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Preparation of Fatty Acid Methyl Esters for Gas-Chromatographic Analysis of Marine Lipids: Insight Studies

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Assays for fatty acid composition in biological materials are commonly carried out by gas chromatography, after conversion of the lipid material into the corresponding methyl esters (FAME) via suitable derivatization reactions. Quantitative derivatization depends on the type of catalyst and processing conditions employed, as well as the solubility of said sample in the reaction medium. Most literature pertinent to derivatization has focused on differential comparison between alternative methods; although useful to find out the best method for a particular sample, additional studies on factors that may affect each step of FAME preparation are urged. In this work, the influence of various parameters in each step of derivatization reactions was studied, using both cod liver oil and microalgal biomass as model systems. The accuracies of said methodologies were tested via comparison with the AOCS standard method, whereas their reproducibility was assessed by analysis of variance of (replicated) data. Alkaline catalysts generated lower levels of long-chain unsaturated FAME than acidic ones. Among these, acetyl chloride and BF_3 were statistically equivalent to each other. The standard method, which involves alkaline treatment of samples before acidic methylation with BF_3, provided equivalent results when compared with acidic methylation with BF_3 alone. Polarity of the reaction medium was found to be of the utmost importance in the process: intermediate values of polarity [e.g., obtained by a 1:1 (v/v) mixture of methanol with diethyl ether or toluene] provided amounts of extracted polyunsaturated fatty acids statistically higher than those obtained via the standard method.

KEYWORDS: Fish oil; derivatization; methylation; microalgae; polarity index; polyunsaturated fatty acids; transmethylation

INTRODUCTION

The nutritional value of a food sample is strongly dependent on its fatty acid profile; therefore, methods that allow one to estimate the fatty acid profile of a sample, based on gas chromatography (GC), are preferred. However, it is difficult to proceed directly via GC because of the high polarity, low volatility, and high tendency to form hydrogen bonds of typical lipid samples (*1*). Therefore, derivatization is usually a requirement prior to implementation of this technique. This process increases the volatility of lipid components, thus providing a better separation and reducing the time to carry out the analysis (*1*, *2*).

Although several derivatization procedures have been described in the literature, most involve conversion of fatty acid components into their corresponding esters. Because lipids are mainly a mixture of esters, preparation of fatty acid methyl esters (FAME) consists essentially on conversion of one ester to another (i.e., transesterification), by cleavage of an ester bond via an alcohol; when such an alcohol is methanol, the reaction is referred to as methanolysis or transmethylation (*2*). When FAME are formed as products of the reaction between fatty acids and methanol, the process is termed methylation. Both methylation and transmethylation are reversible reactions, normally accomplished in the presence of a catalyst; however, either an acid or a base can catalyze the latter, whereas methylation occurs only in the presence of an acid.

Reactions involving acidic catalysts require heat to accelerate the process. Three commonly used acidic catalysts are (the Brønsted−Lowry acids) HCl and H_2SO_4 and (the Lewis acid) BF_3. HCl has been considered a mild and useful derivatization reagent (*3*), because it produces almost quantitative yields; although it has an intermediate methylating power, its transmethylating capacity is low, thus requiring reaction times usually above 30 min. In addition to this constraint, the use of H_2SO_4 has been reported to lead to decomposition of polyunsaturated fatty acids (PUFA) under certain conditions (*4*). BF_3 exhibits a high methylating power and a low transmethylating one, hence requiring a shorter time period to react. Although it has been

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adopted by the American Oil Chemists' Society as the official method to determine the fatty acid composition of marine oils (*5*), several reports (*1*, *6*, *7*) have claimed the appearance of artifacts associated with its use, probably because of the high concentration usually employed [12% (v/v), versus 1−5% (v/v) for the other acids]. Furthermore, BF_3 leads to irreversible damage of the GC column, because traces of this compound migrate to the organic extracting phase (*8*).

The presence of water in the initial sample interferes with both transmethylation and methylation processes, because it promotes hydrolysis of the esters meanwhile formed. Hydrolysis, the reverse reaction of methylation, can occur in the presence of either acid or alkali; however, acid hydrolysis is a reversible reaction, whereas alkaline hydrolysis is essentially irreversible. Consequently, alkali-catalyzed reactions require strict anhydrous conditions, which may be a requirement difficult to fulfill in the case of biological samples. However, when compared with acid catalysts, alkaline counterparts transmethylate lipids at a much faster rate, although they are unable to methylate free fatty acids. Among alkaline catalysts, sodium methoxide, sodium hydroxide, and potassium hydroxide are the most popular; the first is usually selected (and will be employed in our study) because both hydroxides are more susceptible to promote irreversible hydrolysis reactions.

The aforementioned reactions occur in the presence of methanol (as a methylation/transmethylation reagent); therefore, their rate depends not only on the nature of the catalyst and the reaction conditions employed, but also on the solubility of the lipid sample in the methanol-containing medium. Hence, for such polar lipids as free fatty acids and phospholipids (which are easily dissolved in methanol), the reactions can proceed rapidly, whereas such nonpolar lipids as triglycerides require addition of another solvent, to ensure achievement of a single phase (*2*).

Most analytical methods published to date have focused on comparative analysis between existing derivatization methods, to define, for a particular sample, which method enhances the amount of fatty acids extracted. Nevertheless, if the type of sample under analysis is changed, the method that previously provided the best results will likely not do so anymore. Therefore, instead of performing blind optimization for a particular type of sample, it would be more useful to ascertain the relative importance of each parameter on the whole derivatization process. In this way, the eventual optimization of an existing derivatization method might proceed more rationally. This research effort was thus focused on the influence of processing factors in each step of FAME preparation. The study was divided in three main parts, viz.: (i) identification of the main effects involved in the alkali-catalyzed procedure, by studying the influence of catalyst concentration, operating temperature, and reaction time; (ii) characterization of the effects of polarity of the medium where the reaction takes place, as well as of postreaction steps in acid-catalyzed transmethylation/methylation; and (iii) comparative statistical analyses of the various methods employed as referred to the AOCS standard method (for determination of fatty acid composition of marine oils). Because of the increasing demand for analytical methods suitable to determine nutritionally relevant PUFA in their original biological sources (i.e., fish oil and microalgae), our study was performed using both cod liver oil and microalgal biomass as model systems. These two matrixes permitted one to study the derivatization parameters when pure extracted lipids (i.e., cod liver oil) or a mixture of both lipid and nonlipid material (i.e., biological cells) are employed. After the weight of each processing parameter on derivatization processes is determined, it will be possible to improve existing methods to measure lipids in biological materials, so to guarantee high accuracy, high efficiency, and minimum costs.

MATERIAL AND METHODS

Sample Matrices. Cod (*Gadus morhua*) liver oil was purchased in gelatine capsules from Bioarga (Lisbon, Portugal). The contents of several capsules were mixed together; each experiment was performed with ca. 25 mg (with a weight accuracy of no less than four decimal places) of oil as a sample and tested using a randomized design. Algal samples consisted of freeze-dried biomass of the Haptophyceae *Pavlova lutheri*, a marine microalga currently used in aquaculture; each experiment was performed with ca. 100 mg (again with a weight accuracy of no less than four decimal places).

Derivatization. *Alkali-Catalyzed Transmethylation.* The method by Rozés et al. (*9*), which consists of using sodium methoxide (in methanol) as the catalyst was adapted accordingly. The sample (either fish oil or microalga) was accurately weighed into a screw-capped tube, and 2 mL of a (0.5, 1.0, or 2.0 M) solution of sodium methoxide dissolved in methanol was added, together with 1 mg of tricosanoic acid methyl ester (used as a standard). After vigorous homogenization in a vortex for 5 s, the tube was placed at a given temperature (25, 50, or 65 °C) for a specific period (1, 5, or 20 min), to allow the reaction to proceed (the combinations of the experimental conditions tested are described in **Table 1**). Then, 1 mL of hexane containing 0.01% (w/v) butylated hydroxytoluene (BHT) as an antioxidant was added, which led to phase separation; the supernatant (organic phase) was recovered, filtered with anhydrous Na_2SO_4, and injected in GC.

Acid-Catalyzed Transmethylation/Methylation. The method of Lepage and Roy (*8*), modified by Cohen et al. (*10*), was used, using tricosanoic acid as an internal standard. Samples were dissolved in 2 mL of a freshly prepared mixture of acetyl chloride and methanol, at a ratio of 5:100 (v/v), together with 1 mg of tricosanoic acid. The aforementioned reagents were placed in Teflon-capped Pyrex tubes, and the reaction proceeded at 100 °C for 1 h, under pure nitrogen and darkness. After cooling to 30−40 °C, 1 mL of extracting solvent (hexane or isooctane, both containing 0.01% BHT) was added and the FAME-solvent solution was mixed in the vortex for a specific period of time (5 or 30 s). Purification of the solution was achieved either by salting out (using 1 mL of saturated sodium chloride solution) or washing (using 1 mL of water); the combinations of the experimental conditions tested in the postreaction steps are described in **Table 2**. This caused formation of two immiscible phases, which were then allowed to separate; the upper extracting solvent phase was recovered, dried over anhydrous Na_2SO_4, and injected in GC.

The polarity of the derivatization medium was tested with solvent mixtures of toluene, diethyl ether, and chloroform in methanol, in the proportions depicted in **Table 3**. For these experiments, the extracting solvent used was hexane; the FAME-solvent solution was mixed in the vortex for 30 s and purified by addition of 1 mL of water.

Standard Method. The method chosen to perform comparative analysis was determination of fatty acid composition by GC in marine oils as described in the AOCS official methods, method Ce 1b-89 (*5*); it consists of a preliminary alkaline hydrolysis of fats (with 0.5 M sodium hydroxide, at 100 °C for 5 min), followed by acidic transmethylation with 12% (v/v) BF_3 in methanol (at 100 °C for 30 min). Phase separation was achieved with a solution of saturated sodium chloride, using isooctane as an extracting solvent (dried on anhydrous Na_2SO_4 before injection).

Analysis. *Chemical Analysis.* The assay of FAME was carried out with a gas chromatograph AutoSystem XL from Perkin−Elmer (Norwalk, CT), equipped with a flame ionization detector. Separation was achieved in a column Supelcowax-10 (60 m, 0.32 mm, and 0.25 μm) from Supelco (Bellefonte, PA). The temperature was programmed to increase from 170 to 220 °C at a rate of 1 °C min^{-1}; the injector and detector temperatures were 250 and 270 °C, respectively. Injections were performed under split mode for oil samples and splitless mode for microalgal samples, using helium as a carrier gas. Data acquisition and analysis used the Turbochrome software from Perkin−Elmer.

Table 1. Effects of Reaction Parameters (in Alkali-Catalized Reactions) on Fatty Acid Residues (Average ± Standard Deviation) of (a) Cod Liver Oil and (b) *P. lutheri* Biomass[a]

a

temperature (°C)	reaction time (min)	catalyst concentrated (M)	Fatty Acid Residue (mg/g of Oil)					
			14:0	16:0	16:1 (*n*-7)	18:0	18:1 (*n*-7)	18:1 (*n*-9)
25	1	0.5	**61.126 ± 2.621**	**152.803 ± 2.562**	**73.661 ± 2.157**	**32.387 ± 0.843**	**31.441 ± 0.588**	**183.322 ± 0.296**
25	1	1	47.270 ± 0.029*	114.606 ± 0.199*	55.892 ± 0.084*	23.267 ± 0.124*	22.822 ± 0.388*	133.590 ± 0.942*
25	1	2	45.535 ± 1.957*	109.105 ± 2.926*	54.428 ± 1.933*	21.020 ± 0.445*	20.798 ± 0.398*	122.877 ± 2.496*
25	5	0.5	46.101 ± 2.659*	114.332 ± 1.642*	52.976 ± 0.774*	23.559 ± 0.378*	22.270 ± 0.261*	129.716 ± 1.546*
25	5	1	46.564 ± 0.143*	110.879 ± 0.743*	53.331 ± 0.158*	22.021 ± 0.075*	21.293 ± 0.092*	125.428 ± 0.624*
25	5	2	44.550 ± 1.005*	107.171 ± 2.788*	53.469 ± 1.372*	20.852 ± 0.428*	20.835 ± 0.625*	121.377 ± 3.280*
25	20	0.5	44.255 ± 0.530*	110.865 ± 1.251*	51.582 ± 0.521*	23.691 ± 0.257*	22.102 ± 0.597*	129.455 ± 2.021*
25	20	1	46.320 ± 0.240*	110.630 ± 4.102*	53.825 ± 1.867*	21.539 ± 0.723*	21.616 ± 1.585*	124.868 ± 5.534*
25	20	2	45.639 ± 1.173*	105.222 ± 1.388*	52.370 ± 1.068*	20.846 ± 0.109*	20.547 ± 0.247*	119.876 ± 1.317*
50	1	0.5	48.835 ± 4.726*	120.822 ± 10.184*	56.209 ± 5.134*	25.075 ± 1.541*	23.878 ± 1.442*	139.692 ± 9.376*
50	1	1	44.507 ± 0.955*	104.553 ± 5.489*	51.790 ± 2.825*	22.450 ± 0.039*	21.858 ± 0.401*	125.679 ± 1.372*
50	1	2	42.002 ± 1.155*	102.114 ± 2.735*	51.222 ± 1.253*	22.761 ± 0.306*	21.879 ± 0.072*	126.607 ± 0.837*
50	5	0.5	40.700 ± 0.877*	102.642 ± 1.795*	47.542 ± 0.682*	21.576 ± 0.150*	20.207 ± 0.350*	118.234 ± 0.889*
50	5	1	41.446 ± 0.492*	100.172 ± 1.628*	48.950 ± 1.829*	21.875 ± 0.871*	20.950 ± 0.454*	122.601 ± 4.012*
50	5	2	40.517 ± 1.430*	100.342 ± 0.011*	49.857 ± 0.820*	21.976 ± 0.472*	20.992 ± 0.153*	122.588 ± 0.626*
50	20	0.5	42.828 ± 1.405*	103.038 ± 0.291*	48.625 ± 1.397*	21.431 ± 0.679*	20.496 ± 0.180*	119.095 ± 0.905*
50	20	1	43.547 ± 0.985*	110.280 ± 2.507*	52.588 ± 1.143*	24.597 ± 0.932*	23.688 ± 0.914*	137.513 ± 5.288*
50	20	2	42.255 ± 1.292*	102.236 ± 4.518*	50.398 ± 0.798*	21.651 ± 0.716*	20.794 ± 1.288*	121.381 ± 7.050*
65	1	0.5	49.653 ± 8.409*	126.194 ± 19.501*	58.801 ± 9.471*	26.748 ± 4.683*	25.070 ± 3.997*	146.944 ± 24.795*
65	1	1	45.258 ± 0.211*	110.014 ± 0.317*	53.245 ± 0.192*	22.774 ± 0.182*	22.150 ± 0.037*	129.155 ± 0.223*
65	5	0.5	42.206 ± 0.128*	107.721 ± 0.428*	49.727 ± 0.426*	23.163 ± 0.126*	21.770 ± 0.022*	126.394 ± 0.032*
65	5	1	43.132 ± 2.491*	104.678 ± 2.752*	50.556 ± 1.856*	23.561 ± 0.097*	22.265 ± 0.207*	129.517 ± 0.877*
65	5	2	42.579 ± 1.377*	107.104 ± 4.192*	53.150 ± 2.169*	23.849 ± 1.081*	23.017 ± 1.084*	133.795 ± 5.885*
65	20	0.5	45.063 ± 1.783*	109.347 ± 2.877*	51.319 ± 2.370*	23.323 ± 0.477*	22.111 ± 0.251*	128.268 ± 2.548*
65	20	1	41.916 ± 2.379*	104.053 ± 0.588*	49.591 ± 0.197*	22.985 ± 0.164*	21.978 ± 1.249*	126.630 ± 4.715*
65	20	2	27.916 ± 0.012*	78.729 ± 0.111*	34.812 ± 0.130*	23.322 ± 0.121*	18.420 ± 0.023*	106.503 ± 0.011*

a

temperature (°C)	reaction time (min)	catalyst concentrated (M)	Fatty Acid Residue (mg/g of Oil)					
			18:2 (*n*-6)	18:3 (*n*-3)	18:4 (*n*-3)	20:1 (*n*-9)	20:5 (*n*-3)	22:6 (*n*-3)
25	1	0.5	**34.955 ± 1.068**	5.825 ± 0.823*	**15.889 ± 0.432**	**69.728 ± 1.462**	**65.286 ± 0.249**	**91.797 ± 6.396**
25	1	1	27.445 ± 0.976*	9.053 ± 0.117	12.352 ± 0.047*	48.058 ± 0.759*	47.593 ± 0.260*	54.882 ± 3.999*
25	1	2	25.389 ± 0.947*	8.671 ± 0.338	11.891 ± 0.376*	40.139 ± 0.327*	44.001 ± 0.822*	42.234 ± 0.853*
25	5	0.5	24.102 ± 0.399*	7.889 ± 0.649*	10.253 ± 0.526*	46.855 ± 3.007*	38.903 ± 1.983*	43.031 ± 1.090*
25	5	1	24.922 ± 0.115*	8.264 ± 0.085*	10.751 ± 0.204*	42.365 ± 0.269*	41.593 ± 0.398*	48.302 ± 1.561*
25	5	2	25.997 ± 0.919*	8.807 ± 0.024	12.148 ± 0.061*	39.454 ± 1.123*	43.505 ± 1.348*	40.431 ± 2.016*
25	20	0.5	25.451 ± 0.435*	8.197 ± 0.001*	10.349 ± 0.028*	49.472 ± 0.017*	41.252 ± 0.761*	44.806 ± 0.505*
25	20	1	24.319 ± 0.265*	8.380 ± 0.148*	10.938 ± 0.333*	41.422 ± 1.496*	41.434 ± 0.333*	43.105 ± 4.752*
25	20	2	25.297 ± 1.418*	8.768 ± 0.235	11.977 ± 0.303*	39.601 ± 0.469*	42.880 ± 0.985*	40.733 ± 4.016*
50	1	0.5	26.108 ± 0.927*	8.652 ± 0.586	11.085 ± 0.799*	49.915 ± 1.172*	42.727 ± 2.658*	48.384 ± 7.730*
50	1	1	26.154 ± 2.419*	8.689 ± 0.785	11.751 ± 0.221*	47.243 ± 2.029*	48.249 ± 2.083*	55.331 ± 3.814*
50	1	2	25.138 ± 0.035*	**9.435 ± 0.156**	13.219 ± 0.273*	49.096 ± 0.136*	53.235 ± 0.815*	63.590 ± 2.078*
50	5	0.5	23.140 ± 1.711*	7.364 ± 0.263*	9.307 ± 0.397*	43.665 ± 2.778*	36.962 ± 1.831*	38.083 ± 5.375*
50	5	1	25.210 ± 0.287*	8.729 ± 0.549	11.887 ± 1.121*	48.496 ± 2.119*	47.600 ± 4.904*	55.184 ± 6.448*
50	5	2	24.976 ± 0.270*	9.067 ± 0.870	12.674 ± 0.559*	47.228 ± 2.669*	50.395 ± 2.906*	59.037 ± 4.803*
50	20	0.5	23.731 ± 0.982*	7.537 ± 0.088*	10.266 ± 0.412*	43.965 ± 2.336*	30.324 ± 1.591*	45.172 ± 3.313*
50	20	1	26.014 ± 0.816*	8.902 ± 0.016	12.135 ± 0.742*	55.657 ± 2.965*	50.716 ± 2.022*	62.343 ± 9.597*
50	20	2	24.477 ± 0.339*	9.072 ± 1.196	12.278 ± 0.578*	44.773 ± 3.434*	44.903 ± 2.810*	48.808 ± 1.271*
65	1	0.5	27.085 ± 1.107*	9.287 ± 1.994	11.884 ± 2.666*	55.741 ± 2.707*	47.578 ± 1.941*	51.917 ± 5.778*
65	1	1	24.554 ± 0.561*	8.608 ± 0.128*	11.418 ± 0.322*	47.087 ± 2.050*	45.713 ± 2.162*	49.087 ± 5.781*
65	5	0.5	23.212 ± 0.089*	7.995 ± 0.050*	10.216 ± 0.190*	48.809 ± 0.754*	22.193 ± 1.635*	51.222 ± 1.244*
65	5	1	24.813 ± 1.056*	7.482 ± 0.163*	12.066 ± 0.924*	28.789 ± 1.380*	47.463 ± 2.077*	56.347 ± 5.993*
65	5	2	26.056 ± 0.756*	6.057 ± 0.539*	12.925 ± 0.449*	53.593 ± 2.650*	53.002 ± 1.704*	61.386 ± 0.528*
65	20	0.5	23.476 ± 0.943*	7.830 ± 0.630*	10.107 ± 1.225*	49.248 ± 0.290*	40.410 ± 2.279*	45.733 ± 0.847*
65	20	1	23.510 ± 1.249*	8.409 ± 0.464	10.370 ± 0.493*	49.893 ± 4.552*	39.680 ± 3.519*	37.666 ± 6.911*
65	20	2	23.321 ± 0.015*	5.343 ± 0.011*	12.971 ± 0.010*	47.666 ± 0.121*	33.678 ± 0.032*	31.917 ± 0.012*

b

temperature (°C)	catalyst concentrated (M)	Fatty Acid Residue (mg/g of AFDW)					
		14:0	16:0	16:1 (*n*-7)	18:0	18:1 (*n*-7)	18:1 (*n*-9)
25	0.5	5.123 ± 0.280	**5.573 ± 0.421**	**7.841 ± 0.351**	0.356 ± 0.086*	0.869 ± 0.100	0.791 ± 0.215
25	1	4.678 ± 0.264*	4.959 ± 0.307	7.018 ± 0.055	0.262 ± 0.062*	0.756 ± 0.067*	0.662 ± 0.141*
25	2	5.690 ± 0.390	4.614 ± 0.384	6.311 ± 0.090*	**0.789 ± 0.134**	0.800 ± 0.019	1.030 ± 0.111
50	1	4.366 ± 0.112*	4.745 ± 0.311	6.782 ± 0.205	0.208 ± 0.055*	0.807 ± 0.047	0.661 ± 0.176*
50	2	**7.071 ± 0.416**	5.477 ± 0.217	7.394 ± 0.126	0.611 ± 0.366	**0.943 ± 0.030**	**1.209 ± 0.023**
65	0.5	4.426 ± 0.404*	5.290 ± 0.242	6.969 ± 0.268	0.302 ± 0.191*	0.845 ± 0.100	0.788 ± 0.286
65	1	6.171 ± 0.518	5.456 ± 0.210	6.732 ± 0.130	0.269 ± 0.057*	0.845 ± 0.030	0.916 ± 0.100

Table 1 (Continued)

b

temperature (°C)	catalyst concentrated (M)	Fatty Acid Residue (mg/g of AFDW)				
		18:2 (*n*-6)	18:3 (*n*-3)	18:4 (*n*-3)	20:5 (*n*-3)	22:6 (*n*-3)
25	0.5	0.837 ± 0.225	0.547 ± 0.256	2.244 ± 0.355	5.460 ± 0.443	2.492 ± 0.340
25	1	0.903 ± 0.152	0.449 ± 0.148	2.158 ± 0.138	4.966 ± 0.091	2.332 ± 0.047
25	2	**1.403 ± 0.085**	**0.558 ± 0.229**	**2.740 ± 0.062**	5.331 ± 0.231	2.427 ± 0.117
50	1	0.828 ± 0.287	0.402 ± 0.036	2.194 ± 0.341	**5.119 ± 0.112**	**2.436 ± 0.035**
50	2	0.991 ± 0.033	0.405 ± 0.019	2.497 ± 0.335	4.633 ± 0.260	1.495 ± 0.059*
65	0.5	0.849 ± 0.116	0.379 ± 0.054	1.938 ± 0.304*	4.363 ± 0.455	1.722 ± 0.450
65	1	1.038 ± 0.067	0.406 ± 0.012	1.855 ± 0.213*	4.072 ± 0.111*	1.198 ± 0.289*

[a] Values in bold correspond to the highest amounts of each fatty acid obtained; when the remaining treatments generated statistically different results from the highest amounts, such results are followed by an asterisk. Results pertaining to the experiment at 65 °C, 1 min, and 2.0 M were inadvertently lost and thus are not presented. Because of the extension of the table, only the more concentrated fatty acids are presented.

Table 2. Effects of Postreaction Steps (in Acid-Catalyzed Reactions) on Fatty Acid Residues (Average ± Standard Deviation) of (a) Cod Liver Oil and (b) *P. lutheri* Biomass[a]

a

	Extraction Solvent	Hexane	Hexane	Hexane	Hexane	Isooctane	Isooctane	Isooctane	Isooctane
	extraction time (s)	5	5	30	30	5	5	30	30
	phase separation	NaCl	H_2O	NaCl	H_2O	NaCl	H_2O	NaCl	H_2O
fatty acid residue (mg/g of oil)	14:0	49.671 ± 0.167	51.383 ± 2.092	49.412 ± 2.855	**52.398 ± 1.080**	47.101 ± 0.086*	49.367 ± 0.417	*48.880 ± 0.215*	50.157 ± 3.348
	15:0	3.607 ± 0.094	3.683 ± 0.010	3.720 ± 0.267	**3.865 ± 0.161**	3.477 ± 0.099*	3.602 ± 0.040	*3.633 ± 0.002*	3.628 ± 0.240
	16:0	107.350 ± 1.541	109.185 ± 0.081	106.933 ± 1.500	**114.250 ± 3.474**	103.787 ± 2.336*	107.977 ± 0.432	*106.441 ± 1.382*	107.253 ± 3.624
	16:1 (*n*-7)	56.618 ± 1.112	57.757 ± 0.090	56.777 ± 0.993	**60.329 ± 1.651**	54.796 ± 1.387*	56.794 ± 0.059	*56.474 ± 0.863*	56.787 ± 4.644
	18:0	19.786 ± 0.460	20.062 ± 0.160	20.259 ± 0.168	**21.459 ± 2.283**	20.307 ± 0.318	20.615 ± 1.069	*19.825 ± 0.671*	21.076 ± 1.974
	18:1 (*n*-7)	21.190 ± 0.298	21.465 ± 0.046	21.422 ± 0.095	**22.681 ± 2.086**	21.496 ± 0.341	21.817 ± 1.083	*21.088 ± 0.795*	22.160 ± 1.749
	18:1 (*n*-9)	121.502 ± 2.184*	123.193 ± 0.662	123.137 ± 0.027	**130.808 ± 5.026**	123.191 ± 1.735	125.457 ± 5.808	*120.948 ± 4.214**	126.655 ± 3.962
	18:2 (*n*-6)	24.596 ± 0.332	24.984 ± 0.157	25.346 ± 0.571	**26.490 ± 2.577**	24.944 ± 0.365	25.544 ± 0.900	*24.515 ± 0.879*	26.294 ± 2.926
	18:3 (*n*-3)	10.155 ± 0.417	9.552 ± 0.503	9.380 ± 0.618	9.614 ± 0.989	9.003 ± 0.293	9.670 ± 0.673	*9.800 ± 0.868*	**10.409 ± 0.025**
	18:4 (*n*-3)	13.404 ± 0.336	13.604 ± 0.092	13.797 ± 0.484	14.009 ± 1.231	13.406 ± 0.125	13.641 ± 0.632	*13.474 ± 0.213*	**14.122 ± 1.545**
	20:0	1.187 ± 0.017*	1.228 ± 0.043	1.467 ± 0.198	1.163 ± 0.157*	1.833 ± 0.187	2.219 ± 0.168	***1.863 ± 0.258***	1.446 ± 0.034
	20:1 (*n*-9)	39.971 ± 2.241	40.918 ± 1.060	42.143 ± 1.681	43.370 ± 2.437	44.312 ± 0.840	43.873 ± 2.080	*41.196 ± 2.736*	**45.462 ± 2.959**
	20:2 (*n*-6)	2.722 ± 0.089*	2.809 ± 0.110	2.916 ± 0.160	2.740 ± 0.047*	3.018 ± 0.099	3.024 ± 0.106	*2.838 ± 0.270*	**3.194 ± 0.199**
	20:3 (*n*-3)	4.814 ± 0.145*	5.162 ± 0.087	5.090 ± 0.159	4.941 ± 0.543	5.374 ± 0.075	5.312 ± 0.397	*5.060 ± 0.418*	**5.528 ± 0.213**
	20:4 (*n*-6)	1.315 ± 0.056*	1.384 ± 0.140*	1.471 ± 0.048	1.288 ± 0.165*	1.425 ± 0.155	1.286 ± 0.049*	*1.400 ± 0.117*	**1.455 ± 0.240**
	20:5 (*n*-3)	51.219 ± 2.203	53.000 ± 0.500*	53.528 ± 0.586	54.300 ± 1.378	55.313 ± 0.847	55.031 ± 2.678	*52.800 ± 2.382*	**58.033 ± 2.412**
	22:1 (*n*-9)	6.543 ± 2.314	6.567 ± 2.642	5.700 ± 1.154	3.872 ± 0.034*	7.457 ± 2.949	7.276 ± 3.473	*5.556 ± 0.441*	**8.064 ± 2.210**
	22:6 (*n*-3)	63.582 ± 3.906*	66.044 ± 1.289*	70.447 ± 1.457	64.258 ± 3.225*	74.265 ± 2.756	72.010 ± 3.089	*67.559 ± 3.143*	**78.199 ± 2.517**

b

	Extraction Solvent	Hexane	Hexane	Hexane	Hexane	Isooctane	Isooctane	Isooctane	Isooctane
	extraction time (s)	5	5	30	30	5	5	30	30
	phase separation	NaCl	H_2O	NaCl	H_2O	NaCl	H_2O	NaCl	H_2O
fatty acid residue (mg/g of AFDW)	14:0	6.752 ± 0.400	**7.138 ± 0.208**	6.195 ± 0.087*	6.597 ± 0.103	6.758 ± 0.133	6.573 ± 0.367	*7.038 ± 0.009*	6.807 ± 0.314
	15:0	0.222 ± 0.003*	0.208 ± 0.001*	**0.317 ± 0.040**	0.244 ± 0.039*	0.224 ± 0.000*	0.201 ± 0.004*	*0.217 ± 0.004**	0.233 ± 0.024*
	16:0	10.666 ± 0.130	11.009 ± 0.602	10.098 ± 0.451	10.561 ± 0.042	10.641 ± 0.066	10.225 ± 0.256	*10.855 ± 0.420*	**11.223 ± 0.740**
	16:1 (*n*-7)	11.194 ± 0.033	**11.945 ± 0.805**	10.933 ± 0.021	11.349 ± 0.344	11.163 ± 0.155	10.847 ± 0.287	*11.706 ± 0.542*	11.376 ± 0.421
	18:0	2.514 ± 0.223	**2.733 ± 0.158**	1.835 ± 0.105*	2.040 ± 0.299	2.403 ± 0.079	2.402 ± 0.081	*2.152 ± 0.239*	2.096 ± 0.548
	18:1 (*n*-7)	2.782 ± 0.022	2.740 ± 0.057	2.670 ± 0.011	2.841 ± 0.040	2.762 ± 0.006	2.685 ± 0.045	*2.718 ± 0.064*	**2.864 ± 0.149**
	18:1 (*n*-9)	4.002 ± 0.097	3.815 ± 0.095	2.931 ± 0.012*	3.566 ± 0.500	4.021 ± 0.108	3.709 ± 0.050	*3.807 ± 0.119*	**4.862 ± 0.481**
	18:2 (*n*-6)	1.276 ± 0.006	1.287 ± 0.076	1.069 ± 0.006	0.629 ± 0.888	**1.454 ± 0.030**	1.357 ± 0.111	*1.307 ± 0.117*	1.420 ± 0.208
	18:3 (*n*-3)	0.809 ± 0.019	0.796 ± 0.008	0.798 ± 0.002	0.842 ± 0.079	0.802 ± 0.015	0.783 ± 0.034	*0.778 ± 0.088**	**0.908 ± 0.021**
	18:4 (*n*-3)	3.587 ± 0.121	**3.702 ± 0.260**	3.294 ± 0.045	3.511 ± 0.088	3.479 ± 0.037	3.424 ± 0.090	*3.581 ± 0.358*	3.500 ± 0.021
	20:3 (*n*-3)	0.221 ± 0.020	0.204 ± 0.013	**0.251 ± 0.014**	0.149 ± 0.054	0.221 ± 0.007	0.194 ± 0.072	*0.213 ± 0.023*	0.194 ± 0.099
	20:5 (*n*-3)	8.896 ± 0.192	9.187 ± 0.873	8.759 ± 0.213	**9.211 ± 0.370**	8.687 ± 0.087	8.533 ± 0.121	*9.015 ± 0.735*	9.061 ± 0.253
	22:6 (*n*-3)	4.183 ± 0.129	4.371 ± 0.615	3.562 ± 0.032	3.983 ± 0.368	4.097 ± 0.132	4.125 ± 0.030	*4.431 ± 0.543*	**4.771 ± 0.603**

[a] Values in italic correspond to the postreaction parameters employed in the reference method. Values in bold correspond to the highest amounts of each fatty acid obtained; when the remaining treatments generated statistically different results from the highest amounts, such results are followed by an asterisk.

Calculations were performed according to the AOCS official method Ce 1b-89. Pure standards (Sigma) were used for fatty acid identification, which was based on a comparison of peak retention times between samples and standards.

Results from microalgal samples were presented in ash-free dry weight (AFDW) basis. Fatty acids were named using the code i:j (n-k), where i indicates the total number of carbon atoms, j indicates the number of double bonds, and k indicates the position of the last double bond counted from the terminal methyl group.

Statistical Analysis. All methods described above were tested following a randomized experimental design, replicated at least 4 times. Analyses of variance (ANOVA) of the overall dataset and Fisher's

Table 3. Effect of Medium Polarity (in Acid-Catalyzed Derivatization) on Fatty Acid Residues (Average ± Standard Deviation) of (a) Cod Liver Oil and (b) *P. lutheri* Biomass[a]

a

	Medium Solvents	Me + T	Me + E	Me + T	Me + E	Me + C	Me + C	Me
	proportion (v/v)	1:1	1:1	3:2	3:2	1:1	3:2	
	polarity index	3.75	3.95	4.02	4.18	4.60	4.70	5.10
fatty acid residue (mg/g of oil)	14:0	60.468 ± 3.435	**62.184 ± 1.722**	60.063 ± 1.027	57.204 ± 2.911	50.541 ± 1.697*	61.763 ± 1.045	*52.398 ± 1.080**
	15:0	4.369 ± 0.281	**4.581 ± 0.269**	4.344 ± 0.342	4.216 ± 0.375	3.778 ± 0.118	3.279 ± 0.207	*3.865 ± 0.161*
	16:0	130.772 ± 5.183	**138.810 ± 5.875**	129.667 ± 4.793	128.993 ± 5.717	117.006 ± 2.060*	132.676 ± 2.595	*114.250 ± 3.474**
	16:1 (*n*-7)	69.240 ± 1.331	**71.877 ± 2.046**	67.559 ± 2.239	67.595 ± 2.881	61.094 ± 0.916*	69.260 ± 1.909	*60.329 ± 1.651**
	18:0	24.640 ± 2.776	**27.082 ± 1.623**	25.013 ± 2.394	25.911 ± 1.734	23.978 ± 0.110*	26.126 ± 1.193	*21.459 ± 2.283**
	18:1 (*n*-7)	25.846 ± 2.843	**28.175 ± 3.511**	26.381 ± 1.305	27.077 ± 1.842	25.001 ± 0.194	27.251 ± 1.321	*22.681 ± 2.086*
	18:1 (*n*-9)	149.985 ± 6.375	**163.531 ± 9.387**	152.647 ± 8.221	156.931 ± 6.113	144.849 ± 5.086	158.322 ± 5.582	*130.808 ± 5.026*
	18:2 (*n*-6)	30.388 ± 3.327	**32.962 ± 3.779**	30.708 ± 1.746	31.661 ± 3.278	29.243 ± 2.203	31.974 ± 2.108	*26.490 ± 2.577**
	18:3 (*n*-3)	11.915 ± 1.162	**12.579 ± 1.333**	11.803 ± 0.981	8.261 ± 1.236*	11.270 ± 0.204	12.118 ± 1.325	*9.614 ± 0.989*
	18:4 (*n*-3)	16.510 ± 1.630	**18.006 ± 2.102**	16.552 ± 1.252	16.963 ± 1.851	15.762 ± 0.243	17.306 ± 1.203	*14.009 ± 1.231*
	20:0	1.708 ± 0.144	**1.909 ± 0.211**	1.700 ± 0.062	1.695 ± 0.138	1.736 ± 0.016	1.770 ± 0.111	*1.163 ± 0.157**
	20:1 (*n*-9)	52.200 ± 3.010	**58.771 ± 3.817**	54.195 ± 3.273	57.273 ± 2.355	54.588 ± 0.283	57.525 ± 1.158	*25.370 ± 2.437**
	20:2 (*n*-6)	3.862 ± 0.238	**4.277 ± 0.123**	3.747 ± 0.463	3.923 ± 0.218	3.889 ± 0.133	4.141 ± 0.170	*2.740 ± 0.047**
	20:3 (*n*-3)	5.708 ± 0.372	**6.514 ± 0.325**	6.254 ± 0.052	6.429 ± 0.178	5.917 ± 0.041	6.509 ± 0.229	*4.941 ± 0.543**
	20:4 (*n*-6)	1.345 ± 0.184	**1.508 ± 0.258**	1.440 ± 0.107	1.650 ± 0.237	1.437 ± 0.008	1.567 ± 0.278	*1.288 ± 0.165*
	20:5 (*n*-3)	66.850 ± 3.674	**74.880 ± 5.573**	68.708 ± 4.698	72.134 ± 4.295	68.997 ± 2.235	73.111 ± 3.614	*54.300 ± 1.378**
	22:1 (*n*-9)	5.566 ± 0.425	**6.253 ± 0.271**	5.876 ± 0.358	6.086 ± 0.052	5.908 ± 0.254	6.170 ± 0.376	*3.872 ± 0.034**
	22:6 (*n*-3)	88.072 ± 7.345	**100.213 ± 9.870**	93.124 ± 7.190	96.567 ± 6.384	95.779 ± 2.740	99.510 ± 9.513	*64.258 ± 3.225**

b

	Medium Solvents	Me + T	Me + E	Me + C
	proportion (v/v)	1:1	1:1	1:1
	polarity index	3.75	3.95	4.60
fatty acid residue (mg/g of AFDW)	14:0	**5.319 ± 0.229**	4.637 ± 0.460	4.865 ± 0.380
	15:0	**0.076 ± 0.003**	0.058 ± 0.002*	0.057 ± 0.004*
	16:0	**6.171 ± 0.185**	5.733 ± 0.221	5.844 ± 0.284
	16:1 (*n*-7)	**8.675 ± 0.215**	8.373 ± 0.111	8.479 ± 0.158
	18:0	**0.290 ± 0.013**	0.248 ± 0.023	0.233 ± 0.052
	18:1 (*n*-7)	**1.084 ± 0.016**	1.020 ± 0.007	1.042 ± 0.088
	18:1 (*n*-9)	**1.174 ± 0.067**	0.699 ± 0.024*	0.742 ± 0.134*
	18:2 (*n*-6)	**0.857 ± 0.010**	0.768 ± 0.145	0.791 ± 0.135
	18:3 (*n*-3)	**0.542 ± 0.007**	0.535 ± 0.002	0.537 ± 0.104
	18:4 (*n*-3)	2.915 ± 0.012	2.954 ± 0.003	**3.001 ± 0.019**
	20:3 (*n*-3)	0.094 ± 0.015	0.076 ± 0.022	**0.106 ± 0.044**
	20:5 (*n*-3)	7.472 ± 0.023	7.526 ± 0.004	**7.496 ± 0.011**
	22:6 (*n*-3)	**3.608 ± 0.125**	3.338 ± 0.145	3.264 ± 0.256

[a] Values in italic correspond to the reference method. Values in bold correspond to the highest amounts of each fatty acid obtained; when the remaining treatments generated statistically different results from the highest amounts, such results are followed by an asterisk. Me, T, E, and C denote methanol, toluene, (diethyl) ether, and chloroform, respectively.

protected least significant difference (PLSD) test for pairwise comparison were performed using the software StatView (Abacus Concepts, Piscataway, NJ).

RESULTS AND DISCUSSION

Alkali-Catalyzed Transmethylation. Transmethylation of oils with alkaline catalysts has been claimed (*11*) to be accomplished in a few minutes, under mild temperature conditions. To ascertain the magnitude of each processing parameter (temperature, reaction time, and concentration of catalyst) upon the derivatization process, their independent and combined effects were studied. The fatty acid contents of both cod liver oil and microalgal biomass samples, after transmethylation following the various experimental conditions under study, are presented in **Table 1** (reaction time was not tested in microalgae, because of the scarcity of sample). On the basis of ANOVA, it could be concluded that the concentration of catalyst was the most relevant parameter for microalgae, whereas the three parameters played statistically significant roles for oil samples; interaction between parameters also played significant roles for most fatty acids. Among oil samples, the temperature was the most important parameter for 14:0, 15:0, 16:0, 16:1 (*n*-7), 18:1 (*n*-9), 18:2 (*n*-6), 20:2 (*n*-6), and 22:1 (*n*-9), whereas the concentration was the main parameter for 14:0, 15:0, 16:0, 16:1 (*n*-7), 18:1 (*n*-7), 18:1 (*n*-9), 18:4 (*n*-3), and 20:2 (*n*-6); the reaction time was significant for 14:0, 15:0, 16:0, 16:1 (*n*-7), 18:1 (*n*-7), 18:1 (*n*-9), 18:2 (*n*-6), 18:4 (*n*-3), 20:5 (*n*-3), and 22:6 (*n*-3). The ideal temperature range for the reaction to take place should lie in the vicinity of 25 °C, because higher temperatures generated lower amounts of fatty acids (probably because of degradation of the FAME meanwhile formed). Such a result suggests that the temperature is not important in terms of enhancing the extent of the transmethylation reaction, although it may promote undesirable side reactions above a certain threshold; this rationale is in agreement with previous observations (*11*).

In regard to the effect of the concentration, our studies pointed at (significantly) higher fatty acid recoveries when the catalyst concentration was 0.5 M, when compared with 1.0 or 2.0 M. When using sodium methoxide to prepare FAME from bacterial

biomass, Rozes et al. (*9*) claimed increased fatty acid conversion when the catalyst concentration was raised from 0.5 to 1 M. Nevertheless, when the concentration was further raised above 1 M, small decreases in monounsaturated fatty acid recoveries could be observed, thus suggesting that higher concentration values lead to lower conversion rates. Other reports (*12*) even recommend the use of 0.1 M NaOH as adequate to promote quantitative derivatization reactions. Therefore, in our matrices, a concentration of 0.5 M appears adequate for alkali-catalyzed transmethylation.

Hydrolysis is the reverse reaction of methylation; however, unlike methylation, it is catalyzed by either acid or alkali. Whereas acid hydrolysis is reversible, its alkaline counterpart is essentially irreversible. This happens because the carboxylate anion formed reacts preferentially with sodium or potassium (present in the mixture as catalyst components) than with alcohol, thus forming a stable salt (soap); such a reaction is accordingly referred to as saponification. Some reports (*13*) describe partial saponification of previously formed esters as a function of time or temperature, which lead to conversion into free fatty acid soaps. Because the conversion of fatty acid residues from triacylglycerol lipids to methyl esters by a methanolic solution of NaOH has been reported to take as little as 3 min to achieve a plateau of ca. 90% transformation (*11*), increasing time intervals may lead to the occurrence of saponification. Because a small time interval (1 min) seems sufficient to provide an essentially complete reaction, it can thus be stated that, under our experimental conditions, time does not have a significant effect when enhancing alkali-catalyzed transmethylation.

Acid-Catalyzed Transmethylation/Methylation. *Postreaction Procedures.* The preparation of FAME is finalized with a few postreaction procedures, immediately after the transmethylation/methylation reactions. Those procedures are intended to (i) quantitatively transfer the FAME formed in the reaction mixture into an organic solvent, and (ii) purify the FAME-rich organic solvent solution by salting out with a saline solution, washing with water, or both (to prevent production of artifacts and damage of the chromatographic column). Most errors associated with FAME preparation are closely related to the postreaction steps (*2*). Therefore, careful evaluation of the impact of these crucial steps is required, when aiming at accurate and reliable analyses.

When performing postreaction steps in microalgal biomass, the amounts of the various fatty acids obtained were enhanced under several experimental conditions, but no specific trend could be unfolded (**Table 2**). Statistical treatment and analysis of those results revealed that purification of the FAME-solvent solution (with either a saline solution or water) provided equivalent results for all fatty acids studied, whereas the amounts of some fatty acids were affected by the extracting solvent (15:0) or the mixing time (15:0 and 18:0). Aside from the effect of purification, the highest average results in terms of most fatty acid amounts were obtained by 30 s of mixing in the vortex; in regard to the organic solvents under study, because only 15:0 shows different values for isooctane and hexane, both solvents seem adequate for extraction of FAME from the reaction medium.

In what concerns results of the postreaction steps upon cod liver oil, the purification step was, again, not statistically significant. The influence of the organic extracting solvent and the time of mixing was significant for most fatty acids [14:0, 15:0, 16:0, 16:1 (*n*-7), 20:0, 20:1 (*n*-9), 20:2 (*n*-6), 20:3 (*n*-3), 20:4 (*n*-6), 20:5 (*n*-3), 22:1 (*n*-9), and 22:6 (*n*-3)]. Increased

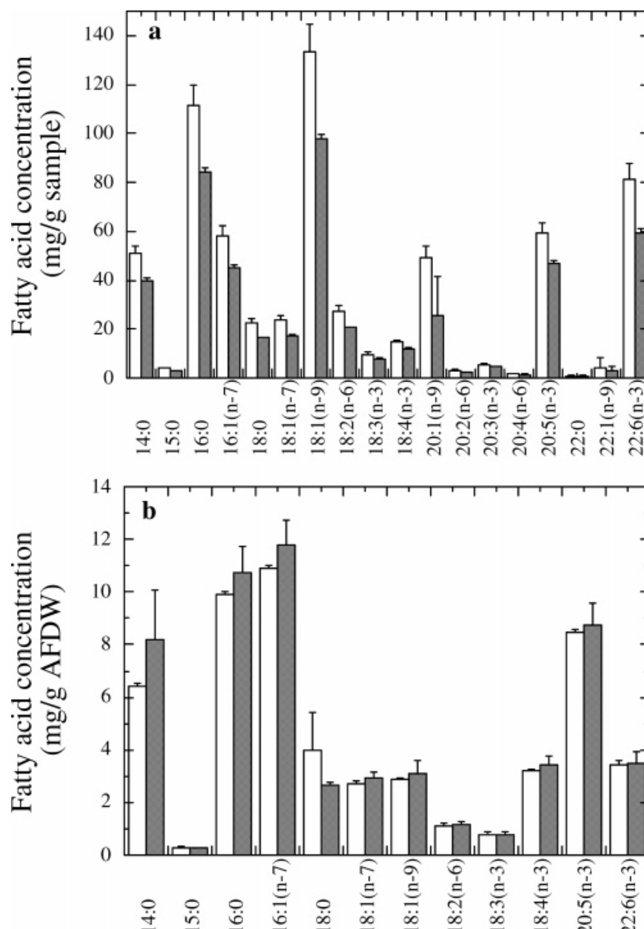
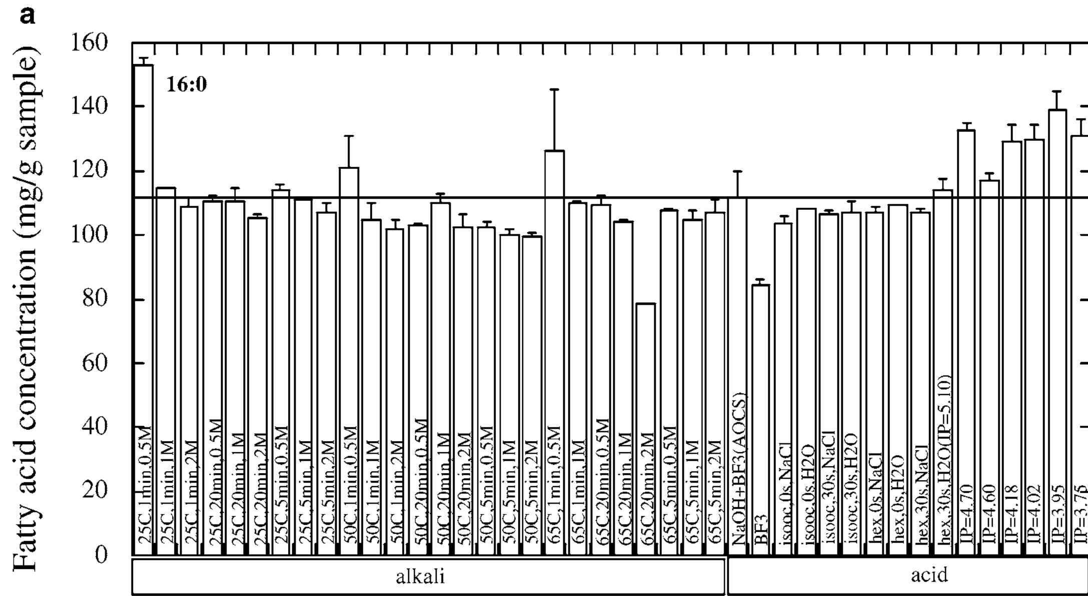


Figure 1. Quantitative profiles of fatty acids (average ± standard deviation) obtained following the two-step (NaOH–methanol and BF_3–methanol) treatment (white bars), as well as the one-step (BF_3–methanol) treatment (gray bars), in both oil (a) and microalgal (b) samples.

mixing times enhanced the amounts of fatty acids. Generally, hexane enhanced the amounts of the low-chain fatty acids [from 14:0 to 18:2 (*n*-6)], whereas the remaining fatty acids were enhanced by extraction with isooctane. The organic extracting solvent should provide similar solubilities for all FAME formed in the reaction mixture, so that the extract would represent the actual composition of the mixture (*2*); besides, its addition has been claimed to reduce peroxidation during storage (*14*). As isooctane [polarity index (PI) = 0.4] is more polar than hexane (PI = 0), such reasoning might explain its higher extracting power toward such long-chain PUFA as 20:5 (*n*-3) and 22:6 (*n*-3). The decrease in 22:6 (*n*-3) amounts when extracted with hexane (and concomitant suggestion of use of another solvent such as isooctane) has also been reported elsewhere (*15*).

Polarity of the Reaction Medium. The type of lipid under analysis determines the polarity of the derivatization medium. If free fatty acids alone are to be methylated or if such polar lipids as phospholipids are to be transmethylated, no other solvent than methanol is required in the reaction medium (*4*, *6*). Otherwise, to solubilize nonpolar lipids as cholesterol esters or triacylglycerols, an additional solvent needs to be added to the reaction mixture. The effectiveness of such a solvent depends on its ability to solubilize lipids, coupled with its miscibility with methanol. In addition, the tendency to generate artifacts, as well as potential risks of explosion and poisoning should also be considered (*2*). Benzene has frequently been cited in the literature as a valuable solvent in lipid analysis, because it


a
Fatty acid concentration (mg/g sample)
160
140
120
100
80
60
40
20
0
16:0
25C,1min,0.5M
25C,1min,1M
25C,1min,2M
25C,20min,0.5M
25C,20min,1M
25C,20min,2M
25C,5min,0.5M
25C,5min,1M
25C,5min,2M
50C,1min,0.5M
50C,1min,1M
50C,1min,2M
50C,20min,0.5M
50C,20min,1M
50C,20min,2M
50C,5min,0.5M
50C,5min,1M
50C,5min,2M
65C,1min,0.5M
65C,1min,1M
65C,20min,0.5M
65C,20min,1M
65C,20min,2M
65C,5min,0.5M
65C,5min,1M
65C,5min,2M
NaOH+BF3(AOCS)
BF3
isooc,0s,NaCl
isooc,0s,H2O
isooc,30s,NaCl
isooc,30s,H2O
hex,0s,NaCl
hex,0s,H2O
hex,30s,NaCl
hex,30s,H2O(IP=5.10)
IP=4.70
IP=4.60
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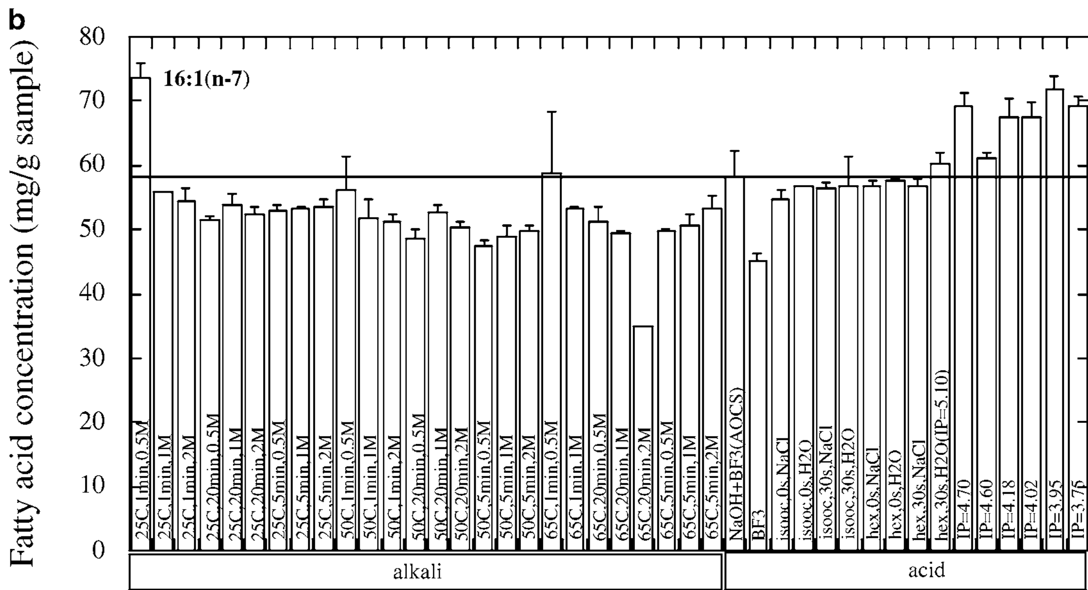
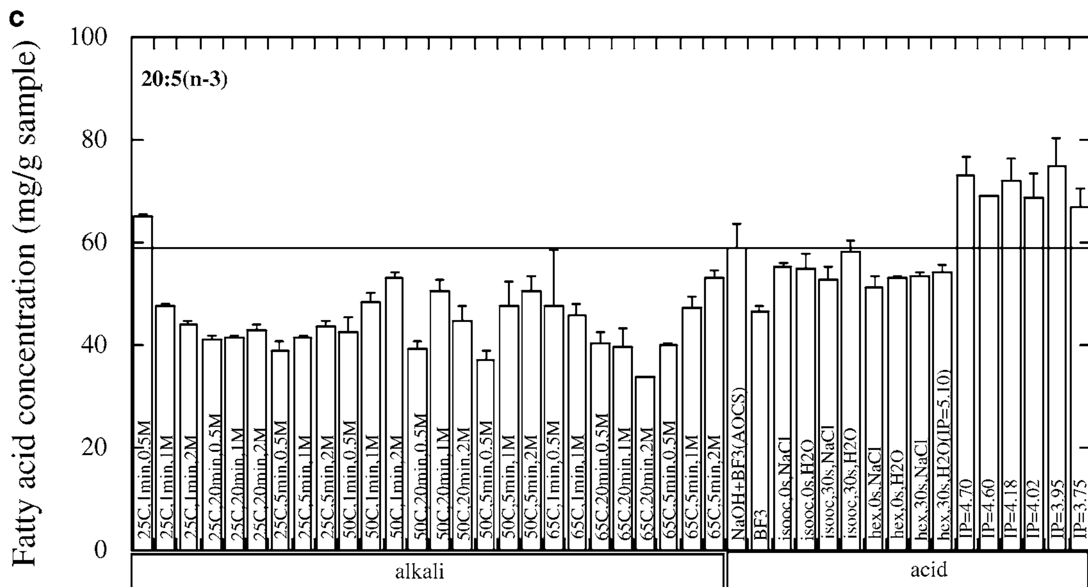
IP=4.02
IP=3.95
IP=3.75
alkali
acid


b
Fatty acid concentration (mg/g sample)
80
70
60
50
40
30
20
10
0
16:1(n-7)
25C,1min,0.5M
25C,1min,1M
25C,1min,2M
25C,20min,0.5M
25C,20min,1M
25C,20min,2M
25C,5min,0.5M
25C,5min,1M
25C,5min,2M
50C,1min,0.5M
50C,1min,1M
50C,1min,2M
50C,20min,0.5M
50C,20min,1M
50C,20min,2M
50C,5min,0.5M
50C,5min,1M
50C,5min,2M
65C,1min,0.5M
65C,1min,1M
65C,20min,0.5M
65C,20min,1M
65C,20min,2M
65C,5min,0.5M
65C,5min,1M
65C,5min,2M
NaOH+BF3(AOCS)
BF3
isooc,0s,NaCl
isooc,0s,H2O
isooc,30s,NaCl
isooc,30s,H2O
hex,0s,NaCl
hex,0s,H2O
hex,30s,NaCl
hex,30s,H2O(IP=5.10)
IP=4.70
IP=4.60
IP=4.18
IP=4.02
IP=3.95
IP=3.75
alkali
acid


c
Fatty acid concentration (mg/g sample)
100
80
60
40
20
0
20:5(n-3)
25C,1min,0.5M
25C,1min,1M
25C,1min,2M
25C,20min,0.5M
25C,20min,1M
25C,20min,2M
25C,5min,0.5M
25C,5min,1M
25C,5min,2M
50C,1min,0.5M
50C,1min,1M
50C,1min,2M
50C,20min,0.5M
50C,20min,1M
50C,20min,2M
50C,5min,0.5M
50C,5min,1M
50C,5min,2M
65C,1min,0.5M
65C,1min,1M
65C,20min,0.5M
65C,20min,1M
65C,20min,2M
65C,5min,0.5M
65C,5min,1M
65C,5min,2M
NaOH+BF3(AOCS)
BF3
isooc,0s,NaCl
isooc,0s,H2O
isooc,30s,NaCl
isooc,30s,H2O
hex,0s,NaCl
hex,0s,H2O
hex,30s,NaCl
hex,30s,H2O(IP=5.10)
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IP=4.02
IP=3.95
IP=3.75
alkali
acid

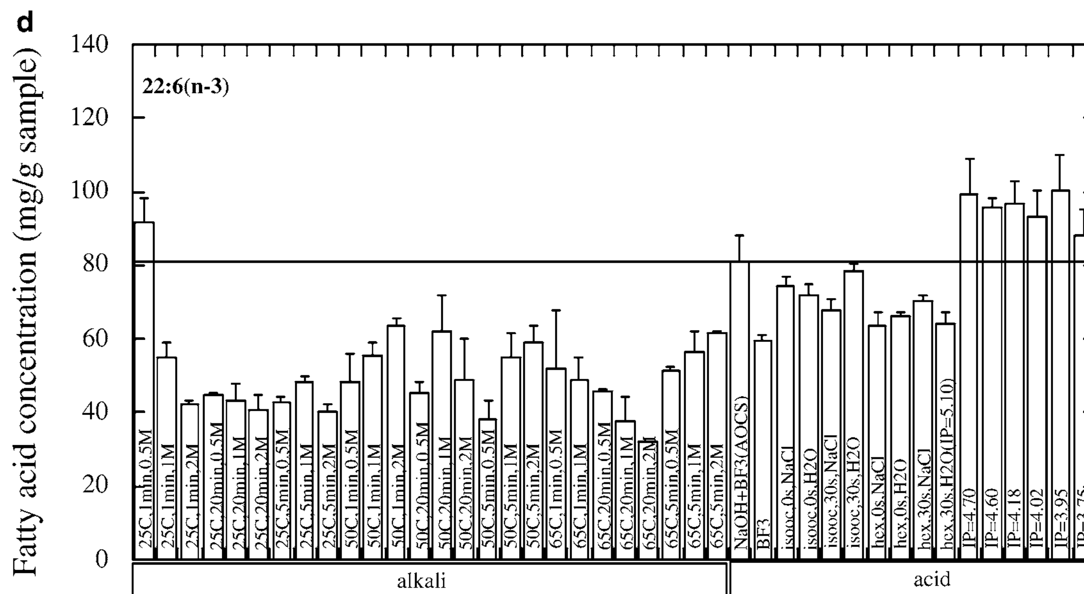
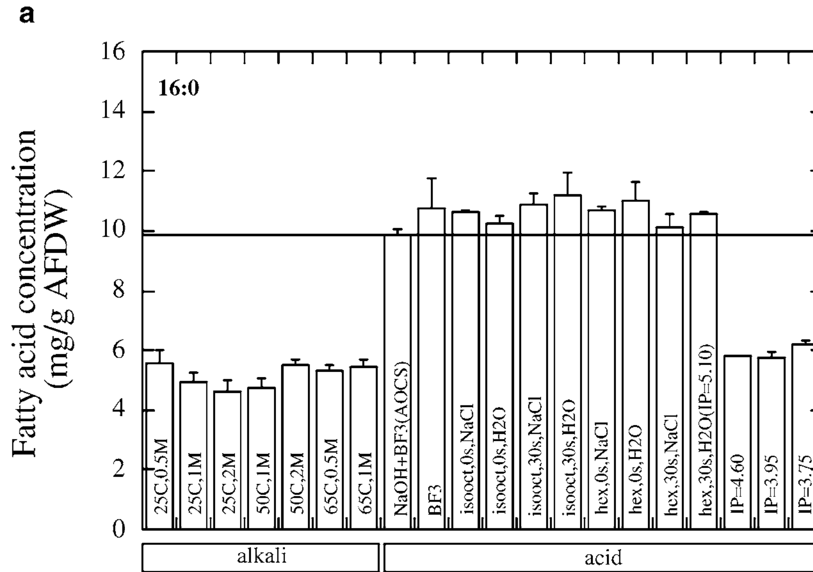


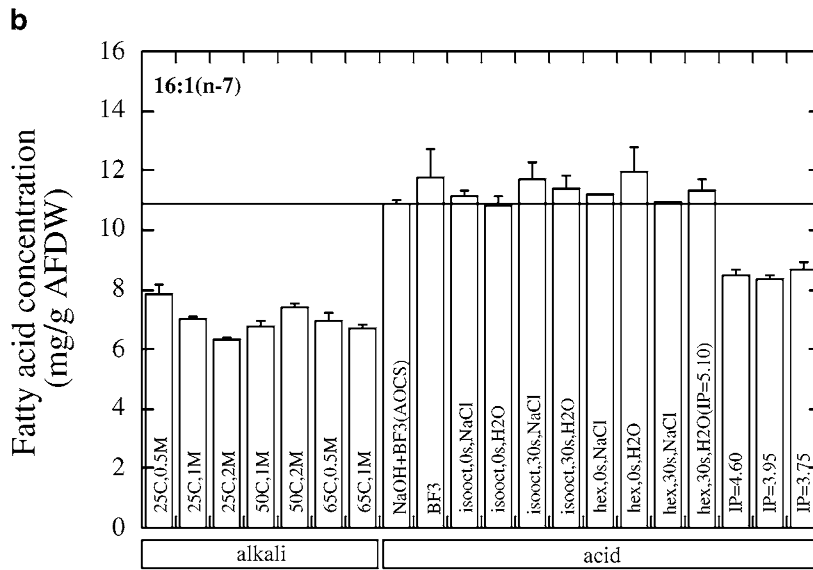
Figure 2. FAME concentrations (average ± standard deviation) in fish oil, pertaining to 16:0, 16:1 (*n*-7), 20:5 (*n*-3), and 22:6 (*n*-3), when submitted to the various treatments under study.

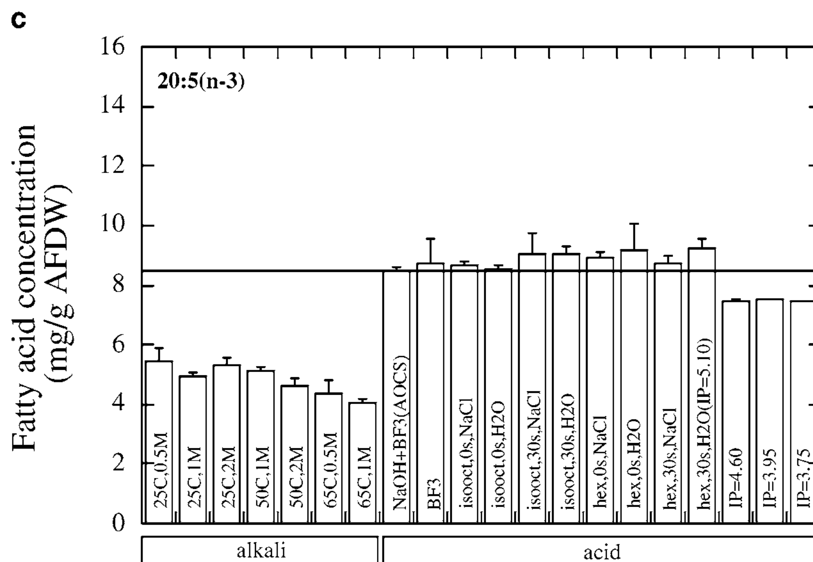
improves solubility of triglycerides (*6*); unfortunately, it is also known to be extremely toxic. An alternative solvent is toluene, which has comparable chromatographic properties and is much less hazardous (*16*). Hexane has also been used as a cosolvent to improve oil solubilization (*17*), although it was found to slow the transesterification process (*18*). After toluene, diethyl ether, and chloroform were mixed to different volumetric proportions with methanol, it was possible to obtain a wide range of medium polarities. The nature of the solvents and their relative amounts were chosen to guarantee miscibility with methanol, as well as fast and clear separation from the nonpolar hexane phase. Results obtained thereby are depicted in **Table 3**, together with results from derivatization with plain methanol, for comparison purposes. Such a procedure was employed only for oil samples, because microalgal samples derivatized with plain methanol and mixtures of solvents came from different cultures, and hence should not be directly comparable.

The solvent mixtures that generated the higher amounts of FAME depended on the matrix in question: microalgal fatty acids attained their highest values when transmethylation/methylation was carried out in a mixture of methanol and toluene 1:1 (v/v); on the other hand, cod oil fatty acids exhibited higher amounts when the reaction was carried out in a mixture of methanol and diethyl ether 1:1 (v/v). ANOVA, including both the type of solvent and its proportion (in the mixture with methanol), indicated that both parameters were significant. Hence, to combine these two parameters in a single one, the PI of the resulting mixture was calculated. The PI of a solution is a weighted average of the individual PIs of the solution components; it is useful to determine the solubility of a particular sample in said solution. Subsequent analysis of the fatty acid amounts recovered at the various PIs studied confirmed that those amounts were enhanced when the PI of the solution decreased. In conclusion, one can state that the use of an adequate mixture of solvents is beneficial, because it increases the amounts of FAME obtained.

Comparison between Methods. The profiles of fatty acids obtained following the two-step (NaOH−methanol and BF_3−methanol) treatment as well as the one-step (BF_3−methanol) treatment alone are depicted in **Figure 1**, for both cod liver oil and microalgal biomass. To check whether the differences between the procedures were statistically significant, ANOVA was performed on each fatty acid. One found that the amounts of fatty acids generated via both methods were statistically equivalent in microalgal samples, whereas most of them were significantly different between treatments in oil samples. From those significantly different fatty acids, the higher amounts were obtained with the two-step treatment. A pure lipid mixture, as cod liver oil, does not dissolve in plain methanol; thus, the interfacial contact area is rather small, which will lead to low reaction rates that compromise the success of the method. Because the two-step treatment involved an extended period of reaction, it generated higher conversion rates than the use of BF_3−methanol alone, in oil samples. Nevertheless, these results do not indicate that the two-step treatment should be preferred to derivatize samples. In microalgal biomass (which was previously freeze-dried, so that it had no problems of solubilization), use of BF_3−methanol alone provided statistically equivalent results (in terms of FAME production) compared to use of the two-step method. Hence, one may conclude that both methods generate equivalent results, provided that the samples may be completely solubilized in the reaction medium. Tande et al. (*19*) have also described similar peak areas for both saturated and unsaturated fatty acids [16:0, 18:0, 20:0, 20:5 (*n*-3), and 22:6 (*n*-3)], following treatment with the two-step official method and BF_3 alone, in highly concentrated ethyl ester mixtures. The alkaline step of the procedure may generate excessive amounts of free fatty acids that, in the following acidic step, will require longer reaction times and higher catalyst concentrations for conversion into their corresponding FAME (*11*); therefore, it is not surprising that elimination of said step and use of the BF_3−methanol step alone, heated for an appropriate time interval, would provide equivalent results. After such an approach, those free fatty acids originally present in the sample, or those that would be produced during the alkaline step cease to be of concern and would automatically be converted into their corresponding FAME. Ackman (*11*) described BF_3−methanol treatments alone as a satisfactory basis for marine oil analysis; he suggested use of a 3.5% (v/v) BF_3 in methanol and hexane, with heating for 1 h at 100 °C.


a
16:0
Fatty acid concentration (mg/g AFDW)
0
2
4
6
8
10
12
14
16
25C,0.5M
25C,1M
25C,2M
50C,1M
50C,2M
65C,0.5M
65C,1M
NaOH+BF3(AOCS)
BF3
isooct,0s,NaCl
isooct,0s,H2O
isooct,30s,NaCl
isooct,30s,H2O
hex,0s,NaCl
hex,0s,H2O
hex,30s,NaCl
hex,30s,H2O(IP=5.10)
IP=4.60
IP=3.95
IP=3.75
alkali
acid


b
16:1(n-7)
Fatty acid concentration (mg/g AFDW)
0
2
4
6
8
10
12
14
16
25C,0.5M
25C,1M
25C,2M
50C,1M
50C,2M
65C,0.5M
65C,1M
NaOH+BF3(AOCS)
BF3
isooct,0s,NaCl
isooct,0s,H2O
isooct,30s,NaCl
isooct,30s,H2O
hex,0s,NaCl
hex,0s,H2O
hex,30s,NaCl
hex,30s,H2O(IP=5.10)
IP=4.60
IP=3.95
IP=3.75
alkali
acid


c
20:5(n-3)
Fatty acid concentration (mg/g AFDW)
0
2
4
6
8
10
12
14
16
25C,0.5M
25C,1M
25C,2M
50C,1M
50C,2M
65C,0.5M
65C,1M
NaOH+BF3(AOCS)
BF3
isooct,0s,NaCl
isooct,0s,H2O
isooct,30s,NaCl
isooct,30s,H2O
hex,0s,NaCl
hex,0s,H2O
hex,30s,NaCl
hex,30s,H2O(IP=5.10)
IP=4.60
IP=3.95
IP=3.75
alkali
acid

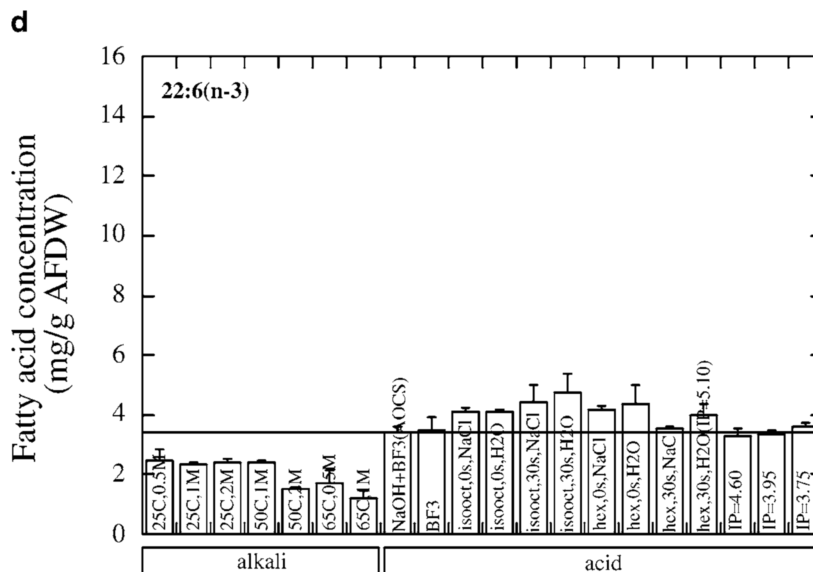


Figure 3. FAME concentrations (average ± standard deviation) in microalga biomass, pertaining to 16:0, 16:1 (*n*-7), 20:5 (*n*-3), and 22:6 (*n*-3), when submitted to the various treatments under study.

Despite its general use, a few adverse effects arising from BF_3 esterification of sensitive fatty acids require special care. Among the most frequently quoted are rearrangement of cyclopropene and cyclopropane fatty acids, destruction of squalene, and dehydration of hydroxylated and conjugated dienoic fatty acids (*11*). Furthermore, use of BF_3 has been associated with production of artifacts and loss of reasonable amounts of PUFA during the assay (*4*). Hence, alternative catalysts should be explored, in attempts to provide suitable alternatives. Use of acetyl chloride has several advantages, viz. lower cost, longer shelf life (without the need for refrigeration), smaller amounts of catalyst needed [5 instead of 12% (v/v)], and complete removal of catalyst in the final extraction step (which does not occur with BF_3, hence leading to its appearance in the GC column, with consequent irreversible damage) (*8*). Published results (*9*, *20*, *21*) on comparative performances of acetyl chloride and BF_3 in various matrices are consistent regarding the similarity of results obtained via both methods. Unfortunately, those studies do not encompass results pertaining to PUFA; therefore, extrapolations are to be done carefully.

To focus on the effects of each experimental method tested upon the various fatty acids present in the samples and because of the huge amount of data produced, only a few fatty acids were selected and thoroughly studied. These included a saturated (16:0), a monounsaturated [16:1 (*n*-7)], and two PUFA with different degrees of unsaturation [20:5 (*n*-3) and 22:6 (*n*-3)]. Such a selection was based on their distinct fractions in the lipid samples and, especially for the latter two, also on their economic relevance.

Experimental results in terms of FAME amounts pertaining to the aforementioned four fatty acids, after having been submitted to the various methodologies under study, are depicted in **Figures 2** and **3** (concerning oil and microalgae, respectively). From inspection of these figures, coupled with statistical analysis of the results obtained with BF_3 and acetyl chloride (when using isooctane as an extracting solvent, mixing for 30 s, and using a saline solution to promote phase separation, so that the sole factor under assessment would be the type of catalyst), it is possible to conclude that the two methods generate statistically equivalent results in microalgal samples. These conclusions agree with results published elsewhere (*9*, *20*, *21*), hence permitting their validation also for some PUFA.

One of the requirements for a reaction to proceed at a fast rate is fast solubilization of either the sample lipids or the FAME formed in the medium. Fatty acids are significantly affected by such items; therefore, use of a reaction medium with an intermediate polarity may improve the yield; in fact, mixtures of methanol and diethyl ether (PI = 3.95) or toluene (PI = 3.75) led to amounts of extracted PUFA that were statistically above those obtained with the standard method (in which the reaction medium is composed mainly of methanol, with PI = 5.10).

The methods employing sodium methoxide provided lower levels of polyunsaturated FAME than the acidic ones, as expected. Alkaline transmethylation has indeed been reported (*1*) to promote higher recovery yields only in terms of short-chain fatty acids. In addition, it cannot be used in samples containing large amounts of free fatty acids, which happens with most raw fish oils (*11*).

A combination of all pieces of experimental and statistical information generated indicates that acidic derivatization using acetyl chloride should be selected for assays of total PUFA. Furthermore, the desired polarity of the reaction medium should be obtained by employing a 1:1 (v/v) solvent mixture of methanol with diethyl ether or toluene, provided that a solvent other than hexane is used in the extraction step.

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