APPENDICES

In the following procedures, all glassware must have Teflon-lined caps and both should be first solvent-rinsed with either $CHCl_3$ or methylene chloride before use. $CHCl_3$ is carcinogenic, thus all work should be carried out in a fume hood when used. We recommend the use of $CHCl_3$ in extraction procedures over the less-toxic methylene chloride until it can be verified that the superior lipid recoveries obtained using $CHCl_3$ can be duplicated with other solvents. See Table 1 for appropriate storage procedures. Below we assume samples are freshly collected and analyzed. If samples have been stored in $CHCl_3$ (Table 1), then corrections must be made for the amount of $CHCl_3$ already present. The addition of BHT as an antioxidant is necessary and it does not interfere with separation of lipid classes in the solvent system we have recommended for isolating fatty alcohols and DMA, nor in the GC analysis of FA if accounted for. However, its use may complicate some TLC applications that are not discussed here.

APPENDIX 1---EXTRACTION OF LIPIDS FROM FISH AND DETERMINATION OF FAT CONTENT

1. Homogenize an individual whole fish in a blender and accurately weigh an approximate 1.5 g aliquot and place it in a 125 mL glass jar.
2. Add 10 mL of MeOH containing 0.01% BHT, shake the mixture and let sit for 10 min. Add 20 mL of $CHCl_3$, shake the mixture again and let sit for several hours or overnight.
3. Filter the mixture through Whatman #1 filter paper into a 40 mL centrifuge tube. Rinse the jar twice with 2 mL of 2:1 $CHCl_3$/MeOH. Rinse the filter paper with the same solvent to bring the final volume to 33 mL.
4. Add 7 mL of 0.9% NaCl to the filtrate, cap the tube and shake for approximately 30 s.
5. Centrifuge the tube for 20 min at 100 × g. Two phases should be obvious. Remove the top phase and discard.

6. Collect the entire lower phase and filter through approximately 1 g of solvent-rinsed anhydrous Na_2SO_4. Rinse the tube and filter three times with approximately 1 mL of $CHCl_3$ each time and collect the filtrate in a second centrifuge tube.
7. Evaporate the solvent under a stream of nitrogen in a water bath at 25°--30°C. When only a few mL remain, transfer the sample to a pre-weighed 10 mL centrifuge tube with several rinsings of the first tube and continue evaporating until all solvent has evaporated.
8. To remove any residual solvent, place the sample under vacuum (established with a vacuum pump).
9. Accurately weigh the sample and add methylene chloride to make up a final concentration of 100 mg/mL.

APPENDIX 2---ISOLATION OF CHYLOMICRONS IN PLASMA OR SERUM

1. Overlay plasma or serum samples (3--4 mL) with a salt solution (0.196 M NaCl) so that there is a 1:1 v/v ratio of sample to saline.
2. Centrifuge the samples for 20 min at 15°C and 26,000 × g in an ultracentrifuge. Carefully withdraw the top layer containing chylomicrons.
3. Repeat the procedure twice with the same volume of saline and polyethylene glycol. Pool the top layers and store under nitrogen at −35°C until extraction.

APPENDIX 3---EXTRACTION OF LIPIDS FROM ADIPOSE TISSUE, BLUBBER, MILK AND CHYLOMICRONS

1. For a 1 g sample of adipose tissue and blubber, add 21 mL of 2:1 $CHCl_3$:MeOH containing 0.01% BHT and, using a glass rod, grind the blubber against the side of a 40 mL centrifuge tube until it is crushed and no longer releasing visible lipid. Cetacean blubber is normally so

structured that it cannot be crushed. In that situation, the blubber should be left to sit in the solvent overnight with occasional mixing. Add 5.2 mL of 0.9% NaCl and then shake and centrifuge the mixture, as above.

2. Recover the lower layer and transfer it to a second large centrifuge tube containing approximately one gram of anhydrous Na_2SO_4. Shake this and allow it to settle. Transfer the liquid with several rinses of the Na_2SO_4 to a third pre-weighed tube.
3. Evaporate the solvent, weigh the sample and make up to 100 mg/mL with methylene chloride as described above.

Note: For extraction of 1 mL of milk or chylomicrons, follow the same protocol but use 18 mL of $CHCl_3$: MeOH and 4 mL of 0.9% NaCl, and shake the mixture initially for 30 seconds. To dry and clean the lipid extract, filter through Na_2SO_4 as described for the fish extraction.

APPENDIX 4---FAME SYNTHESIS

Note: We recommend only using acidic transesterification procedures, as base-catalyzed procedures are subject to a number of problems. Although acidic boron trifluoride (BF_3) in MeOH has been used by many lipid labs, recently the companies providing it do not guarantee that it is anhydrous, which causes incomplete transesterification of samples. This results in inaccurate results and can ruin GC columns. We therefore no longer recommend its use.

1. Place 100 mg of lipid in a leak-proof 10 mL centrifuge tube and add 1.5 mL of methylene chloride and 3 mL of Hilditch reagent. The Hilditch reagent is prepared by dissolving 1.5 mL of concentrated H_2SO_4 in 100 mL of MeOH that has been dried over anhydrous Na_2SO_4. This reagent should be made up weekly or more often to ensure that it is anhydrous.
2. Flush the centrifuge tube with nitrogen, cap, and vortex.
3. Place in a heating block or oven at 100°C for 1 h.

4. Cool to room temperature and add 3 mL of hexane and 1 mL of distilled H_2O. Cap, vortex, and centrifuge for 2--5 min at 100 × g.
5. Remove the top layer to a second tube being careful to not collect any of the interface. Repeat this hexane extraction twice using 1 mL of hexane each time.
6. Add 2 mL of distilled H_2O to the pooled extract. Cap, vortex and centrifuge for 2 min.
7. Again remove the top layer to a third tube. Add approximately 0.5 g of anhydrous Na_2SO_4, cap, shake gently, and let settle.
8. Transfer the solvent to a pre-weighed tube and evaporate under nitrogen in water bath.
9. Weigh the tube and add hexane to the concentration needed for GC analysis, commonly 50 mg FAME per 1 mL hexane.
10. Flush with nitrogen, cap, and vortex.

APPENDIX 5---FABE SYNTHESIS (FOR ANALYSIS OF SAMPLES CONTAINING SHORT-CHAIN FA)

1. Place 50 mg of lipid or less in a leak-proof 10 mL centrifuge tube and add 1 mL of hexane and 1 mL of 10% BF_3 in butanol.
2. Flush with nitrogen, cap, and vortex.
3. Heat at 100˚C for 1 h.
4. Cool to room temperature and add 3 mL of distilled H_2O. Cap, vortex, and centrifuge for 2--5 min at 100 × g.
5. Remove the bottom layer and discard.
6. Repeat this aqueous wash two times using 3 mL of H_2O. Over time, butanol will degrade the GC column and the two H_2O washes are necessary to ensure all traces of butanol are removed.

7. With the last wash, do not discard the lower phase. Rather, remove the upper phase to a second tube and add a scoop of anhydrous Na_2SO_4, cap, shake gently, and let settle.
8. Transfer solvent to GC vial or storage vial.

APPENDIX 6---ISOLATION AND OXIDATION OF FATTY ALCOHOLS AND DMA

TLC

1. Activate general-purpose TLC plates (250 μm layer thickness, glass backed, 5 × 20 cm) by heating at 110°C for 1 h. Let the plates cool to room temperature in a desiccator.
2. Spread in a band approximately 10 mg of sample (15 mg maximum) in 100--200 μl of hexane approximately 2 cm from bottom of plate using 40 μl capillary tubes.
3. If only alcohols and FAME are to be separated, use a solution of 70:30:1 hexane: diethyl ether: acetic acid as the developing solvent. If DMA must be separated from FAME, use instead 100% toluene.
4. Dip the plate into a solution of the appropriate developing solvent approximately 0.5 cm deep. Let the solution run up the plate until the solvent reaches the top of the band. Dry under a stream of nitrogen. Repeat this focusing until a narrow band is created.
5. Place the plate in a developing chamber containing 100 mL of the same solution. Let the solution climb the plate until it almost reaches the top (30--60 min).
6. Remove plate from the tank and dry in a desiccator.
7. Spray with a 0.1% solution of 2,4-dichlorofluorescein in ethanol and view under UV light. Two bands should be present (or three if DMA are present in addition to alcohols). The upper band (which has migrated further) contains FAME. The lower band consists of alcohols. A third band just above the alcohols will contain DMA.

8. Outline all bands with a pencil. Remove the bands from the plate by carefully scraping the silica gel into a small vial using a funnel.
9. Add approximately 3 mL of 1:1 hexane: diethyl ether to extract the lipids from the silica. Shake and let sit for 10 min (while processing other samples). This vial may be centrifuged if it is necessary to make the silica settle.
10. Remove the solution, being careful not to disturb the silica. Repeat the extraction twice, using 3 mL of solvent each time.
11. Set aside the vial containing the FAME. If it was necessary to divide the sample into small portions for TLC separation, the fractions may be recombined at this point.

Oxidation

1. Evaporate the alcohol or DMA solution (containing less than 50 mg) to dryness. It is imperative that all traces of hexane and ether be removed.
2. Add 2 mL of acetone and 10 drops of oxidation reagent to the vial and vortex the mixture for 1 min. Let sit for 10 min and then vortex again for 1 min. A precipitate will form if saturated alcohols are present. The oxidation reagent is made by dissolving 13.5 g of CrO_3 in 50 mL of distilled H_2O and slowly adding 6.4 mL of concentrated H_2SO_4. The reaction is designed to oxidize approximately 80 mg of fatty alcohols with 10 drops (approximately 0.25 mL) of reagent. Volumes should be scaled up or down as necessary.
3. Add 1 mL of water, followed by 2 mL of hexane. The precipitate should dissolve upon adding hexane.
4. Collect the upper hexane layer and extract twice more with 2 mL of hexane.
5. Combine the hexane fractions, dry over anhydrous Na_2SO_4 (as in the FAME synthesis) and evaporate to dryness.
6. Add 1.5 mL of methylene chloride and synthesize FAME as described in Appendix 4.

7. Combine these FAME with the FAME set aside earlier and analyze by GC to obtain the effective fatty acid signature.

APPENDIX 7---OPERATING PARAMETERS FOR FAME ANALYSIS WITH GC-FID

The following descriptions assume the use of a polar capillary column, specifically a 30 m × 0.25 mm ID flexible fused silica column coated with 50% cyanopropyl polysiloxane (0.25 μm film thickness, DB-23, Agilent Technologies, U.S.A.).

Split Injection

A syringe is used to deliver 1 μl of a sample with a concentration of 50 mg/mL to an injector held at a constant temperature of 250°C. The helium split flow is set at a rate of 100 mL/min to generate a split ratio of approximately 1:100. The carrier gas flow rate (He) is 1 mL/min and the flow rates of air and hydrogen to the detector are 450 mL/min and 45 mL/min, respectively. The detector is held at 250°C and the oven temperature program begins at 153°C. It is held at that temperature for 2 min and then ramps at a rate of 2.3°C/min to 174°C. That temperature is maintained for 0.2 min and then ramped at 2.5°C to 210°C. This final temperature is held for 2 min or until just after 24:1 elutes. This program should produce a runtime of approximately 32 min.

Splitless Injection

Injection volume remains the same but a reduced sample concentration of 0.5 mg/mL is used. The temperatures of the injector and detector remain the same. Flow rates of gases to the column and detector are also unchanged. The split flow rate is set at 30 mL/min and the temperature program is modified as follows: the initial temperature is set at 50°C and held for 1 min. The temperature is then rapidly ramped at 45°C/min to 153°C. The program described above is then followed. This results in an approximate 3 min increase in the runtime.

APPENDIX 8---OPERATING PARAMETERS FOR FABE ANALYSIS WITH GC-FID

The following assume the use of a polar capillary column, specifically a 30 m × 0.25 mm ID flexible fused silica column coated with 50% cyanopropyl polysiloxane (0.25 μm film thickness; DB-23; Agilent Technologies, U.S.A.).

The same operating conditions are used as described above for split injection of FAME except the following temperature program is employed: the initial temperature of 65˚C is held for 2 min, and then ramps to 165˚C at a rate of 20˚C /min. It is maintained at this temperature for 0.4 min and then ramps at a rate of 2˚C/min to 215˚C. This temperature is held for 6.6 min and then ramps at a rate of 5˚C /min to 240˚C where the temperature is held for 1 min.

This program should produce a runtime of approximately 45 min.