Sample Preparation Protocols for Stable Isotope Analysis

Updated September 2021

Required PPE

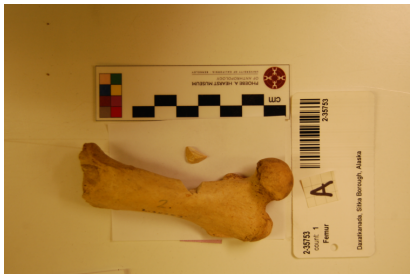
- Long Pants
- Closed-Toe Shoes
- Lab Coat
- Safety Glasses
- Gloves

Required Equipment/Chemicals

- Glass Vials – use 20ml glass scintillation vials with foil-lined caps, as other materials like plastic may react poorly with solvents and contaminate your samples.
- Disposable Glass Pasteur Pipets
- Dremel Tool
- Hydrochloric Acid – Original ACS grade 37%
- Chloroform – >99.5% assay
- Methanol – >99.5% assay
- Deionized (DI) Water
- Drying Oven
- Refrigerator
- Freezer
- Freeze Dryer
- Fume Hood
- 70% Ethanol Wash Bottle
- Kimwipes
- Forceps
- Scoopula
- Tin Capsules
- Silver Capsules

Protocol #1: Demineralization (Bones, Soils)

1) Sample 50-100 mg (ideally, but no less than 25 mg) of bone:
    - Do this using a handheld Dremel Tool. Taking a “V” shaped cut is usually easiest/least damaging for the bones (see photo below).
    - When cutting bone from jaws, try to avoid mixing any tooth material with your sample.



2) Transfer your samples to 20ml glass scintillation vials with foil-lined caps. Carefully label the vial and the cap (archival pens seem to stay on better than Sharpie).

3) Add weak HCl (0.25-0.5 N) to each sample vial (just enough to cover the sample entirely). Put the caps on and then loosen them slightly so air can flow – about a ¼ turn. Place your samples in the fridge (not freezer) for at least 12-24 hours.
4) After 12 to 24 hours (for poorly persevered bones, check sooner), check to see if your bones are fully demineralized by assessing their 'sponginess'. A bone is 'spongy' if it will bend easily while still retaining its integrity. It will also become translucent.
    - If a bone is not spongy, carefully remove the HCl (either by pouring or pipetting off) and replace it with fresh HCl. Put your samples back in the fridge for another 24 hours.
5) **Repeat this process every 24 hours until the bone is fully demineralized.** *Depending on the initial size and density of a sample, the demineralization process can take anywhere from 12 hours to several days.*
6) Once your samples are fully demineralized, rinse them GENTLY with DI water *one at a time*:
    - Carefully remove the HCl (either by pouring or pipetting off), disposing of it in a labeled waste beaker. This waste DI water should be disposed of using the drain in the fume hood.
    - Using a DI water wash bottle, completely fill the vial with DI water. When adding the water, aim it down the side of the vial, making sure not to directly hit your sample with the stream of water, as this may break up the bone. Repeat this process five times for a total of five DI water rinses.
7) Dry out your sample vials by removing as much DI water as possible using a pipet or by tapping the vial onto a paper towel, making sure not to let the sample fall out. Move on to lipid extraction.

Protocol #2: Lipid Extraction (Proteinaceous Tissues: Muscle, Bone Collagen, Whole Organisms): ALWAYS DONE IN A FUME HOOD

1) Transfer your samples to 20ml glass scintillation vials with foil-lined caps. Carefully label the vial and the cap (if spilled, the solvent will remove anything written in Sharpie).
2) In the fume hood, add a 2:1 Chloroform:Methanol solution to your sample vials (just enough to cover the sample entirely) and screw the foil-lined cap on tightly to prevent evaporation. Leave the closed vials in the fume hood for 24 hours.
3) After 24 hours, remove (either by carefully pouring or pipetting off) and replace the 2:1 Chloroform:Methanol in each sample vial. Leave the closed vials in the fume hood for another 24 hours.
    - C:M WASTE DISPOSAL – Be sure to dump used C:M in a labeled waste beaker in the fume hood. *DO NOT* pour C:M down the drain.
4) Repeat step 3 two more times for a total of three 24-hour 2:1 Chloroform:Methanol soaks.
5) After the three-day lipid extraction, rinse your samples with DI water *one at a time*:
    - Remove the 2:1 Chloroform:Methanol, dumping it into the appropriate waste beaker.
    - Using a DI water wash bottle, carefully rinse your sample 6-8 times. Dump the waste DI water in a sperate, labeled waste beaker. This waste DI water should be disposed of using the drain in the fume hood.
6) Once your samples are rinsed, transfer them to new, labeled microcentrifuge tubes. Place your samples in the freezer until you can freeze dry them.

Protocol #3: Solvent Wash (Keratins: Feathers, Hair, Whiskers): ALWAYS IN A FUME HOOD

1) Place your samples in glass test tubes and cover with a 2:1 Chloroform:Methanol solution. Leave the test tubes open in the fume hood for 3–5 minutes.

2) After 3–5 minutes, remove (either by carefully pouring or pipetting off) and replace the 2:1 Chloroform:Methanol in each test tube. Leave the test tubes open in the fume hood for another 3–5 minutes.
    - C:M WASTE DISPOSAL – Be sure to dump used C:M in a labeled waste beaker in the fume hood. *DO NOT* pour C:M down the drain.
3) After this second 2:1 Chloroform:Methanol rinse, rinse your samples with DI water *one at a time*:
    - Remove the 2:1 Chloroform:Methanol, dumping it into the appropriate waste beaker.
    - Using a DI water wash bottle, carefully rinse your sample 6-8 times. Dump the waste DI water in a sperate, labeled waste beaker. This waste DI water should be disposed of using the drain in the fume hood.
4) Let your samples dry in the fume hood. Once they are dry, transfer them into new, labeled vials or microcentrifuge tubes.

Protocol #4: Freeze Drying

1) Check the freeze dryer. Make sure the inside and coils are completely dry (use paper towels to mop up excess water). Replace the top part containing the ports and make sure all the black valves are closed (they are closed when the flat side is facing opposite of the opening).
2) Turn on the freeze dryer. #1 Flip on the black switch on the left side of the machine. #2 Press the temperature (blue) button and wait until ALL of the temperature lights are on (-80°C). #3 Press the vacuum (green) button and wait until ALL of the vacuum lights are on.
3) Prepare to load your samples. Place parafilm over the closed caps of your microcentrifuge tubes and poke two holes in the cap so that water can escape.
4) Carefully arrange your samples in a vacuum flask (round bottom beaker). Cover the flask with a blue top connected to a glass hose. Connect the hose to one of the vacuum ports and turn the black valve slowly until it is completely open (it is opened to the flask when the flat side is on the same side as the hose).
5) Make sure the vacuum goes back down and leave your samples on overnight.
6) When your samples are completely dry, close the black valve slowly and remove the vacuum flask.
7) To turn off the freeze dryer, first turn off the vacuum (green button), then turn off the temperature (blue button), then turn off the machine (black switch).
8) Your samples are now ready to be weighed for SIA analysis.

Protocol #5: Weighing Samples for SIA Analysis

Tin capsules for $\delta^{13}C$ & $\delta^{15}N$ analysis
Silver capsules for $\delta^{2}H$ & $\delta^{18}O$ analysis

1) Get a weighing sheet and a clean sample tray (96-well plate).
    - You can clean trays using Q-tips, 70% Ethanol, and pressurized air.
2) Turn on the microbalance. If needed, calibrate the microbalance with the door closed.
    - '*' button opens/closes the door to the weighing chamber.
    - Tare button zeroes the scale.
3) Clean the glass, forceps, razor blade, scoopula, and any other equipment you are using before you start and between each sample using a 70% Ethanol and kimwipes.
4) Record your first sample ID in the first row labeled 'A1'.
5) Open the weighing chamber (by pressing the '*' button), carefully place a capsule on the scale, and close the chamber. Wait until 'mg' appears on the digital readout (this means the reading has stabilized) and then press 'tare'.

6) Once the reading hits 0.000 mg, open the weighing chamber and remove the capsule. Cut, grab, or scoop your sample and carefully put it inside the capsule.
7) Place the capsule containing the sample material back on the scale and close the door to get an exact weight. Record the weight on weighing sheet, as well as any additional details you may want to keep track of (e.g., double-wrapped).
8) After recording the weight, open the door and remove the capsule. On the glass plate, carefully close the capsule by pinching it with forceps and folding it back over itself. Then continue to press and fold the capsule into a small ball or square. Once your capsule is adequately compressed (with no flaps and no sample pieces poking out), place it into your sample tray in the appropriate well.
9) Once you have weighed all your samples, place a notecard on top of the samples and secure the lid with tape. Make sure the sample sheet and tray are labeled with the same information (e.g., your name, project name, sample type, etc.).
10) Fill out the SIA Sample Submission form found on the CSI website. E-mail this form to Viorel and Laura (cc'ing Seth and Alexi) and take your samples over to the CSI in PAÍS for submission.

SAMPLE TARGET MASSES

The amount of sample material you weigh out will depend on the sample type and isotope system.

Carbon/Nitrogen (Tin Capsules)

Protein: 0.5 – 0.6 mg
Plants/Algae/Fungi: 3 – 4 mg (double-check with Viorel and Laura)
Whisker/Hair Segments: 0.2 – 0.3 mg

Hydrogen (Silver Capsules)

0.2 – 0.3 mg

Oxygen (Silver Capsules)

0.30 – 0.35 mg