalent to 1 mL of 0.5 M NaOH solution (as determined in Reagents, 6)

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NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

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AOCS

AOCS Official Method Da 22-48

Revised 2011

Sulfates

DEFINITION

This method determines all of the water-soluble inorganic sulfates in the sample as sodium sulfate.

SCOPE

Applicable only to soaps and soap products free from sulfonated oils, synthetic detergents, and compounds containing sulfur in organic form. Replaces Da 22-42.

APPARATUS

1. Beakers—400 mL.
2. Ceramic fiber—which may be purchased from Porter-Warner, 303 Church Street, P. O. Box 10710, Rock Hill, SC 29731 USA, phone (803) 324–2964.
3. Gooch crucible—with a pad of ceramic fiber (see Procedure, 1).
4. Muffle furnace.
5. Steam bath/water bath.

REAGENTS

1. Hydrochloric acid (HCl)—reagent grade, concentrated, sp. gr. 1.19 (see Notes, *Caution*).
2. Hydrochloric acid (HCl)—approximately 0.5 M.
3. Barium chloride ($BaCl_2$)—10% solution in distilled water (see Notes, *Caution*).
4. Methyl orange indicator solution—0.1% in distilled water.

PROCEDURE

1. To prepare ceramic fiber, blend about 150 g of ceramic fiber with 250 mL distilled water in a Waring blender at high speed for 5 min. Store in plastic jar. Individual mats may be prepared by slowly pouring 25–50 mL of mixed slurry into the Gooch crucible (without vacuum) and allowing it to settle before turning on the vacuum.

   Alternate preparation—Stack U.S. #6 mesh sieve on top of #10 mesh sieve. Place a suitable amount of ceramic fiber on #6 mesh sieve and rub across sieve until all of the fiber has passed through the openings. Discard the small amount left on #6 sieve. Shake material on #10 mesh sieve for about 1 min, and discard fines passing through the sieve. Save and store the fiber remaining on the #10 mesh sieve. Any method of preparation is acceptable if it results in the making of a satisfactory mat in crucible. Discarding the fines will reduce the magnitude of the blank determination.
2. For the test portion, use the dry residue remaining after the removal of alcohol soluble matter as directed in AOCS Official Method Da 3-48, Procedure, 4.
3. Char over a burner and then ignite in a muffle furnace at 850–950°C until the combustible matter is destroyed and all carbon is removed.
4. Wash the residue into a 400 mL beaker with hot distilled water, using about 100 mL. Cool to room temperature and add 1 or 2 drops of methyl orange indicator. Neutralize with 0.5 M HCl to a red end point and then add 5 mL of concentrated HCl (sp. gr. 1.19) in excess.
5. Heat to boiling and evaporate to dryness and then heat for 30 min at 110–120°C. Take up the residue in 5 mL of concentrated HCl (sp. gr. 1.19), add 50 mL of distilled water and stir thoroughly.

   Note—If silicates and polyphosphates are both absent, Procedure, 5 may be omitted.
6. Filter and wash the residue thoroughly with hot distilled water, collecting the filtrate in a 400 mL beaker. The total volume of the combined filtrate and washings should be about 200 mL.
7. Bring to a boil and slowly add, with dropwise addition, 15-20 mL of $BaCl_2$ solution.
8. Boil until the precipitate of barium sulfate ($BaSO_4$) is well formed, or digest on a steam or water bath overnight. Then allow to stand at room temperature for a few hours (or overnight).
9. Filter through the tared Gooch crucible, wash thoroughly with hot distilled water, and ignite gently to constant weight. Cool to room temperature in a desiccator and weigh.

CALCULATIONS

$$\text{Sodium sulfate } (Na_2SO_4),\ \% = \frac{\text{mass of } BaSO_4,\ g \times 60.86}{\text{mass of test portion, g}}$$

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NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

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AOCS Official Method Da 23-56

Revised 2011

Free Glycerol Iodometric-Periodic Acid Method

DEFINITION

This method determines free glycerol in soap.

SCOPE

Applicable to the determination of free glycerol in soap. Replaces Da 23-48.

APPARATUS

1. Burette—50 mL, accurately calibrated.
2. Meniscus magnifier—suitable to permit reading the burette to 0.01 mL.
3. Flask—1 L volumetric with glass stopper.
4. Pipets—volumetric, 10, 25, 50 and 100 mL. The 25 and 50 mL must conform to National Institute of Testing tolerances and be accurately calibrated to deliver 25 and 50 mL.
5. Beakers—400 mL, with watch glasses to serve as covers.
6. Variable-speed electric stirrer—with glass stirrer.
7. Graduated cylinders—100 and 1000 mL.

REAGENTS

1. Periodic acid (H_5IO_6)—reagent grade, purchased from a chemical supplier (see Notes, *Caution*).
2. Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$)—reagent grade.
3. Potassium iodide (KI)—reagent grade.
4. Glacial acetic acid—reagent grade, 99.5% (see Notes, *Caution*).
5. Starch solution—prepared as noted in Solutions, 4; tested for sensitivity as follows: Place 5 mL of starch solution in 100 mL of water and add 0.05 mL of *freshly prepared* 0.1 M KI solution and one drop of a 50 ppm chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution to 1000 mL. The deep blue color produced must be discharged by 0.05 mL of 0.1 M sodium thiosulfate.
6. Chloroform—reagent grade (see Notes, *Caution* and Recommendations). Blank tests with periodic acid with and without chloroform must agree within 0.5 mL. If they do not, a fresh supply of chloroform must be obtained.
7. Potassium dichromate—reagent grade. The potassium dichromate is finely ground and dried to constant weight at 105–110°C before using (see Notes, *Caution*).
8. Hydrochloric acid (HCl)—reagent grade, concentrated, sp gr 1.19 (see Notes, *Caution*).

SOLUTIONS

1. Periodic acid solution—Dissolve 5.4 g of periodic acid in 100 mL of distilled water; then add 1900 mL of glacial acetic acid and mix thoroughly. Store the solution in a dark, glass-stoppered bottle or store in the dark in a clear, glass-stoppered bottle.

   Note—Only glass-stoppered bottles should be used. Do not use rubber or other stoppers under any circumstances.
2. Sodium thiosulfate solution—0.1 M, prepared by dissolving 24.8 g of sodium thiosulfate in distilled water and diluting to 1 L. The solution is standardized as follows: Pipet 25 mL of the standard dichromate solution (Solutions, 5) into a 400 mL beaker. Add 5 mL of concentrated HCl, 10 mL of KI solution (Solutions, 3), and rotate to mix (a magnetic stirrer and stirring bar may be used). Allow to stand for 5 min without mixing and then add 100 mL of distilled water. Titrate with the sodium thiosulfate solution, stirring continuously until the yellow color has *almost* disappeared. Add 1–2 mL of starch solution and continue the titration, adding the thiosulfate solution *slowly,* until the blue color just disappears. The strength of the thiosulfate solution is expressed in terms of molarity:

$$K_2Cr_2O_7 + 6KI + 14HCl \rightarrow 2CrCl_3 + 8KCl + 7H2O + 3I2$$

$$6Na_2S_2O_3 + 3I_2 \rightarrow 6NaI + 3Na_2S_4O_6$$

$$\text{Molarity of } Na_2S_2O_3 = \frac{\text{Molarity of } K_2Cr_2O_7 \times 25 \text{ mL} \times 6 \text{ moles } Na_2S_2O_3 \text{ per mole } K_2Cr_2O_7}{\text{volume of } Na_2S_2O_3}$$

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3. Potassium iodide (KI) solution—prepared by dissolving 150 g of KI in distilled water and diluting to 1 L. Protect this solution from the light.
4. Starch indicator solution—prepared by making a homogeneous paste of 10 g of soluble starch in cold distilled water. Add this paste to 1 L of rapidly boiling distilled water, stir rapidly for a few seconds, and cool. Salicylic acid (1.25 g/L) may be added to preserve the starch. The solution should be stored in a refrigerator (4–10°C) when not in use. Fresh indicator must be prepared when the end point of the titration fails to be sharp or if the starch solution fails the sensitivity test (see Reagents, 5).
5. Standard potassium dichromate solution— ~0.0167 M prepared by dissolving about 4.9 g, accurately measured, of finely ground and dried potassium dichromate in distilled water in a 1 L volumetric flask and making up to volume at 25°C. The molarity of the $K_2Cr_2O_7$ solution is calculated as follows:

$$\text{Molarity of } K_2Cr_2O_7 = \text{mass of } K_2Cr_2O_7, \text{ g} \div 294.2$$

PROCEDURE

1. Weigh approximately 10 g of test sample to the nearest 0.01 g.
2. Add 91 mL of chloroform (see Notes, *Caution*), measured from a burette to within ± 0.2 mL to a 1 L volumetric flask. Then add 25 mL of glacial acetic acid with a graduated cylinder (see Notes, 1).
3. Transfer the test portion quantitatively to the volumetric flask and add approximately 500 mL of distilled water. Stopper and shake until sample is dissolved. If the soap does not react readily, warm contents of the flask and shake (see Notes, 2). If warmed, cool to room temperature before proceeding.
4. Add distilled water to the mark, stopper, and mix thoroughly by inverting. Set aside until the aqueous and chloroform layers separate.
5. Pipet 50 mL of periodic acid reagent into a series of 400 mL beakers, and prepare two blanks by adding 100 mL of distilled water to each.
6. Pipet 100 mL of the aqueous layer obtained in Procedure, 4 into a 400 mL beaker containing 50 mL of the periodic acid reagent; shake gently to effect thorough mixing. Cover with a watch glass and allow to stand for 30 min (see Notes, 3).
   Note—If the aqueous solution contains suspended matter, filter before pipetting the sample for the test.
7. Add 20 mL of KI solution, mix by gently shaking, and allow to stand 1 min (but never more than 5 min) before titrating. Do not allow to stand in bright light or direct sunlight.
8. Dilute the sample to approximately 200 mL with distilled water and titrate with the standardized sodium thiosulfate solution. Continue the titration until the brown iodine color has *almost* disappeared. Add 2 mL of starch indicator solution and continue the titration until the blue iodine-starch complex color just disappears.
9. Read the burette to nearest 0.01 mL.
10. The blank determinations are handled in exactly the same way as the samples (see Procedure, 7 and 8).
11. If the titration of the sample (Procedure, 9) is less than 0.8 of the titration of the blank (Procedure, 10), then
    (a) Repeat the test using smaller portions (50, 25, 10, and 5 mL in Procedure, 6) until the titration of the sample is more than 0.8 of the blank, or
    (b) If 10 mL (or less) of the sample solution is necessary to bring the titration with the limit noted in Procedure, 11, (a), repeat the test starting at the beginning (Procedure, 6) with a sample of 2 g or less.

CALCULATIONS

1. Report the free glycerol to the nearest 0.1%.

$$\text{Free glycerol, \%} = \frac{(B - S) \times M \times 2.302}{W}$$

Where—
S = volume of titrant, mL of sample
B = volume of titrant, mL of blank
M = molarity of sodium thiosulfate solution
W= mass of test portion, g represented by aliquot (mL) taken for sample analysis in which,

$$W = \frac{\text{mass of test portion, g}^a \times \text{mL of sample}^b}{900}$$

[a]Procedure, 1; [b]Procedure, 6

PRECISION

Collaborative studies have determined the variability of the method as applied to soaps containing approximately 0.5% glycerol as follows—

1. The between-laboratory standard deviation was 0.01.
2. Separate determinations in different laboratories should not differ by more than approximately 0.03.
3. The averages of duplicate determinations in different laboratories should not differ by more than approximately 0.02.

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NOTES

Caution

Periodic acid is an oxidizing agent and is dangerous in contact with organic materials. It is a strong irritant. It decomposes at 130°C. Do not use cork or rubber stoppers on storage bottles containing periodic acid.

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature, forming phosgene gas when heated to decomposition temperatures. Can react explosively with aluminum, lithium, magnesium, sodium, potassium, disilane, N_2O_4, and a mixture of sodium hydroxide + methanol. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Acetic acid in the pure state is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air is 10 ppm.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcino genicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

RECOMMENDATIONS

1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

NUMBERED NOTES

1. If the soap sample contains more than 10% moisture, adjust the amount of chloroform added so the total volume of fatty acid and chloroform will equal 100 ± 1 mL.
2. If the aqueous phase is alkaline due to large amounts of builder in the soap, add concentrated sulfuric acid (see Notes, Caution), sp gr 1.84, in 0.5 mL increments until the solution is definitely acid to litmus test paper.
3. Samples may be allowed to stand up to 1.5 hr at room temperature before titrating, but never longer than 1.5 hr. Do not allow to stand in bright light or direct sunlight.

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AOCS

AOCS Official Method Da 24-48

Reapproved 2009

Sugars, Munson and Walker Method

DEFINITION

This method determines the total sugars in the sample, but does not distinguish between reducing and non-reducing sugars.

SCOPE

Applicable to all soaps and soap products. Replaces Da 24-42.

APPARATUS

1. Beakers—400 and 600 mL.
2. Separatory funnels—500 mL.
3. Volumetric flasks—500 mL.
4. Pipet—50 mL.
5. Ceramic fiber—which may be purchased from Porter-Warner, 303 Church Street, P. O. Box 10710, Rock Hill, SC 29731 USA, phone (803) 324–2964.
6. Gooch crucible—with a 5–6 mm pad of ceramic fiber (see Procedure, 1).
7. Vacuum pump or aspirator.

REAGENTS

1. Sulfuric acid (H_2SO_4)—1:4, v/v with distilled water (see Notes, *Caution*).
2. Sodium hydroxide (NaOH)—reagent grade (see Notes, *Caution*).
3. Modified Fehling's solution, prepared as follows—
   (a) Dissolve 34.639 g of copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) in distilled water, dilute to 500 mL and filter.
   (b) Dissolve 173 g of sodium potassium tartrate tetrahydrate ($NaKC_4H_4O_6 \cdot 4H_2O$) and 50 g of sodium hydroxide (NaOH) (see Notes, *Caution*) in distilled water. Dilute to 500 mL, allow to stand for 2 days, and filter. Mix equal volumes of (a) and (b) just prior to use.
4. Petroleum ether—reagent grade (see Notes, *Caution*).
5. Ethyl alcohol—95%. SDA Formulas 30 and 3A are permitted (see Notes, *Caution*).

PROCEDURE

1. To prepare ceramic fiber, blend about 150 g of ceramic fiber with 250 mL distilled water in a Waring blender at high speed for 5 min. Store in plastic jar. Individual mats may be prepared by slowly pouring 25–50 mL of mixed slurry into the Gooch crucible (without vacuum) and allowing it to settle before turning on the vacuum.

   Alternate preparation—Stack U.S. #6 mesh sieve on top of #10 mesh sieve. Place a suitable amount of ceramic fiber on #6 mesh sieve and rub across sieve until all of the fiber has passed through the openings. Discard the small amount left on #6 sieve. Shake material on #10 mesh sieve for about 1 min, and discard fines passing through the sieve. Save and store the fiber remaining on the #10 mesh sieve. Any method of preparation is acceptable if it results in the making of a satisfactory mat in crucible. Discarding the fines will reduce the magnitude of the blank determination. After preparing the pad, wash the pad thoroughly with distilled water to remove fine particles of fiber and follow with 10 mL of 95% ethyl alcohol and then 10 mL of petroleum ether (see Notes, *Caution*). After all of the petroleum has evaporated, dry in an air oven at 105 ± 2°C for 30 min and cool in a desiccator to room temperature and weigh.
2. Accurately weigh 10 g of test sample into a 600 mL beaker and add 200 mL of distilled water. Add 25 mL of dilute (1:4) H_2SO_4 and boil gently for at least 20 min to invert all of any sucrose present. Cool until the fatty acids are solid, remove and wash the cake thoroughly, saving the washings.

   Note—If starch is present, remove the alcohol-insoluble and water-insoluble matter as directed in AOCS Official Methods Da 3-48 and Da 6-48. Use the alcohol and water solutions for the determination of sugars. Evaporate the alcohol with a rotary evaporator. Combine the alcohol-soluble matter with the water solution, evaporate to a volume of about 200 mL, add 25 mL of dilute (1:4) H_2SO_4, and proceed to invert the sucrose as directed in step 1.
3. Combine the washings and acid liquor in 500 mL separatory funnel and wash once with 25 mL of petroleum ether (see Notes, *Caution*) to remove all of the fat. Allow the phases to separate, draw off all of the lower aqueous phase into a 400 mL beaker, and neutralize with a dilute sodium hydroxide solution. Discard the petroleum ether layer.
4. Transfer into a 500 mL volumetric flask, filtering if any insoluble matter is present, wash the filter paper thoroughly with distilled water and then make to volume. Mix thoroughly and pipet 50 mL into a 400 mL beaker. This should contain no more than 0.25 g of reducing sugars; otherwise, reduce the size of the aliquot and add sufficient distilled water to bring the total volume to 50 mL. Add 50 mL of mixed Fehling's solution.

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5. Cover the beaker with a watch glass and heat, either with an electric hot plate or gas burner, so that boiling begins within 4 min. Continue boiling for exactly 2 min.
6. Filter immediately through the prepared Gooch crucible with the aid of a vacuum filtration apparatus. Wash the beaker thoroughly to transfer all of the cupric oxide (Cu_2O) to the crucible and then wash the residue several times with distilled water at 60°C. Finally, wash with 10 mL of alcohol and 10 mL of petroleum ether.
7. Dry the crucible and contents in an air oven at 105 ± 2°C for 30 min. Cool to room temperature in a desiccator and weigh.

Note—Make certain that all of the petroleum ether has evaporated before placing the sample in the oven.

CALCULATIONS

1. Refer to standard Munson and Walker Tables (see Reference, 1) to find the milligrams of invert sugar corresponding to the weight of Cu_2O found.
2. Invert sugar, $\% = \dfrac{\text{mg of invert sugar} \times 0.1}{\text{mass of test portion in aliquot, g}^a}$
3. Sucrose, % = % invert sugar × 0.95

[a]from Procedures, 4

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

Sodium hydroxide, like all alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide, always add pellets to water and not the reverse. This alkali is extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

REFERENCE

1. *Official Methods of Analysis of the Association of Official Analytical Chemists,* 43.012 (10th edn.).

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AOCS

AOCS Official Method Da 25-48

Reapproved 2009

Starch, Munson, and Walker Method

DEFINITION

This method determines the starch in the sample.

SCOPE

Applicable to all soaps and soap products. Replaces Da 25-42.

APPARATUS

1. Beakers—400 mL.
2. Volumetric flasks—250 mL.
3. Erlenmeyer flasks—500 mL.
4. Reflux condenser—water-cooled.
5. pH meter.

REAGENTS

1. Hydrochloric acid (HCl)—sp. gr. 1.125, prepared by diluting concentrated HCl with distilled water to a sp. gr. of 1.125 at 15°C (see Notes, Caution).
2. Sodium hydroxide (NaOH) solution—approximately 0.5 M.

PROCEDURE

1. Weigh a test portion such that it will contain not more than 1 g of starch. Proceed as directed in AOCS Official Method Da 6-48 to remove the water-soluble matter, using water at 0–10°C to wash the alcohol-insoluble matter.

   Note—If the sample contains starch and sugars, remove the alcohol-soluble matter as directed in AOCS Official Method Da 3-48, and use the alcohol-insoluble matter for the determination of starch. Continue as directed in Procedure, 1 to remove the water-soluble matter.
2. Transfer the insoluble matter on the filter paper without drying to a 500 mL Erlenmeyer flask. Attach a reflux condenser, add 20 mL of dilute HCl (sp. gr. 1.125), 200 mL of distilled water, and boil for 2.5 hr.
3. Cool and add NaOH solution until the solution is almost neutral.

   Note—The solution must not become alkaline at any time. Transfer the solution to a 250 mL volumetric flask and make to volume at room temperature (20–25°C) with distilled water. Mix well.
4. Filter, discard the first 10 mL through the paper, and pipet 50 mL of the filtrate into a 400 mL beaker.
5. Determine the reducing sugars as directed in AOCS Official Method Da 24-48, beginning in Procedure, 4, with "This should contain not less than . . ."

CALCULATIONS

1. Refer to standard Munson and Walker Tables (see References, 1) to find the milligrams of dextrose (d-glucose) corresponding to the weight of Cu_2O found.
2. $$\text{Dextrose, \%} = \frac{\text{mg of dextrose} \times 0.1}{\text{mass of test portion in aliquot, g}}$$
3. Starch, % = % dextrose × 0.93

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

REFERENCE

1. *Official Methods of Analysis of the Association of Official Analytical Chemists,* 43.012 (10th ed.).

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AOCS

AOCS Official Method Da 26-42

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Volatile Hydrocarbons

DEFINITION

This method determines hydrocarbons which are volatile with steam under the conditions of the test.

SCOPE

Applicable to all soaps and soap products.

APPARATUS

1. Steam distillation apparatus constructed and assembled as shown in Figure 1, and consisting of the following—
   (a) Steam trap (Fig. 1, —) a 1 L, round bottom, ring-neck flask, equipped with a siphon tube to drain from the bottom of the flask and provided with a means of regulating the steam flow into the flask. The inlet for the steam into the evolution and scrubber flasks should extend nearly to the bottom of the flasks and should be bent at right angles.
   (b) Sample flask (Fig. 1, b—) a 1 L, round bottom, ring-neck flask. In case large samples are desirable, the size of this flask may be increased.
   (c) Caustic scrubber flask (Fig. 1,—) a steam-jacketed metal flask is preferred, but a 1 L Florence flask provided with a steam coil of 3.2 mm (0.125 in.) copper tubing around the upper half may be used. If a glass flask is used, it should be provided with a safety bucket below and it should be renewed frequently, because the strong caustic weakens the glass rather rapidly. This flask should be connected to the condenser by a Kjeldahl trap (Fig. 1, e), or similar safety device.
   (d) Condenser (Fig. 1, f—) a spiral condenser 350 mm (12 in.) long and of sufficient bore to prevent stop page by the condensate. The cooling water must be 15°C or colder. Ice water may be necessary for low boiling hydrocarbons.
   (e) Measuring burette (Fig. 1, h—) a 10 mL burette calibrated to 0.1 mL and connected to a bulb (Fig. 1, i), of about 100 mL capacity at the lower end. An ordinary 10 mL Mohr type burette may be used, attached preferably by a direct seal, otherwise by rubber tubing to a bulb of specified capacity. An ordinary burette funnel may be placed in the top of the burette in place of the specially flared out top shown in Figure 1.
   (f) Automatic overflow (Fig. 1, i,—) any convenient arrangement such as an inverted wide-mouth bottle fitted with a two-hole stopper. Glass tubing (about 5 mm i.d. and 100-150 mm long, or other convenient size) is inserted through each hole of the stopper. One glass tube is connected with rubber tubing to the bulb (Fig. 1, i). The other serves as an overflow and is arranged to discharge to a drain.
   (g) Stoppers—rubber stoppers must be of good grade and thoroughly cleaned and free from any surface sulfur. They are placed in position and subjected to steam distillation for several hours before use with a sample. All stoppers must fit tightly and be held in place with wire.
   (h) Adapter siphon (Fig. 1, g).
2. A source of dry, oil-free steam.

   Note—Insulating the flasks and tubing to reduce condensation aids control of distillation. Do not use rubber tubing connections between the flask (Fig. 1, a) and the condenser (Fig. 1, f).
3. Specific gravity tube or bottle, sized to accommodate the volume of hydrocarbon removed. A small Nicol or Sprengle type tube made of 3 mm glass tubing is convenient.

ASSEMBLY OF APPARATUS

1. Place 150 mL of NaOH solution and about 5–10 g of NaOH pellets in the caustic scrubber.
2. Rinse the burette and condenser with acetone (see Notes, *Caution*). Attach the automatic overflow (Fig. 1, J) to the bottom of the burette with rubber tubing. Fill the burette with water and adjust so that the water just overflows from the overflow when the level in the burette is near the top of the graduated scale of the 305 mm burette. All connections must be tight and all air bubbles removed from within the tubing.
3. Place the condenser in position so that the lower end extends directly into the upper end of the burette just above the water level or into an adapter siphon (Fig. 1, g).

REAGENTS

1. Sodium hydroxide (NaOH) solution—sp. gr. 1.47 (see Notes, *Caution*).
2. Sodium hydroxide pellets—reagent grade.
3. Sulfuric acid H_2SO_4—1:3, v/v with distilled water (see Notes, *Caution*).

PROCEDURE

1. Weigh 100 ± 0.5 g of the test sample (cut into cubes with about 10 mm edges), or 50 ± 0.3 g of soap powder and transfer to the sample flask (see Notes, 1).

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2. Place the flask in position with 100 mL of dilute (1:3) H_2SO_4 in the dropping funnel (Fig. 1, c). Connect and pass a small amount of steam through to wash the flasks and condensers.
3. Add the acid to the sample slowly to avoid excessive foaming. Turn on the steam cautiously while adding the acid, adjusting the pressure of the steam by a bleeder valve so that just enough steam flows to prevent any liquid from backing into the steam trap flask.
4. When all the acid has been added, turn on enough steam to cause brisk distillation, taking care that no liquid is carried over from the evolution and scrubber flasks and that the condenser water does not become warm.
5. Continue the distillation until there is no increase in the volume of the upper layer for 45 min, or until no droplets can be seen in the condensate.
6. When distillation is complete, shut off and drain the condenser water and allow the steam to heat up the condenser to drive out the last traces of volatile hydrocarbons. Shut off the steam as soon as vapor begins to issue from the lower end of the condenser. Immediately open the stopcock of the dropping funnel to prevent caustic from being drawn into the sample flask.
7. Stopper the burette and allow its contents to come to room temperature or bring it to a definite temperature by immersing the burette in a water bath held at 25°C for 1–2 hr.
8. Read the volume of the upper layer to the nearest 0.01 mL.

CALCULATIONS

$$\text{Volatile hydrocarbons, \%} = \frac{\text{volume of hydrocarbon, mL} \times \text{sp. gr.}}{\text{mass of test portion, g}} \times 100$$

NOTES

Caution

Sodium hydroxide, like all strong alkalies, can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide, always add pellets to water and not the reverse. Strong alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

Acetone is highly flammable. It forms explosive peroxides with oxidizing agents. Use an effective fume-removal device. Do not mix with chloroform.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

NUMBERED NOTES

1. In some cases, the volatile hydrocarbon content may be so low that a test portion larger than 50–100 g is necessary. The size of sample flask may need to be increased if larger samples are used. If the sample size is increased, use correspondingly larger portions of water and acid in the sample flask.

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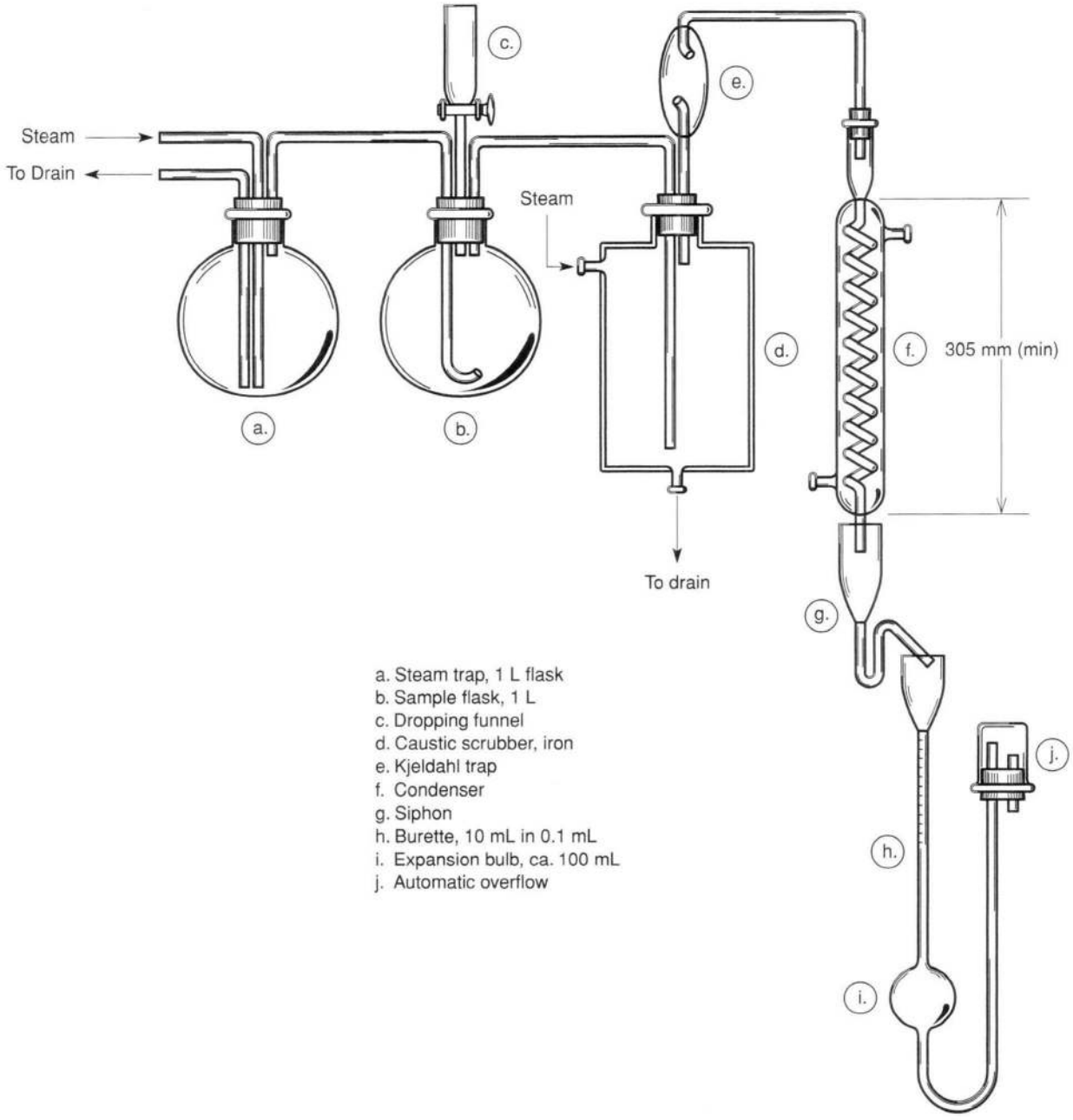

Figure 1. Volatile hydrocarbon apparatus.

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Combined Sodium and Potassium Oxides

DEFINITION

This method determines the potassium in the sample as potassium oxide. The sodium is determined by subtracting the Na_2O equivalent to potassium from the total alkali calculated as Na_2O.

SCOPE

Applicable to all soaps and soap products.

APPARATUS

1. Platinum evaporating dish—50 mL.
2. Volumetric flask—100 mL and 10 mL.
3. Beaker—100 mL.
4. Pipet—10 mL.
5. Filter paper—quantitative grade.
6. Litmus paper or pH paper.
7. Ceramic fiber—which may be purchased from Porter-Warner, 303 Church Street, P. O. Box 10710, Rock Hill, SC 29731 USA, phone (803) 324-2964.
8. Gooch crucible—with a 5-6 mm pad of ceramic fiber, prepared and brought to constant weight (Procedure, 1).
9. Muffle furnace.
10. Steam bath.

REAGENTS

1. Hydrochloric acid (HCl)—concentrated, sp. gr. 1.19 (see Notes, *Caution*).
2. Hydrochloric acid (HCl)—1:1, v/v concentrated HCl with distilled water (see Notes, *Caution*).
3. Ethyl alcohol—80%, prepared by diluting 84 mL of 95% ethyl alcohol with 16 mL of distilled water. SDA Formulas 30 and 3A are permitted (see Notes, *Caution*).
4. Ammonium chloride (NH_4Cl) solution—prepared by dissolving 100 g of NH_4Cl in 500 mL of distilled water. Add 5-10 g of pulverized K_2PtCl_6 and shake at intervals for 6-8 hr. Allow the mixture to settle overnight and filter. The residue may be used for the preparation of an NH_4Cl solution.
5. Platinum solution—prepare a solution containing the equivalent of 1 g of metallic platinum in each 10 mL of solution. (Dissolve 2.1 g H_2PtCl_6 in distilled water to yield 10 mL of solution.) For materials containing less than 15% of K_2O, a solution containing 0.2 g of metallic platinum (0.42 g of H_2PtCl_6) in each 10 mL of solution is recommended. (Dissolve 0.42 g H_2PtCl_6 in distilled water to yield 10 mL of solution.)
6. 10% silver nitrate solution—dissolve 1 g $AgNO_2$ in 9 mL of distilled water.

PROCEDURE

1. To prepare ceramic fiber, blend about 150 g of ceramic fiber with 250 mL distilled water in a Waring blender at high speed for 5 min. Store in plastic jar. Individual mats may be prepared by slowly pouring 25-50 mL of mixed slurry into the Gooch crucible (without vacuum) and allowing it to settle before turning on the vacuum.

   Alternate preparation—stack U.S. #6 mesh sieve on top of #10 mesh sieve. Place a suitable amount of ceramic fiber on #6 mesh sieve and rub across sieve until all of the fiber has passed through the openings. Discard the small amount left on #6 sieve. Shake material on #10 mesh sieve for about 1 min, and discard fibers passing through the sieve. Save and store the fiber remaining on the #10 mesh sieve. Any method of preparation is acceptable if it results in the making of a satisfactory mat in the crucible. Discarding the fibers will reduce the magnitude of the blank determination. After preparing the pad, wash the pad thoroughly with distilled water to remove fine particles of fiber and follow with 20 mL of 80% ethyl alcohol. Dry to constant weight at 105 ± 2°C before using.
2. Weigh 10 ± 0.01 g of the test sample into an evaporating dish and heat over a burner or in a muffle furnace below a red heat (350-450°C) until the mass is well-carbonized. Add hot distilled water and filter.
3. Wash the paper with four to five portions of 5-10 mL each of hot distilled water. Collect the filtrate in a 250 mL beaker. Return the paper and contents to the evaporating dish and continue heating as before until all carbonaceous matter is burned off.

   Note—Excessive heating must be avoided. Removal of most of the alkali by thorough washing with hot water, as directed above, will assist in preventing overheating.
4. Add a few drops of 1:1 HCl and wash with hot distilled water through a filter paper, catching the washings in the beaker containing the filtrate from Procedure, 3. If the filtrate is not already acid to litmus paper, make it acid with a few drops of

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1:1, v/v HCl. Evaporate to a volume of approximately 50 mL and quantitatively transfer to a 100 mL volumetric flask. Cool to room temperature and make to volume with distilled water. Mix thoroughly (see Notes, 1).

5. Pipet 10 mL into a 100 mL beaker and add a few drops of concentrated HCl. Add 10 mL of the platinum solution and evaporate in a water bath or steam bath to the consistency of a thick paste which will become solid on cooling to room temperature.

   Note—Avoid exposure to ammonia fumes during heating.

6. Add about 5 mL of 80% alcohol and 0.6 mL of concentrated HCl. Decant through the tared Gooch crucible and then transfer the precipitate to the crucible. Wash thoroughly with 80% alcohol. Continue washing until the filtrate is colorless.
7. Wash the residue five or six times with 25 mL portions of the NH_4Cl solution and then wash again thoroughly with the 80% ethyl alcohol solution until the washings give no positive test for chloride ions with 10% silver nitrate ($AgNO_3$). Dry in an air oven for 30 min at 105 ± 2°C. Cool to room temperature in a desiccator and weigh.

CALCULATIONS

1. Potassium oxide, K_2O, % = $\dfrac{\text{mass of } K_2PtCl_6\text{, g} \times 19.38}{\text{mass of test portion, g}}$
2. Sodium oxide, Na_2O, % = A – (% K_2× 0.6582)
   Where—
   A = % combined alkali as Na_2, as determined in AOCS Official Method Da 8-48

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

NUMBERED NOTES

1. If silicates are present, evaporate the acidified solution to dryness and then dry in an air oven at 110-120°C for 30 min. Cool and add 5 mL concentrated HCl and 50 mL of distilled water. Filter into a 100 mL volumetric flask, wash filter paper thoroughly, and then add distilled water to the 100-mL mark.

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AOCS

AOCS Official Method Da 28-39

Reapproved 2009

Screen Test

DEFINITION

This method determines the relative degree of fineness of the sample.

SCOPE

Applicable to soap powders.

APPARATUS

1. U.S. Standard (or Tyler equivalent) sieves no. 12, 40 and 100—8 in. dia (20.3 cm), with cover and bottom pan. Additional sieves of different sizes may be used if specified or desired.
2. Rubber cushion—rubber stopper or thick pieces of flat rubber.
3. Camel hair brush.
4. Shaking machine (optional)—Rotap, or equivalent.

PROCEDURE USING HAND SHAKING

1. Nest the sieves in order with the no. 12 on top. Sieves must be clean and *thoroughly* dry.
2. Weigh a 100 ± 0.01 g of well-mixed test portion (without previous drying) and transfer into the top screen.
3. Shake the sieves simultaneously and occasionally tap the bottom sieve on the rubber cushion. When the portion passing through the bottom sieve appears to be negligible (less than 0.1 g per minute of shaking), remove the top sieve and shake this sieve separately over a clean dark pan to be sure that no more of the sample will pass through.
4. Transfer the residue remaining on each sieve to a convenient container with the camel hair brush and weigh each separately. Record the individual weights. Calculate as directed below.

PROCEDURE USING SHAKING MACHINE (OPTIONAL)

1. The procedure is as described above except that the sieves are nested with the cover and bottom pan attached and then shaken in the machine for 10 min.
2. Test each portion as noted in Procedure, 3. Collect and weigh as directed in Procedure, 4 and 5. Calculate as directed under below.

CALCULATIONS

Based on 100 g test portion—

% retained on No. 12 sieve = mass retained on no. 12 sieve, g

% retained on No. 40 sieve = mass retained on no. 12 and 40 sieve, g

% retained on No. 100 sieve = mass retained on no. 12, 40 and 100 sieve, g

NOTE

1. The sum of the percentages remaining on each sieve plus the percentage passing the no. 100 sieve should equal 100.
2. Alternately, the results may be expressed as four fractions. Each fraction would be expressed in terms of the sieve they passed (+) and the sieve they did not pass (–).

   For example, the material collected on sieve no. 40 is material that passed no. 12 but did not pass no. 40. Call this fraction the "+12, -40 fraction."

   Therefore, the four fractions collected would be:

   (A) –12
   (B) +12, –40
   (C) +40, –100
   (D) +100

   Finally, A + B + C + D = 100%.

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AOCS

AOCS Official Method Da 31-58

Reapproved 2009

Copper

DEFINITION

This method determines trace quantities of copper in soap and soap products.

SCOPE

Applicable to soap and soap products.

APPARATUS

1. Spectrophotometer capable of making the required readings. Follow the manufacturer's instructions to set up, operate, calibrate, and maintain the instrument. A photoelectric colorimeter may be used, provided it is equipped with a proper interference filter.
2. Spectrophotometer cuvettes—1 cm, for use at 440 nm.
3. Beakers—100 and 50 mL.
4. Separatory funnels—with glass stoppers, 125 mL.
5. Funnels—25 mm dia.
6. Mixing cylinders—with glass stoppers, 10 mL, or 10 mL volumetric flasks with glass stoppers.
7. Volumetric flasks—with glass stoppers, 500 mL.
8. Pipets—volumetric, 5 mL.
9. Burette—10 mL.
10. Watch glasses—to fit beakers (see Apparatus, 3).

REAGENTS

Note—Laboratory distilled or deionized water may be used, provided it gives a low blank (absorbance <0.030). If this criteria cannot be met, the water should be distilled in borosilicate glass and stored in borosilicate glass containers with glass stoppers.

1. Electrolytic copper—sheet or light turnings, reagent grade or equivalent.
2. Nitric acid (HNO_3)—1:1, v/v with distilled water (see Notes, *Caution*).
3. Citric acid solution (30%)—prepared by dissolving 30 g of reagent grade citric acid in 100 mL of distilled water.
4. Hydrochloric acid (HCl)—reagent grade, concentrated, sp. gr. 1.19 (see Notes, *Caution*).
5. Ammonium hydroxide (NH_4OH)—reagent grade, concentrated, sp. gr. 0.90 (see Notes, *Caution*).
6. Ethyl alcohol solution—3:1, prepared by mixing 750 mL of 95% ethyl alcohol with 250 mL of distilled water (see Notes, *Caution*).
7. Absorbent cotton.
8. Carbon tetrachloride—reagent grade (see Notes, *Caution*).
9. Sodium diethyldithiocarbamate solution—0.2%, prepared by dissolving 1 g sodium diethyldithiocarbamate in 500 mL distilled water. Extract the solution once with 200 mL of carbon tetrachloride and discard the carbon tetrachloride extract. The solution should be prepared fresh weekly and stored at 4-10°C.
10. Phenol red indicator solution—prepared by dissolving 0.10 g of powered phenol red in 20 mL of 95% ethyl alcohol and 80 mL of distilled water.
11. Stopcock lubricant.

PROCEDURE

1. Preparation of the standard curve—
   (a) Weigh 0.2 ± 0.0001 g of clean electrolytic copper into a 50 mL beaker. Add 10 mL of 1:1 HNO_3 and cover the beaker with a watch glass. After cessation of reaction, add about 20 mL of water and transfer the solution to a 500 mL volumetric flask. Wash the beaker five times with 200 mL portions of water, adding the washings to the volumetric flask. Bring to volume with distilled water at room temperature and mix the solution by inverting the flask several times. This stock solution contains 0.4 mg of copper per mL. Prepare a working standard solution by pipetting 10 mL of the stock solution into a 500 mL volumetric flask and bringing to volume with water at room temperature. Invert the flask several times to mix the solution. This solution contains 8 µg (0.008 mg) copper per mL.
   (b) Add 50 mL of 3:1 ethyl alcohol to each of five 125 mL separatory funnels and add to each funnel, respectively, 0 (blank), 1, 3, 6 and 10 mL of the working standard copper solution.
   (c) Add 2 mL of 30% citric acid solution to each funnel and extract the contents with two successive 25 mL volumes of carbon tetrachloride. Shake for about 30 sec each time and discard the extracts.
   (d) Add 2 drops of phenol red indicator solution to the extracted solutions and add concentrated NH_4OH dropwise until the red end point is reached. Add 2 mL of the 0.2% sodium diethyldithiocarbamate solution.

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(e) Shake the mixture with 3 mL (measured accurately with a pipet) of carbon tetrachloride for *at least* 30 sec. Drain the lower layer through a wisp of absorbent cotton, contained in a 25 mm funnel, into a 10 mL glass-stoppered mixing cylinder (or 10 mL volumetric flasks). The cotton is rolled to give a compact thin plug about 2-3 cm long and is inserted into the stem of the funnel so that only a small portion projects into the conical part of the funnel. Shake the contents of the separatory funnel with two more 3 mL portions of carbon tetrachloride, again collecting the extracts in the mixing cylinder. Wash the cotton with carbon tetrachloride, removing the funnel at the instant that the dilution to the 10 mL mark occurs.

(f) Thoroughly mix the contents of the mixing cylinder. If the solution is perfectly clear, add directly to a 1 cm cuvette, otherwise filter through a cotton plug. Cover the cuvette and immediately measure the absorbance vs. carbon tetrachloride at 440 nm in the spectrophotometer.

(g) If the absorbance of the blank vs. carbon tetrachloride is >0.030, the laboratory water should be redistilled as noted in Reagents.

(h) Subtract the absorbance of the blank from the absorbance of the standard solutions and plot on linear graph paper, the corrected absorbance of the standard (y-axis) vs. the µg of copper in the standard solutions (x-axis).

2. Determination of copper in the sample—

(a) Weigh 2.00 ± 0.10 g of test sample and transfer to a 100 mL beaker.

(b) Add 40 mL of 3:1 ethyl alcohol solution and 1 mL of concentrated HCl to the sample. Heat with stirring to dissolve the sample. Pour while hot into a 125 mL separatory funnel and wash the beaker with a small volume (10–15 mL) of 3:1 alcohol solution. Cool in tap water and add 25 mL of carbon tetrachloride. Add 2 mL of 30% citric acid solution and shake the stoppered funnel for 30 sec. Discard the lower carbon tetrachloride layer. Extract with a second 25 mL portion of carbon tetrachloride and discard the lower layer.

(c) From this point on, follow the procedure for the preparation of the standard curve (see Procedure 1, d-g).

CALCULATIONS

$$\text{Copper (Cu) in } \mu g/g = \frac{(S - B)}{2}$$

Where—

S = mass, µg of copper equivalent to the absorbance of the test portion obtained from the standard curve
B = mass, µg of copper equivalent to the absorbance of the reagent blank obtained from the standard curve

PRECISION

1. 3 ppm level—

(a) Two single determinations performed in one laboratory should not differ by more than 0.03 ppm.

(b) Single determinations performed in two different laboratories should not differ by more than 0.4 ppm.

2. 10 ppm level—

(a) Two single determinations performed in one laboratory should not differ by more than 0.4 ppm.

(b) Single determinations performed in two different laboratories should not differ by more than 0.5 ppm.

NOTES

Caution

For nitric acid, use an effective acid-resistant fume-removal device whenever heating acids or performing reactions which liberate acid fumes. When diluting acids, always add acid to water unless otherwise directed in a method. Keep acids off skin and protect eyes when working with acids. If acids come in contact with skin or eyes, wash immediately with large amounts of water. Do not store oxidizing acids (perchloric, nitric, or sulfuric) near organic materials. Mixing organic materials with these acids, particularly perchloric, could result in an explosion.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

Carbon tetrachloride is a known carcinogen. It is toxic by ingestion, inhalation, and skin absorption, causing irritation, headaches, and nausea on mild exposure. More severe exposure can lead to liver and kidney damage. It is a narcotic. It should not be used to extinguish fires. It decomposes to phosgene gas at high temperature. The TLV is 5 ppm in air. A fume hood should be used at all times when using carbon tetrachloride.

1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent the official approval by the AOCS Uniform Methods Committee.

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AOCS

AOCS Official Method Db 1-48

Reapproved 2009

Moisture and Volatile Matter, Air Oven Method

DEFINITION

This method determines the moisture and any materials volatile under the conditions of the test (see Notes, 1).

SCOPE

This method is applicable to soap and soap products containing synthetic detergents. It is not applicable to products containing appreciable amounts of sodium silicate, over 1% glycerol and soaps produced from oils, such as linseed oil, that tend to oxidize readily. The distillation method must be used for these products. Replaces Da 2a-42.

APPARATUS

1. Air oven—maintained at 105 ± 2°C, AOCS Specification H 3-45.
2. Porcelain or glass moisture dishes—60–80 mm dia and 20–40 mm depth.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.

PROCEDURE

1. Weigh 5 ± 0.01 g of sample into a tared moisture dish which has been previously dried for 1 hr at 105 ± 2°C and cooled to room temperature in a desiccator.
2. Dry the sample and moisture dish to constant weight in an air oven at 105 ± 2°C, cool to room temperature in a desiccator, and weigh.
3. Constant weight is attained when successive 1 hr heating periods show a loss (or gain) of not more than 0.1%.

CALCULATIONS

$$\text{Moisture and volatile matter, \%} = \frac{\text{loss in mass, g}}{\text{test portion mass, g}} \times 100$$

NOTES

1. If sodium bicarbonate is present, it will undergo partial decomposition under the conditions of this test and yield water and CO_2.

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AOCS

AOCS Official Method Db 2-48

Reapproved 2009

Alcohol-Soluble and Alcohol-Insoluble Matter

DEFINITION

This method determines the alcohol-soluble and alcohol-insoluble matter in the sample.

1. The alcohol-soluble matter will contain all of the anhydrous (real) soap, and some inorganic salts. Most, if not all, of the sodium chloride will be included. This method is satisfactory for the approximate determination of soap (when buyer and seller agree) with products which contain a high percentage of alcohol-insoluble matter such as cleansers, soap powders, scouring compounds, and pastes.
2. The alcohol-insoluble matter will contain most of the alkali salts such as carbonates, borates, silicates, and phosphates as well as sulfates and starch. This method may be used for the approximate determination of these constituents. These salts are not entirely insoluble in alcohol so for the most accurate results, use separate portions of the sample and follow the specific method for each constituent.

SCOPE

Applicable to all soap and soap products containing synthetic detergents. Replaces Da 3-42.

APPARATUS

1. Beakers or Erlenmeyer flasks—250 mL.
2. Filter paper—the filter paper must impart no acidity or alkalinity to neutral alcohol when poured through it. Place filter paper in a weighing bottle or metal container (seamless container with tight-fitting slipover cover) and dry in an air oven at 105 ± 2°C with cover removed. Remove from the oven, replace cover, cool to room temperature in a desiccator, and weigh. Reweigh after filtration (see Procedure, 7) in the same container.
3. Watch glasses.
4. Air oven—maintained at 105 ± 2°C. AOCS Specification H 3-45.
5. Filter flask—500 mL.

REAGENTS

1. Ethyl alcohol—95% or absolute. SDA Formulas 30 and 3A are permitted (see Notes, *Caution*). Add 3–5 drops of phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1–0.2 M solution, to a faint pink color just before using.
2. Phenolphthalein indicator solution—1% in 95% alcohol.

PROCEDURE

1. Weigh a test portion of such size that it will yield 0.1–1.0 g of alcohol-insoluble matter. Place in 250 mL flask or beaker, add 200 mL of alcohol, cover with a watch glass, and heat on a steam bath with frequent stirring until the soap is dissolved.
2. Filter into a filter flask through the tared filter paper with the aid of vacuum, retaining in the beaker as much of the residue as possible. Repeat this extraction and decantation three times, using 25 mL of hot alcohol each time.
3. After decantation, evaporate (see Notes, *Caution*) any alcohol remaining in the beaker on a steam or water bath and then dissolve the residue in the smallest possible amount of distilled water. Usually 5 mL is sufficient.
4. Add 500 mL of absolute alcohol slowly, stirring vigorously, to reprecipitate the insoluble matter. Heat to boiling on a steam or water bath and filter through the paper or crucible.
5. Wash all of the residue into the filter and wash the filter several times with hot 95% alcohol to remove all of the alcohol-soluble matter.
6. Combine the alcohol filtrate (see Procedure, 2) and the washings (see Procedure, 5) in a tared 250 mL beaker and evaporate on a steam or water bath. Dry to constant weight in an oven at 105 ± 2°C. Cool to room temperature in a desiccator and weigh. See Calculations, 1.
7. Dry the residue on the tared filter paper in an oven at 105 ± 2°C for 3 hr. Cool to room temperature in a desiccator and weigh. See Calculations, 2.

CALCULATIONS

1. Alcohol-soluble matter, $\% = \dfrac{\text{mass, g of residue in beaker}}{\text{mass test portion, g}} \times 100$

2. Alcohol-insoluble matter, $\% = \dfrac{\text{mass, g of insoluble matter}}{\text{mass test portion, g}} \times 100$

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use a properly operating fume hood when heating or evaporating.

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AOCS

AOCS Official Method Db 3-48

Revised 2011

Free Acid or Free Alkali

DEFINITION

This method determines the free (uncombined) acid as oleic acid or alkali in the sample.

SCOPE

Applicable to all soda soaps and soda products containing synthetic detergents. Replaces Da 4a-42.

APPARATUS

1. Erlenmeyer flasks—250 mL.
2. Filter paper—the filter paper must impart no acidity or alkalinity to neutral alcohol when poured through it. The filter is washed with distilled water, alcohol, and petroleum ether before using.
3. Watch glasses.

REAGENTS

1. Ethyl alcohol—95%, SDA Formulas 30 and 3A are permitted. Add a 3–5 drops phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1–0.2 M solution, to a faint pink color just before using.
2. Either hydrochloric (HCl) or sulfuric acid (H_2SO_4) solution, 0.1 M and 0.125 M—accurately standardized. See AOCS Specifications, either H 14-52 or H 13-52, respectively.
3. Sodium hydroxide (NaOH) solution, 0.1 M and 0.25 M—accurately standardized. See AOCS Specification H 12-52.
4. Phenolphthalein indicator solution—1% in 95% alcohol.

PROCEDURE

1. Weigh a test portion of such size that it will yield 0.1-1.0 g of alcohol-insoluble matter. Place in 250 mL flask or beaker, add 200 mL of alcohol, cover with a watch glass, and heat on a steam bath with frequent stirring until the soap is dissolved.
2. Filter into a filter flask through the tared filter paper with the aid of vacuum, retaining in the beaker as much of the residue as possible. Repeat this extraction and decantation three times, using 25 mL of hot alcohol each time.
3. Collect the filtrate and washings in a 250 mL Erlenmeyer flask, heat to incipient boiling on a steam or water bath, and add 0.5 mL of indicator solution.
4. Titrate immediately with standard acid if alkaline or with standard alkali if acid. Use 0.1 M for samples containing small amounts of free acid or free alkali (10% or less) and 0.25 M for all other samples.

CALCULATIONS

1. If free alkali is present and HCl is the titrant:

$$\text{Free alkali as NaOH, \%} = \frac{\text{volume of HCl, mL} \times \text{M of HCl} \times 4}{\text{mass of test portion, g}}$$

2. If free alkali is present, and H_2SO_4 is the titrant:

$$\text{Free alkali as NaOH, \%} = \frac{\text{volume of } H_2SO_4\text{, mL} \times \text{M of } H_2SO_4 \times 8}{\text{mass of test portion, g}}$$

3. If free acid is present:

$$\text{Free acid as oleic, \%} = \frac{\text{volume of alkali, mL} \times \text{M of alkali} \times 28.25}{\text{mass of test portion, g}}$$

where—
M = molarity of standard acid or base

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use a properly operating fume hood when heating or evaporating.

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NUMBERED NOTES

1. Soaps containing little or no alkali need not be filtered as directed in AOCS Official Method Db 2-48, Procedure, 2, before proceeding with determination of free acid or free alkali. However, when substances such as silicates, phosphates, borates, or other similar salts that influence the titration are present, filtration is essential. Carbonates up to 0.5% (as Na_2CO_3) do not affect the determination appreciably and may be omitted, providing a carbonate determination on a separate sample indicates the quantity not to be in excess of 0.5%.
2. Avoid exposure to carbonation at any time since this will seriously affect results.

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AOCS

AOCS Official Method Db 4-48

Reapproved 2009

Water-Insoluble Matter

DEFINITION

This method determines the water-insoluble matter.

SCOPE

Applicable to all soaps and soap products containing synthetic detergents. Replaces Da 6-42.

APPARATUS

1. Beakers or Erlenmeyer flasks—250 mL.
2. Filter paper—the filter paper must impart no acidity or alkalinity to neutral alcohol when poured through it. Place filter paper in a weighing bottle or metal container (seamless container with tight-fitting slip-over cover) and dry in an air oven at 105 ± 2°C with cover removed. Remove from the oven, replace cover, cool to room temperature in a desiccator, and weigh. Reweigh after filtration (Procedure, 9) in the same container.
3. Watch glasses.
4. Air oven—maintained at 105 ± 2°C. AOCS Specification H 3-45.
5. Filter flask—500 mL.

REAGENTS

1. Ethyl alcohol—95% or absolute. SDA Formulas 30 and 3A are permitted (see Notes, *Caution*). Add 3–5 drops of phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1-0.2 M solution, to a faint pink color just before using.
2. Phenolphthalein indicator solution—1% in 95% alcohol.

PROCEDURE

1. Weigh a test portion of such size that it will yield 0.1–1.0 g of alcohol-insoluble matter. Place in 250 mL flask or beaker, add 200 mL of alcohol, cover with a watch glass, and heat on a steam bath with frequent stirring until the soap is dissolved.
2. Filter into a filter flask through the tared filter paper with the aid of vacuum, retaining in the beaker as much of the residue as possible. Repeat this extraction and decantation three times, using 25 mL of hot alcohol each time.
3. After decantation evaporate (see Notes, *Caution*) any alcohol remaining in the beaker on a steam or water bath and then dissolve the residue in the smallest possible amount of distilled water. Usually 5 mL is sufficient.
4. Add 500 mL of absolute alcohol slowly, stirring vigorously, to reprecipitate the insoluble matter. Heat to boiling on a steam or water bath and filter through the paper or crucible.
5. Wash all of the residue into the filter and wash the filter several times with hot 95% alcohol to remove all of the alcohol-soluble matter.
6. Proceed without drying the filter.
7. After the residue is thoroughly washed with alcohol, change receivers and wash the residue with five 50 mL portions of distilled water at 60°C (see Notes, 1).
8. Reserve the water solution for the determination of total alkalinity as directed in AOCS Official Method Db 5-48.
9. Dry the filter paper or crucible containing the water insoluble residue in an air oven for 3 hr at 105 ± 2°C. Cool to room temperature in a desiccator and weigh (see Notes, 2).

CALCULATIONS

$$\text{Water-insoluble matter, \%} = \frac{\text{insoluble matter mass, g}}{\text{mass of test portion, g}} \times 100$$

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use a properly operating fume hood when heating or evaporating.

NUMBERED NOTES

1. If protein or starch is present, use ice water (0–10°C).
2. The nature of the water insoluble material may be determined by further examination if that information is desired.

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AOCS

AOCS Official Method Db 5-48

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Total Alkalinity of Alcohol-Insoluble Matter

DEFINITION

This method determines the alkalinity of the alcohol-insoluble matter, expressed as Na_2O or Na_2CO_3, as required.

SCOPE

Applicable to all soda soaps and soda soap products containing synthetic detergents. Replaces Da 7-42.

REAGENTS

1. Either hydrochloric (HCl) or sulfuric acid (H_2SO_4) solution, 0.5 M—accurately standardized. See AOCS Specifications, either H 14-52 or H 13-52, respectively.
2. Methyl orange indicator solution—0.1% in distilled water.

PROCEDURE

1. Use the water solution obtained from AOCS Official Method Db 4-48, Procedure, 3, or a test solution prepared in an identical manner.
2. Add a few drops of indicator solution and titrate at room temperature with 0.5 M acid.

CALCULATIONS

1. Total alkalinity of alcohol-insoluble matter as Na_2O, $\% = \dfrac{\text{volume, mL acid} \times M \times 3.1}{\text{mass, g of test portion for water-insoluble matter}}$

2. Total alkalinity of alcohol-insoluble matter as Na_2CO_3, $\% = \dfrac{\text{volume, mL acid} \times M \times 5.1}{\text{mass, g of test portion for water-insoluble matter}}$

Where—

M = molarity of the standard acid

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AOCS

AOCS Official Method Db 6-48

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Total Anhydrous Soap

DEFINITION

This method determines the total anhydrous soap.

SCOPE

Applicable to soap and soap products containing synthetic detergents. Replaces Da 8b-42.

APPARATUS

1. Beakers—400 mL or Soxhlet flask, 500 mL.
2. Separatory funnel—500 mL.
3. Extraction cylinder or Stokes flask—
   (a) The extraction cylinder has a 250 mL capacity, is glass-stoppered, about 39 mm dia and 355 mm high.
   (b) The Stokes flask is a 100 mL round-bottom flask sealed onto a 150 mL Erlenmeyer flask (see Fig. 1 in AOCS Official Method Da 8-48).
4. Siphon—a two-hole rubber stopper fitted with small diameter glass tubing constructed to permit removal of the upper layer from the extraction cylinder or Stokes flask.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
2. Ethyl alcohol—absolute. SDA Formulas 30 and 3A are permitted (see Notes, *Caution*). Add 3–5 drops phenolphthalein indicator solution. Boil and neutralize with dilute sodium hydroxide (NaOH), 0.1–0.2 M solution, to a faint pink color just before using.
3. Hydrochloric acid (HCl)—1:1, v/v with distilled water (see Notes, *Caution*).
4. Sodium hydroxide, 0.5 M—accurately standardized. See AOCS Specification H 12-52.
5. Phenolphthalein indicator solution—1.0% in 95% alcohol.
6. Methyl orange indicator solution—0.1% in distilled water.
7. Sodium chloride (NaCl) solution—saturated solution of NaCl in distilled water.

PROCEDURE

1. Weigh a test portion of such size that it will yield 1–5 g of fatty acids. The sample may be placed directly in the Stokes flask, extraction cylinder or into a volumetric flask. If a volumetric flask is used, make up to volume with distilled water, mix thoroughly, and transfer with a pipet a quantity such that it will yield 1–5 g of fatty material to the extraction cylinder or Stokes flask.
2. Add equal volumes of distilled water and 95% alcohol to bring the total volume to about 75 mL. Warm to promote solution but do not exceed a temperature of 60°C.
3. Cool to room temperature (20–25°C). Add HCl (1:1, v/v) until the solution is just acid to methyl orange and then add 10 additional mL.
4. Add sufficient saturated NaCl solution to bring the volume to 160 mL in the cylinder or to bring the upper level of solution within the constricted portion of the Stokes flask.
5. Add 50 mL of petroleum ether without inverting or mixing. Allow to stand until the acids have completely dissolved in the ether.
6. Proceed as directed in AOCS Official Method Da 8-48, Procedure, 5-10, but use petroleum ether instead of diethyl ether in every case.

CALCULATIONS

Calculate as directed in AOCS Official Method Da 8-48, but substitute % fatty matter from AOCS Official Method Db 10-48 for % unsaponified plus unsaponifiable matter.

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Ethanol (ethyl alcohol) is flammable. Use a properly operating fume hood when heating or evaporating.

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AOCS

AOCS Official Method Db 7-48

Reapproved 2009

Chlorides

DEFINITION

This method determines the chlorides in the sample.

SCOPE

Applicable to all soaps and soap products containing synthetic detergents. Replaces Da 9-42.

APPARATUS

1. Beaker—500 mL.

REAGENTS

1. Silver nitrate ($AgNO_3$) solution, 0.1 M—accurately standardized against reagent grade sodium chloride which has been dried at 105°C. The standardization titration is exactly as noted in Procedure, 3, except that accurately weighed portions of sodium chloride are used in place of the sample.
2. Sulfuric acid (H_2SO_4) solution—approximately 0.05 M.
3. Potassium chromate (K_2CrO_4) indicator solution—dissolve 5 g of K_2CrO_4 (see Notes, *Caution*) in distilled water and add $AgNO_3$ solution until a slight orange-red precipitate is formed. Filter and dilute to 100 mL with distilled water.
4. Phenolphthalein indicator solution—1% in 95% alcohol.
5. Magnesium nitrate solution—20% $Mg(NO_3)_2 \cdot 6H_2O$ in distilled water. Solution must be neutral and chloride free.
6. Calcium carbonate—reagent grade.

PROCEDURE

1. Weigh accurately 5 g (2-3 g for samples containing more than 5% NaCl) of the sample into a 500 mL beaker and add 300 mL of chloride-free distilled water. Boil if necessary to effect solution.
2. Add an excess of $Mg(NO_3)_2$ solution (usually about 25 mL of 20% solution), filter and wash thoroughly with chloride-free distilled water to remove all chlorides.
3. Cool to room temperature (20–25°C). If the solution is alkaline, add a little phenolphthalein and then 0.05 M H_2SO_4 until just colorless, avoiding an excess of more than 1 drop of acid. Add 1 mL of K_2CrO_4 indicator for every 100 mL of solution and titrate with 0.1 M $AgNO_3$ to the first permanent reddish tinge.
4. Prepare a blank in a 500 mL beaker containing the same volume of distilled water, $Mg(NO_3)_2$ solution, and indicator. Add sufficient $CaCO_3$ to the blank to make the turbidity equivalent to that of the sample when both are swirled.
5. Using the blank for comparison, continue the titration of the sample until a faint but distinct color change occurs. At the end point, the color of the sample solution should not be dark, but just distinctly different from that of the blank.
6. Add sufficient 0.1 M $AgNO_3$ to the blank so that the colors of the blank and sample match.

CALCULATIONS

1. Chlorides, as NaCl, $\% = \dfrac{(S - B) \times M \times 5.85}{\text{mass of test portion, g}}$
2. Chlorides as KCl, $\% = \dfrac{(S - B) \times M \times 7.46}{\text{mass of test portion, g}}$

Where—

S = volume of titrant, mL of sample
B = volume of titrant, mL of blank
M = molarity of $AgNO_3$

NOTES

Caution

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

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AOCS

AOCS Official Method Db 7b-55

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Potentiometric Determination of Chlorides

DEFINITION

This method determines the chlorides in the sample by potentiometric titration.

SCOPE

Applicable to soaps and synthetic detergents and their products.

APPARATUS

1. Auto-titrator with suitable electrodes (see manufacturer's instructions).
2. Burette—50 mL.
3. Stirrer—electric, with built-in rheostat control. A magnetic stirrer cannot be used.

REAGENTS

1. Dilute HNO_3—approximately 6 M (see Notes, *Caution*).
2. $AgNO_3$ solution, 0.1 M—accurately standardized.
3. Methyl orange indicator solution—0.1% in water.

PROCEDURE

1. Preparation of soap samples—
   (a) Accurately weigh the required amount of test sample, selected as noted in Table 1, and dissolve in 250 mL of hot water. Add 2 drops of methyl orange indicator and acidify with dilute HNO_3. Add several drops in excess (see Numbered Notes, 2).
   (b) Filter through a wet filter paper and wash the fatty acids with hot water. Cool the filtrate to room temperature.
2. Preparation of synthetic detergent samples—
   (a) Accurately weigh the required weight of test sample and dissolve in 250 mL of hot water (see Table 1). Add 2 drops of methyl orange indicator and acidify with dilute HNO_3 (see Numbered Notes, 2).
3. Titration of sample—
   (a) Follow manufacturer recommended instructions for setting up the auto-titrator.
   (b) Titrate with 0.1 M standard $AgNO_3$ solution until the needle has been brought back to the original reference point at 1. Record the volume of 0.1 M $AgNO_3$ solution required in the titration.

CALCULATIONS

$$\text{Chlorides as NaCl, \%} = \frac{(S - B) \times M \times 5.85}{\text{mass of test portion, g}}$$

Where—

S = volume of titrant, mL of sample

B = volume of titrant, mL of blank

M= molarity of the $AgNO_3$solution

NOTES

Caution

For nitric acid, use an effective acid-resistant fume-removal device whenever heating acids or performing reactions which liberate acid fumes. When diluting acids, always add acid to water unless otherwise directed in a method. Keep acids off skin and protect eyes when working with acids. If acids come in contact with skin or eyes, wash immediately with large amounts of water. Do not store oxidizing acids (perchloric, nitric, or sulfuric) near organic materials. Mixing organic materials with these acids, particularly per-chloric, could result in an explosion.

NUMBERED NOTES

1. It is important that the pH meter electrodes be cleaned periodically according to the manufacturer's instructions.
2. The HNO_3 concentration of the solution to be titrated must be kept low to avoid damaging electrode.

REFERENCES

1. Callen and Horrobin, *J. Soc. Chem. In. 47:*T337 (1928).

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Table 1
Test portion weight should be selected as follows:

Percent NaCl expected	Approximate weight of test portion, g
4% or less	5.0
4–8%	2.5
8–16%	1.3
16–32%	0.6
32–64%	0.3
64% and over	0.1

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AOCS Official Method Db 8-48

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Saponification Value

DEFINITION

The saponification value is the mg of potassium hydroxide required to saponify 1 g of the sample.

SCOPE

Applicable to the fatty acids of all soaps and soap products containing synthetic detergents. Replaces Da 16-45.

APPARATUS

1. Erlenmeyer flask—Corning Alkali Resistant, Kimble Resistant, or equivalent, 250 or 300 mL.
2. Air condensers—min 650 mm long.
3. Water bath or a hot plate—with variable heat control.

REAGENTS

1. Hydrochloric acid (HCl), 0.5 M—accurately standardized. See AOCS Specification H 14–52.
2. Alcoholic potassium hydroxide (KOH)—place 5–10 g of KOH in a 2 L flask and from 1–1.5 liters of 95% ethyl alcohol (SDA Formulas 30 and 3A are permitted) and boil in a water bath under a reflux condenser for 30–60 min. Distill and collect the alcohol. Dissolve 27 g of potassium hydroxide, low in carbonate, in 1 L of the distilled alcohol, keeping the temperature below 15.5°C while the alkali is being dissolved (see Notes, *Caution*). This solution should remain clear.
3. Phenolphthalein indicator solution—1% in 95% alcohol.

PROCEDURE

1. Fatty acids are prepared as indicated in AOCS Official Method Da 12-48, Procedure. The temperature of the fatty acids during filtration is not to exceed 15°C above the titer point of the fatty acids. It is essential that the acids be clear and dry, if not, refilter.
2. Weigh a test portion of such size that the back titration is 45–55% of the blank. This will usually require a 3–4 g sample. Add 50 mL of the alcoholic KOH with a pipet and allow the pipet to drain for a definite period of time.
3. Prepare and conduct blank determinations simultaneously with the sample.
4. Connect an air condenser and boil gently but steadily, until the sample is completely saponified. This usually requires about 30 min for normal samples. Be careful that the vapor ring in the condenser does not rise to the top of the condenser or there may be some loss of sample.
5. After the flask and contents have cooled somewhat, but not sufficiently to gel, wash the inside of the condenser, add about 1 mL of indicator, and titrate with 0.5 M HCl until the pink color has just disappeared.

CALCULATIONS

$$\text{Saponification value} = \frac{28.05(\text{volume mL, blank titration} - \text{volume mL, sample titration})}{\text{mass of test portion, g}}$$

NOTES

Caution

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

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Alkaline Silicates

DEFINITION

This method determines the alkaline silicates in the sample.

SCOPE

Applicable to all soaps and soap products containing synthetic detergents. Replaces Da 18-42.

APPARATUS

1. Platinum evaporating dish—100 mL.
2. Platinum crucible—30 mL.
3. Watch glasses.
4. Filter paper—ashless grade, small size, about 90 mm.

REAGENTS

1. Hydrochloric acid (HCl)—reagent grade, concentrated (sp. gr. 1.19) (see Notes, *Caution*).
2. Hydrofluoric acid (HF)—technical grade, 47% solution (see Notes, *Caution*).
3. Sulfuric acid (H_2SO_4)—reagent grade, concentrated (sp. gr. 1.48) (see Notes, *Caution*).

PROCEDURE

1. Sample contains no mineral matter that is insoluble in water—
   (a) Weigh a size of test portion such that it will contain not more than 0.2 g of silicate (SiO_2) into a platinum evaporating dish and ignite over a burner or in a muffle furnace below a dull red heat (350–400°C) until the mass is charred. Add distilled water and filter through ashless filter paper. Wash the dish and residue on the filter paper several times with distilled water so as to extract all water-soluble material.
   (b) Return the filter paper containing the charred residue to the platinum dish and continue the ignition at a bright red heat (850–950°C) until all carbon is removed. Combine the residue and all of the water extract, neutralize carefully with HCl, and then add from 5–10 mL of HCl in excess. Keep the dish covered with a watch glass during this addition to prevent loss from spattering.
   (c) Evaporate to dryness on steam bath or hot plate at a temperature not exceeding 120°C. Cool the residue and moisten with HCl, allow to stand for 5–10 minutes and then break up all lumps with a stirring rod.
   (d) Add about 25 mL of hot distilled water to the residue, heat for a few minutes, and filter through ashless filter paper. Wash the paper and contents thoroughly with hot water. Repeat Procedure, 1, c and d. Filter through a second ashless filter paper.
   (e) Place the two filter papers in a tared platinum crucible and ignite carefully, first at a low temperature until the paper is burned off and then over a burner or in a muffle furnace at a bright red heat (850–950°C). Cool to room temperature in a desiccator and weigh. Repeat the weighing until constant weight is obtained.
   (f) For the most accurate results, moisten the weighed contents of the crucible with water, add 10 mL of HF, 4 drops of H_2SO_4, and evaporate to dryness over a low flame. Ignite as directed in Procedure, 1, e above, cool to room temperature in a desiccator and, weigh. Repeat the weighing until constant weight is obtained.
2. Sample contains mineral matter insoluble in water, or when a determination of the highest accuracy is not required—
   (a) Use a portion of the solution after titrating the matter insoluble in alcohol, but soluble in water (AOCS Official Method Da 7-48, Procedure, 2), such that it will contain not more than 0.2 g of silicate (SiO_2).
   (b) Add from 5–10 mL of HCl and proceed as directed in Procedure, 1, beginning with c.

CALCULATIONS

1. When SiO_2 is weighed:

$$\text{Sodium silicate (1 } Na_2O\text{:3.25 } SiO_2\text{), \%} = \frac{\text{mass, g of residue (Procedure 1, e)} \times 131.8}{\text{mass of test portion, g}}$$

2. When SiO_2 is volatilized by HF:

$$\text{Sodium silicate (1 } Na_2O\text{:3.25 } SiO_2\text{), \%} = \frac{(A - B) \times 131.8}{\text{mass of test portion, g}}$$

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Where—

A = mass, g of crucible and contents from Procedure, 1, e.

B = mass, g of crucible and contents from Procedure, 1, f.

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Hydrofluoric acid is toxic by ingestion and inhalation. It is highly corrosive to skin and mucous membranes. Protective clothing should be worn at all times when working with this acid.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

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AOCS Official Method Db 10-48

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Fatty Matter

DEFINITION

This method determines the substances soluble in petroleum ether in that portion of the sample which is soluble in alcohol.

SCOPE

Applicable to soaps containing synthetic detergents. Replaces Da 29-45.

APPARATUS

1. Beakers—400 mL, or Soxhlet flasks, 500 mL.
2. Separatory funnel—500 mL.
3. Extraction cylinder, or Stokes flask—
   (a) Extraction cylinder—250 mL, with glass stopper, about 39 mm dia and 355 mm high.
   (b) The Stokes flask is a 100 mL round-bottom flask sealed onto a 150 mL Erlenmeyer flask (see Fig. 1 in AOCS Official Method Da 8-48).
4. Siphon—a two-hole rubber stopper fitted with small diameter glass tubing constructed to permit removal of the upper layer from the extraction cylinder or Stokes flask.

REAGENTS

1. Ethyl alcohol—95% SDA Formulas 30 and 3A are permitted (see Notes, *Caution*).
2. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
3. Sodium hydroxide (NaOH) solution—approximately 0.2 M.
4. Phenolphthalein indicator solution—1.0% in 95% ethyl alcohol.
5. Sodium sulfate (Na_2SO_4)—anhydrous, reagent grade.

PROCEDURE

1. Obtain the alcohol-soluble matter as directed in AOCS Official Method Db 2-48, Procedure, 6, although the residue need not be weighed.
2. Dissolve the residue in a mixture of 25 mL of distilled water and 25 mL of alcohol. Warm, if necessary, to promote solution.
3. Transfer to the extraction cylinder of Stokes flask. Wash the beaker alternately with equal volumes of hot distilled water and hot alcohol, adding the washings to the cylinder or flask. Wash the beaker with a few mL of petroleum ether and add to the cylinder or flask. The total volume should be at 160 mL in the cylinder or within the constricted portion of the Stokes flask. If not, add a sufficient amount of equal volumes of distilled water and alcohol to bring to a volume of 160 mL.
4. Cool to 25–30°C. Add 50 mL of petroleum ether and mix by inverting. In case of emulsion formation, add about 10 g of Na_2SO_4 and allow to settle until the layers separate.
5. Remove the upper layer with the siphon and collect the ether extract in a 500 mL separatory funnel.
6. Add 50 mL of petroleum ether, shake vigorously, and allow to separate. Again remove the petroleum ether layer with the siphon. Make five more extractions in the same manner and collect all extracts in the separa tory funnel.
7. Add 10 mL of 0.2 M NaOH to the separatory funnel and shake. Allow to separate and draw off the lower layer. Repeat three more times.
8. Add 10–15 mL of distilled water to the funnel and shake. Allow to separate and draw off the lower water layer. Repeat until the washings are neutral to phenolphthalein.
9. Draw the petroleum ether solution into a tared 400 mL beaker or 500 mL Soxhlet flask. Evaporate the petroleum ether on a steam or water bath, using a gentle stream of nitrogen, until the odor of solvent is no longer perceptible. Dry in an air oven for 30 min at 105 ± 2°C. Cool to room temperature in a desiccator and weigh.

CALCULATIONS

$$\text{Fatty matter, \%} = \frac{\text{mass, g of extract}}{\text{mass, g of test portion}} \times 100$$

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

Petroleum ether is extremely flammable. Use effective fume removal device. Avoid static electricity.

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AOCS Official Method Db 11-48

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Rosin, McNicoll Method

DEFINITION

This method determines the rosin in the fatty acids.

SCOPE

Applicable to soaps containing synthetic detergents. Replaces Da 12-42.

APPARATUS

1. Esterification flask—150 mL round-bottom, or Erlenmeyer type.
2. Reflux condenser—water-cooled. Esterification flask and condenser with ground-glass joints 24/40 are preferred.
3. Extraction cylinder, or Stokes flask—
   (a) Extraction cylinder—250 mL, with glass stopper, about 39 mm dia and 355 mm high.
   (b) The Stokes flask is a 100 mL round-bottom flask sealed onto a 150 mL Erlenmeyer flask (see Fig. 1 in AOCS Official Method Da 8-48).
4. Siphon—a two-hole rubber stopper fitted with small diameter glass tubing constructed to permit removal of the upper layer from the extraction cylinder or Stokes flask.
5. Burettes—50 mL.
6. Separatory funnel—500 mL.
7. Graduated cylinders—50 and 100 mL.
8. Glass beads.

REAGENTS

1. Ethyl alcohol—95%. SDA Formulas 30 and 3A are permitted (see Notes, *Caution*).
2. Sulfuric acid (H_2SO_4), 0.25 M.
3. Sulfuric acid (H_2SO_4)—1:1, v/v with distilled water (see Notes, *Caution*).
4. Sodium chloride (NaCl) solutions—saturated solution in water and 10% solution in water, using reagent grade NaCl.
5. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
6. Methyl orange indicator solution—0.01%, prepared by dissolving 0.1 g of methyl orange indicator in 100 mL of distilled water.
7. Filter paper—qualitative grade, rapid filtering.
8. Alcoholic potassium hydroxide (KOH) solution, 0.2 M—accurately standardized. See AOCS Specification H 15-52. Due to the volatility of the alcohol, this solution should be standardized frequently.
9. Naphthalene-β-sulfonic acid solution—prepared by dissolving 40 g of naphthalene-β-sulfonic acid in 1 L of absolute methyl alcohol.
10. Phenolphthalein indicator solution—1.0 % in 95% ethyl alcohol.

PREPARATION OF FATTY AND ROSIN ACIDS

1. Weigh a test portion of such size that it will yield about 5 g of fatty acids. The sample may be placed directly in the extraction cylinder or Stokes flask.
2. Add equal volumes of distilled water and 95% ethyl alcohol to bring the total volume to about 75 mL. Warm on a steam bath to promote solution, but do not exceed a temperature of 60°C.
3. Cool to room temperature (20–25°C). Add 5 drops of methyl orange indicator and titrate with 0.25 M H_2SO_4 to a pink color and add 5 mL of 1:1 H_2SO_4 in excess.
4. Add sufficient saturated NaCl to bring the volume in the extraction cylinder to 160 mL, or to bring the upper level within the constricted portion of the Stokes flask.
5. Add 50 mL of petroleum ether without inverting the extraction cylinder or mixing the contents of the Stokes flask. Allow to stand until the acids have completely dissolved in the petroleum ether layer.
6. Remove the upper petroleum ether layer with a siphon, transferring into a 500 mL separatory funnel. Add 50 mL of petroleum ether and shake vigorously. Allow to stand until the layers separate clearly and siphon the petroleum ether layer into the separatory funnel.
7. Make three more extractions in a similar manner, using 50 mL of petroleum ether each time.
8. Wash the ether extract in the separatory funnel with several portions of 10% NaCl until the washings are neutral to methyl orange.
9. If any insoluble matter is present in the ether extract, filter through a filter paper and wash the paper with ether to remove all fatty matter. Transfer into a tared beaker or Soxhlet flask and evaporate on a steam or water bath to remove all of the ether.

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PROCEDURE

1. Weigh accurately about 2 g of fatty acids into the esterification flask and add 25 mL of the naphthalene-β-sulfonic acid solution. Add a few glass beads to prevent bumping during boiling, attach the reflux condenser and boil for 30 min.
2. Prepare and conduct a blank determination simultaneously and identical in all respects, except without any sample present.
3. At the end of the boiling period, cool the contents of the flask, add 0.5 mL of phenolphthalein indicator, and titrate immediately with 0.2 M alcoholic KOH to the first pink color which persists for 30 sec.

CALCULATIONS

1. Rosin in fatty acids, uncorrected, $\% = \dfrac{(S - B) \times M \times 34.6}{\text{mass, g of test portion}}$
2. Rosin in fatty acids, corrected, % = % rosin, uncorrected (Calculations, 1) − 1.0
3. Rosin basis original sample, % = % rosin in fatty acids, corrected (Calculations, 2) $\times \dfrac{C}{100}$
4. Rosin soda (soap basis) in original test portion, % = % rosin basis original sample × 1.064.

Where—

S = volume of titrant, mL of test portion

B = volume of titrant, mL of blank

M = molarity of alcoholic KOH solution

C = % fatty anhydride + % unsaponified and unsaponifiable + (D × 0.2905)

D = % combined alkali from AOCS Official Method Da 8a-42, calculated as Na_2O.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1-6%. A fume hood should be used at all times when using petroleum ether.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

NUMBERED NOTES

1. Cooperative studies have shown that the McNicoll method gives results about 1% higher than the amount of actual rosin present. The Technical Committee recommends, therefore, that 1% be deducted from the percentage rosin found in the fatty acids.
2. Whenever less than 5% rosin is found, the qualitative Liebermann-Storch (following) test should be applied to confirm the actual presence or absence of rosin.

   Liebermann-Storch Test—Place 1-2 mL of the fatty acids into a test tube, add 5-10 mL of acetic anhydride, and warm on a steam bath. Cool, pour 1-2 mL into a white porcelain dish, and allow 1 or 2 drops of H_2SO_4 (sp. gr. 1.53) to run down the inside of the dish. If rosin is present, a fleeting violet color is produced immediately at the point of contact. The color changes to a brownish tinge. Check the test using a sample of fatty acids to which has been added a small amount of rosin. Cholesterol and some other substances give a similar test.

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AOCS

AOCS Official Method Dc 1-59

Revised 2011

Sampling and Preparation of Laboratory Sample

SCOPE

Applicable to paste, powder, or liquid detergent fatty alkyl sulfates.

APPARATUS

1. Trier for paste soap—a half-round metal tube 125–250 mm in diameter. The length may be 60–120 cm, depending upon the size of package to be sampled. One end is tapered to a point, the taper not to be more than 2.5 cm long. The other end is attached to a D- or T-shaped handle.
2. Sample tube for liquid product—a glass tube 90–125 mm i.d. The length may be convenient for the size of packages to be sampled. One end is constricted by a short taper (not more than 2.5 cm long) to about 60 mm. The other end is constricted sufficiently so that it can be used as a finger valve.
3. Airtight and watertight sample containers—about 2 kg capacity.
4. Airtight and watertight sample containers—sufficient capacity to hold samples taken as directed in Procedure, 1–3.
5. Spatula—with 25–30 cm blade.

PROCEDURE—SAMPLING

Note—Place all samples in airtight and watertight containers, cover, and seal to prevent any moisture change. Label completely for identification and send to the laboratory for preparation and analysis.

1. Powders and flakes packed in cans or cartons—one can or carton should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 22.7 kg (50 lbs). In the case of smaller containers, a can or carton should be taken at random from each lot of containers, totaling not more than 2,268 kg (5,000 lbs) or fraction thereof. The gross sample should in all cases consist of not less than three cans or cartons taken at random from separate containers. In the case of very large lots where the sample drawn as above will amount to more than 9.1 kg (20 lbs), the percentage of packages sampled should be reduced so that the amount drawn will not exceed 9.1 kg (20 lbs). The individual cans or cartons should be tightly wrapped at once in parafin paper and sealed by rubbing the edges with a heated iron. The inspector should accurately weigh each wrapped can or carton and record its weight and the date of sampling and weighing on the sample package. The wrapped cans or cartons should then be placed in an airtight container, filled as full as possible, then sealed, marked, and sent to the laboratory for testing. Samples should be kept cool until tested.
2. Powders and flakes in bulk—a grab sample of not less than 227 g (0.5 lb) should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 45.4 kg (100 lbs). In the case of smaller containers, a grab sample of not less than 227 g (0.5 lb) should be taken at random from each lot of containers totaling not more than 4,536 kg (10,000 lbs) or fraction thereof. The gross sample should, in all cases, consist of not less than three grab samples of 227 g (0.5 lb), each taken at random from separate containers. In the case of very large lots where the sample drawn as above will amount to more than 9.1 kg (20 lbs), the percentage of packages sampled should be reduced so that the amount drawn will not exceed 9.1 kg (20 lbs). The inspector must rapidly mix the gross sample and place in an airtight container, filled as full as possible, which then should be sealed, marked, and sent to the laboratory for testing. Samples should be kept cool until tested.
3. Liquids—a sample of not less than 240 mL (0.5 pt) should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 37.9 L (10 gal). In the case of smaller containers, a sample of not less than 240 mL (0.5 pt) should be taken at random from each lot of containers totaling not more than 3,785 L (1,000 gal) or fraction thereof. The gross sample should, in all cases, consist of not less than three samples of 240 mL (0.5 pt), each taken at random from separate containers. Before drawing the sample from the container selected, the contents of the container should be thoroughly agitated. The inspector should thoroughly mix the gross sample, place it in clean, dry cans or bottles, which then should be sealed, marked, and sent to the laboratory for testing. Samples should be kept cool until tested.
4. Pastes—
   (a) Pastes packed in cans or cartons of 2.27 kg (5 lbs) or less—one can or carton should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 22.7 kg (50 lbs). In the case of smaller containers, a can or carton should be taken at random from each lot of containers, totaling not more than 2,268 kg (5,000 lbs) or fraction thereof. The gross sample should in all cases consist of not fewer than three cans or cartons taken at random from separate containers. In the case of very large lots where the sample drawn as above will amount to more than 9.1 kg (20 lbs), the percentage of packages sampled should be reduced so that the amount drawn will not exceed

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9.1 kg (20 lbs). The inspector should wrap, seal, mark, and send the samples to the laboratory for testing. Samples should be kept cool until tested.

(b) Pastes packed in bulk—a "trier" sample (see Notes, 1) of not less than 227 g (0.5 lb) should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 22.7 kg (50 lbs). In the case of smaller containers a trier sample of not less than 227 g (0.5 lb) should be taken at random from each lot of containers totaling not more than 2,268 kg (5,000 lb) or fraction thereof. The gross sample shall, in all cases, consist of not less than three 227 g (0.5 lb) samples, each taken at random from separate containers. With very large lots where the sample drawn as noted in this section will amount to more than 4.5 kg (10 lb), the percentage of packages sampled should be reduced so that the amount drawn will not exceed 4.5 kg (10 lbs). The inspector should promptly place the gross sample in a clean, dry, airtight and watertight container, and then wrap, seal, mark, and send the sample containers to the laboratory for testing. Samples should be kept cool until tested.

PREPARATION OF TEST SAMPLES

1. Powders and flakes—minimizing exposure to air, rapidly disintegrate and mix the sample powder, flake, or chip product. If desired, quarter the sample to about 454 g (1 lb). Weigh immediately all test portions for analysis, preserving the remainder in an airtight container and store in a cool, dry place.
2. Liquids—no special preparation of a sample of liquid, other than a thorough mixing, is necessary, unless it is received during cold weather, in which case it should be allowed to stand, before it is tested, for at least 1 h after the sample has come to room temperature (20–25°C). This is particularly true if the sample is to be tested for its lathering qualities (see Notes, 2).
3. Pastes—store in glass containers. If crystals separate during storage, warm carefully in a water bath.

NUMBERED NOTES

1. A trier sample is obtained by inserting the "trier" sampler (see Apparatus, 1) into the material to be sampled. A trier is a half round cylinder about 13–20 mm in diameter, 15–90 cm in length, pointed at one end and having a handle grip at the opposite end from the point. After insertion, the trier is turned two or three times and, upon removal, a core of the material being sampled is obtained.
2. If pastes or liquids are known to be acidic and decomposition of the sample can result from heating, the sample should be adequately labeled for precautionary treatment and carefully warmed to room temperature, or other maximum temperature, as agreed upon for mixing and sampling.

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Moisture by Distillation Method

DEFINITION

This method determines moisture by distillation with an immiscible solvent.

SCOPE

This method is applicable to all fatty alkyl sulfate products except those containing water-miscible volatile substances.

APPARATUS

1. Glass distillation apparatus—with ground-glass connections, constructed and assembled in accordance with the specifications shown in Figure 1.
   (a) Flask—short neck, round bottom type, 1000 mL.
   (b) Condenser—water-cooled, the lower tip of which should be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established.
   (c) Trap—constructed of well-annealed glass. For greatest accuracy several trap sizes are allowable, depending upon the percentage of moisture expected; see Table 1. Traps made of well-annealed glass, constructed essentially as shown in Figure 1, and graduated to contain one of the following specified volumes at 20°C shall be used: 5 mL trap—subdivided into 0.1 mL divisions with each 1 mL line numbered (5 mL at top). The error in any indicated capacity may not be greater than 0.05 mL.
      10 mL trap—subdivided from 0–2 mL divisions and from 2–10 mL in 0.2 mL divisions.
      25 mL trap—subdivided from 0–2 mL divisions and from 2–25 mL in 0.2 mL divisions.
   (d) Distillation may be better controlled by wrapping the tube which leads from the flask to the receiver with glass-fiber tape. It is advantageous to wrap the flask also, especially if there are drafts in the vicinity of the distillation apparatus.
   (e) Calibrate the receiver as follows: add an accurately measured portion, 1 ± 0.01 mL, of distilled water to 100 mL of toluene in the distillation flask. Conduct the distillation as described in Procedure, 2–5, and calculate as directed in Calculation. Cool the apparatus, add another mL of water and repeat the distillation. Continue in this manner up to the capacity of the receiving tube.
2. Pipet—measuring type, 1.0 mL, graduated in 0.01 mL.
3. Heat source—usually an electric heating mantle provided with a suitable means of control.
4. Copper or Nichrome wire—long enough to extend through the condenser with one end twisted into a spiral. The diameter of the spiral is such that it will fit snugly within the receiver and yet can be moved up and down.

REAGENTS

1. Xylene or toluene—reagent grade (see Notes, *Caution*). Use toluene for soaps containing 1% or more of glycerin. Saturate the xylene or toluene with water by shaking with a small quantity of water and distill. Use the distillate for the determination.
2. Absolute ethyl alcohol (ethanol)—(see Notes, *Caution*).

PROCEDURE

1. The entire apparatus must be completely clean. Clean with potassium dichromate-sulfuric acid cleaning solution to minimize the adherence of water droplets to the sides of the condenser and receiver. Rinse thoroughly and dry completely before using.
2. Transfer to the 1,000 mL flask, equipped with the size of the trap specified in Apparatus, an amount of test sample (see Notes, 1) according to the percentage of moisture expected, as shown in Table 2. Add immediately about 100 mL of xylene or toluene. Place a small, thin sheet of long fiber, chemical-resistant glass wool on the surface of the toluene. The glass wool should be thoroughly dried in the oven and held in the desiccator before use.
3. Assemble the apparatus and fill the receiver with solvent by pouring through the condenser until the solvent begins to overflow into the distillation flask. Insert a loose cotton plug in the top of the condenser to prevent condensation of atmospheric moisture within the tube.
4. Heat the flask so that the distillation rate will be about 100 drops per minute. When the greater part of the water has distilled, increase the distillation rate to about 200 drops per minute and continue until the water level in the receiver remains unchanged for 30 min.
5. Discontinue heating, dislodge any drops of water which may be adhering to the inside of the condenser tube or receiver with a wire spiral, and wash down with about 5 mL of toluene or xylene.
6. Wash down condenser with 1 mL of absolute ethanol. Adjust the temperature of the distillate to 20°C and read the volume of water.

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7. Immerse the receiver in water at 25°C for at least 15 min or until the toluene or xylene layer is clear, then read the volume of water.

CALCULATIONS

$$\text{Moisture, \%} = \frac{\text{volume, mL of water} \times 99.8}{\text{mass of test portion, g}}$$

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 0.28 at the 10% moisture level, or 0.83 at the 50% moisture level.
2. Single determinations performed in two different laboratories should not differ by more than 0.52 at the 10% moisture level, or 1.88 at the 50% moisture level.

NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air.

Xylene is flammable and a dangerous fire risk. The TLV is 100 ppm in air.

Ethanol (ethyl alcohol) is flammable.

A fume hood should be used at all times when using the solvents specified in this method.

NUMBERED NOTES

1. If sodium bicarbonate is present it will undergo partial decomposition under the conditions of this test and yield water and CO_2.

Table 1

Moisture expected, %	Size of trap, mL
0–5, incl.	5
Over 5–17, incl.	10
Over 17–30, incl.	10
Over 30–50, incl.	25
Over 50–70, incl.	25
Over 70–85, incl.	25

Table 2

Moisture expected, %	Mass of test portion to be used, g*
0–5, incl.	50 ± 5
Over 5–17, incl.	50 ± 5
Over 17–30, incl.	30 ± 5
Over 30–50, incl.	30 ± 5
Over 50–70, incl.	30 ± 5
Over 70–85, incl.	25 ± 5

*Weighed to the nearest 0.25 g.

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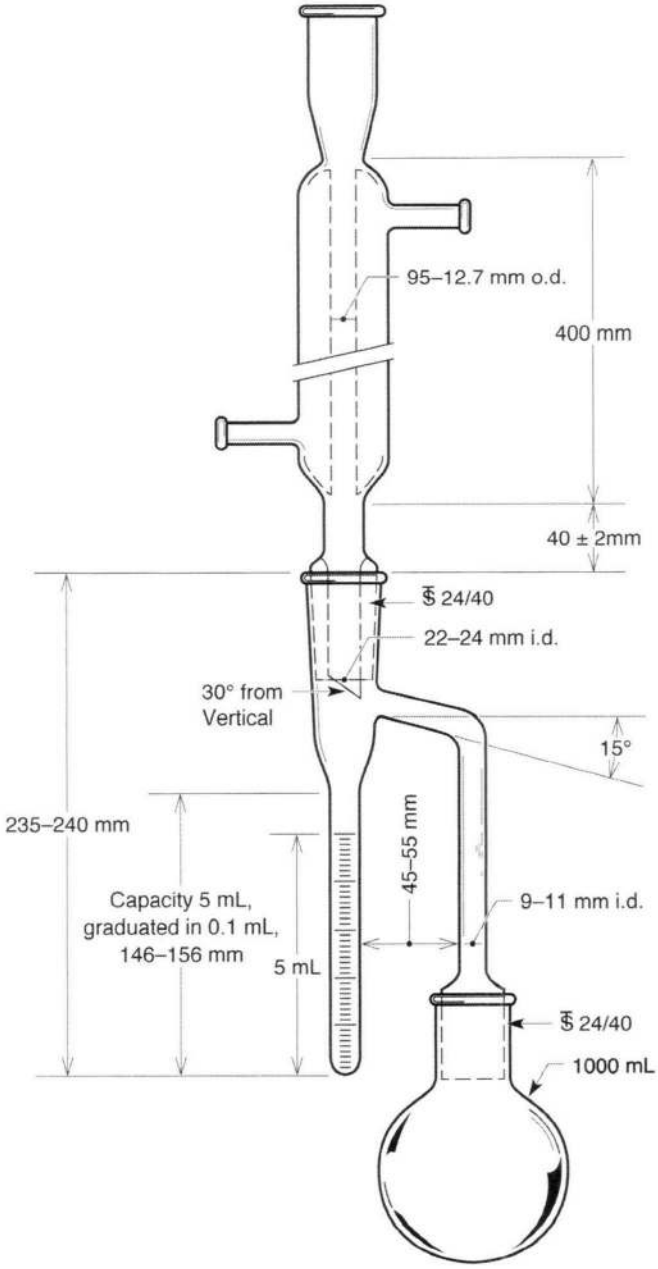

Figure 1. Moisture distillation apparatus.

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Alcohol-Soluble Matter

DEFINITION

This method determines the total alcohol-soluble matter in alkyl sulfates without regard to correction for alcohol-soluble unsulfonated materials.

SCOPE

Applicable to all fatty alkyl sulfates.

APPARATUS

1. Beakers—400 mL.
2. Watch glasses—for beakers.
3. Volumetric flasks—1,000 mL.
4. Soxhlet flasks.
5. Gooch crucible—or sintered-glass crucible or 15 cm dia rapid filter paper.
6. Aluminum dish—the aluminum moisture dish specified in AOCS Official Method Ca 2c-25 is satisfactory, for drying the filter paper, if this option is used.
7. Air oven—maintained at 90 ± 2°C.

REAGENTS

1. Ethyl alcohol—95%, SDA Formulas 30 or 3A are permitted. The alcohol should not be neutralized, however, if the alkali titration with phenolphthalein indicator is more than 0.2 mL, the alcohol should be redistilled (see Notes, *Caution*).
2. Ethyl alcohol—absolute, freshly boiled, conforming to SDA Formulas 30 or 3A (see Notes, *Caution*).

PROCEDURE

1. For pastes or liquids, weigh out a test portion of approximately 20 ± 0.001 g and wash into a 400 mL beaker with absolute ethyl alcohol. For powders, weigh 10 ± 0.001 g and transfer to a 400 mL beaker.
2. Add 300–350 mL of hot absolute ethyl alcohol. Cover with a watch glass and heat on a steam bath for at least 2 hr, stirring frequently to disperse solids and break up any lumps. Have ready either a tared 15 cm rapid filter paper that has been dried in an open aluminum dish at 105 ± 2°C for 1 hr, cooled in a desiccator, and weighed in the dish; a tared Gooch crucible with appropriate filtration medium (ceramic fiber or filter paper); or a tared sintered-glass crucible.
3. At the end of the 2 hr heating period, remove the beaker from the bath and decant the alcohol solution rapidly through the weighed filter medium, retaining as much as possible of the residue in the beaker. Add 50 mL of hot 95% ethyl alcohol to the residue in the beaker. Heat to boiling on a hot plate, breaking up any lumps of the residue. Again decant the alcohol through the tared filter as before. Repeat again with another 50 mL portion of hot 95% alcohol.
4. Evaporate the residual alcohol from the residue in the beaker on the steam bath, stirring at intervals, especially near the end of the evaporation. Dissolve the residue in the beaker with 10 mL of hot water, heating on the steam bath until solution is effected. Dilute the water solution with 200 mL of hot absolute ethyl alcohol, bring to a boil on the steam bath, and filter through the original filter paper. Finally, transfer the precipitate to the paper with the aid of hot absolute alcohol and a policeman.
5. Wash the residue with hot 95% ethyl alcohol. Three or four washings will be required. Combine the filtrate and washings in a 1 L volumetric flask, cool, make up to volume with 95% alcohol, and mix thoroughly.
6. Transfer a 200 mL aliquot to a tared Soxhlet flask. Evaporate on a steam bath, using a gentle stream of clean, dry nitrogen. Swirl the flask to bring the residue onto the sides of the flask to aid in the removal of solvent and moisture. Dry 2 hr in an air oven at 90 ± 2°C. Cool in a desiccator and weigh. Break up the residue with a glass stirring rod, return to the oven for 0.5 hr, and weigh again. Repeat until constant mass is obtained.

CALCULATIONS

Calculate the uncorrected percentage of alcohol-soluble matter as follows:

$$\text{Alcohol-soluble matter (uncorrected), \%} = \frac{A}{B} \times 100$$

Where—

A = mass, g of residue, determined by subtracting the tared mass, g of the filtration medium from the mass, g of the filtration medium + sample.

B = mass of test portion, g, represented by the aliquot used.

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PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 0.72 at a 25% level.
2. Single determinations performed in two different laboratories should not differ by more than 1.55 at a 25% level.

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

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Alcohol-Insoluble Matter

DEFINITION

The residue from the alcohol-soluble determination (AOCS Official Method Dc 3a-59) is used for the total alcohol-insoluble determination.

SCOPE

Applicable to the determination of alcohol-insoluble matter in all fatty alkyl sulfates.

PROCEDURE

1. Place the filter paper containing the residue from the alcohol-soluble matter determination (AOCS Official Method Dc 3a-59) in an appropriate container with slip cover (alternately, a Gooch crucible, or a sintered-glass crucible, that has been tared and brought to constant weight, may be used). Dry the filter for 2 hr at 105 ± 2°C, cool in a desiccator, and weigh. Repeat the drying and weighing as necessary to bring to constant mass. Consecutive weighings should agree to within ± 0.1%.

CALCULATION

Calculate the percentage of alcohol-insoluble matter as follows:

$$\text{Alcohol-insoluble matter, \%} = \frac{A - B}{C} \times 100$$

Where—

A = mass, g of residue and filter
B = mass, g of filter
C = mass, g of test portion

PRECISION

1. Two determinations performed on the same test sample in one laboratory should not differ by more than 0.72 at a 25% level.
2. Single determinations performed in two different laboratories should not differ by more than 1.08 at a 25% level.

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Ester Sulfites

DEFINITION

The determination of ester SO_3 is dependent upon the hydrolysis of the sulfate ester and subsequent measurement of sulfuric acid (H_2SO_4) formed. If no interfering substances are present, the H_2SO_4 can be determined by titration. If other hydrolyzable substances are present in the sample, the H_2SO_4 must be precipitated with barium chloride ($BaCl_2$) after the removal of organic matter and weighed as barium sulfate ($BaSO_4$). In general, when SO_3 is to be determined by titration, the original sample should be used. When the gravimetric determination of SO_3 is necessary (i.e., as $BaSO_4$), the determination must be made on the alcohol-soluble portion of the sample.

SCOPE

Applicable to all fatty alkyl sulfates.

APPARATUS

1. Soxhlet flasks—250 mL, or Erlenmeyer flasks, 250 mL with ℨ ground-glass opening and heat resistant glass.
2. Reflux condenser—about 30 cm in length, water-cooled with ℨ ground-glass joint to fit flask.
3. Burettes—50 mL.
4. Filter paper—ashless, 90 mm dia.
5. Glass beads.
6. Pipets—100 mL.
7. Porcelain crucibles—about 30 mL.
8. Muffle furnace—equipped with pyrometer and suitable controls to maintain a temperature of 900–1,000°C. A Meeker burner may also be used, but is not recommended.

REAGENTS

1. Barium chloride solution—10%, prepared by dissolving 100 g of barium chloride ($BaCl_2 \cdot 2H_2O$) in distilled water and diluting to 1 L.
2. Hydrochloric acid (HCl), 1 M—accurately standardized. See AOCS Specification H 14-52.
3. Hydrochloric acid (HCl)—concentrated, sp. gr. 1.19 (see Notes, *Caution*).
4. Methyl orange indicator solution—0.1% in distilled water.
5. Silver nitrate ($AgNO_4$) solution—1%, prepared by dissolving 1 g of reagent grade $AgNO_3$ in 100 mL of distilled water.
6. Sodium hydroxide (NaOH), 1 M—accurately standardized. See AOCS Specification H 12-52.

PROCEDURE

1. Titration method—
   (a) Weigh 5 ± 0.005 g of the test sample into a 250 mL flask. When using alcohol-soluble material, weigh 1.000 ± 0.001 g of the test sample. Dissolve the sample in 50 mL of distilled water and add 2–3 drops of the methyl orange indicator. Titrate with 1 M HCl (weaker solutions may be used, but see Notes, 1) to the methyl orange end point, then add 35 mL of 1 M HCl in excess. Add several glass beads, attach a water-cooled condenser to the flask, and boil gently for at least 2 hr after foaming has ceased (or at least reduced to a minimum) and the sample appears to be completely hydrolyzed (see Notes, 2).

   Note—It is recommended that the flask be connected to a reflux condenser and the contents boiled for at least 0.5 hr, regardless of the remainder of the hydrolysis carried out. After the hydrolysis is complete, cool the solution and titrate with 1 M NaOH to the methyl orange end point.
2. Gravimetric method (in the presence of interfering substances)—
   (a) Weigh 1.000 ± 0.001 g of alcohol-soluble material (see Notes, 3, obtained as directed in AOCS Official Method Dc 3b-59) in a suitable 250 mL flask. Add 50–100 mL of water and 5–10 mL of concentrated HCl (sp. gr. 1.19). Hydrolyze as directed in Procedure, 1, a. Do not neutralize before hydrolysis. After the hydrolysis is complete, wash the contents of the flask while still hot into a 250-mL volumetric flask. Allow to cool to room temperature and dilute to the mark with distilled water at room temperature. Mix thoroughly and allow to settle. The fat layer should be above the volume mark on the flask. Pour off (or carefully pipet off) the fat layer and discard. The entire sample may be used by filtering off the fat and washing free of sulfates.
   (b) Pipet 100 mL of the aqueous solution into a 400 mL beaker and make neutral to methyl orange. Add 0.5 mL of 1 M HCl and hot water as necessary, to bring the volume to 175–200 mL. Bring to a boil and, while boiling, add 20 mL of the $BaCl_2$ solution. Continue boiling gently for a few minutes. Cover with a watch glass, place on a steam bath (or other suitable heat source), and keep the contents at a temperature of 70°C for 1 hr until the precipitate is well settled.

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(c) Decant the supernatant liquid through an ashless 90 mm filter paper. Finally, transfer the residue of barium sulfate ($BaSO_4$) in the beaker onto the filter paper by means of a stream of hot water from a wash bottle and the aid of a rubber-tipped spatula. Wash the precipitate and paper thoroughly with hot water until the washings, when tested with the 1% silver nitrate ($AgNO_3$) solution, are shown to be free from chlorides.

(d) Transfer the filter paper and precipitate to a tared porcelain crucible (see Notes, 4), with filter paper folded in such a way that the precipitate is inside. Ignite, uncovered, at a low temperature in a muffle furnace (see Notes, 5), or over a Meeker burner, to consume the filter paper without a flame. Burn off the carbon at as low a temperature as possible. After the carbon is burned off, bring to a higher temperature (about 900–1,000°C) until completely ignited. Allow to cool, but while still warm, transfer to a desiccator and bring to constant mass.

CALCULATIONS

1. Titration method (see Notes, 6)—

(a) Ester SO_3, $\% = \dfrac{(A - B)}{C} \times 8.0$

Where—

A = volume, mL of 1 M NaOH required for the titration of the sample
B = volume, mL of excess 1 M HCl added to the sample
C = mass of test portion, g

(b) If a 1 g test portion of alcohol-soluble material is used: Combined ester SO_3, % = (A –B) × 8.0

2. Gravimetric method—

Calculate the percentage of ester SO_3 as follows—

(a) $A_1 = \dfrac{34.3 \times B}{C}$

(b) $A_2 = \dfrac{A_1 \times D}{100}$

Where—

A_1 = % ester SO_3 on the basis of alcohol-insoluble matter
B = mass of $BaSO_4$, g
C = mass of test portion, g
A_2 = % ester SO_3 on the basis of the original material
D = % alcohol-soluble matter in the original material

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 0.14 at a 7% SO_3 level.
2. Single determinations performed in two different laboratories should not differ by more than 0.39 at a 7% SO_3 level.

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. Calculate the mL of the weaker solution in terms of the mL equivalent to 1 M HCl. If this is not done, the calculations will not give the correct answer.
2. Samples that are known to hydrolyze readily may be hydrolyzed overnight by setting the flask covered with watch glass on a steam bath.
3. When the gravimetric method is used, the alcohol-soluble portion of the sample must be used.
4. When determining the tared weight of the crucible, it should be treated in the same manner as for sample ignition.
5. If a muffle furnace is used, the sample should be placed in a cold muffle and the temperature raised slowly or the paper burned off over a burner before placing the crucible in a hot muffle. If a burner is used, care must be taken to avoid loss due to drafts.
6. The mL HCl mentioned are those used to hydrolyze the sample after neutralization to methyl orange. If the molarity of either the HCl or NaOH is other than 1.0 M, the factor of 8.0 may still be used provided the mL obtained are converted to the equivalent of either 1 M HCl or 1 M NaOH, using the relationship:

$$(\text{molarity}_1 \times \text{mL}_1) = (\text{molarity}_2 \times \text{mL}_2)$$

Alternately, a new factor must be calculated, based on the new molarity, to replace the factor of 8.0.

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Combined Alcohols

DEFINITION

This method determines total fatty alcohols and permits estimation of combined fatty alcohols.

SCOPE

Applicable to all fatty alkyl sulfates.

APPARATUS

1. Reflux condenser—either Allihn or Liebig type, water-cooled, at least 30 cm long.
2. Flask—round-bottom, either 250 or 300 mL, with ground-glass connection to the condenser.
3. Burettes—50 mL.
4. Miscellaneous equipment—250 mL extraction cylinder, separatory funnel, Soxhlet flask, and siphon.

REAGENTS

1. Water-alcohol wash solution—prepared by mixing 1 volume of 95% ethyl alcohol with 9 volumes of distilled water.
2. Hydrochloric acid (HCl)—concentrated, reagent grade, sp. gr. 1.19 (see Notes, *Caution*).
3. Hydrochloric acid (HCl), 1 M—accurately standardized. See AOCS Specification H 14-52.
4. Indicator solutions—
   (a) Phenolphthalein—1.0% in 95% ethyl alcohol.
   (b) Methyl orange—0.1% in distilled water.
5. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
6. Potassium hydroxide (KOH), 0.5 M—accurately standardized. See AOCS Specification H 15-52.

PROCEDURE

1. Weigh a test portion of the test sample estimated to yield 1–3 g of alcohol into a 250 mL round-bottom flask or other hydrolyzing flask. Dissolve in 50–60 mL of water. Add 2–3 drops of methyl orange indicator and carefully titrate with either standard HCl or standard alcoholic KOH solution to the methyl orange end point, depending on the starting pH of the solution (see Notes, 1).
2. Connect the flask to a reflux condenser, place on the steam bath overnight, and then boil at least 0.5 hr, or until the sample is completely hydrolyzed. The sample may be hydrolyzed directly (avoiding the overnight step), although a preliminary heating on the steam bath will reduce foaming.
3. When hydrolysis is complete, cool the flask and contents to about 50°C, drain the cooling water from the condenser, and wash down the condenser into the flask with a small amount of water, followed by a small amount of petroleum ether (see Notes, 2). Transfer the contents of the hydrolyzing flask into the extraction cylinder and extract with several 40–50 mL portions of petroleum ether. Collect the extracts in a separatory funnel. In making the first extraction, mix the solvent by gentle rocking, avoiding vigorous shaking to prevent foaming and forming an emulsion. Later extracts may be shaken thoroughly. Each extract should be shaken a minimum of 30 sec. A minimum of five extractions should be made.
4. Wash the combined petroleum ether extract with 30 mL portions of water-alcohol wash solution in order to remove any acid. Care should be taken in the water washing of the extracts to avoid any vigorous shaking to prevent the formation of a difficult-to-break emulsion. A few crystals of either sodium chloride or anhydrous sodium sulfate will aid in breaking an emulsion. The final wash solution should be neutral to methyl orange.
5. If the ester SO_3 is to be determined on this sample, the water washing should be combined with the acid solution for ester SO_3 by titration or gravimetric SO_3. Transfer the washed extract to a tared Soxhlet flask. Evaporate most of the solvent carefully, avoiding the use of air (nitrogen may be used). Continue to dry on top of the steam bath, without exposure to direct steam, until all solvent is removed. Run an evaporation blank, using a known mass of fatty alcohol and 200 mL of petroleum ether, each time the analysis is performed, to ensure that the technique used by the analyst will neither lose fatty alcohol nor leave petroleum ether in the total fatty alcohols weighed.

CALCULATIONS

Calculate the percentage of total and combined fatty alcohols as follows:

1. Total fatty alcohols, $\% = \frac{A}{B} \times 100$

   Where—

   A = mass of residue, g

   B = mass of test portion, g

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2. Combined fatty alcohols, % = D – E
   Where—
   D = % total alcohols
   E = % unsulfated material of free alcohols

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 0.69% at a 10% level.
2. Single determinations performed in two different laboratories should not differ by more than 1.049% at a 10% level.

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. The titration may be used to calculate the alkalinity.
2. Titration for ester SO_3 may be made at this point (see AOCS Official Method Dc 4-59).

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Alkalinity

DEFINITION

This method determines the alkalinity or acidity of fatty alkyl sulfates.

SCOPE

Applicable to all fatty alkyl sulfates free of added alkaline builder.

APPARATUS

1. Burette—50 mL.
2. Beaker—400 mL.

REAGENTS

1. Either hydrochloric acid (HCl) 1 M—or sulfuric acid (H_2SO_4), 0.5 M—accurately standardized. See AOCS Specifications H 14-52 or H 13-52, respectively. Hydrochloric acid is preferred. Standard acid weaker than 1 M may be used and titrations calculated to the equivalent in terms of 1 M solution.
2. Indicator solutions—
   (a) Phenolphthalein—1.0% in 95% ethyl alcohol.
   (b) Methyl orange—0.1% in distilled water.
3. Sodium hydroxide (NaOH), 1 M—accurately standardized. See AOCS Specification H 12-52. A 0.5 M NaOH solution may be used with proper corrections in the calculation.

PROCEDURE

1. Weigh 5 ± 0.001 g of the test sample into a 400 mL beaker. Dissolve in 100-150 mL of warm, distilled water (about 35°C). Add 2 drops of methyl orange indicator and 2 drops of phenolphthalein indicator and titrate as noted in Procedure, 2–4.
2. If the sample solution is acid to methyl orange, titrate with 1 M NaOH solution to the methyl orange end point.
3. If the sample solution is alkaline to methyl orange and acid to phenolphthalein, titrate with 1 M acid to the methyl orange end point.
4. If the sample solution is alkaline to phenolphthalein, titrate with 1 M acid first to the phenolphthalein end point and then to the methyl orange end point.

CALCULATIONS

Calculate the alkalinity to the appropriate basis as follows—

1. If the sample solution was acid to methyl orange (Procedure, 2), calculate the alkalinity to sodium bisulfate ($NaHSO_4$):

   $NaHSO_4$, % = $M_b \times 2.402$

   Where—
   M_b = volume, mL of 1 M NaOH solution required for titration of the sample (see Notes, 1)
2. If the test solution was alkaline to methyl orange and acid to phenolphthalein (Procedure, 3), calculate the alkalinity to sodium bicarbonate ($NaHCO_3$):

   $NaHCO_3$, % = $A \times 1.68$

   Where—
   A = volume, mL of 1 M acid required for titration of the sample
3. If the test solution was alkaline to phenolphthalein (Procedure, 4), calculate the alkalinity to either sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), or sodium bicarbonate ($NaHCO_3$):
   (a) If twice the amount of acid required for titration to the phenolphthalein end point, minus the amount of acid required for titration to the methyl orange end point, is more than zero, then:

   NaOH, % = $(2P - M_a) \times 0.80$
   Na_2CO_3, % = $2(M_a - P) \times 1.06$

   (b) If twice the amount of acid required for titration to the phenolphthalein end point, minus the amount of acid required for titration to the methyl orange end point, is zero or less than zero, then:

   Na_2CO_3, % = $2P \times 1.06$
   $NaHCO_3$, % = $(M_a - 2P) \times 1.68$

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Where—

M_a = volume, mL of 1 M acid required for titration of the sample to the methyl orange end point
P = volume, mL of 1 M acid required for titration of the sample to the phenolphthalein end point

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 0.14 at a 1% level.
2. Single determinations performed in two different laboratories should not differ by more than 0.22 at a 1% level.

NUMBERED NOTES

1. If other than either 1 M NaOH or 1 M acid is used, the mL of NaOH or acid used must be calculated to the equivalent mL in terms of either 1 M NaOH or 1 M acid, for the equations to be valid.

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Sodium Sulfate

DEFINITION

This method determines the sodium sulfate in fatty alkyl sulfates by precipitation of the sulfate with barium chloride.

SCOPE

Applicable to fatty alkyl sulfate free from added alkaline builder.

REAGENTS

1. Barium chloride ($BaCl_2$) solution—100 g/L. Dissolve 100 g of ($BaCl_2 \cdot 2H_2O$) in distilled or deionized water and dilute to 1 L.
2. Hydrochloric acid (HCl)—sp. gr. 1.19, concentrated hydrochloric acid (see Notes, *Caution*).
3. Sulfuric acid (H_2SO_4)—sp. gr. 1.84, concentrated sulfuric acid (see Notes, *Caution*).

PROCEDURE

1. Use the entire alcohol-insoluble portion of the test portion or weigh a suitable size part thereof into a 600 mL flask.
2. Add 200 mL of water to the beaker and neutralize to a methyl orange end point with HCl, adding about 0.5 mL in excess.
3. If the solution is not clear, filter and wash the filter paper until free of chlorides when tested with a dilute solution of silver nitrate. The volume of solution at this point should be about 250–300 mL.
4. Heat to boiling. While boiling, add all at once 15–20 mL of the $BaCl_2$ solution. Continue boiling for a few minutes and then cover with a watch glass. Place on the steam bath and keep the contents of the beaker hot (approximately 70–80°C) for 1 hr, or until the precipitate is well-coagulated and settled. Often, leaving the beaker on the steam bath overnight is an advantage.
5. Test the upper layer with a few drops of $BaCl_2$ solution to ensure that precipitation is complete. If so, decant the clear layer through a 9 mm ashless filter paper. Then transfer the barium sulfate ($BaSO_4$) from the beaker to the paper by means of a stream of hot water from a wash bottle and with the aid of a small spatula. Wash thoroughly with hot water until the washings, when tested with dilute $AgNO_3$ solution, show no chlorides.
6. Transfer the chloride-free residue and filter paper to a tared porcelain crucible. Ignite the paper at a low temperature so that the paper chars without flaming. Burn off the carbon at as low a temperature as possible with the crucible uncovered. After the carbon is burned, raise the temperature to about 800°C. If a muffle furnace is used, place the sample in a cold muffle furnace and raise the temperature slowly or burn off the paper before placing the crucible in a hot muffle furnace. If a burner is used, care must be taken to avoid loss due to drafts.
7. After ignition, moisten the precipitate with 2 drops of concentrated H_2SO_4 and reheat in the muffle furnace to remove the excess acid. Cool and weigh.

CALCULATIONS

Calculate the percentage of sodium sulfate as follows:

$$Na_2SO_4, \% = \frac{A \times 60.86}{B}$$

Where—

A = mass, g of $BaSO_4$

B = mass, g of test portion

$$60.86 = \text{ratio of } \frac{\text{Fw of } Na_2SO_4}{\text{Fw of } BaSO_4} \times 100$$

Fw = formula weight

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

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Unsulfated Material

DEFINITION

This method determines unsulfated fatty alcohols in fatty alkyl sulfates.

SCOPE

Applicable to all fatty sulfates.

APPARATUS

1. Extraction cylinder or Stokes flask, 250 mL—
   (a) The extraction cylinder has a 250 mL capacity, is glass-stoppered, about 39 mm dia and 355 mm high.
   (b) The Stokes flask is a 100 mL round-bottom flask sealed onto a 150 mL Erlenmeyer flask (see Fig. 1 in AOCS Official Method Da 8-48).
2. Separatory funnel—250 mL.
3. Soxhlet flask—250 mL.

REAGENTS

1. Ethyl alcohol—Denatured ethyl alcohol conforming to SDA Formulas 30 or 3A (see Notes, *Caution*).
2. Petroleum ether—(see Notes, *Caution*). See AOCS Specification H 2-41.
3. Potassium hydroxide (KOH) solution—50%. Dissolve KOH in an equal weight of water (see Notes, *Caution*).
4. Monosodium phosphate (NaH_2PO_4).

PROCEDURE

1. Use the alcohol-soluble matter from AOCS Method Dc 3a-59, by selecting an aliquot of suitable size; or weigh, to the nearest 1 mg, approximately 20 g of the test sample matter for pastes and liquids or 10 g for powder. For a dry product, dissolve in about 50 mL of hot alcohol and transfer to the 250 mL extraction apparatus, using sufficient alcohol to bring the volume to 80 mL. When the product is liquid or paste, wash from the weighing dish with hot alcohol until the volume of alcohol equals 80 mL. Add sufficient 50% KOH solution to make distinctly alkaline to phenolphthalein. Then continue washing with hot water. Add sufficient water to bring to the 150 mL mark. Cool well below boiling point of the solvent.
2. Extract with petroleum ether at least five times using 50 mL portions. Shake each extraction vigorously for 0.5 min. Allow to settle well between extractions. Addition of a few grams of NaH_2PO_4 crystals will eliminate emulsion difficulties (see Notes, 1). Draw off the petroleum ether layers into the separatory funnel. Add 30 mL of water and shake to mix the two layers. Draw off the water layer. Transfer a portion of the petroleum ether layer to a tared 150 mL Soxhlet flask and reduce the volume by evaporation in a gentle current of dry nitrogen on top of the steam bath, but do not dry. Complete the transfer of the petroleum ether layer and continue evaporation until no odor of petroleum ether remains. The Soxhlet flask must be removed at once as soon as the petroleum ether has evaporated. A white mist in the neck of the flask is often noticeable when the solvent has evaporated.

   Note—Prolonged heating must be avoided, because the residue is readily volatilized. Cool in a desiccator and weigh.

CALCULATIONS

Calculate the percentages of unsulfated material as follows—

1. If a portion of the alcohol soluble matter is used,

$$A = \frac{B \times C}{D}$$

Where—

A = % petroleum ether extract in the alcohol-soluble matter on the basis of the original sample

B = % alcohol-soluble matter

C = mass, g of residue

D = mass, g of test portion used (or mass, g of the aliquot used, on the basis of alcohol-soluble matter)

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2. If a fresh sample is used:

$$A = \frac{B}{C} \times 100$$

Where—

A = % petroleum ether extract of free alcohols in sample

B = mass, g of residue

C = mass, g of test portion

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 0.11 at a 1% level.
2. Single determinations performed in two different laboratories should not differ by more than 0.22 at a 1% level.

NOTES

Caution

Ethanol (ethyl alcohol) is flammable. Use an effective fume-removal device when heating or evaporating.

Petroleum ether is extremely flammable. Use an effective fume-removal device. Avoid static electricity.

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. Disodium phosphate should not be used if any kind of soaps are present.

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Sampling and Preparation of Sample

SCOPE

Applicable to paste, powder, or liquid alkylbenzene sulfonates.

APPARATUS

1. Trier for paste soap—a half-round metal tube 125-250 mm in diameter. The length may be 60-120 cm, depending upon the size of package to be sampled. One end is tapered to a point, the taper not to be more than 2.5 cm long. The other end is attached to a D- or T-shaped handle.
2. Sample tube for liquid product—a glass tube 90-125 mm i.d. The length may be convenient for the size of packages to be sampled. One end is constricted by a short taper (not more than 2.5 cm long) to about 60 mm. The other end is constricted sufficiently so that it can be used as a finger valve.
3. Airtight and watertight sample containers—about 2 kg capacity.
4. Airtight and watertight sample containers—sufficient capacity to hold samples taken as directed in Procedure, 1-3.
5. Spatula—with 25–30 cm blade.

PROCEDURE

Note—Place all laboratory samples in airtight and watertight containers, cover and seal to prevent any moisture change. Label completely for identification and send to the laboratory for preparation and analysis.

1. Powders and flakes packed in cans or cartons—one can or carton should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 22.7 kg (50 lbs). In the case of smaller containers, a can or carton should be taken at random from each lot of containers, totaling not more than 2,268 kg (5,000 lbs) or fraction thereof. The gross sample should in all cases consist of not less than three cans or cartons taken at random from separate containers. In the case of very large lots where the sample drawn as above will amount to more than 9.1 kg (20 lbs), the percentage of packages sampled should be reduced so that the amount drawn will not exceed 9.1 kg (20 lbs). The individual cans or cartons should be tightly wrapped at once in parafin paper and sealed by rubbing the edges with a heated iron. The inspector should accurately weigh each wrapped can or carton and record its mass and the date of sampling and weighing on the sample package. The wrapped cans or cartons should then be placed in an airtight container, filled as full as possible, then sealed, marked, and sent to the laboratory for testing. Samples should be kept cool until tested.
2. Powders and flakes in bulk—a grab sample of not less than 250 g (0.5 lb) should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 45.4 kg (100 lbs). In the case of smaller containers, a grab sample of not less than 250 g (0.5 lb) should be taken at random from each lot of containers totaling not more than 4,500 kg (10,000 lbs) or fraction thereof. The gross sample should, in all cases, consist of not less than three grab samples of 250 g (0.5 lb), each taken at random from separate containers. In the case of very large lots where the sample drawn as above will amount to more than 9.1 kg (20 lbs), the percentage of packages sampled should be reduced so that the amount drawn will not exceed 9.1 kg (20 lbs). The inspector must rapidly mix the gross sample and place in an airtight container, filled as full as possible, which then should be sealed, marked and sent to the laboratory for testing. Samples should be kept cool until tested.
3. Liquids—a sample of not less than 240 mL (0.5 pt) should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 37.9 L (10 gal). In the case of smaller containers, a sample of not less than 240 mL (0.5 pt) should be taken at random from each lot of containers totaling not more than 3,785 L (1,000 gal) or fraction thereof. The gross sample should, in all cases, consist of not less than three samples of 240 mL (0.5 pt), each taken at random from separate containers. Before drawing the sample from the container selected, the contents of the container should be thoroughly agitated. The inspector should thoroughly mix the gross sample, place it in clean, dry cans or bottles, which then should be sealed, marked, and sent to the laboratory for testing. Samples should be kept cool until tested.
4. Pastes
   (a) Pastes packed in cans or cartons of 2.27 kg (5 lbs), or less—one can or carton should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 22.7 kg (50 lbs). In the case of smaller containers, a can or carton should be taken at random from each lot of containers, totaling not more than 2,268 kg (5,000 lbs) or fraction thereof. The gross sample should in all cases consist of not fewer than three cans or cartons taken at random from separate containers. In the case of very large lots where the sample drawn as above will amount to more than 9.1 kg (20 lbs), the percentage of packages sampled should be reduced so that the amount drawn will not exceed 9.1 kg (20 lbs). The inspector should wrap, seal, mark, and send the samples to the laboratory for testing. Samples should be kept cool until tested.

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(b) Pastes packed in bulk—a "trier" sample (see Notes, 1) of not less than 250 g (0.5 lb) should be taken at random from not less than 1% of the shipping containers, provided each package contains not less than 22.7 kg (50 lbs). In the case of smaller containers a trier sample of not less than 250 g (0.5 lb) should be taken at random from each lot of containers totaling not more than 2,268 kg (5,000 lb), or fraction thereof. The gross sample shall, in all cases, consist of not less than three 250 g (0.5 lb) samples, each taken at random from separate containers. With very large lots where the sample drawn as noted in this section will amount to more than 4.5 kg (10 lb), the percentage of packages sampled should be reduced so that the amount drawn will not exceed 4.5 kg (10 lbs). The inspector should promptly place the gross sample in a clean, dry, airtight and watertight container, and then wrap, seal, mark, and send the sample containers to the laboratory for testing. Samples should be kept cool until tested.

PREPARATION OF TEST SAMPLES

1. Powders and flakes—minimizing exposure to air, rapidly disintegrate and mix the sample powder, flake, or chip product. If desired, quarter the sample to about 454 g (1 lb). Weigh immediately all test portions for analysis, preserving the remainder in an airtight container and store in a cool, dry place.
2. Liquids—no special preparation of a sample of liquid, other than a thorough mixing is necessary, unless it is received during cold weather, in which case it should be allowed to stand, before it is tested, for at least 1 h after the sample has come to room temperature (20-25°C). This is particularly true if the sample is to be tested for its lathering qualities (see Notes, 2).
3. Pastes—store in glass containers. If crystals separate during storage, warm carefully in a water bath.

NUMBERED NOTES

1. A trier sample is obtained by inserting the "trier" sampler (see Apparatus, 1) into the material to be sampled. A trier is a half-round cylinder about 13-20 mm in dia, 15-90 cm in length, pointed at one end and having a handle grip at the opposite end from the point. After insertion, the trier is turned two or three times and, upon removal, a core of the material being sampled is obtained.
2. If pastes or liquids are known to be acidic and decomposition of the sample can result from heating, the sample should be adequately labeled for precautionary treatment and carefully warmed to room temperature, or other maximum temperature, as agreed upon for mixing and sampling.

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Moisture by the Distillation Method

DEFINITION

This method determines moisture by distillation with an immiscible solvent.

SCOPE

This method is applicable to all alkylbenzene sulfonate products except those containing water-miscible volatile substances.

APPARATUS

1. Glass distillation apparatus—with ground-glass connections, constructed and assembled in accordance with the specifications shown in Figure 1.
   (a) Flask—short neck, round-bottom type, 1000 mL.
   (b) Condenser—water-cooled, the lower tip of which should be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established.
   (c) Trap—constructed of well-annealed glass. For greatest accuracy several trap sizes are allowable, depending upon the percentage of moisture expected, see Table 1. Traps made of well-annealed glass, constructed essentially as shown in Figure 1, and graduated to contain one of the following specified volumes at 20°C should be used:
   5 mL trap—Subdivided into 0.1 mL divisions with each 1 mL line numbered (5 mL at top). The error in any indicated capacity may not be greater than 0.05 mL.
   10 mL trap—subdivided from 1–2 mL divisions and from 2–10 mL in 0.2 mL division.
   25 mL trap—subdivided from 0–2 mL divisions and from 2–25 mL in 0.2 mL divisions.
   (d) Distillation may be better controlled by wrapping the tube which leads from the flask to the receiver with glass-fiber tape. It is advantageous to wrap the flask also, especially if there are drafts in the vicinity of the distillation apparatus.
   (e) Calibrate the receiver as follows: add an accurately measured portion, 1 ± 0.01 mL, of distilled water to 100 mL of toluene in the distillation flask. Conduct the distillation as described in Procedure, 2-5, and calculate as directed in Calculations. Cool the apparatus, add another mL of water, and repeat the distillation. Continue in this manner up to the capacity of the receiving tube.
2. Pipet—measuring type, 1.0 mL, graduated in 0.01 mL.
3. Heat source—usually an electric heating mantle provided with a suitable means of control.
4. Copper or nichrome wire—long enough to extend through the condenser with one end twisted into a spiral. The diameter of the spiral is such that it will fit snugly within the receiver and yet can be moved up and down.

REAGENTS

1. Xylene or toluene—reagent grade (see Notes, *Caution*). Use toluene for soaps containing 1% or more of glycerin. Saturate the xylene or toluene with water by shaking with a small quantity water and distill. Use the distillate for the determination.
2. Absolute ethyl alcohol (ethanol) (see Notes, *Caution*).

PROCEDURE

1. The entire apparatus must be completely clean. Clean with potassium dichromate-sulfuric acid cleaning solution to minimize the adherence of water droplets to the sides of the condenser and receiver. Rinse thoroughly and dry completely before using.
2. Transfer to the 1,000 mL flask, equipped with the size of the trap specified in Apparatus, an amount of test sample (see Notes, 1) according to the percentage of moisture expected, as shown in Table 2. Add immediately about 100 mL of xylene or toluene. Place a small, thin sheet of long-fiber, chemical-resistant glass wool on the surface of the toluene. The glass wool should be thoroughly dried in the oven and held in the desiccator before use.
3. Assemble the apparatus and fill the receiver with solvent by pouring through the condenser until the solvent begins to overflow into the distillation flask. Insert a loose cotton plug in the top of the condenser to prevent condensation of atmospheric moisture within the tube.
4. Heat the flask so that the distillation rate will be about 100 drops per minute. When the greater part of the water has distilled, increase the distillation rate to about 200 drops per minute and continue until the water level in the receiver remains unchanged for the 30 min.
5. Discontinue heating, dislodge any drops of water which may be adhering to the inside of the condenser tube or receiver with a wire spiral, and wash with about 5 mL of toluene or xylene.
6. Wash down condenser with 1 mL of absolute ethanol. Adjust the temperature of the distillate to 20°C and read the volume of water.

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7. Immerse the receiver in water at 25°C for at least 15 min or until the toluene or xylene layer is clear then read the volume of water.

CALCULATIONS

$$\text{Moisture, \%} = \frac{\text{volume, mL of water} \times 99.8}{\text{mass, g of test portion}}$$

PRECISION

1. Two single determinations performed in one laboratory should not differ by more than 0.28 at the 10% moisture level, or 0.83 at the 50% moisture level.
2. Single determinations performed at two different laboratories should not differ by more than 0.52 at the 10% moisture level, or 1.88 at the 50% moisture level.

NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation and skin absorption. The TLV is 100 ppm in air.

Xylene is flammable and a dangerous fire risk. The TLV is 100 ppm in air.

Ethanol (ethyl alcohol) is flammable.

A fume hood should be used at all times when using the solvents specified in this method.

NUMBERED NOTES

1. If sodium bicarbonate is present it will undergo partial decomposition under the conditions of this test and yield water and CO_2.

Table 1

Moisture expected, %	Size of trap, mL
0–5, incl.	5
Over 5–17, incl.	10
Over 17–30, incl.	10
Over 30–50, incl.	25
Over 50–70, incl.	25
Over 70–85, incl.	25

Table 2

Moisture expected, %	Mass of test portion to be used, g*
0–5, incl.	50 ± 5
Over 5–17, incl.	50 ± 5
Over 17–30, incl.	30 ± 5
Over 30–50, incl.	30 ± 5
Over 50–70, incl.	30 ± 5
Over 70–85, incl.	25 ± 5

*Weighed to the nearest 0.25 g.

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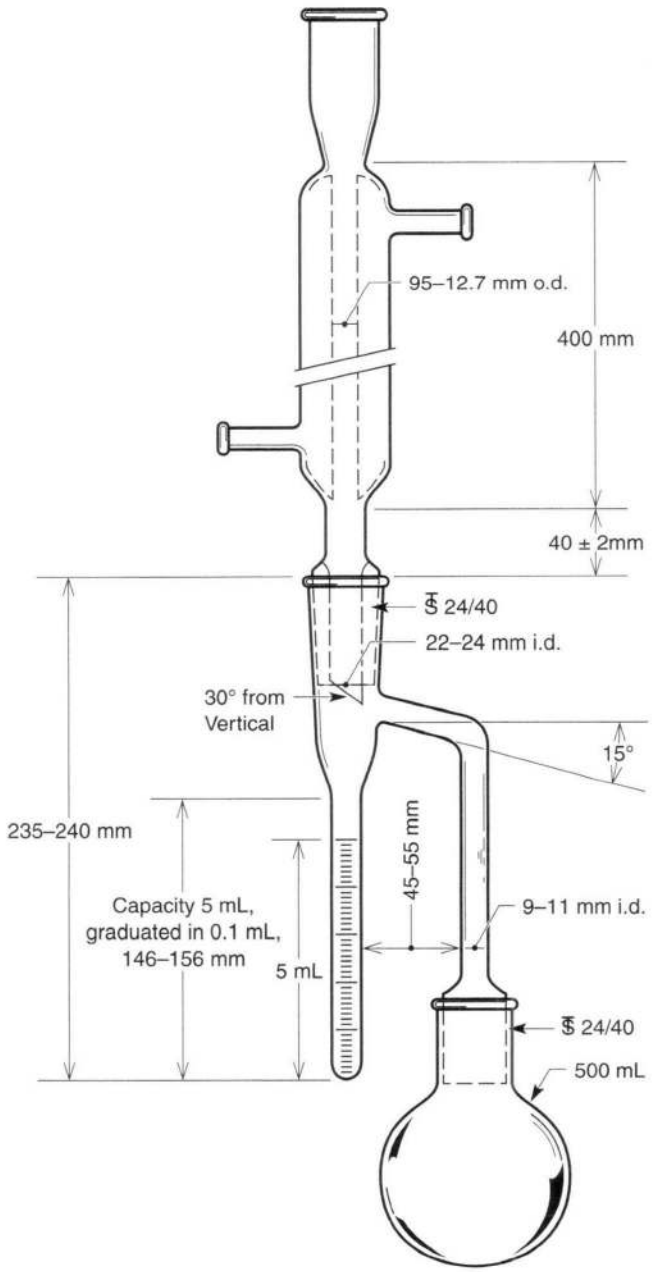

Figure 1. Moisture distillation apparatus.

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Sodium Alkylbenzene Sulfonate by Ultraviolet Absorption

DEFINITION

This method determines sodium alkylbenzene sulfonate by ultraviolet absorption measurement.

SCOPE

This method is applicable to commercial synthetic detergents and built detergent formulations that do not contain organic additives such as amides or alkyl aryl sulfonate such as those of xylene or toluene. Optical dyes and materials normally found in formulated detergents do not interfere; however, materials other than sulfonates that possess strong ultraviolet absorptions must be absent. The indicated sample size and aliquoting scheme is based on samples containing 30–40 percent of active ingredient. The method involves measurement of the ultraviolet absorption of the sample at the maximum absorbance point for sodium alkylbenzene sulfonate (224 nm) and at a selected background point of 270 nm in order to determine the amount of active ingredient present. The percentage of active ingredient is calculated from the above absorptions and data on the pure active ingredient.

APPARATUS

1. A spectrophotometer—equipped for liquid samples and for measurements in the ultraviolet (UV) region. The instrument should be capable of measuring UV absorbance with a repeatability of ± 0.5% (or better) on average at the 0.4 absorbance level in the spectral region between 220 and 270 nm. The spectral positions should be reproducible to within 0.1 nm.
2. Quartz absorption cuvettes—suitable for use in the UV spectral region, matched pairs of path length 1.000 ± 0.005 cm.
3. Volumetric flasks—250, 500, and 1,000 mL, with glass stoppers.
4. Pipets—Class A, 5 and 50 mL.

REAGENTS

1. Ethyl alcohol—95%. SDA Formulas 30 or 3A are permitted (see Notes, *Caution*).
2. A previously analyzed sample, solution or suitable reference sample—used to check the performance of the cuvettes and instrument. Protect such reference samples from contamination and renew periodically.

CALIBRATION AND STANDARDIZATION OF CUVETTES

1. The cuvettes in a pair, when filled with distilled water, should match within 1% transmittance at 224 nm. If not, calibrate the cuvettes as directed in the manufacturer's literature and use the correction factor obtained for each cuvette.
2. Before each day of operation, fill the quartz cuvettes with distilled water and make certain that the cuvettes match within 1% transmittance. This practice is necessary to check the cleanliness of the cuvettes. Clean cuvettes, if necessary, using dichromate cleaning solution, until the proper transmittance is obtained (see Notes, 1).

DETERMINATION OF THE ABSORPTIVITY VALUE

1. Determine the percentage of organic alcohol soluble matter in the sample under test in triplicate (see Notes, 2). The results should agree within 0.5%.
2. Conduct the spectrophotometric determination (using properly calibrated instrument and cuvettes as noted above) on samples containing the active ingredient expected in triplicate as described in Procedure and record the absorbance readings at 224 nm and 270 nm. The absorptivity value is based on commercially available dodecylbenzenes.
3. Calculate the absorptivity value, "a," for each spectrophotometric determination (as directed in Calculations, 1) and use the average of the three for the analysis of unknown samples.

PROCEDURE

1. Powders and solid samples—weigh 0.9-1.1 ± 0.0001 g of a representative test sample. Dissolve the sample in water (see Notes, 3) and dilute to 500 mL in a volumetric flask with water at room temperature. Mix well.
2. Slurry samples—weigh approximately 20 ± 0.001 g of a representative test sample. Add 50 mL of 95% ethyl alcohol and mix to disperse. Dissolve the test portion in water (see Notes, 3) and dilute to 1000 mL in a volumetric flask with water at room temperature. Mix well. Pipet a 50 mL aliquot into a 500 mL volumetric flask, dilute to volume with water at room temperature. Mix well.
3. Pipet a 5 mL aliquot of the test solution prepared in either Procedure, 1 or 2 into a 250 mL volumetric flask and dilute to volume with water at room temperature. Mix well.

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4. Using 1 cm cuvettes in the UV spectrophotometer, measure the absorbance at 224 nm and 270 nm vs. a water blank. There is danger of contamination of the sample with regular production detergent dust in laboratory air, therefore it is essential that the absorbance measurements be made immediately after the step noted in Procedure, 3. Contamination is evident when a high reading (>0.1 absorbance) at 270 nm is obtained. Discard such contaminated samples and start with a new test portion, prepared as noted in Procedure, 3 (see Notes, 4, 5).

CALCULATIONS

1. Absorptivity value, a:

$$\text{Absorptivity value, a} = \frac{(A_{224}) - (A_{270}) \times 100}{M_{as} \times b \times c}$$

Where—

A = observed absorbance, with correction for blank

b = cuvette length in cm

c = concentration of final dilution in g/1000 mL

M_{as} = average percent organic alcohol-soluble matter (see Notes, 3)

2. Percent active ingredient in sample (see Notes, 6):

$$\text{Active ingredient (sodium sulfonate), \%} = \frac{(A_{224}) - (A_{270}) \times 25}{W \times a} \times 100$$

Where—

A = observed absorbance, with correction for blank

W = mass, g of test portion used (Procedure, 1) or g test portion represented in aliquot used (Procedure, 2)

a = absorptivity value for the particular alkylbenzene sulfonate being determined (see Determination of Absorptivity Value)

NOTES

Caution

Ethyl alcohol (ethanol) is flammable. Use a fume hood when evaporating or heating this solvent.

NUMBERED NOTES

1. All glassware must be cleaned and rinsed with high quality distilled or deionized water just prior to use. *Do not dry* the rinsed glassware. This is necessary, especially after the first dilution, to avoid errors due to contamination.
2. The organic alcohol-soluble matter (active ingredient by difference) may be calculated from the results obtained on the original sample by the following determinations:
   (a) Moisture—Distillation Method, AOCS Official Method Da 2b-42.
   (b) Alcohol-insoluble matter—Alcohol-Soluble and Alcohol-Insoluble Matter, AOCS Official Method Da 3-48.
   (c) Sodium chloride—Potentiometric Determination of Chlorides, AOCS Official Method Db 7b-55.
   (d) Unsulfonated material—Neutral Oil, AOCS Official Method Dd 4-60.

   From the results obtained in the above determinations, the percentage of alcohol-soluble matter (sodium alkylbenzene sulfonate) may be calculated as follows:

$$\text{Alcohol-soluble matter (sodium alkylbenzene sulfonate), \%} = 100 \times (M_{ai} + B + C + D)$$

Where—

M_{ai} = % of alcohol-soluble matter (from AOCS Offical Method Da 3-48)

B = % of moisture (from AOCS Official Method Da 2b-42)

C = % of sodium chloride (from AOCS Official Method Db 7b-55)

D = % of neutral oil (from AOCS Official Method Dd 4-60)

3. The test portion must be completely dissolved if satisfactory results are to be obtained. Suggested procedures for dissolving the material are:
   (a) Transfer the weighed test portion to a volumetric flask and dilute to volume with water at room temperature. Carefully insert a Teflon™-covered stirring magnet and agitate vigorously on a magnetic stirrer for 15–20 min. Carefully invert the flask several times to ensure thorough mixing. Continue with the procedure, beginning with Procedure, 3.
   (b) Transfer the weighed test portion to a 600 mL beaker. Add 200 mL of water and place on a steam bath or hot plate for about 10 min with occasional stirring. Cool to room temperature, dilute to volume, and continue with the determination,

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beginning with Procedure, 3. A fine stream of water or a few drops of alcohol will aid in breaking any foam persisting in the neck of the volumetric flask.

4. The observed absorbance readings should be between 0.2 and 0.9, otherwise prepare a new sample. If high absorbance is not related to sample contamination, then a smaller size aliquot may be taken in Procedure, 3 (see Notes, 6).
5. When analyzing materials of doubtful origin, the absorption maximum at 224 nm should be checked. Measure the absorbance at 220, 224, 228, and 270 nm. The absorbance at 224 nm should be greater than the absorbance at either 220 nm or 228 nm. If the absorbance at 224 nm is not the maximum of all the absorbance measurements, alkylbenzene sulfonate is not responsible for the observed absorption and this spectrophotometric method is invalid.
6. The calculation as written is based on the diluting and aliquoting scheme as directed in Procedure and the absorptivity value "a" of products made from commercially available dodecylbenzenes. The calculation may be adapted for general use as follows:

$$\text{Active ingredient (sodium sulfonate), \%} = \frac{(A_{224}) - (A_{270}) \times 25}{c \times a} \times \frac{100}{a}$$

Where—

A = observed absorbance, corrected for blank

b = cuvette length in cm

c = concentration of final dilution in g/1000 mL

a = absorptivity value

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Neutral Oil (Unsulfonated Material) in Alkylbenzene Sulfonates

DEFINITION

This method determines unreacted or neutral oil in alkylbenzene sulfonates.

SCOPE

This method is applicable to powder, paste, and liquid alkylbenzene sulfonates and represents the unsulfonated material remaining in the sample.

APPARATUS

1. Separatory funnels—250 mL, with glass stopper.
2. Erlenmeyer flasks—wide-mouth, 250 mL.

REAGENTS

1. Acetone—reagent grade (see Notes, *Caution*).
2. Ethyl alcohol—95%. Freshly boiled ethyl alcohol, neutralized to the phenolphthalein end point. SDA Formula 30 or 3A ispermitted (see Notes, *Caution*).
3. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
4. Ethyl alcohol/water solution—1:1, v/v with distilled water.

PROCEDURE

1. Accurately weigh a test portion equivalent to 3–5 g of active ingredient and transfer to a 250 mL extraction cylinder. Add 50 mL of neutralized ethyl alcohol and 50 mL of water. Shake to disperse.
2. Add 50 mL of petroleum ether (see Notes, 1), stopper the separatory funnel (see Notes, 2), and shake up and down vigorously for 1 min. Remove the stopper and wash it and the internal wall of the funnel with a small volume of petroleum ether. Allow to stand until the emulsion breaks and both layers are completely clear.
3. Draw off the lower (alcohol-water) layer into a dry, 250 mL wide-mouth Erlenmeyer flask (see Notes, 2). Rinse the stem of the funnel with a small volume of the 1:1, ethyl alcohol-water solution and allow the stem of the funnel to drain well.
4. Drain the petroleum ether layer remaining in funnel into a tared 250 mL wide-mouth Erlenmeyer flask (see Notes, 2).
   Note—The flask should be treated as noted in Procedure, 9 and 10.
5. Transfer the alcohol-water layer from the extraction back into the separatory funnel, using 50 mL of petroleum ether to rinse the contents of the Erlenmeyer flask containing the alcohol-water layer.
6. Remove the petroleum ether by evaporation in a fume hood, using a controlled temperature water bath at 60–70°C (see Notes, 3).
7. Repeat the extraction noted in Procedure, 2–5, so that a total of five extractions are performed on the alcohol-water solution of the sample. Add each of the petroleum ether extracts to the original Erlenmeyer flask and evaporate as noted in Procedure, 6 (see Notes, 4).
8. Evaporate as much water as possible from the petroleum ether in the combined extracts, using the water bath. Remove the flask from the water bath when the petroleum ether no longer distills from the flask.
9. Place the flask on a steam bath and remove the last traces of solvent with the aid of a stream of clean, dry nitrogen. Do not leave in the water bath for any time longer than absolutely necessary to volatilize the petroleum ether. This should require no more than 5 min (see Notes, 5).
10. Add 2 mL of acetone and repeat Procedure, 9. Wipe the flask with a clean cloth, cool in a desiccator and weigh.

CALCULATION

Calculate the results as follows:

$$\text{Neutral oil (unsulfonated materials), \%} = \frac{A}{B} \times 100$$

Where—

A = mass, g of residue, obtained by subtracting the tared weight of the Erlenmeyer flask (see Procedure, 4) from the mass of the Erlenmeyer flask + residue (see Procedure, 10).

B = mass, g of test portion

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NOTES

Caution

Acetone is a flammable solvent. It should not be used near an open flame. The use of a fume hood is recommended when using this solvent.

Ethyl alcohol (ethanol) is flammable. Use a properly operating fume hood when heating or evaporating.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Use of diethyl ether yields high results, because of increased solubility of sulfones and related compounds.
2. The same separatory funnel and Erlenmeyer flasks should be used throughout the determination.
3. The use of a regulated, steam-heated water bath is recommended. An electrically heated sand bath may be used in place of the water bath, provided certain safety measures are carried out. The switch control should be some distance away from the hot plate and place of work. A sand bath of such size as to fit on the top of the hot plate should be provided. The temperature of the sand bath should be automatically maintained between 60 and 70°C by a suitable regulator.
4. The petroleum ether should be evaporated to a small volume after each extraction, permitting the total volume of petroleum ether from the next extraction to be held conveniently in the 250 mL flask. Do not attempt to evaporate more than 125 mL of petroleum ether extract at one time, as losses due to spattering may occur.
5. If the operation described in Procedure, 9 requires more than 5 min, either too great a volume of solvent remained in the flask from the procedure described in Procedure, 8, or the stream of nitrogen was of insufficient volume, or both conditions were abnormal. Take the proper steps to correct either or both sources of error.

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AOCS Standard Procedure Dd 5-92

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Determination of Sodium Linear Alkylbenzene Sulfonate (LAS) Color

DEFINITION

This method determines the color of LAS by visible absorption spectrophotometry.

SCOPE

This method covers the measurement of color of sodium linear alkylbenzene sulfonate (LAS), using platinum-cobalt (Pt-Co) standards and a Klett-Summerson colorimeter. The instrument is calibrated each day with dilutions of platinum-cobalt standard no. 500 APHA. The Pt-Co number for the sample is calculated using an average response factor determined from measurement of dilutions of the Pt-Co standard.

APPARATUS

1. Klett-Summerson Photoelectric Colorimeter—industrial model 900-3 Klett; available from Manostat Corp., 519 8th Avenue, New York, NY 10018 USA. The instrument being used should be calibrated according to the manufacturer's literature (see Notes, 1).
2. Klett blue filter—no. KS-42, available from Klett Manufacturing Co. (see Notes, 2).
3. Colorimeter cuvettes—matched pairs, with a light path length of 4 cm.
4. Volumetric flasks—100 mL.
5. Volumetric pipets—to deliver 5.00 mL, 40.00 mL, and 75.00 mL.

REAGENTS

1. Pt-Co standard no. 500 (available from Fisher Scientific Co., Fisher catalog no. SP 120-1).

PROCEDURE

1. Prepare the following dilutions (see Notes, 3) of Pt-Co. *Caution*—solution is acidic. Use volumetric pipets to measure the specified volumes, then dilute to the mark with deionized water in 100 mL volumetric flasks:

mL	Pt-Co no.
5.00	25
40.00	200
75.00	375

2. Prepare a 5% active material solution of sodium alkylbenzene sulfonate in distilled water (see Notes, 4). If sample has stratified, it may be necessary to stir and/or gently warm (at about, but no higher than, 50°C) to obtain a homogenous aliquot. Dilute an appropriate amount of material (e.g., for a 40% w/w active material sample, 12.5 g) to 100 mL using a volumetric flask (Apparatus, 4). A few drops of ethanol may be used to eliminate any foam near the mark. Mix the solution well and, if necessary, filter to obtain a clear solution for color readings.
3. Before turning on the instrument, insert the blue Klett no. KS-42 filter (Apparatus, 2) between the lamp housing and the instrument.
4. Turn on the instrument, make certain the lamp is on, and wait several minutes for the instrument to equilibrate. Fill the sample cuvette (Apparatus, 3) with distilled water and zero the instrument (see Notes, 1).
5. Remove the cuvette and replace the blank solution with the Pt-Co standard no. 25 prepared in Procedure, 1. Insert the cuvette and measure the solution absorbance. It is not necessary to adjust the zero setting for successive color readings using the same filter or wavelength setting.
6. Repeat Procedure, 5, for the Pt-Co standard nos. 200 and 375 prepared in Procedure, 1.
7. Determine an average response factor for the standard solutions as follows:

   (a) For each standard: $RFi = \frac{\text{Pt-Co no.}}{Ai}$

   Where—

   RFi = response factor for a particular standard "i"
   Ai = Klett measurement for a particular standard "i"

   (b) The average response factor: $RFx = \frac{\text{Sum of RFs}}{n}$

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Where—
RFx = average response factor
n = number of standards

8. Perform the calibration described in Procedure, 5–7, each day that color is to be determined.
9. Using Procedure, 5, measure the absorbance of the 5% LAS solution prepared in Procedure, 2.

CALCULATIONS

1. To obtain the Pt-Co number for the sample

$$\text{Pt-Co no. of sample} = A_{sample} \times RFx$$

Where—
A_{sample} = observed Klett measurement of sample (Procedure, 9)
RFx = average response factor for standards (Procedure, 7)

2. To convert Pt-Co number to Klett, if desired—

$$Klett_{sample} = \text{Pt-Co no.}_{sample} \times 0.633$$

Where—
$\text{Pt-Co no.}_{sample}$ = Pt-Co no. for the sample (obtained from Calculations, 1)

PRECISION

1. Repeatability limit—the absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r), which can generally be inferred by linear interpolation from Table 1.
2. Reproducibility limit—the absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R), which can generally be inferred by linear interpolation from Table 1.

NUMBERED NOTES

1. Specific instructions for the operation of the Klett-Summerson 900-3 can be found in the manual supplied with each instrument.
2. The CIE color scale values for the Klett no. KS-42 blue filter are:
   Instrument used: Pacific Scientific Spectrogard
   Observer angle: 2°
   CIE L*a*b*

   Where—

L* =	2.67	x =	0.1622
a* =	84.63	y =	0.0136
b* =	–74.50	T% =	30

   CIE values were determined using a Pacific Scientific Spectrogard instrument, currently manufactured by BYK Gardner. The original manufacturer of the Spectrogard instrument was HIAC-ROYCO, division of Pacific Scientific Co., 11801 Tech Road, Silver Spring, MD 20904 USA.
3. When properly sealed and stored, these standards are stable for at least one year (see References, 1, 2).
4. The percentage of active material is based on a methylene blue titration (see References, 3).

REFERENCES

1. American Society for Testing and Materials, Philadelphia, PA, Method D-1209.
2. Scharf, W., K. H. Ferber and R. G. White, *Mater. Res. Stand.* 6:302 (1966).
3. American Society for Testing and Materials, Philadelphia, PA, Method D-3673-89.

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Table 1
Statistical results of the interlaboratory test carried out in 1990–91 by the AOCS Technical Committee on Soaps and Detergents[a].

	Sample[b]				
	C	B	AC	BC	A
Number of labs after removal of outliers	9	9	9	9	9
n	64	62	64	61	62
Outliers	0	0	0	3	2
Mean	105	171	93	141	63
Repeatability, S_r	7.53	10.54	7.39	7.75	7.78
RSD_r	7.15	6.15	7.93	5.49	12.42
r ($2.8 \times S_r$)	21.09	29.51	20.69	21.70	21.78
Reproducibility, S_R	12.88	14.32	12.13	15.45	10.01
RSD_R	12.22	8.35	13.02	10.94	15.98
R ($2.8 \times S_R$)	36.07	40.10	33.96	43.26	28.03

[a]Nine laboratories participated, each obtaining four test results for each sample. Statistical results evaluated in accordance with ISO 5725-1986.
[b]5% active material solutions.

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Sampling

SCOPE

Applicable to crude glycerin in drums, tank cars, or trucks (see Notes, 1).

APPARATUS

1. Drum sampler—consisting of two brass tubes, one fitting closely inside the other (see Fig. 1). The ports in the sides of the tubes are located and spaced in such a way that when the tubes are turned to "Open," a continuous slot is formed which permits taking a continuous section of the product throughout the drum or similar container. Ports are also cut at the bottom of both tubes to permit drawing a portion of loose salt or sediment which may have collected at the bottom. A pointer and dial are constructed at the handle end of the sampler, so arranged as to indicate whether the ports are open or closed. A tube 2.5 cm in diameter will withdraw about a 280 g sample from a 415 L drum and a 1.58 cm tube will withdraw about 140 g.
2. Core tank sampler—for tank cars or trucks, consisting of a hollow tube of about 5.08 cm uniform internal diameter and a length sufficient to take a test portion through the entire depth of liquid. The bottom is closed by a tight valve or cock, which when open, allows an unrestricted opening the full size of the tube and which does not leak when closed. This valve is operated by a rod from the top and is so constructed that a test sample can be taken within 1.27 cm of the bottom of the tank (see Fig. 2).
3. Dipper—with handle of about .55 L capacity for taking flow samples during loading (see Notes, 2).
4. Cans—with tight lids for mixing-tube or core samples or accumulating flow samples.
5. Airtight containers—such as pint bottles, for preservation of mixed test samples.

PROCEDURE

Caution—Glycerin is very hygroscopic and must at all times be carefully protected from moisture changes caused by contact with moist air.

1. Drums and similar containers—store in a warm place for several hours prior to sampling, if possible. Mix contents of drum by rolling through several complete revolutions. Introduce the drum sampling tube vertically through bung opening with ports closed until it reaches the bottom. Turn the handle to "Open" position long enough for the sampler to fill and then close. Withdraw the sampler and wipe off surplus glycerin clinging to outside of tube with suitable laboratory tissue wipe. Empty into mixing can. Sample each drum unless all parties concerned have agreed to a lesser number. Keep the mixing can covered at all times except when mixing the combined test samples. Rapidly mix contents of can by stirring thoroughly with a clean wooden paddle and immediately transfer a portion of the glycerin to an airtight container, such as a pint bottle. Label final test sample with complete identification.
2. Tank cars or trucks—lower the core sampler for tank cars or trucks vertically through the tank contents, with the bottom closure wide open, at a uniform rate slow enough to permit the tube to fill as it is lowered, i.e., the tube should not be lowered faster than the glycerin can flow in, so that the levels inside and outside of the sampler are nearly equal at all times. This precaution must be carefully observed. Allow sampler to rest on bottom of tank for a short time before closing valve. Withdraw the sampler slowly, wiping off glycerin clinging to outside of tube with suitable laboratory tissue wipe, and discharge into mixing can. Repeat this operation if necessary to obtain a test sample of sufficient size. Keep the mixing can covered at all times except when actually discharging test samples into it and when mixing the combined withdrawals. Rapidly mix contents of can by stirring thoroughly with a clean wooden paddle and immediately transfer a portion of the glycerin to an airtight container, such as a pint bottle. Label final test sample with complete identification.
3. Flow sample at time of loading—

   Note—Tank cars and other large shipments, by mutual agreement between buyer and seller, may be sampled as follows:

   The total gross test sample to be accumulated should not be less than 0.1% by weight of the total shipment sampled, and must represent not less than 25 withdrawals of uniform size by dipper from the flowing glycerin at the discharge end of the pipe as the glycerin enters the tank car. Determine the approximate number of such withdrawals to be taken and space them as uniformly as possible throughout the loading operation. Wipe the dipper immediately after each withdrawal or rinse and dry completely before reusing, otherwise the glycerin remaining on the dipper may absorb a significant quantity of moisture. Accumulate withdrawals in a can large enough to permit thorough mixing and keep can covered tightly at all times except when actually discharging test samples into it and when mixing the combined withdrawals. Rapidly mix contents of can by stirring thoroughly with a clean wooden paddle and immediately transfer a portion of the glycerin to an airtight container, such as a pint bottle. Label final test sample with complete identification.

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NOTES

1. The foregoing sampling instructions in Procedure 1 and 2 are applicable only to crude glycerin from which little or no salt or other sediment has separated. If much salt has been deposited, there is no satisfactory method of taking a representative test sample. This is particularly true of tank car shipments. These are best sampled by taking a flow sample at the time of loading. It is strongly recommended that such an agreement be made a part of the purchase contract between buyer and seller.
2. Experience has shown that a continuous drip or flow sample taken from a petcock in the loading line is not a very satisfactory or reliable method. Small orifices clog easily and, unless they are under almost continuous observation, there may be intervals during which there is no flow through them. For this reason, the dipper method with sampling at pre-determined intervals is preferred and recommended.

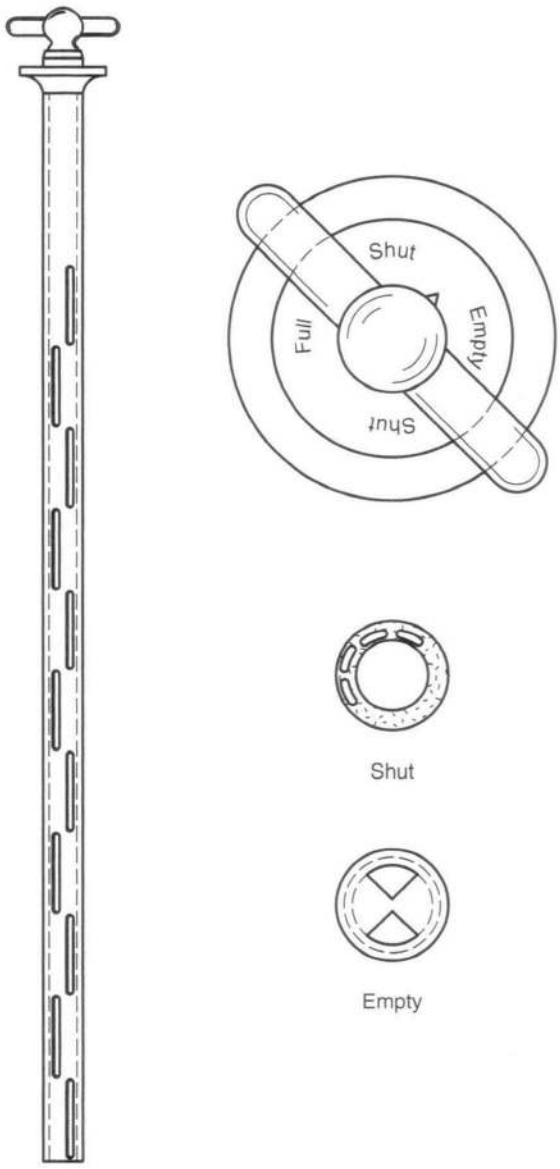

Figure 1. Drum sampler for glycerin

Figure 2. Core sampler for glycerin.

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Glycerol, Sodium Periodate Oxidation Method

DEFINITION

This method determines glycerol and other polyalcohols containing three or more adjacent hydroxyl groups. The glycerol reacts with the sodium periodate in an acid solution, forming aldehydes and formic acid. The latter is a measure of the glycerol in the sample. The sodium periodate method replaces the acetin method and the dichromate method because it has been found to be more accurate and more specific for the determination of glycerol as well as being simpler and more rapid.

SCOPE

Applicable to any glycerin solution, but particularly useful in analyses of samples containing oxidizable organic impurities and certain hydroxylated compounds which interfere with the dichromate and acetin procedures. Trimethylene glycol and other polyalcohols in which the hydroxyl groups are not adjacent do not react at room temperature. This method is not applicable to samples that contain, in addition to glycerol, polyhydric compounds with three or more adjacent hydroxyl groups, unless these compounds are removed prior to testing with the sodium periodate reagent.

APPARATUS

1. Burette—50 mL, calibrated to meet National Institute of Testing specifications. Delivery time must not be less than 90 sec for 50 mL.
2. Meniscus magnifier—which will permit reading the burette to 0.01 mL.
3. Pipet—50 mL, drainage time standardized so that the same quantity will be delivered to sample and blanks.
4. Variable speed stirrer—electrical or mechanical, with glass stirrer.
5. Electrometric pH meter assembly with glass electrode—adjust pH meter to read pH 4.0 when electrodes are placed in the standard buffer solution.
6. Beakers—600 mL.
7. Volumetric flask—250 mL.

REAGENTS

1. Sodium periodate solution—
   (a) Dissolve 60 g of sodium meta periodate ($NaIO_4$), ACS grade, in distilled water containing 120 mL of 0.1 N sulfuric acid, total volume 1 L. Do not heat to dissolve sodium periodate. If the solution is not clear, filter through a sintered-glass filter. Store in a dark, glass-stoppered bottle (see Notes, 1). The acidity of this reagent may change slowly with time so a blank must be run each day when analyses are made. If sodium meta periodate does not dissolve, it is not reagent grade quality and anew supply must be obtained.
   (b) Test for quality—pipet 10 mL of sodium periodate solution into 250-mL volumetric flask, dilute to mark and mix thoroughly. To 0.5–0.6 g of reagent grade glycerine in 50 mL of distilled water add 50 mL of diluted sodium periodate solution with the 50-mL pipet (Apparatus, 3). Prepare a blank using only 50 mL of distilled water. Allow to stand 30 min, add 5 mL of hydrochloric acid, 10 mL of 15% potassium iodide solution and swirl to mix. Allow to stand 5 min and then add 100 mL of distilled water. Titrate with 0.1 N sodium thiosulfate solution, shaking continuously until the yellow color has almost disappeared. Add 1–2 mL of starch indicator and continue titration, adding the thiosulfate solution slowly until blue color has just disappeared. The sodium periodate is satisfactory when the titration of solution containing glycerol divided by titration of the blank is between 0.750 and 0.765.
2. Sodium hydroxide solution, 0.1250 N—accurately standardized with potassium acid phthalate using phenolphthalein indicator (see AOCS Specification H 12-52).
3. Sodium hydroxide solution—about 0.05 N.
4. Sulfuric acid solution, 0.2 N (see Notes, *Caution*).
5. Potassium acid phthalate—primary standard grade, dried at 105°C.
6. Phenolphthalein indicator solution—1% in 95% alcohol.
7. Bromthymol blue indicator solution—0.1% in distilled water prepared by dissolving 0.1 g of dry indicator in 16 mL of 0.01 N NaOH by grinding indicator with the alkali in a mortar. Transfer to a 100-mL volumetric flask, dilute to volume with distilled water and mix thoroughly.

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8. Ethylene glycol solution—mix 1 volume of ethylene glycol (bp 195–197°C) and 1 volume of distilled water.
9. Sodium thiosulfate solution, 0.1 N—accurately standardized.
10. Hydrochloric acid—sp. gr. 1.19, ACS grade (see Notes, *Caution*).
11. Starch indicator solution—make a homogeneous paste of 10 g of potato starch in cold distilled water. Add to this 1 L of boiling distilled water, stir rapidly and cool. Salicylic acid (1.25 g/L) may be added to preserve the indicator. If long storage is required, the solution must be kept in a refrigerator at 4–10°C. Fresh indicator must be prepared when the end point of the titration fails to be sharp from blue to colorless.
12. Potassium iodide (KI) solution—dissolve 150 g KI in distilled water and make to 1 L.
13. Standard buffer solution may be obtained commercially.

PREPARATION OF SAMPLE

Samples containing separated salt, sediment or suspended matter must be warmed and thoroughly mixed to ensure uniform distribution. Some sediment tends to cling to the bottom of the container and the viscosity of glycerin retards rapid dispersion. Any mixing procedure which will secure positive distribution is satisfactory. Careful preparation of sample is necessary to obtain an accurate analysis.

PROCEDURE

1. Make all weighings accurately and rapidly. This is conveniently done from a weighing pipet into the 600-mL beaker, when the sample contains more than 20% glycerol. When the sample contains less than 20% glycerol, it may be weighed into a tared dish and then washed into the 600-mL beaker with distilled water. When the sample is less than 50 mL, dilute to 50 mL with distilled water. For the most accurate results, the sample tested must contain between 0.32 and 0.50 g of glycerol. The approximate weight of sample to be taken for analysis, based upon glycerol content, is given in Table 1. Smaller samples give slightly higher and less precise results.

   When analyzing salt with approximately 2.5% glycerol, use an 18 g sample and dissolve in 100 mL of distilled water. When salt contains 1% or less glycerol, use a 25 g sample and dissolve in 150 mL of distilled water. When the glycerol content is not known, make a single preliminary test using the amount specified for 100% glycerol. From the results of this test the proper sample weight can be selected quite accurately.
2. Add 5–7 drops of bromthymol blue indicator to the sample in the 600-mL beaker and acidify, with 0.2 N H_2SO_4, to a definite green or greenish yellow color (see Notes, 2). Neutralize with 0.05 N NaOH, to the indicator end point, sharp green to blue transition. When the color of the solution interferes (see Notes, 3) with the detection of the color change of the indicator, use the pH meter and adjust to pH 8.1 ± 0.1.
3. At this point, prepare a blank containing 50 mL of distilled water, but no glycerol, and carry through simultaneously with the sample in an identical manner, using the indicator to adjust the pH before adding the sodium periodate solution.
4. Add 50 mL of sodium periodate solution (see Notes, 4) with a pipet, swirl gently to ensure thorough mixing, cover with a watch glass and allow to stand for 30 minutes at room temperature in the dark. If the room temperature exceeds 35°C (95°F), both the reagent and solution of the sample must be cooled to 35°C or less before mixing and must be maintained at 35°C until the titration.
5. At the end of the 30 min period, add 10 mL of 50% ethylene glycol-water solution and allow to stand 20 min.
6. Dilute to approximately 300 mL and titrate, using a pH meter to determine the end point, pH 6.5 ± 0.1 for the blank and 8.1 ± 0.1 for the sample. When approaching the end point, add the titrant a drop (or part of a drop) at a time. Read burette to 0.01 mL and record the reading.

CALCULATIONS

$$\text{Glycerol, \%} = \frac{(S - B) \times N \times 9.209}{W}$$

Where—

S = volume, mL of NaOH solution to titrate sample
B = volume, mL of NaOH solution to titrate blank
Note—B must not be less than 4.5 mL.
N = normality of NaOH
W = mass of sample, g

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PRECISION

Collaborative studies have shown that the following 95% confidence limits may be expected:

	Glycerol present at levels of 70–95%	30–50%	10%
Duplicate determinations on the same day by a single analyst should not differ more than approximately	0.20	0.15	0.10
Single determinations in two different laboratories should not differ more than approximately	0.60	0.45	0.30
Averages of duplicate determinations in two different laboratories should not differ by more than approximately	0.56	0.42	0.28

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended.

NUMBERED NOTES

1. Cork must not be used to stopper any flask used in this determination or in any other way allowed to come in contact with any of the sodium periodate solution used in or for the analysis.
2. If the crude glycerine being analyzed contains more than 0.1% carbonate, expressed in terms of Na_2O, add 0.2 N H_2SO_4 until the pH is 3.0 or less, heat to boiling and cool to room temperature. Then neutralize with 0.05 N NaOH, as directed in Procedure 2, using bromthymol blue indicator.
3. If the sample contains an appreciable amount of buffering material, the pH must be adjusted with a pH meter to the end point to which the sample will be titrated. In some instances, buffering action may be sufficiently great to prevent good reproducibility of results.
4. The presence of a sufficient excess of sodium periodate in a given test can be checked as follows—test the sample using 75% of the sample size used in the original test. This can be done when running duplicate tests by weighing one approximately 75% of the weight of other. If analysis of the smaller sample agrees with that of the larger sample, there is sufficient excess sodium periodate in both cases. If the analysis of the smaller sample shows a higher glycerol content, above what would be expected by experimental error, then there was not sufficient excess of sodium periodate for oxidation of the larger sample and another test must be run using a smaller sample. When the glycerol content is unknown, weigh the amount specified for 100% glycerol, and, from the results of this test, select the proper size sample, taking into account that the results will tend to be high because the sample weighed is smaller than that required for the most accurate results.

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Table 1
Weight of sample to be taken for analysis based upon the glycerol content.

Glycerol in products to be analyzed, %	Sample to be weighed, g
100 or less	0.40–0.53
90 or less	0.45–0.55
80 or less	0.50–0.60
70 or less	0.55–0.75
60 or less	0.65–0.85
50 or less	0.80–1.00
40 or less	0.90–1.30
30 or less	1.20–1.80
20 or less	1.80–2.60
10 or less	4.00–5.00
5 or less	7.0–11.0
2.5 or less	16.0–20.0
1.0 or less	40.00
0.5 or less	80.00

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AOCS Official Method Ea 6-94

Revised 2013

Determination of Crude Glycerin, Titrimetric Method

DEFINITION

This method determines the glycerol content of industrial glycerins. Glycerol is oxidized by sodium periodate in a strongly acidic medium. The formic acid produced by the reaction is titrated with a standard solution of sodium hydroxide. The glycerol content is expressed as a percentage (m/m).

SCOPE

Applicable to industrial glycerins. This method is not applicable in the presence of organic compounds containing more than two hydroxyl groups on adjacent carbon atoms, such as sugars. These compounds may also give rise to formic acid and will interfere in the glycerol determination.

APPARATUS

1. Conical flasks—500 mL.
2. Beakers—tall form, 600 mL.
3. Buret—50 mL, with the jet drawn out so as to allow delivery of 30 drops per mL and calibrated to meet National Institute of Testing specification.
4. Volumetric flasks—500 mL and 1000 mL.
5. Pipet—50 mL graduated.
6. pH meter—fitted with a glass electrode (see Notes, 1).
7. Stirrer—preference should be given to a magnetic stirrer. In this case, check that it does not influence the pH meter

REAGENTS

1. Distilled water—or water of equivalent purity, free from carbon dioxide.
2. Ethylene glycol (1,2-ethanediol, free from glycerol)—solution in distilled water (Reagents, 1) 1:1 (v/v).
3. Sulfuric acid—aqueous solution, approximately 0.05 M.
4. Sodium hydroxide—aqueous solution, approximately 0.05 M.
5. Sodium metaperiodate—neutral, titer at least 98% (m/m), aqueous acid solution 60 g/L (see Notes, 2).
6. Sodium formate—aqueous solution, approximately 1 M.
7. Sodium hydroxide—0.1 M aqueous solution, accurately standardized.
8. Sodium hydroxide—0.125 M aqueous solution, accurately standardized, free from carbon dioxide (see Notes, 3).
9. Potassium hydrogen phthalate $C_6H_4(COOK)(COOH)$—0.05 M aqueous solution (10.12 g/L), pH 4.00 at 20°C (see Notes, 4).
10. Disodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)—0.01 M aqueous solution (3.81 g/L, pH 9.22 at 20°C) (see Notes, 4).
11. Phenolphthalein—5 g/L solution in 95% (v/v) ethanol.
12. Phenol red.

PROCEDURE

Note—As the presence of carbon dioxide can introduce errors, it is advisable to cover the vessels containing the test solutions with a clock glass during the standing periods and also to avoid enriching the carbon dioxide content of the laboratory atmosphere by other operations carried out at the same time.

1. In the case of alkaline laboratory samples or laboratory samples giving a tarry precipitate on acidification, the procedure indicated under the particular case is to be used.
2. If the approximate glycerol content is not known, it should be determined by a preliminary test on 0.50 g of the laboratory sample.
3. If the glycerin content is < 75%: Weigh, to the nearest 0.0001 g, 0.30–0.50 g of the glycerol. Transfer quantitatively to a beaker (Apparatus, 2) for determination.
4. If the glycerin content is > 75%: Weigh, to the nearest 0.0001 g, 5 ± 0.1 g of the test sample. Transfer quantitatively to a volumetric flask (Apparatus, 4). Dilute to 500 mL with distilled water (Reagents, 1) and mix. Transfer by pipet, 50 mL of this solution to a beaker (Apparatus, 2) for determination.
5. Particular case: Transfer the test portion prepared as indicated if the glycerol content is not known, to an appropriate vessel. Dilute to 50 mL, if necessary. In the presence of 2 drops of the phenolphthalein solution (Reagents, 11), neutralize just to decoloration by means of the sulfuric acid solution (Reagents, 3). Add a 5 mL excess of the sulfuric acid solution (Reagents, 3). Fit a reflux condenser. Bring to a boil and maintain boiling for 5 min. Allow to cool. If necessary, filter

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and wash with distilled water (Reagents, 1). Quantitatively transfer the solution obtained to a beaker (Apparatus, 2) for determination.

6. Blank Test: Carry out, at the same time as the determination and under the same conditions, a blank test without the test portion using the same quantities of reagents and diluting water as for the determination.
7. Determination: Dilute the test sample in the beaker (Apparatus, 2) with distilled water (Reagents, 1) to a volume of about 250 mL. Insert the glass electrode and start stirring with the stirrer (Apparatus, 7). Neutralize by means of the sodium hydroxide solution (Reagents, 4) to pH 7.9 ± 0.1 checked by the pH meter (Apparatus, 6). With a pipet (Apparatus, 5) add 50 mL of the sodium metaperiodate solution (Reagents, 5). Mix with moderate agitation. Cover the beaker with a clock glass and allow to stand for 30 min in the dark at a temperature not exceeding 35°C.
8. Add 10 mL of the ethylene glycol solution (Reagents, 2). Mix. Allow to stand for 20 min in the dark at a temperature not exceeding 35°C.
9. Add 5.00 mL of the sodium formate solution (Reagents, 6) with a graduated pipet (Apparatus, 5). With the buret (Apparatus, 3), titrate the acidity present with the sodium hydroxide solution (Reagents, 8) to a pH 7.9 ± 0.2, checked with the pH meter (Apparatus, 6).

CALCULATIONS

1. The glycerol content, expressed as a percentage (m/m), is given by the formula:

$$(V_1 - V_2) \times T \times 0.0921 \times \frac{100}{m}$$

Where—

V_1 = number of mL of the sodium hydroxide solution (Reagents, 8) used for the determination
V_2 = number of mL of the sodium hydroxide solution (Reagents, 8) used for the blank test
T = exact molarity of the sodium hydroxide solution (Reagents, 8) used
m = mass, in g, of glycerin subjected to the reaction with the sodium metaperiodate solution (Reagents, 5)

NOTES

1. The pH meter should be checked by means of two buffer solutions (Reagents, 9 and 10).
2. Verification of the sodium metaperiodate neutrality: In a conical flask (Apparatus, 1), dissolve 5 g of sodium metaperiodate in 150 mL of distilled water (Reagents, 1). Add 5 mL of ethylene glycol (Reagents, 2). Allow to stand in the dark and protected from atmospheric carbon dioxide for 30 min. Under these conditions, it should be possible to neutralize the solution in the presence of phenol red (Reagents, 12) with a sodium hydroxide (Reagents, 7) volume of less than 1 mL and preferably less than 0.2 mL.

   Prepare the acidic solution by weighing, to the nearest 0.1 g, 60 g of sodium metaperiodate (Reagents, 5). Dissolve, in the cold sodium metaperiodate, with 120 mL sulfuric acid (Reagents, 3), diluted in 500 mL of water (Reagents, 1). Transfer to a volumetric flask (Apparatus, 4), dilute to 1000 mL with water (Reagents, 1) and mix. If necessary, filter through a sintered glass filter.

   Verify the solution acidity—The volume of sodium hydroxide (Reagents, 8) used during the blank test (Procedures, 6) should be not less than 4.5 mL, corresponding to the acidity required for reaction. Store the solution in a dark glass flask fitted with a ground glass stopper.
3. Standard acid—until a primary standard acid can be established internationally, it is recommended to interested parties that the standardization of the solution (Reagents, 8) be carried out by means of the same batch of primary standard.
4. Use standard materials especially prepared for pH measurements.
5. This method is identical to IUPAC 3.121, which is also the same as ISO 2879-1975. In addition, the APAG uses ISO 2879-1975.

IUPAC = International Union of Pure and Applied Chemistry
ISO = International Organization for Standardization
APAG = Association Européenne des Producteurs d'Acides Gras

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AOCS Official Method Ea 7-95

Reapproved 2009

Determination of the Relative Density 20/20 in Air and of the Conventional Mass per Unit Volume of Industrial Glycerins

DEFINITION

This method describes the determination of the relative density 20/20 in air and of the conventional mass per unit volume of industrial glycerins (see Notes, 1). The relative density 20/20 in air of glycerins is the ratio of the mass in air of a given volume of the glycerins at 20°C (see Notes, 2). The conventional mass per unit volume of glycerins is the quotient of its mass in air by its volume. It is expressed in g/mL or in kg/m^3. The conventional mass per unit volume of a given substance will vary with temperature.

SCOPE

This method is suitable for any solution of glycerol in water. The relative density 20/20 in air is intended primarily for purified glycerins. The method may not be applicable if substances other than water and glycerin are present.

The general procedures are as follows: (a) Calibration of the pycnometer by measurement of the conventional mass of the water contained in the pycnometer at a given temperature; measurement of the conventional mass of the test sample contained in the pycnometer at the same temperature; calculation of the relative density 20/20 in air by dividing the conventional mass of the test sample by the conventional mass of an equal volume of water when both of them are measured at 20°C. (b) Calculation of the volume of the pycnometer at a given temperature from the conventional mass of the water contained in the pycnometer at that temperature and from the table of conventional mass per unit volume of water at different temperatures (Table 1). (c) Calculation of the conventional mass per unit volume of the test sample at a given temperature by dividing the conventional mass of the test sample at that temperature by the volume of the pycnometer at the same temperature. (d) If desired, conversion of the relative density in air to glycerol content (see Notes, 1) Replaces Ea 7-50.

APPARATUS

1. Pycnometer, Gay-Lussac type or any other suitable pycnometer, preferably 50 mL capacity. The pycnometer should be equipped with a thermometer graduated in 0.1°C with a range of from 10°C to 40°C (see Figure 1). The thermometer should be calibrated against a National Institute of Standards and Technology (NIST) certified thermometer over the working range of the thermometer.
2. Water bath regulated at the required temperature ± 0.1°C.

REAGENTS

1. Water, freshly distilled and then cooled to a temperature slightly below the temperature at which the determination will be made.
2. Acetone.

PROCEDURE

1. Carry out each weighing to the nearest 0.1 mg.
2. Bring the substance (either water or glycerin) to a temperature slightly below the temperature at which the determination will be made.

   Caution—During all testing operations, the test portion must be protected against loss of moisture by evaporation or gain of moisture due to the hygroscopic nature of glycerin.
3. Empty the pycnometer (Apparatus, 1), rinse with acetone (Reagents, 2) and dry. After 2–3 minutes weigh it empty, but with stopper and thermometer in place.
4. Fill the pycnometer with the substance (either water or glycerin), avoiding the formation of bubbles (see Notes, 3). Insert the thermometer. Rinse the outside with distilled water (Reagents, 1). Immerse the pycnometer in the water-bath (Apparatus, 2) at the temperature of measurement to a depth half way up the ground portion of the neck, leaving it there for 30 minutes.
5. While keeping the pycnometer in the water-bath, remove any excess liquid from the neck so that the level is exactly flush with the end of the capillary tube in the neck of pycnometer.
6. Place the stopper on the neck of the pycnometer and remove the pycnometer from the water-bath and hold it for 1 minute in a stream of water at a temperature below the required temperature. Dry the outside of the pycnometer with a lint-free cloth. Leave to cool if necessary and wipe with a cloth lightly soaked in acetone.

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7. After 2–3 minutes, reweigh the pycnometer. Calculate, by difference, the conventional mass of substance (water or glycerin) it contains (see Notes, 4).

CALCULATIONS

1. Relative density 20/20 in air—The relative density 20/20 in air ($d_{20/20}$) is given by the formula:

$$d_{20/20} = \frac{m_2}{m_1}$$

Where—

m_1 = the conventional mass in g, of water (Reagents, 1) contained in the pycnometer (Apparatus, 1) at 20°C as determined by Procedure;

m_2 = the conventional mass, in g, of glycerin contained in the pycnometer at 20°C, as determined by Procedure.

2. Mass per unit volume at temperature *t*—The mass per unit volume at temperature *t*(p_t), g/mL, is given by the formula:

$$p_t = \frac{m_3}{V_t}$$

Where—

m_3 = the conventional mass of glycerin, in g, contained in the pycnometer at temperature *t*, as determined by Procedure;

V_t = the volume of the pycnometer at temperature *t*, in mL (see Notes, 5).

Note—If the mass per unit volume has to be expressed in kg/m^3 (SI units), the value found has to be multiplied by 10^3.

3. Express the results to three decimal places.

PRECISION

1. Repeatability—Duplicate determination on the same day by the same analyst using the same apparatus should not differ by more than 0.0002.
2. Reproducibility—Single determinations made in different laboratories should not differ by more than 0.0005.

NOTES

1. The relative density 20/20 in air is in current use for the qualification of purified glycerins. It is also a measure of glycerol content. To convert the relative density in air to glycerol content, the Table of Bosart and Snoddy has been included. (Table 2). The conventional mass per unit volume of glycerins is used to convert volumes to masses (not taking into account buoyancy due to air).
2. The expressions "relative density" (*d*) and "specific gravity" are equivalent. When the term "relative density" includes, for example, the notation 20/4, the measurement has been carried out at 4°C for the reference substance (water) and at 20°C for the substance to be examined (glycerins). The conversion $d_{20/20}$ to $d_{20/4}$ may be calculated using the following formula:

$$d_{20/4} = d_{20/20} \times 0.99823$$

3. Water and test sample must be free of air bubbles. If bubbles are present, they may be removed by centrifuging.
4. If it is necessary to carry out numerous determinations, it may be practical not to calibrate the pycnometer at each measurement, but merely to check that its mass and volume remain constant with time. The volume of the pycnometer (Apparatus, 1) at a given temperature t (V_t), in mL, is given by the formula:

$$Vt = \frac{mt}{p_t(H_2O)}$$

Where—

m_1 = the conventional mass, in g, of water (Reagents, 1)
contained in the pycnometer (Apparatus, 1) at the temperature *t*, as determined in Procedure;

p_t (H_2O) = the conventional mass per unit volume of water at temperature *t*, in g/mL

5. If the pycnometer is calibrated at 20°C, the volume the pycnometer at *t*°C can be calculated using the formula:

$$V_t = V_{20}[1 + \alpha(t - 20)]$$

Where—

V_t = the volume of the pycnometer at *t*°C, in mL;

V_{20} = the volume of the pycnometer at 20°C, in mL;

α = the cubic expansion coefficient of the glass of the pycnometer, in °C^{-1}.

Some values of α:

Boro-silicate glass D50: 10×10^{-6} °C^{-1}
Boro-silicate glass G20: 16×10^{-6} °C^{-1}
Sodium glass: $15 - 30 \times 10^{-6}$ °C^{-1}

REFERENCE

1. *Industrial and Engineering Chemistry 19:*506 (1927), Bosart and Snoddy Table.

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Table 1
Water, conventional mass per unit volume p_t (H_2O) at temperature *t*. (From Draft Proposal ISO/DP 6883-Doc. ISO/TC34/SC 11N 186)

t(°C)	p_tH_2O(g/mL)	*t*(°C)	p_tH_2O(g/mL)	*t*(°C)	p_tH_2O(g/mL)
15	0.99807	32	0.99399	49	0.98746
16	0.99791	33	0.99367	50	0.98701
17	0.99774	34	0.99334	51	0.98656
18	0.99756	35	0.99300	52	0.98609
19	0.99737	36	0.99265	53	0.98563
20	0.99717	37	0.99230	54	0.98515
21	0.99696	38	0.99193	55	0.98467
22	0.99674	39	0.99156	56	0.98419
23	0.99651	40	0.99119	57	0.98369
24	0.99627	41	0.99080	58	0.98319
25	0.99601	42	0.99041	59	0.98269
26	0.99575	43	0.99001	60	0.98213
27	0.99548	44	0.98960	61	0.98166
28	0.99520	45	0.98918	62	0.98114
29	0.99491	46	0.98876	63	0.98061
30	0.99461	47	0.98834	64	0.98007
31	0.99431	48	0.98790	65	0.97953

Table 2
Specific Gravity vs. Glycerol Concentration by Bosart and Snoddy[a]

Glycerol	Apparent specific gravity				True specific gravity			
	15/15°C	15.5/15.5°C	20/20°C	25/25°C	15/15°C	15.5/15.5°C	20/20°C	25/25°C
100	1.26557	1.26532	1.26362	1.26201	1.26526	1.26501	1.26331	1.26170
99	1.26300	1.26275	1.26105	1.25945	1.26270	1.26245	1.26075	1.25910
98	1.26045	1.26020	1.25845	1.25685	1.26010	1.25985	1.25815	1.25655
97	1.25785	1.25760	1.25585	1.25425	1.25755	1.25730	1.25555	1.25395
96	1.25525	1.25500	1.25330	1.25165	1.25495	1.25470	1.25300	1.25140
95	1.25270	1.25245	1.25075	1.24910	1.25240	1.25215	1.25045	1.24800
94	1.25005	1.24980	1.24810	1.24645	1.24975	1.24950	1.24780	1.24615
93	1.24740	1.24715	1.24545	1.24380	1.24710	1.24685	1.24515	1.24350
92	1.24475	1.24450	1.24280	1.24115	1.24445	1.24420	1.24250	1.24085
91	1.24210	1.24185	1.24020	1.23850	1.24185	1.24155	1.23985	1.23825
90	1.23950	1.23920	1.23755	1.23585	1.23920	1.23895	1.23725	1.23560
89	1.23680	1.23655	1.23490	1.23320	1.23655	1.23625	1.23460	1.23295
88	1.23415	1.23390	1.23220	1.23055	1.23390	1.23360	1.23195	1.23025
87	1.23150	1.23120	1.22955	1.22790	1.23125	1.23095	1.22930	1.22760
86	1.22885	1.22855	1.22690	1.22520	1.22860	1.22830	1.22670	1.22495
85	1.22620	1.22590	1.22420	1.22255	1.22595	1.22565	1.22395	1.22230
84	1.22355	1.22325	1.22155	1.21990	1.22330	1.22300	1.22120	1.21965
83	1.22090	1.22055	1.21890	1.21720	1.22060	1.22030	1.21860	1.21695
82	1.21820	1.21790	1.21620	1.21455	1.21795	1.21765	1.21595	1.21430
81	1.21555	1.21525	1.21355	1.21190	1.21520	1.21500	1.21330	1.21165
80	1.21290	1.21260	1.21090	1.20925	1.21265	1.21235	1.21065	1.20900
79	1.21015	1.20985	1.20815	1.20655				
78	1.20740	1.20710	1.20540	1.20380				
77	1.20465	1.20440	1.20270	1.20110				
76	1.20190	1.20165	1.19995	1.19840				

continued

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Table 2 *(continued)*

Glycerol	Apparent specific gravity				True specific gravity			
	15/15°C	15.5/15.5°C	20/20°C	25/25°C	15/15°C	15.5/15.5°C	20/20°C	25/25°C
75	1.19915	1.19890	1.19720	1.19565	1.19890	1.19865	1.19700	1.19540
74	1.19640	1.19615	1.19450	1.19295				
73	1.19365	1.19340	1.19175	1.19025				
72	1.19090	1.19070	1.18900	1.18755				
71	1.18815	1.18795	1.18630	1.18480				
70	1.18540	1.18520	1.18355	1.18210	1.18515	1.18495	1.18330	1.18185
69	1.18260	1.18240	1.18080	1.17935				
68	1.17985	1.17965	1.17805	1.17660				
67	1.17705	1.17685	1.17530	1.17385				
66	1.17430	1.17410	1.17255	1.17110				
65	1.17155	1.17130	1.16980	1.16835	1.17135	1.17110	1.16960	1.16815
64	1.16875	1.16855	1.16705	1.16560				
63	1.16600	1.16575	1.16430	1.16285				
62	1.16320	1.16300	1.16155	1.16010				
61	1.16045	1.16020	1.15875	1.15735				
60	1.15770	1.15745	1.15605	1.15460	1.15750	1.15725	1.15585	1.15445
59	1.15490	1.15465	1.15325	1.15185				
58	1.15210	1.15190	1.15050	1.14915				
57	1.14935	1.14910	1.14775	1.14640				
56	1.14655	1.14635	1.14500	1.14365				
55	1.14375	1.14355	1.14220	1.14090	1.14360	1.14340	1.14205	1.14075
54	1.14100	1.14080	1.13945	1.13815				
53	1.13820	1.13800	1.13670	1.13540				
52	1.13540	1.13525	1.13395	1.13265				
51	1.13265	1.13245	1.13120	1.12995				
50	1.12985	1.12970	1.12845	1.12720	1.12970	1.12955	1.12830	1.12705
49	1.12710	1.12695	1.12570	1.12450				
48	1.12440	1.12425	1.12300	1.12185				
47	1.12165	1.12150	1.12030	1.11915				
46	1.11890	1.11880	1.11760	1.11650				
45	1.11620	1.11605	1.11490	1.11380	1.11605	1.11595	1.11475	1.11365
44	1.11345	1.11355	1.11220	1.11115				
43	1.11075	1.11060	1.10950	1.10845				
42	1.10800	1.10790	1.10680	1.10575				
41	1.10525	1.10515	1.10410	1.10310				
40	1.10255	1.10245	1.10135	1.10040	1.10240	1.10235	1.10125	1.10030
39	1.09985	1.09975	1.09870	1.09775				
38	1.09715	1.09705	1.09605	1.09510				
37	1.09445	1.09435	1.09335	1.09245				
36	1.09175	1.09165	1.09070	1.08980				
35	1.08905	1.08895	1.08805	1.08715	1.08895	1.08885	1.08790	1.08705
34	1.08635	1.08625	1.08535	1.08455				
33	1.08365	1.08355	1.08270	1.08190				
32	1.08100	1.08085	1.08005	1.07925				
31	1.07830	1.07815	1.07735	1.07660				
30	1.07560	1.07545	1.07470	1.07395	1.07550	1.07535	1.07460	1.07385
29	1.07295	1.07285	1.07210	1.07135				
28	1.07035	1.07025	1.06950	1.06880				
27	1.06770	1.06760	1.06690	1.06625				
26	1.06510	1.06500	1.06435	1.06370				

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Table 2 *(continued)*

Glycerol	Apparent specific gravity				True specific gravity			
	15/15°C	15.5/15.5°C	20/20°C	25/25°C	15/15°C	15.5/15.5°C	20/20°C	25/25°C
25	1.06250	1.06240	1.06175	1.06115	1.06240	1.06230	1.06165	1.06110
24	1.05985	1.05980	1.05915	1.05860				
23	1.05725	1.05715	1.05655	1.05605				
22	1.05450	1.05455	1.05400	1.05350				
21	1.05200	1.05195	1.05140	1.05095				
20	1.04935	1.04915	1.04880	1.04840	1.04930	1.04925	1.04875	1.04830
19	1.04685	1.04660	1.04630	1.04590				
18	1.04435	1.04430	1.04380	1.04345				
17	1.04180	1.04160	1.04135	1.04100				
16	1.03930	1.03925	1.03885	1.03850				
15	1.03675	1.03675	1.03635	1.03605	1.03670	1.03670	1.03630	1.03600
14	1.03425	1.03420	1.03390	1.03360				
13	1.03175	1.03170	1.03140	1.03100				
12	1.02920	1.02920	1.02890	1.02865				
11	1.02670	1.02665	1.02640	1.02620				
10	1.02415	1.02415	1.02395	1.02370	1.02415	1.02410	1.02390	1.02370
9	1.02175	1.02175	1.02155	1.02135				
8	1.01935	1.01930	1.01915	1.01900				
7	1.01690	1.01690	1.01675	1.01660				
6	1.01450	1.01450	1.01435	1.01425				
5	1.01210	1.01205	1.01195	1.01185	1.01205	1.01205	1.01195	1.01175
4	1.00965	1.00965	1.00955	1.00950				
3	1.00725	1.00725	1.00700	1.00710				
2	1.00485	1.00485	1.00480	1.00475				
1	1.00240	1.00240	1.00240	1.00235				

[a] References, 1.

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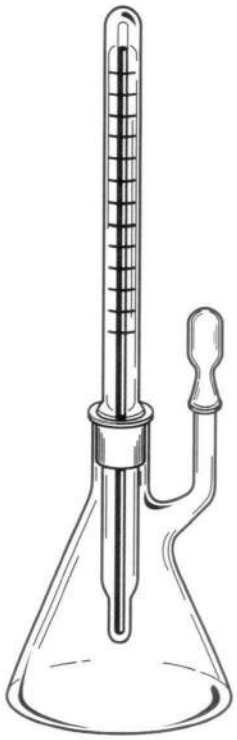

Figure 1. Specific gravity bottle.

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AOCS

AOCS Official Method Ea 8-58

Reapproved 2009

Moisture, Karl Fischer Volumetric Method

DEFINITION

This method determines the actual water content of glycerol by reaction of the water with a pyridinesulfur dioxide solution and titration with an iodine-methanol solution.

SCOPE

Applicable to distilled glycerin. May be used with any glycerol-water solution which contain no interfering substances.

APPARATUS

1. See Notes, *Recommendations*, and Numbered Notes, 2
2. Bottle-burette assemblies—each consisting of a storage bottle and 50 mL burette with side filling tube, properly vented and protected against moisture absorption with drying tubes filled with Drierite, indicating type (see Numbered Notes, 3). The delivery tip of each burette is equipped with a stopper so that the solution is protected from the moisture in the air during the measurement of Solution B (Reagents, 7) and titration with Solution A (Reagents, 6). The rubber stopper on the burette tip is fitted with a capillary tube which allows air to escape from the flask when titrating, thus assuring a continuous and even flow from the burette without loosening the flask from the stopper to relieve pressure which would otherwise develop.
3. Erlenmeyer flasks—narrow neck, 3,000 mL, with rubber stoppers. Insert a drying tube filled with Drierite in the stopper fitting the 3,000 mL flask.
4. Flask—150 mL, Soxhlet extraction type with rubber stoppers to fit opening.
5. Drying tubes—straight form. Insert glass fiber in the bulb of the tube, fill with Drierite almost to the top and cover with glass fiber.
6. Neoprene tubing—6.35 mm i.d., length about 1 m.
7. Glass tubing—76 mm o.d., wall thickness 1 mm., length about 30 cm.
8. Bottle—glass-stoppered, 2,000 mL.
9. Weighing bottle—glass-stoppered dropping bottle or Lunge weighing bottle.

REAGENTS

1. Drierite—indicating type, 8 mesh.
2. Pyridine—anhydrous, reagent grade.
3. Sulfur dioxide—anhydrous, liquefied gas in cylinder.
4. Methanol—anhydrous, reagent grade.
5. Iodine—reagent grade.
6. Solution A, Iodine-Methanol Solution—dissolve 60 g of iodine, reagent grade, in 1,000 mL of methanol, protecting so that solution does not absorb moisture from any outside source.
7. Solution B, Pyridine-Methanol-Sulfur Dioxide Solution—Karl Fischer reagent (see Numbered Notes, 1), stabilized single solution, available commercially or prepared as follows. Add 900 mL of anhydrous pyridine and 900 mL of anhydrous methanol to a 3 L Erlenmeyer flask. Stopper and cool to 4°C. Tare on a scale or balance (sensitive to 0.5 g), add 180 g sulfur dioxide at a fairly rapid rate by discharging from a cylinder through a tube into and beneath the surface of the solution. If the solution becomes hot, interrupt the addition of sulfur dioxide, close flask with a stopper fitted with a drying tube, and cool the solution. When the addition of sulfur dioxide is complete, close flask with a rubber stopper fitted with a drying tube.

   Caution—Do not cool at this or any subsequent stop while tightly stoppered.

   Note—In case the SO_2-pyridine solution is to be used immediately after preparation, cool if necessary, under tap water. Do not cool below room temperature.

CALIBRATION OF SOLUTION A

1. Run 25.0 mL of Solution B into a *clean dry flask.*
2. Attach flask to stopper on delivery tube of burette filled with Solution A. Shake with a rotary motion and titrate with Solution A to first appearance of a red color that persists after 10 sec shaking. Add Solution A in increments of 0.2 mL when approaching the end point. Remove flask and stopper.
3. Add 1 drop of water from a weighed weighing bottle to the solution. Adjust the meniscus in the buret to the "0" mark and titrate with Solution A as directed in Calibration, 2.

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4. Weigh the weighing bottle and determine weight of the water added. Repeat Calibration, 3 and 4 using titrated solution from Calibration, 3.
5. Water factor for Solution A (g water titrated per mL) = $\frac{\text{mass, g of water from Calibration, 4}}{\text{volume, mL solution from Calibration, 3}}$

   Use the average of two single determinations as the factor if they agree within 0.0001. If the factors do not agree within 0.0001, repeat calibration.

PROCEDURE

Caution—Glycerin absorbs water very rapidly so that the sample must be protected from the atmosphere at all times.

1. Measure from burette 25 mL of Solution B (Reagents, 7) into a *clean, dry flask.*
2. Attach flask to stopper on delivery tube of burette filled with Solution A. Shake with a rotary motion and titrate with Solution A to first appearance of a red color that persists after 10 sec shaking. Titrate 0.2 mL at a time when nearing the end point (yellow to red).
3. Remove flask and stopper.
4. Weigh into the flask a test portion of such size that from 10–40 mL of Solution A will be required. This flask must not be left unstoppered except when introducing the test portion. Table 1 is a convenient guide of the size of test portion to weigh and the accuracy with which the test portions are weighed.
5. Immediately stopper the flask after introducing the test portion and keep closed until ready for the next step.
6. Fill the burette with Solution A and adjust meniscus to "0", attach flask to stopper on delivery tube and titrate. Add reagent slowly, but steadily, rotating flask just enough to keep the color dispersed.
7. Titrate 0.2 mL at a time when nearing the end point (yellow to red).
8. Titrate to the first appearance of a red color, which persists for 5 min when the flask is stoppered immediately after reading the end point.

CALCULATIONS

$$\text{Moisture, \%} = \frac{\text{volume of titrant, mL of test portion} \times F}{\text{mass, g of test portion}} \times 100$$

Where—

F = g of water titrated by 1 mL of Solution A (water factor as determined in Calibration of Solution A, 4).

PRECISION

Collaborative studies have shown that the following 95% confidence limits may be expected (see Table 2):

NOTES

Recommendations

Responses to the industry survey carried out by the Associate Methods Editor of Section E in 1989, showed that the Karl Fischer method for determining moisture in glycerin is outdated and no longer used routinely. The survey indicated that the preferred method is the use of commercial automatic coulometric titrators, e.g., the Aqua Star C-1000 or equivalent. Methods based on coulometric instrumentation have not been studied collaboratively within the AOCS.

NUMBERED NOTES

1. Because of its toxicity, pyridine is an undesirable constituent of the Karl Fischer reagent. Both one- and two-component Karl Fischer reagents, based on aliphatic amines and heterocyclic compounds and containing no pyridine, are available commercially.
2. The procedure employing a visual end point is not intended to preclude the use of any satisfactory potentiometric apparatus, of which several are available. The use of a closed system with mechanical stirring is to be encouraged, although it is not essential for accurate analysis. The method prescribed will give satisfactory results on any distilled glycerin not too dark in color to obscure the visual end point.
3. In humid climates or during humid seasons, the drying tubes used to protect the reagent against moisture in the air must be watched closely. The Drierite must be changed at the first evidence of color change.

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Table 1

Expected % moisture	mass of test portion, g
0–1.5	10.000 ± 0.01
1.5–3	5.000 ± 0.001
3–8	2.000 ± 0.0001

Table 2

	Moisture present at level of	
	5.0%	0.5%
Duplicate determination on the same day by a single analyst should not differ more than approximately	0.1	0.02
Single determinations made in two different laboratories should not differ more than approximately	0.25	0.07
Averages of duplicate determinations made in two different laboratories should not differ more than approximately	0.23	0.07

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AOCS

AOCS Official Method Ea 9-65

Reapproved 2009

Color (APHA Scale)

DEFINITION

This method describes a procedure for the measurement of color by comparison with artificial empirical standards.

SCOPE

Applicable to refined glycerin.

APPARATUS

1. Spectrophotometer.
2. Spectrophotometer cuvettes, matched, 10 mm light path.
3. Nessler APHA tall form color comparison tubes—100 mL. The tubes should be approximately 390 mm long with 24 mm o.d. The 100 mL graduation mark should be between 275 and 295 mm above the bottom of the tube. In practice, the tubes should be selected and used in sets that possess the same depth of solution (within 2–3 mm) when filled to the 100 mL mark.
4. A color comparator rack—constructed to permit visual comparison of light longitudinally transmitted through the Nessler tubes. The comparator should be constructed so that white light is passed either through or reflected off a white glass plate and directed vertically with equal intensity through the tubes. The color comparator should be shielded so that no light can enter the tubes horizontally.
5. Light source—daylight-type fluorescent bulb.
6. APHA color standards—the APHA color standards should cover the range 5–100 in steps of 5 units (see Numbered Notes 1, 2).
7. Whatman filter paper—no. 4 or equivalent.

PROCEDURE

1. Filter the laboratory sample if it exhibits any visible turbidity.
2. Fill a color tube to the 100 mL mark with test portion and place in the color comparison rack between the two consecutive standards it most nearly matches in intensity. View the test portion and standards simultaneously.

REPORTING

1. Report as the color the number of the standard that most nearly matches the test portion. If the color lies midway between two standards, report the darker of the two (see Numbered Notes, 3).
2. If differences in hue preclude a definite match of test portion and standard, report the range over which an apparent match is obtained and report the test portion as "off-hue."

PRECISION

1. Agreement within one laboratory—two single determinations performed within one laboratory should not differ by more than 12.0% of the mean color value.
2. Agreement among laboratories—single determinations performed in two different laboratories should not differ by more than 15.9% of the mean color value.
3. Averages of duplicate determinations in two different laboratories should not differ by more than 13.5% of the mean color value.

NOTES

Caution

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended.

NUMBERED NOTES

1. To prepare the platinum-cobalt stock solution, dissolve 1.245 g of K_2PtCl_6, CP grade and 1.000 g of $CoCl_2 \cdot 6H_2O$, ACS grade in distilled water. Add 100 mL of hydrochloric acid, sp. gr. 1.19, ACS grade (see Notes, *Caution*) and dilute to 1 L with distilled water. This solution has an APHA color of 500. The absorbance of the stock solution must fall within the limits outlined in Table 1.

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The measurements shall be made in a 1 cm cuvette against distilled water in a matching cuvette.

Using the stock solution, prepare Color Standards no. 5–100 APHA, in steps of 5, by diluting 1 mL of the stock solution to 100 mL with water for each increment of five in the color standard number. Thus, 1 mL diluted to 100 mL comprises Color Standard no. 5 and 12 mL of stock solution diluted to 100 mL with water comprises Color Standard no. 60.

2. Color standards may be prepared in smaller steps if the color of the test portion requires a closer match than can be obtained with standards differing by five units.
3. Glycerol test samples that are darker than color standards may be diluted as directed in Table 2 before matching. The color standard number for the diluted solution must be multiplied by the appropriate factor.

Table 1

Wavelength, nm	Absorbance
430	0.110–0.120
455	0.130–0.145
480	0.105–0.120
510	0.055–0.065

Table 2
Dilution of glycerol test sample

For color standard	mL test sample	Dilute to (mL)	Multiply color standard number by
100–200	50	100	2
200–400	25	100	4
400–1000	10	100	10

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AOCS Official Method F 1a-44

Reapproved 2009

Moisture, Distillation Method

DEFINITION

This method determines the moisture by distillation with an immiscible solvent.

SCOPE

This method is applicable to sulfonated and sulfated oils except those that contain any of the following: mineral acids, free sulfonic acids, free sulfuric acid esters; alkali hydroxides, carbonates, or acetates; alcohol, glycerol, diethylene glycol, acetone, or other water-miscible volatile compounds.

APPARATUS

1. Glass distillation apparatus—constructed as shown in Figure 1, with the following specifications—
   (a) Flask—500 mL, Erlenmeyer or short-neck, round-bottom type.
   (b) Condenser—water-cooled, the lower tip of which shall be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established.
   (c) Trap—constructed of well-annealed glass and graduated to contain 5 mL at 20°C. It shall be subdivided into 0.1 mL divisions with each 1 mL line numbered and the 5 mL line at the top. The error at any indicated capacity shall not exceed 0.05 mL.
   (d) Distillation is better controlled by wrapping the tube which leads from the flask to the receiver with 100% fiberglass cloth tape. It is advantageous to wrap the flask also, especially if there are drafts in the vicinity of the distillation apparatus.
   (e) See Procedure for the calibration of the distillation apparatus.
2. Pipet—measuring type, capacity 1.0 mL, graduated in 0.01 mL.
3. Heat source—preferably an electric heater provided with a suitable means of control.
4. Copper or nichrome wire—long enough to extend through the condenser with one end twisted into a spiral. The diameter of the spiral should be such that it will fit snugly within the receiver and yet can be moved up and down.

REAGENTS

1. Xylene—reagent grade (see Notes, *Caution*).
2. Oleic acid—USP grade or equivalent. Heat for 5–10 min at a temperature not exceeding 130–135°C before using.

PROCEDURE

Note—The entire apparatus must be entirely clean and completely dry before using. It is recommended that the apparatus be first washed with soap and warm water, rinsed well and then treated with dichromate/sulfuric acid cleaning solution.

1. Calibrate the receiver by the following procedure: Add an accurately measured portion, 1 ± 0.01 mL, of distilled water to 100 mL of xylene in the distillation flask. Conduct the distillation as described in Procedure, 3–6, and calculate as directed in Calculations. Cool the apparatus, add another 1 ± 0.01 mL of water and repeat the distillation. Continue in this manner up to the capacity of the receiving tube.
2. Weigh a sufficient quantity of the test sample into the distillation flask to yield about 4 mL of water. This is conveniently done using a weighing bottle. Add 100 mL of xylene and an amount of oleic acid equivalent to about 2.5 times the weight of the dry test portion. This is done to prevent the test portion from foaming and forming a jelly-like mass.
3. Introduce some glass beads to prevent bumping and mix the contents of the flask thoroughly by swirling. Assemble the apparatus and fill the receiver with solvent by pouring through the condenser until the solvent begins to overflow into the distillation flask. Insert a loose cotton plug in the tip of the condenser to prevent condensation of atmospheric moisture within the tube.
4. Heat the flask so that the distillation rate will be about 100 drops per minute. When the greater part of the water has distilled, increase the distillation rate to about 200 drops per minute and continue until the water level in the receiver remains unchanged for 30 min.
5. Discontinue heating, dislodge any drops of water which may be adhering to the inside of either the condenser tube or receiver with the wire spiral, and wash down with 5 mL of xylene.
6. Immerse the receiver in water at 40°C for 15 min or until the xylene layer is clear. Read the volume of water and determine the temperature of the water. Refer to Table 1 for the specific gravity (sp. gr.) of water at the temperature determined.

CALCULATIONS

$$\text{Moisture},\% = \frac{\text{vol water, mL} \times \text{sp. gr.}}{\text{mass of test portion}} \times 100$$

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NOTES

Caution

Xylene is flammable and a dangerous fire risk. The TLV is 100 ppm in air. Use a fume hood when heating or evaporating.

Table 1

Temperature, °C	Specific Gravity
4	1.000
35	0.994
36	0.994
37	0.993
38	0.993
39	0.993
40	0.992
41	0.992
42	0.991
43	0.991
44	0.991
45	0.990

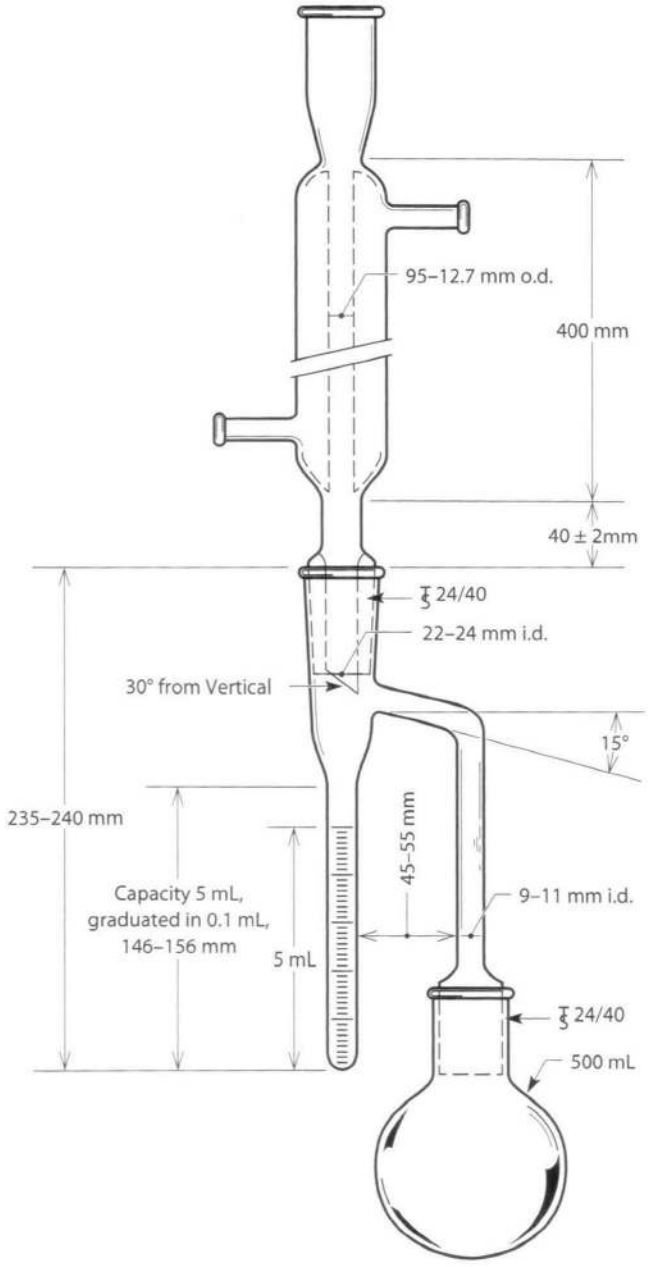

Figure 1. Moisture distillation apparatus.

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Moisture and Volatile Matter, Hot Plate Method

DEFINITION

This method determines the moisture and other substances volatile at about 100°C.

SCOPE

This method is applicable to sulfonated and sulfated oils that do not contain mineral acids, free sulfonic acids, or free sulfuric acid esters; ammonia; acetic acid or similar volatile acids; alkali hydroxides, carbonates, acetates, or similar salts which may react with oleic acid at elevated temperatures, liberating volatile acids; glycerol, diethylene glycol, xylene, or other compounds of similar volatility.

APPARATUS

1. Weighing bottle—glass-stoppered, 10–15 mL.
2. Beaker—Griffin low form, 150 mL, 50 mm dia.
3. Electric hot plate—which may be covered with a heat resistant material to spread the heat, if desired.
4. Thermometer—graduated from 90–150°C, about 76 mm long and substantially constructed.
5. Desiccator—containing an efficient desiccant. Calcium chloride may not be reliable. See AOCS Specification H 9-87 for desiccants.

REAGENTS

1. Oleic acid—USP grade or equivalent.

PROCEDURE

1. Weigh about 5 g of oleic acid into the beaker and insert the thermometer. Heat gradually on the hot plate, stirring with the thermometer until the temperature reaches 130°C.
2. Place the beaker in an oven at 105–110°C for 15 min, cool in the desiccator to room temperature and weigh. Repeat the heating on the hot plate and in the oven until two successive weighings differ by less than 1.5 mg.
3. Weigh accurately about 6 g of the test sample into the beaker containing the oleic acid. This is conveniently done from a weighing bottle. Heat the mixture exactly as described in Procedure, 1 and 2. Cool in a desiccator to room temperature and weigh.

CALCULATION

$$\text{Moisture and volatile matter, \%} = \frac{\text{loss in mass}}{\text{mass of test portion}} \times 100$$

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AOCS Official Method F 2a-44

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Organically Combined Sulfuric Anhydride, Titration Method

DEFINITION

This method determines the organically combined sulfuric anhydride.

SCOPE

Applicable to sulfated oils that split off their combined SO_3 upon boiling with mineral acids and that do not contain compounds which cannot be accurately titrated in water solution with methyl orange as the indicator. Only that portion of sulfur bound through oxygen to carbon is determined. Sulfur bound directly to carbon, i.e., a true sulfonate, is not determined.

APPARATUS

1. Erlenmeyer flask—300 mL, with air condenser 915 mm long, glass stopper, and T 24/40.
2. Glass beads—made of chemically resistant glass, about 4 mm dia and perforated. Before using the beads, boil them thoroughly in several portions of distilled water until the wash water is neutral to methyl orange.

REAGENTS

1. Sodium hydroxide (NaOH), 1.0 M—accurately standardized (see AOCS Specification H 12-52 for standardization procedure).
2. Sulfuric acid (H_2SO_4)—about 0.5M (see Notes, *Caution*).
3. Diethyl ether—reagent grade (see Notes, *Caution*).
4. Sodium chloride (NaCl)—reagent grade.
5. Methyl orange indicator—0.1% in distilled water.

PROCEDURE

1. Determine the alkalinity as directed in AOCS Official Method, F 7-44.
2. Accurately weigh exactly 10 g of the test sample into a 300 mL Erlenmeyer flask. Add sufficient 0.5 M H_2SO_4 to neutralize exactly the total alkalinity as determined by method F 7-44, plus 25 mL in excess. Add a few glass beads. Attach the air condenser and boil for 1.5 h or until the oil and water layers are perfectly clear. Regulate the heating so that the solution boils vigorously, but with very little evaporation.
3. Cool the contents of the flask and wash down the condenser with a spray of distilled water from a wash bottle. Disconnect the condenser and add 30 g of NaCl, 25 mL of diethyl ether, 50 mL of distilled water, and 2 or 3 drops of indicator.
   Note—The titration should be carried out in a properly operating fume hood.
4. Titrate with 0.5 M NaOH to the same end point as in the alkalinity titration. Stopper the flask and shake thoroughly several times during the titration. Wait 3 min before reading the burette.
5. Conduct a blank determination using 25 mL of 0.5 M H_2SO_4 simultaneously with the test portion and similar in all respects.
6. Reserve the titrated solution for the determination of total desulfated fatty matter by AOCS Official Method F 3-44.

CALCULATIONS

Organically combined sulfuric anhydride, % = $(T - B) \times 0.8007$

Where—

T = volume, mL of 1.0 M NaOH required by test portion
B = volume, mL of 0.5 M NaOH required by blank

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

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Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using ethyl ether.

NUMBERED NOTES

1. The factor of 0.8007 is based on the use of NaOH molarity and of the test portion weight as specified in Reagents and Procedure. Any deviation from these specifications will produce erroneous results.

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AOCS

AOCS Official Method F 2b-44

Reapproved 2009

Organically Combined Sulfuric Anhydride, Extraction-Titration Method

DEFINITION

This method determines the organically combined sulfuric anhydride.

SCOPE

Applicable only to sulfated oils that split off their combined SO_3 upon boiling with mineral acids, including test samples containing sodium acetate or other compounds that cannot be accurately titrated in water solution with methyl orange as the indicator. Only that portion of sulfur bound through oxygen to carbon is determined. Sulfur bound directly to carbon, i.e., a true sulfonate, is not determined.

APPARATUS

1. Erlenmeyer flask—300 mL, with glass stopper.
2. Separatory funnel—250 mL.

REAGENTS

1. Sodium hydroxide (NaOH), 0.5 M—accurately standardized (see AOCS Specification H 12-52 for standardization procedure). Also see Notes, 1.
2. Sulfuric acid (H_2SO_4)—about 0.5 M (see Notes, *Caution*).
3. Diethyl ether—reagent grade (see Notes, *Caution*).
4. Sodium chloride (NaCl)—reagent grade.
5. Methyl orange indicator—0.1% in distilled water.
6. Sodium chloride solution—saturated in distilled water.

PROCEDURE

1. Separation of purified oil—
   (a) Weigh 5–10 g of the test sample, depending upon the concentration of the fatty matter, into a 250 mL separatory funnel containing 50 mL of saturated NaCl solution, a few grams of solid NaCl, 2 or 3 drops of indicator and 50 mL of diethyl ether.

   Note—This operation should be carried out in a properly operating fume hood.

   (b) Shake the mixture and neutralize with 0.5 M H_2SO_4 until the lower layer is distinctly pink (about 0.2 mL in excess). At this stage, highly sulfated oils may form three layers instead of two. In such cases, use a fat solvent consisting of two parts diethyl ether and one part 95% alcohol, v/v, instead of diethyl ether.
   (c) Allow the mixture in the separatory funnel to settle for at least 5 min, draw off the lower layer into another separatory funnel, and continue washing the diethyl ether layer with 25 mL portions of saturated NaCl solution until 1 drop of 0.5 M NaOH turns the wash water strongly alkaline when methyl orange indicator is used.
   (d) Allow all separations to settle at least 5 min. Combine the water layers and extract with two 25 mL portions of diethyl ether. Combine the last two ether extracts and wash with saturated NaCl solution until free from acid, as in the case of the diethyl ether layer in the funnel.
   (e) Combine all the diethyl ether layers in the 300 mL Erlenmeyer flask and evaporate on either a steam or a water bath.
2. Determination of increase in acidity upon boiling, designated as "F"—
   (a) Determine the increase in acidity upon boiling as described in AOCS Official Method, F 2a-44, Procedure 2–5. (See Notes, 1).
   (b) Conduct a blank determination simultaneously with the test portion and similar in all respects.
   (c) Reserve the titrated solution for the determination of total desulfated fatty matter by AOCS Official Method, F 3-44.

CALCULATIONS

Organically combined sulfuric anhydride, % = $0.1426 \times F$

Where—

0.1426 = 0.1 of the molecular ratio of SO_3/KOH

F = increase in acidity in mg KOH/g.

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NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using ethyl ether.

NUMBERED NOTES

1. Although sodium hydroxide is used in the procedure, results are expressed based on mg KOH/gram. The factor used in the calculation is based on the fact that 1.0 mL of 1.0 M NaOH solution is equivalent to 56.1 mg of KOH.

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Organically Combined Sulfuric Anhydride, Ash-Gravimetric Method

DEFINITION

This method determines the organically combined sulfuric anhydride.

SCOPE

Applicable to all types of sulfonated and sulfated oils, including true sulfonic acid oils and those containing sodium acetate or similar partially titratable compounds.

APPARATUS

1. Crucible—50 mL, high form.
2. Beaker or Soxhlet flask—150 mL.
3. Beaker—250 mL.
4. Separatory funnel—250 mL.

REAGENTS

1. Diethyl ether—reagent grade (see Notes, *Caution*).
2. Sodium chloride (NaCl)—reagent grade.
3. Sodium sulfate—anhydrous Na_2SO_4, reagent grade.
4. Methyl orange indicator—0.1% in distilled water.
5. Hydrogen peroxide—30% (see Notes, *Caution*).
6. Sodium hydroxide (NaOH)—approximately 1 M for Procedure, 2.

PROCEDURE

1. Absence of ammonia—
   (a) Proceed as described in Separation of Purified Oil, AOCS Official Method, F 2b-44, Procedure, 1, combining the diethyl ether layers in the first funnel rather than in the Erlenmeyer flask.
   (b) Carefully remove any water that may settle and dehydrate the diethyl ether layer by adding 5 g of anhydrous Na_2SO_4, shaking vigorously for 5 min, and filtering directly into a 150 mL beaker or flask, placed in bath of warm water.
   Note—This operation should be carried out in a properly operating fume hood.
   (c) Wash the flask and filter with diethyl ether until free from fat (absence of oil stains on the filter paper after drying) and add the filtrate to the beaker. To prevent the oil from creeping, the volume in the beaker or flask should at no time during the filtering and washing exceed 50 mL.
   (d) Evaporate the diethyl ether solution until the volume has been reduced to about 20 mL and transfer the residue to a tared 50 mL crucible.
   (e) Place the crucible in a beaker containing warm water until practically all of the diethyl ether has evaporated. Rinse the beaker with two 10 mL and three 5 mL portions of diethyl ether, or until all of the oil has been transferred to the crucible. Allow each portion of diethyl ether to evaporate before adding the next.
   (f) Stir the solution with a glass rod to hasten evaporation and to prevent the oil from creeping. Wipe the glass rod with ashless filter paper and add the paper to the crucible before the oil is ignited.
   (g) Ignite the solvent-free residue gently and finally ignite at a dull red heat (500–550°C) to constant weight.
   (h) Moisten the ash with 30% hydrogen peroxide and again ignite carefully to constant weight. Cool in a desiccator containing an efficient desiccant to room temperature and weigh (see Notes, 1).
   (i) See Calculations, 1.
2. Presence of ammonia—
   (a) Dissolve 5–8 g of the test portion in 80 mL of water in a 250 mL beaker. Add 10 mL of 1 M NaOH and boil the solution gently until wet litmus paper no longer indicates ammonia.
   (b) Cool the solution, transfer to a 250 mL separatory funnel, and add about 25 g of solid NaCl.
   (c) Add 2 or 3 drops of methyl orange indicator and proceed with the neutralization, extraction, and following steps as directed in Procedure, 1, a–h.
   (d) See Calculations, 2.

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CALCULATIONS

1. Extracted Ash, $\% = \frac{\text{mass of ash}}{\text{mass of test portion}} \times 100$

2. Organically combined sulfuric anhydride, % = 1.1267 × % extracted ash.

   Where—

   1.1267 = the molecular ratio of $2(SO_3)/Na_2SO_4$

NOTES

Caution

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Hydrogen peroxide is a strong oxidizing agent and a dangerous fire and explosion risk. Concentrated solutions can cause burns and are highly toxic and strongly irritating. The TLV in air is 1 ppm.

NUMBERED NOTES

1. The addition of hydrogen peroxide is for the purpose of oxidizing traces of carbon or sodium sulfide that may form.

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AOCS Official Method F 3-44

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Total Desulfated Fatty Matter

DEFINITION

This method determines the total desulfated fatty matter in the test sample.

SCOPE

Applicable to sulfated oils, but not to test samples that are not completely decomposed upon boiling with mineral acids.

APPARATUS

1. Separatory funnels—250 mL.
2. Beaker or Soxhlet flask—150 mL.

REAGENTS

1. Sulfuric acid (H_2SO_4)—approximately 0.5 M.
2. Diethyl ether—reagent grade (see Notes, *Caution*).
3. Methyl orange indicator—0.1% in distilled water.

PROCEDURE

1. Use the titrated solution obtained from the determination of organically combined sulfuric anhydride by AOCS Official Method, F 2a-44 or F 2b-44, or a test solution prepared in a similar manner.
2. Cool and transfer the titrated solution to a 250 mL separatory funnel, add 50 mL of diethyl ether, and shake.
3. Allow the layers to separate and then draw off the lower water layer into another similar separatory funnel. Extract the water solution two more times using a 25 mL portion of diethyl ether each time.
4. Combine the diethyl ether extracts into one funnel and wash with 15 mL portions of distilled water until the wash water is neutral to methyl orange.
5. Transfer the diethyl ether extracts into a tared 150 mL beaker or flask which has been dried and cooled in a desiccator.
6. Evaporate the diethyl ether in a water bath under a gentle stream of clean, dry nitrogen.

   Note—This operation should be carried out in a properly operating fume hood. Make certain that all traces of diethyl ether are removed before transferring to the drying oven.
7. Dry in an air oven at 105 ± 2°C for 30 min. Cool in a desiccator to room temperature and weigh. Repeat until constant weight is obtained.
8. Reserve the extracted fatty matter for the subsequent determination of unsaponifiable matter by AOCS Official Method, F 5-44.

CALCULATIONS

$$\text{Total desulfated fatty matter, \%} = \frac{\text{mass of residue}}{\text{mass of test portion}} \times 100$$

NOTES

Caution

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48% The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

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Total Active Ingredients

DEFINITION

This method determines the total active ingredients as they exist in the original test sample.

SCOPE

Applicable to sulfonated or sulfated oils. Free alkali or alkali bound as soap is not included. Unsulfated and unsulfonated oil and unsaponifiable fatty matter are included.

Note—In the case of sulfated oils only, the total active ingredients may be estimated by calculation, because they are equivalent to the sum of the desulfated fatty matter and neutralized organically combined sulfuric anhydride (see Calculations, 2).

REAGENTS

1. Diethyl ether—reagent grade (see Notes, *Caution*).
2. Sodium chloride (NaCl)—reagent grade.
3. Sodium sulfate (Na_2SO_4)—anhydrous, reagent grade.
4. Methyl orange indicator—0.1% in distilled water.
5. Alcoholic potassium hydroxide (KOH) solution, 0.5 M— accurately standardized (see AOCS Specification H 15-52 and Notes, *Caution*).

PROCEDURE

1. Proceed as directed in AOCS Official Method, F 2c-44, Procedure, 1, retaining the separated active ingredients in the 150 mL beaker instead of transferring to a crucible. In this case, the beaker must be previously dried, cooled in a desiccator to room temperature, and weighed.
2. Evaporate to a volume of about 20 mL and then add exactly 2 mL of alcoholic KOH solution. Mix by swirling and then evaporate until free from diethyl ether (see Notes, *Caution* and 1). The diethyl ether may be evaporated in a water bath under a gentle stream of clean, dry nitrogen.

   Note—This operation should be carried out in a properly operating fume hood. Make certain that all traces of diethyl ether are removed before transferring to the drying oven.
3. Dry in an air oven at 105 ± 2°C for 1.5 h. Cool in a desiccator to room temperature and weigh. Repeat drying for 30 min periods until a constant weight is obtained.

CALCULATIONS

1. Total active ingredients, $\% = \dfrac{\text{mass of residue} - \text{alkali correction}}{\text{mass of test portion}} \times 100$

   Where—

   Alkali correction = 0.0006791 × (mL KOH × S)

   0.0006791 = molecular ratio of (K–H)/KOH divided by 1000

   S = strength of KOH solution in mg KOH per mL
2. Sulfated oils only—

   Total active ingredients (by calculation), % = P + 1.4871 × Y

   Where—

   1.4871 = molecular ratio of $NaSO_4^-/SO_3$

   P = % total desulfated fatty matter from AOCS Official Method, F 3-44

   Y = % combined SO_3 from AOCS Official Method, F 2c-44

   The factor 1.4871 is based on the assumption that the desulfated fatty matter becomes polymerized at the point where the SO_3 splits off and that no hydroxyl groups are formed. If the latter are formed, the factor becomes the molecular ratio of $(NaSO_3 - 1)/SO_3 = 1.275$. Calculated results were in better agreement withactual determinations when the higher factor was used.

NOTES

Caution

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty

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containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Potassium hydroxide can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fumeremoval device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalis are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. With highly sulfonated or sulfated oils, it may be necessary to add 5–10 mL of the alcoholic KOH in order to stabilize the residue.

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Unsaponifiable Nonvolatile Matter

DEFINITION

This method of analysis determines the unsaponifiable, nonvolatile (above 80°C) matter.

SCOPE

Applicable to sulfated oils.

APPARATUS:

1. Erlenmeyer flask—300 mL, with air condenser 915 mm long, glass stopper, and Ʇ 24/40.
2. Separatory funnels—250 mL.
3. Beaker or Soxhlet flask—150 mL.

REAGENTS

1. Aqueous potassium hydroxide (KOH), 0.5 M—accurately standardized (see Notes, *Caution*).
2. Alcoholic potassium hydroxide—approximately 0.5 M, but not less than 0.5 M (see Notes, *Caution*).
3. Diethyl ether—reagent grade (see Notes, *Caution*).
4. Hydrochloric acid (HCl)—reagent grade, sp. gr. 1.19 (see Notes, *Caution*).
5. Phenolphthalein indicator—1.0% in 95% ethyl alcohol.

PROCEDURE

1. Use the residue of extracted fatty matter obtained from the determination of total desulfated fatty matter by AOCS Official Method, F 3-44, or a test sample prepared in the same manner.
2. Weigh accurately 2–2.5 g of the fatty matter into the flask and add 25 mL of the alcoholic KOH (see Notes, 1). Attach the condenser and heat at a low temperature for 1 hr.

   Note—The temperature should be such that the test sample is hot, but without boiling and with no loss of alcohol during the 1 hr heating period. Swirl occasionally.
3. Transfer the contents of the flask to a 250 mL separatory funnel and wash the flask several times with distilled water, using a total of 50 mL. Pour the water from the flask into the separatory funnel.
4. While the solution is still warm (about 30°C), add 50 mL of diethyl ether. Wash the saponification flask with the diethyl ether before adding it to the funnel. Shake vigorously for about 1 min and then allow the layers to separate (see Notes, 2).
5. Draw off the lower layer into another similar separatory funnel. Extract this solution two more times, using 50-mL portions of diethyl ether each time (see Notes, 3).
6. Combine the diethyl ether extracts into a separatory funnel and add 20 mL of distilled water. Turn the separatory funnel and contents over *gently* about six times and then allow the layers to separate. Draw off and discard the lower layer.
7. Wash the diethyl ether layer twice more with 20 mL portions of distilled water, shaking vigorously after each addition.
8. Add 20 mL of aqueous 0.5 M KOH to the diethyl ether and again turn the separatory funnel and contents over *gently* about six times. Allow the layers to separate, draw off and discard the lower layer.
9. Wash the diethyl ether layer with 20 mL of distilled water followed by 20 mL of aqueous 0.5 M KOH, as described in Procedure, 7 and 8. Repeat this procedure until the alkaline layer is only faintly opalescent after it has been made strongly acid with HCl (sp. gr. 1.19) and allowed to settle for a few minutes.
10. Finally, wash the diethyl ether layer with 20 mL portions of distilled water until the wash water is no longer pink to phenolphthalein.
11. Transfer the diethyl ether layer to a tared 150 mL beaker or flask which has been dried and cooled in a desiccator. Evaporate in a water bath under a gentle stream of clean, dry nitrogen.

    Note—This operation should be carried out in a properly operating fume hood. Make certain that all traces of diethyl ether are removed before transferring to the drying oven.

12 Heat the beaker at 75–80°C in an air oven. Cool in a desiccator to room temperature and bring to constant weight (see Notes, 4).

CALCULATIONS

$$\text{Unsaponifiable nonvolatile matter, \%} = \frac{\text{mass, g of residue} \times \text{\% desulfated fatty matter}}{\text{mass, g of desulfated fatty matter}}$$

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NOTES

Caution

Potassium hydroxide can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use an effective fume-removal device or gas mask to protect respiratory tract against alkali dusts or vapors. When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended.

NUMBERED NOTES

1. A 30% alcoholic KOH solution by volume is the most satisfactory solution from which to extract the unsaponifiable matter.
2. If emulsions form, add 5 mL of alcohol by pouring it down the side of the funnel.
3. It is important to maintain the volume of diethyl ether at not less than 150 mL, otherwise small quantities of unsaponifiable matter may be lost.
4. The presence of fatty acids in the unsaponifiable matter is determined by dissolving the residue in 10 mL of freshly boiled neutral alcohol and titrating with 0.1 M alcoholic KOH solution, using phenolphthalein as the indicator. Not more than 0.1 mL should be required for neutralization. If more is required, the determination has not been carried out effectively and must be repeated.

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Inorganic Salts

DEFINITION

This method determines the inorganic sulfates, chlorides and all other salts that are insoluble in a mixture of oleic acid and carbon tetrachloride.

SCOPE

Applicable to sulfonated and sulfated oils.

APPARATUS

1. The filtration must be performed with tared, ashless filter paper. Use Whatman no. 40, 9 cm, or equivalent grade of ashless paper.
2. Porcelain ignition crucible—tared.
3. Beaker—250 mL.
4. Hot plate—temperature controlled.

REAGENTS

1. Oleic acid—USP grade, or equivalent.
2. Carbon tetrachloride (CCl_4)—reagent grade (see Notes, Caution and 2).
3. Diethyl ether—reagent grade (see Notes, *Caution*).

PROCEDURE

1. The filter paper should be brought to constant weight by washing with 75 mL of CCl_4 and drying at 125–130°C for 45 min.
2. Weigh 3–5 g of the oil laboratory sample into a 250 mL beaker, add an equal amount of oleic acid and heat on a temperature-controlled hot plate with constant stirring at 105–110°C until practically free from water.
3. Continue the heating until the temperature reaches 118–120°C and hold at this temperature for 5 min. If the test sample does not remain liquid on cooling, add oleic acid in small amounts until the test sample remains liquid.
4. Dissolve the dehydrated test sample in 100 mL of warm (50–55°C) CCl_4 (see Notes, *Caution* and 2) and filter through the specified filter paper.
5. Wash the residue with three 15 mL portions of a 2% solution of oleic acid in CCl_4 and then with six 15 mL portions of hot CCl_4. Wash again with two 15 mL portions of diethyl ether (see Notes, *Caution*) or until the residue is free from oil. Make certain that the top of the filter is thoroughly washed and free from any oil.
6. Transfer the last traces of any residue remaining in the beaker to the filter by allowing the solvent to evaporate when the salts become free flowing.
7. Dry the residue at 125–130°C on the filter paper for 45 min, cool in a desiccator and weigh.

   Note—If ammonium salts are present, do not proceed with the ignition in Procedure, 8. Dry at 125–130°C for 45 min and repeat to constant weight.
8. In the absence of ammonium salts, transfer the filter paper with residue to a tared crucible, ignite at a dull red heat (500–550°C) for 15 min, cool in a desiccator to room temperature and weigh. Repeat the ignition until constant weight is obtained (see Notes, 1).

   Note—The ignition should be carried out in a properly operating fume hood.

CALCULATIONS

1. When ammonium salts are present—

$$\text{Inorganic sulfates and chlorides, including ammonium salts, \%} = \frac{\text{mass of dried residue}}{\text{mass of test portion}} \times 100$$

2. When ammonium salts are absent—

$$\text{Nonvolatile inorganic sulfates and chlorides, \%} = \frac{\text{mass of ignited residue}}{\text{mass of test portion}} \times 100$$

NOTES

Caution

Carbon tetrachloride is a known carcinogen. It is toxic by ingestion, inhalation and skin absorption, causing irritation, headaches and nausea on mild exposure. More severe exposure can lead to liver and kidney damage. It is a narcotic. It should not be used to extinguish fires. It decomposes to phosgene gas at high temperature. The TLV is 5 ppm in air. A fume hood should be used at all times when using carbon tetrachloride.

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Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

NUMBERED NOTES

1. In the *absence* of ammonium salts, the difference between the percentage of the dried residue and the percentage of the ignited residue shall not be greater than 0.25%.
2. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for carbon tetrachloride in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

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Total Ammonia

DEFINITION

This method determines total ammonia in the laboratory sample.

SCOPE

Applicable to sulfonated and sulfated oils.

APPARATUS

1. Beaker—500 mL.
2. Erlenmeyer flask—250 mL, with glass stopper.

REAGENTS

1. Sodium hydroxide (NaOH) solution, 0.5 M—accurately standardized (see AOCS Specification H 12-52 for procedure) (see Notes, 1).
2. Sulfuric acid (H_2SO_4) solution, 0.25 M—accurately standardized (see AOCS Specification H 13-52 for procedure).
3. Methyl orange indicator—0.1% in distilled water.
4. pH paper.
5. Sodium chloride (NaCl)—reagent grade.
6. Diethyl ether—reagent grade (see Notes, *Caution*).

PROCEDURE

1. Total alkalinity—
   (a) Determine the total alkalinity as directed in AOCS Official Method F 7-44.
2. Alkalinity after boiling—
   (a) Weigh 10 g of laboratory sample into a 500 mL beaker and add 100 mL of distilled water. Add 25 mL of 0.5 M NaOH and boil for 30 min or until all the ammonia is expelled. Test with moistened pH paper.
   (b) Cool, add 2 or 3 drops of indicator, and titrate to an approximate end point. Transfer the contents to a glass-stoppered flask and add 30 g of NaCl and 25 mL of diethyl ether.
   Note—This procedure should be carried out in a properly operating fume hood.
3. Titrate to the same end point as in the titration of total alkalinity in Procedure, 1, a. Stopper the flask and shake thoroughly several times during the titration. Wait 3 min before reading the burettes.

CALCULATIONS

1. Total ammonia, % = 0.0303 T

Where—

$$T = \frac{A + (B - C) \times 28.05}{\text{mass of test portion, g}}$$

A = total alkalinity in mg KOH/g from Procedure, 1, a
B = volume, mL of 0.5 M alkali added
C = volume, mL of 0.25 M acid added
0.0303 = molecular ratio of NH_3/KOH

NOTES

Caution

Follow the precautions for handling sodium hydroxide and sulfuric acid as noted in AOCS Specifications H 12-52 and H 13-52.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is a dehydrating agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

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NUMBERED NOTES

1. Although sodium hydroxide is used in the procedure, results are expressed based on mg of KOH/g. The factor used in the calculation is based on the fact that 1.0 mL of 1.0 M NaOH solution is equivalent to 56.1 mg of KOH or 1.0 mL of 0.5 M NaOH solution is equivalent to 28.05 mg of KOH. If the normalities of the acid and/or alkali solutions are not exactly 0.5 M, the values for mL (B and C in the calculation) can be calculated on the basis of 0.5 M, e.g., 10.0 mL of 0.45 M NaOH is equivalent to 9.0 mL of 0.50 M NaOH.

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Acidity, Method A

DEFINITION

This method determines free (uncombined) fatty acids, which are calculated in terms of mg of KOH necessary to neutralize the fatty acids in 1 g of test sample.

SCOPE

Applicable to sulfonated or sulfated oils except in the presence of ammonium or triethanolamine soaps or salts or other compounds which do not react neutral to phenolphthalein when dissolved in alcohol.

APPARATUS

1. Erlenmeyer flask—250 mL.
2. Burette—Class A, 50 mL.

REAGENTS

1. Diethyl ether—reagent grade (see Notes, *Caution*).
2. Ethyl alcohol—95% (SDA Formulas 30 and 3A are permitted). Add 2–3 drops of phenolphthalein indicator solution and neutralize with alkali to a faint, but permanent pink color, just before using.
3. Phenolphthalein indicator—1% in 95% ethyl alcohol.
4. Sodium hydroxide (NaOH) solution, 0.5 M—accurately standardized (see AOCS Specification H 12-52) (see Notes, 1).

PROCEDURE

1. Weigh 10 g of test sample into an Erlenmeyer flask and add 50 mL of neutralized alcohol, 25 mL of diethyl ether, and 5 drops of phenolphthalein indicator.
2. Titrate with the standardized NaOH solution until a pink color persists after shaking vigorously for 30 sec.

CALCULATIONS

1. Acidity as mg KOH/g $= \dfrac{\text{volume, mL of alkali} \times 28.05}{\text{mass of test portion, g}}$

NOTES

Caution

Follow the precautions for handling sodium hydroxide as noted in AOCS Specification H 12-52.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

NUMBERED NOTES

1. Although sodium hydroxide is used in the procedure, results are expressed based on mg of KOH/g. The factor used in the calculation is based on the fact that 1.0 mL of 1.0 M NaOH solution is equivalent to 56.1 mg of KOH or 1.0 mL of 0.5 M NaOH solution is equivalent to 28.05 mg of KOH. If the normality of the alkali solution is not exactly 0.5 M, the value for mL of alkali in the calculation can be calculated on the basis of 0.5 M, e.g., 10.0 mL of 0.45 M NaOH is equivalent to 9.0 mL of 0.50 M NaOH.

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Acidity, Method B

DEFINITION

This method determines the free (uncombined) fatty acids, which are calculated in terms of mg of KOH necessary to neutralize the fatty acids in 1 g of test sample.

SCOPE

Applicable to dark colored sulfonated or sulfated oils, except in the presence of ammonium or triethanolamine soaps.

APPARATUS

1. Erlenmeyer flask—250 mL, with glass stopper.
2. Burette—Class A, 50 mL.

REAGENTS

1. Sodium chloride (NaCl)—reagent grade.
2. Diethyl ether—reagent grade (see Notes, *Caution*).
3. Phenolphthalein indicator—1% in 95% ethyl alcohol.
4. Ethyl alcohol—95% (SDA Formulas 30 and 3A are permitted). Add 2–3 drops of phenolphthalein indicator solution and neutralize with alkali to a faint pink color just before using.
5. Sodium hydroxide (NaOH) solution, 0.5 M—accurately standardized (see AOCS Specification H 12-52 for procedure) (see Notes, 1).

PROCEDURE

1. Weigh 10 g of test sample into Erlenmeyer flask containing 100 mL of distilled water. Warm, if necessary, to promote solution. Cool and add 30 g of NaCl, 25 mL of diethyl ether, and 50 mL of neutralized alcohol.
2. Add 5 drops of indicator and titrate with 0.5 M NaOH until the first pink tinge appears in the water layer. Shake vigorously after each addition of alkali.

CALCULATIONS

1. Acidity as mg KOH/g $= \dfrac{\text{volume, mL of alkali} \times 28.05}{\text{mass of test portion}}$

NOTES

Caution

Follow the precautions for handling sodium hydroxide as noted in AOCS Specification H 12-52.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

NUMBERED NOTES

1. Although sodium hydroxide is used in the procedure, results are expressed based on mg of KOH/g. The factor used in the calculation is based on the fact that 1.0 mL of 1.0 M NaOH solution is equivalent to 56.1 mg of KOH or 1.0 mL of 0.5 M NaOH solution is equivalent to 28.05 mg of KOH. If the normality of the alkali solution is not exactly 0.5 M, the value for mL of alkali in the calculation can be calculated on the basis of 0.5 M, e.g., 10.0 mL of 0.45 M NaOH is equivalent to 9.0 mL of 0.50 M NaOH.

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AOCS Official Method F 9c-44

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Acidity, Method C

DEFINITION

This method determines the free (uncombined) fatty acids which are calculated in terms of mg of KOH necessary to neutralize the fatty acids in 1 g of test sample.

SCOPE

Applicable to sulfonated or sulfated oils in the presence of ammonium or triethanolamine soaps, or both.

APPARATUS

1. Erlenmeyer flask—250 mL, with glass stopper.
2. Burette—Class A, 50 mL.

REAGENTS

1. Sodium hydroxide (NaOH) solution, 0.5 M—accurately standardized (see AOCS Specification H 12-52 for procedure) (see Notes, 1).
2. Ethyl alcohol—95% (SDA Formulas 30 and 3A are permitted). Add 2–3 drops of phenolphthalein indicator solution and neutralize with alkali to a faint, but permanent, pink color just before using.
3. Phenolphthalein indicator—1% in 95% ethyl alcohol.
4. Sodium chloride (NaCl) solution—saturated in distilled water.

PROCEDURE

1. Total alkalinity—
   (a) Determine the total alkalinity as directed in AOCS Official Method, F 7-44.
2. Free- and alkali-combined fatty acids—
   (a) Proceed as directed in AOCS Official Method, F 2b-44, Procedure, 1, for separation of purified oil.
   Note—In the presence of acetates, wash the diethyl ether (see Notes, *Caution*) layer carefully with saturated NaCl solution until the wash water is neutral to phenolphthalein when 1 or 2 drops of 0.5 M NaOH are added.
   (b) Add exactly 0.5 mL of 0.5 M NaOH to the extract and evaporate the diethyl ether (see Notes, *Caution*) to a total volume of 25 mL. Add 50 mL of neutral alcohol and titrate the mixture with 0.5 M NaOH to a faint, but permanent pink color. Include the initial 0.5 mL of 0.5 M NaOH in the mL of 0.5 M NaOH required in the titration.

CALCULATIONS

1. Acidity as mg KOH/g = (B – A)

   Where—

   A = total alkalinity from AOCS Official Method, F 7-44

   $$B = \frac{\text{volume, mL NaOH}^* \times 28.05}{\text{mass of test portion}}$$

   *mL NaOH as required in titration in Procedure, 2, b.

NOTES

Caution

Follow the precautions for handling sodium hydroxide as noted in AOCS Specification H 12-52.

Follow the precautions noted in AOCS Offical Method, F 2b-44 for the safe handling of diethyl ether.

NUMBERED NOTES

1. Although sodium hydroxide is used in the procedure, results are expressed based on mg of KOH/g. The factor used in the calculation is based on the fact that 1.0 mL of 1.0 M NaOH solution is equivalent to 56.1 mg of KOH or 1.0 mL of 0.5 M NaOH solution is equivalent to 28.05 mg of KOH. If the normality of the alkali solution is not exactly 0.5 M, the value for mL of alkali in the calculation can be calculated on the basis of 0.5 M, e.g., 10.0 mL of 0.45 M NaOH is equivalent to 9.0 mL of 0.50 M NaOH.

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AOCS Official Method F 10-44

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Water-Immiscible Organic Solvents

DEFINITION

This method determines water-immiscible organic solvents that are volatile with steam (see Notes, 1–4).

SCOPE

Applicable to sulfonated and sulfated oils that do not contain volatile compounds that are mutually soluble in water.

APPARATUS

1. Glass distillation apparatus, constructed as shown in Figure 1, with the following specifications—
   (a) Flask—500 mL, short-neck, round-bottom type.
   (b) Condenser—water-cooled.
   (c) Traps for solvents heavier than water—constructed of well-annealed glass and graduated to contain 5 mL at 20°C. It shall be subdivided into 0.1 mL divisions with each 1 mL line numbered and the 5 mL line at the top. The error at any indicated capacity shall not exceed 0.05 mL.
   (d) Traps for solvents lighter than water—constructed of well-annealed glass and graduated to contain 5 mL at 20°C. It shall be subdivided into 0.1 mL divisions with each 1 mL line numbered and the 5 mL line at the top. The error at any indicated capacity shall not exceed 0.05 mL.
2. Heat source—an electric hot plate with a suitable means of control.
3. Glass beads or pumice stone.

REAGENTS

1. Sodium (NaOH) or potassium hydroxide (KOH) solution—approximately 1 M.
2. Phenolphthalein crystals.
3. Sodium carbonate (Na_2CO_3)—reagent grade.
4. Calcium chloride ($CaCl_2$)—reagent grade.
5. Xylene—reagent grade (see Notes, *Caution*)
6. Oleic acid—USP grade or equivalent.

PROCEDURE

1. Calibration of receivers—
   (a) For solvents heavier than water, calibrate the receiver by the following procedure—add about 1 g of distilled water to a mixture of 80 g of xylene and 10 g of oleic acid. Conduct the distillation as described in AOCS Official Method F 1a-44. Calibrate the receiver up to its maximum capacity at intervals of 1 mL.
   (b) For solvents lighter than water, calibrate the receiver by the following procedure—insert a one-hole stopper containing a short piece of glass tubing into the lower opening of the trap. Attach a separatory funnel to the glass tube by means of rubber tubing. Fill the funnel with some liquid heavier than and non-miscible with water, open the stopcock and raise the funnel carefully until the liquid flows into the receiver to approximately the 0.3 mL mark. Introduce exactly 1 mL of water with a pipet into the graduated arm followed by a drop of pine oil to protect the surface from evaporation. Measure the volume of the water in the tube. Raise the level of the solvent in the graduated tube. Continue in this manner until the entire tube has been calibrated.
2. Introduce 50 mL of distilled water into the distillation flask and a sufficient amount of the test sample to yield about 4 mL of solvent. This is conveniently done with a weighing bottle. If highly volatile solvents are suspected of being present, it is preferable to remove the top of the weighing bottle and to drop the bottle and contents into the distillation flask. If cresylic acid is present, the size of test sample should be such as to yield about 2.5 mL of solvent. If cresylic acid is present, add about 1.5 mL of pine oil, exactly weighed, to the distillation flask to reduce the gravity of the distillate.
3. Rapidly neutralize the solution in the distillation flask with either NaOH or KOH, approximately 1 M, until slightly alkaline to phenolphthalein, added as crystals. Add 2 g of Na_2CO_3, enough $CaCl_2$ to prevent foaming (usually 3–5 g) and a few glass beads or pumice stones to prevent bumping.
4. Attach the receiver to the condenser and fill the receiver with water by pouring through the condenser. Immediately attach the distillation flask and begin the distillation.
5. Heat at such a rate that refluxing will start 7–10 min after the heat is applied, the water being at room temperature initially. Read the volume of solvent collected in the receiver 2 hr after refluxing starts and every hour thereafter until the test is complete. The test is complete when the volume of solvent increases by not more than 0.1 mL in any two consecutive 1 hr periods. The source of heat is removed from the distillation flask and the flask cooled for exactly 3 min before taking readings. The distillation is continued for an additional 15–30 min with the water in the condenser tube emptied as an additional precaution.

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6. After completion of the test, allow the receiver to stand for at least 40 min in order to cool to room temperature and to allow the distillate to become clear and then read the volume of solvent.
7. Remove about 2.5 mL of solvent from the receiver with a pipet and determine the specific gravity (sp. gr.) in a 2 mL pycnometer. In the case of solvents heavier than water the former is separated by means of a small separatory funnel.

CALCULATIONS

$$\text{Water-immiscible organic solvents, \%} = (\text{mass of solvent} - \text{mass of pine oil*})/\ \text{mass of test portion} \times 100$$

Where—

mass of solvent = (total mL distilled × sp. gr.**)

*If present.

**As determined in Procedure, 7.

NOTES

Caution

Xylene is flammable and a dangerous fire risk. The TLV is 100 ppm in air.

NUMBERED NOTES

1. In the presence of water-miscible solvents (such as alcohol) that are also miscible with water-immiscible solvents, some of the former will be found in the solvent layer. When there is an appreciable difference in the boiling points and specific gravities of the two types of solvents, they may be separated qualitatively by fractional distillation and the specific gravities of the fractions determined, from which, together with the specific gravity of the solvent layer, the amount of water-immiscible solvents may be calculated.
2. In the case of pine oil or cresylic acid mixed with alcohol, the alcohol may be volatilized by heating over a hot plate with constant stirring to a temperature of about 150°C, after which the specific gravity of the residue is determined. The quantity of the water-immiscible solvent may now be calculated as in Notes, 1. This method may be applied to all such mixtures where there is a considerable difference in boiling points and specific gravities and the specific gravity of one of the solvents is known.
3. When there is a difference in the specific gravities, but not in the boiling points, and the specific gravity of the water-miscible solvents is known, the water-immiscible solvent is extracted with diethyl ether over water, the ether evaporated, and the specific gravity of the residue determined. In this procedure, it is assumed that the boiling point of the water-immiscible solvent is considerably higher than that of ether. In all other cases, to obtain the water-immiscible solvent, it will be necessary to resort to a quantitative fractional distillation or to chemical means.
4. With test portions yielding distillates that are both heavier and lighter than water, the distillation is first made with the trap for solvents heavier than water and, after flushing all the solvent floating on top of the water layer back into the distillation flask and substituting the trap for solvents lighter than water, the distillation is continued to completion.

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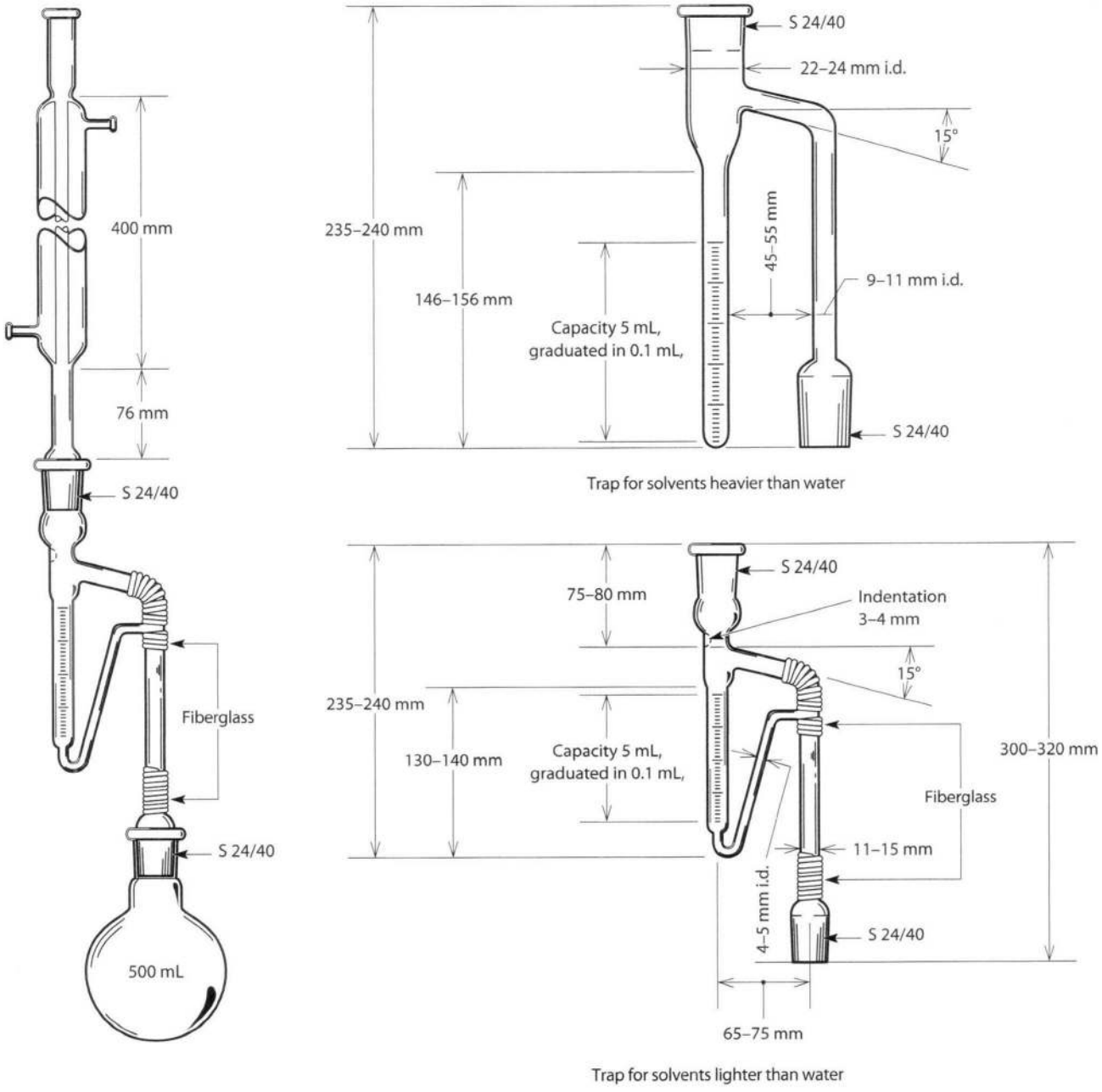

Figure 1. Apparatus for water-immiscible organic solvents.

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AOCS Official Method G 1-40

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Sampling

SCOPE

Applicable to raw and acidulated soapstock from all types of fats and oils.

APPARATUS

1. Metal drum, can or other container with tight fitting cover—at least 1.50 L (120 lb) capacity.
2. Ladle with handle.
3. Sample cans with tight fitting covers— 500 or 1000 mL (1 or 2 lb) capacity.

PRESERVATIVE

1. Oil of cassia.

PROCEDURE

1. Raw soapstock is sampled as it is loaded, because filled or partly filled tank cars, drums, or barrels cannot be properly sampled after standing for any length of time. Acidulated soap stock may be sampled by this method or by any of the methods specified for oils or liquid fats as directed in AOCS Official Method, C 1-47. The test sample is drawn from the flowing stock at the discharge end of the pipe, as the material enters the car or other container.
2. Take test samples with the ladle from the flowing stock at regular and frequent intervals, so that not less than 25 individual portions of uniform size are collected.
3. Place these portions in a drum, can or other metal container with tight fitting cover. Keep the container tightly covered, except during the actual sampling, so as to prevent the loss of moisture and to prevent contamination with water or other foreign material. This is particularly true if the flow is interrupted for any reason.

   Caution—If the flow is interrupted and it is necessary to clear the lines, air is preferable to steam. If it becomes necessary to blow the line with steam, the steam must be blown back from the discharge end of the line into or towards the supply tank and *not* towards the sampling end of the line.
4. The total laboratory sample accumulated shall amount to 0.1–0.2% by weight of the product sampled, i.e., 25–50 L (60–120 lb) per tank car.
5. Add 0.1% of oil of cassia to the composite test sample of raw soapstock (not acidulated stock), mix thoroughly, fill four 500 g–1 kg (1 or 2 lb) cans and seal them hermetically. The cans must be plainly marked for identification.

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AOCS

AOCS Official Method G 3-53

Reapproved 2009

Total Fatty Acids, Oxidized Fatty Acids, Wet Extraction Method

DEFINITION

The procedure for total fatty acids of this method determines the total fatty acids in the test sample, whether present as neutral or saponified oil. Any unsaponifiable fatty matter in the test sample will be included. The procedure for oxidized acids of this method determines petroleum ether-insoluble, but diethyl ether-soluble material, commonly referred to as oxidized fatty acids.

SCOPE

Applicable to raw and acidulated soapstock, except from coconut, palm kernel, and similar lauric-type oils.
Replaced G 3-39

APPARATUS

1. Cylinder—250 mL, glass-stoppered.
2. Erlenmeyer flask—300 mL.
3. Filter paper—Reeve Angel no. 230, Eaton and Dikeman no. 617, or equivalent.
4. Beaker—400 mL.
5. Separatory funnel—500 mL, glass-stoppered.
6. Soxhlet flasks—250 mL.

REAGENTS

1. Alcoholic potassium hydroxide (see Notes, *Caution*)— 50 g of solid KOH in 1 L of 95% ethanol. SDA formulas 30 and 3A are permitted.
2. Hydrochloric acid (1:1) (see Notes, *Caution*)—Mix one volume of HCl (sp. gr. 1.19) with one volume of distilled water.
3. Methyl orange indicator solution—0.1% in distilled water.
4. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
5. Diethyl ether—ACS grade (see Notes, *Caution*).

PROCEDURE FOR TOTAL FATTY ACID DETERMINATION

1. Mix the test sample thoroughly and weigh immediately.
2. Weigh 8–10 g of raw soapstock (4–5 g of acidulated soapstock) into a 300 mL Erlenmeyer flask or 400 mL beaker (see Notes, 1). Add 50 mL of alcoholic alkali, cover with a watch glass, and heat in a water bath to saponify (see Notes, 2). Agitate frequently and heat for at least 30 min or until saponification is complete.
3. Remove the watch glass and continue heating in a water bath with occasional agitation to evaporate the alcohol. To avoid oxidation, do not evaporate beyond a paste. If necessary, add a small amount of water when most of the alcohol has evaporated.
4. Add 100 mL of distilled water and heat until the soap has completely dissolved. Wash the contents into a glass-stoppered cylinder with hot distilled water, taking care not to exceed a total volume of 130 mL in the cylinder.
5. Add 3–5 drops of indicator and neutralize with dilute HCl to a pink methyl orange end point. Then add 1 mL of excess acid. Rotate the cylinder gently to mix the contents.
6. Cool to at least 35°C and add 125 mL of petroleum ether. The fatty acids do not need to have separated completely before adding the ether. Stopper the cylinder, shake gently, and allow to stand until the petroleum ether layer separates.
7. Siphon the petroleum ether layer into a 500 mL separatory funnel, making sure that as little as possible of the insoluble matter which gathers at the ether-water interface is carried over into the separatory funnel. If any appreciable amount of insoluble matter does siphon over into the separatory funnel, it will usually settle to the bottom and can be drained back into the extraction cylinder. Make at least four more similar extractions, using 25–30 mL of petroleum ether each time and shaking the cylinder vigorously for 30 sec with each extraction. Extractions should be continued until the petroleum ether layer is practically colorless. Save the acid water in the extraction cylinder if oxidized fatty acids are to be determined (as noted in the next Procedure).
8. Dilute the combined extracts with petroleum ether to 450 mL and mix thoroughly by gently shaking the separatory funnel.
9. Filter the diluted petroleum ether in a water bath as necessary to permit collection of all the extracts and subsequent washings. Wash the filter paper thoroughly with about 75 mL of petroleum ether, taking care to remove the yellow ring of fatty acids which usually forms near the upper edge of the filter paper. This is best accomplished by washing the paper while its upper edge is lifted away from the filtering funnel with a small spatula or tweezers.

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10. Evaporate the petroleum ether in a water bath under a gentle stream of *clean* dry air. Finally, dry the fatty acids in an air oven at 105 ± 2°C for 30 min. Cool in a desiccator to ambient temperature and weigh. Repeat until constant weight is attained. Constant weight is attained when the loss or gain in weight does not exceed 0.1% in successive 30 min drying periods. This is the weight of total fatty acids.

PROCEDURE FOR OXIDIZED ACID DETERMINATION

1. Add 25–30 mL of diethyl ether to contents of the cylinder after Procedure, 7 for total fatty acids, stopper, shake gently, and allow to stand until the diethyl ether layer separates.
2. Siphon the diethyl ether layer through the same filter paper used previously, into another tared 250 mL Soxhlet flask which has been dried and cooled in a desiccator. Make at least four similar extractions using 25–30 mL of diethyl ether each time, and shake the cylinder vigorously for 30 sec with each extraction. The last diethyl ether extract should be practically colorless.
3. Filter all extracts through the same filter paper and finally wash this filter paper thoroughly with diethyl ether to recover all of the oxidized acids.
4. Evaporate the diethyl ether in a fume hood in a water bath under a gentle stream of clean dry air. Finally, dry the oxidized fatty acids in an air oven at 105 ± 2°C for 30 min. Cool in a desiccator to ambient temperature and weigh. Repeat until constant weight is attained. Constant weight is attained when the loss or gain in weight does not exceed 0.1% in successive 30 min drying periods. This is the weight of oxidized fatty acids.

CALCULATIONS

1. Total Fatty Acids, $\% = \dfrac{\text{mass of total fatty acids}}{\text{mass of test portion, g}} \times 100$
2. Oxidized Fatty Acids, $\% = \dfrac{\text{mass of oxidized fatty acids}}{\text{mass of test portion, g}} \times 100$

NOTES

Caution

When working with extremely caustic materials like potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

When diluting acids, always add acid to water unless otherwise directed in a method. Keep acids off skin and protect eyes when working with acids. If acids come in contact with skin or eyes, wash immediately with large amounts of water.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. On raw soapstock it is advisable to weigh the test portion into the beaker or Erlenmeyer flask from a weighing bottle, because this material loses moisture rapidly. Because a 400 mL beaker is bulky to weigh on an analytical balance, it is advisable to weigh both raw and acidulated test portions from a weighing bottle when a beaker is used.
2. Fifty mL of 95% ethanol and 2–3 g of solid KOH may be used instead of the alcoholic solution, if desired.

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AOCS Official Method G 4-40

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Total Fatty Acids

DEFINITION

This method determines the total fatty acids in the test sample, whether present as neutral or saponified oil. Any unsaponifiable fatty matter in the test sample will be included.

SCOPE

Applicable to raw soapstock or acidulated soapstock from coconut, palm kernel, or other lauric-type oils.

APPARATUS

1. Beakers—250 and 600 mL.
2. Beaker or Soxhlet flask—250 mL, tared with a stirring rod.
3. Cylinder—250 mL, glass-stoppered.
4. Filter paper—Reeve Angel no. 230, Eaton & Dikeman no. 617, or equivalent.
5. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.
6. Separatory funnel—500 mL.

REAGENTS

1. Alcoholic sodium or potassium hydroxide solution—50 g of the solid caustic (see Notes, *Caution*) in 1 L of 95% ethyl alcohol. SDA Formulas 30 and 3A are permitted.
2. Hydrochloric acid (1:1)—mix (see Notes, *Caution*) one volume of HCl (sp. gr. 1.19) with one volume distilled water.
3. Methyl orange indicator solution—0.1% in distilled water.
4. Phenolphthalein indicator solution—1% in 95% ethyl alcohol.
5. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
6. Sodium hydroxide solution, 1.0 M—accurately standardized and free from carbonates (see AOCS Specification H 12-52). This solution should be as free from impurities as possible in order to keep the correction at a minimum. Carbonates are the usual impurities contributing to this factor, but other sodium salts may be present.

   Note—If the presence of impurities is suspected, determine a correction factor "C" by exactly neutralizing 20 mL of the 1.0 M NaOH solution (containing a few drops of phenolphthalein) with 1.0 M HCl solution. Evaporate the neutral solution on a water bath and then dry for 2 hr in an air oven at 105 ± 2°C. Cool in a desiccator to room temperature and weigh. Repeat until constant weight is attained. Constant weight is attained when the loss (or gain) in weight does not exceed 0.1% in successive 1 hr drying periods. Correction factor per mL of 1.0 M NaOH =

$$C = \frac{\text{mass of residue} - 1.1691}{20}$$

PROCEDURE

1. Mix the test sample thoroughly and weigh immediately.
2. Weigh an amount of test sample which will yield about 5 g of fatty acids into a 250 mL beaker. Add 50 mL of alcoholic alkali and heat in a water bath to saponify (see Notes, 1). Cover with a watch glass. Stir frequently and heat for at least 30 min or until saponification is complete.
3. Remove the watch glass and continue heating in a water bath with occasional stirring to evaporate the alcohol. To avoid oxidation, do not evaporate beyond a paste. If necessary, add a small amount of water when most of the alcohol has evaporated.
4. Add 100 mL of distilled water and heat until the soap has completely dissolved. Wash the contents into a glass-stoppered cylinder with hot distilled water, taking care not to exceed a volume of 130 mL in the cylinder.
5. Add 3–5 drops of methyl orange and a small, but definite excess of dilute HCl. Rotate the cylinder gently to mix the contents.
6. Cool to at least 35°C and add 125 mL of petroleum ether. The fatty acids do not have to have completely separated before adding the petroleum ether. Stopper the cylinder, shake gently, and allow to stand until the petroleum ether layer separates.
7. Siphon the petroleum ether layer through a filter paper into a 600 mL beaker or separatory funnel. If this extract is cloudy, it should be refiltered, but any subsequent cloudiness may be disregarded.
8. Make at least four more similar extractions using 25–30 mL of petroleum ether each time and shake vigorously for 30 sec with each extraction.
9. Filter each extract through the same filter paper as the first and finally wash this filter paper thoroughly with petroleum ether to remove all of the fatty acids.

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10. Evaporate the petroleum ether in a fume hood in a water bath under a gentle stream of clean dry air to a volume of 50–75 mL. Add 50 mL of neutral ethyl alcohol and 0.5 mL of phenolphthalein. Titrate with 1.0 M NaOH solution to the first permanent pink color.
11. Evaporate the remaining petroleum ether and concentrate the solution in a water bath under a current of clean dry air until the volume is reduced to about 25 mL.
12. Use hot ethyl alcohol to transfer into a 250 mL beaker or Soxhlet flask which has been dried, cooled in a desiccator, and tared with a stirring rod.
13. Complete the evaporation and then dry in an air oven at 105 ± 2°C for 2 hr. Cool in a desiccator to ambient temperature and weigh. Repeat drying for 30 min intervals until constant weight is obtained. Stir the mass occasionally while evaporating and drying. Constant weight is attained when the loss (or gain) in weight does not exceed 0.1% in successive 30 min drying periods.

CALCULATIONS

1. Total Fatty Acids, % = $\dfrac{\left[W-(0.022+C)T\right]}{\text{mass of test portion}} \times 100$

Where—

W= mass of sodium soap from Procedure, 13
C = Correction for impurities from Reagents, 6
T = volume of titrant, mL of fatty acids from Procedure, 10

NOTES

Caution

When working with extremely caustic materials like sodium or potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

When diluting acids, always add acid to water unless otherwise directed in a method. Keep acids off skin and protect eyes when working with acids. If acids come in contact with skin or eyes, wash immediately with large amounts of water.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

NUMBERED NOTES

1. Fifty mL of 95% alcohol and 2–3 g of solid KOH may be used instead of the alcoholic solution if so desired.

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AOCS Official Method G 5-40

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Neutral Oil

DEFINITION

This method determines the total neutral oil, i.e., unsaponified oil in the test sample. Any unsaponifiable fatty matter in the test sample will be included.

SCOPE

Applicable to all types of soapstock.

APPARATUS

1. Cylinder—250 mL, glass-stoppered.
2. Separatory funnel—500 mL.
3. Beaker—400 mL, or Soxhlet flask, 500 mL.

REAGENTS

1. Aqueous potassium hydroxide (KOH)—14% KOH by weight (see Notes, *Caution*).
2. Ethyl alcohol—SDA Formulas 30 and 3A are permitted, 50% by volume. Mix 10 volumes of 95% alcohol with 9 volumes of distilled water.
3. Ethyl alcohol—as above, 10% by volume. Mix 2 volumes of 95% alcohol with 17 volumes of distilled water.
4. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PROCEDURE

1. Mix the test sample thoroughly and weigh immediately.
2. Weigh 8–10 g of the test sample (4–5 g acidulated soapstock) into an extraction cylinder. Add 125 mL of 50% alcohol, 50 mL of petroleum ether and shake until a homogeneous mixture is formed.
3. Cool to 20–25°C and add 10 mL of the aqueous KOH (Reagents, 1). Agitate gently until thoroughly mixed, but avoid vigorous shaking.
4. Add 25 mL of 50% ethyl alcohol and agitate gently until thoroughly mixed. Allow to settle until the two layers are completely separated, but do not allow the ether/oil layer to remain in contact with the alcohol/alkali layer longer than 30 min.
5. Siphon the ether/oil layer into a 500 mL separatory funnel. Make at least four more similar extractions using 50 mL of petroleum ether for each extraction. Combine all of the extracts in the separatory funnel.
6. Wash the ether extract in the separatory funnel with 25 mL portions of 10% alcohol, shaking vigorously each time, until the washings are neutral to phenolphthalein. Three washings are usually sufficient. Filter through filter paper and wash the paper free from fatty matter with petroleum ether.
7. Draw the ether extract into a tared beaker or Soxhlet flask which has been previously dried and cooled in a desiccator. Evaporate the ether in a water bath under a gentle stream of clean, dry nitrogen. Dry in an air oven at 105 ± 2°C for 30 min. Cool in a desiccator to ambient temperature and weigh. Repeat until constant weight is attained. A constant weight is attained when the loss (or gain) in weight does not exceed 0.1% in successive 30 min drying periods.

CALCULATIONS

1. Neutral Oil, % = $\frac{\text{mass of neutral oil}}{\text{mass of test portion, g}} \times 100$

NOTES

Caution

When working with extremely caustic materials like sodium or potassium hydroxide, always add pellets to water and not the reverse. Alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1–6%. A fume hood should be used at all times when using petroleum ether.

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Titer Test

DEFINITION

This method determines the solidification point of the fatty acids.

SCOPE

Applicable to soapstock from animal and vegetable fats and oils.

APPARATUS

1. Beaker—2 L Griffin low-form. See Figure 1 for diagram of apparatus.
2. Bottle—wide-mouth, 450 mL, height 190 mm, 38 mm i.d. of neck.
3. Test tubes—length 100 mm, 25 mm dia, with or without rim. These tubes may have an etched mark extending around the tube at a distance of 57 mm from the bottom to show the height to which the tube is to be filled.
4. Saponification vessel—this may be a flask, beaker, or casserole of a convenient capacity. The form of this vessel is not important as long as it is satisfactory for saponification, i.e., alkali resistant.
5. Stirrer—2–3 mm o.d., with one end bent in the form of a loop of 19 mm o.d. glass, nichrome, stainless steel, or monel wire may be used. The upper end can be formed to accommodate hand stirring or attached to a mechanical stirrer.
6. Laboratory thermometer—0–150°C.
7. Titer test thermometer—AOCS Specification H 6-40. This thermometer should be calibrated against a National Institute of Standard & Testing standard thermometer.
8. Filter paper—qualitative, rapid filtering grade.

REAGENTS

1. Glycerol-caustic solution—dissolve with the aid of heat, 250 g of solid potassium hydroxide (see Notes, 1) in 1250 g of glycerin (reagent grade). To avoid foaming, do not heat above 135–145°C.
2. Dilute sulfuric acid—30% by weight of H_2SO_4. This is prepared by adding 16 mL of sulfuric acid (sp. gr. 1.84) to 70 mL of distilled water (see Notes, *Caution*).

PROCEDURE

Preparation of the fatty acids—

1. Weigh about 110 g of the glycerol-caustic solution into the saponification vessel. Stir and heat to 150°C. Add a sufficient amount of well-mixed test sample of soap stock to yield at least 40 g of fatty acids (usually 150–200 g) and reheat to 140–150°C. In some cases a small additional amount of the glycerol-caustic solution may be necessary to ensure complete saponification.
2. Continue stirring until the saponification is complete (see Notes, 2).

   Caution—Do not heat above 150°C.
3. Cool slightly, add 200–300 mL of distilled water, stir the mass well and heat until the soap is dissolved. Carefully add, while stirring, 50 mL of dilute H_2SO_4, or a sufficient amount to make the solution acid (test with pH paper). Boil until the fatty acids are completely melted and clear. Additional water may be added before (or during) boiling if desired. Keep the water level high enough to prevent overheating and burning on the side of the vessel.
4. Remove the aqueous layer containing the H_2SO_4. This may be done with an appropriate siphoning device or by any other convenient means. Again add water and boil for 2–3 min or until the fatty acids are entirely melted and clear. Acids of high melting point fats are sometimes slow to melt and clear. Inspect the fatty acid layer while it is quiet to be certain it is completely melted.
5. Remove the water again and, if necessary, repeat the washing as directed in Procedure, 4 until the wash water is neutral to methyl orange indicator.
6. Transfer the fatty acids to a filter paper carefully so as not to include any water. The filter paper may be supported in a small beaker without a funnel. The acids must remain completely melted until entirely filtered.
7. Heat the filtered acids on a hot plate to more than 130°C to remove traces of moisture and fill a titer test tube to a height of exactly 57 mm from the bottom.

   Caution—The test sample is *not* to be held at 130°C, nor reheated to this temperature more than once. If excessive moisture is present, allow the water to settle, decant the fatty acids, and then refilter and reheat. The acids must be thoroughly dry.

Solidification of the fatty acids—

1. Fill the water bath of the titer assembly to the designated level (see Fig. 1) and adjust the temperature to 20 ± 1°C for all test samples having titers of 35°C or higher, and 15–20°C below the titer point for all test samples with titers below 35°C.

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2. Place the test tube containing the fatty acids in the assembly as shown in Figure 1. Insert the titer thermometer to the immersion mark so that it is equidistant from the sides of the tube.
3. Stir with the stirring rod in a vertical manner, at the rate of 100 complete up and down motions per minute (see Notes, 3). The stirrer should move through a vertical distance of 38 mm. The agitation should be started while the temperature is at least 10°C above the titer point.
4. Stir at the directed rate until the temperature remains constant for 30 sec, or begins to rise in less than a 30 sec interval. Discontinue stirring immediately, remove the stirrer (at least raise it out of the test portion) and observe the increase in temperature. The titer point is recorded as the highest temperature indicated by the thermometer during this rise.

PRECISION

Duplicate determinations should agree to within ± 0.2°C.

NOTES

Caution

When diluting acids, always add acid to water unless otherwise directed in a method. Keep acids off skin and protect eyes when working with acids. If acids come in contact with skin or eyes, wash immediately with large amounts of water. Do not store oxidizing acids (perchloric, nitric, and sulfuric) near organic materials. Mixing organic materials with these acids, particularly perchloric, could result in an explosion.

NUMBERED NOTES

1. Sodium hydroxide cannot be substituted for potassium hydroxide in the glycerol-caustic saponification method.
2. The technical committee investigated a number of tests for complete saponification, but none was found which was reliable under all circumstances. Familiarity with the changes which take place in the appearance and character of the test portion usually enables the analyst to determine the proper end point, which is usually indicated by a change in the physical appearance of the test portion (increase in viscosity and thickening). When this occurs, the solution will then become less viscous and the reaction is complete when the test portion assumes a homogenous appearance. The most common characteristic is that of soap bubbles forming and rising from the surface. There are cases in which none of these criteria are evident, so considerable care must be observed at all times to ensure complete saponification.
3. Stirring may be performed mechanically by attaching a small motor with a suitable speed-reducing mechanism to the stirring rod.

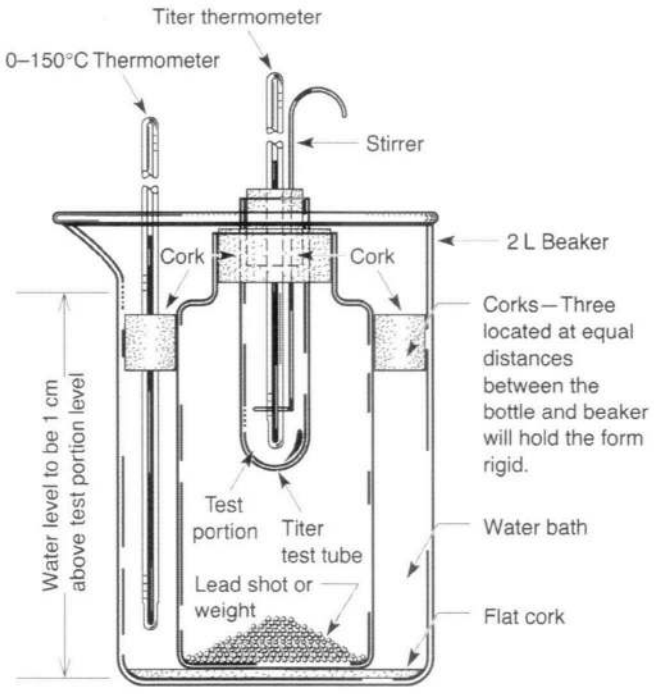

Figure 1. Titer stirring assembly.

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AOCS Official Method G 7-56

Reapproved 2009

pH of Acidulated Soap Stocks

DEFINITION

This method determines the pH of acidulated soap stock by measuring the pH of a water extract. Its value lies in the fact that it is an indicator of the mineral acidity of the acidulated soap stock.

SCOPE

Applicable to all acidulated soap stocks, regardless of type.

APPARATUS

1. pH meter, having a sensitivity capable of ±0.05 pH unit readings, equipped with external electrodes as follows—
   (a) Calomel electrode—range 5–100°C.
   (b) Glass electrode—range 5–100°C.
2. Beakers—50 and 250 mL, low form Pyrex™ is suitable.

REAGENTS

1. Buffer solutions, pH of 4.0, 7.0, and 10.0. Either of the following is satisfactory—
   (a) Prepared from Coleman buffer tablets certified to produce exact pH solutions when used as per full instructions.
   (b) Beckman standard buffer solutions.

STANDARDIZATION OF PH METER

Carefully follow the manufacturer's instructions for the particular meter used.

PROCEDURE

1. Pipet into a 250 mL beaker 50 mL of distilled water.
2. Heat to a temperature between 70 and 80°C.
3. Pour into the hot water 50 g of the test portion to be tested.
   Note—Preheating the test portion sample and the water reduces the tendency to emulsify.
4. Add a few boiling chips or glass beads, place a thermometer in the mixture, and heat slowly to 95°C, stirring the entire time the test portion is being brought to temperature.
5. When the temperature has reached 95°C, remove the beaker with its contents from the heat and allow to stand until phases separate.
6. After the phases have separated, with the aid of a suitable pipet, draw off the aqueous layer and transfer to a 50 mL beaker.
7. Determine the pH to the nearest 0.1 unit in the aqueous phase at 25°C.

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AOCS Specification H 1-39

Reapproved 2009

Forced Draft Oven

Forced draft (mechanical convection) ovens which will operate at and maintain the temperature between the limits specified by the method with which it is to be used.

The rate and direction of air flow must be such that the proper drying results without the danger of loss of finely divided material.

The temperature should be uniform, ±1°C (±1.8°F) over all the area of the shelves when the oven is loaded to half of its capacity and the load uniformly distributed. No portion of any shelf is used that does not conform to this requirement.

The temperature specified in any method is the temperature actually attained by the sample (or samples) on the shelf (or shelves) and is determined with an accurately calibrated thermocouple or maximum reading thermometer inserted in the sample.

After having determined the temperature on the shelves, routine observation and regulation may be made with a calibrated thermometer, traceable to a National Institute of Standards & Technology (NIST) certified thermometer, inserted in the provided place in the top of the oven with the bulb situated nearly at shelf level. The indicated temperature of this thermometer should be correlated with actual sample temperature on the shelf.

The drying time specified in any method is the actual time of drying at the specified temperature. Thus, allowance must be made for any temperature drop due to opening the door or for any other reason.

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AOCS Specification H 2-41

Reapproved 2009

Petroleum Ether

Specific gravity at 15.5°C (60°F): 0.630–0.660.

Color: Water white.

Doctor test: Sweet.

Saponification value: 1.0 mg KOH per 100 mL of petroleum ether.

Distillation test performed as directed in ASTM designation No. 216-54:

1. Initial boiling temperature, not less than 35°C and not over 38°C.
2. Dry flask end point, not less than 52°C and not over 60°C.
3. At least 95% distilling under 54°C.
4. Not more than 69% distilling under 40°C.
5. Residue in distillation flask, neutral to methyl orange.

Copper strip corrosion test: Insert a small strip of polished copper in the distillation flask containing petroleum ether. There should be no appreciable darkening of the copper during distillation.

Evaporation residue: 100 mL of petroleum ether only, not over 0.0011 grams. Add 0.25 g of stearin or similar hard fat (previously dried to constant weight at 101 ± 1°C) to 250 mL of petroleum ether. Evaporate the ether on a steam or water bath and dry the residue to constant weight at 101 ± 1°C. The increase in weight shall not exceed 0.003 g.

Unsaturated compounds: Determined as directed on page 154 of the Analytical Edition of I. & E.C., March, 1938. A trace only is permitted.

Odor test: Immerse a strip of white unglazed blotting paper (2.54 cm × 10.16 cm × 0.422 cm or 1 in. × 4 in. × 0.166 in.) in petroleum ether for 30 sec. Dry at room temperature in still air for 12 min. The paper should remain odorless.

Aromatic compounds: Add 5 drops of petroleum ether to 40 drops of H_2SO_4 (sp. gr. 1.84) and 10 drops of HNO_3 (sp. gr. 1.42) in a test tube. Warm for 10 min and then cool for 30 min. Transfer to a shallow dish and dilute with distilled water. There should be no nitrobenzene odor.

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AOCS Specification H 3-45

Reapproved 2009

Air Oven

The air oven should be one which will operate at and maintain the temperature between the limits specified by the method with which it is to be used.

The oven must be provided with openings at the top and bottom for adequate ventilation.

The temperature should be uniform, ±1°C (± 1.8°F) over all the area of the shelves when the oven is loaded to half of its capacity and the load uniformly distributed. No portion of any shelf is used that does not conform to this requirement.

After having determined the temperature on the shelves, routine observation and regulation may be made with a calibrated thermometer, traceable to an National Institute of Standards & Technology (NIST) certified thermometer, inserted in the provided place in the top of the oven with the bulb situated nearly at shelf level. The indicated temperature of this thermometer should be correlated with actual sample temperature on the shelf.

The drying time specified in any method is the actual time of drying at the specified temperature. Thus, allowance must be made for any temperature drop due to opening the door or for any other reason.

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AOCS Specification H 4-45

Reapproved 2009

Vacuum Oven

The vacuum oven should be one which will operate at and maintain the temperature between the limits specified by the method with which it is to be used.

The oven may be fitted with appropriate connections so it may be flushed with an inert gas if desired.

The temperature should be uniform, ± 1°C (±1.8°F) over all the area of the shelves when the oven is loaded to half of its capacity and the load uniformly distributed. No portion of any shelf is used that does not conform to this requirement.

After having determined the temperature on the shelves, routine observation and regulation may be made with a calibrated thermometer, traceable to an National Institute of Standard & Technology (NIST) certified thermometer, inserted in the provided place in the top of the oven with the bulb situated nearly at shelf level. The indicated temperature of this thermometer should be correlated with actual sample temperature on the shelf.

The drying time specified in any method is the actual time of drying at the specified temperature. Thus, allowance must be made for any temperature drop due to opening the door or for any other reason.

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AOCS Specification H 5-40, H 6-40, H 7-45, H 8-45, H 10-55, H 11-58

Reapproved 2009

Thermometer Specifications

AOCS No.	ASTM Equivalent (a)	Range	Subdivisions	Immersion	Scale Error When Standardized	Standardization
H 5-40	11°C Open Flash	-6–400°C	2°C	25 mm	1–260°C, 2°C above 260°C	See Notes, 1
	11°F	20–760°F	5°F	25 mm	2.5–500°F, 3.5°F above 500°F	
H 6-40	36°C Titer Test	-2–68°C	0.2°C	45 mm	0.2°C	See Notes, 2
H 7-45	15°C Low Softening Point	-2–80°C	0.2°C	Total	0.2°C	See Notes, 2
H 8-45	5°C Cloud & Pour	-38–50°C	1°C	109 mm	0.5°C	See Notes, 2
H 10-55	88°C Vegetable Oil Flash	10–200°C	1°C	57 mm	1°C	See Notes, 2 and 3
	88°F	50–392°F	2°F	57 mm	2°F	See Notes, 2 and 3
H 11-58	42°C Solvent Distillation	95–255°C	0.5°C	100 mm	0.5°C	See Notes, 2
	34°C Aniline Point	25–105°C	0.2°C	51 mm	0.2°C	See Notes, 2

1. The thermometer shall be standardized at the ice point and at intervals of approximately 50°C (100°F) for 25 mm (1 in.) immersion and for the following temperatures of the emergent mercury column according to Table 1.
2. The thermometer shall be standardized at the ice point, if possible, and at intervals of about 20°C (40°F). Use the specified immersion and record the temperature of the emergent column of mercury at each calibration point. The standardization is valid for the stem temperatures found in your laboratory.

   In keeping with good laboratory practice, all laboratory thermometers should be calibrated against a National Institute of Standards & Technology (NIST) certified thermometer. The calibrated laboratory thermometers should be tagged and numbered. A laboratory thermometer calibration chart, showing the thermometer number, location, use in the laboratory and the necessary corrections, should be maintained. A copy of the NIST certificate for the certified thermometer should be kept with the laboratory calibration chart.
3. Thermometers 88°C and 88°F are usually not standard stock items. They may have to be specially ordered, requiring a delivery time of up to 6 wk. One supplier is VWR Scientific.

Table 1.

Thermometer Reading	Average Temp. of Emergent Mercury Column	Thermometer Reading	Average Temp. of Emergent Mercury Column
100°C	44°C	200°F	110°F
150°C	54°C	300°F	129°F
200°C	64°C	400°F	150°F
250°C	77°C	500°F	175°F
300°C	91°C	600°F	205°F
350°C	108°C	700°F	240°F

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AOCS Specification H 9-87
Replaced H 9-45 • Reapproved 2009

Desiccants

1. Drierite™ anhydrous calcium sulfate, 4–8 mesh size.This is a satisfactory desiccant and may be obtained in the indicating variety which changes color from blue to pink as the water is absorbed. It may be regenerated to the original blue color by heating in an oven at 204°C for 1 hr. It is suitable for drying gases and for use in desiccators. It will absorb up to 10–14% of its weight in water when used for drying gases.
2. Activated alumina, 8–14 mesh, may be used for drying air, in desiccators, balances, and closed appliances that must be kept free from moisture. It can be regenerated and reused. It will absorb about 15% of its weight in water.
3. Magnesium perchlorate is a satisfactory desiccant which will absorb up to 50% of its weight in water.
4. Silica gel is a satisfactory desiccant that will absorb up to 30% of its weight in water. It may be used in balances and closed appliances. It is available in an indicating type.
5. Anhydrous sodium sulfate may be used to remove moisture from an organic phase obtained from the extraction of an aqueous phase or when an organic phase is washed with water, e.g., the heptane phase containing methyl esters prepared as noted in method Ce 2-66.
6. Other desiccants have been proposed, however, their use is not recommended because of hazards associated with handling:
   Concentrated sulfuric acid, sp. gr. 1.84
   Phosphoric anhydride powder.

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AOCS

AOCS Specification H 12-52

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Standard Sodium Hydroxide Solution

APPARATUS

1. Erlenmeyer flask—300 mL.
2. Burette—accurately calibrated to meet National Institute of Standards & Technology (NIST) specifications. Because alkali will dissolve glass, to avoid volumetric errors it should not be stored in calibrated apparatus. Burettes used continuously should be recalibrated periodically.
3. Absorption tower or drying tube of adequate capacity—packed with an efficient absorbent for carbon dioxide such as Ascarite or soda lime.

REAGENTS

1. Carbon dioxide-free distilled water—prepared by one of the following methods:
   (a) Boil water for 20 min and cool with absorption tower protection against CO_2 absorption;
   (b) Bubble through water for 12 hr a vigorous stream of air, freed from CO_2 by passing through a tower with Ascarite or soda lime (see Notes, 1).
2. Sodium hydroxide—(1 + 1), to one part by weight of NaOH (containing less than 5% Na_2CO_3) in an alkali-resistant flask or bottle add one part of H_2O. Swirl until solution is complete. Close the container tightly with a rubber stopper. Set aside until Na_2CO_3 has settled, leaving perfectly clear supernatant liquid (see Notes, *Caution*).
3. Acid potassium phthalate, $KHC_8H_4O_4$, National Institute of Standards & Technology (NIST) standard sample for acidimetry. Crush sample to a fineness of approximately 100 mesh and dry for 2 hr at 120°C. Allow to cool in an efficient desiccator.
4. Ethyl alcohol—95% by volume (SDA Formulas 30 or 3A are permitted).
5. Phenolphthalein indicator—dissolve 1.0 g of phenolphthalein in 100 mL of alcohol.

PROCEDURE

1. The following table gives the approximate quantities of NaOH solution (1 + 1) necessary to make 10 L of standard solution of the indicated normality:

Approximate normality	Sodium hydroxide solution to be diluted to 10 L	
	mL	g
0.1 N	54	82
0.25 N	135	205
0.5 N	270	410
1.0 N	540	820

2. Add the required quantity of NaOH solution (1 + 1) to enough CO_2-free distilled water to give a total volume of 10 L and mix well. Protect the solution from CO_2 by stoppering tightly or by means of absorption tube or tower.
3. Weigh accurately sufficient dried $KHC_8H_4O_4$ to require 40 mL of the sodium hydroxide solution to be standardized and transfer it to a 300 mL flask that has been swept free from CO_2. Add 50 mL of cool CO_2 free H_2O. Stopper the flask and swirl gently until the $KHC_8H_4O_4$ is dissolved.
4. When the $KHC_8H_4O_4$ is in solution, add 3 drops of phenolphthalein indicator, and titrate to the first persistent faint pink color with the solution to be standardized taking precautions to exclude CO_2.
5. Determine the quantity of sodium hydroxide solution required to produce the same pink phenolphthalein end point in another flask containing the indicator and the same volume of CO_2-free water. Subtract the amount required from that used in the first titration and calculate the normality of the standard solution by the following equation:

$$\text{Normality} = \frac{\text{mass of } KHC_8H_4O_4, \text{ g}}{\text{volume, mL of NaOH} \times 0.20444}$$

NOTES

Caution

As an alternate method for removing carbonate, a barium hydroxide solution may be added to the undiluted sodium hydroxide solution. If the barium hydroxide solution is used, make certain that the sodium hydroxide solution is cooled prior to the addition of barium hydroxide. Failure to do so will result in a violent splattering of the sodium hydroxide solution and could result

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in a serious injury. See the Laboratory Safety section at the beginning of this edition for precautions regarding the handling of strong alkalies.

NUMBERED NOTES

1. When the standard sodium hydroxide is prepared as directed, it is essentially free of carbon dioxide. It is therefore possible to correct for the amount of alkali required to produce the end point with phenolphthalein as described in the method. The resulting solution is expected to have true normality with respect to the hydrogen ion concentration.

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AOCS Specification H 13-52
Reapproved 2009

Standard Sulfuric Acid Solution

APPARATUS

1. Erlenmeyer flask—300 mL.
2. Burette—accurately calibrated to meet National Institute of Standards & Technology (NIST) specifications.
3. Absorption tower or drying tube of adequate capacity—packed with an efficient absorbent for carbon dioxide such as Ascarite or soda lime.

REAGENTS

1. Carbon dioxide-free distilled water—prepared by one of the following methods:
   (a) Boil water for 20 min and cool with absorption tower protection against CO_2 absorption;
   (b) Bubble through water for 12 hr a vigorous stream of air, freed from CO_2 by passing through a tower with Ascarite or soda lime.
2. Sulfuric acid—reagent quality, approximately 95% H_2SO_4 (see Notes, *Caution*).
3. Sodium hydroxide solution—prepared and standardized as directed by AOCS Specification H 12-52.
4. Ethyl alcohol—95% by volume (SDA Formulas 30 or 3A are permitted).
5. Phenolphthalein indicator—dissolve 1.0 g of phenolphthalein in 100 mL of alcohol.
6. Methyl red indicator—dissolve 100 mg of methyl red in 60 mL of alcohol and dilute with distilled water to 100 mL.
7. Methyl orange indicator—dissolve 100 mg of methyl orange in 100 mL of distilled water.

PROCEDURE

1. The following table gives the approximate quantities of reagent grade H_2SO_4 (95%) necessary to make 10 L of standard solution of the indicated molarity:

Approximate molarity	H_2SO_4 to be diluted to 10 L
	mL
0.05 M	28
0.125 M	70
0.25 M	140
0.5 M	280

2. Add the required quantity of H_2SO_4 solution to enough CO_2-free distilled water to give a total volume of 10 L and mix well.
3. Into a 300 mL flask measure accurately, by means of a burette, 40 mL of the H_2SO_4 solution to be standardized. Add 3 drops of phenolphthalein indicator and titrate to the first persistent faint pink color with standardized sodium hydroxide solution of about the same concentration as the acid being standardized. Calculate the molarity of the acid solution by the following formula:

$$\text{Molarity} = \frac{\text{volume, mL of standard alkali} \times \text{molarity of alkali}}{\text{volume, mL of } H_2SO_4}$$

4. If the standard acid is to be used with methyl orange or methyl red indicators, determine the correction for the volume of acid required to pass from the end point of phenolphthalein to that of methyl orange or methyl red. Add 3 drops of phenolphthalein indicator to 100 mL of CO_2- free water and then add sufficient sodium hydroxide solution to give the faint pink end point with phenolphthalein. Disregard this quantity of sodium hydroxide solution. Add 3 drops of methyl orange or methyl red indicator and sufficient standard acid to give the red acid color of the indicator. Then titrate back with standard sodium hydroxide of the same strength as the standard acid to the methyl orange or methyl red end point, that is to the disappearance of orange or pink color. Subtract the mL of sodium hydroxide from the mL of acid to obtain the correction for the volume of acid required to pass from the end point of phenolphthalein to that of methyl orange, or methyl red. This correction should be added to the mL of acid used in the standardization for calculating the normality for use with methyl orange or methyl red indicator.

NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

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AOCS

AOCS Specification H 14-52

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Standard Hydrochloric Acid Solution

APPARATUS

1. Erlenmeyer flask—300 mL.
2. Burette—accurately calibrated to meet National Institute of Standards & Technology (NIST) specifications.
3. Absorption tower or drying tube of adequate capacity—packed with an efficient absorbent for carbon dioxide such as Ascarite or soda lime.

REAGENTS

1. Carbon dioxide-free distilled water—prepared by one of the following methods:
   (a) Boil water for 20 min and cool with absorption tower protection against CO_2 absorption;
   (b) Bubble through water for 12 hr a vigorous stream of air, freed from CO_2 by passing through a tower with Ascarite or soda lime.
2. Hydrochloric acid—reagent quality, 35–37% HCl.
3. Sodium hydroxide solution—prepared and standardized as directed by AOCS Specification H 12-52.
4. Ethyl alcohol—95% by volume (SDA Formulas 30 or 3A are permitted).
5. Phenolphthalein indicator—dissolve 1.0 g of phenolphthalein in 100 mL of alcohol.
6. Methyl red indicator—dissolve 100 mg of methyl red in 60 mL of alcohol and dilute with distilled water to 100 mL.
7. Methyl orange indicator—dissolve 100 mg of methyl orange in 100 mL of distilled water.

PROCEDURE

1. Table 1 gives the approximate quantities of reagent grade HCl (35–37%) necessary to make 10 L of standard solution of the indicated molarity.
2. Add the required quantity of HCl to enough CO_2 free distilled water to give a total volume of 10 L and mix well.
3. Into a 300 mL flask measure accurately, by means of a burette, 40 mL of the HCl solution to be standardized. Add 3 drops of phenolphthalein indicator and titrate to the first persistent faint pink color with standardized sodium hydroxide solution of about the same concentration as the acid being standardized. Calculate the molarity of the acid solution by the following formula:

$$\text{Molarity} = \frac{\text{volume, mL of standard alkali} \times \text{molarity of alkali}}{\text{volume, mL of HCl}}$$

4. If the standard acid is to be used with methyl orange or methyl red indicators, determine the correction for the volume of acid required to pass from the end point of phenolphthalein to that of methyl orange or methyl red. Add 3 drops of phenolphthalein indicator to 100 mL of CO_2-free water and then add sufficient sodium hydroxide solution to give the faint pink end point with phenolphthalein. Disregard this quantity of sodium hydroxide solution. Add 3 drops of methyl orange or methyl red indicator and sufficient standard acid to give the red acid color of the indicator. Then titrate back with standard sodium hydroxide of the same strength as the standard acid to the methyl orange or methyl red end point, that is to the disappearance of orange or pink color. Subtract the mL of sodium hydroxide from the mL of acid to obtain the correction for the volume of acid required to pass from the end point of phenolphthalein to that of methyl orange, or methyl red. This correction should be added to the mL of acid used in the standardization for calculating the normality for use with methyl orange or methyl red indicator.

Table 1

Approximate molarity	HCl to be diluted to 10 L
	mL
0.1 M	89
0.25 M	222
0.5 M	445
1.0 M	890

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AOCS

AOCS Specification H 15-52

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Standard Alcoholic Potassium Hydroxide

APPARATUS

1. Erlenmeyer flask—300 mL.
2. Burette—accurately calibrated to meet National Institute of Standards & Technology (NIST) specifications.
3. Absorption tower or drying tube of adequate capacity—packed with an efficient absorbent for carbon dioxide such as Ascarite or soda lime.

REAGENTS

1. Potassium hydroxide—reagent grade.
2. Ethyl alcohol—95% by volume (SDA Formulas 30 or 3A are permitted).
3. Acid potassium phthalate ($KHC_8H_4O_4$)—National Institute of Standards & Technology standard sample for acidimetry. Crush sample to a fineness of approximately 100 mesh and dry for 2 hr at 120°C. Allow to cool in an efficient desiccator.
4. Phenolphthalein indicator—dissolve 1.0 g of phenolphthalein in 100 mL of alcohol.

PROCEDURE

1. Dissolve the appropriate amount of KOH (35 g for 0.5 M; 14 g for 0.2 M) in 20 mL of distilled water and dilute to 1 L with alcohol in an alkali-resistant bottle. Mix well and allow to stand overnight. Decant the clear supernatant solution into a brown glass bottle with a rubber stopper without exposure to carbon dioxide (see Notes, *Caution*).
2. Weigh accurately sufficient dried $KHC_8H_4O_4$ to require about 40 mL of the potassium hydroxide solution to be standardized and transfer it to a 300 mL flask that has been swept free of CO_2. Add 50 mL of cool CO_2 free water. Stopper the flask and swirl gently until the $KHC_8H_4O_4$ is dissolved.
3. When the sample is in solution, add 3 drops of phenolphthalein indicator and titrate to the first persistent faint pink color with the solution to be standardized, taking precautions to exclude CO_2.
4. Determine the quantity of the potassium hydroxide solution required to produce the same pink phenolphthalein end point in another flask containing the indicator and the same volume of CO_2 free water. This amount is the blank correction. Subtract this amount from that used in the first titration.
5. Calculate the molarity of the standard solution by the following equation:

$$\text{Molarity} = \frac{\text{mass of } KHC_8H_4O_4, \text{ g}}{\text{volume, mL of KOH} \times 0.20444}$$

NOTES

Caution

As an alternate method for removing carbonate, a barium hydroxide solution may be added to the undiluted potassium hydroxide solution (see Specification H 12–52). If the barium hydroxide solution is used, make certain that the potassium hydroxide solution is cooled prior to the addition of barium hydroxide. Failure to do so will result in a violent splattering of the potassium hydroxide solution and could result in a serious injury. See the Laboratory Safety section at the beginning of this edition for precautions regarding the handling of strong alkalies.

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AOCS Specification H 16-56

Reapproved 2009

Commercial Hexane

Specific gravity at 15.5°C (60°F): 0.678–0.692.

Color: Water white.

Doctor test: Sweet.

Saponification value: Less than 1.0 mg KOH per 100 mL of commercial hexane.

Distillation test: Performed as directed in ASTM designation no. 216–54.

Initial boiling temperature: Not less than 60°C.

Dry flask end point: Not over 70°C.

Residue in distillation flask: Neutral to methyl orange.

Copper strip corrosion test: Insert a small strip of polished copper in the distillation flask containing commercial hexane. There should be no appreciable darkening of the copper during distillation.

Evaporation residue: 100 mL of commercial hexane only should not yield over 0.0011 grams. Add 0.25 g of stearin or similar hard fat (previously dried to constant weight at 101 ± 1°C) to 250 mL of commercial hexane. Evaporate the commercial hexane on a steam or water bath and dry the residue to constant weight at 101 ± 1°C. The increase in weight should not exceed 0.003 g.

Unsaturated compounds: Determined as directed on page 154 of the Analytical Edition of *I.* and *E. C.*, March, 1938. Note that only a trace of unsaturated compounds is permitted.

Odor test: Immerse a strip of white unglazed blotting paper (2.54 cm × 10.16 cm × 0.422 cm or 1 in. × 4 in. × 0.166 in.) in commercial hexane for 30 sec. Dry at room temperature in still air for 12 min. The paper should remain odorless.

Aromatic compounds: Add 5 drops of commercial hexane to 40 drops of H_2SO_4 (sp. gr. 1.84) and 10 drops of HNO_3 (sp. gr. 1.42) in a test tube. Warm for 10 min and then cool for 30 min. Transfer to a shallow dish and dilute with distilled water. There should be no nitrobenzene odor.

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AOCS Specification H 17-58

Reapproved 2009

Synthetic Methyl Alcohol

Specific gravity at 20/20°C: 0.792–0.793

Color: Water white.

Distillation test: Performed as directed in ASTM designation no. 268–53, none boiling below 64°C and none boiling above 65.5°C.

Nonvolatile matter: Not more than 0.005 g per mL.

Odor: Mild and nonresidual.

Moisture: 0.15% by weight by iodine reagent method.

Acidity: Not more than 0.003% by weight equivalent to 0.028 mg of potassium hydroxide (KOH) per g of sample (free acid calculated as acetic acid).

Potassium permanganate test: Color of added $KMnO_4$ to be retained at least 30 min at 15 ± 0.5°C.

Acetone: Not more than 0.03%.

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AOCS Specification H 18-58

Reapproved 2009

Isopropyl Alcohol

Specific gravity at 20/20°C: 0.785–0.790.

Color: Water white.

Distillation test: Performed as directed in ASTM designation no. 268–53, not greater than 1.5°C and this range shall include 82.3°C.

Nonvolatile matter: Not more than 0.005 g per mL.

Odor: Mild and nonresidual.

Moisture: Miscible without turbidity with 19 volumes of 10 degree heptane.

Acidity: Not more than 0.02 mg of KOH per g of sample.

Hydrocarbons: Miscible without turbidity with 10 volumes of distilled water at 20°C.

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AOCS

AOCS Specification H 19-58

Reapproved 2009

Industrial Toluene

Specific gravity at 20/20°C: 0.860–0.870.

Color: Water white.

Distillation test: Performed as directed in ASTM designation no. 268–53, none boiling below 107.5°C and none boiling above 112.5°C, with the total distillation range of any specific sample being not greater than 3.0°C.

Nonvolatile matter: Not more than 0.005 g per mL.

Odor: Characteristic aromatic hydrocarbon and nonresidual.

Moisture: Not sufficient to show turbidity at 20°C.

Acidity: None.

Sulfur as H_2S or SO_2: None.

Corrosion by copper test: Copper should show no iridescence.

Corrosion by mercury test: Mercury should show no discoloration.

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AOCS

AOCS Specification H 20-11

Revised 2011

Standard Test Sieves

Sieve Designation			Nominal Sieve Opening, inches	Nominal Wire Diameter, mm
International Standard[a] (ISO)		U.S.A. Standard		
12.5	mm[b]	½ in.[b]	0.500	2.67
11.2	mm	7/16 in.	0.438	2.45
9.5	mm	⅜ in.	0.375	2.27
8.0	mm	5/16 in.	0.312	2.07
6.7	mm	0.265 in.	0.265	1.87
6.3	mm[b]	¼ in.[b]	0.250	1.82
5.6	mm	No. 3 ½	0.223	1.68
4.75	mm	No. 4	0.187	1.54
4.00	mm	No. 5	0.157	1.37
3.35	mm	No. 6	0.132	1.23
2.80	mm	No. 7	0.111	1.10
2.38	mm	No. 8	0.0937	1.00
2.00	mm	No. 10	0.0787	0.900
1.70	mm	No. 12	0.0661	0.810
1.40	mm	No. 14	0.0555	0.725
1.18	mm	No. 16	0.0469	0.650
1.00	mm	No. 18	0.0394	0.580
850	µm[c]	No. 20	0.0331	0.510
710	µm	No. 25	0.0278	0.450
600	µm	No. 30	0.0234	0.390
500	µm	No. 35	0.0197	0.340
425	µm	No. 40	0.0165	0.290
355	µm	No. 45	0.0139	0.247
300	µm	No. 50	0.0117	0.215
250	µm	No. 60	0.0098	0.180
212	µm	No. 70	0.0083	0.152
180	µm	No. 80	0.0070	0.131
150	µm	No. 100	0.0059	0.110
125	µm	No. 120	0.0049	0.091
106	µm	No. 140	0.0041	0.076
90	µm	No. 170	0.0035	0.064
75	µm	No. 200	0.0029	0.053
63	µm	No. 230	0.0025	0.044
53	µm	No. 270	0.0021	0.037

[a]These standard designations correspond to the values for test sieve apertures recommended by the International Organization for Standardization, Geneva, Switzerland.

[b]These sieves are not in the standard series but they have been included because they are in common usage.

[c]1000 µm = 1 mm

Reference

Official Methods of Analysis, Association of Official Analytical Chemists, 15th ed., Vol. 1, Arlington, VA, 1990, p. xvi.

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AOCS

AOCS Official Method Ja 2a-46

Reapproved 2009

Moisture

DEFINITION

This method determines the moisture by distillation with an immiscible solvent.

SCOPE

This method is applicable to lecithin from soybeans and corn for the determination of moisture. It is not applicable to test samples containing water-miscible volatile substances.

APPARATUS

1. Glass distillation apparatus with ground-glass connections, constructed and assembled according to the specifications shown in Figure 1.
   (a) Flask—short-neck, round bottom type, 500 mL.
   (b) Condenser—water-cooled, the lower tip of which should be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established.
   (c) Trap—constructed of well-annealed glass and graduated to contain 5 mL at 20°C. It shall be subdivided into 0.1 mL divisions with each mL line numbered and the 5 mL line at the top. The error at any indicated capacity shall not exceed 0.05 mL.
   (d) Distillation may be better controlled by wrapping the tube which leads from the flask to the receiver with 100% fiberglass cloth tape. It is advantageous to wrap the flask also especially if there are drafts in the vicinity of the distillation apparatus.
   (e) Calibrate the receiver by the following procedure—add an accurately measured portion, 1 ± 0.01 mL, of distilled water to 100 mL of toluene in the distillation flask. Conduct the distillation as described in Procedure, 2–5, and calculate as directed. Cool the apparatus, add another mL of water, and repeat the distillation. Continue in this manner up to the capacity of the receiving tube.
2. Pipet—measuring type, capacity 1.0 mL, graduated in 0.01 mL.
3. Heat source—either an oil bath or an electric heater provided with a suitable means of control.
4. Copper or Nichrome wire—long enough to extend through the condenser with one end twisted into a spiral. The diameter of the spiral is such that it will fit snugly within the receiver and yet can be moved up and down.

REAGENTS

1. Toluene—ACS grade (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Warm the test sample to soften the product (but do not heat over 60°C) and then mix thoroughly.

PROCEDURE

1. The entire apparatus should be cleaned with potassium dichromate-sulfuric acid cleaning solution to minimize the adherence of water droplets to the sides of the condenser and receiver. Rinse thoroughly and dry completely before using.
2. Weigh a 100 ± 0.1 g test portion of the well-mixed test sample into the distillation flask (see Notes, 1).
3. Add 100 mL of solvent and swirl to mix. Assemble the apparatus and fill the receiver with solvent by pouring through the condenser until the solvent begins to overflow into the distillation flask. Insert a loose cotton plug in the top of the condenser to prevent condensation of atmospheric moisture within the tube.
4. Heat the flask so that the distillation rate will be about 100 drops per minute. When the greater part of the water has distilled, increase the distillation rate to about 200 drops per minute and continue until the water level in the receiver increases no more than 0.1 mL in 30 min, or until the distillation has proceeded for 2 hr, whichever occurs first.
5. Discontinue heating, dislodge any drops of water which may be adhering to the inside of the condenser tube or receiver with a wire spiral, and wash with about 5 mL of toluene.
6. Immerse the receiver in water at 25°C for at least 15 min or until the toluene layer is clear and then note the volume of water.

CALCULATION

$$\text{Moisture, \%} = \frac{\text{volume, mL of water} \times 99.7}{\text{mass, g of test portion}}$$

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NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

NUMBERED NOTES

1. If the test portion contains more than 5% moisture use 50 or 75 g of the test sample with 100 mL of toluene.

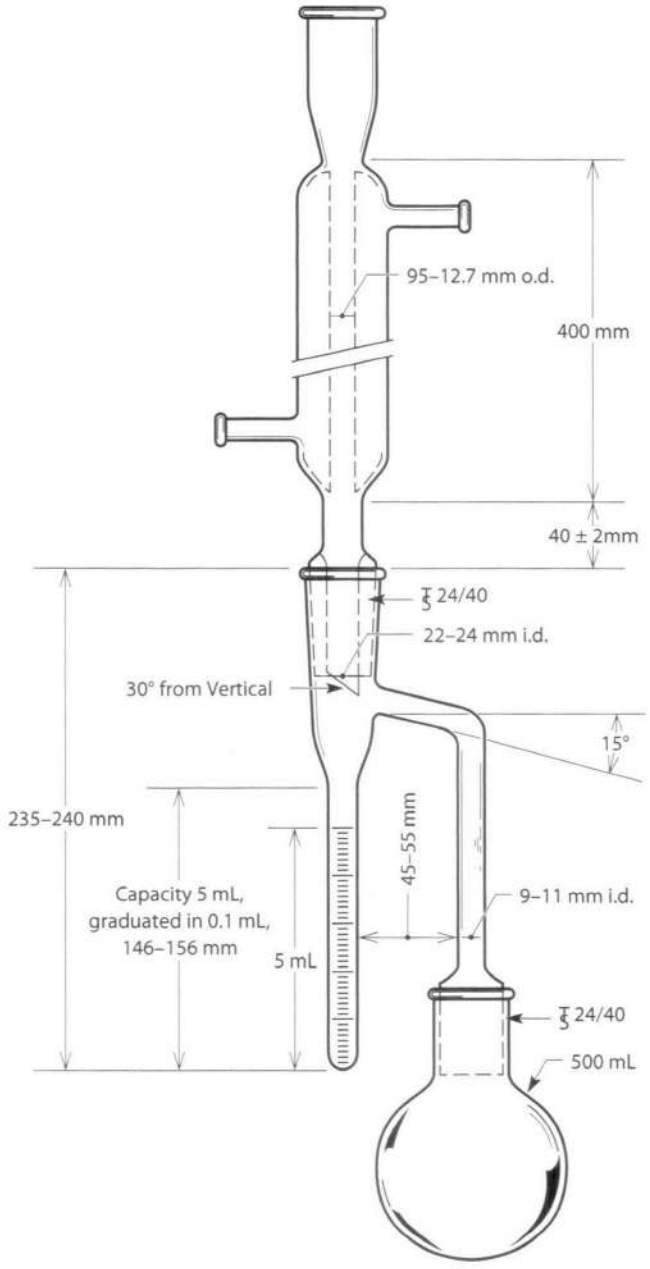

Figure 1. Moisture distillation apparatus.

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AOCS

AOCS Official Method Ja 2b-87

Reapproved 2009

Moisture, Karl Fischer Reagent

DEFINITION

This method determines the actual water content of lecithins by titration with Fischer reagent which reacts quantitatively with water.

SCOPE

Applicable to all lecithins that do not react with and are soluble in the reagents and which do not contain impurities leading to secondary reactions. Such impurities are alkaline compounds and peroxides which react with the reagent and, therefore, show high results.

APPARATUS

1. Karl Fischer titration assembly—manual or automatic, with stirrer.

REAGENTS

1. Methanol—not containing more than 0.05% (w/w) water. If the reagent contains more than this quantity of water, dry it by distillation with magnesium turnings activated with iodine. Collect the distillate in a receiver protected from atmospheric moisture by means of a guard tube fitted with desiccant (Reagents, 9).
2. Chloroform—ACS reagent grade (see Notes, *Caution*, and Notes, 1).
3. 2–Methoxyethanol (ethylene glycol monomethyl ether)—not containing more than 0.05% (w/w) water. If the reagent contains more than this quantity of water, dry it by distillation, rejecting the first portion of distillate, which contains the water.
4. Pyridine—not containing more than 0.05% (w/w) water. If the reagent contains more than this quantity of water, dry it by distillation, rejecting the first portion of distillate, which contains the water (see Notes, *Caution*).
5. Sample solvent, chloroform–methanol (see Notes, *Caution*)—thoroughly mix 800 mL of chloroform with 200 mL of methanol. Store in an airtight container.
6. Sulfur dioxide—anhydrous liquified gas in cylinder.
7. Iodine—ACS grade.
8. Karl Fischer reagent—stabilized single solution, available commercially or prepared as follows (see Notes, 2)—
   (a) Place 670 mL of the methanol or the 2–methoxyethanol in a dry brown-glass flask, fitted with a ground–glass stopper and having a capacity slightly greater than 1 L.
   (b) Add about 85 g of iodine. Stopper the flask and shake it occasionally until the iodine is completely dissolved. Then add approximately 270 mL of pyridine, stopper the flask again, and mix thoroughly. Using the method described below, dissolve 65 g of sulfur dioxide in this solution, cooling to ensure that the temperature of the liquid does not exceed 20°C.
   Note—As the reaction is exothermic, it is necessary to cool the flask from the beginning and to maintain it at about 0°C, for example by immersing it in an ice bath or in crushed carbon dioxide.
   (c) Replace the ground–glass stopper with an attachment for introducing sulfur dioxide, consisting of a cork bearing a thermometer and a glass inlet tube 6 mm × 8 mm, reaching to within 10 mm of the bottom of the flask, and a small capillary tube for connecting to the atmosphere.
   (d) Place the whole assembly with the ice bath on a balance and weigh to the nearest 1 g. Connect the inlet tube to a cylinder of sulfur dioxide by means of a flexible connection and a drying tube filled with the desiccant (Reagents, 9) and gently open the tap on the cylinder.
   (e) Adjust the rate of flow of sulfur dioxide so that all the gas is absorbed without the liquid showing any sign of rising in the inlet tube.
   (f) Maintain the equilibrium of the balance by gradually increasing the tare, and ensure that the temperature of the liquid does not rise above 20°C. Close the tap on the cylinder as soon as the increase in mass reaches 65 g.
   (g) Immediately remove the flexible connection and reweigh the flask and its inlet attachment. The mass of dissolved sulfur dioxide should be between 60 and 70 g. A slight excess is not harmful.
   (h) Stopper the flask, mix the solution, and leave at least 24 hr before using it. In fact, as the result of imperfectly understood reactions which occur in the fresh reagent, the water equivalent of the reagent initially decreases rapidly and then tapers off slowly.
   (i) The water equivalent should be between 3.5 and 4.5 mg/mL. It should be determined daily if methanol has been used, but may be determined less frequently if 2–methoxyethanol has been used.
   (j) It is possible to prepare Karl Fischer reagent having a lower water content by diluting the solution prepared as described above with the sample solvent (Reagents, 5).
   (k) Store the reagent in the dark and protected from atmospheric moisture. It should preferably be stored in the reagent bottle.

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9. Aluminum sodium silicate—anhydrous, in the form of 1.7 mm dia granules, for use as a desiccant. The granules may be regenerated by washing with water and drying at 350°C for at least 48 hr. Alternatively, activated silica gel may be used as a desiccant.

WATER EQUIVALENT OF THE KARL FISCHER REAGENT

1. Accurately weigh to the nearest 0.0001 g, a drop of water, approximately 50 mg, and transfer to the titration vessel. For this calibration of the reagent, 250 mg of crystalline sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) may also be used instead of a drop of water.
2. Add the stirring rod by means of a pair of tweezers and connect the titration vessel with the apparatus.
3. Add 20 mL of methanol by means of a pipet or a burette. Close the aperture and dissolve by stirring gently.
4. Titrate with the Karl Fischer reagent up to the electrometric end point. This point is reached when the deflection remains constant for 30 sec after dropwise adding the Karl Fischer reagent. Determine a blank.
5. The amount of solvent used must allow the electrode to dip 2–3 mm below the surface of the solvent.
6. For direct-reading apparatus, the calibration factor is programmed into the equipment according to the manufacturer's instructions and a blank determination is not required.

PROCEDURE

1. Introduce 0.5–5.0 g of test sample weighed accurately to the nearest 0.1 mg into a dry titration vessel.
2. Add 100–120 mL of 80:20 chloroform/methanol and introduce with tweezers a stirring bar. The solvent volume may be adjusted in the apparatus as long as the ratio between the test sample and the solvent remains within the ranges identified. Cap off the solution to minimize changes in moisture content of the test sample or the solvent.
3. Stir to dissolve the test sample with a stirring-bar rate slow enough to avoid mixing air into the solution. If the test sample will not completely dissolve, repeat using a smaller test sample size.
4. When the test sample has completely dissolved, titrate with Karl Fischer reagent to the electrometric end point. Dilution of the Karl Fischer solution in the case of low water content test samples or adjustment of the test sample weight may be necessary to obtain a sufficient titration. If a slow response time exists, a reduction of the test sample weight may be necessary.
5. Conduct a blank test following the protocols outlined above using the same quantities of all reagents as used for the determination, but omitting the test sample. Average a minimum of three replicates for the blank titration. If the blank produces an excessive titration, the use of dryer solvents, drying solvents by distillation, or pretitration of the solvent with the Karl Fischer reagent to the end point are all available options.

   Note—This procedure may vary for automated titrators. In that case, follow the instructions as outlined in the instrument instruction manual following the precautions as outlined above to minimize exposure that may cause moisture changes in the test sample or the solvent.

CALCULATIONS

1. The water equivalent, T, of the Karl Fischer reagent in mg H_2O/mL is given by the formula:

$$T = \frac{m_1}{(V_0 - V_1)}$$

   Where—

   m_1 = mass of the water added, mg
   V_0 = volume of the Karl Fischer reagent used for the determination of the water equivalent of the Karl Fischer reagent, mL
   V_1 = volume of the Karl Fischer reagent used for the blank test, mL

2. The water content of the test sample is given by the formula:

$$\%\ H_2O,\ (w/w) = \frac{(V_2 - V_3) \times T \times 100}{m_2}$$

   Where—

   V_2 = volume of the Karl Fischer reagent used for the determination (Procedure, 4), mL
   V_3 = volume of the Karl Fischer reagent used for the blank test (Procedure, 5), mL
   m_2 = mass of the test portion (Procedure, 1), mg

3. Take as a result the arithmetic mean of two determinations.

PRECISION

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same operator on the same test sample shall not exceed 0.6% relative.

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NOTES

Caution

Chloroform is a known carcinogen. Use of a fume hood is recommended. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Pyridine is flammable and a dangerous fire risk. The explosive limits in air are 1.8–12.4%. It is toxic by ingestion and inhalation. The TLV is 5 ppm in air. The danger from crude pyridine is greater than from pure pyridine, the associated homologs and impurities being even more toxic than pyridine itself.

NUMBERED NOTES

1. 1,1,1–Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.
2. Because of its toxicity, pyridine is an undesirable constituent of the Karl Fischer reagent. Both one– and two–component Karl Fischer reagents, based on aliphatic amines and heterocyclic compounds and containing no pyridine, are available commercially.

REFERENCES

Adopted from the published collaborative study by Bernetti, R., S.J. Kochan, and J.J. Pienkowski, *J. Assoc. Off. Anal. Chem. 67:*299 (1984).

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AOCS

AOCS Official Method Ja 3-87

Reapproved 2009

Hexane-Insoluble Matter

DEFINITION

This method determines the substances insoluble in hexane under the conditions of the test.

SCOPE

Applicable to lecithin from soybeans and corn.

APPARATUS

1. Erlenmeyer flasks—wide-mouth, 250 mL.
2. Filter—Corning "C" porosity, 30 mL.
3. Filter flasks of convenient size—these flasks should be fitted with one-hole stoppers which will hold the stem of filter funnel.
4. Forced-draft oven—AOCS Specification H 1-39.

REAGENT

1. Hexane—ACS grade (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Warm the test sample to soften the product (but do not heat above 60°C) and then mix thoroughly.

PROCEDURE

1. Weigh 10 g of the well-mixed test sample into 250 mL flask. Add 100 mL of hexane and shake until dissolved.
2. Filter through a filter funnel which has been previously heated at 105°C for 1 hr, stored in a desiccator until coming to ambient temperature, and weighed. Wash the flask twice with 25 mL portions of hexane and pour both washings through the funnel.
3. Place the funnel in a forced-draft oven and dry for 1 hr at 105 ± 2°C. Remove from oven, cool to ambient temperature in a desiccator, and weigh.

CALCULATIONS

$$\text{Hexane-insoluble matter, \%} = \frac{\text{gain in mass, g of funnel}}{\text{mass, g of test portion}} \times 100$$

NOTES

Caution

Hexane is flammable and a dangerous fire risk. The TLV is 50 ppm in air. A properly operating fume hood should be used when working with hexane.

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AOCS

AOCS Official Method Ja 4-46

Revised 2011

Acetone-Insoluble Matter

DEFINITION

This method determines the acetone-insoluble matter under the conditions of the test, free from sand, meal, and other petroleum ether-insoluble material. The phosphatides are included in the acetone-insoluble fraction.

SCOPE

Applicable to lecithin from soybean and corn oils.

APPARATUS

1. Centrifuge—motor-driven with head to hold specified centrifuge tubes, and capable of operating at 1900 (±100) rpm.
2. Centrifuge tubes—Pyrex™, heavy wall, round bottom with lip, 40 mL capacity; Corning no. 8240, or equivalent.
3. Stirring rods—5 mm diameter, convenient length.
4. Burette—50 mL capacity.
5. Beakers—250 and 400 mL capacities.
6. Forced-draft oven—AOCS Specification H 1-39.

REAGENTS

1. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).
2. Acetone—ACS grade (see Notes, *Caution*), presaturated with purified acetone-insoluble material as follows:
   (a) Purification of phosphatides—Purify phosphatides from previous acetone-insoluble determinations by dissolving 5 g in 10 mL of petroleum ether and reprecipitating with 25 mL of acetone. Be sure the precipitate is free from conglomerates before further processing. Transfer the precipitate to two centrifuge tubes, using additional acetone to facilitate the transfer. Stir thoroughly and make to volume of 40 mL, stir, chill for 15 min in ice bath at 0–5°C, stir again, and centrifuge for 5 min. Decant the acetone solution, break up solids with stirring rod, refill tubes with acetone, stir, chill, centrifuge, and decant as before. The solids after the second centrifugation require no further processing and may be used, as is, for presaturating acetone.
   (b) Saturation of acetone—Add purified phosphatides to acetone at 5°C. Five grams of the phosphatides are sufficient for about 16 L of acetone. Maintain at 5°C for 2 hr, shaking vigorously at 15 min intervals. Decant through a rapid filter paper, avoiding transfer of any of the solids to the paper and conducting the filtration under refrigerated conditions (0-5°C) so as to maintain the same conditions for saturation as described in Procedure.

PREPARATION OF TEST SAMPLE

1. Mix thoroughly. If the test sample is plastic, it may be heated to facilitate mixing, but do not heat above 60°C and avoid prolonged heating.

PROCEDURE

1. Weigh 1.9–2.0 g (± 0.0005 g) of well-mixed test sample into a centrifuge tube which has been tared previously with a stirring rod.
2. Add 15 mL of saturated acetone from a burette. Warm in a water bath until lecithin melts, but avoid evaporation of acetone. Stir until the material is completely disintegrated. Place in ice-water bath and chill for 5 min.
3. Remove the tube from the bath and add about one half of the final required volume of chilled (0–5°C) saturated acetone. Stir well to complete dispersion of remaining particles. Make to volume of 40 mL with chilled (0–5°C) acetone, stir, and return tube and contents to ice bath at 0–5°C for 15 min.
4. At the end of the 15 min chilling period, stir again while in the bath; remove rod (see Notes, 1) and centrifuge immediately at 1900 ± 100 rpm for 5 min. Decant the acetone-soluble material into a clean beaker (see Notes, 2).
5. Break up the centrifuged solids with the assigned stirring rod and refill the centrifuge tube to the 40 mL mark with chilled (0-5°C) acetone; stir well and repeat as directed in Procedure, 3 and 4.
6. Following the second centrifugation and pouring off of solvent, return the assigned stirring rod to the tube, and break up the solids. Place the tube and contents in a horizontal position on a laboratory bench until the excess of acetone evaporates. Mix again and place in a forced-draft oven at 105 ± 2°C until constant weight is obtained, usually 30–45 min. Cool to ambient temperature in an efficient desiccator and weigh immediately.

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CALCULATIONS

$$\text{Acetone-insoluble matter, \%} = \left[\frac{\text{mass, g of residue}}{\text{mass, g of test portion}} \times 100\right] - \text{\% hexane insolubles.}^{*}$$

*as determined by AOCS Official Method Ja 3-87.

REPRODUCIBILITY

The average variance of components calculated from the collaborative data obtained by the investigating committee indicates the following 95% probability limits—

1. The difference between duplicate determinations made within a laboratory should not exceed 1.02.
2. The difference between the average of duplicate determinations made in different laboratories should not exceed 1.17.

NOTES

Caution

Both petroleum ether and acetone are extremely flammable. Avoid static electricity. The explosive limits in air for petroleum ether are 1–6%. A fume hood should be used at all times when using these solvents.

NUMBERED NOTES

1. A wooden block containing a suitable number of holes is convenient to support stirring rods in a vertical upside-down position during centrifugation of test sample(s).
2. The acetone may be recovered for reuse by distillation from the acetone solubles. Before reuse, pass over anhydrous sodium sulfate to remove moisture, but avoid reuse of the drying agent. Presaturate the recovered acetone as described in Reagents, 2, (b).

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AOCS Official Method Ja 6-55

Revised 2011

Acid Value

DEFINITION

The acid value is the mg of potassium hydroxide necessary to neutralize the acids in 1 g of test sample.

SCOPE

Applicable to all vegetable lecithins.

APPARATUS

1. Erlenmeyer flask—250 mL.
2. Magnetic stirrer—optional.

REAGENTS

1. Sodium hydroxide (NaOH), 0.1 M—accurately standardized, carbonate free. See AOCS Specification H 12-52.
2. Ethyl alcohol—95% (SDA Formulas 30 and 3A are permitted). Add a little phenolphthalein and neutralize with alkali to a faint but permanent pink color just before using.
3. Phenolphthalein indicator—1% in 95% ethyl alcohol.
4. Petroleum ether—AOCS Specification H 2-41 (see Notes, *Caution*).

PREPARATION OF TEST SAMPLE

1. Warm the test sample to soften the product (do not heat over 60°C) and then mix thoroughly.

PROCEDURE

1. Weigh accurately about 2 g of test sample into a 250 mL Erlenmeyer flask. Dissolve in 50 mL of petroleum ether by mixing gently. Then add 50 mL of neutral alcohol and mix.
2. Add 4 drops of indicator and while mixing, titrate with 0.1 M NaOH to the first pink color which persists for 5 sec. The end point is fairly easily ascertained by adding the bulk of the alkali solution rapidly until near the end point, and then slowing down to about 4, then 1–2 drops at a time.

CALCULATIONS

$$\text{The acid value, mg KOH per g of test portion} = \frac{\text{volume, mL titrant} \times M \times 56.1}{\text{mass, g of test portion}}$$

Where—

M = exact molarity of the standard NaOH solution

NOTES

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air for petroleum ether are 1–6%. A fume hood should be used at all times when using this solvent.

NUMBERED NOTES

1. The acid value is rountinely quoted as KOH, however this method uses NaOH as the titrant.

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AOCS Recommended Practice Ja 7-86
Reapproved 2009

Phospholipids in Lecithin Concentrates by Thin-Layer Chromatography

DEFINITION

Analysis is performed by fractionation with two-dimensional TLC followed by acid digestion and reaction with molybdate to measure total phosphorus for each fraction at 310 nm.

SCOPE

Applicable to all animal or vegetable lecithin preparations. This method is semiquantitative for phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), lysophosphatidylcholine (LPC), and lysophosphatidylethyanolamine (LPE).

APPARATUS

1. Fume hood for perchloric acid.
2. Glass digestion tubes—heavy walled, 19 mm i.d., 100 mm length and marked at 15 mL level.
3. Heater block—for above digestion tubes, capable of 150 ± 2°C.
4. Glass beads.
5. Centrifuge tubes—stoppered, 16–20 mL, Sorvall #00771, or equivalent.
6. Centrifuge—clinical, capable of (1470 × g) (5,700 rpm).
7. U.V. spectrophotometer—capable of measurement at 310 nm.
8. Quartz cuvettes—1 cm, matched within 0.01 absorbance units.
9. Three chromatography development tanks.
10. Volumetric flasks—glass-stoppered, Class A, 100, 250, and 500 mL.
11. Volumetric pipets—class A, 50 and 100 µL and 1.0, 2.0, and 10 mL.
12. TLC plates—silica gel G, 0.25 mm, 20 × 20 cm glass.
13. TLC spot scrapers—Applied Science #7626 or equivalent.
14. Volumetric pipets—Class A, 40, 80, 120, 160, and 200 µL; fixed or adjustable 200 µL; and 1.0, 2.0, and 10 mL.
15. Pasteur pipets—disposable.
16. Large glass marbles, ≥ 20 mm i.d.
17. Laboratory hood required when solvents are used.

REAGENTS

1. Glacial acetic acid—reagent grade.
2. Chloroform—unstabilized, reagent grade (see Notes, *Caution*).
3. Methanol—reagent grade.
4. Ammonium hydroxide—reagent grade, 28–30%.
5. *n*–Butyl acetate—spectro grade. A suitability test is performed by obtaining less than 0.1 absorbance units when read at 310 nm.
6. Nitric acid—reagent grade.
7. Perchloric acid—reagent grade, 70% (see Notes, *Caution*).
8. *n*–Hexane—reagent grade (see Notes, *Caution*).
9. Ammonium molybdate, tetrahydrate $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$—crystal, formula weight 1235.86, reagent grade.
10. Potassium phosphate—monobasic, reagent grade.
11. Iodine, crystals—reagent grade.
12. Hydrochloric acid—concentrated, reagent grade (see Notes, *Caution*).

SOLUTIONS

1. Phosphorus stock solution (1 mg/mL)—Dissolve 1.0982 g potassium phosphate monobasic in distilled water and dilute to 250 mL.
2. Phosphorus working solution (50 µg P/mL)—Dilute 5.0 mL potassium phosphate stock solution to 100 mL with distilled water.
3. Molybdate solution—Dissolve 29.0 g ammonium molybdate in 100 mL concentrated hydrochloric acid and dilute to 500 mL with distilled water. This reagent should be prepared 24 hr before used. Stable up to six months when refrigerated.
4. Ammonium hydroxide (7N)—Pipet 47 mL of concentrated ammonium hydroxide (15N) into a 100 mL volumetric flask containing distilled water and dilute to the mark. Use pipettor or bulb for pipetting.

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5. TLC solvent A—130 mL chloroform, 60 mL methanol, and 8 mL of 7M ammonium hydroxide.
6. TLC solvent B—170 mL chloroform, 25 mL methanol, 25 mL glacial acetic acid, and 6 mL water.

THIN-LAYER CHROMATOGRAPHY

1. Accurately weigh approximately 500 mg of test sample into a 50 mL volumetric flask. Dissolve in and dilute to the mark with n–hexane.
2. Apply 100 µL of test solution to the lower right-hand corner, 2 cm from both edges of a dry plate.
3. Develop the plate in a tank containing TLC solvent A for 90 min or until the solvent travels nearly the full length of the plate. Remove the plate and dry for 30 min at room temperature.
4. Turn the plate 90° to the right and develop again in a second tank containing TLC solvent B for an additional 90 min. Again remove and allow the plate to dry. See Figure 1.
5. Transfer the plate to a third tank containing iodine crystals, until clear visualization of the spots has occurred.
6. Outline the spots with a needle or sharp pencil. See Figure 1. Moisten each spot with water or ethanol to make it plastic.
7. Carefully roll off the spot with a TLC spot scraper and transfer the material into a digestion tube. Repeat, using a separate tube for each isolated phospholipid fraction.

CALIBRATION STANDARDS

Pipet 0 (blank), 20, 40, 80, 120, 160, and 200 µL of the 50 µg/mL working phosphorus standard into separate digestion tubes. Each tube should contain 0 (blank), 1, 2, 4, 6, 8, and 10 µg of phosphorus, respectively. Prepare each in duplicate. Beginning at the digestion stage, carry the standards through the workup identical to the test portion (see Notes, 1).

PHOSPHORUS QUANTITATION

1. Add two glass beads, 1 mL perchloric acid, and 2–3 drops nitric acid to each of the standard and test portions digestion tubes (see Notes, *Caution*, perchloric acid).
2. Place the digestion tubes into a block heater that has been preheated to 140°C. Digest the test portions for 60 min. A glass marble may be placed on the top of each tube to minimize loss of test portion during digestion.
3. Remove and cool. Add distilled water, by rinsing down the sides of the tube, to the 15 mL mark. Mix.
4. Accurately pipet 10 mL of the solution into a stoppered centrifuge tube. Add 1 mL molybdate reagent. Mix vigorously.
5. Accurately pipet 5 mL n–butyl acetate and mix.
6. Centrifuge at 5700 rpm for 3 min to obtain a clear phase separation.
7. Transfer the clarified upper phase to a 1.0 cm quartz cuvette and measure the absorption of the standards and test portion against the reagent blank (standard 0) at 310 nm.

CALCULATIONS

1. Using a linear regression statistic, or by manually plotting µg phosphorus per standard tube vs. absorbance, determine the µg of phosphorus for each fraction obtained. (See Notes, 2.)
2. Determine the test portion weight in µg used as follows—

$$\frac{0.1\text{ mL spot}}{50\text{ mL}} \times W \times \frac{10\text{ mL}}{15\text{ mL}} \times 1000$$

Where—

W = initial test portion mass in mg, or µg test portion = $W \times 1.333$

3. The phosphorus content for each fraction in mg/g is determined as follows—

$$\text{mg P/g} = \frac{U}{S \times 1000}$$

Where—

U = mass, µg of phosphorus from linear regression curve
S = mass, µg test portion used

4. The conversion factors from phosphorus based on the average molecular weight for soy-based phospholipids are as follows (see Notes, 2)—

P = Phosphorus concentration in mg/g	
% PC = P × 2.494	% PA = P × 2.203
% PE = P × 2.336	% PI = P × 2.703

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NOTES

Caution

Chloroform is a known carcinogen. Use of a fume hood is recommended. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is non-flammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Perchloric acid is a potentially hazardous chemical that should be used in an appropriate fume hood. Avoid all contact with organic materials and perchloric acid.

Hexane is extremely flammable. Avoid static electricity. The explosive limits in air for hexane are approximately 1 to 6%. A fume hood should be used at all times when using this solvent.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

NUMBERED NOTES

1. All glassware must be carefully cleaned with sulfuric acid/sodium dichromate cleaning solution and thoroughly rinsed with distilled water before use.
2. Due to the possible variation in molecular weights for the phospholipid fractions, the results based on an average molecular weight should not be viewed as absolute, but rather as semiquantitative (see References, 8).

PRECISION

Based on IUPAC collaborative study WG 3/80, 1984

STATISTICAL ANALYSIS OF RESULTS

		Within Lab (mg/g)		Between Lab (mg/g)	
	x, mg/g	Std Dev	CV %	Std Dev	CV %
PC	5.1–5.6	0.16–0.43	3.2–7.7	0.37–0.76	7.1–14.9
	26.9	0.56	2.1	6.29	23.4
PI	2.7–3.4	0.18–0.34	6.4–10.3	0.25–1.54	8.8–46.2
PE	4.0–5.1	0.24–0.46	5.9–9.0	0.40–1.12	9.9–22.1
PA	1.4–2.2	0.14–0.25	6.5–16.1	0.31–0.53	13.9–36.1
LPC	0.6–1.7	0.9–0.25	7.7–14.6	0.33–0.98	52–110

REFERENCES

1. Erdahl, W.L., A. Stolyhwo, and O.S. Privett, *J. Am. Oil Chem. Soc. 50:*513 (1973).
2. Lowry, R.R., and I.J. Tinsley, *Lipids 9:*491 (1974).
3. Dittmer, J.C., and R.L. Lester, *J. Lipid Research 5:*126 (1964).
4. Dixon, W.J., *Biometrics 9:*74 (1953).
5. Cohran, W.G., *Biometrics 3:*22 (1947).
6. Qualitative and Quantitative Determination of Phospholipids, IUPAC Commission on Oils, Fats, and Derivatives, Report of Working Group 3/80, Working Report of June 1984, pp. A1–A5.
7. This method is identical to IUPAC Standard Method 5.301. *IUPAC Standard Methods for the Analysis of Oils, Fats, and Derivatives,* 1st Supplement to the 7th edn., Blackwell Scientific Publications, 1992.
8. Chapman, G.W., Jr., *J. Am. Oil Chem. Soc. 57:*299 (1980).

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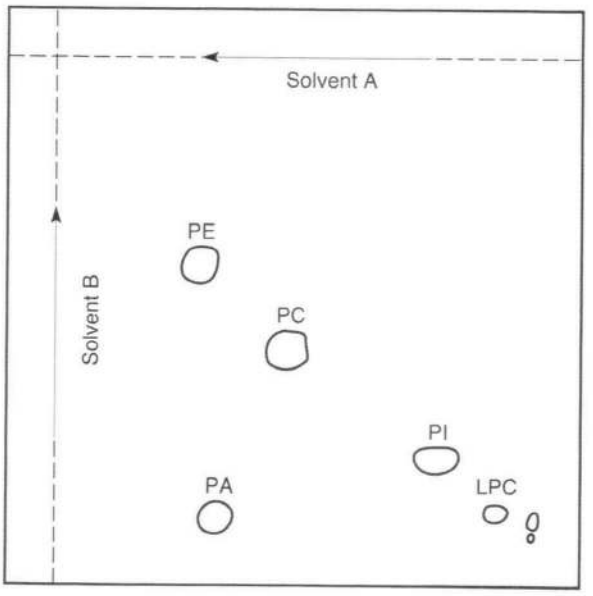

Figure 1. Diagram of TLC of phospholipids of commercial lecithin (soybean).

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AOCS Official Method Ja 7b-91

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Determination of Lecithin Phospholipids by HPLC

DEFINITION

This method is for the direct determination of single phospholipids (PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine) in lecithin by high-performance liquid chromatography (References, 1).

SCOPE

Applicable to oil-containing lecithins, deoiled lecithins, lecithin fractions; not applicable to lyso-PC and lyso-PE.

APPARATUS

1. HPLC basic equipment—
   (a) Sample feeding system—with sample loop, corresponding to the injection volume (10 µL).
   (b) High-pressure pump.
   (c) UV detector—for determination at 206 nm.
   (d) Suitable integrator.
   (e) HPLC separation column—Microparticulate spherical silica, 5 µm particle size, 200 × 4.6 mm i.d. (Alltech Associates, Inc., Deerfield, IL, USA), or equivalent.
2. Microliter syringe—25 µL, graduated in 1 µL.
3. Volumetric flasks—10, 500, and 1000 mL.

REAGENTS

1. *n*–Hexane—HPLC grade (see Notes, *Caution*).
2. 2–Propanol—HPLC grade.
3. Water—HPLC grade.
4. Sodium acetate · $3H_2O$—reagent grade.
5. Acetic acid—reagent grade.
6. Phospholipid standards—PE (#P5274), PA (#P2767), PI (#P0639), PC (#P6263); available from Sigma Chemical Co., St. Louis, MO, USA.
7. 0.2 M sodium acetate solution—prepared by dissolving 27.22 g sodium acetate · $3H_2O$ in 1000 mL HPLC-grade water.
8. 0.2 M acetic acid—prepared by dissolving 6.005 g glacial acetic acid in 500 mL HPLC-grade water.
9. Walpole acetate buffer (pH 4.2)—prepared by mixing 73.5 mL 0.2 M acetic acid (Reagents, 8) to 26.5 mL 0.2 M sodium acetate solution (Reagents, 7), adjusted to exactly pH 4.2 with the aid of a pH meter.
10. Mobile phase—*n*–hexane/2–propanol/acetate buffer pH 4.2 (8:8:1, v/v/v).

PROCEDURE

1. Pretreatment of column—To achieve a stable separating performance of the column (see Notes, 1), it must be charged with a certain quantity of the buffer. This is done by rinsing the column for at least 3 days with the mobile phase at a flow rate of 0.2 mL/min. Then a separation of the baseline and a stable retention time should be achieved.
2. After conditioning the column, flow rate is adjusted to 2 mL/min.
3. Calibration curve—To prepare the calibration curve, dissolve 10, 20, 30, and 40 mg of each single phospholipid in 10 mL of the mobile phase. Prepare four solutions of mixed phospholipid standards.

   Note—The solution time may require up to 3 hrs. Accurately inject 10 µL of these calibration solutions. Plot the peak areas against mass in a graph.
4. Test sample pretreatment—Dissolve 50–250 mg of the lecithin in 10 mL of the mobile phase (see Notes, 2). Prepare in the same way as the calibration substances. Inject exactly 10 µL of the test solution into the column.
5. Rinsing of pump head/system—Wash the column alternately with acetone, water, acetone at low flow rates, about 0.2 mL/min.

CALCULATIONS

1. Read from the calibration curves the amount of phospholipids in the injected volume of the solution of the lecithin test sample (Procedure, 3). Typical chromatograms are shown in Figure 1.

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2. The phospholipid content (P), expressed as percent (mass/mass), is determined by the formula:

$$P = \frac{A \times 100}{m}$$

Where—

A = amount of phospholipid in mg determined from the calibration curve (Calculations, 1).
m = mass of test portion in mg (Procedure, 4).

PRECISION

1. Statistics relating to the determination of PA, PC, PE, and PI are shown in Table 1.

NOTES

Caution

n-Hexane is extremely flammable. Avoid static electricity. The TLV in air is 50 ppm. A fume hood should be used at all times when using *n*–hexane.

NUMBERED NOTES

1. Decreasing separation performance is indicated by a bad separation of the PE/N–acyl–PE peak. The column should then be replaced to ensure good reproducibility. Use of a column oven is recommended for temperature stability; temperature changes will affect analyte retention times.
2. In some lecithins a sedimentation (usually suspended) may form after a certain solution time. It has no influence on the analysis, but will affect the column. Centrifuging is recommended, followed by decanting or filtering through a 0.45 µm filter.

REFERENCES

1. *Standard Methods for the Analysis of Oils, Fats, and Derivatives,* International Union of Pure and Applied Chemistry, 7th edn., Blackwell Scientific Publications, 1987, IUPAC Method 5.302.
2. Beare-Rogers, J.L., A. Bonekamp-Nasner, and A. Dieffenbacher, *Pure Appl. Chem. 64:*447 (1992).

OTHER REFERENCES

IUPAC Commission on Oils, Fats, and Derivatives, Working Report (VI.3), International Union of Pure and Applied Chemistry, July 12, 1989, pp. A7, M1.

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Table 1
Summary of statistics from IUPAC phospholipid collaborative study[a]

	Sample	n[b]	Mean[c]	S_r	CV_r	S_R	CV_R
PA	1	11	12.58	0.27	2.14	1.55	12.28
	2	11	11.68	0.17	1.45	1.12	9.56
	3	10	4.68	0.21	4.58	0.96	20.61
	4	10	12.18	0.28	2.34	2.51	20.63
	5	10	12.09	0.27	2.23	2.54	21.01
	6	11	7.18	0.17	2.37	0.61	8.46
PC	1	10	17.24	0.20	1.19	0.97	5.61
	2	10	19.01	0.49	2.60	1.12	5.89
	3	11	14.44	0.34	2.44	0.86	5.95
	4	8	9.64	0.13	1.40	0.22	2.25
	5	11	9.33	0.41	4.35	0.66	7.04
	6	11	14.74	0.40	2.68	0.83	5.66
PE	1	11	14.04	0.17	1.18	0.74	5.29
	2	10	15.80	0.15	0.98	1.10	6.98
	3	11	10.24	0.23	2.29	0.88	8.55
	4	10	9.45	0.09	0.98	0.77	8.10
	5	10	9.49	0.09	1.00	0.59	6.18
	6	11	11.18	0.17	1.54	1.19	10.65
PI	1	9	11.60	0.49	4.20	1.07	9.20
	2	10	12.43	0.52	4.22	1.47	11.80
	3	9	7.67	0.27	3.52	1.55	20.18
	4	9	7.84	0.32	4.08	0.85	10.88
	5	10	8.86	0.56	6.28	3.45	38.89
	6	8	9.16	0.19	2.11	0.63	6.89

[a] References, 2.
[b] Abbreviations: n, number of labs remaining after elimination of outliers; S_r, standard deviation of repeatability; CV_r, coefficient of variation for repeatability; S_R, standard deviation of reproducibility; CV_R, coefficient of variation for reproducibility; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.
[c] g/100 g sample

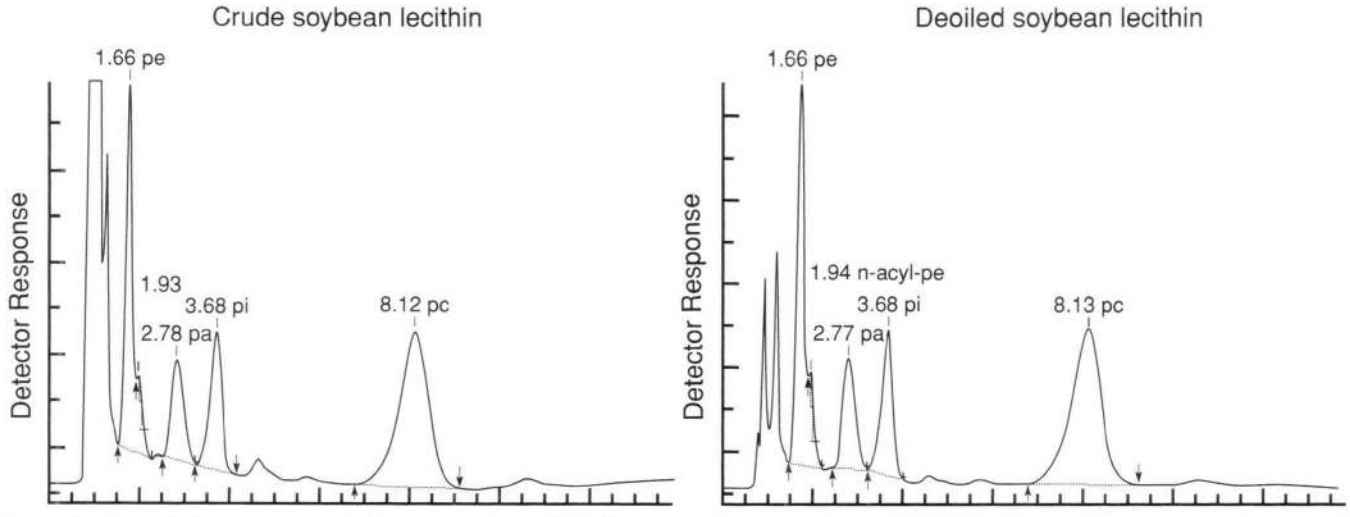

Figure 1. Typical chromatograms of soybean phospholipids.

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AOCS Official Method Ja 7c-07

Revised 2013

Determination of Lecithin Phospholipids by HPLC-LSD

DEFINITION

This method describes a procedure for quantitative determination of phospholipids by high performance liquid chromatography (HPLC) using a diol column and light scattering detection (LSD). The main phospholipid components in lecithin are N–Acyl-phosphatidylethanolamine (N–Acyl–PE), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), and lysophosphatidylcholine (LPC). This method determines the individual main phospholipids in gram per 100 grams of product.

SCOPE

This method can be used for oil-containing and oil-free lecithins and lecithin fractions; not applicable to lyso–PE.

APPARATUS AND APPARATUS CONDITIONS

Instruments

1. HPLC basic equipment—e.g., Merck-Hitachi.
   (a) Intelligent pump L 6200 (low pressure gradient mixing) or additional pump L 6000 (high pressure gradient mixing).
   (b) Auto sampler—AS 2000A.
   (c) Column—(250 mm × 4.0 mm) with pre-column (length/internal diameter: 20 mm/4.0 mm) packed with spherical microparticles (5 µm) of diol bonded silica, e.g. LiChrospher 100 Diol 5. Type, age, and history of the column, packing of the column filling material, and the temperature may influence the separation.
   (d) HPLC column oven—adjustable at 55°C.
   (e) Detector—light scattering detector, e.g., Sedex 45 (S.E.D.E.R.E).
   (f) Degasser—e.g., ERC 3612 (ERC).
   (g) PC chromatography evaluation system.
   (h) Laboratory balance.
2. Microliter syringe—25 µL, graduated in 1 µL units.
3. Filter—for filtration of external standard and sampling solutions, e.g., Millex HV.
4. Volumetric flasks—50, 100, and 2500 mL.

REAGENTS

1. Crude lecithin—(reference mixture with precisely defined N-acyl-PE, PA, PE, PC, PI, and LPC contents) e.g., ILPS-LE01 "Mixed Soy Phospholipid Reference Standard"
2. *n*–Hexane—HPLC quality
3. 2–Propanol—HPLC quality
4. Acetic acid—100% p.a.
5. Triethylamine—p.a.
6. Purified water

SOLUTIONS

1. Solvent—*n*–hexane/2–propanol—80:20 (v/v)
2. Eluent A—*n*–hexane/2–propanol/acetic acid/triethylamine—81.42:17.00:1.50:0.08 (v/v/v/v)
   To obtain a reproducible elution solvent composition it is recommended that the solvents be weighed out taking their densities into account. For a batch size 2.5 L use the following weights: *n*–hexane 1341.4 g, 2–propanol 331.5 g, acetic acid 39.4 g, triethylamine 1.45 g.
3. Eluent B—2–propanol/purified water/acetic acid/triethylamine—mixed in a volume ratio 84.42:14.00:1.50:0.08. For a batch size 2.5 use the following weights: 2–propanol 1646.2 g, water 350.0 g, acetic acid 39.4 g, triethylamine 1.45 g.
4. External reference solutions—
   Prepare 3 different external reference solutions.
   For this purpose accurately weigh out in three 100 mL volumetric flasks ca:
   550 mg crude phospholipid ref. substance—designation: R1 (5.5 mg/mL)
   850 mg crude phospholipid ref. substance—designation: R2 (8.5 mg/mL)
   1150 mg crude phospholipid ref. substance—designation: R3 (11.5 mg/mL)

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Dissolve in *n*–hexane/2–propanol 80:20 mixture and make up to 100.00 mL with the same solvent. Filter the standards before injection in the HPLC.

PROCEDURE

1. General

The separation is carried out by gradient elution using a mobile phase mixture of *n*–hexane, 2–propanol, water, acetic acid, and triethylamine (Eluent A and Eluent B).

N–Acyl–PE, PA, PE, PC, PI, and LPC are identified by comparison of their retention times in the high-pressure liquid chromatogram with those of a reference crude lecithin (see Fig. 1).

N–Acyl–PE, PA, PE, PC, PI, and LPC are quantified by determination of the relationship between the area under the particular peak in the chromatogram and the area under the peak of a reference crude lecithin of precisely defined content of individual phospholipids. Use calibration ranges of the individual phospholipids where the signals behave in a linear manner.

2. Test sample pretreatment—

Heat the test sample at maximum 60°C and stir thoroughly. Weigh out accurately approximately 425 mg crude lecithin in duplicate, place each in a separate 50 mL volumetric flask, dissolve in *n*-hexane/2-propanol 80:20 mixture, and make up to 50.00 mL with the same solvent (final concentration: 8.5 mg/mL)—Designation: S1a, S1b, etc.). Filter test samples before use.

3. HPLC Analysis—

Adjust the working conditions of the chromatograph using the reference samples to get a separation as shown in Figure 1. Optimize the separation for the type of column used by adjusting the gradient parameters. The following conditions are recommended:

Detector parameters sensitivity: gain 5–6
temperature: 50°C
pressure: 2.0 bar

Flow: 1.0 mL/min (2.0 mL/min for column rinsing)

Gradient

Time (min)	Proportion B (%)	Flow (ml/min)
0.0	5	1.0
5.0	20	1.0
8.5	40	1.0
15.0	100	1.0
17.5	100	1.0
17.6	5	1.0
21.0	5	1.0
22.0	5	2.0
27.0	5	2.0
29.0	5	1.0

Use 20 µL injection volumes for calculation of the regression lines.

4. Calibration—

Use the following analytical sequence for the quantitative determination of phospholipids:
R1, R2, R3 (1 injection each); S1a, S1b (2 injections each), etc.; R1, R2, R3 (1 injection each).

5. Evaluation—

Identify by comparing the retention times of the substances in the chromatograms of the reference solutions and the test samples.

CALCULATIONS

Calculate the content of individual phospholipids using appropriate linear regression analysis of the six calibration runs.

The content of the individual phospholipid (P), expressed as percent (mass/mass), is determined by the formula:

$$P = \frac{A \times 100}{M}$$

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where

P = amount of the individual phospholipid in g/100g of the test portion
A = amount of phospholipid in mg determined from the calibration curve
M = mass of test portion in mg

The result of the determination is expressed to 2 decimal places.

PRECISION

The information on the repeatability and reproducibility of this method are the result of an interlaboratory test carried out in 1993–1996 by the ILPS (International Lecithin and Phospholipid Society) and evaluated in accordance with ISO 5725:1994 and DGF unified method A-II 1, giving the data and results in Table 1.

NOTES

Caution

n–Hexane is extremely flammable. Avoid static electricity. The TLV in air is 50 ppm. A fume hood should be used at all times when using *n*–hexane.

NUMBERED NOTES

Peak separation of LPE, LPI, and LPA is often insufficient for enzymatically hydrolyzed lecithins.

REFERENCES

1. Herslöf, B., U. Olsson, and P. Tingvall, in Phospholipids, eds. J. Hanin and G. Pepeu, Plenum Press, New York, pp. 295–298 (1990).
2. Bonekamp, A., and H.-J. Fiebig, Fett/Lipid 101:80–83 (1999). This proposal was accepted as official unified method DGF F-I 6a (00).
3. Balazs, P., P. Schmit, and B. Szuhaj, *J. Am. Oil Chem. Soc. 73*:193–197 (1996).

Table 1
Summary of statistics from an ILPS phospholipid collaborative study.

	Sample	n[a]	Mean[b]	S_r	CV_r	S_R	CV_R
N–Acyl–PE	1	8	1.52	0.04	6.43	0.11	19.57
	2	8	1.59	0.05	8.99	0.20	34.49
PA	1	11	4.67	0.17	9.89	0.24	14.34
	2	10	6.23	0.20	9.16	1.17	52.63
PE	1	9	11.74	0.17	3.98	0.34	8.01
	2	10	12.14	0.21	4.84	0.37	8.49
PC	1	11	14.70	0.25	4.69	0.46	8.74
	2	11	13.45	0.27	5.66	0.46	9.49
	3	12	94.70	1.01	2.98	2.14	6.34
PI	1	11	9.36	0.17	5.13	0.30	8.88
	2	11	9.87	0.20	5.68	0.33	9.39
LPC	1	10	0.71	0.06	23.72	0.15	58.29
	2	7	0.46	0.03	19.45	0.11	69.69

[a] Abbreviations: n, number of labs remaining after elimination of outliers; S_r, standard deviation of repeatability; CV_r coefficient of variation for repeatability; S_R, standard deviation of reproducibility; CV_R, coefficient of variation for reproducibility; N–Acyl–PE, N–Acyl–Phosphatidylethanolamine; PA, phosphatidic acid; PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PI, Phosphatidylinositol; LPC, lysophosphatidylcholine.

[b] g/100g sample.

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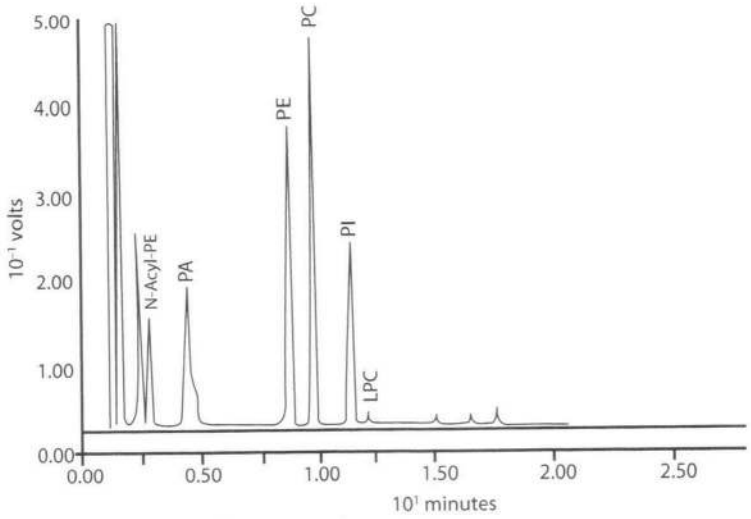

Figure 1. HPLC Chromatogram of separation of phospholipid species.

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AOCS

AOCS Official Method Ja 8-87

Revised 2013

Peroxide Value

DEFINITION

This method determines all substances, in terms of meq peroxide per 1000 g of test sample, which oxidize potassium iodide under the conditions of the test. These are generally assumed to be peroxides or other similar products of fat oxidation.

SCOPE

Applicable to all lecithin products. This method is highly empirical and any variation in procedure may result in variation in results.

APPARATUS

1. Pipet—Mohr, measuring type, 1 mL.
2. Erlenmeyer flasks—glass-stoppered, 250 mL.
3. Burette—50 mL, Class A.
4. Graduated cylinders—50 and 100 mL.
5. Volumetric flask—1000 mL.

REAGENTS

1. Acetic acid-chloroform solution—(see Notes, *Caution* and *Recommendations*) mix 3 parts by volume of glacial acetic acid, reagent grade, with 2 parts by volume of chloroform, USP grade.
2. Potassium iodide (KI) solution—saturated solution of KI, ACS grade in recently boiled distilled water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals. Store in the dark. Test daily by adding 2 drops of starch solution to 0.5 mL of the potassium iodide solution in 30 mL of acetic acid-chloroform solution. If a blue color is formed which requires more than 1 drop of 0.1 M sodium thiosulfate solution to discharge, discard the iodide solution and prepare a fresh solution.
3. Sodium thiosulfate solution, 0.1 M—accurately standardized.
4. Sodium thiosulfate solution, 0.01 M—accurately standardized. This solution may be prepared by accurately pipetting 100 mL of the 0.1 M solution into a 1000 mL volumetric flask and diluting to volume with recently boiled distilled water.
5. Starch indicator solution, 1.0% in distilled water prepared as follows—to 1 g of potato starch add slowly and with constant stirring 10 mL of cold water to make a homogeneous slurry. Add this slurry slowly and with stirring to 90 mL gently boiling distilled water. Continue to boil for 1 min, cool, and store in a stoppered bottle. Discard the preparation when it becomes cloudy or when it gives a reddish-blue color with iodine. Refrigerate when not in use.
6. Test for starch sensitivity—Place 5 mL of starch solution in 100 mL of water and add 0.05 mL of freshly prepared 0.1 M KI solution and one drop of a 50 µg/g chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution to 1000 mL. The deep blue color produced must be discharged by 0.05 mL of 0.1 M sodium thiosulfate.

PROCEDURE

1. Weigh 5.00 ± 0.05 g of test sample into a 250 mL glass-stoppered Erlenmeyer flask and then add 30 mL of the acetic acid-chloroform solution. Swirl the flask until the test sample is dissolved in the solution. Add 0.5 mL of saturated potassium iodide, preferably using Mohr type measuring pipet.
2. Allow the solution to stand with occasional shaking for exactly 1 min and then add 30 mL of distilled water and 0.5 mL of starch indicator solution.
3. Stir the solution rapidly using a magnetic stirrer and titrate with 0.1 M sodium thiosulfate. Continue the titration until the blue color has just disappeared.

   Note—If the titration is less than 0.5 mL, repeat the determination using 0.01 M sodium thiosulfate solution.
4. Conduct a blank determination of the reagents daily. The blank titration must not exceed 0.1 mL of the 0.1 M sodium thiosulfate solution.

CALCULATIONS

1. Peroxide value as meq of peroxide per 1000 g of test portion $= \dfrac{(S - B)(M)(1000)}{\text{mass of test portion, g}}$

   Where—

   B = volume of titrant, mL of blank
   S = volume of titrant, mL of test portion
   M = molarity of sodium thiosulfate solution

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NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

RECOMMENDATIONS

Isooctane has been reported to be a suitable replacement for chloroform in peroxide value method Cd 8-53. Isooctane may be a suitable replacement for chloroform in Ja 8-87; however, this has not been validated by collaborative study.

1,1,1–Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

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AOCS

AOCS Official Method Ja 9-87

Reapproved 2009

Gardner Color

DEFINITION

This method determines lecithin color by comparison of an undiluted test sample to standards of a specified color.

SCOPE

Applicable to all nongranular lecithin products that can be fluidized under the conditions of the test.

APPARATUS

1. Glass standards—eighteen, numbered separately, having the transmittance characteristics given in Table 1. Glass standards may be obtained from Byk Gardner, Inc., Columbia, MD 21045, USA; Tintometer Ltd., Salisbury, UK.
2. Clear glass tubes—10.65 ± 0.025 mm i.d., and about 114 mm outside length. Viscosity tubes, as described in AOCS Official Method Tq 1a-64, are satisfactory. Kimble EXAX test tubes, #46463-1075, may also be used.
3. Suitable apparatus for comparing test portions and standards—the apparatus may be of any design, but for the highest precision, should have the following characteristics:
   (a) Illumination—CIE Illuminant C.
   (b) Surrounding field—Should not differ significantly in brightness from the test portions and standards, and should be essentially achromatic.
   (c) Field of view—Two standards and the test portion should subtend a visual angle of about 2°. Two standards and a test portion should always be in the field of view.
   (d) Separation of standard and test portion—There should be a perceptible separation between test portion and standard, but this should be as small as mechanically possible.

   Another type of apparatus may be used, if it can be demonstrated to give the same results.

PROCEDURE

1. Plastic and very high-viscosity lecithins should be heated gently to 60°C.

   Note—Heat darkens all lecithins. Prolonged heating at temperatures near 60°C should be avoided. Do not heat the test sample above 60°C.
2. Transfer the test portion to a color tube until it is filled to about 2.5 cm from the top. Avoid entrapment of air bubbles by holding the tube at a slight angle, thereby allowing the test portion to flow gently down the side. If bubbles are present, allow them to rise to the surface, or remove by centrifugation before testing.
3. The color of the test portion is reported as the number of the standard most closely matching the test portion. If the test portion falls between two standards, it will be reported as "+" or "–" depending on whether it is darker (+) or lighter (–) than the standard it most closely approaches. Thus, between colors 5 and 6, the steps will be 5, 5+, 6–, and 6.

NUMBERED NOTES

1. The values of the chromaticity coordinates may be obtained by spectrophotometric measurements, in accordance with ASTM Designation E308, or by visual comparison with authentic standards. The tolerance of individual standards shall not depart from the coordinates given in Table 1 by more than ⅓ of the distance to the next color. In any one set, no two standards shall be closer together than ⅔ of the nominal value of the color difference between them.

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Table 1
Chromaticity coordinates of reference standards.

	Chromaticity coordinates[a]	
Standard number	X	Y
1	.3177	.3303
2	.3233	.3352
3	.3329	.3452
4	.3437	.3644
5	.3558	.3840
6	.3767	.4061
7	.4044	.4352
8	.4207	.4498
9	.4343	.4640
10	.4503	.4760
11	.4842	.4818
12	.5077	.4638
13	.5392	.4458
14	.5646	.4270
15	.5857	.4089
16	.6047	.3921
17	.6290	.3701
18	.6477	.3521

[a] See Notes, 1.

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AOCS

AOCS Recommended Practice Ja 10-87

Reapproved 2009

Brookfield Viscosity

DEFINITION

This method determines the viscosity in centipoises of a fluid lecithin at a specified temperature.

SCOPE

Applicable to lecithin products that are fluid at the specified temperature.

APPARATUS

1. Brookfield dial reading viscometer model RVT, or digital display viscometers RVDV-E, RVDV-I+, RVDV-II+, or RVDV-III Ultra.
2. Beaker—600 mL, Griffen type (see Notes, 1).
3. Heating bath—capable of maintaining within ± 0.5°C of the specified temperature.

PROCEDURE

1. Using the heating bath, heat 500 mL of the test sample in a 600 mL Griffen-type beaker to the specified temperature, normally 25°C, and allow the test sample to equilibrate for 3 hr.
2. Refer to the instruction manual for the viscometer operating instructions. Use a spindle number at an rpm setting of 10, 20, or 50, which results in a dial reading between 30 and 70 on the fifth revolution. If this dial range cannot be obtained, a different spindle may be used. Start with spindle number 4 at 20 rpm for unknown products.
3. Using the dial reading, calculate viscosity as outlined below.

CALCULATIONS

1. Viscosity (centipoises) = viscometer reading × Brookfield conversion factor.

NOTES

1. Brookfield recommends the use of a guard, which cannot be used with the 250 mL tall form beaker previously recommended in this method. The 600 mL Griffen-type beaker is preferred when there is sufficient test sample. If there is a limited amount of test sample, a 300 mL tall form beaker may be used.

   The manufacturer's recommendations for calibrating the viscometer should be followed when using beakers smaller than the 600 mL Griffen-type beaker.

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AOCS Official Method Ja 11-87
Reapproved 2009

Viscosity of Transparent Liquids by Bubble Time Method

DEFINITION

The viscosity of a liquid is the resistance experienced by one portion of the liquid moving over another portion of the liquid. The absolute unit of viscosity in the cgs system is the poise which is expressed as dyne-seconds per square centimeter. Stokes are equal to poises divided by density.

SCOPE

1. This method determines the viscosity in bubble seconds by timing or comparison. The bubble seconds are approximately equal to stokes for most liquids.
2. This method is applicable to transparent liquids that are free from crystalline or gel particles.

APPARATUS

1. Bath—constant-temperature, circulating. Lauda Model MT–3 or any suitable circulating bath capable of maintaining a temperature at 25 ± 0.1°C with water as the heat transfer medium.
2. Standard viscosity tubes—ASTM D1545 specifications (see Notes, 1) of clear glass and with flat bottoms, 10.65 ± 0.025 mm i.d.; 114 ± 1 mm outside length. Plainly legible lines shall be located as follows (see Notes, 1)—27 ± 0.5 mm; 100 ± 0.5 mm; 108 ± 0.5 mm. The distance between the first and second lines should be 73 ± 0.5 mm. All distances should be measured from the outside bottom of the tube.
3. Reference standards (see Notes, 2)—a series of standard viscosity tubes (Apparatus, 2) filled with transparent liquids having predetermined viscosities in stokes and bubble seconds and spaced in logarithmically even increments of about 26% (log 1.260 = 0.100), as listed in Table 1. The standards are to be marked numerically as shown in Table 1 without reference to stokes or bubble time.

   It is suggested that the reference standards be marked in three series—light series, 15 tubes marked 0.22–8.0; heavy series, 14 tubes marked 10–200; and very heavy series, 7 tubes marked 250–1000. Also listed in Table 1, for general and historical reference only, is the long established series of Gardner-Holdt letter standards, in tubes that do not have the three lines and are shorter than the standard viscosity tubes described in Apparatus, 2.
4. Timing device—stopwatch or electric stop clock capable of being read to a precision of 0.1 sec.
5. Tube racks—inverting rack, capable of inverting one or more viscosity tubes 180° to within 1° of a vertical position, while rack and tubes are immersed in the constant temperature bath.
6. Viscosity tube corks—no. 2 short.

PROCEDURE

1. Fill a standard viscosity tube with the material to be tested to approximately level with the 108 mm line.
2. Transfer the tube to a constant 25°C temperature bath with the cork loosely inserted. Hold at this temperature for 10 min.
3. At the end of 10 min, adjust the level of the liquid so that the bottom meniscus will be level with the 100 mm line. Insert the cork so that the bottom of the cork is on the level with the 108 mm line. This will ensure a bubble of suitable and uniform size.
4. Insert the tube upside down in the rack and immerse in the 25°C water bath. If the comparison method is to be used, the proper standards are inserted in the rack alongside the liquid to be tested. Allow the tube to stand with cork down in the bath a minimum of 20 min before beginning the time travel for the bubble.
5. To read, invert the tube quickly and determine the time required for the bubble to rise in seconds or compare against a standard. When determining the time in seconds, start the timing device when the top of the bubble becomes tangent to the 27 mm line on the tube. Stop the timing when the top of the bubble becomes tangent with the 100 mm line. This gives a 73 mm timed bubble travel. All timings and comparisons shall be made with the tube in an exact vertical position.

REPORT

The viscosity obtained by either the timing or comparator method may be expressed as "bubble seconds" or "approximate stokes."

Note—The numerical tube number is an approximate measurement of stokes when the Bubble Time Method is applied to most raw materials and finished products encountered by the paint, varnish, and lacquer industries. This relationship does not hold for silicones or water dispersions of gums or similar materials.

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PRECISION

1. For viscosities of liquids which have a timed bubble travel of 4 sec or less, more precise results can be obtained by comparison against reference standards having a predetermined viscosity of timed bubble travel (see Apparatus, 3).
2. Adequate control of the temperature bath is essential. A variation of ± 0.1°C in the temperature of the bath will cause a 1% variation in the timed bubble travel.
3. Positioning of the tube in a vertical position is mandatory. A tube one radian off the vertical will give an error of approximately 10% in the time of bubble travel.
4. The precision of the method was determined with ten laboratories participating (see Notes, 3) as follows:

Relative Standard Deviation	
Within Lab	Between Lab
2.5%	4.5%

Liquids used ranged from 4.46–440.2 stokes.

NUMBERED NOTES

1. Empty standard viscosity tubes are available from Wilkens-Anderson Co., 4525 W. Division St., Chicago, IL 60651, USA, Phone (312) 384–4433.
2. Viscosity Reference Standards are available from Hiac Royco, Pacific Scientific Instruments, 11801 Tech Road, Silver Spring, MD 20904, USA, Phone (301) 680-7000.
3. For further information on Precision of this method, see K.E. Holt, "Proceedings," *Am. Soc. Testing Mats.,* Vol. 57, pp. 297–300 (1957), and the Federation of Paint and Varnish Production Clubs, "Official Digest," Vol. 30, May 1958, pp. 540–543.

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Table 1
RECOMMENDED NUMERICAL STANDARDS FOR COMPARATOR VISCOSITY TUBES *
(Arranged to show relationship between stokes, bubble seconds, and Gardner-Holdt letters. Stokes shown in logarithmic progression).

Bubble time sec*	Tube number	Stokes	Gardner–Holdt letter	Tube number	Stokes	Gardner–Holdt letter
		0.10		10	10	W
		0.13	A3	13	13	X
		0.16		16	16	Y
		0.20		20	20	Z
0.75	.22		A2			
		0.25		25	25	Z1
		0.32	A1	32	32	Z2
0.81	.34					
		0.40		40	40	Z3
0.90	.50	0.50	A	50	50	Z4
		0.63	B	63	63	
1.00	.68					
		0.80	C	80	80	Z5
1.15	.92					
		1.00	D	100	100	Z6
1.30	1.15					
		1.25	E	125	125	Z7
			F			
1.55	1.45					
		1.60	G	160	160	Z8
1.85	1.80					
		2.00	H	200	200	Z9
			I			
2.20	2.15					
		2.50	J	250	250	Z10
			K			
2.65	2.65					
			L			
			M			
3.20	3.20	3.20		320	320	
			N			
			O			
4.00	4.00	4.00	P	400	400	
			Q			
			R			
5.00	5.00	5.00	S	500	500	
			T			
6.30	6.30	6.30	U	630	630	
8.00	8.00	8.00		800	800	
			V			
10.00	10.00	10.00		1000	1000	

*The bubble time in seconds of the numerical tubes under 4 sec was determined by a technique employing a movie camera. Above 2.65 the bubble seconds as measured by the Bubble Time Method and stokes as measured by the Kinematic Method are approximately equivalent for most products. Below 2.65 this relationship does not hold.

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AOCS

AOCS Analytical Guidelines Ja 12-89

Revised 2011

Analysis of Lecithin Co-Products

DEFINITION

These guidelines identify standard methods that are recommended for the analysis of lecithin co-products.

SCOPE

This procedure is applicable to the following co-products related to lecithin manufacture: deodorizer distillate, soapstock, clabberstock/pit skimmings, and spent bleaching earth.

The standard methods recommended for the characterization of lecithin related co-products and by-products are shown in Table 1.

NOTES

1. Methods for the analysis of pesticide residues in fats, oils, and lecithin are too complex and elaborate to reproduce in the Official Methods and Recommended Practices of the AOCS. The analyst should refer to methodology issued by the United States Department of Agriculture (USDA).

Table 1

Co-Product	Recommended tests	Recommended methods
Deodorizer distillate	Tocopherols and sterols	AOCS Ce 7-87
	Pesticide residues	See Notes, 1
	Total fatty acids	AOCS G 3-53
	Unsaponifiables	AOCS Ca 5b-71
	Moisture	AOCS Ja 2b-87
Soapstock	Unsaponifiables	AOCS Ca 5b-71
	Total fatty acids	AOCS G 3-53
	pH	AOCS G 7-56
	Moisture	AOCS Ja 2b-87
Clabberstock & pit skimmings	Pesticide residues	See Notes, 1
	Peroxide value	AOCS Cd 8b-90
	Free fatty acids	AOCS Ca 5a-40
	Moisture	AOCS Ja 2b-87
	Fatty acid composition	AOCS Ce 1-62
Spent bleaching earth	Oil content	AOCS Ba 3-38
	Peroxide value	AOCS Cd 8b-90
	Trace metals	AOCS Ca 15-75

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AOCS Recommended Practice Ja 13-91
Reapproved 2009

Determination of Tocopherols in Lecithin by HPLC

DEFINITION

This method quantitates the individual tocopherols in lecithin by HPLC, utilizing a test sample cleanup and fluorescence detection (References, 1, 2).

SCOPE

This method is applicable to lecithins, deodorizer distillates, and shell drains.

APPARATUS

1. Volumetric flasks—actinic, class A, 50 and 100 mL.
2. Volumetric pipet—class A, 10 mL.
3. Syringes—glass Luerlock, 10 mL and 10 µL.
4. Filter assembly—Acrodisc-CR, 0.45 µm pore size.
5. Filter assembly—for HPLC mobile-phase filtration.
6. Analytical balance—accurate to ± 0.0001 g.
7. HPLC system—including:
   (a) Analytical column—4 mm × 15 cm, packed with microparticulate silica with a mean diameter of 5 µm. Baseline resolution of the tocopherol isomers should be obtained (see Notes, 1, 2, and 3).
   (b) Silica guard column—see Notes, 4.
   (c) Injection volume—10 µL.
   (d) Flow rate—1 mL/min.
   (e) Fluorescence detector—excitation wavelength, 290 nm; emission wavelength, 330 nm (or UV detector at 290 nm). Adjust range and sensitivity to obtain peak heights between 30 and 60% of full-scale response for 10 µL injections of working standards (see Notes, 5 and AOCS Official Method Ce 8-89).
   (f) Recorder or recording integrator.
8. Silica Sep-Pak—Waters Associates, Milford, MA, USA (see Notes, 6).

REAGENTS

1. Isooctane—HPLC grade (see Notes, *Caution*).
2. Isopropanol (IPA)—HPLC grade (see Notes, *Caution*).
3. Methanol—HPLC grade (see Notes, *Caution*).
4. Mobile phase—consisting of isopropanol (1%, v/v) in isooctane, prepared by pipetting 10.0 mL of isopropanol into approximately 900 mL of isooctane and diluting to 1 L with isooctane (see Notes, 7). The mobile phase should be filtered and degassed before using (see Apparatus, 4, 5).
5. Dilution solvent—isopropanol (5%, v/v) in isooctane.
6. α–, β–, δ–, and γ–tocopherol standards (see Notes, 8).

PROCEDURE

Note—Using actinic glassware, protect test samples and standards from light, especially UV light, to avoid degradation.

1. Preparation of standard—
   (a) Stock solution—Accurately weigh 10 mg (± 0.01 mg) of each tocopherol standard(Reagents, 6) into a 100 mL volumetric flask and dilute to volume with dilution solvent (Reagents, 5).
   (b) Working standard solution—Pipet 15 mL of standard stock solution [Procedure, 1, (a)] into a 50 mL volumetric flask and dilute to volume with dilution solvent (Reagents, 5). Calculate concentration of each tocopherol isomer based on its purity (see AOCS Official Method Ce 8-89 for a discussion of UV determination of tocopherol purity). Prepare fresh daily, and store standards at 0–5°C under nitrogen.
2. Preparation of test sample—
   (a) Weigh 5 g of lecithin, 100 mg distillate, or 500 µg shell drain into a 100-mL actinic-glass volumetric flask. The concentration of tocopherols in the test solution should give on-scale peaks. If necessary, further dilute the test solution to obtain on-scale peaks if concentrations are low or increase the test sample weight up to the 5 limit (see Notes 6 and 9).

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(b) Dissolve the test sample in the dilution solvent (Reagents, 5) and dilute to volume. Mix well. Lecithin test samples require additional cleanup, step (c); otherwise proceed to step (d).
(c) Remove phospholipids from lecithins using a silica Sep-Pak. Prewash a silica Sep-Pak with 3–4 mL of dilution solvent (Reagents, 5). Discard the wash. Pass about 10 mL of test sample in the dilution solvent (Reagents, 5) through the silica Sep-Pak, discard the first 3 mL, collect the next 3 mL for analysis, and discard the remaining sample solution (see Notes, 6).
(d) Filter the test sample with the 0.45 mm pore size filter and inject 10 mL for analysis.
Note—The test samples should be analyzed on the same day they are prepared to avoid loss of tocopherol.
(e) Elution times may vary, depending on the column used, but the order of elution for tocopherols is as follows: α–, β–, γ–, and δ–tocopherol.

CALCULATIONS

1. For individual tocopherols (α, β, γ, δ):

$$\text{mg/kg (ppm)} = \frac{Ps}{Pr} \times Cr \times \frac{100}{Ws}$$

Where—
Ps = peak height of tocopherol in the test portion; α, β, δ, or γ
Pr = average peak height of tocopherol in standard
Cr = concentration of working standard solution in mg/mL
100 = total volume in mL of test solution
Ws = mass of test sample in grams

2. The β–, γ–, and δ–tocopherol contents of the test sample are calculated in the same way, using the data from the chromatography of the corresponding tocopherol standard (see Notes, 9). If only the α-tocopherol standard is available, refer to AOCS Official Method Ce 8-89, Procedure, 1, (b) and Notes 4, 10, and 11.
3. The total tocopherol concentration may then be obtained by a summation of the individual isomers.

PRECISION

1. Reproducibility of all isomers: 2.3% RSD.
2. Spike recoveries of isomers: α, 96.8 ± 5.6%; γ, 97.0 ± 5.1%.

NOTES

Caution

Isopropanol (isopropyl alcohol) is flammable and a dangerous fire risk. The explosive limits in air are 2–12%. It is toxic by ingestion and inhalation. The TLV in air is 400 ppm.

Isooctane is flammable and a dangerous fire risk. The explosive limits in air are 1.1–6%. It is toxic by ingestion and inhalation.

Methanol (methyl alcohol) is flammable. Use effective fume-removal device when heating or evaporating. Methanol is toxic. Avoid contact with eyes. Avoid breathing vapors. Can react vigorously with sodium hydroxide + chloroform, potassium hydroxide + chloroform, and perchloric acid.

NUMBERED NOTES

1. A Waters Resolve 5 µm silica column is suitable. See also AOCS Official Method Ce 8-89 for additional suitable columns.
2. After 75–100 test sample injections, the column should be flushed with methanol.
3. BHA coelutes and interferes with δ–tocopherol measurement using the designated HPLC parameters.
4. In order to maximize the HPLC column life, the guard column should be used at all times and replaced after 20–25 test samples have been analyzed.
5. Test sample cleanup protocol was developed using the Waters Sep-Pak and has not been validated for other HPLC silica cleanup cartridges. Other silica cartridges may be employed for phospholipid removal, but would require a study to verify tocopherol recovery. Elution volume and/or solvent strength (dilution solvent; Reagents, 5) may have to be adjusted. Deodorizer distillates and shell drains do not require Sep-Pak cleanup.
6. Do not contact test sample or solvents with rubber or plastics, because these materials may contain extractable tocopherols and other antioxidants.
7. Various suppliers carry α–tocopherol. β– and γ–, as well as α–tocopherol, are available from Matreya, Inc., Pleasant Gap, PA, USA. δ–tocopherol, as well as the other tocopherol isomers, may be obtained from Merck (Darmstadt, Germany). Purity of some commercially available tocopherol standards may vary between 80 and 100% (see AOCS Official Method Ce 8-89, Notes, 4).
8. Response of α–tocopherol was shown to be linear over the range of 4–60 µg/mL. High concentrations of tocopherols could cause self-absorption, resulting in nonlinear response. Adjustment of the test sample concentration to obtain a tocopherol response as close as possible to the response of the standard minimizes the chance of error due to nonlinearity.

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9. Distillates and shell drain test samples may contain interfering compounds, which will be detected by UV. These interferences are not detected using a fluorescence detector.

REFERENCES

1. Carpenter, Jr., A.P., *J. Am. Oil Chem. Soc.* 56:668 (1979).
2. Ryan, D.J., *Tocopherols in Oils and Lecithins by HPLC,* Central Soya Food Research Lab Report, April 27, 1982.

Table 1
Recommended test sample weights.

Test sample	Test sample weight
Shell drain	500 µg
Distillates	100 mg
Lecithin	5 g

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AOCS Recommended Practice Ja 14-91

Reapproved 2009

Iodine Value, Wijs Method

DEFINITION

The iodine value (IV) is a measure of the content of unsaturated fatty acids. The test sample is reacted with Wijs iodine trichloride solution; the unreacted excess reagent is determined by titration with standard sodium thiosulfate solution. The iodine value indicates the g iodine which reacts with 100 g lecithin.

SCOPE

Applicable to all vegetable lecithins.

APPARATUS

1. Iodine value flask—300 mL.
2. Burette—class A 50 mL.
3. Analytical balance.

REAGENTS

1. Wijs iodine solution—available commercially.
2. Chloroform—reagent grade (see Notes, *Caution* and 3).
3. Potassium iodide (KI) solution—10%, w/v.
4. Sodium thiosulfate solution, 0.1 M—properly standardized (see Notes, 1).
5. Starch indicator solution (see Notes, 2).
6. Deionized water.
7. Potassium dichromate ($K_2Cr_2O_7$)—primary standard, for standardization of sodium thiosulfate (see Notes, *Caution*).
8. Concentrated hydrochloric acid (HCl)—reagent grade, for standardization of sodium thiosulfate (see Notes, *Caution*).

PROCEDURE

1. Accurately weigh 0.2 g of lecithin ± 0.001 g into the iodine value flask.
2. Dissolve the test sample in 10 mL of chloroform.
3. Add 25 mL of the iodine solution (Reagents, 1), briefly swirl the mixture and allow to stand for exactly 1 hr in the dark.
4. Immediately add 20 mL of 10% potassium iodide solution (Reagents, 3) and 100 mL of water.
5. Immediately titrate the free iodine with the 0.1 M standardized sodium thiosulfate (Reagents, 4) until the yellow color has almost disappeared. Add a few drops of the starch indicator solution (Reagents, 5) and continue the titration just to the disappearance of the blue color.
6. Carry out a blank determination similar in all respects.

CALCULATION

$$\text{Iodine value (IV)} = \frac{(B - S) \times 1.269}{W}$$

Where—

S = volume, mL thiosulfate solution consumed by titration of the test portion

B = volume, mL thiosulfate solution consumed by titration of the blank

W = original test sample mass, g

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Hydrochloric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is toxic by ingestion and inhalation and a strong irritant to eyes and skin. The use of a properly operating fume hood is recommended. When diluting the acid, always add the acid to the water, never the reverse.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

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NUMBERED NOTES

1. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) solution, 0.1 M, accurately standardized vs. potassium dichromate primary standard as follows:
   (a) Sodium thiosulfate solution approximately 0.1 M, prepared by dissolving 24.9 g of sodium thiosulfate in deionized water and diluting to 1 L.
   (b) The potassium dichromate primary standard ($K_2Cr_2O_7$) should be finely ground, dried at 105°C for 2 hr and cooled in a desiccator. Accurately weigh 0.16–0.22 g ± 0.0001 g of potassium dichromate into a 500 mL flask by difference from a weighing bottle. Dissolve in 25 mL of water, add 5 mL of concentrated hydrochloric acid (HCl), 20 mL of 10% potassium iodide solution (Reagents, 3), and rotate to mix. Allow to stand for 5 min and then add 100 mL of water. Titrate with the sodium thiosulfate solution, shaking continuously until the yellow color has almost disappeared. Add 1–2 mL of the starch indicator solution and continue the titration, adding the thiosulfate solution slowly until the blue color just disappears. The strength of the sodium thiosulfate solution is expressed in terms of its normality.

$$\text{Molarity of } Na_2S_2O_3 \cdot 5H_2O \text{ solution} = \frac{20.394 \times \text{mass } K_2Cr_2O_7, \text{ g}}{\text{volume, mL sodium thiosulfate}}$$

2. Make a paste with 1 g of starch and a small amount of cold distilled water. Add, while stirring, to 200 mL of boiling water and immediately remove from heat source. Cool. Test for sensitivity by placing 5 mL of the starch solution in 100 mL of water and adding 0.05 mL of 0.1 M iodine solution. The deep blue color produced must be discharged by 0.05 mL of 0.1 M sodium thiosulfate. Salicylic acid (1.25 g/L) may be added to preserve the indicator. If long storage is required, the solution must be stored in a refrigerator at 4–10°C. Fresh indicator must be prepared when the end point of the titration from blue to colorless fails to be sharp. If stored under refrigeration, the starch solution should be stable for about 2–3 wk.
3. 1,1,1–Trichloroethane (TCE) has been suggested as a replacement for carbon tetrachloride in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied by the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

REFERENCES

1. Lucas Meyer method, 080688/AM-37/300. Adopted with Lucas Meyer approval June 18, 1991.

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AOCS Procedure M 1-92
Replaces M 1-59 • Reapproved 2009

Determination of Precision of Analytical Methods

The former AOCS Procedure M 1-59, "Determination of Precision of Analytical Test Methods," was declared a surplus (i.e., outdated) method in 1989. Current guidelines for the statistical analysis of collaborative study data have resulted from harmonization efforts between the AOAC International and International Union of Pure and Applied Chemistry (IUPAC) and the International Organization for Standardization (ISO).

AOCS Procedure M 4-86, revised and amended in 1988 and 1992, explains collaborative study procedures and reviews statistical parameters in detail.

The accepted statistical method for the analysis of collaborative study data is ISO 5725:1994. The minimum statistics to be determined and reported for any collaborative study, and which should be included in the AOCS method, are repeatability (r) statement, reproducibility (R) statement, and table of statistical parameters (as noted in Table 1). Specifically, the statements and table that should appear in the collaborative study report and the AOCS method based on the collaborative study are as follows:

Repeatability limit—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) as calculated from the formulas in Table 1.

Reproducibility limit—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as calculated from the formulas in Table 1.

The reader may consult the references cited at the end of AOCS Procedure M 4-86. The ISO 5725:1994 protocol, summarized in References 2 and 3, includes sequential application of the Cochran and Grubbs tests at 2.5% probability until no further outliers are flagged or until a drop occurs of 22.2% (2/9) of the original number of laboratories providing data. The AOACI Inter-laboratory Statistical Program is available from AOAC International (www.aoac.org).

REFERENCES

1. Pocklington, W.D., *Pure Appl. Chem. 62:*149 (1990).
2. Horwitz, W., *Pure Appl. Chem. 67:*331 (1995).
3. Collaborative Study Guidelines, (www.aoac.org)

Table 1
The interlaboratory study completed by the AOCS [Name of Technical Committee] in [year], in which [number] laboratories participated, each obtaining [number of determinations] test results for each sample analyzed at [note any special conditions, e.g., temperature], gave the statistical results (evaluated in accordance with ISO 5725-1994) summarized in the following table.

	Sample(s)[a]	
	A	B
Number of laboratories retained after eliminating outliers		
Number of outlying laboratories removed		
Mean value(s), x [Note units associated with mean value, e.g., %, µg/kg, etc.]		
True or accepted value, if known		
Repeatability standard deviation (S_r)		
Repeatability relative standard deviation (RSD_r)		
Repeatability value, r = ($2.8 \times S_r$)		
Reproducibility standard deviation (S_R)		
Reproducibility relative standard deviation (RSD_R)		
Reproducibility value, R = ($2.8 \times S_R$)		

[a] Sample key: A, [description of sample]; B, [description of sample]; (list all samples analyzed, both in Table and in sample key).

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AOCS Procedure M 2-09

Revised 2013

Writing and Approval of Methods

PART I—ADOPTION AND PUBLICATION OF METHODS

Article XII, Section 1 of the Bylaws of the American Oil Chemists' Society reads as follows: "Official Methods and Recommended Practices. The Society investigates, adopts, and publishes such methods of analysis in the field of oils, fats, and related materials as may appear to be in the public interest, convenience or necessity. This publication shall be called *Official Methods and Recommended Practices of the American Oil Chemists' Society.*"

To facilitate careful consideration and orderly adoption of methods of analysis, the Uniform Methods Committee (UMC) has established the following procedure:

1. Every method must be assigned to a technical committee, subcommittee, or task group and have committee approval before the method can be recommended to the Uniform Methods Committee.
2. The technical committee chairperson will obtain this approval by a ballot of all committee members, furnishing the members with a copy of the method and supporting data.
3. Approval shall consist of a two-thirds affirmative vote of those voting on the method. A negative ballot must be accompanied by a reason for the negative vote. To be a legal ballot, 60% of the ballots sent out must be returned.
4. The technical committee chairperson will submit to the AOCS Technical Services copies of all methods and supporting data sent out with the committee ballot. These will be sent to the members of the Uniform Methods Committee. Any comments by UMC members will be sent to the AOCS Technical Services, who will pass them on to the technical committee chairperson for consideration.
5. A method receiving no negative ballots shall immediately be recommended to the Uniform Methods Committee.
6. If a method receives the necessary two-thirds affirmative ballots but has one or more negative ballots, the committee chairperson may (a) recommend the method (accompanied by all negative ballots) be forwarded to the Uniform Methods Committee or (b) hold the method for immediate discussion and resolution of the negative ballots. All members casting negative ballots shall be contacted by the committee chairperson.
7. The Uniform Methods Committee members shall have one month to consider and return the ballots. All negative ballots must be accompanied by reasons for negative vote.
8. Methods receiving no negative ballots will be submitted directly to the editor of *Official Methods and Recommended Practices of the American Oil Chemists' Society* for publication without further discussion by the Uniform Methods Committee.
9. Methods receiving one or more negative ballots will be reviewed with the committee chairperson.
10. A negative ballot may be resolved by (a) withdrawal of the negative ballot by the member casting the ballot or (b) a vote of two-thirds of the committee members to override the negative ballot.
11. If the negative ballots are resolved by the Uniform Methods Committee, the method is submitted to the editor for publication. If the negative ballots are not resolved, the method is returned to the technical committee for further study.
12. To achieve its objectives of developing and validating analytical methods, the society may encourage publication of reports of its validation studies in either the *Journal of the American Oil Chemists' Society* or *INFORM.* Officially adopted methods are published in the *Official Methods and Recommended Practices of the AOCS.*

PART II—REVIEW AND UPDATING OF EXISTING METHODS

"All Official Methods and Recommended Practices are updated through Additions and Revisions to Methods. In addition, all methods are completely reviewed at regular intervals by qualified persons (known as 'associate method editors')."

The methods published by the society are used for referee purposes and, therefore, must be the most accurate and precise methods available. To keep the Official Methods in line with the changing technology in analytical determinations, the Uniform Methods Committee has established the following procedure:

1. Every method is assigned to either a technical committee or an associate methods editor.
2. Each year, the technical committee chairperson (or the designated subcommittee chairperson) or the associate methods editor will review the assigned methods to ascertain their status in light of current technological developments.
3. A revision in a method must be submitted to the AOCS Technical Services for submission to the Uniform Methods Committee.
4. All AOCS methods shall be reviewed prior to the publication of a new *Official Methods and Recommended Practices of the AOCS.* The Uniform Methods Committee and the editor shall "reapprove" the methods. The reapproval date is to be noted on the method.

PART III—DEFINITIONS

1. Official Method—an official method is a method that has been approved by the sponsoring technical committee and the Uniform Methods Committee and adopted by the Governing Board of the AOCS.

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2. Recommended Practice—a Recommended Practice is a method that may be of interest or value, but does not have official method status.
3. AOCS Standard Procedure—A Standard Procedure is a method that relies on the use of specific apparatus in accordance with the manufacturer's instructions. These procedures are reviewed and studied to meet official method status. In cases where the technology may be used in place of an official method, parallel equivalence studies are also performed.
4. AOCS Analytical Guidelines—Analytical Guidelines provide both a list of methods applicable to the determination of key parameters in a matrix and a description of their applicability together with pertinent notes and comments.
5. Quality Assurance—assurance is the overall concept by which the quality of analytical results generated by a laboratory is assured. This concept encompasses such mechanisms as choice of methods, skills of personnel, calibration of equipment, sample handling and preservation, maintenance of records, reporting of results, quality control considerations, laboratory facilities, etc.
6. Quality Control—control is one of the mechanisms of quality assurance and encompasses such laboratory activities as maintenance of quality control charts, standard additions, calibration curves, recovery studies by additions and dilutions, participation in proficiency testing or collaborative studies, replication of analyses, split sampling, equivalency testing, preservation of samples, use of certified standards, editing of results, etc.
7. Precision—the precision of a method usually is associated with inherent random errors and is expressed in terms of its standard deviation or, preferably, relative standard deviation (coefficient of variation). Precision usually refers to the repeatability of a result, or the "grouping" of results that would reveal "scatter" around a mean value which, with close grouping, would indicate a method of high precision (see AOCS Procedure M 1-92).
8. Accuracy—the accuracy of a method usually is described in terms of its systematic error but is measured in terms of its ability to reflect the "true" value of a system, or its ability to hit the targeted value. An analytical method may have poor precision and yet be accurate; conversely, it may be inaccurate and have good precision. However, the ideal method would have relatively high precision grouped around the targeted value and limited random and systematic errors.

PART IV—FORMAT FOR AOCS METHODS

Methods proposed for inclusion in *Official Methods* should conform in style and be in keeping with the foregoing definitions and the guidelines which follow. An outline of methods format follows the guideline section.

Format Guidelines

1. Method numbers—each method should carry a page heading to show the section of the book for which it is intended. Specific method numbers will be assigned by the editor, but if the method revises or replaces an existing method, the present number should be indicated.
2. Title—each method should have a specific, descriptive title.
3. Definition—the definition should indicate what analyte is determined by the test, how the determination is made (GC, HPLC, AA, solvent extraction, etc.) and any special principle(s) involved that may be necessary for a better understanding of the method (balanced equations, color changes, wavelengths, special chromatographic columns, etc.).
4. Scope—the scope should describe the product and/or matrix to which the method applies and any similar products or matrices. Limitations, applicable concentrations and any known interferences (matrix, spectral, etc.) should be noted.
5. Apparatus—sources of apparatus and equipment must be indicated and, with the exception of a sole source supplier, all appearances of endorsement must be avoided. A general description of the apparatus may be used and is preferred. Cases of custom-made equipment and apparatus should be avoided if at all possible, but, if these are necessary, full specifications, drawings or diagrams should be provided.
6. Reagents—all reagents used in the method must be listed. In some methods, special solutions and/or standards may be prepared. This should be noted in separate lettered and numbered sections titled "Preparation of Solutions" and "Preparation of Standards." Grades of chemicals should be indicated in the method in keeping with both Section H of *Official Methods* and the guidelines that follow.

   Chemicals are made in several grades based on quality and purity. These qualities are designated by names which vary somewhat among manufacturers. In general, the quality of chemicals used in testing must be high. It will be necessary for the purchaser to read the quality designations in the catalog being used, as designations vary considerably with manufacturers. Although not complete, the following list will give some idea of the grades of chemicals available:
   (a) Primary standards—primary standards of high precision assay are suitable as references and in the preparation of standard solutions.
   (b) ACS reagent grade—for some chemicals, the American Chemical Society has established standards. Most manufacturers making chemicals meeting these standards list them as "ACS Reagent Grade."
   (c) Reagent grade—this designation is used by some manufacturers to label chemicals of high quality. Many chemicals of reagent grade carry the manufacturer's analysis on the label.
   (d) USP grade—USP-grade chemicals meet the specifications of the *United States Pharmacopeia,* a publication establishing standards for chemicals and drugs used as pharmaceuticals.

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(e) Other grades—there are other designations of quality such as "Spectro," "Highest Purity," or the manufacturer's name grade, all of which may be very high quality, but will necessitate reading the quality grade designations in the preface of most catalogs to determine the qualities listed.

7. Procedure—instructions in the procedure should be clear and succinct, usually with no more than one thought to a line and following in logical continuity to the conclusion.
8. Sample requirements—where appropriate.
9. Calculations—all calculations should be shown, including explanation of derivation of all factors, whether they be chemical or mathematical.
10. Precision—All AOCS methods require precision data. The precision (and accuracy if possible) of the method must be presented as shown in Table 1 in AOCS Procedure M 1-92. The precision and accuracy (see Part III, 5 and 6) data must be generated during the collaborative study. The method should contain statements on repeatability and reproducibility limits, similar to the examples given in AOCS Procedure M 1-92. If a method is adopted from another organization, citation must be given to the publication containing the statistical analysis of the organization's collaborative study.
11. Notes—the notes should contain information of particular importance to the method, such as
    (a) Safety considerations—practices that may involve hazards of a carcinogenic nature, flammability, use of fume hoods, or dangers from ingestion or inhalation, etc., should be prefaced "*Caution.*"
    (b) Sensitivity—response per unit concentration.
    (c) Detection level—minimum measurable concentration above the background level.
    (d) Reporting—manner of reporting results, such as units of measurement and significant figures, should be explained.
    (e) Interferences—interferences are stated briefly in the Scope of the method; however, if methodology exists for either reducing or eliminating the interference, it should be noted.
12. References—references should be included for purposes of background information, with due credit given to the original publication of the method and in consideration of proprietary information. References must include published results of collaborative studies, whether performed by the AOCS or other organizations. If the method is an official method of another organization, reference must also be made to the organization's official methods publication.
13. Other considerations—
    (a) Authorship—self-explanatory, but is used to provide a contemporary source of background information.
    (b) Style—the method should be written in the imperative voice.
    (c) Figures—scaleable electronic format.
    (d) Standards for accuracy—if standards exist for checking the accuracy of the method, they should be noted in the method write-up, along with information on where the standards may be obtained.
14. Committee responsibility—the technical committee proposing the method is responsible for its accuracy. It is important that numerical values, spelling, calculations, and all laboratory directions be checked carefully. The method should be written in AOCS Methods format prior to the collaborative study (see AOCS Procedure M 4-86).

PART V—DESCRIPTION OF AOCS METHODS

AOCS Official Methods

A method will be granted AOCS Official Method status only after (a) an acceptable collaborative study, (b) proper statistical criteria have been met, (c) approval of the technical committee, (d) approval of the Uniform Methods Committee, and (e) the collaborative study results have been published, or at least submitted for publication. In lieu of (a) and/or (b), a method may be adopted, but only as a Recommended Practice.

AOCS Recommended Practices

A recommended practice is a method that may be of interest, but does not have Official Method status. The method is still subject to the approval process noted in this procedure. A recommended practice may or may not be based on a collaborative study. For example, the statistical evaluation of an attempted collaborative study (or repeat studies of the same method) may reveal the variation of the method to be outside the acceptable performance range (assuming that all other obvious errors have been corrected). If there is a high degree of interest, the method may be adopted still, but only as a Recommended Practice. The interest in such a method may be based on having a simpler, more rapid or qualitative procedure either as an adjunct to an official method or for use in general.

AOCS Standard Procedures

AOCS Standard Procedures are guidance documents or specific methodology that requires the use of specific equipment according to the manufacturer's instructions. Standard Procedures require both pre-collaborative and collaborative study and require approval by an AOCS Methods Subcommittee and the Uniform Methods Committee.

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AOCS Analytical Guidelines
AOCS Analytical Guidelines are guidance documents detailing the AOCS methods suitable for analysis of key parameters in a particular matrix and giving a description of their applicability together with pertinent notes and comments.

PART VI—ADOPTION OF METHODS FROM OTHER ORGANIZATIONS

Methods of other organizations may be adopted as AOCS Official Methods, provided criteria are met as follows:

1. The method must be based on a published collaborative study.
2. The collaborative study must, at a minimum, be based on the guidelines noted in AOCS Procedure M 4-86, with acceptable supporting statistical data.
3. The method must be rewritten into AOCS Official Methods format, with supporting statistics included.
4. The AOCS methods write-up must include the journal reference to the collaborative study, as well as the appropriate reference to the other organization's official methods publication.
5. After being rewritten into AOCS format, the method must be approved by the procedure outlined in Part I.

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AOCS Procedure M 3-82

Revised 2013

Surplus Status of Methods

As an essential part of the regular exercise of issuing the Additions and Revisions to the *Official Methods and Recommended Practices of the AOCS,* a mechanism is hereby adopted for the purpose of declaring a method surplus:

1. Consideration of a method for surplus status may be suggested by any member through the appropriate technical committee for ultimate review by the Uniform Methods Committee. Such methods, although having initially been subjected to Collaborative Study and therefore having met AOCS approval, are concluded not to be of interest due to one or more of the following reasons:
   (a) obsolescence;
   (b) replaced by another method;
   (c) the product is no longer marketed.
2. Upon approval for the declaration of a surplus status, the method will be affixed with the symbol "*" to indicate that it is being considered in this status for a period of 1 yr and will continue to be printed and carried, during this time, in the *Official Methods and Recommended Practices of the AOCS.* Surplus status information will be made available on the website and other media for comments prior to the final withdrawal of any Official Method, Recommended Practice, Analytical Guideline, or Standard Procedure.
3. At the end of the 1 yr period these methods will continue their official status but will be carried in future editions only by reference.
4. Also at the end of the 1 yr period, methods in a surplus status will be retained in the archives of AOCS Headquarters for historical purposes so that future copies may be obtained upon request, at nominal charge.
5. Members who question whether a method should be placed in surplus status, or wish that the text be retained or reprinted in the next edition, should do so through the appropriate Technical Committee or through the Editor of Analytical Methods.
6. A listing of surplus methods appears at the end of the alphabetical index.

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AOCS

AOCS Procedure M 4-86

Revised 2012

Collaborative Study Procedures

PART I—GENERAL ORGANIZATION

The collaborative study is organized and directed by a technical committee for a specific method under investigation. The technical committee reviews the literature and selects an appropriate analytical method, modifying it if necessary. When the method is finalized, it is written in Official Methods format before any further action is taken. The technical committee should select a qualified analyst to try the method before a full-scale collaborative study is initiated. The results of the trial, as well as the design of the collaborative study, are then reviewed with both the chairperson of the Uniform Methods Committee (UMC) and the AOCS technical director. The chairperson of the UMC must approve the proposed experimental design before the full-scale collaborative study is begun.

The materials used for the collaborative study are prepared and distributed to the participants by either a technical committee member or a qualified person designated by the technical committee. No fewer than eight laboratories should participate in the collaborative study, with a minimum of five test samples sent to each laboratory (References, 1). This is a minimum criterion; in actual practice, both the number of laboratories and the number of test samples should be exceeded. In addition, a reference, practice or performance evaluation sample should be included with instructions not to analyze the unknowns until a specified degree of recovery, repeatability and/or other attribute has been achieved. If possible, the study should be international, with no fewer than six countries represented.

Laboratories with experience in the general subject matter of the method are selected as collaborators through solicitation by the technical committee. Because the objective of the study is to evaluate the method and not the analyst (References, 4), all analysts must be instructed to follow the method exactly as written. The content of the analyte in the test samples is unknown to the participants.

All individual results obtained by the collaborators are reported to the technical committee chairperson, who compiles and evaluates them. Statistical treatment of the data is essential for evaluation of the method for accuracy, precision, sensitivity, and specificity. Statistical guidelines are provided in AOCS Procedure M 1-92. Other sources are the *Statistical Manual of the Association of Official Analytical Chemists* (References, 5) and *Guidelines for the Development of Standard Methods by Collaborative Study* (References, 6).

A technical committee must make the initial judgment on the performance of the method. If the collaborative study is deemed satisfactory on the basis of statistical analysis, the technical committee may then proceed with the approval process (AOCS Procedure M 2-09). If the collaborative performance is unsatisfactory, every effort must be made to locate possible sources of errors such as incorrect transcription of values, failure to follow the procedure exactly, ambiguous directions, use of incorrect equipment, improper standards, contaminated reagents, etc. Depending on the nature of the errors and the number of laboratories involved, it may be necessary to repeat the collaborative study to obtain acceptable performance. Approved methods may be modified and restudied collaboratively as needed, should feedback from general use reveal flaws in the method or in its written set of directions. Approved methods are updated on a periodic basis (AOCS Procedure M 2-09).

The collaborative study provides the basic information on the performance of analytical methods. The extent of the information will depend on the number of test samples provided, the number of replicate analyses performed and the number of laboratories participating. The data should be unbiased because the composition of the test samples is known only to the administrator of the study. Some of the requirements of the study and their relationship to the characteristics and attributes of the method are as follows:

1. Accuracy (where possible)—test samples must be of defined composition (by spiking, formulation, or analytical consensus).
2. Specificity—test samples should contain related analytes.
3. Sensitivity—test samples should differ from each other, or from negative samples, by a known amount, when possible.
4. Applicability—test samples should include the concentration range and matrix components of interest.
5. Blanks—the design should include different matrices with "none" of the component of interest.
6. Precision—instructions should request replicate analyses, preferably on different days. By far a better procedure is to include blind (unknown to the analyst) replicate test samples or split levels (Youden pairs) in the series.
7. Practicability—instructions should request information as to the actual and elapsed time required for the analyses; the availability of reagents, equipment, and standards; and any necessary substitutions. When practice samples are included, the number of analyses required to achieve the stated recovery and repeatability should be reported.

As numerous beginners in this field have discovered, much preliminary work must be done before conducting the collaborative study.

1. The method must be chosen and demonstrated to apply to the matrices and concentrations of interest.
2. The crucial variables in the method should be determined, and the need for their control must be emphasized [a ruggedness test (References, 7) is useful for this purpose].
3. The method should be written in detail, in AOCS *Official Methods* format, by the technical committee and tested by an analyst not previously connected with its development.
4. Unusual standards, reagents, and equipment must be available from usual commercial sources of supply, or sufficient quantities must be prepared or obtained to furnish to the participants.

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5. The test samples must be identical and homogeneous so that the sampling error is only a negligible fraction of the expected analytical error.
6. A sufficient number of test samples must be prepared to cover typical matrices and the concentration range of interest (tolerance, maximum or minimum specifications, likely levels of occurrence, etc.)
7. A minimum of eight laboratories and sufficient test samples must be included to provide a minimum of 40 data points. Additional laboratories and test samples are recommended.
8. The test samples must be stable and capable of surviving the rigors of commercial transportation.
9. Reserve test samples should be prepared and preserved to replace lost shipments and to permit an analytical check when outliers occur in the collaborative results, to attempt to discover the cause of the abnormal results. Reserved test samples should be analyzed on a periodic basis to determine if any degradation of the test samples is occurring during the study.
10. The instructions must be clear. They should be reviewed by someone not connected with the study to uncover potential misunderstandings and ambiguities.
11. If the analyte is subject to change, provision must be made for all participants to begin the analysis at the same time.
12. Practice samples of a known and declared composition should be accompanied by instructions not to analyze the unknowns until a specified degree of recovery and repeatability (or other attribute) has been achieved.
13. Provision should be made, when necessary, for submission of standard curves, calculations, or chromatograms in order to assist in determining possible causes of error.
14. If either primary or secondary standards are available, their use is required in the collaborative study as a check on both the accuracy and performance of the method. If standards are available, it should be noted in the method write-up along with complete information on the source of the standards.

PART II—SPECIFIC PROTOCOL FOR THE DESIGN, CONDUCT, AND INTERPRETATION OF COLLABORATIVE STUDIES (REFERENCES, 8, 9, 10)

This part of the document summarizes the minimum requirements for a collaborative study, based upon the recommendations accepted by consensus of the 27 participants at the International Union of Pure and Applied Chemistry (IUPAC) Workshop on the Harmonization of Collaborative Analytical Studies, held in Geneva, Switzerland, May 4–5, 1987, Lisbon, Portugal, August 4, 1993, and Delft, Netherlands, May 9, 1994. If a collaborative study is to be indicated as complying with the "IUPAC Protocol," it must be in conformity with the minimum rules that follow. Additional requirements may be imposed by other organizations for their specific needs.

These harmonized requirements are the result of efforts begun by the late Dr. Harold Egan, Laboratory of the Government Chemist, United Kingdom, who organized a meeting of interested international organizations in London, England, in March 1978. This was followed by symposia in Helsinki, Finland, in 1981 and Washington, DC, USA, in 1984, and the workshops in Geneva, 1987, Lisbon, 1993, and Delft, 1994.

PROTOCOLS AND DESIGN

Preliminary work

Collaborative studies require considerable effort and should be conducted only on methods that have received adequate prior testing. Such within-laboratory testing should include, as applicable, information on the following:

1. Preliminary estimates of precision—estimates of the total within-laboratory standard deviation of the analytical results over the concentration range of interest; as a minimum at the upper and lower limits of the concentration range, with particular emphasis on any standard or specification value (see Notes, 1 and 2).
2. Systematic error (bias)—estimates of systematic error of the analytical results over the concentration range and in the commodities of interest; as a minimum at the upper and lower limits of the concentration range, with particular emphasis on any standard or specification value. The results obtained by applying the method to relevant reference materials should be noted.
3. Recoveries—the recovery of "spikes" added to real materials and to extracts, digests or other treated solutions thereof.
4. Applicability—the ability of the method to identify and measure the physical and chemical forms of the analyte likely to be present in the materials.
5. Interference—the effect of other substances that are likely to be present at appreciable concentrations in matrices of interest and that may interfere in the determination.
6. Method comparison—the results of comparison of the application of the method with existing tested methods intended for similar purposes.
7. Calibration procedures—the procedures specified for calibration and for blank correction must not introduce important bias into the results.
8. Method description—the method must be clearly and unambiguously written.
9. Significant figures—the initiating laboratory should indicate the number of significant figures to be reported, based on the output of the measuring instrument (see Notes, 3).
10. Familiarization (practice) samples—with new or unfamiliar techniques, materials of stated composition for practice should be provided to collaborators to demonstrate that the stated value can be reproduced prior to analysis of collaborative test samples.

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Design of the collaborative study

1. Number of materials (Material = analyte/concentration level/matrix combination)—at least five materials must be used; only when a single-level specification is involved for a single matrix may this minimum required number of materials be as low as three. For this design parameter, the two portions of a split-level and the two individual portions of blind replicates per laboratory are considered as a single material.

   A single split-level (Youdan pair) statistically analyzed as a pair is a single material. If statistically analyzed and reported as single test samples, they are 2 materials. Also, the pair can be used to calculate within-laboratory standard deviation,

$$S_r = \sqrt{([\Sigma d_{i^2}])/2n} \text{ for duplicates, blind or open.}$$

$$S_r = \sqrt{([\Sigma d_i - d]^2/2[n - 1])} \text{ for Youden pairs,}$$

   where d_i is the difference between the 2 individual values from the split level for each laboratory and n is the number of laboratories, and d is the average of the d's. In the special case in which the split levels are treated as single test samples (Youden pairs), the among-laboratories standard deviation, S_R is the average of the two S_R values calculated from the individual components of the split level.

   The blank or negative control may or may not be a material, depending on the usual purpose of the analysis. For example, in trace analyses, where very low levels (near the limit of quantification) are often sought, the blanks are considered as materials and are necessary to determine certain statistical "limits of measurement." However, if the blank is merely a procedural control, as in macrolevel analyses (e.g. oil in oilseeds) it would not be considered a material.

   Materials should be representative of commodities usually analyzed. Furnish only enough test sample to provide the number of test portions specified in the instructions.
2. Number of laboratories—at least eight laboratories must report results for each material; only when it is impossible to obtain this number (e.g., very expensive instrumentation or specialized laboratories required) may the study be conducted with fewer, but with an absolute minimum of five laboratories. If the study is intended for international use, laboratories from different countries should participate.
3. Number of replicates—the repeatability precision parameters must be estimated by using one of the following sets of designs (listed in approximate order of desirability):
   (a) Split-level (Youden pair)—for each level that is split and which constitutes only a single material for purposes of design and statistical analysis, use two nearly identical test samples that differ only slightly in analyte concentration (e.g., < 1–5%) obtained either naturally or by diluting (or by fortifying) one portion of the material with a small amount of diluent (or of analyte). Both portions are supplied to the participating laboratories as test samples under a random code number, and each test sample should be analyzed only once.
   (b) Combination blind replicates and split-level—use split-levels for some materials and blind replicates for other materials in the same study (single values from each submitted portion).
   (c) Blind of replicates—for each material, use blind identical replicates; when data censoring is impossible (e.g., automatic input, calculation and printout), nonblind identical replicates may be used.
   (d) Known replicates—for each material, use known replicates (two or more analyses of portions from the same test sample), but only when it is not practical to use one of the preceding designs.
   (e) Independent replicate analyses—use only a single portion from each material (i.e., do not replicate) in the collaborative study, but rectify the inability to calculate repeatability parameters by quality control parameters and other within-laboratory data obtained independently of the collaborative study.

Statistical analysis (see flowchart).

In the statistical analysis of the collaborative study data, the required statistical procedures listed below must be performed and the results reported. Supplemental procedures are not precluded.

1. Valid data—only valid data should be reported and subjected to statistical treatment. Valid data are those data that would be reported as resulting from the normal performance of laboratory analyses; they are not marred by method deviations, instrument malfunctions, unexpected occurrences during performance, or by arithmetic, clerical, or typographical errors.
2. One-way analysis of variance—one-way analysis of variance and outlier treatments must be applied separately to each material to estimate the components of variance and repeatability and reproducibility parameters.
3. Initial estimate—calculate the mean, × (average of laboratory averages), repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R), with no outliers removed, but using only data that has been determined to be valid.
4. Outlier treatment—the estimated precision parameters that must also be reported are based on the initial valid data purged of all outliers flagged by the harmonized 1994 outlier removal procedure. This procedure essentially consists of sequential application of the Cochran and Grubbs tests (at 2.5% probability (*P*) level, 1-tail for Cochran, 2-tail for single Grubbs) until no further outliers are flagged, or until a drop of more than 22.2% (2 of 9 laboratories) in the original number of laboratories providing valid data would occur. Prompt consultation with a laboratory reporting suspect values can result in correction of mistakes or discovering conditions that lead to invalid data. Recognizing mistakes and invalid data is much preferred to relying upon statistical tests to remove deviate values.

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5. Cochran test—first apply the Cochran outlier test (1-tail test at $P = 2.5\%$), and remove any laboratory whose critical value exceeds the tabular value given in Table 1, Cochran critical values, for the number of laboratories and replicates involved.
6. Grubbs tests—apply the single-value Grubbs test (2- tail) and remove any outlying laboratory; if no laboratory is flagged, then apply the pair-value test (two values at the same end and one value at each end; $P = 2.5\%$ overall). Remove any laboratory(ies) flagged by these tests, using Table 2, but stop removal if more than 22.2% (2 of 9 laboratories) would be removed (see Notes, 4).
7. Final estimation—recalculate the parameters listed in the initial estimate section after the laboratories flagged by the preceding procedure have been removed. If no outliers were removed in the Cochran-Grubbs sequence, terminate testing. Otherwise, reapply the Cochran-Grubbs sequence to the data purged of the flagged outliers until no further outliers are flagged, or until more than a total of 22.2% (2 of 9 laboratories) would be removed in the next cycle. (See flowchart for outlier removal on page 4.)

Final Report

The final report should be published and should include all valid data. Other information and parameters should be reported in a format similar (with respect to the reported items) to the following (as applicable):

"[x] collaborative tests carried out at the international level in [year(s)] by [organization] in which [y and z] laboratories participated, each performing [k] replicates, gave the statistical results summarized in Table [t]." (For example of Table [t] parameters, see Table 2.)

Symbols (see Symbols and Terms section).

Definitions (see Definitions section).

Miscellaneous

1. Recovery—recovery of added analyte as a control on method or laboratory bias should be calculated as follows:

$$\text{Marginal Recovery, \%} = \frac{(\text{Total analyte found} - \text{analyte originally present})}{(\text{analyte added})} \times 100$$

   Although the analyte may be expressed as either concentration or amount, the units must be the same throughout. When the amount of analyte is determined by analysis, it must be determined in the same way throughout. Analytical results should be reported uncorrected for recovery. Report recoveries separately.
2. When S_L is negative—by definition, S_R is greater than or equal to S_r in collaborative studies; occasionally the *estimate* of S_r is greater than the estimate of S_R (the range of replicates is greater than the range of laboratory averages, and the calculated S_{L2} is then negative). When this occurs, set $S_L = 0$ and $S_R = S_r$.

SYMBOLS AND TERMS

Use the symbols and terms in Table 3 for designating parameters developed by a collaborative study. If other symbols are used, their relationship to the recommended symbols should be explained fully.

DEFINITIONS

1. Method performance study—a method performance (collaborative) study is an interlaboratory study in which each laboratory uses the defined method of analysis to analyze identical portions of homogeneous materials to assess the performance characteristics obtained for that method of analysis.
2. Laboratory performance study—a laboratory performance study is an interlaboratory study consisting of one or more assays conducted by a group of laboratories on one or more identical materials, by whatever method is in use in each laboratory, for the purpose of comparing the results of each laboratory with those of other laboratories, with the objective of evaluating or improving laboratory performance.
3. Material performance study—a material performance study is an interlaboratory study in which a group of selected laboratories analyze a candidate reference material by methods judged most likely to provide the least biased estimates of concentration (or of a property) and the smallest associated uncertainty, for the purpose of providing a reference value of the analyte concentration (or property) in the material.
4. Repeatability value (r)—when the mean of the values obtained from two single determinations, performed simultaneously or in rapid succession by the same operator, using the same apparatus under the same conditions for the analysis of the same test sample, lies within the range of the mean values cited in the final report, the difference between the two values obtained should not be greater than the repeatability value (r), which can generally be inferred by linear interpolation of S_r from the report.
5. Reproducibility value (R)—when the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions for the analysis of the same laboratory sample, lie within the range of the mean values cited in the final report, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility value (R), which can generally be inferred by linear interpolation of S_R from the report (see Notes, 5–7).
6. One-way analysis of variance—one-way analysis of variance is the statistical procedure for obtaining the estimates of within-laboratory and between-laboratory variability on a material-by-material basis. Examples of the calculations for the single-level and single-splitlevel designs can be found in ISO 5725:1994.

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COCHRAN AND GRUBBS CRITICAL VALUES

Table 1 presents critical values for the Cochran maximum variance test, 1-tail, at the $P = 2.5\%$ level, expressed as a critical variance ratio; and critical values for the Grubbs tests, at the $P = 2.5\%$ level, expressed as the percent reduction in standard deviation caused by removal of the suspect value(s), where L = number of laboratories for the given material.

Although the table is strictly applicable only to a balanced design (same number of replicates from all laboratories), it can be applied to an unbalanced design without too much error, if there are only a few deviations.

1. Calculation of Cochran outlier test value—compute the within-laboratory variance for each laboratory and divide the largest of these by the sum of all of the variances and multiply by 100. The resulting quotient is the Cochran statistic, which indicates the presence of a removable outlier if this quotient exceeds the critical value listed in Table 1 for the number of replicates and laboratories specified.
2. Calculation of the Grubbs test values—to calculate the single Grubbs test statistic, compute the average for each laboratory and then calculate the standard deviation (SD) of these L averages (designate as the original S). Calculate the SD of the set of averages with the highest average removed (S_H); calculate the SD of the set of averages with the lowest average removed (S_L). Then calculate the percentage decrease in SD as follows:

$$100 \times [1 - (S_L/S)] \text{ and } 100 \times [1 - (S_H/S)]$$

The higher of these two percentage decreases is the single Grubbs statistic, which signals the presence of an outlier to be omitted at the $P = 2.5$ level, 2-tail, if it exceeds the critical value listed in the single-value column, column 2, for the number of laboratory averages used to calculate the originals (see Table 2).

To calculate the paired Grubbs test statistics, calculate the percentage decrease in standard deviation obtained by dropping the two highest averages and also by dropping the two lowest averages, as above. Compare the higher of the percentage changes in standard deviation with the tabular values in column 3 in Table 2 (Grubbs Critical Values) and proceed with (1) or (2): (1) If the tabular value is exceeded, remove the responsible pair. Repeat the cycle, starting at the beginning with the Cochran extreme variance test, the Grubbs extreme value test, and the paired Grubbs extreme value test. (2) If no further values are removed, then calculate the percentage change in standard deviation obtained by dropping both the highest extreme value and the lowest extreme value together, and compare with the tabular values in the last column of Table 2 (Grubbs critical values). If the tabular value is exceeded, remove the highlow pair of averages, and start the cycle again with the Cochran test until no further values are removed In all cases, stop the outlier testing when more than 22.2% (2/9) of the averages are removed.

NOTES

1. The total within-laboratory standard deviation is a more inclusive measure of imprecision than the ISO repeatability standard deviation initial estimate (see *Statistical analysis*, 3 [Initial estimate]). This parameter is the maximum within-laboratory standard deviation to be expected from the performance of a method, at least on different days and preferably with different calibration curves. It includes between-batch as well as within-batch variations. In this respect, it can be considered as a measure of within-laboratory reproducibility. Unless this value lies well within acceptable limits, it cannot be expected that the between-laboratory standard deviation (reproducibility standard deviation, S_R) will be any better. This precision term is not estimated from the minimum collaborative study described in this protocol.
2. The total within-laboratory standard deviation may also be estimated from ruggedness trials that indicate how tightly controlled the experimental factors must be and what their permissible ranges are. These experimentally determined limits should be incorporated into the description of the method.
3. In making statistical calculations from the reported data, the full power of the calculator or computer is to be used, with no rounding or truncating until the final reported mean and standard deviations are achieved. At this point, the standard deviations are rounded to two (2) significant figures, and the mean and relative standard deviations are rounded to accommodate the significant figures of the standard deviations. For example, if $S_R = 0.102$, the mean (x) is reported as 0.147, not as 0.1473 or 0.15, and RSD_R is reported as 8.2% (symbols are defined in the Symbols and Terms section). If standard deviation calculations must be conducted manually in steps with the transfer of intermediate results, the number of significant figures to be retained for squared numbers should be at least 1 plus 2 times the number of figures in the data.
4. The Grubbs tests are to be applied one material at a time to the set of replicate means from all laboratories, and not to individual values from replicated designs, because the distribution of all the values taken together is multimodal, not Gaussian, i.e., their differences from the overall mean for that material are not independent.
5. When the results of the interlaboratory test make it possible, the value of r or R can be indicated as a relative value, i.e., as a percentage of the determined mean value, or as an absolute value.
6. When the final reported result is an average derived from more than a single value, i.e., k is greater than 1, the value of R must be adjusted according to the following formula:

$$R' = [R^2 + r^2 (1 - [^1/_k])]^{1/2}$$

Similar adjustments must be made for replicates constituting the final values for S_R and RSD_r, if these will be the reported parameters used for quality control purposes.

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7. The repeatability value, r, may be interpreted as the amount by which two determinations should agree with each other within a laboratory 95% of the time. The reproducibility value, R, may be interpreted as the amount by which two separate determinations conducted in different laboratories should agree with each other 95% of the time.

REFERENCES

1. Reprinted with permission from *Anal. Chem. 50:*337A (1978), with revisions.
2. *Official Methods of Analysis,* Association of Official Analytical Chemists, 14th edn., 1990, p. XXII.
3. Youden, W. J., *J. Assoc. Off. Agric. Chem. 45:*169 (1962).
4. Egan, H., *Ibid. 60:*260 (1977).
5. Youden, W. J. and E.H. Steiner, in *Statistical Manual of the Association of Official Analytical Chemists,* Association of Official Analytical Chemists, Arlington, VA, 1975.
6. *Guidelines for the Development of Standard Methods by Collaborative Study,* Pocklington, W.D., ed. Laboratory of the Government Chemist Library, Queens Road, Teddington, Middlesex TW11 0LY, England.
7. Youden, W. J., *J. Assoc. Off. Agric. Chem. 46:*55 (1963).
8. Horwitz, W., *Pure Appl. Chem. 60:*855 (1988).
9. Horwitz, W., *Pure Appl. Chem. 67:*331 (1995).
10. Collaborative Study Guidelines, *J. AOAC International 78:*143A (1995).
11. Pocklington, D.W., *Pure Appl. Chem. 62:*149 (1990).
12. Kelly, P.C., *J. Assoc. Off. Anal. Chem. 73:*58 (1990).

Table 1

Critical values for the Cochran maximum variance ratio at the 2.5% (1-tail) rejection level, expressed as the percentage the highest variance from an individual laboratory is of the total variance (sum of variances from all laboratories).

	r = number of replicates per laboratory				
L = Number of laboratories at a given level (concentration)	*r* = 2	*r* = 3	*r* = 4	*r* = 5	*r* = 6
4	94.3	81.0	72.5	65.4	62.5
5	88.6	72.6	64.6	58.1	53.9
6	83.2	65.8	58.3	52.2	47.3
7	78.2	60.2	52.2	47.3	42.3
8	73.6	55.6	47.4	43.0	38.5
9	69.3	51.8	43.3	39.3	35.3
10	65.5	48.6	39.9	36.2	32.6
11	62.2	45.8	37.2	33.6	30.3
12	59.2	43.1	35.0	31.3	28.3
13	56.4	40.5	33.2	29.2	26.5
14	53.8	38.3	31.5	27.3	25.0
15	51.5	36.4	29.9	25.7	23.7
16	49.5	34.7	28.4	24.4	22.0
17	47.8	33.2	27.1	23.3	21.2
18	46.0	31.8	25.9	22.4	20.4
19	44.3	30.5	24.8	21.5	19.5
20	42.8	29.3	23.8	20.7	18.7
21	41.5	28.2	22.9	19.9	18.0
22	40.3	27.2	22.0	19.2	17.3
23	39.1	26.3	21.2	18.5	16.6
24	37.9	25.5	20.5	17.8	16.0
25	36.7	24.8	19.9	17.2	15.5
26	35.5	24.1	19.3	16.6	15.0
27	34.5	23.4	18.7	16.1	14.5
28	33.7	22.7	18.1	15.7	14.1
29	33.1	22.1	17.5	15.3	13.7
30	32.5	21.6	16.9	14.9	13.3
35	29.3	19.5	15.3	12.9	11.6
40	26.0	17.0	13.5	11.6	10.2
50	21.6	14.3	11.4	9.7	8.6

Cochran statistic = (largest individual within laboratory variance)/(sum of all the within-laboratory variances)

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Table 2
Critical values for the Grubbs extreme deviation outlier tests at the 2.5% (2-tail), 1.25% (1-tail) rejection level, expressed as the percent reduction in the standard deviations caused by removal of the suspect value(s) (see text for calculating Grubbs statistics)

L = Number of laboratories at a given level (concentration)	One highest or lowest	Two highest or two lowest	One highest and one lowest
4	86.1	98.9	99.1
5	73.5	90.3	92.7
6	64.0	81.3	84.0
7	57.0	73.1	76.2
8	51.4	66.5	69.6
9	46.8	61.0	64.1
10	42.8	56.4	59.5
11	39.3	52.5	55.5
13	33.8	46.1	49.1
14	31.7	43.5	46.5
15	29.9	41.2	44.1
16	28.3	39.2	42.0
17	26.9	37.4	40.1
18	25.7	35.9	38.4
19	24.6	34.5	36.9
20	23.6	33.2	35.4
21	22.7	31.9	34.0
22	21.9	30.7	32.8
23	21.2	29.7	31.8
24	20.5	28.8	30.8
25	19.8	28.0	29.8
26	19.1	27.1	28.9
27	18.4	26.2	28.1
28	17.8	25.4	27.3
29	17.4	24.7	26.6
30	17.1	24.1	26.0
40	13.3	19.1	20.5
50	11.1	16.2	17.3

The critical values in Table 1 were calculated by R. Albert (October 1993) by computer simulation involving several runs of approximately 7000 cycles each for each value, and then smoothed. Although the table of critical values for the Cochran maximum variance ratio strictly applicable only to a balanced design (same number of replicates from all laboratories) it can be applied to an unbalanced design without too much error, if there are only a few deviations.

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Table 3
Symbols and terms.

Mean (of laboratory averages)	–	
Standard deviations (estimates):		
Repeatability	S_r	
"Pure" between-laboratory	S_L	
Reproducibility	S_R	
Variances ($S_{R2} = S_{L2} + S_{r2}$)	S_2	(with subscripts r, L, R)
Relative standard deviations	RSD	(with subscripts r, L, R)
Maximum tolerable differences (as defined by ISO 5725-1986; Definitions section)		
Repeatability value	r	$(2.8 \times S_r)$
Reproducibility value	R	$(2.8 \times S_R)$
Number of replicates per laboratory	k	(general)
Average number of replicates per laboratory	k	(for a balanced design)
Number of replicates for laboratory i	ki	
Number of laboratories	L	
Number of materials	m	
Total number of values in a given assay	n	(= kL for a balanced design)
Total number of values in a given study	N	(= kLm for an overall balanced design)

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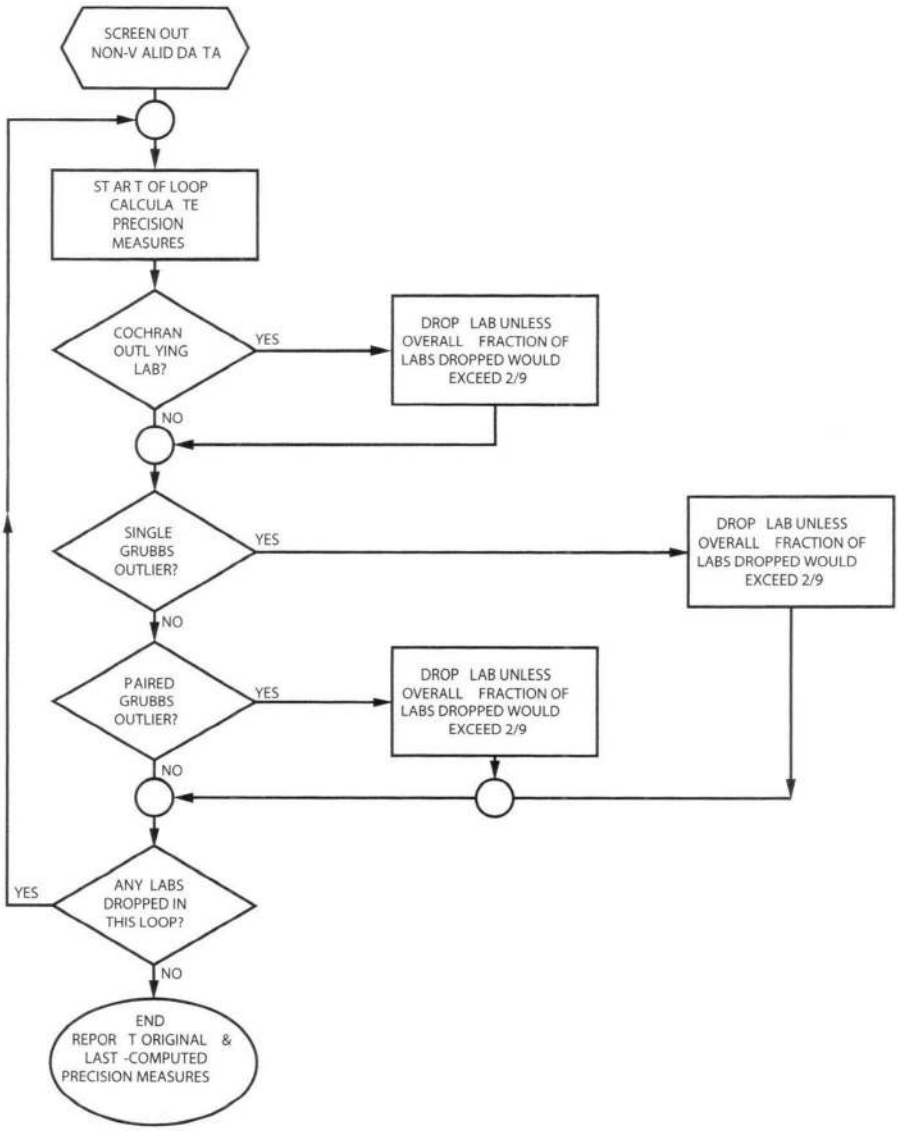
IUPAC-1987 harmonized statistical procedure.



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AOCS

AOCS Criteria M 5-09

Revised 2011

Approved Chemists

DEFINITION

The AOCS Examination Board Approved Chemist is a chemist recognized as proficient in the use of AOCS methodologies utilized in the Laboratory Proficiency Program. The chemist must participate in the Program series in which approval is requested. Approval entitles the chemist to act as a referee for those analyses consistent with the Laboratory Proficiency Program series for which Approved Chemist status is granted.

SCOPE

All chemists/technicians employed by independent and industrial laboratories (there must be no conflict of interest when analyses are performed at industrial laboratories) may elect to pursue initial and continued Approved Chemist status. Approval is granted for one year and the chemist/technician must both apply for approval and demonstrate satisfactory performance on a yearly basis.

PROCEDURE

1. Application—
   (a) Any chemist wishing to be considered for Approved Chemist status must first be able to demonstrate the ability to perform the required analyses. This includes:
   —Having an existing facility in which to perform the appropriate analyses *at the time of application.*
   —Having all apparatus and reagents required by AOCS methodology *at the time of application.*
   —Being willing to allow members of the AOCS Examination Board (or designated representatives) to inspect the laboratory facilities.
   (b) Completion of the application form, check list, and submission of the application and administrative fees (see Notes, 1). Request for applications must be made in writing to: AOCS Headquarters, 2710 S. Boulder Dr., Urbana, IL 61802 USA.
   Applications must be received at AOCS Headquarters by the final date.
2. Categories of approval, required analyses and number of samples
   Applicants may request approval in any of the Laboratory Proficiency Program series. The required analyses on each sample in each series must be completed.
3. Criteria for approval—
   (a) Successful completion of the proficiency series in which approval is sought is determined by meeting the following criteria: A score on an individual series must be either less than 1.4 with no outliers or less than 1.3 with only one outlier.
   —Absolutely no missing sample results. If there are mitigating circumstances surrounding missing samples and results, it will be necessary to send a letter to the AOCS Examination Board chairperson, indicating the circumstances so that special consideration may be given.
   (b) The initial failure to meet the criteria will result in a probationary period of one year. If the problem is not resolved during that year, approval will not be granted the following year.
4. Approval is granted *only* to the individual named in the application, who must also be the same individual who participates in the Laboratory Proficiency Program series in which approval is requested. Approved Chemist status is not transferable from one individual to another. Approval does not necessarily imply automatic transfer when an Approved Chemist moves from one facility to another either within the same organization or with a new organization.
5. Transfers—If the Approved Chemist transfers before the end of a certification period, the approved status changes to approved on probation. In these cases, the AOCS Examination Board chairman must be notified, in writing, regarding the following:
   —Name and address of new location.
   —Explanation of reason for change of location.
   —That the new location has the necessary facilities, apparatus, and reagents to perform the analyses for which the chemist is approved.
   If there are concerns about the new facility to which the Approved Chemist has transferred, it will be necessary for members of the AOCS Examination Board (or designated representatives) to inspect the laboratory facilities before continued approval can be granted.
6. Foreign approval—Individuals in foreign countries wanting to become Approved Chemists must comply with the criteria outlined in this procedure. However, if circumstances warrant special consideration, the need for special consideration will be determined by consensus of the AOCS Examination Board. Special consideration will be given only by consensus of the AOCS Examination Board and only to the extent that the integrity of the Program is not compromised.

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7. Participant results will be made available by June 1 of each year. After June 1, those chemists on probation or disapproved may appeal to the AOCS Examination Board between June 1 and July 1, but no later than July 1. The finalized listing of Approved Chemists will be made public by August 1 (see Notes, 2).

NOTES

1. The application and administrative fees are subject to change at the discretion of the AOCS Examination Board.
2. Approved Chemist listings are published yearly in *INFORM* magazine, the *AOCS Directory*, and on the AOCS home page (www.aocs.org).

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AOCS

AOCS Criteria M 6-09

Revised 2011

Certified Laboratories

DEFINITION

AOCS Certified Laboratories are those laboratories recognized to be proficient in the use of AOCS methodologies for the analysis of soybean meal, as determined by criteria established by the AOCS Examination Board. Certification entitles a laboratory to recognition as a National Oilseed Processors Association (NOPA) referee for soybean meal analyses.

SCOPE

This procedure applies to any laboratory seeking AOCS certification. Both independent and industrial laboratories may apply for certification. There must be no conflict of interest when analyses are performed at industrial laboratories.

PROCEDURE

1. Application
   (a) Any laboratory wishing to be considered for Certified Laboratory status must first be able to demonstrate the ability to perform the required analyses. This includes:
   —Having an existing facility in which to perform the appropriate analyses *at the time of application.*
   —Having all apparatus and reagents required by AOCS methodology *at the time of application.*
   —Being willing to allow members of the AOCS Examination Board (or designated representatives) to inspect the laboratory facilities.
   (b) An AOCS Examination Board Approved Chemist, approved in oilseed cake and meal analyses, must be retained on the laboratory staff.
   (c) Completion of the application form and submission of the application and administrative fee (see Notes, 1). Request for application must be made in writing to: AOCS Headquarters, 2710 S. Boulder Dr., Urbana, IL 61802 USA. Applications must be received at AOCS Headquarters by **May 31,** annually.
2. Certification
   (a) The initial laboratory certification will be based both on the acceptance of a satisfactory application and the Approved Chemist staff position criteria. Subsequent certification will include the Approved Chemist criteria as well as successful participation in the AOCS Examination Board blind check sample program for soybean meal.
   (b) The AOCS Examination Board blind check sample program provides each laboratory with twelve (12) samples of soybean meal. The samples will be distributed in NOPA-approved sample bags with specific instructions requesting a "NOPA Referee" or "AOCS Certified" analysis.
   (c) The twelve (12) blind check samples will be comprised of duplicates of six (6) unique samples distributed at different times. The average and standard deviation of the six unique samples will be used to assess the performance of each individual laboratory. Outliers will be detected and eliminated at 3.0 standard deviation units.
   (d) For individual sample assessment, a single sample is considered deficient if any or all of the laboratory values fall outside ± 2.0 standard deviation units from the grand mean with outliers detected and eliminated. A laboratory will be decertified if six or more samples are found to be deficient at 95%.
   (e) For average laboratory bias assessment, the average bias for each laboratory and sample constituent will be calculated and bias values determined on constituents for all twelve samples. The standard error of the bias will be determined from the pooled standard deviation for all of the laboratories for the twelve samples. Any laboratory which has any average constituent bias outside the 95% confidence limit will be placed on probation. A laboratory which is on probation and has an average constituent bias outside the 99.7% confidence limit will be decertified.
   (f) Laboratory performance is evaluated using both the individual samples and the average constituent bias. The criteria for each evaluation is based on placing a laboratory on probation at the 95% confidence limit and decertifying a laboratory currently on probation at the 99.7% confidence limit.
   (g) A laboratory cannot be decertified without first being placed on probation. Any laboratory that has been decertified can return to the program after one (1) year, but will be certified on probation. If a laboratory is subsequently decertified a second time, it can return to the program after two (2) years. Likewise, after a third decertification, a lab cannot reapply for three (3) years.
   (h) A laboratory can remain on probation indefinitely until either both criteria are less than the 95% confidence limit or until either criteria exceeds the 99.7% confidence limit.
   (i) All new laboratories that comply with the program requirements will be considered certified on probation for the first year of participation.

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(j) Participant results will be made available by July 1 of each year. After July 1, those laboratories on probation or decertified may appeal to the AOCS Examination Board between July 1 and July 15, but no later than July 15. The finalized listing of Certified Laboratories will be made public by September 1 (see Notes, 2).

(k) A laboratory whose AOCS Approved Chemist leaves the employ of the Certified Laboratory will remain certified for a period of up to one year. During this time, they must have a chemist participate in the AOCS Approved Chemist program and become approved in their laboratory.

3. Transferability of laboratory certification—AOCS Laboratory Certification is not transferable. Should an independent laboratory have more than one facility, each individual facility must pursue certification on an independent basis.
4. Foreign approval—Laboratories outside the USA wanting to become certified must comply with the criteria outlined in this procedure. However, if circumstances warrant special consideration, the need for special consideration will be determined by consensus of the AOCS Examination Board. Special consideration will be given only by consensus of the AOCS Examination Board and only to the extent that the integrity of the Program is not compromised.

NOTES

1. The application and administrative fee is subject to change at the discretion of the AOCS Examination Board.
2. Approved Chemist listings are published yearly in the *NOPA Year Book and Trading Rules, INFORM,* and the AOCS web page (www.aocs.org).

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AOCS Analytical Guidelines S 1-64
Reapproved 2009

Methods for Testing Commercial Fatty Acids

SCOPE

This method covers the selection and application of procedures for testing commercial fatty acids from animal, vegetable and marine origin within the scope of each method and the following recommended practices. The test methods included are listed in Table 1.

SAMPLING

Sample material in accordance with AOCS Method Ta 1-09. Fatty acids are frequently solid at room temperature and shall be completely liquefied and blended prior to testing. Temperatures during liquefying of the fatty acids should not exceed 10–15°C above the melting point of the sample.

MOISTURE

The hot plate method AOCS Method Tb 1a-64 will determine moisture and any other materials that are volatile under the conditions of the test. Hydroxylated acids that tend to esterify or highly unsaturated acids that tend to oxidize should not be run by this method.

ACID VALUE

Acid value is a measure of the quantity of free fatty acids and is defined as the mg of potassium hydroxide required to neutralize the fatty acids in 1 g of sample. Acid value determination is useful in establishing the identity and purity of a fatty acid. Determine acid value by AOCS Method Te 1a-64, which is the referee method. Other solvents should be checked out carefully before using for control purposes.

UNSAPONIFIABLE MATTER

The term of unsaponifiable matter includes all of those substances that are not saponified by alkali and are soluble in the ordinary fat solvents. Included are the higher aliphatic alcohols, sterols, pigments, and hydrocarbons. Determine unsaponifiable matter in accordance with AOCS Method Tk 1a-64. Normal fatty acids contain small percentages of unsaponifiable matter, usually less than 2%. When excessive amounts of unsaponifiable matter are found, low quality is indicated.

ASH

The ash in fatty acids is determined by igniting the acids under specified conditions and determining the percentage of noncombustible material. Carry out this procedure in accordance with AOCS Method Tm 1a-64. The ash content of normal fatty acids should be very small.

TITER

Titer is the solidification point of fatty acids under specified conditions. Determine titer in accordance with AOCS Method Tr 1a-64. The titer value can be correlated with the degree of unsaturation of a fatty acid and is useful in determining the identity and purity of a fatty acid.

SAPONIFICATION VALUE

Saponification value is a measure of the alkali-reactive groups in fatty acids and is expressed as the mg of potassium hydroxide that will react with 1 g of sample. The saponification value on a good grade of vegetable fatty acids is normally only 1 or 2 points higher than the acid value as determined in accordance with AOCS Method Te 1a-64. The difference between the saponification value and the acid value is frequently referred to as the ester value. Saponification value can be used to calculate the average molecular weight of the fatty acid. Determine the saponification value in accordance with AOCS Method Tl 1a-64.

UNSATURATION

Iodine value is a convenient test for expressing the degree of unsaturation of a fatty acid. By definition, iodine value is the cg of iodine absorbed per g of sample. Determine the iodine value in accordance with AOCS Method Tg 1a-64, which gives fairly good accuracy and precision when applied to normal fatty acids.

Note—The analyst is cautioned to follow the method exactly as written since small deviations in sample size, reagents, and time of reaction will definitely affect the accuracy and precision of the method.

When Method Tg 1a-64 is applied to fatty acids containing conjugated double bonds such as dehydrated castor acids, an empirical figure is obtained giving only a relative measure of the total unsaturation. When Method Tg 1a-64 is used for dehydrated castor acids, a sample weight of 0.11–0.13 g of fatty acids must be used to get comparative results between laboratories.

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The iodine value of tall oil fatty acids containing appreciable amounts of rosin acids is not a reliable measure of the fatty acid unsaturation because of the unsaturation of the rosin acids.

REFRACTIVE INDEX

Refractive index is not a very useful means of specifying fatty acids. In some instances it may be useful in detecting adulteration. Refractive index will vary with iodine value and is occasionally used to calculate a quick approximation of iodine value. Determine refractive index using AOCS Method Tp 1a-64.

SPECIFIC GRAVITY

Specific gravity is useful in translation of volume to weight and the reverse and must be determined with care. Specific gravity is not a measure of quality and a fatty acid that deviates slightly in specific gravity but conforms otherwise is normally satisfactory. Determine specific gravity using AOCS Method To 1a-64.

Note—Temperature is critical in the determination of specific gravity and must be closely controlled. In reporting specific gravity the temperature must be given.

FLASH POINT AND FIRE POINT

Flash point of fatty acids is normally quite high, unless they contain traces of volatile inflammable material. The flash point is useful for determining that hazardous amounts of solvent are not present. The flash point is determined by AOCS Method Tn 1a-64.

FATTY ACID COMPOSITION

The use of chromatography for the separation of fatty acids by chain length and conjugation has developed very rapidly. AOCS Method Ce 1-62/Ce 1a-13 is applicable to fatty acids. Work done to date shows the method to be simple and accurate with good precision.

COLOR

AOCS Method Td 2a-64 uses the Photometric Index (spectrophotometric color) to characterize the color of commercial fatty acids. It is expressed as 100 times the absorbance at each of two wavelengths, 440 and 550 nm. Gardner color method (Td 1a-64) may also be used. For light colored samples, Pt-Co method (Td 1b-64) may be used.

ROSIN ACID

Fatty acids derived from tall oils will normally contain varying percentages of rosin acids. When the amount of the rosin acids does not exceed 15%, determine their content in accordance with AOCS Method Ts 1a-64. In method Ts 1a-64 the rosin acids are determined by titration, after esterification of the fatty acids with methanol in the presence of sulfuric acid as a catalyst, followed by separation of the acids. This method requires use of an experimentally determined correction factor, dependent upon the amount of rosin acids present.

COLOR CHANGE AFTER HEATING

The color stability of fatty acids when subjected to elevated temperatures is of importance to many commercial users of fatty acids. The conditions under which color stability is determined such as temperature, time of heating, time required to reach temperature and exposure to air all affect final results. Use the Test for Color after Heating of Fatty Acids, AOCS Method Td 3a-64, to measure the color of normal fatty acids after heating. The method is applicable to all normal fatty acids. It has been shown to have good precision within and between laboratories and should be used as a referee method.

APPEARANCE

Observation for appearance of fatty acids should be made on the liquefied sample. Transfer the sample to a Gardner-Holdt viscosity tube as described in the Test for Viscosity of Transparent Liquids, AOCS Method Tq 1a-64, and check for haze, dirt, skins, or other material that might indicate contamination of the sample.

HYDROXYL VALUE

Hydroxyl value is defined as the mg of potassium hydroxide equivalent to the hydroxyl (OH) content of 1 g of sample. Hydroxystearic acid is the only common fatty acid to which this test is applicable, and is determined by AOCS Method Cd 13-60.

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Table 1

Test	AOCS Method
Sampling	Ta 1-09
Moisture	
Hot Plate Method	Tb 1a-64
Acid Value	Te 1a-64
Unsaponifiable Matter	Tk 1a-64
Ash	Tm 1a-64
Titer	Tr 1a-64
Saponification Value	Tl 1a-64
Iodine Value	Tg 1a-64
Refractive Index	Tp 1a-64
Specific Gravity	To 1a-64
Flash Point and Fire Point, Cleveland Open Cup	Tn 1a-64
Fatty Acid Composition	
Gas Chromatography	Ce 1-62/Ce 1a-13
Color	
Photometric Index	Td 2a-64
Gardner Color	Td 1a-64
Platinum-Cobalt Scale	Td 1b-64
Rosin Acid in Fatty Acids	Ts 1a-64
Color Test after Heating	Td 3a-64
Hydroxyl Value (direct titration)	Cd 13-60

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AOCS Analytical Guidelines S 2-64
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Methods for Testing Drying Oils

SCOPE

This method covers the selection and use of procedures for testing drying oils commonly used in paints, varnishes, and related products. The test methods included are listed in Table 1.

SAMPLING

Sample the material in accordance with Sampling of Industrial Oils and Derivatives, AOCS Method Ta 1-09.

PREPARATION OF SAMPLE

Melt the sample if it is not already completely liquid. The temperature during melting should not exceed 10–15°C above the melting point of the sample. Mix the laboratory sample thoroughly by shaking, stirring or pouring from one vessel to another. Take the sample for the individual tests from this thoroughly mixed sample.

ACID VALUE

The acid value of an oil is an indication of the condition of the seed from which the oil is extracted and of the refining to which it has been subjected. It is not useful for the identification of the type of oil. The Acid Value of Fatty Acids, AOCS Method Te 2a-64, is generally most satisfactory as to precision and reproducibility. There is no choice between sodium and potassium hydroxides except personal preference. If the percentage of free fatty acids calculated as oleic is required, the following equation may be used for the transformation:

$$\text{Free fatty acids, \%} = 0.503 \times \text{Acid Value}$$

UNSATURATION

The drying properties of fats and oils are indicated by the amount and nature of unsaturation they contain. The amount is conventionally expressed as the iodine value, cg of iodine absorbed per g of sample (percentage by weight of iodine absorbed). The iodine value is a fairly satisfactory measure of the relative drying time and speed of heat-polymerization among a group of oils of the same type. However, both drying time and heat-polymerization are affected by the kind of distribution of fatty acids in the oil, and these methods are not as useful in comparing oils of different types. The measure of unsaturation is an alternative to the determination of the fatty acids for the identification of natural oils, since each natural oil has its own range of unsaturation values.

Determine the unsaturation of natural drying oils that do not contain conjugated double bonds by the Wijs method as described in the Test for Iodine Value of Drying Oils and Fatty Acids, AOCS Method Tg 1-64, which gives fairly good accuracy and precision. It has largely superseded the Hanus and other methods which tend to give high results. When the Wijs method is applied to oils containing conjugated double bonds, such as tung oil and dehydrated castor oil, an empirical figure is obtained that is indicative of the relative amount of unsaturation present, but is not a measure of the total unsaturation. With careful control of the reaction conditions, however, reproducible and useful results may be obtained. Where the total unsaturation is required, make the determination using a modification of the Rosenmund-Kuhnhenn method (ASTM D1541-60). This method gives an accurate measure of total unsaturation in conjugated oils and is also satisfactory for nonconjugated oil, although somewhat more difficult to run than the Wijs method (AOCS method Tg 1-64). Quantitative hydrogenation will also yield an accurate measure of the total unsaturation. There is no standard satisfactory method for oils containing acetylenic bonds, such as isano oil.

The iodine value is useful for the identification of linseed, soybean, safflower, and similar natural oils. The amount of conjugated diene (or triene) is useful for identifying tung and oiticica oils, as well as dehydrated castor oil, although it is not the only measure that should be used.

Determine the amount of conjugated diene by reaction with maleic anhydride in accordance with the diene value, AOCS Method Th 1a-64, or by spectrophotometric measurement using the Spectrophotometric Determination of the Conjugated Dienoic Acid, AOCS Method Ti 1a-64. The spectrophotometric method is more rapid and subject to less interference but requires equipment that is not always available. The maleic anhydride method is easier to run, but encounters interference from hydroxyl groups, peroxides, and other oxidation products, as well as from certain stearic configurations, which may be encountered in processed oils.

SAPONIFICATION VALUE

The saponification value is, essentially, a measure of the molecular weight of the fatty acid portion of the glyceride, varying inversely with the weight except for certain modified oils. It is not a measure of the quality or identity of the oil. The value is useful for certain calculations in the use of the oil, such as in the manufacture of alkyd resins.

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Determine the saponification value in accordance with the Test for Saponification Value of Drying Oils, AOCS Method Tl 1a-64, which is satisfactory for all normal oils and for many special and synthetic products. As indicated in the method, longer saponification times are required by certain synthetic oils, and some special products may require the use of a higher boiling solvent, such as ethylene glycol, for complete saponification.

Saponification value is not changed significantly by polymerization but increases rapidly with oxidation. A saponification value significantly higher than normal indicates the presence of oxidized or brown oils or else modification with chemicals such as maleic or fumaric acids.

UNSAPONIFIABLE MATTER

The unsaponifiable matter is a measure of the materials present in the oil that are oil-soluble and are not converted to water-soluble soaps by the saponification condition used. A small amount of unsaponifiable matter is characteristic of all natural oils, varying with the extraction and refining conditions. Within the limits of the individual oil specifications, the amount of unsaponifiable matter is no measure of the quality or identity of the oil. An excessive amount of unsaponifiable matter indicates contamination with nonglyceride matter such as mineral oil, hydrocarbon resins, etc.

Determine the unsaponifiable matter in accordance with the Test for Unsaponifiable Matter in Drying Oils and Fatty Acids, AOCS Method Tk 1a-64. Since the exact amount of unsaponifiable matter obtained is governed by the partition coefficient of the matter between the soap solution and the solvent, different results may be expected if some other solvent, such as ethyl ether, is used. A rapid, qualitative test for excessive unsaponifiable matter consists of saponifying a small quantity of the oil and diluting with water. A milky emulsion indicates excess unsaponifiable matter.

ASH

Ash is determined by igniting the oil, under specified conditions, and weighing the noncombustible material. Most natural and processed oils contain small amounts of ash, but the amount is insignificant. Certain synthetic drying oils may contain residual catalyst or other materials, thus giving larger amounts of ash. Although the ash and metal content of boiled oils may be specified, the trend with new and improved driers is toward the specification of drying time, allowing the manufacturer to obtain this in any way desired.

Determine the ash in accordance with the Test for Ash in Drying Oils and Fatty Acids AOCS Method Tm 1a-64.

ACETONE TOLERANCE

The acetone tolerance is a measure of the amount of higher polymer in a heat-bodied oil when no non-fatty material is present. It is only applicable to heat-bodied oils and should only be used to assure uniformity of deliveries. There is no correlation between the acetone tolerance and the usefulness of an oil, but, if acetone tolerance and other properties of an oil are the same as those of an accepted sample, the two probably have been produced by the same technique.

Determine the acetone tolerance in accordance with the Test for Acetone Tolerance of Heat-Bodied Drying Oils, AOCS Method Tt 1a-64. The method consists of adding acetone until a cloudy dispersion persists. Temperature is extremely important and must be controlled very closely. This determination may be made as a "cloud point," measuring the temperature at which cloudiness appears for a given acetone concentration but this is a more difficult technique, experimentally, for this particular solvent-solute combination. Quantities greater than the smallest traces of water in the acetone also affect the result significantly, and the amount must be kept within the prescribed limits.

COLOR

The color of an oil, in bulk, is usually relatively useful in predicting how it will behave in use in comparison with other similar oils. However, since some oils darken on heating or oxidation and others bleach, the color in bulk is rarely helpful in comparing oils of different types.

Determine the color by comparison with standards, either liquid or glass, as described in the Test for Color of Transparent Liquids (Gardner Color Scale), AOCS Method Td 1a-64, which is the fastest method and, in general, is sufficiently accurate. For extremely light-colored oils (Gardner color No. 3 or lower) use the method as described in the Method of Test for Color of Clear Liquids (Platinum-Cobalt Scale) (AOCS Method Td 1b-64). Since this method uses a much thicker layer of oil than Method Td 1a-64, light colors may be judged more precisely. However, because of this thickness difference, there is no accurate correlation between the two methods, and the method specified must be used.

Note—For edible oils, methods based on Lovibond glasses (AOCS Method Cc 13b-45) or rarely, on spectrophotometric measurements (AOCS Method Cc 13c-50) are used. For tallows and certain other inedible oils the FAC Method (AOCS Method Cc 13a-43) is commonly specified. These methods should not be used for drying oils.

In making visual comparisons of the color of sample and standard, careful control of the conditions of illumination and view are necessary if high precision is called for. Care must be taken that the observer has normal color perception.

Standards darker than Gardner color no. 18 are not very useful, since comparison of sample and standard, under the conditions specified, is difficult. When it is necessary to specify the color of very dark oils it is usually more satisfactory to specify the color of the oil diluted with a standard amount of solvent sufficient to bring the color into range of the standard color-measuring methods. The nature of the solvent and the dilution must be specified exactly.

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SPECIFIC GRAVITY

Specific gravity of an oil is a useful measure, since translation from volume to weight, or the reverse, is often required. For this reason, it should be determined with care. Specific gravity is not a measure of the quality of the oil, and an oil that deviates slightly from the specified limits in specific gravity, but otherwise conforms, is usually completely satisfactory. Specific gravity increases with polymerization or oxidation in a regular manner, and for every bodied or blown oil of a given viscosity there is an appropriate specific gravity.

Determine the specific gravity in accordance with the Test for Specific Gravity of Drying Oils, Varnishes and Related Materials at 25/25°C, AOCS Method To 1b-64, which is capable of high precision. Specific gravity is very sensitive to temperature, and the temperature of measurement must be controlled with high precision. If measurements are made at other than the standard temperature or if the value of the specific gravity is required at some temperature other than the standard, the approximate value may be calculated as described in AOCS Method To 1b-64. A "weight per gallon" cup may be used to determine specific gravity; however, it should not be used when accuracy is required.

REFRACTIVE INDEX

Refractive index is a scientifically defined property, and numerous accurate instruments are available for its determination. Since the method used depends upon the instrument, no method for its determination is given. Any one of a large number of instruments operated according to the manufacturer's instructions will give satisfactory results.

Refractive index is not a very useful means of specify ing drying oils. It is useful in detecting adulteration in oils containing substantial amounts of conjugation, such as tung, oiticica, and dehydrated castor oils. Since refractive index varies with iodine value, it can be used as a quick approximation of iodine value. Use AOCS Method Tp 1a-64.

VISCOSITY

Viscosity is the resistance experienced by one portion of a liquid flowing over another portion. It is expressed in poises, the absolute unit, or in stokes, equivalent to poises divided by density. The viscosity of most natural oils is very low, and its specification serves no useful purpose. Tung, oiticica and many processed oils have measurable (by the tube method) viscosities, but in the case of processed oils the viscosity is a result of the method of processing and is in no way related to the merit of the oil.

Determine the viscosity of drying oils in accordance with the Test for Viscosity of Transparent Liquids by Bubble Time Method, AOCS Method Tq 1a-64. This method describes a means for measuring the travel of an air bubble in a cylindrical tube either by timing or by comparison with standard tubes of known viscosity. The results are very close to the true viscosity in stokes, but are not exactly correct, so that viscosity determined by this method should be reported in "approximate stokes" or "bubble seconds." More precise results may be obtained for viscosities of less than 4 sec by comparison with standards, and for more viscous oils by timing. If higher precision is required, use a capillary viscometer such as one of those described in the Test for Kinematic Viscosity, ASTM Method D 445. Results obtained in poises should be divided by the density to give stokes.

When tube methods are used, results are affected by the size and shape of the air bubble. Therefore, if precise results are required, tubes conforming exactly to the standard must be used, and the air bubble must be adjusted to the correct size. A difference in tube diameter of 0.05 mm will result in an error of approximately 2%. All viscosity measurements are very sensitive to temperature and extremely close temperature control (±0.1°C) is necessary for precise results.

DRY PROPERTIES

Since drying oils, by definition, set to a solid film, the time required for this to take place is an important property of all such oils. Unfortunately the time required is greatly affected by a number of variables including temperature, drier content, light, humidity, film thickness, air circulation, etc. All these must be controlled with great care to assure reproducible results. Furthermore, results obtained under one set of conditions do not, necessarily, allow prediction of results which might be obtained under other conditions.

Empirical measures of drying properties are useful in comparing one oil with others of the same general type, but must be used cautiously otherwise. Because of the effect of pigmentation and other variables, it is difficult to predict the drying time of a paint from the drying time of the oil used in its manufacture.

Select a set of generally acceptable conditions (except for drier content which must be specified). These conditions may, of course, be varied as required, but any variation must be outlined carefully if agreement is to be obtained. Numerous other methods of measuring drying time have been proposed. Most of these use some mechanical device to determine the end point. However, many of these devices interfere, in an unpredictable manner, with the circulation of air, the amount of oxygen, and the amount of light available to the film, so that results are likely to be erratic. The most widely used device, the Sanderson machine, which drops sand on the film, gives results between the "set-to-touch" and "dry" times.

Since the drying of oils is a continuous process that goes on indefinitely, it is difficult to select sharp end points that may be measured precisely. The "set-to-touch" point, where the internal cohesion of the film exceeds its adhesion to the finger, is probably the sharpest. This point coincides very closely with the point where the film changes from a liquid to a gel. The "dry time" is more subjective, and it is difficult to get close agreement between laboratories, especially for oils with relatively long drying times.

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FLASH POINT

The flash point of a liquid is the temperature indicated on the thermometer when flame application produces a distinct flash. It is an indication of the temperature at which inflammable gases are produced by the liquid.

Most natural and synthetic drying oils have very high flash points of about 260°C (500°F), unless they contain traces of volatile, flammable materials. If the contaminating solvent is known, it is possible to set up a relationship between the solvent content and the flash point.

Flash point of vegetable oils is helpful in determining that no hazardous amounts of solvents have been left in solvent-extracted oils or that the oils have been contaminated with such solvents. If no inflammable solvents are present, the flash point of natural oils is meaningless as far as specifications are concerned.

Determine the flash point in accordance with the Test for Flash Point of Drying Oils, AOCS Method Tn 1a-64, which uses the Cleveland Open Cup. The exact flash point obtained is empirical, depending upon the rate of heating and other factors set forth in the method. These must be followed carefully if reasonable precision is to be obtained.

NONVOLATILES (SOLIDS)

Determine the nonvolatiles (solids) content of solutions of drying oils in organic solvents in accordance with the Test for Nonvolatiles, AOCS Method Tc 1a-64.

CLARITY

This requirement provides for the quick rejection of natural oils which are obviously contaminated by solid matter, such as dirt or water in excess of the solubility limit. Most natural oils contain some saturated glyceride which may crystallize out at low temperatures, giving a cloudy appearance. If the cloudiness disappears on warming, it is probably due to the saturated glyceride and should be disregarded.

Some processed oils are naturally hazy, as a result of the processing methods used, and a clarity requirement should not be included in specifications for processed oils unless it is known that properly processed oils of the type desired will meet the requirements.

Table 1

Test	AOCS Method
Sampling	Ta 1-09
Acid Value	Te 2a-64
Unsaturation	
Iodine Value	Tg 1-64
Diene Value	Th 1a-64
Dienoic Acids	Ti 1a-64
Saponification Value	Tl 1a-64
Unsaponifiable Matter	Tk 1a-64
Ash	Tm 1a-64
Acetone Tolerance Test	Tt 1a-64
Color	
Gardner	Td 1a-64
Platinum-Cobalt Scale	Td 1b-64
Specific Gravity	To 1b-64
Refractive Index	Tp 1a-64
Viscosity, Bubble Time Method	Tq 1a-64
Dry Time	No Method
Flash Point and Fire Point, Cleveland Open Cup	Tn 1a-64
Nonvolatiles (Solids)	Tc 1a-64
Clarity	No Method

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AOCS Analytical Guidelines S 3-64

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Methods for the Testing of Epoxidized Oils

SCOPE

These methods are applicable to epoxidized fatty materials and epoxy compounds in general. The test methods included are listed in Table 1.

SAMPLING

While sampling procedures have not been studied, the procedures listed in the general methods section should be adequate. Sample material in accordance with AOCS Method Ta 1-09.

PREPARATION OF TEST SAMPLE

Data on epoxidized oils are obtained from tests on materials which have been subjected to a drying treatment. Solvents must also be removed prior to analysis.

ACID VALUE

The acid value of an epoxidized oil is a measure of the free fatty acids present. Since acetic and formic acid are used in the manufacture of epoxidized oils, trace amounts of these acids may also be present. The acid value of an epoxidized oil is defined as the mg of potassium hydroxide necessary to neutralize 1 g of sample. AOCS Method Te 2a-64 is used to determine the acid value.

COLOR

The color of epoxidized oils may be measured by the Gardner color system. AOCS Method Td 1a-64, Gardner Color, is rapid and generally sufficiently accurate for determining the color level of epoxidized oils. When more precise determinations are required on the very light colored materials, the Platinum-Cobalt Scale, AOCS Method Td 1b-64, may be used.

HYDROXYL VALUE

The hydroxyl value of epoxidized oils measures the amount of side-reactions which occurred during preparation of the epoxidized oil. The particular side-reaction involved is the cleavage of the epoxy groups to form hydroxylated derivatives. AOCS Method Tx 1a-66 is suitable for measuring the hydroxyl value of epoxidized oils.

OXIRANE OXYGEN

The epoxidized oils are useful as plasticizer-stabilizers for polyvinyl chloride resins because of the presence of the oxirane oxygen function. Other unique properties of the epoxidized oils as compared with the unepoxidized precursor can also be attributed to the epoxy oxygen group. The amount of epoxy oxygen in epoxidized oils is indicated by the oxirane oxygen content. This is determined by the AOCS Method Cd 9-57. The result is expressed as weight percent oxirane oxygen based on epoxidized oil. The method comprises a direct titration of epoxy oxygen groups with hydrogen bromide.

SAPONIFICATION VALUE

Saponification value is not a measure of quality or identity of an epoxidized oil although it may be useful for certain calculations in the use of the oil. If the saponification value of epoxidized oils is required it may be determined by AOCS Method Tl 1a-64.

SPECIFIC GRAVITY

The specific gravity is useful for conversion between weight and volume. Determine this property by AOCS Method To 1b-64. Specific gravity is affected by temperature and the temperature at which the measurement is made must be closely controlled and always be reported. Specific gravity is not a measure of quality and oils deviating slightly in specific gravity but otherwise conforming are usually completely satisfactory.

UNSATURATION

The degree of unsaturation of epoxidized oils is usually an important specification. The amount of unsaturation is conventionally expressed as the iodine value, cg of iodine absorbed per g of sample. The iodine value of epoxidized oils is determined by AOCS Method Tg 1-64 using a 10 g sample.

VISCOSITY

No method has been considered for reporting this property of epoxidized oils. However, AOCS Method Tq 1a-64 is applicable in the majority of cases.

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Table 1

Test	AOCS Method
Sampling	Ta 1-09
Acid Value	Te 2a-64
Color	
Gardner	Td 1a-64
Platinum-Cobalt Scale	Td 1b-64
Hydroxyl Value	Tx 1a-66
Oxirane Oxygen	Cd 9-57
Saponification Value	Tl 1a-64
Specific Gravity	To 1b-64
Unsaturation (Iodine, Value)	Tg 1-64
Viscosity, Bubble Time Method	Tq 1a-64

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Methods for Testing Fatty Amines

SCOPE

Fatty amines are defined as industrial products containing essentially RNH_2, R_2NH, and R_3N, where R is a fatty chain derived from animal, coco, cotton, palm, soybean oil, tall oil, and fats. The methods in this section have been tested on fatty primary amines, fatty secondary amines and fatty tertiary amines. Use of these methods has also been extended to other classes of fatty amines, such as methylated fatty amines. Some of the methods, however, have been found not to be applicable to amidoamines and short chain polyamines. The test methods included are listed in Table 1.

SAMPLING

While sampling procedures have not been studied, the procedures listed in the general methods section should be adequate. These products will not stratify while completely liquid, but near their freezing point, the more saturated components may precipitate first. Fatty amines will absorb carbon dioxide to form carbonates and will absorb water from the air. Samples held at higher temperatures will discolor and degrade. In sampling and handling, do not heat higher nor longer than is necessary to obtain a uniform sample. Sample material in accordance with AOCS Method Ta 1-09.

Caution—Fatty amines are strong organic alkalies which can damage, irritate, or sensitize body tissues. Flush from body surfaces and clothing immediately upon contact, neutralize with 4% acetic acid and flush with water again.

COLOR

The Platinum-Cobalt Scale system is specified for determining color of very light products in the range of Platinum-Cobalt Scale 0-500. The Gardner system is used for reporting darker colors from Gardner 1-18. The two systems overlap to the extent that Platinum-Cobalt Scale 500 is about a Gardner 2 or 3. Determine Platinum-Cobalt Scale Color by AOCS Method Td 1b-64, determine Gardner Color by AOCS Method Td 1a-64.

MOISTURE

Water is determined by reaction with an excess of Karl Fischer reagent, which is then back-titrated with a water-methanol solution. The usual direct titration with Karl Fischer reagent results in higher values for these products. Determine Moisture by AOCS Method Ca 2e-84.

AMINE VALUE

Total, primary, secondary, and tertiary amine values are the mg of potassium hydroxide equivalent to the total, primary, secondary, and tertiary amine basicities in 1 g of sample. These empirical values may be converted to % Total, % Primary, % Secondary, and % Tertiary amines if the average molecular weights of these components in the sample are known, or by AOCS Method Tf 3a-64. Determine amine values by AOCS Method Tf 1a-64 and Tf 2a-64 (Potentiometric), or by AOCS Method Tf 1b-64 and Tf 2b-64 (Indicator).

IODINE VALUE-MODIFIED WIJS

Iodine value is a measure of the unsaturation of the alkyl groups, defined as the cg of iodine absorbed per g of sample. In this modification of the standard Wijs method, acetic acid is used as the solvent and mercuric acetate as a catalyst. Determine Iodine Value by AOCS Method Tg 2a-64.

NONAMINES

The nonamines in these materials are generally fatty amides, substituted fatty amides, fatty nitriles, fatty alcohols, and unsaponifiables from the fatty acids from which the amines were produced. Nonamines are determined by dissolving the sample in solvent, passing through an ion exchange column which will absorb the amines, evaporating the solvent, and weighing the residue. Determine Nonamine by AOCS Method Tw 1a-64.

PRIMARY, SECONDARY, TERTIARY AMINES

The primary, secondary, and tertiary amine contents of a sample can be calculated from the primary, secondary, and tertiary amine value, and its nonamine content if:

1. The sample contains only fatty primary amines, fatty secondary amines, and fatty tertiary amines and nonamines.
2. The average lengths of the alkyl chains are the same for the fatty primary amines, fatty secondary amines, and fatty tertiary amines. This is generally true for technical grades of coco, tallow, and other types of fatty amines. It is less true for distilled

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grades of fatty amines. It will not work for mixtures such as a blend of primary coco amines with secondary ditallow amines.

Calculate the primary, secondary, and tertiary amine contents by AOCS Method Tf 3a-64.

Table 1

Test	AOCS Method
Sampling	Ta 1-09
Color	
Gardner	Td 1a-64
Platinum-Cobalt Scale	Td 1b-64
Moisture	Ca 2e-84
Amine Value-Total	
Potentiometric	Tf 1a-64
Indicator	Tf 1b-64
Amine Values-Primary, Secondary, and Tertiary	
Potentiometric	Tf 2a-64
Indicator	Tf 2b-64
Iodine Value—Modified Wijs	Tg 2a-64
Nonamine	Tw 1a-64
Primary, Secondary, Tertiary Amines	Tf 3a-64

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Methods for Testing Fatty Amidoamines

SCOPE

Fatty amidoamines are defined as industrial products with amine values generally over 50, derived by reacting fatty acids with an excess of polyamines, which may or may not contain other functional groups such as hydroxyl. Varying proportions of the free amine groups in the product may be converted to the imidazoline form. The methods in this section have been tested on such products as N-oleylamido ethyl ethanolamine, 1-hydroxy-2-heptadecenyl imidazoline, and N (tallowyl) amido ethyl ethanolamine. The test methods included are listed in Table 1.

SAMPLING

While sampling procedures have not been studied, the procedures listed in the general methods should be adequate. These products will not stratify while completely liquid, but near the freezing point, the more saturated components may precipitate out first. Fatty amidoamines will absorb atmospheric water which then may hydrolyze imidazoline components back to the amidoamine form. Sample material in accordance with AOCS Method Ta 1–09.

Caution—Fatty amidoamines with high amine values are strong organic alkalies which can damage, irritate or sensitize body tissues. Flush from body surfaces and clothing immediately upon contact, neutralize with 4% acetic acid, flush with water again.

MOISTURE

Water is determined by reaction with an excess of Karl Fischer reagent, which is then back-titrated with watermethanol solution. The usual direct titration with Karl Fischer reagent sometimes results in high values for these products. Determine the % moisture by AOCS Method Ca 2e–84

COLOR: GARDNER

The Gardner color system was chosen as best suited for reporting the colors of these products. Determine the color by AOCS Method Td 1a–64.

AMINE VALUE—TOTAL

The total amine value is the mg of potassium hydroxide equivalent to the basicity in g of sample, and normally is a measure of the free amine groups in the sample. Determine the Amine value by AOCS Method Tf 1a–64 and Tf 2a–64.

IODINE VALUE

Iodine value is a measure of the unsaturation of the alkyl groups, and is defined as the cg of iodine absorbed per g of sample. In this modification of the standard Wijs method, acetic acid is used as the solvent and mercuric acetate as a catalyst. Determine the iodine value by AOCS Method Tg 2a–64.

NONAMINE

The nonamines in these materials are generally fatty amides, substituted fatty amides, fatty nitriles, fatty alcohols, and unsaponifiables from the fatty acids from which the amines were produced. Nonamines are determined by dissolving the sample in solvent, passing through an ion exchange column which will absorb the amines, evaporating the solvent from the nonamines, and weighing. Determine Nonamine by AOCS Method Tw 1a–64.

Table 1

Test	AOCS Method
Sampling	Ta 1–09
Moisture	Ca 2e–84
Color:	
Gardner	Td 1a–64
Amine Value—Total Potentiometric	Tf 1a–64
Amine Values – Primary, Secondary, and Tertiary Potentiometric	Tf 2a-64
Iodine Value—Modified Wijs	Tg 2a–64
Nonamine	Tw 1a–64

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Methods for Testing Fatty Quaternary Ammonium Chlorides

SCOPE

Fatty quaternary ammonium chlorides are defined as industrial products containing essentially $R_1R_2R_3R_4NCl$, usually in solvents, where $R_1R_2R_3R_4$ may be short alkyl or fatty chains derived from animal, coconut, cotton, palm, soy and tall oils, and fats. The methods in this section have been tested on trimethyl fatty and dimethyl difatty quaternary ammonium chlorides. The test methods included are listed in Table 1.

SAMPLING

While sampling procedures have not been studied, the procedures listed in the general methods should be adequate. These products will not normally stratify while completely liquid, but near the freezing point, some components may separate out. The solidified material may also be non-uniform. Industrial products normally contain volatile solvents. Avoid losing these components during sampling and handling. Prolonged storage at elevated temperatures will discolor and degrade. Sample material in accordance with AOCS Method Ta 1–09.

COLOR

The Platinum-Cobalt Scale system is specified for determining color of very light color products in the range of Platinum-Cobalt Scale 0-500. The Gardner system is used for reporting darker colors. The two systems overlap to the extent that Platinum-Cobalt Scale 500 is about a Gardner 2 or 3. Determine Platinum-Cobalt Scale color by AOCS Method Td-1b, determine Gardner color by AOCS Method Td 1a–64.

MOISTURE

Water is determined by reaction with an excess of Karl Fischer reagent, which is then back-titrated with water-methanol solution. The usual direct titration with Karl Fischer reagent results in high values for these products. Determine moisture by AOCS Method Tb Ca 2e–84.

ACID VALUE AND AMINE VALUE

Acid Value is the mg of potassium hydroxide needed to neutralize 1 g of sample, and is usually due to amine hydrochloride. Amine Value is the mg of potassium hydroxide equivalent to the amine basicity in 1 g of sample, and is usually due to fatty amine. Most industrial quaternaries contain a small amount of methyl chloride, slow hydrolysis of which causes a gradual increase in acid value, a gradual decrease in amine value. Acid value and amine value can be converted to % Fatty Amine Hydrochloride and % Fatty Amine if the Average Molecular Weights of these components are known for the sample. Determine the acid value and the amine value by AOCS Method Te 3a–64.

ASH

Ash in quaternaries is essentially sodium chloride, unless free caustic is found in the Acid Value and Amine Value procedure. Determine the ash by AOCS Method Tm 2a–64.

FLASH POINT

Flash points are run to determine if the product should be classified "Flammable" under ICC regulations. The method listed is not suitable for high flash point quaternaries. Determine the flash point by AOCS Method Tn 2a–84.

IODINE VALUE

Iodine value is a measure of the unsaturation of the alkyl group, defined as the cg of iodine absorbed per g of sample. In this modified Wijs method, chloroform is used as the solvent and sodium lauryl sulfate is added to keep the free iodine in the aqueous phase. Determine the iodine value by AOCS Method Tg 3a–64.

AVERAGE MOLECULAR WEIGHT

The average molecular weight of an industrial product will vary from lot to lot and from producer to producer because of small variations in the alkyl groups. Where this variation is important, the average molecular weight of the quaternary can be calculated by titrating the quaternary with perchloric acid, determining its nonvolatile content and correcting for amine, amine hydrochloride and salt (% Ash). Determine the average molecular weight by AOCS Method Tv 1a–64.

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PH

pH is dependent upon the amines and amine hydrochlorides present in the material. Most industrial quaternaries will exhibit a slow drop in pH with time because of hydrolysis of methyl chloride dissolved in the product. Determine the pH by AOCS Method Tu 1a–64.

NONVOLATILES (SOLIDS)

Industrial quaternaries normally contain quaternary compounds, water, alcohol, and minor amounts of salt, amine, and amine hydrochloride. The simplest method of determining the quaternary compounds content of a sample is to determine its % nonvolatiles, which for most purposes is equivalent to % quaternary compounds. Determine the nonvolatiles (solids) by AOCS Method Tc 2a–64.

Table 1

Test	AOCS Method
Sampling	Ta 1–09
Color:	
Gardner	Td 1a–64
Platinum-Cobalt Scale	Td 1b–64
Moisture	Ca 2e–84
Acid Value and Amine Value	Te 3a–64
Ash	Tm 2a–64
Flash Point and fire points	Tn 2a–84
Iodine Value—Modified Wijs	Tg 3a–64
Average Molecular Weight	Tv 1a–64
pH	Tu 1a–64
Nonvolatiles (Solids)	Tc 2a–64

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Methods for Testing Fatty Diamines

SCOPE

Fatty diamines are defined as industrial products, the main component of which is $RNHCH_2CH_2CH_2NH_2$, where R is a fatty chain derived from animal, coco, cotton, soya and tall oils, and fats. Other components may be RNH_2, R_2NH, R_3N, $RN(CH_2CH_2CH_2NH_2)_2$, CH_3-CH_2-CH_2-NH_2, and small percentages of nonamines such as fatty nitriles, fatty alcohols, fatty amides, substituted fatty amides, hydrocarbons, and $RNHCH_2CH_2CH_2CN$. The test methods adopted to date are shown below in Table 1.

SAMPLING

While sampling procedures have not been studied, the procedures listed in the general methods should be adequate. These products will not stratify while completely liquid, but near the freezing point, the more saturated components may titer out first. Fatty diamines will absorb carbon dioxide from the air to form carbonates and will absorb water from the air. Samples held at higher temperatures will discolor and degrade. In sampling and handling, do not heat higher or longer than is necessary to obtain a uniform sample. Sample material in accordance with AOCS Method Ta 1-09.

Caution—Fatty amidoamines with high amine values are strong organic alkalies which can damage, irritate, or sensitize body tissues. Flush from body surfaces and clothing immediately upon contact, neutralize with 4% acetic acid, flush with water again.

COLOR

Gardner method Td 1a-64 was chosen as the best suited for reporting the colors of these products.

MOISTURE

Water is determined by reaction with an excess of Karl Fischer reagent, which is then back-titrated with water-methanol solution. The usual direct titration with Karl Fischer reagent results in high values for these products. Determine moisture by AOCS Method Ca 2e-84.

AMINE VALUE

Total, primary, secondary and tertiary amine values are the mg of potassium hydroxide equivalent to the total, primary, secondary, and tertiary amine basicities in 1 g of sample. Since some of the components contain both primary and secondary amine groups, or secondary and tertiary amine groups, and since more than one component can have a primary, or a secondary or a tertiary amine group, it is impossible to calculate compositions from amine value without determining by another method what some of the components are. The best possibility for determining compositions seems to be a gas chromatographic method. Determine total amine value by AOCS Method Tf 1a-64 and primary, secondary and tertiary amine values by AOCS Method Tf 2a-64. Both methods are potentiometric.

IODINE VALUE

Iodine value is a measure of the unsaturation of the alkyl groups, defined as the cg of iodine absorbed per g of sample. In this modification of the standard Wijs method, acetic acid is used as the solvent and mercuric acetate as a catalyst. Determine Iodine Value by AOCS Method Tg 2a-64.

NONAMINE

The nonamines in these materials are generally fatty amides, substituted fatty amides, fatty nitriles, fatty alcohols, and unsaponifiables from the fatty acids from which the amines were produced. Nonamines are determined by dissolving the sample in solvent, passing through an ion exchange column which will absorb the amines, evaporating the solvent from the nonamines and weighing. Determine Nonamine by AOCS Method Tw 1a-64.

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Table 1

Test	AOCS Method
Sampling	Ta 1-09
Color:	
Gardner	Td 1a-64
Moisture	Ca 2e-84
Amine Value—Total Potentiometric	Tf 1a-64
Amine Value—Primary, Secondary, Tertiary—Potentiometric	Tf 2a-64
Iodine Value—Modified Wijs	Tg 2a-64
Nonamine	Tw 1a-64

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Methods for Testing Polymerized Fatty Acids

SCOPE

Polymerized fatty acids are defined as polycarboxylic acids produced by polymerizing fatty acids from animal or vegetable fats and oils, in either an ester or free acid state, by means of heat alone or catalytically. The test methods included are listed in Table 1.

SAMPLING

While sampling procedures have not been studied, the procedures listed in the general methods should be adequate. Some grades of these acids are color sensitive to heat and oxygen, particularly if trace amounts of heavy metals are present. Sample material in accordance with AOCS Method Ta 1–09.

ACID VALUE

Acid value is defined as the mg of potassium hydroxide needed to neutralize 1 g of sample. It is a measure of the free acids present in the sample. If the average molecular weight of the acids is known, the percentage of free acids present can be calculated. Determine the acid value by AOCS Method Te 1a–64.

SAPONIFICATION VALUE

Saponification value is defined as the mg of potassium hydroxide which will react with 1 g of sample. It is a measure of free acids, esters and other alkali-reactive groups in the sample. Determine the saponification value by AOCS Method Tl 1a–64.

UNSAPONIFIABLES

Unsaponifiables are those components which will not react with potassium hydroxide to form water-soluble materials. They include hydrocarbons, fatty alcohols, and sterols. Determine the unsaponifible matter by AOCS Method Tk 1a–64.

COLOR, GARDNER

The Gardner color system was chosen as best suited for reporting the colors of these products. Determine the color by AOCS Method Td 1a–64.

MOISTURE

Water is determined by the Karl Fischer method, in which water is reacted with Karl Fischer reagent. Determine the Moisture by AOCS Method Ca 2e–84

UNSATURATION

Standard methods of determining unsaturation, such as halogen addition, high-pressure hydrogenation or ozone absorption, will not give satisfactory results on these compounds. For example, a Wijs iodine value will give erroneously high values because of substitution reactions which occur.

TITER

Most samples will not give sharp, reproducible titer values because of the presence of polymeric acids.

COMPOSITION

The percentage of mono-, di-, and polycarboxylic acids are obtained by high pressure liquid chromatography method. This method as a recommended practice is found as Tf 5-91.

Table 1

Test	AOCS Method
Sampling	Ta 1–09
Acid Value	Te 1a–64
Saponification Value	Tl 1a–64
Unsaponifiables	Tk 1a–64
Color:	
Gardner	Td 1a–64
Moisture	Ce 2e–84
Unsaturation	No Method
Titer	No Method
Composition	Tf 5–91

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AOCS Official Method Ta 1-09

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Sampling of Industrial Oils and Derivatives

DEFINITION

This method covers the procedure for obtaining representative samples of industrial oils and derivatives from various types of containers.

SCOPE

Applicable to certain industrial oils and derivatives that are in either a liquid or solid state when shipped. For a specific class or product, refer to the AOCS methods for testing that class. Incorporates Ta 1-64, Ta 1a-64, Ta 16-64, Ta 1c-64, and Ta 1d-64.

SIZE AND NUMBER OF TEST SAMPLES

1. The general procedure is to draw a number of portions from the bulk quantity or a number of portions from all or several packages, composite these, mix thoroughly, and distribute representative portions into suitably sized airtight containers for the laboratory sample. Thus the number of samples to prepare depends on the circumstances.
2. A gross sample in the proportion of not less than 9 kg per 27,300 kg cargo is required of all tank cars.
3. A gross sample in the proportion of not less than 9 kg for each 100 drums or equivalent quantity is required when drums and other packages are sampled.
4. When sampling drums and packages, each container is to be sampled, unless by agreement of parties concerned, in which case the schedule in Table 1 may be used.
5. In the case of all bulk shipments, whether in drums, tank cars, or tank wagons, the minimum size for each laboratory sample is 1 L.

TEST SAMPLE CONTAINERS

1. Test sample containers should have closely fitting covers and be made of glass, stainless steel, aluminum, enameled ware, or tin-plated steel.

GENERAL SAMPLING PROCEDURE

1. Sampling of tank cars and tank wagons—
   (a) Core sampling—the core sampler is an aluminum or stainless steel tube approximately 3 m long and approximately 5 cm in diameter. One end of the sampler is fitted with a tight valve which allows an unrestricted opening 5 cm in diameter when fully opened and is free from leaks when closed. The valve is opened and closed by means of a rod from the top of the sampler.

   Procedure for core sampling—

   (1) Open the slide valve and insert into the tank at such a rate that the level of material inside the tube is always approximately the same level outside the tube. This is necessary in order not to have the tube only partially filled when it reaches the bottom of the tank, or the balance of the sample will be from the bottom, and therefore not representative.
   (2) Close the slide valve when the tube has reached the bottom of the tank and withdraw the tube.
   (3) Empty the contents of the tube by opening the slide valve and allowing the contents to drain into a suitable container.

   (b) Petcock sampling—If free water or suspended material is present, a representative sample should be taken by means of a petcock sampler (pumping line sample). If a petcock sample is required, the shipper should be notified before unloading so that an agreement for settlement may be made.

   The petcock sampler is at a minimum a 1 cm petcock inserted in a vertical section of the pumping line and so arranged that the discharge may be collected in a suitable container.

   Note—A solenoid-actuated valve controlled by an electric timer or a valve operated by means of a spring lever and cam shaft is sometimes preferred instead of a petcock.

   Procedure for petcock sampling—

   (1) Adjust petcock so that a continuous stream will flow without dripping during the entire pumping period.
   Caution—Do not collect sample while line is being steamed out before or after pumping.
   (2) Collect samples in a clean, dry container.
   (3) Mix contents thoroughly after initial sample has been taken and immediately transfer to smaller container for delivery to the laboratory.

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2. Sampling of drums
   (a) Sampling tube—The sampling tube is glass with a 1.5 cm bore. One end is constricted by a short taper to avoid leakage, while the other end is constricted sufficiently to act as a finger valve. The sampling tube (drum thief) may be purchased from Fisher Scientific Co.
   (b) Procedure for drum sampling—
      (1) Mix contents thoroughly by rolling the drum to and fro. Contents of a drum must be thoroughly mixed before sampling since water and sediment will settle to the bottom of the drum.
      (2) Insert the tube in the top of the drum so that the level inside the tube is approximately the same as the level outside the tube.
      (3) Place finger over the finger valve and withdraw the tube.
      (4) Empty the contents of the tube into suitable containers by releasing the finger from the valve.
3. Sampling of storage tanks
   (a) Bomb sampler—The Bacon bomb sampler (average model) is a tube made of fatty acid resistant metal ca. 7 cm diameter and ca. 25 cm length. It has a threaded cover plate which is removable and a weighted bomb-shaped nose to keep the sampler in an upright position while sampling. A 1 cm tube is threaded through the nose of the sampler from the inside so that the end of the tube is flush with the outside of the nose. The other end projects inward to ca. 1.5 cm from the inside of the cover plate. As the liquid enters the tube and is trapped by the sampler, the air is displaced through a hole in the cover plate. A needle valve in the cover plate permits control of the filling speed by throttling the escaping air. The Bacon bomb sampler (average model) may be purchased from Precision Scientific Company, Chicago, IL USA.
   (b) Procedure for sampling storage tanks—
      (1) Agitate thoroughly if possible.
      (2) Arrange cord or chain so that the sampler may be lowered into the liquid at a uniform rate.
      (3) Adjust the needle valve so that the sampler will not be quite full when it is withdrawn. If, when the cover plate is removed, the sampler is full, the sample should be discarded, the needle valve adjusted, and a new sample taken.
      (4) Lower sampler into the liquid and withdraw at a uniform rate.
      (5) Remove the cover plate and transfer the sample to a suitable container.
4. Sampling of solids in cartons and bags
   (a) Procedure for sampling—
      (1) Take grab samples of uniform and proportional size from packages at random, the number of packages being sampled conforming to the number specified in Table 1.
      (2) Mix sampled material thoroughly before distributing into laboratory containers.

SPECIAL CONSIDERATIONS FOR INDIVIDUAL PRODUCT TYPES

1. Commercial Fatty Acids (formerly Ta 1a-64)

   Caution—Metals such as copper, bronze, or brass should not be allowed to come in contact with the sample. Cover liners must also be resistant to fatty acids. The contests should be completely liquid, if necessary, heat with indirect steam or a suitable heater in case of drums. Fatty acids should not be heated more than is necessary to completely liquefy, otherwise discoloration may occur.
2. Epoxidized Oils (formerly Ta 1b-64)

   Caution—Make certain that the contents are completely liquid and warm, but do not overheat. Mix thoroughly before sampling the material.
3. Fatty Acid Amines, Fatty Amidoamines, Fatty Quaternary Ammonium Chlorides, and Fatty Diamines (formerly Ta 1c-64)

   Caution—These compounds must be protected from the air (carbon dioxide and/or water) and the sample containers are to be sealed immediately and tightly. The same precautions apply to fatty quaternary ammonium chlorides, but in this case, it is to prevent the loss of a volatile solvent when present. Fatty amines, fatty diamines, and fatty amidoamines with high amine values are strong, organic alkalis and the necessary safety devices and extreme care should be used when handling these materials. Materials must be completely liquid before sampling. Warm if necessary, but do not overheat. Mix thoroughly before sampling the material. Prolonged storage at elevated temperature will not only discolor, but degrade these products.
4. Polymerized Fatty Acids (formerly Ta 1d-64)

   For sampling drums, a glass sampling tube of 2–2.5 cm i.d. and approximately 100 cm long, with one end contrasted sufficiently so that it can be used as a finger valve, should be used. The material may be heated to the minimum temperature required to facilitate adequate flow before sampling.

   Caution—Heavy metals should not be allowed to come in contact with the sample. Cover liners must also be resistant to fatty acids. Prolonged storage at elevated temperatures are to be avoided because some grades of these acids are color sensitive to heat and oxygen, particularly if the trace amounts of heavy metals are present.

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Table 1

Number of containers in shipment	Number of packages[a] to be sampled
1–10	1–3
10–25	2–4
25–50	3–6
50–75	6–8
75–100	8–10

[a]In no case shall less than 10% of the packages be sampled.

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AOCS Official Method Ta 1e-70

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Sampling of Dibasic Acids

DEFINITION

The method covers representative sampling of dibasic acids derived from the fatty series of organic compounds which are being produced on a commercial scale and the precautions required in handling these materials.

SCOPE

Applicable to dibasic acids in pourable solid form, during production, or at storage in various types of containers.

GENERAL SAMPLING PROCEDURE

1. Sampling of bags and barrels is to be done as described in AOCS Official Method Bc 1-50 for sampling soya flour (see Notes, 1).
2. Sampling of cartons, tote boxes, hoppers, etc., is to be done as described in AOCS Official Method Bc 1-50 (as above) with the exception that the sampling trier must be made long enough to sample the full depth of the container.
3. Size, number of test samples, and sample containers are to be as described in AOCS Official Method Bc 1-50.

NOTES

1. In the presence of small amounts of moisture, dibasic acids readily attack metals such as iron and copper and become discolored. It is recommended, therefore, that sampling tools and containers be constructed of materials such as stainless steel, glass, plastic, or paper.

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AOCS Official Method Tb 1a-64

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Moisture and Volatile Matter Fatty Acids Hot Plate Method

DEFINITION

This method determines the moisture and any other volatile under the conditions of the test.

SCOPE

Applicable to most fatty acids. It is not applicable to hydroxylated fatty acids, which tend to esterify; or to highly unsaturated fatty acids, such as linseed fatty acids, which tend to oxidize; or to fatty acids lower than C_{14}, such as coconut fatty acids, which will volatilize under the conditions of this test. Replaces L 2a-57.

APPARATUS

1. Electric hot plate—the surface should have a high polish, otherwise cover with a heat-resistant pad to prevent wear on the moisture dish.
2. Glass beakers—100–150 mL, or other similar and convenient dish.
3. Desiccator—containing an efficient desiccant. Calcium chloride is not satisfactory. See AOCS Specification H 9-87.

PREPARATION OF TEST SAMPLE

1. Since water tends to settle in test samples that have been softened or melted, care must be taken to mix the test samples thoroughly so as to distribute the water uniformly.

PROCEDURE

1. Weigh accurately 5–20 g of well-mixed test sample into a tared beaker which has been previously dried and cooled in a desiccator.
2. Heat the test sample on a hot plate, rotating the beaker gently by hand to avoid splattering which will result from a rapid moisture loss.
3. The approach of the end point may be judged by the absence of foam. Another good method of judging the end point is to place a clean, dry watch glass on top of the beaker. The evaluation of steam is indicated by condensation on the watch glass. The temperature of the test sample is at no time allowed to exceed 130°C, except at the end of the test.
4. When the apparent end point has been reached, heat momentarily to the point of incipient smoking, but use caution not to overheat.
5. Cool to room temperature in a desiccator and weigh.

CALCULATIONS

1. Moisture and volatile matter, $\% = \dfrac{\text{loss in mass}}{\text{mass of test portion, g}} \times 100$

PRECISION

1. Repeatability—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) as calculated from the formulas in Table 1.
2. Reproducibility—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as calculated from the formulas in Table 1.

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Table 1
The interlaboratory study carried out by Lincoln D. Metcalfe in 1975, in which eight laboratories participated, each obtaining four test results for each test portion analyzed by AOCS Official Method Tb 1a-64, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarized in the table.

	Test Sample[a]	
	A	B
Number of labs after removal of outliers	8	8
n	31	30
Outliers	1	2
Mean, %[b]	0.03	0.14
Repeatability, S_r	0.02	0.02
RSD_r	51.70	16.25
r ($2.8 \times S_r$)	0.056	0.056
Reproducibility, S_R	0.03	0.04
RSD_R	80.81	24.80
R ($2.8 \times S_R$)	0.084	0.112

[a] Key to test samples: A, industrial tallow; B, corn oil.
[b] Mean value for moisture, %.

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Nonvolatiles (Solids) Solutions of Drying Oils, Hot Plate Method

DEFINITION

This method determines the nonvolatiles (solids) content under the specific conditions of the test.

SCOPE

Applicable to solutions of natural and synthetic drying oils in organic solvents. Replaces Ka 14-60.

APPARATUS

1. Electric hot plate—equipped with controls necessary to maintain a temperature of 150 ± 2°C.
2. Thermometer—AOCS Specification H 5-40, range 6–400°C.
3. Thermometer well—see Figure 1. Insert the thermometer in well, seal with suitable heat transfer agent, e.g., vegetable oil or high temperature silicon-based oil, place on the hot plate, and use to adjust the surface temperature of the hot plate.
4. Moisture dishes—aluminum foil with flat bottom and small tab on top edge for handling, dia 58 mm, depth 18 mm (see Notes, 1).
5. Desiccator—containing an efficient desiccant. Calcium chloride is not efficient for this application. See AOCS Specification H 9-87.
6. Dropping bottles—for adding benzene and for weighing highly volatile test samples.

REAGENTS

1. Toluene—$C_6H_5CH_3$, ACS grade (see Notes, *Caution*).

PROCEDURE

1. Dry the moisture dish in an oven for 1 hr at 130°C. Cool in a desiccator and weigh to the nearest 0.0005 g (see Notes, 2).
2. From a dropping bottle, weigh by difference, 0.45–0.55 g of test portion to the nearest 0.0005 g into the moisture dish.
3. Add about 1.5 mL of toluene with the dropping bottle. Rotate the dish until the test portion is completely dissolved and uniformly spread over the bottom of the dish. With some high viscosity materials it may be necessary to heat momentarily to affect solution.
4. Place the dish on the hot plate which has been previously adjusted to 150 ± 2°C. After 10 min remove from the hot plate and place on a clean, dry surface. Allow to cool for 5 min and weigh. If weighing cannot be done within 5 min, place the dish in a desiccator.

CALCULATIONS

$$\text{Nonvolatiles (Solids) content, \%} = \frac{\text{mass of residue, g}}{\text{mass of test portion, g}} \times 100$$

PRECISION

1. Agreement within laboratory, 95% confidence limit, two single determinations performed in one laboratory shall not differ by more than 0.64%.
2. Agreement between laboratories, 95% confidence limit, single determinations performed in two different laboratories shall not differ by more than 0.80%.
3. Precision was determined in a collaborative study involving four laboratories with three test samples and a total of 48 results reported.

NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

NUMBERED NOTES

1. Use tab on moisture dish for identification purposes—never inscribe on the bottom of the dish as it must be perfectly flat for reliable results.
2. The balance must be rapid acting, well adjusted, and in good operating condition. Handle all weights and dishes at all times with forceps.

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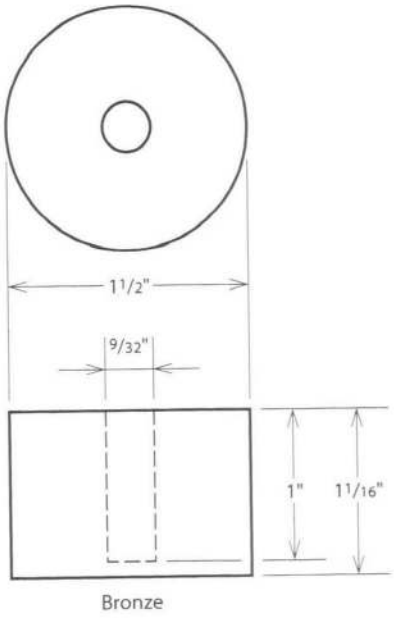

Figure 1. Thermometer well.

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Nonvolatiles (Solids) of Fatty Quaternary Ammonium Chlorides, Vacuum Oven Method

DEFINITION

This method determines the nonvolatiles (solids) content under the specific conditions of the test.

SCOPE

Applicable to fatty quaternary ammonium chlorides.

APPARATUS

1. Vacuum oven—with suitable controls for maintaining a temperature of 100°C.
2. Air oven—with suitable controls for maintaining a temperature of 105°C.
3. Erlenmeyer flask—50 mL.
4. Desiccator—containing an efficient desiccant. Calcium chloride is not efficient for this application. See AOCS Specification H 9-87.

PROCEDURE

1. Melt the test sample, if not liquid, in a water bath, mix thoroughly and accurately weigh 0.7–1.0 g in a dry, tared, 50 mL flask.
2. Place the flask in an air oven for 1 hr at 105 ± 3°C. After about 15 min of oven time rotate the flask gently to coat the test portion over the entire bottom of the flask.
3. Place the flask in a vacuum oven for 8 hr at 100 ± 3°C and an internal vacuum of 27–29 in. of mercury.
4. Cool in desiccator for ½ hr, and reweigh.

CALCULATIONS

$$\text{Nonvolatiles (Solids) content, \%} = \frac{\text{mass of residue, g}}{\text{mass of test portion, g}} \times 100$$

PRECISION

At a confidence level of 95%, in the range of values of 51–77:

1. Two single determinations performed in one laboratory shall not differ from the true mean value by more than ± 2%.
2. Single determinations performed in different laboratories shall not differ from the true mean value by more than ± 5%.

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Color
Gardner 1963 (Glass Standards)

DEFINITION

This method determines the color by comparison with standards of specified color.

SCOPE

Applicable to natural and synthetic drying oils, fatty acids, and oil derivatives that do not differ in hue appreciably from the standards.

APPARATUS

1. Glass standards—eighteen, numbered separately, having the transmittance characteristics given in Table 1 and the tolerance specified in Notes, 2. Details of suppliers of glass standards may be obtained from The Tintometer Limited, Waterloo Road, Salisbury, Wiltshire, SPI 2JY, UK. Tel: +44 1722 327242, Fax: +44 1722 412322, E-mail: sales@tintometer.com
2. Clear glass tubes—10.65 ± 0.025 mm i.d. and about 114 mm outside length. (Viscosity tubes, as described in AOCS Official Method Tq 1a-64, are satisfactory. Kimble EXAX test tubes, #46463–1075, may also be used.)
3. Suitable apparatus for comparing test portion and standards—The apparatus may be of any design, but, for the highest precision, should have the following characteristics:
   (a) Illumination—CIE Illuminant C.
   (b) Surrounding field—Should not differ significantly in brightness from the test portion and standards, and should be essentially achromatic.
   (c) Field of view—Two standards and the test portion should subtend a visual angle of about 2°. Two standards and a test portion should always be in the field of view.
   (d) Separation of standard and test portion—There should be a perceptible separation between test portion and standard, but this should be as small as mechanically possible.

   Other conditions may be used, if they can be demonstrated to give the same results.

PROCEDURE

1. All hazy material should be filtered at 25°C.
2. Fill a sample tube (Apparatus, 2).
3. Compare with standards, bringing the next lightest and next darkest into the field of view at the same time.

REPORTING

1. The color of the test portion is reported as the number of the standard most closely matching the test portion. If the test portion falls between two standards, it will be reported as "+" or "–" depending on whether it is darker (+) or lighter (–) than the standard it most closely approaches. Thus, between colors 5 and 6, the steps will be 5, 5+, 6–, and 6.

COLOR STANDARDS

1. The standard shall be of glass, with the color produced by the glass component only, and shall have the color characteristics given in Table 1.

NOTES

1. The values of the chromaticity coordinates may be obtained by spectrophotometric measurements, in accordance with ASTM standards.
2. Tolerance—individual standards shall not depart from the coordinates given in Table 1 by more than $\frac{1}{3}$ of the distance to the next color. In any one set, no two standards shall be closer together than $\frac{2}{3}$ of the nominal value of the color difference between them.

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Table 1
Chromaticity coordinates of reference standards.

	Chromaticity coordinates[a]	
Standard number	X	Y
1	.3177	.3303
2	.3233	.3352
3	.3329	.3452
4	.3437	.3644
5	.3558	.3840
6	.3767	.4061
7	.4044	.4352
8	.4207	.4498
9	.4343	.4640
10	.4503	.4760
11	.4842	.4818
12	.5077	.4638
13	.5392	.4458
14	.5646	.4270
15	.5857	.4089
16	.6047	.3921
17	.6290	.3701
18	.6477	.3521

[a]See Notes, 1.

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Color, Platinum-Cobalt Scale

DEFINITION

This method determines the color of products that are completely liquid and clear by comparison with artificial standards.

SCOPE

Applicable to industrial oils and derivatives with light colors, such as certain fatty nitrogen compounds. For a specific class or product refer to AOCS methods for that product class. Replaces Na 1-C2.

APPARATUS

1. Nessler Platinum-Cobalt Scale high form color comparison tubes—100 mL with ground-on optically clear glass caps, approximately 390 mm long with 24 mm o.d. The 100 mL graduation should be between 275 and 295 mm above the bottom of the tube.
2. A color comparator rack to hold up to 12 tubes—constructed to permit visual comparison of light transmitted through the above tubes longitudinally, in which white light is passed through or reflected off a white glass plate and directed with equal intensity through the tubes and which is shielded so no light enters the tubes from the side.
3. Light source—daylight type fluorescent bulb.
4. Platinum-Cobalt Scale color standards—this set consists of color standards of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, and 500. (See Notes, 1).
5. Whatman Filter—no. 4 paper or equivalent.

PROCEDURE

1. All hazy test samples should be filtered while completely melted, at 25°C for normally liquid materials, and not more than 10°C above its melting point for a solid material. Maintain this temperature throughout the color determination.
2. Fill a color tube to the 100 mL mark with test portion, put on cap, and place in comparator rack between the 2 standards it most nearly matches in intensity, looking down into the tubes with the rack set properly beside the light source. View test portion and standards simultaneously.

REPORTING

1. Reporting the test portion color as NDT (not darker than) the standard which it matches or the darker of the 2 standards between which it falls. Report test portions lighter than 10 standards as LT 10. Report test portions darker than the 500 standard as DT 500.

NOTES

1. To prepare the platinum-cobalt stock solution, dissolve 1.245 g of K_2PtCl_6, C.P. grade and 1.000 g of $CoCl_2 \cdot 6H_2O$, ACS grade, in distilled water. Add 100 mL of HCl, sp. gr. 1.19, ACS grade, and dilute to 1 L with distilled water. This solution has a Platinum-Cobalt Scale of 500.
2. To prepare the color standard, add the amount indicated in Table 1 of the Platinum-Cobalt 500 solution to a color tube, fill the tube to the 100 mL mark with distilled water, cap and seal. Keep in dark when not in use.
3. Platinum-Cobalt 500 stock solution is available from chemical supply houses.

Table 1

Platinum-Cobalt Color	mL of Platinum-Cobalt 500 Solution	Platinum-Cobalt Color	mL of Platinum-Cobalt 500 Solution
10	2	100	20
20	4	150	30
30	6	200	40
40	8	250	50
50	10	300	60
60	12	350	70
70	14	400	80
80	16	450	90
90	18	500	100

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Color, Photometric Index

DEFINITION

This method measures the color of commercial fatty acids and expresses the color as percent transmission at 440 and 550 nm (percent transmittance at 440/550 nm). Alternately, the method can be used to designate a photometric index to characterize the color which is expressed as 100 × the absorbance at each of the two wavelengths, 440 and 550 nm.

SCOPE

Applicable to all commercial fatty acids. Replaces L 13a-57.

APPARATUS

1. Spectrophotometer—(see Notes, 2) *capable of adjustment* to give the following readings on a standard nickel sulfate solution (prepared as noted in Apparatus, 3) at 25–30°C, after setting the zero point and after adjusting the 100% transmittance point (0 absorbance) against carbon tetrachloride in a cuvette having the specification outlined in Apparatus, 2 below:

Nanometers/Transmittance	
400	Less than 4.0%
460	26.2 ± 2.0%
510	73.9 ± 1.0%
550	54.8 ± 1.0%
620	5.2 ± 0.5%
670	1.1 ± 0.5%
700	Less than 2.0%

2. Matched glass cylindrical cuvettes—(see Notes, 1) approximately 21.8 mm i.d.; approximately 24.5 mm o.d.. All cuvettes to be used with a given instrument should be checked with carbon tetrachloride and the nickel sulfate solution at 550 nm and given within ± 0.6% of the same transmittance. The cuvettes should be clean and free from scratches.
3. Standard nickel sulfate solution. Dissolve 200 g of $NiSO_4 \cdot 6H_2O$ analytical reagent grade, in distilled water. Add 10 mL of concentrated HCl. Dilute to exactly 1,000 mL in a volumetric flask. The temperature of the solution should be between 25 and 30°C. The nickel content of the solution must fall between 4.40 and 4.46 g of nickel per 100 mL at 25–30°C.
4. Filter paper—fine porosity such as E and D no. 192, Whatman no. 12, Reeve Angel no. 871, or S and S No. 596, or equivalent.

REAGENTS

1. Carbon tetrachloride—(see Notes, *Caution,* 3), redistilled if the transmittance differs from distilled water by 0.5% or more at 440 nm.
2. Diatomaceous earth—Hy-Flo Supercel grade, available from Manville, or equivalent.

CALIBRATION OF SPECTROPHOTOMETER

1. Turn on the spectrophotometer and allow at least a 20 min warm-up period before standardizing or making any measurements.
2. After the initial warm-up period, rotate both of the control knobs on top of the instrument counterclockwise to their stop position. Adjust the galvanometer by means of the galvanometer adjustment or by sliding the scale so that an exact zero reading is obtained.
3. Set the wavelength dial at 460 nm. Recheck the zero reading of the instrument, insert a cuvette filled with carbon tetrachloride in the instrument and set the 100% transmittance point exactly.
4. Fill a cuvette with the standardizing nickel sulfate solution and measure the transmittance of the solution. The reading must fall between 24.2 and 28.2.
5. In a like manner set the instrument at 550 nm and measure the transmittance of the nickel sulfate solution. The reading must fall between 53.8 and 55.8.
6. If the 460 reading is above 26.2, the 550 reading should be above 54.8; if the 460 reading is below 26.2 the 550 reading should be below 54.8; otherwise adjust the wavelength knob underneath the instrument until both readings are in the same direction above or below the median values established, but within the specified limits.
7. Set the instrument at 510 nm and measure the transmittance of the nickel sulfate solution. The 510 reading must fall between 72.9 and 74.9.

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8. Read the other specified values. All must fall within the limits noted in Apparatus, 1.

PROCEDURE

1. Test sample must be absolutely clear. Suspended material, even if colloidal in size, will cause light scattering. If not perfectly clear, filter with diatomaceous earth using 0.5 g of earth per 300 g of fatty acid.
2. Adjust the temperature of the fatty acid to 25–30°C (or 10°C above the melting point if necessary) and fill a cuvette, using a sufficient amount of test sample to ensure a full column in the light beam.
3. Set the instrument to 440 nm and insert a cuvette filled with carbon tetrachloride. Adjust the % transmission to exactly 100%.
4. Replace the cuvette containing carbon tetrachloride with the one containing the test sample. Read % transmission (% T) and/or absorbance (A) (see Notes, 4).
5. In a like manner, set the instrument at 550 nm and read % transmission (% T) and/or the absorbance (A) as in Procedure, 3 and 4.

CALCULATIONS

Express the color as:

1. % Transmission at 440/550 nm (% T 440/550 nm), or
2. Calculate a Photometric Index as:

$$\text{Photometric Index} = 100 \times A_{440} \text{ and } 100 \times A_{550}$$

Where—

A = absorbance.

NOTES

Caution

Carbon tetrachloride is a known carcinogen. It is toxic by ingestion, inhalation and skin absorption. It is a narcotic. It should not be used to extinguish fires. It decomposes to phosgene gas at high temperature. The TLV is 5 ppm in air. A fume hood should be used at all times when using carbon tetrachloride.

NUMBERED NOTES

1. The cuvettes may be standard test tubes, which are clean and free from scratches. Test tubes of the dimensions specified are available from glassware suppliers, e.g., Kontes and Kimball.
2. The Coleman Junior Spectrophotometer, manufactured by Coleman Instruments, Inc., Maywood, IL, USA meets the specifications of the spectrophotometer required.
3. Methylisobutylketone (MIBK) may be a replacement for carbon tetrachloride in this method.
4. Absorbance (A), formerly called optical density, is defined as the common logarithm of the reciprocal of the transmittance (T):

$$A = \log_{10} \frac{1}{T}$$

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Color after Heating

DEFINITION

This method measures the color stability of fatty acids after heating under the specified conditions of the test.

SCOPE

Applicable to all normal fatty acids with a boiling point of less than 200°C. Replaces L 15a-58

APPARATUS

1. Oil bath—5000 mL stainless steel beaker equipped with a mechanical stirrer and containing any light-colored alkali-refined oil. Hydrogenated cottonseed oil is suitable for this purpose.
2. Heat source—Tirrill burners (3) are satisfactory, but electric immersion heaters with suitable thermostatic control may be used.
3. Thermometer—partial immersion, 95–255°C, AOCS Specification H 11-58.
4. Tubes—2.5 × 24 cm with ground-glass joint (see Fig. 1). Available from Kontes, Vineland, NJ, USA, or any other manufacturer of special glassware (see Notes, 1).
5. Test tube holder—see Figure 2.
6. Timer—interval timer capable of measuring intervals of time up to and including 120 min.
7. Filter paper—fine porosity such as Eaton and Dikeman no. 512, Whatman no. 12, Reeve-Angel no. 871, S and S no. 596, or any equivalent filter paper.
8. Nitrogen—source of nitrogen capable of being regulated to 1–2 psi pressure.

PROCEDURE

1. Bring the bath to a temperature of 205 ± 2°C and maintain the temperature within the specified range.
2. If the test sample to be tested is not liquid at room temperature, liquefy by heating on a water bath to a temperature not more than 10°C above the titer of the test sample.
3. Fill a clean, dry tube to a depth of 12.7 cm with the test sample, and place in the oil bath.
4. Adjust the level of the oil in the bath to 14 cm from the bottom of the tube(s). When placing several tubes in the bath, the bath temperature should not drop more than 5°C below the minimum bath temperature, and the recovery time to reach 205°C should not exceed 5 min.
5. Adjust the flow of nitrogen so that the surface of the test sample is blanketed by inert gas at all times.
6. Set the timer for 60 min if the test sample has an iodine value >15, or for 120 min if the test sample has an iodine value of ≤15.
7. Remove the tube(s) from the bath and read the color immediately using AOCS Official Method Td 1a-64 and Gardner color standards.

NOTES

1. Glassware cleanliness is of utmost importance. The suggested method is washing with detergent and rinsing with reagent-grade acetone or alcohol. Dichromate solutions should not be used.

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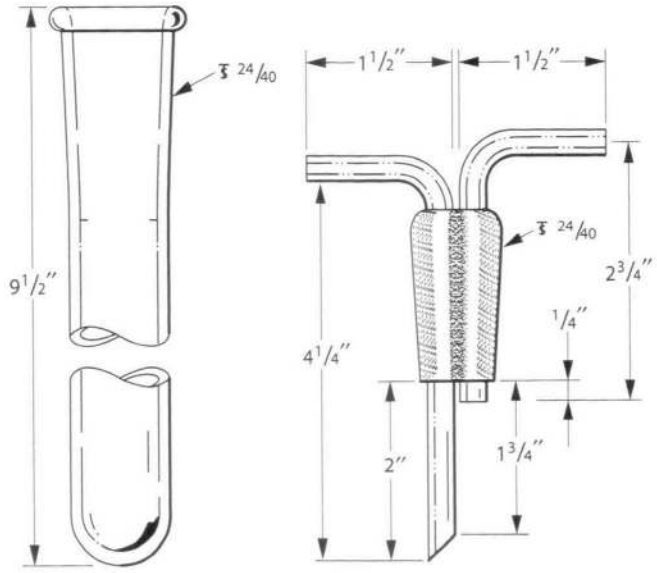

Figure 1. Details of color stability tube.

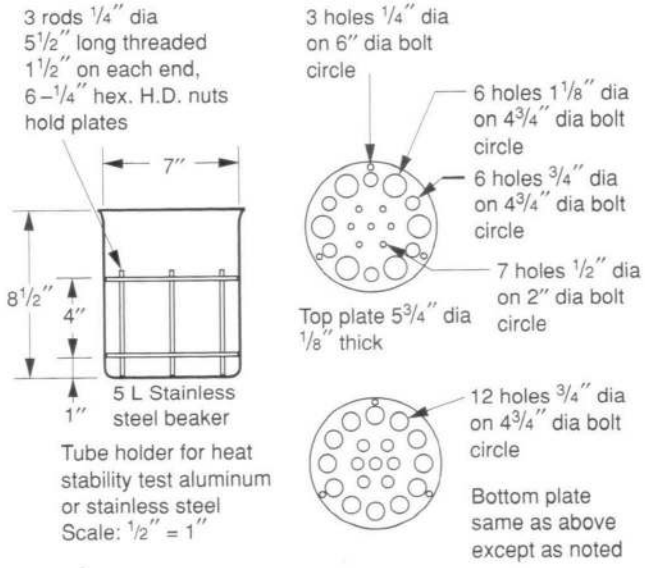

Figure 2. Details of tube holder for heat stability test.

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Acid Value

DEFINITION

The acid value is the mg of potassium hydroxide necessary to neutralize fatty acids in 1 g of test sample.

SCOPE

Applicable to all commercial fatty acids and polymerized fatty acids. The method is also satisfactory for vegetable deodorizer distillates and distillation residues from hydrolyzed vegetable oil acid soapstock.

APPARATUS

1. Erlenmeyer flask—500 mL.

REAGENTS

1. Sodium hydroxide solution, 0.5 M—accurately standardized. See AOCS Specification H 12-52.
2. Ethyl alcohol—95%, SDA Formula 30 and 3A are permitted. Add a few drops of phenolphthalein indicator solution. Boil and neutralize with NaOH solution to a faint pink color just before using.
3. Phenolphthalein indicator solution—1.0% in 95% alcohol.

PROCEDURE

1. Weigh accurately 5 g of fatty acids into 500 mL Erlenmeyer flask and add 75–100 mL of hot neutral alcohol. Agitation and further heating may be necessary to bring the fatty acids into complete solution.
2. Add 0.5 mL of indicator and titrate immediately, while shaking, with 0.5 M NaOH to the first pink color which persists for 30 sec.

CALCULATIONS

$$\text{Acid value, expressed as mg KOH per g of fatty acids} = \frac{\text{volume of titrant, mL} \times \text{M} \times 56.10}{\text{mass of test portion, g}}$$

Where—

M = molarity of accurately standardized sodium hydroxide solution.

PRECISION

At a confidence level of 95%:

Material	Fatty Acids Polymerized	Distillates Residues	Fatty Acids Tallow Coconut Soybean Linseed
Range of Values:	162–195	24–64	195–275
Two single determinations performed in 1 laboratory shall not differ from the mean value by more than:	± 0.8%	± 1.0%	± 1.2%
Single determinations performed in different laboratories shall not differ from the mean value by more than:	± 1.3%	± 1.9%	± 1.4%

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Acid Value

DEFINITION

The acid value is the mg of potassium hydroxide necessary to neutralize the free acids in 1 g of test sample.

SCOPE

Applicable to all neutral and synthetic drying oils, epoxidized oils, fatty alcohols, and fatty esters. Replaces Ka 2-58.

APPARATUS

1. Erlenmeyer flask—250 or 300 mL.

REAGENTS

1. Sodium hydroxide—approximately 0.1 M in methyl alcohol (see AOCS Specification H 17-58), accurately standardized with potassium hydrogen phthalate (see AOCS Specification H 12-52), using 1% phenolphthalein as the indicator.
2. Isopropyl alcohol-toluene mixture—(see Notes, *Caution*) prepared by mixing equal volumes of reagent grade isopropyl alcohol with reagent grade toluene. The mixture must be neutralized with 0.1 M methanolic sodium hydroxide to a pink color that persists for 1 min, using 1% phenolphthalein as the indicator.
3. Phenolphthalein indicator solution—1% in methyl alcohol.

PROCEDURE

1. Determine the size of the test portion from the following table:

Acid value	Approx. mass of test portion, g	Accuracy of weighing
0–5	20	± 0.05 g
5–15	10	± 0.05 g
15–30	5	± 0.05 g
30–100	2.5	± 0.001 g
100 and over	1.0	± 0.001 g

2. Weigh the designated size of test portion into an Erlenmeyer flask. Add approximately 100 mL of neutralized solvent and 1 mL of indicator and mix until test portion is completely dissolved. Warming may be necessary for bodied oils.
3. Titrate with the standard alkali solution, shaking vigorously to the appearance of the first permanent pink color of the same intensity as that of the neutralized solvent before addition. The color must persist for 1 min.

CALCULATIONS

$$\text{Acid value, mg KOH per g of test portion} = \frac{\text{volume of alkali, mL} \times M \times 56.1}{\text{mass of test portion, g}}$$

Where—

M = molarity of alkali determined by standardization

NOTES

Caution

This is a flammable mixture. Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene or solvent mixtures containing toluene.

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AOCS Official Method Te 3a-64

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Acid Value and Free Amine Value of Fatty Quaternary Ammonium Chlorides

DEFINITION

The acid value is the mg of potassium hydroxide necessary to neutralize the acidity in 1 g of test sample. The free amine value is the mg of potassium hydroxide equivalent to the free amine basicity in 1 g of test sample.

SCOPE

Applicable to fatty quaternary ammonium chlorides (QACs). Replaces Ne 1-62.

APPARATUS

1. Erlenmeyer flasks—300 mL.
2. Microburette—10 mL capacity graduated to 0.02 mL.

REAGENTS

1. Isopropyl alcohol—reagent grade. Prior to running the acid value, the isopropyl alcohol must be neutralized with 0.1 M methanolic sodium hydroxide to a pink color that persists for 1 min, using 1% phenolphthalein as the indicator.
2. Sodium hydroxide—approximately 0.1 M in reagent grade methyl alcohol, accurately standardized with potassium hydrogen phthalate, using 1% phenolphthalein as the indicator (see AOCS Specification H 12-52).
3. Hydrochloric acid—approximately 0.1 M and 0.2 M in isopropyl alcohol, accurately standardized with sodium carbonate, using 0.1% bromcresol green as the indicator, or by AOCS Specification H 14-52.
4. Phenolphthalein indicator solution—1% in 95% ethyl alcohol.
5. Bromphenol blue indicator solution—0.2% in isopropyl alcohol.

PROCEDURE

1. Acid value and free amine value—
   (a) Melt test sample, if it is not already liquid, in a water bath, mix thoroughly, and accurately weigh 5–20 g into each of two 300 mL flasks.
   (b) For the acid value, add 100 mL of neutralized solvent to the first flask, and swirl to dissolve the test sample. Heat if necessary.
   (c) Add 1 mL phenolphthalein indicator. If the solution remains colorless, titrate while swirling, with 0.1 M NaOH to the appearance of the first permanent pink color of the same intensity as that of the neutralized solvent before addition. If the solution turns pink, the presence of a free caustic is indicated. Proceed with the "Free Caustic" in Procedure, 2.
   (d) For the amine value add 100 mL of solvent and swirl to dissolve. Heat if necessary.
   (e) Add 1 mL of bromphenol M blue indicator and titrate, while swirling, with 0.2 M HCl to the yellow endpoint (see Notes, 1).
2. Free Caustic—
   (a) Titrate the first test portion as noted in Procedure, (d) and (e) for amine value. This titration includes free caustic and free amine. From this titration calculate the mL of 0.2 M HCl to neutralize the second test portion:

$$\text{volume, mL of 0.2 M HCl} = \frac{\text{volume of HCl, mL (1st titration} \times \text{mass of 2nd test portion, g)}}{\text{mass of 1st test portion, g}}$$

   (b) Add 100 mL of neutralized solvent, and the calculated amount of 0.2 M HCl to the second flask. Add 1 mL phenolphthalein indicator and titrate, while swirling, with 0.1 M NaOH, to the appearance of the first permanent pink color of the same intensity as that of the neutralized solvent before addition.
   (c) Use this titration to calculate the amine value, using the second test portion weight.
   (d) Convert this caustic titration on the second test portion over to the first test portion weight:

$$\text{volume, mL of 0.2 M NaOH} = \frac{\text{volume of NaOH, mL (titration of 2nd test portion)} \times \text{mass of 1st test portion, g}}{\text{mass of second test portion, g}}$$

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CALCULATIONS

1. Acid value = $\frac{mL \times M \times 56.1}{\text{mass of test portion, g}}$

2. Free amine value = $\frac{mL \times M \times 56.1}{\text{mass of test portion}}$

3. Free sodium hydroxide, % = $\frac{mL \times M \times 56.1}{\text{mass of test portion, g}}$

4. % Amine hydrochloride and % free amine can be calculated as follows:

$$\text{Amine hydrochloride, \%} = \frac{\text{acid value} \times \text{molecular wt of amine hydrochloride}}{561}$$

$$\text{Free amine, \%} = \frac{\text{free amine value} \times \text{molecular wt of free amine}}{561}$$

Caution—Molecular weights listed above are averages of products from several producers. They are suitable for use with this test only, since normal variations between different products do not seriously affect the calculated percentages. They are not suitable in other tests, such as the determination of % Quaternary by titration. In addition, there is a current trend toward the use of methylated amines.

PRECISION

At a confidence level of 95%, in the ranges of value indicated:

Value	Acid Value	Amine
Range of Values: Two single determinations performed in 1 laboratory shall not differ from the mean value by more than:	0.1–0.2 ± 53%	0.2–0.3 ± 21%
Range of Values: Single determinations performed in different laboratories shall not differ from the mean value by more than:	0.1–0.2 ± 170%	0.2–0.3 ± 82%

NOTES

1. If the amine value is extremely low, 0.1 M HCl should be used for better accuracy.

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Fatty Quaternary Ammonium Chloride(QAC)	Free Amine Molecular Weight	Amine Hydrochloride Molecular Weight
Trimethyl Lauryl QAC	212	248
Trimethyl Myristyl QAC	243	280
Trimethyl Palmityl QAC	276	312
Trimethyl Stearyl QAC	302	338
Trimethyl Oleyl QAC	296	332
Trimethyl Tall Oil Fatty QAC	310	346
Trimethyl Coco QAC	238	374
Trimethyl Tallow QAC	288	324
Trimethyl Cotton QAC	296	332
Dimethyl Dilauryl QAC	364	400
Dimethyl Dimyristyl QAC	415	441
Dimethyl Dipalmityl QAC	468	504
Dimethyl Distearyl QAC	553	589
Dimethyl Di(tall fatty oil) QAC	518	554
Dimethyl Dicoco QAC	401	437
Dimethyl Di(hydrogenated tallow) QAC	519	555
Dimethyl Furfuryl Hydrogenated Tallow QAC	368	404

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AOCS Official Method Te 4a-70

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Purity by Total Acidity and Acid Value, Dibasic Acids

DEFINITION

This method describes purity as percent total acidity and acid value which is the mg of potassium hydroxide necessary to neutralize the free acids in 1 g of test sample.

SCOPE

Applicable to all dibasic acids being produced on a commercial scale from fatty raw materials.

APPARATUS

1. Erlenmeyer flask—500 mL.
2. Burette—100 mL, calibrated, with TFA-fluorocarbon resin stopcock plug.

REAGENTS (see Notes, 1)

1. Water—unless otherwise indicated, references to water shall be understood to mean carbon dioxide free distilled water or water of equal purity.
2. Isopropanol—neutralized, add 8–10 drops phenolphthalein indicator to 1 L of isopropanol. Boil and neutralize to a faint pink color by the dropwise addition of 0.5 M sodium hydroxide solution.
3. Phenolphthalein indicator solution—1% in methyl alcohol.
4. Sodium hydroxide—standard solution (0.5 M), accurately standardized with potassium hydrogen phthalate, using 1% phenolphthalein as the indicator (see AOCS Specification H 12-52).

PROCEDURE

1. Determine range of test portion size by using the formula:

$$\text{Weight} = \frac{(4.5 \pm 0.2) \times M}{B \times P}$$

Where—

M = molecular weight of acid

B = number of reacting groups in acid

P = approximate expected percent acidity of test portion

2. Weigh test portion to the nearest 0.1 mg in 500 mL Erlenmeyer flask. Add 100 mL of hot neutralized isopropanol and 3–4 drops of phenolphthalein indicator. Apply additional heat if necessary to attain complete solution.
3. Titrate with standard of 0.5 M sodium hydroxide solution to the first pink end point persistent for 15 sec. Read and record the volume of titrant to the nearest 0.02 mL.

CALCULATIONS

1. Percent purity by total mass = $\dfrac{\text{volume, mL of alkali} \times \text{molarity} \times \text{mol. wt. of acid}}{10 \times \text{test portion mass, g} \times \text{no. of reacting groups in acid}}$

Report to nearest 0.1%

2. Acid value = $\dfrac{\text{volume, mL of alkali} \times \text{molarity} \times 56.1}{\text{mass of test portion, g}}$

PRECISION

1. Repeatability (single analyst)—the standard deviation of results (each the average of duplicates), obtained by the same analyst on different days, has been established to be 0.04% absolute at 27 degrees of freedom. Two such values should be considered suspect (95% confidence level) if they differ by more than 0.13% absolute.
2. Reproducibility (multilaboratory)—the standard deviation of results (each the average of duplicates), obtained by different analysts in different laboratories, has been estimated to be 0.14% absolute at 6 degrees of freedom. Two such values should be considered suspect (95% confidence level) if they differ by more than 0.47% absolute.

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NOTES

1. Reagent grade chemicals or the equivalent shall be used throughout, including water.
2. Established by ASTM Subcommittee B4 of Committee E-15 when developing Tentative Method of Test for Total Acidity of Organic Acids—ASTM designation E301-68.

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Total Amine Value of Fatty Amines, Potentiometric Method

DEFINITION

The total amine value is the mg of potassium hydroxide equivalent to the basicity in 1 g of test sample.

SCOPE

Applicable to fatty amines. Replaces Nb 1-62

APPARATUS

1. pH meter—equipped with external electrodes and having a sensitivity capable of ± 0.05 pH unit reading or similar potentiometric titrator.
   (a) Calomel electrode—range 5–100 C.
   (b) Glass electrode—range 5–100 C.
   (c) Combination electrode
2. Magnetic stirrer—with Teflon™-coated stirring bar.
3. Beakers—250 mL, low form, Pyrex™ is suitable.
4. Graduated cylinders—10 and 100 mL.
5. Microburette—10 mL capacity, graduated to 0.02 mL

REAGENTS

1. Isopropyl alcohol—99%, AOCS Specification H 18-58.
2. Hydrochloric acid—approximately 0.5 M—in 99% isopropyl alcohol, accurately standardized with sodium carbonate to a pH of 3.5, or standardized according to AOCS Specification H 14-52.
3. Isopropyl alcohol solution—prepared by mixing 5 mL water with 95 mL of 99% isopropyl alcohol.
4. Chloroform, ACS or AR grade (see Notes, *Caution* and 1).

STANDARDIZATION OF pH METER

Carefully follow the manufacturer's instructions for the particular meter used, and standardize at pH of 4.0, 7.0, and 10.0.

PROCEDURE

1. Melt the test sample if it is not already liquid, mix thoroughly and weigh 0.5 g into 250 mL beaker.
2. Add 90 mL chloroform, 10 mL isopropyl alcohol solution and boil for 1 min on a hot plate.
3. Cool to room temperature. Insert the stirring bar into the beaker and stir while titrating with 0.5 M HCl using the millivolt scale.
4. Record the millivolt reading every mL, but in the vicinity of the end point record the millivolt reading every 0.1 mL. Plot a graph, showing the millivolts against the titration volume. The end point is the midpoint of the inflection on the titration curve.

CALCULATIONS

$$\text{Total amine value} = \frac{\text{volume, mL} \times \text{M} \times 56.1}{\text{test portion mass, g}}$$

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

NUMBERED NOTES

1. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. The production of TCE is projected to continue until the year 2002. Cyclohexane and isooctane may also be considered

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as alternative solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

PRECISION

At a confidence level of 95%, in the range of values:	Amines 75–268	Amido-Amines 143–156	Diamines 305–406
Two single determinations performed in one laboratory shall not differ from the mean value by more than:	± 2.7%	± 4.0%	± 2.6%
Single determinations performed in different laboratories shall not differ from the mean value by more than:	± 4.0%	± 6.2%	± 3.3%

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Total Amine Value of Fatty Amines, Indicator Method

DEFINITION

The total amine value is the mg of potassium hydroxide equivalent to the basicity in 1 g of test sample.

SCOPE

Applicable to fatty amines. Replaces Nb 4-62.

APPARATUS

1. Erlenmeyer flask—300 mL.

REAGENTS

1. Isopropyl alcohol—reagent grade.
2. Bromphenol blue indicator solution—0.2% in isopropyl alcohol.
3. Hydrochloric acid—approximately 0.2 M, in isopropyl alcohol, accurately standardized with sodium carbonate, using 0.1% bromcresol green as the indicator, or standardized according to AOCS Specification H 14-52.

PROCEDURE

1. Melt the test sample if it is not already liquid, mix thoroughly, and weigh 0.5 g into 250 mL beaker.
2. Add 50 mL of isopropyl alcohol, and boil for 1 minute to drive off any free ammonia that may be present. Cool to room temperature.
3. Add 5–10 drops of indicator, and while swirling the flask, titrate with 0.2 M HCl to the yellow end point.

CALCULATION

$$\text{Total amine value} = \frac{\text{volume, mL} \times \text{M} \times 56.1}{\text{test portion mass, g}}$$

PRECISION

At a confidence level of 95%, in the range of values of:	Amines 75–267
1. Two single determinations performed in 1 lab shall not differ from the true mean value by more than:	±2.8%
2. Single determinations performed in two different labs shall not differ from the true mean value by more than:	±3.1%

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Primary, Secondary, and Tertiary Amine Values of Fatty Amines, Potentiometric Method

DEFINITION

The primary amine value is the mg of potassium hydroxide equivalent to the primary amine basicity in 1 g of test sample. The secondary amine value is the mg of potassium hydroxide equivalent to the secondary amine basicity in 1 g of test sample. The tertiary amine value is the mg of potassium hydroxide equivalent to the tertiary amine basicity in 1 g of test sample.

SCOPE

Applicable to fatty amines. The method is not applicable to fatty amines containing reactive groups and structures, e.g., diethylene-(DETA)-diamides and 3,3'-iminobispropylamine-(DPTA)-diamides. Replaces Nb 3-62.

APPARATUS

1. pH meter—equipped with external electrodes and having a sensitivity capable of ± 0.05 pH unit reading or similar potentiometric titrator.
   (a) Calomel electrode—range 5–100°C.
   (b) Glass electrode—range 5–100°C.
   (c) Combination electrode
2. Magnetic stirrer—with Teflon™-coated stirring bar.
3. Graduated cylinders—10, 50, 100, and 1000 mL.
4. Erlenmeyer flasks—Corning Alkali Resistant, Kimble Resistant or equivalent, 300 mL.
5. Air condenser—minimum 650 mm long.
6. Beakers—250 mL and 200 mL, low form, Pyrex™ is suitable.
7. Burette—25 mL.
8. Microburette—10 mL capacity, graduated to 0.02 mL.
9. Hot plate—with variable heat control.
10. Volumetric flask—2000 mL.
11. Glass funnel.

REAGENTS

1. Glacial acetic acid—ACS or AR grade.
2. Chloroform—ACS or AR grade (see Notes, *Caution*).
3. Acetic anhydride—ACS or AR grade.
4. Isopropyl alcohol—reagent grade.
5. Salicylaldehyde—AR grade.
6. Perchloric acid—ACS grade.
7. Acid potassium phthalate—NBS primary standard.

SOLUTIONS

1. Isopropyl alcohol solution—prepared by mixing 5 mL water with 95 mL of isopropyl alcohol.
2. Perchloric acid, approximately 0.1 M—in glacial acetic acid, add 28.4 g perchloric acid to 1000 mL of glacial acetic acid in a 2000 mL beaker while stirring. Carefully add 46.6 g of acetic anhydride while stirring. Carefully pour the solution through the funnel into a 2000 mL volumetric flask, and dilute to mark with glacial acetic acid. Mix the solution and allow to stand for 24 hr before standardizing.

   Standardization—weigh accurately 0.31–0.38 g of finely ground and dried potassium acid phthalate into a 250 mL beaker. Add 50 mL of glacial acetic acid and warm gently to dissolve the test sample. Cool, and add an additional 50 mL of glacial acetic so as to wash down the sides of the beaker. Insert the stirring bar into the beaker and while stirring titrate with the perchloric acid solution, using the millivolt scale. Record the millivolt reading every mL, but in the vicinity of the end point record the millivolt reading every 0.1 mL. Plot the graph showing the millivolts against the titration. The end point is the midpoint of the inflection on the titration curve. The strength of the perchloric acid is expressed in terms of its normality:

$$\text{Normality of perchloric acid solution} = \frac{\text{g acid potassium phthalate} \times 4.8967}{\text{mL of perchloric acid solution}}$$

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3. Hydrochloric acid approximately 0.5 M—in isopropyl alcohol, accurately standardized by titrating sodium carbonate to a pH of 3.5, or standardized by AOCS Specification H 14-52.

STANDARDIZATION OF pH METER

Carefully follow the manufacturer's instructions for the particular meter in use and standardize at pH values of 4.0, 7.0, and 10.0.

PROCEDURE

1. Determine the total amine value of AOCS Method Tf 1a-64.
2. Determine the size of the test portion from the following table:

	Mass of test portion	
Type of Amine	Secondary + Tertiary Amine Value	Tertiary Amine Value
Primary	5 g	5 g
Secondary	1 g	5 g
Tertiary	1 g	1 g

3. Melt the test sample if it is not already liquid, mix thoroughly, and accurately weigh the designated size of the test sample into a 250 mL beaker for the secondary plus tertiary amine value and into a 300 mL flask for the tertiary value. Mark the beaker S and the flask T.
4. To test portion S add 90 mL chloroform (see Notes, *Caution* and 1), and boil for 1 min on a hot plate to drive off any free ammonia that may be present.
5. Cool to room temperature, add 5–6 mL of salicylaldehyde, and allow to stand for 30 min.
6. After 30 min add 10 mL of the isopropyl alcohol solution. Insert the stirring bar into the beaker and while stirring titrate with 0.5 M HCl using the millivolt scale.
7. Record the millivolt reading every mL but in the vicinity of the end point record the millivolt reading every 0.1 mL. Plot a graph showing the millivolts against the titration. The end point is the midpoint of the inflection on the titration curve.
8. To test portion T add 25 mL acetic anhydride, 2 mL acetic acid, and 2–3 glass beads.
9. Attach an air condenser, and reflux on a hot plate for 45 min, allowing the condensate to rise only 5 cm in the condenser.
10. Rinse down the condenser and quantitatively transfer the solution with about 50 mL of glacial acetic acid into a 250 mL beaker.
11. Cool to room temperature. Insert the stirring bar into the beaker and while stirring titrate with 0.1 M $HClO_4$. Plot a graph showing the millivolts against the titration. The end point is the midpoint of the inflection on the titration curve.

CALCULATIONS

1. Total amine value of primary, secondary, and tertiary amine groups—determine by AOCS Method Tf 1a-64.
2. Titration S:

$$\text{Amine value of secondary and tertiary amine groups} = \frac{\text{mL} \times \text{M} \times 56.1}{\text{test portion mass, g}}$$

3. Titration T:

$$\text{Amine value of tertiary amine groups} = \frac{\text{mL} \times \text{M} \times 56.1}{\text{test portion mass, g}}$$

4. Primary amine value = Total amine value 1 – amine value 2
   Secondary amine value = Amine value 2 – amine value 3
   Tertiary amine value = Amine value 3

PRECISION

At a confidence level of 95%, in the range of values indicated (see Table 1).

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 µg/g in air. A fume hood should be used at all times when using chloroform.

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NUMBERED NOTES

1. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 µg/g) is lower than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternative solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

Table 1
Precision

Type of Amine	Fatty Amines					Fatty Diamines		
	Primary		Secondary	Tertiary		Primary	Secondary	Tertiary
Range of values	0.2	5–257	9–105	0.8–1.2	67	161–197	136–198	6–11
Two single determinations performed in one laboratory shall not differ from the true mean value by more than:	—	± 2.4%	± 6.3%	± 55%	± 4.3%	± 6.4%	± 4.3%	± 33%
Single determinations performed in different laboratories shall not differ from the true mean value by more than:	—	± 2.6%	± 8.3%	± 57%	± 4.4%	± 7.7%	± 4.3%	± 33%

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AOCS Official Method Tf 2b-64

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Primary, Secondary and Tertiary Amine Values of Fatty Amines, Indicator Method

DEFINITION

The primary amine value is the mg of potassium hydroxide equivalent to the primary amine basicity in 1 g of test sample. The secondary amine value is the mg of potassium hydroxide equivalent to the secondary amine basicity in 1 g of test sample. The tertiary amine value is the mg of potassium hydroxide equivalent to the tertiary amine basicity in 1 g of test sample.

SCOPE

Applicable to fatty amines. The method is not applicable to fatty amines containing reactive groups and structures, e.g., diethylene-(DETA)-diamides and 3,3'-iminobispropylamine-(DPTA)-diamides. Replaces Nb 4-62.

APPARATUS

1. Erlenmeyer flask—300 mL or beaker, 150 mL.
2. Magnetic stirrer—with Teflon™-coated stirring bar.

REAGENTS

1. Hydrochloric acid approximately 0.2 M and 0.1 M in isopropyl alcohol—accurately standardized with sodium carbonate, using 0.1% bromcresol green as the indicator, or standardized according to AOCS Specification H 14-52.
2. Salicylaldehyde—AR grade.
3. Phenyl isothiocyanate—AR grade.
4. Chloroform—ACS or AR grade (see Notes, *Caution* and 2).
5. Bromphenol blue indicator solution—0.2% in 99% isopropyl alcohol.

PROCEDURE

1. Determine the total amine value of AOCS Method Tf 1b-64.
2. Determine the size of the test sample from the following table:

Amine Value	Mass of Test Sample
≤ 10	5 g
> 10	2 g

3. Melt the test sample if it is not already liquid, mix thoroughly and accurately weigh the designated size of the test portion into two 250 mL beakers or 300 mL flasks. Mark them S and T.
4. Add 50 mL chloroform (see Notes, *Caution* and 2) to each flask (or beaker) and boil for 1 min on a hot plate to drive off any free ammonia that may be present.
5. Cool to room temperature. Add 3 mL of salicylaldehyde to test portion S, and allow to stand for 5 min. Add 5 mL of phenyl isothiocyanate to test portion T and allow to stand for 30 min.
6. Add 1 mL of indicator to each test portion and while swirling the flasks or by using the magnetic stirrer, titrate with 0.2 M HCl to the yellow end point (see Notes, 1). The yellow may fade back to green upon standing, but this is to be disregarded if the yellow color is bright and the addition of another mL of 0.2 M HCl does not change the yellow color.

CALCULATIONS

1. Total amine value of primary, secondary, and tertiary amine groups—determined by AOCS Method Tf 1b-64.
2. Titration S:

$$\text{Amine value of secondary and tertiary amine groups} = \frac{\text{mL} \times \text{M} \times 56.1}{\text{test portion mass, g}}$$

3. Titration T:

$$\text{Amine value of tertiary amine groups} = \frac{\text{mL} \times \text{M} \times 56.1}{\text{test portion mass, g}}$$

4. Primary amine value = Total amine value 1 – amine value 2

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5. Secondary amine value = Amine value 2 – amine value 3
6. Tertiary amine value = Amine value 3

PRECISION

At a confidence level of 95%, in the range of values indicated:

Type of Amine	Primary		Secondary	Tertiary	
Range of Values	0.1	5–256	8–105	0.9–1.5	68
Two single determinations performed in one laboratory shall not differ from the true mean value by more than:	± 180%	± 2.9%	± 6.3%	± 67.5%	± 3.1%
Two single determinations performed in different laboratories shall not differ from the true mean value by more than:	± 187%	± 2.9%	± 8.0%	± 176	± 10.4%

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

NUMBERED NOTES

1. In the case of titrating the tertiary content of a primary amine it is advisable to use a microburette as the titration will be extremely small. Errors in titration are greatly magnified because of the high molecular weight involved. 0.1 M HCl may be used if the quantity of tertiary amine is very low.
2. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less that those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

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Percent Primary, Secondary, and Tertiary Amines in Fatty Amines

DEFINITION

The percentages of primary, secondary, and tertiary amines in the test sample can be calculated from determinations of primary, secondary and tertiary amine values and percent non-amine.

SCOPE

Applicable to materials containing only fatty primary amines, difatty secondary amines, trifatty tertiary amines and non-amine components. This method will not work for blends such as a mixture of coco primary amines and tallow difatty secondary amines.

METHODS

Determine the primary, secondary, and tertiary amine values and percent non-amine by the following methods:

Primary, Secondary, and Tertiary Amine Values AOCS Method Tf 1a-64 and AOCS Method Tf 2a-64. Non-Amine AOCS Method Tw 1a-64.

CALCULATIONS

1. Correct the primary, secondary, and tertiary amine values for non-amine and convert all values to primary amine values from the following (see Notes, 1 for *):

$$\text{Corrected primary amine value} = \frac{\text{primary amine value} \times 100}{100 - \%\ \text{non-amine}}$$

$$\text{Corrected and converted secondary amine value} = \frac{\text{secondary amine value} \times 1.93^* \times 100}{100 - \%\ \text{non-amine}}$$

$$\text{Corrected and converted tertiary amine value} = \frac{\text{tertiary amine value} \times 2.86^*}{100 - \%\ \text{non-amine}}$$

2. The total corrected primary amine value is obtained by summing the corrected and converted amine values.

   Total corrected primary amine value = corrected primary amine value + corrected and converted secondary amine value + corrected and converted tertiary amine value.

3. Calculate the average molecular weight of primary amine.

$$\text{Average molecular weight of primary amine} = \frac{56{,}100}{\text{total corrected primary amine value}}$$

4. Calculate the average molecular weights of secondary and tertiary amine in the following manner:
   Average molecular weight of secondary amine = (2 × average molecular wt of primary amine) -17.
   Average molecular weight of tertiary amine = (3 × average molecular wt of primary amine) -34.
5. Calculate the percent primary amine, percent secondary amine, and percent tertiary amine using the originally determined amine values and the calculated average molecular weights (Calculations, 4):

$$\%\ \text{Primary amine} = \frac{\text{primary amine value} \times \text{average molecular wt of primary amine}}{561}$$

$$\%\ \text{Secondary amine} = \frac{\text{secondary amine value} \times \text{average molecular wt of secondary amine}}{561}$$

$$\%\ \text{Tertiary amine} = \frac{\text{tertiary amine value} \times \text{average molecular wt of tertiary amine}}{561}$$

NOTE

1. Conversion factors (*) are based upon the assumption that the average chain lengths of the alkyl chains in the monofatty, difatty, and trifatty amines are the same. This will be generally true, but will not be valid if dissimilar amines are blended after manufacture.

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AOCS

AOCS Official Method Tf 4a-77

Revised 2009

Primary Amine Composition By Gas Chromatography

DEFINITION

Trifluoracetyl derivatives of primary amines are prepared and separated using gas chromatography.

SCOPE

This method is applicable to animal and vegetable fatty primary amines having 6–24 carbon atoms. Saturated and unsaturated primary amines are separated by this method. The method is not suitable for undistilled primary amines unless they are of high purity.

APPARATUS

1. Oven or hot plate—capable of maintaining a temperature of 75–100°C.
2. Vials—2 mL and 5 mL, with screw-top Teflon™-lined caps.
3. Medicine droppers or disposable dropping pipets.
4. Syringe—10 µL Hamilton or equivalent, labeled in 1 µL increments, which are graduated to 0.1 µL.
5. Gas chromatograph having the minimum following characteristics:
   (a) Sample inlet port—capable of a constant temperature of 275°C.
   (b) Column oven—that can be programmed to at least 210°C, and kept at a constant temperature.
   (c) Detector—flameionization (FID) preferable, capable of being temperature regulated and held constant at 275°C.
   (d) Recorder—1 mV span, chart speed 0.5 in. per min.
6. GC column—6 ft (183 cm) by ⅛ in. (3.2 mm) o.d. or ¼ in. (6.3 mm) o.d. stainless steel. Packed with 15% Silar-7 on 80/100 mesh Gas Chrom Q (see Notes, 1).

REAGENTS

1. Chloroform—AR Grade (see Notes, *Caution* 2 and 3).
2. Trifluoracetic anhydride—Eastman Organics, or equivalent.
3. Gas supplies for chromatograph—all gases should have a minimum of 99.5 mol% purity.
4. Column packing—Silar 7, 15% by weight coated on 80/100 mesh Gas Chrom Q.
5. Test sample of individual primary amines of known purity—to serve as standards.

PROCEDURE

1. Melt the test sample if solid and mix thoroughly. Transfer approximately 5 drops (0.2–0.3 g, accurately weighed) of test portion to a 5 mL vial.
2. Add 1 mL of chloroform (see Notes, *Caution* and 3) and warm if necessary to dissolve the test portion. Mix thoroughly.
3. Transfer approximately 5 drops (0.1 g) of the test portion solution to a 2 mL vial.
4. Add 15 drops of trifluoracetic anhydride and cap immediately. Shake vigorously and allow to stand at least 5 min.
5. Add 5 drops of distilled water and shake to neutralize any remaining anhydride.
6. Inject approximately 2 µL of test portion of bottom layer. Dilution may be advisable dependent on sensitivity and noise level of the GC instrument. If necessary to dilute, add more chloroform (see Notes, *Caution* and 3) before drawing test portion.

GAS CHROMATOGRAPHIC CONDITIONS

1. A new column should be conditioned at 200–250°C for a minimum of 24 hr prior to use. Column flows should be optimized for the particular column size, usually 25–30 mL for ⅛ in. columns and 100–120 mL for ¼ in. column.
2. Sample inlet—275°C.
3. Detector temperature—275°C.
4. Column temperature—initial 150°C, programmed increase 3–4°C per min to 200°C.
5. Carrier gas—nitrogen or helium, with flow optimized by a Van Dempter plot of linear gas velocity vs. plate height for methyl stearate.
6. Hydrogen flow—adjust for maximum response of methyl stearate.
7. Air flow—optimize for maximum response as with hydrogen flow.

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CALCULATIONS

1. Identify the peaks by relative position on the chart. The amine derivatives appear in order of increasing number of carbon atoms and of increasing unsaturation. Identities are established using a known mixture of standards at the same conditions as for the test portions.
2. Determine the area for each peak. This may be accomplished by taking the height times the width at half height of each peak or by use of a mechanical or electronic integrator. Sum the areas of all the peaks and calculate the percentage represented by each:

$$\text{Area } \% = \frac{A_i \times 100}{TA}$$

Where—

A_i = area of individual peak

TA = sum of all peak areas

3. The flame detector (FID), if peaked for hydrogen flow vs. carrier gas flow, is relatively linear within a homologous series and as such, response factors are not normally required. For extremely accurate work, however, response factors may be required.
4. Instrument and column performance are monitored by noting the separation of the C18 and C18:1 peaks. If this remains adequate and the peaks in general show little or no tailing, the column can be considered satisfactory.

PRECISION

1. Two single determinations of major components greater than 5% performed in one laboratory shall not differ by more than 2.5%.
2. Two single determinations of major components greater than 5% performed in different laboratories shall not differ by more than 3.6%.

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

NUMBERED NOTES

1. The packing and/or packed columns are available from Alltech Associates, 2051 Waukegan Road, Deerfield, IL 60015 USA or Supelco, Inc., Bellefonte, PA 16823 USA.
2. Chloroform as a solvent for TFA derivatives may gradually strip the liquid phase from the front end of the GLC column.
3. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride and methylene chloride. Cyclohexane and isooctane may also be considered as alternative solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official recommendation by the AOCS Uniform Methods Committee.

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AOCS Recommended Practice Tf 5-91
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Composition of Dimer Acids by High-Performance Liquid Chromatography (HPLC)

DEFINITION

This analytical method describes the separation and quantification of monobasic, dibasic, and polybasic acids in "dimer acids" manufactured by the catalytic polymerization of fatty acids. The method utilizes HPLC and laser mass detection.

SCOPE

This analytical method is applicable to finished dimer acids derived from tall oil, oleic acid, and mixed vegetable oils, and dimer acids that have been bleached, distilled, or hydrogenated. The components of the dimer acids are separated according to their polarity by normal-phase HPLC. Historically, area percents from this method are within ±0.6% w/w of dimer acid standards whose compositions were determined by semipreparative HPLC (gravimetrically). Within the linear ranges of the components as described in this method, the response factors of the mono-, di-, and polybasic acids are assumed to be equal and, therefore, uncorrected area percents are reported (see Precision, 1).

APPARATUS

1. High-performance liquid chromatography system—capable of gradient mixing (e.g., Waters Powerline 600 or Hewlett-Packard 1040 system).
2. Column—an 8 µm axially compressed silica gel column (Rainin no. 80-100-G3), 4.6 mm i.d. × 25 cm.
3. Guard column—consisting of 5 µm axially compressed silica gel (Rainin no. 80-100-G3), 4.6 mm i.d. × 5 cm.
4. Autoinjector—capable of delivering a 30 µL injection accurate to within ±0.1 µL.
5. Temperature control module—capable of heating the column to 45°C (Waters TCM, or equivalent).
6. Detector—laser light-scattering mass detector from Varex Corporation (Model LLSD-MK II). The settings for the detector are: heater, 70°C; carrier gas (nitrogen), 55 mL/min (see Notes, 1).
7. Chromatography computer workstation or a suitable integrator—for data handling.
8. Volumetric flasks—10 mL.
9. Syringe filter—0.2 µm.

REAGENTS

Note—All solvents must be of HPLC-grade quality (see Notes, *Caution* and 2).

1. Cyclohexane—e.g., Baxter B & J catalog no. 053-4.
2. Isopropyl alcohol (IPA)—e.g., EM Science catalog no. PX1834-1.
3. Acetic acid—glacial, e.g., EM Science catalog no. AX0073-1.
4. Solvent A—99.8% cyclohexane and 0.2% glacial acetic acid.
5. Solvent B—84.8% cyclohexane, 15.0% IPA, and 0.2% acetic acid.
   Note—Prepare enough solvent B for 2 d of analyses, then discard.
6. Solvent C—86.3% cyclohexane, 13.5% IPA, and 0.2% glacial acetic acid.
7. Column conditioning solvent—0.75% IPA in cyclohexane containing 0.2% glacial acetic acid.

PREPARATION OF TEST SAMPLE AND STANDARD

1. This method requires that two dimer acid standards be used at least once each day to verify that the instrumentation is operating correctly. Primary standards are dimer acid samples whose compositions have been determined gravimetrically by semipreparative HPLC. See appendix, immediately following this method for gravimetric procedure for preparation of primary standards. Dimer acids intended for use as primary standards should be hydrogenated and/or distilled. Dimer primary standards are available from Henkel Corp., Emery Group, 4900 Este Avenue, Cincinnati, OH 45232 USA.

   See Table 1 for a comparison of results obtained in analysis of several dimer acids by both the gravimetric method and the laser mass detector method.
2. To prepare either the dimer acid standard solutions or the test solutions, weigh 0.500 ± 0.005 g of dimer acid into a tared 10 mL volumetric flask, dilute carefully to volume with solvent B (Reagents, 5), stopper, and shake for 4 min or longer, until the test sample is completely dissolved.

   Note—Particular care must be taken in this step because a poor dissolution will result in low polybasic acid results and high monobasic acid results. In addition, the refractive index of some dimer acids nearly equals that of solvent B, making

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assessment of complete dissolution a difficult task. After shaking, carefully examine the bottom of the flask for remains of any film of undissolved dimer acid. Continue shaking, if necessary, until the dimer acid is completely dissolved.

3. The prepared solutions should be transferred into a 5 mL syringe, filtered through a 0.2 µm HPLC filter into the autosampler containers, and capped.

Note—The standard/test sample solution lifetime is *24 hr.*

DETECTOR RESPONSE

1. The laser light-scattering mass detector was selected on the basis of universal response to each solute and the response of each solute being proportional to the mass of the solute present. In this application, it is very important that the response be proportional to the mass of each component, regardless of the functionality of the component, and that nonlinearized responses from the detector be compared to the results from the gravimetric analysis.

PROCEDURE

1. Condition the column, using the column conditioning solvent (Reagents, 7) (see initial setting for the gradient profile in Table 2), for 30 min after the column heater has been turned on.
2. Inject 30 µL of test sample (see Notes, 4) and begin the pump gradient (Apparatus, 1), using solvents A and B (Reagents, 4 and 5, respectively), according to Table 2.
3. Elute neutral components first with 0.75% IPA in 2.5 min. Neutral components can be detected, but quantitation may be difficult due to their volatility at detector heater temperature and carrier gas flow-rate employed. Commercial dimer acids analyzed by the gravimetric method were found to contain 0.08–0.2% by weight of neutral components, so the neutral content can be assumed to be negligible.
4. Elute monobasic acids with 0.75% IPA in 4.5–5.0 min.
5. Elute dibasic acids with 1.0% IPA in 11.5–13.5 min with the step profile at 2–4 min. The time window depends upon the dead volume of the HPLC system. e.g., Waters Powerline 600 has a dead volume of about 1.5 mL due to low-pressure mixing.
6. Elute polybasic acids with 13.5% IPA in 24.5 min with the step profile at 18.0 min. A smaller band (assumed to be a high molecular weight polymeric band) may occasionally elute after the primary polybasic band. The first band is assumed to contain primarily trimer acids, whereas the other band probably comprises tetramer and high molecular weight polymeric acids (see Fig. 1 for sample chromatogram).
7. At 25 min, linearly ramp the IPA strength from 13.5% IPA to 0.75% IPA over a 7 min period. Hold the IPA strength at 0.75% IPA from 32 min to 40 min to equilibrate the column so that it is ready for the next injection.
8. Chromatograph appearance: The peaks should be sharp with baseline separation (see Notes, 5 and Fig. 1). The elution times for the components should fall within the following time limits: monobasic acids 4.0 ± 0.3 min; dibasic acids 11.5–13.5 min; polybasic acids 24–25 min.

CALCULATIONS

1. Application of this methodology to the analysis of the dimer acids listed in the Scope of this method gives compositions with a relative error (worst case reported) per component of 15.4% for monobasic, 0.9% for dibasic, and 12.9% for polybasic acids, as determined by semipreparative HPLC gravimetric analysis. The expected accuracy of the method would be less than or equal to these values on a component basis. Using this basis, the response factors of the mono-, di-, and polybasic acids are assumed to be equal, and the area percents by the HPLC/laser mass dimer acid method are reported uncorrected. In addition, it is assumed that all components of the dimer acid samples to be analyzed via this method are eluted and detected under the conditions described. All neutral components are neglected, especially because their nebulization rates vary substantially from the mono-, di-, and polybasic acids.
2. Area percent calculations:
   monobasic % = (area of monobasic/total area) × 100
   dibasic % = (area of dibasic/total area) × 100
   polybasic % = (area of polybasic/total area) × 100
3. The accuracy of the method should be computed on a mono-, di-, and polybasic component basis as illustrated in the following equation:

$$\%\ \text{accuracy}\ \% = \frac{(A_{sc}\ \% - \ TC\%)}{TC\%}$$

Where—

A_{sc} = average of standard component (%)
TC = theoretical component (%)

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Also, the precision of the standard and test sample results should be reported on a component basis. Because the analysis time is long, no more than duplicate analyses are specified in this method. The precision should be reported as a % deviation from the average of the two runs:

$$\% \text{ precision} = \frac{(C_1 - C_2)}{AC_{1+2}}$$

Where—

C_1 = component value (%) from run 1

C_2= component value (%) from run 2

AC_{1+2} = average of components (%) from runs 1 and 2

Note— $(C_1 - C_2)$ must be expressed as an absolute value. If the accuracy of the experimental compositions of the standards differs by more than the relative error per component, as described in Table 1, from their respective theoretical compositions, or the standard or test sample duplicate run exceeds the respective component average by more than the relative error per component, either one or more instrumental problems exist or there are problems with the standard solutions (poor dissolution, old, etc.) (see Notes, 5).

PRECISION

1. Table 1 shows a comparison of results obtained with the gravimetric method (see Appendix) vs. the laser mass detector method (triplicate analyses in one laboratory).
2. Table 3 summarizes the results of a 1990 collaborative study of the laser mass detector method.

NOTES

Caution

Cyclohexane is flammable and a dangerous fire risk. It is moderately toxic by inhalation and skin contact. The TLV in air is 300 µg/g.

Isopropyl alcohol is flammable and a dangerous fire risk. The explosive limits in air are 2–12%. It is toxic by ingestion and inhalation. TLV in air is 400 µg/g.

NUMBERED NOTES

1. Settings for temperature of the heater and the carrier gas flow-rate allow tuning of the detector to achieve maximum sensitivity by minimizing solute evaporation of the analyte.
2. Solvents may be used without further purification.
3. If tailing problems are encountered with this gradient pump table method, an alternate gradient method could be used. If the instrument is capable of mixing three solvents, then the conditions in Table 4 can be used.
4. The linear ranges established were 20–70 µg for the monobasic acids, 200–2000 µg for the dibasic acids, and 20–250 µg for the polybasic acids. Higher levels of monobasic acids were not investigated. Based on an injection volume of 30 µL or 1500 µg of test sample, the lower end of the linear range would be 2.0% for the mono- and polybasic acids regardless of feedstock used or treatment, e.g., distillation and/or hydrogenation. However, some dimer acid samples contain less than 2% polybasic acids and, therefore, an injection volume of 40 µL or 2000 µg of total test sample would be needed. In this case, the only adjustment to the chromatography needed would be to equilibrate the column with 0.6% IPA instead of 0.75% IPA. This will ensure baseline resolution between the mono- and dibasic fractions. The increased loading on the column will neither saturate the detector nor affect the polybasic acid peak shape.

   Studies have shown that the guard module and column will remain effective for about six months of use with minimal gradient changes as specified in the method. No evidence of column degradation was observed after more than 200 injections.
5. Data handling: To make certain there is baseline resolution between separated bands, use a computer data system to expand the chromatogram from 0 to 25 millivolts. There should be a 1–2 millivolt difference between the baseline and the valley of the di- and polybasic bands. If the difference is greater than 1–2 millivolts, adjust the dibasic band so that it elutes earlier by increasing the IPA composition by 1% in the conditioning step. Follow this with 1% increase in IPA composition at the second step of the table. There should be a 2% difference between the initial conditions and the elution step for the dibasic bands (e.g., if initial conditions are 95% A and 5% B, the second step would be 93% A and 7% B).

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Table 1
Laser mass method vs. gravimetric method.

Feedstock[a]	Component	Gravimetric[b] wt%	Gravimetric[b] SD	Laser[c] Area %	Laser[c] SD	% Relative error
1	Mono	4.6	0.26	4.6	0.05	0
	Di	79.5	0.21	79.5	0.04	0
	Poly	15.4	0.31	16.0	0.13	4.0
2	Mono	4.1	0.09	3.7	0.02	9.8
	Di	78.6	0.07	79.2	0.19	0.8
	Poly	16.9	0.03	17.1	0.20	1.2
3	Mono	4.5	0.01	4.3	0.04	4.5
	Di	82.0	0.03	81.6	0.32	0.5
	Poly	13.5	0.03	14.1	0.28	0.5
4	Mono	3.9	0.19	3.3	0.01	15.4
	Di	89.6	0.21	90.4	0.11	0.9
	Poly	6.4	0.32	6.4	0.01	0.0
5	Mono	3.9	0.05	3.7	0.09	5.2
	Di	93.0	0.07	93.6	0.20	0.7
	Poly	3.1	0.12	2.7	0.01	12.9

[a]Feedstock key: 1, Empol-1018 (mixed vegetable oil); 2, Empol-1016 (oleic acid); 3, Versadyne-228 (tall oil); 4, Empol-1014 (distilled, mixed vegetable oil); 5, Empol-1008 (hydrogenated, distilled oleic acid).
[b]Gravimetric method, triplicate analysis
[c]Laser mass detector method, triplicate analysis..

Table 2
Gradient mixing and flow-rates for the resolution of dimer acid components[a].

Time, min	Flow, mL/min	A%	B%	Profile	% IPA
Initial	1.2	95	5	Linear	0.75
2–4.0	1.2	93	7	Step	1.0
18.0	1.2	10	90	Step	13.5
25.0	1.2	10	90	Linear	13.5
32.0	1.2	95	5	Linear	0.75
40.0	1.2	95	5	Linear	0.75

[a]See Notes, 3 and Table 4 for gradient option.

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Table 3
Dimer acid collaborative study of laser mass detector method, 1990.

Test sample	Component	x	Number of labs	RSD_r %	RSD_R %
1	Mono	3.5	8	3.5	21.8
	Di	93.9	7[b]	0.1	0.6
	Poly	2.6	7[b]	2.0	20.2
2	Mono	2.7	7[b]	2.7	8.2
	Di	94.7	7[b]	0.2	0.2
	Poly	2.7	7[b]	4.3	6.4
3	Mono	4.1	7[b]	2.1	13.1
	Di	81.4	8	0.4	2.8
	Poly	14.6	8	1.6	13.8
4	Mono	4.5	7[b]	1.5	7.3
	Di	81.0	7[b]	0.3	1.0
	Poly	14.5	7[b]	1.7	7.0
5	Mono	10.2	7[b]	1.5	7.4
	Di	77.8	7[b]	0.3	0.5
	Poly	12.0	8	1.1	10.1
6	Mono	3.1	6[b]	1.8	2.7
	Di	91.2	6[b]	0.3	0.5
	Poly	5.7	6[b]	3.5	19.6

[a]Test sample description: 1, Empol-1008 (oleic acid based, hydrogenated and distilled); 2, Empol-1061 (mixed vegetable oil, hydrogenated and distilled); 3, Empol-1016 (oleic acid based); 4, Empol-1018 (mixed vegetable oil); 5, Empol-1020 (tall oil); 6, Empol-1014 (mixed vegetable oil, distilled).
[b]One outlier removed.

Table 4
Altemate gradient method.

Time, min	Flow, mL/min	Solvent			Profile	% IPA
		A	B	C		
0	1.2	100	0	0	Step[a]	0.75
2.0	1.2	0	100	0	Step	1.0
17.0	1.2	0	0	100	Step	13.5
25.0	1.2	0	0	100	Step	13.5
32.0	1.2	100	0	0	Linear[b]	0.75
40.0	1.2	100	0	0	Linear	0.75

[a]Step: Go to solvent B immediately.
[b]Linear: Gradient changes one solvent to another over a time interval.

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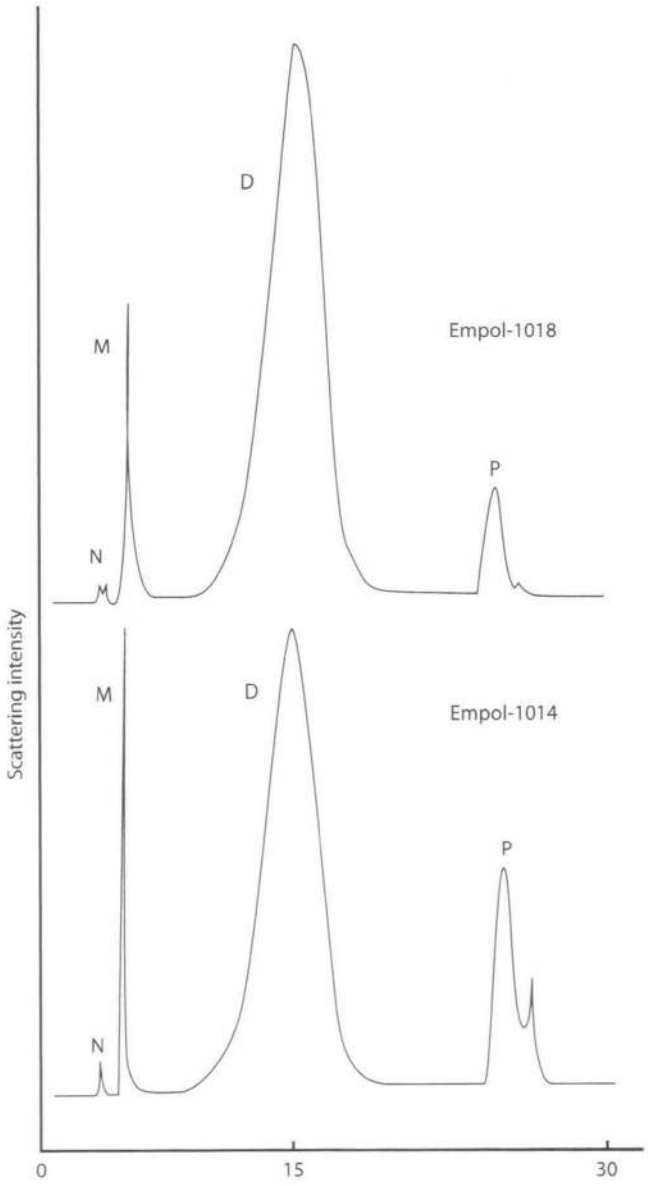

Figure 1. Chromatogram of Henkel Corporation, Emery Group samples of 1014 (distilled mixed vegetable oil) and 1018 (mixed vegetable oil) dimer acids. N, neutral components; M, monobasic components; D, dibasic components; P, polybasic components.

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Appendix Primary Standard Preparation/Gravimetric Procedure

DEFINITION

This analytical method describes the separation and gravimetric quantification of neutral, monobasic, dibasic, and polybasic acids in "dimer acids" manufactured by the catalytic polymerization of fatty acids. The method utilizes HPLC with ultraviolet detection.

SCOPE

This analytical procedure is applicable to dimer acids derived from tall oil, oleic, and mixed vegetable oils that are either distilled or nondistilled, hydrogenated, or nonhydrogenated. The components of the dimer acid are separated according to their polarity by normal-phase semipreparative HPLC with quantification by direct weighing of the collected vacuum-dried fractions.

APPARATUS

1. High-performance liquid chromatography system—capable of gradient mixing (e.g., Waters powerline 600 or Hewlett-Packard 1040 system).
2. Column—a 60A, 8 µm axially compressed silica gel column, 10 mm i.d. × 25 cm (Rainin catalog no. 83-111-C, with guard 10 mm i.d. × 5 cm, Rainin catalog no. 83-111-G).
3. Manual injector—with a 2 mL loop (e.g., Waters U6K or Rheodyne 6 port position valve with loop filler part).
4. Detector—UV, set for 254 nm (e.g., Waters 484 or 490 detector).
5. Fraction collector—minimum of six ports with $^{1}/_{16}$ in. (1.6 mm) tubing connections and capable of receiving a contract closure from an external source (e.g., Valco Instrument Company electronic actuator ten port position valve).
6. Data system—chromatography workstation capable of programming the fraction collector through contact closures.

REAGENTS

Note—All solvents must be of HPLC-grade quality (see Notes, *Caution*).

1. Cyclohexane—e.g., Baxter B & J catalog no. 053-4.
2. Isopropyl alcohol (IPA)—e.g., EM Science catalog no. PX1834-1.
3. Acetic acid—glacial, e.g., EM Science catalog no. AX0073-1.
4. Solvent A—99.5% cyclohexane, 0.3% IPA, and 0.2% acetic acid.
5. Solvent B—89.8% cyclohexane, 10% IPA, and 0.2% acetic acid.

PREPARATION OF TEST SAMPLE

1. Weigh 0.50 g of test sample into a 10 mL volumetric flask and dilute to the mark with solvent A (Reagents, 4). Sonication or vigorous shaking is required to completely dissolve the sample. The test sample should be filtered through a 0.25 µm HPLC syringe filter prior to injection.

   Note—The test solution lifetime is *24 hr.*

PROCEDURE

1. Condition the column with 100% solvent A (Reagents, 4).
2. Inject 2 mL of the test solution (overfill the loop, e.g., fill the syringe with 3.0 mL) and begin the gradient pump (see Table 5).
3. Trigger the inject start signal to activate the external events (see Table 6) that will control the fraction collector from the data station or pump control module. This is normally part of a method and would automatically start after the injection (e.g., Powerline 600).
4. The fractions are to be collected in thoroughly clean 250 mL beakers and placed in hood.

   Note—Handle beakers with tongs.
5. Concentrate the fractions with a steady stream of nitrogen while keeping the beaker warm (70°C) (e.g., beakers may be placed in a sand box on a steam table in the hood with a thermometer embedded in the sand).
6. After the volume of the test solution has been reduced to ≤5 mL, remove the nitrogen and add 2 mL of methylene chloride, rinse the sides of the beaker, swirl and transfer via glass pasteur pipet to a 10 mL beaker.

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Note—The 10 mL beakers should be clean, dried in vacuum oven at 70°C for 30 min and allowed to cool to room temperature in a desiccator. Handle beakers *with tongs only,* and weigh them on a five place balance.

7. Add another 2 mL of methylene chloride, rinse the sides of the beaker and transfer to the 10 mL beaker. Repeat the washing one more time, and then evaporate the solvent from the 10 mL beakers with a steady stream of nitrogen. After all the solvent appears to be evaporated, transfer *with tongs* to the vacuum oven set at 70°C for 30 min.
8. Cool the beakers in a desiccator for 30 min and weigh.
9. Repeat Procedure steps 1–8 two more times to give a total of three runs per test sample.
10. See Figure 2 for test sample chromatogram.

METHOD PERFORMANCE

1. Table 7 summarizes the results of a 1990 collaborative study of the gravimetric analysis technique.

NOTES

Caution

Cyclohexane is flammable and a dangerous fire risk. It is moderately toxic by inhalation and skin contact. The TLV in air is 300 ppm.

Isopropyl alcohol is flammable and a dangerous fire risk. The explosive limits in air are 2–12%. It is toxic by ingestion and inhalation. TLV in air is 400 ppm.

Table 5
Gradient mixing and flow-rates for the resolution of dimer acid components for gravimetric analysis.

Time, min	Flow, mL/min	%A	%B	Profile	%IPA
Initial	5.0	100	0	Linear	0.3%
6.0	5.0	100	0	Linear	0.3%
6.1	5.0	100	0	Linear	0.3%
36.0	5.0	95	5	Linear	3.4%
37.0	5.0	0	100	Linear	10.0%
47.0	5.0	0	100	Linear	10.0%
55.0	5.0	100	0	Linear	10.0%
65.0	5.0	100	0	Linear	0.3%

Table 6
Conditions for collecting dimer acid fractions for gravimetric analysis.

Time	Action	Position	Direction
0.0	Off	1	Waste
6.0	On	2	Collect neutrals fraction
8.5	On	3	Collect monobasic fraction
12.5	On	4	Collect dibasic fraction
38.0	On	5	Collect polybasic fraction
55.0	On	1	Waste

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Table 7
Gravimetric analysis.[a]

Dimer acid feedstock[b]	Component	wt%	SD
1	Mono	4.6	0.26
	Di	79.5	0.21
	Poly	15.4	0.31
2	Mono	4.1	0.09
	Di	78.6	0.07
	Poly	16.9	0.03
3	Mono	4.5	0.01
	Di	82.0	0.03
	Poly	13.5	0.03
4	Mono	3.9	0.19
	Di	89.6	0.21
	Poly	6.4	0.32
5	Mono	3.9	0.05
	Di	93.0	0.07
	Poly	31	0.12

[a]Gravimetric analysis was done in triplicate, with the average values reported.
[b]Test sample description: 1, Empol-1018 (mixed vegetable oil); 2, Empol-1016 (oleic acid); 3, Versadyne-228 (tall oil); 4, Empol-1014 (distilled mixed vegetable oil); 5, Empol-1008 (hydrogenated/distilled oleic acid).

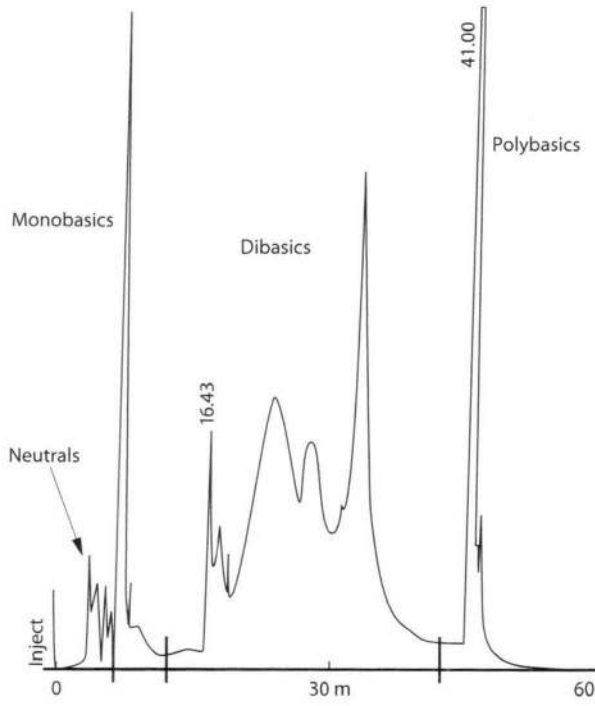

Figure 2. Typical chromatogram (gravimetric method) of Empol-1014 dimer acid. The large marks through the base-line denote when the fractions were collected.

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AOCS Official Method Tg 1-64

Revised 2012

Iodine Value, Wijs Method

DEFINITION

The iodine value is a measure of the unsaturation of industrial oils and derivatives and is expressed in terms of the number of cg of iodine absorbed per g of test sample (% iodine absorbed).

SCOPE

Applicable to normal industrial oils and derivatives, such as natural or synthetic drying oils and their fatty acids. For a specific class or product, refer to the AOCS method for testing the particular product (see Notes, 1). Replaces Ka 9-51.

APPARATUS

1. Glass-stoppered bottles or wide mouth Erlenmeyer flask—500 mL.
2. Glass-stoppered volumetric flask—conforming to National Institute of Standards & Technology (NIST) tolerances and accurately calibrated to contain 1000 mL.
3. Pipets—5, 20, and 25 mL.
4. Filter paper—Whatman #541, or equivalent.

REAGENTS

1. Glacial acetic—ACS grade. The permanganate test should be applied to be sure that this specification is met.
   (a) Permanganate test—dilute 2 mL of the acid with 10 mL of distilled water and add 0.1 mL of 0.2 M $KMnO_4$. The pink color must not be entirely discharged within 2 hr.
2. Potassium iodide—ACS or AR grade.
3. Chlorine—99.8% (see Notes, *Caution*). Satisfactory commercial grades are available in cylinders, but gas must not be dried by passing through sulfuric acid (sp. gr. 1.84) before introducing into the iodine solution.

   Note—Prepared Wijs (see Notes, *Caution* and 2) solution is available commercially and may be used in place of laboratory-prepared solution.
4. Soluble starch—tested for sensitivity. Make a paste with 1 g of starch and a small amount of cold distilled water. Add, while stirring, 200 mL of boiling water. Place 5 mL of this solution in 100 mL of water and add 0.05 mL of 0.1 M iodine solution. The deep blue color produced must be discharged by 0.05 mL of 0.1 M sodium thiosulfate.
5. Potassium dichromate—ACS grade. The potassium dichromate is finely ground and dried to constant weight at about 110°C before using.

   Note—A standard sample of potassium dichromate with a certificate of analysis may be obtained from the National Institute of Standards Testing in Washington, D.C. This sample, or equivalent, is strongly recommended for the primary standard for this method. Treat as directed in the certificate of analysis accompanying the sample.
6. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)—ACS grade.
7. Iodine—ACS grade.
8. Carbon tetrachloride—ACS grade (see Notes, *Caution*, Recommendations and 5).
9. Wijs solution, AR grade (see Notes, *Caution*).

SOLUTIONS

1. Potassium iodine solution—dissolve 150 g in distilled water and make up to 1 L.
2. Starch indicator solution—make a homogeneous paste of 10 g of potato starch in cold distilled water. Add to this 1 L of boiling distilled water, stir rapidly and cool. Salicylic acid (1.25 g/L) may be added to preserve the indicator. If long storage is required, the solution must be kept in a refrigerator at 4–10°C (40–50°F). Fresh indicator must be prepared when the end point of the titration from blue to colorless fails to be sharp. If stored under refrigeration, the starch solution should be stable for about 2–3 wk.
3. Sodium thiosulfate solution, 0.1 M—dissolve 24.9 g of sodium thiosulfate in distilled water and dilute to 1 L.
   (a) Standardization—weigh 0.16–0.22 g of finely ground and dried potassium dichromate into a 500 mL flask or bottle by difference from a weighing bottle. Dissolve in 25 mL of water, add 5 mL of hydrochloric acid, 20 mL of potassium iodine solution and rotate to mix. Allow to stand for 5 min and then add 100 mL of distilled water. Titrate with sodium thiosulfate solution, shaking continuously until yellow color has almost disappeared. Add 1–2 mL of starch indicator and continue the titration, adding the thiosulfate solution slowly until the blue color has just disappeared. The strength of the sodium thiosulfate solution is expressed in terms of its molarity.

$$\text{Molarity of } Na_2S_2O_3 \text{ solution} = \frac{20.394 \times \text{mass of } K_2Cr_2O_7, \text{ g}}{\text{volume, mL of sodium thiosulfate solution}}$$

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4. Wijs solution (see Notes, *Caution*) can be purchased (see Reagents) or prepared in the laboratory. Dissolve 13.0 g of iodine in 1 L glacial acetic acid. Gentle heating may be necessary to promote solution. Cool remove a small quantity (100–200 mL) and set aside in a cool place for future use. Pass dry chlorine gas into the iodine solution until the original titration is not quite double. A characteristic color change takes place in the Wijs solution when the desired amount of chlorine has been added. This may be used to assist in judging the end point. A convenient procedure is to add a small excess of chlorine and immediately bring back to the desired titration by addition of some of the original iodine solution which was taken out at the beginning. The original solution and finished Wijs solution are both titrated with $Na_2S_2O_3$ solution as directed in Procedure, 6 and 7. The Wijs solution may be prepared from commercial iodine monochloride as follows:
   (a) Stock Solution—Add 317 ± 0.1 g of iodine monochloride to 1 L of glacial acetic acid and filter through Whatman #541 filter paper or equivalent into a clean and dry actinic glass bottle. Filter rapidly to prevent contamination with moisture and store in a cool place. Discard the solution if a precipitate forms on standing.
   (b) Wijs Solution—Using a graduated cylinder pour 117.0 ± 0.1 mL of the stock solution into a standard 5 lb bottle of glacial acetic acid and mix well by shaking.
5. The I/Cl ratio of the Wijs solution shall be within the limits of 1.10 ± 0.1. The procedure for determining the ratio is as follows:
   (a) Iodine content—
      (1) Pour 150 mL of saturated chlorine water into a 500 mL Erlenmeyer flask and add a few glass beads.
      (2) Pipet 5 mL of the Wijs solution into the flask containing the saturated chlorine water. Shake, and heat to boiling.
      (3) Boil briskly for 10 min, cool and add 30 mL of 2% sulfuric acid and 15 mL of 15% potassium iodide solution.
      (4) Mix well, and titrate immediately with 0.1 M sodium thiosulfate solution to a starch end point.
   (b) Total halogen content—
      (1) Pour 150 mL of recently boiled distilled water into a clean, dry 500 mL Erlenmeyer flask.
      (2) Add 15 mL of 15% potassium iodine solution.
      (3) Pipet 20 mL of Wijs solution into the flask, and mix well.
      (4) Titrate with 0.1 M sodium thiosulfate solution to a starch end point.

CALCULATIONS

$$\text{Halogen Ratio } R = \frac{2A}{3B - 2A}$$

Where—

A = volume of titrant, mL, of iodine content as mL sodium thiosulfate

B = volume of titrant, mL, of total halogen content as mL sodium thiosulfate

PROCEDURE

1. Melt the test sample, if it is not already liquid, and filter through filter paper to remove any solid impurities and the last traces of moisture. The temperature during melting and filtering should not exceed the melting point of the test sample by more than 10°C. The test sample must be absolutely dry.
   Note-All glassware must be absolutely clean and dry.
2. Weigh the test sample (see Notes, 3) accurately into a 500 mL flask or bottle to which has been added 20 mL of carbon tetrachloride (see Notes, *Caution*) or other solvent (see Notes, Recommendations and 5). The weight of the test sample must be such that there will be an excess of Wijs solution of 100–150% over the amount absorbed. Table 1 is a guide to the size of test portion to weight.
3. Pipet the 25 mL of Wijs solution into flask containing the test sample, stopper the flask, and swirl to ensure an intimate mixture.
4. Prepare and conduct at least two blank determinations with each group of test samples simultaneously and similar in all respects to the test samples.
5. Store the flasks in a dark place for 30 min at a temperature of 25 ± 5°C. For conjugated oils such as tung, oiticica or dehydrated castor oil, allow the absorption to proceed for 1 hr at 25 ± 1°C.
6. Remove the flasks from storage and add 20 mL of KI solution, followed by 100 mL of distilled water.
7. Titrate with 0.1 M $Na_2S_2O_3$ solution, adding it gradually and with constant and vigorous shaking (see Notes, 4). Continue the titration until the yellow color has almost disappeared. Add 1–2 mL of starch indicator solution and continue the titration until the blue color has just disappeared.

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CALCULATION

$$\text{Iodine Value} = \frac{(B - S) \times M \times 12.69}{\text{mass of test portion, g}}$$

Where—

B = volume of titrant, mL of blank

S = volume of titrant, mL of test portion

M = molarity of $Na_2S_2O_3$ solution

NOTES

Caution

Carbon tetrachloride is a known carcinogen. It is toxic by ingestion, inhalation and skin absorption. It is a narcotic. It should not be used to extinguish fires. It decomposes to phosgene gas at high temperature. The TLV is 5 ppm in air. A fume hood should be used at all times when using carbon tetrachloride.

Chlorine is a poisonous gas. The TLV is 1 ppm in air. It is a strong oxidizing agent and should not be allowed to come in contact with organic materials, hydrogen, powdered metals and reducing agents. A fume hood should be used at all times when using chlorine.

Wijs solution causes severe burns and vapors can cause lung and eye damage. Use of a fume hood is recommended. Wijs solution without carbon tetrachloride is available commercially.

Potassium dichromate is toxic by ingestion and inhalation. There is sufficient evidence in humans for the carcinogenicity of chromium [+6], in particular lung cancer. It is a strong oxidizing agent and a dangerous fire risk in contact with organic chemicals.

RECOMMENDATIONS

Cyclohexane has been proposed as a replacement for carbon tetrachloride in the iodine value method—see AOCS Recommended Practice Cd 1-87 (89), AOCS Official Method Cd 1d-92 and *JAOCS 65:*745 (1988). Isooctane has been reported to be a suitable replacement for chloroform in the peroxide value method, and therefore could be tried as a substitute for carbon tetrachloride in the iodine value method. These recommendations do not represent official approval by the AOCS Uniform Methods Committee. Laboratories that use these solvents may report their findings to the AOCS Technical Director.

NUMBERED NOTES

1. When the iodine value is determined on materials having conjugated systems, the result is not a measure of total unsaturation, but rather is an empirical value indicative of the amount of unsaturation present. Reproducible results are obtained which afford a comparison of total unsaturation. When the iodine value is required on the fatty acids of natural and synthetic drying oils, the preparation and separation are performed as directed in AOCS Official Method Cd 6-38.
2. All Wijs solutions are sensitive to temperature, moisture and light. Store in a cool and dark place and never allow to come to a temperature above 25-30°C.
3. In the case of dehydrated castor oils, weigh 0.11–0.13 g.
4. Mechanical stirring is satisfactory for agitating during the addition of thiosulfate.
5. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and cyclohexaneacetic acid may also be considered as alternate solvents. See AOCS Methods Cd 1b-87 and Cd 1d-92.

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Table 1
Test portion mass, g

Iodine Value	Normal oils 100–150% excess of reagent	Conjugated oils 115–135% excess of reagent
< 3	10 –10	
3	8.4613–10.576	
5	5.0770–6.3460	
10	2.5384–3.1730	
20	0.8461–1.5865	
40	0.6346–0.7935	
60	0.4321–0.5288	
80	0.3175–0.3969	0.3377–0.3691
90	0.2822–0.3528	0.3002–0.3281
100	0.2540–0.3175	0.2702–0.2953
110	0.2309–0.2886	0.2456–0.2684
120	0.2117–0.2646	0.2252–0.2461
130	0.1954–0.2442	0.2078–0.2271
140	0.1814–0.2268	0.1930–0.2109
150	0.1693–0.2116	0.1801–0.1969
160	0.1587–0.1984	0.1689–0.1846
170	0.1494–0.1868	0.1589–0.1737
180	0.1411–0.1764	0.1501–0.1640
190	0.1337–0.1671	0.1422–0.1554
200	0.1270–0.1587	0.1351–0.1476
210	0.1210–0.1547	0.1287–0.1406
220	0.1155–0.1443	0.1228–0.1374

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AOCS Official Method Tg 1a-64

Revised 2012

Iodine Value of Fatty Acids, Wijs Method

DEFINITION

The iodine value is a measure of the unsaturation of fatty acids and is expressed in terms of the number of g of iodine absorbed per 100 g of test sample (% iodine absorbed).

SCOPE

Applicable to all normal fatty acids (see Notes, 1).

APPARATUS

1. Glass-stoppered bottles or iodine flasks or wide mouth Erlenmeyer flask—500 mL.
2. Glass-stoppered volumetric flask—conforming to National Institute of Standards & Technology (NIST) tolerances and accurately calibrated to contain 1000 mL.
3. Pipets—5, 20, and 25 mL.
4. Filter paper—Whatman #541, or equivalent.

REAGENTS (See Numbered Notes)

1. Glacial acetic—ACS grade. The permanganate test should be applied to be sure that this specification is met.
   (a) Permanganate test—dilute 2 mL of the acid with 10 mL of distilled water and add 0.1 mL of 0.2 M N $KMnO_4$. The pink color must not be entirely discharged within 2 hr.
2. Potassium iodide—ACS or AR grade.
3. Chlorine—99.8% (see Notes, *Caution*). Satisfactory commercial grades are available in cylinders, but gas must not be dried by passing through sulfuric acid (sp. gr. 1.84) before introducing into the iodine solution.

   Note—Prepared Wijs (see Notes, *Caution* and Numbered Notes, 2) solution is available commercially and may be used in place of laboratory-prepared solution.
4. Soluble starch—tested for sensitivity. Make a paste with 1 g of starch and a small amount of cold distilled water. Add, while stirring, 200 mL of boiling water. Place 5 mL of this solution in 100 mL of water and add 0.05 mL of 0.1 M iodine solution. The deep blue color produced must be discharged by 0.05 mL of 0.1 M sodium thiosulfate.
5. Potassium dichromate—ACS grade. The potassium dichromate should be finely ground and dried to constant weight at about 110°C before using.

   Note—A standard sample of potassium dichromate with a certificate of analysis may be obtained from the NIST in Washington, D.C. This sample, or equivalent, is strongly recommended for the primary standard for this method. Treat as directed in the certificate of analysis accompanying the sample.
6. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)—ACS grade.
7. Iodine—ACS grade.
8. Cyclohexane-acetic acid solution—1:1 (v/v). Make fresh daily (see Recommendations).
9. Wijs solution, AR grade (see Notes, *Caution*).

SOLUTIONS

1. Potassium iodine solution—dissolve 150 g in distilled water and make up to 1 L.
2. Starch indicator solution—make a homogeneous paste of 10 g of potato starch in cold distilled water. Add to this 1 L of boiling distilled water, stir rapidly and cool. Salicylic acid (1.25 g/L) may be added to preserve the indicator. If long storage is required, the solution must be kept in a refrigerator at 4–10°C (40–50°F). Fresh indicator must be prepared when the end point of the titration from blue to colorless fails to be sharp. If stored under refrigeration, the starch solution should be stable for about 2–3 wk.
3. Sodiu.m thiosulfate solution, 0.1 M—dissolve 24.9 g of sodium thiosulfate in distilled water and dilute to 1 L.
   (a) Standardization—weigh 0.16–0.22 g of finely ground and dried potassium dichromate into a 500-mL flask or bottle by difference from a weighing bottle. Dissolve in 25 mL of water, add 5 mL of hydrochloric acid, 20 mL of potassium iodine solution and rotate to mix. Allow to stand for 5 min and then add 100 mL of distilled water. Titrate with sodium thiosulfate solution, shaking continuously until yellow color has almost disappeared. Add 1–2 mL of starch indicator and continue the titration, adding the thiosulfate solution slowly until the blue color has just disappeared. The strength of the sodium thiosulfate solution is expressed in terms of its molarity.

$$\text{Molarity of } Na_2S_2O_3 \text{ solution} = \frac{20.394 \times \text{mass of } K_2Cr_2O_7, \text{ g}}{\text{volume, mL of sodium thiosulfate solution}}$$

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4. Wijs solution (see Notes, *Caution*) can be purchased (see Reagents, 3, 9) or prepared in the laboratory. Dissolve 13.0 g of iodine in 1 L glacial acetic acid. Gentle heating may be necessary to promote solution. Cool, remove a small quantity (100–200 mL) and set aside in a cool place for future use. Pass dry chlorine gas into the iodine solution until the original titration is not quite double. A characteristic color change takes place in the Wijs solution when the desired amount of chlorine has been added. This may be used to assist in judging the end point. A convenient procedure is to add a small excess of chlorine and immediately bring back to the desired titration by addition of some of the original iodine solution which was taken out at the beginning. The original solution and finished Wijs solution are both titrated with $Na_2S_2O_3$ solution as directed in Procedure, 6 and 7. The Wijs solution may be prepared from commercial iodine monochloride as follows:
   (a) Stock solution—Add 317 ± 0.1 g of iodine monochloride to 1 L of glacial acetic acid and filter through Whatman #541 filter paper or equivalent into a clean and dry actinic glass bottle. Filter rapidly to prevent contamination with moisture and store in a cool place. Discard the solution if a precipitate forms on standing.
   (b) Wijs solution—Using a graduated cylinder pour 117.0 ± 0.1 mL of the stock solution into a standard 5 lb bottle of glacial acetic acid and mix well by shaking.
5. The I/Cl ratio of the Wijs solution shall be within the limits of 1.10 ± 0.1. The procedure for determining the ratio is as follows:
   (a) Iodine content—
      (1) Pour 150 mL of saturated chlorine water into a 500 mL iodine flask and add a few glass beads.
      (2) Pipet 5 mL of the Wijs solution into the flask containing the saturated chlorine water. Shake, and heat to boiling.
      (3) Boil briskly for 10 min, cool and add 30 mL of 2% sulfuric acid and 15 mL of 15% potassium iodide solution.
      (4) Mix well, and titrate immediately with 0.1 M sodium thiosulfate solution to a starch end point.
   (b) Total halogen content—
      (1) Pour 150 mL of recently boiled distilled water into a clean, dry 500-mL iodine flask.
      (2) Add 15 mL of 15% potassium iodine solution.
      (3) Pipet 20 mL of Wijs solution into the flask, and mix well.
      (4) Titrate with 0.1 M sodium thiosulfate solution to a starch end point.

PROCEDURE

1. Melt the test sample, if it is not already liquid, and filter through filter paper to remove any solid impurities and the last traces of moisture. The temperature during melting and filtering should not exceed the melting point of the test sample by more than 10°C. The test sample must be absolutely dry.
   Note—All glassware must be absolutely clean and completely dry.
2. Weigh the test sample (see Notes, 3) accurately into a 500 mL flask or bottle to which has been added 15 mL of cyclohexane:acetic acid, 1:1 (v/v). The weight of the test sample must be such that there will be an excess of Wijs solution of 100–150% over the amount absorbed. Table 1 is a guide to the size of the test sample to weight.
3. Pipet the 25 mL of Wijs solution into the flask containing the test sample, stopper the flask and swirl to ensure an intimate mixture.
4. Prepare and conduct one blank determination with each group of test samples simultaneously and similar in all respects to the test samples.
5. Store the flasks in a dark place for 30 min at a temperature of 25 ± 5°C (see Notes, 4).
6. Remove the flasks from storage and add 20 mL of KI solution, followed by 100 mL of distilled water added to the lip of the iodine flasks to absorb any iodine that may leak past the stoppers while they are waiting to be titrated.
7. Titrate with 0.1 M $Na_2S_2O_3$ solution, adding it gradually and with constant and vigorous shaking (see Notes, 5). Continue the titration until the yellow color has almost disappeared. Add 1–2 mL of starch indicator solution and continue the titration until the blue color has just disappeared.

CALCULATION

$$\text{Iodine value} = \frac{(B - S) \times M \times 12.691}{\text{mass of test portions, g}}$$

Where—

B = volume of titrant, mL of blank

S = volume of titrant, mL of sample

M = molarity of $Na_2S_2O_3$ solution

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PRECISION

At a confidence level of 95%:

	Fatty acids	
Materials:	Soybean Linseed	Tallow Coconut
Range of values:	130–200	7–55
1. Two single determinations performed in one laboratory should not differ from the mean value by more than:	± 1.8%	± 4.6%
2. Single determinations performed in different laboratories shall not differ from the mean value by more than:	± 2.1%	± 5.2%

NOTES

Caution

Chlorine is a poisonous gas. The TLV is 1 ppm in air. It is a strong oxidizing agent and should not be allowed to come in contact with organic materials, hydrogen, powdered metals and reducing agents. A fume hood should be used at all times when using chlorine.

Wijs solution causes severe burns and vapors can cause lung and eye damage. Use of a fume hood is recommended. Wijs solution without carbon tetrachloride is available commercially.

RECOMMENDATIONS

Cyclohexane has been proposed as a replacement for carbon tetrachloride in the iodine value method—see AOCS Recommended Practice Cd 1b-87 and *JAOCS 65*:745 (1988). Cyclohexane-acetic acid, 1:1, v/v has been shown to be a suitable replacement for carbon tetrachloride—see AOCS Official Method Cd 1d-92.

NUMBERED NOTES

1. When the iodine value is determined on materials having conjugated systems, the result is not a measure of total unsaturation, but rather is an empirical value indicative of the amount of unsaturation present. Reproducible results are obtained which afford a comparison of total unsaturation. When the iodine value is required on the fatty acids of natural and synthetic drying oils, the preparation and separation are performed as directed in AOCS Official Method Cd 6-38.
2. All Wijs solutions are sensitive to temperature, moisture and light. Store in a cool and dark place and never allow to come to a temperature above 25–30°C.
3. In the case of dehydrated castor oil fatty acids, weigh 0.11–0.13 g.
4. Store test portions with an iodine value greater than 150 for 1 hr in the dark at 25 ± 5°C.
5. Mechanical stirring is satisfactory for agitating during the addition of thiosulfate.
6. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied by the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

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TABLE 1
Test portion mass, g

Iodine Value	Normal oils 100–150% excess of reagent	Conjugated oils 115–135% excess of reagent
< 3	10 –10	
3	8.4613–10.576	
5	5.0770–6.3460	
10	2.5384–3.1730	
20	0.8461–1.5865	
40	0.6346–0.7935	
60	0.4321–0.5288	
80	0.3175–0.3969	0.3377–0.3691
90	0.2822–0.3528	0.3002–0.3281
100	0.2540–0.3175	0.2702–0.2953
110	0.2309–0.2886	0.2456–0.2684
120	0.2117–0.2646	0.2252–0.2461
130	0.1954–0.2442	0.2078–0.2271
140	0.1814–0.2268	0.1930–0.2109
150	0.1693–0.2116	0.1801–0.1969
160	0.1587–0.1984	0.1689–0.1846
170	0.1494–0.1868	0.1589–0.1737
180	0.1411–0.1764	0.1501–0.1640
190	0.1337–0.1671	0.1422–0.1554
200	0.1270–0.1587	0.1351–0.1476
210	0.1210–0.1547	0.1287–0.1406
220	0.1155–0.1443	0.1228–0.1374

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AOCS

AOCS Official Method Tg 2a-64

Reapproved 2009

Iodine Value of Fatty Amines, Modified Wijs Method

DEFINITION

The iodine value is a measure of the unsaturation of the alkyl group or groups and is expressed in terms of the number of cg of iodine absorbed per g of test sample (% iodine absorbed).

SCOPE

Applicable to all fatty amines, fatty diamines, and fatty amidoamines except those containing conjugated unsaturation (See Notes, 1). Replaces Nb 5-62.

APPARATUS

1. The equipment is exactly the same as described in AOCS Official Method Tg 1-64.

REAGENTS

The reagents are the same as described in AOCS Official Method Tg 1-64, under Reagents, with the following additions:

1. Chloroform—ACS or AR grade (see Notes, *Caution*, Recommendations and 5).
2. Mercuric acetate—ACS or AR grade (see Notes, *Caution* and 2).

SOLUTIONS

The solutions (see Notes, 2) are the same as described in AOCS Official Method Tg 1-64, under Solutions, with the following addition:

1. Mercuric acetate—dissolve 2.5 g of mercuric acetate in glacial acetic acid and make up to 100 mL.

PROCEDURE

1. Melt the test sample, if it is not already liquid, and filter through filter paper to remove any solid impurities and the last traces of moisture. The temperature during melting and filtering should not exceed the melting point of the test sample by more than 10°C. The test sample must be absolutely dry.

   Note—All glassware must be absolutely clean and dry.
2. Accurately weigh the test sample into a 500 mL flask according to the following guide:

Iodine Value	Test Sample Mass, g
<10	1.5
20	0.8461–2.0576
40	0.6346–0.7935
60	0.4321–0.5288
80	0.3175–0.3966
90	0.2822–0.3528
100	0.2538–0.3173
110	0.2309–0.2880
120	0.2115–0.2646
130	0.1954–0.2646
140	0.1813–0.2266
160	0.1587–0.1983
180	0.1410–0.1762
200	0.1269–0.1586

3. Add 20 mL of glacial acetic acid.
4. If a test sample does not go into solution, the flask can be warmed slightly at this point, until test sample is dissolved, but the flask must be allowed to cool to room temperature before proceeding to next step.
5. Pipet 25 mL of iodine solution into the test sample flask, allow pipet to drain in the same manner for both the test samples and the blanks.
6. Add 10 mL of mercuric acetate solution (see Notes, Caution and 2) directly after adding the iodine solution.

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7. Stopper the flask immediately and moisten the stopper with the potassium iodide solution so as to prevent the loss of iodine or chlorine, but guard against the use of a quantity sufficient to run down the inside of the flask. Swirl flask to obtain good mixture.
8. Prepare and conduct at least 2 blanks with each group of test sample simultaneously and similar in all respects to the test samples.
9. Store the flask in a dark place for 3 min (see Notes, 3) at a temperature of 25 ± 5°C. Swirl the flask occasionally, being careful not to allow the solution to crawl up the side of the flask over halfway.
10. Remove the flask from storage, add 20 mL of potassium iodide solution, 50 mL of distilled water, and 30 mL of chloroform.
11. Titrate with 0.1 M thiosulfate solution adding it gradually and with constant and vigorous shaking (see Notes, 4) until the yellow color has almost disappeared.
12. Add 2 mL of starch indicator solution rinsing the neck of the flask therewith, and continue the titration until the blue to brown color begins to disappear. Near the end of the titration it is necessary to shake violently, with the stopper inserted, in order to free any iodine that is dissolved so that it may be taken up with the potassium iodine solution. Only at this stage is the stopper allowed to come into contact with the liquid. Titrate to a colorless end point.

CALCULATIONS

$$\text{Iodine Value} = \frac{(B - S) \times M \times 12.69}{\text{mass of test portion, g}}$$

Where—

B = volume of titrant, mL of blank
S = volume of titrant, mL of sample
M = molarity of $Na_2S_2O_3$ solution

PRECISION

	Amines	Amido Amines	Amido Amines	Diamines
At a confidence level of 95%, in the range of values of:	3–81	2–3	73–79	8–71
1. Two single determinations performed in one laboratory should not differ from the mean value by more than:	± 4.6%	± 28%	± 9.5%	± 8.3%
2. Single determinations performed in different laboratories shall not differ from the mean value by more than:	± 11.3%	± 55%	± 12.4%	± 13.1%

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Mercury and mercury compounds are highly toxic by ingestion, inhalation, and skin absorption. The TLV (as mercury) is 0.05 mg/m^3 of air. See the Laboratory Safety section in Volume 1 of this Edition for a procedure to contain environmental contamination of mercury waste from Kjeldahl digestions. Disposal of any mercury waste must be in accordance with local and EPA regulations.

Chlorine is a poisonous gas. The TLV is 1 ppm in air. It is a strong oxidizing agent and should not be allowed to come in contact with organic materials, hydrogen, powdered metals, and reducing agents. A fume hood should be used at all times when using chlorine.

Wijs solution causes severe burns and vapors can cause lung and eye damage. Use of a fume hood is recommended. Wijs solution without carbon tetrachloride is available commercially.

RECOMMENDATIONS

Cyclohexane has been proposed as a replacement for chloroform in the iodine value method—see AOCS Recommended Practice Cd 1-87 (89), AOCS Official Method Cd 1d-92 and *JAOCS 65:*745 (1988). Isooctane has been reported to be a suitable replacement for chloroform in the peroxide value method, and therefore could be tried as a substitute for carbon tetrachloride in the

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iodine value method. These recommendations do not represent official approval by the AOCS Uniform Methods Committee. Laboratories that use these solvents should report their findings to the AOCS Technical Director.

NUMBERED NOTES

1. When the iodine value is determined on materials having conjugated systems, the result is not a measure of total unsaturation, but rather is an empirical value indicative of the amount of unsaturation present. Reproducible results are obtained which afford a comparison of total unsaturation. When the iodine value is required on the fatty acids of natural and synthetic drying oils, the preparation and separation are performed as directed in AOCS Official Method Cd 6-38.
2. All Wijs solutions are sensitive to temperature, moisture, and light. Store in a cool and dark place and never allow to come to a temperature above 25–30°C.
3. A mercuric compound is added as a catalyst to accelerate the rate of addition of iodine to double bonds. A longer reaction time may be used instead of adding mercuric compounds. Mercuric acetate can be omitted for test samples of lower iodine value. If mercuric acetate is not used for higher iodine value test samples, the reaction time will have to be increased.
4. Mechanical stirring is satisfactory for agitating during the addition of thiosulfate.
5. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and cyclohexane-acetic acid may also be considered as alternate solvents. See AOCS Methods Cd 1b-87 and Cd 1d-92.

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AOCS Official Method Tg 3a-64

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Iodine Value of Fatty Quaternary Ammonium Chlorides, Modified Wijs Method

DEFINITION

The iodine value is a measure of the unsaturation of the alkyl group or groups and is expressed in terms of cg of iodine absorbed per g of test sample (% iodine absorbed).

SCOPE

Applicable to fatty quaternary ammonium chlorides. (See Notes, 1 and 2). Replaces Nc 4-62.

APPARATUS

1. The equipment is the same as described in AOCS Official Method Tg 1-64.

REAGENTS

The reagents are the same as described in AOCS Official Method Tg 1-64, under Reagents, 1-8, with the following additions—

1. Chloroform—ACS or AR Grade (see Notes, *Caution*, and 6).
2. Sodium dodecyl sulfate—see Notes, 3.

SOLUTIONS

The solutions (see Notes, 4) are the same as described in AOCS Official Method Tg 1-64, under Solutions.

PROCEDURE

1. Melt the test sample, if it is not already liquid, in a water bath, mix thoroughly, and weigh into the glass stoppered flask 1.5 g of the test sample by difference to an accuracy of 0.0005 g. The test sample should not be heated more than 10°C above its melting point.

   Note—All glassware must be clean and dry.
2. Add 20 mL of chloroform (see Notes, *Caution*) or other solvent (see Notes, Recommendations and 6), and swirl to dissolve test sample.
3. Pipet 25 mL of iodine solution into the sample flask, allowing the pipet to drain in the same manner for both the test samples and the blanks.
4. Stopper the flask immediately and moisten the stopper with the potassium iodide solution so as to prevent the loss of iodine or chlorine, but guard against the use of a quantity sufficient to run down the inside of the flask. Swirl flask to obtain good mixture.
5. Prepare and conduct at least 2 blanks with each group of test samples simultaneously and similar in all respects to the test samples.
6. Store the flask in a dark place for 30 min at a temperature of 25 ± 5°C. Swirl the flask occasionally, being careful not to allow the solution to crawl up the side of the flask over halfway.
7. Remove the flask from storage, add 20 mL of potassium iodide solution, followed by approximately 2.2 g of sodium dodecyl sulfate and 40 mL of distilled water.

   Note—Sodium dodecyl sulfate is added to prevent the free iodine from being tenaciously held by the nonaqueous phase, giving a sharper end point.
8. Titrate with 0.1 M thiosulfate solution adding it gradually and with constant and vigorous shaking (see Notes, 5) until the yellow color has almost disappeared.
9. Add 2 mL of starch indicator solution rinsing the neck of the flask therewith, and continue the titration until the blue to brown color begins to disappear. Near the end of the titration it is necessary to shake violently, with the stopper inserted, in order to free any iodine that is dissolved so that it may be taken up with the potassium iodide solution. Only at this stage is the stopper allowed to come into contact with the liquid. Titrate to a colorless end point.

CALCULATIONS

$$\text{Iodine Value} = \frac{(B - S) \times M \times 12.69}{\text{mass of test portions, g}}$$

Where—

B = volume of titrant, mL of blank
S = volume of titrant, mL of test sample
M = molarity of $Na_2S_2O_3$ solution

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PRECISION

At a confidence level of 95%, in the range of values of 0.5–0.8:

1. Two single determinations performed in one laboratory shall not differ from the true mean value by more than ± 28%.
2. Single determinations performed in different laboratories shall not differ from the true mean value by more than ± 58%.

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Chlorine is a poisonous gas. The TLV is 1 ppm in air. It is a strong oxidizing agent and should not be allowed to come in contact with organic materials, hydrogen, powdered metals, and reducing agents. A fume hood should be used at all times when using chlorine.

Wijs solution causes severe burns and vapors can cause lung and eye damage. Use of a fume hood is recommended. Wijs solution without carbon tetrachloride is available commercially.

RECOMMENDATIONS

Cyclohexane has been proposed as a replacement for chloroform in the iodine value method—see AOCS Recommended Practice Cd 1-87 (89), AOCS Offical Method Cd 1d-92 and *JAOCS 65*:745 (1988). Isooctane has been reported to be a suitable replacement for chloroform in the peroxide value method, and therefore could be tried as a substitute for carbon tetrachloride in the iodine value method. These recommendations do not represent official approval by the AOCS Uniform Methods Committee. Laboratories that use these solvents should report their findings to the AOCS Technical Director.

NUMBERED NOTES

1. When the iodine value is determined on materials having conjugated systems, the result is not a measure of total unsaturation, but rather is an empirical value indicative of the amount of unsaturation present. Reproducible results are obtained which afford a comparison of total unsaturation. When the iodine value is required on the fatty acids of natural and synthetic drying oils, the preparation and separation are performed as directed in AOCS Official Method Cd 6-38.
2. There is some evidence that the use of mercuric acetate in this procedure will give erroneous results. See Notes, 3 in AOCS methods Tg 2a-64 regarding the use of mercuric acetate.
3. Suitable sodium dodecyl sulfate may be purchased from: Fluka Chemical Corp., 980 South Second Street, Ronkonkoma, NY 11779, USA, or Fisher Scientific, 711 Forbes Avenue, Pittsburgh, PA 15219-9919, USA.
4. All Wijs solutions are sensitive to temperature, moisture, and light. Store in a cool and dark place and never allow to come to a temperature above 25–30°C.
5. Mechanical stirring is satisfactory for agitating during the addition of thiosulfate.
6. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride, and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

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AOCS

AOCS Official Method Th 1a-64

Revised 2011

Diene Value

DEFINITION

The diene value is a measure of conjugation of unsaturated bonds, expressed in terms of the equivalent number of grams of iodine per 100 grams of test sample (% iodine absorbed).

SCOPE

Applicable to all normal drying oils. Replaces Ka 12-55.

APPARATUS

1. Pipet—volumetric, 25 mL.
2. Erlenmeyer flasks—with ground glass joints, 250 mL, with condensers to fit.
3. Erlenmeyer flask—500 mL.
4. Separatory funnels—short-stem, with ground glass stoppers, 250 mL capacity.

REAGENTS

1. Maleic anhydride-toluene solution—dissolve 6 g of finely powdered maleic anhydride, reagent grade, m.p. 52–54°C, in 94 g of dry, reagent grade toluene (see Notes, *Caution*), which, if necessary, has been distilled after drying over anhydrous cupric sulfate.
2. Standard sodium hydroxide solution, 1.0 M—accurately standardized (see AOCS Specification H 12-52).
3. Diethyl ether—ACS grade (see Notes, *Caution*).
4. Ethyl alcohol—95%, SDA Formulas 30 and 3A are permitted.
5. Methyl orange indicator solution—1% in freshly boiled and cooled distilled water.
6. Phenolphthalein indicator solution—1.0% in 95% alcohol.

PROCEDURE

1. Weigh 3 g (± 0.001 g) of test sample into a dry Erlenmeyer flask, and add with a pipet 25.0 mL of the maleic anhydride-toluene reagent. Add a small boiling stone and attach an air condenser. Place on a hot plate, and allow to reflux gently for 3 hr.
2. Prepare and conduct blank determination similar to and simultaneously with the test sample.
3. After refluxing, add 5 mL of water (see Notes, 1) through the condenser and allow to reflux for an additional 15 min.
4. Remove the flask and attach condenser from the hot plate, allow to cool, and then add 5 mL portions of diethyl ether (see Notes, *Caution*) and 10 mL water through the condenser. Disconnect the condenser and wash down the condenser joint with 10 mL water, allowing the washings to run into the flask.
5. Carefully transfer the contents of flask into separatory funnel. Wash flask with three 15 mL portions of ether followed by three 15 mL portions of water, adding both diethyl ether (see Notes, *Caution*) and water rinsings to the separatory funnel.
6. Stopper the funnel, shake the contents vigorously for 1 min, remove the stopper, and allow the layers to separate. If any evidence of emulsion is present, add a few drops of alcohol, swirl gently and then allow to stand (see Notes, 2).
7. When the two clear layers have formed, draw off the lower aqueous layer into a 500 mL Erlenmeyer flask. Repeat the extraction with one 25 mL portion of water followed by 4 subsequent and separate 10 mL portions. Break up any formation of emulsion with a few drops of alcohol (see Notes, 2). Test a portion of the sixth extract for acidity with methyl orange indicator. If there is any evidence of acidity, continue extracting until the extract is neutral. Titrate the combined extracts with 0.1 M sodium hydroxide solution using phenolphthalein indicator.

CALCULATIONS

Report to the nearest whole unit, see Precision section below—

$$\text{Diene Value} = \frac{(B - S) \times M \times 12.69}{\text{mass of test portion, g}}$$

Where—

M = molarity of NaOH solution
B = volume of titrant, mL of blank
S = volume of titrant, mL of test sample

PRECISION

Collaborative tests indicate that five different operators should obtain values agreeing within 5 units and the same operator should agree within 2 units.

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NOTES

Caution

Toluene is flammable and a dangerous fire risk. Explosive limits in air are 1.27–7%. It is toxic by ingestion, inhalation, and skin absorption. The TLV is 100 ppm in air. A fume hood should be used at all times when using toluene.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

NUMBERED NOTES

1. Use only freshly boiled and cooled distilled water throughout the procedure.
2. Water containing about 8 drops of 2-octanol per 100 mL will prevent emulsion formation.

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AOCS Official Method Ti 1a-64

Reapproved 2009

Spectrophotometric Determination of Conjugated Dienoic Acid

DEFINITION

This method determines the diene conjugation of unsaturated linkages present, expressing this value as a percentage of conjugated dienoic acid.

SCOPE

Applicable to dehydrated castor oil, dehydrated castor fatty acids, and their methyl or ethyl esters. Replaces Ka 13-64.

APPARATUS

1. Ultraviolet spectrophotometer—covering a spectral range of 220–350 nm with wavelength scale readable to 0.1 nm, equipped with absorption cuvette compartment for holding 1.000, 2.500, and 5.000 cm cuvettes.
2. Absorption cuvettes—quartz, matched pairs of length 1.000 ± 0.005 cm, 2.500 ± .005 cm, and 5.000 ± 0.005 cm. The cuvettes in a pair when filled with water or isooctane must match within 0.01 absorbance unit. Use 1.000 cm non-demountable type consisting of Pyrex™ glass cuvette body of 22 mm o.d. with centered ground glass stopper, threaded metal caps, polished crystalline quartz windows, and cork gaskets.
3. Filter tube—80 cm by 4.5 cm with glass stopcock sealed at one end.
4. Cork stopper—covered with aluminum foil to fit loosely in the top of the filter tube.
5. Dropping bottle—stoppered, for weighing the test samples.
6. Volumetric flasks—glass-stoppered, having capacities of 100, 50, and 25 mL.
7. Volumetric pipets—precision grade, assorted sizes, for making dilutions when required.

REAGENTS

1. Silica gel—40–60 mesh, Code 11-08, Davison Chemical Co., Baltimore, MD, USA, or equivalent.
2. Glass wool.
3. Isooctane (2,2,4 trimethyl pentane)—Pure or Spetro grades of isooctane are satisfactory and should be used. Check the absorbency of a 1 cm layer of the isooctane against distilled water at 233 nm. The absorbency compared with distilled water set at zero absorbency shall not be more than 0.070 at 233 nm. If the isooctane does not meet these requirements, purify it as follows:

   Place about 8 cm of glass wool above the lower end of the filter tube. Add about 50 cm of silica gel and about 5 cm of glass wool. Fasten the tube vertically to a ring stand and place a funnel and 2 L amber bottle under the tube. Pour the isooctane slowly into the tube, filling about three-fourths full, and allow the isooctane to filter through the silica gel. Renew the silica gel in the tube as often as necessary to yield isooctane conforming to the transmission limit given above. A constant flow can be maintained by feeding the isooctane from a 500 mL glass-stoppered separation funnel, the tip of which is below the surface of the isooctane in the filter tube. The 2 L amber bottle used for storing the purified isooctane should be rinsed with a little of the first eluate from the column, and the rinse should be discarded. An aluminum-lined screw cap should be used to close the bottle for storage.

PROCEDURE

1. Weigh by difference into a 100 mL volumetric flask, to the nearest 0.1 mg, 70–100 mg of the test sample. Add about 75 mL of purified isooctane.
2. Rotate the flask and warm the contents, if necessary, to completely dissolve the test sample. Cool to room temperature, and allow to stand at least 15 min to attain temperature equilibrium. Dilute to volume with purified solvent and mix thoroughly.
3. Make two 10 to 100 mL successive dilutions to give a final concentration of about 0.01 mg of test sample per mL (see Notes, 1).
4. Measure the absorbance by scanning from 200–250 nm and record the maximum absorbance near 233 nm. Use a matching cuvette containing purified solvent only for the blank cuvette. Take several readings to obtain an average value.
5. The observed absorbance reading should lie between 0.2 and 0.8; otherwise change the weight of the test sample or the dilution to give the required reading.

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CALCULATIONS

Report to three significant figures; see Precision, 1 and 2.

$$\text{Conjugated dienoic acid, \%} = 0.84\left(\frac{As}{bc} - K_0\right)$$

Where—

K_0 = absorptivity by acid or ester groups
= 0.07 for esters
= 0.03 for acids
A_s = observed absorbance at 233 nm
b = cuvette length in cm
c = concentration of test sample, g/L of the final dilution used for the absorption measurement

PRECISION

1. Repeatability results determined by the same operator and the same apparatus should not differ from the mean by more than ±0.39%.
2. Reproducibility results in different laboratories by different operators should not differ from the mean by more than ±1.26%.

NOTES

1. When making dilutions, allow sufficient time for solutions to reach room temperature.

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AOCS Official Method Tk 1a-64

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Unsaponifiable Material

DEFINITION

Unsaponifiable matter includes those substances frequently found dissolved in fatty acids and drying oils which cannot be saponified by caustic treatment, but which are soluble in the normal fat solvents. Included are the higher aliphatic alcohols, sterols, pigments, and hydrocarbons.

SCOPE

Applicable to all fatty acids, drying oils, and polymerized fatty acids.

APPARATUS

1. Glass-stoppered extraction cylinder graduated at 40, 80, and 130 mL capacity—height about 300 mm, about 35 mm dia.
2. Erlenmeyer or Soxhlet flasks—100–200 mL.
3. Separation funnels—500 mL.
4. Glass siphon—see Procedure 3.

REAGENTS

1. Ethyl alcohol—95%, SDA Formula 30 and 3A are permitted.
2. Aqueous potassium hydroxide solution—50% KOH by weight.
3. Petroleum ether—AOCS Specification H 2-41.
4. Sodium hydroxide solution, 0.02 M—accurately standardized, see AOCS Specification H 12-52.
5. Phenolphthalein indicator solution—1.0% in 95% alcohol.

PROCEDURE

1. Weigh accurately about 5 g of well-mixed test sample into an Erlenmeyer or Soxhlet flask. Add 30 mL of alcohol and 5 mL of aqueous KOH. Boil gently but steadily under a reflux condenser for 1 hr or until completely saponified. Complete saponification is essential.
2. Transfer to the extraction cylinder and wash to the 40 mL mark with alcohol. Complete the transfer with warm and then cold distilled water until the total volume is 80 mL. Wash out the flask with a little petroleum ether and add to the cylinder. Cool the cylinder and contents to room temperature (20–30°C), and then add 50 mL of petroleum ether.
3. Insert the stopper and shake vigorously for at least 1 min and allow to settle until both layers are clear. Use a glass siphon to remove the upper layer as completely as possible without including any of the lower portion.
4. The petroleum ether fractions are drawn into and accumulated in a 500 mL separator funnel containing 5 mL of 10% ethyl alcohol in order to minimize possibility of leakage of petroleum ether.
5. Repeat the extraction using 50 mL portions of petroleum ether each time, at least six more times, shaking vigorously with each extraction.

   Note—There are some cases in which seven extractions may not be sufficient. This is best judged by making another extraction and evaporating this separately. Extraction should be discontinued when a single extraction shows less than 0.005 g of residue.
6. Wash the combined extracts in the separatory funnel with 25 mL portions of 10% alcohol in distilled water, shaking vigorously and drawing off the alcohol layer after each wash. Washing should be discontinued when the wash water is neutral to phenolphthalein. Be careful not to remove any of the ether layer.
7. Transfer the ether extract to a tared beaker and evaporate to dryness in a water bath under a gentle stream of clear, dry air. Complete the drying to constant weight, preferably in a vacuum oven at 75–80°C, and an internal pressure of not more than 200 mm of mercury, or in an air oven at 105°C for 15 min intervals. Cool in a desiccator and weigh.
8. Run a blank exactly as the test sample, Procedure 1–6.
9. After weighing both the residue from the test samples and the residue from the blank, take up both in 50 mL of warm (50°C) 95% alcohol containing indicator and previously neutralized to a faint pink color. Titrate with 0.02 M NaOH to same pink color.

CALCULATIONS

1. Weight of fatty acids in test portion residue and blank residue = $mL \times M \times 0.282$,
   Where—
   mL = mL of NaOH
   M = molarity of NaOH determined by standardization

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2. % Uncorrected Unsap = $\frac{\text{mass of test portion residue, g}}{\text{mass of test portion}} \times 100$

3. % Unsap corrected for fatty acids = $\frac{\text{mass of test portion residue, g} - \text{mass of fatty acids in test portion residue, g}}{\text{mass of test portion}} \times 100$

4. % Unsap corrected for fatty acids and blank =

$$\frac{\left(\text{mass residue, g} - \text{mass of blank residue, g}\right) - \left(\text{mass of test portion residue FA, g} - \text{mass of blank residue FA, g}\right)}{\text{mass of test portion, g}} \times 100$$

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AOCS Official Method Tl 1a-64

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Saponification Value

DEFINITION

The saponification value is a measure of the alkali reactive groups in industrial oils and derivatives and is expressed as the mg of potassium hydroxide which react with 1 g of the test sample.

SCOPE

Applicable to industrial oils and derivatives.

APPARATUS

1. Erlenmeyer flasks—Corning Alkali Resistant, Kimble Resistant or equivalent, 250–300 mL.
2. Air condensers—minimum 650 mm long.
3. Water bath or a hot plate—with variable heat control.
4. Pipet—50 mL, volumetric.

SOLUTIONS

1. Hydrochloric acid, 0.5 M—accurately standardized (see AOCS Specification H 14-52).
2. Alcoholic potassium hydroxide (KOH)—place a few grams (5–10) of KOH in a 2 L flask and add from 1–1.5 liters of 95% ethyl alcohol (SDA Formulas 30 and 3A are permitted) and boil in a water bath under a reflux condenser for 30–60 min. Distill and collect the alcohol. Dissolve 27 g of potassium hydroxide, low in carbonate, in 1 L of the distilled alcohol.

   Caution—There is considerable evolution of heat during the dissolution. Place in a water bath, keeping the temperature below 15.5°C (60°F) while the alkali is being dissolved. The solution should remain clear.
3. Phenolphthalein indicator solution—1% in 95% alcohol (see Notes, 1).

PROCEDURE

1. Melt the test sample if it is not already liquid and filter through filter paper to remove solid impurities and traces of moisture. The test sample must be completely dry (see Notes, 2 and 3).
2. Weigh a test sample of such size that the back titration is 45–55% of the blank. Add 50 mL of the alcoholic KOH with the pipet and allow the pipet to drain for a definite period of time.
   (a) For natural and synthetic drying oils and their fatty acids weigh approximately 4–5 grams.
   (b) For commercial fatty acids, weigh approximately 3–4 grams.
   (c) A 20 g test sample should be used for materials with a saponification value in the 0–10 range and a melting point above 30°C.
3. Prepare and conduct blank determinations simultaneously with the test sample, similar in all respects to the test sample.
4. Connect the air condenser and boil gently, but steadily until the test sample is completely saponified (see Notes, 4–6). Be careful that the vapor ring in the condenser does not rise to the top of the condenser or there may be some loss of solvent.
5. After the flask and condenser have cooled somewhat, but not sufficiently to jell, wash down the inside of the condenser with a little distilled water. Disconnect the condenser, add about 1 mL of indicator, and titrate with 0.5 M HCl until the pink color has just disappeared.
6. Return flask to hot plate, heat to boiling, and again titrate to disappearance of whatever pink color may have developed in bringing the contents of the flask to a boil. Add this titration volume to that obtained in obtained in Procedure 5.

CALCULATIONS

$$\text{Saponification Value} = \frac{56.10 \times M \left(\text{volume of titrant, mL of blank} - \text{volume of titrant, mL of test portion}\right)}{\text{mass of test portion, g}}$$

Where—

M = molarity of standardized HCl solution

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PRECISION

	Polymerized Fatty Acids	Fatty Acids Tallow Coconut Soybean Linseed
At a confidence level of 95% in the range of values of:	187–197	195–275
1. Two single determinations performed in one laboratory shall not differ from the mean value by more than:	± 5.5%	± 1.6%
2. Single determinations performed in different laboratories shall not differ from the mean value by more than:	± 5.7%	± 1.7%

NOTES

1. A "masked phenolphthalein indicator" may be used with off-color material. Prepare by dissolving 1.6 g of phenolphthalein and 2.7 g of methylene blue in 500 mL of alcohol (SDA Formula 30 or 3A). Adjust the pH with alcoholic alkali solution so that the greenish-blue color is faintly tinged with purple. Color change is from green to purple when going from acid to alkali.
2. Drying oils of high viscosity should not be filtered.
3. For preparation of fatty acids from oils see AOCS Official Method Cd 6-38.
4. Some test samples, particularly those difficult to saponify, may require more than 1 hr. This can only be determined by trial. Clarity and homogeneity of the test solution are partial indicators of complete saponification, but they are not necessarily absolute criteria.
5. For normal materials, reflux for about 1 hr.
6. For certain synthetic and chemically modified drying oils, run in duplicate using 1 and 2 hr heating periods. If 2 hr heating gives appreciably higher results than the 1 hr run, additional determinations for 4 and 6 hr should be run to establish the time required for complete reaction.

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AOCS Official Method Tm 1a-64

Revised 2011

Ash

DEFINITION

This method determines the residues remaining after incineration under the specified conditions of the test.

SCOPE

Applicable to drying oils, their fatty acids, and commercial fatty acids. Not applicable to boiled oils (see Notes, 1). Replaces Ka 10-55 and L 5a-57.

APPARATUS

1. Crucible—porcelain or high-silica-content glass, 50 mL capacity (see Notes, 2).
2. Electric muffle furnace.
3. Desiccator—containing an efficient desiccant (see AOCS Specification H 9-87).
4. Oil sample bottle—125 mL.
5. Triangle—nichrome or clay.

PROCEDURE

1. Ignite crucible in muffle furnace at 550–650°C, cool slightly, hold in desiccator for 1 hr, and weigh to 0.0001 g.
2. Fill 125 mL sample bottle with well-mixed test sample and weigh to 0.05 g. Pour about 20 g of the oil from the bottle into crucible supported on a triangle, using care so that no oil runs down the outside of the crucible or bottle.
3. Heat gently (see Notes, 3) by moving a flame on the bottom and sides of the crucible until the oil ignites. Reduce the size of the flame until the heat is just sufficient to keep the test sample burning.
4. When the first batch of oil has burned off, add about 20 g more of oil, and continue in the same manner until all of the oil in the 125 mL bottle has been added. Reweigh sample bottle to obtain total weight of oil used, which will be somewhat over 100 g.
5. Continue heating the test sample to a black char, and then transfer the crucible to the muffle furnace. Heat at 800 ± 25°C for 1 hr.
6. Remove crucible from the furnace, cool slightly, place in desiccator, and cool to room temperature. Weigh and repeat heating in the furnace to constant weight.

CALCULATIONS

1. $$\text{Ash, \%} = \frac{\text{mass, g of residue}}{\text{mass, g of test portion}} \times 100$$

2. $$\text{Ash, ppm} = \frac{\text{mass, g of residue}}{\text{mass, g of test portion}} \times 10^6$$

PRECISION

1. Repeatability—The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) as calculated from the formulas in Table 1.
2. Reproducibility—The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as calculated from the formulas in Table 1.

NOTES

1. Boiled oils do not give reproducible results. Drying oils containing lead are partially reduced by the carbon to give metallic lead and oxides of variable composition, and zinc oxide is relatively volatile at the furnace temperature. Ash determinations on boiled oils are consequently empirical and not highly reproducible.
2. Platinum crucibles are not recommended. Boiled oils or oils contaminated with drying oils containing lead may ruin platinum by alloy formation.
3. The entire burning and charring procedure should be carried out in a properly operating fume hood.

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Table 1
The interlaboratory study carried out by Lincoln D. Metcalfe in 1975, in which eight laboratories participated, each obtaining four test results for each test sample analyzed by AOCS Official Method Tm 1a-64, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarized in the following table.

	Test Sample[a]	
	A	B
Number of labs after removal of outliers	8	8
n	31	30
Outliers	1	2
Mean, ppm[b]	77	16
Repeatability, S_r	13.28	3.26
RSD_r	17.14	20.20
r ($2.8 \times S_r$)	37.18	9.13
Reproducibility, S_R	29.20	6.13
RSD_R	37.68	38.01
R ($2.8 \times S_R$)	81.76	17.16

[a] Key to test samples: A, industrial tallow; B, corn oil.
[b] Value for ash in ppm.

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Ash of Fatty Quaternary Ammonium Chlorides

DEFINITION

This method determines the residues remaining after incineration under the specified conditions of the test.

SCOPE

Applicable to fatty quaternary ammonium chlorides. Replaces Nc 2-62.

APPARATUS

1. Crucible—porcelain or high silica content glass, 250 mL capacity.
2. Electric muffle furnace.
3. Desiccator—containing an efficient desiccant (see AOCS Specification H 9-87).
4. Triangle—nichrome or clay.

PROCEDURE

1. Ignite crucible in muffle furnace at 550–650°C, cool slightly, hold in desiccator for 1 hr, and weigh to 0.0001 g.
2. Melt the test sample, if it is not already liquid, in a water bath, mix thoroughly, and accurately weigh 20 g into the crucible.
3. Heat gently (see Notes, 1) by moving a flame on the bottom and sides of the crucible until the oil ignites. Reduce the size of the flame until the heat is just sufficient to keep the test sample burning.
4. Continue the heating of the test sample to a black char and transfer the crucible to the muffle furnace. Heat at 550–650°C for 1 hr.
5. Remove the crucible from the furnace, cool slightly, place in desiccator, and cool to room temperature. Weigh and repeat heating in the furnace to constant weight (see Notes, 2).

CALCULATIONS

$$\text{Ash, \%} = \frac{\text{mass, g of residue}}{\text{mass, g of test portion}} \times 100$$

NOTES

1. The entire burning and charring procedure should be carried out in a properly operating fume hood.
2. The ash can be concluded to be essentially sodium chloride, unless free caustic is found by using AOCS Method Te-3a-64

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AOCS Official Method Tn 1a-64

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Flash and Fire Points, Cleveland Open Cup Method

DEFINITION

This method determines the temperature at which the test sample will flash and burn.

SCOPE

Applicable to drying oils, their fatty acids and commercial fatty acids except those which for any reason flash below 149°C (300°F). Replaces Ka 7-55 and L 11a-57.

APPARATUS

1. Thermometer—AOCS Specification H 5-40.
2. Cleveland open flash cup—ASTM Designation D 92-72, constructed of brass and conforming to the dimensional requirements prescribed in Table 1. The beveled edge of the cup is at an angle of approximately 45 degrees. There may be a fillet of about 3.97 mm ($^5/_{32}$ in.) in radius inside the bottom of the cup.
3. Heating plate—constructed of brass, cast iron, wrought iron, or steel, 6.35 mm ($^1/_4$ in.) thick and 156.4 mm (6 in.) in dia. There is a plane depression 0.79 mm ($^1/_{32}$ in.) deep in the center of the plate with diameter just sufficient to fit the cup and centered with a circular opening cut through the plate, 55.0 mm (2 $^3/_{16}$ in.) in dia. The plate is covered with a sheet of fireproof material 6.35 mm thick and of the same shape as the metal plate.
4. Heat source—gas burner, alcohol lamp, or electric heater with rheostat control. Whatever form of heat is used, under no circumstances are the products of combustion or free flame allowed to come up around the cup. If a flame heater is used, it may be protected from drafts or excessive radiation with any suitable shield that does not project above the upper surface of the asbestos board. The heat source is centered under the plate opening and must not produce local super heating.
5. Metal flame test burner.

PROCEDURE (see Notes, *Caution*)

1. Flash point—
   (a) The flash and fire point determinations are carried out in a room or compartment free from air drafts and darkened sufficiently so that the flash is readily discernible. Avoid breathing over the surface of the test sample.
   (b) Fill the cup with oil or melted fat test sample so that the top of the meniscus is exactly at the filling line. Suspend or secure the thermometer in a vertical position with the bottom of the bulb about 6.35 mm ($^1/_4$ in.) from the bottom of the cup and in a position halfway between the center and back of the cup.
   (c) Heat the test sample at a rate not to exceed 16.7°C (30°F) rise per min to within 55.6°C (100°F) of the flash point. Thereafter regulate the rate of heating so that the temperature of the test sample increases 5–6.1°C (9–11°F) per min.
   (d) Apply the test flame which is about 3.17 mm ($^1/_8$ in.) in dia. as the temperature reaches each successive 2.8°C (5°F) mark. Pass the flame in a straight line or on the circumference of a circle having a radius of at least about 150 mm (6 in.) across the center of the cup and at right angles to the dia. passing through the thermometer. The test flame shall, while passing across the surface of the test sample, be in the plane of the upper edge of the cup. The time for passage of the test flame across the cup shall be about 1 sec.
   (e) The flash point is the temperature indicated by the thermometer when a flash point appears at any point on the surface of the test sample. The true flash must not be confused with a bluish halo that sometimes surrounds the test flame.
2. Fire point—
   (a) Continue the heating, after the flash point determination, as directed in Procedure, 1, (c) in the manner prescribed for the flash point until the fire point is reached.
   (b) The fire point is the temperature indicated by the thermometer when application of the test flame causes burning for a period of at least 5 sec.

NOTES

Caution

Protective fireproof clothing should be worn at all times when performing this test. Suitable fire-extinguishing equipment should be available nearby. Persons performing this test for the first time should do so only under the supervision of someone familiar with the test method.

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TABLE 1
Dimensional Requirements for Cleveland Open Flash Cup

	Inches			Millimeters		
	Minimum	Normal	Maximum	Minimum	Normal	Maximum
Inside diameter immediately below filling mark	$2\frac{15}{32}$	$2\frac{1}{2}$	$2\frac{17}{32}$	62.7	63.5	64.3
Outside diameter below flange	$2\frac{21}{32}$	$2\frac{11}{16}$	$2\frac{23}{32}$	67.5	68.3	69.1
Inside height from center to bottom of rim	$1\frac{9}{32}$	$1\frac{5}{16}$	$1\frac{11}{32}$	32.5	33.3	34.1
Thickness of bottom	$\frac{7}{64}$	$\frac{1}{8}$	$\frac{9}{64}$	2.8	3.2	3.6
Distance from rim to filling mark	$\frac{23}{64}$	$\frac{3}{8}$	$\frac{25}{64}$	9.1	9.5	9.9
Distance, lower surface flange to bottom of cup	$1\frac{7}{32}$	$1\frac{1}{4}$	$1\frac{9}{32}$	31.0	31.8	32.6
Vertical distance, upper surface flange to rim	$\frac{7}{64}$	$\frac{1}{8}$	$\frac{9}{64}$	2.8	3.2	3.6
Thickness of rim	$\frac{5}{64}$	$\frac{3}{32}$	$\frac{7}{64}$	2.0	2.4	2.8
Width of lower surface of flange	$\frac{9}{16}$	$\frac{19}{32}$	$\frac{5}{8}$	14.3	15.1	15.9

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Flash Point of Fatty Quaternary Ammonium Chloride, Closed Cup Method

(Modified Closed Cup Method, ASTM Designation D 93-80)

DEFINITION

This method determines the temperature at which the test sample will flash when a test flame is applied under the conditions specified for the test.

SCOPE

Applicable to fatty quaternary ammonium chlorides and their solutions that are liquids at temperatures 11°C (20°F) below their expected flash points. Replaces Tn 2a-64.

APPARATUS

1. Pensky-Martens closed cup flash tester—ASTM Designation D 93-52. Either hand or motor stirring is allowable, but the latter is preferred. The specified lowrange ASTM P.M. and high-range ASTM P.M. centigrade and Fahrenheit thermometers are replaced by a single centigrade or Fahrenheit thermometer as described below.
2. Thermometers—AOCS Specification H 10-55, Closed Cup Flash Point Thermometers.

CALIBRATION OF TESTER

1. Make a determination in triplicate on the flash point of standard p-xylene and standard isopropanol (see Notes, 1 and 2). Average these values for each material. If the difference between and values for the two materials are less than 8.4°C (15°F) or more than 15.0°C (27°F), repeat the determinations on fresh standards. Calculate a correction factor as follows:

For °C:	For °F:
$X = 33.3 - A$	$X = 92 - A$
$Y = 21.7 - B$	$Y = 71 - B$

$$\text{Correction} = \frac{X + Y}{2} \qquad \text{Correction} = \frac{X + Y}{2}$$

Where—

A = observed flash point of p-xylene
B = observed flash point of isopropanol.

Apply this correction to all determinations.

PROCEDURE

1. Melt the test sample, if it is not already liquid, in a water bath and mix thoroughly. Do not heat the test sample excessively (see Notes, 3).
2. Fill the cup with the quaternary test sample (see Notes, 4) so that the top of the meniscus is exactly at the filling line; place the lid on the cup and properly engage the locating devices. Insert the thermometer and suspend so that the bottom of the bulb is exactly 4.445 cm ($1\frac{3}{4}$ in.) below the level of the rim of the cup, which corresponds to the level of the lower surface of the portion of the lid inside the rim.
3. Light the test flame and adjust so that it is the size of a bead 3.97 mm ($\frac{5}{32}$ in.) in dia.
4. Heat the test sample and adjust it so that the temperature of the test sample increases at a rate of 0.8–1.3°C (1.5–2.5°F) per min. During the heating, turn the stirring device at a rate of 1–2 revolutions per sec.
5. At every 1.1°C (2°F) rise in temperature, discontinue stirring and apply the test flame by operating the device which controls the shutter and lowers the test flame into the shutter opening. Lower the test flame in $\frac{1}{2}$ sec and leave in a lowered position for 1 sec, then quickly return to the high position and resume stirring.
6. The flash point is the temperature indicated on the thermometer at the time of the flame application that causes a distinct flash in the interior of the cup. The true flash must not be confused with the bluish halo that sometimes surrounds the test flame.

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PRECISION

At a confidence level of 95%, in range of values of 21°–87°C (69°–188°F)—

1. Two single determinations performed in one laboratory shall not differ from a true mean value by more than ± 5.5%.
2. Single determinations performed in different laboratories shall not differ from a true mean value by more than ± 10.4%.

NOTES

1. Each unit of apparatus should have its calibration checked about once a week if in constant use, or on each occasion of use if used only occasionally.
2. Specifications for p-xylene and isopropanol—
   (a) p-Xylene shall conform to the following requirements:
   Specific gravity, 0.860–0.866 at 15.56°C/15.56°C.
   Boiling range, 2°C max from start to dry point.
   The range shall include the boiling point of pure p-xylene, which is 138.35°C (281.03°F). Purity, 95% min.
   (b) Isopropanol shall conform to the following requirements:
   Specific gravity, 0.8175–0.8135 at 20/20°C, as determined by means of a calibrated pycnometer.
   Distillation range, shall entirely distill within a 1.0°C range which shall include the temperature 80.4°C.
3. If the quaternary is a solid at room temperature, it should be liquefied by heating at a temperature not to exceed 5.5°C (10°F) over its melting point. The flash point determination will then start at this elevated temperature.
4. Remove bubbles from the surface of the test portion liquid before starting a determination.

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Specific Gravity

DEFINITION

This method determines the ratio of the weight of a unit volume of the test sample at 25° or 70°C to the weight of a unit volume of water at 25°C.

SCOPE

Applicable to all fatty acids.

APPARATUS

1. Specific gravity bottle with a side arm cap and ground-glass pycnometer thermometer. (pycnometer)—with well-fitting ground-glass joints (see Notes, 1 and 2), 50 mL capacity.
2. Water baths—maintained at 25 ± 0.2°C and 70 ± 0.2°C. (See Notes, 3.)
3. Thermometer—any convenient thermometer of suitable range with 0.1 or 0.2 subdivisions. Standardize carefully, preferably by comparison with a thermometer calibrated by the NIST (see AOCS Methods Section H for specifications on thermometers).

PROCEDURE

1. Specific gravity bottle calibration—
   (a) Clean and dry thoroughly and then fill with recently boiled and cooled distilled water at 20–23°C.
   (b) Fill the bottle to overflowing by holding the bottle on its side in such a manner as to prevent the entrapment of air bubbles.
   (c) Insert the stopper and immerse in water bath at 25 ± 0.2°C. Keep the entire bulb completely covered with water and hold at 25°C for 30 min.
   (d) Carefully remove any water which has exuded from the side opening. Remove the bottle from the bath and wipe completely dry.
   (e) Weigh the bottle and contents. Calculate the weight of water in the flask by subtracting the weight of the empty bottle from the weight of bottle plus water.
2. Specific gravity at 25/25°C—
   (a) Determine mass, g of bottle; weigh the bottle with cap and thermometer attached before adding the test sample.
   (b) Melt the test sample and filter through filter paper to remove any impurities and the last traces of moisture. The test sample must be completely dry.
   (c) Cool the test sample to 20–23°C (see Notes, 4) and fill the bottle to overflowing, holding the bottle on its side in such a manner as to prevent the entrapment of air bubbles. Reweigh the test sample after proper filling and adjusting the temperature.
   (d) Insert the stopper, immerse, and hold in the water bath at 25 ± 0.2°C for 30 min (see Notes, 3).
   (e) Carefully wipe off any fatty acid which has come through the capillary opening and remove from bath. Clean and dry thoroughly.
   (f) Weigh the bottle and contents immediately to avoid any temperature change. Calculate the specific gravity (see Calculations, 1).
3. Specific gravity at 70/25°C—
   (a) The procedure is the same as that for 25/25°C above, except that the melted fat is poured into the specific gravity bottle at 66–68°C.
   (b) The bottle containing the test portion is allowed to remain in a bath at 70 ± 0.2°C for 30 min. Cool to room temperature and weigh the bottle and contents. Calculate the specific gravity (see Calculations, 2).

CALCULATIONS

1. $$\text{Specific gravity at } 25/25°C = \frac{\text{mass, g of bottle and test portion} - \text{mass, g of bottle}}{\text{mass, g of water at } 25°C}$$

2. $$\text{Specific gravity at } 70/25°C = \frac{F}{W[1+(0.000025 \times 45)]}$$

Where—

F = mass, g of test portion at 70°C

W= mass, g of water at 25°C

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NUMBERED NOTES

1. Unless the specific gravity bottles are protected with caps, care must be taken so that no fatty acid or water is lost in the interval between removal from the bath and weighing. If the temperature of the room is above 25°C, this is very apt to happen. Even the warmth of a hand surrounding the bottle is sufficient to cause expansion of the contents of the flask.
2. Unless the specific gravity bottles are made of glass with a very low coefficient of expansion, a correction must be made for the expansion of the glass. The coefficient of expansion of ordinary glass is ca. 0.000025 (see Calculations, 2).
3. This method can be performed without a water bath. Cool the bottle with test portion in it slightly below 25°C, add a little more test portion and then adjust the temperature carefully keeping the test portion just at the top of the side arm. Hold the bottle in hand with a tissue to protect it from hand oil and moisture. Then warm the bottle and test portion with hand heat.
4. Fatty acids not liquid at 25°C may be tested at 70/25°C.

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Specific Gravity

DEFINITION

This method determines the ratio of weight of a unit mass volume of the test sample at 25°C to the weight of a unit mass volume of water at 25°C.

SCOPE

Applicable to natural and synthetic drying oils and oil derivatives.

APPARATUS

1. Specific gravity bottle, pycnometer—with well-fitting ground glass joints (see Notes, 1 and 2), 50 mL capacity. To calibrate these bottles, clean and dry thoroughly and then fill with recently boiled and cooled distilled water at 20–23°C. Fill the bottle to overflowing by holding the bottle on its side in such a manner as to prevent the entrapment of air bubbles. Insert the stopper and immerse in water bath at 25 ± 0.2°C. Keep the entire bulb completely covered with water and hold at specified temperature for 30 min. Carefully remove any water which has exuded from the side opening. Remove the bottle from the bath and wipe completely dry. Weigh the bottle and contents. Calculate the weight of water in the flask by subtracting the weight of empty bottle from the weight of bottle plus water.
2. Water baths—maintained at 25 ± 0.2°C and at 70 ± 0.2°C.
3. Thermometer—any convenient thermometer of suitable range with 0.1 or 0.2°C subdivisions. Standardize carefully, preferably by comparison with a thermometer calibrated by the United States National Institute of Testing (see AOCS Methods Section H for specifications on thermometers).

PROCEDURE

1. Specific gravity of oils and oil derivatives with viscosities up to 40 stokes—
   (a) Cool the test sample to 20–23°C and fill the specified specific gravity bottle to overflowing, holding the bottle on its side in such a manner as to prevent the entrapment of air bubbles (see Notes, 3 and 4).
   (b) Insert the stopper, immerse, and hold in the water bath at 25 ± 0.2°C for 30 min.
   (c) Carefully wipe off any fatty acid which has come through the capillary opening and remove from bath. Clean and dry thoroughly.
   (d) Weigh the bottle and contents and calculate the specific gravity as directed in Calculations, 1.
2. Specific gravity of oils and derivatives with viscosities over 40 stokes—
   (a) Place the test sample in the Hubbard specific gravity bottle, using extra care to prevent entrapment of air bubbles (see Notes, 3 and 4).
   (b) Cool to 20–23°C, stopper, and continue as directed in Procedure, 1.
3. Specific gravity of materials that are highly viscous or semi-solid—
   (a) Carefully pour a 10 to 15 g test sample in the Hubbard specific gravity bottle, using extra care to prevent entrapment of air bubbles (see Notes, 3 and 4) and weigh accurately.
   (b) Cool to 20–22°C and add recently boiled and cooled (23–25°C) distilled water by pouring gently down the side of the bottle.
   (c) Insert the stopper, immerse, and hold in the water bath at 25 ± 0.2°C for 30 min.
   (d) Carefully remove any water which has come through the capillary opening. Remove from bath, clean, and dry thoroughly.
   (e) Weigh the bottle and contents, and calculate the specific gravity as directed in Calculations, 2. 4. See Notes, 5 for calculations to approximate changes in specific gravity with changes in temperature.

CALCULATIONS

1. Specific gravity at 25/25°C for materials determined by one or the other of the first two procedures above =

$$\frac{C - A}{(B - A)}$$

2. Specific gravity at 25/25°C for material determined by the third procedure above =

$$\frac{C - A}{(B - A) - (D - C)}$$

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Where—

A = mass of empty specific gravity bottle, g

B = mass of specific gravity bottle plus water, g

C = mass of specific gravity bottle plus test portion, g

D = mass of specific gravity bottle plus test portion, plus water, g

PRECISION

The precision of the method was determined from Smalley Drying Oil Check Samples submitted 1961–1966, a total of 380 results. For agreement between laboratories, single determinations performed in one laboratory shall not differ by more than 0.0008.

NOTES

1. Unless the specific gravity bottles are protected with caps, care must be taken so that no fatty acid or water is lost in the interval between removal from the bath and weighing. If the temperature of the room is above 25°C, this is very apt to happen. Even the warmth of the hand surrounding the bottle is sufficient to cause expansion of the contents of the flask.
2. Unless the specific gravity bottles are made of glass with a very low coefficient of expansion, a correction must be made for the expansion of the glass. The coefficient of expansion of ordinary glass is about 0.000025 (see Method To 1a-64, Calculations, 2).
3. In the determination of the specific gravity of the high-viscosity materials, special care must be taken to avoid entrapment of air bubbles. The oil should be poured slowly down the side of the specific gravity bottle, and the uncapped test portion should stand until free of air bubbles. This will take ½–1 hr. Alternately, the test portion may be centrifuged for a few minutes at 1000 rpm.
4. In order to remove entrapped bubbles, the test portion should be centrifuged at approximately 1000 rpm for 5 min. Do not attempt to remove by standing because the time required is so long that there is danger of change in gravity due to absorption of oxygen.
5. Using 0.00068 as thermal coefficient of gravity for drying oil and table values for the weight of 3.785 L of water at various temperatures, approximate specific gravity changes can be made from a specific gravity at one temperature to a specific gravity at another temperature as follows:

   (a) To change from sp. gr. @ $\frac{t_1}{t°C}$ to $\frac{t_2}{t°C}$

   $$\text{sp. gr. @ } \frac{t_2}{t°C} = \text{sp. gr. @ } \frac{t_1}{t°C} = [0.00068 \times (t_1 - t_2°C)]$$

   (b) To change from sp. gr. @ t_1°C to t_2°C

   $$\text{sp. gr. @ } \frac{t}{t_2°C} = \frac{(\text{sp. gr. @ } \frac{t}{t_1°C})(\text{wt. 4 L } H_2O \text{ @ } t_1°C)}{\text{wt 4 L } H_2O \text{ @ } t_2°C}$$

   (c) Some common factors calculated from above for drying oils—to change sp. gr. @ 25/25°C to sp. gr. @ 25/15.5°C subtract 0.0018. to change sp. gr. @ 25/25°C to sp. gr. @ 15.5/15.5°C add 0.0047.

   (d) If large changes in temperature are involved, corrections for the expansion of the pycnometer must be made (see method To 1a-64, Notes, 2).

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Refractive Index

DEFINITION

The refractive index of a substance is the ratio of the speed of light in a vacuum to the speed of light in the substance. For practical measurements, including this method, the scales of standard instruments indicate refractive indices with respect to air rather than vacuum (see Notes, 1 and 2).

SCOPE

Applicable to drying oils and all fatty acids. Replaces Ka 4-55 and L 9a-57.

APPARATUS

1. Refractometer—Abbé or dipping type. The temperature of the refractometer prisms must be controlled to within ± 0.1°C and for this purpose it is preferably provided with a thermostatically controlled water bath and motor-driven pump to circulate water through the instrument. The instrument is standardized, following the manufacturer's instructions, with a liquid of known purity and refractive index or with a glass prism of known refractive index. Distilled water, which has a refractive index of 1.33299 at 20°C, and 1.33251 at 25°C is satisfactory in some cases.
2. Light source—if the refractometer is equipped with a compensator, a tungsten lamp, or daylight bulb is permissible. Otherwise, a monochromatic light such as an electric sodium vapor lamp is required.

REAGENTS

1. Ethanol—100%.
2. Lens tissue, uncoated facial tissue, or cotton cloth— recommended for cleaning and drying the prisms in order to avoid damage to them.

PROCEDURE

1. Melt the test sample if it is not already liquid, and filter through filter paper to remove any impurities and the last traces of moisture. Do not exceed the melting point by more than 10°C. The test sample must be completely dry.
2. Make certain that the temperature of the refractometer is 25.0°C for fatty acids liquid at room temperature, or 65°C for fatty acids solid at room temperature and that the prisms are clean and completely dry. Place a drop or two of the test sample on the prism of the refractometer. Close the prisms and tighten firmly with a screw head. Allow to stand for approximately one minute so that the test sample comes to the temperature of the instrument. If the dipping refractometer is used, immerse the prism in the test sample which must be maintained at the designated temperature and allow 3 min for temperature adjustment before reading.
3. Adjust the instrument and light to obtain the most distinct reading possible and then determine the refractive index. Take several readings and calculate the average of all readings.

NUMBERED NOTES

1. Approximate temperature corrections may be made by the following calculations:

Abbé refractometer— $R = R' + K(T' - T)$

Where—

R = the reading reduced to temperature T

R' = the reading at T′ C

T = the standard temperature

T' = the temperature at which the reading R' is made

K = 0.000385

2. The index of refraction of oils is characteristic within certain limits for each kind of oil. It is related to the degree of saturation, particularly to the extent of conjugation, but it is affected by other factors such as free fatty acid content, oxidation, and heat treatment.

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AOCS Official Method Tq 1a-64

Revised 2011

Viscosity of Transparent Liquids by Bubble Time Method

DEFINITION

The viscosity of a liquid is the resistance experienced by one portion of a liquid moving over another portion of the liquid. The absolute unit of viscosity in the cgs system is the poise which is expressed as dyne-seconds per square centimeter. Stokes are equal to poises divided by density.

SCOPE

This method determines the viscosity in bubble seconds by timing or comparison. The bubble seconds are approximately equal to stokes for most liquids. This method is applicable to transparent liquids which are free from crystalline or gel particles.

APPARATUS

1. Constant temperature bath—any suitable bath capable of maintaining temperature at 25 ± 0.1°C with water as the heat exchange medium.
2. Standard viscosity tubes—(see Notes, 1) of clear glass and with flat bottoms, 10.65 ± 0.025 mm i.d.; 114 ± 1 mm outside length. Plainly legible lines shall be located as follows—
   (a) 27 ± 0.5 mm.
   (b) 100 ± 0.5 mm
   (c) 108 ± 0.5 mm.

   The distance between lines (a) and (b) shall be 73 ± 0.5 mm. (All distances shall be measured from the outside bottom of the tube.)
3. Reference Standards—a series of standard viscosity tubes, with lines as noted in Apparatus, 2, filled with transparent liquids having predetermined viscosities in stokes and bubble seconds and spaced in logarithmically even increments of about 26% (log 1.260 = 0.100) as listed in Table 1. The standards are to be marked numerically as shown in Table 1 without reference to stokes or bubble time.

   It is suggested that the reference standards be marked in three series—
   (a) Light series—15 tubes marked 0.22–8.0;
   (b) Heavy series—14 tubes marked 10–200;
   (c) Very heavy series—7 tubes marked 250–1,000.

   Also listed in Table 1, for general and historical reference only, is the long established series of Gardner-Holdt letter standards, in tubes which do not have the three lines and are shorter than the standard viscosity tubes described in Apparatus, 2.
4. Timing device—stopwatch or electric stopclock capable of being read to a precision of 0.1 sec.
5. Tube racks—inverting rack, capable of inverting one or more viscosity tubes from 180 degrees to within 1 degree of a vertical position while the rack and tubes are immersed in the constant temperature bath.
6. Viscosity tube corks—no. 2 short.

PROCEDURE

1. Fill a standard viscosity tube with the material to be tested to approximately level with the 108 mm line.
2. Transfer the tube to a constant 25°C temperature bath with the cork loosely inserted. Hold at this temperature for 10 min.
3. At the end of 10 min, adjust the level of the liquid so that the bottom meniscus will be level with the 100 mm line. Insert the cork so that the bottom of the cork is on the level with the 108 mm line. This will ensure a bubble of suitable and uniform size.
4. Insert the tube in the rack and immerse in the 25°C water bath. If the comparison method is to be used, the proper standards are inserted in the rack alongside the liquid to be tested. Allow the tubes to stand with corks fully in place in the bath a minimum of 20 min before reading the viscosity.
5. To read, invert the tube quickly and determine the time required for the bubble to rise in seconds or compare against a standard. When determining the time in seconds, start the timing device when the top of the bubble becomes tangent to the 27 mm line on the tube. Stop the timing when the top of the bubble becomes tangent with the 100 mm line. This gives a 73 mm timed bubble travel. All timings and comparisons shall be made with the tubes in an exact vertical position.

REPORTING

The viscosity obtained by either the timing or comparison method may be expressed as "bubble seconds" or "approximate stokes."

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Note—The numerical tube number is an approximate measurement of stokes when the Bubble Time Method is applied to most raw materials and finished products encountered by the paint, varnish, & lacquer industries. This relationship does not hold for silicones or water dispersions of gums or similar materials.

PRECISION

1. For viscosity of liquids which have a timed bubble travel of 4 sec or less, more precise results can be obtained by comparison against reference standards having a predetermined viscosity or timed bubble travel (see Reference Standards in Apparatus, 3).
2. Adequate control of the temperature bath is essential. A variation of 0.1°C in the temperature of the bath will cause a 1% variation in the timed bubble travel.
3. Positioning of the tube in a vertical position is mandatory. A tube one radius off vertical will give an error of approximately 10% in the time of bubble travel.
4. The precision of the method was determined with ten laboratories participating as follows:

Within laboratory (68%)	2.5%
Within laboratory (95%)	4.9%
Between laboratory (68%)	4.5%
Between laboratory (95%)	9.0%

Liquids used ranged from 4.46 to 440.2 stokes.

NOTES

1. Empty standard viscosity tubes are available from Wilkens-Anderson Co., 4525 W. Division St., Chicago, IL 60651 USA. Phone (312) 384–4433.
2. For further information on precision of this method see References.

REFERENCES

1. Holt, K.E., "Proceedings," *Am. Soc. Testing Mats., 57:*297 (1957).
2. *Federation of Paint and Varnish Production Clubs, Official Digest, 30:*540 (1958)

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Table 1
Recommended Numerical Standards for Comparator Viscosity Tubes* (Arranged to show relationship between stokes, bubble seconds, and Gardner-Holdt letters; shown in logarithmic progression).

Bubble time sec*	Tube number	Stokes	Gardner-Holdt letter	Tube number	Stokes	Gardner-Holdt letter
		0.10		10	10	W
		0.13	A3	13	13	X
		0.16		16	16	Y
0.75	.22	0.20	A2	20	20	Z
		0.25		25	25	Z1
0.81	.34	0.32	A1	32	32	Z2
		0.40		40	40	Z3
0.90	.50	0.50	A	50	50	Z4
1.00	.68	0.63	B	63	63	Z5
		0.80	C	80	80	Z6
1.15	.92	1.00	D	100	100	Z7
1.30	1.15	1.25	E	125	125	Z8
1.55	1.45		F	160	160	Z9
1.85	1.80	1.60	G	200	200	Z10
2.20	2.15	2.00	H	250	250	
2.65	2.65	2.50	I	320	320	
3.20	3.20	3.20	J	400	400	
4.00	4.00	4.00	K	500	500	
5.00	5.00	5.00	L	630	630	
6.30	6.30	6.30	M	800	800	
8.00	8.00	8.00	N	1000	1000	
10.00	10.00	10.00	O			
			P			
			Q			
			R			
			S			
			T			
			U			
			V			

[a] The bubble time in seconds of the numerical tubes under 4 sec was determined by a technique employing a movie camera. Above 2.65 the bubble seconds (as measured by the bubble time method) and stokes (as measured by the kinematic method) are approximately equivalent for most products. Below 2.65 this relationship does not hold.

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Titer Test

DEFINITION

This method determines the solidification point of fatty acids.

SCOPE

Applicable to all fatty acids. Replaces L 6a-59.

APPARATUS

1. For titer above 35°C—use a Griffin low-form beaker, 2 L capacity, and wide-mouth bottle capacity 450 mL, height 190 mm and neck i.d. 38 mm. Assemble as shown in illustration in AOCS method G 6-40.
2. For a titer of 35°C and below—see illustration in AOCS method Cc 12-59 for details. A glass beaker may be used instead of stainless steel, if it is desired to observe the test sample during the test. In this case, the 5800 and 8000 mL stainless steel beakers with the ground cork insulation between them may be replaced with a single 8000 mL glass beaker.
3. Test tubes—length 100 mm, dia 25 mm with or without rim. These tubes may have an etched mark extending around the tube at a distance of 57 mm from the bottom to show the height to which the tube is to be filled (see Notes, 1).
4. Stirrer—2–3 mm o.d., with one end bent in the form of a loop of 19 mm o.d. Glass, nichrome, stainless steel, or monel wire may be used. The upper end can be formed to accommodate hand stirring or attached to a mechanical stirrer (see Notes, 2).
5. Laboratory thermometer—0–150°C.
6. Titer test thermometer—AOCS Specification H 6-40. This thermometer should be calibrated against a National Institute of Standards and Technology certified thermometer (see AOCS thermometer specifications). If titer is between 68 and 80°C, thermometer H 7-45 should be used.

REAGENTS

1. Ethylene glycol.
2. Dry ice (solid carbon dioxide).

PROCEDURE

1. Heat the fatty acids on a hot plate to 130°C to remove traces of moisture, and fill a titer test tube to a height of 57 mm from the bottom.

   Caution—The test sample is not to be held at 130°C, nor reheated to this temperature more than once. If excessive moisture is present, allow the water to settle, decant the fatty acids and then refilter and reheat. The acids must be dry and free of suspended matter.
2. For titer above 35°C—fill the water bath to the designated level (see illustration in AOCS Method Cc 12-59) and adjust the temperature 15–20°C below the expected titer point.
3. For titer 35°C and below—fill the bath with ethylene glycol to the designated level. Insert the stainless steel screen and place dry ice just beneath the ethylene level (see illustration in AOCS Method Cc 12-59 for details). Crush the dry ice to about ½ in. cubes and add slowly and cautiously to reach the desired temperature. Maintain the temperature of the bath at 15–20°C below the titer point of the test sample.
4. Place the tube containing the fatty acids in the assembly, as shown in the illustration in method Cc 12-59. Insert the titer thermometer to the immersion mark so that it will be equidistant from the sides of the tube.
5. Stir with the stirring rod in a vertical manner at the rate of 100 complete up and down motions per min. The stirrer should move through a vertical distance of about 38 mm. The agitation is started while the temperature is at least 10°C above the titer point.
6. Stir at the directed rate until the temperature remains constant for 30 sec, or begins to rise for two 30-sec interval. Discontinue stirring immediately, remove the stirrer or raise it out of the test sample, and observe the increase in temperature. The titer point is the highest temperature indicated by the thermometer, during this rise. Duplicate determinations are usually expected to agree within 0.2°C.

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PRECISION

At a confidence level of 95%—

	Fatty Acids		
Materials:	Soybean Linseed	Tallow	Coconut
Range of Values:	17–26	39–44	21–24
1. Two single determinations performed in one laboratory shall not differ from the mean value by more than:	± 2.9%	± 1.0%	± 1.1%
2. Single determinations performed in different laboratories shall not differ from the mean value by more than:	± 7.0%	± 1.0%	± 3.4%

NOTES

1. Titer test tubes may be obtained from Fisher Scientific, catalog number 14-963A.
2. Stirring may be performed mechanically by attaching a small motor with suitable speed-reducing mechanism to the stirring rod.

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Rosin Acids in Fatty Acids

DEFINITION

This method determines the amount of rosin acids in fatty acids.

SCOPE

Applicable to fatty acids containing not more than 15% rosin acids. Replaces L 14a-58.

APPARATUS

1. Flask— 250 mL Erlenmeyer, of chemically resistant glass with a Ʇ 24/40 neck.
2. Condenser— water cooled, with Ʇ 24/40 joint fitting the flask described in Apparatus, 1.
3. Separatory funnel— 500 mL, pear-shaped, fitted with a glass stopper.
4. Burette—50 mL capacity with 0.1 mL divisions.

REAGENTS

1. Methanol—99.5%.
2. Sulfuric acid—sp. gr. 1.84 (see Notes, *Caution*)
3. Sodium sulfate solution, 10%—dissolve 100 g of anhydrous sodium sulfate in 1 L of water.
4. Diethyl ether—reagent grade (see Notes, *Caution*).
5. Methyl orange indicator—dissolve 0.1 g of methyl orange in 100 mL of water.
6. Ethyl alcohol—natural 95% ethyl alcohol or neutral denatured alcohol conforming to SDA formula 30 or 3A.
7. Phenolphthalein indicator—dissolve 1 g of phenolphthalein in 100 mL of methanol.
8. Standard alcoholic potassium hydroxide solution, 0.2 M or 0.5 M.—dissolve (see Notes, *Caution*) 13.3 g of KOH (preferably in pellet form) for a 0.2 M solution or 33.3 g of KOH for a 0.5 M solution in methanol. Dilute to 1 L with methanol. Standardize solution to ± 0.001 M (see AOCS Specification H 12-52). The standardized solution should be protected against evaporation and the absorption of CO_2 from the air, and should be standardized frequently.

PROCEDURE

1. Dissolve 40 ± 0.1 g of the test sample in 100 mL of methanol in a 250 mL Erlenmeyer flask. Swirl the flask to dissolve the oil and add a clean boiling chip.
2. Add slowly to 5 mL of H_2SO_4 while swirling the flask vigorously. Connect the flask to the condenser, apply heat and reflux the contents for 10 min.
3. Cool the flask to room temperature with cold water.
4. Add 250 mL of the Na_2SO_4 solution to a 500 mL separatory funnel. Pour the contents of the flask into the funnel and complete the quantitative transfer of the flask contents with 100 mL of diethyl ether.
5. Thoroughly shake the mixture in the funnel. Allow to settle, draw off the salt layer and discard.
6. Wash contents of the funnel twice again with 250 mL portions of Na_2SO_4 solution. The last washing should not react with methyl orange indicator to give a pink color.
7. After removing the last wash, drain the contents of the separatory funnel into a 500 mL Erlenmeyer flask. Rinse the funnel with 20 mL of diethyl ether and add the rinsing to the flask.
8. Add 20 mL of ethyl alcohol and 1 mL of the phenolphthalein indicator. Titrate to the appearance of a pink-red color using 0.2 M alcoholic KOH if the rosin acids content is less than 5% and 0.5 M if more than 5%.

CALCULATIONS

Calculate the percentage of rosin acids as follows (see Notes, 1)—

$$\%\text{Rosin Acids} = \frac{\left(1.031 \times \text{titration, mL} \times \text{M} \times 30.2\right)}{\text{mass of test portion, g}} - \left(0.74\right)$$

Where—

M = molarity of standardized potassium hydroxide

PRECISION

1. Agreement within laboratory—two single determinations performed in one laboratory shall not differ by more than 0.20%.
2. Agreement between laboratories—single determinations performed in two different laboratories shall not differ by more than 0.34%.

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NOTES

Caution

Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. It is an oxidizing agent and should not be stored in the vicinity of organic materials. Use great caution in mixing with water due to heat evolution that can cause explosive spattering. Always add the acid to water, never the reverse.

Diethyl ether is highly flammable and is a severe fire and explosion hazard when exposed to heat or flame. It is a central nervous system depressant by inhalation and skin absorption. It will form explosive peroxides upon exposure to light. Handle empty containers, particularly those from which ether has evaporated, with extreme caution. Explosive limits in air are 1.85–48%. The TLV is 400 ppm in air. A fume hood should be used at all times when using diethyl ether.

When working with extremely caustic materials like sodium hydroxide and potassium hydroxide, always add pellets to water and not the reverse. These alkalies are extremely exothermic when mixed with water. Take precautions to contain the caustic solution in the event the mixing container breaks from the extreme heat generated.

NUMBERED NOTES

1. For further information on this method, including derivation of correcting factors, see *JAOCS 29*:342 (1952).

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Acetone Tolerance

DEFINITION

This method measures the higher polymer in heat-polymerized drying oils by determining the solubility in acetone under the specific conditions of the test. The acetone number is the weight of acetone in g required to produce a cloudiness in 100 g of oil at 25°C.

SCOPE

Applicable to heat-bodied drying oils. Replaces Ka 11-55.

APPARATUS

1. Glass-stoppered Erlenmeyer flasks—200 mL capacity.
2. Burette—50 mL.
3. Drying tube.
4. Water packet for burette—shown in illustration in this method.
5. Constant temperature bath—capable of maintaining a temperature of 25 ± 0.1°C.
6. Glass beads—approximately $^1/_{16}$–$^1/_8$ in dia.

REAGENTS

1. Acetone—ACS grade (see Notes). Store the acetone in a bottle fitted with a drying tube.
2. Determine the specific gravity of the dried acetone at 25 C/25°C, using method To 1a-64, before proceeding with the acetone number determination. The acetone should have a specific gravity of 0.7864–0.7872 corresponding to a water content of 0.00 to a maximum of 0.24%.

PROCEDURE

1. Weigh 30 g of the test sample with an accuracy of 0.01 g into a glass-stoppered Erlenmeyer flask containing 3–4 glass beads. Clamp the flask in the constant temperature bath so that the level of the water in the bath is higher than the test sample level.
2. Fill the burette with acetone and place a drying tube at the top of the burette to protect against moisture. Allow at least 10 min for the test sample and acetone to attain a temperature of 25°C.
3. Disconnect the flask from the clamp and remove the stopper from the flask. Add a small amount of acetone, restopper and swirl gently to dissolve the contents, keeping the flask immersed in the water bath. Continue addition of acetone, taking care not to splash the contents of the flask over the upper part of the flask during mixing. Each addition of acetone produces a dense "cloud" in the liquid layer, which is discharged by agitation. Near the end point, the cloudiness persists for longer periods of time. Add the reagent dropwise at this point until the addition of one drop produces a permanent dense cloud in the liquid layer.
4. Remove the stoppered flask from the bath and wipe dry with a clean cloth. Weigh the flask and its contents and record the weight of the added acetone.

CALCULATIONS

$$\text{Acetone Number} = \frac{\text{mass of acetone, g}}{\text{mass of oil, g}} \times 100$$

NOTES

The acetone should be dry and kept over molecular sieves.

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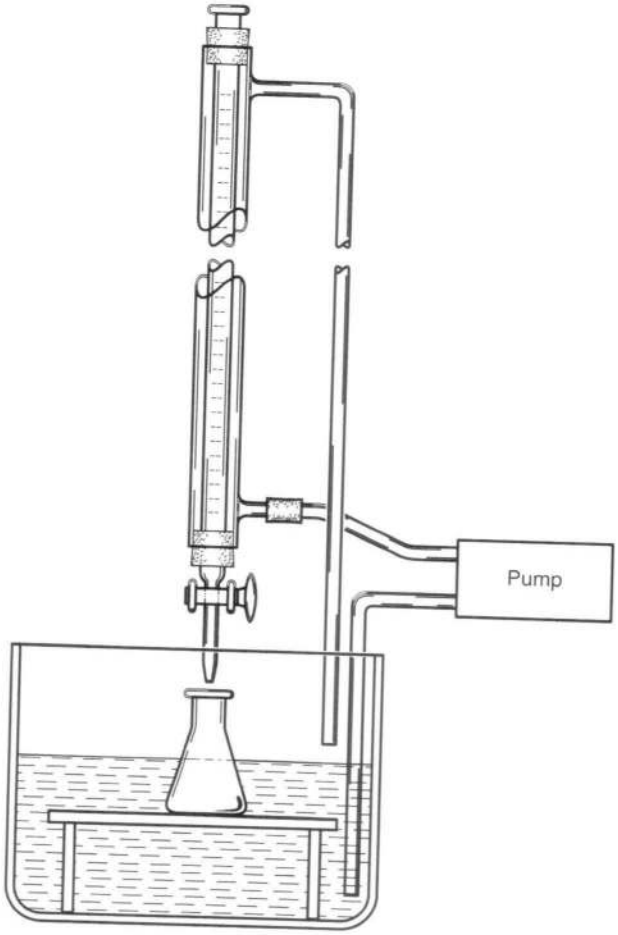

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pH of Fatty Quaternary Ammonium Chlorides

DEFINITION

This method determines the pH of the test sample in water under the conditions of the test.

SCOPE

Applicable to fatty quaternary ammonium chlorides. Replaces Nc 6-62.

APPARATUS

1. pH meter—equipped with external electrodes and having a sensitivity capable of ± 0.05 pH unit readings.
   (a) Calomel electrode—range 5–100°C.
   (b) Glass electrode—range 5–100°C.
   (c) Combination electrode
2. Beakers—250 mL, low-form, Pyrex™ brand or equivalent is suitable.
3. Magnetic stirrer—with Teflon™-coated stirring bar.

REAGENTS

1. Buffer solutions—pH 4.0, pH 7.0 and pH 10.0, prepared from standard buffer concentrate, to produce exact pH solution when used per instructions, for standardizing the pH meter.
2. Isopropanol solution—made by mixing equal volumes of reagent grade isopropanol and distilled water.

STANDARDIZATION OF PH METER

1. Using standard buffer solutions (Reagents, 1), carefully follow the manufacturer's instructions for the particular meter on hand.

PROCEDURE

1. Determine percent solids by AOCS Method Te 2a-64
2. Determine the weight of test sample based on percent solids needed to make 200 g of 5% solution using the following formula:

$$\frac{200 \times 5}{\%\ \text{solids}}$$

3. Melt the test sample in a water bath if it is not already liquid, mix thoroughly and weigh the proper amount into a 250 mL beaker. Do not exceed the melting point of the test sample by more than 10°C.
4. Add 100 g of the isopropanol solution and warm, if necessary, to dissolve the test sample.
5. Cool to room temperature and add enough isopropanol solution into the beaker to give a total of 200 g of isopropanol solution and test sample.
6. Insert the stirring bar into the 250 mL beaker, and while stirring, determine the pH to the nearest 0.1 unit in the same manner as the standardization referred to in Standardization of pH Meter, 1.

PRECISION

At a confidence level of 95%, in the range of values of 6.4–8.5—

1. Two single determinations prepared in one laboratory shall not differ from the true mean value by more than ± 4.0%.
2. Single determinations performed in different laboratories shall not differ from the true mean value by more than ± 8.1%.

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Average Molecular Weight of Fatty, Quaternary Ammonium Chlorides

DEFINITION

This method determines the average molecular weight of a fatty quaternary ammonium chloride by converting to the acetate, titrating potentiometrically and correcting for the non-quaternary components.

SCOPE

Applicable to fatty quaternary ammonium chlorides.

APPARATUS

1. pH meter—equipped with external electrodes and having a sensitivity capable of ± 0.05 pH unit reading or similar potentiometric titrator.
   (a) Calomel electrode—range 5–100°C.
   (b) Glass electrode—range 5–100°C.
2. Magnetic stirrer—with Teflon™-coated stirring bar.
3. Beakers—250 mL and 2000 mL, low form, Pyrex™ is suitable.
4. Graduated cylinders—25 and 2000 mL.
5. Volumetric flask—2000 mL.
6. Glass funnel.
7. Burette—25 mL.

REAGENTS

1. Glacial acetic acid—ACS or AR grade.
2. Chloroform—ACS or AR grade (see Notes, *Caution* and 1).
3. Mercuric acetate—ACS or AR grade (see Notes, *Caution*).
4. Acetic anhydride—ACS or AR grade (see Notes, *Caution*).
5. Perchloric acid—ACS or AR grade (see Notes, *Caution*).
6. Potassium acid phthalate—primary standard.
7. Buffer solutions—pH 4.0, pH 7.0 and pH 10.0, prepared from standard buffer concentrate, to produce exact pH solution when used per instructions, for standardizing the pH meter.

SOLUTIONS

1. Mercuric acetate solution—dissolve 6 g in 100 mL of glacial acetic acid. Prepare fresh for each determination.
2. Perchloric acid, approximately 0.1 N in glacial acetic acid—add 28.4 g perchloric acid to 1000 mL of glacial acetic acid in a 2000 mL beaker while stirring. Carefully add 46.6 g of acetic anhydride while stirring. Carefully pour the solution through the funnel into a 2000 mL volumetric flask and dilute to mark with glacial acetic acid. Mix the solution, and allow to stand for 24 hr before standardizing.
   (a) Standardization—accurately weigh 0.31–0.39 ± 0.0001 g of finely ground and dried potassium acid phthalate into a 250 mL beaker, using analytical balance. Add 50 mL of glacial acetic acid, and warm gently to dissolve the test sample. Cool, and add an additional 50 mL of glacial acetic acid washing down the sides of the beaker. Insert the stirring bar into the beaker. While stirring, titrate with the perchloric acid solution using the millivolt scale. Record the millivolt reading every 0.1 mL. Plot a graph showing the millivolts against the titration. The end point is the midpoint of the inflection on the titration curve. The strength of the perchloric acid is expressed in terms of its molarity:

$$\text{Molarity of perchloric acid solution} = \frac{\text{mass, g of acid potassium phthalate} \times 4.8967}{\text{volume, mL of perchloric acid solution}}$$

STANDARDIZATION OF pH METER

Using standard buffer solutions (Reagents, 7), carefully follow the manufacturer's instructions for the particular meter on hand.

PROCEDURE

1. Determine the acid value, free amine value, percent free amine, and percent amine hydrochloride by AOCS Method Te 3a-64.
2. Determine the percent ash by AOCS Method Tm 2a-64.

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3. Determine the percent nonvolatiles by AOCS Method Tc 2a-64.
4. Melt the test sample, if not already liquid, in a water bath, mix thoroughly and accurately weigh 1.0–1.5 ± 0.0001 g into a 250 mL beaker.
5. Add 100 mL glacial acetic acid, and heat gently, if necessary, to affect solution. Add 15 mL of mercuric acetate solution. Insert the stirring bar into the beaker and stir for 5 min.
6. Add 20 mL chloroform (see Notes, *Caution* and 1), and titrate, while stirring, with 0.1 M $HClO_4$ using the millivolt scale.
7. Record the millivolt reading every mL but in the vicinity of the end point, record the millivolt readings every 0.1 mL. Plot a graph showing the millivolt readings against the titration. The end point is the midpoint of the inflection on the titration curve.

CALCULATIONS

$$\text{Average molecular weight} = \frac{\%\text{ nonvolatiles} - \%\text{ ash} - \%\text{ amine hydrochloride} - \%\text{ free amine}}{\left[\frac{\text{mL of titration} \times \text{N}}{\text{mass of test portion, g} \times 10} - \frac{\%\text{ ash}}{58.5} - \frac{\text{acid value}}{561} - \frac{\text{amine value}}{561}\right]}$$

PRECISION

At a confidence level of 95%, in the range of 349–561:

1. Two single determinations performed in one laboratory shall not differ from the true mean value by more than ± 3.1%.
2. Single determinations performed in different laboratories shall not differ from the true mean value by more than ± 6.4%.

NOTES

Caution

Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Avoid contact with the skin. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal. It is nonflammable, but will burn on prolonged exposure to flame or high temperature. The TLV is 10 ppm in air. A fume hood should be used at all times when using chloroform.

Mercury and mercury compounds are highly toxic by ingestion, inhalation, and skin absorption. The TLV (as mercury) is 0.05 mg/m^3 of air. See the Laboratory Safety section in Volume 1 of this Edition for a procedure to contain environmental contamination of mercury waste from Kjeldahl digestions. Disposal of any mercury waste must be in accordance with local and EPA regulations.

Acetic anhydride is a strong irritant and corrosive. It can cause burns and eye damage. Eyes and hands should be protected when working with this compound. Work should be carried out in a properly operating fume hood.

Perchloric acid is a strong acid and strong oxidizing agent. It can ignite or detonate by shock or heat when in contact with organic materials. It is toxic by ingestion and inhalation and is a strong irritant. It should not be stored near any type of organic material. See OSHA regulations for the proper storage of hazardous chemicals.

NUMBERED NOTES

1. 1,1,1-Trichloroethane (TCE) has been suggested as a replacement for chloroform in this method. Although TCE is a halogenated hydrocarbon, the TLV (350 ppm) is less than those of chloroform, carbon tetrachloride and methylene chloride. Cyclohexane and isooctane may also be considered as alternate solvents. These solvents have not been collaboratively studied within the AOCS technical committees. This recommendation does not represent official approval by the AOCS Uniform Methods Committee.

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AOCS Official Method Tw 1a-64

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Percent Non-Amines in Fatty Amines and Diamines

DEFINITION

Non-amines are those substances not absorbed on a column of ion exchange resin under the conditions of the test. In fatty amines, they are normally composed of fatty amides, substituted fatty amides, fatty nitriles, fatty alcohols, and unsaponifiables derived from the original fatty acids or fatty alcohols.

SCOPE

Applicable to fatty amines and diamines.

APPARATUS

1. Chromatographic columns—made by attaching 500 mL bulbs with Ʇ 24/40 joints to chromatographic tubes, available from Scientific Glass Apparatus Co., Catalog # JC-235011 with attached fritted-glass discs inside a Ʇ 19/22 joint (see illustration in this method).
2. Beakers—Griffin, low-form, 150, 250 and 1000 mL.
3. Sintered glass funnel—coarse, 350 mL.

REAGENTS

1. Dowex 50W-X4—500–100 mesh reagent grade resin, or equivalent (see Notes, 1).
2. Isopropyl alcohol—reagent grade.

SOLUTIONS

1. Hydrochloric acid approximately 0.2 M in isopropyl alcohol—accurately standardized (see AOCS Specification H 14-52).

PREPARATION OF CHROMATOGRAPHIC COLUMNS

1. Place the Dowex 50W-X4 resin in a 250 mL beaker, pour 100 mL of isopropyl alcohol on the resin and stir.
2. Filter with suction through a coarse sintered glass funnel.
3. Repeat until there is no residue left upon evaporation of the isopropyl alcohol used to wash the residue. Transfer the washed resin to a bottle and add enough isopropyl alcohol to just cover the resin.
4. To fill the chromatographic column, first place approximately 1 cm of glass wool on top of the fritted glass disc, then pour a slurry of resin in isopropyl alcohol on top of the glass wool. For fatty amines use enough resin to just fill the column after the resin has settled. For fatty diamines, a 500 mm column should be packed with 450 mm of resin. Once the resin has been placed in the column, keep it covered with isopropyl alcohol at all times.

PROCEDURE

1. Accurately weigh into a 150 mL beaker approximately 5 g of test sample for fatty amines or 2.5 g of test sample for fatty diamines.
2. Add 75 mL of isopropyl alcohol and warm to dissolve the test sample.
3. Allow the level of isopropyl alcohol to fall to the top of the resin column and quantitatively transfer the amine solution onto the column so as not to disturb the resin. Wash the beaker with a small quantity of hot (55–60°C) isopropyl alcohol and add to the column (see Notes, 2).
4. Place a 1 L beaker under the column to collect the eluate.
5. When the test sample solution has run down to the top of the resin, slowly add 500 mL of isopropyl alcohol so as not to disturb the resin.
6. When the alcohol level is low enough, add another 200 mL of isopropyl alcohol to the bulb.
7. Collect the eluate until the flow stops.
8. Evaporate the eluate on the steam bath to about 75 mL and quantitatively transfer it to a tared 250 mL beaker containing several glass beads.
9. Evaporate the concentrated eluate to dryness on the steam bath.
10. Dry the residue in a vacuum oven at 70°C for 2 hr with 686–737 mm Hg (27–29 in.) of vacuum.
11. Weigh the beaker containing the dried residue.
12. Dissolve the residue in 50 mL of isopropyl alcohol.
13. Add 5–10 drops of 0.2% bromphenol blue in isopropanol and while stirring, titrate, using a microburette, with 0.2 M HCl in isopropanol to a yellow end point (see Notes, 3).

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CALCULATIONS

$$\% \text{ Non-Amines} = \left[1.00 - \left(\frac{\text{mL} \times \text{M} \times 56.1}{\text{mass residue, g} \times \text{amine value of test portion}}\right)\right] \times \frac{\text{mass residue, g}}{\text{mass test portion}} \times 100$$

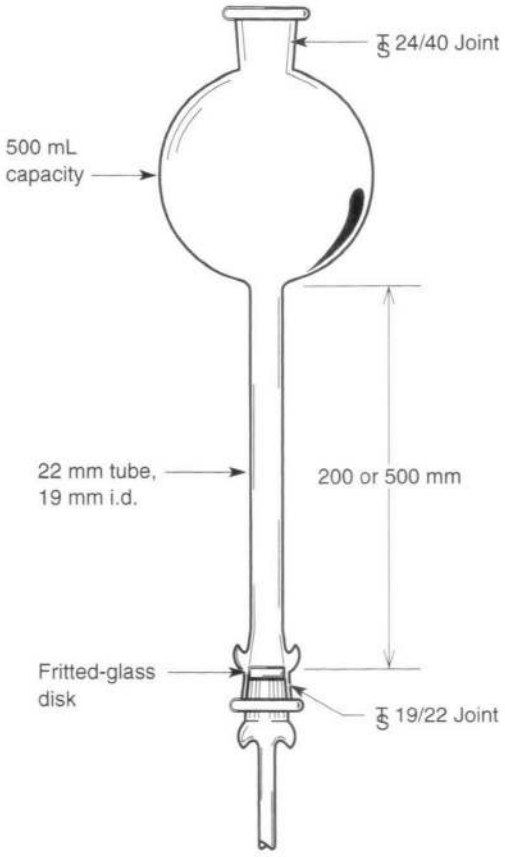
Where—

mL = volume, mL of standardized HCl

M = Molarity of standardized HCl amine value determined by AOCS Method Tf 1a-64 or Tf 1b-64.

NOTES

1. Blanks should be run on each new batch of resin. Use 80 g of resin for duplicate determinations of fatty amines using 200 mm columns and 200 g of resin for duplicate determinations using 500 mm columns for fatty diamines. Experience has shown that the total quantity of isopropyl alcohol used for washing the resin requires 400 mL for 80 g batches and 900 mL for 200 g batches of resin.
2. Since some amines are not very soluble in isopropyl alcohol, it is necessary to heat the chromatographic column bulb so that the amine solution is maintained at 55–60°C until it has passed onto the resin. The heating can be discontinued when the 500 mL of isopropyl alcohol are added. The bulb can be conveniently heated by suspending the bulb through a heating mantle top, sized to fit a 500 mL flask, which is connected to a variable transformer. The titration is performed to determine the small amount of fatty amine that may be present in the residue. It is assumed that amines that pass through the column have the same average molecular weight as those in the original test sample.



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AOCS Official Method Tx 1a-66

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Hydroxyl Value of Epoxidized Oils

DEFINITION

The hydroxyl value is defined as the mg of potassium hydroxide equivalent to the hydroxyl content of 1 g of test sample.

SCOPE

Applicable to oils and their derivatives containing an oxirane ring structure.

APPARATUS

1. Erlenmeyer flasks—250 mL, with Ʇ 24/40 glass stopper.
2. Pipet—5 mL, volumetric.
3. Burette—50 mL, graduated in 0.1 mL divisions.

REAGENTS

1. Pyridine—C.P. or reagent grade distilled at 114–115°C (see Notes, *Caution*).
2. Acetic anhydride—ACS grade, fresh (see Notes, *Caution).*
3. Pyridine-acetic anhydride reagent—freshly prepared before use by mixing 3 volumes of pyridine with 1 volume of acetic anhydride.
4. *n*-butyl alcohol—C.P. or reagent grade neutralized with 0.5 M ethanolic KOH to faint pink phenolphthalein end point.
5. Ethanolic potassium hydroxide solution, 0.5 M or 1.0 M—standardized, using AOCS Specification H 15-52 (see Notes, 1–3).
6. Phenolphthalein indicator solution—1% in ethanol.

PROCEDURE

1. Weigh to the nearest 0.1 mg into the Erlenmeyer flask the indicated amount of the test sample for acetylation—

Expected Hydroxyl Value	Test Sample Mass
0–25	10 g ± 0.1 g
25–50	5 g ± 0.1 g
50–100	2.5 g ± 0.1 g
100–200	1.25 g ± 0.1 g

2. Pipet 5.0 mL of the pyridine-acetic anhydride reagent into the flask containing the test sample for acetylation. For test samples having a 0–25 hydroxyl value, add an additional 5 mL of pyridine (not neutralized) to the flask. Thoroughly mix the contents by swirling. Stopper flask using a small amount of stop-cock grease for seal. Let stand at 25°C for 24 hr.

   Note—If 1.0 M alcoholic KOH is available for titrating, then use 10 mL of pyridine-acetic anhydride reagent.
3. Pipet 5.0 mL of pyridine-acetic anhydride reagent into an empty flask for the reagent blank (add the same volume of any extra pyridine, not neutralized, that was added to test sample). Stopper the same as the test sample and let stand at 25 ± 5°C for 24 hr.
4. Determine the Acid Value by AOCS Method Te 2a-64.
5. After the test sample and blank flasks have stood for 24 hr add 10 mL of water. Use water to wash stoppers and sides of flasks. Add 10 mL of pyridine (not neutralized), again washing stoppers and sides of flasks. Stopper and mix thoroughly by swirling. Let flasks stand for 30 min after mixing with occasional swirling of the flask before proceeding to next step.
6. Add 25 mL of n-butyl alcohol (neutralized to phenolphthalein end point) to both test sample and blank, again rinsing off stoppers and sides of the flask.
7. Add 1 mL of phenolphthalein indicator solution to both test sample and blank. Titrate to a faint pink end point with 0.5 M alcoholic KOH solution.

CALCULATIONS

1. $$\text{Uncorrected Hydroxyl Value} = \frac{(B - S) \times M \times 56.1}{W}$$
2. Corrected Hydroxyl Value = Uncorrected Hydroxyl Value + Acid Value
3. $$\%\ OH = \frac{\text{hydroxyl value}}{32.98}$$

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Where—

B = volume, mL KOH solution required for the reagent blank, Procedure, 7
S = volume, mL KOH solution required for the ± 0.1 titration of the acetylated test portion, Procedure, 7
W = mass of test portion used for acetylation, Procedure, 1 Acid Value determined by AOCS Method Te 2a-64

PRECISION

The method precision was determined in accordance with AOCS Method M 1-59 (surplus method, 1989), using five laboratories and three types of epoxidized products. Precision at the 95% confidence level for each product type is:

Type of Epoxidized Oil	Epoxy Soybean	Epoxy Tallate	Epoxy Castor
Average hydroxyl value:	7.57	29.6	180.5
Agreement Within a Laboratory - Two single determinations performed within a laboratory should not differ by more than:	1.90	2.20	6.68
Agreement Among Laboratories - Single determinations performed in two different laboratories should not differ by more than:	2.31	4.50	8.53

NOTES

Caution

Pyridine is flammable and a dangerous fire risk. The explosive limits in air are 1.8–12.4%. It is toxic by ingestion and inhalation. The TLV is 5 ppm in air. The danger from crude pyridine is greater than from pure pyridine, the associated homologs and impurities being even more toxic than pyridine itself.

Acetic anhydride is a strong irritant and corrosive. It can cause burns and eye damage. Eyes and hands should be protected when working with this compound. Work should be carried out in a properly operating fume hood.

NUMBERED NOTES

1. The caustic strength should not be less than 0.5 M (or 1.0 M if 10 mL of acetic anhydride reagent is used) in order that the blank titration will not require more than 50 mL. Refilling of the burette during any single titration is to be avoided.
2. Ethanolic KOH should be prepared from Formula 30 ethanol (denatured with methanol).
3. Methanolic KOH may be substituted for ethanolic KOH.

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AOCS Official Method Ty 1a-76

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Dicarboxylic Acid Composition by Gas-Liquid Chromatography of Dimethyl Esters

DEFINITION

This method relates standard conditions for the separation and identification of dimethyl esters of dicarboxylic acids by gas-liquid chromatography (GLC).

SCOPE

The method is applicable to dimethyl esters of dicarboxylic acids having 5–10 carbon atoms. The use of the FFAP liquid phase facilitates the separation of dicarboxylic acid dimethyl esters (see Notes, 1 and 2). Separation of dimethyl esters of unsaturated or chain-substituted derivatives of dicarboxylic acids has not been investigated by this method.

APPARATUS

1. Gas chromatographic instrument having the following minimal characteristics—
   (a) Column oven-capable of constant temperature operation to ± 1.0°C at a temperature from 130–210°C.
   (b) Sample inlet port—with independent temperature operation to ± 1.0°C at a temperature from 130–210°C.
   (c) Columns— 1.5–3.0 m (5–10 feet) long, 0.32–0.63 cm (⅛–¼ in.) o.d., made of stainless steel or glass tubing. Columns of 0.32 cm (⅛ in.) o.d. are packed with 5–10% FFAP liquid phase coated on 80-100 mesh Chromosorb W-AW or equivalent. The 0.63 cm (¼ in.) o.d. columns are packed with 5–12% FFAP liquid phase coated on 60–80 mesh Chromosorb W-AW or the equivalent. Prepacked FFAP columns are available from Supelco, Inc,, Supelco Park, Bellefonte, PA 16823 USA.
   (d) Detectors—either thermal conductivity (TC) or flame-ionization (FID) type. If separately thermostated, they should be maintained at column temperature or up to 50°C hotter.
   (e) Recorder—0–1.0, 2.5 or 5.0 mv range. One sec full scale deflection with a chart speed of ½–1 in./min. Attenuator switch to change recorder range as required. The 0–1.0 mv recorder is used when a thermal conductivity detector is employed; any one of the three may be used with the flame ionization detector.
   (f) Gases—Carrier gas for thermal conductivity (TC) detection, use helium, minimum purity of 99.95 mol%; for flame-ionization detection, use helium, nitrogen, or argon, minimum purity of 99.95 mol%.

   For flame-ionization detector (FID), use hydrogen, minimum purity 99.95 mol% and dry air, dry dew point -75 F max.
2. Syringe for injecting test sample—a 1 or 5 µL capacity with known and reproducible volume. A Hamilton 7000 Series syringe or equivalent is recommended.

REAGENTS

1. Column packing—FFAP (free fatty acid phase, Carbowax 20M reacted with nitroterephthalic acid). Available from Analabs, Inc., 80 Republic Drive, North Haven, CT 06473 USA and Supelco, Inc., Supelco Park, Bellefonte, PA 16823 USA.
2. Dicarboxylic acid dimethyl ester standard—prepare a synthetic mixture of known weight percent diesters of glutaric, adipic, pimelic, suberic, azelaic, and sebacic having a composition similar to the type and amount of diesters present in the test sample to be analyzed. Pure dimethyl esters are available from Analabs, Inc., 80 Republic Drive, North Haven, CT 06473 USA.

CALIBRATION

1. Relative response factors should be determined for each component to correct for nonlinearity of instrument response due to molecular weight differences. These factors are determined by analyzing a dimethyl ester standard mixture, preferably having a composition similar in type and weight percent of components to that of the test sample. The units of area per weight percent are calculated by dividing the area of each peak in the standard mixture by its weight percent. Then relative response values can be calculated by dividing this number by the units of area per weight percent obtained for the major component.

$$\text{Relative response factor} = \frac{\left(A_X/W_X\right)}{\left(A_Y/W_Y\right)}$$

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Where—

A_X = area % of each peak

A_Y = area % of the major component

W_X = wt % of each peak

W_Y = st % of the major component

PROCEDURE

1. With carrier gas flowing through the apparatus, adjust the inlet port, column oven, and detector to operating temperature. A column temperature either in the range of 130–175°C for analysis of lower molecular weight esters as glutaric, adipic, and pimelic or in the range of 170–210°C for higher molecular weight esters is recommended. A new column should be conditioned at about 10°C above the maximum isothermal column operating temperature with helium flowing for at least 24 hr. The column should be disconnected from the detector during the conditioning period. With FID, adjust the hydrogen and air flow-rates, ignite the flame and operate the apparatus at medium sensitivity rather than near its maximum sensitivity. Record a baseline to check for stability of the instrument.
2. The proper gas flow-rate and temperature will permit elution of dimethyl sebacate and shorter chain length dimethyl esters in less than 30 min. A constant gas flow should be maintained throughout the analysis to give a linear signal response.
3. Measure the dimethyl ester standard using the syringe. When using a TC detector, the usual test sample size is between 0.5–4 μL. For FID, about 0.01–0.1 mg of actual esters are measured, but this is usually in a suitable solvent so that 0.1–1.0 μL of solution is injected. Pierce the septum of the test sample inlet port and quickly discharge the test sample. Withdraw the needle and note on the recorder chart the small peak due to air when using TC or solvent when using FID, which marks the test sample introduction reference point. Test sample size must be adjusted so that the major peak is not attenuated more than four times, preferably less.
4. Determine the instrument and column performance by noting the separation of the ester peaks which differ by one carbon atom in chain length in the dimethyl ester standard. Complete separation of each of the esters present must be obtained.
5. Having determined the optimum conditions, inject a second test sample of the dimethyl ester standard and watch the recorder pen to see that the peaks do not go off scale. Change the setting of the attenuator if necessary to keep the peaks on the chart paper. Note the attenuation on the chart at the point that changes are made.
6. Record the chromatogram of the test sample(s) of dibasic acid dimethyl esters to be analyzed such that each principle component peak is recorded full scale by means of the attenuator. After all the peaks have been traced and the pen has returned to baseline, remove the chart for calculation.

CALCULATIONS

1. Identify the peaks by relative position on the chart. The diesters appear on the chromatogram in order of increasing number of carbon atoms. With constant operating conditions, the retention time (or chart distance) from the air peak to various test portion component peaks can be used for identification. However, relative retentions are more reproducible. Relative retentions are determined by dividing the observed retention time for each peak by the retention time observed for the main peak of the product in question (or other peak if some other basis is desired). Identification must be established with known mixtures chromatographed under the same conditions before or after the unknown test portions are analyzed.
2. Determine the area of each peak. If the instrument is equipped with an electromechanical or electronic integrator, the area is best measured by following the manufacturer's instructions. When an integrator is employed, the baseline must remain constant or the integrator must be equipped with a baseline corrector. Otherwise, the area is obtained by drawing lines tangent to the sides of the peak and intersecting the baseline. Determine the area of the resulting triangle by multiplying the height by one half the base. For an attenuated peak the outer sides of the peak must be full chart span and the tangents drawn intersecting the base-line should utilize the upper two-thirds to obtain the peak width. Determine the area by multiplying the height by one half the base. The height must be corrected for attenuation including the correction required if the baseline is not the recorder zero:

$$\text{Area} = \tfrac{1}{2} \text{ base} \times \text{height} \times \text{attenuation factor}$$

Divide the area of each component by its relative response factor obtained experimentally in accordance with the Calibration Section, to obtain the corrected area:

$$A_X \text{ (Corrected Area)} = \frac{\text{peak area}}{\text{relative response factor}}$$

The percentage of each component is calculated from the ratio of each corrected area to the sum of the corrected areas under all the component peaks and reported as percent by weight—

$$\% X = \frac{A_X}{\Sigma A_N} \times 100$$

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Where—

$\%_X$ = mass % of each component

A_X = corrected area of each component

ΣA_N = sum of the corrected areas under all of the component peaks

PRECISION

1. Two single determinations of major components (>5%) performed in one laboratory shall not differ by more than 1.49%.
2. Two single determinations performed in different laboratories shall not differ by more than 4.3%.

NOTES

1. Diethylene glycol succinate liquid phase columns of the same length, diameter, phase loading level, and support as described for FFAP under Apparatus, 1 also facilitate the separation of dicarboxylic acid dimethyl esters. The additional advantage of the FFAP column is that it will separate methylethyl esters of dicarboxylic esters. The additional advantage of the FFAP column is that it will separate methylethyl esters of dicarboxylic acids from dimethyl esters in the event methylethyl esters are present in the test portion. Prepackaged diethylene glycol succinate columns are available from Supelco, Inc., Supelco Park, Bellefonte, PA 16823 USA.
2. It is recommended that chromatographers read *Tentative Recommended Practice for General Gas Chromatography Procedures.* ASTM Designation: E260-65T, issued 1965.

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AOCS Recommended Practice Tz 1a-78

Reapproved 2009

Activity of Hydrogenation Catalysts

DEFINITION

This recommended practice determines the activity of a catalyst relative to a standard catalyst under standardized conditions of this test (see Notes, 1).

SCOPE

Applicable to all hydrogenation catalysts for the hydrogenation of vegetable derived oils. Formerly Ca 17-78.

APPARATUS

1. Hydrogenator—capable of holding up to 2 L of vegetable oil. Unit must be capable of temperature control (both heating and cooling), pressure control and the adjustment of agitation rate.
2. Standard hydrogenation catalyst—tested and approved by the designated Committee of the AOCS and available from the AOCS Office, Urbana, IL USA.
3. A quality refined and bleached soybean oil meeting the following specifications—
   (a) <0.1% FFA as determined by AOCS Method Ca 5a-40.
   (b) <1.5 ppm sodium as determined by atomic absorption spectrophotometry or calibrated by other official or tentative methods.
   (c) <0.01% phosphatides as determined by AOCS Method Ca 12-55.
4. Sample jars.

STANDARDIZATION OF THE REACTOR

1. The agitation rate required to obtain an 80 IV product with soybean oil in 40 min, using the standard catalyst establishes the standard condition for the particular reactor used. Hence, that rate, along with the 177°C (350°F) and 6.82 kg/cc (15 psig) of hydrogen and 0.05% nickel in the oil, become fixed conditions for the comparison of catalyst for that reactor.
   (a) Rinse the reactor before each use with a sample of the oil and then discard the rinse oil. If the rinse is to be made after the reactor has been idle more than 5 d, the oil should be brought up to the temperature of the proposed hydrogenation, 177°C (350°F), with agitation and under vacuum or in the presence of 9.9 kg/cc (20 psig) of nitrogen.
   (b) Separate a test sample of soybean oil (meeting the specifications shown under Apparatus, 3) into two portions equal in total quantity to approximately 50% of capacity of the reactor. Thus, for a 2 L reactor, a total charge of 1000 g is to be used. To one portion of the oil, add the full complement of 0.05% nickel by weight of the oil (the standard catalyst has a nominal nickel content of 25%). Thus, for a 1000 g charge of oil, 0.2% or 2 g of the standard catalyst is utilized. Add the portion of the oil containing the catalyst to the reactor with the second half of the oil test sample. Seal the reactor and flush with nitrogen or evacuate and heat to 177 ± 3°C (350 ± 5°F) under agitation. Replace the nitrogen with hydrogen at 6.82 kg/cc (15 psig) and proceed to hydrogenate.
   (c) The hydrogen pressure and the temperature of the oil should be checked frequently (at 5 min intervals is appropriate) during each run and adjustments made if necessary.
   (d) After 15 min, the reaction is stopped by flushing the hydrogen out with nitrogen and a small test sample of the oil is drawn for the determination of iodine value or refractive index. On the basis of the reaction rate determined, the reaction is continued until the determined end point (time or hydrogen quantity) has been established. The reaction need not be stopped if the reactor used permits drawing out the test samples without changing pressure. Calculate the kg/cc (psig) of hydrogen per unit IV drop or the IV drop per min. This is the hydrogenation factor. If refractive index is the monitoring tool, a 1.0 IV drop equals a 0.000123 drop in the refractive index. It is important to note all parameters—weight of oil, agitation rate (rpm), pressure 6.82 kg/cc (15 psig), temperature 177 ± 3°C (350 ± 5°F), and reaction time.
   (e) From the data obtained above, predict the amount of time needed to reach exactly an 80 IV. This might be achieved by preparation of a graph of the change in IV or refractive index vs. time. Increase or decrease the agitation rate of succeeding runs to achieve a hydrogenation time of 40 ± 4 min to reach an 80 IV with a fresh oil test sample. Use may also be made of the amount of hydrogen reacting with the oil for determining the reaction end point if a low-pressure tank and gauge are available. For example, if the hydrogenation factor is 0.157 kg/cc (0.345 lbs) for each unit IV drop and a 55 IV drop is required, then stop the reaction when 8.64 kg/cc (19 lbs) – (0.157 × 55 = 8.64) or (0.345 × 55 = 18.98) gas has reacted with the oil. Confirm with iodine value or refractive index determined on the final test portion.
   (f) Repeat the adjustment of the agitation rate with succeeding test sample, maintaining the standard temperature of 177°C (350°F), 6.82 kg/cc (15 psig) pressure and 0.05% nickel by weight catalyst charge until the desired reaction time is achieved for the standard product. After each successful hydrogenation with the standard catalyst, recalculate the hydrogenation factor (either kg/cc or lbs hydrogen per IV drop or IV change per min) and use an average of all runs to date with the standard catalyst for the best estimate of the rate factor.

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PROCEDURE FOR CATALYST ACTIVITY

1. Rinse equipment with a portion of each new oil test sample to be introduced to the reactor.
2. Recharge reactor in the same manner and with the same weight of oil used for the standard runs. Use the same level (0.05%) of nickel by weight of the oil contributed by the test catalyst. The level of nickel in the test catalyst may have to be analyzed in advance if it is not known. Bring the reactor temperature to 177 ± 3°C (350 ± 5°F) with agitation under nitrogen or under vacuum. Flush nitrogen out with hydrogen and start timing reaction immediately. Seal off reactor and bring hydrogen to a pressure of 6.82 kg/cc (15 psig).
3. The hydrogen pressure and the temperature of the oil should be checked frequently (at 5 min intervals is appropriate) during each run and adjustments made if necessary.
4. After the reaction has progressed 10–15 min, the reaction is stopped by flushing the hydrogen out with nitrogen and a small test sample of the oil is drawn for the determination of iodine value or refractive index. On the basis of the reaction rate determined, the reaction is continued until the determined end point (time of addition of predicted amount of hydrogen) has been established. The reaction need not be stopped if the reactor used permits drawing out test samples without changing pressure.
5. At the predetermined end point, stop the agitation and flush the reactor several times with nitrogen. Analyze the finished test sample to confirm that an 80 ± 2 IV has been obtained.
6. A check of the standard catalyst should be made periodically (with each batch if batches are spaced more than a week apart) to confirm that no shift has occurred in the mass transfer of the reactor.
7. Final results of this procedure are the number of minutes required to hydrogenate with the test catalyst under the same conditions under which the standard catalyst required 40 min to attain an 80 IV for the same oil.

NOTES

1. While this procedure provides the activity of a test catalyst under the specified reaction conditions, it does not mean that the test catalyst will have the same relative activity to the standard catalyst at some other operating conditions. The test only provides a means for establishing a relationship which may be used for specifying the relative properties of a number of catalysts.

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AOCS Recommended Practice Tz 1b-79

Revised 2011

Selectivity of Hydrogenation Catalysts

DEFINITION

This recommended practice determines the linoleic acid selectivity (SL) and/or linolenic acid selectivity (SLn) of a catalyst, either by graphical expression of the fatty acid composition of the hydrogenated oil or with the help of a computer program which solves the kinetic equations of consecutive first order reactions.

SCOPE

Applicable to all hydrogenation catalysts for the hydrogenation of vegetable derived oils.

APPARATUS

1. The equipment is exactly the same as described in AOCS Method Tz 1a-78, Activity of Hydrogenation Catalysts, Recommended Practice, Apparatus.
2. IBM 1130 computer system—or services of a programmer for conversion of the Fortran IV program into a form acceptable to an alternate computer system.
3. Gas chromatograph—to provide fatty acid composition of oil in accordance with AOCS Method Ce 1-62.

PROCEDURE

1. Proceed as directed in AOCS Method Tz 1a-78, Activity of Hydrogenation Catalysts, Standardization and Procedure Sections.
2. Determine the fatty acid composition of the initial oil test sample and oil samples withdrawn from the reactor when the IV is approximately 110 and at 80 IV. Abbreviations for individual fatty acids which are used in this method are Linolenic, Ln; Linoleic, L; Oleic, Ol; and Stearic, S.

CALCULATIONS

1. Graphic calculation procedure—
   The graphic calculation procedures are adaptations of those published by Albright (References, 1) and Allen (References, 2) and use the graphs shown in Figures 1 and 2. These figures are based on the same equations shown in Calculations, 3, for the computer program. Fatty acid values for constructing the figures were obtained by simulating the hydrogenation of a typical soybean oil with the different selectivities shown in the figures.

   To calculate selectivities, determine the fatty acid composition of the oil before and after hydrogenation. For the linoleate selectivity calculate S/S_O. Also calculate L/L_O by division of the linoleic content of the hydrogenated oil by that of the original oil. Read the linoleic selectivity from the graph shown in Figure 1.

   To calculate linolenate selectivity, calculate Ln/Ln_O by division of the linolenic content of the hydrogenated oil by the linolenic content of the original oil. Along with L/L_O calculated earlier, read the linolenic selectivity on the graph shown in Figure 2. Interpolation between selectivities on the graph may be made as necessary (Figs. 1 and 2 may be enlarged for greater ease of use).
2. Interpretation of graphical calculation—
   The graphical and computer calculations give values for linolenic selectivity which are quite similar, especially over the most useful ranges of fatty acid composition. A comparison of the methods has been published (References, 4).

   Within the limitation of a consecutive order reaction, the computer program rapidly determines, if data permit, the linolenate and linoleate selectivities of any initial oil composition. The accuracy of the determination is 0.1%, which generally is better than the experimental data warrant.
3. Computer calculating procedure—
   The following computer program was first explained in a publication by Butterfield and Dutton (References, 3) and is presented with the permission of the authors and the Agriculture Research Service, United States Dept. of Agriculture.
   (a) Description of computer program—
   To determine linolenate and linoleate selectivities, the computer must determine A, B and C from the following equations:

   (1) $Ln = Ln_O e^{-A}$

   (2) $L = Ln_O \frac{\quad}{B-A}(e^{-A} - e^{-B}) + Loe^{-B}$

   (3) $Ol + Ln_O \frac{\quad}{B-A} \frac{\quad}{C-A}(e\text{-}A - e\text{-}C) - Lno \times$

   (4) $\frac{A}{B-A} \frac{B}{C-A}(e^{-B} - e^{-C}) + Lo\frac{B}{C-B}(e\text{-}B - e\text{-}C) + Oloe\text{-}c$

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Where—

Ln = mol% linolenate in hydrogenated test sample

L = mol% linoleate in hydrogenated test sample

Ol = mol% oleate in hydrogenated test sample

Ln_O = mol% linolenate in initial test sample

L_O = mol% oleate in initial test sample

Ol_O = mol% oleate in initial test sample

These three equations are equivalent to those published by Albright (References, 1) if simple substitutions are made—A = k_1t, B = k_2t, C = k_3t, where k_1, k_2 and k_3 are rate constants and t is time.

Equation 1 is solved for A directly, but equations 2 and 3 have no formal solutions for B and C. Thus, the computer approximates these values by initializing B as a fraction of A, calculating an L value, comparing it to the experimental L value and then incrementing B so as to converge on the correct value. The convergence process is stopped when B is determined within 0.1%. If experimental accuracy warrants, a more precise determination of B can be made. C is then determined by the same convergence process. Linolenate and linoleate selectivities are the ratios A/B and B/C, respectively, which are printed out by the computer.

The program proceeds by entering the molar composition of the initial oil and the hydrogenated oil into the computer. To prevent the possibility of division by zero, data are then checked to see whether either or neither of the selectivities can be calculated. Linoleate selectivity cannot be calculated if there has been no increase in saturates; neither selectivity can be calcu- lated if linolenate is present in the initial oil, but not in the hydrogenated oil. Printout consists of test sample identification, the composition of the initial oil and of the hydrogenated oil and the two selectivities or a statement that either or both could not be calculated. A typical printout is shown in Figure 3.

REFERENCES

1. Albright, L. F., *JAOCS 42:*250 (1965).
2. Allen, R. R., *JAOCS 44:*466 (1967).
3. Butterfield, R. O., and H. J. Dutton, JAOCS 44:549
4. Scholfield, C. R., R. O. Butterfield and H. J. Dutton, *JAOCS 56:*664 (1979).

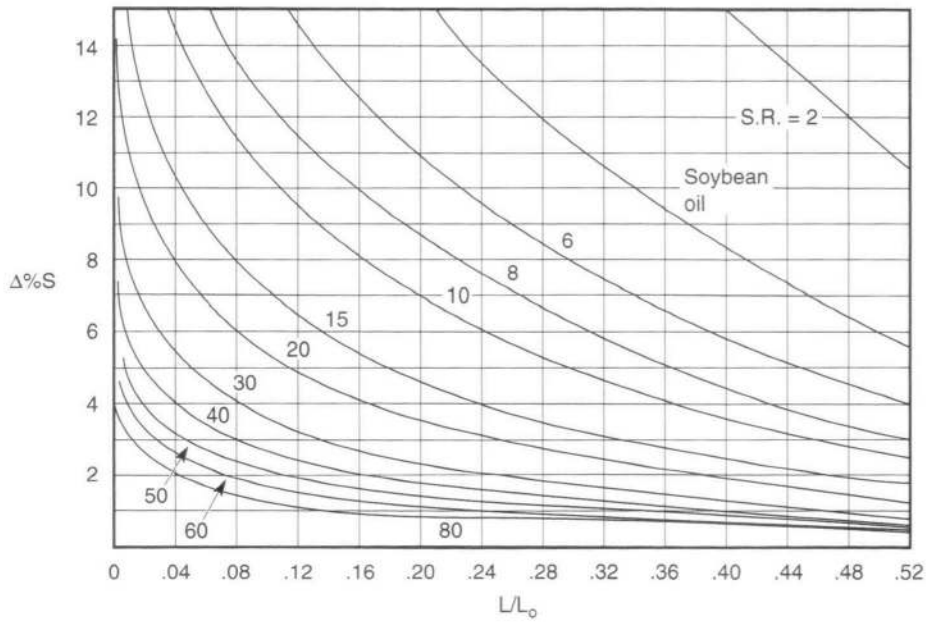

Figure 1. Graph for calculation of linoleate selectivity, see References, 1.

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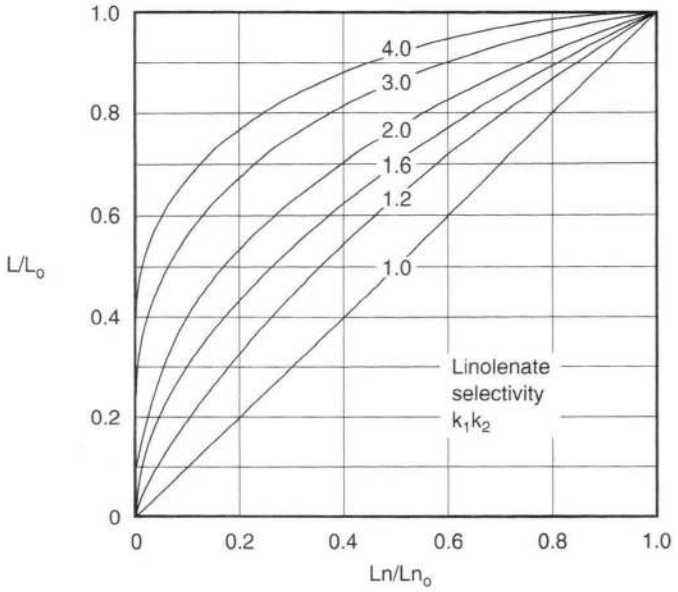

Figure 2. Graph for calculation of linolenate selectivity, see References 2.

Figure 3. Computer Program Tz 1b-79:

```
// JOB
// * CATALYST SELECTIVITY PROGRAM
// FOR SELK
* ONE WORD INTEGERS
* IOCS (CARD, 1132 PRINTER)
DIMENSION Z(3)
CIF I IS _, IC DATA. IF I IS 0, END OF DATA. IF I IS +, H2 DATA.
5 READ (2,10) Z,T,D,XM,S,I
10 FORMAT (3A4,4F6.2,12)
CNORMALIZATION OF DATA

TOT=T+D+XM+S
CINITIAL CONDITION, END OF DATA, DATA SET TEST
IF (I) 15,95,19

CENTERING OF INITIAL CONDITIONS
15 TO=T
DO=D

XMO=XM
SO=S
TOT I=TOT
WRITE (3,18)
18 FORMAT (1H1)
N=0
GO TO 5
CTEST FOR INITIAL TRIENE
19 IF (TO -.01*T) 300,300,20
CTEST FOR TRIENE DECREASE
20 IF (TO -1.01*T) 200,200,21
CTEST FOR DATA SET TRIENE
```


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Figure 3. Computer Program Tz 1b-79: (continued)

```
21 IF (T -.001) 200,200,22
CCALCULATION OF TRIENE HYDROGENATION RATE 22 A=ALOG(TO/T)
NT=1
EA=EXP (-A)
B=0
CCALCULATION OF DIENE HYDROGENATION RATE
BD=.3*A
25 EB = EXP(-B)
CTEST TO PREVENT NEGATIVE ANSWERS
IF (B) 200,27,27
27 DC=TO*A*(EA-EB)/(B-A)+DO*EB
IF (DC-D) 35,45,30
30 B=B+BD
GO TO 25
35 B=B-BD
CCONVERGENCE TERMINATION TEST
IF (B/1000.-BD) 40,45,45
40 BD=BD/2.
GO TO 30
45 EB=EXP(-B)
C=0
CCALCULATION OF MONOENE HYDROGENATION RATE
CD=.3*B
CTEST FOR SATURATE INCREASE
IF (S-(SO+.01*SO)) 75,75,50
50 EC=EXP(-C)
CTEST TO PREVENT NEGATIVE ANSWERS
IF (C) 75,52,52
52 XMC=TO*A*B*(EA-EC)/((B-A)*(C-A))-TO*A*B* (EB-
EC)/((B-A)*(C-B))+DO*B1*(EB-EC)/(C-B)+XMO*EC IF
(XMC-XM) 60,70,55
55 C=C+CD
GO TO 50
60 C=C-CD
CCONVERGENCE TERMINATION TEST
IF (C/1000.-CD) 65,70,70
65 CD=CD/2
GO TO 55
CRATIO CALCULATIONS
70 SD=B/C
ND=1
GO TO 80
75 ND=2
80 ST=A/B
85 N=N+1
CTEST TO PREVENT PRINTOUT BEING SPLIT BETWEEN PAGES
IF (N-6) 87,87,86
86 N=1
WRITE (3,18)
CPRINTOUT DEPENDS UPON WHAT CALCULATIONS
WERE PERMITTED
87 WRITE (3,90) Z,TO,DO,XMO,SO,T,D,XM,S
90 FORMAT (//8H SAMPLE,3A4,5X,6HTRIENE,5X,
5HDIENE,4X, 7HMONOENE,13X,8HSTEARATE//13H INITIAL OIL,13X,F6.2,3
(4X,F6.2)/217H HYDROGENATED OIL,8X,F6.2,3(4X,F6.2)) IF (ABS(TOTI-TOT)-1.) 96,92,92
92 WRITE (3,94)
94 FORMAT ('*** WARNING *** SUM OF INITIAL AND
SAMPLE PERCENTAGES 2VARY BY GREATER THAN 1 PERCENT.')
```


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Figure 3. Computer Program Tz 1b-79: (continued)

```
96 GO TO (100,110),NT
100 WRITE (3,105)ST
105 FORMAT (/24H LINOLENATE SELECTIVITY,F9.2)
GO TO 120
110 WRITE (3,115)
115 FORMAT (/59H LINOLENATE SELECTIVITY CAN NOT
BE DETERMINED FROM THE 1E DATA)
120 GO TO (125,135),ND
125 WRITE (3,130)SD
130 FORMAT (24H LINOLEATE SELECTIVITY,F9.2)
GO TO 5
135 WRITE (3,140)
140 FORMAT (58H LINOLEATE SELECTIVITY CAN NOT BE
DETERMINED FROM THE 1DATA)
GO TO 5
CDATA DOES NOT PERMIT CALCULATION OF EITHER
SELECTIVITIES
200 NT=2
ND=2
GO TO 85
300 NT=2
CTEST FOR DIENE DECREASE
IF (DO-1.01*D) 200,200,305
CTEST FOR DATA SET DIENE
305 IF (D-.001) 200,200,310
CCALCULATION OF MONOENE HYDROGENATION RATE
WHEN TRIENE IS NOT PRESENT
310 B=ALOG(DO/D)
EB=EXP(-B)
C=0
CCALCULATION OF MONOENE HYDROGENATION RATE
WHEN TRIENE IS NOT PRESENT
CD=.3*B
CTEST FOR SATURATE INCREASE
IF (S-(SO+.01*SO)) 200,200,325
325 EC=EXP(-C)
CTEST TO PREVENT NEGATIVE ANSWERS
IF (C) 200,327,327
327 XMC=DO*B*(EB-EC)/(C-B)+XMO*EC
IF (XMC-XM) 335,345,330
330 C=C+CD
GO TO 325
335 C=C-CD
CCONVERGENCE TERMINATION TEST
IF (C/1000.-CD) 340,345,345
340 CD=CD/2
GO TO 330
345 SD=B/C
ND=1
GO TO 85
95 CALL EXIT
END
```


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Figure 4. Example of Job Entry:

Job
XEQ SELK

Original	SBO	7.7	53.7	23.7	4.2	−1
NI-SBO	150C	2.3	35.3	45.1	6.2	1
NI-SBO	170C	1.1	27.8	53.4	7.0	1
NI-SBO	190C	.5	23.5	58.0	6.8	1

Computer Printout

Sample NI-SBO 150C	Triene	Diene	Monoene	Stearate
Initial Oil	7.70	53.70	23.70	4.20
Hydrogenated Oil	2.30	33.30	45.10	6.20
Linolenate Selectivity	2.24			
Linoleate Selectivity	7.93			

Sample NI-SBO 170C	Triene	Diene	Monoene	Stearate
Initial Oil	7.70	53.70	23.70	4.20
Hydrogenated Oil	1.1	27.80	53.40	7.00
Linolenate Selectivity	2.39			
Linoleate Selectivity	11.79			

Sample NI-SBO 190C	Triene	Diene	Monoene	Stearate
Initial Oil	7.70	53.70	23.70	4.20
Hydrogenated Oil	0.50	23.50	58.00	6.80
Linolenate Selectivity	2.70			
Linoleate Selectivity	14.06			

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