METHODS

# An Improved Method for Determining Medium- and Long-Chain FAMEs Using Gas Chromatography

Zhidong Xu · Kevin Harvey · Thomas Pavlina ·
Guy Dutot · Gary Zaloga · Rafat Siddiqui

**Abstract** The existing protocols for analyzing fatty acid methyl esters (FAMEs) using a one-step acetyl chloride (AC) catalyzed transesterification and extraction procedure cannot accurately determine the medium- and long-chain fatty acids simultaneously in clinical (enteral, parenteral) formulations. For example: (1) addition of AC at room temperature generates an exothermic reaction that often results in loss of sample and possible injury to the analyst; (2) certain polyunsaturated fatty acids (PUFAs) are less stable at elevated temperatures during the transesterification and contribute to the over-estimation of the C16:0 and C18:1 fatty acids; and (3) the flame-ionization detector (FID) response varies depending on the carbon chain length of the fatty acids, that consequently impacts the underestimation of medium-chain fatty acid (C6–C10) recoveries. To overcome these deficiencies and accurately determine FAMEs, we have developed an improved one-step transesterification method that employs the addition of AC in tubes kept on a dry ice bath, the transesterification at room temperature, and the data analysis using relative response factors. Using this modified protocol, we determined the fatty acid composition of lipid emulsions (Omegaven® and Lipidem®) on a Shimadzu GC2010 gas chromatography (GC) system using a capillary GC column (Zebron ZB-WAX plus, 30 m, 0.25 mm ID, 0.25 μm). Our data suggest that the improved method can be easily used to accurately determine fatty acids (C6–C24) in functional foods and lipid emulsions.

**Abbreviations**

| | |
|---|---|
| AC | Acetyl chloride |
| AOAC | Association of Analytical Chemists |
| Calc. | Calculations |
| Exp | Experimental |
| FAME | Fatty acid methyl esters |
| FAs | Fatty acids |
| FID | Flame ionization detector |
| GC | Gas chromatography |
| IS | Internal standard |
| PUFAs | Polyunsaturated fatty acids |
| REF | Relative response factor |
| Theo | Theoretical |

Z. Xu · K. Harvey · R. Siddiqui
Methodist Research Institute, Clarian Health,
Indiana University School of Medicine,
Indianapolis, IN 46202, USA

T. Pavlina · G. Zaloga
Baxter Healthcare Corporation, Deerfield, IL, USA

G. Dutot
Baxter SAS, Maurepas Cedex, France

R. Siddiqui
Department of Medicine, Indiana University School
of Medicine, Indianapolis, IN 46202, USA

R. Siddiqui (✉)
Cellular Biochemistry Laboratory,
Methodist Research Institute,
E504D, 1800N Capitol Ave, Indianapolis, IN 46202, USA
e-mail: rsiddiqui@clarian.org

## Introduction

Absolute accuracy is important when analyzing the fatty acid (FA) composition of functional foods and clinical dietary formulations. Lipids in functional food and dietary

formulations contain a wide variety of fatty acids differing in chain length, degree of unsaturation and position, and configuration of double bonds. Although a gas chromatography/mass spectrometry method has been developed to quantitatively determine C8–C26 chain length fatty acids [1], the GC analysis of FAMEs with FID remains the most frequently used method [2, 3]. The results from GC/FID are often expressed as a relative percentage of total fatty acids, which may potentially contribute to error in data interpretation [1]. The accurate quantification of FAs in biological samples depends on proper extraction, methylation of FAs into FAMEs, optimized GC run conditions and calculation of their concentration using internal and external standards [4–6].

The one-step digestion, extraction and esterification of biological samples, which is referred to as "direct transesterification," is widely used because of its simplicity, rapidity and higher accuracy [7–9]. This method has some complications. For example: addition of acetyl chloride at room temperature generates an exothermic reaction that often results in loss of sample and possible injury to the analyst; adding the acetyl chloride slowly with stirring is often cumbersome for large numbers of samples; certain polyunsaturated fatty acids are less stable at temperatures of 100 °C during the transesterification process; and the generation of molecular species that may contribute to the over-estimation of fatty acids [10]. Furthermore, the FID response varies with carbon-chain variation. The FID response is based on ionization of alkyl carbon entities, whereas the carboxyl carbon is not ionized during the combustion [11, 12]. To accurately determine FAMEs with a FID, a correction factor is normally applied based on a theoretical relative response factor ($RRF_{(Theo)}$) to compensate for the unionized carboxyl carbon [11, 13]. The widely used $RRF_{(Theo)}$ was originally calculated based on "active atom" theory without considering the instrument conditions [6, 14]. However, it has been realized that the instrument parameters, sample running conditions and the fatty acid composition in samples can influence the RRF and hence may affect the analysis [15–20].

To overcome these deficiencies and accurately determine medium- and long-chain FAMEs, we have developed an improved one-step transesterification method that employs the addition of acetyl chloride in a dry ice bath, transesterification at room temperature and data analysis using the experimental relative response factor ($RRF_{(Exp)}$) based on relative peak area ratios of the individual fatty acid to the internal standard (IS), C23:0, under optimized GC run conditions. Our data suggest that our improved protocol for FAME analysis accurately determines both medium- and long-chain fatty acids in a one-step transesterification and extraction procedure. This procedure can easily be adopted to perform an accurate FAME analysis on large numbers of clinical and research samples on a routine basis in less time and without any threat of hazards to the analyst.

## Materials and Methods

### Materials

Fatty acid standards, the internal standard, and FAME mixture (GLC-461A) were purchased from Nu-Chek (Nu-Chek Prep, Inc., USA). The purity of all fatty acid standards was at least 99%. Benzene (CHROMASOLV® Plus, for HPLC, ≥99.9%), hexane (CHROMASOLV®, for HPLC, ≥97.0%), chloroform (anhydrous, ≥99%), methanol (anhydrous, 99.8%) and acetyl chloride (Puriss. p.a., ≥99.0%) were purchased from Sigma–Aldrich (St Louis, MO, USA). A FocusLiner for the GC-2010 system was purchased from Supelco (Sigma–Aldrich), and a Zebron ZB-WAX plus (30 m, 0.25 mm ID, 0.25-μm film thickness) capillary GC column was purchased from Phenomenex (Torrance, CA, USA). Lipid emulsions: Lipidem® and Omegaven®, were from B. Braun Melsungen AG (Germany) and Fresenius Kabi Deutschland GmbH (Germany), respectively. The internal standard of C23:0-M (tricosanoate-methyl ester) was dissolved in the hexane solution at a concentration of 0.982 mg/ml, and the internal standard of C23:0-acid (C23:0-A) was dissolved in the methanol-benzene (4:1, v/v) solution at a concentration of 0.989 mg/ml. All fatty acid standards were flushed with $N_2$ and stored at −20 °C.

### Instrumentation

GC chromatography was performed with a Shimadzu GC2010 chromatography system (Shimadzu Scientific Instruments, Columbia, MA, USA) equipped with an auto sampler and a flame ionization detector. Helium was used as carrier and make-up gas. The injection volume was 1 μl, which was used with a split ratio of 1:50, or alternative ratios as reported elsewhere in the text. The injection port and detector temperatures were 240 and 250 °C, respectively. The column temperature program was as follows: temperature was held at 30 °C for 2 min, increased to 180 °C at 20 °C/min, held at 180 °C for 2 min, increased to 207 °C at 4 °C/min, held at 207 °C for 3 min, increased to 220 °C at 2 °C/min, held at 220 °C for 2 min, and then increased to 240 °C at 2 °C/min before finally being held at 240 °C for 2 min.

### Optimization of the One-Step Extraction Transesterification Process
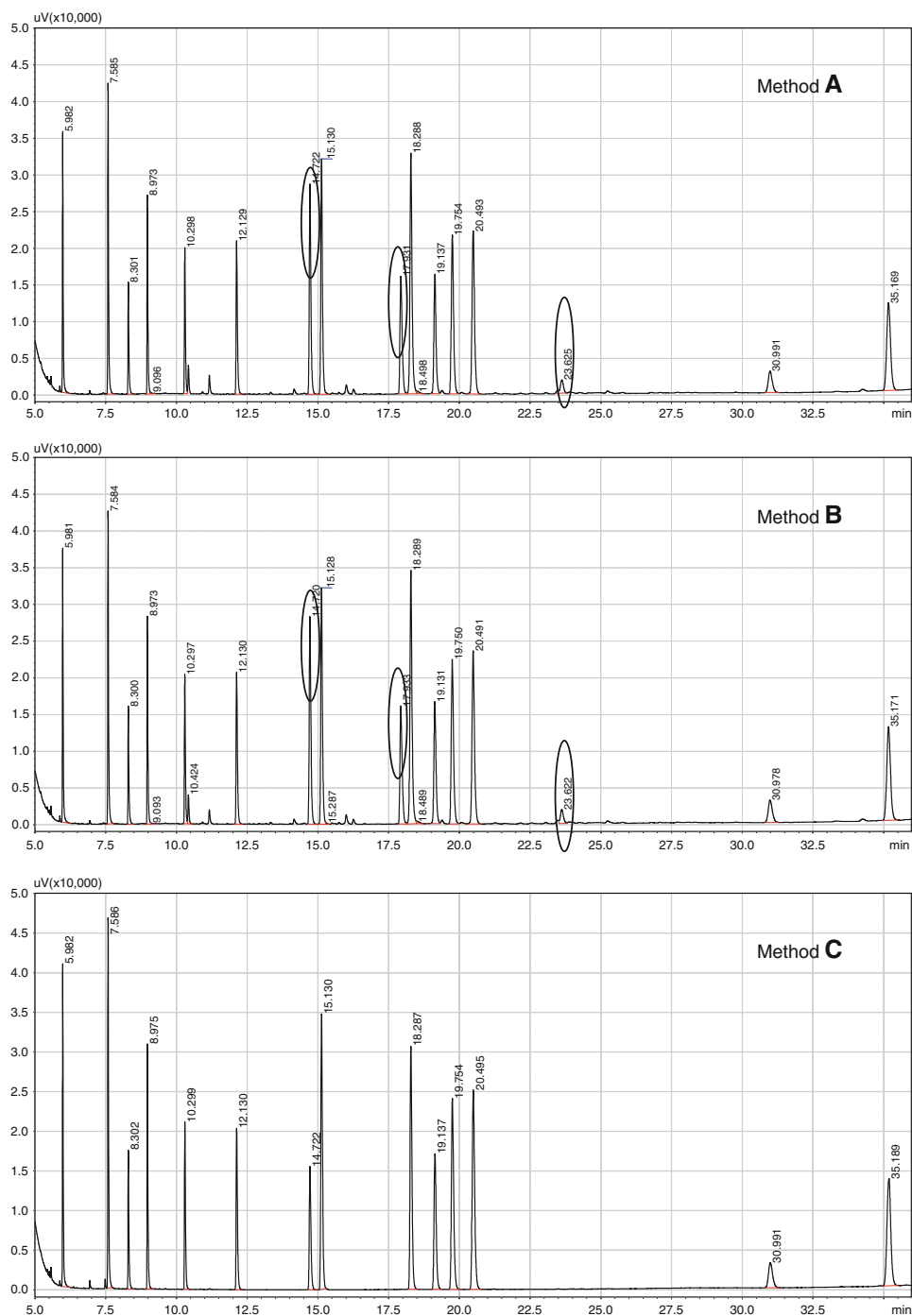
#### Method A

A 50-μl lipid sample was placed in 10 × 130 mm Pyrex tubes (10 ml) which had Teflon-lined screw caps, and then mixed with 40 μl of the internal standard and 2 ml of methanol-benzene (4:l, v/v). A small magnetic stirring bar

was placed in each tube and, while stirring, 200 µl of acetyl chloride was slowly added over a period of 1 min. The tubes were tightly closed and subjected to transesterification at 100 °C for 1 h. The tubes were allowed to cool in an ice bath, and then 5 ml of 6% $K_2CO_3$ solution was added slowly to stop the reaction and neutralize the mixture. The tubes were briefly vortexed and then centrifuged at $900 \times g$ for 10 min (at 25 °C). The top layer was collected and transferred to a sample vial for GC analysis.

**Table 1** Fatty acid standard mixture

| FA | (mg/ml) | FA | (mg/ml) |
|---|---|---|---|
| C6:0 | 1.67 | C16:1n-7 | 2.38 |
| C8:0 | 1.77 | C18:1n-9 | 2.38 |
| C9:0 | 0.71 | C18:2n-6 | 1.50 |
| C10:0 | 1.18 | C18:3n-6 | 2.17 |
| C12:0 | 0.96 | C18:3n-3 | 2.49 |
| C14:0 | 1.15 | C22:6n-3 | 2.30 |
| C16:0 | 1.04 | | |



**Fig. 1** Comparison of transesterification methods. Standard mixture (listed in Table 1) is used to prepare FAMEs using transesterification Methods A, B or C, as described in the text. The major extra peaks are marked by *black circles*. The GC chromatogram is a representative of three experiments performed in triplicate (*n* = 9)

## Method B

A 50-µl sample of the lipid mixture was placed in 10 × 130 mm Pyrex tubes (10 ml) which had Teflon-lined screw caps, and then mixed with 40 µl of the internal standard and 2 ml of methanol-benzene (4:1, v/v). The tubes were gently vortexed, and then placed in a dry ice bath for 10 min. Then 200 µl of acetyl chloride was added to the tubes quickly; the tubes were flushed with N$_2$, tightly closed, and subjected to transesterification at 100 °C for 1 h. The samples were further processed as described in "Method A".

## Method C

The samples were prepared and acetyl chloride was added to each tube as described in "Method B". The tubes were then kept in the dark at room temperature for 24 h. The samples were further processed as described in "Method A".

## Data Quantification

The concentration of individual fatty acids ($C_{(GC)}$) were calculated using the expression $C_{(GC)} = A_{(FA)}/A_{(IS)} \times C_{(IS)}$, where $A_{(FA)}$ is the GC peak area of the fatty acid whose concentration is to be determined, $A_{(IS)}$ is the GC peak area of the internal standard, and $C_{(IS)}$ is the concentration (mg/ml) of the internal standard.

## Determination of the Relative Response Factor

### Theoretical Relative Response Factor

Theoretical relative response factor for each fatty acid (C6–C24) relative to the C23:0 internal standard was calculated according to the active carbon theory [12].

### Experimental Relative Response Factor

For determining the RRF$_{(Exp)}$, 130 µl of the GLC-461A reference standard in hexane was mixed with 20 µl of internal standard (C23:0-M; 0.982 mg/ml in hexane) and subjected to the GC analysis. The ratio of the fatty acid to the internal standard, and the instrument parameters, were as indicated elsewhere in the text. Each experiment was done in triplicate. The relative response factor was calculated based on the equation: $RF = C_{(Theo)}/C_{(GC)}$, where $C_{(Theo)}$ is the concentration of the fatty acid based on its weighed amount, and $C_{(GC)}$ is the concentration of the fatty acid calculated based on the GC peak area and internal standard (C23:0) concentration.

## Results and Discussion

### Optimization of GC Parameters

Based on the recommendations proposed in the official method of the association of analytical chemists (AOAC) [21], we employed C23:0-M as the internal standard, and a wax-type capillary gas chromatograph column to perform the fatty acid analysis. We initially compared the GC analysis of fatty acids with the splitless and split injection modes, using different inlet liners (including no wool, wool-packed, gooseneck and FocusLiner). Our results (not shown) demonstrated that GC analysis with split injection at 250 °C using a FocusLiner inlet yields optimal fatty acid resolution. Most of the analyses were performed using a 1:50 split ratio based on optimization described below.

### Optimization of a One-Step Transesterification

In order to develop a safe, simple and highly reproducible transesterification method for accurately determining FA concentrations for routine analysis, we modified and optimized the original Lepage and Roy one-step transesterification method [8, 9]. A standard mixture containing C6 to C22 fatty acids, as shown in Table 1, was used to optimize the transesterification using three different approaches, referred to as Methods A, B and C. "Method A" is the original Lepage and Roy method wherein the addition of acetyl chloride for transesterification is usually done very

**Table 2** Recovery of fatty acids using different transesterification methods

| FA | RRF$_{(Exp)}$ | Method A %, mean ± SD | Method B %, mean ± SD | Method C %, mean ± SD |
|---|---|---|---|---|
| C6:0 | 1.44 | 94.1 ± 1.4 | 92.2 ± 2.7 | 100.4 ± 2.3 |
| C8:0 | 1.20 | 91.6 ± 2.0 | 90.1 ± 2.9 | 97.4 ± 2.4 |
| C9:0 | 1.19 | 93.4 ± 1.8 | 92.3 ± 2.6 | 97.7 ± 2.2 |
| C10:0 | 1.16 | 93.1 ± 1.9 | 92.0 ± 2.8 | 98.5 ± 2.2 |
| C12:0 | 1.15 | 94.8 ± 1.6 | 93.9 ± 3.6 | 99.8 ± 2.1 |
| C14:0 | 1.14 | 104.4 ± 3.9 | 105.4 ± 4.3 | 104.2 ± 2.5 |
| C16:0 | 1.12 | 151.1 ± 38.8 | 168.8 ± 19.6 | 105.3 ± 3.6 |
| C16:1n-7 | 1.12 | 99.1 ± 0.7 | 98.1 ± 2.3 | 103.1 ± 2.4 |
| C18:1n-9 | 1.07 | 110.3 ± 14.8 | 114.6 ± 8.4 | 101.2 ± 2.8 |
| C18:2n-6 | 1.07 | 97.9 ± 0.6 | 97.5 ± 2.5 | 102.0 ± 2.2 |
| C18:3n-6 | 1.07 | 97.3 ± 1.7 | 96.5 ± 2.3 | 102.2 ± 2.2 |
| C18:3n-3 | 1.08 | 95.9 ± 1.5 | 95.3 ± 2.4 | 100.8 ± 2.2 |
| C22:6n-3 | 1.16 | 94.1 ± 2.5 | 93.0 ± 2.4 | 100.1 ± 2.1 |

The concentration of the fatty acid was calculated based on the internal standard peak area and corrected with RRF$_{(Exp)}$, as describe in the text. The recovery was calculated by formula [$C_{(Exp)}/C_{(Theo)} \times 100$]
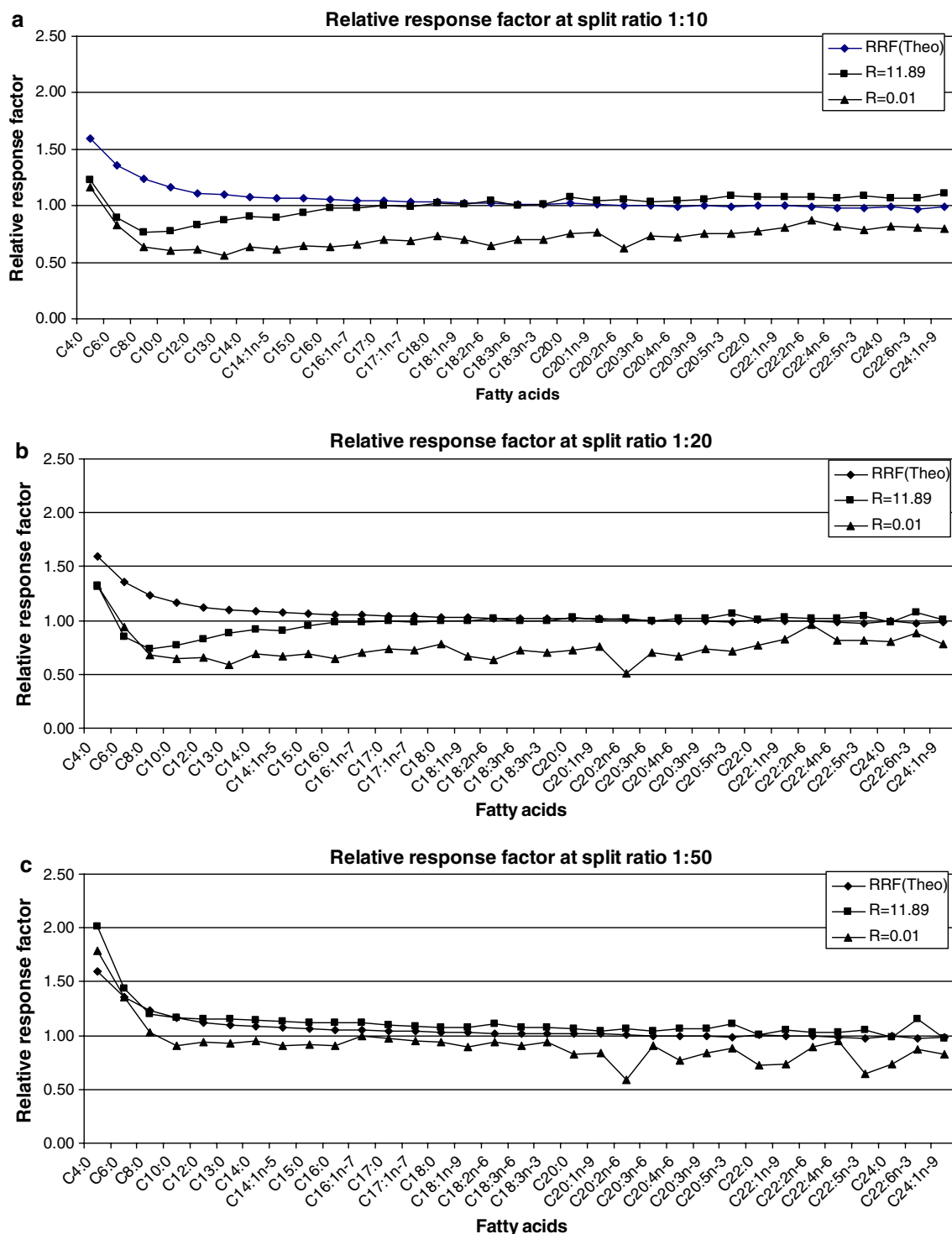
**Fig. 2** Effect of injector split ratio on the relative response factor in the presence of variable fatty acid-to-internal standard (FA$_{(Sample)}$/FA$_{(IS)}$) ratio. Fatty acid-methyl esters from GLC-461A standard mixture (FA$_{sample}$) and C23:0-M (FA$_{IS}$) internal standard (0.982 mg/ml) was prepared in hexane to obtained variable (0.01–11.86) FA$_{(Sample)}$/FA$_{(IS)}$ ratios ($R$) as shown in Table 3. Each mixture (FA$_{(Sample)}$/FA$_{(IS)}$) was applied for GC analysis using an injector split ratio of **a** 1:10, **b** 1:20, or **c** 1:50, while keeping all other GC run parameters conditions constant. The curves representing the lowest (0.01) and the highest (11.89) $R$ are shown for simplicity. All other curves (data not shown) fall within the lowest and the highest $R$ curves. Each *point* represents a mean of three experiments

**Relative response factor and split ratio**
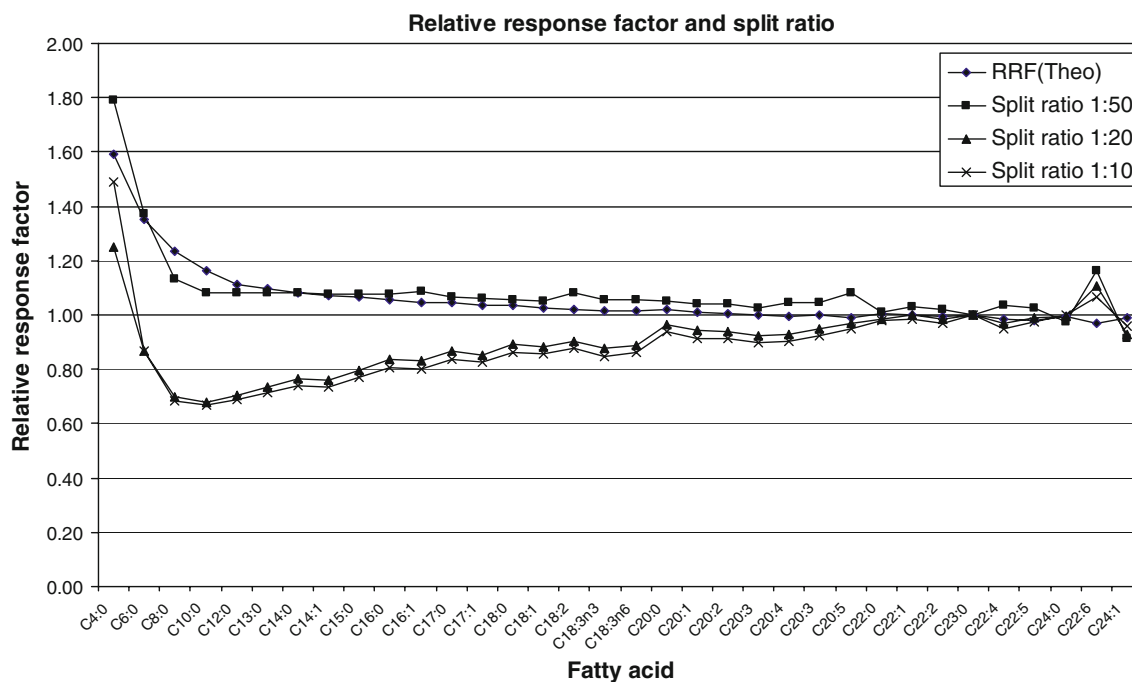


**Fig. 3** Effect of injector split ratio on the relative response factor in the presence of a fixed fatty acid-to-internal standard (FA$_{(Sample)}$/FA$_{(IS)}$) ratio. The GLC-461A standard mixture and internal standard (C23:0-M, 0.982 mg/ml) was prepared in hexane to obtained a fixed (0.44) FA$_{(Sample)}$/FA$_{(IS)}$ ratio. Samples were run using a split ratio of 1:5, 1:10, 1:20, or 1:50, while keeping all other GC run parameters constant. Each *point* represents a mean of three experiments

carefully and slowly to avoid an exothermic reaction. This is usually done while the reaction contents are kept stirring, which takes approximately 1 min to complete. The exothermic nature of the reaction sometimes results in loss of sample because of the splatter, and may also pose a threat of possible injury to the analyst. This procedure is not only cumbersome when a large number of samples are to be analyzed, but transesterification using "Method A" also resulted in the formation of extra peaks, as shown in Fig. 1a. We therefore modified the method and incubated the tubes for transesterification on a dry ice bath for 10 min prior to adding acetyl chloride ("Method B"). The results shown in Fig. 1b also resulted in the formation of extra peaks, as in "Method A". We further modified the procedure, and in "Method C", after adding acetyl chloride in the reaction mixture on a dry bath, the transesterification was performed at room temperature for 24 h. Results shown in Fig. 1c clearly indicate that the extra peaks were no longer present on the chromatogram. These results suggest that the extra peaks may have resulted from side reactions at an elevated temperature. The recoveries of the individual fatty acids using Methods A, B, or C are shown in Table 2. The data demonstrate that the presence of extra peaks induced by transmethylation at 100 °C (Methods A and B) interfere with the estimation of fatty acids, and resulted in overestimation of particularly C16:0 and C18:1n-9. Comparing the data from Methods A and B, it appears that there are no significant differences between acetyl chloride added at

room temperature or at low temperature; however, addition of acetyl chloride at low temperature avoids the need of its dropwise addition and constant stirring. We also evaluated the effect of the amount of water present (1–5%) in the transesterification reaction using "Method C". Our results demonstrate that the water content (up to 5% in the transesterification) does not significantly affect fatty acid analysis of the standard mixture or biological samples (data not shown).

Relative Response Factor

Accurate determination of fatty acid concentration by GC using FID not only depends on the optimized extraction/transesterification and instrument parameters, but is also contingent on the signal response of the FID detector to FAMEs of different composition. The early pioneer work by Ackman and Sipos [12], and Bannon [13], proposed using a theoretical relative response factor (RRF$_{(Theo)}$), based on the ionized carbon atoms, to correct the FID response to FAMEs of different composition. We realized from the previously published results [16–20], and also from our preliminary analysis, that the RRF for different FAMEs can be varied depending upon the GC run conditions, and therefore may cause an error in the determination of fatty acid concentrations in a given sample. The data shown in Fig. 2 indicates that when the FA$_{(Sample)}$:FA$_{(IS)}$ is varied, there was a variation in RRF$_{(Exp)}$, and this variation

**Table 3** Relative response factor ($RRF_{(Exp)}$) with variable $FA_{(Sample)}/FA_{(IS)}$ ratios at a split ratio 1:50

| FA | $RRF_{(Theo)}$ | $FA_{(Sample)}/FA_{(IS)}$ | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | 11.89 | 5.95 | 2.97 | 1.49 | 0.74 | 0.37 | 0.19 | 0.09 | 0.05 | 0.02 | 0.01 |
| | | $RRF_{(Exp)}$ | | | | | | | | | | |
| C4:0 | 1.59 | 2.01 | 2.02 | 2.04 | 2.09 | 2.03 | 2.03 | 2.10 | 1.99 | 1.92 | 1.73 | 1.79 |
| C6:0 | 1.35 | 1.44 | 1.43 | 1.44 | 1.43 | 1.40 | 1.40 | 1.40 | 1.35 | 1.34 | 1.23 | 1.36 |
| C8:0 | 1.23 | 1.20 | 1.19 | 1.20 | 1.17 | 1.12 | 1.10 | 1.07 | 1.05 | 1.02 | 0.93 | 1.03 |
| C10:0 | 1.16 | 1.16 | 1.15 | 1.16 | 1.15 | 1.11 | 1.03 | 0.99 | 0.98 | 0.96 | 0.90 | 0.91 |
| C12:0 | 1.11 | 1.15 | 1.14 | 1.15 | 1.16 | 1.13 | 1.05 | 0.99 | 0.96 | 0.95 | 0.89 | 0.94 |
| C13:0 | 1.10 | 1.15 | 1.15 | 1.16 | 1.17 | 1.14 | 1.05 | 0.99 | 0.94 | 0.90 | 0.80 | 0.93 |
| C14:0 | 1.08 | 1.14 | 1.13 | 1.14 | 1.16 | 1.14 | 1.06 | 1.01 | 0.99 | 0.96 | 0.91 | 0.95 |
| C14:1n-5 | 1.07 | 1.13 | 1.13 | 1.14 | 1.15 | 1.14 | 1.06 | 1.02 | 0.97 | 0.95 | 0.90 | 0.91 |
| C15:0 | 1.07 | 1.12 | 1.12 | 1.13 | 1.15 | 1.14 | 1.06 | 1.02 | 0.98 | 0.95 | 0.91 | 0.92 |
| C16:0 | 1.05 | 1.12 | 1.11 | 1.12 | 1.14 | 1.14 | 1.06 | 1.02 | 0.96 | 0.93 | 0.88 | 0.90 |
| C16:1n-7 | 1.05 | 1.12 | 1.12 | 1.13 | 1.15 | 1.15 | 1.07 | 1.03 | 0.98 | 0.96 | 0.86 | 1.00 |
| C17:0 | 1.04 | 1.10 | 1.09 | 1.10 | 1.12 | 1.12 | 1.06 | 1.02 | 0.98 | 0.94 | 0.89 | 0.97 |
| C17:1n-7 | 1.04 | 1.09 | 1.08 | 1.09 | 1.11 | 1.12 | 1.05 | 1.01 | 0.96 | 0.96 | 0.89 | 0.95 |
| C18:0 | 1.03 | 1.07 | 1.07 | 1.08 | 1.09 | 1.10 | 1.04 | 1.00 | 0.97 | 0.96 | 0.90 | 0.94 |
| C18:1n-9 | 1.03 | 1.07 | 1.07 | 1.08 | 1.09 | 1.10 | 1.03 | 1.00 | 0.94 | 0.90 | 0.81 | 0.89 |
| C18:2n-6 | 1.02 | 1.11 | 1.10 | 1.11 | 1.12 | 1.13 | 1.06 | 1.00 | 0.95 | 0.94 | 0.79 | 0.94 |
| C18:3n-6 | 1.01 | 1.08 | 1.07 | 1.08 | 1.09 | 1.10 | 1.03 | 1.00 | 0.94 | 0.93 | 0.90 | 0.91 |
| C18:3n-3 | 1.01 | 1.08 | 1.07 | 1.08 | 1.09 | 1.10 | 1.04 | 1.00 | 0.96 | 0.94 | 0.87 | 0.94 |
| C20:0 | 1.02 | 1.06 | 1.06 | 1.07 | 1.06 | 1.05 | 1.02 | 0.99 | 0.93 | 0.90 | 0.84 | 0.83 |
| C20:1n-9 | 1.01 | 1.04 | 1.04 | 1.05 | 1.06 | 1.07 | 1.02 | 0.99 | 0.94 | 0.90 | 0.90 | 0.84 |
| C20:2n-6 | 1.01 | 1.06 | 1.05 | 1.06 | 1.05 | 1.07 | 0.99 | 0.95 | 0.87 | 0.80 | 0.74 | 0.59 |
| C20:3n-6 | 1.00 | 1.04 | 1.03 | 1.04 | 1.04 | 1.06 | 1.01 | 0.98 | 0.94 | 0.89 | 0.86 | 0.90 |
| C20:4n-6 | 0.99 | 1.06 | 1.05 | 1.06 | 1.06 | 1.08 | 1.01 | 0.99 | 0.93 | 0.89 | 0.80 | 0.77 |
| C20:3n-9 | 1.00 | 1.06 | 1.05 | 1.06 | 1.06 | 1.08 | 1.03 | 0.98 | 0.93 | 0.91 | 0.80 | 0.84 |
| C20:5n-3 | 0.99 | 1.11 | 1.10 | 1.11 | 1.11 | 1.12 | 1.06 | 1.01 | 0.96 | 0.94 | 0.83 | 0.88 |
| C22:0 | 1.01 | 1.01 | 1.01 | 1.02 | 1.02 | 0.99 | 0.97 | 0.93 | 0.90 | 0.85 | 0.82 | 0.72 |
| C22:1n-9 | 1.00 | 1.05 | 1.04 | 1.05 | 1.04 | 1.05 | 0.99 | 0.96 | 0.90 | 0.86 | 0.84 | 0.74 |
| C22:2n-6 | 0.99 | 1.03 | 1.02 | 1.03 | 1.03 | 1.05 | 1.00 | 0.97 | 0.93 | 0.90 | 0.89 | 0.89 |
| C23:0 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| C22:4n-6 | 0.98 | 1.03 | 1.02 | 1.03 | 1.04 | 1.06 | 1.00 | 1.00 | 0.98 | 0.95 | 0.95 | 0.95 |
| C22:5n-6 | 0.98 | 1.05 | 1.04 | 1.05 | 1.05 | 1.06 | 1.00 | 0.95 | 0.90 | 0.85 | 0.79 | 0.64 |
| C24:0 | 0.99 | 0.98 | 0.97 | 0.98 | 0.98 | 0.96 | 0.95 | 0.91 | 0.89 | 0.86 | 0.83 | 0.73 |
| C22:6n-3 | 0.97 | 1.15 | 1.15 | 1.16 | 1.15 | 1.18 | 1.07 | 1.02 | 0.97 | 0.89 | 0.88 | 0.87 |
| C24:1n-9 | 0.99 | 0.97 | 0.94 | 0.95 | 0.96 | 0.95 | 0.91 | 0.88 | 0.83 | 0.82 | 0.79 | 0.83 |

in $RRF_{(Exp)}$ was also influenced by the split ratio used for injection (Fig. 2a–c). It is obvious from these results that there are differences between $RRF_{(Exp)}$ and $RRF_{(Theo)}$ values, which are more pronounced for medium-chain FAs, and become smaller as the carbon-chain length increases. The effect of injection split ratios on $RRF_{(Exp)}$ was also determined using a fixed (0.44) fatty acid to internal standard ($FA_{(Sample)}/FA_{(IS)}$) ratio. The data reported in Fig. 3 indicate that as the split ratio increases, the $RRF_{(Exp)}$ value becomes closer to that of $RRF_{(Theo)}$. Data from Figs. 2 and 3 demonstrated that the optimal $RRF_{(Exp)}$ could

be obtained when a split ratio of 1:50 was used; however, the $RRF_{(Exp)}$ was still influenced by the $FA_{(Sample)}:FA_{(IS)}$ ratio. We therefore determined $RRF_{(Exp)}$ over a wide range of $FA_{(Sample)}:FA_{(IS)}$ ratios for each fatty acid. The data presented in Table 3 indicate that as the $FA_{(Sample)}:FA_{(IS)}$ ratio varied, the RRF is also varied. Short- to medium-chain fatty acids (C4–C10), in particular, have much variation between $RRF_{(Theo)}$ and $RRF_{(Exp)}$, and may have potential for underestimation when only the $RRF_{(Theo)}$ is used to calculate their concentration. Based on these results, we suggest the GC analyst should first determine the $RRF_{(Exp)}$

value under optimum GC run conditions for each fatty acid, and should use the $RRF_{(Exp)}$ closest to the $FA_{(Sample)}:FA_{(IS)}$ ratio for accurately computing each FA concentration.

Comparison of Methods for Data Calculation

Different approaches are used to quantify fatty acid compositions from the GC chromatograph. The concentration calculation based on the internal standard concentration and peak area offers reasonably accurate values, particularly for long-chain saturated and unsaturated fatty acids; but, this method would not give an accurate concentration for medium-chain fatty acids. One way to overcome this problem is to use multiple internal standards for FAs of different carbon chain lengths [6, 22, 23]. These data can be further corrected using a $RRF_{(Theo)}$ to get an estimation of fatty acid concentrations, including those of the medium-chain fatty acids. However, we realized that if an accurate estimation of short- to long-chain fatty acids is desired in a given sample, and accuracy is the ultimate priority, then concentrations corrected with a $RRF_{(Exp)}$ and/ or calculated with an external standard curve combined with the using of internal standard would be superior to calculating FA concentrations, as described above. We, therefore, compared FA data analysis methods inclusive of: Calc. I Fatty acid concentration calculated based on internal standard and peak area; Calc. II Fatty acid concentration calculated with Calc. I and corrected with the theoretical RRF; Calc. III Fatty acid concentration calculated with Calc. I and corrected with the experimental RRF; and Calc. IV Fatty acid concentration calculated based on the internal standard and external standard curves. Data representing fatty acid recovery were calculated with

the four data calculation methods (shown in Table 4). The data indicate that Calc. III and IV calculations produced similar results, which are the most consistent relative to the theoretical concentration of fatty acids.

Total Fatty Acid Analysis of Two Lipid Emulsions

Based on our current data, we suggest that our modification of a one-step transesterification process ("Method C") and use of Calc. III ($RRF_{(Exp)}$ instead of $RRF_{(Theo)}$) would yield a superior analysis of FAs. In order to confirm our data, we used two known lipid emulsions. Omegaven® contains 10% lipids as triglycerides enriched in n-3 PUFAs, whereas Lipidem® contains 20% lipids as triglycerides enriched in medium-chain fatty acids. Using these emulsion we further evaluated Methods A, B and C for transesterification. Fatty acid concentrations were calculated based on Calc. III, using $RRF_{(Exp)}$ obtained from Table 3 according to the $FA_{(Sample)}:FA_{(IS)}$ ratio of the samples. From the data presented in Table 5, we found that transesterification using Methods A and B resulted in a higher C16:0, C18:0 and C18:1n-9 concentration than that using "Method C". The "Method C" also produced a better recovery of n-3 PUFA. These results further confirmed that transesterification at 100 °C produces extra peaks that interfere with the estimation of some fatty acids. Furthermore, heating during transesterification also resulted in loss of the PUFAs, particularly C20:5n-3 and C22:6n-3.

In conclusion, an improved protocol collectively using "Method C" for a one-step transesterification and $RRF_{(Exp)}$ for data analysis accurately determined both medium- and long-chain fatty acids in commercial lipid emulsion

**Table 4** Comparison of calculation methods for fatty acid recovery

| FA | Calc. I | Calc. II | | Calc. III | | Calc. IV |
|---|---|---|---|---|---|---|
| | %, mean ± SD | $RRF_{(Theo)}$ | %, mean ± SD | $RRF_{(Exp)}$ | %, mean ± SD | %, mean ± SD |
| C6:0 | 69.7 ± 2.3 | 1.35 | 94.4 ± 2.3 | 1.44 | 100.4 ± 2.3 | 99.6 ± 2.8 |
| C8:0 | 81.2 ± 2.4 | 1.23 | 100.2 ± 2.4 | 1.20 | 97.4 ± 2.4 | 97.3 ± 3.2 |
| C9:0 | 82.1 ± 2.2 | 1.19 | 97.5 ± 2.2 | 1.19 | 97.7 ± 2.2 | 98.7 ± 2.3 |
| C10:0 | 84.9 ± 2.2 | 1.16 | 98.8 ± 2.2 | 1.16 | 98.5 ± 2.2 | 98.3 ± 3.3 |
| C12:0 | 86.7 ± 2.1 | 1.12 | 96.7 ± 2.1 | 1.15 | 99.8 ± 2.1 | 98.9 ± 3.2 |
| C14:0 | 91.4 ± 2.5 | 1.08 | 98.7 ± 2.5 | 1.14 | 104.2 ± 2.5 | 103.0 ± 3.4 |
| C16:0 | 94.0 ± 3.6 | 1.06 | 99.2 ± 3.6 | 1.12 | 105.3 ± 3.6 | 104.2 ± 3.7 |
| C16:1n-7 | 92.1 ± 2.4 | 1.05 | 96.4 ± 2.4 | 1.12 | 103.1 ± 2.4 | 105.2 ± 3.3 |
| C18:1n-9 | 94.6 ± 2.8 | 1.03 | 97.2 ± 2.8 | 1.07 | 101.2 ± 2.8 | 100.9 ± 3.2 |
| C18:2n-6 | 95.4 ± 2.2 | 1.02 | 97.4 ± 2.2 | 1.07 | 102.0 ± 2.2 | 101.2 ± 3.1 |
| C18:3n-6 | 95.6 ± 2.2 | 1.01 | 96.9 ± 2.2 | 1.07 | 102.2 ± 2.2 | 102.7 ± 3.2 |
| C18:3n-3 | 93.3 ± 2.2 | 1.01 | 94.6 ± 2.2 | 1.08 | 100.8 ± 2.2 | 102.1 ± 3.0 |
| C22:6n-3 | 86.3 ± 2.1 | 0.97 | 83.9 ± 2.1 | 1.16 | 100.1 ± 2.1 | 101.2 ± 2.9 |

Fatty acid mixture was transesterified using "Method C", and run at a split ratio of 1:50

**Table 5** Total fatty acid analysis of Omegaven and Lipidem

| Emulsion | Omegaven | | | Lipidem | | |
| --- | --- | --- | --- | --- | --- | --- |
| Method (mg/ml) | A Mean ± SD | B Mean ± SD | C Mean ± SD | A Mean ± SD | B Mean ± SD | C Mean ± SD |
| C6:0 | 0.22 ± 0.01 | 0.23 ± 0.01 | 0.16 ± 0.01 | 0.32 ± 0.00 | 0.34 ± 0.03 | 0.27 ± 0.00 |
| C8:0 | – | – | – | 49.75 ± 1.25 | 49.55 ± 2.74 | 50.45 ± 0.36 |
| C10:0 | – | – | – | 34.98 ± 0.84 | 34.87 ± 1.83 | 35.25 ± 0.31 |
| C12:0 | 0.09 ± 0.01 | 0.10 ± 0.01 | 0.08 ± 0.00 | 0.28 ± 0.01 | 0.28 ± 0.02 | 0.25 ± 0.02 |
| C14:0 | 4.48 ± 0.39 | 4.56 ± 0.29 | 4.25 ± 0.01 | 0.76 ± 0.03 | 0.53 ± 0.29 | 0.18 ± 0.01 |
| C15:0 | 0.36 ± 0.05 | 0.36 ± 0.04 | 0.32 ± 0.00 | – | – | – |
| C16:0 | 14.41 ± 3.64 | 14.69 ± 3.23 | 10.95 ± 0.09 | 17.85 ± 0.55 | 15.33 ± 2.91 | 11.04 ± 0.13 |
| C16:1n-7 | 7.00 ± 0.11 | 7.19 ± 0.14 | 7.08 ± 0.01 | 0.54 ± 0.00 | 0.45 ± 0.08 | 0.34 ± 0.01 |
| C16:2n-4 | 1.48 ± 0.38 | 1.47 ± 0.34 | 0.92 ± 0.02 | 0.71 ± 0.02 | 0.40 ± 0.31 | 0.06 ± 0.02 |
| C17:0 | 0.42 ± 0.17 | 0.43 ± 0.15 | 0.27 ± 0.01 | 0.37 ± 0.02 | 0.28 ± 0.14 | 0.10 ± 0.00 |
| C16:3n-4 | 0.85 ± 0.19 | 0.86 ± 0.21 | 0.81 ± 0.02 | 0.03 ± 0.00 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| C17:1n-7 | 0.22 ± 0.12 | 0.25 ± 0.13 | 0.28 ± 0.01 | 0.08 ± 0.00 | 0.07 ± 0.01 | 0.05 ± 0.00 |
| C16:4n-1 | 1.70 ± 0.01 | 0.65 ± 0.92 | 1.80 ± 0.01 | – | – | – |
| C18:0 | 8.33 ± 5.46 | 8.31 ± 5.05 | 2.58 ± 0.16 | 14.68 ± 0.50 | 10.97 ± 5.07 | 4.57 ± 0.17 |
| C18:1n-9 | 11.08 ± 2.12 | 11.35 ± 1.88 | 9.46 ± 0.10 | 21.57 ± 0.68 | 20.29 ± 0.97 | 18.34 ± 0.14 |
| C18:1n-7 | 3.03 ± 0.32 | 2.98 ± 0.23 | 2.75 ± 0.05 | 2.48 ± 0.56 | 2.24 ± 0.21 | 1.72 ± 0.04 |
| C18:2n-6 | 2.73 ± 0.11 | 2.81 ± 0.06 | 2.75 ± 0.01 | 41.34 ± 0.96 | 41.40 ± 2.04 | 41.76 ± 0.32 |
| C18:3n-3 | 1.18 ± 0.06 | 1.18 ± 0.03 | 1.15 ± 0.06 | 4.85 ± 0.08 | 4.84 ± 0.21 | 4.84 ± 0.05 |
| C18:4n-3 | 4.34 ± 0.07 | 4.40 ± 0.04 | 4.50 ± 0.02 | 0.39 ± 0.01 | 0.36 ± 0.02 | 0.32 ± 0.01 |
| C20:0 | – | – | – | 0.36 ± 0.02 | 0.32 ± 0.03 | 0.26 ± 0.01 |
| C20:1n-11 | 0.26 ± 0.06 | 0.26 ± 0.04 | 0.22 ± 0.01 | 0.14 ± 0.02 | 0.11 ± 0.03 | 0.05 ± 0.00 |
| C20:1n-9 | 1.09 ± 0.03 | 1.11 ± 0.01 | 1.10 ± 0.01 | 0.41 ± 0.04 | 0.41 ± 0.03 | 0.38 ± 0.01 |
| C20:1n-7 | 0.18 ± 0.07 | 0.17 ± 0.05 | 0.11 ± 0.00 | – | – | – |
| C20:4n-6 | 1.66 ± 0.16 | 1.67 ± 0.11 | 1.60 ± 0.05 | 0.90 ± 0.04 | 0.79 ± 0.07 | 0.66 ± 0.00 |
| C20:4n-3 | 0.91 ± 0.04 | 0.91 ± 0.06 | 0.99 ± 0.02 | 0.20 ± 0.01 | 0.21 ± 0.03 | 0.24 ± 0.01 |
| C20:5n-3 | 18.85 ± 0.08 | 19.23 ± 0.38 | 19.94 ± 0.10 | 5.59 ± 0.13 | 5.63 ± 0.30 | 5.50 ± 0.06 |
| C22:0 | – | – | – | 0.36 ± 0.01 | 0.35 ± 0.01 | 0.29 ± 0.00 |
| C22:1n-11 | 1.01 ± 0.09 | 0.99 ± 0.02 | 0.97 ± 0.02 | 0.28 ± 0.02 | 0.30 ± 0.02 | 0.28 ± 0.02 |
| C22:1n-9 | 0.18 ± 0.03 | 0.17 ± 0.02 | 0.17 ± 0.02 | 0.07 ± 0.01 | 0.06 ± 0.02 | 0.05 ± 0.01 |
| C21:5n-3 | 0.65 ± 0.01 | 0.65 ± 0.02 | 0.67 ± 0.00 | 0.25 ± 0.02 | 0.27 ± 0.02 | 0.26 ± 0.00 |
| C22:4n-6 | 0.14 ± 0.02 | 0.17 ± 0.00 | 0.17 ± 0.01 | 0.05 ± 0.01 | 0.06 ± 0.02 | 0.08 ± 0.00 |
| C22:5n-6 | 0.39 ± 0.02 | 0.42 ± 0.00 | 0.40 ± 0.00 | 0.17 ± 0.02 | 0.18 ± 0.01 | 0.18 ± 0.00 |
| C22:5n-3 | 2.09 ± 0.18 | 2.13 ± 0.14 | 2.02 ± 0.01 | 1.10 ± 0.06 | 0.98 ± 0.10 | 0.83 ± 0.01 |
| C24:0 | – | – | – | 0.09 ± 0.00 | 0.09 ± 0.01 | 0.08 ± 0.01 |
| C22:6n-3 | 18.38 ± 0.15 | 18.69 ± 0.39 | 19.52 ± 0.12 | 4.22 ± 0.11 | 4.26 ± 0.28 | 4.39 ± 0.02 |
| Total | 110.16 ± 14.15 | 110.83 ± 14.00 | 100.47 ± 0.96 | 207.63 ± 6.03 | 198.69 ± 17.86 | 185.58 ± 1.75 |

The lipid emulsions were analyzed at a split ratio of 1:50. The concentration of the fatty acid was calculated based on the internal standard peak area, and subsequently corrected with the RRF$_{(Exp)}$

samples. Transesterification with Methods A and B resulted in higher C16:0 and C18:1n-9 concentrations than using "Method C", due to side reactions. Furthermore, "Method C" also produced a better recovery of n-3 polyunsaturated fatty acids (EPA and DHA). This procedure can be easily adopted for analyzing large quantities of lipid samples for routine analysis in less time and without any threat of hazards imparted to the analyst.

## References

1. Lagerstedt SA, Hinrichs DR, Batt SM, Magera MJ, Rinaldo P, McConnell JP (2001) Quantitative determination of plasma

C8–C26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. Mol Genet Metab 73:38–45

2. Eder K (1995) Gas chromatographic analysis of fatty acid methyl esters. J Chromatogr B Biomed Appl 671:113–131

3. Shantha NC, Napolitano GE (1992) Gas chromatography of fatty acids. J Chromatogr 624:37–51

4. Nota G, Naviglio D, Romano R, Sabia V, Musso S (1998) Evaluation and improvement of transesterification methods of triglycerides. Anal Lett 31:2499–2512

5. Holman RT, Johnson SB, Mercuri O, Itarte HJ, Rodrigo MA, De Tomas ME (1981) Essential fatty acid deficiency in malnourished children. Am J Clin Nutr 34:1534–1539

6. Schreiner M (2005) Quantification of long chain polyunsaturated fatty acids by gas chromatography. Evaluation of factors affecting accuracy. J Chromatogr A 1095:126–130

7. 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

8. Lepage G, Roy CC (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res 25:1391–1396

9. Lepage G, Roy CC (1986) Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 27:114–120

10. Fournier V, Destaillats F, Juaneda P, Dionisis F, Lambelet P, Sebedio JL et al (2006) Thermal degradation of long-chain polyunsaturated fatty acids during deodorization of fish oil. Eur J Lipid Sci Technol 108:33–42

11. Ulberth F, Gabernig RG, Schrammel F (1999) Flame-ionization detector response to methyl, ethyl, propyl, and butyl esters of fatty acids. J Am Oil Chem Soc 76:263–266

12. Ackman RG, Sipos JC (1964) Application of specific response factors in the gas-chromatographic analysis of methyl esters of fatty acids with flame ionization detectors. J Am Oil Chem Soc 41:377–380

13. Bannon CD, Craske JD, Hilliker AE (1986) Analysis of fatty acid methyl esters with high accuracy and reliability. V. Validation of theoretical relative response factors of unsaturated esters in the flame ionization detector. J Am Chem Soc 63:105–110

14. Ulberth F, Schrammel F (1995) Accurate quantitation of short-, medium-, and long-chain fatty acid methyl esters by split-injection capillary gas-liquid chromatography. J Chromatogr A 704:455–463

15. Olsson U, Kaufman P, Herslof BG (1990) Multivariate optimization of a gas–liquid chromatographic analysis of fatty acid methyl esters of blackcurrant seed oil. J Chromatogr 505:385–394

16. Eder K, Reichlmayr-Lais AM, Kirchgessner M (1991) Gas chromatographic analysis of fatty acid methyl esters: avoiding discrimination by programmed temperature vaporizing injection. J Chromatogr 588:265–272

17. Albertyn DE, Bannon CD, Craske JD, Hai NT, O'Rourke KL, Szonyi C (1982) Analysis of fatty acid methyl esters with high accuracy and reliability. I. Optimization of flame-ionization detectors with respect to linearity. J Chromatogr 247:47–61

18. Bannon CD, Craske JD, Felder DL, Garland IJ, Norman LM (1987) Analysis of fatty acid methyl esters with high accuracy and reliability. VI. Rapid analysis by split injection capillary gas–liquid chromatography. J Chromatogr 407:231–241

19. Schreiner M, Hulan HW (2004) Determination of the carbon deficiency in the flame ionization detector response of long-chain fatty acid methyl esters and dicarboxylic acid dimethyl esters. J Chromatogr A 1045:197–202

20. Slemr J, Slemr F, D'Souza H, Partridge R (2004) Study of the relative response factors of various gas chromatograph-flame ionisation detector systems for measurement of C2–C9 hydrocarbons in air. J Chromatogr A 1061:75–84

21. Cunniff P (1995) AOAC official method 991.04 DDT in technical products and pesticide formulations. AOAC International, Gaithersburg

22. Armenta RE, Scott SD, Burga AM, Radianingtyas H, Barrow CJ (2009) Optimization of fatty acid determination in selected fish and microalgal oils. Chromatographia 70:629–636

23. Sonnichsen M, Muller BW (1999) A rapid and quantitative method for total fatty acid analysis of fungi and other biological samples. Lipids 34:1347–1349