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Bone and tooth collagen extraction for stable isotope analysis and radiocarbon dating

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Abstract

Several collagen extraction protocols are described in the literature, but most lack detailed descriptions of the laboratory manipulations and the specific material used.
This collagen extraction protocol described here largely follows Longin's (1971) with a few changes. Shortly; The full bone is demineralised using diluted HCl (0.2 to 0.5M). This is followed by removing humic acids using a short NaOH rinse, and by solubilisation and filtration of the collagen.

This protocol aims to provide collagen of appropriate quality for both stable isotope analysis and ^{14}C dating, at a low cost and with a decent duration. This protocol was found to also be suitable, with minor adaptations, to badly preserved samples.

Image Attribution

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Guidelines

We suggest reading carefully the guidelines and every step of the protocol before starting because some steps and equipment may have to be adapted to your samples.

Numerous bone and dentine collagen extraction protocols are readily available, but they often lack detailed instructions regarding the process and the material needed. This protocol is tailored for researchers willing to implement stable isotope analysis on skeletal material but, for whom the significant amount of collagen extraction protocols published and tested make their pre-treatment choice uneasy. It can be adapted to fragile archaeological remains (e.g. poorly preserved skeletal material or fish bones).
The protocol has been designed to consider multiple factors simultaneously :

- Ensuring a reliable and satisfactory collagen purity suitable for both ^{14}C dating and stable isotope analysis. Discussions with experts such as Mathieu Boudin from The Royal Institute of Belgium (KIK-IRPA) have influenced this aspect, drawing inspiration from Wojcieszak et al. (2020).
- Providing a minimally destructive and flexible pre-treatment approach, considering the condition, mass and age of the samples (e.g. Reducing the HCl concentration and temperature used during the demineralisation of highly degraded samples or lightweight samples).
- Emphasising affordability by utilising inexpensive materials and equipment, making the protocol accessible to researchers with limited budgets.
- Assuring a relatively short duration for the extraction process.

Materials

Equipment

- Drill tool (e.g. Dremel 3000; Proxxon)
- Heater (e.g. Oven; heating bath; -Ideally with a program function).
- Ultrasonic bath
- Freeze-dryer
- Fume hood
- Freezer
- Analytical balance
- Timer

Consumables

- Aluminium foil
- Mini-saw (Dremel; Proxxon): For most mammifère bones, we advise a maximum diameter of 22mm to avoid leaving traces in unexpected places. The width should not be over 0.7 mm to avoid the risk of losing too much sample in the form of a powder.
- Diamond-tipped drill bits (e.g. Dremel- 7103 2mm diamond)
- PP tubes with screw caps - Tubes resistant to heat (holding 13-15 ml and with a diameter of 16 mm; e.g. Böttger® - round-bottom tubes 100*16mm with screw caps). Ezee filters fit in these sample tubes.
- Disposable pasteur pipettes.
- Ezee filters (Elkay laboratory products® - 9ml Ezee Filter separator (127-3193-000))
- pH paper
- Glass vials with well-sealing screw caps (e.g. Wheaton® - liquid scintillation vial with attached cap (DWK986541))
- Parafilm

Chemicals

- 70% ethanol solution
- 'DI-water'. It refers to deionised and/or ultrapure water (e.g. MQ water) because ultrapure water is also deionised.
- Diluted HCl solution in ultrapure water. For most samples, 0.5M is used. For more degraded samples a lower concentration is advisable (see protocol).
- 0.25M NaOH.
- "pH3 solution" made using ultrapure water and diluted HCl. pH paper is sufficiently precise for its use in this protocol.

Safety warnings

This protocol involves harmful machines and chemicals, please wear the PPE equipment (a lab coat, gloves, goggles and a mask when sampling bones especially).

Before start

400 mg of dry bone sample or half a tooth root (app. 200 mg) is usually sufficient to obtain enough collagen for ^{14}C dating and carbon, nitrogen and sulphur isotope analysis. However, the quantity and quality of the collagen obtained at the end of this extraction protocol remain largely sample-dependent.

Sample acquisition and cleaning

1 Place the skeletal material to sample on a clean sheet of aluminium foil in a well-ventilated space.

2 With a clean mini diamond saw mounted on a rotary multifunction tool (e.g. Dremel 3000 or Proxxon), carefully cut a piece of the skeletal material aiming for the appropriate mass. The bone powder created during this step can be kept in the foil for further analysis of the material.

3 With a diamond drill bit, mechanically clean the bone/tooth surface by removing approximately 2mm of its outer surface.

> **Note**
>
> Thoroughly clean the surfaces with ethanol between each sample and replace the mini saw and the drill bit with clean ones.

4 The saws and drill bits can be cleaned by being soaked in an ultrasonic bath in DI-water, then in ethanol, then in water again (Repeat until the water is clear), 5 minutes each time.

5 Place the samples in a small container. Label the container with the sample ID on several surfaces, and then add DI-water. Sonicate the samples in the container for approximately 3 minutes, avoiding temperature increase. The water may become murky.

> **Note**
>
> To prevent warming the samples, avoid sonication for extended periods.

6 Remove the water from the container, rinse the samples 1 to 3 times and add fresh DI-water. Repeat go to step #5 until the water in the container remains clear. In general, two sonification steps suffice.

7 Let the samples dry Overnight in the container placed in a fume hood.

8 Weight the sample once it is dry.

Demineralisation

9 Insert the bone or dentin sample in a reaction tube with a screw cap*.

10 In a fume hood, add approximately 10 mL of the diluted HCl solution to the reaction tube, ensuring the sample is fully submerged. For well-preserved samples, a 0.5M HCl solution at room temperature can be used.
If the sample seems fragile or highly degraded, consider working at low temperatures (e.g. 4 °C) and/or with a more dilute acid solution (e.g. 0.2M HCl solution).
Do not close the tubes tightly as CO_2 forms during the demineralisation.

Note

The demineralisation might take longer when using a lower temperature and/or a lower HCl concentration.

11 Every two to three days, replace the HCl solution in the tube with fresh HCl. If no bubbling is observed within 30 minutes after the addition of fresh HCl at room temperature (01:00:00 if HCl is stored at 4 °C), the sample can be considered as demineralised.

Note

Often, the bone or dentin sample, once demineralised, is flexible, transparent and/or floating in the solution.
However, it is not mandatory to wait until the sample is fully flexible or slushy, the lack of reaction between the HCl solution and the material is sufficient to consider the sample adequately demineralised.

12 Once the skeletal material is demineralised (i.e. no bubbling), rinse it in the tube three times with DI-water. Rinse also the edges of the tube. If the sample is fragmented, use an Ezee filter.

Note

*To use appropriately an Ezee filter, the sample tube should have a diameter of 16 mm (See 'Material' section).

13 If the NaOH step is not directly following, the rinsed demineralised sample can be preserved in the fridge in DI- water. If the sample is crumbly after the demineralisation, it is also possible to remove the DI-water and let the sample sit in the freezer.

Note

Different samples within the same batch are likely to demineralise at different rates. Therefore, you may want to store the samples that are ready first in the fridge or freezer while the others are still demineralising.

Removal of lipids and humic acids

14 Remove any DI-water from the tubes with the samples. If the sample was frozen, wait for it to thaw and remove the excess of DI-water.

15 Add 0.25 M NaOH solution making sure to cover the sample.

16 Let the sample in contact with the NaOH solution for 00:15:00 . Discard the NaOH solution from the tube using a Paster pipette or an Ezee filter if the sample consists of small fragments. 15m

Note

The correct timing of the contact time with the NaOH is important. For this, it is advisable to space the addition of the NaOH enough (e.g. 2 minutes) between each sample and to keep track of the NaOH addition time.

Note

*To appropriately use an Ezee filter, the sample tube should have a diameter of 16 mm (See 'Material' section).

17 Rinse the sample and the edges of the tube 3 times with DI-water.

Neutralising the NaOH

18 Add the diluted solution of HCl (same concentration as for the demineralisation step). Make sure the solution covers the sample.

> **Note**
>
> This step is important because the NaOH solution may absorb atmospheric CO_2 and thus, bias the radiocarbon dates.

19 Let the sample and the solution react for 00:05:00 . 5m

20 Rinse the sample three times with DI-water.

Solubilisation of the collagen

21 Add the pH 3 HCl solution to the tubes. Make sure it largely covers the samples.

> **Note**
>
> Adding a generous amount of pH 3 HCl solution helps to prevent significant collagen loss during go to step #25

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22 Transfer the tubes to a heat-resistant rack.

23 Heat the sample in pH 3 HCL solution at 70 °C for 48:00:00 . 2d

> **Note**
>
> For that step, using a programmable oven is handy.

24 While waiting for the samples to solubilise, label the glass vials for each sample and weigh them. Also, prepare small pieces of parafilm to cover each glass vial.

Filtration

25 Once the samples are solubilised, filter the liquid samples by slowly pressing the Ezee filters into the sample tubes. Then transfer the filtered liquid to the pre-weighed glass vials designated for each sample.

Note

*To appropriately use an Ezee filter, the sample tube should have a diameter of 16 mm (See 'Material' section).

26 After the initial filtration, some of the collagen solution (pH 3 HCl solution) may remain at the bottom of the tube. To maximise recovery, add more of the pH 3 HCl solution to the tube. Then, proceed to 25 to filter the solution again.

Note

This will ensure that as much collagen as possible is collected.

27 Add a piece of parafilm on top of each glass vial and pierce it several times with a sharp tool (e.g. a clean toothpick for each sample).

Freezing

28 Put a cap above the parafilm and place the glass vials with the filtered samples in a freezer overnight.

Lyophilisation

29 Remove the caps from the vials and place the frozen samples in a freeze-dryer for at least 24 hours.

Note

When transferring samples between the freezer and the freeze-drier, do so quickly to prevent the samples from thawing.

Weighing and storing

30 Once you obtained fully dry collagen, remove the samples from the freeze-drier.

Expected result

A well-preserved collagen should look fluffy and white/beige.

Note

Ice crystals can resemble dry collagen.
It is important not to thaw a partially dried collagen sample as this might complicate the following processing.

If unsure whether the collagen is dry after some time in the freeze-drier, let the sample in for a longer time.

If unsure whether the sample is fully dehydrated after removing it from the freeze-drier, quickly weigh the collagen and place it back in the freeze-drier for an additional time, e.g., 12 hours. Then, weigh it again. If the sample was not fully dehydrated, the mass must have decreased. Repeat until the mass of the collagen is constant.

31 After removing the parafilm, place the caps on the glass vials to prevent the collagen from taking up humidity.

Note

Freeze-dried collagen is highly hygroscopic.

32 Weight the dry collagen in the glass vials and calculate the collagen yield.

Note

A collagen yield of ≥ 1 % is generally considered the threshold for determining well-preserved collagen in skeletal remains (van Klinken, 1999).

CITATION

G.J. van Klinken (1999). Bone Collagen Quality Indicators for Palaeodietary and Radiocarbon Measurements. Journal of Archaeological Science.
LINK
https://doi.org/10.1006/jasc.1998.0385

Storage

33 The collagen can be stored in a constantly dry environment (e.g. a freezer).

Protocol references

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Citations

Step 32

G.J. van Klinken. Bone Collagen Quality Indicators for Palaeodietary and Radiocarbon Measurements
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