Get Everything Together

Wash glassware in PO_4-free detergent; muffle @ 450C,4hr (Peacock et al 2001)

2 bottles of N_2
Acetone (Fisher # A929-4)
Chloroform (Fisher # C297-4)
Hexane (Fisher # H303-4)
Methanol (Fisher # A454-4)
Muffled borosilicate Pasteur pipettes, 9", 7 per sample
One disposable 25 mL glass pipette per sample (Fisher #13-678-36D)
Two muffled 50 mL borosilicate glass centrifuge tubes per sample
One muffled 250 mL separatory funnel per sample
Three 16 x 100 mm muffled screw-cap tubes per sample
One 16 x 125 mm screw-cap tube per sample
One silicic acid chromatography column per sample (VWR cat # BJ5054)
White quartz sand, 50-70 mesh (Sigma cat # S-9887) for extraction blank
Soil of choice for positive control standard
gc vials (Supelco cat # 27066-U or VWR # HP-5181-3376)
caps (Supelco cat # 33233-U or VWR # 66011-200)
100 µL inserts for gc vials (Alltech cat # 98031)
Surrogate standard: Prepare fresh for each batch. Syringe 40 µL of di-19:0 PC (Avanti #850367C; 25 mg/mL) into a muffled TOC vial. Gravimetrically bring to 10 mL final volume in $CHCl_3$ (15.0 g $CHCl_3$).
Phosphate buffer: Dissolve 8.7 g K_2HPO_4 in Milli-Q water, and bring to 1000 mL volume. Acidify to pH 7.4. Add in about 20 mL of $CHCl_3$ to keep anything from growing in it.
0.2 *M* methanolic KOH: Using acetone-washed tweezers, quickly weigh two pellets of KOH, rinse them with a few drops of chloroform and drop them into a muffled TOC tube. KOH is hydroscopic and water will interfere with the methylation, hence the need for speed. Tare the TOC tube with the KOH pellets, and add enough methanol (**Lot # 010771**) to achieve a ratio of 0.28 g KOH : 25 mL methanol. Swirl to dissolve. (Shelf-life 6 months)
Acetic acid, 1.0 *M*: Add 5.8 mL concentrated acetic acid to water, and bring to 100 mL final volume.
Internal standard: (Prepare immediately before reconstituting samples for GC analysis.) Measure approx. 15 mg (but record the exact weight!) of 10:0 FAME (methyl decanoate, Aldrich cat # 299030-2.5G) into a muffled 100 mL volumetric flask, and bring to volume with hexane. This has a very short shelf life; do not keep it longer than a day or two.
Pipet-Aid
Vortexer

Extraction of Soil Lipids

Preparation

- Glassware needed:
  - One 250 mL separatory funnel per sample
  - Two 50 mL muffled centrifuge tubes per sample
  - One muffled Pasteur pipette per sample
  - One disposable 25 mL glass pipette per sample
- Tighten the nuts on the stopcocks of the separatory funnels (so they won't leak).

Procedure

1. Label a set of 50 mL tubes (green tape).
2. Using the dedicated syringe, add 0.5 mL of surrogate standard solution to each sample (usually 8.00 g of soil). Get as much of the liquid into the soil (as opposed to on the walls of the tube) as possible. Rinse syringe with chloroform 3 times.
3. Using the repipets, add 12 mL methanol, 6 mL chloroform, then 5 mL phosphate buffer to each sample and vortex. Vent after vortexing.
4. Sonicate samples for 15 minutes.
5. Centrifuge samples for 3 minutes at 1000 rpm, room temperature is fine.
6. Using a disposable 25 mL glass pipette and a Pipet-Aid, transfer extractant from each sample into separatory funnels.
7. Repeat steps 3 – 6 two more times.
8. Add 18 mL $CHCl_3$ and 17 mL Milli-Q water to each sep funnel to break phase. Shake each funnel for 2 minutes. Let stand overnight.
9. After phase separation, drain lower phase into clean 50 mL centrifuge tubes.
10. Dry at 37-40º in the N-EVAP. Samples can either be stored in the freezer or moved directly to the next step of silicic acid chromatography.

Silicic Acid Chromatography

Preparation

- Glassware needed:
  - One 16 x 125 mm tube per sample to collect waste solvents
  - One 16 x 100 mm muffled tube per sample to collect the polar lipid fraction
  - One small muffled beaker for holding chloroform.
  - Two muffled 9" Pasteur pipettes per sample
- Place the needed number of chromatography columns into a 120° - 130° oven for at least one hour (manufacturer recommends 3 to 6 hours).
- Switch on the water bath on the N-EVAP (37C). Install clean Pasteur pipettes. Fill the water bath to the correct level if necessary.
- Set the acetone and chloroform repipets to 5 mL, and methanol repipet to 2.5 mL.
- Remove samples from freezer and allow to rise to room temperature.

Procedure

Note: Use syringe and rubber stopper to push the solvents through the chromatography columns. It is important that the columns are not allowed to become dry once the procedure has started.

1. Place silicic acid columns on chromatography rack and allow to cool.
2. Condition each column with 5 mL acetone x 2 followed by 5 mL chloroform x 2. You'll need to stop after the first rinse of chloroform to pour out the solvents in the waste tubes.
3. Add about 150 μL of chloroform to each sample and swirl to dissolve total lipids.
4. Load the column with a Pasteur pipette, being careful to drip the liquid directly into the center of the column. Use a new pipette for each sample.
5. Repeat steps 3 and 4 two more times for each sample.
6. Set chloroform and acetone repipets to 2.5 mL.
7. Elute with 2.5 mL of chloroform twice to remove the neutral lipid fraction.
8. Elute with 2.5 mL of acetone twice to remove the glycolipid fraction.
9. Switch to the labeled, muffled 16 x 100 mm screw-cap tubes.
10. Elute with 2.5 mL methanol x 4.
11. Evaporate the solvents from the polar lipids in the N-EVAP, 37°C. Store frozen.

Methylation of Polar Lipids

Glassware needed

- 2 Pasteur pipette per sample
- 1 muffled 16 x 100 mm tube per sample

Procedure

1 . Label a set of small tubes (orange tape) before putting on gloves. Turn on N-EVAP heat unit and set temperature at 37º. Remove samples from freezer. Allow these to thaw.
2. Add 0.5 mL chloroform and 0.5 mL methanol (**Lot #010771**) to each sample.
3. Attach a Pastuer pipette to a 1000 µL Rainin pipetter and add 1.0 mL methanolic KOH to each sample. Cap tubes TIGHTLY. Swirl to mix.
4. Place samples in 37º C N-EVAP bath for 30 minutes. Leave caps on. Remove and allow to cool.
5. Add 2 mL hexane to each sample and swirl to mix.
6. Using the Rainin pipetter, add 200 µL of 1 *N* acetic acid to each sample. Swirl to mix. You will start to see phase separation taking place.
7. Set Milli-Q repipetter to 2.0 mL.
8. Add 2 mL Milli-Q water to each sample to break phase.
9. Vortex samples for 30 seconds.
10. Centrifuge samples for 5 minutes @ 2000 rpm.
11. Using a separate Pasteur pipette for each sample, transfer the top phase to a clean tube. Be careful not to take up any of the lower phase. Save these pipettes (you'll be using them again) by placing them in order on the foil strip with tips upright in the hood.
12. Re-extract the samples with 2 mL of hexane twice, following the same procedures as laid out in steps 9-11.
13. Evaporate the solvent in the N-EVAP at room T. Store samples in freezer.

Analysis

On the day of analysis, reconstitute by resuspending the FAMEs in 50 µL of the internal standard and transferring to the gc vials with inserts.

Preparation of Phosphate Buffer

Muffled glassware needed: 1000-mL volumetric flask, 1000-mL beaker, 1000-mL vacuum flask, brown storage bottle (might need 2), graduated cylinder (to measure chloroform into storage bottle) OR, INSTEAD OF FILTERING, MAKE UP FRESH BUFFER FOR EACH EXPERIMENT.

Sonicated in acetone-. filter tweezers, two stir bars

Calibrate a 1000-mL volumetric flask gravimetrically. Muffle the flask. Weigh 8.7 g of Potassium Phosphate Dibasic into it. Add in enough of the nanopure water to fill the bulb of the flask and swirl until all the powder dissolves. Once it's all dissolved, top up with "nanopure" water to the mark. Pour into a large beaker for the titration and add a clean (sonicated in acetone) stir bar. Standardize the pH meter (see below) then titrate the buffer with 3M HCl to a pH of 7.4. Be careful not to overshoot; that said, a pH of 7.39 is acceptable.The 3M HCl is in a small glass bottle under the hood in the room with the pH meter. (Note: when needing to make more of this, be sure to use nanopure water and a muffled bottle.) Prepare the microfilter apparatus by inserting the funnel part tightly into the top of a muffled vacuum flask, placing a piece of Millipore filter paper on top, then clamping the glass reservoir tightly onto the funnel. When preparing the vacuum apparatus, use the special tweezers in the drawer behind the hood to handle the filter paper. Acetone rinse the tweezers, but don't acetone rinse the **white** (not blue) Millipore filter paper!! When inserting the funnel, be sure that the long edge of the neck is flush to the vacuum connection of the flask. This will help to prevent liquid from being sucked into the pump. Connect the flask to the pump and turn it on. Check for leaks by filtering a small amount of buffer, and then, assuming all looks good, filter the buffer. Pour the buffer into a muffled brown storage bottle, which has 50 mL of chloroform already added, and add the stir bar from the beaker. Stir the buffer vigorously to mix the chloroform thoroughly into it, then let sit overnight before using. There might be more buffer than will fit in the bottle; any excess can be stored in a different brown bottle until needed. Be sure after adding this excess buffer to the original bottle that you chloroform extract again by placing on a stir plate.

The Millipore filter apparatus should be rinsed ten times in Di, allowed to dry, wrapped in foil, and stored in the cupboard.