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Determination of Total Lipid and Lipid Classes in Marine Samples

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Abstract

Lipids are largely composed of carbon and hydrogen and, therefore, provide a greater specific energy than other organic macromolecules in the sea. Being carbon- and hydrogen-rich they are also hydrophobic and can act as a solvent and absorption carrier for organic contaminants and thus can be drivers of pollutant bioaccumulation in marine ecosystems. Their hydrophobic nature facilitates their isolation from seawater or biological specimens: marine lipid analysis begins with sampling and then extraction in non-polar organic solvents, providing a convenient method for their separation from other substances in an aquatic matrix.

If seawater has been sampled, the first step usually involves separation into operationally defined 'dissolved' and 'particulate' factions by filtration. Samples are collected and lipids isolated from the sample matrix typically with chloroform for truly dissolved matter and colloids, and with mixtures of chloroform and methanol for solids and biological specimens. Such extracts may contain several classes from biogenic and anthropogenic sources. At this time, total lipids and lipid classes may be determined. Total lipid can be measured by summing individually determined lipid classes which customarily have been chromatographically separated. Thin-layer chromatography (TLC) with flame ionization detection (FID) is regularly used for the quantitative analysis of lipids from marine samples. TLC-FID furnishes synoptic lipid class information and, by summing classes, a total lipid measurement.

Lipid class information is especially useful when combined with measurements of individual components e.g., fatty acids and/or sterols, after their release from lipid extracts. The wide variety of lipid structures and functions means they are used broadly in ecological and biogeochemical research assessing ecosystem health and the degree of influence by anthropogenic impacts. They have been employed to

measure substances of dietary value to marine fauna (e.g., aquafeeds and/or prey), and as an indicator of water quality (e.g., hydrocarbons).

Introduction

The methods described here concern substances that are defined operationally as marine lipids. This definition is based on their amenability to liquid-liquid extraction in non-polar organic solvents, and it provides a convenient method for their separation from other substances in an aquatic matrix. Their hydrophobic nature facilitates their isolation from seawater or biological specimens, as well as their enrichment, and the removal of salts and proteins.

The measurement of lipid content and its composition in marine organisms has been of great interest in food web ecology, aquaculture nutrition, and food science for decades. Lipids are universal components in living organisms, acting as essential molecules in cell membranes, as major sources of bioavailable energy, providing thermal insulation and buoyancy, and serving as signaling molecules. Although procedures for lipid determination in other fields have been described well, their use with marine samples commonly necessitates modification to adapt to field conditions as well as to sample type[1].

For seawater samples, the first step usually requires separation into the operationally defined 'dissolved' and 'particulate' fractions, normally by filtration (Protocol step 1). The particulate fraction is what is retained by the filter, and size of the pores is important in defining the cut-off[2]. Often when we are sampling particulate matter, we would like to relate lipid concentrations to total mass concentrations, in which case a separate, smaller, sample (e.g., 10 mL) has to be taken for this purpose (Protocol step 1, note). To get an accurate mass determination it is important to add ammonium formate (35 g/L) at the end of the filtration.

The seawater filtrate from the larger sample should amount to between 250 mL and 1 L depending on sample type and is subjected to liquid-liquid extraction in a separatory funnel (Protocol step 2). The hydrophobic nature of lipids means they can be separated from other compounds by extraction in a nonpolar solvent such as chloroform. A two-layer system is created where lipids partition into the organic layer while water soluble components stay in the aqueous layer.

Particulate samples on a filter, or biological specimens are extracted with a modified Folch et al. extraction[3], also involving chloroform (Protocol step 3). Again, an organic/aqueous system is created in which lipids partition into the organic phase, while water soluble molecules remain in the aqueous phase, and proteins are precipitated. In fact, for solids, most laboratories use some variation of the Folch et al. extraction[3] procedure involving chloroform and methanol. For filters, the first step is to homogenize in 2 mL of chloroform and 1 mL of methanol.

During extraction, care should be taken to protect lipids from chemical or enzymatic modification, by keeping samples and solvents on ice to reduce ester bond hydrolysis or carbon-carbon double bond oxidation. Tissues and cell lipids are quite well protected by natural antioxidants and by compartmentalization[4]; however, following the homogenization of samples, cell contents are combined rendering lipids more disposed to alteration, chemically

or enzymatically. Some lipids, such as most sterols, are very stable, while others, such as those containing polyunsaturated fatty acids, are more susceptible to chemical oxidation. Others, such as sterols with conjugated double bonds, are prone to oxidation catalyzed by light[5]. Following extractions, lipids are much more susceptible to chemical oxidation, and samples should be stored under an inert gas such as nitrogen. A gentle stream of nitrogen would also be used to concentrate extracts.

After concentration, lipids would then normally be quantified in bulk as they are an important component of marine ecosystems providing a high concentration of energy, more than twice the kJ/g of carbohydrates and proteins. Invariably they would next be quantified as individual components: the comprehensive analysis of lipids generally involves separation into simpler categories, according to their chemical nature. Thus, a full analysis involves measuring total lipids, lipid classes and individual compounds.

Total lipid can be determined by taking the sum of individually measured lipid classes separated by chromatography[6]. A marine lipid extract may contain more than a dozen classes from biogenic and anthropogenic sources. The wide variety of lipid structures means much information can be gained by determining individual groupings of structures. Lipid classes individually, or in certain groups, have been used to signal presence of certain types of organisms, as well as their physiological status and activity[2]. They have also been used as an indicator of the origins of organic material, including dissolved organic matter (DOM) as well as hydrophobic contaminants.

Triacylglycerols, phospholipids and sterols are among the more important biogenic lipid classes. The first two are biochemically related as they possess a glycerol backbone to which two or three fatty acids are esterified (**Figure 1**). Triacylglycerols, together with wax esters are very important storage substances, while other fatty acid-containing lipid classes such as diacylglycerols, free fatty acids, and monoacylglycerols are generally minor constituents. Free fatty acids are present at lower concentrations in living organisms, as the unsaturated ones can be toxic[7]. Sterols (both in their free and esterified forms) and fatty alcohols are also included among the less polar lipids, while glycolipids and phospholipids are polar lipids. Polar lipids have a hydrophilic group, which allows for the formation of lipid bilayers found in cell membranes. Free sterols are also membrane structural components, and when taken in ratio to triacylglycerols they provide a condition or nutritional index (TAG : ST) which has been widely used[8]. When taken in ratio to phospholipids (ST : PL) they can be used to indicate plant sensitivity to salt: higher values maintain structural integrity and decrease membrane permeability[9]. The inverse of this ratio (PL : ST) has been studied in bivalve tissues during temperature adaptation[10].

Marine lipid classes can be separated by thin-layer chromatography (TLC) on silica gel coated rods (Protocol step 4) and then detected and quantified by flame ionization detection (FID) in an automatic FID scanner. TLC/FID has become routinely used for marine samples as it rapidly furnishes synoptic lipid class data from small samples, and by taking the sum of all classes, a value for total lipids. TLC/FID has been subjected to a quality-assurance (QA) assessment and was found to meet standards required for consistent external calibration, low blanks, and precise replicate analysis[11]. Coefficients of variation (CV) or relative standard deviations are around 10%, and FID scanner total lipid data are normally around 90% of those obtained by gravimetric and other methods[2]. Gravimetry gives higher total

lipids likely because the FID scanner measures only non-volatile compounds, and also as a result of possible inclusion of non-lipid material in gravimetric measurements.

The information provided by lipid class analysis is especially useful when combined with determinations of fatty acids as individuals, or sterols, or the two in combination. The first step towards these analyses involves the release of all component fatty acids together with sterols in the lipid extracts (Protocol step 5). The wide variety of lipid structures and functions means they have seen broad use in ecological and biogeochemical studies assessing ecosystem health and the extent to which they have been influenced by anthropogenic and terrestrial inputs. They have been used to measure biosynthesis of substances of dietary value to marine fauna as well as to indicate the quality of water samples. Measuring lipids in sediment core samples helps show the sensitivity of sediments to changes in human land use near the land-sea margin.

The primary tool for identifying and quantifying individual lipid compounds has traditionally been gas chromatography (GC) with FID. Before analysis however, these compounds are made more volatile by derivatization. Fatty acids are released in the presence of an acidic catalyst (H_2SO_4) from acyl lipid classes (**Figure 1**). In organic chemistry, the acyl group (R-C=O) is usually derived from a carboxylic acid (R-COOH). They are then re-esterified to fatty acid methyl esters (FAME) which gives better separations on GC columns (Protocol step 5).

Protocol

NOTE: To clean glassware, instruments and filters for lipid analyses, wash them 3 times with methanol followed by 3 washes with chloroform, or heat them to 450°C for at least 8 hours.

1. Filtration procedure for seawater dissolved and particulate lipids

NOTE: The particular fraction of interest is operationally defined by the filtration procedure. In this case the pore size is 1.2 µm.

1. Set up the filtration manifold without a filter and rinse the setup with filtered seawater.
2. Using clean forceps place a 47 mm glass fiber (GF/C) filter, that has been washed, into the clean filtration system.
3. Take the sample and gently swirl to resuspend any material that may have settled on the bottom of the collection container. Accurately measure out a known volume, in this case 1 L, and filter this through the filter.
   NOTE: The volume will depend on the amount of particulate material in the sample: usually between 250 mL and 5 L depending on season and location.
4. When the sample has been measured out, gently wet the filter with filtered seawater. Add the entire sample slowly to the filtration system and rinse the graduated cylinder with filtered seawater to ensure all particles are added to the filter. Rinse the setup with filtered sea water on the sides to ensure all particles are rinsed down onto the filter.
   1. Allow all the seawater to pass through the filter but do not allow the filter to dry out completely as it can disrupt the cells on the filter; break suction as the last of the water disappears.

5. Using clean forceps and a clean pipette, fold the filter in half, then in thirds and then in half lengthwise to roll the filter into a tube. Place it into a clean, labeled 10 mL glass vial.

6. Cover the filter with 2 mL of chloroform. Fill the head space with nitrogen and seal with PTFE tape. Place in a rack in a -20 °C freezer. The samples will be stable at this temperature up to a year.

   NOTE: To relate lipid concentrations to total mass concentrations, a dry weight measurement is also needed. This involves putting a 24 mm pre-weighed filter into a dry weight filtration setup, stirring the sample and taking a small subsample which is filtered onto the small filter. When the filter is nearly dry, about 10 mL of 3.5% ammonium formate is added to the filter. The filter is folded in half, returned to a labeled Petri dish and placed in a freezer.

2. Liquid-liquid extraction of seawater or liquid samples

1. **Prepping the sample**

   1. Measure a known volume of filtrate into a lipid clean glass graduated cylinder. Place this sample in a clean 1 L separatory funnel. Then add 20 mL of chloroform to the sample and shake for 2 min, venting frequently.

2. **Removal of first extract and addition of acid after first separation**

   1. Wrap funnel in aluminum foil and wait 5 to 10 min for separation to occur. Peel back the bottom of the foil to see the two layers. Collect the bottom, organic layer through the stopcock into a clean round bottomed flask, being careful not to include any of the top layer. Cap the round bottomed flask under nitrogen and place in the freezer.

   2. Add 0.25 mL of concentrated H_2SO_4 for each liter of sample, to the sample in the separatory funnel and shake the funnel gently.

   3. Add 10 mL of chloroform and shake vigorously for 2 min while venting frequently. Allow the separation to take place.

3. **Second and third separations**

   1. Wait for the separation and add the bottom layer into the round bottomed flask.

   2. Add a third 10 mL of chloroform, and shake for 2 minutes, again venting frequently. After separation, add the bottom layer to the round bottomed flask.

   3. Transfer the extract in the round bottomed flask to a rotary evaporator, evaporate and transfer into a 2 mL vial.

3. Extraction protocol for solids (modified Folch et al.[3] extraction)

1. **Setup**

   NOTE: The extraction setup requires an insulated container filled with ice. Solvents include methanol, chloroform extracted water and 2:1 chloroform:methanol. All solvents should be placed on ice so that they are cold by the time the extractions are started. The samples also go on ice, so that everything remains cold.

   1. Rinse all tubes and PTFE lined caps 3 times with methanol and then 3 times with chloroform. One centrifuge tube and one 15 mL vial with a cap is needed for each extraction.

2. Place the sample in a centrifuge tube containing 2 mL of chloroform. The size of the sample depends on the amount of lipid present, approximately 5 to 10 mg of lipid preferred.

2. **Grinding and extraction**

1. Add approximately 1 mL of ice-cold methanol and grind the sample into a pulp quickly with a clean homogenizer or PTFE/metal-ended rod.

2. Wash the rod back into the tube with approximately 1 mL of ice-cold 2:1 chloroform: methanol and then with 1/2 mL of ice-cold chloroform-extracted water. If necessary, use a clean set of forceps to force particles back into the vial before washing. Cap the tube and sonicate the mixture for 4 minutes in an ultrasonic bath (35 - 42 kHz).

3. **Double pipetting**: Centrifuge the sample for 2 min at 1800 × *g*. Collect the entire organic layer (bottom layer) using the double pipetting technique in which a 5 ¾" pipette with the bulb on loosely is gently pushed through the two layers, while squeezing the bulb, causing bubbles to come out.

1. When the bottom of the second layer is reached, use the thumb to remove the bulb without drawing the organic layer into the pipette. Place a 9" pipette inside the 5 ¾" one and remove the bottom layer through the 5 ¾" pipette.

2. Place the extract in a clean vial. Continue to take off the bottom layer until it is all removed.

4. **Washing pipettes**: Rinse the 9" pipette into the vial containing the organic layer using 1.5 mL of ice-cold chloroform to wash down the outside of the pipette and 1.5 mL to wash down the inside. Gently turn that pipette while washing and make sure that all the chloroform runs down the pipette into vial.

1. Rinse the 5 ¾" pipette into the tube containing the aqueous layer in the same way.

5. Sonicate and centrifuge the samples and double pipette when separated, using new pipettes each time. Repeat at least three times and pool all organic layers. After removing the organic layer for the third time, wash both pipettes into the vial containing the organic layer.

6. Using a gentle stream of nitrogen, concentrate down to volume, then seal with PTFE tape and store in a freezer.

4. Developing systems and steps for rod TLC separation of marine lipid classes

1. **Prepping the rods for TLC**

1. Blank scan the rods in the automatic FID scanner three times

2. **Spotting a sample**: Apply samples and standards with a syringe to the rods at or just below the origin. Dispense 0.5 µL and touch the drop to the rod. Allow to dry before placing the next drop on the same spot. Spot all samples in a line on rods.

3. **Focusing in acetone**: Focus samples twice (three times if samples are very concentrated) in 70 mL of acetone. Watch the solvent front as it climbs the rod until the bottom of the spot merges with the top. Remove the rods, dry them for around 5 s, then repeat the procedure to produce a narrow band of lipid material near the bottom of the rod.

4. Dry and condition the rods in a constant humidity chamber for 5 min. A constant humidity chamber is a desiccator with a saturated solution of calcium chloride under the plate.

2. **Sequence leading to the first chromatogram (hydrocarbon to ketone)**
    1. **First development system**: The first development system is hexane:diethyl ether:formic acid, 98.95:1:0.05. Use a syringe to add the formic acid but first rinse the syringe 3× with formic acid. Rinse the formic acid out of the syringe immediately afterwards with chloroform. Use 30 mL of the mixture to wet the paper and rinse the tank. Discard the rinse solution and add the remaining 70 mL to the tank.
        1. Take the racks and gently lower them into the tank. Watch until the solvent front reaches the sample spots, then start the timer. After 25 min, remove the rods from the development chamber, dry in the constant humidity chamber for 5 min, and redevelop in the same solution for another 20 min.
    2. Dry the rods for 5 min in the automatic FID scanner and then scan to the lowest point behind the ketone peak using a PPS scan of 25.
3. **Sequence leading to the second chromatogram (triacylglycerol to diacylglycerol):**
    1. Condition the rods for 5 min in the constant humidity chamber.
    2. **Second development system**: The second development system is hexane:diethyl ether:formic acid, 79:20:1. Add ~30 mL to the development tank to wet the paper and rinse the tank. Then discard and develop the rods for 40 min in the remaining 70 mL. For the best separation between the TAG (saturated) and the TAG (polyunsaturated) peaks use a mixture of 79.9:20:0.1, but for separation of the ST and DAG peaks use a mixture of 79:20:1.
    3. Dry and scan to lowest point behind the diacylglycerol peak in a second partial scan using a PPS scan 11.
4. **Sequence leading to the third chromatogram (acetone-mobile polar lipid and phospholipid)**
    1. Condition the rods for 5 minutes in the constant humidity chamber.
    2. Develop the rods twice for 15 minutes in 70 mL of acetone. Between developments air dry the rods for about 30 seconds.
    3. Condition the rods for 5 minutes in the constant humidity chamber.
    4. **Third development system**: The third development system is a mixture of chloroform, methanol, and chloroform-extracted water, 50:40:10. Develop the rods twice for 10 minutes in 70 mL of the mixture. Between developments, air dry the rods for about 30 seconds.
    5. Dry and scan entire length of rods.

5. FAME derivatization with H_2SO_4 in MeOH

1. **Making the Hilditch reagent**
    1. **Preparing the methanol**: Place 100 mL of MeOH in a clean volumetric flask and then gently sprinkle in anhydrous $NaSO_4$ until the bottom of the flask is covered. Once covered, invert twice so that any water in the methanol is absorbed by the $NaSO_4$. After inverting and shaking, let it sit for at least 5 min.
    2. **Adding the acid**: Slowly decant the methanol into a glass jar (by now the $NaSO_4$ is a hard lump in the bottom of the flask) and the H_2SO_4 is added. Slowly add 1.5 mL sulphuric acid to the methanol using a

pipette. Add a few drops at a time and once all the acid has been added, cap and gently stir to mix. The solution is now ready to be used for derivatives, but it must be made up on a weekly basis.

2. **Making the derivatives**

    1. Transfer approximately 200 µg of lipids from an extract vial that has had the volume brought up to a known amount, into a clean, 15 mL vial and evaporate under nitrogen to dryness. The amount removed will be determined by the concentration of the sample from TLC/FID. Use a clean pipette to remove the sample.
    2. When the sample has dried, add 1.5 mL of dichloromethane and 3 mL of the newly made Hilditch reagent.
    3. Vortex the sample, and sonicate it in an ultrasonic bath for 4 min to remove lipids that have adhered to the glass vial. Fill the vial with nitrogen, cap, and seal it with PTFE tape and heat at 100°C for 1 hour.

3. **Stopping the reaction**

    1. Allow the samples to cool completely to room temperature for 10 min after removal from the oven, then open the vials carefully.
    2. Slowly add approximately 0.5 mL saturated sodium bicarbonate solution (9 g/100 mL chloroform-extracted water), then 1.5 mL hexane. Shake or vortex the vial, then let stand so that it separates into 2 layers.

4. **Collecting the FAME's**

    1. **Removing the top layer**: Once the derivatization has been halted, and there is clear separation, remove the upper, organic phase and place in a lipid clean 2 mL vial.
    2. Evaporate the solvent in the 2 mL vial to dryness and refill it with hexane to approximately 0.5 mL.
    3. Fill the head space of the vial with nitrogen, cap and seal the vial with PTFE tape, sonicate for another 4 minutes to re-suspend the fatty acids, and then it is ready to go to the GC.

        NOTE: If fatty acid concentrations are required, the aqueous layer must be washed three times with hexane and all the organic layers pooled into the 2 mL vial. This involves adding 2 mL of hexane, vortexing the sample, centrifuging, and removing the organic layer, all repeated 3 times.

Representative Results

As the fastest growing food production sector, aquaculture is evolving in terms of technological innovations and adaptations to meet changing requirements. One of these is to reduce the dependence on wild-sourced fishmeal and fish oil, which provide feed ingredients for many aquaculture species. Terrestrial plant oils are being investigated as sustainable and economical replacements for fish oil in aquafeeds, and the liver is a target tissue for analysis because it is the primary site for lipid metabolism[12]. **Figure 2** shows the raw TLC-FID chromatograms obtained from our nine-component standard, a diet we formulated with fish oil at 7% and rapeseed oil at 5%, and liver tissue from an Atlantic salmon fed that diet. **Table 1** shows the data obtained after analyzing dietary replicates and samples from different fish. These data were obtained after constructing standard curves from scanner FID responses to quantify the lipid classes in the extracts using Peak Simple software (version 4.54). The data show the prevalence of triacylglycerols in the diets and the

livers and also the importance of membrane phospholipids in the liver.

Continental margins generally feature very high biological productivity and they are especially important in the cycling of carbon. Surface primary productivity reaches the seabed more so in shallower water, and so measuring quantity and quality of particles settling from the upper mixed layer into the benthic food web is of great interest. Being rich in carbon and having a very high energy content, lipids are important components of the productivity of continental shelves. Historically, waters adjacent to Newfoundland and Labrador supported one of the greatest fisheries in the world for about five centuries, and we have been studying production and transfer of lipids in this system[13]. **Figure 3** shows TLC-FID chromatograms obtained from our standard, lipids in settling particulate matter collected at 220 m off the coast of Newfoundland, and lipids in a small mysid, *Erythrops erythrophtalma* collected near the same depth. This time the chromatograms have been processed through plotting software and the two partial scans have been combined with the final complete scan. **Table 2** shows the data obtained after analyzing replicate samples of settling particulate matter and the mysid. Among 19 taxa from 5 phyla, the small mysid had, on average, the highest lipid concentration (6% of wet weight)[13].

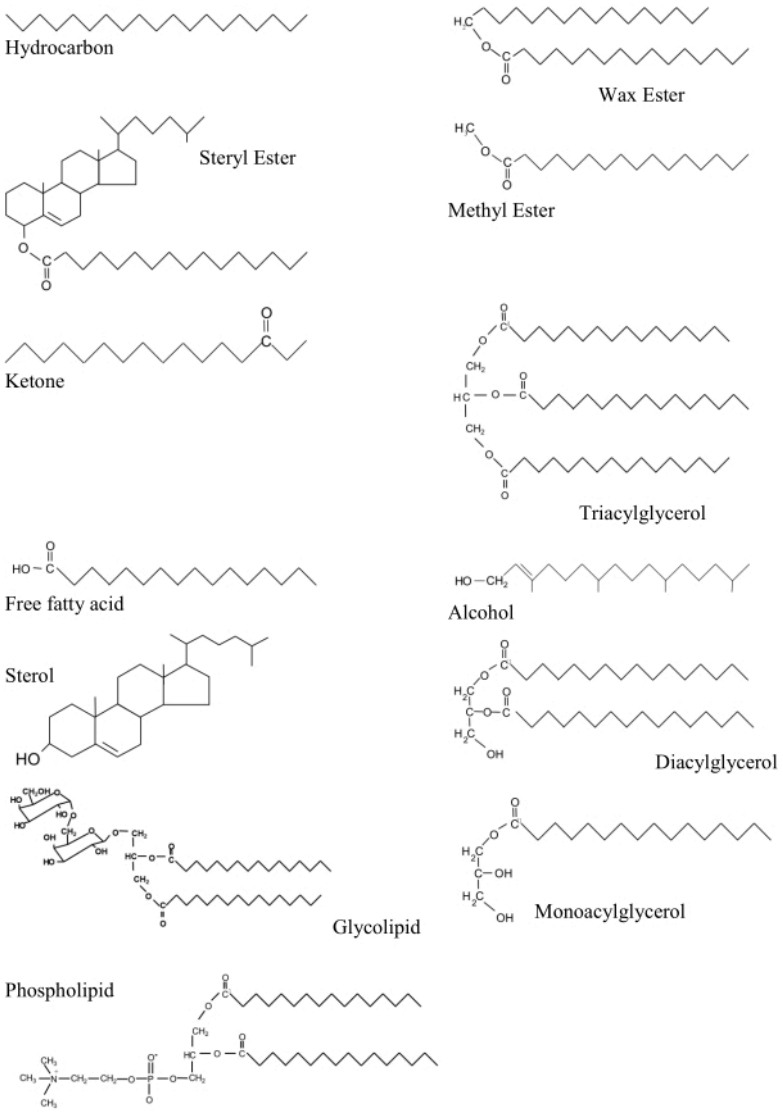

Figure 1: Principal lipid classes in marine samples in an approximate order of increasing polarity. Each structure is drawn with the most hydrophobic part of the molecule pointing towards the right of the Figure. Representative compounds for lipid classes are:- hydrocarbon: nonadecane; wax ester: hexadecyl palmitate; steryl ester: cholesteryl palmitate; methyl ester: methyl palmitate; ketone: 3-hexdecanone; triacylglycerol: tripalmitin; free fatty acid: palmitic acid; alcohol: phytol; sterol: cholesterol; diacylglycerol: dipalmitoyl glycerol; monoacylglycerol: monopalmitoyl glycerol; glycolipid: digalactosyl diacylglycerol; phospholipid: dipalmitoyl phosphatidylcholine. Please click here to view a larger version of this figure.

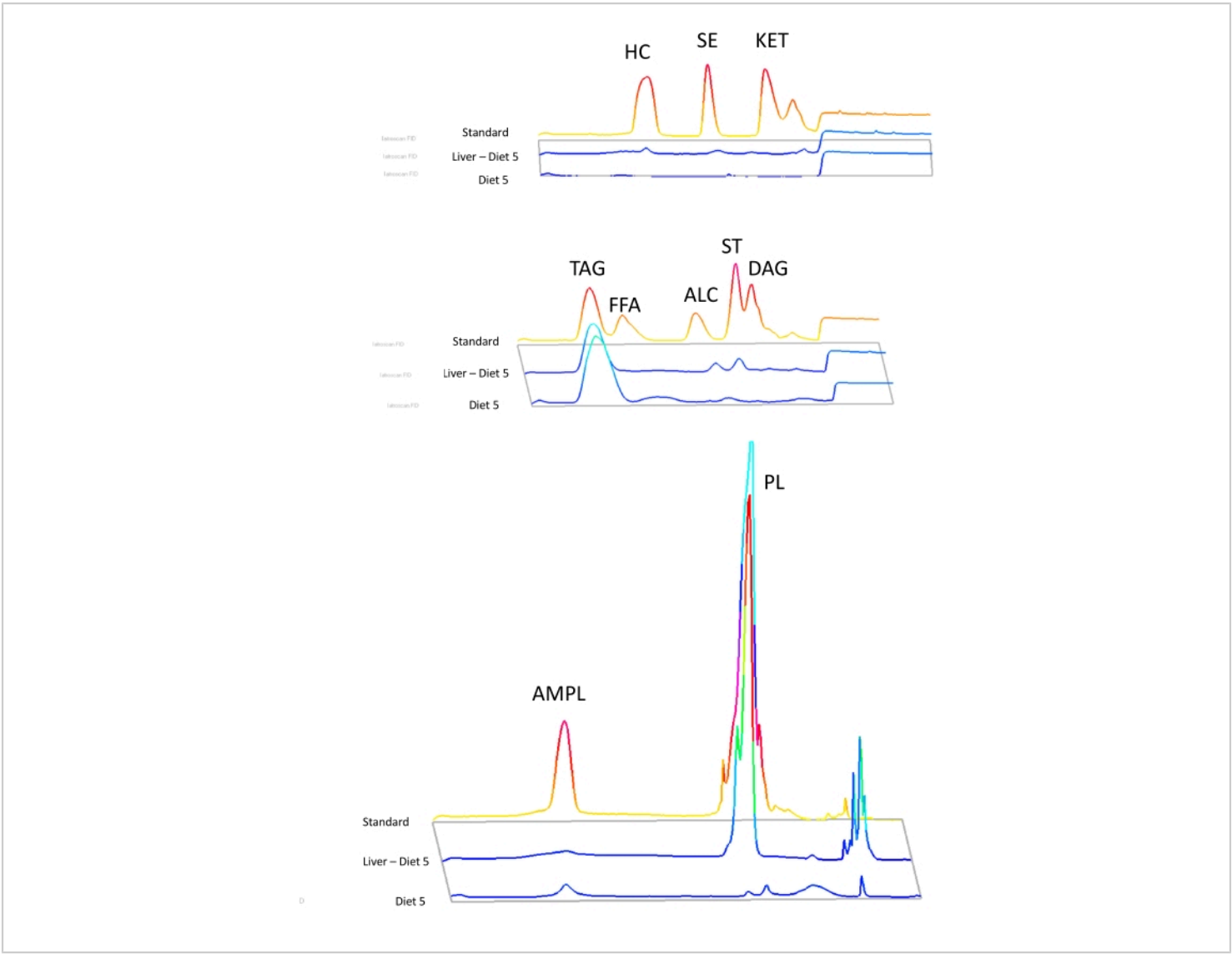

Figure 2: TLC-FID chromatograms of lipid composition from an aquaculture feeding experiment. Extracts were spotted on silica gel-coated TLC rods and a three-stage development system was used to separate lipid classes. The first and second development systems were hexane:diethyl ether:formic acid (98.95:1:0.05) and (79.9:20:0.1) respectively in order to separate neutral lipids including triacylglycerol, free fatty acid, and sterol for scanning in the automatic FID scanner. The third development systems consisted of 100% acetone prior to chloroform:methanol:water (5:4:1) in order to separate acetone-mobile polar lipids and phospholipids. Standard curves (i.e., nonadecane, cholesteryl palmitate, 3-hexdecanone, tripalmitin, palmitic acid, cetyl alcohol, cholesterol, monopalmitoyl glycerol, dipalmitoyl phosphatidylcholine) were used to quantify the lipid classes in the extracts using Peak Simple software (version 4.54). Please click here to view a larger version of this figure.

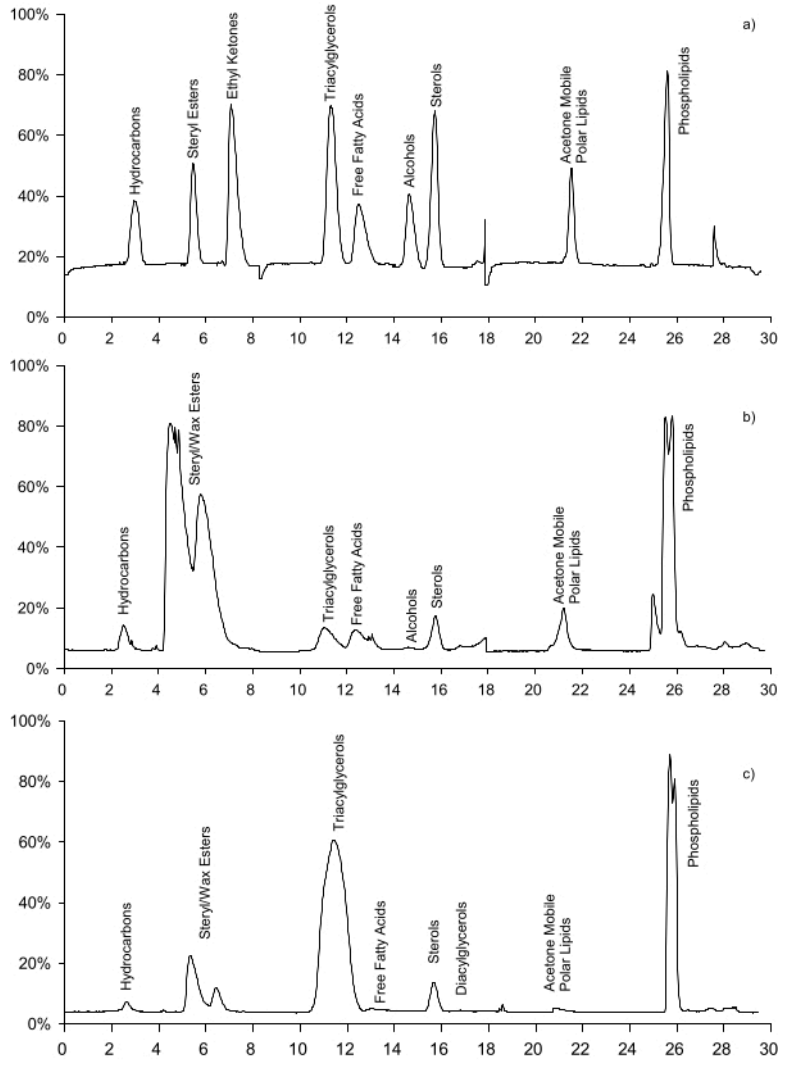

Figure 3: TLC-FID chromatograms of lipid composition of near-bottom samples from coastal Newfoundland. a) nine component standard, b) 220 m settling particulate matter from Conception Bay, Newfoundland, c) lipid classes in the mysid, *Erythrops erythrophtalma*. Please click here to view a larger version of this figure.

	Fish oil/rapeseed oil diet	Atlantic salmon liver
Hydrocarbons	1.3±0.9	0.5±0.2
Steryl Esters/Wax Esters	0.4±0.6	0.6±0.3
Ethyl Esters	0	0
Methyl Esters	0	0
Ethyl Ketones	0	0.3±0.2
Methyl Ketones	0	0
Glyceryl Ethers	0	0
Triacylglycerols	145.0±26.3	16.9±8.1
Free Fatty Acids	21.9±2.2	1.2±0.9
Alcohols	0	1.4±0.4
Sterols	6.8±2.1	2.6±0.2
Diacylglycerols	0	0
Acetone Mobile Polar Lipids	14.0±2.5	2.2±0.6
Phospholipids	12.5±4.0	22.0±2.0
Total Lipids	201.8±27.4	47.7±11.8

Table 1: Lipid composition in an aquaculture feeding experiment. Data are (mean±standard deviation) of an experimental diet containing 6.80% fish oil and 4.80% rapeseed oil, as fed (mg g^{-1} wet weight), and of livers of Atlantic salmon (mg g^{-1} wet weight) after feeding this diet for 12 weeks.

	Settling particulate matter	*Erythrops erythrophtalma*
Steryl Esters/Wax Esters (% total lipid)	10.2±8.28	8.85±1.67
Triacylglycerols (% total lipid)	19.7±5.35	58.5±9.19
Phospholipids (% total lipid)	16.2 ± 3.51	21.4±5.35
Neutral Lipids (% total lipid)	12.5±4.0	73.4±5.46
Lipolysis index (%)	18.1±5.20	2.77±2.78
Total Lipids	0.57±0.25	5.86±1.44
Neutral lipids: hydrocarbons, wax and steryl esters, ketones, triacylglycerols, free fatty acids; (FFA), alcohols (ALC), sterols, diacylglycerols; LI: lipolysis index [(FFA + ALC) (acyl lipids + ALC)$^{-1}$]; Total lipid (sum of TLC/FID determined lipid classes) particulate matter - % dry weight, Mysid - % wet weight		

Table 2: Lipid composition of near-bottom samples from coastal Newfoundland. Data are (mean±standard deviation) of 220 m settling particulate matter from Conception Bay Newfoundland, and of the mysid, *Erythrops erythrophtalma.*

Footnote: Neutral lipids: hydrocarbons, wax and steryl esters, ketones, triacylglycerols, free fatty acids; (FFA), alcohols (ALC), sterols, diacylglycerols; LI: lipolysis index [(FFA+ ALC) (acyl lipids + ALC)$^{-1}$]; Total lipid (sum of FID determined lipid classes) particulate matter - % dry weight, Mysid - % wet weight.

Discussion

The speed with which the TLC-FID system provides synoptic lipid class information from small samples makes TLC-FID an able tool for screening marine samples before undertaking more involved analytical procedures. Such analyses usually require release of component compounds from lipid extracts and derivatization to increase volatility in the case of gas chromatography. TLC-FID combined with GC-FID has been found to be a powerful combination for extracts of seafood and other foodstuffs[14]. For successful marine lipid analyses it is critical that samples are protected against degradation and contamination throughout and that great care is taken with the application of the sample to the rod. One approach is to apply the entire marine sample to the rod using a microcapillary pipettor[15], and an innovation in marine sample types is to add sea surface microlayer and aerosol samples to seawater samples[16].

The FID system in the automatic scanner provides rapid microgram quantitation without derivatization or clean-up; however, it is not as sensitive, precise or linear as found in gas chromatographs. This means that calibration curves have to be constructed, and that occasionally it may be necessary to analyse samples at two different loads in order to keep both smaller and larger lipid class peaks within calibration ranges.

By using the partial scan facility in the FID scanner, it is possible to separate multiple classes of lipids from a single sample application to a rod. However, chromatography on silicic acid fails to resolve wax esters (WE) and steryl esters (SE), and a few classes can be included in the "acetone-

mobile polar lipid" (AMPL) peak[17]. WE-SE was the major lipid class in bonefish oocytes and it is suggested they are used to support buoyancy and/or energy storage[18].

In AMPL from photosynthetic organisms, the glycoclycerolipids often elute together with monoacylglycerols and pigments in acetone. This may present a quantitation concern as chlorophyll *a* and glycolipids monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) have different FID responses in the scanner; however, we use, 1-monopalmitoyl glycerol as the standard for the AMPL class, and this has a response intermediate among them[17].

While some FID scanner peaks can contain more than one lipid class, it is sometimes useful to functionally regroup separated lipid classes. For example, AMPL and PL have been grouped into polar lipids and then into structural lipids with the addition of sterol[19]. Such groupings were used to study critical periods for lipid use during development in invertebrates[19]. Other groupings involving free fatty acids and alcohols can be used as degradation indicators such as the lipolysis index (**Table 2**) or the hydrolysis index[1]. LI is the lipolysis index of all acyl lipids while HI is the hydrolysis index of non-polar acyl lipids. LI values are always lower than those for HI for any sample because all acyl lipids are included.

Occasionally peak splitting occurs in rod separations of extracts of marine samples due to the presence of high levels of polyunsaturated species which can make identification difficult. This has been observed with wax esters (**Figure 3**), triacylglycerols and free fatty acids[20,21], and necessitates co-spotting with authentic standards and/or confirmation with other chromatographic techniques. Similarly, peak splitting may occur in the polar lipid region (**Figure 2** and **Figure 3**), and further developments may be undertaken to separate out component glycolipids and pigments[17,22] and phospholipid classes[22,23].

Disclosures

The authors have no competing financial interests.

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