Phase I: Extraction of Soil Lipids

Preparation
Calibrate the methanol repipetter to dispense 10 ml
Calibrate the chloroform repipetter to dispense 5 ml

Consumable supplies and glassware
All glass must be very clean (i.e., 4 hrs @ 450 °C)
2 beakers (150 ml)
2 beakers (50 ml)
Each sample needs:
2 large (50 ml) test tubes – blue-labeled
1 small (15 ml) test tube – green-labeled
2 Pasteur pipettes
4 ml of phosphate buffer
5 ml nanopure water
250 µl internal standard

Procedure
1. Add 5 g of freeze-dried soil to large test tube and label with blue tape.
2. Add 4 ml phosphate buffer.
3. With the repipetter, add 10 ml methanol followed by 5 ml chloroform.
4. Add 250 µl internal standard 19:0.
5. Vortex each sample briefly and vent.
6. Sonicate samples for two minutes, and then set for three hours.
7. Centrifuge samples for 10 minutes @ 3000 rpm.
8. Label second set of large test tubes with blue tape.
9. Pour clear supernatant into second set of large test tubes.
10. Add 5 ml chloroform to first test tube, vortex, & centrifuge for 10 min @ 3000 rpm.
11. Decant supernatant into second set of large test tubes.
12. Add 5 ml nanopure water to second set of large test tubes.
13. Vortex each sample briefly and vent.
14. Allow mixture to sit overnight (best) or proceed to step 15.

Day Two: Turn on water bath (37°C) for the N-EVAP. Make sure the bath is nearly full with distilled water.
15. Centrifuge samples for 10 minutes @ 3000 rpm.
16. With the pasture pipette, transfer the bottom organic phase for each sample into small test tube. To prevent uptake of unwanted material, blow small bubbles through the pipette while passing through the top phase.
17. Evaporate the solvent in the N-EVAP until standing liquid is gone, but still looks wet. Process will take ~30 minutes.
18. Store samples at -20°C.
Phase II: Silicic Acid Chromatography

Preparation
Calibrate the chloroform and methanol repipetter to dispense 2.5 ml
Turn on water bath (37°C) for the N-EVAP. Make sure the bath is full with distilled water.

Consumable supplies & glassware
A set (i.e., 6) waste test tubes for unwanted lipid fraction
1 beaker (50 ml)

Each sample:
1 SPE chromatography column
1 small (15 ml) test tubes – yellow-labeled
1 pasture pipette

Procedure
Note: In order for the chromatography columns to work, they must not dry out. Keep the columns in contact with an organic solvent at all times.

1. Remove samples from freezer and while still cold, add ~150 μl chloroform.
2. Place SPE columns on vacuum manifold.
3. Condition each column with ~10 ml acetone then with ~10 ml chloroform.
4. Load the column with a Pasteur pipette. Drip the sample directly into the center of the column.
5. Add another 150 μl of chloroform to the green-labeled test tube and load that into the column.
6. Add another 150 μl of chloroform to the green-labeled test tube and swirl the chloroform to get lipids from the side of the test tube. Load that into the column.
7. Elute column with 2.5 ml chloroform twice to remove the neutral lipid fraction.
8. Elute column with 2.5 ml acetone twice to remove the glycolipid fraction.
9. Switch to the yellow-labeled test tubes and elute with 2.5 ml methanol four times.
10. Evaporate the solvent for the polar lipids in the N-EVAP until standing liquid is gone, but still looks wet. Process will take 2 hours.
11. Store samples at -20°C.
Phase III: Methylation of Polar Lipids

**Preparation**
Turn on water bath (60°C). Make sure the bath is full.

**Consumable supplies & glassware**
3 beakers (100 ml) - methanolic KOH, hexane, water
5 beakers (50 ml) - methanol, chloroform, acetic acid, cleaning, hexane-MTBE

Each sample:
- 1 small (15 ml) test tubes – orange-labeled
- 4 pasture pipette

**Reagents**
0.2 M methanolic KOH
10 ml of methanol in a 100 ml beaker
Quickly weigh 2-3 pellets of KOH and record weight
Immediately add pellets to the methanol
Add enough methanol to achieve the correct concentration (0.28 g KOH : 25 ml methanol)
Sonicate to dissolve. Shelf life is 6 months, so make enough for the whole session.

**Procedure**
1. Remove samples from freezer and while still cold, add **500 µl chloroform** and **500 µl methanol**.
2. Add **1 ml methanolic KOH**, then vortex briefly.
3. Place samples in the 60°C water bath for 30 minutes; allow samples to cool before the next step.
4. Add **2 ml hexane** and vortex briefly.
5. Add **200 µl 1N acetic acid** and swirl to mix.
6. Add **2 ml nanopure water** to break phase.
7. Vortex samples for 30 seconds.
8. Centrifuge samples for 5 minutes @ 3000 rpm.
9. With a Pasteur pipette, transfer the top phase into orange-labeled test tubes.
10. Add **2 ml hexane** to the yellow-labeled test tube and vortex for 30 seconds.
11. Centrifuge samples for 5 minutes @ 3000 rpm.
12. With a Pasteur pipette, transfer the top phase into orange-labeled test tubes.
13. Perform steps 10 and 12 **ONE** more time.
14. Check to see if bubbles (i.e., water) appear at the bottom of the orange-labeled test tubes. Remove bubbles with a Pasteur pipette.
15. Evaporate the solvent in the N-EVAP until standing liquid is gone, but still looks wet. Process will take ~15 minutes.
16. Store samples at -20°C or add 300 µl hexane-MTBE and transfer into GC vial for analysis.