



The Open Nutrition Journal

Content list available at: www.benthamopen.com/TONUTRJ/

DOI: 10.2174/1874288201711010017



LETTER

A Rapid and Simple Method for Fatty Acid Profiling and Determination of ω -3 Index in Red Blood Cells

Olufunmilola Akinyemi¹, Geza Bruckner¹, John Johnson³, Terry A. Lennie² and David Hildebrand^{3,*}¹Department of Clinical Sciences, University of Kentucky, Lexington, KY, USA²College of Nursing, University of Kentucky, Lexington, KY, USA³Department of Plant & Soil Sciences, University of Kentucky, Lexington, KY, USA

Received: July 15, 2016

Revised: December 23, 2016

Accepted: December 25, 2016

Abstract: Fatty acid profiling has become a very useful and effective tool in the diagnosis, prevention and treatment of several diseases with cardiovascular disease being particularly important. In order to arrive at accurate conclusions that would help promote the health of individuals plagued by such diseases, not only excellent laboratory methods are required, but also very important monitoring responses to treatment. Improvements in methods of fatty acid profiling in biological systems regarding safety of extraction, precision and time for analysis are valuable. The ω -3 index (a measure of the amount of eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA, in Red Blood Cell membranes expressed as the percent of total fatty acids) is of growing interest because it has been reported to provide prognostic information regarding the risk for heart diseases. Sodium methoxide has been widely used for the determination of ω -3 fatty acids in food samples. This study demonstrates that sodium methoxide can be used effectively in RBC fatty acid profiling and determination of the ω -3 index. Briefly, the fatty acid profiles and ω -3 index of red blood cell samples were analyzed and results compared using three different methods: a two- step extraction and methylation method described by Hara and Radin, a single step extraction and methylation method described by Harris *et al.* and the sodium methoxide method.

Our results revealed that there were no statistically significant differences ($p < 0.05$) between the three methods for the representative fatty acids, [16:0 ($p = 0.10$), 18:0 ($p = 0.40$), 18:1(ω 9) ($p = 0.29$), 18:2(ω 6) ($p = 0.95$), 18:3(ω 3) ($p = 0.50$), 20:5(ω 3) ($p = 0.56$), 22:6(ω 3) ($p = 0.06$)] and ω -3 index ($p = 0.11$) except for 20:4(ω 6), ($P = 0.02$). In conclusion, we show that sodium methoxide can be used effectively in a one-step extraction and methylation procedure for high throughput analysis of fatty acids in red blood cell membranes. It is rapid (10 minute extraction and methylation), simple, safer than and as accurate as other commonly reported methods.

Keywords: Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), Omega-3 index, Sodium methoxide, Erythrocytes.

INTRODUCTION

Fatty acids constitute an important structural element of biological membranes, serving many vital functions in biological systems such as providing energy sources and acting as signaling molecules [1].

The fatty acid composition of the red blood cell membrane has been reported to be an invaluable tool in providing prognostic information regarding the risk for coronary heart disease, cancers, neuropsychiatric diseases, accelerated cellular aging and early mortality [2 - 7].

Of growing interest is the omega-3 Index (a measure of the amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in Red Blood Cell membranes expressed as the percent of total fatty acids) which has been shown to be inversely proportional to the risk for sudden cardiac death [2, 8, 9]. This has led to the increased

* Address correspondence to this author at the Department of Plant & Soil Sciences, University of Kentucky, Lexington, KY 403 PSB, USA; Tel: 859/218-0760, Fax: 859/2577848; E-mail: dhild@uky.edu

awareness of the health benefits of foods rich in EPA and DHA with an ultimate aim of increasing blood levels of these fatty acids and hence increasing the omega-3 Index [10, 11].

Without a full knowledge of the total fatty acid composition of Red Blood Cell membranes, it would be almost impossible to calculate the omega-3 index. This requires the need for efficient laboratory methods that would enable high throughput analysis of fatty acids in biological systems.

FATTY ACID COMPOSITION IN BIOLOGICAL SYSTEMS

Studies determining the fatty acid composition of a myriad of biological samples have previously been reported. Samples studied for example include: skin, breast milk, sperm, cheek cells, monocytes, neutrophils, T-lymphocytes, B-lymphocytes, red blood cells and whole blood [12 - 16]. However, the most often reported biological samples used in determination of fatty acid composition in relation to nutritional status are adipose tissue, plasma, platelets and erythrocytes [12].

A vivid picture of the long-term dietary intake, with particular emphasis on long-chain omega-3 polyunsaturated fatty acids, can be obtained by deducing the concentrations of fatty acids in red blood cells (RBCs) [17, 18]. The fatty acids present in erythrocyte membrane phospholipids can be incorporated during *de novo* synthesis or *via* exchange from the pool of fatty acids in the plasma [12]. The membrane fatty acid composition is a dynamic system, one that keeps changing based on selective recruitment of fatty acids from precursor pools [19]. The fatty acid composition of plasma phospholipids poorly reflect long-term dietary intake (as a result of the fast turnover) while those of red blood cells portray dietary fat intake during the preceding months.

The fatty acid composition of phospholipids contributes considerably to membrane properties and also, is peculiar to which class it belongs [12]. Studies have been carried out to determine the total erythrocyte membrane fatty acids as well as the proportions that make up each phospholipid class. Agren *et al.* [20] and Hodson *et al.* [12] reported data showing the percentages of fatty acids that comprise the erythrocyte membranes and phospholipid classes (Table 1).

Table 1. Total erythrocyte membrane fatty acid compositions and percentages of erythrocyte phospholipid classes. Results are expressed in mol%, mean \pm SD.

Fatty Acid	ET ¹	ET ²	PE ²	PC ²	PS ²
16:0	25.6	23.0 \pm 1.1	15.7 \pm 1.2	34.7 \pm 1.5	3.5 \pm 0.9
18:0	14.3	15.0 \pm 0.6	8.1 \pm 1.0	10.0 \pm 1.1	39.4 \pm 1.2
18:1 n-9	14.3	14.0 \pm 0.6	16.8 \pm 1.3	16.3 \pm 0.4	6.6 \pm 0.7
18:2 n-6	11.2	11.6 \pm 1.1	6.6 \pm 1.2	22.6 \pm 2.0	2.2 \pm 0.2
18:3 n-3	0.1	0.1 \pm 0.1	0.2 \pm 0.1	0.3 \pm 0.1	-
20:3 n-6	1.0	1.5 \pm 0.2	1.3 \pm 0.3	2.0 \pm 0.5	1.9 \pm 0.4
20:4 n-6	14.1	13.7 \pm 1.0	23.8 \pm 1.8	5.8 \pm 1.2	23.1 \pm 1.7
20:5 n-3	0.8	1.4 \pm 0.4	2.9 \pm 0.9	1.1 \pm 0.4	0.9 \pm 0.2
22:5 n-3	1.6	2.5 \pm 0.2	5.1 \pm 0.5	0.6 \pm 0.1	4.0 \pm 0.6
22:6 n-3	3.9	6.7 \pm 0.9	12.6 \pm 1.6	3.0 \pm 0.6	14.1 \pm 1.4

Abbreviations: ET, Erythrocyte Total; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; ¹[12]; ²[20]

The data presented in Table 1 are based on results from two studies [12, 20]. The amounts reported for total erythrocyte membrane fatty acids from the Hodson *et al.* study are comparable to those from Agren *et al.* There are similar amounts of total fatty acids 16:0, 18:0, 18:1 n-9, 18:2 n-6, 18:3 n-3, 20:5 n-3 with 16:0 being the most abundant fatty acid. When comparing total erythrocyte fatty acid composition with the fractions PE, PC and PS, there are some remarkable differences. PE has a lower abundance of 16:0, 18:0, 18:2 n-6 and 20:3 n-6 compared to the total fatty acid composition. PC has higher abundance of 16:0 and 18:2 n-6. These data are in agreement with other reported studies [21 - 24].

METHODS OF DETERMINING FATTY ACID COMPOSITION

Scientists have worked extensively to improve laboratory methods used to measure the fatty acid content of biological samples with a goal to developing newer methods that would; (i) improve the extraction and derivatization efficiency of lipids, (ii) be less time-consuming, (iii) cost-effective and, (iv) most importantly be safe.

Conventionally, fatty acid analysis involves procedures that consist of an initial extraction step, followed by derivatization of fatty acids (FAs) to fatty acid methyl esters (FAMES), and subsequent chromatographic determination of FAMES (1).

Previous studies have shown that fatty acid analysis of human red blood cell can be determined by a two-step extraction and methylation or a combined one-step extraction and methylation procedure [17, 20, 25, 26].

METHYLATION PROCEDURES

Several different methylating agents have been used in determining the fatty acid composition of RBC. Of notable interest is the use of 14% boron trifluoride/methanol which is widely used in the transmethylation of fatty acids in red blood cells [1, 17, 27, 28]. Other methylating reagents used include acetyl chloride [29], 2% sulfuric acid in methanol [30] and sodium methoxide [16, 31].

In recent years, various studies have suggested that a combined one-step extraction and methylation procedure is faster and simpler in many ways when compared to the conventional method. Kang and Wang described a simplified method for analysis of polyunsaturated fatty acids in which they compared the conventional two-step method (prior extraction with chloroform/methanol and subsequent methylation with 14% boron trifluoride/methanol) to a combined extraction-methylation procedure. It was concluded from his study that prior lipid extraction in lipid analysis can be omitted without affecting the recovery of long chain FAs [32]. K. Eder, in his review, stated that direct transesterification proceeds at a faster rate than saponification, with hydrolysis and esterification taking place in a single step, requiring only one reagent [26]. However, accuracy of any laboratory method should be checked with the use of primary lipid standards prior to the analysis of biological samples. Incomplete recovery of FAMES from lipid standards would necessitate optimization of analytical conditions before trans-esterification of the sample [26].

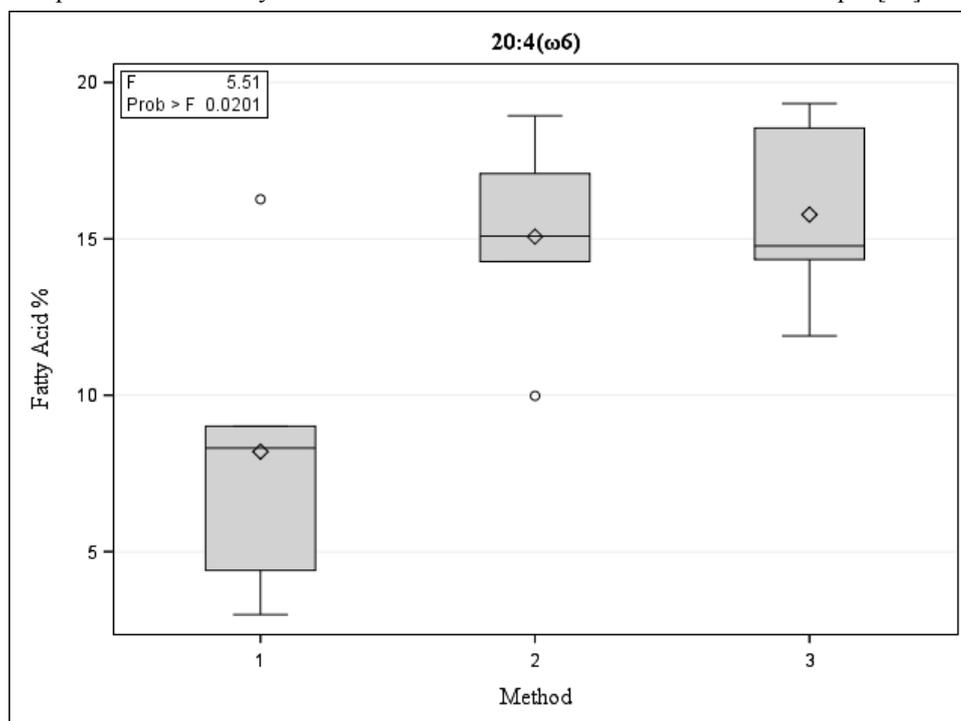


Fig. (1). Box plot showing comparison of fatty acid 20:4 (ω 6) for the three methods, $p=0.02$.

Direct trans-esterification of lipids can either be acid or base-catalyzed. Reagents commonly used for acid catalyzed trans-esterification are: methanolic hydrochloric and sulfuric acid [33]; and boron trifluoride in methanol [10, 34, 35] whereas reagents that have been used for base-catalyzed trans-esterification of biological samples are sodium methoxide [16, 31] and potassium hydroxide in methanol [6]. Boron trifluoride in methanol (12-14%) is the most often used for transesterification of RBC lipids [26]. Several other reagents used for trans-esterification of human and plant samples include acetyl chloride [17, 26, 36, 37] and aluminum chloride [26, 38].

The aim of this study was to determine if the sodium methoxide method can be used effectively in a one-step extraction and methylation procedure for high throughput analysis of fatty acids in red blood cell membranes when compared with the notable Hara and Radin [39] and the Harris *et al.* method [10].

MATERIALS AND METHODS

Materials

- FAME standard mixtures were obtained from Nu-Chek Prep, Inc. of Elysian, MN. Standard mixtures used were: GLC-538, GLC-455, GLC-480 and O8C. Standards were prepared by initially dissolving them in chloroform followed by serial dilution with isooctane. The selected GC conditions were sufficient to provide baseline resolution of all components of the above standard mixtures except for 24:0, 22:3n3 (co-elution) and 22:4n6 (partial resolution).
- Sodium methoxide prepared in our lab. Briefly, stock solution was prepared by dissolving 18 g of sodium (stored under toluene) in 1L of methanol. Care was taken to avoid moisture contamination due to the ability of sodium metal to react exothermically with water to produce sodium hydroxide and the flammable hydrogen gas.
- Methanol containing 14% boron trifluoride, hexane (Fisher Scientific).

Laboratory Methods

Whole blood was centrifuged at 1,300 g for 10 minutes. Red blood cells (RBC) were isolated, washed twice with phosphate buffered saline and aliquoted into 10 centrifuge tubes (0.5 mL each). 100 μ L of 2.5% BHT (Butylated hydroxytoluene) was added, for each mL of blood, as an antioxidant. Thereafter, the packed red blood cells were stored under argon gas at -80°C prior to analysis. The fatty acid profiles as well as the ω -3 index of the red blood cells were determined and compared using three methods.

Method 1: The Hara and Radin Method (Hara and Radin, 1978; (39-41) (Two-Step Extraction and Methylation)

Extraction was accomplished using 3:2 hexane isopropanol and the subsequent methylation carried out with 14% boron trifluoride in methanol*. Briefly, to 50- μ L of RBC*, 2mL of hexane:isopropanol followed by 2mL of hexane was added. The mixtures were vortexed well and upper hexane layer was transferred to screw capped tubes. Volume of upper hexane layer was reduced to \leq 0.25mL by blowing with nitrogen gas. Methylation was carried out as in Method 2 with 14% boron trifluoride in methanol.

Method 2: The Described Method of Harris et al. (10) (One-Step Extraction and Methylation)

14% boron trifluoride was used*.

To 50- μ L RBC* in screw capped tubes, 250- μ L 14% boron trifluoride in methanol followed by 250- μ L hexane was added. Tubes were flushed with argon and sealed. Mixture was briefly vortexed and heated at 100°C for 10 minutes. After cooling, 2mL isooctane + 0.001% BHT was added and vortexed. Thereafter, upper isooctane layer was transferred into GC vials for analysis.

Method 3: Sodium Methoxide Method (One-Step Extraction and Methylation)

To 50- μ L aliquot of RBC* in open glass tubes, 0.5 mL sodium methoxide was added. The mixtures were vortexed for about 10-20 seconds and placed on a shaker for 10 minutes. Thereafter, 2 mL isooctane + 0.001% BHT was added and then vortexed well. The upper isooctane layer was transferred into GC vials for analysis.

Fatty Acid Composition Analysis

FAMES were analyzed using a Varian 3800 Gas Chromatograph equipped with a model 1177 split/splitless injector and FID detector. The injector temperature was 240°C and the detector temperature was 260°C. Sample volumes of between 1.5 μ L and 2.0 μ L were injected in splitless mode using a Varian model 8400 autoinjector. The chromatographic column used was an Agilent VF-23ms of length 30m, inside diameter of 0.25mm and a film thickness of 0.25 microns. The carrier gas was hydrogen held at a constant flow of 1.6 mL/min using electronic flow control. The column oven temperature profile was: 80°C initial hold for 1.0 min, then programmed to 120°C at 10°C/min, then to 204°C at 4°C/min and then to 250°C at 20°C/min with a final hold of 7 min (total run time of 35.3 min). The total run time was sufficient to elute large sterol peaks. Data were compared by peak area against the peak area of the standard mixture of fatty acids characteristic of RBCs. Results are expressed as means \pm SE.

*0.2 μ g/ μ L RBC 1,2-diheptadecanoyl phosphatidylcholine (di-17:0-PC) was added to all the RBC samples as a standard for quantitative determinations.

Calculation of the ω 3 index: Amounts of EPA + DHA/Total fatty acids x 100%.

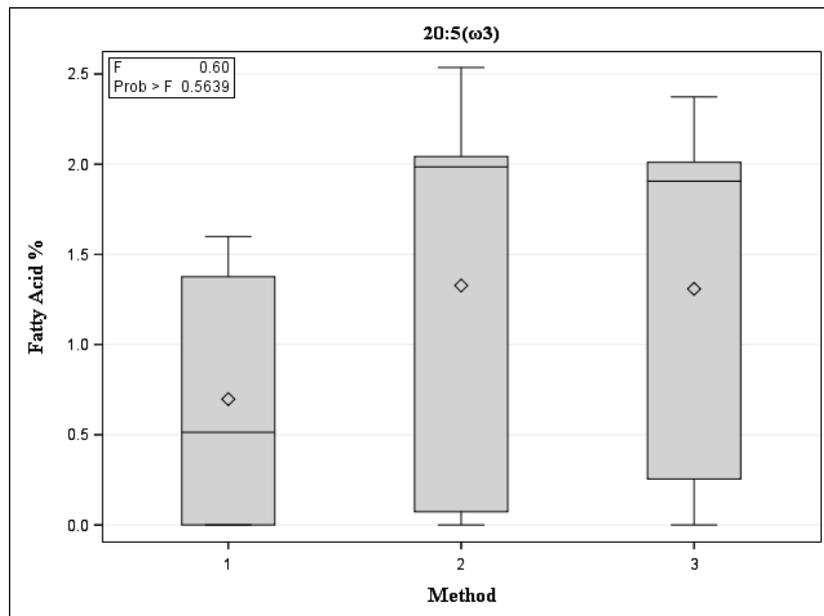


Fig. (2). Box plot showing comparison of fatty acid 20:5 (ω 3) for the three methods, $p=0.56$.

STATISTICAL ANALYSIS

Statistical analysis to determine differences between methods was investigated using SAS version 9.4 by subjecting collected data to analysis of variance (ANOVA) and two-sample t-tests were used to compare the means. P values of < 0.05 were considered significant.

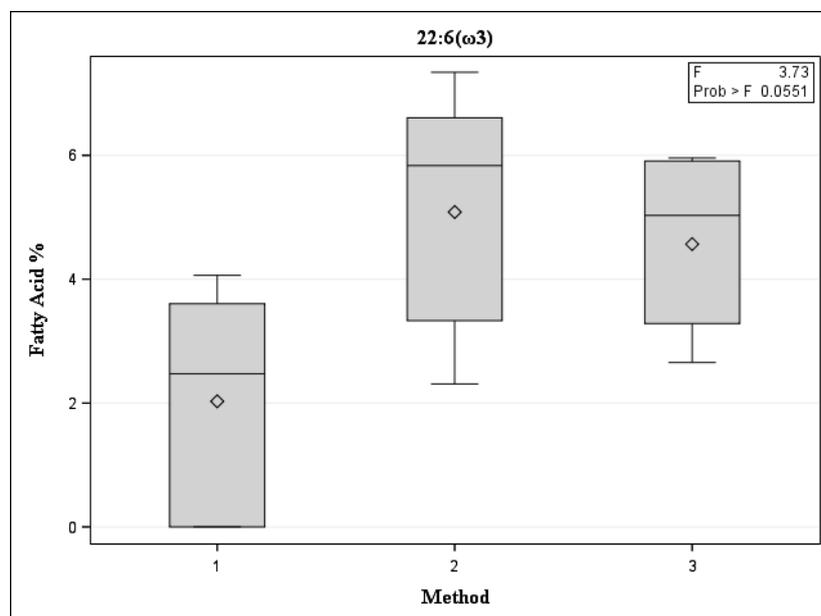


Fig. (3). Box plot showing comparison of fatty acid 22:6 (ω 3) for the three methods, $p=0.06$.

RESULTS AND DISCUSSION

The results show that there were no statistically significant differences between the three methods for the fatty acids, [16:0 ($p=0.10$), 18:0 ($p=0.40$), 18:1(ω 9) ($p=0.29$), 18:2(ω 6) ($p=0.95$), 18:3(ω 3) ($p=0.50$), 20:5(ω 3) ($p=0.56$), 22:6(ω 3) ($p=0.06$)] and ω -3 index ($p = 0.11$) except for 20:4(ω 6), ($P=0.02$). Our results are in agreement with previously reported data of total erythrocyte membrane fatty acid composition [12, 20, 21]. Although there were no statistically significant differences between the three methods, Method 1 yielded lower values for the ω -3 fatty acids 20:5(ω 3) and 22:6(ω 3)

when compared with Methods 2 and 3 (Figs. 1-3). We also observed wide variability between the measured values for fatty acids 20:5(ω 3) and 22:6(ω 3). This accounts for the low ω -3 index value reported for Method 1 because the omega-3 index value is calculated using these two fatty acids (Fig. 4).

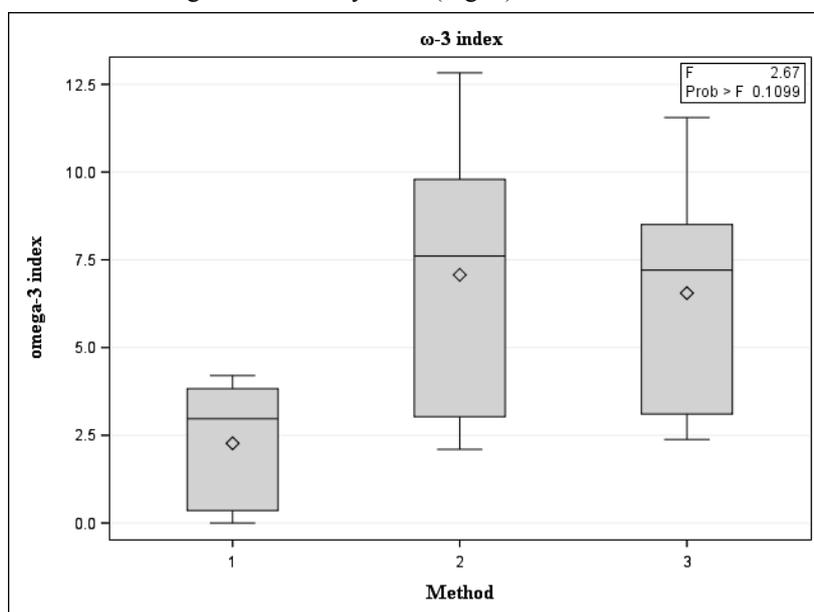


Fig. (4). Box plot showing comparison of ω -3 index values for the three methods, $p=0.11$.

From the comparison of results obtained by measuring a representative set of red blood cell fatty acids and ω -3 index, we observed that there were no significant differences between the Hara and Radin [39], Harris *et al.* [10] and the sodium methoxide methods as shown in Table 2. The use of sodium methoxide for methylation of the red blood cells has not in any way altered the derivatization of fatty acid methyl esters as there was no difference in relative fatty acid composition between the three methods. Our results also agree with the findings of Kang and Wang [32] that a prior extraction step is not necessary in fatty acid profiling of red blood cells and can be conveniently omitted to yield desirable outcomes.

Table 2. Quantitative results of fatty acid analysis by the three methods. The percent of each fatty acid was calculated by dividing its absolute amount by the total amount of fatty acids. Percent values are expressed as means \pm SE of three ($n=5$) measurements.

Fatty Acids	Method 1	Method 2	Method 3
16:0	31.8 \pm 2.0 ^a	27.7 \pm 2.0	25.2 \pm 2.0
18:0	24.4 \pm 1.7 ^a	21.7 \pm 1.7 ^a	21.2 \pm 1.7 ^a
18:1 (9)	17.1 \pm 1.1 ^a	16.6 \pm 1.1 ^a	19.1 \pm 1.1 ^a
18:2 (6)	12.2 \pm 0.9 ^a	12.0 \pm 0.9 ^a	12.3 \pm 0.9 ^a
18:3 (3)	0.8 \pm 0.3 ^a	0.4 \pm 0.3 ^a	0.3 \pm 0.3 ^a
20:4 (6)	8.2 \pm 1.8 ^a	15.1 \pm 1.8 ^b	15.8 \pm 1.8 ^b
20:5 (3)	0.7 \pm 0.4 ^a	1.3 \pm 0.4 ^a	1.3 \pm 0.4 ^a
22:6 (3)	2.0 \pm 0.8 ^a	5.1 \pm 0.8 ^a	4.6 \pm 0.8 ^a
ω -3 index	2.3 \pm 1.6 ^a	7.1 \pm 1.6 ^a	6.6 \pm 1.6 ^a

^{a,b}Values for each fatty acid with the same letter do not differ significantly between the methods.

Desirable aspects of Method 3 that makes it highly recommended and a better choice over Methods 1 and 2 are:

- It proceeds at room temperature and does not require heating.
- It is safer and can be used in very inexpensive open test tubes. Methods 1 and 2 require use of screw-capped tubes for the methylation step because 14% boron trifluoride is highly volatile and toxic if inhaled. Also the tubes can burst due to the pressure buildup.
- It is rapid with extraction and methylation in 10 minutes.
- It costs less because sodium methoxide has a longer shelf life when compared with 14% boron trifluoride-methanol (about 4 months and has to be refrigerated at all times) [40 - 42] and sodium methoxide is much less expensive especially when prepared from sodium and methanol as described herein.

- Sodium methoxide does not liberate aldehydes from plasmalogens as compared with the use of boron trifluoride in methanol [26, 43]. Hence, dimethyl acetals (DMAs) are not formed during esterification. This is especially important when working with Red Blood Cells [26, 31, 43 - 45].
- Using the sodium methoxide method should yield more consistent results in comparison with Method 1 as depicted by the high variability seen with results from Method 1. This could be attributed to the use of a single tube in the Method 2 and 3 experiments as opposed to Method 1 that requires two steps and the changing of tubes in between extraction and methylation phases. During the change of tubes, there may be partial loss of the samples, which can lead to incomplete recovery of FAMES.
- Method 2 described by Harris [10] is very similar to the simplified method for analysis of polyunsaturated fatty acids which was described by Kang and Wang [32]. However, the major difference in the protocol is the length of time required for the heating phase. Kang and Wang heated the samples at 100°C for 1 hour while Harris *et al.* heated at the same temperature for 10 mins. The differences in samples analyzed might be accountable for the differences in the heating times.
- Our results not only show that sodium methoxide is an effective methylating agent in RBC fatty acid analysis, they also further validate that a prior extraction step can be conveniently omitted to achieve the desired outcome. An overview of common reagents that have been used to analyze RBC fatty acids over the past 20 years is summarized in Table 3.

Table 3. Studies with common transesterification agents used for RBC fatty acid profiling over 20 years.

References	Prior extraction	Transesterification agent
[34]	Yes	14% boron trifluoride in methanol
[35]	Yes	14% boron trifluoride in methanol
[10]	No	14% boron trifluoride in methanol
[33]	Yes	1% sulfuric acid in methanol
[25]	Yes	14% borontrifluoride in methanol
[44]	No	20% boron trifluoride in methanol or 14% boron trichloride in methanol
[6, 32]	Yes	Potassium hydroxide in methanol
[32]	No	14% boron trifluoride in methanol
[45]	Yes	5% sulfuric acid in methanol
[20]	Yes	14% boron trifluoride in methanol

CONCLUSION

In conclusion, our results demonstrate that sodium methoxide can be used effectively in a single step extraction and methylation process for red blood cell fatty acid analysis. This was validated *vs.* the notable Hara and Radan [39] method and the Harris *et al.* method [10]. Furthermore, we have described in detail the steps involved in using sodium methoxide not previously reported. This simplified sodium methoxide method is less time consuming, safer, less expensive and yields results not significantly different from the well-known use of boron trifluoride-methanol. It allows for high throughput analysis of fatty acids including long chain fatty acids which are of utmost importance in calculating the omega-3 index in erythrocytes.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by the National Institute of Nursing Research, 5R01NR013430-03. We gratefully acknowledge the expert assistance and advice of Scott Serdoz, Hirotada Fukushima, Maythem AL-Amery and Will Serson. Special thanks to Sarah Janse of the Statistics Department, University of Kentucky for her immense help.

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