This collection details the different established protocols for Zooarchaeology by Mass Spectrometry (ZooMS) for use on archaeological bone. ZooMS allows for taxonomic identification by the peptide mass fingerprinting of collagen type I. These protocols can be used individually or combined depending on the preservation, sample size, and ability to do destructive analysis. All the protocols are optimized for bone as the starting material.

In the acid insoluble protocol bone is pretreated with hydrochloric acid. The resulting collagen bone shaddow is washed to remove the acid and then heated in ammonium bicarbonate to gelitinize the collagen. The extracted collagen is then digested with trypsin and the peptides are purified using C18 ZipTips. This protocol can be used in conjunction with the acid soluble protocol which analyzes the acid used to pretreat the samples.

This protocol is suitable for a wide range of preservation conditions from very good to poor preservation. The ideal starting volume is 10-20mg of bone as a powder or a bone chip. However, smaller sample sizes have yielded high quality results depending on preservation conditions. This protocol can be modified for high throughput in 96 well plates.

If you are using this protocol, please cite the DOI for the protocol and the following two papers on which it is based:
This protocol can be performed in a standard wet chemistry laboratory setting.

2. Wear your personal safety equipment while conducting this protocol (lab coat, chemical resistant gloves and safety glasses).

3. Be aware of your specific lab guidelines regarding sample handling and storage.

4. Be aware of your country and facility specific guidelines regarding the dispose of chemical waste.
MATERIALS TEXT

MATERIALS

- **Seq Grade Modified Trypsin, 100µg (5 x 20µg)** Promega Catalog #V5111
- **Trifluoracetic acid for HPLC** ACROS Organics Fisher
- **Acetonitrile ROTISOLV® HPLC Gradient** Carl Roth Catalog #HN44.1
- **Ammonium hydrogen carbonate 98 % pure** ACROS Organics Fisher Catalog #10364072
- **Hydrochloric acid fuming 37%** for analysis EMSURE® ACS ISO Reag. Ph Eur Merck
- **Sodium hydroxide Pellets** EMSURE® for Analysis Supelco® Millipore Catalog #106498
- **Pierce™ C-18 Tips** Thermo Fisher Scientific Catalog #87784
- **Pierce™ Trypsin-Protease MS grade** Thermo Fisher Scientific Catalog #90057

- Both listed trypsin products are suitable for ZooMS applications. The resuspension buffer indicated in that protocol is used from the Kit manufactured by Promega.
- Standard glass bottles in different sizes (500 ml, 250 ml, 100 ml, 50 ml, 20 ml) e.g. Laboratory bottles, round, clear, with PP-screw cap and pouring ring, Borosilicate glass 3.3 from VWR + 20 ml brown glass bottle e.g. DURAN®, borosilicate glass 3.3, brown
- Pipette tips for different volumina (0.5 µl - 5 ml) e.g. from STARLAB or Eppendorf
- Pipettes with different volumina ranges (0.5- 10 µl, 2-20 µl, 20-200 µl, 100-1000 µl, 0.5-5 ml), e.g. Eppendorf Research plus
- Microcentrifuge tubes e.g. 1.5 ml and/or 2.0 ml, safe lock, Eppendorf
- 15 ml & 50 ml centrifuge tubes: e.g PP-screw cap 50 ml or 15 ml, SARSTEDT
- Standard tube racks for microcentrifuge, 15 ml and 50 ml tubes
- pH strips, e.g. MColopHast (ranges from 0-6, 4-7, 0-14), Merck Millipore
- Scale for laboratory use, e.g. Fisherbrand™ analytical scale, FisherScientific
- Centrifuge with a rotor for 1.5 ml/2.0 microcentrifuge tubes, e.g. Eppendorf 5424 with rotor FA-45-24-11
- Reagent reservoirs, e.g. disposable reagent reservoirs, WVR
- Ultrapure water system, e.g. Milli-Q 7000, MerckMillipore
- Ethanol for cleaning, e.g. ROTIPURAN® ≥ 99.8 %, p.a., denatured, Carl Roth
- Vacuum centrifuge, e.g. Rotation Vacuum Concentrator RVC 2-18 CDplus, Christ
- Incubator, e.g. VWR, Incubator, INCU Line Prime

SAFETY WARNINGS

Be aware of your country and facility specific safety guidelines. This protocol uses several solvents, acids and other chemicals which need special precaution. Please be aware of the international GHS hazard statements (listed below) and follow your country and institute specific precautions/guidelines.

The GHS hazard (H-) and precautionary (P-) statements for the chemicals used in this protocol, are:

**Ethanol** (for cleaning):
- H225 (Highly Flammable liquid and vapor), H319 (Causes serious eye irritation)
- P210 (Keep away from heat, hot surface, sparks, open flames and other ignition sources. - No smoking), P264 (Wash ... thoroughly after handling), P280 (Wear protective gloves/protective clothing/eye protection/face protection), P303+P361+P353 (IF ON SKIN (or hair): Take off Immediately all contaminated clothing. Rinse SKIN with water or shower), P337+P313 (IF eye irritation persists: Get medical advice/attention)

Citation: Samantha Brown, Sandra Hebestreit, Naihui Wang, Nicole Bolvin, Katerina Douka, Kristine Korzow Richter (06/29/2020). Zooarchaeology by Mass Spectrometry (ZooMS) for bone material - Acid insoluble protocol. https://dx.doi.org/10.17504/protocols.io.bf43jqyn

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Acetonitrile:
- H225 (Highly Flammable liquid and vapor), H302+H312+H332 (Harmful if swallowed, in contact with skin or if inhaled), H319 (Causes serious eye irritation)
- P210 (Keep away from heat, hot surface, sparks, open flames and other ignition sources. - No smoking), P280 (Wear protective gloves/protective clothing/eye protection/face protection), P305+P351+P338 (IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing), P403+P235 (Store in a well-ventilated place. Keep cool.)

Trifluoracetic acid:
- H318 (Causes serious eye damage), H314 (Causes severe skin burns and eye damage), H412 (Harmful to aquatic life with long lasting effects), H332 (Harmful if inhaled), H290 (May be corrosive to metals)
- P273 (Avoid release to the environment), P280 (Wear protective gloves/protective clothing/eye protection/face protection), P301+P330+P331 (IF SWALLOWED: Rinse mouth. Do NOT induce vomiting), P304+P340 (IF INHALED: Remove person to fresh air and keep comfortable for breathing), P305+P351+P338 (IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing), P310 (Immediately call a POISON CENTER or doctor/physician)

Ammonium bicarbonate (AmBic):
- H302 (Harmful if swallowed)
- P264 (Wash thoroughly after handling), P270 (Do not eat, drink or smoke when using this product), P301+P312 (IF SWALLOWED: call a POISON CENTER/doctor/... IF you feel unwell), P330 (Rinse mouth)

Trypsin
- H315 (Causes skin irritation), H319 (Causes serious eye irritation), H334 (May cause allergy or asthma symptoms or breathing difficulties if inhaled), H335 (May cause respiratory irritation)
- P261 (Avoid breathing dust/fume/gas/mist/vapors/spray), P264 (Wash thoroughly after handling), P271 (Use only outdoors or in a well-ventilated area), P272 (Contaminated work clothing should not be allowed out of the workplace), P280 (Wear protective gloves/protective clothing/eye protection/face protection), P302 + P352 (IF ON SKIN: wash with plenty of water), P304 + P340 (IF INHALED: Remove person to fresh air and keep comfortable for breathing), P305+P351+P338 (IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing), P332+P313 (IF SKIN irritation occurs: Get medical advice/attention), P310 (Immediately call a POISON CENTER or doctor/physician), P403+P233 (Store in a well-ventilated place. Keep container tightly closed), P501 (Dispose of contents/container to an authorised landfill)

Sodium hydroxide
- H290 (May be corrosive to metals), H314 (Causes severe skin burns and eye damage)
- P280 (Wear protective gloves/protective clothing/eye protection/face protection), P301+P330+P331 (IF SWALLOWED: Rinse mouth. Do NOT induce vomiting), P305+P351+P338 (IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing), P308+P310 (IF exposed or concerned: Immediately call a POISON CENTER or doctor/physician)

Hydrochloric acid:
- H290 (May be corrosive to metals), H314 (Causes severe skin burns and eye damage), H335 (May cause respiratory irritation)
- P280 (Wear protective gloves/protective clothing/eye protection/face protection), P301+P330+P331 (IF SWALLOWED: Rinse mouth. Do NOT induce vomiting), P305+P351+P338 (IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing), P308+P310 (IF exposed or concerned: Immediately call a POISON CENTER or doctor/physician)

BEFORE STARTING
1. We recommend the preparation and storage of chemicals in glass bottles only and no long-term storage of chemicals and buffers in plastic tubes/containers.
2. Preparation of buffers and chemicals at least 24 hrs prior to start (please find detailed instructions on solution preparation in section 1 of this protocol).
3. We recommend the following cleaning protocol for surfaces and equipment: Ultrapure water first (e.g. MilliQ water) and then disinfection with 70% (v/v) ethanol.

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Reagent preparation

1

**General Information:**
1. We recommend to use glassware for buffers and solutions and no long-term storage in plastic containers and/or tubes.
2. TFA, HCl, NaOH and ACN stock solutions (chemicals should be handled under a fume hood only.
3. Always label all chemicals with the compound name, concentration and the date.
4. Chemicals are prepared with water from an ultrapure water system (e.g. Milli-Q water, see materials list)

2

**0.6 M Hydrochloric acid (HCl)** (Shelf life: 6 months at 4 °C)

*Handling of HCl should be performed under a fume hood because of its corrosive properties. HCl can cause severe skin burns, eye and respiratory irritations. Don’t touch your skin or eyes with contaminated gloves and change them immediately.*

2.1 For 1000 ml of 0.6 M HCl, add 50 mL of 37 % (v/v) HCl to 950 mL ultrapure water carefully. Mix gently.

*Always calculate the needed amount of HCl stock solution you need since this is dependent of the specific compound density.*

3

**0.1 M Sodium Hydroxide (NaOH)** (Shelf life: 6 months at 4 °C)

*Handle NaOH carefully because it can lead to severe skin burns and eye damage after exposure. Don’t touch your skin or eyes with contaminated gloves and change them immediately.*

3.1 For 1000 ml total volume, weigh out 4 g of stock NaOH pellets and transfer into a clean glass bottle.

3.2 Dissolve the NaOH pellets in 800 mL of ultrapure water and mix by gently inverting.

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3.3 After the NaOH pellets are fully dissolved, fill up to \(1000 \text{ mL}\).

4 \[50 \text{ mM}\] Ammonium Bicarbonate (AmBiC) buffer, \(pH 8\) (Shelf life: 6 months at \(4 \degree C\))

Please check the pH value regularly since AmBic decomposes over the time which might cause changes in the pH value.

4.1 For 1000 ml, weigh out \(3.95 \text{ g}\) of AmBiC powder and transfer into a clean glass bottle.

4.2 Dissolve the AmBiC in \(800 \text{ mL}\) of ultrapure water and mix by gently inverting.

4.3 Fill up to \(1000 \text{ mL}\) with ultrapure water and mix by gently inverting.

5 \(5 \% (v/v)\) Trifluoroacetic acid (TFA) (Shelf life: 6 months at \(\text{Room temperature}\))

\(\text{Trifluoroacetic acid (TFA) is very hazardous (corrosive)! Handling should only be performed under a fume hood.}\)

\(\text{Prepare this solution in a brown glass bottle since TFA is slightly light sensitive.}\)

\(\text{Always pipette water first, then add the acid slowly!}\)

\(\text{Repeated used of plastic pipettes in TFA stock solutions causes plastics to build up in the TFA stock solution and can interfere with mass spectrometry, therefore ALWAYS use a glass pipette/syringe for TFA.}\)

5.1 Dilute \(1 \text{ mL}\) TFA stock solution using a glass syringe/pipette (no plastic) in \(19 \text{ mL}\) ultrapure water and mix carefully.

6 Wash solution: \(0.1 \% (v/v)\) TFA in ultrapure water (Shelf life: 6 months at \(\text{Room temperature}\))
For 1000 ml, add 1 mL of TFA to 1000 mL of ultrapure water and mix by gently inverting.

7 Conditioning solution: 0.1 % (v/v) TFA in 50 % (v/v) Acetonitrile (ACN), (Shelf life: 3 months at Room temperature)

**ACN is a volatile compound** and if there is too much headspace in the storage bottle the concentration of ACN in solution goes down over long-term storage. We recommend not to store small solution amounts in large bottles over a long period of time. Either prepare new conditioning solution or store the remaining solution in smaller bottles.

7.1 For 1000 ml, mix 500 mL ACN with 500 mL ultrapure water.

7.2 Add 1 mL of TFA carefully and mix by gently inverting.

8 Trypsin solution (Stable once resuspended for 1 month at -20 °C)

8.1 Freeze dried Trypsin (20 µg) is resuspended in 50 µl of Trypsin resuspension buffer (included in Trypsin Kit or per manufactures instructions). The final concentration is now 0.4 µg/µl.

8.2 Transfer the solution in a labelled 0.5 ml tube and store at -20 °C.

Sample preparation and collagen extraction

9 Weighing out the sample

**General information**
1. All steps should take place in a dedicated wet chemistry laboratory.
2. Wipe down all surfaces with 1. ultrapure water, 2. 70% (v/v) Ethanol).
3. Aliquot out the reagents you will need (HCl).
4. Needed after proper bone material demineralization (see step 13): Aliquot out the reagents you will need (AmBic, NaOH).

9.1 **Archaeological samples:** Weigh out 10 mg - 20 mg of bone chips or ~ 10 mg bone powder into a microcentrifuge tube labelled with the sample name, date and your name. Record the weight.
2. **Modern samples:** Weigh out 5 mg - 15 mg of bone chips or ~ 7 mg bone powder into a microcentrifuge tube labelled with the sample name, date and your name. Record the weight.

10

**Acid Demineralization (destructive)**

Add 500 µl of cold (4 °C) 0.6 M HCl to the sample (or enough acid to cover the sample completely) and place the tubes in the fridge at 4 °C. Leave until demineralization is complete (bone becomes flexible/ spongy and no bubbling is visible anymore).

*This may take 1 day to 2 weeks for specific sample types. Less time will be needed for bone powders (4-10 h).*

For samples with good collagen preservation, complete demineralization is not necessary. Please be aware that the more the collagen remains in acid the more it breaks down. Hence monitor samples carefully at this stage to reduce protein loss.

*Don’t forget to prepare blanks at this point! This should be empty tubes with only HCl added. Perform every step with the blanks, which you do with the actual samples.*

11

Centrifuge the sample at 20000 rpm, Room temperature, 00:01:00, or at high speed to settle the bone and (heavier) collagen to the base of the tube.

12

Remove the acid supernatant by pipetting.

*Do not disturb your sample during pipetting.*

*If you want to analyse the acid solubel collagen fraction (please see the protocol "Zooarchaeology by Mass Spectrometry - Acid soluble protocol") you should keep that acid supernatant and transfer it into a new microcentrifuge tube. We recommend to label this tube with the suffix 'AC' and the sample name. The acid supernatant can be stored at -20 °C.*
13

**Washing and humic acid removal**

Add \(200 \mu l\) of \(50 \text{ mM}\) AmBic to each sample, briefly mix by pipetting or inverting the sample, centrifuge, and then discard. Repeat for a total of 3 times.

*If the sample is large, use enough AmBic to cover the whole sample.*

This step is to wash the sample back to neutral pH. If the sample is very small or there is little collagen, less washes can be performed to avoid collagen loss.

13.1

Add \(200 \mu l\) of \(0.1 \text{ M}\) NaOH, mix by pipetting or inverting the sample, and incubate for \(00:05:00\) at Room temperature. Centrifuge briefly. Remove and discard the NaOH wash.

This step is performed to remove humic acids which interfere with the MALDI-TOF MS. If the sample is believed to be highly degraded already, this step can be skipped as it may further damage the remaining collagen.

13.2

Add \(200 \mu l\) of \(50 \text{ mM}\) AmBic, briefly mix by pipetting or inverting the sample, centrifuge briefly, and then discard. Repeat for a total of 3 times

This step is to wash the sample back to neutral pH after NaOH washing. If you skipped that step, you can skip those washes. If the sample is very small or there is little collagen, less washes can be performed to avoid collagen loss.

14

**Gelatinization**

Add \(100 \mu l\) of AmBic to the sample and incubate for \(01:00:00\) at \(65 \, ^\circ\text{C}\).

If your sample amount is limited or if less sample than recommended was available, use \(50 \mu l\) of AmBic for...
If your sample amount is limited or if less sample than recommended was available, use 50 µl of AmBic for gelatinization. If your bone sample is large, use as much AmBic as needed to cover the whole bone for gelatinization.

14.1 During incubation, label two new sets of microcentrifuge tubes. These tubes are to collect the AmBic supernatant after gelatinization. We recommend to label one set with the suffix "EXT" (extract) and the sample name and the second set with the suffix "SE" (second extract) and the sample name. If you used 50 µl of AmBic for gelatinization, only the EXT tube is needed.

14.2 Centrifuge your samples at 20000 rpm, Room temperature, 00:01:00, (or at highest speed) after incubation. If the sample is high precious or heavily degraded, you can use a lower centrifuge speed to avoid sample destruction.

14.3 Transfer now 50 µl of the AmBic supernatant to the "EXT" labelled tube and the remaining 50 µl supernatant to the "SE" labelled tube. The "SE" labelled tube serves as back up and can be stored at -20 °C. Adjust the amounts of "EXT" and "SE" if less AmBic was used for gelatinization.

If the bone was large and required more than about 200 µl of AmBic to cover it, the volume will need to be reduced by evaporation (either open tubes on a heat block or a vacuum centrifuge) until it is a suitable volume (usually around 100 µl - 200 µl) to continue with the protocol as written.
If the original bone sample needs to be stored, freeze sample at \(-20 \, ^{\circ}C\) or dry the sample at Room temperature.

**Digestion and peptide clean up**

15

**Trypsin Digestion**

Add 1 µl of 0.4 µg/µl trypsin solution to the "EXT" labelled tube.

15.1

Incubate sample overnight at \(37 \, ^{\circ}C\) (approx. 12:00:00 - 18:00:00).

15.2

Centrifuge your samples at 20000 rpm, Room temperature, 00:01:00, (or at highest speed) after incubation.

15.3

Aliquot out the TFA you need and add 1 µl of 5 % (v/v) TFA to stop the trypsin.

*The peptides can be purified using a C18 ZipTip. If not extracting immediately, the samples can be stored in the freezer at \(-20 \, ^{\circ}C\) at this point.*

16

**Preperations for C18 ZipTipping**

For each sample you must prepare separate tubes of wash and conditioning solution. Prepare as follows:

1. One tube per sample containing 250 µl conditioning solution (or you can just use a common reagent reservoir - label with “C”)
2. One tube per sample containing 450 µl wash solution (label with "W")
3. One tube per sample with 50 µl conditioning solution- this tube is to collect your purified peptides. We recommend to label it with the prefix “COL” and the sample name.

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**General information:**
During your extraction your peptides are bound to the C18 filter sitting in the pipette tip (ZipTip). The peptides then need to be washed and eluted. Therefore, subsequent wash and conditioning solutions will be ‘contaminated’ with your sample and so solutions after the sample has been bound to the C18 filter and cannot be used for more than one sample.

## C18-ZipTipping

Before use, each tip needs to be conditioned. A separate tip is used for each blank and sample. The pipette should be set at 100 µL and the volume NOT changed during the time. Be careful to avoid pulling air through the filter, especially at the steps with only 50 µL of solution. We recommend to use an extra ZipTipping Only pipette as repeated use of the filters pulls the pipettes out of calibration.

Purify your peptides using the C18 ZipTip from the digested “EXT” sample as follows:

1. Rinse tip twice with 100 µl of conditioning solution and discard.
2. Rinse tip twice with 100 µl of wash solution and discard.
3. Resuspend the sample back and forth over the tip, at least 5 times.
4. Rinse tip twice with 100 µl of wash solution and discard.
5. Elute sample into the prepared “COL” labelled tube with the prepared 50 µl of conditioning solution and pass through tip at least 5 times.

*Be careful not to push air through the C18 filter (never go past the first stop point of the pipette)*![

The sample(s) are now ready to be spotted on the MALDI plate. If not spotting immediately, store sample(s) in freezer at -20 °C.

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