Jun 29, 2020

Zooarchaeology by Mass Spectrometry (ZooMS) for bone material - AmBiC protocol

In 1 collection

DOI
dx.doi.org/10.17504/protocols.io.bffdjji6

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.bffdjji6

Protocol Citation: Samantha Brown, Sandra Hebestreit, Naihui Wang, Nicole Boivin, Katerina Douka, Kristine Korzow Richter 2020. Zooarchaeology by Mass Spectrometry (ZooMS) for bone material - AmBiC protocol. **protocols.io https://dx.doi.org/10.17504/protocols.io.bffdjji6**

Manuscript citation:
Wang, N; Brown, S; Richter, K. K; Ditchfield, P; Hebsetreit, S; Kozilikin, M; Luu, S; Wedage, O; Grimaldi, S; Chazen, M; Horwitz, K. L; Spriggs, M; Summerhayes, G; Shunkov, M; Douka, K. (2020). Testing the efficacy and comparability of ZooMS protocols on archaeological bone. Under review.

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Protocol status: In development
We are still developing and optimizing this protocol

Created: April 22, 2020

Last Modified: June 29, 2020

Protocol Integer ID: 36037

Keywords: ZooMS, zooarchaeology, archaeology, mass spectrometry, MALDI, peptide mass fingerprinting, collagen, protein extraction, bone, non-destructive,

Abstract

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.

The AmBic protocol can be used on samples where distructive analysis cannot be undertaken. Samples are pretreated by soaking in ammonium bicarbonate at room temperature followed by a brief heating step to melt a small amount of collagen out of the bone. The bone can then be dried. The extracted collagen is then digested with trypsin and the peptides are purified using C18 ZipTips.

This protocol is best suited for well preserved remains with good collagen preservation. The protocol suggests 10-20mg of bone material, but as it is non-destructive larger bones or objects can be used. This method is more likely to fail than the acid based methods as collegen preservation decreases.

If you are using this protocol, please cite the DOI for the protocol and the following two papers on which it is based:

Buckley, M., Collins, M., Thomas-Oates, J., & Wilson, J. C. (2009). Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry: RCM*, *23*(23), 3843–3854. https://doi.org/10.1002/rcm.4316

van Doorn, N. L., Hollund, H., & Collins, M. J. (2011). A novel and non-destructive approach for ZooMS analysis: ammonium bicarbonate buffer extraction. *Archaeological and Anthropological Sciences*, *3*(3), 281. https://doi.org/10.1007/s12520-011-0067-y

Image Attribution

Image created by Kristine Korzow Richter. Photo by Ayushi Nayak. Bone icon by iconfield at thenounproject.com. Collagen image adapted from smart.servier.com.

Guidelines

1. This protocol can be performed in a standard wet chemistry laboratory setting.
2. Wear your personal safety equipemt 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

MATERIALS

Seq Grade Modified Trypsin, 100ug (5 × 20ug) **Promega** **Catalog #**V5111

Trifluoracetic acid for HPLC ACROS organics **Fisher Scientific** **Catalog #** Product Code. 11904951

Acetonitrile ROTISOLV® HPLC Gradient **Carl Roth** **Catalog #**HN44.1

Ammonium hydrogen carbonate 98 % pure ACROS Organics **Fisher Scientific** **Catalog #**10364072

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. MColorpHast (ranges from 0-6, 4-7, 0-14), Merck Millpore
- Scale for labarotory 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, VWR
- Ethanol for cleaning (e.g. ROTIPURAN® ≥99,8 %, p.a., denatured, Carl Roth)
- Ultrapure water system, e.g. Milli-Q 7000, MerckMillipore
- Vaccum 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/guiedelines.

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)

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)

Before start

1. We recommend the preperation and storage of chemicals in glass bottles only and no long-term storage of chemicals and buffers in plastic tubes/containers.
2. Preperation of buffers and chemicals at least 24 hrs prior to start (please find detailed instrutctions on solution preperation 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 desinfection with 70% (v/v) Ethanol

Reagent preparartion

1

Note

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 and ACN stock solutions should be handled under a fume hood only.*
3. *Always label all chemicals with the compound name, concentraion and the date.*
4. *All chemicals are prepared with water from an ultrapure water system (e.g. Milli-Q water, see materials list)*

2 [M] 50 mM **Ammonium Bicarbonate (AmBiC) buffer**, pH 8 (Shelf life: 6 months at 4 °C)

2.1 For 1000 ml, weigh out 3.95 g of AmBiC powder and transfer it into a clean glass bottle.

2.2 Dissolve the AmBiC in 800 mL of ultrapure water and mix by genty inverting.

2.3 Fill up to 1000 ml with ultrapure water and mix by gently inverting.

3 [M] 5 % (v/v) **Trifluoroacetic acid (TFA)** (Shelf life: 6 months at Room temperature)

Safety information

Triflouroacetic acid (TFA) is very hazardous (corrosive)! Handling should only be performed under a fume hood.
Prepare this solution in a brown glass bottle since TFA is slightly light sensitive.
Always pipette water first, then add the acid slowly!
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.

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

4 **Wash solution:** [M] 0.1 % (v/v) **TFA in ultrapure water** (Shelf life: 6 months at Room temperature)

4.1 For 1000 ml, add 1 mL of TFA to 1000 mL of ultrapure water and mix by gently inverting.

5 **Conditioning solution:** [M] 0.1 % (v/v) **TFA in** [M] 50 % (v/v) **Acetonitrile (ACN)**, (Shelf life: 3 month at Room temperature)

Note

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 stoorage. 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.*

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

5.2 Add 1 mL of TFA carefully and mix by genty inverting.

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

6.1 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 [M] 0.4 µg/µl .

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

Sample preparation and collagen extraction

7 **Weighing out the sample**

Note

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. *Alliquot out the reagents you will need (AmBic).*

7.1
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.

8 **Sample pretreatment**

Add 100 µL of [M] 50 mM AmBic to each sample.

Note

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

Note

Don´t forget to prepare blanks at this point! This should be empty tubes with only AmBic added. Perform every step with the blanks, which you do with the actual samples.

8.1 Leave samples at Room temperature Overnight to clean and remove any soluble contamination.

Note

You can leave the samples at this stage for up to a week before proceeding with this protocol.

8.2 Centrifuge the sample at 20000 rpm, Room temperature, 00:01:00 , or high speed .

Note

If the sample is high precious or heavily degraded, you can lower the centrifuge speed or skip this step to avoid sample destruction.

8.3 Remove and discard the supernatant.

9 Gelatinization

Add 100 µL of [M] 50 mM AmBic to the sample and incubate for 01:00:00 at 65 °C .

Note

If your sample amount is limited or if less sample then 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.

9.1 During incubation, label two new sets of microcentrifuge tubes.

Note

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 50µl of AmBic for gelatinization, only the EXT tube is needed.

9.2 Centrifuge your samples at 20000 rpm, Room temperature, 00:01:00 or high speed after incubation.

Note

If the sample is high precious or heavily degraded, you can use a lower centrifuge speed to avoid sample destruction.

9.3 Transfer now 50 µL of the AmBic supernatant to the “EXT” labelled tube and the remaining 50 µL supernatant to the “SE” labelled tube.

Note

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 (go to step #9 and see the note).

Note

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.

Note

If the original bone sample needs to be stored, dry the bone/sample at Room temperature.

Digestion and peptide clean up

10 **Trypsin Digestion**

Add 1 µL of [M] 0.4 µg/µl trypsin solution to the “EXT” labelled tube.

10.1 Incubate sample overnight at 37 °C (approx. 12:00:00 - 18:00:00).

10.2 Centrifuge your samples for 00:01:00 at 20000 rpm, Room temperature, 00:01:00 , or at high speed after incubation.

10.3 Aliquot out the TFA you need and add 1 µL of [M] 5 % (v/v) TFA to stop the trypsin.

Note

The peptides can be purified using a C18 ZipTip. At this point the samples can be stored in the freezer at -20 °C .

11 **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 that tube with the prefix “COL” and the sample name.

Note

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.

12 **C18-ZipTipping**

Note

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.

Note

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

Note

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 *.*