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Bone/tooth sampling and cleaning for collagen extraction

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Maciej T. Krajcarz[1,2], Magdalena Krajcarz[2,3], Dorothée G. Drucker[4], Hervé Bocherens[2,4]

[1]Institute of Geological Sciences, Polish Academy of Sciences (Warsaw, Poland);
[2]Department of Geosciences, Biogeology, University of Tübingen (Tübingen, Germany);
[3]Institute of Archaeology, Nicolaus Copernicus University in Toruń (Toruń, Poland);
[4]Senckenberg Centre for Human Evolution and Palaeoenvironment, University of Tübingen (Tübingen, Germany)

Maciej T. Krajcarz
Institute of Geological Sciences, Polish Academy of Sciences...

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Abstract

This protocol presents the procedure of collecting a sample of bone or tooth that is planned for further collagen extraction, prior to stable isotope analysis, radiocarbon dating, and/or proteomic identification. The protocol includes notes about estimating the sample size and selecting the sampling area, and details of taking a sample, cleaning a sample, drying a sample, and storing it after the sampling is completed. General notes on the destructiveness of the procedure and important issues to be considered before sampling are also provided, as well as basic health safety practices. This protocol has been established and tested in the Biogeology working group of the Department of Geosciences, University of Tübingen (Tübingen, Germany) and the Bioindicator and Stable Isotope labs of the Institute of Geological Sciences of the Polish Academy of Sciences (Warsaw, Poland).

Guidelines

General notes

This is a protocol for collecting a sample of fossil, subfossil, or modern bone or tooth for collagen extraction, prior to stable isotope analysis, radiocarbon dating, and/or proteomic identification.

The procedure includes a set of several activities that must be done in order to ensure further storing of a sample. These includes: i) collecting a sample by cutting it off from the bone/tooth specimen; ii) cleaning the sample after sampling, i.e., removal of dust and any remnants of sediment, soil, mineral precipitates, and/or other tissues; iii) drying the sample after cleaning; and iv) further storing.

This protocol has been established and tested in the Biogeology working group of the Department of Geosciences, University of Tübingen (Tübingen, Germany) and the Bioindicator and Stable Isotope labs of the Institute of Geological Sciences of the Polish Academy of Sciences (Warsaw, Poland). Daniel Billiou (Sorbonne University, Paris, France) contributed to the start of the establishment of this protocol from 1989 to 2001.

Time taken

Estimating the sample size and selecting the sampling area

Unpredictable. Time for estimating the sample size depends on the knowledge of the collagen content in the specimen. If known, it takes few minutes; if unknown, it takes up to several days.

Time for selecting the sampling area depends on the sample type, the operator's skills in bone/tooth anatomy, and the collaborations with specialists. For experienced specialists, it may take several minutes. For unexperienced beginners or non-specialists, it may take weeks.

Sampling

Around 10-30 minutes per sample. Additional 5-10 minutes must be counted for preparing and cleaning the working space.

Cleaning

At least 40 minutes per batch of samples (maximum number of samples in one batch depends on the size of an ultrasonic cleaner and usually is between 10 and 50). If a sample has large amount of sediment and produces a lot of dirt, the cleaning procedure can be prolonged by multiplicity of 10 minutes (rarely longer than 70 minutes in total). Additional 5-10 minutes must be counted for preparing and cleaning the working space.

Drying

48.5 h. Additional around 5 minutes must be counted for labelling and packing for each sample and placing it in a storage cabinet. Additional 5-10 minutes must be counted for preparing and cleaning the working space.

Storing

This is a post-procedure activity that does not consume an operator's time.

Total

Total time depends on the number of samples prepared in one batch and the operator's manual skills and experience (especially important at step 2). The drying step can be done at once for a number of samples, which significantly shortens the total time. For a single sample it usually takes between 49.5 h and 53 h.

Anticipated results

Expected result is a clean (sediment-free) bone or tooth fragment that is ready for collagen extraction (see the protocol **dx.doi.org/10.17504/protocols.io.8epv5rrp4g1b/v1**). Please note that the following collagen extraction may include

additional pre-treatment steps, such as dividing a sample into subsamples (for variable analyses), crushing, and defatting.

Troubleshooting

This procedure is relatively straightforward and not troubleshooting. The rare problems include the following ones:

1. The sampled specimen has broken when sampled (steps: 2, 4). Possible reason: Too much force was applied during cutting. Solution: Avoid to apply too much force when cutting; allow the blade to cut in its speed.
2. The blade got stuck in the specimen while sampling (steps: 2, 4). Possible reason: Speed of the blade was too low or cutting angle was changed during the cutting. Solution: Immediately stop the cutting (switch the engine off), remove gently the blade from the specimen and inspect it for any damage; replace if necessary and continue sampling. Adjust the speed (generally by increasing it) and keep the same angle through the entire cutting process (the blade must not bend during cutting).
3. The collected sample is too small (step 3). Possible reason: The sample size was underestimated at the step of selection the sampling area. Solution: Predict carefully the sample size taking in consideration the weight of the entire specimen and its thickness within the sampled area. If necessary and agreed with the sample's curators, continue sampling.
4. The sample splits down into pieces during sampling, cleaning or drying (steps: 2, 4, 11–16, 19). Possible reason: The specimen was initially cracked. Solution: No worries; collect all the largest pieces and treat them in the following steps and store them together as one sample.
5. The sample became discolored or covered with mold during storing (step 21). Possible reason: The final drying time was too short or drying temperature was too low, or a sample was cooled down after drying in the open air that allowed it resorbing the atmospheric humidity. Solution: Keep the 40°C drying temperature for 48 hours drying time or more; cool the sample down immediately after drying in a desiccator. For any measures, the altered sample must be regarded contaminated and degraded, and should not be taken for collagen extraction nor other analyses.

Materials

Reagents

- Acetone (several mL per sample)
- Milli-Q water (<0.055 µS or >18.2 MOhm; around 100 mL per sample)

Equipment

- Laboratory balance (at least 0.01 mg accuracy);
- Hand-operated engine for replaceable rotary blades, with adjustable speed and easy moveable handle (for example, with elastic connection)
- Lancet (optional, only if attached remains are to be removed)
- Air compressor with a movable hand-operated gun (optional, only if attached remains are to be removed or the blade is to be dried through the compressed air blow)
- Beaker for cleaning the rotary blade, diameter larger than the rotary blade's diameter
- Hood with ventilation and illumination, and a sink with sediment trap
- Glass vials for ultrasonic cleaning (volume several mL), 1 per sample
- Glass or ceramic container for drying in the oven (volume several mL), 1 per sample (optional, the vials for ultrasonic cleaning can be used)
- Ultrasonic cleaner
- Oven with adjustable temperature (40°C) and ventilation
- Desiccator container
- Stainless steel pliers
- Wash bottle for Milli-Q water
- Desiccator cabinet for storing

Consumables

- Waterproof marker (to label glass/ceramic containers)
- Weighing papers/containers, 1 per sample
- Plastic zip bags for storing or another plastic containers, such as jars or vials (2-ml Eppendorf vials are recommended if samples are not too large), 1 per sample
- Printed *Bone sampling protocol form* sheet and pencil (or a computer and an editable file of *Bone sampling protocol form*)
- Replaceable diamond-coated rotary blades, 22.0 mm diameter, thickness 0.19 mm, 1 per sample (possibly less, as they can be re-used)
- Replaceable diamond-coated or corundum-coated rotary bits (optional, only if attached remains are to be removed)

Safety warnings

Health safety issues

Health security issues must be considered, such as:

- hand protection (against splinters, rotating sharp elements) – particularly at steps 2, 6, 7;
- eye protection (against splinters) – particularly at steps 2, 6, 7;
- skin protection (against contact with biological tissues; possible biological hazard!) – all steps;
- cloth protection (against dust; possible biological hazard!) – all steps;
- breath protection (against dust; possible biological hazard!) – particularly at steps 2, 6, 7, 8, 9;
- hearing protection (against ultrasounds) – particularly at steps 12, 16;
- risk of contact with chemicals (acetone) – particularly at steps 11, 12, 13, 14, 18;
- risk of electric shock (especially when electric devices and water are used at the same time) – particularly at steps 6, 12, 16;
- risk of thermic burns (by the oven) – particularly at steps 17, 19.

It is necessary to use gloves, googles, anti-dust mask, lab coats, ear protection muffs, well-ventilated hood, and good illumination.

Bio-hazard!

Modern and fossil bones and teeth are biological material and as so they constitute biological hazard! Avoid direct contact with skin, eyes, and clothes; avoid inhalation of dust; no eating or drinking in the same room; use disinfectants to clean the work station and all surfaces around.

Before start

This procedure is destructive. Thus, before making a decision about sampling, particular concern must be given to the need for sampling this particular specimen, the scientific justification of this kind of sampling for resolving research questions, and to undertake any other planned analyses before the sampling. The shape and size of selected specimen must be first well documented for further analyses that base on shape, size, and color. This may include a variety of actions, depending on the specimen type, taxonomy, preservation, size, and other characteristics. The most typical documentation includes one or several of the following: written and/or graphical description; measurements; photographs of different sides and from different angles; 3D scan; computer tomography; microscope photographs of surface modifications. Consultation with specialists (such as: paleontologists, zooarchaeologists, taphonomists, pathologists, archaeologists) is recommended. It is also important to communicate with persons responsible of collection and/or other analysts about parallel studies envisioned, such as aDNA, which may influence the choice of part to sample, the quantity and workflow. For ethical purpose, the adapted documentation (pictures, measurements, scans) has to be discussed with specialists and checked out.

Estimating the sample size

It must be regarded that most typical analyses of bone/dentine collagen usually demand: around 2 mg for isotopic analysis of $\delta^{13}C$ and $\delta^{15}N$ (typically 0.4 mg per one EA/IRMS run, but this amount must be multiplied in case of repeated runs, for example if the analysis is conducted in a triplicate; also, it is usually very difficult to collect all collagen from the vial, so its total amount should exceed the demanded analytical aliquot); around 5 mg for isotopic analysis of $\delta^{34}S$ (typically only 2-3 mg per one EA/IRMS run, but more is recommended likewise); around 5 mg for radiocarbon dating; and around 1 mg for ZooMS proteomic analysis. This gives around 13 mg of collagen in total, that should be rounded up (to 20 mg) due to potential loss during further transfers, extraction, drying, subsampling, etc. Taking the
usual collagen content in bone/dentine, represented by the collagen yield after extraction (usually between 4% and 20%, or between 40 mg and 200 mg of collagen per 1 g of bone/dentine sample; see e.g., Krajcarz et al. 2018, 2023), the sample size is then estimated to between around 0.10 g and 0.45 g (100 mg and 450 mg). Note that other potential analyses should be also considered. It is recommended to collect one larger sample and store it for future analyses rather than undertake multiple sampling, which is more damaging to the specimen.

In modern specimens or specimens coming from well-known and previously studied stratigraphic contexts, the collagen content can usually be predicted. In the case of unknown or unpredictable collagen content, it is recommended to estimate of sample size through the analysis of nitrogen content (nitrogen wt% or N%) in a bone (Iacumin et al. 1996, Bocherens et al. 1997). The CHN elemental analyzer is used for that and the analysis typically demands around 5–10 mg of a bone/dentine, that can be scratched off the specimen within the planned sampling area or drilled off. A table below can be used to determine the sample size basing on the nitrogen content. It is recommended to take a larger sample than the minimum estimated from bone's %N, especially for lower quantities (i.e., rather 0.12 g than 0.10 g), because at the low quantity the loss during extraction becomes disproportionate.

	A	B
	%N in bone	minimum sample size to get ca. 20 mg of collagen

	A	B
	<0.4	not suitable
	0.4 – 1.0	0.45 g
	1.0 – 1.5	0.40 g
	1.5 – 2.0	0.35 g
	2.0 – 2.5	0.30 g
	2.5 – 3.0	0.25 g
	3.0 – 3.5	0.20 g
	3.5 – 4.0	0.15 g
	4.0 – 4.5	0.12 g
	4.5 – 5.0	0.10 g
	>5.0	0.10 g

Table for estimation the bone sample size (g) to get ca. 20 mg of collagen after the collagen extraction

Selecting the sampling area

The area of a specimen to be sampled is considered independently for each specimen and depends on the specimen type and the sample size. It must be considered that the sample size is most usually around 0.12 g to 0.45 g, so depending on the specimen thickness the sampling will usually remove around 0.5 sq. centimeter up to several sq. centimeters of a specimen.

It is recommended to avoid sampling those areas that are important for taxonomy, morphometrics, pathology, tooth wear, or modification analysis, such as: tooth crowns, bone epiphyses, joints, processes, muscle attachments, pathological changes, post-mortem modifications. Consultation with specialists (such as: paleontologists, zooarchaeologists, taphonomists, pathologists) is recommended. If possible, it is recommended to sample within the already-damaged area (such as excavation damage, storage damage, previous sampling).

Sampling

Cutting a sample off the specimens is the most difficult task in the procedure. It requires manual skills and experience. It is recommended to train the cutting first on test specimens that don't have heritage or museal value. Sampling should make as minimum damage to the specimen as possible.

Cleaning

It is very important to remove all remnants left after the sampling (such as dust) and all other mechanical contaminants from the sample before the collagen extraction. The manual mechanical cleaning is not necessary in some cases, i.e., if the specimen was already well cleaned before. The acetone bath is for breaking down clay mineral aggregates coming from burial environment (soil or sediment). It is not necessary in the case of modern specimens that were never buried in the ground. The water bath is to remove all dirt and contaminants, including the dust from sampling, and should be done for each specimen.

Drying

It is extremely important to dry well the sample before packing it and storing, to prevent any decay and microbial activity.

Sampling

1 Prepare the cutting work station. The work must be done under the hood, at a well-ventilated and well-illuminated work station. Respect the health security issues.

2 Carefully cut off a fragment of bone or tooth of around 0.12 g to 0.45 g from the selected area of the specimen using a rotary saw with diamond-coated blade mounted on the engine handle. Respect the health security issues. Low speed (below 20,000 rpm) is recommended to avoid burning of the material and collagen degradation. Usually the best results are achieved when the specimen is hold in one hand and the blade handle is operated by the other hand of the operator. Please note the risk of hurting the holding hand by the blade! Extreme caution must be given to this task!

3 Check the weight of the sample on a lab balance (±0.01 mg); use a weighting paper/container when weighing (tare the balance with weighing paper/container before placing a sample in); note the weight in the Bone sampling protocol form.

4 If necessary, continue sampling until the appropriate weight is obtained.

5 Place the remaining bone in its original container. Add a note on the container or in the attached label about the sampling (including: sample ID, type of analysis, sample size, date, operator's name).

6 Clean or replace the blade after each sampling. Cleaning is done by carefully submerging the rotating blade in a clean Milli-Q water for several seconds. If after this there is any dust or remains still visible on the blade, it should be replaced. The blade is then dried by rotating it for several seconds above the water (in air; optionally, it can be dried by carefully blasting with compressed air).

Cleaning

7 Remove mechanically all macroscopically visible remnants of soil, sediment, mineral precipitates, other tissues than compact bone/dentine (such as cancellous bone, dental cementum, dental enamel) from the sample, using a lancet (by scratching) or a rotary diamond-coated or corundum-coated bit mounted on the engine handle (by drilling off). Respect the health security issues. Try to remove all remnants, but to save as much of compact bone or dentine tissue as possible.

8 Carefully remove the remaining dust with compressed air.

9 Tidy up the cutting work station. Remember to remove all the dust from the equipment, tools, working table, and all surfaces around. Disinfect all surfaces.

10 Prepare the cleaning work station. The work must be done under the hood, at a well-ventilated and well-illuminated work station. Respect the health security issues. The sample cannot be touched by skin, cloth, or nitrile gloves, to prevent contamination. It is recommended to touch the sample only with clean stainless metal or plastic (PP, PE) tools.

11 Place the sample in a glass vial suitable for ultrasonic cleaner, labelled with sample ID using a water-proof marker (it is recommended to protect the ID symbol with a piece of transparent scotch, in other case it can be washed down by the acetone) and add acetone. The acetone level must fully cover the sample.

12 Place the vial in an ultrasonic cleaner (the cleaner must be filled up with water up to the security level, but not above the vial opening) and turn it on for 10 min. in room temperature. 10m

13 After bathing, dispose carefully the acetone by decantation. Please note your local chemical disposal regulations!

14 If there is any dust or dirt visible in the vial, wash it out with Milli-Q water using a wash bottle over a sink (it is recommended to do the washing over a beaker that would catch the sample if it slipped down from the vial).

15 Take the sample out from the vial using the clean pliers and carefully rinse it with Milli-Q water over the sink, and place again into the vial (it is recommended to do the rinsing over a beaker that would catch the sample if it slipped down from the grab). Clean the pliers by rinsing it with acetone.

16 Repeat the bathing step several times using Milli-Q water instead of acetone, to remove any remained sediment and dirt. Bathing is repeated at least 3 times or more, until the water is visually clean after the bath. It is recommended to use a low-power ultrasonic cleaner (below 600 Watt – does not warm up substantially when working) or to let the ultrasonic cleaner cool down between each bath step, to avoid overheating the samples and collagen degradation. 30m

Drying

17 Immediately after cleaning, placed the sample in an open glass or ceramic container suitable for oven heating and marker-labelled accordingly, and place it in the oven at 40°C for at least 48 h. 2d

18 Tidy up the work station. Wash the tools (vials, blades, bits, lancets, pliers); the vials can be re-used, but remember to remove the scotch and the ID symbol using acetone; you may use

tap water for washing, but at the end rinse all the tools with Milli-Q water several times; leave them for drying. Remove water from the ultrasonic cleaner.

19 After 48h of drying, move immediately the container with sample to the desiccator and leave there for 30 minutes to cool down to the room temperature.

20 Place the sample in an ID-labelled plastic zip bag or another plastic container (2-ml Eppendorf vials are recommended if samples are not too large) and store it in a desiccator cabinet.

Storing

21 The sample placed in a labelled plastic container is stored in a desiccator cabinet before the collagen extraction step (see **dx.doi.org/10.17504/protocols.io.8epv5rrp4g1b/v1**). Upon dry and cool conditions (room temperature or below, humidity <5%) the sample can be stored for years.

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