One-Step Extraction/Methylation Method for Determining the Fatty Acid Composition of Processed Foods

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In a one-step extraction/methylation (OSM) method for determining individual fatty acids (FA) in processed food products, freeze-dried samples, containing 10–50 mg fat, were transmethylated without prior fat extraction with a mixture of methanol-HCl/toluene. After washing the organic phase the formed FA methyl esters were ready for separation by gas-liquid chromatography (GLC). The relative standard deviation for total FA content was <3.5%, regardless of the food type analyzed. Furthermore, the FA composition of selected fatty foods as obtained by the OSM procedure was almost identical with the FA composition of the pure fats extracted by the Soxhlet procedure and with chloroform-methanol, respectively. The OSM method is inexpensive and simple to perform and is, therefore, well suited for nutrient labeling studies, especially in situations where many samples have to be analyzed for their total FA content.

KEY WORDS: Extractive methylation, fatty acid composition, GLC, processed foods.

The fat content and fatty acid (FA) composition of foods are of major concern to the consumer as well as the scientist because of the nutrition/health issue in connection with the consumption of fatty foods. Kinsella (1) stressed the problem of collecting relevant and quantitative data for these important dietary components. Extraction of a particular food sample with organic solvents followed by transesterification of the fat to form FA methyl esters and subsequent gas-liquid chromatography (GLC) analysis represents the classical solution for this task. Although the sequence of analytical steps is well established, it is time-consuming, laborious and uneconomical. In addition, the term "crude fat" is often methodologically defined, *i.e.*, that proportion of a given sample that is soluble in an organic solvent or solvent mixture. Crude fat is a heterogenous material, consisting of a mixture of acylglycerols, phospholipids, FA, sterols, waxes, pigments, etc. It is the FA constituents of the crude fat that are nutritionally important in many foods. Therefore, it is more appropriate to estimate the total FA composition rather than the amount of total fat ("ether extract") of the sample (2).

In light of these findings, methods for the direct estimation of the total FA composition without prior fat extraction have been devised for feedstuffs, digesta, feces (3), cornmeal, soymeal, buttermilk powder (4), spinach leaves (5), soybean seed (6), rapeseed (7), plasma (8) and biological tissues (9). In these assays, acid-catalyzed (2,3,5,6,8,9) as well as alkali-catalyzed (4,7) transesterification of lipids was employed. It would represent a considerable advantage if such a rapid method could also be applied to processed foods for a national food survey study and for nutritional labeling purposes.

The direct determination of the total and individual FA contents of selected food commodities is reported following the protocol outlined by Sukhija and Palmquist (2). The accuracy of the proposed method was checked by means of a reference fat with a known FA composition. The results of the one-step methylation (OSM) method were compared with values obtained with the fat extraction-transesterification-GLC procedure.

MATERIALS AND METHODS

Reagents, solvents and methyl esters of myristic, palmitic, stearic, oleic, linoleic and linolenic acid were of analytical reagent-grade quality (E. Merck, Darmstadt, Germany) and were used without further purification. Nonadecanoic acid, nonadecanoic acid methyl ester, trilaurin, trimyristin and tristearin were obtained from Sigma Chemical Co. (Munich, Germany). The certified reference material BCR No. 162 (soya-maize oil blend) was supplied by the Community Bureau of Reference (Commission of the European Communities, Brussels, Belgium).

Pure fats and oils, donated by Unilever-Austria, Vienna, Austria, were warmed to 50°C and filtered to remove traces of moisture. Processed food samples were purchased in local supermarkets.

The internal standard (IS) solution used for quantitation of the total FA content contained 3 mg nonadecanoic acid/mL toluene. For the recovery studies, 3 mg nonadecanoic acid methyl ester/mL toluene was used as IS.

Sample preparation. Food samples were homogenized with an Osterizer-blender and, for the OSM, freeze-dried overnight (Edwards Modulyo 4K; Edwards, Kniese & Co., Marburg, Germany). After the drying step, the material was carefully ground with a glass pestle.

One-step methylation (OSM). Samples were processed as described by Sukhija and Palmquist (2). In brief, 100–500 mg test material, containing 10–50 mg fat, was accurately weighed in a 16 × 160-mm Pyrex tube fitted with a PTFE-lined screw cap. Then, 1 mL of C19:0 IS solution, 1 mL toluene and 3 mL freshly made 5% methanolic HCl were added. The methanolic HCl was prepared from acetyl chloride and methanol according to Christie (10). After carefully mixing the contents and flushing the headspace of the vial with N_2, the tubes were tightly stoppered and heated for 2 hr in a water bath at 70°C. To the cooled tubes, 5 mL 6% K_2CO_3 and 2 mL toluene were added and thoroughly vortexed. Phase separation was accomplished by centrifugation for 5 min at 1100 rpm in a Funke-Gerber centrifuge. The organic layer was transferred to a 16 × 100-mm Pyrex tube and dried with anhydrous Na_2SO_4. One microliter of the supernatant was analyzed for FA content by GLC.

Chloroform:methanol extraction (CM). The CM procedure was done according to Daugherty and Lento (11). For FA analysis by GLC, an aliquot of the fat-containing chloroform extract was taken, and the solvent was removed by a gentle stream of N_2. The residue was transmethylated as described above.

Acid hydrolysis-Soxhlet extraction (AHS, Weibull-Stoldt method). The AHS procedure was done according to

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Walstra and Mulder (12). Light petroleum ether was the extractant. The FA analysis was performed as described for the CM method.

GLC separation and quantitation of individual FA. A Shimadzu GC 9A fitted with a SPL G9 split/splitless injector and a flame-ionization detector (FID) was used. Individual FA were separated with a 25-m × 0.32-mm fused silica column coated with 0.2 μm CP Sil 88 (Chrompack, Middelburg, The Netherlands). Peak areas were processed with a Shimadzu CR 3A computing integrator. Samples (1 μL) were injected at a column temperature of 140°C by the "hot needle" technique (13). One minute after injection, the temperature was raised at 3°C/min to 180°C. The injector/detector was set at 230°C. Hydrogen at 0.5 bar was the carrier gas. The split ratio was *ca.* 1:50. The FA were tentatively identified by comparison of their retention times with authentic standards. The concentrations of total and individual FA (mg/g sample dry weight) were calculated by means of the IS and the sample weight (2).

Statistical analysis. The ANOVA and LSD tests were performed as described by Sachs (14).

RESULTS

Accuracy of the OSM method. It is generally assumed that long-chain FA (>12 carbon atoms) separated by GLC produce an FID response that is directly proportional to the mass of the FA (15). To verify this assumption, a soya/maize oil blend with certified FA composition (BCR reference material No. 162) was transesterified by the OSM method, and the content of FA was determined by GLC. The mass fractions of individual FA were calculated without applying other correction factors. Mean values of individual FA were well within the limits of the certified values ± 2 standard deviations, which is the interval containing about 95% of the population of the laboratory means found in the certification exercise (16) (Table 1). Therefore, the FID response did not differ from unity to a great extent for the tested FA, irrespective of chainlength and degree of unsaturation. For fats consisting of FA with a wider range of chainlength or unsaturation, *e.g.*, milk fat or fish oil, this simple assumption may not hold true.

The accurately weighed masses of triacylglycerol standards and pure fats and oils, which were processed by the OSM method, and the corresponding FA contents as estimated by GLC are listed, in Table 2, along with the calculated total triacylglycerol values. A factor of 0.94 (3 × MW of FA divided by the MW of the monoacidic triacylglycerol) was used to convert total FA to total triacylglycerol content. In general, the known and the experimental values agreed well within the lipid mass range studied (10–50 mg). With pure triacylglycerols, the total triacylglycerol values tended to be somewhat higher than the given masses. Possible contamination of the monoacidic triacylglycerols with small amounts of free FA may explain these differences.

Influence of water and sample size on the methylation of FA. Because the water content of processed foods may interfere with the transesterification reaction, the influence of residual moisture was tested separately in a series of experiments. Water was added directly to known amounts of the BCR reference fat. The transesterification reaction was not hindered, provided the added water was below 100 mg. Beyond this critical limit, the recovery rate of total FA dropped sharply (Fig. 1). Additional tests were performed with vacuum oven-dried liver sausage. Up to 28.3 mg water added directly to the test portion (*ca.* 40 mg), corresponding to a moisture content of 40.7%, did not interfere with the formation of methyl esters from acyl glycerols. One series of experiments was carried out with C19:0 as IS, and the other with C19:0 FA methyl ester. No differences between the total FA values were observed, regardless of the nature of the IS.

Determination of total lipid and individual FA content by the OSM method. Table 3 shows the FA composition, expressed as mass fraction of individual FA methyl ester/total FA methyl esters, of the crude fat of a freeze-dried liver sausage, obtained by 3 different methods. Only the major FA (C14:0, C16:0, C18:0, C18:1 and C18:2) are reported. The crude fat was extracted by the AHS and the CM method, and an aliquot of the total extract was subsequently methylated. For the OSM method, the

TABLE 1

Accuracy of the OSM Method Tested by Means of the BCR Reference Fat No. 162 (n = 6)

Fatty acid	Certified values (mass %) mean	Certified values (mass %) SD	Found values (mass %) mean	Found values (mass %) SD
C16:0	10.65	0.279	10.52	0.092
C18:0	2.87	0.123	2.73	0.088
C18:1	24.14	0.392	23.91	0.139
C18:2	56.66	0.760	57.50	0.315
C18:3	4.68	0.309	4.38	0.095

TABLE 2

Total Fatty Acid (TFA) and Equivalent Total Triacylglycerol (TTG) Content of Synthetic Triacylglycerols and Pure Fats Estimated with the OSM Method

Fat source	Amount (mg)[a]	TFA (mg)	TTG (mg)[b]	Diff[c]
Trilaurin	12.1	11.75	12.50	−0.40
	17.8	17.02	18.11	−0.32
Trimyristin	12.0	11.49	12.22	−0.22
	41.4	38.99	41.48	−0.08
Tristearin	13.9	12.88	13.70	0.20
	25.2	24.27	25.82	−0.62
	32.2	30.36	32.30	−0.10
	48.7	45.88	48.81	−0.11
Lard	12.2	11.44	12.17	0.03
	28.6	26.85	28.56	0.04
	47.0	45.03	47.90	−0.90
Coconut	10.4	9.37	9.96	0.44
	28.3	26.37	28.05	0.25
	45.3	43.59	46.37	−1.07
Sunflower	10.6	9.49	10.10	0.50
	22.2	20.98	22.32	−0.12
	29.6	27.67	29.44	0.16
	41.4	39.02	41.51	−0.11
	49.1	46.62	49.60	−0.50

[a]Results are of single determinations.

[b]TTG equivalent = TFA/0.94.

[c]Known amount—TTG.

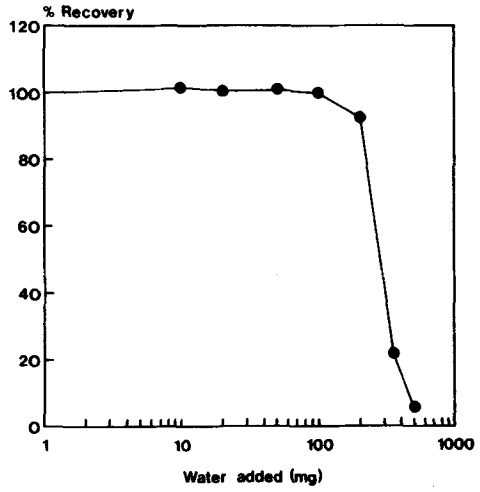

FIG. 1. Effect of water added (mg) to the transesterification mixture on the recovery of total FA.

TABLE 3

Fatty Acid Composition (mass fraction of individual FA methyl ester/total FA methyl ester) of Fat Extracted from Canned Liver Sausage by the Acid Hydrolysis-Soxhlet (AHS), Chloroform: methanol (CM) and the One-Step Method (OSM)[1]

	Fatty acid composition (mass %)		
Fatty acid	AHS (n = 4)	CM (n = 4)	OSM (n = 6)
14:0	1.93^b	2.03^a	1.90^b
16:0	26.46^a	26.17^a	25.77^b
18:0	15.61^a	$15.42^{a,b}$	15.15^b
18:1	40.73^a	40.55^a	40.26^a
18:2	9.01^c	9.48^b	10.06^a

[1]Values in a row with different superscripts differ significantly (P<0.05).

whole sample was taken without prior fat isolation. Although the mean FA percentage values for the 3 methods tested agreed well, statistically significant differences among the results were detected by using ANOVA, except for C18:1. This facet was also observed with other food samples (data not shown). When the content of individual FA/g sample dry matter was computed from the known lipid content, the absolute differences between the results were more pronounced (Table 4). In general, the results of the AHS procedure were closer to the results of the OSM than to the values of the CM extraction (Table 4).

Various food products were analyzed for their fat and individual FA contents by means of the (i) AHS procedure, (ii) CM extraction and (iii) OSM method (Table 5). The fat content of the materials by means of the OSM method is expressed as total triacylglycerol equivalent (total FA/0.9). These experiments served two purposes. First, the precision of the proposed OSM method (4 independent analyses per sample) was tested with products that differed considerably in fat content (35.8–948.5 mg/g dry matter). The repeatability of the overall OSM method, including sample preparation and GLC analysis, expressed as relative standard deviation (RSD), ranged between 1.01 and 3.38%. Dependence of RSD on the fat content was not observed, as the regression analysis of fat content *vs* RSD resulted in an insignificant correlation coefficient ($r = 0.559$, $P<0.05$). The ANOVA and LSD tests indicated that the results of the AHS extraction and the OSM method did not differ significantly, except for the red cabbage meal, potato chips, dry soup and dry gravy, whereas generally a higher amount of lipo-soluble compounds was extracted by CM (Table 5).

TABLE 4

Content of Individual Fatty Acids/g Canned Liver Sausage (dry matter). Test Material Containing 630 mg Fat/g by Means of the AHS and 683 mg/g by Means of the CM Procedure, Respectively[1]

Fatty acid	AHS	CM	OSM
		mg/g	
14:0	12.2^b	13.9^a	12.5^b
16:0	166.7^b	178.7^a	169.1^b
18:0	98.3^b	105.3^a	99.2^b
18:1	256.6^c	277.0^a	264.4^b
18:2	56.8^b	64.7^a	66.0^a

[1]Values in a row with different superscripts differ significantly (P<0.05).

TABLE 5

Fat Content of Processed Foods Measured by AHS and CM Extractions and by the OSM Method (total triacylglycerol equivalents); Mean Values of Duplicates Reported[1]

Product	AHS	CM	OSM
		mg fat/g dry matter	
Frankfurter sausage	664.9^b	716.9^a	670.0^b
Dressed fish sticks[d]	205.1^b	245.3^a	208.4^b
Red cabbage meal[d]	144.8^c	195.3^a	184.1^b
Noodles	33.2^b	42.4^a	35.8^b
Potato chips	354.7^c	401.1^a	377.3^b
Dry soup	148.2^c	174.9^b	179.5^a
Dry gravy	299.7^c	335.7^a	320.3^b
Veal goulash[e]	274.7^b	341.4^a	280.2^b
Mayonnaise	959.8^a	983.5^a	948.5^a

[1]Values in a row with different superscripts (a–c) differ significantly (P<0.05).
[d]Frozen product.
[e]Canned product.

DISCUSSION

With increased evidence of the relationship between diet and heart disease, the need for accurate labeling of the FA composition of multicomponent foods was put forward. A simple method, which eliminates needlessly complex pretreatment of the sample before final GLC analysis, is desirable. Such a method was proposed by Outen *et al.* (3) and later modified by Sukhija and Palmquist (2) for the analysis of animal feeds, digesta and feces. Unfortunately, the protocol given by these authors has not been applied to the analysis of multicomponent foods.

In principle, acid- or alkali-catalyzed transesterification

can be applied to form FA methyl esters from acyl glycerols. Both methods have some shortcomings. Inherent to alkali-catalyzed transesterification is the fact that free FA are not methylated. Thus, with alkali-catalyzed alcoholysis, care must be taken to exclude water from the reaction mixture to prevent the formation of free FA as a result of hydrolysis of lipids (10). Bearing in mind this limitation, the alkali-catalyzed direct-derivatization methods proposed by Long *et al.* (4) and by Hougen and Bodo (8), respectively, were excluded from our considerations.

The accuracy of the OSM method was demonstrated by analyzing a reference fat with a known FA composition. Quantitative recovery of the polyunsaturated FA is demonstrated in Table 1, suggesting that no decomposition of these FA occurs during the applied transesterification reaction. The excellent properties of methanolic HCl for the transesterification of lipids, as found in this experiment, are in concordance with the literature (2,3), although negative side reactions of this reagent were also reported (10).

Synthetic triacylglycerol standards and pure fats and oils showed excellent agreement between the known lipid mass and the corresponding total FA (Table 2). Neither the sample mass (10–50 mg) nor the fat type influenced the final outcome. In a similar procedure, in which 1% H_2SO_4 in methanol was used for transesterification, the methyl ester yield from pure triacylglycerols was only 70% for a 5-mg sample (6). Probably, the relatively short heating time used by the authors (only 20 min) failed to esterify the FA. Since a reaction time of 2 hr was chosen in the present study, it is unlikely that substantial amounts of free and bound FA remained unreacted.

Browse *et al.* (5) suggested the addition of a water scavenger (2,2′-dimethoxypropane) to improve FA methyl ester recovery in a combined digestion/methylation procedure applied to leaf lipids. In the current study, this provision seems unnecessary, because up to 100 mg water added deliberately to the reaction mixture did not interfere with the formation of methyl esters (Fig. 1); a fact, which was also observed by Sukhija and Palmquist (2). Moreover, unreacted dimethoxypropane was shown to produce spurious peaks on the FA chromatogram (9), which in turn will interfere severely with the calculation of the total FA values. Further experiments with food samples substantiated that small water amounts were not harmful to the transesterification reaction. Substituting C19:0 methyl ester for the C19:0 acid as IS did not affect the recovery of total FA, even in the presence of water.

When the FA composition of the fat contained in foods is expressed as mass fraction of the individual FA methyl ester/total FA methyl esters, the FA spectrum remained virtually unchanged, whether the fat had been extracted prior to transesterification or not (Table 3). Although the values given in Table 3 agreed well, ANOVA indicated significant differences. We believe that only the higher proportion of C18:2 in the chromatograms of the OSM method is responsible for these differences. Whether this is due to an artifact or a contaminant (*e.g.*, phthalates) not resolved from true C18:2, or due to a more efficient recovery of C18:2 by the OSM method was not separately investigated.

Normally, the gravimetrically determined fat content of a food sample and the FA composition of the extracted fat are the basis for the calculation of the content of individual FA/g of the food tested. It is well known that different methods for fat extraction applied to the same foodstuff produce different results (17). This discrepancy leads automatically to deviating figures for individual FA/g sample. In particular, no significant difference between the C16:0 proportion in the FA spectrum of the AHS- and the CM-extracted fat was identifiable (Table 3), but after conversion to mg/g sample, the results were quite different (Table 4). The direct estimation of FA by the OSM method is much better for nutrient labeling purposes, because no conversion factor is needed. Furthermore, a crude lipid extract consists not only of acyl glycerols (mainly triacylglycerols), which present the principle energy supply, but also of nonnutritive components. Only the nutritionally important *O*-acyl lipids are directly estimated as FA with the OSM method.

To compare the total FA content of a sample with a gravimetrically determined fat content, total triacylglycerol equivalents could be computed by (total FA)/0.9 (2). The values obtained this way were consistent, but not identical, with the results of the AHS and the CM procedures (Table 5). Foodstuffs that contain significant levels of phospholipids and/or partial acylglycerols would require special conversion factors to enable computation of triacylglycerol equivalents that correspond to the results of conventional methods for the determination of crude fat.

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