FA Determination in Cold Water Marine Samples

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ABSTRACT: The determination of FA in cold water marine samples is challenging because of the presence of large proportions of a variety of labile PUFA. This study was undertaken to establish optimal methods for FA analysis in various sample types present in the marine environment. Several techniques used in FA analysis, including lipid fractionation, FAME formation, and picolinyl ester synthesis, were examined. Neutral lipids, acetone-mobile polar lipids, and phospholipids (PL) were readily separated from each other on columns of activated silica gel, but recoveries of PL were reduced. Deactivation of the silica gel with 20% w/w water produced variable recoveries of PL (66 ± 22%). FAME formation with BF_3 gave optimal recoveries, and a method to remove hydrocarbon contamination from these samples before GC analysis using column chromatography was optimized. Picolinyl derivatives of FA are useful in structural determinations with MS, and a new base-catalyzed transesterification method of their synthesis from FAME was developed. Finally, a series of calculations, combining FA proportions with acyl lipid class concentrations, was designed to estimate FA concentrations. In algae and animal samples, these estimates were in good agreement with actual FA concentrations determined by internal standards.

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FA analysis is important in fields such as biochemistry, oceanography, biogeochemistry, and aquaculture. For instance, in biogeochemical studies, FA may act as signature compounds of organisms to determine sources and sinks of organic material; such information enhances our knowledge of carbon cycling in the marine environment (1–4). In aquaculture the interest is in FA nutrition and the establishment of optimal levels of EFA in fish diets (5). Accurate quantification of these FA is a challenge, particularly with samples from cold-water environments that contain elevated levels of oxygen-sensitive PUFA. The widespread interest in FA applications has led to the development of a variety of techniques for their analyses, including, for example, several fractionation and methylation procedures. Ideally, all research groups conducting FA research should adopt standard methods. However, even uniformity in technique may not be sufficient to guarantee accurate results in separate laboratories. For example, Roose and Smedes (6) compared the efficiency of a lipid extraction technique in a number of laboratories and found that, although all groups claimed to follow the Bligh and Dyer (7) technique, very few actually used conditions that conformed to the original method. Modifications of this and other techniques so as to generate results more quickly and easily are commonplace. To allow comparisons of results acquired by different techniques, one must have some knowledge of the effects of variations in methodology on FA recovery.

Before FA analysis can be carried out, lipids must first be extracted from the matrix in which they are encountered. A number of studies have examined variations in lipid recovery with differing solvent systems (8–11) and further comparison is not necessary. In this report, the methods of both Folch *et al.* (12) and Bligh and Dyer (7), depending on sample type, are used. To determine FA qualitatively and quantitatively, methyl ester derivatives must be formed. A variety of methods to transesterify lipid extracts are available (13–17), and a quantitative evaluation of these transesterification methods will determine if all procedures, as assumed, produce equivalent results. Additional techniques, such as fractionation of the lipid extract (18), removal of contaminants, and formation of derivatives for MS (16,19), often are performed, and new methods for those steps are described and critically evaluated here. Finally, a simple method to approximate FA concentrations using acyl lipid data is discussed and applied to marine samples. In most cases, results of comparisons are evaluated by application to typical marine samples: *Nannochloropsis* sp. (green algae), *Isochrysis galbana* (flagellate), and *Mytilus edulis* (bivalve).

EXPERIMENTAL PROCEDURES

Samples. Blue mussels (*M. edulis*) were taken from stocks maintained at the Ocean Sciences Centre (OSC) in Logy Bay, Newfoundland, Canada. The shells were removed, and the bodies were blotted dry. Each mussel was immediately weighed and extracted. Algal samples of *Nannochloropsis* sp. and *I. galbana* were taken from cultures in logarithmic phase grown at the OSC. Known volumes of phytoplankton samples were filtered onto precombusted GF/C filters. Filters were then placed in $CHCl_3$ and stored under nitrogen in the dark at –20°C.

Extraction. Blue mussels were extracted according to the procedure of Bligh and Dyer (7). Typically, each sample of approximately 4 g wet weight was homogenized with a Trinitron homogenizer in 12 mL of 1:2 $CHCl_3$/MeOH. The homogenate was filtered, and the tissue and the filter were rehomogenized

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Abbreviations: AMPL, acetone-mobile polar lipid; NL, neutral lipid; NMID, nonmethylene-interrupted dienoic; OSC, Ocean Science Centre; PL, phospholipid; SE/WE, steryl ester/wax ester; TIC, total ion chromatogram.

with another 4 mL of $CHCl_3$. Following a second filtering, the filtrate was mixed with 4 mL of water, allowed to separate, and the $CHCl_3$ layer was recovered.

Phytoplankton samples were extracted with a simplified Folch *et al.* (12) procedure (20). Samples were homogenized with both sonication and grinding with a steel rod in 4 mL of 2:1 $CHCl_3$/MeOH and 0.5 mL of water. After centrifuging at $100 \times g$ for 2 min, the lower $CHCl_3$ layer was collected. At least three washes of the aqueous phase with 3 mL of $CHCl_3$ were carried out.

Quantification of lipid classes. Lipid classes were determined using the Iatroscan TLC-FID system according to the method described in Parrish (20). Samples of lipid extract were applied to Chromarods SIII, which were then developed twice in 99:1:0.05 hexane/diethyl ether/formic acid for 25 and 20 min. The rods were scanned from the top to just after the ketone peak. The second development was for 40 min in 80:20:0.1 hexane/diethyl ether/formic acid. Rods were then scanned to just after the DAG peak. Finally, the rods were developed twice in acetone (2×15 min), followed by a double development in 5:4:1 $CHCl_3$/MeOH/H_2O (2×10 min). The entire length of the rods was then scanned. This yielded three partial chromatograms that were joined to produce one full chromatogram.

Fractionation of lipid extract. Lipids were fractionated into neutral lipids (NL), acetone-mobile polar lipids (AMPL), and phospholipids (PL) using column chromatography on silica gel. A small amount of precombusted glass wool was placed in the tapered end of a Pasteur pipet, and the pipet was packed with approximately 0.8 g of silica gel that had been activated by heating at 110°C for 1 h. The column was then rinsed with 2 bed vol each of MeOH and $CHCl_3$. Approximately 5 mg of lipid extract in $CHCl_3$ was placed at the top of the column, and NL were recovered with 2 bed vol (approximately 6 mL) of 98:1:0.5 $CHCl_3$/MeOH/formic acid at a flow of approximately 1 mL min^{-1}. AMPL was eluted with 2 bed vol of acetone. One bed volume of $CHCl_3$ was then passed through the column to return it to a more neutral polarity, and PL were eluted with 2 bed vol of MeOH. Up to 20 mg of lipids were fractionated in this way but using proportionally greater solvent volumes.

FAME preparation. To evaluate the efficiency of FAME formation, five different procedures for their synthesis were applied to identical lipid extract samples (approximately 1 mg) of *I. galbana* with 23:0 FAME added as internal standard. In all cases, lipid extracts were evaporated to near dryness before any derivatization reagents were added. The five procedures are described briefly as follows: (i) BF_3 (14): hexane (0.5 mL) and 10% BF_3 in MeOH (1 mL) were added to the extract, the mixture was shaken, covered with N_2, and then heated at 80–85°C for 1 h. The samples were allowed to cool, 0.5 mL of water was added, and the samples were again shaken. Hexane (2 mL) was added and the mixture was shaken, then centrifuged. The upper hexane layer, containing the FAME, was removed and concentrated. (ii) HCl (16): acetyl chloride (2 mL) was added slowly to MeOH (18 mL) to make methanolic HCl. Hexane (1 mL) and methanolic HCl (2 mL) were added to the extract and heated for 2 h at 80°C. Samples were allowed to cool, and 3 mL of 5% aqueous NaCl was added. Hexane (2 mL) was added, the sample was shaken, and the hexane layer was withdrawn; (iii) H_2SO_4 (13): 2 mL of 6% H_2SO_4 in MeOH and 5 mg of hydroquinone were added to the sample and the mixture was heated at 70°C for 5 h. The samples were allowed to cool, and 1 mL of water and 1.5 mL of hexane were added. The mixture was shaken and centrifuged and the upper hexane layer was collected; (iv) AOCS Official Method Ce 1b-89 (17): to the sample was added 0.5 mL of 0.5 mol L^{-1} NaOH in MeOH; the mixture was heated at 100°C for 7 min and then allowed to cool. Next, 1 mL of 10% BF_3 in MeOH was added, and the mixture was heated at 100°C for 5 min. The sample was again allowed to cool, 0.5 mL of iso-octane was added, and the mixture was shaken for 30 s. Saturated NaCl solution (2 mL) was added, and the solution was shaken and centrifuged. The upper iso-octane layer was collected; (v) NaOMe (15): 2 mL of 0.25 mol L^{-1} sodium methoxide in 1:1 MeOH/diethyl ether was added to the sample, and the mixture was covered with nitrogen and heated at 100°C for 30 s. The sample was cooled, 1 mL of iso-octane and 5 mL of saturated NaCl were added, and the mixture was shaken vigorously for 15 s. The upper iso-octane layer was then collected.

Hydrocarbon cleanup. Several algal samples containing hydrocarbon pollutants from shipboard activity were collected. These hydrocarbon contaminants interfered with a number of FAME peaks and had to be removed before analysis. This was done using a Pasteur pipet packed with activated silica gel in a modification of a procedure recommended by Christie (16) for removal of sterols. The column was rinsed with 1 bed vol (approximately 3 mL) each of $CHCl_3$ and hexane, and the FAME sample (approximately 1 mg) was placed at the head of the column. Iso-octane (1 bed vol) was used to elute the contaminating hydrocarbons. The FAME were then recovered with 2 bed vol of 80:20 hexane/diethyl ether. In addition to hydrocarbon pollutants, this technique removed biogenic hydrocarbons, such as phytane and pristane.

Argentation TLC. Silver nitrate-impregnated plates were prepared by dipping silica gel-coated plates (coating thickness of 250 μm) in 20% $AgNO_3$ in acetonitrile. Plates were activated for 1 h at 110°C immediately prior to use. Approximately 10 μg of FAME was applied in a concentrated spot using a Hamilton syringe. Plates were then developed in a closed chamber in 90:10 hexane/diethyl ether (21) until the solvent front reached the top of the plate (approximately 20 min). Spots were visualized with 2′,7′-dichlorofluorescein under UV light, and the esters were recovered from the silica with 1:1 hexane/diethyl ether.

Picolinyl ester preparation. Picolinyl esters of FA were prepared following two separate procedures. FAME (approximately 10 mg) were hydrolyzed by reaction with 2 mL of 0.5 mol L^{-1} NaOH in MeOH at 70°C for 30 min, followed by acidification with 1 mol L^{-1} of HCl and extraction with hexane (22). The FA in hexane solution were evaporated to near dryness, 0.5 mL of trifluoroacetic anhydride was added, and the mixture was heated at 50°C for 30 min. Excess reagent was evaporated, and 0.5 mL of a solution containing both 20 mg of

3-(hydroxymethyl)pyridine and 4 mg of 4-dimethylaminopyridine in dichloromethane was added. The solution was then allowed to stand at room temperature for 3 h. The resulting picolinyl esters were extracted with hexane (16).

Alternatively, picolinyl esters were formed by transesterification based on the method of Roelofsen *et al.* (23), originally developed for the transesterification of methyl esters to butyl esters. Approximately 10 mg of freshly cut Na was dissolved in 10 mL of 3-(hydroxymethyl)pyridine by sonication, and 1 mL of this solution was added to the FAME sample (10 mg) that had been concentrated to near dryness. Twenty beads of precombusted molecular sieve (Type 3A) were added, and the mixture was covered with N_2 and heated at 80°C for 1 h. The sample was allowed to cool, and 2 mL of hexane was added to extract the picolinyl esters.

Chromatographic conditions. FAME were analyzed using a Varian 3400 gas chromatograph (GC) equipped with a temperature-programmable injector and a Varian 8100 autosampler. A flexible fused-silica column (30 m × 0.32 mm i.d.) coated with Omegawax 320 (Supelco, Mississauga, Canada) was used for general FAME separation. Hydrogen (flow rate 2 mL min^{-1}) was used as the carrier gas, and the gas line was equipped with an oxygen scrubber. The temperature program and flow rates used are described in Budge *et al.* (24). Theoretical response factors for FAME, as recommended by Craske and Bannon (25) and presented in Christie (16), were used in all analyses.

Picolinyl esters were analyzed on a Hewlett-Packard 5890/5971A GC–MS. A 70 eV ionization potential was used, and the mass range was 50–500 *m/z*. The esters were separated on a CP-Sil 5CB coated column (25 m × 0.25 mm i.d.; Chrompack, Middelburg, The Netherlands) using the following temperature program: 190°C for 0.5 min, followed by a ramp to 295°C at a rate of 3.0°C min^{-1} and hold at 295°C for 9.5 min. Helium was used as the carrier gas at a flow of 2 mL min^{-1}. The injector was isothermal at 250°C, and the mass analyzer interface was held at 280°C.

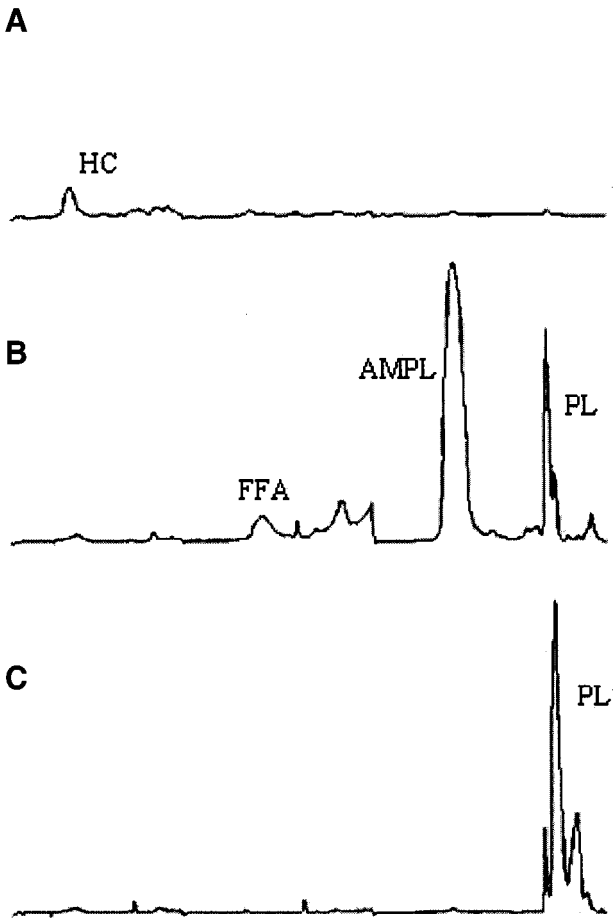


FIG. 1. TLC-FID chromatogram of fractionated lipid extracts of *Nannochloropsis* sp. (A) Neutral fraction; (B) acetone-mobile polar lipid (AMPL) fraction, (C) phospholipid (PL) fraction. Peak attentuation is the same for all chromatograms. HC, hydrocarbons.

RESULTS AND DISCUSSION

Fractionation of lipid extracts. The column chromatography separation of lipid extracts was evaluated by examining the resulting fractions by TLC-FID. Chromatograms of those fractions from typical algal and bivalve samples are shown in Figures 1 and 2. In both samples, both the NL and PL fractions contained predominantly the desired lipid classes with a small amount of pigment (AMPL) present in the NL fraction of *M. edulis*. However, the AMPL fraction in both samples appeared to contain a portion of the NL and PL fractions. For example, in *M. edulis*, sterols (ST) eluted from the column with acetone, suggesting that $CHCl_3$/MeOH/formic acid (98:1:0.5) was not polar enough to recover this mildly polar lipid within the NL fraction. DAG are slightly more polar than ST and can also be expected to elute from the column with acetone. DAG are rare in most marine samples and would normally make only a small contribution to total acyl lipids.

The other commonly encountered NL peak in the AMPL fraction was FFA. This was unexpected, as the 0.5% formic acid was included in the NL solvent specifically to recover FFA. The presence of FFA and acetone-insoluble PL in the AMPL fraction suggests that both peaks were the result of degradation of an AMPL species either on the silica gel column or during evaporation and concentration. Degradation of a glycolipid, for example, could produce a molecule of FFA and some more polar compound containing both a sugar moiety and an acylated FA. However, for the purposes of determining the FA composition of each fraction, this degradation will have little impact on the utility of the column separation if changes in FA structure with this breakdown are not apparent. In a separate study with sediments, where AMPL comprised more than 50% of total lipids, we found no significant differences in total FAME concentrations before and after fractionation. Of individual FA, only the concentrations of 16:1n-5, 17:0, and 20:1n-7, present in amounts less than 3% of total FA, were found to be significantly different ($P < 0.05$). Concentrations of the major FA remained unchanged. Thus, alteration of FA structure with breakdown of AMPL is not a concern.

Of the three fractions, recoveries of individual components in the NL and AMPL fractions were close to 100%. Recoveries of PL, however, were reduced on silica gel. PC, commonly

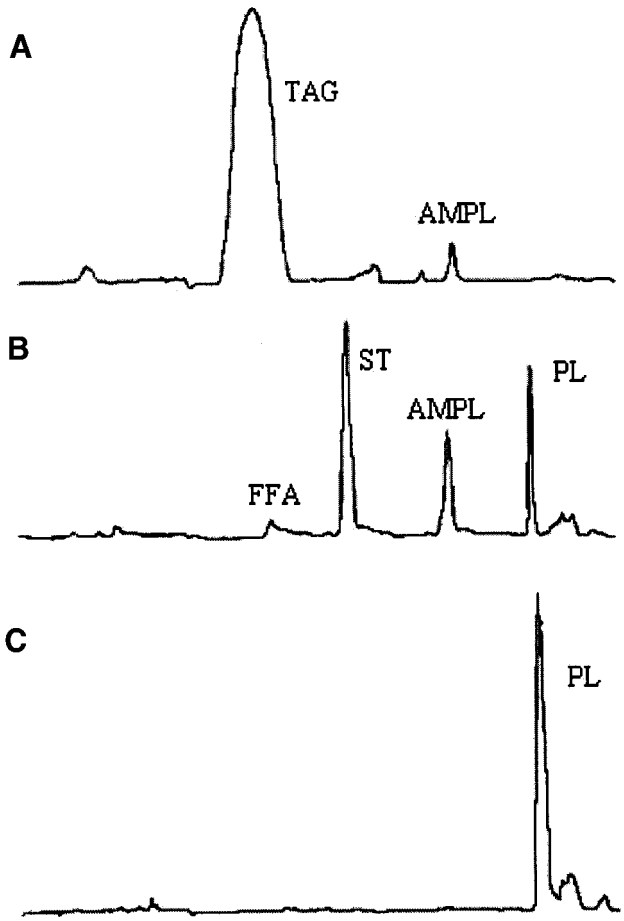


FIG. 2. TLC-FID chromatogram of fractionated lipid extracts of *Mytilus edulis*. (A) Neutral fraction; (B) AMPL fraction; (C) PL fraction. Peak attentuation is the same for all chromatograms. ST, sterols; for other abbreviations see Figure 1.

a major component of PL, was used as a model compound to determine the extent of this problem: Only 73 ± 4% of the PC was recovered. In an attempt to improve recovery, the silica gel was deactivated and equilibrated with 20% water by weight for 3 h. In one instance, this gave a recovery of 89%, but results were variable and, on average, only 66 ± 22% was recovered. It is generally assumed that recovery from silica gel is quantitative, and this on-column loss would certainly result in inaccurate PL and FA concentrations. However, these losses seem to be equivalent across the lipid class so that proportions of individual PL may still be accurately determined. In all situations, caution should be employed when using silica gel to separate PL from other lipid classes.

Methylation techniques. One-way ANOVA of the FAME concentrations (Table 1) revealed only a few significant differences in the results. Those differences were apparent in total branched, total monounsaturated, total polyunsaturated, and total FAME. With the AOCS method, significantly more ($P < 0.05$) branched-chain FAME were present than with any other method, suggesting that this method may be particularly useful in the esterification of FA with those structures. Of all five procedures, the NaOMe-catalyzed method produced the smallest concentration of total FAME. This was expected, as basic catalysts such as NaOMe are unable to methylate any FFA that are present, resulting in lower total FAME concentrations. While not statistically significant, the BF_3 method did produce the largest PUFA and total FAME concentrations of any acidic catalyst, despite highly cited reports that the use of BF_3 may lead to lowered PUFA yields (16,26).

Evaluated as percent total FAME (Table 2), the AOCS method produced significantly higher ($P < 0.001$) proportions of both branched-chain FAME, likely derived from bacteria, and monounsaturated FAME than all other methods. This method also generated significantly lower ($P = 0.008$) proportions of saturated FAME. These same trends were evident in the absolute concentration data, but the differences only became significant when expressed as proportions. These differences were due to differences in individual FAME proportions, specifically the presence of significantly more ($P < 0.001$) 16:1n-9 and significantly less ($P < 0.001$) 14:0 in the AOCS data. The higher yield of branched-chain FA obtained with the AOCS method and the suggestion that 16:1n-9 is derived from freshwater bacteria (27) implies that this method may be particularly effective at esterifying bacterial lipids. The only other technique producing 16:1n-9 methyl ester was the BF_3 method. A stronger catalyst such as BF_3 may be better able to esterify FA of those lipids. However, there were few significant differences in proportions among the other four methods, and if data are to be reported only as a percentage of the total, the most convenient method may be used.

Removal of hydrocarbon contamination. By using silica gel column chromatography, it was possible to remove hydrocarbon contamination from FAME samples and recover those FAME in proportions equivalent to the FAME levels prior to cleanup (Fig. 3). However, the proportion of diethyl ether in the solvent used to elute the FAME was critical. Table 3 contains ratios of peak areas before and after hydrocarbon removal in two mixed algal FAME samples for a variety of methyl esters relative to 16:0 as 1.0. Values near 1.0 represent close to 100% recovery, but values much less than 1.0 represent losses of FAME. Initial attempts to elute FAME with 99:1 hexane/diethyl ether did recover methyl esters, but proportions of PUFA were significantly reduced (Table 3). Clearly, recovery was a function of double bond number rather than chain length, and mixtures of 90:10 hexane/diethyl ether also suffered from reduced PUFA recovery. Mixtures of 80:20 hexane/diethyl ether, however, recovered all PUFA, yielding ratios of peak areas before and after cleanup that were very near unity (Table 3) with coefficients of variation less than 10% for all FAME. This equivalent recovery before and after cleanup is particularly important if an internal standard has been added before hydrocarbon removal.

Picolinyl ester preparation. The transesterification procedure reported here was applied to the methyl esters of *M. edulis* tissue, and examination of the total ion chromatogram (TIC) of the hexane-extractable reaction products revealed only picolinyl esters (Fig. 4A). There was no evidence of unreacted

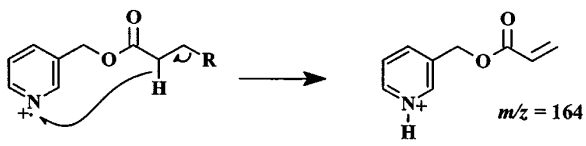
TABLE 1
Variation in FA Concentration (mg/mL culture) in Cultures of *Isochrysis galbana* with Differing Derivatization Procedures (mean ± SD, *n* = 4 or 5)[a]

	BF_3	HCl	H_2SO_4	AOCS	NaOMe
Branched					
i-15:0	0.06 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02
ai-15:0	0.01 ± 0.03	0.04 ± 0.07	0.00 ± 0.00	0.11 ± 0.02	0.00 ± 0.00
ai-16:0	0.09 ± 0.05	0.09 ± 0.03	0.00 ± 0.00	0.20 ± 0.00	0.00 ± 0.00
Subtotal	0.16 ± 0.06[a]	0.13 ± 0.09[a]	0.00 ± 0.00[b]	0.32 ± 0.02[c]	0.01 ± 0.02[b]
Saturates					
14:0	3.10 ± 0.67	2.98 ± 0.49	2.95 ± 0.52	1.99 ± 0.30	2.07 ± 0.37
15:0	0.06 ± 0.02	0.03 ± 0.05	0.06 ± 0.01	0.04 ± 0.01	0.10 ± 0.03
16:0	1.49 ± 0.34	1.32 ± 0.29	1.38 ± 0.33	1.11 ± 0.21	1.03 ± 0.23
18:0	0.03 ± 0.03	0.05 ± 0.03	0.03 ± 0.02	0.03 ± 0.00	0.00 ± 0.00
Subtotal	4.68 ± 1.03	4.39 ± 0.80	4.42 ± 0.86	3.18 ± 0.52	3.21 ± 0.61
Monounsaturates					
16:1n-9	0.15 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	0.55 ± 0.16	0.00 ± 0.00
16:1n-7	1.11 ± 0.24	1.03 ± 0.23	1.00 ± 0.19	0.84 ± 0.15	0.75 ± 0.12
18:1n-9	1.58 ± 0.25	1.54 ± 0.23	1.55 ± 0.26	1.48 ± 0.23	1.11 ± 0.23
18:1n-7	0.31 ± 0.03	0.32 ± 0.04	0.28 ± 0.03	0.16 ± 0.04	0.23 ± 0.11
Subtotal	3.16 ± 0.60[a]	2.89 ± 0.49	2.83 ± 0.47	3.03 ± 0.47	2.10 ± 0.30[b]
Polyunsaturates					
16:2n-4	0.23 ± 0.03	0.26 ± 0.03	0.21 ± 0.03	0.15 ± 0.04	0.15 ± 0.04
16:3n-4	0.06 ± 0.02	0.09 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.11 ± 0.07
16:4n-1	0.07 ± 0.01	0.04 ± 0.03	0.07 ± 0.01	0.05 ± 0.01	0.03 ± 0.03
18:2n-6	3.63 ± 0.59	3.39 ± 0.51	3.39 ± 0.55	2.97 ± 0.41	2.72 ± 0.36
18:3n-6	0.34 ± 0.06	0.32 ± 0.06	0.31 ± 0.06	0.27 ± 0.03	0.27 ± 0.04
18:3n-3	1.63 ± 0.19	1.51 ± 0.19	1.53 ± 0.20	1.36 ± 0.15	1.20 ± 0.11
18:4n-3	1.80 ± 0.25	1.62 ± 0.23	1.66 ± 0.25	1.43 ± 0.18	1.40 ± 0.17
20:2n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.04
20:4n-6	0.04 ± 0.02	0.01 ± 0.03	0.05 ± 0.01	0.05 ± 0.01	0.02 ± 0.03
20:5n-3	0.05 ± 0.02	0.02 ± 0.03	0.06 ± 0.01	0.05 ± 0.02	0.03 ± 0.03
22:5n-6	0.38 ± 0.04	0.38 ± 0.05	0.38 ± 0.07	0.36 ± 0.06	0.27 ± 0.06
22:6n-3	1.84 ± 0.13	1.78 ± 0.18	1.85 ± 0.22	1.80 ± 0.19	1.37 ± 0.24
Subtotal	10.09 ± 1.34[a]	9.42 ± 1.27	9.56 ± 1.41	8.53 ± 1.05	7.64 ± 0.96[b]
Total	18.12 ± 2.97[a]	16.83 ± 2.59	16.81 ± 2.73	15.05 ± 2.05	12.96 ± 1.81[b]

[a]*Note*: Means with different superscript roman letter designations (a,b,c) are significantly different ($P < 0.05$) from each other.

methyl esters, implying complete conversion of methyl esters to picolinyl esters. For comparison, a second TIC of reaction products generated by hydrolysis of methyl esters, followed by esterification (16), also is included (Fig. 4B). Although some picolinyl esters were formed, a variety of unreacted methyl esters were clearly present, indicating that incomplete hydrolysis was a problem. Undoubtedly, hydrolysis could be forced through the use of stronger base, higher reaction temperatures, and longer reaction times, but FA structures may be modified under such harsh conditions. However, the transesterification procedure developed here produced quantitative conversion of FAME to picolinyl esters without compromising lipid structure.

A fragmentation pattern of the picolinyl ester of an unusual FA is presented in Figure 5. This type of nonmethylene-interrupted dienoic (NMID) FA has been reported in bivalves (28), but confirmation of its structure is always difficult because authentic standards do not exist, making a mass spectral identification necessary. Harvey (19) has outlined mechanisms for the formation of various ions to be expected from a variety of picolinyl esters, but no information specifically applicable to FA containing double bonds separated by more than two methylene groups is provided. In Figure 5, a molecular ion at *m/z* 399 is obvious, allowing the FA to be assigned a length of 20 carbon atoms with two double bonds. A prominent ion, apparent at *m/z* 164, is the McLafferty rearrangement of the picolinyl ester, formed through the following mechanism:

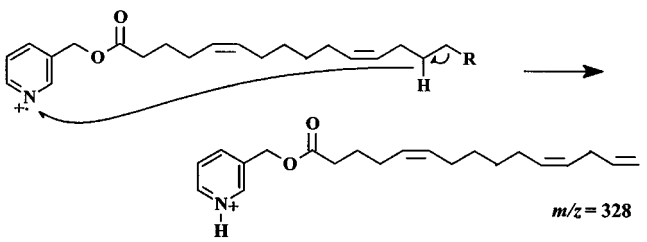


A series of diagnostic ions, such as those of *m/z* 328, 342, 356, and 370, is also generated by the following mechanism:

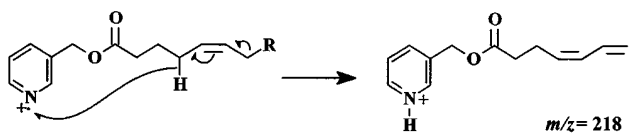
TABLE 2
Variation in Proportions of FA (% total FA) in Cultures of *Isochrysis galbana* with Differing Derivatization Procedures (mean ± SD, *n* = 4 or 5)[a]

	BF_3	HCl	H_2SO_4	AOCS	NaOMe
Branched					
i-15:0	0.33 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.13
ai-15:0	0.07 ± 0.16	0.23 ± 0.37	0.00 ± 0.00	0.76 ± 0.10	0.00 ± 0.00
ai-16:0	0.48 ± 0.28	0.55 ± 0.13	0.00 ± 0.00	1.37 ± 0.18	0.00 ± 0.00
Subtotal	0.87 ± 0.31[a]	0.78 ± 0.47[a]	0.00 ± 0.00[b]	2.13 ± 0.19[c]	0.11 ± 0.13[b]
Saturated					
14:0	17.02 ± 1.17[a]	17.69 ± 0.37[a]	17.54 ± 0.70[a]	13.20 ± 0.38[b]	15.89 ± 0.82[a]
15:0	0.32 ± 0.05	0.17 ± 0.25	0.34 ± 0.03	0.29 ± 0.01	0.80 ± 0.21
16:0	8.17 ± 0.73	7.81 ± 0.62	8.13 ± 0.69	7.32 ± 0.52	7.91 ± 0.73
18:0	0.15 ± 0.15	0.30 ± 0.16	0.17 ± 0.10	0.22 ± 0.03	0.00 ± 0.00
Subtotal	25.70 ± 1.92[a]	25.97 ± 0.86[a]	26.18 ± 1.13[a]	21.04 ± 0.84[b]	24.60 ± 1.60[a]
Monounsaturated					
16:1n-9	0.82 ± 0.49[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	3.66 ± 0.79[b]	0.00 ± 0.00[a]
16:1n-7	6.12 ± 0.39	6.09 ± 0.54	5.91 ± 0.23	5.54 ± 0.32	5.79 ± 0.27
18:1n-9	8.76 ± 0.21	9.13 ± 0.10	9.20 ± 0.14	9.81 ± 0.37	8.57 ± 1.24
18:1n-7	1.72 ± 0.13	1.91 ± 0.10	1.69 ± 0.10	1.06 ± 0.30	1.83 ± 0.89
Subtotal	17.42 ± 0.90[a]	17.12 ± 0.49[a]	16.80 ± 0.12[a]	20.07 ± 0.63[b]	16.19 ± 0.66[a]
Polyunsaturated					
16:2n-4	1.29 ± 0.05	1.55 ± 0.14	1.23 ± 0.06	0.99 ± 0.30	1.18 ± 0.24
16:3n-4	0.34 ± 0.03	0.56 ± 0.11	0.35 ± 0.02	0.31 ± 0.03	0.85 ± 0.52
16:4n-1	0.40 ± 0.03	0.22 ± 0.20	0.44 ± 0.01	0.36 ± 0.02	0.21 ± 0.20
18:2n-6	20.09 ± 0.28	20.14 ± 0.24	20.15 ± 0.23	19.72 ± 0.14	21.00 ± 0.29
18:3n-6	1.88 ± 0.04	1.88 ± 0.07	1.85 ± 0.06	1.82 ± 0.04	2.11 ± 0.09
18:3n-3	9.06 ± 0.42	9.01 ± 0.32	9.15 ± 0.34	9.05 ± 0.24	9.32 ± 0.44
18:4n-3	9.99 ± 0.42	9.64 ± 0.24	9.87 ± 0.14	9.50 ± 0.13	10.84 ± 0.38
20:2n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.49 ± 0.33
20:4n-6	0.22 ± 0.12	0.07 ± 0.16	0.33 ± 0.02	0.30 ± 0.02	0.17 ± 0.24
20:5n-3	0.29 ± 0.07	0.12 ± 0.17	0.33 ± 0.03	0.32 ± 0.09	0.20 ± 0.20
22:5n-6	2.14 ± 0.18	2.28 ± 0.12	2.24 ± 0.08	2.41 ± 0.11	2.09 ± 0.36
22:6n-3	10.32 ± 1.06	10.67 ± 0.73	11.09 ± 0.70	11.98 ± 0.46	10.64 ± 1.74
Subtotal	56.04 ± 1.82[a]	56.13 ± 1.52[a]	57.02 ± 1.20[a]	56.76 ± 0.79[b]	59.10 ± 2.18[a]
Total	100	100	100	100	100

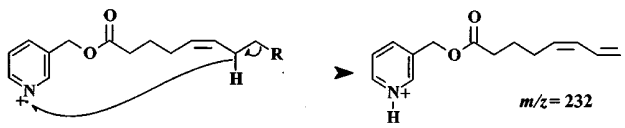
[a]*Note*: Means with different superscript roman letter designations (a,b,c) are significantly different ($P < 0.05$) from each other.



The 40-unit gap between *m/z* 178 and 218 and between 246 and 286 is due to suppression of the reaction in the first reaction and suggests that the double bonds are located in that area. The bonds are fixed at the Δ5 and Δ11 positions, identifying the FA as 20:2Δ5,11, by the presence of ions of *m/z* 218 and 300 generated by the following mechanism:



Ions at *m/z* 232 and 314 are also indicative of bond position owing to the following reaction:



Using these diagnostic ions and mechanisms, the identity of other NMID FA including 20:2Δ5,13, 22:2Δ7,13, and 22:2Δ7,15 was confirmed in samples of *M. edulis*.

Approximation of FA concentrations using acyl lipid data. Internal standards are not commonly used in FA analysis of marine samples because their FA composition is complex, and an initial GC analysis is necessary to ensure that the internal standard does not co-elute with any FAME. Because of this, it has become convention to report FA data as weight percentages of total FA, especially in biological samples, giving no information about actual concentrations. However, lipid class data are invariably expressed as concentrations, making it possible to

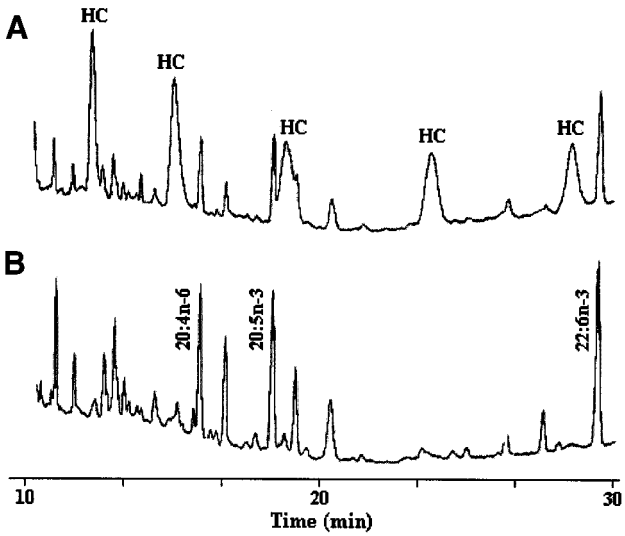


FIG. 3. GC-FID chromatograms of FAME before (A) and after (B) removal of HC contamination in a mixed algal sample. For abbreviation see Figure 2.

estimate FAME concentrations from acyl lipid data provided by TLC-FID. This concept of combining FAME and lipid data may be particularly relevant in light of the U.S. Food and Drug Administration's food labeling regulations insisting that a theoretical TAG, assembled from FA in the lipid extract, be defined as the "fat content" on food labels (29).

First, weight percent data of total FA are converted to mole percent data. These mole percent data allow calculation of an average number of double bonds and carbon atoms in a particular sample. This, in turn, allows calculation of an average FA M.W., which may then be applied to the acyl lipid data to determine a molar mass for each acyl lipid class. With that molar mass, the FA contribution to mass for each acyl lipid class may be determined. These calculations are performed within a spreadsheet, and Table 4 illustrates the results at each step. This calculation produces a very accurate approximation for lipid classes when the nonacyl portion of the molecule is known with certainty, such as with TAG, FFA, and PL. Inaccuracy increases with lipid classes such as steryl/wax esters (SE/WE) and AMPL. This is a particular problem with the AMPL group, which contains nonacylated pigments in addition to glycolipids and MAG. AMPL separations on Chromarods were originally conceived as a means of purifying the PL peak (30), but the AMPL peak itself has been important in some studies. For example, it was a dominant class in a cold ocean tunicate (31),

TABLE 3
Recoveries of FAME After Hydrocarbon Removal in a Mixed Algal Sample Using Different Proportions of Hexane/Diethyl Ether[a]

	Hexane/diethyl ether	
	99:1	80:20
Saturated		
14:0	1.05	0.99
16:0	1.00	1.00
22:0	1.02	—
Mean	1.02 ± 0.03	1.10 ± 0.10
Monounsaturated		
16:1n-9	0.95	1.06
16:1n-7	0.96	1.00
17:1	0.70	—
18:1n-9	0.97	—
18:1n-7	0.96	1.03
Mean	0.91 ± 0.12	1.04 ± 0.02
Polyunsaturated		
16:2n-6	0.62	0.98
16:3n-4	—	0.95
18:2n-6	0.85	1.08
18:3n-3	0.55	1.02
18:4n-3	0.23	1.02
20:5n-3	—	1.03
22:4n-6	0.37	—
22:6n-3	0.15	1.00
Mean	0.46 ± 0.26	0.99 ± 0.06
Mean total	0.76 ± 0.31	1.04 ± 0.08

[a]Expressed as normalized ratios of peak areas before and after cleanup. *Note*: Peak areas were normalized to the peak area of 16:0 by the following equation:

$$\frac{(\text{FA area})_{\text{after}}/(\text{FA area})_{\text{before}}}{(16:0\ \text{area})_{\text{after}}/(16:0\ \text{area})_{\text{before}}}$$

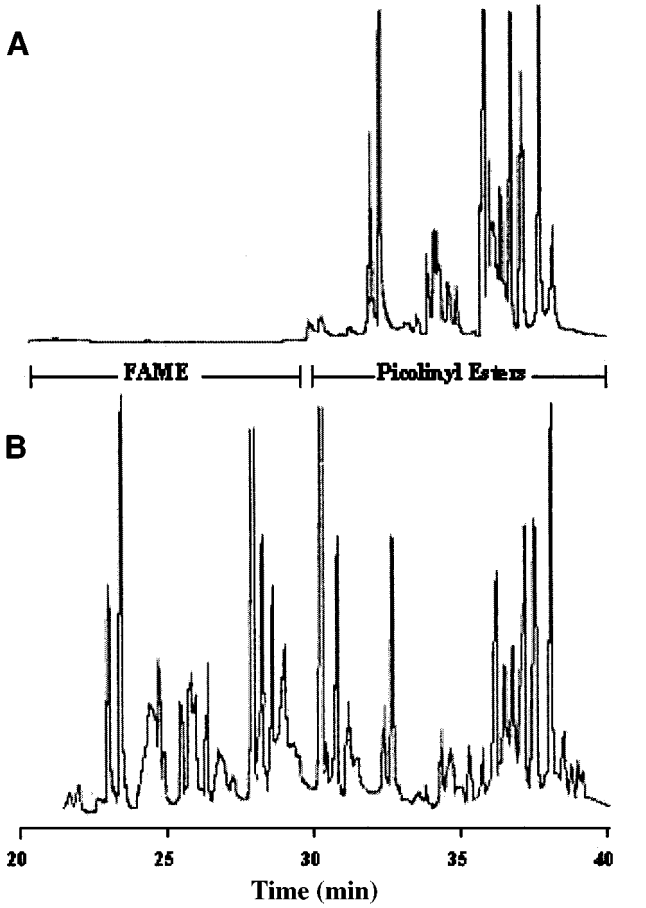


FIG. 4. Total ion chromatograms of the hexane-extractable material recovered after formation of picolinyl esters. (A) Picolinyl esters formed by transesterification of FAME; (B) picolinyl esters formed by hydrolysis of FAME, followed by reaction with 3-(hydroxymethyl)pyridine.

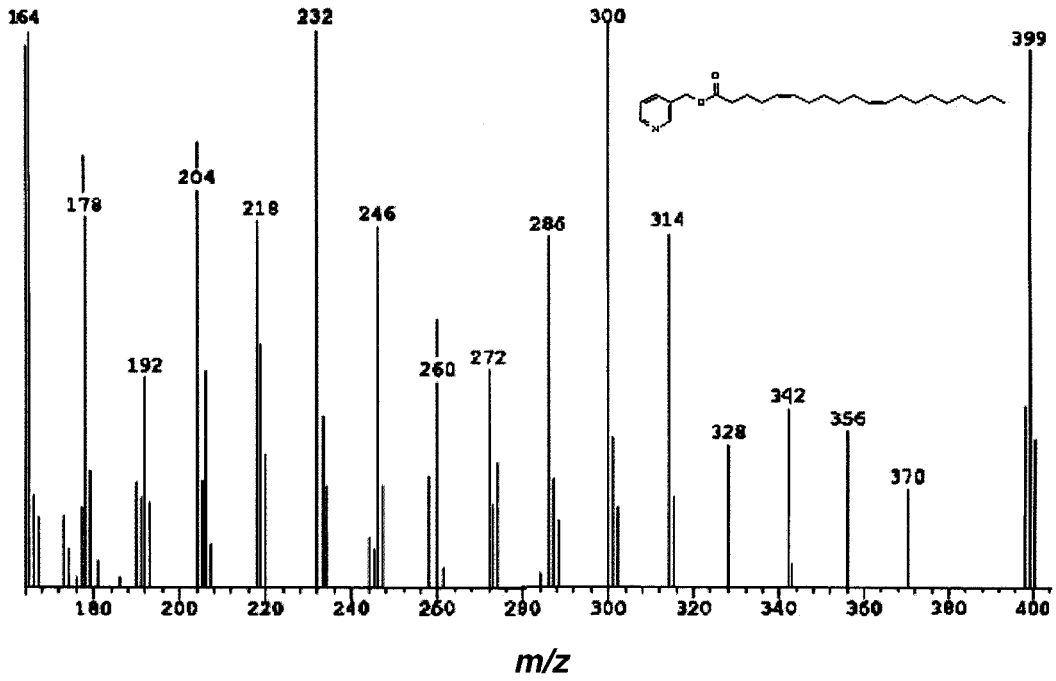


FIG. 5. Partial mass spectrum of the picolinyl ester of 20:2Δ5,11.

its contents were high in stressed scallops (32), and its concentrations in lake filtrates peaked during the decomposition of algal blooms (33). Likely structures for the backbones of AMPL can be proposed, but estimates of FA concentrations can be expected to be less reliable as AMPL portions increase.

In Table 5, the nonacyl lipid structures used to calculate molar mass are shown. In algae, approximately one-third of the AMPL peak was assumed to consist of digalactosyl diacylglycerol (34,35). A similar assumption was made with the calculation for animal tissue except that a cerebroside structure was substituted. SE/WE were determined as one peak and usually constituted a very small portion of total lipid (<5%). For ease of calculation, a 1:1 ratio of SE/WE was assumed with a 24-methylcholesta-5,22-dien-3β-yl ester in the SE portion (36). A C_{16} hydrocarbon chain in WE (36), primarily derived from zooplankton, was used to calculate the contribution of that class.

To evaluate the accuracy of these estimates, two marine samples (*Nannochloropsis* sp. and *M. edulis*) were analyzed for both lipid class concentrations and FAME proportions, and FAME concentrations were estimated from those data. FAME concentrations were also determined in the same samples using an internal standard (23:0 FAME). The results of these comparisons, as well as concentrations of TAG, FFA, and PL, are shown in Table 6. For both sample types, estimated and actual values were quite similar and, in fact, there was not a significant difference in the two values for the algal sample. The values for the animal tissue, however, were significantly different ($P = 0.002$), with the calculation slightly overestimating the actual amount. This suggests that some assumption within the calculation is incorrect. Better knowledge of the actual composition of the AMPL and PL fractions would undoubtedly improve the accuracy of this estimation. This calculation also makes the false assumption that FA structures are uniformly distributed throughout all lipid classes. Although this assumption simplifies the calculation, it must also be contributing to the error in the calculated method.

Before FA data can be applied to any problem, there must be some confidence that the results are accurate and reproducible. For that purpose, the methods and techniques typically used to generate FA data were critically evaluated in this work. A column chromatographic separation of the lipid extract can be performed to produce neutral, AMPL, and PL fractions. However, whereas deactivation of the silica gel with 20% w/w water improves recovery, only approximately 75% of PL can be recovered from these silica gel columns. Relative proportions of individual PL remain constant, but caution should be exercised when quantitative data are required. The extract can then be transesterified with the most convenient acid-catalyzed procedure to form FAME. In this laboratory, 10–14% BF_3 in MeOH is routinely used as a catalyst without any evidence of PUFA loss. A simple method to remove hydrocarbon contamination from FAME samples with column chromatography on silica gel also can be applied when necessary without selective loss of FAME on the column. A new transesterification method for the formation of picolinyl esters for use in the mass spectral identification of FA was also developed. This new method is quantitative and offers the advantage of avoiding hydrolysis of the lipid extract and formation of artifacts associated with that procedure.

TABLE 4
Results of Calculations to Produce FAME Concentrations from FAME Proportions and Lipid Class Concentrations in *Nannochloropsis* sp.

FAME	Molar mass	Weight %	Moles (×1000)	Mole %
14:0	242.400	5.61	23.14	6.94
14:1n-5	240.384	0.31	1.29	0.39
i-15:0	256.427	1.11	4.33	1.30
15:0	256.427	0.30	1.17	0.35
ai-16:0	270.454	1.40	5.18	1.55
16:0	270.454	16.20	59.90	17.95
16:1n-9	268.438	3.43	12.78	3.83
16:1n-7	268.438	19.35	72.08	21.61
i-17:0	284.481	0.19	0.67	0.20
ai-17:0	284.481	0.38	1.34	0.40
16:2n-4	266.422	0.29	1.09	0.33
17:0	284.481	0.22	0.77	0.23
16:3n-4	264.406	0.76	2.87	0.86
16:4n-1	262.391	0.24	0.91	0.27
18:0	298.508	0.33	1.11	0.33
18:1n-9	296.492	3.30	11.13	3.34
18:1n-7	296.492	0.50	1.69	0.51
18:2n-6	294.476	4.28	14.53	4.36
18:3n-6	292.461	0.43	1.47	0.44
18:3n-3	292.461	0.19	0.65	0.19
20:4n-6	318.498	4.14	13.00	3.90
20:5n-3	316.483	32.45	102.53	30.73
23:0				
Sum moles			333.63	100
Average chain length		17.41		
Average double bonds		2.14		
Average M.W.		285.97		

Lipid class	Amount (µg in extract)	FAME from	Amount (µg in extract)
Hydrocarbons	64		
Steryl/wax esters	0	Steryl/wax esters	0
Methyl esters	1	Methyl esters	1
Ketones	7		0
TAG	134	TAG	134
FFA	15	FFA	16
Alcohols	2		
Pink pigment	0		
Sterols	39		
DAG	6	DAG	6
AMPL[a]	370	AMPL	76
Polar lipids	335	Phospholipids	250
Total	973	Total lipids	483

[a]AMPL, acetone-mobile polar lipid.

FA data are rarely reported as absolute amounts, perhaps because there is hesitancy in many laboratories to add an internal standard that may coelute with a naturally occurring FA. Lipid class data, generated by TLC-FID, however, are commonly reported as concentrations. A series of calculations, designed to incorporate FA proportions with acyl lipid class concentrations to arrive at FA concentrations, was developed. These estimates, which will increase in accuracy as knowledge of the nonacyl structures of acyl lipids increases, were in good agreement with actual FA concentrations, determined using internal standards, in algae and animal samples.

TABLE 5
Nonacyl Structures of Acyl Lipids Used to Estimate FAME Concentrations from Acyl Lipid Data

Lipid class	Nonacyl structure
Steryl esters	24-Methylcholesta-5,22-dien-3β-yl ester
Wax esters	C_{16} alcohol
Methyl esters	—
TAG	Glycerol
FFA	Add CH_3
DAG	Glycerol
AMPL	DGDG[a] in algae, cerebroside in animal tissues
Phospholipids	Glycerol and choline

[a]DGDG, digalactosyl diacylglycerol; for other abbreviation see Table 4.

TABLE 6
Estimated and Actual FAME Concentrations[a] in Marine Samples (mean ± SD, *n* = 3 to 6)

		Nannochloropsis sp. (µg/40 mL culture)	*Mytilus edulis* (µg/mL extract)
Lipids	TAG	63 ± 48	1420 ± 160
	AMPL	318 ± 49	183 ± 24
	PL	307 ± 66	435 ± 86
FAME	Estimated	371 ± 95	2220 ± 210
	Actual	333 ± 59	1870 ± 160
% Discrepancy		11 ± 4	18 ± 1

[a]For abbreviations see Table 4.

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