Sep 14, 2019

Stable isotopes in avian research: a step by step protocol to feather sample preparation for stable isotope analysis of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and hydrogen ($\delta^{2}H$). Version 1.1

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1 Works for me dx.doi.org/10.17504/protocols.io.z2uf8ew

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ABSTRACT

Summary

Using a 2:1 chloroform methanol solution and Versa^{tm} detergent diluted with deionized water we cleaned feather samples of oil/debris for stable isotope analysis of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and hydrogen ($\delta^{2}H$). Prepared samples were then measured, cut, and packaged in tin or silver capsules for analysis of carbon($\delta^{13}C$) and nitrogen($\delta^{15}N$) or hydrogen($\delta^{2}H$), respectively. We aim to standardize the laboratory procedure across institutions and research groups focused on avian ecology and conservation and to improve result accuracy and reproducibility.

Background

Stable isotope analysis of relatively abundant elements such as carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and hydrogen ($\delta^{2}H$) have been widely implemented, particularly over the past two decades, in ecology, conservation, and forensic studies (Vander Zanden et al. 2018.). For example, stable isotope analyses of biological tissues can reveal useful information on diet, trophic level interactions, and large scale animal movements (Bridge et al. 2011; Contina et al. 2013; Zenzal et al. 2018). However, laboratory sample preparation for stable isotope studies requires a standardized protocol to ensure accuracy and repeatability of the results (Paritte and Kelly 2009).

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Useful references:

Zenzal T.J. et al. 2018. Temporal migration patterns between natal locations of ruby-throated hummingbirds (*Archilochus colubris*) and their Gulf Coast stopover site. Movement ecology, 6(1), p.2.

Vander Zanden et al. 2018. Application of isoscapes to determine geographic origin of terrestrial wildlife for conservation and management. Biological conservation, 228, pp.268-280.

Contina et al. 2013. Using geologgers to investigate bimodal isotope patterns in Painted Buntings (*Passerina ciris*). The Auk, 130(2), pp.265-272.

Bridge et al. 2011. Causes of bimodal stable isotope signatures in the feathers of a molt-migrant songbird. Canadian Journal of Zoology, 89(10), pp.951-959.

Paritte and Kelly 2009. Effect of cleaning regime on stable-isotope ratios of feathers in Japanese Quail (*Coturnix japonica*). The Auk, 126(1), pp.165-174.

GUIDELINES

Anytime work with samples is being performed, latex or nitrile gloves should be worn to prevent contamination from outside substances. All work should be performed on a flat, cleaned surface.

Some steps in this protocol, such as standard weights and data sheets, might be idiosyncratic to our lab and equipment used at the time

of the analysis. Please contact andrea.contina@ucdenver.edu if you have any questions or comments.

MATERIALS

NAME	CATALOG #	VENDOR
Chloroform		
Ethanol		
Methanol		
double distilled water (ddH2O)		
Versa-Clean Multi-Purpose Cleaner	04-342	Fisher Scientific
Kimwipes	34120	Kimberly-Clark

MATERIALS TEXT

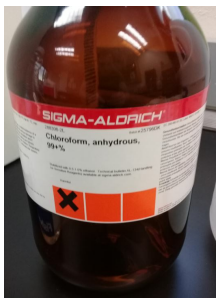
These are the standards used for Hydrogen analysis in our lab: KHS (Kudu Horn Standard), CBS (Caribou Hoof Standard), BHCO (*Brown-headed Cowbird).* Standards used for Carbon and Nitrogen analysis in our lab: USGS40, USGS41a, and USGS 42. USGS 42 can be used for the reference curve and QAQC.**Note: different standards, capsules, and weights are used for Carbon and Nitrogen (see Table 1 below). Other materials:** Staples Compressed-gas duster.

SAFETY WARNINGS

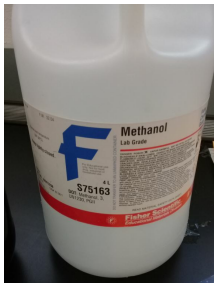
When combining Chloroform and Methanol, only open containers under a fume hood. Latex or Nitrile gloves should be worn when handling substances. Protective eyewear should also be worn.

Preparation of Cleaning Solution: 2:1 Chloroform Methanol Solution

1 Under fume hood, pour 100mL **chloroform** into clean 250 mL beaker.



2 Pour 50mL **methanol** into beaker.



3 Stir contents with a stir rod, or carefully swirl contents of container **under fume hood** to mix contents.

4 If desired for repeated use, pour 2:1 chloroform methanol solution into at least 250mL container with sealable, non-plastic lid.

Preparation of Cleaning Solutions: 30:1 Deionized Water Detergent Solution

5 In clean 250mL beaker, pour 150mL **deionized water**, and 5mL **detergent**.

6 Gently swirl content of container.

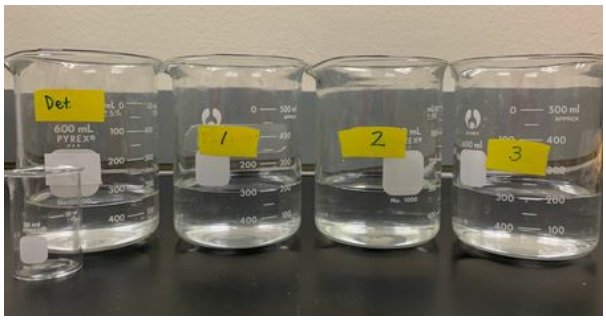
6.1 Avoid agitating solution in any manner which might generate bubbles.

7 Mark beaker DETERGENT.

8 Into three clean 250mL beakers, pour 200mL deionized water each.

9 Mark Beakers 1, 2, and 3.

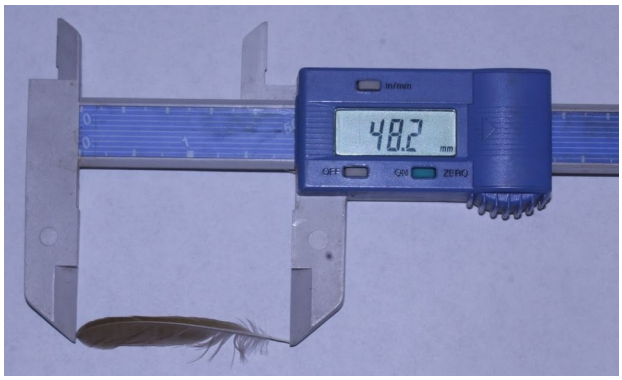
10 Place beakers **in order** DETERGENT, 1, 2, 3. Feathers will be cleaned in this order to avoid final prepared feather being contaminated with detergent.



Beakers

Preparing of Feathers

11 Using calipers, measure the length of the feather, starting from the tip of the quill (calamus) to the end of the rachis or farthest barb. Record the weight of the feather with balance.

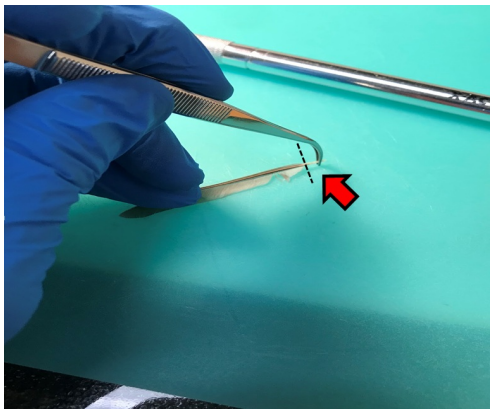


Measuring the length of the feather

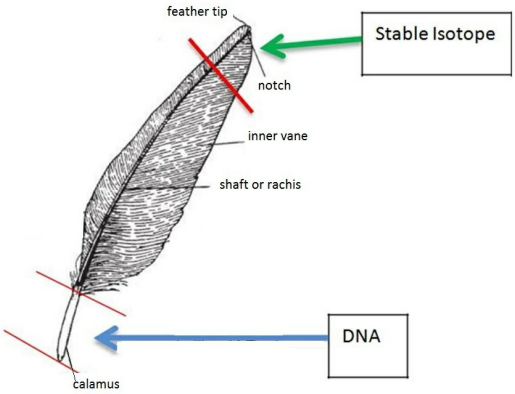
Weighting the feather

12

Using a razor that has been cleaned with ethanol, cut off the calamus of the feather as indicated below:



The dashed line indicates where the calamus should be cut.



Schematic feather parts

12.1 It is useful to use one hand to hold down the feather by placing the gloved base of your hand against the end of the feather and holding the calamus with forceps when cutting the calamus, this prevents the calamus from being lost. (technique pictured above)

12.2 The calamus is removed to later acquire **DNA** from the feather.

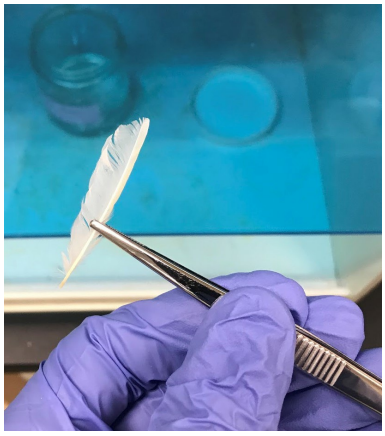
13

Place separated calamus into marked and **clean paper envelope**, to absorb humidity, and seal envelope with adhesive to ensure calamus is retained. Label the clean envelope with banding ID number, location, species, sex, and date of collection.

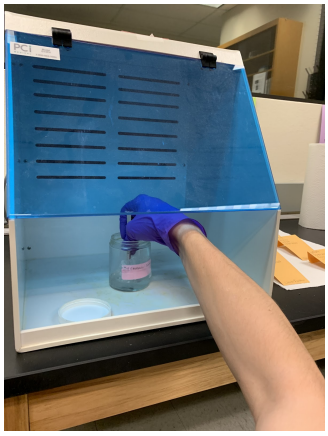
14 With clean paper towels, prepare in advance a drying “bed” for feathers by layering the paper towels over one another.

15

Using forceps, hold the rachis of the feather so that the feather is perpendicular to the forceps. Under a fume hood, dip the feather into a **2:1 (chloroform methanol) solution.**



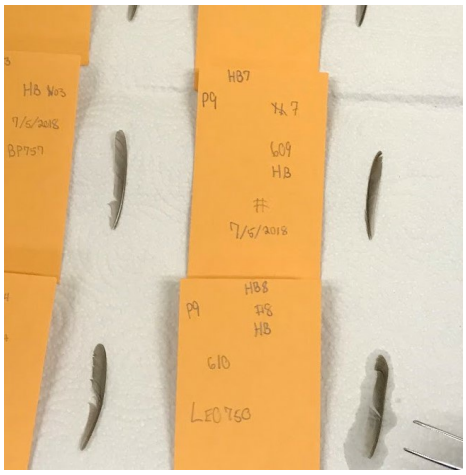
Holding the rachis of the feather with forceps



Working under a fume hood

16 Agitate the feather to make stirring motions with the feather for **30 seconds.**

17 After each feather has been **washed in 2:1 (chloroform methanol) solution**, place each feather on dry cloth or paper towel, next to clean envelopes showing feather details and individual IDs.



Drying feathers on paper towel next to clean envelopes showing feathers ID

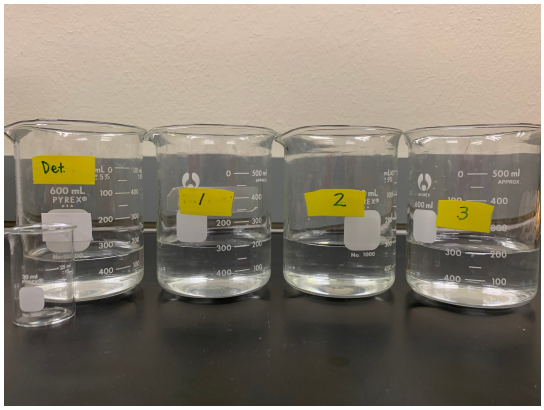
18

Allow feather to dry for **24-36 hours or until completely dry.**

18.1 Please note: 24-36 hours was at room temperature of 26C near the room's heating system. This may have reduced drying time in our lab.

19

With clean forceps, pick up each feather from the drying bed and dip into solution of **30:1 water to detergent (prepared from Steps 5-10 above.)**



Beakers

20 Agitate each feather for 30 seconds to ensure thorough cleaning.

21 After 30 seconds of cleaning remove the feather from **30:1 water to detergent** solution with forceps, and place each feather into the container of deionized water (first beaker, labelled "1.")

22 Agitate feather for 30 seconds.

23 Repeat step 21-22 two additional times with separate beakers of deionized clean water (beakers "2", and "3.")

23.1

Note: ensure **the order of containers (beakers) is kept the same** for every feather to ensure all detergent is removed from each feather.

24 Place each cleaned feather onto paper towel to dry, and transfer marked envelopes from Step 6 to place adjacent to each feather to keep track of samples.

24.1 Ensure every feather is next to its respective envelope (label), and that the placement of each feather relative to its labelled envelope is kept the same for every feather, to ensure the feathers details are not mistaken.

25

Allow feather to dry for **24-36 hours or until completely dry.**

26 Place each feather into its respective clean labelled envelope, and store cleaned envelopes into separate container.

Weighing standards for stable isotope analysis (Hydrogen)

27 Clean three sets of forceps and a spatula with **95% ethanol** by staurating a kimwipe™ with ethanol and rubbing all used surfaces of the instrument. Place instrument under lamp or in direct light to evaporate ethanol.

28 Using air duster, or similar cleaning product, clear surface of microbalance, and remove any particles from previous uses. Ensure chamber is clean, **no residuals remain from previous uses.**

28.1 Air duster will remove particles from chamber.

29 Using forceps, place a 3.5x5mm silver capsule, open end up, into the center of a microbalance.

29.1 Bent tip forceps are preferred for this step.

30 Close microbalance chamber and tare microbalance to empty silver capsule mass.

31 With spatula, pick up a very small quantity (as shown in the photo) of BHCO, KHS, or CBS standard.

Small quantity of BHCO standard

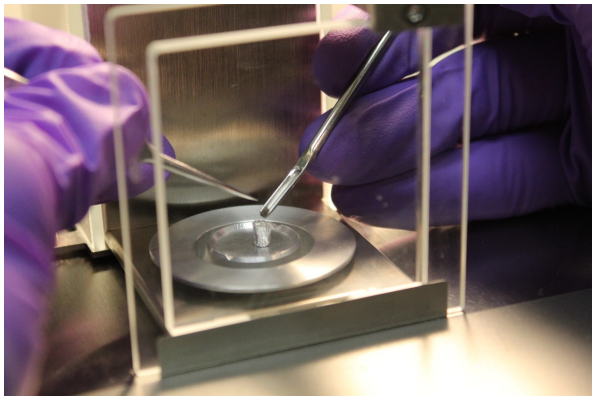
31.1 ⚠

REMEMBER: silver capsules are used for hydrogen isotope analysis, tin capsules are used for carbon and nitrogen isotope analysis. Please see **Table 1** below for further details on standards and weights for different isotopes. **This is crucial!**

Isotope	Standard	Standard Weight	Sample Weight	Capsule
Hydrogen	CBS	200 µg (+/-10µg)	200 µg (+/-10µg)	silver
	BHCO	200 µg (+/-10µg)	200 µg (+/-10µg)	silver
	KHS	200 µg (+/-10µg)	200 µg (+/-10µg)	silver
Carbon	USGS40	390 µg (+/-10µg)	390 µg (+/-10µg)	tin
	USGS42	390 µg (+/-10µg)	390 µg (+/-10µg)	tin
	USGS41a	390 µg (+/-10µg)	390 µg (+/-10µg)	tin
Nitrogen	USGS40	390 µg (+/-10µg)	390 µg (+/-10µg)	tin
	USGS42	390 µg (+/-10µg)	390 µg (+/-10µg)	tin
	USGS41a	390 µg (+/-10µg)	390 µg (+/-10µg)	tin

Table 1. *Details on standards, capsules and weights for different isotopes used at the University of Oklahoma.* ***Weights might need to be adjusted depending on species and laboratory equipment.***

32 Open microbalance chamber, and hold spatula with standard over open capsule. Use second pair of forceps to push small quantities of standard off of the end of the spatula into the capsule.



Microbalance chamber with open silver capsule being filled with BHCO standard

33 Remove spatula and forceps from balance chamber, and close draft shield doors.

34 If mass of standard is below required standard mass, repeat steps 32-33, else proceed to step 35.

35 If mass of standard is above required mass, use bent tip forcepts to hold capsule, and second pair of forceps to remove tiny excess of standard and place into waste receptacle.

36 **Once desired mass is achieved**, **record mass on data sheet (see below for data sheet template)**, and use bent tip forceps to remove capsule from microbalance. Place capsule on clean flat surface.

37 Using the tip of a third pair of forceps, push the opened end of the capsule so it gets closed (sealed), and continue to hold end closed with forceps.

38 With a gloved hand **very gently** squeeze the base of the capsule between thumb and forefinger until capsule is completely flat. If you squeeze too quickly, you might damage the capsule and disperse the standard.

39 Using forceps holding the end of the capsule, place the capsule flat end down on a clean, dry surface, and rotate forcepts 180 degrees in the direction of the rest of the capsule, creating a curved section of the capsule in the place of the opening.

40 Gently ungrasp the end of capsule with forceps, and regrasp the capsule with the inside of one arm of the forceps on outside of the end of the curved portion of capsule, and the inside portion of the other arm of the forceps on the nearest flat portion of the capsule to the newly formed curved section. Squeeze the forceps to make a fold in the capsule.

41 Repeat steps 39 and 40 until capsule is approximately twice the width of the forceps in length. Fold any remaining capsule over the folded portion of the capsule.

42 Place the now folded capsule on a flat, clean, dry surface, and place the forceps around the capsule such that the inside of the arms fo the forceps have greater hight than the capsule, and the angle between the capsule sides and the forcep arms is minimized. Squeeze the arms of the forceps gently, to compact the capsule along its width. **Make the capsule as round-ish as possible.**

43 If necessary, rotate the capsule 90 degrees, and repeat step 42. Repeat this step until every oientation of the caspule has been pressed an equal number of times.

43.1 This ensures the capsule maintains a relatively cubic or round-ish shape.

44 If necessary, pick up the foceps and place the outside surface of one arm of the forceps onto the topmost surface of the capsule. Press the forceps so the capsule is pressed evenly along its topmost surface.

45 Repeat steps 43-44 until capsule has relatively smooth surfaces and is cubic or round-ish. **There should not be tears in the surface of the capsule** or any sections of the capsule that extend out beyond the rest of the cubic or round-ish shape.

45.1 ⚠

If any tearing occurs, or if the capsule is dropped onto an uncleaned surface at any time, place the compromised capsule into a waste receptacle, and restart the process with a fresh capsule.

46 When the capsule is to the desired dimensions, use a pair of forceps to place the capsule into its designated well on the tray. Record what **well** the capsule has been placed into (e.g. **A6**, or **C5**, etc.) on a data sheet, along with the **final weight**, **sample ID**, and any other relavant information.

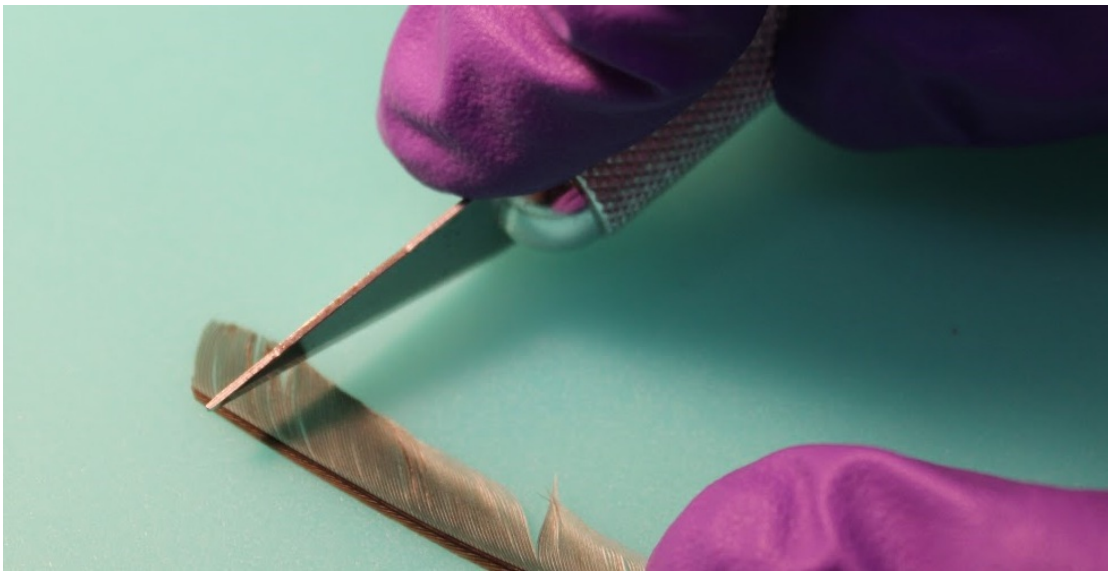
Weighing Feather Samples for Stable Isotope Analysis (Hydrogen)

47 Clean two sets of forceps and a razor blade with 95% ethanol by saturating a kimwipe™ with ethanol and rubbing all used surfaces of the instruments. Place instruments under lamp or another direct light source to evaporate ethanol.

48 Using air duster, or similar cleaning product, clear surface of microbalance.

49 ⚠

With clean razor blade, cut off a portion of the tip of the now cleaned and dried feather (the point opposite the calamus) to be sampled for **stable isotope analysis**.



Cutting off a portion of the tip of the cleaned feather

49.1 The method of cutting is not important, but how the feather is cut should remain consistent between feather samples. We recommend cutting from the rachis, and using the barbs attached to the incised rachis. Note that **different sections of the rachis might introduce variation stable isotope values** (particularly for longer feathers) and we recommend to be consistent across feather samples with the tip and rachis section being used for analysis.

50 Using forceps, gently separate the clipped part of the rachis and any attached barbs from the rest of the feather.

51 Using clean forceps, place feather on tared balance, and close balance shields.

52 If the feather is above desired mass, use clean razor to cut off an additional portion of the feather. Using same forceps as in the previous step, place the feather on the balance. Repeat as needed until weight is within desired parameters (**+/- 10 µg.**)

53 If the feather is above desired mass, using same forceps as step 51, remove the feather from balance, and use a clean razor to remove small portions of the sample.

53.1 If sample is close to desired mass, barbs can be used for finer adjustment of mass, while removal of rachis will produce a larger change in mass.

54 If sample is within desired mass, remove from balance with forceps, and place onto clean surface.

55 Using clean bent forceps, place empty capsule onto balance and tare balance.

56 Remove capsule from balance, with clean forceps, place sample inside the capsule.

56.1 It may be necessary to separate the feather sample into smaller subsections with a razor in order to fit into capsule.

56.2 Be careful that portions of the sample are **not protruding** from the capsule by static electricity.

57 When all portions of sample are inside capsule, use bent forceps to place capsule with sample into balance.

58 If readout of balance does not match original mass, check razor, forceps, and cutting surface for portions of sample that may have been ejected form capsule or that may have adhered to instruments. Use forceps to transfer these sample portions into capsule.

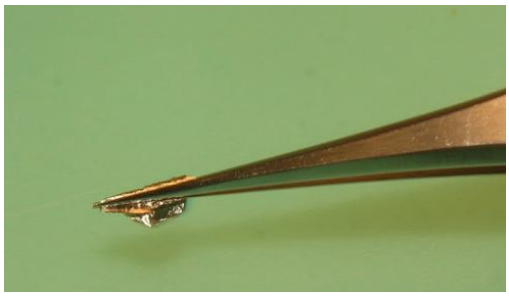
59 Once desired mass is acheived, record mass on data sheet together with sample ID, and use bent tip forceps to remove capsule from microbalance. Place capsule on clean flat surface.

60 Using the tip of a pair of forceps, push the open end of the capsule closed, and continue to hold end closed with forceps.

Closing the silver capsule from the top

61 With a gloved hand **gently** squeeze the base of the capsule between thumb and forefinger until the capsule is flat. **Do not damage the capsule**.

62 Using forceps, holding the end of the capsule, place the capsule flat end down on a clean, dry surface, and rotate forceps 180 degrees to form a curved section of the capsule.



Folding the silver capsule

63 Gently ungrasp end of capsule with forceps, and regrasp capsule with the inside of one arm of the forcep on the end of the curved portion of the capsule, and the inside protion of the other arm of the forcep on the nearest flat portion of the capsule to the newly formed curved section. Squeeze the forceps to make a fold in the capsule.

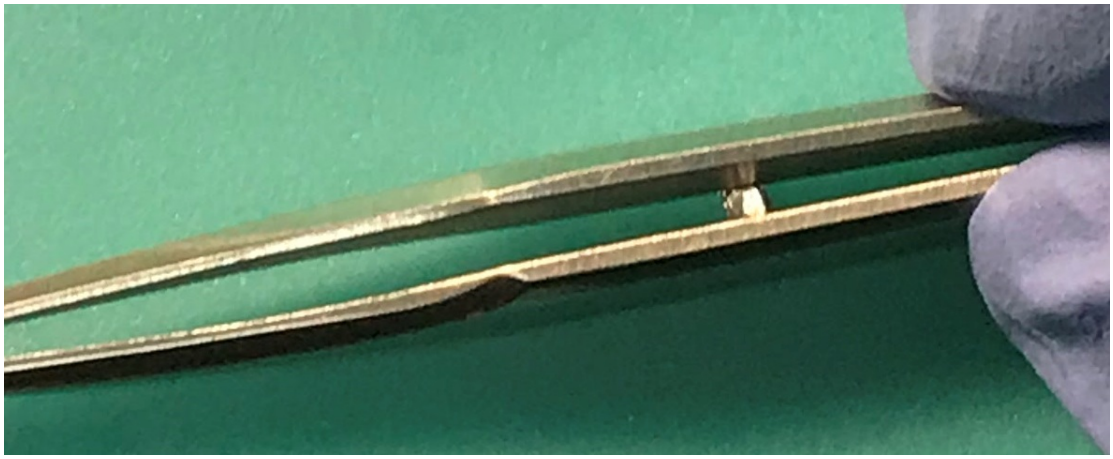
64 Repeat steps 62 and 63 until capsule is approximately twice the width of the forceps in length. Fold any remaining capsule over the folded portion of the capsule.

65 Using a third pair of forceps, grab one of the ends of the capsule and repeat steps 64 along the width of the capsule until capsule approaches desired shape for analysis.

Schematic representation of the folding capsule process

66 ⚠

Place now foldd capsule on a flat, clean, dry surface, and place the forceps around the capsule such that the inside of the arms of the forceps have a greater height thtan the capsule, and the angle between the capsule sides and the forceps is a minimum. Squeeze the arms of the forceps gently, to **compact the capsule** along its width.



Final folded round-ish capsule

67 Rotate the capsule 90 degrees, and repeat step 66.

67.1 Rotate capsule after every compression fo ensure a relatively cubic or rectangular or **round-ish shape.**

68 Pick up the forceps and place the outside surface of one arm of the forceps onto the topmost surface of the capsule. Press the forceps so that the capsule is pressed evenly along its topmost surface. See photo above.

69 Repeat steps 67-68 until the capsule has relatively smooth sides, and is cubic or round-ish in shape. There should be **no tears in the surface of the capsule.**

70 If tearing occurs, or if the capsule is droppe donto an uncleaned surface at any time, place the compromised capsule into a waste receptacle, and restart the process with a fresh capsule.

71 ⚠

Once the capsule is completely pressd, pick up the capsule with clean forceps and place the capsule into designated well. Record well position (e.g. **A6**, or **C7**, etc.), next to sample weight, sample ID, and tray identification number and species.

REMEMBER: Print this data sheet and **always keep it attached to the plastic tray** that will be used to transport the samples to the mass-spec lab. Keep track of the samples! **IMPORTANT**: the sequence of the samples and standards matters, we recommend to follow the examples below.

Example of data sheet for Hydrogen (see Table 1)

well	well n.	sample ID	sample weight	Feather type (P1/P9/R)	Feather length	Feather weigth	notes	Tray number_____
A	1	BHCO						
A	2	BHCO						
A	3	CBS						
A	4	BHCO						
A	5	KHS						
A	6	**sample**						
A	7	**sample**						
A	8	**sample**						
A	9	**sample**						
A	10	**sample**						
A	11	**sample**						
A	12	**sample**						
B	1	CBS						
B	2	BHCO						
B	3	KHS						
B	4	**sample**						
B	5	**sample**						
B	6	**sample**						
B	7	**sample**						
B	8	**sample**						
B	9	**sample**						
B	10	**sample**						
B	11	**sample**						
B	12	CBS						
C	1	BHCO						
C	2	KHS						
C	3	**sample**						
C	4	**sample**						
C	5	**sample**						

Data sheet template (for Hydrogen) used at the University of Oklahoma. Alternate standards and samples following the order indicated on the data sheet. Label everything as best as you can and keep track of the samples, standards, and trays. ***Weights might need to be adjusted depending on species and laboratory equipment.***

Example of data sheet for Carbon and Nitrogen (see Table 1)

AS slot	Tray	Well	Identifier 1	Identifier 2	target weight (mg)	actual weight (mg)	Target N (ug)
0	1	A1	nothing	machine blank			0.0
1	1	A2	empty tin	tin blank			0.0
2	1	A3	USGS42	reference curve	0.050		7.7
3	1	A4	USGS42	reference curve	0.100		15.3
4	1	A5	USGS42	reference curve	0.200		30.6
5	1	A6	USGS42	reference curve	0.300		45.9
6	1	A7	USGS42	reference curve	0.600		91.8
7	1	A8	USGS42	reference curve	1.000		153.0
8	1	A9	USGS42	reference curve	1.500		229.5
9	1	A10	USGS40	standard 1	0.600		57.1
10	1	A11	USGS42	QAQC	0.375		57.4
11	1	A12	USGS41a	standard 2	0.580		55.2
12	1	B1		**sample**			**55-60**
13	1	B2		**sample**			**55-60**
14	1	B3		**sample**			**55-60**
15	1	B4		**sample**			**55-60**
16	1	B5		**sample**			**55-60**
17	1	B6		**sample**			**55-60**
18	1	B7		**sample**			**55-60**
19	1	B8		**sample**			**55-60**
20	1	B9		**sample**			**55-60**
21	1	B10		**sample**			**55-60**
22	1	B11	USGS40	standard 1	0.600		57.1
23	1	B12	USGS42	QAQC	0.375		57.4
24	1	C1	USGS41a	standard 2	0.600		57.1
25	1	C2		**sample**			**55-60**
26	1	C3		**sample**			**55-60**
27	1	C4		**sample**			**55-60**
28	1	C5		**sample**			**55-60**
29	1	C6		**sample**			**55-60**
30	1	C7		**sample**			**55-60**
31	1	C8		**sample**			**55-60**

Data sheet template (for Carbon and Nitrogen) used at the University of Oklahoma. Alternate standards and samples following the order indicated on the data sheet. Label everything as best as you can and keep track of the samples, standards, and trays. Note "**nothing**" and "**empty tin**" in tray wells A1 and A2, respectively. ***Weights might need to be adjusted depending on species and laboratory equipment.***