Title

Microwave Energy Increases Fatty Acid Methyl Ester Yield in Human Whole Blood Due to Increased Sphingomyelin Transesterification

Journal

Lipids, 50(9)

ISSN

0024-4201

Authors

Metherel, Adam H
Henao, Juan J Aristizabal
Ciobanu, Flaviu
et al.

Publication Date

2015-09-01

DOI

10.1007/s11745-015-4053-5

Peer reviewed

Lipids
DOI 10.1007/s11745-015-4053-5

ORIGINAL ARTICLE

Microwave Energy Increases Fatty Acid Methyl Ester Yield in Human Whole Blood Due to Increased Sphingomyelin Transesterification

Adam H. Metherel[1] · Juan J. Aristizabal Henao[1] · Flaviu Ciobanu[1] · Ameer Y. Taha[2] · Ken D. Stark[1]

Received: 13 April 2015 / Accepted: 10 July 2015

Abstract Dried blood spots (DBS) by fingertip prick collection for fatty acid profiling are becoming increasingly popular due to ease of collection, minimal invasiveness and its amenability to high-throughput analyses. Herein, we assess a microwave-assisted direct transesterification method for the production of fatty acid methyl esters (FAME) from DBS. Technical replicates of human whole blood were collected and 25-μL aliquots were applied to chromatography strips prior to analysis by a standard 3-h transesterification method or microwave-assisted direct transesterification method under various power (variable *vs* constant), time (1–5 min) and reagent (1–10 % H_2SO_4 in methanol) conditions. In addition, a standard method was compared to a 5-min, 30-W power microwave in 1 % H_2SO_4 method for FAME yield from whole blood sphingomyelin, and sphingomyelin standards alone and spiked in whole blood. Microwave-assisted direct transesterification yielded no significant differences in both quantitative (nmol/100 μL) and qualitative (mol%) fatty acid assessments after as little as 1.5- and 1-min reaction times, respectively, using the variable power method and 5 % H_2SO_4 in methanol. However, 30-W power for 5 min increased total FAME yield of the technical replicates by 14 %. This increase appears largely due to higher sphingomyelin-derived FAME yield of up to 109 and 399 % compared to the standard method when determined from whole blood or pure standards, respectively. In conclusion, microwave-assisted direct transesterification of DBS achieved in as little as 1-min, and 5-min reaction times increase total fatty acids primarily by significantly improving sphingomyelin-derived fatty acid yield.

Keywords Fingertip prick · Whole blood · Microwave · Direct transesterification · Sphingomyelin · Fatty acid methyl ester · Sulfuric acid

Abbreviations

BF_3	Boron trifluoride
DBS	Dried blood spots
DHA	Docosahexaenoic acid, 22:6n-3
EPA	Eicosapentaenoic acid, 20:5n-3
FAME	Fatty acid methyl ester(s)
FTP	Fingertip prick
HUFA	Highly unsaturated fatty acid(s)
H_2SO_4	Sulfuric acid
MUFA	Monounsaturated fatty acid(s)
PUFA	Polyunsaturated fatty acid(s)
SFA	Saturated fatty acid(s)

Introduction

Dried blood spots (DBS) collected by fingertip prick provide fatty acid determinations similar to venous whole blood sampling [1, 2], and in recent years have gained in acceptance and been adopted for use in a variety of populations including infants [3, 4], the elderly [5] and military personnel [6], among others [2, 7–9]. In addition, the % sum of eicosapentaenoic acid (EPA, 20:5n-3) + docosahexaenoic acid (DHA, 22:6n-3) in whole blood can be used as a predictor for risk of sudden cardiac death [10].

✉ Ken D. Stark
kstark@uwaterloo.ca

[1] Department of Kinesiology, University of Waterloo, 200 University Avenue, Waterloo, ON N2L 3G1, Canada

[2] Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California, Davis, 1 Shields Avenue, Davis, CA 95616, USA

Published online: 02 August 2015

Although erythrocytes and plasma are the more commonly used blood fractions for fatty acid analysis, DBS whole blood collection is simpler, less invasive and can be directly transesterified into fatty acid methyl esters (FAME) which bypasses the traditionally required lipid extraction step [2]. However, this process still requires between one and three hours to complete with boron trifluoride (BF_3) [11] and sulfuric acid (H_2SO_4) [12], respectively. Furthermore, rapid collection and subsequent pressures placed on storage capabilities, particularly in the absence of −80 °C storage [13], increases the importance of employing more rapid analytical techniques to ease the storage burden.

The use of fingertip prick blood collection for fatty acid composition analysis of plasma was initially performed in 1990 [14], with microwave-assisted direct transesterification techniques on brain phospholipid extracts reported in 1992 [15]. More recently, microwave-assisted direct fatty acid transesterifications have been reported for the conversion of DBS by fingertip prick [1, 6, 9] and serum [16] lipids into FAME. Although microwave-assisted direct transesterification may fail to fully transesterify triacylglycerols when using 14 % boron trifluoride (BF_3) in methanol [1], this may be a limitation of the kitchen-grade microwave utilized in two of the previous studies [1, 9]. This potential limitation may be overcome by systems such as the CEM Discover SP-D single-mode [16] or MARS multi-mode [6, 16] microwaves specifically designed for laboratory processes (CEM Corporation, Matthews, North Carolina, USA). Alternative methods to expedite the transesterification process have been adopted using ultrasonic energy [17–20], with promising results, and microwave-assisted extraction techniques have been implemented previously for the extraction of fats from poultry feeds [21], seeds, meat and bakery products [22], acorns [23] and avocado pulp [24].

The purpose of this study is to assess the implementation of a microwave-assisted direct transesterification with H_2SO_4 in methanol as the transesterification reagents, first, on human whole blood technical replicates, and second, in a small population of low to moderate omega-3 status human subjects using DBS collected on chromatography paper using an advanced laboratory grade microwave. Parameters assessed include power (variable *vs* constant), time (1–5 min) and reagent concentration (1–10 % H_2SO_4). In addition, we assess the role of increased transesterification of sphingomyelin-bound fatty acids on total FAME yield with microwave-assisted reactions.

The results of this study indicate that fatty acid determinations by microwave-assisted transesterification increased FAME yield in as little as one and a half minutes due primarily to increased sphingomyelin-derived fatty acid yield. This provides a method that may in the future enable health practitioners to obtain fast and reliable omega-3 fatty acid profiling for dietary compliance and health assessments.

Methods and Materials

Study Design

The study was designed to develop a microwave-assisted direct transesterification method for the rapid assessment of fatty acid profiles. Whole blood technical replicates samples on chromatography paper collected from a single male participant were used as a reference material to compare numerous analytical parameters of microwave-assisted direct transesterification methods with a transesterification method of 1 % H_2SO_4 in methanol by convection block heating for 3 h at 70 °C serving as a control method [12]. In addition, dried blood spots (DBS) from a small cohort ($n = 7$, 24.1 ± 3.4 years, 2 females, 5 males) of low to moderate omega-3 status were further assessed to determine applicability of the method to a population. Due to preliminary results, the sphingomyelin fraction was isolated from whole blood technical replicates of a second male participant, and separately, sphingomyelin standards were purchased and each sphingomyelin source was compared between microwave-assisted and standard techniques. Pure standards were assessed individually and spiked in whole blood technical replicates ($n = 4$) from a third individual male participant (24 years, high omega-3 status). The overall study protocol received clearance from the University of Waterloo Human Ethics Committee and the participants provided informed consent prior to participation.

Method Development

As a result of pilot work showing a more rapid increase in pressure during microwave-assisted transesterification reactions with BF_3 compared with H_2SO_4, H_2SO_4 was selected as the transesterification reagent for the current study. Based on this pilot work with whole blood samples, baseline microwave-assisted direct transesterification reactions were performed with 1 mL 1 % H_2SO_4 in methanol + 0.5 mL hexane using the CEM Discover® SP system (CEM Corporation, Mathews, NC, USA). The settings for this pilot method were 5 min at 30-W power with a maximum temperature setting of 165 °C. This maximum temperature setting prevents the microwave system from exceeding the reaction tube maximum pressure limit of 300 PSI, at which point the microwave will automatically stop. The microwave settings of 30 W and 165 °C were also tested for 2, 3 and 4 min reaction times. Using this method the maximum pressure of 300 PSI is reached after approximately 2 min of 30-W power application. Subsequently,

Table 1 Fatty acid concentrations as determined by microwave-assisted direct transesterification of whole blood on chromatography strips

Name	1 % H_2SO_4 transesterification				
	Convection	Microwave with 30 W			
	3 h	2 min	3 min	4 min	5 min
SFA	384 ± 15^{ab}	282 ± 20^{c}	359 ± 17^{a}	422 ± 36^{bc}	443 ± 24^{d}
MUFA	161 ± 7^{ab}	107 ± 4^{c}	151 ± 5^{a}	181 ± 12^{c}	187 ± 7^{d}
N-6	293 ± 13^{ab}	191 ± 6^{c}	269 ± 11^{a}	316 ± 20^{bc}	326 ± 12^{d}
N-3	51.5 ± 1.7^{ab}	35.2 ± 1.2^{c}	46.9 ± 3.3^{a}	54.8 ± 3.5^{b}	55.5 ± 2.7^{b}
PUFA	345 ± 15^{ab}	227 ± 7^{c}	316 ± 14^{a}	371 ± 23^{bc}	381 ± 15^{d}
HUFA	156 ± 7^{ab}	107 ± 3^{c}	145 ± 8^{a}	167 ± 10^{b}	169 ± 7^{b}
Total	890 ± 33^{ab}	616 ± 29^{c}	826 ± 30^{a}	973 ± 71^{bc}	1011 ± 41^{d}
Name		Variable power @ 165 °C Max for 2 min with 1–10 % H_2SO_4			
		1 %	2 %	5 %	10 %
SFA	384 ± 15^{ab}	334 ± 40^{a}	380 ± 31^{ab}	388 ± 10^{ab}	397 ± 23^{b}
MUFA	161 ± 7^{a}	133 ± 12^{b}	167 ± 12^{a}	176 ± 5^{a}	162 ± 9^{a}
N-6	293 ± 13^{a}	227 ± 9^{b}	283 ± 16^{a}	290 ± 11^{a}	239 ± 8^{b}
N-3	51.5 ± 1.7^{a}	39.5 ± 1.9^{b}	48.0 ± 2.6^{a}	48.1 ± 2.0^{a}	39.0 ± 1.9^{b}
PUFA	345 ± 15^{a}	267 ± 11^{b}	331 ± 18^{a}	339 ± 12^{a}	278 ± 10^{b}
HUFA	156 ± 7^{a}	121 ± 5^{b}	147 ± 8^{a}	148 ± 5^{a}	122 ± 5^{b}
Total	890 ± 33^{a}	734 ± 61^{b}	878 ± 61^{a}	903 ± 26^{a}	837 ± 39^{a}
Name		Variable power @ 165 °C Max with 5 % H_2SO_4 for 1–3 min			
		1 min	1.5 min	2 min	3 min
SFA	384 ± 15^{ab}	342 ± 25^{a}	422 ± 19^{b}	388 ± 10^{ab}	381 ± 33^{ab}
MUFA	161 ± 7^{ab}	139 ± 16^{a}	174 ± 11^{b}	176 ± 5^{b}	158 ± 8^{ab}
N-6	293 ± 13^{a}	244 ± 26^{b}	300 ± 17^{a}	290 ± 11^{a}	266 ± 12^{ab}
N-3	51.5 ± 1.7^{a}	42.4 ± 4.2^{b}	49.9 ± 2.8^{a}	48.1 ± 2.0^{ac}	44.2 ± 1.1^{bc}
PUFA	345 ± 15^{a}	286 ± 30^{b}	350 ± 20^{a}	339 ± 12^{a}	310 ± 13^{ab}
HUFA	156 ± 7^{a}	129 ± 13^{b}	154 ± 9^{a}	148 ± 5^{ac}	136 ± 4^{bc}
Total	890 ± 33^{a}	767 ± 63^{b}	946 ± 49^{a}	903 ± 26^{a}	850 ± 46^{ab}

Different superscript letters between transesterification methods and within a fatty acid group signify statistical differences as determined by Tukey's HSD *post hoc* test following a significant *F* value by One-way ANOVA. Data are presented as nmol fatty acid per 100 μL blood, mean ± SD, n = 4

SFA saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid and *HUFA* highly unsaturated fatty acid

for reactions longer than 2 min the power is continuously cycled on and off until the end of the transesterification reaction to maintain temperature and pressure just under 165 °C and 300 PSI, respectively. As such, a 4-min reaction represents a constant 30 W of power for the initial 2 min and an additional 2 min of cycling 30 W on and off.

Further methodological adjustments were made by maintaining the same temperature limit of 165 °C and allowing the CEM software program to determine the optimal power output that would reach the temperature limit in the shortest amount of time while not exceeding the pressure limits of 300 PSI. This microwave-assisted method will hereafter be referred to as the variable power method. As a result of higher total FAME yields with 1 % H_2SO_4 in methanol for the 165 °C variable power (734 ± 33, nmol/100 μL) *vs* 30 W constant power (616 ± 29, nnmol/100 μL) method (Table 1), the variable power method was determined to be superior and was assessed under additional time (1, 1.5, 2 and 3 min) and reagent (1, 2, 5 and 10 % H_2SO_4 in methanol) conditions.

Microwave-Assisted Direct Transesterification of Whole Blood on Chromatography Strips

Two vacutainers of approximately 8 mL each were used to obtain whole blood by venipuncture from a single fasted participant (male, 28 years old) on his usual low omega-3 diet, and 500-μL aliquots were stored at −75 °C until time

of analysis. We have previously shown that storage of the whole blood samples under these conditions will not significantly affect the fatty acid profile for up to 6 months [13], and all analyses were performed within 3 months of storage.

For analysis, whole blood samples were removed from storage and allowed to thaw on ice. After thawing, the sample was vortexed and 25 μL of whole blood was aliquoted to pre-washed chromatography paper strips and allowed to fully saturate into the paper prior to microwave analysis. Microwave-assisted (CEM Discover® SP, CEM Corporation, Mathews, NC, USA) direct transesterification reactions were compared to standard direct transesterification reactions of 2 mL 1 % H_2SO_4 in methanol + 1 mL hexane for 3 h ($n = 4$) [12]. To control for the potential effects of different transesterification methods on FAME yields, an internal standard of 10 μg docosatrienoic acid methyl ester (22:3n-3, Nu Check Prep, Inc., Elysian, MN, USA) was added following transesterification and used for quantitation of individual fatty acids.

Microwave-Assisted Transesterification of Dried Blood Spots in a Population

Whole blood samples were collected by venipuncture from a small cohort ($n = 7$) including 2 females and 5 males with low to moderate omega-3 fatty acid status (18.7–36.4, % of n-3 highly unsaturated fatty acids (HUFA) in total HUFA). Aliquots (25 μL) of the whole blood were applied to pre-washed chromatography strips as described previously [13] and allowed to dry in air for approximately 30 min. DBS were then transesterified by the 5-min, 30-W microwave method or 3 h standard 1 % H_2SO_4 method as previously described, or for 1 h at 95 °C with 1 mL 14 % boron trifluoride in methanol with 0.3 mL hexane. Following transesterification 10 μg of 22:3n-3 methyl ester internal standard was added for quantitation of sphingomyelin-derived FAME independent of the effects of the transesterification method.

Microwave-Assisted Transesterification of Whole Blood Sphingomyelin

One vacutainer of approximately 8 mL was used to obtain whole blood which was collected by venipuncture from a second individual participant (male, 23 years old) on his usual low omega-3 diet, and 500-μL aliquots were stored at −75 °C until time of analysis. Total lipids were extracted from 100 μL thawed whole blood using 3 mL of 2:1 chloroform:methanol with 50 μg/mL butylated hydroxytoluene as antioxidant [9]. The lower, organic, lipid-containing phase was pipetted into a new test tube and an additional 2 mL of chloroform was added to the original test tube for a second extraction. The lower organic phase was pipetted and mixed with the first organic extract. Whole blood sphingomyelin was isolated from total lipid extracts by thin layer chromatography [25]. Briefly, total lipid extracts of whole blood were applied to silica gel G plates (Whatman International Ltd, Maidstone, UK) and developed in chloroform:methanol:water (25:10:1, vol:vol:vol). Lipids were visualized under ultraviolet light after treatment with a 0.1 % solution of 2,7-dichlorofluorescin (Sigma, St. Louis, MO) in methanol. The sphingomyelin region was collected, and the fatty acids were transesterified to FAME with either 1 % H_2SO_4 in methanol in a block heater for 3 h or with 1 % H_2SO_4 in methanol by microwave energy for 5 min ($n = 4$), as described above. Following transesterification 10 μg of 22:3n-3 methyl ester internal standard was added for quantitation of sphingomyelin-derived FAME independent of the effects of the transesterification method.

Microwave-Assisted Transesterification of Sphingomyelin Standard

Both chicken egg (predominantly 16:0, palmitic acid) and bovine milk (predominantly 23:0, tricosanoic acid) sphingomyelin standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Aliquots of 75 μg of each were taken from a prepared solution, mixed and solvents were evaporated. These two standards were selected and mixed to compare the potential role of chain length on transesterification fatty acid yield. Microwave-assisted transesterification of sphingomyelin standards (200 nmol) for 5 min at 30 W power was compared to the standard transesterification method of heating 1 % H_2SO_4 in methanol in a heat block at 70 °C for 3 h ($n = 4$). In addition, reaction test tubes were spiked with 0, 10, 25, 50 or 100 nmol of the sphingomyelin standard mixture in chloroform. Following chloroform evaporation, 25-μL aliquots of whole blood from a single 24 year old male participant on his usual high omega-3 dietary intake were added to test tubes. The mixture was then transesterified by the 30-W, 5-min microwave method. Following transesterification 10 μg of 22:3n-3 methyl ester internal standard was added as noted previously.

Gas Chromatography

Fatty acid methyl esters were analyzed on a Varian 3900 gas chromatograph (GC) equipped with a DB-FFAP 15 m × 0.10 mm i.d. × 0.10 μm film thickness, nitroterephthalic acid modified, polyethylene glycol, capillary column (J&W Scientific from Agilent Technologies, Mississauga, ON) with hydrogen as the carrier gas. Samples (1 μL) were introduced by a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) into the injector heated to

250 °C with a split ratio of 100:1. The initial temperature was 150 °C with a 0.25-min hold followed by a 35 °C/min ramp to 200 °C, an 8 °C/min ramp to 225 °C with a 3.2-min hold and then an 80 °C/min ramp up to 245 °C with a 15-min hold at the end [26]. The flame ionization detector temperature was 300 °C with air and nitrogen make-up gas flow rates of 300 and 25 mL/min, respectively, and a sampling frequency of 80 Hz. Peaks were identified by retention times through comparison to an external mixed standard sample (GLC-462, Nu Chek Prep Inc., Elysian, MN, USA).

Statistical Analyses

Qualitative (mol%) and quantitative (nmol/100 μL) values for individual and group FAME were determined and are expressed as means ± SD. All statistical analyses were performed with the SPSS System (SPSS Inc., Chicago, IL, USA). Following significant *F* values by One-way ANOVA, differences in FAME yields using the various transesterification methods from whole blood and DBS were determined by the Tukey's Honestly Significant Different *post hoc* procedure. In addition, differences in FAME yield for the isolated sphingomyelin fraction from whole blood and sphingomyelin standard were determined by an independent *t* test. Significance was inferred for all statistical analyses at $p < 0.05$.

Results

Quantitative Assessment of Microwave-Assisted Direct Transesterification of Whole Blood on Chromatography Strips

Multiple microwave settings were tested for the direct transesterification of whole blood samples on chromatography strips. Manipulated reaction settings included time and power, and major fatty acid subclasses are reported for both quantitative (Table 1) and qualitative (Table 2) results. A constant application of 30 W for 5 min yields significantly higher concentrations (μmol/100 μL) of SFA (15 %), MUFA (16 %), n-6 polyunsaturated fatty acid (PUFA) (11 %), total PUFA (10 %) and total fatty acids (14 %) (Table 1) compared with a 3 h standard 1 % H_2SO_4 direct transesterification reaction. With the exception of increased MUFA yields with 4 min of microwave irradiation, both the 3- and 4-min reaction times show no statistical differences across the major fatty acid groups compared with the standard method (Table 1). When applying 30 W for only 2 min, all fatty acid subclasses are 25–35 % lower compared with control. When utilizing the variable power method of irradiation, total fatty acid content is 19 and 11 % higher compared with 30 W constant power during 2 and 3 min reactions, respectively (data not shown). Therefore, the variable power method was used to test the effect of incremental concentrations of H_2SO_4 in methanol and time on fatty acid yield.

As shown in Table 1, the yield of subclasses with 1 % H_2SO_4 and variable power method remain lower than standard yields with the exception of SFA. Increasing the H_2SO_4 content from 2 to 5 % in methanol abolishes all differences in subclass yields compared with the control. Further increases to 10 % H_2SO_4 in methanol gives 18–24 % lower yields of n-6, n-3, PUFA and HUFA compared with both control and 5 % H_2SO_4 in methanol. The variable power method with 5 % H_2SO_4 in methanol was then examined further with regard to the effect of time required for transesterification. Both the 1.5- and 2-min reaction times with variable power and 5 % H_2SO_4 in methanol are statistically equal to the standard 3-h method. The 3-min reaction produced lower n-3 PUFA (14 %) and HUFA (13 %) compared with the control while the 1-min reaction resulted in lower n-6 PUFA (17 %), n-3 PUFA (18 %), PUFA (17 %), HUFA (17 %) and total fatty acids (14 %).

The large increases in total fatty acid yield, particularly with the 5-min, 30-W microwave-assisted direct transesterification method, appear to be driven primarily by the very-long chain SFA and MUFA (Fig. 1). Specifically, 22:0 increases by 111 % (4.39 nmol), 23:0 by 100 % (1.26 nmol), 24:0 by 135 % (8.61), 24:1n-9 by 137 % (8.86 nmol) and 16:0 by 14 % (29.5 nmol) during the this microwave direct transesterification method compared with the 3-h standard method. Shortening the time of the microwave reaction to 2 min yields lower fatty acid concentrations for 22:0, 24:0 and 24:1n-9 and 16:0 compared to control.

Qualitative Assessment of Microwave-Assisted Direct Transesterification of Whole Blood on Chromatography Strips

Qualitative assessment of the major fatty acid subclasses demonstrates less variation amongst the various microwave direct transesterification methods (Table 2). The 30-W constant power microwave method yields no significant qualitative (mol%) differences during 3, 4 or 5 min of reaction time for all fatty acid subclasses. The 2-min reaction yields 6 % higher SFA, and 6 and 5 % lower n-6 PUFA and PUFA, respectively.

When utilizing the variable power method for 2 min, qualitative differences are demonstrated for all H_2SO_4 concentrations compared with the standard 3-h method (Table 2). The largest differences are shown in lower yields of 8, 25, 30, 26 and 28 % for MUFA, n-6 PUFA, n-3 PUFA, PUFA and HUFA, respectively with 10 % H_2SO_4

Table 2 Qualitative fatty acid profiles as determined by microwave-assisted direct transesterification of whole blood on chromatography strips

Name	1 % H_2SO_4 Transesterification				
	Convection	Microwave with 30 W			
	3 h	2 min	3 min	4 min	5 min
SFA	42.4 ± 0.8^{a}	44.8 ± 1.1^{b}	43.0 ± 1.1^{ab}	42.6 ± 0.5^{ab}	43.0 ± 1.0^{ab}
MUFA	17.8 ± 0.3^{ab}	17.1 ± 0.4^{a}	18.0 ± 0.3^{b}	18.3 ± 0.2^{b}	18.2 ± 0.4^{b}
N-6	32.3 ± 0.6^{a}	30.5 ± 0.7^{b}	31.9 ± 0.7^{ab}	31.9 ± 0.4^{ab}	31.7 ± 0.6^{ab}
N-3	5.68 ± 0.10	5.61 ± 0.16	5.57 ± 0.09	5.54 ± 0.07	5.40 ± 0.15
PUFA	38.0 ± 0.7^{a}	36.1 ± 0.8^{b}	37.5 ± 0.8^{ab}	37.4 ± 0.5^{ab}	37.1 ± 0.8^{ab}
HUFA	17.2 ± 0.3	17.1 ± 0.5	17.2 ± 0.3	16.9 ± 0.2	16.5 ± 0.4
Name		Variable power @ 165 °C Max for 2 min with H_2SO_4			
		1 %	2 %	5 %	10 %
SFA	42.4 ± 0.8^{ab}	44.3 ± 1.6^{a}	42.2 ± 0.3^{ab}	42.0 ± 0.1^{b}	40.2 ± 1.0^{b}
MUFA	17.8 ± 0.3^{a}	17.8 ± 0.7^{a}	18.6 ± 0.2^{ab}	19.0 ± 0.2^{b}	16.4 ± 0.2^{c}
N-6	32.3 ± 0.6^{a}	30.3 ± 1.4^{b}	31.5 ± 0.2^{ab}	31.4 ± 0.5^{ab}	24.3 ± 0.7^{c}
N-3	5.68 ± 0.10^{a}	5.27 ± 0.18^{b}	5.35 ± 0.08^{b}	5.19 ± 0.08^{b}	3.95 ± 0.16^{c}
PUFA	38.0 ± 0.7^{a}	35.6 ± 1.6^{b}	36.8 ± 0.2^{ab}	36.6 ± 0.6^{ab}	28.2 ± 0.8^{c}
HUFA	17.2 ± 0.3^{a}	16.2 ± 0.7^{ab}	16.4 ± 0.2^{ab}	16.0 ± 0.3^{b}	12.4 ± 0.5^{c}
Name		Variable power @ 165 °C Max with 5 % H_2SO_4			
		1 min	1.5 min	2 min	3 min
SFA	42.4 ± 0.8	43.7 ± 2.2	43.7 ± 0.5	42.0 ± 0.1	43.2 ± 1.9
MUFA	17.8 ± 0.3^{a}	17.8 ± 0.8^{a}	18.0 ± 0.1^{ab}	19.0 ± 0.2^{b}	18.0 ± 0.5^{ab}
N-6	32.3 ± 0.6^{a}	31.1 ± 1.3^{ab}	31.0 ± 0.5^{ab}	31.4 ± 0.5^{ab}	30.2 ± 1.0^{b}
N-3	5.68 ± 0.10^{a}	5.41 ± 0.23^{ab}	5.16 ± 0.05^{bc}	5.19 ± 0.08^{bc}	5.02 ± 0.19^{c}
PUFA	38.0 ± 0.7^{a}	36.2 ± 1.5^{ab}	36.2 ± 0.5^{ab}	36.6 ± 0.6^{ab}	35.3 ± 1.2^{b}
HUFA	17.2 ± 0.3^{a}	16.5 ± 0.8^{ab}	15.9 ± 0.3^{bc}	16.0 ± 0.3^{bc}	15.4 ± 0.5^{c}

Different superscript letters between transesterification methods and within a fatty acid group indicate statistical differences as determined by Tukey's HSD *post hoc* test following a significant *F* value by One-way ANOVA. Data are presented as mol% of fatty acid in the total fatty acids, mean ± SD, $n = 4$

SFA saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid, *HUFA* highly unsaturated fatty acid and *H_2SO_4* sulfuric acid in methanol

in methanol. In addition, n-3 PUFA are lower for all four H_2SO_4 concentrations and MUFA are higher in the 2 and 5 % concentrations. Testing the effect of time during the variable power method with 5 % H_2SO_4 yields 7, 12, 7 and 10 % lower values during 3-min reactions for n-6 PUFA, n-3 PUFA, PUFA and HUFA, respectively, and the 2-min reaction time increases MUFA and decreases n-3 PUFA and HUFA. Interestingly, the shortest reaction time of 1 min yields no significant differences for any of the fatty acid subclasses compared with control even though significant quantitative differences are shown (Table 1).

Microwave-Assisted Transesterification of Dried Blood Spots in a Population

The large increases in fatty acid yield and very-long chain SFA and MUFA demonstrated from a single participant (Fig. 1) following a 5-min, 30-W microwave direct transesterification compared to the 3-h standard H_2SO_4 method was confirmed in a small cohort ($n = 7$) (Fig. 2). Interestingly, direct transesterification of DBS from this population by a standard 1 h transesterification with BF_3 was also significantly higher than the 3-h H_2SO_4 method, but showed no significant differences compared to the microwave method. Specifically, 22:0 increased by 61 % (2.93 nmol), 23:0 by 90 % (0.97 nmol), 24:0 by 75 % (5.50 nmol) and 24:1n-9 by 67 % (5.00 nmol) following the microwave direct transesterification method compared with the 3-h standard H_2SO_4 method, and 22:0 increased by 71 % (3.40 nmol), 23:0 by 118 % (1.27 nmol), 24:0 by 82 % (6.01 nmol) and 24:1n-9 by 74 % (5.52 nmol) following the standard BF_3 method compared to the 3-h standard H_2SO_4 method. No significant differences are noted for 16:0 between the three methods.

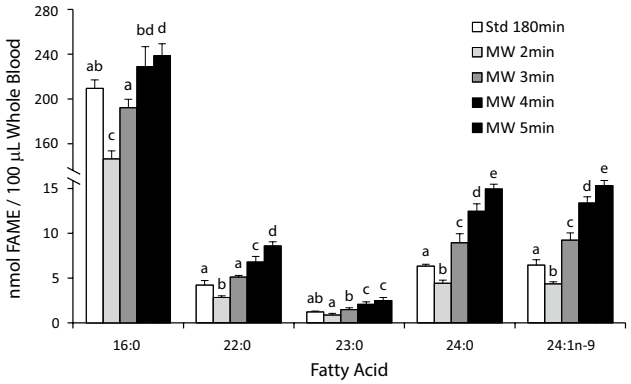

Fig. 1 Fatty acid methyl ester (FAME) yield in 25 µL whole blood on chromatography paper following direct transesterification with 1 % sulfuric acid in methanol. *Different letters* between transesterification methods and within a fatty acid group indicate statistical differences as determined by Tukey's HSD *post hoc* test following a significant *F* value by One-way ANOVA. Data are presented as nmol FAME per 100 µL whole blood, means ± SD, $n = 4$. *Std* standard control method, *MW* microwave

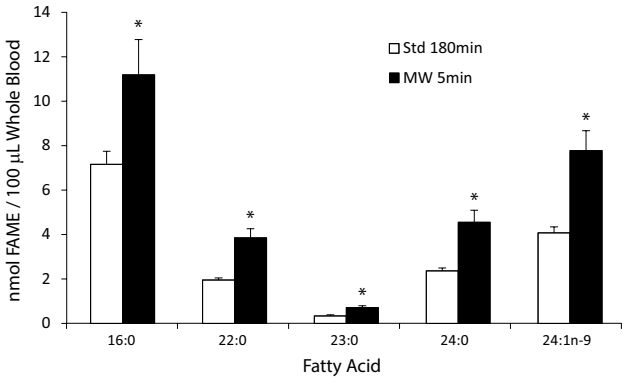

Fig. 3 Fatty acid methyl ester (FAME) yield from sphingomyelin in 100 µL whole blood on chromatography paper following direct transesterification with 1 % sulfuric acid in methanol. *Indicates fatty acid values significantly different than the 3-h control as determined by Tukey's HSD *post hoc* test following a significant *F* value by One-way ANOVA. Data are presented as nmol fatty acid per 100 µL whole blood, means ± SD, $n = 4$. *Std* standard control method, *MW* microwave

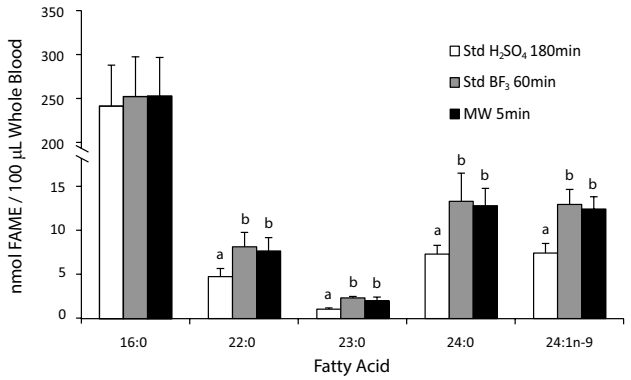

Fig. 2 Fatty acid methyl ester (FAME) yield from 25 µL dried blood spots on chromatography paper following direct transesterification with 1 % sulfuric acid in methanol or 14 % boron trifluoride in methanol. *Different letters* between transesterification methods and within a fatty acid group indicate statistical differences as determined by Tukey's HSD *post hoc* test following a significant *F* value by One-way ANOVA. Data are presented as nmol FAME per 100 µL whole blood, means ± SD, $n = 7$. BF_3 boron trifluoride, H_2SO_4 sulfuric acid, *MW* microwave and *Std* standard control method

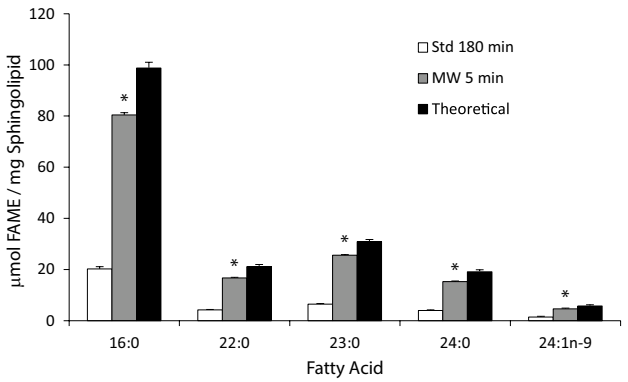

Fig. 4 Fatty acid methyl ester (FAME) yield from 200 nmol sphingomyelin standard following direct transesterification with 1 % sulfuric acid in methanol. *Indicates FAME significantly different than 3-h control values as determined by Tukey's HSD *post hoc* test following a significant *F* value by One-way ANOVA. Data are presented as µmol FAME per mg of standard, means ± SD, $n = 4$. *Std* standard control method, *MW* microwave

Microwave-Assisted Transesterification of Isolated Sphingomyelin and Sphingomyelin Standards

The 5-min 30-W microwave method was further assessed on both isolated sphingomyelin from whole blood (Fig. 3) and on pure sphingomyelin standards alone (Fig. 4) and spiked in whole blood to assess recovery (Fig. 5). Each of the four SFA (16:0, 22:0, 23:0 and 24:0) and one MUFA (24:1n-9) examined increased significantly with microwave-assisted transesterification compared with 3 h of the standard transesterification (Fig. 3). Using microwave energy increased fatty acid concentrations from sphingomyelin isolated from whole blood (nmol FAME/100 µL whole blood) by 56 % for 16:0, 97 % for 22:0, 109 % for 23:0, 92 % for 24:0 and 91 % for 24:1n-9 Similarly, the same fatty acids determined from pure sphingomyelin standards show higher yields with microwave energy (Fig. 4); however, these yields of 399 % for 16:0, 398 % for 22:0, 395 % for 23:0, 382 % for 24:0 and 322 % for 24:1n-9 are much higher compared with the whole blood sphingomyelin (Fig. 3). In addition, microwave-assisted transesterification yields fatty acid concentrations

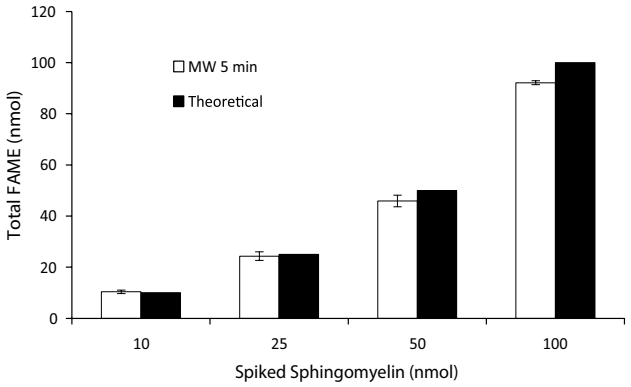

Fig. 5 Fatty acid methyl ester (FAME) yield from 25 µL whole blood spiked with 10, 25, 50 or 100 nmol sphingomyelin standard following direct transesterification with 1 % sulfuric acid in methanol. Data are presented as nmol FAME, means ± SD, $n = 4$. *MW* microwave

from the sphingomyelin standards that are 79–83 % of the calculated theoretical fatty acid concentrations compared to only 20–25 % for the 3 h conventional method.

Based on the results of the pure sphingomyelin standard transesterification, it is clear that under the assessed reaction conditions microwave energy provides significantly higher FAME yield than the standard 3-h method. To further assess the FAME yield from sphingomyelin, 10, 25, 50 and 100 nmol of was spiked in whole blood, transesterified for 5 min with 30 W power and compared to theoretical values. Fame yields are determined to be 104, 97, 92 and 92 % when whole blood was spiked with 10, 25, 50 and 100 nmol, respectively. Theoretical yields fall within one standard deviation for both the 10 and 25 nmol spiked whole blood, and below one standard deviation in both the 50 and 100 nmol spiked whole blood.

Discussion

In the present study, microwave transesterification was tested as a potential method to significantly reduce time and monetary commitment for fatty acid analysis. Presently, microwave-assisted direct transesterification of whole blood on chromatography paper at 30 W with 1 % H_2SO_4 in methanol for 5 min or less is demonstrated to be a viable replacement method to the standard three-hour block-heater method for quantitative and qualitative fatty acid assessments. Microwave-assisted direct transesterification can increase fatty acid yield due to a substantially improved FAME production from sphingomyelin.

Fingertip prick samples are limited to qualitative assessments as volume or mass of blood collected on chromatography paper can be difficult to determine. For this reason, qualitative results have been included to illustrate the increased usefulness of microwave-assisted direct transesterification with whole blood on chromatography paper.

Results of technical replicates from a single subject suggests that transesterification may be further reduced to as little as 1 min with no differences compared to standard methods for qualitative assessments (Table 2), or 1.5 min for quantitative assessments using variable power and 5 % H_2SO_4 (Table 1). However, additional analyses in a larger cohort is required to confirm this. Nevertheless, this suggests that an individual's omega-3 status, including a DBS collection followed by rapid transesterification time of no more than one minute—and potentially less—combined with fast gas chromatography [26] could provide an assessment of cardiovascular disease risk [10] in less than 1 h. This rapid profiling and reduced monetary requirement makes fingertip prick blood collections an exciting tool for future use in clinical settings to provide rapid feedback to patients in need of dietary improvement or compliance confirmation.

Very-long chain SFA and MUFA as well as 16:0 appear to be driving the increase in fatty acid yields during the 4 and 5 min reaction times with the 30 W + 1 % H_2SO_4 reactions in technical replicates, and the 5-min reaction time in a small cohort. Interestingly, 22:0, 23:0, 24:0 and 24:1n-9 represent only 3 % of all SFA and MUFA in these blood samples as determined by the standard 3 h method, however, these same fatty acids are responsible for approximately 27 % of the observed increase in SFA and MUFA during the 5 min microwave method. N-6 PUFA are generally not present in sphingomyelin and the significant increase in total n-6 PUFA representing 27 % of the overall increase with the microwave method cannot be explained solely by an improvement in sphingomyelin transesterification. However, it suggests that improved yields by microwave-assisted direct transesterification may occur in additional lipid fractions such as phospholipids, triacylglycerols or cholesteryl esters, although these contributions appear relatively minor. More research is required to confirm these findings.

Contrary to our findings with sphingomyelin, at least three previous studies have shown a reduced yield for very-long chain SFA and MUFA with the use of microwave-assisted transesterification both in serum/plasma and whole blood [1, 6, 16]. Most recently, two studies from one research group using a similar but multi-modal microwave system from the same company found yields of 70–90 % for 20:0, 22:0, 24:0 and 24:1n-9 [6] and 20–70 % for the same fatty acids in an earlier study [16]. These two studies employed acetyl chloride in methanol (~11 % by volume) as per the method of Lepage and Roy [27], and contrary to what we show in the present study, increasing the reaction time had no effect on fatty acid yield. The discrepancy

between the studies is most likely related to the use of different transesterification reagents and/or microwave settings. These differences can make comparison between studies difficult; however, it appears that the present microwave reaction conditions are more optimal for complete FAME yield, particularly in very-long chain SFA and MUFA derived from sphingomyelin.

Very-long chain SFA and MUFA methyl esters yields appear particularly responsive to transesterification with microwave energy and are located abundantly in sphingolipids. Fatty acids from sphingomyelin are 14 % 22:0, 9 % 23:0, 24 % 24:0 and 13 % 24:1n-9 in rat liver [28], with 16:0 (22 %) also found in large amounts. Reaction temperatures with microwave energy are approximately 95 °C higher than the standard method, and this is likely to play a part in the improved fatty acid yield. Future studies should attempt to control for this and attempt to elucidate the contribution of microwave energy to FAME yield independent of reaction temperature. The finding that these sphingomyelin-related fatty acids are primarily responsible for the improved FAME yield from microwave-assisted direct transesterification led us to test the FAME yields from sphingomyelin isolated from whole blood and sphingomyelin standards. Approximately 5 % of whole blood samples are comprised of the sphingomyelin lipid fraction [29], and this is in relative agreement with our findings that approximately 4–6 % of whole blood fatty acids are derived from sphingomyelin, depending on the transesterification method employed. Interestingly, weight % of EPA + DHA in whole blood by standard 3 h convection (4.34 ± 0.09) methodology appears to be significantly overestimated by 6 % in our technical replicates compared to the 5 min microwave-assisted method (4.13 ± 0.12) (data not shown), reflecting incomplete sphingomyelin transesterification by the standard method. In addition, erythrocyte sphingomyelin content may be as high as 18 % of total lipids [30], and such overestimations may be compounded even further when assessing % EPA + DHA in erythrocytes as is the case with the commonly utilized omega-3 status assessment tool, the omega-3 index [31].

We hypothesized that the improvement in fatty acid yield, particularly in SFA and MUFA, with microwave-assisted direct transesterification techniques may be due to a more effective transesterification of the sphingomyelin fraction in whole blood. In fact, microwave energy with 30 W + 1 % H_2SO_4 for 5 min increased individual FAME yield by 56–109 % in isolated sphingomyelin from whole blood and by 322–399 % in a mixture of chicken egg and bovine milk sphingomyelin standards. While the increase in recovery is much higher with the sphingomyelin standards, numerous plausible explanations exist. The higher total sphingolipids present in the standards (200 nmol) *versus* 100 μL whole blood (40 nmol) may result in a saturation of the standard conductive transesterification process with sphingomyelin standards that is overcome with microwave-assistance. The microwave-assisted transesterification may also become saturated, albeit to a lesser extent and at higher sphingomyelin levels. Sphingomyelin FAME yields from spiked whole blood were 92–104 % of theoretical values, representing complete transesterification of 10 and 25 nmol spikes and near complete transesterification of 50 and 100 nmol spikes, and 200 nmol of pure standard yielded 80 % of total expected FAME. In addition, sphingomyelin bands from silica gel TLC plates—either by co-elution with similar polarity and easily transesterified phospholipids or with endogenous fatty acids that cannot be 'washed' prior to lipid separation—may be contaminated with non-sphingomyelin fatty acids. Due to the relatively low levels of sphingomyelin in whole blood, these contaminant fatty acids may represent significant portions of the isolated sphingomyelin, and due to their ease of transesterification would result in an artificially high FAME determination by conventional methods.

Sphingomyelin-derived fatty acids are bound by an amide (C–N) bond as opposed to an ester bond (C–O–C) in glycerol-bound fatty acids. Sphingolipids have been demonstrated to be more difficult to transesterify [11] which may be due to the need to break the more stable amide bond. This increased amide bond stability compared to ester bonds is due to resonance stabilization in which the double bond between the carbon and oxygen atoms bond is shared between the oxygen and nitrogen (C=O ⟷ C=N) [32]. Presently, the microwave energy applied to the blood samples for transesterification appears sufficiently greater than convectional block heater energy resulting in the breakage of the amide bond and more complete transesterification of these sphingomyelin-derived fatty acids.

Using the variable power microwave method improves fatty acid yield compared with the 30-W constant power method as the variable power method reaches maximum temperature and pressure settings sooner. Increasing the H_2SO_4 concentrations from 1 % to 2, 5, and 10 % H_2SO_4 in methanol generally increases the fatty acid yield up to 5 % H_2SO_4, after which 10 % H_2SO_4 yields significantly lower total n-6 PUFA, n-3 PUFA, PUFA and HUFA. A similar decrease in n-3 PUFA and HUFA yield is also demonstrated using the same variable power method with 5 % H_2SO_4 in methanol and extending the reaction time from 2 to 3 min. The losses in fatty acid yields appear to occur only in PUFA and HUFA and suggests that balancing time and H_2SO_4 composition used in microwave analysis is crucial for the prevention of fatty acid peroxidation during transesterification. It is noted elsewhere that dilute sulfuric acid, such as the 1 and 2 % H_2SO_4 in methanol used here, exhibits no oxidizing properties [33]. However, in concentrated form it is capable of oxidizing many substances, and

under the conditions in the present study H_2SO_4 concentrations of 5 and 10 % in methanol appear capable of oxidizing PUFA in as little as 3 and 2 min, respectively.

This appears to be the first report demonstrating that microwave transesterification increases whole blood fatty acid yield through increased sphingomyelin fatty acid transesterification, and reduced yields with higher H_2SO_4 compositions alone or in combination with longer reaction times through fatty acid degradation. The microwave system utilized here, however, is limited by the microwave apparatus itself as only one sample at a time can be analyzed, and is further limited by internally set pressure limits that reduce the amount of power and heat that the sample may be exposed to. Previous studies have demonstrated that multi-sample microwave-assisted direct transesterification systems can be applied to whole blood samples to significantly increase throughput by increasing the number reactions performed at a given time [6, 16], albeit with different transesterification reagents.

In the present study, methods for the microwave-assisted direct transesterification of whole blood on chromatography was developed and compared to conventional methods. Reaction times are reduced significantly from 3 h to 1 min for microwave-assisted direct transesterification with H_2SO_4 in methanol without any significant changes in either quantitative or qualitative fatty acid profiles. Higher throughput DBS analysis such as the microwave-assisted direct transesterification method tested presently makes omega-3 health screening tests a more realistic pursuit as the cost and time of analysis may continue to drop allowing for high-throughput omega-3 fatty acid profiling for the purpose of disease risk detection assessment. We believe this to be the first study demonstrating that higher fatty acid yields with microwave-assisted direct transesterification is due to higher transesterification of sphingomyelin-bound fatty acids.

Acknowledgments This project was supported by funding from an Early Researcher Award from the Ontario Ministry of Research and Innovation and a Business Investment Program award from Health Technology Exchange to K.D.S. In addition, basic infrastructure was purchased through Canada Foundation of Innovation and the Ontario Research Fund matching grants. Doctoral research funding was provided by the Natural Sciences and Engineering Research Council to A.H.M.

Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest in connection with the manuscript.

References

1. Armstrong JM, Metherel AH, Stark KD (2008) Direct microwave transesterification of fingertip prick blood samples for fatty acid determinations. Lipids 43:187–196
2. Marangoni F, Colombo C, Galli C (2004) A method for the direct evaluation of the fatty acid status in a drop of blood from a fingertip in humans: applicability to nutritional and epidemiological studies. Anal Biochem 326:267–272
3. Agostoni C, Galli C, Riva E, Colombo C, Giovannini M, Marangoni F (2005) Reduced docosahexaenoic acid synthesis may contribute to growth restriction in infants born to mothers who smoke. J Pediatr 147:854–856
4. Agostoni C, Galli C, Riva E, Rise P, Colombo C, Giovannini M, Marangoni F (2011) Whole blood fatty acid composition at birth: from the maternal compartment to the infant. Clin Nutr 30:503–505
5. Fratesi JA, Hogg RC, Young-Newton GS, Patterson AC, Charkhzarin P, Block TK, Sharratt MT, Stark KD (2009) Direct quantitation of omega-3 fatty acid intake of Canadian residents of a long-term care facility. Appl Physiol NutrMetab 34:1–9
6. Lin YH, Hanson JA, Strandjord SE, Salem NM, Dretsch MN, Haub MD, Hibbeln JR (2014) Fast transmethylation of total lipids in dried blood by microwave irradiation and its application to a population study. Lipids 49:839–851
7. Bailey-Hall E, Nelson EB, Ryan AS (2008) Validation of a rapid measure of blood PUFA levels in humans. Lipids 43:181–186
8. Marangoni F, Colombo C, Galli C (2005) A method for the direct evaluation of the fatty acid status in a drop of blood from a fingertip in humans. World Rev Nutr Diet 94:139–143
9. Metherel AH, Armstrong JM, Patterson AC, Stark KD (2009) Assessment of blood measures of n-3 polyunsaturated fatty acids with acute fish oil supplementation and washout in men and women. Prostaglandins LeukotEssentFatty Acids 81:23–29
10. Albert CM, Campos H, Stampfer MJ, Ridker PM, Manson JE, Willett WC, Ma J (2002) Blood levels of long-chain n-3 fatty acids and the risk of sudden death. N Engl J Med 346:1113–1118
11. Morrison WR, Smith LM (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with borontrifluoride-methanol. J Lipid Res 5:600–608
12. Dugan LR Jr, McGinnis GW, Vadehra DV (1966) Low temperature direct methylation of lipids in biological materials. Lipids 1:305–308
13. Metherel AH, Aristizabal Henao JJ, Stark KD (2013) EPA and DHA levels in whole blood decrease more rapidly when stored at -20 °C as compared with room temperature, 4 and -75 °C. Lipids 48:1079–1091
14. Ohta A, Mayo MC, Kramer N, Lands WE (1990) Rapid analysis of fatty acids in plasma lipids. Lipids 25:742–747
15. Banerjee P, Dawson G, Dasgupta A (1992) Enrichment of saturated fatty acid containing phospholipids in sheep brain serotonin receptor preparations: use of microwave irradiation for rapid transesterification of phospholipids. Biochim Biophys Acta 1110:65–74
16. Lin YH, Loewke JD, Hyun DY, Leazer J, Hibbeln JR (2012) Fast transmethylation of serum lipids using microwave irradiation. Lipids 47:1109–1117
17. Alvarez SB, Priego CF, Luque de Castro MD (2008) Ultrasonic enhancement of leaching and *in situ* derivatization of haloacetic acids in vegetable foods prior to gas chromatography-electron capture detection. J Chromatogr A 1201:21–26
18. Li J, Yue Y, Hu X, Zhong H (2009) Rapid transmethylation and stable isotope labeling for comparative analysis of fatty acids by mass spectrometry. Anal Chem 81:5080–5087
19. Li J, Yue Y, Li T, Hu X, Zhong H (2009) Gas chromatography-mass spectrometric analysis of bonded long chain fatty acids in a single zebrafish egg by ultrasound-assisted one-step transmethylation and extraction. Anal Chim Acta 650:221–226
20. Liu L, Li Y, Feng R, Sun C (2010) Direct ultrasound-assisted methylation of fatty acids in serum for free fatty acid determinations. Can J Chem 88:898–905

21. Mahesar SA, Sherazi ST, Abro K, Kandhro A, Bhanger MI, van de Voort FR, Sedman J (2008) Application of microwave heating for the fast extraction of fat content from the poultry feeds. Talanta 75:1240–1244
22. Virot M, Tomao V, Ginies C, Visinoni F, Chemat F (2008) Microwave-integrated extraction of total fats and oils. J Chromatogr A 1196–1197:57–64
23. Perez-Serradilla JA, Ortiz MC, Sarabia L, Luque de Castro MD (2007) Focused microwave-assisted Soxhlet extraction of acorn oil for determination of the fatty acid profile by GC-MS. Comparison with conventional and standard methods. Anal Bioanal Chem 388:451–462
24. Ortiz MA, Dorantes AL, Gallndez MJ, Cardenas SE (2004) Effect of a novel oil extraction method on avocado (Persea americana Mill) pulp microstructure. Plant Foods Hum Nutr 59:11–14
25. Christie WW (2003) Lipid Analysis. The Oily Press Bridgewater, UK
26. Masood A, Stark KD, Salem N Jr (2005) A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. J Lipid Res 46:2299–2305
27. Lepage G, Roy CC (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res 25:1391–1396
28. Fex G (1971) Metabolism of phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin in regenerating rat liver. Biochim Biophys Acta 231:161–169
29. Christie WW (1985) Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. J Lipid Res 26:507–512
30. Talbott CM, Vorobyov I, Borchman D, Taylor KG, DuPre DB, Yappert MC (2000) Conformational studies of sphingolipids by NMR spectroscopy II. Sphingomyelin. Biochim Biophys Acta 1467:326–337
31. Harris WS, Von Schacky C (2004) The Omega-3 Index: a new risk factor for death from coronary heart disease? Prev Med 39:212–220
32. Martin RB (2001) Peptide bond characteristics. MetIons Biol Syst 38:1–23
33. Luque de Castro MD, Luque Garcia JL (2002) Acceleration and automation of solid sample treatment. Elsevier Amsterdam, NED