**Preparative HPLC separation of underivatized amino acids for isotopic analysis**

Jennifer A. Tripp

UCL Institute of Archaeology

31-34 Gordon Square

London WC1H 0PY U.K.

email: j.tripp@ucl.ac.uk

Thibaut Devièse

Oxford Radiocarbon Accelerator Unit

Research Laboratory for Archaeology and the History of Art

University of Oxford

1-2 South Parks Road

Oxford OX1 3TG U.K.

James S. O. McCullagh

Chemistry Research Laboratory

Department of Chemistry

University of Oxford

Mansfield Road

Oxford OX1 3TA U.K.

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## Abstract

Single-compound analysis of stable or radioactive isotopes has found application in a number of fields ranging from archaeology to forensics. Often, the most difficult part of these analyses is the development of a method for isolating the compound(s) of interest, which can derive from a wide range of samples types including hair, nails, and bone. Here we describe three complementary preparative HPLC techniques suitable for separating and isolating amino acids from bone collagen and hair keratin. Using preparative reversed-phase, ion-pair, or mixed-mode chromatography in aqueous carbon-free mobile phases, or those from which carbon can easily be removed, underivatized single amino acids can be isolated and further analyzed using mass spectrometric techniques.



## Key Words

amino acid (AA), high performance liquid chromatography (HPLC), ion-pair chromatography (IP-HPLC), mixed-mode high performance liquid chromatography (MM-HPLC), preparative chromatography, isotope ratio mass spectrometry (IRMS), accelerator mass spectrometry (AMS), liquid chromatography isotope ratio mass spectrometry (LC-IRMS), $\delta^{13}C$, $\Delta^{14}C$, stable isotopes, radiocarbon dating, compound specific isotope analysis (CSIA), compound-specific radiocarbon analysis (CSRA)

## 1. Introduction

Single-compound isotope analysis has emerged as a useful technique in a wide range of disciplines including archaeology, geology, ecology, and forensics, with applications that include radiocarbon dating, dietary analysis, environmental reconstructions, studies of metabolism, and sourcing agricultural products (1-14). Many archaeological applications of isotopic analysis, such as radiocarbon dating and dietary analysis, involve the isolation of collagen from bones. In this chapter, we therefore focus on the separation and isolation of bone collagen amino acids, for the subsequent measurement of single-compound carbon and nitrogen isotope ratios. These methods have seen several applications and updates since the first edition of this chapter and here we have included these, where relevant, to reflect the latest developments in the field (15-21). In addition to collagen, the methodology has also been successfully applied to separate amino acids in hair keratin, so we have included this procedure as well (22).

While mass spectrometric methods for measuring stable and radioisotopes are established, separation techniques for isolating the desired compounds are still being developed and modified. These chromatographic methods often utilize preparative gas or high-performance liquid chromatography (GC or HPLC) for the isolation of single compounds, and subsequent measurement of their isotopic ratios using isotope ratio mass spectrometry or accelerator mass spectrometry (IRMS or AMS). Chromatographic techniques developed for compound specific isotopic analysis must be compatible with the isotope measurement method and provide appropriate analyte resolution. Contamination of isolated compounds with substances containing isotopes of interest must be avoided. For instance, if carbon-13 or carbon-14 is to be measured, carbon-containing components

in the mobile phase are a potential source of contamination and must be avoided (or completely removed prior to the isotope measurement). In addition, the stationary phase must be stable under the separation conditions to prevent, for example, 'column bleed' contaminating the eluent with C18 alkyl-chain carbon derived from the stationary phase. Derivatization of the compounds, especially common in analytical separation of amino acids, must be avoided, reversed, or corrected. Lastly, unless baseline separation is achieved and the entire eluting peak can be isolated, isotopic fractionation during the separation must be avoided or, if possible, corrected. If the edges of the peak are enriched or depleted in the 'heavier' isotope, and the entire peak cannot be collected, the measured isotopic ratio will differ significantly from the true ratio for the compound.

With these considerations in mind, we have developed several preparative HPLC protocols that result in isolation of sufficient amounts of amino acids that can be collected for analysis of their $\delta^{13}C$ and $\delta^{15}N$ by IRMS, or compound-specific radiocarbon analysis (CSRA) using AMS. A two-step separation procedure was initially developed to allow for the isolation of a number of single amino acids from collagen and keratin (15). The first separation in this procedure involves reversed-phase (RP) HPLC using a C18 column and Milli-Q™ (Millipore, USA) water as the mobile phase. While the more polar amino acids elute in the void volume with no separation, the amino acids with non-polar side chains are efficiently separated. The initial peak from the first separation is collected, concentrated, and re-injected into a RP column, which is packed with a perfluorinated stationary phase, using an aqueous mobile phase containing a surfactant for ion-pair (IP) chromatography (23). This surfactant, or IP reagent, possesses a nonpolar tail that interacts strongly with the RP column, and an ionic head group that can exchange with the amino acids. For the

separation, we chose pentadecafluorooctanoic acid (PDFOA) as the IP reagent. Perfluorinated compounds such as this have been widely used as IP reagents in preparative chromatography due to their lack of UV absorption and volatility, which leads to easy removal from the separated compounds using evaporation. The combination of these two chromatographic processes allows for the efficient separation of bone collagen or keratin amino acids and minimizes contamination from allochthonous carbon. One advantage of this separation method is that the initial RP-HPLC procedure results in quick, simple, and nearly complete separation of essential from nonessential amino acids, a useful distinction for dietary studies. Overall, the method provides accurate results for single-compound carbon and nitrogen IRMS measurements, but AMS dates on single amino acids have proved problematic, likely due to small amounts of contamination from column bleed.

Advances in instrumentation, in particular the introduction in 2004 of the IsoLink™ system by Thermo Electron (Thermo Electron, Bremen, Germany), have provided an alternative to preparative chromatography for the isolation of the amino acids followed by off-line measurement of isotopic ratios. The IsoLink is an integrated LC-IRMS system, and isotope ratios of amino acids separated on an analytical scale can be measured by direct injection of the eluent into the IRMS (24). This instrument is suitable for direct measurement of stable carbon isotope ratios ($\delta^{13}C$), but the IP separation procedure is not suitable for use with this system which is restricted to non-carbon containing mobile phases and modifiers. Thus, a mixed mode separation, utilizing a column containing both ionic and nonpolar groups and an aqueous mobile phase, was developed for use with the IsoLink. The analytical-scale method and its application is described in detail elsewhere (22, 24-27), but a preparative version of the mixed-mode separation has also been

reported for use in separating amino acids for radiocarbon dating (28). This preparative method provides accurate $\delta^{13}C$ measurements and AMS dates on amino acids isolated in a single chromatographic separation. While the mixed-mode separation gave accurate results for both radiocarbon dating and stable isotope analysis, the drawback was that it required multiple injections of the same sample in order to collect enough hydroxyproline for AMS analysis. Recently, a new high-capacity separation focused on hydroxyproline, using the same mixed mode stationary phase, has been developed at the Oxford Radiocarbon Accelerator Unit (21). The approach enables the collection of sufficient hydroxyproline for a radiocarbon date after a single separation of 30-50 mg of hydrolysed collagen. This method is now used routinely at the Oxford Radiocarbon Accelerator Unit to produce accurate radiocarbon dates, in particular for highly contaminated bones.

In this chapter we present in detail the three complementary preparative chromatographic methods for collection of amino acids suitable for subsequent isotopic analysis.

## 2. Materials

Preparative chromatography was carried out on a Varian ProStar HPLC system consisting of two 210 isocratic pumps, a 410 autosampler, a 320 dual-pathlength UV detector, and a 701 fraction collector, controlled by Star Workstation PC software (**Note 1**). The autosampler was fitted with a 1-mL syringe and 2-mL sample loop. The entire system was kept at room temperature during separations with the exception of the last method developed in Oxford for which the column is maintained at 30°C in an oven (21). All solutions were made using ultra-pure water from a MilliQ system and glassware used in

sample preparation for radiocarbon dating was cleaned using an acid-base wash and baked at 500$^{o}$C for 3 hours before use.

### *2.1 Extraction of Collagen from Bone*

The materials listed here are for standard collagen extraction. In the case of modern bone, extremely contaminated bone, or bone that has been treated with conservation agents, additional solvent washes are required prior to demineralization. (**Note 2)**

1. 0.5 M hydrochloric acid
2. Ultrapure MilliQ™ deionized water (**Note 3**)
3. 0.1 M NaOH
4. HCl solution at pH 3, prepared by the careful addition of 6M HCl to an excess of water with stirring. An accurate pH meter should be used to adjust the concentration to pH 3.

### *2.2 Preparation of Hair (Keratin) Samples*

1. 2:1 (v/v) methanol:chloroform

2. 1:2 (v/v) methanol:chloroform

### *2.3 Protein Hydrolysis*

1. 6M HCl

2. Nitrogen gas (or another inert gas)

### *2.4 Preparative reversed-phase HPLC*

1. Column: Waters Symmetry C18, 19 x 150 mm, 7 µm particles.
2. Mobile phase: Ultrapure MilliQ™ deionized water
3. Additional solvent: HPLC-grade methanol

### *2.5 Preparative ion-pair HPLC*

1. Column: Supelco Discovery HS F5, 10 x 250 mm, 5 µm particles. (**Note 4**)

2. Mobile phase: 0.5 mM aqueous pentadecafluorooctanoic acid (PDFOA), prepared by mixing 0.207 g of PDFOA with 1 L of water

3. Additional solvents: HPLC-grade methanol and tetrahydrofuran

4. 6 M HCl

### *2.6 Preparative Mixed-Mode Chromatography*

1. Column: Primsep A, 22 x 250 mm, 5µm particle size (SIELC Technologies, Prospect Heights, IL, USA)
2. Mobile phases: (A) purified water and (B) 0.3% (v/v) phosphoric acid

### *2.7 Accelerator mass spectrometry (AMS) and isotope ratio mass spectrometry (IRMS)*

1. Chromosorb
2. Tin capsules to hold samples

## 3. Methods

### *3.1 Extraction of Collagen from Bone*

The following method is used to extract collagen from modern and archaeological bone in preparation for isotopic analysis. Note the different sample preparation procedure for archaeological and modern bone. Modern bone contains lipids that can interfere with isotope measurements if they are not removed, so a solvent wash is typically performed. Archaeological bones, while containing a trivial amount of lipid, normally have sediment stuck to the outer surface that is removed by abrasive cleaning. The collagen extraction procedure after the initial sample preparation is the same for both sample types. For conventional isotopic analysis in triplicate, about 0.2-0.6 g of bone should be sufficient. For preparative scale radiocarbon dating of individual amino acids it is recommended that at least 1 g of bone be used to account for variability in amino acid abundance and collagen yield. The extraction procedure reported here is based on work published by Longin (29) and further refined and described by Brock et al. (30).

**Bone sample preparation**:

1. For archaeological samples: clean bones by abrasive blasting with silica, ensuring that all ink and other surface contaminants are removed. Remove any extraneous cartilage.
2. For modern samples: place the bone in a beaker and submerge in 2:1 methanol:chloroform; place sample in an ultrasonic bath for 30 min. Then, allow to soak for 24 hours. Remove the bone from the solution using tweezers (**Note 5**), and repeat the procedure once more. After the second wash, examine the bottom of the beaker to see if any lipid is present. If some lipid remains, repeat the wash until no

lipids are seen in the beaker. Finally, rinse three times with purified water, ultrasonicating for 30 min each time.

3. For archaeological samples treated with conservation agents a solvent wash is recommended. Protocols vary based on the nature of the conservation agent. Refer to reference (31) for further information about appropriate methods.

**Removal of the mineral phase from bone:**

Within the last decade a quicker and more efficient demineralization method has been developed, and the new method is described here (30). This method is now routine at the Oxford Radiocarbon Accelerator Unit, including for all samples destined for single-compound isotope analysis and radiocarbon dating.

1. Crush 500 to 1000 mg of bone (accurately measured) to small pieces and place in a test tube. Place tubes in a test tube rack.
2. Add 10 mL 0.5 M HCl to cover bone chunks.
3. Cover the tubes with glass caps or aluminum foil and leave at room temperature, changing the acid every 2-4 hours. Samples may be left overnight if necessary. Demineralization typically takes between 8 and 48 hours depending on the amount of bone and the size of the pieces (**Note 6**).
4. When sample is soft, or floats, the bone has been sufficiently demineralized. Decant the supernatant liquor, then rinse the sample with distilled water three times (**Note 7**).

**Gelatinization**:

1. The remaining bone pellets contain mostly insoluble protein. Place these pellets in a glass test tube and submerge them in dilute HCl at pH 3. Heat to 75°C for 20 hours. This process denatures the collagen triple helical structure making it more soluble. The protein pellets should now go into solution (**Note 8**).
2. Filter the supernatant liquor to remove particulates. The solution now contains mostly dissolved collagen and some salts. Freeze-dry the solution overnight in pre-weighed tubes. The resulting material should be mostly denatured collagen. Weigh after drying to determine the collagen yield.

### *3.2 Hair (keratin) preparation*

Hair is mainly composed of protein and the majority of this protein is keratin, hence no protein extraction procedure is required. However, because hair is exposed to the external environment it is liable to contamination. Cleaning hair samples prior to analysis is vital (**Note 9**).

1. Put hair in 10-mL test tube. Add 8 mL purified water and place in an ultrasonic bath for 30 min.
2. Decant water away from sample. Add 8 mL 2:1 chloroform:methanol and place in an ultrasonic bath for 30 min. Decant solution, add fresh 2:1 chloroform:methanol, and repeat the ultrasonication. If sample still seems oily or greasy, this step should be repeated until hair seems clean.
3. Decant the solution. Add 8 mL 1:2 chloroform:methanol and place in an ultrasonic bath for 30 min.

4. Decant solution. Add 8 mL water and place in an ultrasonic bath for 30 min. Decant water, add fresh water, and repeat the ultrasonication. Decant the solution. The hair is now ready for hydrolysis.

### *3.3 Protein Hydrolysis*

The amount of hydrolysate required will depend upon the desired measurement. IRMS requires about 10 mg of collagen or hair which should be ample for triplicate analysis. Dating individual amino acids using accelerator mass spectrometry requires a larger amount of individual amino acids and ultimately between 1 and 2 mg of carbon from the analyte of interest. Aim to isolate approximately 2-4 mg of the amino acid of interest to ensure between 1 and 2 mg of carbon is used for dating. A graph showing the approximate amino acid abundances in collagen and keratin can be found in Figure 1 (32).

1. Add 6M HCl (1 mL per 10 mg of protein) to the sample in a glass hydrolysis tube and seal under vacuum or nitrogen atmosphere. (**Note 10**)
2. Heat the tube to 110°C for 24 hours.
3. Cool the sample under an inert atmosphere, and then remove the HCl by vacuum evaporation.

### *3.4 Preparative reversed-phase HPLC*

1. Dissolve the isolated amino acid residue in purified water to a concentration of 10 mg amino acids per 1 mL of water, then filter with 2 micron filters. The filtrate is now ready for preparative chromatography and isotopic analysis (**Note 11**)

2. Wash the HPLC column at a flow rate of 8 mL/min, first with methanol for 30 min and then with ultrapurified MilliQ™ water for at least 5 hours.
3. Set the detector to 205 nm. (**Note 12**)
4. Inject 1 mL of sample solution into the column. Elute for 45 min, while collecting fractions at 30 second intervals. An annotated chromatogram of hydrolyzed bovine bone collagen is shown in Figure 2.
5. The fractions containing peaks eluting after 5 min contain the amino acids with nonpolar side groups. After identifying these fractions, combine the tubes containing the same amino acid, freeze the solutions and remove the water by lyophilization.
6. The initial one or two peaks on the chromatogram contain the rest of the amino acids. Combine these fractions, lyophilize, and then dissolve the residue in purified water to a concentration of 10 mg/mL. This becomes the sample solution for the subsequent ion-pair separation.

### *3.5 Preparative ion-pair HPLC*

1. Prepare the sample as described above in Step 3.4.6.
2. Wash the column at a flow rate of 8 mL/min with methanol, tetrahydrofuran, then methanol again, for 30 min each.
3. Equilibrate the column by pumping 0.5 mM PDFOA solution through the column at a flow rate of 8 mL/min for at least 48 hours. (**Note 13**)

4. Inject the 1 mL sample solution and allow to elute for 60 min, collecting fractions every 1 min. (**Note 14**) An annotated chromatogram of the ion-pair separation of hydrolyzed bovine bone collagen is shown in Figure 3.
5. Combine fractions containing the same amino acid and add 1 mL of 6 M HCl per 20 mL of collected sample. (**Note 15**)
6. Remove the mobile phase by lyophilization.

### *3.6 Preparative mixed-mode chromatography*

1. Prepare the sample solution by adding 700 µL of 0.1M NaOH to ~30 mg of lyophilized amino acid. (**Note 16**)
2. Load this solution into a 2-mL BP Plastipak™ syringe (distributed by Fisher Scientific UK Ltd, Loughborough, UK) fitted with a 0.2-µm PTFE syringe filter (Thermo Scientific, Rockwood, TN, USA) to remove any insoluble matter.
3. Add 300 µL of MilliQ ™ water to the same filter to maximise the recovery. The resulting mixture is at a concentration of ~30 mg/mL.
4. Equilibrate the column with purified water for at least 40 minutes at a flow rate of 18 mL/min.
5. Inject 1 mL of sample solution, and elute with pure water for 55 minutes, then run a linear gradient from 55 until 56 minutes from 100% water to 100% phosphoric acid (0.3% by volume). A representative chromatogram showing the separation of archaeological bone collagen hydrolysate, annotated with the amino acid labels, is shown in Figure 4.

6. Combine fractions containing the same amino acid into a single flask and remove the water from the sample using rotary evaporation, gyro-vacuum evaporation, or lyophilization. (**Note 17**)
7. Before beginning the next separation, equilibrate column with water for a minimum of 40 minutes.

### *3.7 Isotope ratio mass spectrometry*

Small amounts of amino acid are often difficult to weigh into the tin capsules used to package samples for IRMS measurements. To deal with this, the following procedure was used.

1. Measure a small amount (tip of a spatula) of Chromosorb™ into the tin capsule.
2. Dissolve the amino acid in a minimal amount of purified water. Drop the solution onto the Chromosorb within the tin capsule.
3. Roll the tin to encapsulate the sample and Chromosorb, and transfer to the IRMS instrument for measurement.

Stable isotope ratio measurements ($\delta^{13}C$ and $\delta^{15}N$) for our studies were made using an elemental analyzer (Carlo Erba, Milan, Italy) coupled to a 20-20 isotope ratio mass spectrometer (Sercon Ltd., Crewe, UK). The isotopic reference used was Vienna Pee Dee Belemnite (VPDB) for $\delta^{13}C$ and air for $\delta^{15}N$ (33).

### *3.8 Accelerator mass spectrometry*

Samples were prepared for AMS dating in the same manner as described for IRMS measurements, except the Chromosorb and tin capsules were baked at 500°C for 12 hours

prior to use. $^{14}C$ dates were measured on an AMS system using a cesium ion source for ionization of the solid graphite sample. Graphitized samples were prepared by reduction of $CO_2$ over an iron catalyst in an excess $H_2$ atmosphere at 560°C prior to AMS $^{14}C$ measurement (34). Calibration and statistical analyses were performed on OxCal software (35-37) using the most recent IntCal calibration curve (38). Background corrections for pretreatment, chromatography, and AMS measurement of radiocarbon dates are described in reference (21).

**4. Notes**

1. Any HPLC system consisting of at least two isocratic pumps that can achieve flow rates up to 10 mL/min and a UV detector would be suitable for these methods. The autosampler and fraction collector are not necessary but make the injection of samples and collection of multiple fractions much easier. Detection methods other than UV, such as refractive index or light scattering, have not been investigated but may work as well.
2. Modern bone contains lipids which are removed by extraction with 2:1 (v/v) methanol:chloroform. Bone that has been conserved usually undergoes a solvent wash consisting of sequential extraction with acetone, methanol, and chloroform. If the identity of the conservation agent is known, a different extraction procedure may be used. A recent study describes and compares a number of extraction protocols. (31)
3. All water used for collagen extraction and HPLC separation is ultrapure MilliQ™ deionized water, even if it is simply described as “water”.

4. This separation was also conducted using the same column used in the reversed-phase separation. While offering higher loading capacity, separation on the C18 column between critical pairs of amino acids (such as hydroxyproline-aspartic acid) was not as efficient. Chromatograms can be compared in reference (15).
5. Do not decant the liquid as lipids may have sunk to bottom of the tube.
6. If the bone is powdered instead of left in chunks, the acid demineralisation step will be much faster and only take a few hours.
7. Alternatively, especially for powdered samples, use an inert filter to remove any small particles during the water wash. Note that some filters are coated with organic substances such as glycerin for preservation, and this coating, if present, must be removed by washing thoroughly in MilliQ water. It is also prudent to conduct appropriate analytical tests (such as %C of the final aqueous wash) to ensure that that the cleaning procedure is sufficient to remove contamination due to the coating.
8. Make sure the test tubes are covered to prevent evaporation, and ensure that the covers stay on during heating as pressure can build up. Previous protocols have recommended heating for 24-48 hours, though the additional time does not seem to affect collagen yield.
9. Hair grows at a rate of approximately 1 cm per month on average. As there is no protein turnover associated with hair keratin, a chronological isotopic record of (primarily) dietary history is represented along the length of the hair ending at the present in the hair root. The whole hair may be analyzed or it may be sectioned prior to hydrolysis to provide a chronological record of changes in constituent amino acid isotope ratios.

10. Use clean glassware (not plastic) that can be sealed and heated to 110°C ensuring no evaporation.
11. Careful note of pH limits for filters used should be made to ensure they do not degrade and contaminate the samples.
12. 205 nm was chosen as the detection wavelength because all of the amino acids absorb at that wavelength, but water has very low absorption. Other detection methods, if available, would likely also give suitable results.
13. Monitoring the baseline during the equilibration will enable you to determine when the column is fully equilibrated. The baseline will remain steady as the PDFOA becomes adsorbed onto the column. At the moment of equilibration, once the column is fully saturated with PDFOA, the baseline absorption rises suddenly as the PDFOA breaks through and elutes from the column. At this point the column is ready to be used for the separation.
14. Separation on the C18 column, if that is used, takes 120 min, though this time would of course vary with column dimensions and particle size.
15. Adding HCl protonates the PDFOA to ensure that it is volatile (its anion is not). Fluorine analysis was used to monitor the removal of the PDFOA from the amino acid sample and this method resulted in fluorine levels below detection limits (15).
16. NaOH is used to moderate the acidity of the sample. This is crucial to avoid degradation of the stationary phase of the column.
17. These three methods of water removal were used interchangeably and as available in the laboratory. Each was tested to show that they did not contribute exogenous carbon to the sample.

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