JAN 28, 2021

fatty acid analysis sponges

PLOS One

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OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.bhnpj5dn

External link:
<https://doi.org/10.1371/journal.pone.0241095>

Protocol Citation: Anna de Kluijver 2021. fatty acid analysis sponges . **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.bhnpj5dn>

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Protocol status: Working

Created: Jun 18, 2020

Last Modified: Jan 28, 2021

PROTOCOL integer ID:
 38319

Keywords: PLFA, BDE, lipids, GC, PLFA identification, derivatization, sponges

ABSTRACT

This protocol has been used to analyse fatty acid composition of sponges (Porifera). The original protocol has been developed at NIOZ Yerseke, former NIOO-CEME (Netherlands) (Boschker and Middelburg) and has been used to quantify PLFA in combination with stable isotopes for sediments, plankton, organisms (including sponges). This protocol has been adopted to the procedures of the organic geochemistry lab at Utrecht University.

GUIDELINES

Contamination: work clean and avoid organic contamination, especially plastics throughout the procedure
 glassware: use very clean glassware that has been rinsed in decon, dishwasher washed and oven-dried
 storage: The samples and extracts should be stored in a freezer at -20°C or colder.

SAFETY WARNINGS

- ! This procedure uses hazardous chemicals
 1. Read the MSDS forms for each chemical in the procedure.
 2. Use gloves throughout the procedure.
 3. Wear a lab coat and safety glasses throughout the procedure.
 Dispose of all chemical waste in appropriately labeled containers

BEFORE START INSTRUCTIONS

All sponge material should be (freeze)dried, grinded and homogenized

solution preparation

- 1 Prepare P-buffer: dissolve 8.7 g K_2HPO_4 in 1 L mQ in a 1 L glass bottle, adjust to pH 7-9 with 1 Molarity (M) HCl, check with pH paper
- 2 Prepare Bligh and Dyer extraction (BDE) mix: methanol (MeOH)/ dichloromethane (DCM) / P-buffer in ratio of 2/1/0.8 (v/v/v), e.g. 250 mL MeOH, 125 mL DCM and 100 mL P-buffer in a 500 mL Erlenmeyer with glass stopper
- 3 Prepare standards:
 20 mg $\text{C}_{19:0}$ FAME in 2 mL hexane (100 x stock)
 $\text{20 } \mu\text{L}$ $\text{C}_{12:0}$ FAME in 2 mL hexane (100 x stock)
- 4 Prepare 0.2 Molarity (M) methanolic NaOH by dissolving 1.08 g CH_3NaO in 100 mL MeOH
- 5 Activate silicic acid gel by heating it at $\text{120 }^\circ\text{C}$, for at least 2 hours and allow it to cool. Once activated, it can be kept in a closed bottle for 2 or 3 days.
- 6 Prepare 1 Molarity (M) acetic acid: add 5.742 mL glacial acetic acid ($\text{17.416 Molarity (M)}$) into 25 mL miliQ and fill to 100 mL in a 100 mL volumetric flask.

Total lipid extraction

- 7 Weight 30-50 mg dried sponge sample in extraction tubes (Kimax[®] centrifuge tube).

This amount is enough to do either lipid-class or total lipid analysis, if you want to do both, weight a double amount.

8 Add 28.5 mL Blight and Dyer mix, close with lid and shake/vortex

9 Extract the sample in an ultrasonic bath for 00:10:00

10 Extract for minimal 03:00:00 on a roller table - horizontal shaker. Check that lids are tightly closed!

11 add 7.5 mL DCM, close lid and shake/vortex

12 add 7.5 mL P-buffer, close lid and shake/vortex

- 13** Let layers separate by either leaving the tubes overnight in the freezer or by gentle centrifugation (170 x g, 00:03:00 (room temperature))
- 14** Collect the DCM layer (bottom) with a Pasteur pipette or a glass syringe into 50 mL glass vials. The type of vials depend on the evaporator. Take a bit of air into the syringe or pipette. Bring the tip carefully in the bottom layer. Let a few air bubbles out to clean the tip of the syringe/pipette. Collect the DCM and avoid taking the fluffy layer. When using a glass syringe, clean it in DCM: methanol (1:1) between samples.

courtesy S. Hoetjes/ D. Lankes

- 15** Add again (7.5 mL DCM, let layers separate (centrifugation or freezer) and collect DCM layer into the same vial
- 16** Dry samples completely under a gentle N₂-stream in a turbovap (30 °C , maximum 5 psi pressure, but start with 1-2 psi)

- 17 Add 1 ml 1 mL of DCM and evaporate again.
- 18 Transfer sample in 1 mL (approximately) DCM:MeOH (1:1) to a pre-weighed 4 ml vial and dry in a Flexivap 26 °C under a gentle N₂ stream
- 19 Optional: if your sample contains some residue, it can be cleaned over a pasteur glass pipette with pre-extracted cotton wool in DCM: MeOH (1:1)
- 20 Weigh your vials to obtain total lipid weight /sponge dry weight. Now you have your Bligh and Dyer extract (BDE)

Separation into polarity classes

- 21 Prepare columns: add a ball of pre-extracted cotton-wool into the tip of a 10 mL glass pipette. Add 1 spoon (~0.5 g) on top. The whole column is ~3 fingers width, 3 cm. The column is stored into a cylinder filled with DCM. Make sure the silica layer is soaked in DCM. Gently tap the column to get rid of air bubbles.
- 22 Put the column in a holder, place a vial or beaker glass underneath and wash the column with

DCM

- 23 Dissolve and add your sample in 0.5 mL DCM.
- 24 Don't disturb the coyour neutral lipid fraction. Collect in a 10 mL vial if you want to analyze it or in a waste beaker/vial if it can be discarded.
Don't disturb the column!
- 25 Elute with 7 mL acetone (picograde pestinorm), this is your glycolipid fraction. Collect in a 10 mL vial if you want to analyze it or in a waste beaker/vial if it can be discarded.
- 26 Elute with 15 mL MeOH into a 20 m glass vial , this is your phospholipid fraction that will be used for further analysis.
- 27 Dry the MeOH fraction in the turbovap (30 °C max 5 psi, but start with 1-2 psi)

mild alkaline methylation

- 28 Add to dried sample 1 ml MeOH/toluene (1:1 v/v)
Add 1 ml 0.2 M methanolic NaOH (exactly with glass syringe/pipette)
Add 50 µL 0.1 mg/mL C_{19:0} FAME standard
- 29 Incubate at 37 °C 00:15:00 in a oven
- 30 Stop methylation by adding
2 mL hexane
0.3 mL 1 Molarity (M) acetic acid (exactly, with pipette)

2 mL milliQ

- 31 Mix very well by shaking or vortexing. Let layers separate. The upper (hexane) layer should be clear
- 32 Collect the upper layer with a Pasteur pipette into a 10 ml glass vial. Avoid taking the water layer.
- 33 Add again 2 mL hexane, repeat 31-32, collect in the same vial
- 34 Add again 2 mL hexane, repeat 31-32, collect in the same vial
- 35 Evaporate hexane until complete dryness
Step 35 includes a Step case.
Acidmethylation

GC analysis

step case

Acidmethylation

- Add 0.5 mL DCM:MeOH (1:1) to dry sample vials
- Transfer the samples into a 4 ml glass vial
- N₂ dry samples
- Add 0.5 mL BF₃-methanol (Merck Millipore: product code 801663, 20%)
- Incubate samples at 60°C for 10 minutes
- Add 0.5 mL MilliQ water and 40 µl standard C_{19:0} FAME
- Add 1 mL DCM
- Collect the DCM layer (bottom layer) into another 4 mL glass vial

- Repeat the wash procedure 2 more times
- Dry under N₂

36 Add 50 µL C_{12:0} FAME and ~ 100 µL hexane or ethyl acetate Transfer samples into GC analysis vials.

Store in -20 °C freezer until analysis

Hexane and ethyl acetate give comparable results. Make sure that C_{12:0} FAME is in the same solvent

Step 36 includes a Step case.

DMDS

hydrogenation

Alox separation