Reagent Preparation: Soil PLFA Extraction, Purification, and Analysis by MIDI Software

Rinse all glass used for measuring or storing solvents with DI rinse (3x), then hexane rinse (3x), followed by acetone rinse (1x to dry)

Reagent 1 (Bligh-Dyer extractant)

1) Prepare 50 mM K_2HPO_4 buffer (pH 7.4 ± 0.2):

Add 5.4431 g K_2HPO_4 into approx. 500 mL DI H2O, pH with 0.1 M NaOH or HCl, bring total vol to 625 mL, aliquot into 200 mL volumes for use in Reagent 1. Store in glass bottles.

For 425 mL	For 950 mL
2) Measure out 250 mL of Methanol and add it to the bottle. 3) Measure out 100 mL of the 50 mM K_2HPO_4 buffer and add it to the methanol. 4) Mix the two liquids. 5) Add 125 mL of Chloroform and mix well.	2) Measure out 500 mL of Methanol and add it to the bottle. 3) Measure out 200 mL of the 50 mM K_2HPO_4 buffer and add it to the methanol. 4) Mix the two liquids. 5) Add 250 mL of Chloroform and mix well.

6) Cap the bottle and keep it in a cabinet until use. Phosphate buffer is stable, but Reagent 1 should be prepared within 1 week of use.

7) Internal standard (details below) goes into Reagent 1 immediately prior to use.

Internal standard:

1) Dissolve 20.18 mg internal standard, 19:0 phosphatidylcholine (Avanti Polar Lipids), into 10 ml 1:1 chloroform:methanol. Store at -20 C. Prepare in 16 x 100 mL glass tube with teflon cap.

2) Add to Reagent 1 just before use at a rate of 0.5 ul per ml. (For 950 mL use 475 ul internal standard; For 425 mL use 237.5ul)

Reagent 2- Deionized Water

Reagent 3 and 6- HPLC grade Chloroform

Reagent 4 – Trans-esterification Reagent

1) Dissolve 0.561g of Potassium Hydroxide in 75mL of Methanol.

2) Add 25 mL Toluene

Reagent 5 – Glacial Acetic Acid (0.075M)
If using 99.7% Acetic Acid (17.4 M) add 1.075 mL acid to 248.93 mL DI H_2O

Reagent 7 – 5:5:1 Chromatography Eluent Solution

1) Mix 50 mL of Methanol with 10mL of DI Water

2) Add 50 mL Chloroform

Prepared by Lauren Hale 12/12/16 based on MIDI training and protocol from Buyer Sasser (2102) AES.

Protocol: Soil PLFA Extraction, Purification, and Analysis by MIDI Software

Overview

Extraction and Purification: 4 Part Procedure	**Analysis**
1) Extraction of the PLFA from the Soil	1) Run on the PLFAD1 Method with the PLFAD1 calibration standard
2) Separation of the Organic Molecules	
3) Chromatographic Purification of PLFA	
4) Trans-esterification	

1) **Part 1: Extraction of PLFA from Soil**

1) Samples are dried overnight in the SpeedVac (dark room, change rotor)

2) After drying, the samples are sifted to remove any rocks or large debris

3) The soil is placed into a stock tube

4) 1-2 g of soil is weighed out into a clean 16x100mm Glass test tube (record weight).

5) Blanks should be used to make an even number of samples

6) Add 4mL of Reagent 1 to the tube containing the soil. Cap the tube with a Teflon lined cap.

7) Vortex for ~5 seconds then sonicate for 10 minutes.
Repeat twice more (3 rounds of sonication)

8) Centrifuge tubes at 3500 rpm for 15 minutes.

Part 2: Separation of the Organic Molecules

9) Remove the tubes and pipette the supernatant into a clean 16x100 mm glass test tube.

10) Add 1mL of both Reagent 2 and Reagent 3 to each tube. Vortex for 5 seconds

11) Then centrifuge again at 3500 rpm for 15 minutes.
If the bottom layer is cloudy, centrifuge for an additional 10 minutes

12) Transfer the bottom layer (Chloroform) to a clean 12x75 mm glass test tube.

13) Samples should be dried in SpeedVac set to low/ambient temperature (45-60 mins), until fully dry (dried samples can be stored in freezer overnight).

Part 3: Chromatographic Purification of PLFA

14) Precondition each well to be used in SPE 96-well plate
3 washes with 1 mL Methanol
3 washes with 1 mL Chloroform

15) Dissolve the samples in 1mL of Chloroform and transfer to the appropriate well using a class pipette, retain glass pipette in tube for step 16. Use a clean pipette for each sample.

16) Wash the sample tube with an additional 1mL chloroform and add to the appropriate well
To improve recovery, add chloroform to glass pipette to and allow it to drain into tube, then suspend and collect.

17) Allow the samples to gravity drain into the columns

18) Wash the columns with 1 mL of Chloroform (removes neutral lipids)

19) Wash columns with 1 mL of Acetone (removes glycolipids)

29) Place a clean 1.5 mL Glass Vial below each column and elute the phospholipids with 0.5 mL of Reagent 7 (5:5:1 Chromatography Eluent Solution).

30) Allow to gravity drip

31) Finish eluting the PLFAs by turning on the vacuum pump to pull the remaining solvent through.

32) Decant the sample into a clean 12x75 mm glass test tube

33) Dry the samples in the SpeedVac for approximately 1 hour at ambient temperature.

Part 4: Trans-Esterification

34) Add 0.2 mL of Reagent 4 to each tube

35) Cap each tube and vortex on high for approximately 5 seconds

36) Incubate at 37^0C for 15 minutes

37) Add 0.4mL of Reagent 5 and 0.5mL of Reagent 6 to each tube

38) Cap each tube and vortex for 10 seconds

39) Allow to sit until the phases have separated

40) Transfer the bottom layer (Chloroform) to a GC vial

41) Dry in the SpeedVac for 20-30 minutes at ambient temperature

42) Resuspend the sample by adding 100 uL of Hexane to each GC vial

43) Cap the vial and vortex

44) Transfer the sample to a limited-volume insert and place the insert into the GC vial that the sample came from

Analysis

45) Analyze using the PLFAD1 Method using the PLFAD1 Calibration Standard.

46) Load tray with PLFAD1Calibration Standard 1^{st}, followed by sample vials and blank.

47) Load hexane into wash bottles (spot A and B). Ensure bottles are clean, first wash empty bottles with soap and water, then several DI rinses, then hexane rinses. Allow to dry fully. Add hexane just prior to use (it will evaporate quickly). Fill past fill line, but not to top, ensuring syringe guide does not dip into hexane. Do not add septum, leave bottles with caps and syringe guide.

47) Start Sherlock Sample Processer. Unlock table and enter sample information corresponding to tray number, choose PLFAD1 method, name samples.

48) When all has been entered select “Run Batch”. When prompted “Are you ready”, choose “Yes”. Agilent ChemStation authorization info will be requested, leave empty, hit “enter”