41.1.28A - Oils and Fats

AOAC Official Method 996.06
Fat (Total, Saturated, and Unsaturated) in Foods

Hydrolytic Extraction Gas Chromatographic Method
First Action 1996
Revised 2001

(Applicable to determination of fat in foods.)

Caution: Boron trifluoride may be fatal if inhaled.

See Tables **996.06A-C** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Fat and fatty acids are extracted from food by hydrolytic methods (acidic hydrolysis for most products, alkaline hydrolysis for dairy products, and combination for cheese). Pyrogallic acid is added to minimize oxidative degradation of fatty acids during analysis. Triglyceride, triundecanoin ($C_{11:0}$), is added as internal standard. Fat is extracted into ether, then methylated to fatty acid methyl esters (FAMEs) using BF_3 in methanol. FAMEs are quantitatively measured by capillary gas chromatography (GC) against $C_{11:0}$ internal standard. Total fat is calculated as sum of individual fatty acids expressed as triglyceride equivalents. Saturated and monounsaturated fats are calculated as sum of respective fatty acids. Monounsaturated fat includes only *cis* form.

B. Apparatus

(**a**) *Gas chromatograph (GC).*—Equipped with hydrogen flame ionization detector, capillary column, split mode injector, oven temperature programming sufficient to implement a hold-ramp-hold sequence. Operating conditions: temperature (°C): injector, 225; detector, 285; initial temp, 100 (hold 4 min); ramp, 3°C/min; final temp

240; hold 15 min; carrier gas, helium; flow rate, 0.75 mL/min; linear velocity, 18 cm/s; split ratio, 200:1.

(**b**) *Capillary column.*—Separating the FAME pair of adjacent peaks of $C_{18:3}$ and $C_{20:1}$ and the FAME trio of adjacent peaks of $C_{22:1}$, $C_{20:3}$, and $C_{20:4}$ with a resolution of 1.0 or greater. SP2560 100 m 0.25 mm with 0.20 µm film is suitable.

(**c**) *Mojonnier flasks.*

(**d**) *Stoppers*.—Synthetic rubber or cork.

(**e**) *Mojonnier centrifuge basket.*

(**f**) *Hengar micro boiling granules.*

(**g**) *Baskets.*—Aluminum and plastic.

(**h**) *Shaker water bath.*—Maintaining 70-80°C.

(**i**) *Steam bath.*—Supporting common glassware.

(**j**) *Water bath.*—With nitrogen stream supply, maintaining 40 ± 5°C.

(**k**) *Wrist action shaker*.—Designed for Mojonnier centrifuge baskets.

(**l**) *Mojonnier motor driven centrifuge.*—Optional; maintaining 600 *g*.

(**m**) *Gravity convection oven.*—Maintaining 100 ± 2°C.

(**n**) *Vortex mixer.*

(**o**) *Gas dispersion tubes.*—25 mm, porosity "A," extra coarse, 175 µm.

(**p**) *Three dram vials.*—About 11 mL.

(**q**) *Phenolic closed top caps*.—With polyvinyl liner, to fit vials.

(**r**) *Teflon/silicone septa*.—To fit vials.

C. Reagents

(**a**) *Pyrogallic acid.*

(**b**) *Hydrochloric acid.*—12M and 8.3M. To make 8.3M HCl, add 250 mL 12M HCl to 110 mL H_2O. Mix well. Store at room temperature (20-25°C).

(**c**) *Ammonium hydroxide.*—58% (w/w).

(**d**) *Diethyl ether.*—Purity appropriate for fat extraction.

(**e**) *Petroleum ether.*—Anhydrous.

(**f**) *Ethanol.*—95% (v/v).

(**g**) *Toluene.*—Nanograde.

(**h**) *Chloroform.*

(**i**) *Sodium sulfate.*—Anhydrous.

(**j**) *Boron trifluoride reagent.*—7% BF_3 (w/w) in methanol, made from commercially available 14% BF_3 solution. Prepare in the hood.

(**k**) *Diethyl ether-petroleum ether mixture.*—1 + 1 (v/v).

(**l**) *Triglyceride internal standard solution.*—$C_{11:0}$-triundecanoin; 5.00 mg/mL in $CHCl_3$. Accurately weigh 2.50 g $C_{11:0}$-triundecanoin into 500 mL volumetric flask. Add ca 400 mL $CHCl_3$ and mix until dissolved. Dilute to volume with $CHCl_3$. Invert flask at least 10 additional times. Triglyceride internal standard solution is stable up to 1 month when stored in refrigerator (2-8°C).

(**m**) *Fatty acid methyl esters (FAMEs) standard solutions.*—(*1*) *Mixed FAMEs standard solution.*—Reference mixture containing series of FAMEs, including $C_{18:1}$ *cis* and *trans* (available as GLC-85 from Nu Chek Prep, Elysian, MN 56028, USA, or equivalent). To prepare mixed FAMEs standard solution, break top of glass vial, open, and carefully transfer contents to 3-dram glass vial. Wash original vial with hexane to ensure complete transfer and add washings to 3-dram glass vial. Dilute to ca 3 mL with hexane.

(*2*) *$C_{11:0}$ FAME standard solution.*—$C_{11:0}$-Undecanoic methyl ester in hexane. Use only in preparation of individual FAME standard solutions, (*3*). To prepare $C_{11:0}$ FAME standard solution, break top of glass vial open and carefully transfer contents to 50 mL volumetric flask. Wash original vial with hexane to ensure complete transfer and add washings to 50 mL volumetric flask. Dilute to volume with hexane. $C_{11:0}$ FAME standard solution is stable up to 1 week when stored at 0°C.

(*3*) *Individual FAME standard solutions.*—Standard solutions of each of following FAMEs: $C_{4:0}$-tetranoic methyl ester, $C_{6:0}$-hexanoic methyl ester, $C_{8:0}$-octanoic methyl ester, $C_{10:0}$-decanoic methyl ester, $C_{12:0}$-dodecanoic methyl ester, $C_{13:0}$-tridecanoic methyl ester, $C_{14:0}$-tetradecanoic methyl ester, $C_{14:1}$-9-tetradecenoic methyl ester, $C_{15:0}$-pentadecanoic methyl ester, $C_{15:1}$-10-pentadecenoic methyl ester, $C_{16:0}$-hexadecanoic methyl ester, $C_{16:1}$-9-hexadecenoic methyl ester, $C_{17:0}$-heptadecanoic methyl ester, $C_{17:1}$-10-heptadecenoic methyl ester, $C_{18:0}$-octadecanoic methyl ester, $C_{18:1}$-9-octadecenoic methyl ester, $C_{18:2}$-9,12-octadecadienoic methyl ester, $C_{18:3}$-9,12,15-octadecatrienoic methyl ester, $C_{20:0}$-eicosanoic methyl ester, $C_{20:1}$-8-eicosenoic methyl ester, $C_{20:2}$-11,14-eicosadienoic methyl ester, $C_{20:3}$-11,14,17-eicosatrienoic methyl ester, and $C_{22:0}$-docosanoic methyl ester. Prepare individual FAME standard solutions as follows: Break top of glass vial open and carefully transfer contents to 3-dram glass vial. Wash original vial with hexane to ensure complete transfer and add washings to 3-dram glass vial. Add 1.0 mL $C_{11:0}$ FAME standard solution, (*2*), dilute to total volume of ca 3.0 mL with hexane. Individual FAME standard solutions are stable up to 1 week when stored in refrigerator (2-8°C).

D. Extraction of Fat

Finely grind and homogenize test samples prior to extraction of fat.

[*Note:* With matrixes of unknown composition, it may be necessary to analyze test portion without addition of internal standard to ensure against interferences. Should interfering peak be found, the area of C_{11} internal standard peak must be corrected before performing calculations. Use 2.0 mL chloroform instead of internal standard solution.]

(**a**) *Foods excluding dairy products and cheese.*—Accurately weigh ground and homogenized test portion (containing ca 100-200 mg fat) into labeled Mojonnier flask. Force material into flask as far as possible. Add ca 100 mg pyrogallic acid, **C**(**a**), and 2.00 mL triglyceride internal standard solution, **C**(**l**). Add a few boiling granules to flask. Add 2.0 mL ethanol and mix well until entire test portion is in solution. Add 10.0 mL 8.3M HCl and mix well. Place flask into basket in shaking water bath at 70-80°C set at moderate agitation speed. Maintain 40 min. Mix contents of flask on Vortex mixer every 10 min to incorporate particulates adhering to sides of flask into solution. After digestion, remove flask from bath and allow to cool to room temperature (20-25°C). Add enough ethanol to fill bottom reservoir of flask and mix gently.

(**b**) *Dairy products.*—Accurately weigh ground and homogenized test portion (containing ca 100-200 mg fat) into labeled Mojonnier flask. Force material into flask as far as possible. Add ca 100 mg pyrogallic acid, **C**(**a**), and 2.00 mL triglyceride

internal standard solution, **C**(**l**). Add a few boiling granules to flask. Add 2.0 mL ethanol and mix well until entire test portion is in solution. Add 4.0 mL H_2O and mix well. Add 2.0 mL NH_4OH, **C**(**c**), and mix well. Place flask into basket in shaking water bath at 70-80°C set at moderate agitation speed. Maintain 10 min. Mix contents of flask on Vortex mixer every 5 min to incorporate particulates adhering to sides of flask into solution. After digestion, remove flask from water bath and add a few drops of phenolphthalein. Keep solution basic (pink) with addition of ammonium hydroxide. Add enough ethanol to fill bottom reservoir of flask and mix gently.

(**c**) *Cheese.*—Accurately weigh ground and homogenized test portion (containing ca 100-200 mg fat) into labeled Mojonnier flask. Force material into flask as far as possible. Add ca 100 mg pyrogallic acid, **C**(**a**), and 2.00 mL triglyceride internal standard solution, **C**(**l**). Add a few boiling granules to flask. Add 2.0 mL ethanol and mix well until entire test portion is in solution. Add 4.0 mL H_2O and mix well. Add 2.0 mL NH_4OH, **C**(**c**), and mix well. Place flask into basket in shaking water bath at 70-80°C set at moderate agitation speed. Maintain 20 min. Mix contents of flask on Vortex mixer every 10 min to incorporate particulates adhering to sides of flask into solution. Add 10.0 mL 12M HCl and place flask into boiling steam bath and maintain 20 min. Mix flask contents every 10 min using Vortex mixer. Remove flask from steam bath and allow to cool to room temperature (20-25°C). Add enough ethanol to flask to fill bottom reservoir and mix gently.

Add 25 mL diethyl ether to Mojonnier flask from (**a**), (**b**), or (**c**). Stopper flask and place in centrifuge basket. Place basket in wrist action shaker, securing flask in shaker with rubber tubing. Shake flask 5 min. Rinse stopper into flask with diethyl ether-petroleum ether mixture, **C**(**k**). Add 25 mL petroleum ether, stopper flask, and shake 5 min. Centrifuge flask (in basket) 5 min at 600 *g*. (*Note:* If centrifuge is not available, allow contents to set at least 1 h until upper layer is clear.) Rinse stopper into flask with diethyl ether-petroleum ether mixture. Decant ether (top) layer into 150 mL beaker and carefully rinse lip of flask into beaker with diethyl ether-petroleum ether mixture. Slowly evaporate ether on steam bath, using nitrogen stream to aid in evaporation. Residue remaining in beaker contains extracted fat.

E. Methylation

Dissolve extracted fat residue in 2-3 mL chloroform and 2-3 mL diethyl ether. Transfer mixture to 3 dram glass vial and then evaporate to dryness in 40°C water bath under nitrogen stream. Add 2.0 mL 7% BF_3 reagent, **C**(**j**), and 1.0 mL toluene, **C**(**g**). Seal vial with screwcap top containing Teflon/silicone septum. Heat vial in oven 45 min at 100°C. Gently shake vial ca every 10 min. (*Note:* Evaporation of liquid from vials indicates inadequate seals; if this occurs, discard solution and repeat the entire procedure.) Allow vial to cool to room temperature (20-25°C). Add

5.0 mL H_2O, 1.0 mL hexane, and ca 1.0 g Na_2SO_4, **C**(**i**). Cap vial and shake 1 min. Allow layers to separate and then carefully transfer top layer to another vial containing ca 1.0 g Na_2SO_4. (*Note:* Top layer contains FAMEs including FAME of triglyceride internal standard solution.)

Inject FAMEs onto GC column or transfer to autosampler vial for GC analysis.

F. GC Determination

Relative retention times (vs FAME of triglyceride internal standard solution) and response factors of individual FAMEs can be obtained by GC analysis of individual FAME standard solutions and mixed FAME standard solution. Inject ca 2 µL each of individual FAME standard solutions and 2 µL of mixed FAMEs standard solution. Use mixed FAMEs standard solution to optimize chromatographic response *before* injecting any test solutions. After all chromatographic conditions have been optimized, inject test solutions from **E**.

G. Calculations

Total fat is the sum of fatty acids from all sources, expressed as triglycerides. Expressing measured fatty acids as triglycerides requires mathematical equivalent of condensing each fatty acid with glycerol. For every 3 fatty acid molecules, 1 glycerol ($HOCH_2CHOHCH_2OH$) is required. Essentially, 2 methylene groups and 1 methine group are added to every 3 fatty acids.

Calculate retention times for each FAME in individual FAMEs standard solutions, **C**(**m**)(*3*), by subtracting retention time of $C_{11:0}$ peak from retention time of fatty acid peak. Use these retention times to identify FAMEs in mixed FAMEs standard solution. Use additional FAME solutions (from the same supplier) when necessary for complete FAME identity verification.

(**a**) Calculate response factor (R_i) for each fatty acid *i* as follows:

where Ps_i = peak area of individual fatty acid in mixed FAMEs standard solution; $Ps_{C\ 11:0}$ = peak area of $C_{11:0}$ fatty acid in mixed FAMEs standard solution; $W_{C11:0}$ = weight of internal standard in mixed FAMEs standard solution; and W_i = weight of individual FAME in mixed FAMEs standard solution.

(*Note:* Peaks of known identity with known relative retention times are listed in Table **996.06D**. When peaks of unknown identity are observed during the

chromatographic run, attempt to identify such peaks using MS, FTIR, etc. Peaks of unkown identity should not be included in the summation when quantifying fat in the test sample.)

(**b**) Calculate amount of individual (triglycerides) (W_{TG}) in test sample as follows:

$$W_{FAMEi} =$$

$$W_{TGi} = W_{FAMEi} \quad f_{TGi}$$

where Pt_i = peak area of fatty acid *i* in test portion; $Wt_{C11:0}$ = weight of $C_{11:0}$ internal standard added to test portion, g; 1.0067 = conversion of internal standard from triglyceride to FAME; $Pt_{C11:0}$ = peak area of $C_{11:0}$ internal standard in test portion; and f_{TGi} = conversion factor for FAMEs to triglycerides for individual fatty acids (*see* Table **996.06E**).

(*Note:* If procedure is followed exactly, $Wt_{C11:0}$ should be 0.010 g.)

(**c**) Calculate amount of total fat in test sample (sum of all fatty acids; expressed as triglycerides [including *cis* and *trans* forms of monounsaturated acids]) as follows:

$$\text{Total fat, \%} = (\quad WD_{TG}/W_{\text{test portion}})$$

where $W_{\text{test sample}}$ = weight of test portion, g.

(**d**) Calculate weight of each fatty acid (W_i) as follows:

$$W_i = W_{FAMEi} \quad f_{FAi}$$

where f_{FAi} = conversion factors for conversion of FAMEs to their corresponding fatty acids (*see* Table **996.06E**).

(**e**) Calculate percent of saturated fat in test sample (w/w; expressed as saturated fatty acids; sum of $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, etc.) as follows:

$$\text{Saturated fat, \%} = (\quad \text{saturated } W_i/W_{\text{test portion}}) \quad 100\%$$

(**f**) Calculate amount of monounsaturated fat in test sample (w/w; expressed as sum of only *cis* form of monounsaturated fatty acids [$C_{16:1}$, $C_{17:1}$, $C_{18:1}$ *cis*, $C_{20:1}$, etc.]) as follows:

$$\text{Monounsaturated fat, \%} = \left(\text{monounsaturated } W_i/W_{\text{test portion}}\right) \quad 100\%$$

$$\text{Polyunsaturated fat, \%} = \left(\text{polyunsaturated } W_i/W_{\text{test portion}}\right) \quad 100\%$$

(*Note:* Test samples containing hydrogenated fat will yield complicated chromatograms due to large number of isomers formed during hydrogenation process. One general indication of hydrogenation is presence of $C_{18:1}$ *trans* peak(s). For hydrogenated fat chromatograms, use the following guidelines to calculate FAME peak areas: *trans* peaks elute prior to *cis*, therefore, include all peaks between $C_{18:1}$ *cis*and $C_{18:2}$ *cis*,*cis* in calculation of $C_{18:2}$ peak area. Often $C_{18:1}$ *trans* "peak" consists of broad series of peaks [due to positional isomers from hydrogenation]; include all of these in $C_{18:1}$ *trans* peak area.)

Reference:
J. AOAC Int. **80,** 555(1997); **82**, 1146(1999).

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Table 996.06A: Interlaboratory study results for determination of total fat in food by hydrolytic extraction—gas chromatography

Table 996.06B: Interlaboratory study results for determination of saturated fat in food stuffs by hydrolytic extraction— gas chromatography

Table 996.06C: Interlaboratory study results for determination of monounsaturated fat in food stuffs by hydrolytic extraction— gas chromatography

Table 996.06D: Retention time of fatty acids and methyl ester (FAME)

Table 996.06E: Factors (f_{TG}) for conversion of FAMEs totrygliceride equivalents