

# Determination of Total, Saturated, Unsaturated, and Monounsaturated Fats in Cereal Products by Acid Hydrolysis and Capillary Gas Chromatography: Collaborative Study

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Fifteen laboratories participated in a collaborative study to determine total, saturated, unsaturated, and monounsaturated fats in cereal products by gas chromatographic (GC) analysis of fatty acid methyl esters (FAMES). Cereal products, representing a wide range of cereal grains and processes, were hydrolyzed in 8N HCl and extracted with ethyl and petroleum ethers. FAMES were produced by the reaction of the mixed ether extracts with sodium hydroxide in methanol (NaOH/MeOH) and then with boron trifluoride reagent (14% BF<sub>3</sub> in MeOH). They were quantitatively determined by capillary GC. Total fat was calculated as the sum of individual fatty acids expressed as triglyceride equivalents in accordance with nutrition labeling guidelines. Saturated, unsaturated, and monounsaturated fats were calculated as sums of individual fatty acids. The total fat contents of samples ranged from 0.56 to 12.64%. A split design was used to determine performance parameters of results obtained by 15 laboratories on 24 samples. Of the 24 samples, 7 were blind duplicates and 5 were independent materials. Statistical analysis for total fat yielded a relative standard deviation for repeatability (RSD<sub>r</sub>) range of 1.32 to 13.30% and a relative standard deviation for reproducibility (RSD<sub>R</sub>) range of 4.42 to 22.82%. The goal of this study was to determine total fat, saturated fat, unsaturated, and monounsaturated fat in cereal-based products by complete extraction, methylation, and quantitation of total fatty acids. The acid hydrolysis-capillary GC method for determining total, saturated, unsatu-

rated, and monosaturated fats in cereal products has been adopted by AOAC INTERNATIONAL.

Fat has received increased attention since the Nutritional Labeling and Educational Act (NLEA) of 1990 (1). In the final regulations, the U.S. Food and Drug Administration (FDA) defined total fat as the sum of fatty acids expressed as triglyceride equivalents for nutrition labeling purposes (2).

Currently, no AOAC method yields quantitative recoveries of fatty acids from cereal products that meet the new NLEA labeling regulations (3, 4). This method under collaborative study was designed to yield results consistent with the new definitions for fat as promulgated by FDA regulations.

The method was developed to determine total, saturated, unsaturated and monounsaturated fatty acids in cereal products. A total lipid extract was obtained by digesting the sample with hot 8N hydrochloric acid. The hydrolyzed fat components were extracted into ethyl and petroleum ethers, saponified, and methylated. The resulting fatty acid profile is suitable for expressing fat content in cereal products to a level as low as 0.5%. Samples covered a wide variety of cereal matrixes, including baked, puffed, extruded and frosted cereal products. Samples covered a variety of cereal grains and different fat, carbohydrate, and protein levels.

Fats are released by acid hydrolysis and extracted by ethyl and petroleum ethers. After methylation, the total fatty acid content is determined by capillary gas chromatography (GC). Results are expressed as triglyceride equivalents (5-7).

## Collaborative Study

The 15 collaborators received a practice sample and the protocol. They were instructed to set up the procedure and analyze the sample at least 3 times (or until proficient) and to send to the associate referee the results, along with chromatograms and recommendations for modifications. After the data were

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The recommendation was approved by the Methods Committee on Commodity Foods and Commodity Products and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1996) *J. AOAC Int.* 79, 42A, and "Official Methods Board Actions" (1996) *The Referee*, March issue.

reviewed, each of the 15 collaborators received 24 samples of assorted ready-to-eat (RTE) cereal-based products.

A calibration standard including even-numbered fatty acid methyl esters (FAMES) from C<sub>8</sub> through C<sub>22</sub> and C<sub>13:0</sub>, C<sub>15:0</sub> and C<sub>17:0</sub>, in concentration ranges typifying the fatty acid contents of cereal products, was used for GC calibration. Each collaborator was instructed to spike each weighed sample with 5 mg tritridecanoin (C<sub>13</sub> triglyceride) internal standard before extraction.

Samples included 7 blind duplicates and 5 pairs of independent materials (8) selected on the basis of a total fat content difference of less than 3 standard deviations from each other. Samples consisted of RTE cereals or grain-based products covering different cereal grain types, manufacturers, processing methods, fat contents, and fatty acid profiles.

Samples were ground to pass a 0.5 mm screen, blended, and stored in polyethylene bottles at ambient temperature. Collaborators received 2.5 g of each sample in a polyethylene bottle and a copy of the procedure. They were requested to determine the amount of total, saturated, unsaturated, and monounsaturated fatty acids in each product; record results on a provided spreadsheet; and return results, sample chromatograms, and a completed report form to the associate referee.

### 996.01 Fat (Total, Saturated, Unsaturated, and Monounsaturated) in Cereal Products Acid Hydrolysis—Capillary Gas Chromatographic Method

#### First Action 1996

(Applicable for determination of fat in cereal products containing 0.5–13.0% total fat.)

*Caution:* See Appendix: Laboratory Safety for “Safe Handling of Special Chemical Hazards”—ethanol and petroleum ether; “Safe Handling of Alkalies”—sodium hydroxide; and

“Safe Handling of Acids”—hydrochloric acid. Boron trifluoride may be fatal if inhaled. See Material Safety Data Sheets, or equivalent, for each reagent. Reagents may be either flammable or caustic, or may form explosive peroxides. Work in hood with gloves and safety glasses. Dispose of waste solvents in an appropriate manner compatible with applicable environmental rules and regulations.

Method Performance:

See Tables 996.01A–D for method performance data.

#### A. Principle

Total lipid extract is obtained by digesting sample with hot HCl. Hydrolyzed fat components are extracted into ethyl and petroleum ethers, saponified, and methylated.

Fatty acid methyl esters (FAMES) are quantitatively determined by capillary gas chromatography (GC). Total fat is calculated as sum of individual fatty acids expressed as triglyceride equivalents. Saturated, unsaturated, and monounsaturated fats are calculated as sums of individual fatty acids. Method can be used for quantitation of *trans* isomers when appropriate standards of FAMES are used.

#### B. Apparatus

(a) *Gas chromatograph.*—Equipped with capillary split/splitless injection system and flame ionization detector. Operating conditions: temperature (°C): injector, 250; detector, 275; hydrogen flow, 34 mL/min; air flow, ca 300 mL/min; split ratio, 100:1; carrier gas, helium; linear velocity, 21 cm/s at 175°C; initial temp, 120°C (hold 4 min); rate, 5.0°C/min; final temperature, 230°C; final time, 5.0 min.

(b) *GC column.*—30 m × 0.25 mm id, 0.2 μm film, non-bonded 90% cyanopropyl, 10% phenyl siloxane capillary column. See Figure 996.01A for chromatogram of FAMES standard solution and Figure 996.01B for chromatogram of test sample.

(c) *Roehrig fat extraction tube.*—With 28 mL spout.

Table 996.01A. Method performance for determination of total fat in cereal products by acid hydrolysis—capillary gas chromatography

Sample	No. of labs	Average total fat, %	s <sub>r</sub>	s <sub>R</sub>	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %	r <sup>a</sup>	R <sup>b</sup>
A	15	0.56	0.07	0.13	13.30	22.82	0.20	0.36
B	15	0.71	0.03	0.05	4.27	7.61	0.08	0.14
C	14	1.44	0.05	0.13	3.59	8.69	0.14	0.36
D	13	2.74	0.04	0.14	1.32	5.00	0.11	0.39
E	14	5.23	0.31	0.54	6.01	10.27	0.87	1.51
F	13	10.51	0.20	0.46	1.94	4.42	0.56	1.29
G	15	12.64	0.52	1.12	4.10	8.84	1.46	3.14
H	15	1.26 <sup>c</sup>	0.07	0.12	5.71	9.31	0.20	0.34
I	15	2.13 <sup>c</sup>	0.13	0.18	6.00	8.59	0.36	0.50
J	15	3.92 <sup>c</sup>	0.35	0.35	8.87	8.87	0.98	0.98
K	15	5.47 <sup>c</sup>	0.49	0.55	8.88	9.99	1.37	1.54
L	14	7.03 <sup>c</sup>	0.34	0.44	4.85	6.29	0.95	1.23

<sup>a</sup>  $r = 2.8 \times s_r$ .

<sup>b</sup>  $R = 2.8 \times s_R$ .

<sup>c</sup> Mean value of independent materials.

**Table 996.01B. Method performance for determination of saturated fat in cereal products by acid hydrolysis—capillary gas chromatography**

Sample	No. of labs	Average saturated fat, %	$s_r$	$s_R$	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %	$r^a$	$R^b$
A	15	0.18	0.04	0.14	25.31	27.95	0.11	0.39
B	15	0.21	0.01	0.02	3.53	9.51	0.03	0.06
C	15	0.36	0.02	0.03	4.37	7.45	0.06	0.08
D	15	1.49	0.09	0.11	6.16	7.06	0.25	0.31
E	15	1.45	0.07	0.08	4.48	5.49	0.20	0.22
F	15	3.14	0.03	0.13	0.83	4.23	0.08	0.36
G	15	3.23	0.12	0.17	3.77	5.24	0.34	0.48
H	15	0.35 <sup>c</sup>	0.04	0.04	11.76	11.76	0.11	0.11
I	13	0.49 <sup>c</sup>	0.02	0.03	3.24	6.84	0.06	0.08
J	15	0.98 <sup>c</sup>	0.08	0.08	8.13	8.13	0.22	0.22
K	14	0.77 <sup>c</sup>	0.06	0.07	7.80	9.76	0.17	0.20
L	15	2.06 <sup>c</sup>	0.08	0.10	3.73	4.83	0.22	0.28

<sup>a</sup>  $r = 2.8 \times s_r$ .<sup>b</sup>  $R = 2.8 \times s_R$ .<sup>c</sup> Mean value of independent materials.

(d) *Water baths*.—Capable of (1) maintaining  $80^\circ \pm 2^\circ\text{C}$ ; and (2) ambient temperature.

(e) *Oven*.—Capable of maintaining  $103^\circ \pm 2^\circ\text{C}$ .

(f) *Boiling chips*.—Fat free.

(g) *Cotton*.—Defatted; alternatively, glass wool can be used.

(h) *Glass funnel*.— $60^\circ$  angle, fluted.

(i) *Steam table*.

(j) *Test tubes*.— $22 \times 275$  mm, Pyrex, screw top, with cap.

(k) *Test tube rack*.

(l) *Bottle*.—For dispensing acid, 1000 mL with 15 mL dispensing bulb, or equivalent.

(m) *Vortex mixer*.

(n) *Reaction flasks*.—250 mL, flat-bottom boiling flasks with 24/40 joint.

(o) *Condenser*.—Water-cooled reflux condenser, with 40–50 cm jacket and 24/40 joint.

(p) *Centrifuge tubes*.—50 mL, conical.

(q) *Centrifuge*.—Capable of maintaining  $1875 \times g$ .

(r) *Grinding mill*.—Equipped with 0.5 mm screen.

### C. Reagents

(a) *Ethanol*.

(b) *Methanol*.

(c) *Hydrochloric acid (HCl)*.—8N (25 + 11; v/v).

(d) *Ethyl ether*.—Peroxide free.

(e) *Petroleum ether*.—Peroxide free.

(f) *Boron trifluoride (BF<sub>3</sub>) reagent*.—14% BF<sub>3</sub> in methanol.

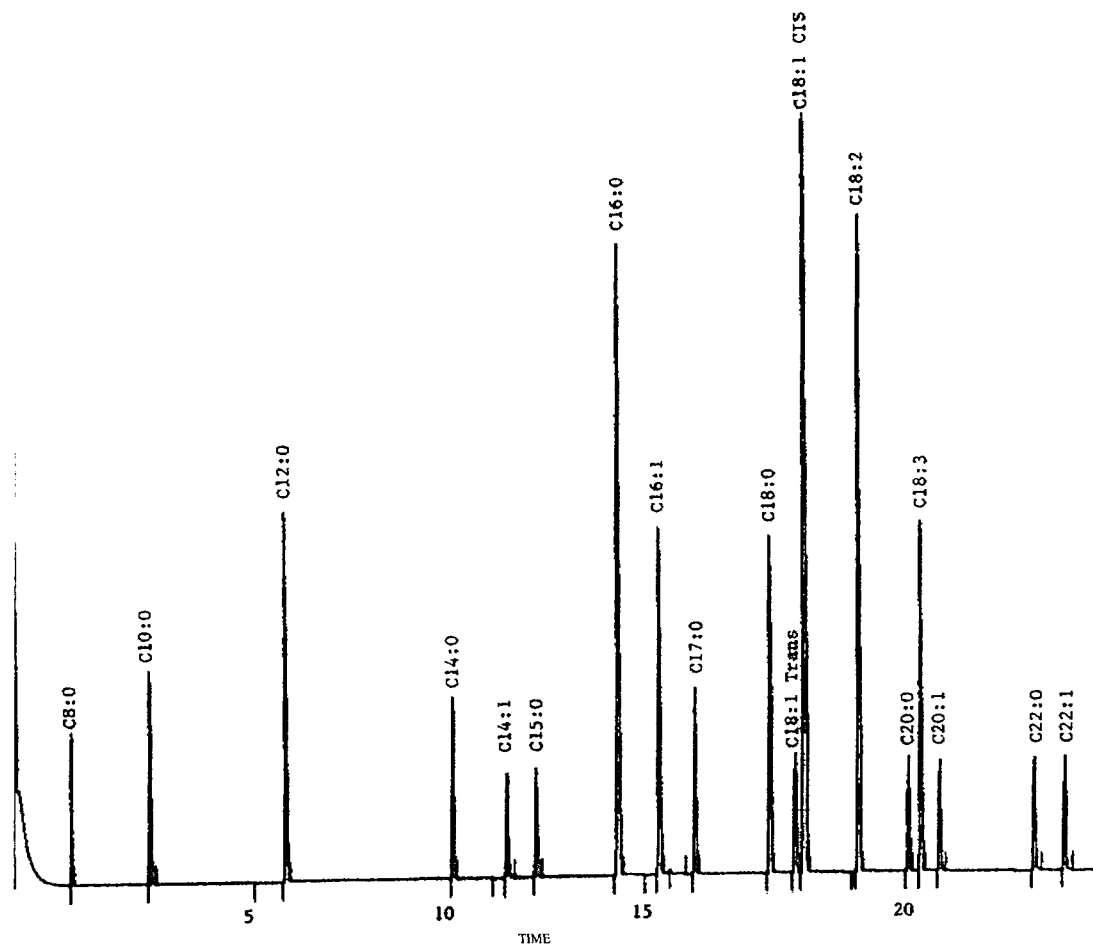
**Table 996.01C. Method performance for determination of unsaturated fat in cereal products by acid hydrolysis—capillary gas chromatography**

Sample	No. of labs	Average unsaturated fat, %	$s_r$	$s_R$	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %	$r^a$	$R^b$
A	12	0.32	0.02	0.04	6.78	12.03	0.06	0.11
B	14	0.48	0.02	0.03	3.37	5.93	0.06	0.08
C	14	1.02	0.06	0.12	5.77	11.32	0.17	0.33
D	11	1.14	0.01	0.05	1.14	4.11	0.03	0.14
E	13	3.60	0.17	0.38	4.80	10.47	0.48	1.06
F	13	6.89	0.18	0.46	2.64	6.72	0.50	1.29
G	15	8.82	0.40	1.07	4.53	12.09	1.12	3.00
H	14	0.87 <sup>c</sup>	0.04	0.06	4.95	6.54	0.11	0.17
I	15	1.53 <sup>c</sup>	0.10	0.13	6.37	8.69	0.28	0.36
J	15	2.76 <sup>c</sup>	0.29	0.29	10.41	10.41	0.81	0.81
K	15	4.47 <sup>c</sup>	0.38	0.43	8.50	9.67	1.06	1.20
L	14	4.66 <sup>c</sup>	0.30	0.35	6.35	7.57	0.84	0.98

<sup>a</sup>  $r = 2.8 \times s_r$ .<sup>b</sup>  $R = 2.8 \times s_R$ .<sup>c</sup> Mean value of independent materials.

**Table 996.01D. Method performance for determination of monounsaturated fat in cereal products by acid hydrolysis–capillary gas chromatography**

Sample	No. of labs	Average monounsaturated fat, %	$s_r$	$s_R$	RSD $_r$ , %	RSD $_R$ , %	$r^a$	$R^b$
A	12	0.08	0.01	0.01	7.61	12.60	0.03	0.03
B	14	0.11	0.01	0.01	5.43	12.31	0.03	0.03
C	14	0.18	0.01	0.01	3.74	6.78	0.03	0.03
D	11	0.44	0.01	0.02	1.19	4.05	0.03	0.06
E	13	2.81	0.15	0.33	5.22	11.81	0.42	0.92
F	13	5.03	0.14	0.45	2.73	9.02	0.39	1.26
G	14	6.93	0.24	0.95	3.42	13.70	0.67	2.66
H	14	0.28 <sup>c</sup>	0.03	0.03	9.52	10.48	0.08	0.08
I	15	0.34 <sup>c</sup>	0.02	0.03	6.98	8.01	0.06	0.08
J	15	1.30 <sup>c</sup>	0.24	0.24	18.75	18.75	0.67	0.67
K	15	2.51 <sup>c</sup>	0.24	0.26	9.59	10.31	0.67	0.73
L	14	2.64 <sup>c</sup>	0.16	0.21	6.21	7.91	0.45	0.59

<sup>a</sup>  $r = 2.8 \times s_r$ .<sup>b</sup>  $R = 2.8 \times s_R$ .<sup>c</sup> Mean value of independent materials.**Figure 996.01A. Chromatogram of FAMES standard solution.**

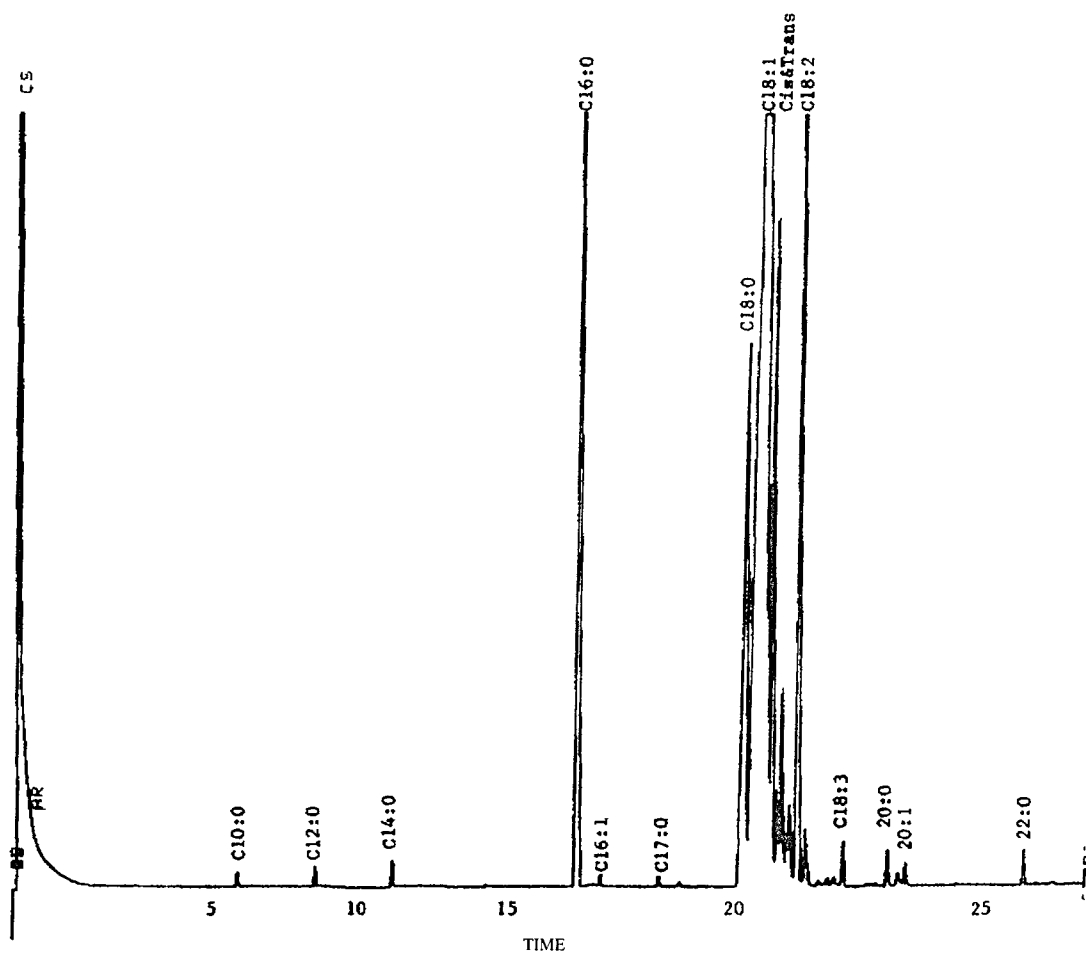


Figure 996.01B. Chromatogram of test sample.

(g) *Methanolic sodium hydroxide (NaOH) solution.*—0.5N NaOH in methanol.

(h) *Sodium chloride (NaCl) solution.*—Saturated aqueous solution (ca 26%, w/v).

(i) *n-Heptane.*—GC grade.

(j) *Fatty acid methyl esters (FAMES) standard solution.*—Mixture of following FAMES prepared in *n*-heptane: C<sub>8:0</sub> - methyl octanoate (caprylate), 0.3 mg/mL; C<sub>10:0</sub> - methyl decanoate (caprate), 0.5 mg/mL; C<sub>12:0</sub> - methyl dodecanoate (laurate), 1.0 mg/mL; C<sub>13:0</sub> - methyl tridecanoate (tridecanoate), 0.5 mg/mL; C<sub>14:0</sub> - methyl tetradecanoate (myristate), 0.5 mg/mL; C<sub>14:1</sub> - methyl tetradecenoate (myristoleate), 0.3 mg/mL; C<sub>15:0</sub> - methyl pentadecanoate (pentadecylate), 0.3 mg/mL; C<sub>16:0</sub> - methyl hexadecanoate (palmitate), 2.0 mg/mL; C<sub>16:1</sub> - methyl 9-hexadecenoate (palmitoleate), 1.0 mg/mL; C<sub>17:0</sub> - methyl heptadecanoate (margarate), 0.5 mg/mL; C<sub>18:0</sub> - methyl octadecanoate (stearate), 1.0 mg/mL; C<sub>18:1</sub> - methyl 9-octadecenoate (elaidate, *trans*), 0.4 mg/mL; C<sub>18:1</sub> - methyl 9-octadecenoate (oleate, *cis*), 3.0 mg/mL; C<sub>18:2</sub> - methyl 9,12-octadecadienoate (linoleate), 2.0 mg/mL; C<sub>20:0</sub> - methyl eicosanoate (arachidate), 0.3 mg/mL; C<sub>18:3</sub> - methyl 9,12,15-octadecatrienoate (linolenate), 1.0 mg/mL; C<sub>20:1</sub> - methyl 11-eicosenoate (gondoate), 0.3 mg/mL; C<sub>22:0</sub> - methyl docosanoate (behenate), 0.3 mg/mL; C<sub>22:1</sub> - methyl 13-docosenoate (erucate), 0.3 mg/mL. Prepared

FAMES standard solution is stable up to 1 month if stored in refrigerator in well-sealed amber bottle. Store commercially made FAMES standard solution according to manufacturer specifications.

(k) *Triglyceride internal standard solution.*—C<sub>13:0</sub> tritridecanoin; 5.0 mg/mL in CHCl<sub>3</sub>. Triglyceride internal standard solution is stable up to 1 week if stored in refrigerator in well-sealed amber bottle.

#### D. Extraction of Fat

(1) Equilibrate one water bath to 80°C. Equilibrate another water bath to ambient temperature.

(2) Weigh  $2 \pm 0.0001$  g sample into test tube, B(j). Add 1 mL triglyceride internal standard solution, C(k). (Note: When running matrixes of unknown composition, it may be necessary to analyze sample without adding internal standard to ensure against interferences. Should interfering peak be found, the area of C<sub>13</sub> internal standard peak must be corrected before performing calculations.)

Add 2 mL ethanol, C(a), while shaking or stirring to ensure adequate wetting of all particles and then add 10 mL 8N HCl. Cover tube with screw cap.

(3) Mix tube contents ca 5 s until thoroughly moistened. Mix on Vortex mixer additional 5 s. Place tube in 80°C water bath and hold 40 min.

(4) Remove test tube from water bath and immediately add 10 mL ethanol, mix on Vortex mixer 5 s, and then cool in another water bath to ambient temperature.

(5) Transfer entire contents of test tube with 15 mL ethyl ether into Roehrig tube, *B(c)*. Rinse test tube with another 15 mL ethyl ether and transfer washes to Roehrig tube. (*Caution:* Sample must be cool to keep pressure from building and blowing out acid out when tube is shaken. Wear protective eye glasses and gloves. Avoid grasping tube with bare hands.)

(6) Shake Roehrig tube gently 1 min by partial inversions. Vent pressure often by removing stopper. (*Note:* Sample may stick to sides of tube while shaking. It is necessary to suspend as much of sample as possible. After venting, shake tube vigorously and rotate from side to side while holding tube at top and bottom.)

(7) Perform steps (5) and (6) with all test samples before performing step (8).

(8) Add 30 mL petroleum ether and shake well 1 min.

(9) Tilt Roehrig tube such that sample in stem is suspended in solvent. Place tube in rack with spout pointing up until settling is complete (ca 30 min).

(10) Decant ether layer through filter consisting of pledget of fat-free cotton or glass wool packed firmly in stem of funnel to allow free passage of ether into flat-bottom flask, *B(n)*, containing boiling chips.

(11) Repeat extraction of fat from each sample with 15 mL ethyl ether and 15 mL petroleum ether. Shake well after each addition of ether.

(12) Repeat steps (9) and (10).

(13) Re-extract fat sample with 15 mL ethyl ether and 15 mL petroleum ether. Shake well after addition of ethers.

(14) Repeat steps (9) and (10).

(15) In ventilated hood, slowly evaporate ethers from 250 mL flat-bottom flask to almost dryness on steam table under stream of dry nitrogen.

(16) Remove flask from steam table and evaporate remaining solvent with nitrogen only.

*Note:* Do not overexpose extracts to air. Complete fat analysis as soon as possible on the same day as extraction.

### E. Methylation

(1) Add 10 mL methanolic NaOH solution, *C(g)*, to extracted fat from *D(16)*.

(2) Attach flask to water-cooled condenser, *B(o)*, seal joint with methanol, and heat. Reflux 10 min.

(3) Add 10 mL BF<sub>3</sub> reagent, *C(f)*, through top of condenser. Continue to reflux additional 5 min.

(4) Add 10 mL *n*-heptane, *C(i)*, through top of condenser and reflux additional 1 min.

(5) Remove flask from heat or turn off heat. (*Note:* Allow flask to cool to ambient temperature [ca 10 min] before removing from condenser.)

(6) Transfer entire mixture to centrifuge tube, *B(p)*. Rinse flat-bottom flask with 5 mL NaCl solution, *C(h)*. Rotate flask

gently several times before transferring washes to centrifuge tube. Mix contents of tube well by shaking gently or by using a Vortex mixer set at low speed and allow layers to separate. If necessary, centrifuge to separate layers. Upper layer contains FAMES in *n*-heptane.

(7) Transfer 1.0 mL upper layer to GC vial and use for analysis.

### F. GC Determination

Inject 1 µL FAMES standard solution, *C(j)*, of known concentration onto GC column, *B(b)*, under conditions described in *B(a)*. Observe chromatogram for any chromatographic artifacts. Make sure all FAMES in standard solution are eluted from capillary column.

Inject 1 µL test solution from *E(7)* onto GC column.

### G. Calculations

*Note:* For any unknown or uncalibrated peaks, use the nearest calibrated fatty acid response factors and conversion factors to calculate total, saturated, unsaturated, and monounsaturated fats.

Calculate the empirical response factors for each fatty acid as follows:

$$R_i = \frac{P_{S_i} \times W_{S_{C_{13:0}}}}{P_{S_{C_{13:0}}} \times W_{S_{i_s}}}$$

where  $R_i$  = response factor for fatty acids  $i$ ;  $P_{S_i}$  = peak area of individual FAME  $i$  in FAMES standard solution;  $W_{S_{C_{13:0}}}$  = mg of C<sub>13:0</sub> FAME in injected FAMES standard solution;  $P_{S_{C_{13:0}}}$  = peak area of C<sub>13:0</sub> FAME in FAMES standard solution; and  $W_{S_{i_s}}$  = mg of individual FAMES  $i$  in injected FAMES standard solution.

**Table 996.01E. Theoretical conversion factors for conversion of FAMES to their corresponding triglycerides ( $f_{TG}$ ) and fatty acids ( $f_{FA}$ )**

Fatty acid	$f_{TG}$	$f_{FA}$
8:0	0.9915	0.9114
10:0	0.9928	0.9247
12:0	0.9937	0.9346
14:0	0.9945	0.9421
14:1	0.9944	0.9417
15:0	0.9948	0.9453
16:0	0.9950	0.9481
16:1 <i>cis</i>	0.9950	0.9477
17:0	0.9953	0.9507
18:0	0.9955	0.9530
18:1 <i>cis</i> or <i>trans</i>	0.9955	0.9527
18:2	0.9954	0.9524
18:3	0.9954	0.9520
20:0	0.9959	0.9570
20:1	0.9959	0.9568
22:0	0.9962	0.9604
22:1	0.9962	0.9602

**Table 1. Collaborative study results (blind duplicates) of total, saturated, unsaturated, and monounsaturated fats (%) by acid hydrolysis and capillary GC analysis**

Laboratory	Total fat	Saturated		Unsaturated		Monounsaturated		
		Saturated	Unsaturated	Saturated	Unsaturated	Saturated	Unsaturated	
Sample A								
1	0.52	0.64	0.16	0.20	0.34	0.41	0.08	0.09
2	0.54	0.51	0.15	0.14	0.36	0.34	0.10	0.09
3	0.52	0.60	0.20	0.24	0.31	0.33	0.07	0.07
4	0.76	0.73	0.19 <sup>a</sup>	0.32 <sup>a</sup>	0.53 <sup>a</sup>	0.38 <sup>a</sup>	0.16 <sup>a</sup>	0.08 <sup>a</sup>
5	0.52	0.55	0.15	0.18	0.34	0.35	0.08	0.08
6	0.51	0.51	0.15	0.15	0.33	0.34	0.08	0.08
7	0.44	0.46	0.15	0.15	0.28	0.32	0.08	0.09
8	0.48	0.50	0.14	0.15	0.32	0.33	0.08	0.08
9	0.78	0.47	0.21 <sup>a</sup>	0.14 <sup>a</sup>	0.53 <sup>a</sup>	0.30 <sup>a</sup>	0.18 <sup>a</sup>	0.07 <sup>a</sup>
10	0.52	0.51	0.16	0.16	0.33	0.33	0.08	0.08
11	0.50	0.42	0.16	0.14	0.32	0.26	0.08	0.06
12	0.52	0.57	0.16	0.19	0.34	0.35	0.10	0.09
13	0.57	0.64	0.17	0.16	0.33	0.32	0.08	0.08
14	0.77	0.95	0.17 <sup>a</sup>	0.35 <sup>a</sup>	0.57 <sup>a</sup>	0.46 <sup>a</sup>	0.09 <sup>a</sup>	0.12 <sup>a</sup>
15	0.40	0.40	0.15	0.16	0.23	0.24	0.07	0.06
Sample B								
1	0.73	0.72	0.21	0.21	0.49	0.48	0.10	0.10
2	0.72	0.75	0.20	0.20	0.49	0.52	0.11	0.13
3	0.68	0.68	0.20	0.21	0.46	0.44	0.10	0.10
4	0.81	0.80	0.24	0.24	0.53	0.53	0.13	0.12
5	0.70	0.72	0.20	0.20	0.47	0.48	0.10	0.10
6	0.71	0.72	0.20	0.20	0.48	0.49	0.11	0.12
7	0.64	0.63	0.20	0.18	0.44	0.44	0.11	0.11
8	0.70	0.72	0.20	0.20	0.48	0.49	0.11	0.11
9	0.71	0.69	0.21	0.20	0.47	0.46	0.10	0.10
10	0.72	0.74	0.21	0.22	0.48	0.49	0.12	0.12
11	0.72	0.65	0.22	0.20	0.47	0.43	0.11	0.10
12	0.81	0.78	0.24	0.24	0.53	0.50	0.14	0.13
13	0.77	0.74	0.22	0.21	0.50	0.48	0.12	0.11
14	0.69	0.60	0.17	0.15	0.48	0.43	0.09	0.08
15	0.70	0.60	0.21	0.21	0.29 <sup>b</sup>	0.37 <sup>b</sup>	0.09 <sup>b</sup>	0.10 <sup>b</sup>
Sample C								
1	1.51	1.41	0.37	0.34	1.06	1.01	0.17	0.16
2	1.52	1.49	0.35	0.36	1.10	1.07	0.17	0.18
3	1.40	1.45	0.35	0.39	0.99	1.00	0.16	0.17
4	1.76	1.63	0.38	0.41	1.38	1.15	0.19	0.20
5	1.42	1.41	0.34	0.34	1.02	1.00	0.17	0.16
6	1.43	1.42	0.35	0.35	1.02	1.01	0.17	0.17
7	1.31	1.35	0.33	0.35	0.98	1.01	0.18	0.19
8	1.46	1.45	0.37	0.36	1.03	1.03	0.19	0.18
9	1.32	1.25	0.32	0.35	0.94	0.84	0.16	0.15
10	1.49	1.46	0.37	0.36	1.06	1.04	0.19	0.18
11	1.67	1.48	0.40	0.37	1.20	1.04	0.19	0.17
12	1.48	1.49	0.38	0.38	1.04	1.05	0.18	0.18
13	1.48	1.45	0.36	0.35	1.02	1.01	0.17	0.17
14	1.30 <sup>a</sup>	1.70 <sup>a</sup>	0.31 <sup>a</sup>	0.28 <sup>a</sup>	0.94 <sup>a</sup>	1.35 <sup>a</sup>	0.16 <sup>a</sup>	0.17 <sup>a</sup>
15	1.20	1.20	0.38	0.36	0.75	0.81	0.17	0.17

Table 1. (continued)

Laboratory	Total fat		Saturated		Unsaturated		Monounsaturated	
	Sample D							
1	2.77	2.76	1.51	1.50	1.13	1.12	0.42	0.41
2	2.77	2.78	1.47	1.48	1.16	1.16	0.45	0.44
3	2.85 <sup>a</sup>	3.13 <sup>a</sup>	1.63 <sup>a</sup>	1.80 <sup>a</sup>	1.09 <sup>a</sup>	1.19 <sup>a</sup>	0.40 <sup>a</sup>	0.46 <sup>a</sup>
4	2.95	2.94	1.58	1.57	1.22	1.23	0.43	0.44
5	2.70	2.71	1.44	1.41	1.12	1.16	0.42	0.42
6	2.66	2.67	1.44	1.44	1.09	1.09	0.43	0.43
7	2.72	2.65	1.58	1.51	1.14	1.14	0.46	0.46
8	2.62	2.65	1.40	1.41	1.09	1.10	0.43	0.44
9	2.85	2.76	1.23	1.52	1.48 <sup>a</sup>	1.10 <sup>a</sup>	0.60 <sup>a</sup>	0.41 <sup>a</sup>
10	2.81	2.82	1.50	1.52	1.16	1.16	0.46	0.46
11	2.87	2.92	1.52	1.57	1.20	1.21	0.46	0.46
12	2.72	2.84	1.46	1.55	1.12	1.16	0.44	0.45
13	2.73	2.68	1.45	1.43	1.08	1.07	0.42	0.41
14	2.55 <sup>a</sup>	3.08 <sup>a</sup>	1.29 <sup>a</sup>	1.64 <sup>a</sup>	1.14 <sup>a</sup>	1.26 <sup>a</sup>	0.40 <sup>a</sup>	0.36 <sup>a</sup>
15	2.40	2.40	1.45	1.46	0.86 <sup>b</sup>	0.83 <sup>b</sup>	0.36 <sup>b</sup>	0.35 <sup>b</sup>

Sample E								
1	5.02	5.09	1.42	1.42	3.39	3.45	2.62	2.72
2	5.42	5.08	1.46	1.39	3.73	3.46	2.96	2.76
3	5.16	5.26	1.56	1.62	3.44	3.42	2.51	2.52
4	3.62	4.89	1.52	1.28	1.94 <sup>a</sup>	3.39 <sup>a</sup>	0.98 <sup>a</sup>	2.62 <sup>a</sup>
5	5.38	5.43	1.40	1.42	3.74	3.75	2.53	2.57
6	5.19	5.14	1.42	1.41	3.54	3.51	2.79	2.76
7	4.93	4.59	1.40	1.40	3.53	3.20	2.80	2.50
8	5.17	5.23	1.44	1.45	3.50	3.56	2.80	2.85
9	5.31	5.16	1.41	1.36	3.67	3.57	2.91	2.87
10	5.85	5.21	1.56	1.48	4.03	3.49	3.22	2.73
11	6.17	6.27	1.55	1.57	4.35	4.42	3.53	3.58
12	6.87 <sup>a</sup>	3.26 <sup>a</sup>	1.40 <sup>a</sup>	1.58 <sup>a</sup>	5.16 <sup>a</sup>	1.53 <sup>a</sup>	2.56 <sup>a</sup>	0.76 <sup>a</sup>
13	5.56	5.06	1.46	1.33	3.37	3.06	2.64	2.35
14	5.74	6.15	1.50	1.53	3.98	4.35	3.11	3.37
15	4.60	4.80	1.44	1.41	2.97	3.19	2.41	2.54

Sample F								
1	10.47	10.44	3.19	3.18	6.80	6.78	5.07	5.03
2	10.28	10.29	3.05	3.05	6.76	6.77	5.05	5.06
3	10.53	10.22	3.36	3.31	6.69	6.45	4.66	4.42
4	10.28	10.07	3.29	3.22	6.52	6.38	4.55	4.47
5	10.62	10.57	3.07	3.05	7.06	7.04	4.53	4.51
6	10.40	10.20	3.12	3.07	6.82	6.69	5.10	4.99
7	10.58	9.97	3.21	3.17	7.36	6.79	5.40	5.00
8	10.11	10.26	2.99	3.04	6.66	6.76	4.97	5.03
9	10.57	10.68	2.86	2.86	7.23	7.34	5.49	5.63
10	12.22 <sup>a</sup>	10.93 <sup>a</sup>	3.18 <sup>a</sup>	3.21 <sup>a</sup>	8.47 <sup>a</sup>	7.22 <sup>a</sup>	6.65 <sup>a</sup>	5.37 <sup>a</sup>
11	11.15	11.42	3.23	3.20	7.40	7.70	5.51	5.72
12	11.00 <sup>a</sup>	13.22 <sup>a</sup>	3.33 <sup>a</sup>	3.33 <sup>a</sup>	7.17 <sup>a</sup>	9.29 <sup>a</sup>	5.44 <sup>a</sup>	5.69 <sup>a</sup>
13	10.41	10.35	3.01	3.04	6.28	6.20	4.58	4.50
14	11.26	11.90	3.24	3.29	7.50	8.07	5.63	6.05
15	10.10	10.10	3.07	3.10	6.54	6.54	4.83	4.88



Table 1. (continued)

Laboratory	Total fat		Saturated		Unsaturated		Monounsaturated	
Sample G								
1	12.38	12.89	3.17	3.33	8.65	8.98	7.09	7.37
2	13.53	13.15	3.27	3.21	9.64	9.35	7.94	7.69
3	12.19	11.90	3.50	3.41	8.15	7.96	6.23	6.10
4	12.77	11.39	3.50	3.09	8.70	7.79	6.71	6.14
5	12.80	12.82	3.12	3.21	9.03	9.11	6.50	6.42
6	12.00	11.90	3.11	3.10	8.64	8.07	6.76	6.51
7	13.43	12.37	3.60	3.26	9.83	9.12	7.98	7.37
8	12.44	12.26	3.10	3.06	8.79	8.65	7.21	7.09
9	12.80	12.40	2.94	2.88	9.28	8.96	7.70	7.37
10	13.09	13.27	3.24	3.26	9.26	9.42	7.57	7.73
11	13.74	14.77	3.26	3.40	9.86	10.72	8.10	8.79
12	10.21	10.12	3.39	3.31	6.35	6.35	4.67	4.55
13	12.73	13.00	3.15	3.18	8.14	8.26	6.56	6.65
14	13.75	15.53	3.22	3.51	9.91	11.32	8.09 <sup>a</sup>	9.57 <sup>a</sup>
15	11.80	11.80	3.13	3.12	8.14	8.11	6.68	6.63

<sup>a</sup> Cochran outlier; removed.

<sup>b</sup> Single Grubbs outlier; removed.

Determine relative retention times for each FAME in FAMES standard solution relative to C<sub>13:0</sub> and use them to identify various FAMES in test samples.

Calculate amount of each fatty acid (as corresponding methyl esters),  $F_{ME}$ , in test sample as follows:

$$F_{ME} = \frac{Pt_i}{Pt_{C_{13:0}}} \times \frac{Wt_{C_{13:0}} \times 1.006}{R_i}$$

where  $Pt_i$  = peak area of individual FAMES  $i$  in test sample;  $Pt_{C_{13:0}}$  = peak area of C<sub>13:0</sub> internal standard in sample; and  $Wt_{C_{13:0}}$  = mg C<sub>13:0</sub> triglyceride internal standard in injected test sample.

Calculate amount of each fatty acid (as corresponding triglyceride),  $F_{TG}$ , in test sample as follows:

$$F_{TG} = F_{ME} \times f_{TG}$$

where  $f_{TG}$  = theoretical conversion factors for conversion of FAMES to their corresponding triglycerides. (See Table 996.01E.)

Calculate amount of each fatty acid (as corresponding fatty acids),  $F_{FA}$ , in test sample as follows:

$$F_{FA} = F_{ME} \times f_{FA}$$

where  $f_{FA}$  = theoretical conversion factors for conversion of FAMES to their corresponding fatty acids. (See Table 996.01E.)

Calculate amount of total fat (sum of all lipid fatty acids; expressed as triglycerides) in test sample as follows:

$$\text{Total fat, \%} = \sum \frac{F_{TG}}{W_{\text{sample}}} \times 100$$

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

Calculate amount of saturated fat (expressed as fatty acid sum of C<sub>8:0</sub>–C<sub>22:0</sub> [all fatty acids with no double bonds]) in test sample as follows:

$$\text{Saturated fat, \%} = \sum \frac{\text{Saturated } F_{FA}}{W_{\text{sample}}} \times 100$$

Calculate amount of unsaturated fat (expressed as fatty acids; C<sub>14:1</sub>, C<sub>16:1</sub>, C<sub>18:1</sub>, *cis* and *trans*, C<sub>18:2</sub>, C<sub>18:3</sub>, C<sub>20:1</sub>, and C<sub>22:1</sub>) in test sample as follows:

$$\text{Unsaturated fat, \%} = \sum \frac{\text{Unsaturated } F_{FA}}{W_{\text{sample}}} \times 100$$

Calculate amount of monounsaturated fat (expressed as fatty acids; C<sub>14:1</sub>, C<sub>16:1</sub>, C<sub>18:1</sub>, *cis* and *trans*, C<sub>20:1</sub>, and C<sub>22:1</sub>) in test sample as follows:

$$\text{Monounsaturated fat, \%} = \sum \frac{\text{Monounsaturated } F_{FA}}{W_{\text{sample}}} \times 100$$

Ref.: *J. AOAC Int.* **80**, 359(1996)

## Results and Discussion

Collaborative study results are presented in Tables 1 and 2. All data were statistically analyzed according to AOAC INTERNATIONAL guidelines (10). After outlying data were removed, the statistical analysis was repeated. Results are shown in Tables 3–6. Total fat for labeling purposes is the sum of all lipid fatty acids expressed as triglyceride equivalents. The total fat content was calculated according to equation provided in section G, *Calculations*. Total fat results are summarized in Table 3. Methyl esters of saturated, unsaturated, and monounsaturated fatty acids are reported as fatty acid equivalents in Ta-

**Table 2. Collaborative study results (independent pairs) of total, saturated, unsaturated, and monounsaturated fats (%) by acid hydrolysis and capillary GC analysis**

Laboratory	Total fat		Saturated		Unsaturated		Monounsaturated	
	Sample pair H		Sample pair I		Sample pair J		Sample pair K	
1	1.23	1.36	0.47	0.30	0.70	0.99	0.39	0.22
2	1.15	1.33	0.36	0.29	0.74	0.98	0.31	0.22
3	1.18	1.34	0.39	0.38	0.74	0.91	0.29	0.22
4	1.50	1.44	0.51	0.32	0.92	1.04	0.43	0.22
5	1.26	1.31	0.41	0.29	0.79	0.97	0.32	0.21
6	1.23	1.27	0.38	0.28	0.79	0.93	0.33	0.23
7	1.07	1.18	0.35	0.28	0.73	0.90	0.32	0.22
8	1.17	1.31	0.36	0.29	0.73	0.96	0.31	0.23
9	1.18	1.26	0.37	0.31	0.76	0.90	0.31	0.20
10	1.27	1.31	0.41	0.30	0.81	0.95	0.34	0.23
11	1.34	1.29	0.41	0.31	0.86	0.92	0.36	0.22
12	1.38	1.37	0.47	0.33	0.85	0.98	0.37	0.24
13	1.19	1.30	0.39	0.29	0.72	0.91	0.30	0.21
14	1.27	1.40	0.32	0.37	0.89	0.98	0.32	0.19
15	1.10	0.90	0.39	0.30	0.61 <sup>a</sup>	0.58 <sup>a</sup>	0.29 <sup>a</sup>	0.16 <sup>a</sup>
Sample pair I								
1	1.96	2.32	0.49	0.50	1.38	1.73	0.31	0.32
2	2.02	2.37	0.48	0.48	1.45	1.78	0.34	0.35
3	1.96	2.49	0.50 <sup>b</sup>	0.61 <sup>b</sup>	1.37	1.77	0.31	0.38
4	2.10	2.18	0.52	0.46	1.49	1.62	0.36	0.30
5	2.02	2.38	0.50	0.52	1.45	1.75	0.33	0.33
6	1.99	2.16	0.49	0.46	1.40	1.60	0.36	0.32
7	1.66	2.01	0.44	0.44	1.22	1.56	0.32	0.33
8	2.00	2.32	0.50	0.49	1.41	1.73	0.34	0.35
9	1.88	2.54	0.46 <sup>b</sup>	0.68 <sup>b</sup>	1.34	1.75	0.32	0.35
10	2.10	2.32	0.52	0.49	1.49	1.73	0.37	0.36
11	2.08	2.60	0.54	0.53	1.45	1.95	0.35	0.37
12	2.07	2.44	0.52	0.54	1.46	1.79	0.35	0.39
13	2.03	2.42	0.51	0.51	1.39	1.76	0.33	0.35
14	1.86	1.82	0.42	0.43	1.36	1.30	0.30	0.27
15	1.60	2.10	0.51	0.51	1.06	1.51	0.30	0.34
Sample pair J								
1	3.75	4.34	0.79	1.24	2.80	2.92	0.57	2.15
2	3.76	4.40	0.70	1.23	2.89	2.98	0.64	2.20
3	3.35	4.32	0.70	1.40	2.49	2.73	0.52	1.85
4	3.45	4.48	0.67	1.41	2.62	2.91	0.55	1.92
5	3.61	4.41	0.70	1.20	2.75	3.01	0.57	1.34
6	3.30	4.24	0.63	1.21	2.58	2.84	0.54	2.09
7	3.07	4.49	0.64	1.33	2.43	3.15	0.55	2.30
8	3.57	4.33	0.70	1.22	2.71	2.92	0.60	2.15
9	3.40	4.18	0.66	1.14	2.59	2.86	0.58	2.14
10	3.84	4.31	0.78	1.25	2.89	2.87	0.65	2.03
11	3.54	5.39	0.68	1.44	2.70	3.71	0.56	2.78
12	3.67	3.89	0.73	1.28	2.77	2.44	0.62	1.60
13	3.81	4.58	0.74	1.25	2.83	2.65	0.62	1.89
14	2.58	4.56	0.54	1.29	1.92	3.07	0.42	2.18
15	3.30	3.80	0.72	1.21	2.39	2.38	0.59	1.76

Table 2. (continued)

Laboratory	Total fat		Saturated		Unsaturated		Monounsaturated	
Sample pair K								
1	4.61	5.92	0.60	0.92	3.81	4.73	2.57	2.18
2	5.10	5.83	0.62	0.84	4.26	4.74	2.92	2.14
3	4.89	5.49	0.75	0.91	3.93	4.34	2.59	1.84
4	5.99	5.58	0.79	0.87	4.94	4.44	3.26	1.89
5	5.49	5.44	0.71	0.81	4.54	4.39	3.00	1.82
6	5.19	5.49	0.65	0.81	4.50	4.44	3.14	2.06
7	4.03	5.65	0.57	0.92	3.47	4.73	2.42	2.08
8	5.17	5.58	0.65	0.84	4.29	4.50	2.95	2.04
9	5.81	4.79	0.75 <sup>b</sup>	0.34 <sup>b</sup>	4.81	4.24	3.27	1.86
10	5.72	5.83	0.73	0.89	4.75	4.68	3.29	2.16
11	6.04	6.75	0.73	0.93	5.05	5.52	3.43	2.45
12	5.28	6.06	0.70	0.93	4.36	4.86	2.96	2.25
13	5.78	6.00	0.74	0.91	4.62	4.64	3.13	2.05
14	4.92	6.31	0.58	0.84	4.12	5.19	2.77	2.34
15	4.40	4.90	0.59	0.64	3.58	4.06	2.53	1.91
Sample pair L								
1	6.67	7.65	1.42	2.81	4.95	4.48	2.39	2.90
2	5.77	8.00	1.22	2.82	4.30	4.81	2.15	3.15
3	6.25	7.46	1.37	2.92	4.62	4.20	2.16	2.61
4	6.54	7.46	1.34	2.82	4.90	4.28	2.27	2.68
5	6.40	7.68	1.29	2.73	4.83	4.58	2.31	2.52
6	6.45	7.38	1.32	2.69	4.85	4.46	2.43	2.96
7	5.89	6.93	1.28	2.69	4.62	4.24	2.32	2.82
8	6.41	7.35	1.33	2.64	4.61	4.37	2.43	2.88
9	6.20	7.60	1.24	2.53	4.69	4.71	2.34	3.12
10	6.50	7.85	1.34	2.84	4.87	4.64	2.46	3.12
11	6.66	8.48	1.32	2.98	5.04	5.10	2.44	3.37
12	2.68 <sup>b</sup>	8.00 <sup>b</sup>	1.51 <sup>b</sup>	2.89 <sup>b</sup>	1.05 <sup>b</sup>	4.74 <sup>b</sup>	0.27 <sup>b</sup>	3.13 <sup>b</sup>
13	6.46	7.95	1.31	2.81	4.68	4.34	2.33	2.77
14	7.80	7.94	1.38	2.89	6.07	4.67	2.89	3.10
15	6.00	7.10	1.34	2.66	4.36	4.12	2.31	2.80

<sup>a</sup> Single Grubbs outlier; removed.<sup>b</sup> Cochran outlier; removed.

bles 4, 5, and 6, respectively, for saturated, unsaturated, and monounsaturated fats.

Once total fat data for any sample were rejected as outliers, corresponding results for saturated and unsaturated fats also were rejected, even if those were not flagged as outliers by AOAC criteria. In the opinion of the associate referee, this is the appropriate way to handle these data because saturated and unsaturated fats are components of total fat. It is possible for the corresponding saturated and unsaturated fatty acid components of an outlying total fat result to go through the AOAC outlier removal procedure without being flagged as outliers.

The total fat contents of samples ranged from 0.56 to 12.64% (Table 3). Repeatability relative standard deviations (RSD<sub>s</sub>) values for total fat ranged from 1.32 to 13.30, and the reproducibility relative standard deviations (RSD<sub>R</sub>s) values ranged from 4.42 to 22.82. Data for sample A data yielded the highest RSD<sub>s</sub> and RSD<sub>R</sub> values: 13.30 and 22.82, respectively.

Sample A also contained the least total fat, 0.56%. At this very low total fats level, small absolute variations of saturated and unsaturated fat show up statistically as outliers. The theoretical critical values for Cochran's test is 0.575 for sample A. The experimental value for this sample is 0.5765. Because of the magnitude of the absolute difference and the concentration and distribution of the fatty acids, these data were not rejected. The rest of the data from other collaborators for sample A passed the outlier criteria for total fat. However, the corresponding saturated, unsaturated, and monounsaturated fats for this sample from 3 collaborators were rejected (Tables 4-6). The specific results rejected as outliers (Cochran, single and double Grubbs) are shown in Table 1.

Data for saturated and unsaturated fatty acids were not flagged as outliers for collaborator 14 for samples C and D; collaborator 12 for samples E and F and sample pair L; collaborator 10 for sample F; and collaborator 3 for sample D (Ta-

**Table 3. Method performance for total fat in cereal products by acid hydrolysis and capillary gas chromatography**

Sample	No. of laboratories	Total fat average, %	S <sub>r</sub>	S <sub>R</sub>	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %
A	15	0.56	0.07	0.13	13.30	22.82
B	15	0.71	0.03	0.05	4.27	7.61
C	14	1.44	0.05	0.13	3.59	8.69
D	13	2.74	0.04	0.14	1.32	5.00
E	14	5.23	0.31	0.54	6.01	10.27
F	13	10.51	0.20	0.46	1.94	4.42
G	15	12.64	0.52	1.12	4.10	8.84
Pair H	15	1.26 <sup>a</sup>	0.07	0.12	5.71	9.31
Pair I	15	2.13 <sup>a</sup>	0.13	0.18	6.00	8.59
Pair J	15	3.92 <sup>a</sup>	0.35	0.35	8.87	8.87
Pair K	15	5.47 <sup>a</sup>	0.49	0.55	8.88	9.99
Pair L	14	7.03 <sup>a</sup>	0.34	0.44	4.85	6.29

<sup>a</sup> Mean value of independent materials.

bles 1 and 2). However, these results were rejected because the corresponding total fat results from these laboratories had been rejected as Cochran outliers. Treating the data this way resulted in a higher number of outlying results.

For labeling purposes, saturated fat is expressed as the sum of all fatty acids containing no double bonds. It was calculated according to equation 6, and results are expressed as the corresponding fatty acids in accordance with labeling regulations. Rejection criteria for saturated fat was in keeping with AOAC guidelines. The saturated fat contents of samples ranged from 0.18 to 3.23%. RSD<sub>r</sub> and RSD<sub>R</sub> values are summarized in Table 4. In addition to excluding the saturated fat components for the samples and laboratories mentioned above (collaborators 10, 12, and 14 for samples D, E, and F and sample pair L), saturated fat results for sample A were rejected from collaborators 4, 9, and 14. Results from collaborator 9 for 2 independent pairs, (K and I); and collaborator 3 for 1 independent pair (I) also were excluded from the final statistical analysis (Table 1).

Declaration of the quantitative amount of monounsaturated and polyunsaturated fats for nutrition labeling is voluntary, unless claims are made about fatty acids and cholesterol (2). Labeling regulations do not define unsaturated fat. Polyunsaturated and monounsaturated fats are defined as *cis,cis*-polyunsaturated, and *cis*-monounsaturated fatty acids respectively. This definition excludes *trans* isomers.

In this study, we included *trans* isomers as part of unsaturated fat. From a chemical perspective, this inclusion is correct. Technically, it does not meet NLEA definitions for monounsaturated and polyunsaturated fats. However, it is easy and straightforward to separate and exclude the *trans* isomer from calculations of monounsaturated fat (for equation, *see* section *G, Calculations*) after the *cis* and *trans* isomers have been adequately and conclusively identified. Some cereal products contain *trans* isomers added during processing. The *trans* isomers reference standards currently are not always available for quantitation purposes. Frequently, it is impossible to resolve

**Table 4. Method performance for saturated fat in cereal products by acid hydrolysis and capillary gas chromatography**

Sample	No. of laboratories	Saturated fat average, %	S <sub>r</sub>	S <sub>R</sub>	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %
A	15	0.18	0.04	0.14	25.31	27.95
B	15	0.21	0.01	0.02	3.53	9.51
C	15	0.36	0.02	0.03	4.37	7.45
D	15	1.49	0.09	0.11	6.16	7.06
E	15	1.45	0.07	0.08	4.48	5.49
F	15	3.14	0.03	0.13	0.83	4.23
G	15	3.23	0.12	0.17	3.77	5.24
Pair H	15	0.35 <sup>a</sup>	0.04	0.04	11.76	11.76
Pair I	13	0.49 <sup>a</sup>	0.02	0.03	3.24	6.84
Pair J	15	0.98 <sup>a</sup>	0.08	0.08	8.13	8.13
Pair K	14	0.77 <sup>a</sup>	0.06	0.07	7.80	9.76
Pair L	15	2.06 <sup>a</sup>	0.08	0.10	3.73	4.83

<sup>a</sup> Mean value of Youden nonduplicate pairs.

**Table 5. Method performance for unsaturated fat in cereal products by acid hydrolysis and capillary gas chromatography**

Sample	No. of laboratories	Unsaturated fat, average, %	S <sub>r</sub>	S <sub>R</sub>	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %
A	12	0.32	0.02	0.04	6.78	12.03
B	14	0.48	0.02	0.03	3.37	5.93
C	14	1.02	0.06	0.12	5.77	11.32
D	11	1.14	0.01	0.05	1.14	4.11
E	13	3.60	0.17	0.38	4.80	10.47
F	13	6.89	0.18	0.46	2.64	6.72
G	15	8.82	0.40	1.07	4.53	12.09
Pair H	14	0.87 <sup>a</sup>	0.04	0.06	4.95	6.54
Pair I	15	1.53 <sup>a</sup>	0.10	0.13	6.37	8.69
Pair J	15	2.76 <sup>a</sup>	0.29	0.29	10.41	10.41
Pair K	15	4.47 <sup>a</sup>	0.38	0.43	8.50	9.67
Pair L	14	4.66 <sup>a</sup>	0.30	0.35	6.35	7.57

<sup>a</sup> Mean value of independent materials.

and quantitate every isomer in a sample. Fortunately, the processor's declaration of unsaturated fat is voluntary and most processors have an excellent idea of what is present in their products. In such situations, exclusion of the *trans* isomer can be easily accomplished.

Treatment of unsaturated and monounsaturated fats was accomplished by the same guidelines outlined for total and saturated fats.

### Associate Referee's Comments

Quality control practices consistent with this method were incorporated into this study. A standard FAMES mixture (Kel-Fim-FAME-5, Cat. No. 4210; Matreya, Inc., Pleasant Gap, PA) was used to determine the relative retention time and empirical relative response factor (RF) for each fatty acid in the mixture. GC conditions were first optimized with methyl stearate (C<sub>18:0</sub>) as the reference FAME (RF = 1). When we were satisfied that

the chromatographic conditions were optimal and that empirical and theoretical relative RFs were in excellent agreement with each other, new empirical RFs were determined with methyl tridecanoate (C<sub>13:0</sub>) as reference.

Tritridecanoic (5.0 mg/mL in CHCl<sub>3</sub>), was the internal standard for all samples. It was used to optimize methylation and extraction. This dual approach of independently optimizing chromatographic and then methylation procedures by using 2 standards ensures method reliability and optimal conditions. The standard FAMES mixture can also be used as an external standard. This mixture ensures that optimum chromatographic conditions are used routinely. It also allows cross-checking of internal standard responses.

Each laboratory was instructed to optimize chromatographic parameters until acceptable response factors and quantitative results were obtained with the standard FAMES mixture prior to sample analysis. No laboratory was rejected because of an improperly optimized gas chromatograph.

**Table 6. Method performance for monounsaturated fat in cereal products by acid hydrolysis and capillary gas chromatography**

Sample	No. of laboratories	Monounsaturated fat, average, %	S <sub>r</sub>	S <sub>R</sub>	RSD <sub>r</sub> , %	RSD <sub>R</sub> , %
A	12	0.08	0.01	0.01	7.61	12.60
B	14	0.11	0.01	0.01	5.43	12.31
C	14	0.18	0.01	0.01	3.74	6.78
D	11	0.44	0.01	0.02	1.19	4.05
E	13	2.81	0.15	0.33	5.22	11.81
F	13	5.03	0.14	0.45	2.73	9.02
G	14	6.93	0.24	0.95	3.42	13.70
Pair H	14	0.28 <sup>a</sup>	0.03	0.03	9.52	10.48
Pair I	15	0.34 <sup>a</sup>	0.02	0.03	6.98	8.01
Pair J	15	1.30 <sup>a</sup>	0.24	0.24	18.75	18.75
Pair K	15	2.51 <sup>a</sup>	0.24	0.26	9.59	10.31
Pair L	14	2.64 <sup>a</sup>	0.16	0.21	6.21	7.91

<sup>a</sup> Mean value of Youden nonduplicate pairs.

## Collaborators' Comments

A number of collaborators reported partial separation of the C<sub>18:1</sub> *cis* and *trans* isomers for a number of samples, resulting in "odd-shaped peaks". This happened typically with high-fat products (>10% fat) containing large amounts of *cis* and *trans* isomers. The problem may be alleviated by sample dilution if the only cause is an abundance of *cis* and *trans* isomers. However, dilution may result in loss of fatty acids that are present in small amounts but contribute to the total fat value. Baseline resolution of *cis-trans* isomers may be improved by using longer columns (60–100 m), but the investment in resources and time may not be worth the effort. Some collaborators expressed concern with inclusion of *trans* fatty acids with unsaturated fat. This inclusion is in accordance with labeling regulation.

## Conclusions

RSD<sub>r</sub> values (a measure of intralaboratory variation) ranged from 1.32 to 13.30 for total fat; from 0.88 to 11.76 for saturated fat; from 1.14 to 10.41 for unsaturated fat; and, from 1.19 to 18.75 for monounsaturated fat. RSD<sub>R</sub> values (a measure of interlaboratory variation) ranged from 4.42 to 22.82 for total fat; 4.19 to 14.59 for saturated fat; 4.11 to 12.09 for unsaturated fat, and 4.05 to 18.75 for monounsaturated fat. This method is suitable for determining total, saturated, unsaturated, and monounsaturated fats in grain-based products.

## Recommendation

On the basis of the results of this study, it is recommended that the acid hydrolysis–capillary GC method for the determination of total, saturated, unsaturated, and monounsaturated fats in cereal products be adopted first action.

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