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Sample preparation methods for the analysis of poly-methylene interrupted fatty acids

Tadej Mezek, Michael T. Arts, Ed Sverko and Aaron T. Fisk

Introduction

Poly-methylene interrupted fatty acids (PMI-FA) are uncommon fatty acids (FA) that have been found in various types of seed oils (Smith et al. 1969, Madrigal & Smith 1975, Plattner et al. 1975), plants or plant oils (Fore et al. 1966, Jamieson Reid 1972), human milk (Murawski et al. 1971), beef and mutton tallow (Hoffmann & Meijboom 1969), and various marine invertebrates (Ackman & Hooper 1973, Jefferts et al. 1973, Paradis & Ackman 1974, Pearce & Stillway 1976, Garrido & Medina 2002, Budge et al. 2007, Saito 2007). In marine systems, PMI-FA are believed to be synthesized *de novo* only in bivalves and carnivorous gastropods (Joseph 1982, Budge et al. 2007, Saito 2007). Although PMI-FA often represent a small portion of total fatty acids, they are potentially valuable as biomarkers in food web studies (Budge et al. 2007). Proportions and values of PMI-FA vary among species. According to Budge et al. (2007) the values of PMI-FA in bearded seals (*Erignathus barbatus*) and Pacific walruses (*Odobenus rosmarus*) approach 1 % of total FA expressed as weight percent. Much higher values of PMI-FA were reported for the shallow water clam (*Mactra chinensis*, at 6.6 %) and the deep-sea clam (*Calyptogena phaseoliformis,* at 30 %) of total FA expressed as weight percent of total FA (Saito 2007). Until recently, a limited number of pretreatment procedures have been available to reduce the complexity of the lipid sample prior to analysis with gas chromatography (GC), particularly an issue with PMI-FA resulting in analyses that typically yield only qualitative estimates. However, new sample preparation procedures, such as solid phase extraction (SPE) and modern analytical equipment, such as capillary columns and gas chromatography/mass spectrometry (GC/MS), have provided increasingly effective methods for precise qualitative and quantitative analysis of PMI-FA. Here we review past, current, and possible future sample preparation procedures for the analysis of PMI-FA.

The more common FA of animal and plant origin usually have even-numbered carbon chains; typically from 12 to 24 carbon atoms and containing 0 to 6 double bonds (predominantly in the *cis* configuration). Despite the numerous possible configurations relating to degree of unsaturation, configuration, and despite position of double bonds, carbon chain length, and constituents attached to the FA molecule, FA found in nature most commonly contain a single methylene group between double bonds. These FA are referred to as methylene interrupted FA (MI-FA; Fig. 1a), whereas PMI-FA contain more than one methylene group between double bonds within the FA molecule (Fig. 1b). Although some authors designate PMI-FA as NMI-FA (non-methylene inter-

Fig. 1a – MI-FA
20:4Δ5,8,11,14 = 20:4ω-6
Arachidonic acid

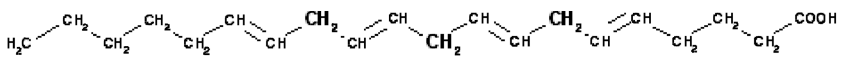

Fig. 1b – PMI-FA
20:4Δ5,11,14 = 20:3ω-6
Sciadonic acid

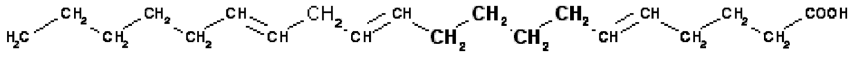

Fig. 1. a. Methylene interrupted FA with single methylene group between double bonds on the aliphatic chain (indicated in bold). **b.** Poly-methylene interrupted FA with more than one methylene groups between at least 2 of the double bonds on the aliphatic chain (indicated in bold).

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rupted fatty acids), we suggest that the term PMI-FA is preferable because it more specifically reflects the possibility of multiple (2 or more) methylene groups between contiguous double bonds. In addition, there is a risk that the use of NMI-FA terminology may be incorrectly interpreted to mean the absence of a methylene group between contiguous double bonds.

Organisms contain complex mixtures of different lipid moieties; the most abundant are those containing FA as a part of their molecular structure. Fatty acids can be analytically isolated from lipid classes (moieties from which FA are released; i.e., glycerol), followed by derivatization. Derivatized fatty acid methyl esters (FAME, including PMI-FAME) can then be directly analyzed by a GC equipped with various types of detectors (most commonly flame ionization detectors; FID). Fatty acids can be further separated and purified before GC analysis by first subjecting the FAME to various preparative procedures, such as TLC (thin layer chromatography) and/or $AgNO_3$-TLC (Silver ion-TLC; Tanaka et al. 1997, 1999, 2007). Despite the possibility of using high performance liquid chromatography (HPLC) to reduce the complexity of the lipid sample, HPLC has not yet found widespread use by analysts, possibly because of the higher cost of HPLC compared to TLC, and because HPLC columns improved considerably only just recently (i.e., higher capacity and reduced amounts of sample residues on the column). Following earlier successes with the $AgNO_3$-TLC method, the technique has been adapted to HPLC ($AgNO_3$-HPLC); however, to our knowledge this new method has not yet been successfully applied to questions of interest to freshwater or marine researchers.

TLC is used for preparative purposes (removal of unsaponifiable material; i.e., glycerol moieties), which can interfere with GC analysis and/or coupled with $AgNO_3$-TLC to separate lipid extract (usually their FAME derivatives) by their degree of unsaturation. The common goal of such preparative procedures is to remove sample-related matrix material, which may confound analysis, and to enhance separation and resolution; however, the increase in analytical steps can result in an increase of analytical variability.

$AgNO_3$-HPLC can, compared to $AgNO_3$-TLC, offer better and more consistent separation, cleaner fractions, and higher sample applications at no loss of resolution on modern novel columns, but at much higher equipment cost (Christie 2003). However, it was recently demonstrated that preparative steps could be omitted to obtain simultaneous chromatographic separation of MI-FA and PMI-FA when the exact position of double bonds of the PMI-FA are known (Budge et al. 2007). For example, Budge et al. (2006, Appendix 7) demonstrated that PMI-FA can be readily revealed and that they elute between more common MI-FA.

Key words: biomarkers, food webs, non-methylene interrupted fatty acids, NMI-FA, PMI-FA, poly-methylene interrupted fatty acids

Analysis of PMI-FA

Gas chromatography-mass spectrometry (GC/MS) with a Quadrupole can be particularly valuable, or even essential, to identify novel FA (e.g., PMI-FA) because the mass spectra can be used to structurally identify the possible PMI-FA. Because FAME derivatives provide little structural information for the FA, other derivatives, which produce indicative ions, should be used. Such ions can elucidate the exact position of double bonds on the aliphatic chain and thus correctly identify the compounds of interest. In the case of MI-FA, identification and quantification are usually based on analytical standards and calibration curves, principly by using external or/and internal standards (i.e., Supelco's 37-component FAME mix, catalog# 47015-U) with known molecular formulae, concentrations, and elution patterns.

Currently, no PMI-FA standards are available. This necessitates different analytical approaches to reveal the exact position of double bonds and to unequivocally quantify the individual PMI-FA. Quantitation could also be obtained by calculating the response factor of representative PMI-FA. These analytical limitations have only allowed the publication of qualitative PMI-FA results (i.e., on a percentage basis, relative to the proportion of all identified FAME). One way to overcome this identification problem is to derivatize the carboxyl group with a reagent containing a nitrogen atom, resulting in a fragmentation pattern that could be specific enough to reveal the position of double bonds in the aliphatic chain. According to Christie (2003), picolynyl esters or dimethyloxazoline (DMOX) derivatives are the derivatives of choice in this regard.

Derivatives of FA

For elution on a GC column, picolynyl esters require an oven temperature 50 °C higher than that routinely used for FAME analyses. A GC column that has a non-polar stationary phase is required; however, this has the undesirably quality of introducing a loss of resolution with FA compounds. Although picolynyl ester sample preparation is relatively simple, DMOX derivatives require more complicated analytical procedures and may degrade quickly during storage (Christie 2003). However, if such derivatives are only used for qualitative analysis, for example to identify the exact position of a double bond, degradation is of little or no importance. For example, Garrido & Medina (2002) used DMOX derivatives to determine the position of double bonds of a number of PMI-FA. Other possible approaches to elucidating the

position of multiple double bonds in PMI-FA include hydrogenation (Tanaka et al. 1997, 1999, 2007) and deuteration (Christie 2003). Deuteration is particularly useful to characterize other moieties on the aliphatic chain. Hydrogenation and deuteration are performed on FAME or nitrogen atom containing derivatives and not on crude lipid extracts.

In previous studies, the exact positions of double bonds were determined by reductive ozonolysis and oxidative ozonolysis by measuring IR (infra-red) spectra or by measuring NMR (nuclear magnetic resonance) spectra (Fore et al. 1966, Smith et al. 1969, Paradis & Ackman 1974, Madrigal & Smith 1975). However, the chemicals and laboratory equipment required for such analyses are expensive and not always readily available; therefore, the use and application of such techniques for aquatic ecologists has been limited. Additionally, these techniques are not sensitive, and if the unknown compounds are not available in milligram amounts, they are unable to structurally identify the unknowns.

Despite the availability of novel GC capillary columns, preparative $AgNO_3$-TLC is still widely used for separation of FAME, which can be subjected to further preparative analysis (e.g., DMOX derivatives). After the plate is developed and the bands of lipids visualized, they can be scraped off the plate, re-extracted and purified to remove the visualization reagent (Tanaka et al. 1997) before further processing. On a developed TLC plate, each band represents a group of FAME having the same number of double bonds. Most of the papers reviewed here from older (Fore et al. 1966, Paradis & Ackman 1974) to more recent (Wilson & Sargent 1992, Garrido & Medina 2002, Budge et al. 2004) utilize such procedures. The most recent publication (Budge et al. 2007) uses this procedure only for qualitative analysis (i.e., to reveal the positions of double bonds).

Conclusions

Based on the literature reviewed we conclude that the separation of PMI-FA simultaneously with separation of MI-FA can be obtained by using modern capillary columns such as Supelco's DB-23. However, the main analytical challenge resides in the identification and location of PMI-FA double bond placement. This is crucial in the quest to better understand the role of PMI-FA as biomarkers. To help identify double-bond location, we propose the use of either nitrogen-containing derivatives of FA (DMOX), or deuteration, directly (without preparative TLC). However, if loss of resolution is an issue we propose using Supelco's Ag-Ion SPE (catalog# 54225-U) rather than using preparative $AgNO_3$-TLC because modern SPE cartridges are small, cost-effective, and provide better quantitative outcomes when used with the appropriate controls. Routine quantitative methods for PMI-FA could be a valuable tool for elucidating trophic relationships in food webs because these unique FA are found in a limited group of organisms (e.g., mollusks), where they are assumed to be synthesized *de novo*. Thus, routine monitoring of PMI-FA may be of great importance in revealing food web interactions, especially if, as described in Budge et al. (2007), their passage up the food web chain occurs in a predictable manner. We suggest that PMI-FA has the potential to; (a) more clearly quantify prey-predator relationships and feeding strategies, (b) help identify species, and (c) more quantitatively measure the impacts of large scale processes such as invasive species and/or climate change on natural ecosystems. Thus, we suggest that the identification of novel PMI-FA will undoubtedly have a significant impact on future studies regarding food web dynamics in both marine and freshwater ecosystems.

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Authors' addresses: T. Mezek, National Institute of Biology, Department of Freshwater and Terrestrial Ecosystem Research, Večna pot 111, SI-1000 Ljubljana, Slovenia; E-mail: Tadej.Mezek@nib.si

M.T. Arts, E. Sverko, National Water Research Institute, Aquatic Ecosystem Management Research Division, 867 Lakeshore Road, P.O. Box 5050, Burlington, Canada ON L7R 4A6.

A.T. Fisk, Great Lakes Institute for Environmental Research, University of Windsor, 401 Sunset Ave, Windsor, Ontario, Canada N9B 3P4.