Chapter 2

Lipid Extraction Techniques for Stable Isotope Analysis and Ecological Assays

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Abstract

Lipid extraction is an important component of many ecological and ecotoxicological measurements. For instance, percent lipid is often used as a measure of body condition, under the assumption that those individuals with higher lipid reserves are healthier. Likewise, lipids are depleted in ^{13}C compared with protein, and it is consequently a routine to remove lipids prior to measuring carbon isotopes in ecological studies so that variation in lipid content does not obscure variation in diet. We provide detailed methods for two different protocols for lipid extraction: Soxhlet apparatus and manual distillation. We also provide methods for polar and nonpolar solvents. Neutral (nonpolar) solvents remove some lipids but few non-lipid compounds, whereas polar solvents remove most lipids but also many non-lipid compounds. We discuss each of the methods and provide guidelines for best practices. We recommend that, for stable isotope analysis, researchers test for a relationship between the change in carbon stable isotope ratio and the amount of lipid extracted to see if the degree of extraction has an impact on isotope ratios. Stable isotope analysis is widely used by ecologists, and we provide a detailed methodology that minimizes known biases.

Key words Lipid extraction, Stable isotope analysis, Polar lipids, Neutral lipids, Soxhlet apparatus, Ecophysiology, Ecotoxicology, Diet reconstruction

1 Introduction

Stable isotope analysis (SIA) can be used to determine what constitutes a consumer's diet [1–3]. As a consumer digests its food, the nutrients are assimilated into the consumer's tissues. Atoms from the food are absorbed in the gut and used for metabolism and repair in all tissues in the consumer's body; thus, if the ratio of heavy to light isotope (e.g., ^{13}C:^{12}C) is different for different prey items, one can deduce what proportion of each prey was eaten by the consumer. In particular, stable isotope ratios are often used to disentangle variation in contaminant levels associated with diet

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from variation associated with environmental factors [4–6]. A continuous-flow stable isotope mass spectrometer measures the weight of heavy and light isotopes of different elements from various tissues relative to a standard reference material, resulting in the stable isotope ratio of that individual [2, 3]. Different types of tissues grow at different rates and during different times of year, so different types of tissues can be used to get diet information from different time periods [2, 3].

^{13}C is depleted in lipids compared to proteins and variation in lipid content can confound interpretation of diet [7–10]. To estimate stable isotope ratios in proteins, it is therefore necessary to chemically extract lipids from samples before measuring stable isotope ratios, or to algebraically account for such effects for most consumer tissues [11–20].

Several methods have been used to chemically extract lipids from tissues. The most common method uses chloroform–methanol as a solvent [21, 22]. However, sometimes petroleum ether, hexane, and ethyl acetate/alcohol are used as alternative solvents [6, 23]. As both chloroform–methanol and ethyl acetate are more polar than petroleum ether or hexane, those compounds extract a greater proportion of polar compounds, including proteins, than petroleum ether or hexane [23–25]. In particular, nonpolar solvents (e.g., hexane, petroleum ether) only remove neutral lipids while polar solvents (e.g., chloroform-methanol) also remove structural lipids, such as phospholipids. As a result, stable isotope values on tissue extracted with polar solvents tend to be more enriched in ^{13}C and ^{15}N than tissue extracted with nonpolar solvents [23, 25].

Here, we provide a detailed protocol for preparing tissues for stable isotope analysis. We provide only the methods up to the point of weighing and encapsulation. Samples are then sent to a stable isotope laboratory for the measurement of stable isotope ratios using an isotope ratio mass spectrometer. Various tissues can be used for stable isotope analysis, and we provide representative methods for muscle and hair. Most soft tissues (i.e., eggs, liver, other internal organs) can be treated virtually identically to muscle. Feathers can be treated very similar to hair. Most tissues can be prepared with minor modification to these methods.

We focus on the lipid extraction step. Apart from stable isotope analysis, lipid extraction is widely used to measure lipid content (*see* **Note 1**). Percent lipid is often used as an index of body condition, under the assumption that fatter individuals are healthier, and our process could also be used to measure body condition. Percent lipid is also used to normalize lipophilic contaminant concentrations, as tissues that have higher lipids are likely to be more contaminated with lipophilic contaminants (e.g., PCBs, DDE, etc.). For each of those applications, Subheading 3.2 or 3.3 can be used to estimate lipid content. The lipid extraction technique (Subheading 3.2 or 3.3) can be used in many ecological applications where lipid content is desired. For instance, such a procedure

can be used to measure lipid content for contaminants analyses or to measure body condition. Regardless, the Soxhlet apparatus is preferable to manual washing because it automates the procedure and improves reproducibility.

The solvent used clearly impacts carbon and nitrogen stable isotope ratios (*see* **Note 2**). Nonpolar solvents (e.g., petroleum ether, hexane) extract only the neutral lipids that store energy while polar solvents (e.g., chloroform-methanol) extract structural lipids, such as phospholipids, as well as neutral lipids. In most cases, the simplest matrix possible is desired, to minimize variation associated with matrix composition. In that case, the chloroform:methanol method is likely preferable, as it completely extracts all lipids. However, lipid extraction also alters nitrogen isotope ratios because amino acids bound to phospholipids on cell membranes, and other lipids are usually naturally depleted in ^{15}N [26]. Thus, accurate measurement of $\delta^{15}N$ requires a separate analysis of nonextracted tissue. As an alternative to chemical lipid extraction, it is possible to account for lipid content using the carbon to nitrogen ratio. Such algebraic approximations have nearly five times as much error as the chemical lipid extraction outlined in this protocol [20].

We provide a detailed, reproducible methodology for preparation of samples for stable isotope analysis, including lipid extraction. With minor modification, our protocol can be used for most tissues. The carbonaceous exoskeleton of arthropods and some other invertebrates must be removed using acid hydrolysis because calcium carbonate is enriched in ^{13}C, causing similar issues as lipids [27]. Likewise, the removal of collagen from bone requires additional work and various washing techniques can be applied to feathers to remove preen oils [28, 29]. However, our methods work well for most tissues.

2 Materials

1. Disposable 2 mL microcentrifuge tubes along with permanent markers to write on the tubes and fiberboard storage boxes and dividers to store tubes, and kimwipes for cleaning.
2. Benchtop freeze dryer, standard lab oven with electronic control, drying chamber, and desiccator cabinet.
3. Vacuum pump with pump oil.
4. Soxhlet apparatus with Allihn condenser.
5. Standard orbital shaker and centrifuge rotary evaporator.
6. Glass microfiber filters (55 mm).
7. High performance cellulose extraction thimble (1 mm wall, 10 × 50 mm).
8. Multi extraction mantles—6 recess, heating only, 250 mL.

9. Hook connector, 3-prong dual adjustment clamp.
10. Refrigerated bath circulator.
11. Microbalance (e.g., 5.1 g × 1.0 μg).
12. Petroleum ether or chloroform (99%) and methanol.
13. Clear PVS tubing 5/16″–50″.
14. 30 mm jar forceps.
15. 20 mL glass screw thread scintillation vials.
16. Tin capsules 5 × 9 mm.
17. Flat-bottom plates with lids.

3 Methods

3.1 Subsampling Tissue

1. The protocol described here is specifically for muscle, but can be used with little alteration for any other soft tissue, such as egg contents, plasma, blood, liver, pancreas, stomach, or any other internal organs. Digestive tract contents would need to be emptied and rinsed prior to any analysis. Liquids (e.g., plasma, blood) are typically freeze-dried prior to any analysis, to simplify homogenization. Subsampling of liquids would occur with a pipette following vortexing.
2. General sanitation. Preparing samples for stable isotope analysis requires certain steps to be followed to ensure that accurate results are obtained. Cross contamination is a serious concern when preparing samples. Samples are measured in micrograms, so contaminants that might be negligible to other procedures can be highly influential to our results. To prevent contamination, clean gloves should be worn at all times when working with samples, to protect both the sample and the researcher. In this protocol, we use a 70% ethanol (alcohol) solution to clean and disinfect our tools, the counter, other surfaces, our gloves, etc. Before working with each sample, the workspace and tools should be wiped with a Kimwipe and ethanol. Do not forget to put everything away, especially samples, which may become contaminated or start to decompose. Tools should be washed with soap and water and dried or left to dry (on the rack) before being put away. Make certain to create an accurate labeling method so that each sample can be uniquely identified throughout the process.
3. Subsampling from muscles. Tools and supplies needed for sampling include gloves, cutting board, scalpel or knife, micro-centrifuge tubes and boxes, forceps, fine point marker, kimwipes, and ethanol. In our lab, muscle samples are often provided as pieces of frozen meat in a small container or bag, usually far more sample than is needed for stable isotope

analysis. Begin by cutting the sample into small (approximately grape-sized) pieces with either a scalpel or a knife. Try to include only lean, red muscle, with as little fat and connective tissue as possible. This may be difficult depending on the quality of the sample, so patience and attention to detail are required. The sample run for stable isotope analysis should consist of a 2 mL micro-centrifuge tube filled about half way with muscle. The tube should be labeled with a fine point marker on the lid and side with the sample ID number. Use forceps to pack the sample into the tube; avoid leaving air pockets underneath the sample, as they may expand and force the sample out of the tube during freeze–drying (next step). Once filled, these tubes should be placed in a micro-centrifuge box to help avoid confusion should the samples come out in the freeze dryer. Leave an empty space between tubes in the micro-centrifuge box to prevent contamination if a sample is forced out of its tube (covering the tube with parafilm with perforations should further ensure that) during freeze–drying. Additional samples can be archived for future use or otherwise disposed appropriately.

4. Freeze–drying. Prior to stable isotope analysis, samples must be dried to remove all traces of water from the sample and prevent decomposition during storage. Freeze–drying is the preferred method for muscle samples or other soft tissues, but hair, feathers, or plant samples can be dried in a drying oven. Samples should be frozen before using the freeze dryer, as any liquid material may foam and expand, causing cross-contamination with neighboring samples. Ensure that all tube lids are open so the vacuum reaches the sample. If necessary, several boxes of samples can be stacked on top of each other. Grease the seals with silicon to create a strong seal. Ensure that the air valve is closed, and that the plastic end cap is inserted into the end of the rubber drain hose attached to the bottom of the dryer. Turn the dryer on. Samples typically need at least 48 h of freeze–drying to completely remove moisture and will not be affected by freeze–drying for longer than is necessary. The freeze dryer can be left running overnight or over the weekend. Once the samples have had at least 48 h in the dryer, end the freeze–drying. Very slowly open the valve on the top to allow air into the dome. It will be possible to hear the air rushing in. Once the air can no longer be heard, take the samples out. Leave the dome off so moisture from the freezer coils does not condense on the inside. Take the end cap out of the drain hose to allow the liquid to drain. Once the frost on the freezer coils has melted, use paper towels to dry the inside of the freeze dryer. The samples are now completely dehydrated and no longer need to be kept frozen, and can be stored in a desiccator cabinet.

3.2 Lipid Extraction by Soxhlet Apparatus

1. The soxhlet apparatus is commonly used in chemistry applications for dissolving soluble substances from a solid matrix. Freeze–drying has removed all the water from the sample, but not the lipids (oils and fats). The Soxhlet removes lipids by bathing the sample in a solvent that will dissolve and remove lipids from the sample (e.g., petroleum ether or chloroform). A heater under each of the Soxhlet extractors boils the solvent, and the vapors rise and condense in the top section, which is cooled by water. The freshly distilled solvent drips down from the condenser to saturate the samples in the extractor, and when the solvent level rises high enough, it fills a siphon and drains back into the reservoir to be boiled again. Thus the samples are constantly bathed in fresh solvent, while the lipids become concentrated in the bottom reservoir. The Soxhlet apparatus should be set up in a fume hood, in case any solvent escapes past the condensers. Before starting, the following tools are needed: gloves, a set of cellulose thimbles in their beakers, the corresponding data sheet, a pencil, a mortar and pestle, 55 mm glass microfiber filter papers, Kimwipes, and ethanol.
2. Identifying thimbles. Each sample will be placed in a cellulose thimble, and each extractor holds a set number of thimbles; in our extractor it is six 10 × 50 mm thimbles. We use notches in the top of each thimble to identify individual samples, which correspond to a sample ID number indicated on the data sheet. Thus, a set of six notched thimbles is needed for each extractor.
3. Breaking up the sample. Crushing the dried sample with mortar and pestle for a few seconds will break up connective tissue in muscle, make the sample easier to wrap in the filter paper, and allow the solvent to penetrate more easily. Take the sample out of the micro-centrifuge tube and put it into the mortar. Crush the sample with the pestle for a few seconds. Ideally the sample will break into small pieces, but some will not come apart as easily. This should not matter, as long as the sample can still be made to fit into the thimble later. Some samples may appear greasy or even wet, most likely because the sample might have excessive fat content; proceed with Soxhlet preparation like any other sample.
4. Addition to filter paper. To prevent bits of cellulose shed from the thimbles from contaminating the samples, each sample will be wrapped in glass microfiber filter paper. Dump the sample out of the mortar onto a filter paper. Carefully wrap the sample in the filter paper by folding over each end and rolling it up like a burrito. There may be more sample than can fit in a single filter paper without ripping it, but one paper will hold plenty for stable isotope analysis. Before inserting the sample, check the thimble for large pieces of paper or sample that may be

stuck in the bottom, remove any such pieces, then slide the rolled paper into the thimble. Forceps may be needed to insert the paper completely. Be careful not to rip the filter paper. If a rip occurs, throw the paper out and rewrap the sample in a new paper. Clean everything with ethanol and a Kimwipe. Make sure the mortar is dry before adding a new sample.

5. Preparing the Soxhlet. To remove neutral lipids a nonpolar solvent such as petroleum ether or hexane can be used. To remove all lipids (including phospholipids and structural lipids) a polar solvent must be used, such as chloroform/methanol. However, the latter will also remove a small amount of non-lipid material. Check the solvent level in the flask. The volume of solvent in the flask should be three to four times the volume of the extractor chamber (usually about half full), with a few boiling chips in the bottom of the flask. The solvent can be reused for multiple extractions and slowly turns yellow as more lipid becomes dissolved in it and eventually will need to be replaced (we keep a waste ether jug stored in a flammables cabinet). The boiling chips can be reused, so try not to dump them out of the flask. Petroleum ether and other solvents are toxic and the fumes are highly flammable, so when adding new solvent to the flask only pour it under the fume hood and be careful not to get it on exposed skin. Use a glass funnel to avoid spills. Fit the Soxhlet sections back together and clamp them back into place. Fill the other extractors in the same manner. Leave each empty beaker beside the tube that contains the corresponding thimbles, and leave the data sheet under the fume hood with the samples to avoid confusion about what is in the Soxhlet.
6. Loading the Soxhlet. Once all samples have been prepared, or all 36 thimbles are full (for a 6-heater unit), begin loading them into the Soxhlet. First unscrew the clamp at the top and carefully slide the condenser (the top section) up and out of the extractor (the middle section), then retighten the clamp to hold the condenser in place. Slide the extractor out of the flask (the bottom section). Use long forceps to slide the six thimbles from one beaker into the extractor, all the way to the bottom. If they are stacked on top of each other they will not become fully immersed in the solvent and the Soxhlet will not be effective. Make sure the thimbles are not blocking the siphon as this will not allow the solvent to drain back into the flask.
7. Starting the Soxhlet. Before turning on the Soxhlet heater, check the water level in the recirculating chiller by taking the hatch off the top. It should be full right to the very top. Add more water if necessary. If the water runs out while running, the coolant will fail and the Soxhlet will boil dry. Turn on the chiller pump. Use the buttons to navigate the screen menu. Adjust the temperature to 10°. High humidity sometimes

causes water to condense inside the Soxhlet, which interferes with the solvent draining. Under these conditions a higher water temperature may help avoid this moisture condensation, but may slow the rate at which the solvent condenses.

8. Extraction in the Soxhlet. Once the water reaches the desired temperature, turn on each of the heaters under the flask holding the solvent (at a setting of approximately 4.5 on our unit). Check the Soxhlet after a little while to ensure that the solvent is at a slow, steady boil and that it is draining properly. Don't forget to close the fume hood to its operational level. Samples need at least 8 h in the Soxhlet. It can be left running overnight, but not for longer than 24 h or the solvent may boil dry.
9. Removal from Soxhlet. After running, turn off the Soxhlet heaters and remove the samples, placing them back into their appropriate beakers with the long forceps. Place the beakers with the samples into the drying oven set at 60 °C for at least 48 h. Keep the data sheet with the samples in the oven to avoid losing it.

3.3 Lipid Extraction with a Shaker (If a Soxhlet Apparatus is Not Available, Lipid Extraction Must be Performed Manually)

1. Weighing the sample. Add freeze–dried and broken up tissue (Subheading 2, **steps 1** and **2**) to a sample tube labeled with the sample ID number. Weigh the tube before and after the sample is added.
2. Extraction. Add the solvent to the sample tubes, typically chloroform/methanol (2/1), which is known as Folch's reagent. The final volume of the solvent should be 20 times the tissue sample (50 mg of dried tissue in 1 mL of solvent mixture). Place the box of sample tubes on a shaker and agitate the whole mixture at room temperature for 15–20 min.
3. Centrifugation. After shaking, centrifuge the samples for 3 min to separate the sample tissue from the solvent. After centrifugation, pour out the solvent and repeat Subheading 3.2. This extraction step should be repeated three times.
4. Evaporation. Evaporate the samples under vacuum in a rotary evaporator for 45 min or under a nitrogen stream if the volume is low. Reweigh the sample (the difference in weight will provide the lipid content of the original sample).

3.4 Unpacking and Homogenization

1. Once the samples have dried, they must be removed from the thimbles or sample tubes and prepared for weighing. Before a sample can be weighed, it must be reduced to a fine powder. The following items will be needed: gloves, forceps, a stainless-steel dissection probe, vials (such as scintillation vials), a black permanent marker, Kimwipes, and ethanol.
2. Label the vial with the sample ID number on the lid and also on the sides of the vial (written twice, 90° apart to reduce the

chance of smudging both labels by pinching between thumb and forefinger). If lipids were extracted using the shaker method, place the sample in the vial and proceed to **step 3.5**. If using the Soxhlet method, continue with **step 3.3**.

3. Carefully remove the sample from the thimble. Be gentle, as the filter paper will be very delicate and can rip easily. Check the thimble to make sure no sample or large chunks of filter paper remain in the bottom. Put the thimble back in the correct beaker.
4. Unwrap the filter paper, empty the sample into the vial, and discard the filter paper. If small pieces of filter paper become stuck to the sample, try to remove these with forceps. It may not be possible to remove all traces of paper, while this is not ideal, it is acceptable since the glass microfiber filter paper contains no nitrogen or carbon that will affect the measured stable isotope ratios (although it could affect the sample weight, which would affect %C and %N estimates).
5. Use the blunt handle end of the probe to homogenize the sample inside the vial. Be careful not to use too much force as the glass vials are prone to breaking. If the vial breaks, transfer the sample into a new vial, and carefully remove all traces of broken glass from the sample. Pounding or grinding the sample should reduce some of the sample into a fine powder, which is what is weighed out and prepared for final analysis. Only a very small amount of powder is needed; even a thin coating on the bottom of the vial should be enough. Notice that the majority of the sample will not become powder. Although this is mainly due to connective tissue, the sample must be broken down as much as possible. The point of homogenizing (crushing) the sample is to have a representative subsample when weighed.
6. Tighten the lid on the vial and place it back in the tray. Clean the workspace and utensils with ethanol and Kimwipes before moving on to other samples.

3.5 Weighing

1. A precise amount of sample must be weighed out on the microbalance and carefully packaged in tin capsules for mass spectrometer analysis. This final step of sample preparation demands a great deal of precision, any mistake at this stage is almost guaranteed to affect the accuracy of the final results. Proper sterilization and cleanliness of workspace and materials are particularly important to avoid contamination. The microbalance is a delicate and expensive instrument. It must be handled carefully; even a vibration in the table may affect the reading. After weighing and wrapping, the samples are sent to a laboratory for analysis using a mass spectrometer. The results are typically emailed back. The following equipment is needed:

gloves, one or two sets of forceps, a well plate, tin capsules, a stable isotope submission spreadsheet, a pencil, a red marker, Kimwipes, and ethanol.

2. Turn the Microbalance on. The balance will probably need to be calibrated when turned on and after every few hours of operation. If the calibration light flashes while weighing a sample, finish weighing it before starting calibration. Touch the tab to begin calibration. Calibration is automatic and usually takes 2 or 3 min. Once finished, press *back* on the screen to return to weighing the sample.
3. Open the balance door. Using forceps carefully place a tin capsule on the raised center tray. Try to place it as close to the center as possible, where the measurement will be the most accurate. Close the balance door to prevent drafts from interfering with the measurement (Yes—slight air drafts in the room can affect the weight of the sample). The weight displayed on the screen will fluctuate first, but it will settle on a weight after a few seconds. In general, it has not settled until *mg* appears after the weight, and it may still change after. Once it has stopped fluctuating, press the *tare* button to zero the weight. From now on, it will display the weight of the sample in the capsule only, not the capsule itself. Every capsule will have a different weight, so it is crucial to tare the balance with each new capsule.
4. Open the door and remove the capsule. Close the door afterward to prevent powder from entering the balance because it may affect the weight or contaminate the sample. Stand the capsule on the sterilized lab bench to add sample. The forceps can be used to gently open the rim of the capsule more. The capsules are made of pure tin so that it does not contaminate the sample. They are very thin and very fragile, so be careful not to crush or tear them. If the tin is torn, discard the capsule and begin again.
5. Very carefully use the forceps to take some of the powdery sample out of the vial and place it in the capsule. Very little is needed (usually 0.4–0.6 mg for muscle, but consult the lab where the stable isotope ratios will be measured). Only add the fine powder, not chunks of sample as these chunks may not be pure tissue (or may over-represent a certain period). When enough sample is in the capsule, open the balance door and place the capsule in the center of the tray like before. Be careful not to spill. If the sample is stuck to the forceps or the outside of the capsule, it should be gently wiped off with a Kimwipe before entering the balance. Different tissues will have different weight windows. If the weight is below this window, the sample may not be detectable by the mass spectrometer, and if it is higher, it may go off the calibrated scale.

6. If the weight is not correct, then add or remove sample. Be very careful when removing the sample, as the point of the forceps can easily tear the bottom of the capsule. If this happens, throw the capsule away and restart.
7. Once the correct weight is obtained, roll or squeeze the capsule into a tight ball or cube using two pairs of forceps or gloved fingers. It is sometimes easiest to crimp the end closed with forceps before rolling to prevent the sample coming out while rolling. The ball or cube should be as small as possible with no cracks, protrusions, or angular edges as these may become caught in the mass spectrometer's autosampler.
8. Place the ball back in the balance, close the door, and record the final weight in the data sheet. The weight will likely be slightly different than the weight previously measured, and it may even fall outside of the target window (typically 0.4–0.6 mg for muscle, but it will depend on the lab). If it is within 0.005 mg of the window, it is usually considered an acceptable error. If it is off by more than 0.005 mg then throw it away and restart (aim for 0.5 mg when weighing muscle).
9. Open the well plate and place the ball into the well indicated by the data sheet. Make sure to replace the plate lid to keep powder and other contaminants out. Use the marker to make a small red dot on the lid of the vial to indicate that this sample has been completed, as it is difficult to tell otherwise.
10. Be sure to wipe down all workspaces and utensils (including hands) before moving on to the next sample. If powder or spilled sample is on the central tray of the microbalance, wipe it off with a Kimwipe. The glass door can be removed by lifting straight up, make sure to line up the notch in the bottom of the door with the peg on the balance when replacing it.
11. Once finished weighing samples, tape the plate lid closed at all four sides, label the tray, and put the plate in the desiccator for storage. Be sure to close the door before turning the microbalance off.

3.6 Processing Hair Samples

1. Most mammals have two types of hairs: guard hair (overhairs), which grow from primary follicles, and underfur (underhairs), which grow from secondary follicles. These hair types often grow at different times of year, so they can be separated to provide more specific dietary information. Guard hair is long, thick, and straight, like the bristles on a paint brush, while underfur is short, thin and may be curly like a cotton ball. We also collect an archive sample containing both types. The following items are needed: gloves, coin envelopes, vials (such as scintillation vials), scissors, a black marker, petri dishes, one or two pairs of forceps, a moustache comb, Kimwipes, and ethanol.

The steps here are specifically for hair, but can be adopted equally well for other external tissues, such as modified scales or feathers. In the case of feathers, they should be washed with detergent and then chloroform-methanol to remove preen oil and other contaminants that would reflect current diet rather than diet when the feather was grown [26].

2. Labeling sample. Label one vial with the sample ID number followed by GH for guard hair, and the other with the ID plus UF for underfur. Label them on the lid and twice on the side. Cut a chunk of hair off a sample as close to the skin as possible. Place it in a petri dish to help contain the hair. The guard hair should stick up above the underfur. Pinch the top of the guard hair between fingers and run the mustache comb, or a set of forceps through the fur. After a few passes, only guard hair will be left in the hand. By the same token the hair that gets caught in the comb will be mostly underfur. A few stray guard hairs may need to be removed one by one.
3. The hair needs to be washed to remove any traces of blood, dirt, feces, or other contaminants. In addition to the tools previously listed, dish soap, a beaker, and a fine mesh tea strainer will also be needed. Use about a tablespoon of dish soap to make some soapy water in a large beaker. Pour about half a vial full of soapy water into the scintillation vial containing the hair. Close the lid and shake the sample vigorously for no less than 1 min. It is optimal to shake multiple samples at the same time to speed the process up.
4. Rinse the sample. Dump the sample into a tea strainer. Rinse out the inside of the vial and lid under high pressure water. Rinse the hair in the strainer under high pressure water. Use the forceps to lift the hair above the strainer and rinse the hair thoroughly. In the past, we have had problems with soap residue being left on the vial or sample, which can confound the stable isotope results. It is vital to rinse the vial, lid, and sample very thoroughly. Once the samples are rinsed, put it back in the appropriate vial and repeat the process, so each sample gets washed and rinsed **twice**. After the second rinse, put the sample back in its vial and place it in the drying oven (place the lid next to the vial in the oven). Let the sample dry for 48 h.
5. Homogenizing. A representative sample of the hair is needed, so the hair needs to be reduced to a powder or very small sections before it can be weighed.
6. Method one: Scissors. Scissors may be used for small numbers of samples, or samples with very little tissue. Simply use a pair of scissors to cut up a hair sample in its scintillation vial. Scissors that pivot at the neck of the vial when the tips reach the bottom are the ideal size. The hair needs to be cut into very small

pieces, preferably into a powder consistency. Static may cause sample to stick to the scissors and vial when humidity is low. It may be necessary to stop homogenizing and come back to finish a sample later when the static has dissipated.

7. Method two: Ball mill. The ball mill is a much more efficient way of homogenizing large numbers of hair samples with adequate sample weight. Samples are placed in a stainless-steel grinding jar with a ball bearing. The mill shakes the cylinder very rapidly and the ball bearing grinds the sample into powder. In addition to the earlier tools, before a spatula, paper towels, Kimwipes, and ethanol are needed. The ball mill runs two samples at once, so a second person speeds up this step.
8. There are several different sizes of cylinders for the ball mill; use the two largest sizes. The ball mill runs grinding jars at once, and it is very important to keep it balanced. Always run two jars of the same size at once, even if one jar is run empty. There are also different sizes of ball bearings; use the largest size.
9. Turn the mill on. Dials control the frequency and duration of shaking. Frequency should be set to 30 Hz and duration depends on the amount of sample, but 2 min is standard.
10. Place the sample inside a jar with a ball bearing and close the lid as tightly as possible. Larger samples should be placed in the larger jars. Make sure someone knows which sample is in which jar as the jars themselves are not labeled. Securely fasten a jar in each clamp and tighten them as much as possible to ensure that the jars do not come out while milling. Close the plastic door to contain the jars. The mill will not run with the door open. Press the start button to begin homogenizing.
11. Once the milling is complete, take the jars out and open them. They may be difficult to open, so use the scoopula as a prying implement. The sample should be reduced to powder, and if not, it may need more time in the mill. Scoop the sample out and place in the correct vial. Some time may be needed to scrape sample off the inside of the jar to get enough. Running a second set of jars while working with this set will allow speed up workflow.
12. Clean the cylinders out with water and dry them with Kimwipes before putting a new sample in. Be sure that the cylinders are clear of all sample residue and other contaminants. Clean the workspace and turn off the mill.
13. Weighing. Follow the same procedure (**step 5**) as weighing muscle samples, except the weight window may be different.

4 Notes

1. Typically, the sample can be weighed before and after lipid extraction (Subheadings 2 or 3). In that case, the difference in weight is the weight of lipids. For stable isotope analysis, the laboratory will report the weight of carbon and nitrogen in the sample, and the stable isotope ratio relative to a standard ($\delta^{13}C$ or $\delta^{15}N$). Lipid extraction typically alters both carbon and nitrogen stable isotope ratios. As the alteration in the nitrogen stable isotope ratio is unrelated to lipid content, it is usually ideal to run two sets of samples: one with lipid extraction for $\delta^{13}C$ and one without lipid extraction for $\delta^{15}N$.
2. The type of solvent impacts lipid extraction (Fig. 1). Neutral solvents, such as petroleum ether or hexane, do not extract all polar lipids. Consequently, they do not increase $\delta^{13}C$ or decrease the C:N ratio as much as polar solvents. In contrast, polar solvents, such as chloroform:methanol, extract more lipids, including polar lipids. However, they also remove a greater proportion of non-lipid compounds.

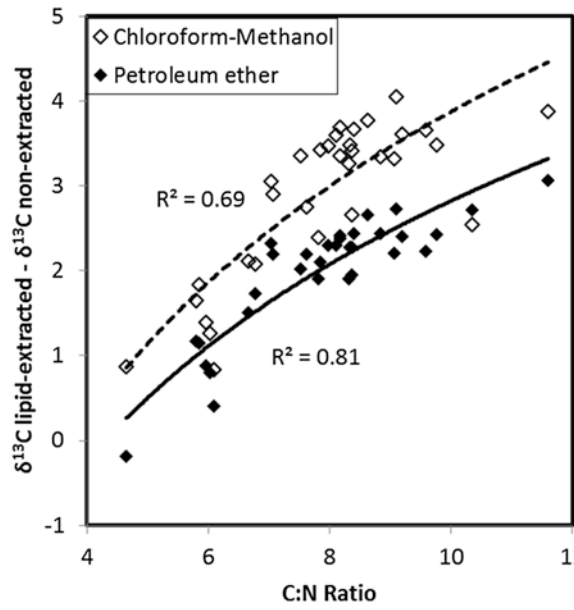

Fig. 1 The change in carbon stable isotope ratio following lipid extraction using petroleum ether as a solvent and using chloroform:methanol as a solvent. *Filled symbols* are for petroleum ether while *unfilled symbols* are for chloroform:methanol

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