Rapid PLFA Extraction Procedure

VERSION 1

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Soil Sieving

Thaw the soils overnight in a refrigerator. Only thaw enough samples for one day of sieving. A sample must be completely thawed before sieving.

Keep the samples in a cooler with blue ice so they remain cold.

Wear latex or nitrile gloves while sieving to prevent contamination of the samples.

Rinse a 2 MM sieve, catch pan and forceps with 95% ethanol before beginning each sample. Also rinse the gloves. Everything must be dry before beginning. A clean kimwipe may be used for drying.

Place the sieve in the catch pan. Gently break up the sample over the sieve. Remove any large roots, rocks and detrital organic matter. Gently work the soil through the sieve into the catch pan.

Pick out any roots larger than 2 mm from the soil. Put the soil back into the bag and put the bag back into the -80 C freezer.

Freeze Drying

Transfer the samples into new, labeled 50 ml plastic centrifuge tubes. Cover each tube with parafilm and poke 6 pin holes in the parafilm. Take the samples out of the freezer in batches of ~10 transfer them and put the batch back in the -80 freezer before starting the next batch. This will minimize sample thawing.

Leave the samples in the freezer a minimum of 2 hours before putting them in the freeze drier.

Follow the directions posted on the freeze drier. The samples should dry for 3 days.

After freeze drying is complete, remove the parafilm, cap the tubes and return them to the -80 freezer.

Reagents

Phosphate Buffer

Dissolve 8.7 grams K2HPO4 in ~950 ml Deionized water. Adjust to pH 7.4 with 1 M NaOH or H3PO4. Bring to 1 liter volume and store in a glass bottle for up to 3 months.

Bligh-Dyer Extractant

100 ml 50 mM PO_4 buffer pH 7.4 (8.7 g K_2HPO_4 per liter)

250 ml methanol

125 ml chloroform

Mix fresh daily, or at least weekly if many runs are anticipated

Transesterification Reagent

Dissolve 0.224 grams KOH in 30 ml methanol, then add 10 ml toluene.

Prepare weekly

0.075 M Acetic Acid

Add ~240 mls deionized water to a 250 ml volumetric flask. Add 1.08 mls glacial acetic acid (wear safety glasses!). Mix and bring to volume with deionized water. Store in a glass bottle for up to 2 months.

5:5:1 Methanol:Chloroform:Water

Add 50 mls methanol to a 125 ml glass bottle. Add 50 mls chloroform and 10 mls deionized water. Store for up to 1 month.

C19:0 standard for final transfer to GC vials. *Store in freezer (-20°C)*

Make a stock solution (1.25 g/L) of C19:0 Fame (methyl nonadecanoate) by dissolving 12.5mg in ~9.5ml Hexane in 10ml volumetric flask. Add hexane to the 10 ml mark and mix. Store stock solution in a 15mL test tube (store under N_2 in -20C freezer). Label the solution with "Stock 19 1250 ng/ul" and the date. Solution is good for 3 months.

Transfer 1mL of the stock to 50mL (clean and hexane rinsed) volumetric flask and add hexane to the 50mL line. Transfer solution to a glass tube, resulting in 25mg/L (= 25ng/uL) of the standard.

Label this bottle "**C19:0 25 ng/ul**" and store under N_2 in -20C freezer. Label with date.

May be made up to one week ahead of use if stored under N2 in -20 freezer. Discard any excess after use.

Extraction efficiency standard C21:0

Weigh 7.335 mg of 1,2-diheneicosanoyl-sn-glycero-3-phosphocholine into a 10 ml volumetric flask. Dissolve in chloroform and bring to the 10 ml mark. Store this stock solution in a 15mL test tube (store under N_2 in -20C freezer with parafilm around the cap). Label the solution with "C21:0 Stock" and the date. Solution is good for 3 months. Be sure to sweep with nitrogen after each use.

Transfer 0.5 mL of the stock to 5mL (clean and hexane rinsed) volumetric flask and add Chloroform to the 5 mL line. Transfer solution to a glass tube, resulting in 25mg/L (= 25ng/uL) of the standard.

Label this bottle "**C21:0 Working**" and store under N_2 in -20C freezer with parafilm around the cap. Label with date.

May be made up to one week ahead of use if stored under N2 in -20 freezer. Discard any excess after use.

Lipid Extraction

Wear Nitrile gloves for all remaining steps of this procedure.

Weigh 1.5 – 2 grams of soil into labeled 13 X100 mm test tubes. Record the weight to 3 decimal places. Freeze dry for 3 days. Add 75 ul of "C21:0 working" standard to the soil in each tube. Include 1 empty tube as a blank with each set.

Leave tubes open for 15 minutes to allow the chloroform to evaporate. Cap the tubes with PTFE lined caps.

Samples may be stored in -20 freezer until ready to extract.

Add 4 ml of Bligh-Dyer extractant to each tube.

Sonicate 10 min in an Ultrasonic Cleaning Bath at room temperature.

Place the tubes on their side on the reciprocal shaker and shake at 60 cycles per minute for 2 hours.

Shake down any residual soil from the cap or sides of the tube.

Centrifuge in the LabConco centrivap vacuum concentrator (hereafter referred to as centrivap) for 10 minutes without vacuum or heat.

Use a transfer pipet (one per sample) to transfer the liquid phase to a clean, labeled 13 X 100 test tube with a PTFE lined cap.

Separation

Turn on the centrivap cold trap.

Add 1 ml chloroform and 1 ml DI water to each tube.

Vortex each tube for 10 seconds. Centrifuge for 10 minutes in the centrivap without vacuum.

Aspirate and discard the top (aqueous) layer.

Dry in the centrivap (with vacuum) at room temperature ~1 hour. Without caps.

The tubes may be capped and stored in a -20 C freezer until proceeding with the chromatography.

Chromatography

Add 1 ml chloroform to each tube using the single channel pipettor to dissolve lipids for chromatography. Replace the cap and vortex for 20 seconds.

Use a 50 mg silica gel SPE 96-well plate. (Phenomenex Part # 8E-S012-DGB – No Substitutes.). Place the plate on top of the 1.5 ml Multi-Tier microplate Labeled non CHCl3 waste.

Wash each well 3 times with 1 ml methanol using the multichannel pipettor. Empty the methanol waste into the container labelled for non CHCl3 waste after each 1 ml has drained through the silica gel.

Move the silica gel plate to the 1.5 ml Multi-Tier microplate Labeled CHCl3 waste. Wash each well 3 times with 1 ml chloroform using the multichannel pipettor. Empty the waste into the CHCl3 container after each 1 ml has drained through the silica gel

Transfer a sample from the tube to the silica gel plate using a transfer pipet. Be sure to record which sample went into each well on the plate map. Add another 1 ml of chloroform to the tube using a single channel pipettor, vortex for 5 seconds and transfer to the same well in the silica gel plate.

Repeat for all the samples in the set. Empty the waste into the CHCl3 container.

Turn on the centrivap cold trap. Set the centrivap to preheat to 70 C.

Add 1 ml chloroform to each well using the multichannel pipettor. Use fresh tips for each row. After the Chloroform has drained through the Silica gel, move the silica gel plate to the Non CHCl3 waste plate and empty the chloroform waste into the CHCl3 waste container.

Add 1 ml acetone to each well using the multichannel pipettor. Use fresh tips for each row.

Place clean 1.5 ml Multi-Tier microplate (E & K Scientific Part # EK-99238) in the bottom the 96-Well Plate Manifold.

After the acetone has drained from the silica gel, move the silica gel plate to the top of the 96-Well Plate Manifold and start the vacuum.

Add 0.5 ml of 5:5:1 methanol:chloroform:H_2O to each well using the multichannel pipettor to elute phospholipids.

Prepare the balance plate by adding 0.5 ml of 5:5:1 methanol:chloroform:H_2O to the same number of wells as the sample plate. Add DI water to wells in the balance plate to bring it to the same weight as the sample plate.

Place the sample and balance plates in the centrivap.

Centrivap to dryness (70°C for 30 min, then 37°C until dry, ~ 2 hrs total). Set centrivap for 120 minutes at 70°C with the heat time at 30 min.

The samples may be stored in a -20 freezer awaiting transesterification.

Transesterification

Turn on the plate heater and set to 37°C.

Use the multi-channel pipettor to add 0.2 ml transesterification reagent to each well. Change pipet tips between each row. Swirl the plate gently for 30 seconds to mix.

Put the plate on the plate heater at 37°C for 15 min.

Turn on the Centrivap cold trap.

Use the multi-channel pipettor to add 0.4 ml of 0.075 M acetic acid and 0.4 ml chloroform. Change pipet tips between each row and between the acetic acid and the chloroform.

Seal with Teflon/silicon cap mat (E & K Scientific # EK-99249), shake vigorously, let separate.

Transfer bottom 0.3 ml from each well into the corresponding well of a new 1 ml Multi-Tier plate (E & K Scientific # EK-99234) using the single channel pipettor.

Repeat with another 0.4 ml chloroform, transferring bottom 0.4 ml this time.

Prepare the balance plate by adding 0.7 ml of chloform to the same number of wells as the sample plate. Add DI water to wells in the balance plate to bring it to the same weight as the sample plate.

Evaporate in SpeedVac concentrator at room temperature-remove as soon as dry (~ 45 min).

Transfer to GC vials

Remove the “C19:0 25 ng/ul” solution from the freezer and allow to come to room temperature.

Label GC vials, place an insert in each one and place a Teflon lined cap loosely on each one.

Use a single channel pipettor to add 75 ul of the “C19:0 25 ng/ml” solution to one well (tube) of the plate. Cap the tube with a silicon cap, tip the tube to about 45 degrees and gently roll the tube 3 revolutions to dissolve the fames on the sides of the tube.

Use a single channel pipettor to mix the sample by drawing it up and dispensing back into the well 3 times. Then transfer it to the GC vial insert.

Gas Chromatography Mass Spectrometry Analysis

FAME Standards

- Supelco 37 Component FAME Mix: as a calibration standard from Sigma-Aldrich (CRM47885), containing 37 FAMES with concentrations 200, 400 and 600 ug/mL
- Bacterial Acid Methyl Ester (BAME) mix: for additional FAME identification from Sigma-Aldrich (47080-U)

Solvents

- Hexane (needle rinse solvent): Hexanes, Optima™, Fisher Scientific (H303-4)

GC Column

Phenomenex ZB-FAME, 30m length, 0.25mm ID, 0.25um film

GC method

Front inlet temperature: 250°C
Inlet split ratio 25:1
Constant He flow: 1mL/min
GC oven program: Initial: 80°C, hold for 1.5min, ramp 1: 40°C/min until 160°C, ramp 2: 5°C/min until 185°C, ramp 3: 30°C/min until 260°C, hold 3 min.

MS method

Transfer line: 265°C
Electron Ionization (EI) source: 280°C
Filament ON: 3 min delay
MS scan: 45-450 m/z
Dwell time: 0.1 sec

Chromeleon Processing method

MS detection: Thermo Cobra peak picking software
MS settings: peak dependent baseline correction (3 spectra bunch)
MS component table: FAME components recognized based on RT window, MS quantitation peak, MS confirming peak 1 and MS confirming peak 2

Sample injection
Sample volume: 1µl
Rinse solvent: Hexane

Sequence setup
BAME 1:10 dilution – qualitative standard mix
FAME37 calibration standards + fixed C19:0 internal standard spiked in at 25 ug/mL (same concentration added to all calibrants; same concentration as in analytical samples)
Cal 12 1:50
Cal 24 1:25
Cal 30 1:20
Cal 40 1:15
Cal 60 1:10
Sample 1-10
Blank
Check standard – Cal 40
Sample 11-20
Blank
Check standard – Cal 40
Sample 21-30
Blank
Check standard – Cal 40
Sample 31-40
Blank
Check standard – Cal 40
Etc. up to 100 samples

Septum and liner are replaced every 200 samples. EI source is cleaned every 500 samples.
GCMS Raw Data Processing

1. Batch export data files to excel
2. Use VLOOKUP script to exclude non-confirmed compounds, due to lack of the quantitation or confirming MS peaks or ratios
3. Calibration of 37FAME standard using relative peak areas compared to internal standard C19:0

4. Quantification of FAMEs by using external calibration. For FAME compounds not in 37FAME standard, we use the response of nearest 37FAME standard peaks in the chromatogram.

Data Processing

Refer to the FAME list for naming conventions, formula weights, and names of each FAME.

Data are received from CSU Central Instrument Facility as concentration of each FAME (μg/ml). Data are converted from extraction concentration to nanomoles of FAME per gram soil using the following equation:

$$\left(\frac{\left(\frac{concentration\ \left(\frac{\mu g}{ml}\right) * \frac{1ml}{1000\mu l} * \frac{1g}{10^6\mu g}\ chloroform\ correction\ \left(\frac{1}{0.95}\right) * final\ extraction\ volume\ (75\mu l)}{FAME\ formula\ weight\ (g/mole)}\right) * \frac{10^9 nanomoles}{mole}}{Soil\ freeze-dried\ mass\ (g)}\right)$$

This equation can be simplified to:

$$\frac{\left(\frac{concentration\ \frac{\mu g}{ml} * chloroform\ correction\ \left(\frac{1}{0.95}\right) * 75\mu l}{FAME\ formula\ weight\ (g/mole)}\right)}{Soil\ dry\ mass\ (g)}$$

Extraction efficiency is determined as follows:

$$\left(\frac{(concentration\ of\ c21{:}0\ standard\ \left(\frac{\mu g}{ml}\right) * chloroform\ correction\ \left(\frac{1}{0.95}\right) * final\ extraction\ volume\ (75\mu l)}{3750\ (expected\ ng\ C21{:}0)}\right) * 100$$

The expected amount of C21:0 standard is calculated from the volume of working c:21:0 solution (75μl) of 1,2-diheneicosanoyl-sn-glycero-3-phosphocholine (25ng/μl) multiplied by 2 as 1 mole of this compound is converted to two moles of heneicosanoic acid methyl ester (the C21:0 FAME).

PLEASE NOTE: Reported concentrations of each FAME do NOT have extraction efficiency taken into account. Extraction efficiency is reported for each sample, but it is up to the end user to incorporate extraction efficiency.

Data QA/QC

Instrument QA/QC is determined from expected concentration of c16:0 in the Supelco 37 component FAME mix, usually at the 40mg/ml concentration. Actual and expected concentrations are reported in the BatchResults files for each run.

Samples are repeated if extraction efficiency is less than 10% or greater than 111%.

Accompanying documentation

There are various conventions that can be used for lipid chemistry nomenclature. While NEON term definitions attempt to conform to conventionally accepted nomenclature, some differences and inconsistencies may exist. For a list of FAME terms used by the Analytical Laboratory and mapping to NEON terms, please refer to the 'EcoCore_FAME_List_v1.xlsx'.