methods

# Quantitative analysis of fatty acid precursors in marine samples: direct conversion of wax ester alcohols and dimethylacetals to FAMEs

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**Abstract** **To apply fatty acid analyses to the study of foraging ecology and diet determination, all compounds that may be deposited as fatty acids in a predator must be quantified in the prey. These compounds include the usual fatty acids in acyl lipids, but also the alcohols of wax esters and the vinyl ethers of plasmalogens. In routine fatty acid analysis, samples are extracted and transesterified (methylated), resulting in the formation of fatty acid methyl esters (FAMEs); however, fatty alcohols and dimethylacetals (DMAs) are also generated if wax esters or plasmalogens are present. Here, we present a new method using a modified Jones' reagent to oxidize these alcohols and DMAs to free fatty acids (FFAs). These FFAs are then easily methylated and quantitatively recombined with FAMEs from the same sample. This generates a fatty acid signature of prey that is equivalent to that which the predator has available for deposition upon digestion of that prey. This method is validated with alcohol and DMA standards. Its application to typical marine samples is also presented, demonstrating the change in effective fatty acid signature after inclusion of fatty acids derived from wax esters and plasmalogens.**—Budge, S. M., and S. J. Iverson. **Quantitative analysis of fatty acid precursors in marine samples: direct conversion of wax ester alcohols and dimethylacetals to FAMEs.** ***J. Lipid Res.*** **2003.** 44: **1802–1807.**

**Supplementary key words** oxidation • plasmalogen • lipid • trophic interactions

In the past three decades, the application of fatty acid signatures (1) has developed from a potential tool for delineating marine food webs (2) into a powerful technique for quantitative diet assessment of predators (S. J. Iverson, C. Field, W. D. Bowen, and W. Blanchard, unpublished observations). A variety of studies using lipid techniques between these two extremes have been conducted by comparing the fatty acids found in predator fat stores with those found in the prey (3–6), allowing qualitative, and potentially quantitative, statements to be made about diets and trophic interactions. These types of studies are possible because the fatty acid signatures of prey items consumed are deposited largely unaltered in most predators, providing an integrated record of diet. However, a potential complication is that some types of prey may contain fatty acid precursors. These are compounds that do not have an acid structure but are metabolized in the predator to form fatty acids that are, in turn, deposited in adipose tissue. This, of course, means that any such compound must be accurately quantified. Without determining these compounds, one may be comparing the fatty acid signature of the predator's fat store with an incomplete and possibly erroneous prey fatty acid signature.

The two most common lipid classes that present these problems in marine ecosystems are wax esters (WEs) and plasmalogens. WEs consist of a fatty acid esterified to a fatty alcohol. Upon digestion by the predator, the WE is hydrolyzed to give one molecule of each of those lipids. The fatty acid enters the pool of fatty acids available for deposition, while the alcohol is oxidized to the corresponding fatty acid, which is then also available for incorporation into fat stores (7). Plasmalogens are a common type of phospholipid that contain a vinyl-ether linked alkyl chain, in addition to an esterified fatty acid and a polar phosphate group. During digestion, the vinyl ether-linked alkyl chain is first oxidized to an aldehyde, then immediately to an alcohol (8). The alcohol is then processed as described above, also entering the predator's fatty acid pool.

Typical sample preparation for fatty acid analysis by gas chromatography (GC) involves the transesterification (methylation) of acyl lipids (usually triacylglycerols and phospholipids) and free fatty acids (FFAs) to form fatty acid methyl esters (FAMEs) using an acid-catalyzed reaction. However, exposure of a lipid extract containing WEs or plasmalogens to this acidic environment will generate several products that are not FAMEs. With WEs, the result is similar to that of digestion, where FAMEs and a fatty al-

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Abbreviations: DMA, dimethylacetal; FAME, fatty acid methyl ester; GC, gas chromatography; WE, wax ester.

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cohols are produced. Depending on their concentration and the GC column employed, these alcohols may appear on the chromatogram simply as a rising baseline or, in the worst case, as peaks, usually broad and unresolved, on which FAMEs elute. With plasmalogens, the vinyl-ether linkage is broken in the presence of acid to generate an aldehyde, which immediately reacts further with the acid to produce fatty dimethylacetals (DMAs). DMAs are a particular problem because the common 16:0 and 18:0 DMA coelute with *i*-16:0 and *i*-18:0 FAME during GC analysis using a typical FAME (polyethylene glycol) column (9). Although transesterification can be accomplished using base-catalyzed procedures that do not produce DMAs, these procedures are generally less reliable, they do not esterify FFAs, and their imprudent use can cause alterations to fatty acids (10).

Thus, since alcohols and DMAs cannot be simultaneously determined with FAMEs, a more complicated procedure must be carried out. Typically, FAME, alcohol, and DMA bands are first isolated by TLC, then extracted and filtered. Each separate type of compound is then determined individually by GC. Quantification of alcohols is particularly inconvenient because it may require a separate derivatization reaction (11). Through the use of internal standards, one must then add back the amounts of corresponding structures of alcohols and DMAs to determine the new effective FAME composition. Here we report an alternative to this procedure. We propose the separation of FAMEs from both alcohols and DMA by TLC, followed by simply oxidizing any alcohol or DMAs to the appropriate FFA, which can then be directly methylated and recombined with the FAME fraction for GC analysis. This produces a fatty acid signature of the prey that is equivalent to the signature that the predator has available for deposition from that prey item. We demonstrate the use of this technique on alcohol and DMA standards, as well as on a variety of marine samples containing these compounds.

## METHODS

### Analyses of standards

FAME and alcohol standards (C16 to C24) were supplied from Nu-Chek Prep (Elysian, MN). A solution consisting of 5.58 mg of saturated FAMEs (20% each of five components) and 6.0 mg of monounsaturated alcohols with the same carbon number as the FAME series (20% each of five components) in hexane was evaporated to dryness. To this was added 2 ml of acetone and 10 drops (~0.25 ml) of Jones' reagent (12), which we modified to: 13.5 g $CrO_3$ and 6.4 ml concentrated $H_2SO_4$ made up to 50 ml with distilled $H_2O$. This modified reagent contained half of the volume of $H_2SO_4$ employed in the original Jones' reagent. While use of the original concentrations of reagents did effectively oxidize alcohols and DMAs, it also generated artifacts in the chromatogram. These artifacts were absent when the modified reagent was employed. The mixture of standards, acetone, and Jones' reagent was vortexed for 1 min, allowed to sit for 30 min, and then vortexed again for 1 min. Heat was released and a precipitate formed when alcohols were present. Then 1 ml of water was added, followed by 2 ml of hexane, vortexing after each addition. The precipitate dissolved upon addition of hexane. After centrifugation, the upper hexane layer was removed and the aqueous layer was extracted twice more with 2 ml each of hexane. Hexane fractions were combined, washed with 2 ml of water, and dried over anhydrous $Na_2SO_4$. The FFAs generated were then methylated with 10% $BF_3$/methanol as described in Iverson et al. (13). Peak areas from GC chromatograms were compared with known amounts of starting material to determine the extent of conversion of alcohols to FAMEs in relation to the original amount of FAME present. This entire procedure was repeated three times on the original standard mixtures.

To assess absolute yields of the reaction, 16:0 and 18:0 alcohol standards (15 mg each) were accurately weighed and dissolved in acetone. The modified Jones' reagent was added and the FFAs generated were methylated as described above. Recovered FAMEs were then determined by GC to ensure no other reaction products were present, and were evaporated to dryness and weighed.

Because commercial standards of DMAs were not available, plasmalogens were isolated from bivalves known to contain substantial amounts of this lipid. First, lipids were extracted from blue mussel (*Mytelis edulis*) tissue using a modified Folch et al. (14) procedure as described in Iverson et al. (15) and transesterified with 10% $BF_3$/methanol. DMAs produced were then separated from FAMEs by TLC on preparative silica gel TLC plates (coating thickness of 250 μm) developed in toluene. Bands of FAMEs and DMAs were visualized with dichlorofluoroscein under UV light. The DMAs were scraped from the plate, extracted with 1:1 hexane-diethyl ether and analyzed by GC. DMAs were then evaporated to dryness and oxidized with modified Jones' reagent as described above. The FFAs generated from DMAs were then methylated and again analyzed by GC for comparison.

### Application to marine samples

To demonstrate the results of the oxidation technique on samples known to contain either alcohols or plasmalogens, lipids were extracted and transesterified from fish and invertebrate samples [white barracudina (*Notolepis rissoi*), myctophids, and euphausiids], as described above. For later comparison, this hexane fraction, containing FAMEs and either alcohols or DMAs, was analyzed by GC to illustrate the results obtained without first oxidizing alcohols and DMAs. This mixture was then separated into FAME, alcohol, and/or DMA components by TLC in toluene. In this solvent, the band containing FAMEs migrates furthest, fatty alcohols remain at the origin, and DMAs migrate just above the origin. After separation, the FAME band was removed from the plate, extracted from the silica gel with 1:1 hexane-diethyl ether, and set aside. The alcohol and DMA bands were collected and also extracted with 1:1 hexane-diethyl ether. If both alcohols and DMAs were present in the same sample, they were collected together. The mixture was then evaporated to dryness and treated with the modified Jones' reagent. The resultant FFAs were methylated and the FAME produced were combined with the FAME fraction that was set aside after TLC. The combined fractions were then analyzed by GC and compared with the results obtained without oxidizing alcohols and DMAs.

### GC conditions

All FAME samples were analyzed using a Perkin Elmer Autosystem II Capillary GC with a flame ionization detector using a flexible fused silica column (30 m × 0.25 mm ID) coated with 50% cyanopropyl polysiloxane (0.25 μm film thickness; J and W DB-23; Folsom, CA). Helium was used as the carrier gas and the gas line was equipped with an oxygen scrubber. The following temperature program was employed: 153°C for 2 min, hold at 174°C for 0.2 min after ramping at 2.3°C $min^{-1}$, and hold at 220°C for 3 min after ramping at 2.5°C $min^{-1}$. Up to 66 FAMEs were identified according to Iverson et al. (13). Response factors for FAMEs (16) were applied, and each FAME was reported as weight percent of total.

FAMEs were described using the shorthand nomenclature of A:Bn-X, where A represents the number of carbon atoms, B the number of double bonds, and X the position of the double bond closest to the terminal methyl group. Data are expressed as mean ± SD throughout.

## RESULTS AND DISCUSSION

A wide variety of marine species are known to contain WEs and plasmalogens (7, 9, 17). In our laboratory, we have detected WEs in zooplankton, squid, and in some teleosts and odontocete cetaceans, while plasmalogens have been found in some euphausiids and bivalves. Here we present a method to efficiently and quantitatively transform alcohols and DMAs into FAMEs, which is necessary to appropriately relate predator and prey fatty acid signatures. We first demonstrated this quantitative conversion using a standard mixture of FAMEs and alcohols. This mixture, containing equivalent proportions of five saturated FAME, was carried through the reaction with the same carbon series of five monounsaturated fatty alcohols in an original average mole ratio of 0.84 ± 0.01 FAME-alcohol for each of the five pairs (**Table 1**). This mole ratio was then compared with the mole ratio derived from peak areas determined by GC after actual oxidation of the alcohols to FAMEs. In each case, the ratio of original FAME-FAME produced from alcohols (mean 0.86) remained very similar to the original ratio of FAME-alcohol (0.84) (Table 1). In addition, after the oxidation reaction, the quantitative proportions of components were consistent with the complete conversion of all alcohols to FAMEs with no losses, as well as retention of the original quantities of FAMEs (**Fig. 1**).

We next assessed absolute yields of the reaction using 16:0 and 18:0 alcohol standards and obtained a reaction yield of 97%. Theoretically, this oxidation reaction should proceed to completion. In the procedure we outlined, the sulphuric acid was limiting and ~0.29 mmol of alcohol (equivalent to 70 mg 16:0 alcohol) or 0.58 mmol DMA (or 165 mg 16:0 DMA) can be oxidized. Since the mass of alcohols used to assess yield (30 mg) was much less than this maximum, we can be confident that the loss of product was not due to insufficient reagent. In addition, neither TLC nor GC revealed any unreacted alcohols in the products. Thus, we can assume that the slight loss of FAME product was due to minor errors in the hexane extraction of the FFAs or FAMEs generated. To ensure the reaction goes to completion when applying this method to marine samples, we recommend methylating ⩽100 mg of lipid. Of course, if greater amounts of alcohol or DMAs must be oxidized, the reaction can be scaled up as necessary. It is convenient that both alcohols and DMA are obvious in the GC chromatogram (e.g., **Fig. 2A**); this allows investigators to determine if the oxidation reaction is necessary for a given species. Likewise, if insufficient amounts of reagent are used in the initial oxidation, the unreacted precursors will be readily visible in the chromatogram, and a second oxidation can be carried out to complete the conversion.

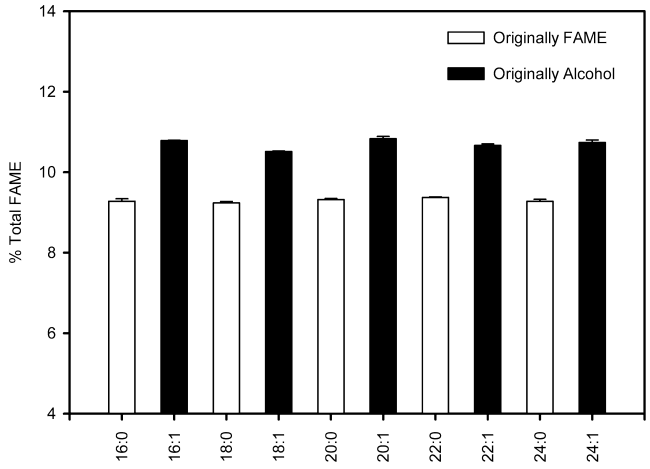


**Fig. 1.** Proportions of saturated fatty acid methyl esters (FAMEs) (originally present as FAMEs) and unsaturated FAMEs (derived from alcohols) analyzed in a standard mixture after treatment with modified Jones' reagent (n = 3, mean ± SD). The original standard mixture contained five saturated FAMEs (16:0–24:0), each at 9.6% (by mass) of total mixture, and five monounsaturated fatty alcohols (16:1–24:1), each at 10.4% (by mass) of total mixture. Expected FAME proportions after adjusting for the differences in mass between a fatty alcohol and FAME of the same alkyl structure are 9.2% (16:0–24:0) and 10.8% (16:1–24:1).

We then used lipids containing plasmalogens, isolated from the blue mussel, to demonstrate the production of DMAs and their subsequent quantitative conversion to FAMEs. Unlike alcohols, DMAs form sharp peaks on polar GC columns, which could be readily mistaken for FAMEs. Thus, it was possible to simply compare peak areas before and after oxidation (Fig. 2A, B). In this particular example, after FAME preparation the two greatest DMA peaks were 18:0 DMA and 20:1n-7 DMA, which corresponded to the nor-

TABLE 1. Mole ratios of saturated fatty acid methyl ester:monounsaturated alcohol before and after conversion of the monounsaturated alcohols to fatty acid methyl ester in a standard mixture

| | 16:0/16:1 | 18:0/18:1 | 20:0/20:1 | 22:0/22:1 | 24:0/24:1 | Mean ± SD |
|---|---|---|---|---|---|---|
| FAME:alcohol[a] | 0.83 | 0.84 | 0.84 | 0.85 | 0.86 | 0.84 ± 0.01 |
| FAME:FAME from alcohol by GC[b] | 0.85 | 0.87 | 0.86 | 0.88 | 0.86 | 0.86 ± 0.01 |

FAME, fatty acid methyl ester; GC, gas chromatography.

[a] Derived from the original total masses of each saturated FAME and each corresponding unsaturated alcohol contained in the standard mixture prior to oxidation.

[b] Calculated from GC chromatogram peak areas after the oxidation reaction that converted the monounsaturated alcohols to FAMEs.

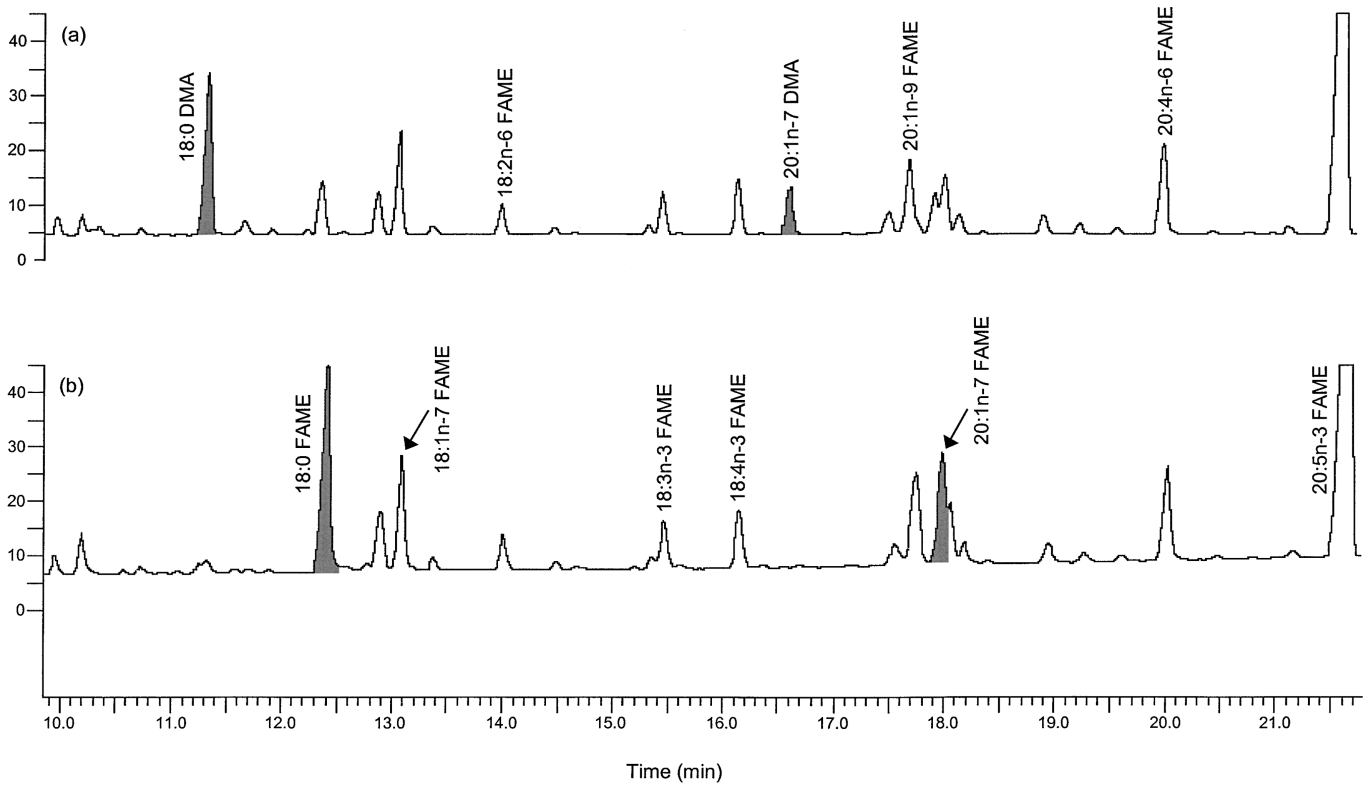


**Fig. 2.** A: Selected portion of gas chromatography chromatogram showing location of 18:0 dimethylacetal (DMA) and 20:1n-7 DMA (gray shading) among FAME peaks. B: Same portion of chromatogram after oxidation of DMAs with modified Jones' reagent. Gray shading here indicates peak areas that have changed with the addition of FAMEs derived from DMAs.

mal elution time of 16:4n-3 and an unknown component, respectively (Fig. 2A). As a result, 16:4n-3 would have been incorrectly quantified at a level of 5.30 ± 0.68% of total fatty acids when it normally averages 0.39 ± 0.34% in all marine prey species in the western Atlantic (18) and only 0.20 ± 0.02% in the blue mussel. Thus, this aberrant level and that of an unknown component (at 1.6 ± 0.25%) that elutes where no other fatty acid is known to elute may serve as indicators of the presence of DMAs on this polar cyanopropyl polysiloxane column. On less polar polyethylene glycol columns, DMAs normally coelute with iso-branched acids of the same carbon number (9) so that unusually large amounts of branched chain acids may indicate the presence of DMAs in that situation. On the polar column employed here, the subsequent changes in levels of 18:0 and 20:1n-7 FAME after oxidation are obvious (Fig. 2B). However, agreement between the levels of all DMAs and the FAMEs produced after oxidation was also excellent (**Fig. 3**), with levels of 16:0 the only minor exception. A blank that was carried through the reaction did not yield any 16:0, suggesting that the slight increase in 16:0 in FAMEs was not a result of contamination. To ensure complete recovery of DMAs from the TLC plate when initially isolating those components, all material near the bottom of the plate was collected. Thus, it is possible that traces of 16:0 alcohol reported to be present in blue mussels (19) were removed along with the DMAs from the TLC plate. Such traces of 16:0 alcohol would be oxidized during the reaction, creating a larger 16:0 FAME peak than expected. Lastly, it should be noted that this oxidation allowed us to identify the unusual 17:0 DMA and *i*-17:0 DMA. To our knowledge, this is the first report of these DMAs in the marine environment.

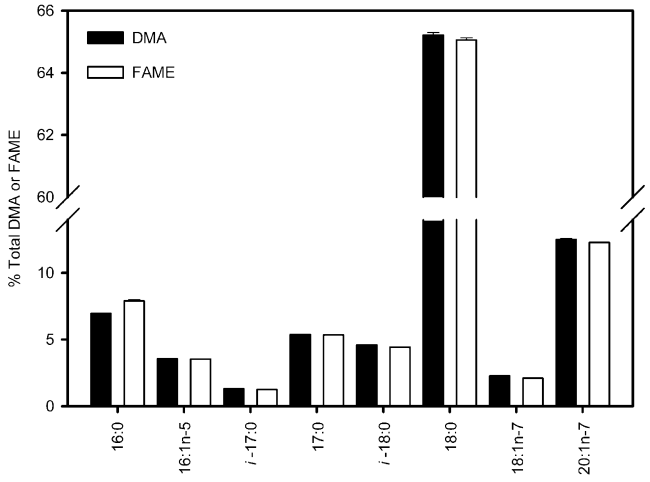


**Fig. 3.** Proportions of DMAs isolated from a marine sample after acid transesterification and TLC in comparison with proportions of FAMEs derived from these DMAs after oxidation with modified Jones' reagent (n = 3, mean ± SD).

A more convenient procedure that we originally attempted was to simply apply the oxidation reaction to the entire FAME sample rather than first separating FAMEs from alcohols and DMAs. The mild Jones' reagent is recommended for the oxidation of labile alcohols (20), making that approach seem reasonable. However, when FAME samples containing long-chain PUFA were carried through the procedure, we found reduced recovery of the polyunsaturates, while levels of saturated and monounsaturated FAMEs were unaffected. We attempted a number of steps to rectify this situation, including the use of an ice bath, excess Jones' reagent, and exclusion of oxygen. Despite these measures, the losses of PUFA persisted. It seems that these long-chain PUFA are too prone to oxidation to be exposed to even such a mild oxidizing agent under any conditions. Conveniently, alcohols and DMAs present in marine samples almost always contain one or no double bonds (7), thus allowing this reagent to be used in the oxidation of alcohols and DMAs, but necessitating their prior TLC separation from the FAMEs. It was this potential of loss of material that made the validation of the method with monounsaturated standards necessary.

It might seem that an obvious remedy to the problem of PUFA loss with exposure to Jones' reagent is to simply use another oxidizing agent. In theory, there are a large number of potential reagents that could be employed to oxidize alcohols to carbonyls (21), but we required an oxidant that was strong enough to carry the oxidation through to the production of FFAs rather than stopping with the generation of aldehydes. Jones' reagent, composed of acidic $CrO_3$, is actually among the mildest of those oxidation agents capable of producing carbonyls, so, in fact, it is probably the best choice of oxidizing agent among the available alternatives. Its use with fatty alcohols had also been previously reported (22), so it seemed a logical choice. We have demonstrated here that DMAs are also effectively oxidized to FFAs with this reagent. The only disadvantage associated with the use of Jones' reagent is the toxicity of chromium compounds and the difficulties with waste disposal.

One should also take note of the importance of the complete evaporation of the solvent in which the alcohols and DMAs are originally dissolved. Usually, this solvent is hexane and its evaporation can be easily accomplished using a stream of $N_2$ in conjunction with a warm water bath (30°C) that also effectively prevents accumulation of condensation. Jones' reagent is an aqueous solution, thus the alcohols and DMAs must be dissolved in a solvent that is miscible with water, such as acetone. If any traces of hexane remain, alcohols and DMAs will stay in solution in hexane and will not mix with the Jones' reagent, and oxidation to FFAs will not occur. This complete removal of solvent, however, effectively prevents the application of this procedure to any sample that may contain short-chain alcohols or DMAs. Such volatile compounds would be lost under evaporation and cannot be quantified with this technique. Although short-chain DMAs are unlikely, this is not the case for WE alcohols and FAMEs (23).

Lastly, we have shown the proportions of major fatty acids before and after oxidation in three different sample types: barracudina (Fig. 1A), euphausiids (**Fig. 4B**), and myctophids (Fig. 4C). These are prey species that are typical of the samples normally processed in our laboratory. In addition to acyl lipids, barracudina and myctophids contained WEs, while euphausiids contained WEs and traces of plasmalogens. Consistent in all three profiles is a dramatic change after treatment with Jones' reagent. In the barracudina, increases in 16:0, 18:0, 18:1n-7, 20:1n-9, and 22:1n-11 are obvious after oxidation (Fig. 4A), and these results are in agreement with the finding that those structures are the major alcohol components of barracudina (24). In several planktivorous fish, the fatty acids 20:1n-9 and 22:1n-11 are very abundant and are particularly important in differentiating prey species and for diet determination (13, 25), thus accurate determination of these compounds and their contribution to fatty acid signatures is essential. Less is known about the DMA composition of euphausiids, but the increase in 14:0 after oxidation indicates that there is an important source of this alkyl structure in this organism (Fig. 4B); however, the contribution of alcohols to these peaks complicates the interpretation of the fatty acid source.

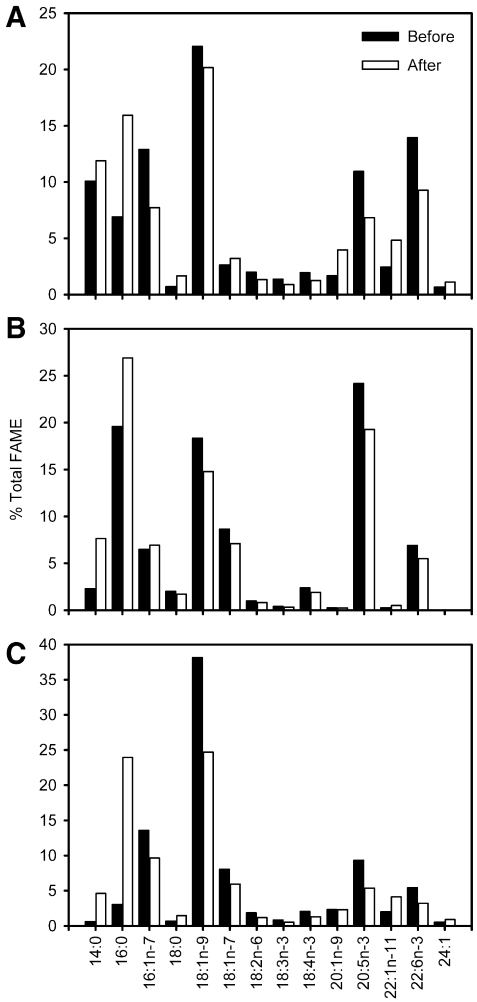


**Fig. 4.** Proportions of selected FAMEs as quantified in typical marine samples containing wax esters and/or plasmalogens before and after oxidation. Barracudina (A), euphausiids (B), and myctophids (C) (n = 1 for each).

With these data, it is not possible to determine whether a 14:0 alcohol or 14:0 DMA, or both, is responsible for this increase. In the myctophid (Fig. 4C), the greatest increases in levels of fatty acids after oxidation occur in 14:0 and 16:0, which also agrees with reports that those structures are among the major alcohols in this fish (26).

Common to all three examples in Fig. 4 are noticeable decreases in several fatty acids, such as 20:5n-3 and 22:6n-3. With proportional data such as that reported here, decreases in levels of particular fatty acids, such as 20:5n-3, will occur with increases in other fatty acids (e.g., 14:0 and 16:0). Additionally, a large increase in one fatty acid (e.g., 16:0) may obscure a smaller increase in another (e.g., 20:1n-9; Fig. 4B, C), making it appear as if there is no change in the second fatty acid. Thus, the addition of an internal standard is necessary to evaluate absolute differences of some components. However, since we are primarily interested in the "global" fatty acid signature available to the predator for lipid deposition (S. J. Iverson, C. Field, W. D. Bowen, and W. Blanchard, unpublished observations), the overall change in signature after oxidation is most important for our purposes. Replicates of barracudina, euphausiid, and myctophid samples not shown here also displayed similar changes in levels of fatty acids and confirmed these results. Clearly, our understanding of the prey fatty acid signature available to the predator would be very different if only the initial FAME results were used without correcting for alcohols and DMAs.

In conclusion, the method we have described provides a convenient and effective means to transform fatty acid-precursors to their corresponding fatty acid structures, which will allow appropriate comparison of the fatty acids available for deposition in a predator's fat stores from that of its prey. While we have concentrated on applications to marine samples, this technique is certainly not limited to those sample types; it can just as easily be applied to the investigation of waxes found in terrestrial plants and seeds. We present this chemical technique as an alternative to the time-consuming procedures that are necessary to determine alcohols and DMAs separately. This method should aid in the interpretation of trophic interactions and diets using fatty acid signatures.

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