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Evaluation of a Rapid Method for the Quantitative Analysis of Fatty Acids in Various Matrices

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Abstract

A simplified method for quantitative analysis of fatty acids in various matrices by gas chromatography is proposed as an alternative to the conventional method and the variables of the protocol examined to optimize the processing conditions. The modified method involves direct methylation of fatty acids in homogenized samples with boron trihalide (BF₃ or BCl₃ in methanol) followed by extraction with hexane. The addition of hexane to the reaction mixture after the methylation process can enhance the efficiency of fatty acid methylation and is critical for those samples that contain high levels of triglycerides. A mechanism underlying this effect is proposed.

Keywords

Methylation; Methylation models; Gas chromatography; High performance thin layer chromatography

1. Introduction

It is well-recognized that cumbersome and time-consuming gas chromatography (GC) methods for the processing and analysis of lipids in a large number of samples are impractical [1,2] and it is unambiguously confirmed that they are responsible for sample loss and contamination [3].

Many laboratories are currently using GC to analyse fatty acid composition in various matrices such as cell membranes and cultures [4], microorganisms [5], plasma [6], tissues [7], etc. These studies have greatly expanded our knowledge in areas such as cellular function, bacterial taxonomy, aquaculture, human and animal nutrition, fatty acid metabolism, etc. They have also provided the analysts with additional criteria for rapid identification of samples.

Several simplified methods for analysis of fatty acid methyl esters (FAME) by GC have been reported [2,3,6,8,9] and compared with the multiple steps conventional method that involves an extraction procedure (generally based on Folch or a modified Folch method), a methylation

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procedure (most often using boron trihalides), a fatty acid methyl esters extraction (commonly using hexane) and the final GC determination. There are some differences between these reported simplified methods in terms of the use of methanol with or without sodium hydroxide in the methylation process, the type of boron trihalide catalyst (BX_3) used and the addition of the extractant solvent before or after the methylation process. Protocols for preparation of FAME using boron trihalides in the absence of a base reagent, (NaOH) and in the presence of an extraction solvent (hexane) prior to the initiation of the methylation process have been proposed [3,6,10,11] as an option to save time, reduce contamination and avoid sample loss. It has been reported that due to the poor solubility of triacylglycerides in methanol, a further solvent is advisable, for instance hexane, if the methylation process is expected to be completed in a reasonable frame of time [12] and that no solvent other than methanol is necessary if free fatty acids alone are to be methylated [12]. In this study, we examined the influence of the type of BX_3 used, namely BF_3 and BCl_3 , before comparing the results of the simplified method with those obtained by the conventional multiple-step protocol in the analysis of certified and non-certified materials. In addition, the presence or absence of NaOH in the reactor and the addition of hexane extraction solvent before or after the preparation of the FAME was investigated by means of a factorial design. Comprehensive models were proposed to explain the factorial design results and high performance thin layer chromatography (HPTLC) was used to confirm the validity of the proposed models.

2. Experimental

2.1 Reagents and samples

Sodium hydroxide, hexane, methanol, boron trifluoride in methanol (20 % w/v) and chloroform were purchased from Merck (Darmsadt, Germany). Butylated hydroxytoluene (BHT) and boron trichloride in methanol (14 %) were purchased from Sigma-Aldrich Co. USA. FAME standards were purchased from Nu-Chek Prep (Elysian, MN), the nonadecanoic acid methyl ester ($C_{19:0}$) internal standard was from Fluka (Buchs, Switzerland). The standard reference material (SRM) NIST 1544 (frozen diet composite) was supplied by the National Institute of Standards and Technology NIST (Gaithersburg, ND, USA), human red blood cells and serum were collected from an anonymous donor. Cod plasma, salmon liver and salmon muscle samples were supplied by the Aquaculture Nutrition program at NIFES. Milk powder sample were from a Proficiency Testing Program for Fatty Acid Analysis Laboratories. Cod liver oil and commercial ethyl ester capsules (Fri Flyt omega-3, Vesterålen Naturprodukter AS, Sortland, Norge) were obtained from a local pharmacy. Brain samples from male Wistar rats (200 g, Tactonic, USA) were kindly donated by Anita Alvheim from the Seafood and Health Program at NIFES.

2.2. Instrumentation

2.2.1 Gas chromatography (GC)—Analysis of the FAME prepared by the above described methods was performed on a Perkin-Elmer AutoSystem XL gas chromatograph (Perkin-Elmer, Norwalk, Connecticut) equipped with a liquid autosampler and a flame ionisation detector. The FAME samples were analysed on a CP-Sil 88 capillary column (50 m $\times$ 0.32 mm I.D. 0.2 μ m film thickness, Varian, Courtaboeuf, France). Data collection was performed by the Perkin-Elmer TotalChrom Data System software version 6.3. The temperature program was as follows: the oven temperature was held at 60 °C for 1 min, ramped to 160 °C at 25 °C/min, held at 160 °C for 28 min, ramped to 190 °C at 25 °C/min, held at 190 °C for 17 min, ramped to 220 °C at 25 °C/min and finally held at 220 °C for 10 min. Direct on-column injection was used. The injector port temperature was ramped instantaneously from 50 to 250 °C and the detector temperature was 250 °C. The carrier gas was ultra-pure helium at a pressure of 82 Kpa. Analysis time was 60 min. This time interval was sufficient to detect FAME with chains

from 10 to 24 carbons in length. The FAME peaks were identified by comparison of their retention times with the retention times of highly purified FAME standards.

2.2.2. High performance thin layer chromatography (HPTLC)—HPTLC separation of triacylglycerol (TAG) and methyl esters was carried out on a silica gel plate (20 cm × 10 cm, 0.2 mm thick, Merck, Darmstadt, Germany). The plate was prewashed in a tank containing 10 ml of a polar solution, dried in a fume cupboard for 15 min and activated at 110 °C for 30 min. The polar solution used for plate prewashing consisted of 25 ml of methyl acetate, 25 ml of 2-propanol, 25 ml of chloroform, 10 ml of methanol and 9 ml of potassium chloride 0.25 %. The chromatographic estimation was performed by applying standards and samples on the activated plates as 6 mm bands with the help of a Camag automatic TLC sampler ATS4 (Camag, Muttenz, Switzerland) and a dosage of 1 µl of lipid (5 mg/ml) dissolved in chloroform with 0.05 % of BHT. The plates were developed to 90 mm using a Camag Automated Multiple Development AMD2 (Muttentz, Switzerland) and a mobile phase consisting of isohexane:diethyl-ether:acetic-acid (75:23.5:1.5 v/v/v). After development, the plates were dried in the AMD2 for 20 min and the bands visualised by dipping the plates into an aqueous solution of 3 % copper acetate and 8 % phosphoric acid for 15 s. Afterwards, the plates were charred in a drying oven at 160 °C for 15 min. The visualised plates were scanned automatically using a densitometric Camag TLC scanner 3 (Muttentz, Switzerland) in the absorption mode at 350 nm using a deuterium lamp as a source of radiation. The slit dimensions were 4.0 × 0.2 mm at a scan speed of 20 mm/s and data resolution of 100 µm per step. Concentrations of the chromatographed compounds were determined from the intensity of the absorption via peak areas using winCATS Planar Chromatography Manager version 1.4.2.8121 (Camag, Muttenz, Switzerland).

2.3. Sample protocol

2.3.1. Conventional method—The sample (0.2 g) is weighed in a 10 ml Sovirell pyrex tube and mixed with 4 ml of chloroform:methanol 2:1 v/v and internal standard (1–3 mg/10 mg fat). It is then vortex-mixed for 30 seconds and left at –20 °C overnight. The sample is filtered and the solvent is rotary-evaporated to dryness. The residue is dissolved in 6 ml of diethylether, transferred to a test tube and dried under a stream of nitrogen. A portion of 1 ml of NaOH in methanol (0.5 M) is added, vortex-mixed and heated for 15 min at 100 °C. After cooling the mixture in water, 2 ml of BX₃/CH₃OH (X = F or Cl) are added, vortex-mixed and heated for 5 min at 100 °C. The mixture is cooled and subsequent portions of 1 ml of hexane and 2 ml of water added, vortex-mixed for 15 seconds, placed in a centrifuge, allowed to reach a speed of 3000 rpm, and then stopped immediately. After collecting the hexane phase (1 ml), an additional aliquot of 1 ml of hexane is added to the mixture, vortex-mixed and centrifuged. After that the hexane phase is collected. Depending upon the fat content, the total 2 ml hexane phase collected are either concentrated or diluted and submitted to GC analysis.

2.3.2. Simplified method—The protocol is based on the above described conventional protocol with some modifications. Briefly, 50 µl (or 50 mg) of sample are mixed with 2 ml BX₃/CH₃OH and internal standard (1 mg/10 mg fat). The mixture is heated at 100 °C for 1 h and cooled down to room temperature. Aliquots of 1 ml of hexane and 2 ml of H₂O are added, vortex-mixed for 15 seconds, placed in a centrifuge at 3000 rpm for 2 min and the methyl esters are then extracted from the upper hexane phase. Depending on the fat content the sample is either concentrated under nitrogen or diluted with hexane and subsequently subjected to GC analysis. It must be mentioned that the reaction time of the simplified method was studied in advance at 15, 30, 45 and 60 min using a SRM (NIST 1544) and it was found that 60 min was the optimum time to obtain results comparable with the conventional method. The type of BX₃ to be used in connection with this method will be selected in the study described in the experimental design section.

2.4. Experimental design

2.4.1. Effect of the boron trihalide type—In the present study, the selection of a suitable boron trihalide, to be used in further comparison studies and evaluation of some of the parameters that might affect the simplified method described above, was carried out by means of the conventional methodology. The effect of the type of boron halide used on the quantitative analysis of fatty acid was evaluated by preparing FAME from a commercial omega 3 ethyl ester capsule formulation, using the conventional method and BF_3 or BCl_3 as catalysts. Six independent experiments were performed ($n = 6$) and expressed as average and standard deviation values. An independent laboratory was used to check the validity of the results.

2.4.2. Conventional method versus simplified method—After selecting an appropriate catalyst (BX_3) to be used with the simplified and conventional method, 7 different certified and non-certified samples (human serum, human red blood cells, cod plasma, salmon liver, salmon muscle, food reference material and milk powder) were treated according to the above described conventional and simplified methods under the same chromatographic conditions. Six independent experiments per sample were performed ($n = 6$) and expressed as average and standard deviation values. The results from both methods were analysed and compared. The hypothesis that there are no differences between the simplified and the conventional methods was tested by three different paired two-samples tests and the Kolmogorov-Smirnov test. The latter statistical test was used to determine whether there are significant differences between the distributions of the FAME prepared by both methods.

2.4.3. Evaluation of the variables sodium hydroxide and hexane—In general, simplified methods for fatty acid analysis are characterised by three well defined steps: 1) methylation, 2) extraction and 3) GC analysis. Different reports have suggested that combining steps 1 and 2 without the need of a base reagent (NaOH) is a valid strategy to save time and material and avoid further sample manipulation [2,3,9].

A factorial design at two levels (2^2) and GC were selected to study the effect of the presence or absence of base reagent (1 ml of $\text{NaOH}/\text{CH}_3\text{OH}$ 0.5 M or sole CH_3OH respectively) and the addition of hexane in the reactor before the preparation of the FAME (combining steps 1 and 2) or the addition of hexane in the reactor after the FAME are formed (without combining steps 1 and 2). The experimental conditions described for the simplified method were used in this study. The description of the factorial design along with the experimental arrangement of the variables is showed in Table 1. The experimental design was applied on human serum, human red blood cells, food reference material, cod liver oil and rat brain. Some models were proposed to explain the factorial design results and their confirmation was achieved by HPTLC using cod liver oil and human red blood cells. Six independent experiments per sample were performed ($n = 6$) and expressed as average and standard deviation values.

2.5. Statistics

The results were expressed as average and standard deviation values of six replicates. Paired two samples tests such as t-test, sign-test, signed rank test and Kolmogorov-Smirnov test were used to compare the performance of the catalysts and the results of the comparison between the conventional and the simplified method. The factorial experiments were analysed by using a multiple-sample comparison test. All the tests were performed at the 95 % confidence level using Statgraphics Plus 5.1 software package (Statistical Graphics Corp., Herndon, USA).

3. Results and Discussion

3.1. Selection of the type of BX_3

Different observations have been reported when BF_3 and BCl_3 are used as catalysts for fatty acid methylation. In a study aiming at comparing six widely used methods for the determination of fatty acid composition in tuna, the presence of an artefact was observed when BF_3 was used [13]. Unidentified peaks have been also recorded when a methanolic solution of BCl_3 was employed in a study intended for comparing BF_3 and BCl_3 performance [2]. Lipids from a commercial omega 3 ethyl ester capsule formulation were extracted and methylated by the conventional method using BF_3 or BCl_3 as catalysts. The results of these experiments (Table 2) expressed as percentage of the total fatty acid content and as mg FAME/g of sample revealed no significant differences between the concentrations of the fatty acids when either BF_3 or BCl_3 were employed. The percentage of unknown peaks averaged at 3.2 % and 3.0 % of the total fatty acid content when BCl_3 and BF_3 were used respectively. These results were confirmed by an independent laboratory.

Although there were no significant differences between the two halides tested, it was decided to use BF_3 in the subsequent experiments in accordance with the Union of Pure and Applied Chemistry (IUPAC) [14], the International Association of Official Analytical Communities (AOAC) [15], the American Oil Chemists' Society (AOCS) [16] and the British Standards Institution (BSI) [17], which recommend this reagent for the preparation of FAME.

3.2. Conventional and simplified method comparison

There was no significant difference between the conventional and simplified method, the coefficients of variation were comparable for both methods. The average percentages of total variance for the former and the latter were 2.7 and 3.2 % respectively. Only 2.9 % of the variation in individual fatty acid content was due to differences between the methods which was comparable with the variation between replicate separations (2.1 %) and between repeat injections onto the column (1.7 %).

The concentrations of the major fatty acids in human red blood cells and human serum (Table 3), in salmon muscle, salmon liver and cod plasma (Table 4), and in a reference material and milk powder (Table 5) prepared by the simplified and the conventional method and analysed by GC under the same chromatographic conditions, are expressed as mg FAME/g of sample. Table 3–Table 5 showed that the analytical data obtained with the conventional and the simplified methods were only slightly different. In all instances, the results from the conventional and simplified method did not reveal any statistical differences at the 95 % confidence level. It is important to mention that sample homogeneity is a crucial factor to the success of the simplified method. Experiments on whole salmon samples (results not shown) revealed differences between the conventional and the simplified method at the level of particular fatty acids such as $C_{18:2n-6}$, $C_{20:5n-3}$ and $C_{20:1n-9}$, which could be attributed to the variable amounts of bones and skin that remain in the reactors after the methylation.

Although the chromatographic analysis time of the proposed simplified method is 3 times longer than the conventional method, it does not require time consuming operations such as extraction, solvent transfer, evaporation, centrifugation, etc, in addition to the bulk of glass material required to perform all these operations.

3.3. Effect of the variables NaOH and hexane on the validated simplified method

The effect of the selected variables (NaOH and hexane) on the major fatty acids found in a human serum sample ($C_{16:0}$, $C_{18:0}$, $C_{18:1n-9}$ and $C_{18:2n-6}$) are shown in Fig 1a. The results revealed clearly that the addition of hexane after the methylation process (bottom quadrants in

Fig 1a) increases dramatically the FAME concentrations when compared to the addition of hexane before the methylation process (top quadrants in Fig 1a). The observed increments were higher than 50 %. Comparison of the left and right top quadrants in Fig 1a (representing the addition of hexane before the methylation step) demonstrated that the absence or presence of NaOH does not affect the FAME concentrations. The slight FAME changes observed when comparing the two top quadrants were not statistically significant at a confidence level of 95 %. This outcome was also observed when the right and left bottom quadrants (adding hexane after the formation of the FAME) were compared, giving further evidence that NaOH at the concentration level used in this study (0.5 M) does not have any influence on the methylation process. The considerable increase in FAME concentrations (> 50 %) when hexane is added after the methylation and regardless of the NaOH presence was consistently observed when the factorial design was applied on food reference material and cod liver oil (results not shown). It is important to mention that the FAME concentrations of these samples (when hexane was added after the methylation) were in accordance with the conventional method.

In contrast, the application of the factorial design on human red blood cells (Fig 1b) and rat brain samples (results not shown) revealed that neither hexane (added before or after the methylation) nor NaOH (absence or presence) have an impact on FAME concentrations. Fig 1b shows that the four quadrants generate the same information. The small differences observed between quadrants were not statistically significant at the 95 % confidence level. The red blood cell FAME concentrations in the four quadrants were in accordance with those estimated by the conventional method.

3.4. Underlying mechanisms: effective methylation area model

In summary, our results have demonstrated that addition of hexane before the methylation step reduces dramatically the FAME concentrations while adding hexane after methylation brings about concentrations close to that with the conventional method. However, in some instances adding hexane before or after the methylation step does not affect the FAME concentrations. Based on these contrasting results and considering that triacylglycerols (TAG) are poorly soluble in methanol and that TAG are the major sources of fatty acids, it could be hypothesised that *a priori* addition of hexane promotes the dissolution of TAG in the hexane phase. The boundary between the hexane phase containing solubilised TAG (top layer) and the methanol phase containing solubilised BF_3 catalyst (bottom layer) define an “effective methylation area” where the actual methylation process is taking place (double head arrows in Fig. 2). A high concentration of TAG in the sample will generate FAME through the “effective methylation area”. As the concentration of FAME starts increasing in the hexane phase the efficiency of the “effective methylation area” begins to diminish, probably because the FAME begin to saturate this particular area or perhaps because the generated FAME protect the remaining TAG, hence preventing the direct contact of TAG with the reagents responsible for the methylation (Fig. 2). Conversely, a very low concentration of TAG in the sample cannot produce enough FAME to saturate the “effective methylation area” or shield the remaining TAG (Fig. 3) hence leading to a more effective conversion of TAG into FAME. The *a posteriori* addition of hexane, independent of the TAG concentration, precludes the formation of the so-called “effective methylation area” and consequently maximizes the interaction of TAG with the methylation reagents, leading to higher FAME concentrations comparable to those obtained with the conventional method.

To assess whether or not the contact area between methanol and hexane, which defined the hypothesized “effective methylation area” has an effect on the FAME yield and consequently is an important factor in the methylation process of samples rich in TAG, some experiments were performed using the simplified method to methylate a cod liver oil sample, using reaction tubes with different diameters (1.5, 2.5 and 5 cm) and keeping constant the volume of the

reactants (2 ml of BF₃/MeOH and 1 ml of hexane added *a priori*). The results (Table 6) demonstrated that a decrease in the volume/area ratios of the phases brings about an increase in the FAME yield. The results obtained with the 5 cm diameter reactor were in accordance with those obtained with the conventional method. These results confirmed the importance of the proposed “effective methylation area” in the methylation process of samples rich in TAG. The model described in Fig. 2 allows to explain why the *a priori* addition of hexane to human serum, with a relative high content of TAG, (top quadrants in Fig 1a) reduces dramatically the FAME concentrations compared to the *a posteriori* addition of hexane (bottom quadrants in Fig 1a). The results for food reference material and cod liver which contain high levels of TAG were also explained by Fig 2.

The results for human red blood cells (Fig 1b) and rat brain samples (rich in phospholipids) containing low levels of TAG and showing minimal changes in FAME concentration despite of the order of hexane addition can be explained by the model described in Fig. 3.

The chromatogram for an unmethylated sample free of TAG such as the ethyl ester omega-3 capsules containing mainly ethyl-eicosapentaenoic acid (ethyl-EPA) and ethyl-docosapentaenoic acid (ethyl-DHA) is presented in Fig. 4a. Addition of hexane before methylation causes a reduction of the FAME signals as a result of the incomplete conversion of the ethyl esters into methyl ester (Fig 4b). The levels of unreacted ethyl-EPA and ethyl-DHA were 13.17 and 18.02 % respectively. The results shown in Fig 4b can be explained in terms of the model depicted in Fig 2 but using the ethyl form instead of the TAG form. On the other hand, Fig 4c shows clearly that a complete conversion of the mixture of ethyl-EPA and ethyl-DHA into methyl-EPA and methyl-DHA respectively, occurs when the ethyl esters are exposed to the methylation reagents and hexane is added only after the formation of the FAME in the methylation reactor.

The proposed models (Fig 2–Fig 3) provide a rational explanation for the observed changes in FAME concentrations estimated by GC when hexane is added *a priori* in systems with high or very low TAG content. Nevertheless, it is necessary to obtain evidence regarding the presence or absence of TAG predicted by the effective methylation area model after the methylation process is completed. To accomplish this aim, cod liver oil (high TAG content) and red blood cells (low TAG content) samples were treated using the simplified method without NaOH and adding hexane before or after the methylation step. The methylation products in the hexane phase were analysed by HPTLC instead of GC to check whether the main product in some instances is a mixture of TAG and FAME (as is predicted in Fig 2) or mainly FAME (as is predicted in Fig 3). The HPTLC chromatograms for cod liver oil show that an *a priori* addition of hexane to the reactor containing methanol, BF₃ and the sample with a high TAG level, yields a mixture of TAG and FAME at the end of the methylation process (Fig 5a). The *a posteriori* addition of hexane when the methylation process of the sample with a high TAG content is completed generates mainly FAME (Fig. 5b). The red blood cells HPTLC chromatograms (not shown) were similar to that showed in Fig. 5b, indicating that samples with a very low TAG content generates mainly FAME independent of the order (*a priori* or *a posteriori*) of addition of hexane in the methylation reactor. The HPTLC results demonstrated unequivocally that the proposed effective methylation area model (Fig 3–Fig 4) offers a comprehensive explanation for the observed changes in FAME concentrations by GC when hexane is added either *a priori* or *a posteriori* in systems with high or very low TAG content.

4. Conclusions

The proposed simplified method has demonstrated to be a rapid alternative to the established conventional protocol for the analysis of fatty acids in samples containing high or low levels

of TAG or very small sample sizes. The type of catalyst (BCl_3 or BF_3) was found to have little effect on the formation of FAME. It was demonstrated that the addition of NaOH to the reaction mixture during methylation process is not necessary. The *a priori* or *a posteriori* addition of hexane to the reactor will have a significant impact on the methylation efficiency of samples containing high levels of TAG. Thus, it is advisable to add hexane after the methylation process regardless of the fat content. The underlying mechanism behind this critical effect was rationally explained and experimentally confirmed by means of an effective methylation area model and TLC analysis respectively.

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a) Human serum

		Hexane before			
Without NaOH	C _{16:0}	0.56 ± 0.00	C _{16:0}	0.57 ± 0.02	With NaOH
	C _{18:0}	0.23 ± 0.01	C _{18:0}	0.21 ± 0.01	
	C _{18:1n-9}	0.41 ± 0.01	C _{18:1n-9}	0.44 ± 0.02	
	C _{18:2n-6}	0.48 ± 0.01	C _{18:2n-6}	0.46 ± 0.02	
	C _{16:0}	1.41 ± 0.02	C _{16:0}	1.37 ± 0.03	
	C _{18:0}	0.48 ± 0.00	C _{18:0}	0.49 ± 0.00	
	C _{18:1n-9}	1.04 ± 0.03	C _{18:1n-9}	1.03 ± 0.02	
	C _{18:2n-6}	1.17 ± 0.05	C _{18:2n-6}	1.14 ± 0.04	
		Hexane after			

b) Human RBC

		Hexane before					
Without NaOH	C _{16:0}	0.43 ± 0.02	C _{16:0}	0.44 ± 0.00	With NaOH		
	C _{18:0}	0.34 ± 0.01	C _{18:0}	0.34 ± 0.01			
	C _{18:1n-9}	0.27 ± 0.01	C _{18:1n-9}	0.26 ± 0.01			
	C _{18:2n-6}	0.35 ± 0.01	C _{18:2n-6}	0.38 ± 0.01			
	C _{20:4n-6}	0.20 ± 0.01	C _{20:4n-6}	0.22 ± 0.01			
	C _{22:6n-3}	0.15 ± 0.01	C _{22:6n-3}	0.17 ± 0.01			
	C _{16:0}	0.44 ± 0.00	C _{16:0}	0.45 ± 0.03			
	C _{18:0}	0.35 ± 0.01	C _{18:0}	0.35 ± 0.01			
	C _{18:1n-9}	0.28 ± 0.02	C _{18:1n-9}	0.29 ± 0.02			
	C _{18:2n-6}	0.38 ± 0.01	C _{18:2n-6}	0.37 ± 0.02			
C _{20:4n-6}	0.21 ± 0.00	C _{20:4n-6}	0.23 ± 0.01				
C _{22:6n-3}	0.17 ± 0.01	C _{22:6n-3}	0.17 ± 0.01				
		Hexane after					

Fig. 1.

Effect of the variables NaOH and hexane on the major fatty acids (mg/g) in human serum and human red blood cells. The values are expressed as mean ± standard deviation of the main fatty acids ($n = 6$)

Fig. 2.
Underlying mechanism for samples with high levels of triacylglycerol (TAG).

Fig. 3.
Underlying mechanism for samples with very low levels of triacylglycerol (TAG).

Fig. 4. GC chromatograms for an omega-3 commercial capsule. a) unmethylated sample; b) addition of hexane before methylation; c) addition of hexane after methylation.

Fig. 5. HPTLC chromatograms for a cod liver oil sample, used to study the effective methylation area model. a) *a priori* addition of hexane; b) *a posteriori* addition of hexane.

Table 1

2^2 factorial design used to study the effect of the variables NaOH and hexane on the methylation process

Variable	Experiment description	Short designation	Experimental arrangement
X_1	Absence of base reagent (–) Presence of base reagent (+)	/MeOH NaOH/MeOH	
X_2	Addition of extractant solvent before methylation (+) Addition of extractant solvent after methylation (–)	Hexane/before Hexane/after	

Table 2Comparison of BCl₃ and BF₃ as catalysts for FAME preparation using the conventional method (*n* = 6)

Sample: Catalyst: Units:	Ethyl ester omega-3 commercial capsules			
	BCl ₃ %	mg/g	BF ₃ %	mg/g
16:0	2.32 (2.25) ± 0.07 (0.07)	20.32 (20.19) ± 0.61 (0.56)	2.78 (2.70) ± 0.08 (0.10)	22.85 (22.76) ± 0.69 (0.59)
18:0	4.21 (4.14) ± 0.13 (0.12)	32.82 (33.02) ± 0.98 (0.89)	3.86 (3.75) ± 0.12 (0.15)	31.68 (31.49) ± 0.95 (0.87)
18:1n-9	8.93 (9.00) ± 0.27 (0.26)	70.28 (68.46) ± 2.11 (1.76)	8.44 (8.36) ± 0.25 (0.30)	69.33 (69.50) ± 2.08 (1.73)
18:1n-7	2.84 (2.76) ± 0.09 (0.07)	23.24 (24.31) ± 0.70 (0.59)	2.96 (2.87) ± 0.09 (0.10)	24.29 (24.34) ± 0.73 (0.61)
18:2n-6	1.00 (0.97) ± 0.03 (0.03)	7.92 (7.64) ± 0.24 (0.20)	0.91 (0.89) ± 0.03 (0.03)	7.53 (7.51) ± 0.23 (0.19)
18:4n-3	2.26 (2.19) ± 0.07 (0.06)	18.03 (17.62) ± 0.54 (0.93)	2.12 (2.10) ± 0.06 (0.07)	17.43 (17.50) ± 0.52 (0.45)
20:1n-9	2.11 (2.13) ± 0.06 (0.06)	16.31 (16.36) ± 0.49 (0.41)	1.92 (1.88) ± 0.06 (0.06)	15.77 (15.78) ± 0.47 (0.38)
20:4n-6	1.79 (1.80) ± 0.05 (0.05)	13.96 (14.05) ± 0.42 (0.38)	1.75 (1.73) ± 0.05 (0.04)	14.42 (14.47) ± 0.43 (0.36)
20:4n-3	1.73 (1.75) ± 0.05 (0.06)	13.44 (13.48) ± 0.40 (0.34)	1.69 (1.70) ± 0.05 (0.05)	13.87 (13.80) ± 0.42 (0.37)
20:5n-3	35.02 (34.26) ± 1.04 (0.98)	270.50 (271.63) ± 8.11 (6.99)	35.87 (35.95) ± 1.08 (0.90)	294.57 (293.43) ± 8.84 (7.57)
22:1n-11	1.49 (1.48) ± 0.04 (0.04)	11.10 (11.08) ± 0.33 (0.27)	1.56 (1.56) ± 0.05 (0.04)	12.79 (12.84) ± 0.38 (0.33)
21:5n-3	1.58 (1.59) ± 0.05 (0.05)	12.10 (12.04) ± 0.36 (0.32)	1.52 (1.56) ± 0.05 (0.04)	12.49 (12.52) ± 0.37 (0.31)
22:5n-3	3.62 (3.59) ± 0.11 (0.11)	28.14 (28.26) ± 0.84 (0.72)	3.35 (3.34) ± 0.10 (0.09)	27.51 (27.63) ± 0.83 (0.72)
22:6n-3	23.17 (22.68) ± 0.70 (0.61)	180.17 (179.88) ± 5.41 (4.64)	23.42 (23.31) ± 0.70 (0.61)	192.23 (193.07) ± 5.77 (5.00)
Total unknown	3.24 (3.18) ± 0.09 (0.14)	23.88 (23.79) ± 0.72 (0.62)	3.00 (2.96) ± 0.09 (0.07)	24.77 (24.60) ± 0.74 (0.69)

Values as mean ± standard deviation

Bracketed values are from an independent laboratory

Table 3

Comparison of fatty acid composition in red blood cells (RBC) and serum using the conventional and the simplified method ($n = 6$)

Sample: Method:	Human			
	RBC	Conventional Serum	RBC	Simplified Serum
16:0	0.44 ± 0.02	1.18 ± 0.05	0.45 ± 0.03	1.37 ± 0.03
18:0	0.34 ± 0.01	0.32 ± 0.01	0.35 ± 0.01	0.49 ± 0.00
18:1n-9	0.29 ± 0.01	1.02 ± 0.05	0.29 ± 0.02	1.03 ± 0.02
18:2n-6	0.38 ± 0.01	1.24 ± 0.06	0.37 ± 0.02	1.14 ± 0.04
20:4n-6	0.21 ± 0.01	0.12 ± 0.00	0.23 ± 0.01	0.12 ± 0.00
22:6n-3	0.17 ± 0.01	0.08 ± 0.00	0.17 ± 0.01	0.08 ± 0.00

Concentrations in mg FAME/g of sample (values as mean ± standard deviation)

Table 4
Comparison of fatty acid composition in fish samples using the conventional and the simplified method ($n = 6$)

Sample: Method:	Fish			Simplified Liver			Plasma
	Muscle	Conventional Liver	Plasma	Muscle	Simplified Liver	Plasma	
14:0	0.9 ± 0.1	0.6 ± 0.0	0.3 ± 0.0	1.0 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
16:0	3.4 ± 0.1	5.4 ± 0.3	2.7 ± 0.1	3.4 ± 0.2	5.6 ± 0.3	2.4 ± 0.1	2.4 ± 0.1
16:1n-7	1.1 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	1.1 ± 0.1	0.8 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
18:0	0.8 ± 0.1	1.9 ± 0.1	0.4 ± 0.0	0.8 ± 0.0	2.1 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
18:1n-9	3.1 ± 0.2	4.5 ± 0.2	1.7 ± 0.1	2.9 ± 0.1	4.7 ± 0.1	1.5 ± 0.0	1.5 ± 0.0
18:1n-7	0.7 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
18:2n-6	0.9 ± 0.0	1.3 ± 0.1	0.5 ± 0.0	0.9 ± 0.0	1.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
20:1n-9	1.2 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	1.0 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
22:1n-11	1.1 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.9 ± 0.1	0.4 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
20:5n-3	1.5 ± 0.1	2.8 ± 0.1	2.1 ± 0.1	1.5 ± 0.1	3.0 ± 0.1	1.9 ± 0.0	1.9 ± 0.0
22:5n-3	0.7 ± 0.0	1.3 ± 0.1	0.2 ± 0.0	0.7 ± 0.0	1.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:6n-3	5.0 ± 0.2	7.9 ± 0.4	3.9 ± 0.2	5.4 ± 0.3	7.6 ± 0.2	4.1 ± 0.1	4.1 ± 0.1

Concentrations in mg FAME/g of sample (values as mean ± standard deviation)

Table 5Comparison of fatty acid composition in food samples using the conventional and the simplified method ($n = 6$)

Sample: Method:	Frozen diet composite (NIST 1544)		Milk powder	
	Conventional	Simplified	Conventional	Simplified
14:0	1.0 ± 0.0	1.1 ± 0.1	7.4 ± 0.3	8.5 ± 0.2
16:0	6.3 ± 0.1	6.3 ± 0.2	67.0 ± 0.4	65.6 ± 2.1
18:0	2.1 ± 0.0	2.0 ± 0.1	6.9 ± 0.0	7.3 ± 0.4
18:1n-9	11.9 ± 0.2	11.7 ± 0.7	53.0 ± 0.4	55.9 ± 1.9
18:2n-6	6.7 ± 0.1	6.9 ± 0.4	15.4 ± 0.1	19.1 ± 0.9

Concentrations in mg FAME/g of sample (values as mean ± standard deviation)

Table 6

Effect of the reaction tube diameter on FAME prepared by the simplified method and with *a priori* addition of hexane. The reactant volumes were kept constant ($n = 3$)

Reactor diameter (cm) → Fatty acid	Conventional method	Simplified method		
		1.50	2.50	5.00
14:00	33.11	12.25 ± 0.29	29.94 ± 0.63	32.82 ± 0.83
15:00	2.95	1.02 ± 0.02	2.53 ± 0.06	2.93 ± 0.04
16:00	82.98	27.41 ± 1.15	71.56 ± 1.43	82.61 ± 0.83
16:1n-9	5.03	1.72 ± 0.04	4.29 ± 0.09	4.99 ± 0.08
16:1n-7	72.2	25.98 ± 0.92	61.75 ± 1.29	72.32 ± 1.1
17:00	1.53	0.46 ± 0.01	1.24 ± 0.02	1.54 ± 0.02
16:2n-4	3.41	1.3 ± 0.02	3.02 ± 0.08	3.4 ± 0.05
18:00	17.42	5.47 ± 0.14	14.27 ± 0.22	17.28 ± 0.26
16:3n-3	1.79	0.72 ± 0.03	1.52 ± 0.03	1.77 ± 0.04
18:1n-11	18.47	6.08 ± 0.13	15.41 ± 0.23	18.44 ± 0.28
18:1n-9	125.59	40.76 ± 0.86	101.28 ± 2.12	124.96 ± 2.5
18:1n-7	38.82	12.92 ± 0.49	31.47 ± 0.66	38.73 ± 0.98
16:4n-3	1.11	0.8 ± 0.02	1.01 ± 0.02	1.1 ± 0.02
18:2n-6	15.17	5.4 ± 0.19	12.94 ± 0.2	15.1 ± 0.3
20:00	0.58	0 ± 0.01	0 ± 0	0.58 ± 0.01
18:3n-3	7.81	2.99 ± 0.06	7.12 ± 0.11	7.87 ± 0.16
20:1n-11	13.47	3.87 ± 0.11	10.57 ± 0.16	13.45 ± 0.2
20:1n-9	103.21	29.39 ± 0.69	77.9 ± 1.19	102.69 ± 1.03
20:1n-7	3.46	0.91 ± 0.02	1.83 ± 0.03	3.44 ± 0.03
18:4n-3	21.99	8.64 ± 0.32	19.64 ± 0.2	22.1 ± 0.34
20:2n-6	2.56	0.74 ± 0.02	1.74 ± 0.03	2.54 ± 0.06
20:4n-6	4.41	1.34 ± 0.05	4.07 ± 0.1	4.39 ± 0.09
22:1n-11	61.14	15.41 ± 0.4	43.07 ± 1.09	61.63 ± 0.93
22:1n-9	6.66	2.01 ± 0.05	4.95 ± 0.08	6.6 ± 0.17
20:4n-3	6.26	2.27 ± 0.08	5.24 ± 0.08	6.25 ± 0.1
20:5n-3	83.77	30.58 ± 0.73	72.96 ± 1.46	83.36 ± 1.67
24:00:00	1.69	0.37 ± 0.02	1.39 ± 0.03	1.67 ± 0.03
24:1n-9	3.15	0.66 ± 0.02	2.14 ± 0.02	3.13 ± 0.05
22:5n-3	11.27	3.7 ± 0.14	10.06 ± 0.1	11.21 ± 0.22
22:6n-3	112.11	35.6 ± 0.77	91.84 ± 0.92	111.17 ± 2.81

Concentrations in mg FAME/g of sample (values as mean ± standard deviation)