A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies

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Abstract Conventional sample preparation for fatty acid analysis is a complicated, multiple-step process, and gas chromatography (GC) analysis alone can require >1 h per sample to resolve fatty acid methyl esters (FAMEs). Fast GC analysis was adapted to human plasma FAME analysis using a modified polyethylene glycol column with smaller internal diameters, thinner stationary phase films, increased carrier gas linear velocity, and faster temperature ramping. Our results indicated that fast GC analyses were comparable to conventional GC in peak resolution. A conventional transesterification method based on Lepage and Roy was simplified to a one-step method with the elimination of the neutralization and centrifugation steps. A robotics-amenable method was also developed, with lower methylation temperatures and in an open-tube format using multiple reagent additions. The simplified methods produced results that were quantitatively similar and with similar coefficients of variation as compared with the original Lepage and Roy method. The present streamlined methodology is suitable for the direct fatty acid analysis of human plasma, is appropriate for research studies, and will facilitate large clinical trials and make possible population studies.—Masood, A., K. D. Stark, and N. Salem, Jr. **A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. *J. Lipid Res.* 2005.** 46: **2299–2305.**

Supplementary key words fatty acid analysis • plasma • transesterification method • robotic chemistry • fast gas chromatographic analysis

The consumption of n-3 polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), is implicated in various aspects of human health (1, 2). Presently, analysis of the fatty acid composition of human blood and plasma samples involves tedious and time-consuming extraction and transesterification procedures (3–8). In addition, gas chromatography (GC), which is the analytical technique of choice for fatty acid methyl ester (FAME) analysis, requires long run times in order to resolve all of the commonly occurring mammalian fatty acids (9–14). Streamlined, cost-effective methodology is needed to facilitate large clinical trials that examine the effects of n-3 PUFAs on chronic health conditions as well as population-based nutritional surveys.

Currently, there is evidence that n-3 PUFA intake reduces the risk of cardiovascular disease. Supplementation of approximately 1 g of EPA/DHA to patients surviving myocardial infarction has been demonstrated to reduce the risk of cardiovascular and total death, with a profound 45% reduction in sudden cardiac death (15). Observational studies have also demonstrated that blood levels of n-3 highly unsaturated fatty acids (HUFAs) are inversely related to the risk of coronary heart disease (16), sudden cardiac death (17), and fatal ischemic heart disease (18). In as much as dietary intakes of n-3 PUFA are well indicated in the blood compartment (19, 20), it has been proposed that n-3 HUFA status in blood may be an important, modifiable, clinical biomarker for the prevention of various chronic diseases (21, 22). Increased blood status of DHA has also been associated with visual and cognitive functions, including improved visual acuity in infants (23) and decreased risk of suicide (24) and postpartum depression (25). A rapid, cost-effective, technique for determining n-3 fatty acids in blood would allow evidence to be collected regarding n-3 PUFA status and several aspects of health, including infant development (26–31), neurodegenerative disease prevention (32), depression and behavioral disorders (33), enhancement of immune defenses (34), and chronic inflammatory diseases (35).

The present study presents two modifications of standard FAME analytical techniques. First, a fast GC method (36) was adapted to FAME analysis of human plasma sam-

Manuscript received 17 May 2005 and in revised form 22 July 2005.

Published, JLR Papers in Press, August 1, 2005.
DOI 10.1194/jlr.D500022-JLR200

Abbreviations: BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; FID, flame ionization detector; GC, gas chromatography; HUFA, highly unsaturated fatty acid; RRF, relative response factor.

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ples to reduce the analysis time from >70 min per sample to <12 min and thereby allow for high throughput. Second, the tedious plasma lipid transesterification and extraction procedure was streamlined to a one-step procedure. These procedures include a simple and convenient "bench-top" procedure to increase the efficiency of FAME analysis in a traditional laboratory, as well as a modification that is amenable to automated robotic transmethylation reactions. The application of the present methodological advances promises the ability to perform FAME analysis on a greatly increased number of clinical and research samples.

MATERIALS AND METHODS

Reagents and samples

Acetyl chloride, 2[6]di-tert-butyl-*p*-cresol [butylated hydroxytoluene (BHT)], potassium carbonate, analytical-grade methanol, hexane, and toluene were purchased from Sigma-Aldrich Chemical Co. Porcine brain ceramide and sphingomyelin were purchased from Avanti Polar Lipids, Inc. BHT was added to methanol (50 μg BHT/ml methanol) to prevent fatty acid oxidation. The internal (23:0 methyl ester) and external (GLC-462) fatty acid standards were purchased from Nu-Chek Prep (Elysian, MN). The internal standard was dissolved in the methanol-BHT solution at a concentration of 100 μg/ml. Blood containing the anticoagulant heparin was collected from an anonymous donor by venipuncture and was immediately centrifuged for 5 min at 2,000 *g*. The resulting plasma was then aliquoted in batches of 5 ml, frozen, and stored at −80°C.

Instrumentation

Samples prepared using the method of Lepage and Roy (6) were analyzed by the conventional GC techniques used in this laboratory (14) and by a fast GC method developed for the present study from a method for the analysis of citrus essential oils (36). Conventional analyses were performed on an Agilent 6890 Plus Gas Chromatograph and fast GC on an Agilent 6890N Network Gas Chromatograph (Agilent Technologies; Palo Alto, CA) equipped with a 7683 automatic liquid sampler and flame ionization detectors (FIDs). Both instruments were controlled and data collection was performed by a GC Chemstation, Rev. A.09.03 (Agilent Technologies). The fast GC was equipped with a 208 V power supply to enable fast temperature ramping. The aspiration and dispensing application procedure was performed with a Freedom Evo Tecan Robot (Research Triangle Park, North Carolina).

Transesterification methods

The transesterification method developed by Lepage and Roy (6) was used as the reference point for comparisons of new methods. Briefly, 100 μl of the internal standard solution (providing 10 μg of 23:0 methyl ester) and 50 μl of plasma were added to 13 mm × 100 mm borosilicate glass tubes containing a 2 ml methanol-hexane (4:1; v/v) mixture. Samples were vortexed, and the tubes were placed on ice. Acetyl chloride (200 μl) was added drop-wise while swirling the tubes. The tubes were capped under nitrogen and transferred to a heating block at 100°C. The samples were heated for 10 min and vortexed briefly, caps were retightened to prevent leakage, and samples were returned to the heating block for another 50 min. Afterwards, the samples were placed on ice to cool, uncapped, and neutralized by an addition of 5 ml of a 6% solution of K_2CO_3. The tubes were recapped and vortexed for 1 min, followed by centrifugation for 2 min at 3,000 rpm to remove emulsion and separate the mixture into two phases. The upper organic phase was collected, and the extraction procedure was repeated on the lower phase by adding 0.5 ml of hexane, vortexing, and centrifuging. The organic phases were combined and evaporated under nitrogen to a volume of 60 μl. This solution was transferred to a GC vial, and the vial was crimped under nitrogen for FAME analysis by GC. All reactions were performed in sextet in a well-ventilated fume hood.

Simplified, one-step transesterification reaction

A stock solution of the reagents used in the method of Lepage and Roy (6) was prepared just prior to each experiment. The 1.9 ml of stock solution required for each sample included 1.7 ml of methanol, 100 μl of acetyl chloride, and 100 μl of the internal standard solution (containing 10 μg of 23:0 methyl ester). Briefly, 50 μl of plasma and 1.9 ml of the stock solution were combined in screw-capped glass tubes. The tubes were capped and heated at 100°C for 60 min. The tubes were allowed to cool to room temperature. Hexane (0.75 ml) was added, and the tubes were vortexed for 30 s. The upper organic phase was collected with a Pasteur pipette. This extraction procedure was repeated as above in order to optimize lipid extraction. The combined hexane solution was evaporated under nitrogen to dryness, and the dry residue was then redissolved in 60 μl of hexane, transferred to GC vials, and capped under nitrogen.

Robotics-amenable transesterification reaction

The stock solution for the robot-amenable reaction differed slightly from that used in the simplified procedure above. The 1.9 ml of stock solution required for each sample contained 1.4 ml of methanol, 100 μl of acetyl chloride, 100 μl of the internal standard solution (containing 10 μg of 23:0 methyl ester), and 0.3 ml of toluene. Toluene was added to prevent complete evaporation of the reaction mixture in the open tubes. The transesterification process was similar to the previous one-step method with the following exceptions. The plasma and stock solution with toluene mixtures were placed on a heating block at 80°C in uncapped tubes for 120 min. The stock solution of reagents (but without internal standard) was then added (1 ml) a total of four times during the subsequent 2 h reaction period, at equal intervals, approximately every 25 min. The tubes were then removed and allowed to cool to room temperature, and then 1 ml of hexane solution was added to each tube. The tubes were then placed on a Tecan robotic deck and the phases mixed using the pipetting arms. The pipette tips on the liquid handling arm were inserted into the top (hexane) phase, and 0.5 ml of hexane was aspirated. This solution was rapidly expelled at the bottom of the tube. This aspiration procedure was repeated five times, followed by a 1 min delay. This aspiration-ejection procedure was repeated three more times for a total of 20 aspirations. The separation and collection of the upper (hexane) phase was identical to the procedure described above for the simplified method.

Amide linkage transesterification experiments

Porcine brain ceramide and sphingomyelin were also analyzed by both the closed-tube and open-tube reaction procedures. The effect of extending heating times from 60 min to 120 min for each transesterification on concentrations of fatty acids as determined by fast GC FAME analysis was also examined. In each of the experiments, 100 μg of either porcine brain ceramide or sphingomyelin was combined with 1.9 ml of the simple stock solution or the stock solution with toluene. Samples containing the stock solution with toluene were heated at 75°C in open tubes for 60 min or for 120 min. Samples containing the stock solution

without toluene were capped and heated at 100°C for 60 min or for 120 min. The samples were allowed to cool to room temperature, and the FAMEs were collected and prepared for fast GC analyses as described above.

Conventional GC analyses

Conventional analyses were performed with a fused silica capillary column (DB-FFAP), 30 m × 0.25 mm inner diameter (ID) × 0.25 μm film thickness (J and W Scientific, Agilent Technologies), a split/splitless injector, a 7683 automatic liquid sampler, and flame ionization detection. Temperature program, initial: 130°C with a 1 min hold; ramp: 4°C/min to 178°C, 1°C/min to 225°C, and then 40°C/min to 245°C, with a 13 min hold. Carrier gas was H_2, with a linear velocity of 60 cm/s; a constant pressure of 102.4 kPa was used. Fatty acid analysis was performed by autoinjection of 1 μl of each sample at a split ratio of 7.5:1. The FID temperature was 250°C, with air and nitrogen make-up gas flow rates of 450 and 10 ml/min. The sampling frequency was 20 Hz. The run time for a single sample was 74 min, with a sample injection-to-injection time of 76 min.

Fast GC analyses

For fast GC analyses, the column used was a DB-FFAP of 15 m × 0.1 mm ID × 0.1 μm film thickness (J and W Scientific from Agilent Technologies). Temperature program was as follows: initial, 150°C with a 0.25 min hold; ramp: 35°C/min to 200°C, 8°C/min to 225°C with a 3.2 min hold, and then 80°C/min to 245°C with a 2.75 min hold. Instrumental conditions were as follows: carrier gas was H_2 at a flow rate of 56 cm/s and a constant head pressure of 344.7 kPa; FID set at 250°C; air and nitrogen make-up gas flow

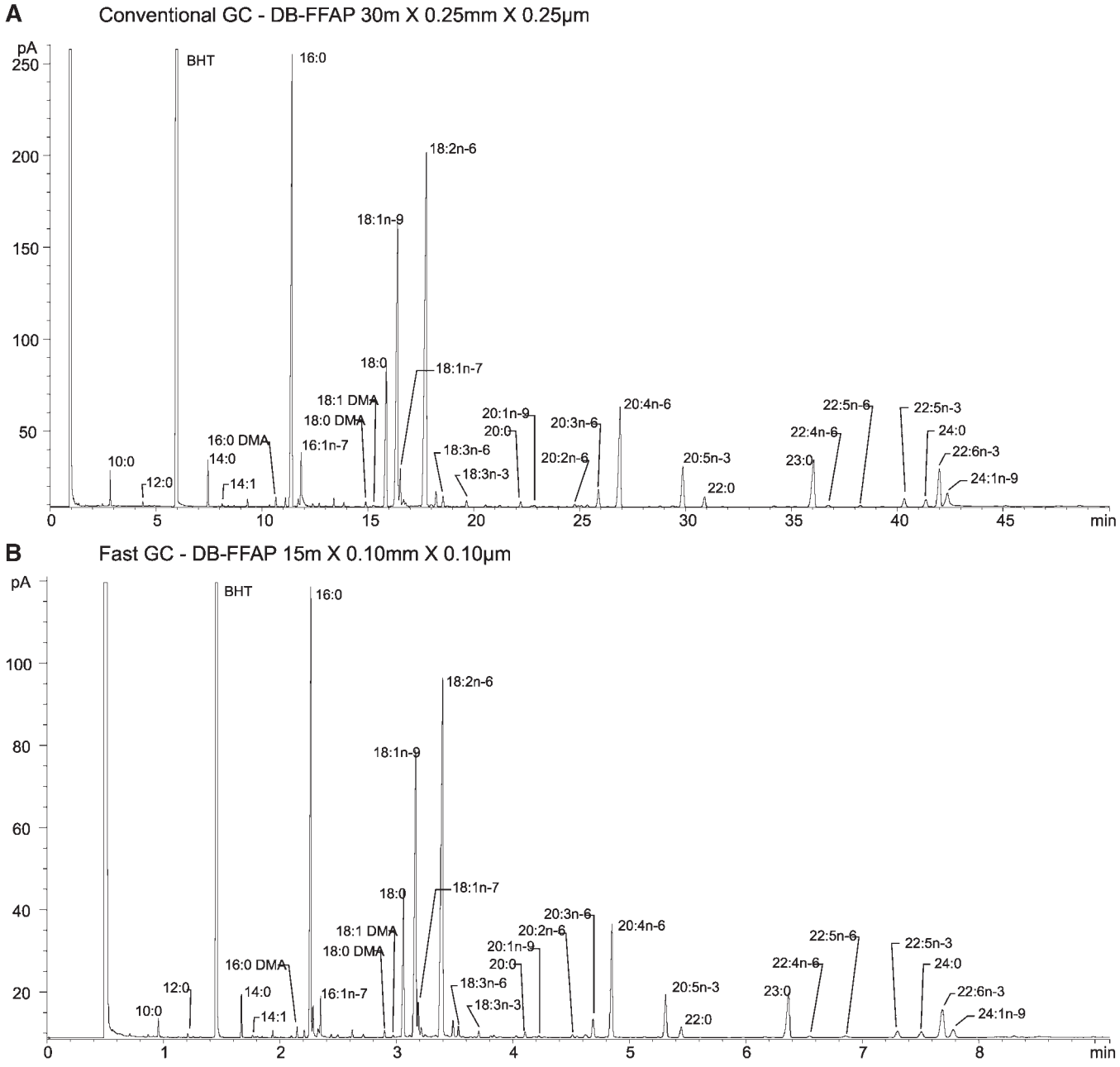

Fig. 1. Gas chromatograms of fatty methyl esters of human plasma, including the internal standard 23:0 methyl ester (10 μg) obtained from the conventional Lepage and Roy (6) transmethylation. BHT, butylated hydroxytoluene; DMA, dimethylacetal.

rates of 450 ml/min and 10 ml/min; split ratio of 200:1; sampling frequency of 50 Hz; autosampler injections of 2 μl volume. Run time for a single sample was 11 min, with a sample injection-to-injection time of 16 min.

Data quantitation

The amounts of individual fatty acids (C_{fa}) were calculated using the expression $C_{fa} = (A_{fa}/A_{is}) \times (C_{is}/V_p)/RRF$, where A_{fa} is the chromatographic area units of the fatty acids whose concentration is to be determined, A_{is} is the chromatographic area units for the internal standard, C_{is} is the concentration of the internal standard used in the reactions in terms of μg/ml, and V_p is the volume in ml of plasma sample used in an experiment. The relative response factor (RRF) for each peak was determined from a commercial, equal-weight standard composed of 28 fatty acids (462 standard, Nu-Chek Prep). The RRF was derived from the deviation from the theoretical response for this standard.

RESULTS AND DISCUSSION

Fast gas chromatography

The application of fast GC techniques demonstrated previously for citrus oil analysis (36) to FAME analysis of human plasma samples was successful in duplicating the resolution achieved with conventional GC techniques used previously in our laboratory (14), as demonstrated in **Fig. 1**. The chromatograms from the conventional and fast GC techniques show similar resolution, with a much faster analysis time for the fast GC. Fast GC techniques can provide the same results in approximately one fifth of the time it takes with conventional GC; run times of this speed would allow the analysis of approximately 100 samples per day. Faster run times can be achieved with this column for FAMEs using faster temperature ramping. For example, an aggressive, single-ramp method of 45°C/min from 135°C to 240°C results in a run time of 5.5 min. However, this resulted in overlaps of 24:1n9 with 22:6n3. Faster programs (approximately 5 min) on the present column may be used for analyses of biological sources that do not contain one of these fatty acid pairs.

The smaller peak areas observed with the fast GC technique were expected, because of the high injector split ratio (200:1 vs. 7.5:1 in conventional GC). The underlying principle of fast GC is miniaturization (37). This is achieved mainly by reducing column diameters, reducing film thickness, and shortening column length, and requires increased pressure capacity and faster temperature ramping. Sample capacity is therefore limited by this miniaturization, and high split ratios are often used to reduce the risk of column overload.

The DB-FFAP 15 m × 0.1 mm ID × 0.1 μm film thickness column was custom ordered from Agilent technologies. An additional benefit of this column is that it is less expensive than the conventional DB-FFAP 30 m × 0.25 mm ID × 0.25 μm film thickness column despite the requirement for custom ordering. The initial fast GC parameters were determined by previous fast GC results and the GC Method Translation Software (Version 2.0.a.c, Agilent Technologies). The final temperature program, which starts from an initial temperature of 150°C rather than 130°C and terminates at 245°C within 11 min rather than 74 min, was obtained by trial and error to mimic results obtained with a conventional GC run, in terms of both peak elution order and quantitation of fatty acids. This trial and error process was facilitated by the fast run times.

The concentrations of fatty acids in human plasma as determined by fast GC and by conventional GC are presented in **Table 1**. Percentage values may be readily calculated from these values, if desired, and may be useful for certain clinical analyses. The mean values and the percentage coefficient of variance (% CV) were similar in both methods. Both the % CV and mean differences were minimal for the major plasma fatty acids (16:0, 18:0, 18:1n-9, 18:2n-6, 20:4n-6, 20:5n-3, and 22:6n-3). The greatest discrepancies were found for minor fatty acids, but these were not greater than would be expected for peaks that comprise only 0.1–1% of the total fatty acids. The absolute differences in concentration, however, were relatively small. These findings demonstrate that the present fast GC method is suitable for fatty acid analyses of human plasma and can significantly reduce analytical run times. The reduction in GC analysis time is such that, without further methodological advances, sample preparation time and effort is clearly the rate-limiting step for fatty acid analysis.

TABLE 1. Comparison of human plasma fatty acid concentrations using conventional and fast GC (n = 6)

	Conventional GC		Fast GC		
Fatty Acids	Mean	CV	Mean	CV	Difference
	mg/ml	*%*	*mg/ml*	*%*	*%*
14:0	61	5.7	57.6	5.8	−5.9
16:0 DMA	21.1	5.3	20.9	4.5	−0.7
16:0	1,041	4.6	1,033	4.0	−0.8
18:0 DMA	15.1	4.6	14.6	4.7	−3.3
18:0	355	4.8	361	4.9	1.7
20:0	13.6	5.8	14.7	7.8	8.2
22:0	33.4	5.6	34.9	4.7	4.5
24:0	29.7	5.7	30.7	4.7	3.2
Total saturates	1,577	4.6	1,574	4.0	−0.2
14:1	4.9	5.5	4.9	7.5	0.0
16:1n-7	70.6	4.0	65.0	3.1	−8.0
18:1 DMA	5.2	5.1	5.3	5.6	1.60
18:1n-7	67.7	4.8	70.4	5.5	4.00
18:1n-9	780	5.2	782	4.9	0.19
20:1n-9	5.7	4.8	5.1	0.2	−10.76
24:1n-9	43.4	4.2	41.6	7.7	−4.21
Monounsaturates	978	4.9	973	4.7	−0.5
18:2n-6	1,133	6.3	1,096	3.1	−3.3
18:3n-6	23.5	6.6	24.1	8.3	2.5
20:2n-6	8.2	5.3	8.3	6.4	1.5
20:3n-6	50.4	4.5	51.3	5.5	1.8
20:4n-6	318	4.2	327	6.3	2.6
22:4n-6	6.8	2.9	7.9	5.3	15.6
22:5n-6	6.0	4.6	6.7	8.6	11.4
Total n-6 PUFA	1,547	5.6	1,521	3.4	−1.7
18:3n-3	15.1	5.2	15.8	6.1	4.7
20:5n-3	130	4.1	131	6.4	0.7
22:5n-3	32.9	4.6	34.0	5.7	3.3
22:6n-3	150	4.2	146	1.6	−2.9
Total n-3 PUFA	329	4.0	327	3.8	−0.5
Total PUFA	1,875	5.3	1,848	3.3	−1.5
Total fatty acid	4,431	4.8	4,395	3.5	−0.8

CV, coefficient of variance; DMA, dimethylacetal; GC, gas chromatography; PUFA, polyunsaturated fatty acid.

TABLE 2. Comparison of fatty acid composition using various transesterification methods (n = 6) by fast GC

	Standard Lepage		Simplified Reaction (Closed Tube)		Robotic Variant (Open Tube)	
Fatty Acids	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
	mg/ml	%	*mg/ml*	%	*mg/ml*	%
14:0	61.2 ± 3.5	5.7	60.8 ± 2.5	4.1	59.7 ± 2.4	4.0
16:0 DMA	21.1 ± 1.1	5.3	19.6 ± 0.6	3.1	19.5 ± 0.9	4.7
16:0	1,041.0 ± 47.9	4.6	998.5 ± 25.3	2.5	991.5 ± 19.9	2.0
18:0 DMA	15.1 ± 0.7	4.6	14.0 ± 0.3	2.3	14.5 ± 1.0	7.2
18:0	355.5 ± 17.0	4.8	341.2 ± 8.5	2.5	360.8 ± 7.6	2.1
20:0	13.6 ± 0.8	5.8	12.9 ± 0.3	2.3	14.7 ± 0.7	4.7
22:0	33.4 ± 1.9	5.6	29.9 ± 1.1	3.8	36.3 ± 1.1	2.9
24:0	29.7 ± 1.7	5.7	27.1 ± 0.9	3.2	30.9 ± 1.0	3.1
Total saturates	1,577.4 ± 73.3	4.6	1,519.1 ± 36.9	2.4	1,541.0 ± 24.7	1.6
14:1	4.9 ± 0.3	5.5	5.1 ± 0.2	3.3	5.0 ± 0.2	3.4
16:1n-7	70.6 ± 2.8	4.0	67.9 ± 2.4	3.5	69.1 ± 2.0	2.9
18:1 DMA	5.2 ± 0.3	5.1	5.1 ± 0.1	1.4	5.1 ± 0.3	5.9
18:1n-7	67.7 ± 3.2	4.8	65.2 ± 2.4	3.6	64.3 ± 1.4	2.2
18:1n-9	780.5 ± 40.8	5.2	779.78 ± 21.3	2.7	759.9 ± 11.3	1.5
20:1n-9	5.7 ± 0.3	4.8	5.7 ± 0.1	1.2	5.5 ± 0.2	4.1
24:1n-9	43.4 ± 1.8	4.2	40.1 ± 1.1	2.8	46.3 ± 2.2	4.8
Monounsaturates	978.3 ± 47.7	4.9	968.9 ± 25.8	2.7	1,010.0 ± 16.3	1.6
18:2n-6	1,133.2 ± 71.2	6.3	1,098.2 ± 31.6	2.9	1,087.3 ± 14.8	1.4
18:3n-6	23.5 ± 1.5	6.6	21.6 ± 0.7	3.2	22.8 ± 0.6	2.5
20:2n-6	8.2 ± 0.4	5.3	7.7 ± 0.1	1.9	7.2 ± 0.6	7.8
20:3n-6	50.4 ± 2.3	4.5	46.6 ± 1.0	2.2	47.7 ± 0.8	1.8
20:4n-6	318.5 ± 13.4	4.2	296.7 ± 7.7	2.6	307.4 ± 5.7	1.8
22:4n-6	6.8 ± 0.2	2.9	6.4 ± 0.4	6.5	6.3 ± 0.1	1.7
22:5n-6	6.0 ± 0.3	4.6	7.2 ± 0.2	2.3	7.9 ± 0.2	2.1
Total n-6 PUFA	1,546.6 ± 86.7	5.6	1,484.3 ± 41.2	2.8	1,486.5 ± 21.7	1.5
18:3n-3	15.1 ± 0.8	5.2	14.6 ± 0.5	3.3	15.3 ± 1.2	7.7
20:5n-3	130.4 ± 5.3	4.1	122.2 ± 3.3	2.7	134.6 ± 4.0	3.0
22:5n-3	32.9 ± 1.5	4.6	29.7 ± 0.7	2.2	37.5 ± 2.0	5.4
22:6n-3	150.5 ± 6.3	4.2	133.3 ± 3.1	2.3	141.8 ± 2.6	1.8
Total n-3 PUFA	328.9 ± 13.2	4.0	299.8 ± 4.4	1.5	329.2 ± 7.8	2.4
Total PUFA	1,875.4 ± 98.5	5.3	1,784.0 ± 44.4	2.5	1,815.8 ± 27.7	1.5
Total fatty acid	4,431.2 ± 211.0	4.8	4,272.0 ± 105.2	2.5	4,366.7 ± 64.4	1.5

Transesterification modifications

Subsequent to the successful development of a fast GC method for mammalian fatty acid analysis, studies designed to simplify the Lepage and Roy method (6) of sample preparation were undertaken. The results from both the simplified (closed tube) and the robotic variant of the simplified method (open tube) for transesterification reactions are compared with the standard Lepage and Roy methodology (**Table 2**). Samples prepared with these three methods were analyzed using the validated fast GC system. The total fatty acid concentrations were similar irrespective of the method of transmethylation. Both the simplified and robotic variant transesterification methods incorporated a common methodological adaptation, in that all reagents were combined into a simple stock solution that could be added in one step. This is a major departure from the classic Lepage and Roy technique of the addition of the sample and the internal standard, followed by methanol-hexane addition and vortexing, and then drop-wise addition of acetyl chloride after chilling the solution to avoid a violent reaction. Samples are then vortexed, heated, and neutralized before the organic phase is collected. Stepwise changes to the Lepage and Roy method were made, eventually demonstrating the suitability of the one-step stock solution method, without the requirement for postheating neutralization or centrifugation to separate phases. A one-container stock solution is very amenable for application to robotics and is cost efficient, in as much as several pipetting steps, with the change of pipette tips, are eliminated.

The fatty acid compositional profile deduced from the simplified reaction experiments produced results identical to those of the standard Lepage and Roy reaction. The open-tube, robotic variant method also gave results substantially similar to those of the standard Lepage and Roy reaction. The additional 60 min of reaction time, together with the multiple additions of reagents/solvents and the use of the less-volatile toluene in the open-tube method, compensated for the lower-temperature reaction (80°C). This method was derived for application to an automated method for preparation of FAMEs using the Tecan robot. The open-tube feature and the elimination of neutralization, vortexing, and centrifugation will greatly simplify a robotic method.

Initial experiments with the open-tube methodology were performed for 60–90 min at 75°C. Results for certain fatty acids, such as 20:0, 22:0, 24:0, and 24:1n-9, exhibited a decline of approximately 40%, as compared with results obtained with either the Lepage and Roy method or the simplified reaction conditions. These longer-chain fatty acids tend to be associated with the sphingomyelin and ceramide fraction of human plasma (38). Sphingomyelin and ceramide contain amide bonds that are more difficult

to hydrolyze and subsequently esterify, and more extreme reaction conditions, such as elevated temperatures and longer times (39), may be required for complete reaction. However, experiments with purified porcine brain ceramide and sphingomyelin at reaction temperatures of 75°C and 100°C and heating times of 1–2 h revealed only marginal differences in the individual fatty acid concentrations of 20:0, 22:0, 24:0, and 24:1n-9, and in the total fatty acid concentration (data not shown). Nevertheless, conditions were sought for which accurate quantification of all fatty acids could be obtained. We speculated that because of the evaporation of reagent in the open tube, the reaction was not proceeding to completion for these fatty acid species. Therefore, we extended the heating period to 120 min and elevated the temperature to 80°C, with addition of reagent four times at intervals of approximately 25 min. These conditions led to complete reaction of all lipid species, so that the concentration profiles obtained were the same as those obtained with the standard Lepage technique (Table 2).

A two-phase extraction method, with mixing by vortexing, one sample at a time, would be extremely time-consuming for a robot. Hence, the feasibility of mixing by vigorous repeated pipetting (aspiration) was tested. This methodology worked very well, because aspiration and dispensing performed by a robot yielded excellent results, as reflected by the comparability of the data from the standard Lepage and robotic variant columns shown in Table 2. In Table 2, the standard Lepage and the simplified method utilized vortexing, whereas the robotic variant method used aspiration. The mean differences in fatty acid concentrations between samples extracted by vortexing or by aspiration were generally within 5% and were nonsignificant.

We present here a simplified and efficient method for the analysis of fatty acids in human plasma. This method was generated by adapting fast GC methodology for human plasma FAME analysis with a DB-FFAP 15 m $\times$ 0.1 mm ID $\times$ 0.1 μm film thickness column. This method displayed the same peak eluting order, better peak integration characteristics, and similar quantitation, therefore successfully replicating the results obtained with conventional GC as has been performed in the present laboratory for many years (14). The present study also demonstrates, for the first time, a simple transesterification procedure that is easy to perform and suitable for high throughput FAME preparation from plasma samples in basic laboratories. The use of a stock solution and the elimination of several postreaction steps remove much of the labor-intensive and scale-limiting steps of previous methods (5, 8, 11). The robotic variant method presented here produced results equivalent to those obtained with traditional methodology. Thus, the method may make possible larger clinical studies and even population-based nutritional analyses.

In conclusion, we have developed a convenient, easy-to-use procedure for transesterification of fatty acids from plasma samples that can be performed in resource-limited laboratories and is amenable to adaptation for high-throughput robotics. When combined with the fast GC analytical method, the reduction in analytical burden, cost, and time promises to greatly impact fatty acid analyses for both clinical research and patient care in the near future.

This research was supported in part by the Intramural Research Program of the National Institutes on Alcohol Abuse and Alcoholism, National Institutes of Health. K.D.S. was supported in part by a GENESIS Young Investigator Award.

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