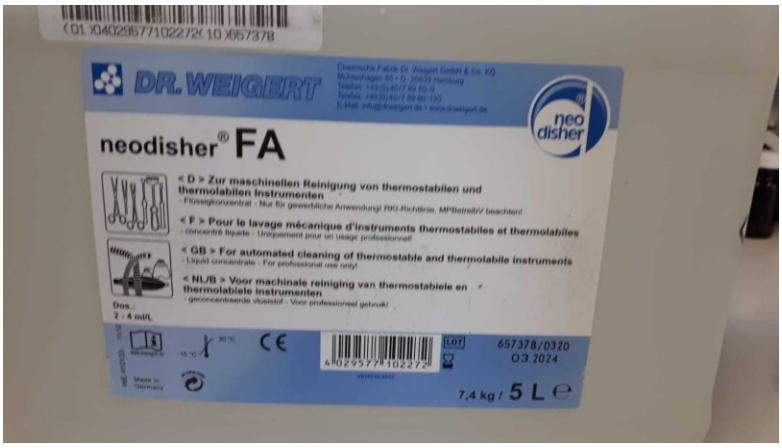
Collagen extraction

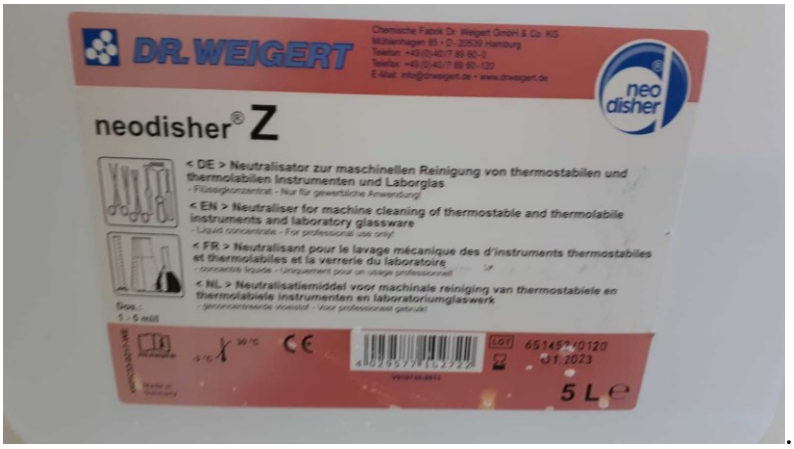
Before starting the extraction

Cleaning glassware

Ensure that the glassware to be used is completely clean of any organics. This can be achieved using a detergent/bleach. In our lab we use the detergent and neutralizer (to neutralize basic conditions) shown below but you can use your own chemicals.

<u>There should be no re-use of glassware that contained solution from one sample to another.</u>





Glassware and other material needed

- 16mm test tubes (glass or Pyrex)
- Glass rods
- Beakers to transfer solutions
- Ezee filters for 16mm test tubes
- Scalpel
- Dremel with different tip options (metal disks to cut or clean bone)

Mechanical cleaning of the sample

Perform the treatments in a clean and dust-free environment. The mechanical cleaning can itself cause dust and so after treating each sample the space should be cleaned. Ideally, perform the mechanical cleaning steps in a separate room from where the chemical steps will be done. Handle the samples at all time with gloves that are free of chalk or other substances that might contaminate the sample. Use a clean electrostatic dissipative lab coat throughout the lab procedures.

Label all necessary glassware with sample ID using permanent ink that does not easily fade.

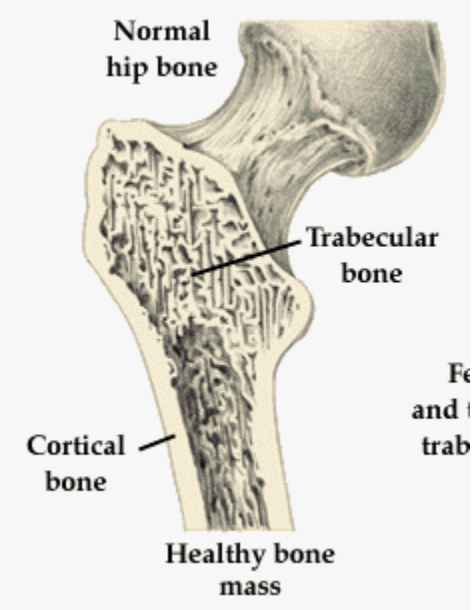
Before bone collagen extraction can start the bone sample needs to be cleaned of any dirt and of cancellous bone. For collagen extraction take c. 500mg to 1000mg of bone. For carbon and nitrogen stable isotope analyses you will need 1 to 2mg of bone. Thus, the given

amount may be excessive. With experience you will gain a better understanding of how much bone should be taken for the extraction.

The sample should have NOT been persevered by applying a chemical substance. In this case a different protocol must be applied.

To cut the bone use a dremel with an attached cutting disk. Try to cut only the amount needed and if possible as a single chunk. In some cases, the bone will be quite brittle and you can just break the amount needed. In other cases, the fragments may already be available.

To clean the bone, you may do it manually using a metal scalpel (may be the only option for brittle samples) or the dremel using the different metal tips. If you have access to an aluminum blaster you can also use this as it makes cleaning considerably faster.

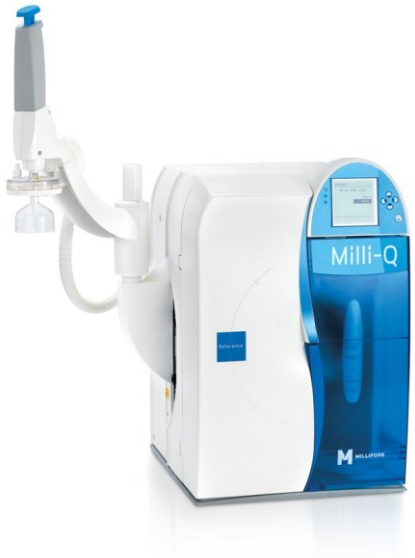


The cancellous (or trabecular) bone should be removed during mechanical cleaning. Only leaving the cortical bone.

Very clean bone. It will not always be possible to have a bone this clean. Especially if it is a thin and brittle bone (e.g. badly preserved rib)

Place the clean bone sample(s) in a glass tube containing acetone for 1 hour (make sure this covers completely the bone. If the bone is very fatty, then you should do this step before starting the mechanical clean). Discard the acetone and add ultrapure water (you need a Milli Q system for this – or similar). Place the tube in a rack and this within an ultrasonic bath for 15min. Repeat this twice. If you do not have an ultrasonic bath, then leave the sample in ultrapure water for one hour at a time.

Example of a sonic bath



Example of Milli Q system

Solutions needed for chemical treatment

All the solutions needed during this procedure should be prepared in advance. Do not use solutions that have been made too long before the use as they may have lost their titer. For this reason, you should prepare a volume of solution that is appropriate for the number of samples treated. Here is a list of all the solution you will need throughout the whole procedure (pure chemicals such as acetone are not included in the list but mentioned later in the protocol, so be sure you have all the materials available before starting). I recommend that solution are at least 99.5% pure (water should be ultrapure).

Table 1: List of solutions required during the treatment.

Solution	Concentration stock soluition	Volumes	Comments
0.2 M HCl	37% HCl	(1 L) 16.4 ml + 983.6 ml dd H_2O	The reaction between acid and water generates a lot of heat and it can be

1% HCl	37% HCl	(250 ml) 6.8 ml + 243.8 ml dd H_2O	very dangerous. As common practice when working with acid, be sure to add the HCl to the water and not the other way around. In this way, the water can dissipate the heat produced and thus reducing the risk of explosion.
pH3 HCl	0.2 M HCl	Add few drops per time until the right pH is reached (pH strips)	
Ultrapure water			E.g. from a MiliQ system

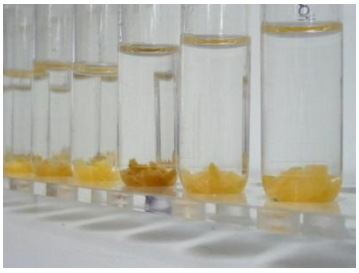
Chemical treatment: protocol

The bone chuck should be in a 16mm test tube labelled using permanent ink. Before the next step, be sure to have removed all the traces of water since concentrated acid can be very reactive in presence of water. Remove residues of water with a pipette or let it dry in the oven at 40 °C for 30 minute or 1h.

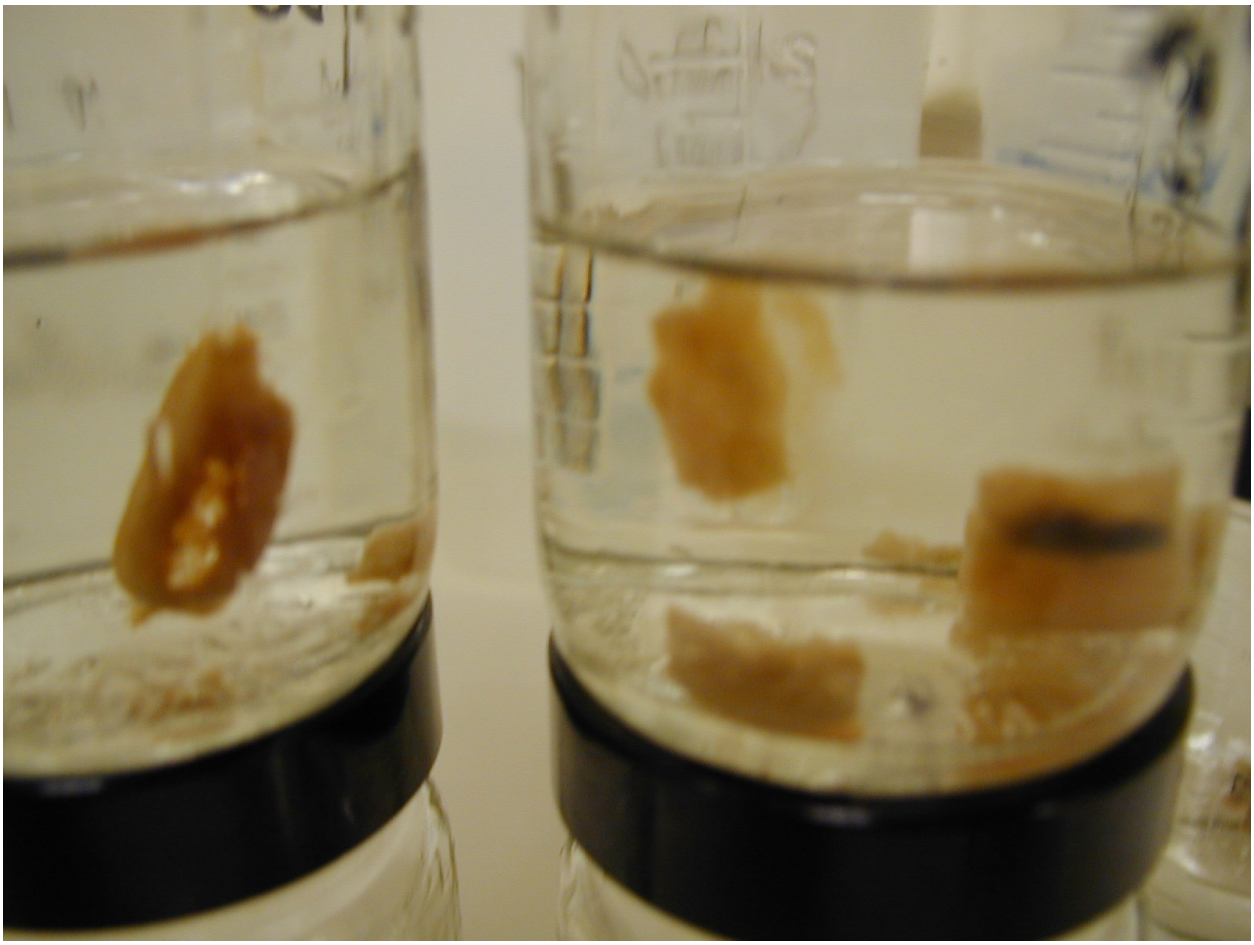
Add few drops (enough to cover the sample) of 37% HCl solution and let it sit for 1h, RT (room temperature).

Add 0.2 M HCl (>20ml) at RT. Cover the tube with aluminum foil to avoid contamination (this is to be employed in all the steps listed below). Change acid every other day until the bone chunks yield 'pseudomorphs', i.e. they are completely decalcified, and appear translucent and flexible. You can press the collagen using a glass rod. If this feels hard the sample has not been demineralized. If the collagen is breaking when pressing with the glass rod and you feel no hard spots then it has been demineralized. Once the sample is fully demineralized wash it 5x in ultrapure water (this should fully cover the sample). Wait 10min in between each wash. When decanting a sample, you may use a glass rod as shown in the figure below to avoid collagen loss.

Using a glass rod while decanting lowers the risk of sample loss



Example I of demineralized collagen



Example II of demineralized collagen

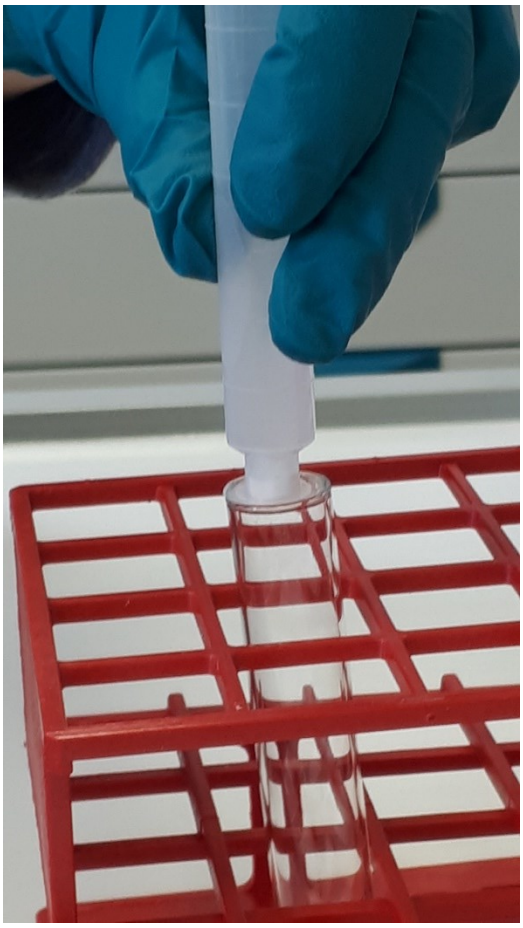
Gelatinization

Fill up the tube up to c. ¾ of its height with ultrapure water. Add 0.2M HCl drops (use a pipette) until pH 3 is reached. This can be checked using pH test paper. Place the tubes in a heat block at a temperature of 70°C and covered by aluminum foil (see figure below). Leave to gelatinize for 24h to 48h. You have to check if the sample is fully gelatinized, that is, the collagen has been fully dissolved. If gelatinization is difficult you can add more acid to bring back the pH to 3 as this will change due to water loss. Notice also that the sample may still contain sediments or humic acids (dark) that will not dissolve.

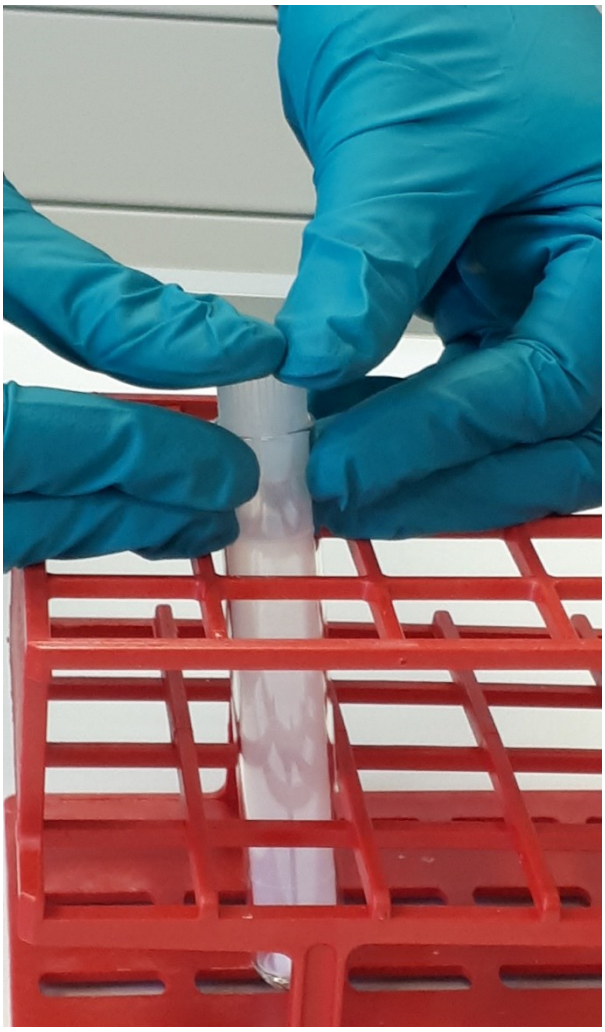
Example of a heating block

Filtration

Centrifuge the tubes at 2500 rpm (or approximately) for 10 minutes. This is to push to the bottom any remaining sediments and non-dissolved contaminant organics. Decant the gelatinized solution into a clean 16mm tube. Avoid (e.g. using a glass rod) the transfer into the clean tube any sediments (if present). Filter the sample using 16mm Ezee filters (9ml capacity). Introduce the Ezee filter into the tube into the gelatinized solution as shown in the figure below (the tube in the figure below is empty but it is supposed to represent the tube with the solution).



Press down the filter until it reaches the bottom of the tube as shown in the figure below.



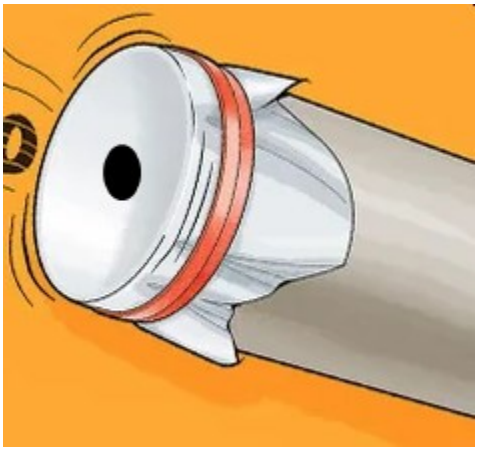
After this decant the filtered solution into a clean and labelled tube.

Sample drying

The best method to dry the sample is to use freeze drying. Freeze dried samples, if well preserved, have a marshmallow like appearance and consistency (see figure below) and this makes the handling/transfer processes considerably easier.

Example of freeze dried collagen

To freeze dry a sample cover the tube containing the filtered solution with aluminum foil and fix it to the tube using a rubber band (as in figure below). Make a small hole on the aluminum foil so that the water can escape. Freeze the sample in a freezer until this is completely solid. If you do not have a freezer, then you can dip the tube into liquid nitrogen. However, this must be done slowly so that the unequal expansion of the water does not cause the tube to break. Once the water is fully frozen put the tubes in the freeze drier and start the machine. Full removal of the water usually takes 24h to 48h.

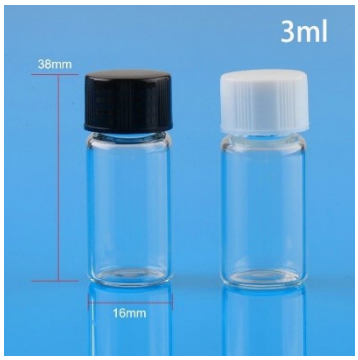


Use aluminum foil with a rubber band to cover the sample before freeze drying. Make a small hole so that water can escape.

If you do not have a freeze drier then you can use a drying oven. However, the sample once dried will not have a marshmallow like structure. The collagen will break, the sample will likely show a yellow coloration, and it may stick to the walls of the tube. If this happens you may have to scratch the collagen out using a scalpel.

Sample storage

Once the sample is dried place it in a clean small container (e.g. small glass tube with cover or Eppendorf tubes). You may also store these containers in a desiccator to avoid exposure to humidity. See figures below.



Sample containers and desiccator

Pre-treatment of bone and teeth for carbonate measurements

All lab material should be thoroughly cleaned. Clean any equipment that may be shared among samples before using it (e.g. scalpels, dremel tips, containers, etc.).

Mechanical cleaning of bone/tooth sample and powdering it

Perform the treatments in a clean, dust-free environment. The mechanical cleaning can itself cause dust and so after treating each sample the space should be cleaned. Ideally, do the mechanical cleaning steps in a separate room from where the chemical steps will be done. Handle the samples at all time with gloves that are free of chalk or other substances that might contaminate the sample. Use a clean electrostatic dissipative lab coat throughout the lab procedures.

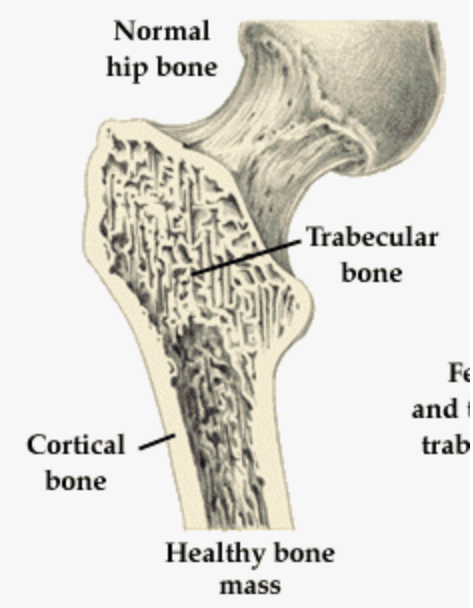
Label all necessary glassware with sample ID using permanent ink that it does not fade.

Before the chemical pre-treatment can start the bone/tooth sample needs to be cleaned of any dirt and of cancellous bone. For carbonate preparation take c. 10-20mg of cortical bone or tooth enamel powder. To clean the bone/tooth you can use a metal scalpel and do it manually (may be the only option for brittle samples) or the dremel using the different metal tips. If you have access to an aluminum blaster you can also use this as it makes cleaning considerably faster.

In the case of teeth be certain that you are only sampling the white exterior enamel and not the dentine (see figure below).

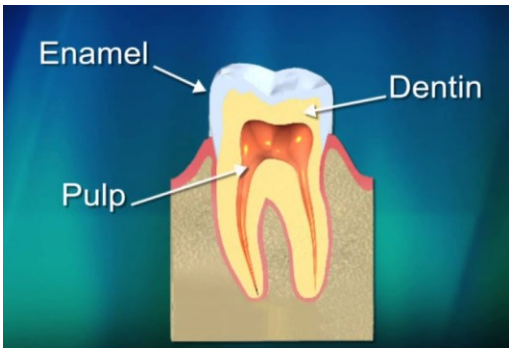
If you are working with bone and this is very fatty you can include an acetone step, as described for the collagen extraction method, before powdering the sample.

To powder the sample, you can use directly an appropriate dremel tip (see figure below). The powder should have approximately the grain size of powdered sugar. Alternatively, you can take a small chunk and powdered it manually using an agate pestle and mortar. If the sample is too hard you can mill the bone/tooth. However, if you use a mechanical mill do not mill the sample beyond the necessary time as overheating can change the isotopic values of the sample. For that, you will have to do some trials and start off by milling the sample for short periods and then gradually increase this as necessary.



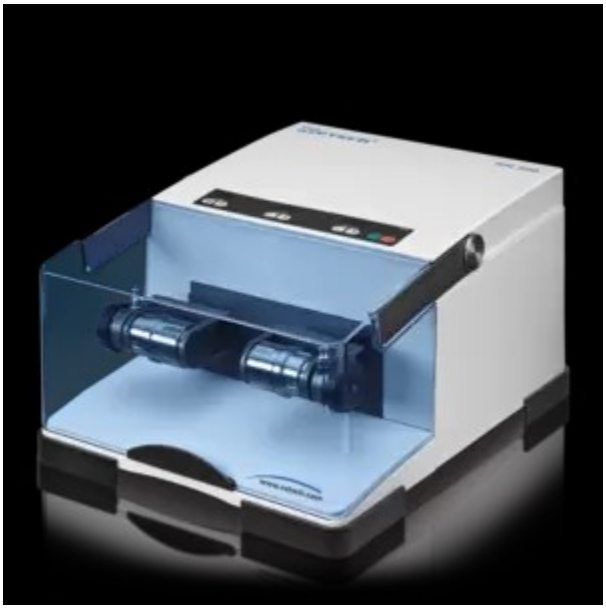
Sample only from the cortical bone for carbonate isotopic analyses.

Once you clean a sample you may use the same dremmel tip (or a different ones) to get bone powder for isotopic analysis. Notices also that you would only need to sample a much smaller part of the bone. 10-20mg of bone powder are needed.



When working with teeth for carbonate analyses sample only the enamel.

An agate mortar and pestle can be used to powder a sample if this is not too hard



A mill may be needed for harder samples. In this case do NOT mill too long as the overheating may alter the isotopic values of the sample. The sample containers for the mills with which I worked were made of high quality stainless steel and grinding balls.

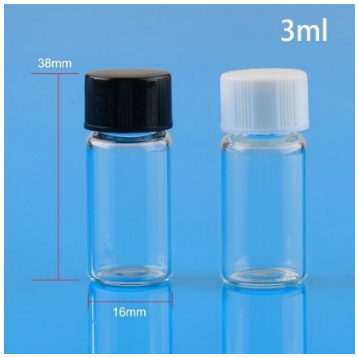
Chemical treatment

Place the powder in a lab tube and add 0.1M acetic acid (c. 0.04ml/mg). Leave in solution for 4h. You need to shake the sample every 30min or alternatively you can use a lab shaker (see figure below). After 4h decant the acetic acid and rinse the sample 5x in ultrapure water. Let the sample rest for c. 10min in between washes. Following this, place the tube in a drying oven and leave it there until the sample is fully dried.

Example of a lab shaker

Sample storage

Once the sample is dried place it in a clean small container (e.g. small glass tube with cover or Eppendorf tubes). You may also store these containers in a desiccator to avoid exposure to humidity. See figures below.



Sample containers and desiccator