Procedures for extraction and purification of leaf wax biomarkers from peats

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SUMMARY

Palaeoecological and palaeoclimate reconstruction, using leaf wax biomarkers, is a relatively new sub-discipline of peatland science. The ability to process large numbers of samples rapidly for biomarkers makes this type of analysis particularly appealing. This review is a guide to the preparation of leaf waxes for analysis by gas chromatography. The main phases of preparation are extraction of soluble organic compounds from sediment, separation of the total extract into fractions of differing polarity, and the derivatisation of polar functional groups. The procedures described here are not meant be exhaustive of all organic geochemical possibilities in peatlands, but a distillation of methods for the preparation of leaf waxes that are commonly and increasingly being used in palaeoecological and palaeoclimatological studies.

KEY WORDS: chemical fossils, lipids, palaeoclimate, palaeoecology, silica gel column chromatography.

1. INTRODUCTION

Peatlands provide a wealth of data for the reconstruction of palaeoclimates and palaeo-environments using the fossil remains of myriad organisms (Blackford 2001). While many types of micro- and macrofossils have been used for palaeoenvironmental reconstruction for decades, the use of chemical fossils for this purpose is a relatively recent development (e.g. Pancost *et al*. 2002). Biomarkers are compounds whose origin can be traced to a particular taxon. They are typically found in the solvent-soluble fraction of peatland sediments and have two principal origins, namely plants (e.g. leaf waxes) and microbes (e.g. membrane lipids). The procedures described here are mainly for the analysis of leaf wax biomarkers. The specificity of biomarkers can range from those representing large groups of organisms to those representing individual genera and species. For example, C_{29} *n*-alkane is used as a biomarker for all vascular plants and 5-*n*-alkylresorcinols indicate family *Cyperaceae* (Avsejs *et al*. 2002).

As for any proxy, biomarker reconstructions have benefits and limitations. The identification and quantification of biomarkers is automated and can be carried out relatively rapidly, placing high-resolution reconstructions closer within reach. An additional benefit is that fossil identification and quantification is standardised. Traditional micro- and macrofossil reconstructions rely heavily on the skill of the person making the fossil identifications. The use of analytical chemical instrumentation and techniques removes much of the inconsistency amongst human technicians. Data provided by analytical instruments provide biomarker abundance indices relating to environmental parameters (Chambers *et al*. 2011). The most important benefit of biomarker analysis is the ability to make compound-specific stable isotope measurements. The hydrogen and carbon isotope ratios of biomarkers are important tools for palaeoclimate reconstruction, and give specific insight into the hydrological and carbon cycles (e.g. Chikaraishi & Naraoka 2002). However, the library of specific organic biomarkers is relatively small for peatlands, and not all important plant taxa have had biomarker compounds assigned to them. Fortunately, the body of work on biomarkers in peatlands is growing, and as it does, so too will the power of biomarker proxy reconstructions.

The procedures described here are for the preparation of leaf wax biomarkers for analysis by gas chromatography. Preparation procedures are divided into three stages: extraction, separation, and derivatisation. Compounds must first be extracted from bulk sediment using organic solvents or solvent mixtures. The resulting total lipid extract is then separated into compound classes by column chromatography or solid phase extraction. Finally, each fraction is either derivatised to remove polar functional groups or purified farther by removing co-eluting compounds. Figure 1 gives a possible workflow diagram starting with the total lipid extract and ending with purified, analysable compounds.

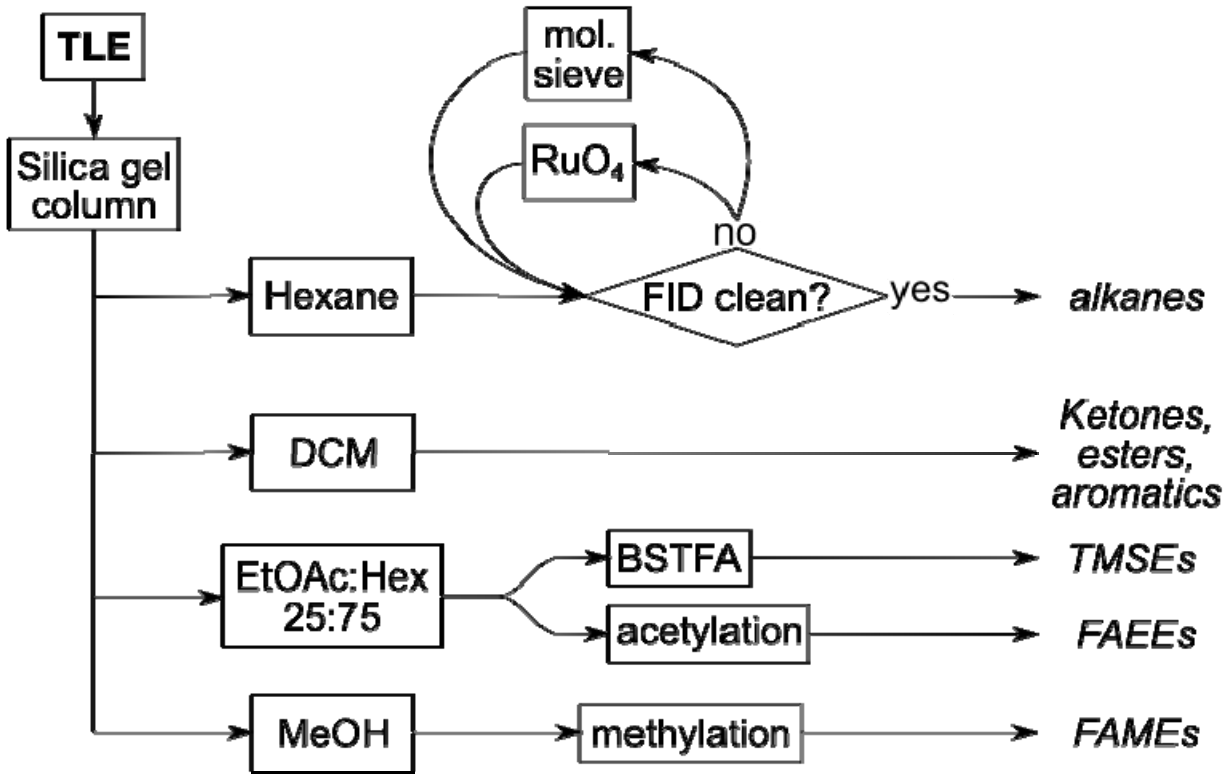

Figure 1. Workflow diagram for purification of the total lipid extract (TLE) into alkanes; ketones, esters, and aromatic compounds; trimethylsilyl ethers (TMSEs) or (fatty alcohol ethyl esters (FAEEs); and fatty acid methyl esters (FAMEs). Each fraction eluted from the silica gel column is noted by the solvent used. The question, "FID clean?" refers to the presence or absence of co-eluting compounds visible in a chromatogram from a GC with flame ionisation detector (FID).

2. HANDLING PEAT SAMPLES AND SUPPLIES FOR ORGANIC ANALYSIS

The goal of sampling and storage techniques is to minimise degradation and contamination of target compounds. Before sampling, peat cores should be kept in cold storage (~4 °C). Samples of cores should be taken using metal tools cleaned with deionised water and a solvent such as acetone to remove organic contaminants. If a solvent is used for cleaning sampling tools, care must be taken to keep the solvent away from any plastic in which samples are stored (e.g. US 'whirl-pak' bags), as plasticisers and other contaminants from these are easily transferred with solvents to the sample. All glassware should be baked in a muffle furnace at 480 °C overnight before use, to remove any organic contaminants. Vials and caps should be handled carefully with forceps whenever possible. Caps should have (solvent resistant) fluoropolymer liners. Remember that nitrile or other laboratory gloves are worn to protect the skin from chemicals, but do not protect the samples from contamination.

3. EXTRACTION OF SOLUBLE LIPIDS

Typically, soluble lipids can be identified and quantified from an extract of 1–2 cm^3 of peat. However, samples of 3–4 cm^3 or larger will yield a more favourable concentration of compounds for stable isotope analysis of lipids. Once subsamples are taken from a core, they should be kept frozen (-20 °C). In preparation for lipid extraction, peat samples must be freeze-dried. Typically, lipids are extracted from lacustrine or marine sediments by Soxhlet, or an automated method such as an Accelerated Solvent Extractor (developed by Dionex). Because they typically lack a mineral component, peatland sediments can be treated like fresh leaf samples; lipids can be extracted by three successive solvent rinses with ultrasonic agitation (e.g. Hou *et al*. 2008). For each rinse, add 10–20 ml of an appropriate solvent or solvent mixture to the freeze-dried peat sample in a 40 ml I-Chem vial or similar vessel, and sonicate for 30 minutes (Nichols *et al*. 2010). The extraction solvent or solvent mixture should approximately match the polarity of

the target compounds. For example, if hydrocarbons are the target, hexane could be used (Nichols *et al*. 2010). For fatty acids or other more polar compounds, a mixture such as 2 : 1 (by volume) dichloromethane : methanol may be appropriate (Hou *et al*. 2008). Combine decanted solvent from all three rinses for the total lipid extract (TLE), and evaporate the solvent under nitrogen.

4. COLUMN CHROMATOGRAPHY

4.1 Silica gel separation

Before analysis by gas chromatography, compounds in the total lipid extract (TLE) are first separated into classes by polarity. This is accomplished by silica gel column chromatography. Chromatographic separation is based on the relative attraction of analyte compounds to either the stationary phase that makes up the column (in this case silica gel) or the mobile phase passing through the column (in this case a series of solvents and solvent mixtures of increasing polarity). Appropriate columns can be hand-packed in Pasteur pipettes. When packing by hand, first insert a small amount of activated quartz (glass) wool from the top of the pipette, then pack 60Å activated silica gel into the column such that there is approximately 5 cm of silica above the glass wool (Figure 2). To activate silica gel, and to keep it active, store both the stock silica gel and packed columns in a drying oven at 50 °C. Any water will interfere with the efficiency of the stationary phase.

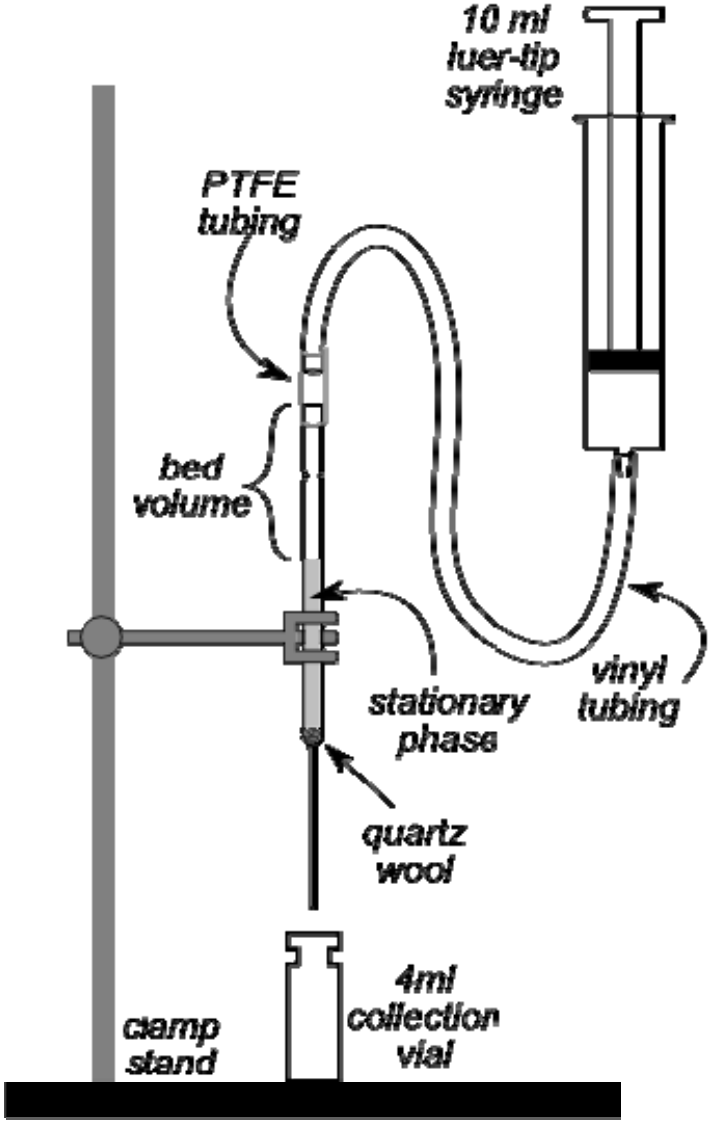

Figure 2. A typical setup for column chromatography. The stationary phase can be active silica gel, aminopropyl material, or zeolite molecular sieve. The space in the pipette above the stationary phase is referred to as the "bed volume".

The solvent scheme for the elution of different compound classes can vary based on the target compounds. A typical elution scheme is described here. Just before adding sample, clean the flash column with 4 ml of each of the solvents used for elution in reverse order. In the Pasteur pipette columns described above, 4 ml is approximately three times the bed volume (the volume inside the pipette above the stationary phase). For added efficiency, solvent can be added to the column using 500 ml PTFE squirt bottles. In this example, the column would be cleaned first with methanol (most polar), then with DCM (moderately polar), and finally hexane (least polar). After cleaning, the total extract is loaded with hexane onto the column. After the extraction solvent has been completely evaporated, transfer the sample onto the column with the smallest volume possible, while still making sure that none of the sample is left in the TLE vial (approximately 0.5 ml). Once the sample has been loaded, hydrocarbons are eluted with 4 ml (about 3 bed volumes) of hexane; aromatic, ketone, and ester compounds are eluted with 4 ml of dichloromethane; optionally, alcohol compounds are eluted with 4 ml of 1 : 3 ethyl acetate : hexane; and the remaining compounds, including those with carboxylic acid groups, are eluted with 4 ml of methanol. If alcohol compounds are not eluted separately, they will elute in the methanol fraction.

4.2 Separating fatty acids

Although fatty acids can be recovered in the methanol fraction described above, acids can be separated from the neutral compounds before silica gel chromatography. This separation can be accomplished using an aminopropyl solid phase extraction (SPE) column. Prepare the SPE column similarly to the silica gel column: pack the pasteur pipette with LC-NH_2 SPE packing material. Pre-clean the column with three bed volumes of a 12 : 1 solution of dichloromethane and isopropyl alcohol (DCM : IPA). Load the TLE onto the SPE column with a small volume of 12 : 1 DCM : IPA. Elute the neutral fraction with 4 ml of this same DCM : IPA solution. Acids will remain bonded to the aminopropyl material. Release these compounds by flushing the column with 4 ml of 4 % acetic acid in ethyl ether.

4.3 Increasing productivity

If compounds are allowed to elute from flash columns by gravity alone, as is typical in an organic chemistry laboratory, each analysis may take hours. To reduce the analysis time, a device can be assembled to force the mobile phase through the column. Parts for this device include a disposable 10 ml luer-tip syringe, about 40 cm of vinyl tubing, and 2–3 cm of fluoropolymer tubing (Figure 2). Connect one end of the vinyl tubing to the syringe and insert the other end inside the fluoropolymer tubing. The fit should be snug. Stretch the fluoropolymer tip of the completed device over the top of a Pasteur pipette to mould it to shape. To use the device, fit the fluoropolymer tip over the top of the flash column after adding mobile phase. While holding the tip onto the top of the column, depress and hold the plunger on the syringe until the mobile phase reaches the top of the stationary phase. Be sure not to allow air into the stationary phase. The next volume of solvent can now be added.

One of the advantages of using biomarkers is the ability to analyse large numbers of samples rapidly. To increase productivity, a simple rack can be constructed to perform many column chromatographic separations simultaneously. An example is shown in Figure 3. Nested holes are drilled in a piece of 2×2 cm fluoropolymer or aluminium stock. The inner hole allows the point of the pipette to fit through, while the outer hole penetrates only halfway through the stock to accommodate the diameter of the pipette without allowing it to slip down. The stock can be suspended by two clamp stands above a tube rack holding the elution vials.

5. HEXANE FRACTION

Hydrocarbons without functional groups elute in the hexane fraction of the chromatographic scheme described above. This fraction is dominated by saturated hydrocarbon leaf waxes. These are generally odd-carbon-numbered from 19 to 33 carbons. The vegetation type comprising the peat determines the distribution of these compounds among the different homologues. For example, the most abundant leaf wax hydrocarbon of *Sphagnum* is C_{23}; while the most abundant in shrubs, trees, grasses and sedges is either C_{29} or C_{31} (Baas *et al*. 2000, Nichols *et al*. 2006, Pancost *et al*. 2002, Bingham *et al*. 2010). In typical analyses, these leaf waxes are the target compounds; however, when analysed by gas chromatography, these important biomarkers can co-elute with other hydrocarbons such as hopanes or other bacterial compounds. Leaf wax hydrocarbons can be further purified in two ways. Ruthenium tetroxide oxidation can be used to remove unsaturated compounds (destructively), or a molecular sieve can be used to separate branched from unbranched compounds (non-destructively). Ruthenium (VIII) oxide reacts with double bonds to form carboxylic acids, which can be removed with

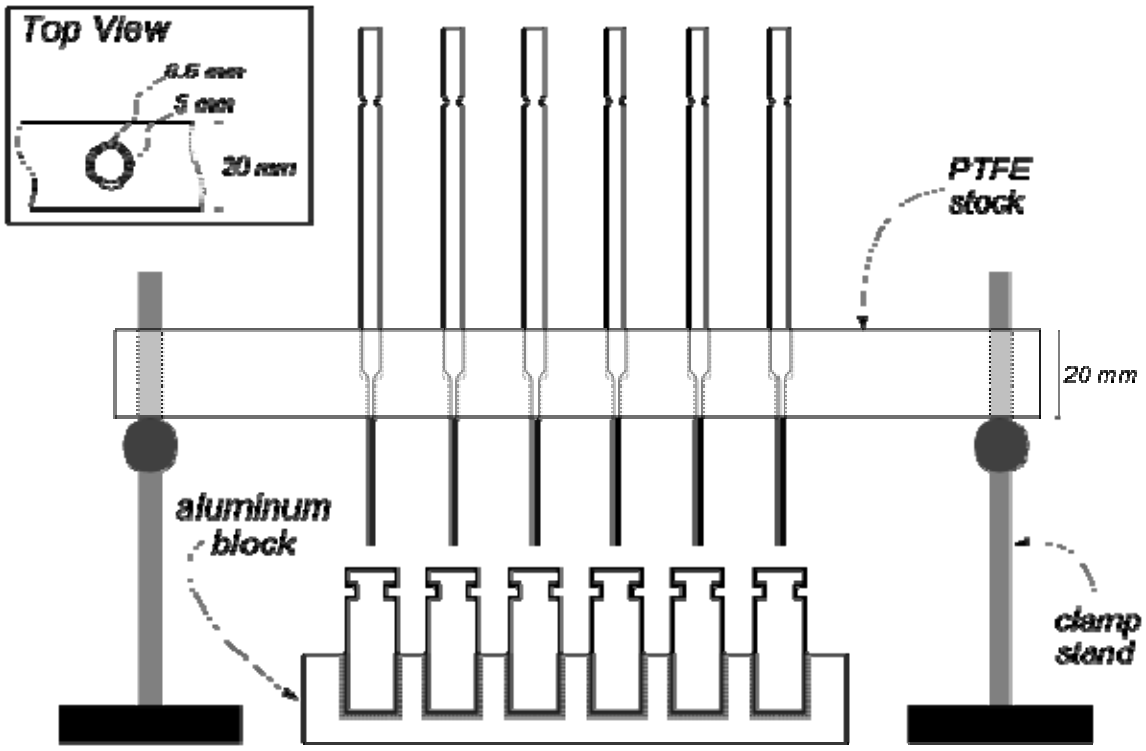

Figure 3. Suggested setup of a multiple-column rack for performing several flash column separations simultaneously.

farther silica gel column chromatography. Zeolite molecular sieves physically separate branched and unbranched hydrocarbons.

5.1 Ruthenium tetroxide oxidation

Occasionally, leaf wax *n*-alkane samples can contain unsaturated compounds that co-elute with the target saturated leaf waxes. These can be removed from the sample by oxidising them with ruthenium tetroxide (RuO_4), which will oxidise the double bonds into carboxylic acid groups, allowing them to be separated from the saturated compounds, which remain unchanged. Ruthenium tetroxide is a highly unstable reagent and must be prepared immediately before use. To prepare 2 ml of RuO_4 solution, add 1 ml of carbon tetrachloride, 1.5 ml of acetonitrile and 2 ml of organics-free water to a screw-top test tube. Weigh out 5 mg of RuO_2 and 50 mg of $NaIO_4$. When ready to perform the oxidation and all solvent has been evaporated from the samples, add the RuO_2 and $NaIO_4$ to the tube and shake vigorously until a yellow-green solution appears. There will be two phases in the tube, the yellow-green organic phase in the bottom of the tube and the water phase above. Use a Pasteur pipette to add the yellow-green RuO_4 solution to the alkane samples after all solvent has been evaporated. As the RuO_4 is consumed, the solution in the sample vial will lose its colour. Continue adding RuO_4 solution to the sample dropwise until the yellow-green colour persists. When completed, saturated compounds can be separated from the newly formed carboxylic acids by eluting them from a silica gel column with hexane, as described above (Huang *et al*. 1999).

5.2 Zeolite sieving

Straight-chained compounds can be separated from branched and cyclic compounds non-destructively using a zeolite molecular sieve. Pack a Pasteur pipette column with zeolite molecular sieve adsorbent. After loading the hexane fraction onto the column, elute the branched and cyclic compounds with 4 ml of hexane. The straight-chained compounds will remain adsorbed. Allow all the solvent to evaporate from the sieve material overnight. To release the straight-chain compounds, place the sieve material in a fluoropolymer (e.g. Teflon) tube and add, dropwise, concentrated hydrofluoric acid (48 %) to dissolve the zeolite. For this procedure, the tube must be resistant to both HF and organic solvents. The alkanes can now be extracted from the solution with three rinses of 1 ml of hexane. Add the hexane to the fluoropolymer tube, agitate, and carefully pipette out the hexane into a new container (McDuffee *et al*. 2004).

Because hydrofluoric acid is a potentially

hazardous reagent, alternative desorption methods have been sought. For example, unbranched compounds can also be desorbed by heating the dried sieve material to 80 ºC in a 12 % solution of cyclohexane in *n*-pentane for eight hours (Grice *et al*. 2008). This method is typically used for petroleum samples, and has not been widely tested in samples with small concentrations of leaf waxes. It is prudent to test the recovery of the target compounds in concentrations close to those of the samples before using this method with samples of unknown concentration.

6. DICHLOROMETHANE FRACTION

Ketones, esters and aromatic compounds elute in the DCM fraction. No further procedures are necessary before GC injection. This fraction contains many triterpenoid compounds, which may be more specific biomarkers than hydrocarbons. Depending on the compound desired for analysis, ruthenium tetroxide oxidation may be appropriate. For example, if the target compounds are *n*-alkan-2-ones, co-eluting compounds can be removed by this method (Nichols & Huang 2007).

7. ALCOHOL FRACTION

Sterols and fatty alcohols elute in the 1:3, Ethyl Acetate:dichloromethane fraction. Because alcohol groups are too polar for normal phase gas chromatography, these groups must be derivatised before analysis. There are two methods for derivatisation, namely BSTFA (bis (trimethylsilyl) trifluoroacetamide) and acetylation. BSTFA derivatisation replaces alcohol groups with trimethylsilyl ethers (TMSEs), and acetylation replaces alcohol groups with ethyl esters. BSTFA derivatisation can be preferable because this procedure will derivatise not only alcohols, but also acids and amines. However, TMSEs are unstable and do not persist for more than a few days. Ethyl esters produced by acetylation are stable indefinitely, but the acetylation reaction is effective on alcohol groups only.

7.1 BSTFA derivatisation

Lipid alcohol groups are derivatised with bis (trimethylsilyl) trifluoroacetamide (BSTFA). To accomplish this, 30 µL of BSTFA and 40 µL of pyridine (a catalyst) are added to the sample. Before capping the vial, the headspace air is replaced with pure N_2 (commercial grade is acceptable) to prevent oxidation. The vial is then heated at 80 °C for two hours or 60 °C for six hours. When it is cool, the sample is ready to analyse (Huang *et al*. 1994, Pancost *et al*. 2002). However, because of the unpleasant smell of pyridine and its damaging effect on GC columns, it is preferable to evaporate the solvent and replace it with toluene before analysis. The result of this procedure is that the alcohol groups are replaced with trimethylsilyl ethers: $(CH_3)_3Si{-}O{-}R$.

7.2 Acetylation

Begin by completely evaporating any solvent in the alcohol fraction. Add to the vial a 1:1 (by volume) solution of acetic anhydride and anhydrous pyridine. Evacuate the air in the headspace of the vial with N_2 and cap tightly. Heat the vial in a block heater at 70 °C overnight (Sauer *et al*. 2001). Cool the vial to room temperature. The resulting ethyl esters are ready for GC injection; however, as in BSTFA derivatisation, it is preferable to evaporate the pyridine and redissolve the sample in a different solvent, such as toluene, before analysis.

8. FATTY ACIDS

The methanol fraction contains a wide variety of polar compounds. If not eluted separately, both alcohols and acids will be present. Before analysis by gas chromatography, carboxylic acids must be esterified by heating with acidified methanol. The resulting methyl esters can be injected directly onto a normal phase GC column. To prepare acidified methanol, slowly introduce acetyl chloride to GC grade methanol (5:95 by volume). It is prudent to chill the methanol before this preparation, because the reaction is exothermic and may be vigorous. For each sample of fatty acids dissolved in about 0.3 ml of toluene, 1 ml of acidified methanol should be added. The mixture is heated at 60 °C for 12 hours, then the methyl esters are separated from the polar components of the mixture by water and hexane extraction. After the sample has cooled, add approximately 1.5 ml of organics-free 5 % NaCl solution and approximately 0.5 ml of hexane. Shake the vial vigorously using a vortex stirrer. When the hexane separates at the top of the vial, carefully pipette the hexane, which has now dissolved the methyl esters, into a new vial. Repeat the extraction twice more by adding additional volumes of hexane to the original sample. The methyl esters may require additional cleanup by elution from a silica gel flash column (similar to the one used for the original separation). Elute unwanted compounds with 4 ml of hexane and the methyl esters with 4 ml of dichloromethane. For compound-specific isotope

analysis, the carbon and hydrogen isotope values for the methanol used in methylation should be known so that the sample can be corrected for the added methyl group (Hou *et al.* 2007).

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