Freeze Drying and Total Lipid Extraction Protocol

Hazardous chemicals used

- Ethanol- flammable SDS – flammables storage
- Methanol -flammable, health hazard, toxic SDS - flammables storage
- Chloroform- health hazard, irritant, toxic SDS -toxics storage
- Hydrochloric acid- irritant, corrosive SDS – acid storage

PPE= gloves, lab coat, safety classes

Ventilation= all work must be done in fume hood. Check for proper operation before use. Volatile gasses are a health hazard.

Spill procedure= chemical spill kit underneath sink in room 242. If chemicals in eyes, wash with eyewash, if chemicals spill on clothing, strip clothing and safety shower. Report large accident to Eh&S. Small chemical spills can be soaked with a paper towel, and towel should go in a ziplock bag in the flammables cabinet with labeled hazardous waste sticker.

All waste for this experiment (which will contain chloroform and methanol) should go into the volatile organics waste bottle. This bottle should be kept in flammables cabinet when not in active use.

Through training by a qualified member of the lab should be done prior to any lipid extraction. All persons must have up to date safety trainings.

Special precaution to not inhale chloroform, or put skin in contact with acids.

Be sure all machines (vortex, sonicator, centrifuge) are in working order and you have been trained on their use.

This experiment is designed to be done in the fume hood of FISH 242. This room contains the specific equipment needed to safely run this protocol.

Supplies

- Glass dram vial (~1 dram)
- Tweezers
- Kimwipes
- Rubberbands
- Small dram vial tube box
- Ball mill supples
- Metal spatula
- Ethanol
- Weigh paper

- 0.35 mL aluminum weigh boat https://www.coleparmer.com/i/cole-parmer-micro-aluminum-weighing-dishes-13mm-dia-x-3-5mm-h-35-ml-100-pk/0101907?searchterm=0101907#
- 12 mL Kimax tube with lid https://www.google.com/url?hl=en&q=https://www.fishersci.com/shop/products/kimax-reusable-tubes-ptfe-faced-rubber-lined-screw-cap-8/1493010b&source=gmail&ust=1484281135097000&usg=AFQjCNEWVYHWbZQM-B-uAdcFk65Nd2pibg
- Methanol
- Chloroform
- Hydrochloric acid
- Nanopure water
- Pasture pipette
- Glass pasture pipette tips 5 ¾ inch http://www.sigmaaldrich.com/catalog/product/aldrich/cls7095b5x?lang=en&region=US
- Pasture pipette bulb

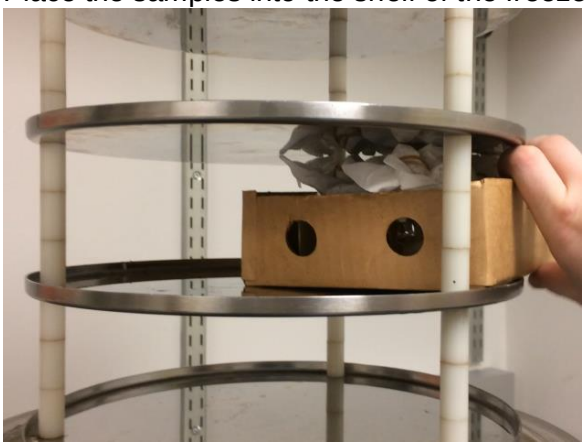
Samples (At least 100g or more. The more the better)

- Kept at -80°C in a glass dram vial until ready to freeze dry
    - Gill
    - Abductor muscle
- Label all caps and vials

Freeze Drying

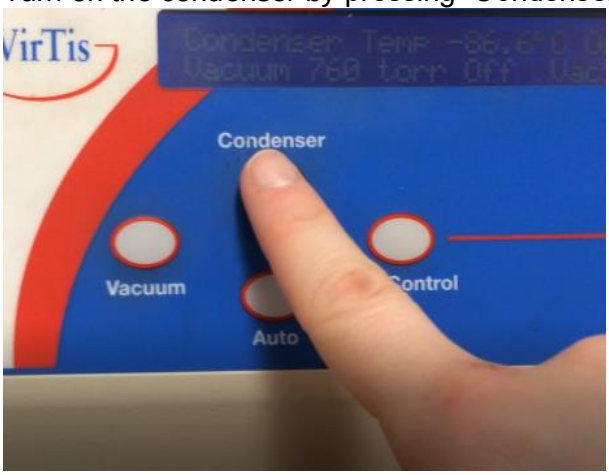
1. Remove caps of dram vials
2. Use a small piece of kimwipe and wrap it around the top of the vial with a rubber band
    a. To prevent the tissue from flying out of the bottle in the vacuum of the freeze dryer

3. Place the samples into the shelf of the freeze dryer



4. Check that vacuum and condenser are OFF.

5. Place the large O-ring onto the freeze dryer acrylic manifold cylinder
    a. Use Dow Corning High Vacuum Grease if O-ring is dry
6. Turn on the condenser by pressing “Condenser”

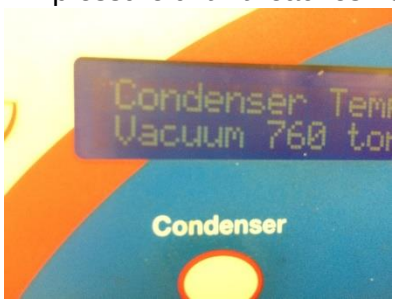


7. Place acrylic manifold over the top of the metal shelving unit, making sure the rubber O-ring stays in place

8. Make sure the flat clear plastic piece on the bottom of the shelves is flush with the round opening of the freeze dryer unit
    a. Use grease if necessary
9. Turn all greased pieces ¼ turn to secure them air-tight
10. Make sure all the brown and white valves on top of the manifold are closed (vertical)
11. Make sure the drain hose is not plugged into the front of the unit
12. Wait until the condenser reaches -40°C
13. Press the “Vacuum” button

14. Wait until the vacuum reaches below 100mTorr
    a. This will take about 10-15 min
    b. If it takes too long, make sure everything is air tight
15. Keep samples in the freeze dryer for 48 hours
16. Turn off the vacuum

   a. Slowly turn one of the brown and white valves at the top to bleed out the pressure until it reaches 760 torr



17. If you are not going to grind the tissue right away
   a. Fill with argon gas
   b. Put lid on

18. Store in dry box

19. Insert the drain hose into the gray slot in the front of the freezedryer

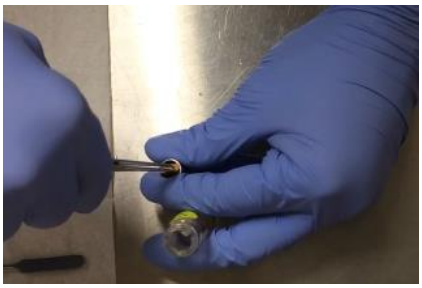
20. Press “Compressor” and “up” at the same time to start defrost mode
   a. 1 hour

21. Use 2 L of DI water to rinse the metal coils
   a. ESPECIALLY because we have salt water in our samples

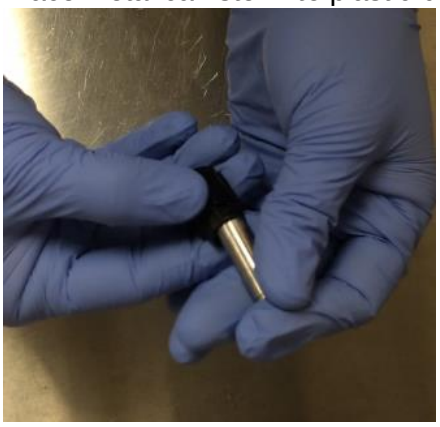
Ball Mill

1. Prepare sterile tweezers, metal spatula, and ball mill supplies
    a. Ball, metal canister, plastic cover canister

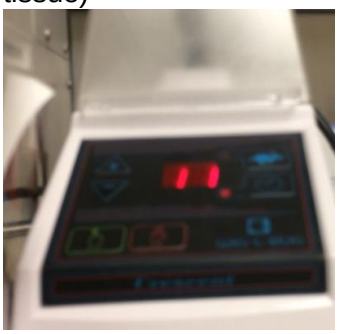
2. Place metal ball into metal canister
3. Place freeze dried tissue into the metal canister and place the metal lid on



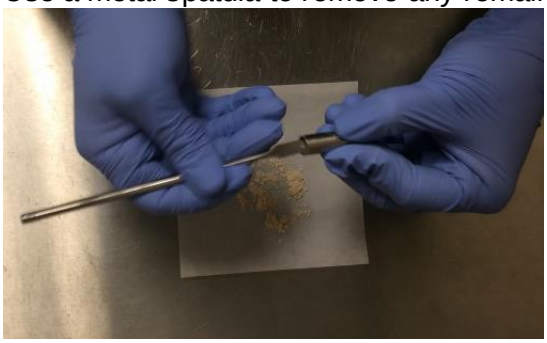
4. Place metal canister into plastic ball mill canister and close it, place it into the ball mill



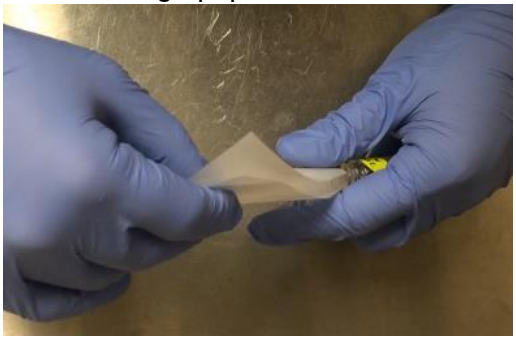
5. Set the ball mill for 10-30 seconds (depending on the amount and consistency of the tissue)



6. Open the canisters and tap out the powder onto a piece of weigh paper
7. Use a metal spatula to remove any remaining powder from the canister



8. Curl the weigh paper into a funnel and return the dust to the dram vial



9. Sterilize all tools and canisters with ethanol between each sample, dry thoroughly
10. If you will not be sampling the powder right away

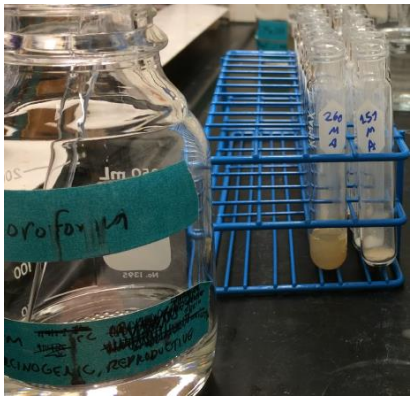
a. Fill the tube with argon gas
b. Place them in a dry box

Total Lipid Extraction

1. Acid wash 48 12 mL Kimax centerfuge tubes
2. Muffle glass tubes for 2-4 hours at 500°C (not the lids, just let the lids air dry)
3. Muffle 5 ¾ cm pasture pipettes
4. Use a wash bottle to rinse all tubes with chloroform and methanol
    a. Empty liquid into hydrogenated waste bottle
    b. Let them dry completely (usually several hours or overnight)
    c. Label all tubes with sharpie (SHARPIE ONLY! No other markers)
    d. Each sample needs 2 tubes one is “tube A” one is “tube b”

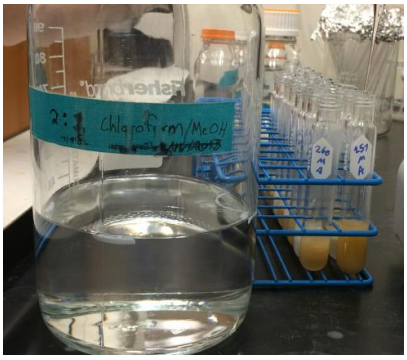
5. Weigh (24 samples) 15 mg of freeze dried powdered tissue on the microbalance in 333. Add to “tube A”.
    a. Use 0.35 mL aluminum weigh boats
    b. Use a new weigh boat for each sample
    c. Make sure to clean any tools used with ethanol between samples
    d. Clean any spilled powder with a vacuum

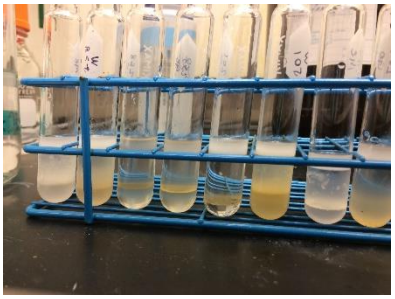
6. Record weight
7. Record any weight remaining in the weigh boat and subtract from tissue weight
8. Use pasture pipette and glass pasture pipette tips for all liquid addition
    a. Use a clean pipette between chemicals and between samples if sample is touched with the glass tip
9. Add 2 mL chloroform and swirl.

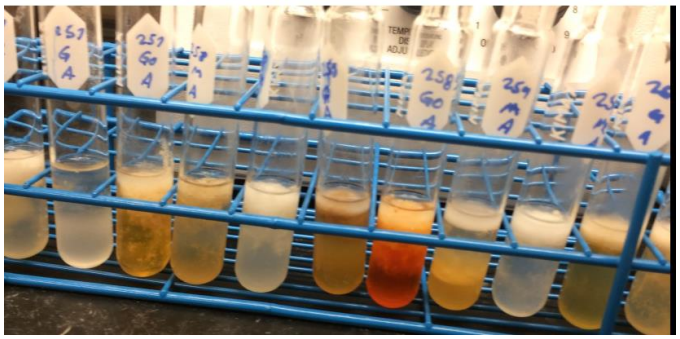


10. Add 1 mL methanol (swirl).

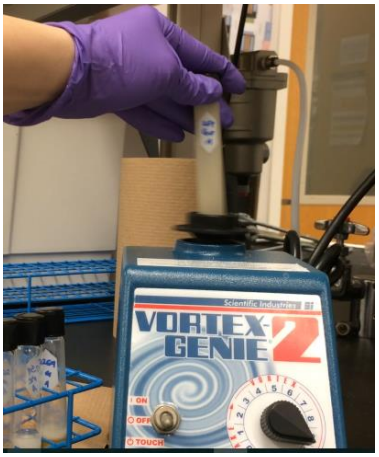
11. Add 1 mL 2:1 cholorform:methanol
    a. 500 ml chloroform
    b. 250 ml methanol



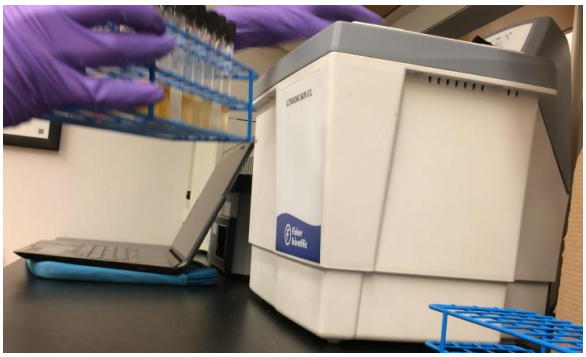




12. Screw on the lids
13. Vortex for 5 seconds to mix



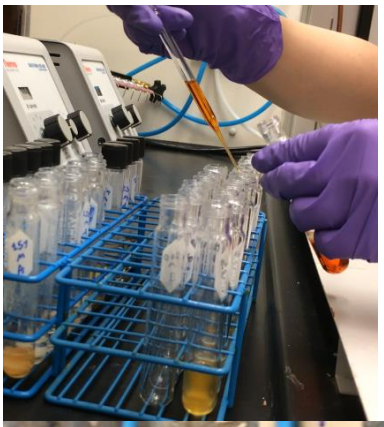
14. sonicate for 20 mins
    a. Make sure the water is covering the tubes, but they are not floating out of the rack



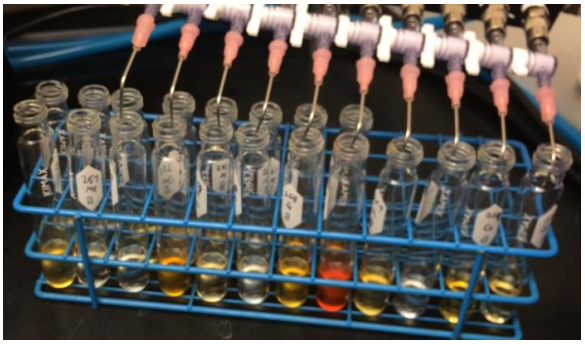
15. Add 500 uL of Nanopure water to each vial

16. Cap and vortex for 30 second (to make an emulsion)
17. Centrifuge for 10 mins at 3000 rpm
    a. Make sure to balance if not all slots are used

18. Prepare a pasture pipette with a bulb
19. Label 24 more tubes with the same labels as your current tubes, these are “tube B”
20. Remove most of bottom “organic” chloroform layer and place it into tube B
    a. This contains the lipids
    b. Use slight pressure when moving the pipette through the upper layer and pellet
    c. Be careful not to put any piece of the hard pellet into tube B
    d. Make small bubbles to push any of the upper layer out of the pipette
    e. It is not necessary to get all of the layer, but get most



21. Add 2.7 mL of 2:1 chloroform:methanol to tube A
22. Add 0.3 mL more Nanopure water If needed to form a pellet
23. Vortex for 5 seconds
24. sonciate for 10 mins
25. Centrifuge for 10 mins at 3000 rpm.
26. remove most of bottom layer into tube B (repeat step 20)
27. Blow B tube to dryness under N2 or over several days in the fume hood



28. Add 1 mL 2:1 chloroform:methanol to B tube and vortex until fully dissolved
29. Transfer two aliquots of 200 uL to two pre-weighed and labeled aluminum weigh boats. (i.e., duplicate measurements)
30. As for the rest of the liquid in tube B, blow to dryness, cover with parafilm and freeze for later fatty acid analysis.
31. Evaporate samples in metal boats in hood.
    a. This may take a few minutes to a hour
    b. You should see no liquid, and a thin sticky layer at the bottom of the boat
32. Reweigh the boats on microbalance to get the weight of the lipids
33. Multiply this value by 0.2 (20% of the 1 ml of dissolved lipids in solution)
34. Average the duplicate values into a single number for that sample
35. Divide by the lipid weight to get the total lipid percentage for each sample