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Based on M. Brett Lab protocol

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PROTOCOL TO EXTRACT AND TRANSMETHYLATE FATTY ACIDS FROM BIOLOGICAL TISSUES FOR QUANTIFICATION AND ISOTOPIC ANALYSIS

INTRODUCTION

This protocol describes the process to extract and transmethylate fatty acids (FAs) from biologic material so that they are suitable for quantification by GC-FID and isotopic analysis by GC-C-irMS. The method is based on that used in the Brett lab at UW as refined by Sami Taipale. The first step follows the Bligh & Dyer method to extract total lipids from tissues. Total lipid content is quantified gravimetrically. The method of acid-catalyzed transesterification using 1% sulfuric acid in methanol is employed to derivitize FAs into fatty acid methyl ester (FAMEs), which are then extracted using 1:1 hexane: diethyl ether. For carbon isotope analysis of the resulting FAMEs, the addition of the methyl group needs to be accounted for and requires samples of the MeOH to be saved for $\delta^{13}C$ analysis.

SAFETY

This method uses numerous chemicals that are considered hazardous for flammability and toxicity. Therefore, most of the work must be conducted in the fume hood. Acutely toxic chemicals include chloroform, methanol (a.k.a., MeOH), hexanes, and diethyl ether. Chloroform and hexanes can affect the respiratory and central nervous systems with repeat and prolonged exposure. Methanol and diethyl ether can induce dizziness and shortness of breath. All of the above chemicals are flammable. Potassium bicarbonate powder can irritate skin and is harmful if inhaled. Concentrated sulfuric acid (H_2SO_4) is used when preparing reagents; sulfuric acid is a very strong acid and can cause severe burns. always wear rubber gloves, a lab coat, and work in the hood with the face shield down when using this chemical. *When working with all chemicals always wear a minimum of nitrile gloves and eye protection. Work in the fume hood when dealing with organic solvents.* Familiarize yourself with the MSDS for all chemicals prior to starting this protocol.

MATERIALS

- Twenty-four to 36 clean 12 mL borosilicate glass tubes plus caps (for 8-12 samples @ 3 per sample).
- Minimum 70 ashed 5 3/4" Pasteur pipettes.
- Wheaton auto-pipetter (fits Pasteur pipettes) and rubber blubs
- Two 5 mL glass gas-tight syringe with attached needle. Clean by rinsing with 2 mL Nanopure water (discarded into waste). Repeat with individual rinses of 2 mL DCM, 2 mL methanol, and 2 mL water, discarding each into waste.

PREPARING REAGENTS

2:1 chloroform:MeOH — Using a clean, ashed 500 mL graduated cylinder and working in the fume hood, first add 500 mL of chloroform to the designated 1 L glass bottle. Rinse cylinder with a few mL of methanol (MeOH) and discard into waste. Next add 250 mL of MeOH to the glass bottle.

2% $KHCO_3$ in water — Weigh 20 g of solid potassium bicarbonate ($KHCO_3$) on the pan balance using an ashed aluminum weigh boat. Transfer to a 1 L volumetric flask using an ashed glass powder funnel. Fill to mark with Nanopure water. Mix thoroughly and transfer to the designated 1 L glass bottle.

1:1 hexanes:diethyl ether — Using a clean, ashed 500 mL graduated cylinder and working in the fume hood, first add 500 mL of hexanes to the designated 1 L glass bottle. Rinse cylinder with a few mL of diethyl ether and discard into waste. Next add 500 mL of diethyl ether to the glass bottle.

1% H_2SO_4 in MeOH — Using a clean, ashed 100 mL graduated cylinder and working in the fume hood, measure 99 mL of methanol (MeOH) and add to the designated glass bottle. Using a new ashed Pasteur pipette and the Wheaton auto-pipetter, pipette 1 mL of sulfuric acid (H_2SO_4) into the MeOH. Never add MeOH to acid. Lastly, using a new Pasteur pipette, pipette 2 mL of straight MeOH (MeOH without H_2SO_4) into a new GC vial. Cap, label with date and lot # from the MeOH bottle, and place in the flammables freezer.

SAMPLE & MATERIALS PREPARATION

****Batch sizes of 8-12 samples are efficient to prepare over 2 days, starting the afternoon of the first day.****

1. Samples should be freeze-dried and ground using the ball mill prior to fatty acid extraction. See the Freeze Dryer and Sample Grinding protocols for detailed instructions.
2. Collect three ashed borosilicate glass centrifuge tubes plus acid washed caps for each sample in the batch. Label tubes with sample ID information and an 'A', 'B', or 'C'.
3. Using the 5 point Metler balance in room 232, weigh the 'B' tubes without the caps. Record weight as "B Tube Weight" in the FA notebook.
4. In the fume hood, rinse each tube with ~1 mL choloroform:MeOH using a new ashed Pasteur pipette. Discard into waste and repeat. Leave tube right-side up in rack and allow solvent to evaporate (or place in N2 evaporator).
5. Working on the counter near the scales, cut one triangle of ashed aluminum foil for each sample in the batch.
6. Place a piece of foil on Metler microbalance in lab and tare. Add 3.5-4 mg of sample to foil and record weight as "sample + foil" in the FA notebook.
7. Transfer sample to Tube A using the foil as a funnel.
8. Reweigh the foil and record as "foil weight." Subtract foil weight from sample and foil weight to obtain sample weight and record.
9. Repeat steps 6-8 for all samples in the current batch.

TOTAL LIPID EXTRACTION & QUANTIFICATION

1. Using an ashed Pasteur pipette and the auto-pipetter, add 2 mL chloroform to Tube A for each sample.
2. Add 1 mL of MeOH to Tube A for each sample.[1]
3. Add 1 mL of pre-mixed 2:1 cholorform:MeOH to Tube A for each sample.[1]
4. Add 500 uL Nanopure water to Tube A for each sample.[1]
5. Cap A tubes and place in wore rack. Sonicate for 10 minutes.
6. Vortex each tube for ~30 seconds.
7. Centrifuge tubes for 3 minutes at 3000 rpm. Make sure centrifuge is balanced; use additional tubes filled with 4 mL 2:1 cholorform:MeOH if necessary.

[1] Use a new ashed Pasteur pipette and the auto-pipetter for this step.

8. Remove the *bottom* 'organic' layer using a new ashed Pasteur pipette for each sample. Transfer to the B tube. Make sure only to transfer the lower layer by keeping sight pressure when moving the pipette through the upper layer. It is not necessary to get 100% of the lower layer in this step (but get close).
9. Using a clean 5 mL glass syringe that has ben pre-rinsed 2X with choloroform, add 2.7 mL of chloroform to the A tubes for each sample. Check that volumes among the A tubes are close to equal (for balance). Add choloroform as necessary.
10. Repeat steps 5-8, adding the lower layer to the sample in Tube B.

GRAVAMETRIC ANALYSIS OF TOTAL LIPDS

1. Evaporate total lipid samples in the B tubes under gentle stream of nitrogen.
2. With the samples completely dry, weigh the B tubes without the cap on the Metler balance. Record weights in the FA notebook as "B Tube + Sample Weight".

TRANSMETHYLATION

1. Turn on full water bath and set to 50 °C.
2. Starting with the fully evaporated samples in the B tubes, add 1 mL toluene to each sample using a new ashed Pasteur pipette and the auto-pipetter.
3. Add 2 mL 1% H_2SO_4 in MeOH to each sample using a new ashed Pasteur pipette and the auto-pipetter.
4. Flush with nitrogen and recap.
5. Vortex for 10 seconds to fully mix.
6. Place samples in a wire rack and incubate in the water bath for 16 hours.

****This is the end of day 1****

7. Remove tubes from the water bath and allow to cool (~5 minutes). Turn off the water bath.
8. Add 2 mL 2% $KHCO_3$ using a new ashed Pasteur pipette and the auto-pipetter.
9. Add 5 mL of 1:1 hexane: diethyl ether using a clean 5 mL glass syringe that has been pre-rinsed 2X with the solvent.
10. Cap tubes and shake gently, then release cap to let out CO_2 until the bubble layer is no longer present.
11. Tighten caps and vortex for 10 seconds.
12. Centrifuge 2 minutes at 1500 rpm.

13. Remove the *top* 'organic' layer using a new ashed Pasteur pipette for each sample. Transfer to the C tube. As before, make sure only to transfer the upper layer. It not necessary to get 100% of the upper layer in this step (but get close).
14. Add another 5 mL 1:1 hexane:diethyl ether to Tube B using a clean 5 mL glass syringe that has ben pre-rinsed 2X with 1:1 hexane:diethyl ether. Check that remaining volumes among the B tubes are close to equal (for balance) and add more solvent as necessary.
15. Repeat steps 10-13, adding the upper layer to the sample in Tube C.
16. Evaporate the solvent from the FAME samples contained in the C tubes under a gentle stream of nitrogen.

STORAGE AND PREPARATION FOR ANALYSIS

1. Add 1.5 mL hexanes to each C tube using a new ashed Pasteur pipette and the auto-pipetter. Wash the sides of the tube with the solvent as it's being added.
2. Vortex for ~10 seconds to fully dissolve FAMEs.
3. Using a new ashed Pasteur pipette for each sample, transfer the sample from the C tubes to new, pre-labeled GC vials. Immediately cap the GC vial after the sample transfer to minimize evaporation.
4. Organize samples into a cryobox for analysis and store in the flammables freezer under the GCs.

WASTE

Label an empty jug with a hazardous waste label and include the following information:

Chemical Composition	%
methanol	
chloroform	
hexanes	
diethyl ether	
potassium bicarbonate ($KHCO_3$)	
sulfuric acid (H_2SO_4)	
water	

Pour leftover solvents from the A and B tubes and any leftover reagents into the waste jug. When the jug is 4/5 full, submit an Online Chemical Waste Collection request. Used Pasteur pipettes should be left in beakers in the hood until solvents have been fully evaporated then disposed of in the Lab Glass box under the sink. See the section on

Non-Hazardous Laboratory Glass and Plastic at the UW EH&S website here.

CLEANING & RECYCLING

Glass tubes and caps should be cleaned following the HEEL Glass- & Plastic-ware Cleaning protocol. After cleaning return to the designated Fatty Acid cabinet above the bench with the sonicator and water bath. The glass syringes should be cleaned by rinsing with 2 mL water (Nanopure), 2 mL DCM, 2 mL methanol, and 2 mL water prior to being returned to the drawer.

REAGENTS & SUPPLIES

- Chloroform (0.75% Ethanol as Preservative/HPLC), Fisher Chemical, 4 L, Fisher Scientific catalogue #: C606SK-4.
- Diethyl Ether
- Hexanes (HPLC), Fisher Chemical, 4 L, Fisher Scientific catalogue #: H302-4.
- Methanol (HPLC), Fisher Chemical, 4 L, Fisher Scientific catalogue #: A452-4.
- Sulfuric Acid (ACS grade), Fisher Chemical, 5000 mL, Fisher Scientific catalogue #: A300-500.
- Disposable Borosilicate Glass Pasteur Pipettes, case of 1440, Fisher Scientific catalogue #: 13-678-20B.
- Borosilicate Glass Tubes and Caps

REFERENCES

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