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Phospholipid fatty acid (PLFA) analysis

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We use this protocol and it's working

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Abstract

Phospholipid fatty acid (PLFA) purification and analysis

Guidelines

NOTES:
All glassware must be fired in aluminum foil at 450°C for at least 4.5 hours to remove all organic substances. Care must be used in organic-free technique to minimize contaminations between samples. Any equipment which cannot be fired must be rinsed with methanol followed by chloroform and allowed to dry to remove any organics. All transfers of solution MUST OCCUR IN A FUME HOOD. Fatty acids are somewhat photosensitive, so care should be taken to minimize exposure to light.

Before start

Supplies and Reagents

Extraction

Supplies: Centrifuge bottles, Pipettes, test tubes, funnels, filter papers, Pasteur pipettes, beakers
Reagents: phosphate buffer, chloroform, methanol, soil samples

Separation/Fractionation

Supplies: vacuum, manifold, silica SPE columns 500 mg, test tubes, pipettes (including capillary pipettes), beakers
Reagents: methanol, chloroform, acetone

Methylation

Supplies: Vortex, water bath, test tubes, pipettes (0.5 mL, 2 mL, 1 mL, 10 mL), Pasteur pipettes, beakers
Reagents: 0.2 N acetic acid, 0.2 N KOH in methanol, chloroform, toluene, chloroform-extracted water

Extraction of Lipids from Soils -First Extraction

1 Add 5 g of soil (fresh mass) to a centrifuge bottle (250 mL).

5 g soil (fresh mass)

2 Add 5 mL of chloroform, 10 mL of methanol, and 4 mL of phosphate buffer* minus the water content of the soil. 1 mL of chloroform for each g of soil, volumes should be 1:2:0.8 chloroform:methanol:buffer.

*50 mM phosphate buffer: 8.7 g K2HPO4 in 1 L D.I. water which has been chloroform extracted. Adjust pH with 3.5 ml 6 N HCl to 7.4

5 mL chloroform

10 mL methanol

4 mL phosphate buffer (50mM, pH 7.4)

3 Swirl. Extract wrapped in foil for 2 hours.

02:00:00 extraction

4 Spin centrifuge bottles at 2500 rpm for 20-30 minutes.

Command

2,500 rpm

CENTRIFUGE

00:20:00

Extraction of Lipids from Soils - Second Extraction

5 Add additional 5 mL of chloroform and 5 mL of buffer to break the phase and achieve a final ratio of 1:1:0.9 chloroform : methanol : buffer.

5 mL chloroform

5 mL phosphate buffer

6 Swirl. Allow to separate overnight (approx 18 hours) covered to protect lipids from light.

18:00:00 extraction in dark

7 Spin centrifuge bottles at 2500 rpm for additional 20-30 minutes.

Command

2,500 rpm

CENTRIFUGE

00:20:00

8 Transfer organic phase to a test tube through a Whatman #2 filter paper and evaporate solvent under N_2.

9 Store in sealed test tubes under N_2 at −20°C.

Extraction of Lipids from Leaf Litter

10 Place 250 mg equivalent dry weight of ground litter in 20 mL test tube.

250 mg dry weight of ground litter

11 Add 1.6 mL potassium phosphate buffer (subtract moisture in litter from the 1.6 mL of buffer), 4 mL methanol, 2 mL cholorform (solvents should be in final ratio of 0.8 : 2 : 1).

1.6 mL potassium phosphate buffer

4 mL methanol

2 mL chloroform

12 Vortex each tube for 30 seconds.

00:00:30 vortex

13 Place in 37°C water bath fro 30 minutes, vortexing every 5 minutes.

37 °C water bath

00:30:00 vortexing every 5 minutes

14 Pipette out liquid (leave litter) into 2nd test tube.

15 Add additional 1.6 mL buffer, 4 mL methanol, and 2 mL chloroform to original test tube.

1.6 mL phosphate buffer

4 mL methanol

2 mL chloroform

16 Incubate at 37 °C for 30 minutes, vortexing every 5 minutes.

37 °C water bath

00:30:00

17 Pipette out as much liquid as possible into 2nd test tube. Discard original test tube with leaf litter.

18 Add additional 4 mL chloroform and 4 mL of buffer to second tube. Vortex for 30 seconds.

4 mL chloroform

4 mL phosphate buffer

Command

30s

VORTEX

19 Allow to separate overnight. Remove lower phase **only** to small test tube by passing through a Whatman #2 filter paper.

20 Store tubes, sealed, under N_2 at −20 °C.

Fractionation of Lipids

21 Prepare columns by flushing them first with *methanol* to dehydrate them, then with *chloroform* to prepare them for the lipid. Pull the solvents through with a very low vacuum*.

*Vacuum should be 5” Hg or less or the column may collapse. The prep rinses and chloroform and acetone extractions may be collected in a waste container in the manifold and discarded

before inserting test tubes for the methanol elution.

22 Allow lipids to reach room temperature. Resuspend the dried lipids in 150 µL of *chloroform* and transfer to the column. Repeat for a total of 4 transfers per sample.

150 µL chloroform

23 Elute neutral lipids with 5 mL of *chloroform*. Pull solvent through with a low vacuum. Discard solvent.

5 mL chloroform

24 Elute glycolipids with 5 mL of *acetone*. Pull solvent through with a low vacuum. Discard solvent.

5 mL acetone

25 Elute phospholipids with 5 mL of *methanol* under vacuum into a test tube.

5 mL methanol

26 Blow off solvent (methanol) with the N_2 Store dried PLFAs under N_2 at –20 °C until methylation.

Methylation- formation of fatty acid methyl esters

27 Remove samples from freezer and allow them to reach room temperature.

28 Make 0.2 N methanolic KOH by dissolving 0.28 g KOH in 25 mL of methanol. KOH absorbs water from the air, so movement from the balance to the methanol is critical. Make fresh every day.

25 mL 0.2N methanolic KOH

29 Make 0.2 N acetic acid if none already made by diluting 1.15 mL glacial acetic acid to 100 mL total volume with chloroform extracted D.I.

100 mL 0.2 N acetic acid

30 Create 1:1 v mix of methanol : toluene.

31 Add 0.5 mL of the methanol : toluene solution to each sample.

0.5 mL methanol : toluene solution

32 Add 0.5 mL of KOH in methanol to each sample. Vortex for 30 seconds.

0.5 mL KOH in methanol

Command

30s

VORTEX

33 Place in water bath heated to 37 °C for 15 minutes. Then cool tubes to room temperature.

37 °C water bath

00:15:00

34 Add 0.5 mL of acetic acid to each tube. May look milky/chunky.

0.5 mL acetic acid

35 Add 2 mL of chloroform to all samples followed immediately by 2 mL of chloroform-extracted DI water. Vortex for 30 seconds.

2 mL chloroform

2 mL chloroform-extracted DI water

Command

30s

VORTEX

36 Centrifuge samples for 5 minutes.

Command

5 minutes

CENTRIFUGE

37 Pipette out bottom (organic) layer into a new test tube being careful not to transfer any of the aqueous phase.

38 Add 1 mL of chloroform to each of the original sample tubes. Vortex for 30 seconds. Centrifuge for 5 minutes. Pipette organic (lower) layer into the new test tube.

1 mL chloroform

Command

30s

VORTEX

Command

5 min

CENTRIFUGE

39 Repeat step 38.

go to step #38

40 Add 1 mL of chloroform to each of the original test tubes but DO NOT VORTEX OR CENTRIFUGE. Transfer chloroform to new test tubes. New test tubes should now contain FAMEs in 5 mL of chloroform Remove chloroform under N_2 blow-down. Store samples at –20 °C until identified by gas chromatography.

1 mL chloroform

Separation, Quantification and Identification

41 Resuspend FAMEs in hexane. Include a known amount of an internal standard.

42 Transfer to GC vial with PTFE septa cap.

43 Identify by retention time/co-elution with standards on a GC

44 Molar concentrations of each fatty acid can be determined by relationship between area of standard peak and known concentration of standard.