Improved recovery of fatty acid through direct transesterification without prior extraction or purification

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Summary Methods currently in use for the quantitative measurement of fatty acids by gas–liquid chromatography after transesterification are usually lengthy and cumbersome. The technique described is a one-step reaction that is carried out in the same tube and bypasses all the extraction and purification steps. Recoveries of fatty acid and triglyceride standards (C6:0 to C24:1) were better than 96%. When the direct transesterification method was compared to the Folch extraction procedure, increases of fatty acid concentration of 11.4% and 15.8% were observed in human milk and adipose tissue, respectively. The method appears to be particularly advantageous for the recovery of the highly volatile medium chain triglycerides and there is no need to add an antioxidant to protect unsaturated lipids.—**Lepage, G., and C. C. Roy.** Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J. Lipid Res.* 1984. **25:** 1391–1396.

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Fatty acid (FA) analysis of biological specimens by gas–liquid chromatography (GLC) requires solvent extraction, purification, and derivatization procedures that are both lengthy and cumbersome (1–4). Attempts to bypass extraction (5, 6) and purification steps (7, 8) have met with varying degrees of success. This report proposes a technique that circumvents most of the preparative steps and consists of a direct transesterification procedure. It leads to more complete recoveries of both medium chain (MCFA) and long chain fatty acids (LCFA).

Abbreviations: GLC, gas–liquid chromatography; MCFA, medium chain fatty acid; LCFA, long chain fatty acid; FA, fatty acid; TG, triglyceride; TLC, thin-layer chromatography.

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MATERIALS AND METHODS

Analytical grade solvents were redistilled in an all-glass system. All glassware was rinsed with chloroform–methanol 2:1 (v/v) and dried under nitrogen. Boron fluoride–methanol (140 g of BF_3 per liter of methanol) obtained from Chromatographic Specialties, Brockville, Ontario and acetyl chloride, purchased from Fisher Scientific Ltd., Montreal, Quebec, were used without further purification.

Fatty acid and fatty acid methyl ester standards (Analabs, North Haven, CT; Terochem, Rexdale, Ontario; Sigma, St. Louis, MO; and Mandel, Montreal, Quebec) as well as triglyceride standards (Sigma, St. Louis, MO) were certified to be >99% pure. Unsaturated lipid standards were bought packaged in ampoules under an inert gas to prevent oxidation.

Recoveries of standards as a function of preparative steps

Certain preparative steps were carried out to test their effects on the recoveries of FA and triglyceride (TG) standards, particularly those of medium chain length known to be vulnerable in view of their volatility and greater solubility (7, 9). FA and TG standards were weighed and diluted with methanol–benzene 3:2 (v/v). After being mixed thoroughly, the two mixtures of standards were separated into individual reaction vials. The organic phase for both FA and TG standards was either dried under a stream of nitrogen or lyophilized prior to esterification. Because methylation greatly increases volatility, recoveries were checked on the methyl ester FA solution that had been dried under nitrogen.

Direct transesterification method

Internal standards, consisting of 500 μg each of nonanoic acid (C9:0) and tridecanoic (C13:0) and tricosanoic acid (C23:0), dissolved in 1 ml of methanol–benzene 3:2, were added to an aliquot of 100 μl of human milk or to approximately 10 mg of adipose tissue. One ml of freshly prepared acetyl chloride–methanol 5:100 (v/v) and a small magnetic stirring bar were then added to each tube which was capped and subjected to methanolysis at 100°C for 1 hr (10). Tubes were weighed before and after heating as a check for leakage. After the contents had cooled to room temperature, the external standard (500 μg of methylated pentadecanoic acid (C15:0) dissolved in 1 ml of hexane) and 1 ml of water were added. The tubes were then shaken, centrifuged, and stored at 4°C until injection into the chromatograph.

Extraction by the method of Folch, Lees, and Sloane Stanley (11)

Two ml of chloroform–methanol 2:1 (v/v) was added to an aliquot of 100 μl of maternal milk or to approximately 10 mg of adipose tissue, and the mixture was mechanically shaken for 10 min (11). After centrifugation, the lower phase was collected and then 2 ml of chloroform–methanol 2:1 (v/v) was added to the precipitate and the same procedure was repeated. The lower phases were pooled and 145 mM NaCl was added in order to separate the methanol and chloroform phase (12). Following centrifugation, the lower phase containing the lipids was evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was solubilized in 1 ml of methanol–benzene 3:2 (v/v) and 1 ml of acetyl chloride–methanol 5:100 (v/v) was added. The mixtures then were subjected to methanolysis at 100°C for 1 hr. The specimens were processed and stored as described above before injection into the chromatograph.

Gas–liquid chromatography

FA were chromatographed as methyl esters on a 10-ft glass column with an internal diameter of 2 mm. The column was packed with 5% SP-2340 on Chromosorb W-AW (100–120 mesh). Analysis was performed on a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector. Nitrogen was used as carrier gas at a flow rate of 28 ml/min. The injection port temperature was 220°C and the detector was 300°C. The column temperature was held at 80°C for 2 min and in a step-wise fashion reached a plateau of 215°C.

The gas chromatograph was calibrated using a standard mixture of FA. A correction factor was applied to compensate for the lower ionization detector response to unsaturated FA relative to corresponding weights of saturated FA. Yields of reactions were determined by adding known amounts of methyl pentadecanoate to lipid methanolysates in the hexane extraction just before GLC.

Calculations and validation of the method

The amount of each of the three internal standards and the multiplier factor for each sample were entered through the keyboard of the gas chromatograph. The peak area for each identified FA was determined by a computer programmed to calculate the amount of each FA recovered in each sample using the internal standard method. The external standard served as an indicator for the completeness of the esterification and of the hexane extraction steps. The ratios of areas between the external standard (C15:0) and the three internal

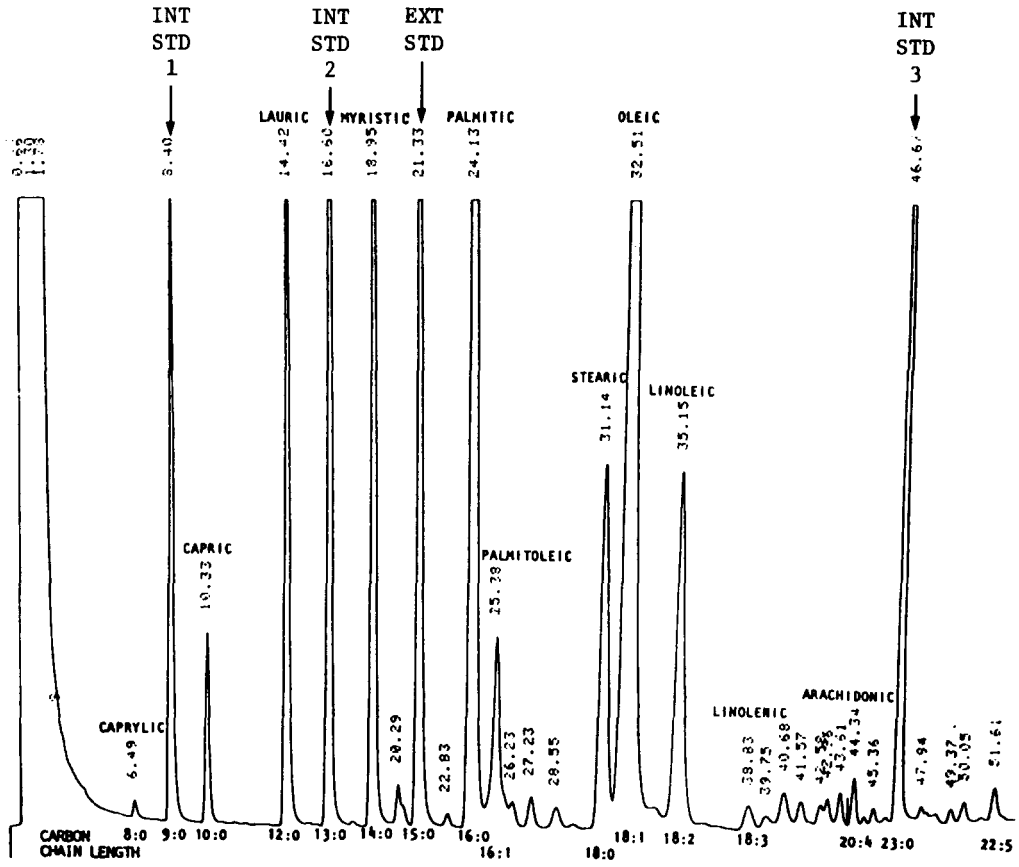

Fig. 1. Typical gas–liquid chromatography profile of fatty acid methyl esters (40–500 ng of each) obtained from maternal milk.

standards (C9:0, C13:0, C23:0) were inspected as a further check on esterification and hexane extraction.

The three internal standards and the external standard gave rise to well-individualized peaks that did not interfere with the FA pattern of human milk (**Fig. 1**).

RESULTS

The effects of various preparative steps on the recovery of FA and of their esterified forms is shown in **Table 1.** When solutions of FA standards were either dried under a stream of nitrogen or lyophilized prior to esterification, there was a decrease in the percentage recovery of MCFA, C_6–C_{12} as defined by Bach and Babayan (13). The reduction was particularly marked with lyophilization, but it was also seen when solvent was removed by nitrogen, and became more pronounced with the shorter chain MCFA. Losses of methylated FA occurring after exposure to a stream of nitrogen were greater than nonmethylated FA and consisted principally of MCFA. In contrast to the results with solutions of

TABLE 1. Recovery (%) of fatty acid standards submitted to different preparative steps

Fatty Acid	Preparative Steps[a] 1	2	3
6:0	12.1	0	0
8:0	84.2	0	4.3
9:0	92.0	1.6	20.1
10:0	95.8	21.6	41.0
12:0	97.9	75.2	74.7
13:0	98.3	85.4	85.2
14:0	98.7	89.6	92.0
16:0	99.6	92.5	98.5
16:1	99.3	92.0	97.6
18:0	99.2	93.0	98.4
18:1 (n-9)	98.3	91.9	99.1
18:2 (n-6)	98.4	92.3	99.2
18:3 (n-3)	99.6	93.5	100.4
20:0	99.9	94.0	100.1
22:0	96.5	91.3	99.4
23:0	95.8	90.1	96.2
24:0	95.8	91.0	97.2
22:6	100.0	93.8	99.9

[a] 1, Drying under a stream of nitrogen prior to esterification; 2, lyophilization before esterification; 3, esterification followed by drying under a stream of nitrogen.

FA standards, neither drying under a stream of nitrogen nor lyophilization prior to transesterification affected the percentage recovery of solutions of TG standards (**Table 2**).

The effect of water on the completeness of hydrolysis and methylation reactions achieved by the transesterification procedure is summarized in **Table 3.** Addition of increasing amounts (10%, 20%, 30%) of water to solutions of FA standards did not interfere with methylation. However, when the solutions of TG standards were reconstituted with the same percentage of water contained in the FA standard solutions, there was impairment of their hydrolysis. Although the recoveries were unchanged with 10% water, they decreased significantly with 20% and 30% water.

Table 4 shows the results of the FA analysis in which the procedure of Folch et al. (11) was compared to the direct transesterification method for human milk and adipose tissue specimens to which methanol–benzene 3:2 (v/v) was added to achieve a final water concentration of less than 10%. The comparative fatty acid content of human milk was higher with the direct transesterification technique. The percentage increase of total FA ($\bar{X} \pm SD$) in human milk aliquots was 11.4 ± 1.5% and 15.8 ± 4.1% in adipose tissue samples. This method seemed to be particularly advantageous over the procedure of Folch et al. (11) for the recovery of octanoic acid (C8:0), the major MCFA in MCT preparations (13).

Recovery of each of the three internal standards was found to be greater than 96%. The stability of the methyl ester FA in the hexane upper phase was satisfactory since serial determinations carried out twice a week over a 2-month period on the same sample gave identical results. Losses of unsaturated FA by oxidative processes, during the direct transesterification reaction or following a 9-week period during which the methylated FA were kept at 4°C in the supernatant hexane, were never observed.

TABLE 2. Recovery (%) of triglyceride standards submitted to different preparative steps

Fatty Acid	Preparative Steps[a] 1	2
6:0	96.6	96.8
8:0	100.4	99.8
9:0	100.2	98.7
10:0	99.6	97.7
12:0	97.6	97.3
13:0	97.2	97.1
14:0	98.9	96.6
16:0	98.9	98.8
17:0	96.8	97.1
18:0	96.9	97.5
18:1 (n-9)	96.1	96.6
18:2 (n-6)	96.2	96.9
20:0	94.8	97.2
22;0	97.9	96.7
24:1	96.3	97.3

[a] 1, Drying under a stream of nitrogen prior to transesterification; 2, lyophilization before transesterification.

TABLE 3. The effect of water on esterification (%) of lipid standards

Fatty Acid	H_2O (%) Added to FA Solution[a] 10%	20%	30%	H_2O (%) Added to TG Solution[b] 10%	20%	30%
6:0	99.7	98.9	92.9	99.3	98.2	94.1
8:0	99.9	93.0	92.2	99.9	97.1	93.8
9:0	100.3	92.6	91.6	100.7	95.8	88.6
10:0	100.1	92.3	90.9	100.6	94.8	85.9
12:0	99.4	92.2	90.7	96.8	91.2	82.1
13:0	99.1	92.5	90.6	99.7	88.6	79.9
14:0	98.6	93.2	90.9	99.8	89.0	70.4
16:0	99.4	94.8	91.8	98.9	86.7	58.1
16:1	99.8	94.6	91.6			
17:0				96.2	86.5	53.6
18:0	99.3	94.8	91.4	97.0	84.4	45.9
18:1 (n-9)	100.2	94.1	90.7	98.3	85.9	57.2
18:2 (n-6)	100.2	93.7	90.2	98.9	87.1	66.6
18:3 (n-3)	100.6	95.4	91.6			
20:0	98.8	95.5	92.2	97.1	80.1	32.7
22:0	99.1	93.3	90.7	96.2	72.9	23.0
22:6	99.3	97.1	96.4			
23:0	96.9	94.1	89.0			
24:0	98.3	93.4	88.9			
24:1				96.5	73.6	23.9

[a] FA, fatty acid.
[b] TG, triglyceride.

DISCUSSION

The technique described is rapid (1.25 vs 5 hr) and the amount of manipulation necessary for each specimen is minimal (3 min vs 45 min) when compared to the procedure of Folch et al. (1, 11, 14). In addition, it has a number of advantages over standard methods where losses of lipids are likely to occur during the multiple extraction and evaporation steps (15). It is a one-step reaction carried out in the same tube and the nascent methyl ester FA are stable for at least 2 months at 4°C. Because fatty acids are directly freed from tissues during the transesterification procedure instead of being subjected to chloroform–methanol homogenization and extraction, a more complete recovery is expected to occur. This point needs to be further assessed but it is the most likely explanation for the fact that a larger discrepancy between the two methods in fatty acid content was found with adipose tissue than with human milk samples (Table 4).

From the external standard, the yield of three internal standards of varying molecular weight and volatility was

TABLE 4. Comparative fatty acid content of biological specimens using the classical Folch technique and the direct transesterification method[a]

Fatty Acid	Human Milk		Adipose Tissue	
	Folch et al. Extraction	Direct Transesterification[b]	Folch et al. Extraction	Direct Transesterification
	mg/dl		*mg/g*	
8:0	9 ± 0.2	14 ± 0.2	0	0
10:0	78 ± 1.0	90 ± 1.0	trace	trace
12:0	345 ± 3.8	384 ± 4.0	2 ± 0.1	3 ± 0.1
14:0	375 ± 3.8	414 ± 3.8	23 ± 0.3	27 ± 0.3
14:1	37 ± 1.0	26 ± 0.3	3 ± 0.2	3 ± 0.1
16:0	736 ± 5.7	819 ± 4.5	140 ± 1.8	161 ± 2.0
16:1	142 ± 0.9	158 ± 0.8	57 ± 2.1	66 ± 2.7
18:0	269 ± 2.2	300 ± 2.5	24 ± 0.2	28 ± 0.3
18:1 (n-9)	1216 ± 10.9	1364 ± 10.7	377 ± 4.0	433 ± 4.2
18:2 (n-6)	319 ± 2.8	358 ± 4.5	82 ± 2.2	93 ± 2.3
18:3 (n-3)	24 ± 0.3	26 ± 0.6	6 ± 0.3	6 ± 0.4
20:0	15 ± 0.7	18 ± 0.3	3 ± 0.2	5 ± 0.2
20:1	16 ± 0.8	19 ± 0.9	6 ± 0.5	7 ± 0.4
20:3	11 ± 0.9	13 ± 0.5	trace	trace
20:4	18 ± 0.5	21 ± 0.5	2 ± 0.3	2 ± 0.2
22:0	6 ± 0.3	5 ± 0.1	trace	trace
22:6	15 ± 1.2	14 ± 0.8	trace	trace
24:0	trace	trace	trace	trace
Total	3633 ± 32.0	4047 ± 31.2	726 ± 16.0	841 ± 17.2

[a] Mean ± SE, three samples from the same specimen.
[b] See text for details.

calculated (Fig. 1). This constitutes an important advantage since each of the three internal standards was used to assess not only the completeness of the transesterification reaction over the entire FA spectrum but also of the migration of the lipid methanolysates into the hexane phase. We have found that weighing the sealed tubes before and after heating in order to check for leakage becomes an essential step in the prevention of a disproportionate loss of the more volatile FA methyl esters or destruction of unsaturated esters.

During the initial phase of the study, transesterification was carried out with boron fluoride–methanol 14:100 (w/v) using the technique described by Morrison and Smith (16). We encountered the same problem as Rogiers (17) in that the solvent peak showed severe tailing and the column was irreversibly damaged due to the adverse effect of traces of BF_3 contained in the hexane phase. Because of the observation of Morrison and Smith (16) that incomplete esterification could be brought about by as little as 0.5% of water, experiments were designed to check various techniques to eliminate water. Tables 1 and 2 show that both nitrogen evaporation of water and lyophilization had adverse effects on the recovery of FA but this decrease was essentially limited to MCFA because of their high volatility as noted by Gerhardt and Gehrke (7). Since volatility of these FA is further enhanced by methylation, it was not surprising that nitrogen evaporation further exaggerated the loss reported by Heckers and Melcher (9). Even if losses by nitrogen evaporation of lyophilization seemed to be restricted to FA as opposed to TG, we must keep in mind the words of caution from Wardell, Hill, and D'Souza (3) that freezing and thawing cause disruption of fat globules and a greater hydrolysis of TG than does the heating process. This brings us back to the problem of the vulnerability of the FA.

In view of these difficulties during the preparative steps, attempts were made to transesterify lipids in a one-step procedure without prior extraction of TG standards containing 10% water. After noting that even when the reaction with boron fluoride–methanol was carried out overnight at 100°C, the maximum degree of transesterification obtainable was 85%, other methanolyzing acids were tested and acetyl chloride was found to be the most appropriate. As noted in Table 3, a 10% water content of the standard solutions did not affect hydrolysis or methylation, but higher concentrations of water did. Consequently, it was decided to process biological samples in a reaction medium with a total water content no greater than 10%.

Autoxidation is known to be a problem for the measurement of unsaturated FA. The addition of an antioxidative agent such as BHT (18, 19) does solve this problem but creates a new one in that there is the presence of a BHT peak on the chromatogram (20). The proposed method of Shimasaki, Phillips, and Privett

(6) for eliminating artifacts by purifying the methyl esters by TLC is not acceptable in our view since we found higher losses of FA after they had been methylated (Table 1). The present method, outlined in this report, is a single-step procedure that does not require the addition of an antioxidant to protect unsaturated FA. Once methylated, unsaturated FA are no longer subject to autoxidation as pointed out by Wren and Szczepanowska (21). We confirmed this observation through repeated injections of the same sample over a 2-month period.

Although few biological samples have been processed so far, they are representative of those in which fatty acid patterns are of interest. Coefficients of variation are small and attest to the reproducibility of the present technique. Since the glassware was thoroughly cleaned and redistilled solvents were used, no peak appeared on the blank chromatogram. After processing hundreds of standard mixtures and specimens and injecting them into the gas chromatograph, no alteration of the chromatographic column has so far been observed. Preliminary exploration of the applicability to other biological specimens (feces, serum) and to other lipid classes (cholesterol esters, phospholipids) appears promising.

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