notes on methodology

Specific methylation of plasma nonesterified fatty acids in a one-step reaction

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Summary Analysis of plasma nonesterified fatty acids (NEFA) by gas–liquid chromatography requires procedures that are both lengthy and cumbersome. A 45-min direct methylation procedure was carried out at 24–29°C on 150 μl of plasma added with an internal standard in 5.0 ml of methanol–acetyl chloride 50:1 (v/v). To stop the reaction, 3 ml of 6.0% K_2CO_3 was added. After addition of 150 μl of hexane, shaking and centrifugation, an aliquot of the upper phase was injected into the gas chromatograph. The specificity of the methylation reaction for NEFA without hydrolysis of other classes of plasma lipids was substantiated with appropriate standards. This one-step specific methylation procedure is superior to currently used methods. — **Lepage, G., and C. C. Roy.** Specific methylation of plasma nonesterified fatty acids in a one-step reaction. *J. Lipid Res.* 1988. **29:** 227–235.

Supplementary key words lipids • triglyceride • cholesteryl ester • phospholipid • sphingomyelin • gas–liquid chromatography • thin-layer chromatography • hydrolysis • transesterification • internal standard

Nonesterified fatty acids (NEFA) are akin to glucose in that they constitute a readily available source of energy for metabolic needs. Their tissue uptake is rapid and they compete with glucose for utilization by insulin-dependent tissues, principally muscle (1). Determination of NEFA is an important component of the plasma lipid profile in that it is a reliable index of the homeostasis between storage and release of fat (2, 3) which may be disturbed in several clinical conditions (4–6).

A number of methods have been proposed for the determination of NEFA in biological fluids. They include: the Dole method (7), Technicon (8), colorimetry (9–11), titrimetry (12), enzymatic determinations (13–15), gas–liquid chromatography (GLC) (16–20) and, more recently, high pressure liquid chromatography (21–28). As Chilliard, Bauchart, and Barnouin (12) pointed out, the reason why there are so many methods for determining NEFA is that there are a number of still unresolved difficulties in obtaining quantitative recoveries with an assay that is specific and unaffected by interfering substances. GLC is a specific method but most systems are too laborious. Some authors have tried to bypass extraction and purification steps with varying degrees of success (29–33). The aims of this study were to improve existing methods by a technique that would not only be more precise but would also save time by circumventing most of the preparative steps.

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. Acetyl chloride, gold label (Aldrich, Milwaukee, WI), was used without further purification. Borosilicate glass tubes with Teflon-lined screw-caps (100 × 13 mm) were bought from Fisher Scientific Ltd., Montreal, Quebec. Methylation was carried out in a Reacti-Therm heating/stirring dry block which controls temperature ± 0.5°C (Pierce Chemical Co., Rockford, IL).

Fatty acid (FA) 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 (TG), cholesteryl ester (CE), phosphatidylcholine (PL), and chicken egg spingomyelin (SP) standards (Sigma) were certified to be 99% pure and used without further purification. Unsaturated lipid standards were bought packaged in ampoules under an inert gas to prevent oxidation.

Lipid standard mixtures

Five different mixtures of lipid standards were prepared. The first consisted of nonesterified fatty acids, while each of the others was made up of esterified lipids. Each of the latter consisted of either four species of TG, seven species of PL, ten species of CE, or chicken egg sphingomyelin, respectively. Those standards were weighed and solubilized in $CHCL_3$–CH_3OH 2:1. Three hundred-μl amounts of solution containing 15 μg of each standard were precisely weighed, placed in borosilicate glass tubes with Teflon-lined screw-caps and frozen at −50°C until processed. In order to further test the reliability of the proposed technique, another set of standards was prepared. It contained a mixture of NEFA, TG, PL, and CE solubilized in $CHCL_3$–CH_3OH 2:1 and placed in tubes that

Abbreviations: NEFA, nonesterified fatty acids; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; FA, fatty acid; TG, triglyceride; CE, cholesteryl ester; PL, phospholipid; SP, sphingomyelin; HPLC, high pressure liquid chromatography; PUFA, polyunsaturated fatty acids.

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were kept at −50°C until analysis. The solutions in the tubes were evaporated to dryness under a gentle stream of nitrogen at 24°C for about 2 min and reconstituted with various solvent mixtures. No internal standard was added to the two sets of lipid standards in order to assess the extent of NEFA methylation. After addition of K_2CO_3 to stop the reaction, 150 μl of hexane containing 15 μg of methylated 13:0 was placed in the tare tube and used as an external standard which made possible calculation of the percentage completeness of the esterification reaction. Precise amounts of substrate and of external standard were recorded in the injection table of the gas chromatograph for each sample.

Specific methylation method (Fig. 1)

To an internal standard consisting of 15 μg of tridecanoic acid (13:0) dissolved in 5 ml of methanol–acetyl chloride 50:1 (v/v), 150 μl of plasma was added in a glass tube. A small magnetic bar was placed in each tube. They were tightly closed with Teflon-lined caps and left at 24° to 29°C for 45 min in a Reacti-Therm heating/stirring dry block. The methylation reaction was stopped with 3 ml of 6% K_2CO_3 solution which was slowly added to the mixture. One hundred fifty μl of hexane was added, and the tubes were then shaken and centrifuged for 10 min at 2000 *g*. About 30 μl of the 150 μl of hexane upper phase was carefully placed into a small injection vial with a 200-μl Pipetman, from which 4 μl was injected into the gas chromatograph.

Tserng et al. method (30)

In a borosilicate glass tube, a 100-μl aliquot of plasma was mixed with 50 μl of internal standard solution containing 15 μg of pentadecanoic acid in methanol, 2 ml of 2,2-dimethoxypropane, and 40 μl of concentrated hydrochloric acid. A small magnetic bar was placed in each tube which was then closed with a Teflon-lined screw-cap and kept at room temperature for 15 min in the Reacti-Therm heating/stirring dry block. Pyridine, 20 μl, was added to stop the reaction, and the mixture was concentrated in a stream of nitrogen to about 100 μl. It was then diluted with 1.0 ml of water. The aqueous mixture was extracted with 1.0 ml of hexane. After centrifugation to separate the layers, the hexane layer was transferred and evaporated to dryness under a gentle stream of nitrogen at room temperature. Residues were dissolved in 100 μl of benzene and an aliquot was injected into the gas chromatograph.

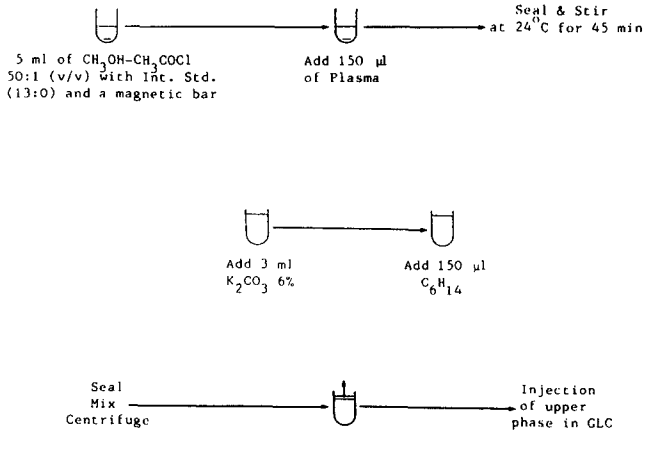

Fig. 1. Outline of the procedure for a biological sample.

Folch et al. method (34) and TLC separation (35)

To an internal standard consisting of 60 μg of pentadecanoic acid dissolved in 12 ml of chloroform–methanol 2:1 (v/v), 400 μl of plasma was added in a glass tube and the mixture was mechanically shaken for 10 min. After centrifugation, 2.6 ml of 145 mM NaCl was added to the supernatant in order to separate the methanol and chloroform phases. After evaporation of the lower phase to dryness at room temperature under a gentle stream of nitrogen, the residue was solubilized in 100 μl of chloroform–methanol 2:1 (v/v) and was applied to a TLC plate of Silica gel 60 (0.2 mm thick) without fluorescent indicator (E. Merck, Darmstadt). A mixture of lipid standards was applied adjacent to the sample. The plate was developed in a solvent system consisting of hexane–diethyl ether–glacial acetic acid 80:20:3 (v/v/v) in an equilibrated tank. After development, the aluminum plate was cut and the lipid standards were visualized by iodine vapor. The silica gel band containing NEFA was scraped off immediately and placed in a glass tube to which 2 ml of methanol–benzene 4:1 (v/v) was added. A small magnetic bar was placed in each tube and the silica-containing NEFA was submitted to the previously described direct transesterification method (36). At the end of the reaction, an aliquot of the benzene upper phase was injected into the gas chromatograph.

Gas–liquid chromatography

Vials were placed in a Hewlett-Packard 7671 automatic injector. FA were chromatographed as methyl esters on a 60-m fused silica column with an internal diameter of 0.32 mm (**Fig. 2**). The column was wall-coated with 0.20 μm SP-2331 (25% bonded phase). Analysis was performed on a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector. Helium was used as carrier gas (1.9 ml/min at 80°C) and nitrogen as make-up gas. The split ratio was 7:1. After an initial isothermal period of 8 min at 80°C, the temperature was programmed to 165°C, rising by 6.5°C/min with a hold at that temperature for 7 min. The second increment was 1.6°C/min to 200°C with a hold at that temperature for

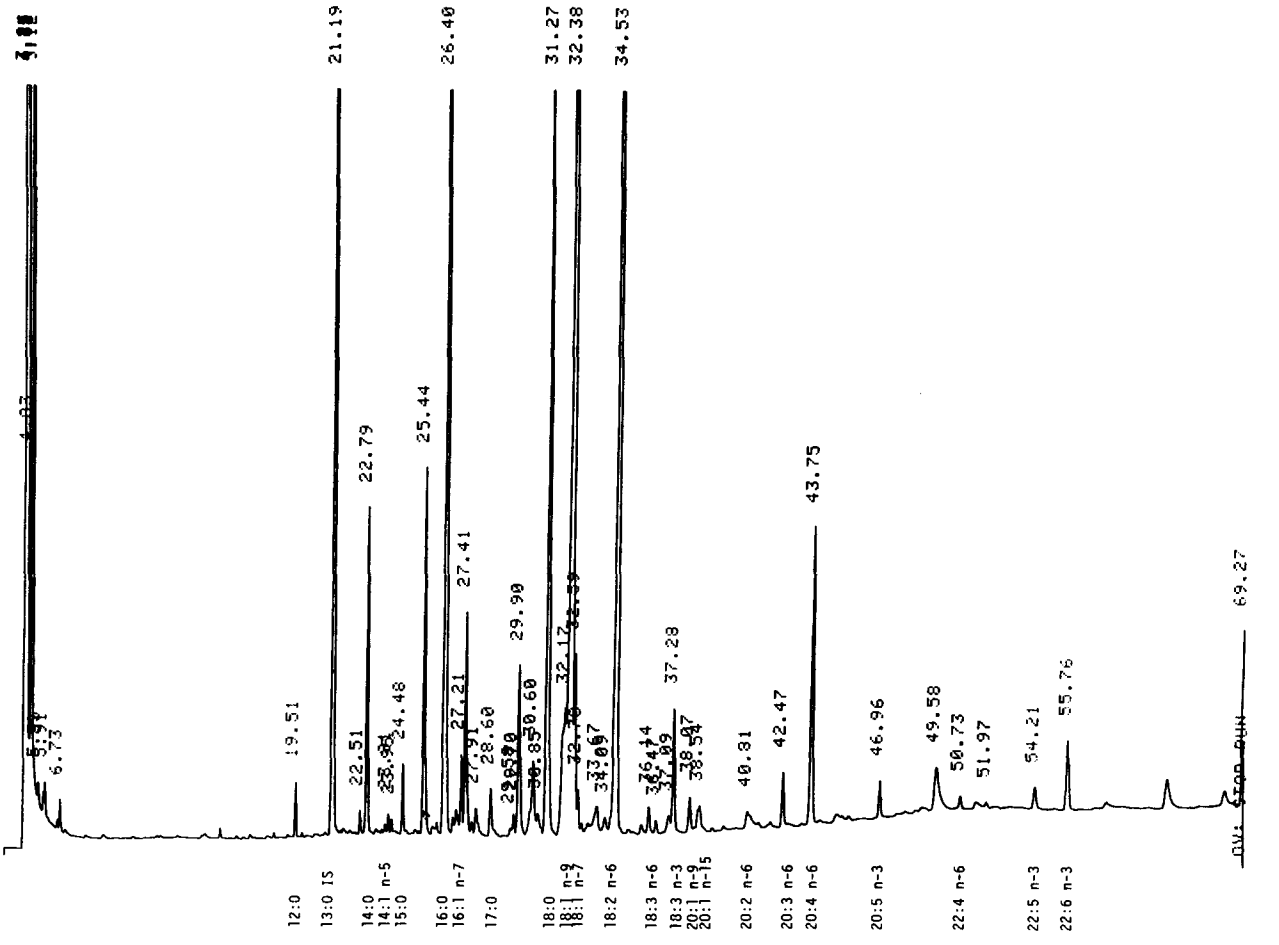

Fig. 2. Gas-liquid chromatography profile of fatty acid methyl esters after specific methylation of NEFA directly on plasma.

5 min. The third increment was 0.3°C/min to 204°C. Temperature was then increased to 220°C with increments of 10°C/min and held at that temperature for 5 min. Finally, the oven temperature was programmed down to 80°C with decreases of 25°C/min, with a final hold of 2 min. Injector and detector temperatures were 200°C and 250°C, respectively. The gas chromatograph was calibrated using standard mixtures of 45 different FA. Response-correcting factors were determined in μg/area ratio.

RESULTS

As shown in **Table 1,** the specific methylation of the NEFA was affected by the methanol–acetyl chloride ratio used as the methylating agent and by the duration of the reaction when it was elected to carry out the reaction at 24° to 29°C in the presence of 150 μl of water. After many trials on mixtures of standards made up of NEFA, TG, CE, PL and SP, as well as on mixed lipid standard matrices, it was found that 5 ml of methanol–acetyl chloride 50:1 (v/v) could methylate 97.2% of the NEFA over a 45-min period without significantly hydrolyzing other lipids at 24°-29°C in the presence of 150 μl of water. With methanol–acetyl chloride 100:1 (v/v), the time of the reaction was prolonged to 90 min and it was only 88.2% complete. With methanol–acetyl chloride 25:1 (v/v) an unacceptable percentage of neutral (TG) and complex lipids (CE, PL) was methylated.

In order to establish a comparison with currently available techniques, five samples of the same plasma were processed using a TLC separation of NEFA after Folch extraction (34), Tserng's specific methylation of NEFA (30), and our proposed method. **Table 2** shows that NEFA totaled 79.7 ± 3.9 μg/ml with the Folch-TLC procedure, 102.3 ± 9.0 with Tserng's, and 214.1 ± 4.2 by our specific methylation technique. Although both the saturated and the unsaturated fatty acid classes of NEFA were lost by the Folch-TLC and the Tserng methods, the discrepancy with the proposed method was more striking for the unsaturated acids. Moreover, variance around the mean was

TABLE 1. Completeness (%) of esterification of lipid standards in the reaction[a]

CH_3OH-CH_3COCl (v/v)	Time	FA	TG	CE	PL	SP
	min			%		
100:1	15	44.5 ± 8.5	0	0	0	0
100:1	30	68.4 ± 3.0	0	0	0.1 ± 0.1	0
100:1	45	73.7 ± 0.3	0.1 ± 0.1	0.1 ± 0	0.4 ± 0.1	0
100:1	60	79.8 ± 0.9	0.3 ± 0.1	0.1 ± 0.1	1.0 ± 0.2	0
100:1	90	88.2 ± 1.7	0.4 ± 0	0.3 ± 0.1	1.5 ± 0.2	0
50:1	15	62.7 ± 6.3	0	0	1.1 ± 0.5	0
50:1	30	77.8 ± 4.1	0.2 ± 0.1	0.2 ± 0	1.6 ± 0.3	0
50:1	**45**	**97.2 ± 0.2**	**1.4 ± 0.2**	**0.6 ± 0.1**	**2.2 ± 0.2**	**0**
50:1	60	99.9 ± 0.1	2.9 ± 0.1	0.9 ± 0.1	2.6 ± 0.1	0
25:1	15	90.6 ± 1.5	2.2 ± 0.2	1.4 ± 0.1	15.3 ± 3.5	0
25:1	30	100.0 ± 0.1	5.2 ± 0.5	2.5 ± 0.4	30.1 ± 0.7	0
25:1	45	100.0 ± 0	7.1 ± 0.3	3.2 ± 0.3	44.2 ± 2.6	0.1 ± 0.1
25:1	60	100.0 ± 0.1	9.2 ± 0.1	3.8 ± 0.4	54.2 ± 1.4	0.1 ± 0.1

[a]Values are expressed as % recovery (mean ± SEM) from three aliquots of lipid standards.

higher with the Tserng procedure as compared with our method.

Table 3 shows recovery of NEFA standards processed with the three methods. One can appreciate that with the Folch-TLC method the loss of unsaturated fatty acids appears to be proportional to the number of unsaturated bonds. Recovery of NEFA standards with the Tserng procedure is as low as could have been predicted from the results shown in Table 2. The percentage of recovery ranged from 24.6 to 78.9%. Complete methylation of NEFA by dimethoxypropane and hydrochloric acid could not be achieved in the presence of water. Many unidentified peaks emerged on the chromatogram and the variance of results was high. Finally, with the proposed method, 150 μl of water did not interfere with methylation carried out with acetyl chloride. Furthermore, the recovery of all NEFA standards approached 100%.

Most of the steps involved for processing biological samples by currently used techniques are circumvented (**Table 4**). One hour after adding the solvent to the plasma sample, an aliquot of the supernatant can be injected into the gas chromatograph.

DISCUSSION

Over the years, many modifications have been made to the technique of determination of NEFA since Dole (37) first described the quantitation of carboxylic groups by titrimetry in 1956. However, there was no unanimity with regards to its reliability when samples contained simple and complex lipids (38–40). For Van der Vusse, Roemen, and Reneman (40), the Hagenfeldt modification (18) produced hydrolysis of FA from PL. In 1969, Lauwerys (9) proposed a colorimetric determination of NEFA. Soloni and Sardina (41), Brunk and Swanson (11), and Greenspan and Schroeder (22) found problems with extraction and interference by albumin. In an attempt to eliminate factors interfering with titrimetric and colorimetric methods, Regouw et al. (10) introduced prior separation with TLC. With the advent of enzymatic methods, an extraction procedure was no longer needed (42). However, Shimizu et al. (42) claimed that the method resulted in lower values (10%), which might be due to the fact that FA with more than 18 carbon atoms were poor substrates for the acyl-CoA synthetase derived from *Pseudomonas aeruginosa* (42). Miles et al. (43) presented a microfluorimetric method as a modification of the enzymatic method. Ramirez found problems with this method (14). Finally,

TABLE 2. Plasma NEFA analysis: comparison of three methods[a]

Fatty Acid	NEFA Fraction		
	Folch + TLC	Tserng Procedure[b]	Proposed Method
		μg/ml	
12:0	1.6 ± 0.1	0.4 ± 0.3	0.7 ± 0.1
14:0	5.1 ± 0.1	2.4 ± 0.5	3.3 ± 0.1
16:0	38.6 ± 2.3	33.5 ± 2.7	66.8 ± 1.0
16:1 (n-7)	1.7 ± 0.9	2.5 ± 0.7	3.2 ± 0.1
17:0	0.5 ± 0.2	0.6 ± 0.2	1.1 ± 0.1
18:0	12.7 ± 0.4	13.6 ± 0.8	31.2 ± 0.6
18:1 (n-9)	12.6 ± 0.6	23.7 ± 3.5	36.2 ± 0.6
18:2 (n-6)	6.3 ± 0.8	21.4 ± 2.0	49.5 ± 1.3
18:3 (n-3)	0	0.4 ± 0.4	1.2 ± 0.1
20:3 (n-6)	0	0.6 ± 0.3	3.3 ± 0.1
20:4 (n-6)	0.6 ± 0.3	3.3 ± 0.1	10.8 ± 0.2
20:5 (n-3)	0	0	1.5 ± 0.1
22:6 (n-3)	0	0	4.4 ± 0.2
Total	79.7 ± 3.9	102.3 ± 9.0	214.1 ± 4.2

[a]Values are expressed as mean ± SEM on five aliquots of the same plasma sample.
[b]Reference 30.

TABLE 3. Recovery of FA standards: comparison of three methods[a]

Fatty Acid	Folch + TLC	Tserng Procedure[b]	Proposed Method
		%	
12:0	74.6 ± 0.8	30.2 ± 0.8	99.1 ± 0.1
14:0	85.2 ± 2.0	69.9 ± 2.0	98.9 ± 0.1
16:0	87.6 ± 0.1	76.9 ± 0.4	96.8 ± 0.2
18:0	84.6 ± 0.8	78.9 ± 0.3	96.0 ± 0.2
18:1 (n-9)	66.7 ± 2.5	49.3 ± 1.0	97.0 ± 0.2
18:2 (n-6)	35.7 ± 2.2	45.1 ± 1.8	98.1 ± 0.5
20:4 (n-6)	23.4 ± 5.1	32.2 ± 1.8	99.0 ± 0.2
22:6 (n-3)	15.1 ± 4.3	24.6 ± 3.7	99.0 ± 0.5

[a]Values are expressed as % recovery (mean ± SEM) from three aliquots of lipid standards.
[b]Reference 30.

Degen and Van Der Vies (15) proposed the addition of paraoxon in order to prevent the spontaneous hydrolysis of esterified FA reported by some investigators (44).

In view of a purported lack of specificity and interference by non-fatty acid compounds, many gas-liquid chromatography methods were then proposed for quantifying individual NEFA in plasma. Sampson and Hensley (16) and Penttila et al. (45) proposed injection of the solvent extractant without derivatization as a screening method. FA are difficult to analyze directly by GLC due to the broad and often tailing peak shapes. This is the reason why most of the proposed techniques methylate FA before injection (17, 20, 29, 38, 46–49). Because current methods, such as the very lengthy one proposed by Hagenfeldt (18), involve solvent extraction, purification, and derivatization they have been claimed to be too laborious and therefore unsatisfactory for clinical use (32). For all these methods the extraction step poses a dilemma. Many of these methods involved simple solvent partitioning which led to losses of NEFA because the solubility of the different FA varies widely (50). Van der Vusse et al. (40) found that the Dole extraction overestimated NEFA through hydrolysis of PL and reported that with the Folch extraction, recovery of the 17:0 standard only amounted to 75%. Tso and Simmonds (51) found that, unless great care was taken in washing the precipitate (denatured protein), one could lose a considerable amount of lipid when using the Bligh and Dyer procedure (51). Consequently liquid-solid partitioning was devised, but erroneous results were obtained when the lipids were hydrolyzed in an alkaline medium (52, 53). If esterified lipids were not removed prior to methylation it was thought safer to refrain from using reagents capable of transesterification (54). Therefore, some investigators preferred to separate NEFA from other lipids on TLC (17, 19, 20). This separation step proved to be not only time-consuming but, moreover, exposed the unsaturated acids to oxidation, which then led lipidologists away from the TLC purification step (6, 33). Some investigators have tried to circumvent some preparative steps but with only limited success (30–32).

Until recently, GLC was the method of choice for separation and quantitation of nanogram quantities of FA, but in the past 5 years many published HPLC methods have been promoted as a good alternative (21–23, 25–28, 55). However, detection has proved to be a problem since saturated FA do not absorb at 210 nm to any appreciable extent, and the preparation of a UV or fluorescent derivative from a crude lipid sample is a time-consuming procedure (24, 56). Furthermore, the sensitivity of the HPLC procedure is limited for FA in the range of 0.5 to 1 μg (22). It is, therefore, not sensitive enough to determine a number of NEFA present in normal plasma at femtomole concentrations.

The foregoing discussion suggests that GLC is the method of choice for separation and quantitation of nanogram quantities of NEFA. While more reliable, GLC still has a number of unresolved difficulties, and most systems are too laborious. We have succeeded in circumventing extraction and purification steps for total fatty acids (36) and have tested the hypothesis that this one-step reaction could be adapted for the dosage of NEFA. In our first attempt to methylate specifically NEFA in the presence of water-methanol-benzene-acetyl chloride 1:4:1:1, results on pure lipid solutions gave excellent results (57). After a closer look at the stoichiometry of the reaction and many experiments carried out with two different mixtures of NEFA, TG, PL, and CE, results showed more significant hydrolysis of PL than with pure solutions of PL. Molarity of the acetyl chloride of this acid-catalyzed reaction had to be lowered from 2 M to 268 mM. While 100 μl of benzene was necessary to prevent extensive (20%) hydrolysis of PL, it was no longer necessary in the presence of 268 mM acetyl chloride. Stoichiometry of this acid-catalyzed methylation of NEFA suggests that it is balanced on a narrow ridge, separating incomplete esterification on the one hand from extensive transesterification of TG, PL, and

TABLE 4. Preparative steps circumvented by the specific methylation method

Steps	Conventional Techniques	Specific Methylation
Lipid extraction with $CHCl_3$–CH_3OH	+	–
Centrifugation	+	–
NaCl (145 mM) added to supernatant	+	–
Separation of the two phases	+	–
Evaporation of lower phase	+	–
Scraping of silica	+	–
Extraction of NEFA from silica	+	–
Evaporation of extractant	+	–
Methylation of NEFA	+	+
Extraction of methyl ester FA	+	–
Evaporation of extractant	+	–
Injection into GLC	+	+

CE on the other. It is true that this method appears to be unforgiving and only strict adherence to protocol will give excellent results. As pointed out in Table 1, specific methylation of the NEFA is dependent on the proportion of methanol–acetyl chloride and the duration of the reaction. A temperature of 24°–29°C was chosen because higher temperatures of 37°C and 50°C led to hydrolysis of 5.2% and 18.4% of TG, 4.9% and 17.2% of PL, and 6.5% and 23.1% of CE, respectively. A limited amount of plasma had to be used because water over a certain percentage inhibits the methylation reaction (58). Completeness of the methylation reaction could not be achieved in a mixture containing a molar ratio of water–acetyl chloride higher than 6. In experiments using 200 μl of plasma, NEFA were methylated to a maximum of 88% after 45 min, while hydrolysis of PL and TG was unacceptably high after 60 min. Methanol is the methyl donor for the COOH group of FA but its prime role is dilution of the acid concentration to 268 mM, thereby preventing hydrolysis of esterified lipids. Selective NEFA derivatization can also be achieved by methylation with diazomethane (59), trimethylsilylation (60), methyl iodide–potassium carbonate (32), and 2,2-dimethoxypropane (30). However, acetyl chloride is the only methylating agent that could achieve a complete reaction in the presence of the molar proportions of plasma catalyzing acid of 5.9:1 without producing artifacts. Five ml of methanol–acetyl chloride 100:1 (v/v) could methylate only 88.2% of NEFA after 90 min, while the reaction was nearly complete after 45 min with methanol–acetyl chloride 50:1 (v/v). However the reaction was too fast with methanol–acetyl chloride 25:1 (v/v) and within 15 min 15.3% of PL were hydrolyzed. Moreover, acetyl chloride is such a strong acid that, if not neutralized with K_2CO_3 at the end of the reaction, it will damage the liquid phase of the GLC column in the event that the hexane supernatant aliquot is contaminated with infranatant. Time was the last variable of this equation and 45 min was selected because this interval of time was sufficient to bring about complete methylation of NEFA without notable hydrolysis of esterified lipids.

After having proved that with this new methodology one could methylate NEFA in a specific fashion without hydrolyzing neutral and complex lipids, we elected to compare the new method to two others that are well established (30, 34). A large discrepancy existed between the three methods for total concentrations of NEFA. There was a twofold increase over the results obtained with the method of Tserng et al. (30) and the increase was 2.7-fold when compared with the method of Folch et al. (34). Attention is drawn to the larger loss of PUFA with both techniques. Is it possible that the proposed method could overestimate NEFA? This is very unlikely in view of our observations with standards of both neutral and complex lipids that undergo very little hydrolysis under the experimental conditions used. Further validation comes from experiments comparing the recovery of NEFA standards with the three methods. With both the Folch and Tserng procedures the loss of NEFA standards with each method was comparable to the loss of plasma NEFA. Moreover, there was a direct correlation between the extent of individual FA and their number of unsaturated bonds. In accordance with our results, Van der Vusse et al. (40) reported that the PUFA, 18:2, 20:4 and 22:6, were found in relatively large amounts when the Dole procedure was employed instead of that of Folch et al. and that recovery of the internal 17:0 standard amounted to only 75%. We observed that many unidentified peaks emerged on the chromatograms obtained with Tserng's procedure. Tserng et al. made this observation in their report calling them "interfering plasma peaks" (30). However Lefèvre et al. (31) stipulated that the lack of purification prior to methylation explained the presence of additional peaks on the chromatograms. We first thought that those "interfering plasma peaks" originated from the biological specimen but we finally came to the conclusion that the most important additional peak with a relative retention time of 1.02 (methylated 15:0 as the reference homolog) was a nonmethylated 15:0. The poor recoveries of FA standards (24.6–78.9%) obtained with Tserng's procedure were the result of poor methylation by 2,2,-dimethoxypropane in the presence of 5% water coupled with the possible loss of FA methyl esters during two successive evaporations in a stream of air (58).

Addition of an internal standard at the beginning of the reaction is of great importance because it is taken through all the steps of the procedure and therefore obviates the need to take into account the completion of full methylation of NEFA and the dilution of the GLC injection volume. Tridecanoic acid (13:0) is, in our experience, the best choice as an internal standard for NEFA because it is present only in trace amounts (0.1 μg/ml) compared to 0.9 μg for pentadecanoic acid and 0.7 μg for heptadecanoic acid. Furthermore, it forms a well-individualized peak in a relatively uncrowded part of the FA methyl ester chromatographic run. However, 15:0 was used instead of 13:0 in this study because the loss of 13:0 methyl ester was too large during the evaporation steps required for the Folch and Tserng methods. A longer chain internal standard would be ill-advised in view of the fact that it chromatographs in a region where a number of peaks are in close proximity.

The choice of tube in which the reaction is taking place is of prime importance. Tubes must be narrow because, after addition of 3 ml of K_2CO_3, 150 μl of hexane will be added and constitutes the supernatant from which an aliquot must be sampled prior to its injection into the GLC. Moreover the tubes should have a Teflon liner because rubber caps used in many laboratories produce artefactual peaks. The same problems may arise from caps previously

used for phospholipid analysis using the Bartlett procedure (61). The magnetic bar plays a significant role for completion of the specific methylation of NEFA in the presence of 150 μl of water. The most difficult step of this procedure is without any doubt the sampling of the aliquot of supernatant hexane for injection. A 2000 *g* × centrifugation for 10 min produces a 2-mm-thick supernatant layer. With a 200-μl Pipetman, one should barely touch the hexane layer with the polypropylene tip and aspirate approximately 30 μl for the automatic injector vial. One hundred and fifty μl of hexane or of benzene can extract methylated FA equally well, but hexane is a better choice. With a density of 0.660 and the lack of solubility in methanol–water as compared to a density of 0.879 and some solubility of benzene in the infranatant, hexane offers a cleaner separation of the supernatant from the infranatant. After centrifugation, proteins form a well-defined pellet, leaving a clean 150 μl of hexane floating on a large methanol–water layer. One could elect to add more hexane than recommended in this report but it should be remembered that the supernatant cannot be concentrated by evaporation without incurring large losses of volatile methylated FA. When the supernatant was injected into the GLC, there was a clean profile of FA methyl esters (Fig. 2) which showed no evidence of damage despite the fact that 1500 injections had been done previously using the same 60-m SP-2331 (25% bonded) capillary column. From these observations one can conclude that no pyrolysis of accumulated neutral lipids and PL had occurred. Neither the injection inlet nor the detector or its jet had been cleaned since the first injection and the baseline shown in Fig. 2 was a very low 4.72 at the start of the run. Pure hexane injections always produce a baseline without any ghost peak or memory effect.

The specific methylation procedure described in this paper circumvents all extraction and purification steps. It is much more accurate than previous methods, faster, easier to perform, and less expensive. In addition, it is much more sensitive. The one-step reaction for NEFA represents a logical extension of our previous efforts (36, 58) to improve the speed and the precision of existing techniques for the measurement of lipids. Plasma NEFA levels and patterns obtained with this method suggest that some quantitative and qualitative data reported so far using other methods will have to be reevaluated in the light of their technical shortcomings.

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