New protocol for compound specific radiocarbon analysis of archaeological bones

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Abstract

RATIONALE: For radiocarbon results to be accurate, samples must be free of contaminating carbon. Sample pre-treatment using an HPLC approach has been developed at the Oxford Radiocarbon Accelerator Unit (ORAU) as an alternative to conventional methods for dating heavily contaminated bones. This approach isolates hydroxyproline from bone collagen, enabling a purified bone-specific fraction to then be radiocarbon dated by accelerator mass spectrometry (AMS).

METHODS: Using semi-preparative chromatography and non-carbon based eluents, this technique enables the separation of underivatised amino acids liberated by hydrolysis of extracted bone collagen. A particular focus has been the isolation of hydroxyproline for single compound AMS dating since this amino acid is one of the main contributors to the total amount of carbon in mammalian collagen. Our previous approach, involving a carbon-free aqueous mobile phase, required a 2-step separation using two different chromatographic columns.

RESULTS: This paper reports significant improvements that have been recently made to the method to enable faster semi-preparative separation of hydroxyproline from bone collagen, making the method more suitable for routine radiocarbon dating of contaminated and/or poorly preserved bone samples by AMS. All steps of the procedure, from the collagen extraction to the correction of the AMS data, are described.

CONCLUSIONS: The modifications to the hardware and to the method itself have reduced significantly the time required for the preparation of each sample. This makes it easier for other radiocarbon facilities to implement and use this approach as a routine method for preparing contaminated bone samples.

Keywords

Compound Specific Radiocarbon Analysis, Semi-preparative chromatography, Hydroxyproline, Accelerator Mass Spectrometry (AMS)

1. Introduction

Radiocarbon dating is the principal method for building chronologies in archaeology. However, for radiocarbon results to be accurate, samples must be free of contaminating compounds that contain carbon. This is even more crucial when dating material approaching the limit of the method (~50,000 years), because trace amounts of contamination will drastically affect the accuracy of the date. As little as 1% contamination will yield results >10,000 years too young for samples at the age limit [1]. Contamination can originate from a wide range of sources in the post-depositional environment. Humic acids, derived from the breakdown of plant organic matter, are one such group of contaminants. These molecules range in size from a few hundred to several thousand Daltons. They may enter the bone matrix after its deposition and become chemically cross-linked with the collagen [2]. Sample contamination may also occur during excavation and post-excavation activities (i.e. with the application of conservation materials) as well as during laboratory handling.

Bone is the most common archaeological material dated at the Oxford Radiocarbon Accelerator Unit (ORAU) and over several decades much work has been undertaken to improve purification techniques prior to AMS measurement. Collagen, which forms the bulk of the bone protein fraction, is commonly targeted for extraction and subsequent radiocarbon dating. The majority of laboratories extract collagen using protocols based partially on the method outlined by Longin [3]. This consists of an acid-base-acid (ABA) treatment. The initial acidification is designed to demineralise the bone. This is followed by a dilute sodium hydroxide wash designed to remove humic acids from the collagen. Re-acidification of the collagen removes any dissolved carbonate formed during the base wash. Following water washes, the extracted collagen is gelatinised to bring the three polypeptide chains of the collagen into solution, and then filtered (at the ORAU we use Ezee-filters™ (Elkay, Basingstoke, UK)) [4]. For samples showing the presence of consolidant or preservative materials, this chemical pretreatment is preceded with organic solvent extractions (i.e. acetone, methanol and chloroform) [4-6]. Since 2000, the ORAU has used an ultrafiltration protocol to further purify the gelatin samples. Ultrafiltration has been tested upon a broad range of samples from the span of the radiocarbon timescale (i.e. modern back to 50,000 BP). The efficiency of ultrafiltration has been questioned by several authors but results obtained from archaeological bones showed that ultrafiltration appeared to remove contaminating carbon more efficiently than other methods, as verified by the atomic C/N ratios. For material >30 ka BP, ultrafiltration often produced dates that were significantly older, with collagen therefore probably containing reduced levels of contamination[6-8]. However, ultrafiltration

will not remove contaminants that are greater than the molecular weight cut-off of the filter which is 30kD. In cases where there is cross-linking or high molecular weight contamination, ultrafiltration will not remove this material [9-11]. In addition, when the remaining collagen is degraded and broken up, there is a risk of losing the sample during the ultrafiltration and/or collecting an insufficient amount of collagen for AMS measurement. In addition, Brock et al have shown that not all of the <30 kD MW material in the collagen is being passed through the ultrafilter [6]. Despite this, the method appears to improve the quality of the collagen isolated, based on the measured atomic C/N ratios and the percentage carbon on combustion. It is therefore quite widely used in radiocarbon facilities.

An alternative to collagen purification techniques is to focus on compound-specific radiocarbon dating approaches. For bones, this has focused upon extracting single amino acids, or tripeptides, from the collagen [12-16]. One amino acid in particular, (2*S*, 4*R*)-4-hydroxyproline (hydroxyproline or HYP), has been favoured. It has a relatively high abundance in mammalian collagen and it is one of the three most abundant contributors to the total amount of carbon in mammalian collagen (12.9% (Table 1)).

The compound -specific approach has not been widely adopted, in large part because of the cost of the required instrumentation and the problems with quantifying background carbon additions due, for example, to column bleed. This chromatography method is also time consuming, which has so far prevented its adoption as a routine technique for isolating amino acids for radiocarbon dating. It has only been used in a limited number of cases for bone samples, which have high levels of contamination that could not be removed by other methods [9, 10, 18]. In this paper, we describe several modifications to the hardware and novel developments to the amino acid dating method designed to reduce the sample preparation time and allow for accurate dating of samples even when heavily contaminated.

2. Materials & Methods

2.1 Reagents and standards

Amino acid standards (Sigma #09416), hydrochloric acid ≥30% (Fluka #08256) and phosphoric acid 85-90% (Fluka #79606) were obtained from Sigma-Aldrich Ltd (Poole, UK) and used for the optimisation of the chromatographic separation. Water was purified using a Milli-Q™ reverse osmosis system, typically 18.2MΩ.cm resistivity and <4 ppb carbon (Millipore, Watford, UK). Chromosorb™ WAW (Supelco #20123) was obtained from Sigma-Aldrich Ltd and was baked in an oven at 500°C before use. All glassware and Chromasorb™ were also baked at 500°C for 3 hours to eliminate any possible carbon-based

contaminants.

2.2 Archaeological and historical bone standards

Three samples for which we know the ^{14}C content were used as standards for this study (Table 2). The first is a pig bone from the shipwreck of the Mary Rose, which sank in AD 1545. Its radiocarbon age is equivalent to 311 ± 8 BP [19]. The second and third are Alaskan permafrost bison bones which date to ~60-80 ka BP and are therefore beyond the ^{14}C age limit of ~50 ka BP [4, 19].These samples were used during the method optimisation and to check modern and background carbon levels.

2.3 Instrumentation

Chromatographic separations were performed using a ProStar HPLC system (Varian Analytical Instruments, , Walnut Creek, CA, USA) equipped with an autosampler (Model 410), two isocratic pumps with titanium heads (Model 210), a column oven set at 30°C, a Primesep A column 22 × 250 mm, particle size 5 μm (manufactured by SIELC Technologies, Wheeling, IL, USA and distributed by Hichrom, Theale, UK), a UV detector (Model 320) set at 205 nm and a fraction collector (Model 701). The system is controlled by Star workstation PC software (Version 6.0) provided by Varian. The autosampler was modified to include a 1-mL glass syringe and a 2-mL sample stainless steel loop, enabling up to 1 mL of sample to be injected. The system was equilibrated with MilliQ™ deionized water prior to injection.

3. Results and Discussion

3.1 Bone pre-treatment and collagen extraction

The initial bone collagen extraction treatment followed the method outlined by Brock et al. [4] Bone samples were shot-blasted with aluminium oxide to clean the surfaces and crushed using a steel pestle and mortar. The samples were then demineralized with three 0.5M hydrochloric acid treatments at room temperature, the first two for 2 hours and the third one overnight. Following demineralization, the acid insoluble fraction (mainly made of collagen) was rinsed three times with ultrapure MilliQ™ deionized water. The samples were then treated with 0.1M sodium hydroxide for 30 min at room temperature and rinsed three times in MilliQ™ deionized water. A final 0.5M HCl wash was used to eliminate atmospheric carbon dioxide incorporated during the NaOH treatment. Once more, this was followed by three MilliQ™ deionized water rinses. After each acid or base treatment and water rinse, the samples were centrifuged and the supernatant discarded. The resultant collagen was

gelatinized at 75°C for 20 hours in a solution of pH 3 water (15 mL, 1mM HCl) and filtered using Ezee-filters™ (60-90 µm). Finally, the samples were freeze dried to a final pressure of 0.1mbar using a VaCo 5 freeze-dryer (Zirbus, Bad Grund, Germany) for approximately 24 hours.

3.2 Collagen hydrolysis

Freeze-dried collagen samples (40-50 mg) were precisely weighed into 11.5-mL screw top glass test tubes and 6M HCl was added via micropipette at a ratio of approximately 1 mL of 6M HCl per 10 mg of collagen. The tubes were flushed (blanketed) with N_2 gas for 5 minutes to provide an inert atmosphere, capped, and set in a heating block at 110°C for 24 hours. THe samples were previously hydrolyzed in an oven but we replaced the oven with a heating block for a better control of the temperature and to avoid the degradation of the lids when kept at 110°C in the oven. During the hydrolysis, the collagen's peptide bonds are broken thus freeing the amino acids. The hydrolysis solution was then evaporated to dryness in a Genevac EZ-2 vacuum evaporator (Genevac Ltd, , Ipswich, UK) to eliminate the hydrochloric acid, but the samples do remain acidic. 700 µl of 0.1M NaOH was then added to re-dissolve and moderate the acidity of the sample. This is crucial to avoid degradation of the stationary phase of the chromatographic column. This solution was loaded 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 and filtered into a Waters® HPLC 1-mL total recovery vial (Waters, Elstree, UK). 300 µL of MilliQ™ water were added to the amino acid residue and filtered into the same HPLC vial to recover as much sample as possible (Figure 1).

3.3 Chromatography optimisation

Two HPLC separation methods with carbon-free aqueous mobile phase were developed at ORAU. The first one (Method 1) involved a single mixed-mode chromatographic column (Primesep A column (22 × 250 mm, particle size 5 µm; SIELC)) [16]. This SIELC column is silica-based and designed for mixed-mode separation. The column has a dual chemistry stationary phase containing a hydrophobic long alkyl chain and an ionisable embedded carboxyl group. With this mixed- mode column, some of the aromatic amino acids, such as phenylalanine, are separated based on a partial interaction of the non-polar side chains with the reverse phase C18 component of the column but the major mechanism, which allows for the separation of the amino acids, is the ionic interaction of the various ionisable base

moieties, within the amino acids, with the embedded ionic group of the column (cation exchange). The cation exchange is controlled by the eluent ionic strength and pH. Adequate separation of amino acids therefore requires careful control of the eluent acidity. The second method (Method 2) involved two steps [20]. The first separated essential amino acids (EAAs) on a reversed-phase chromatographic column (dC18 Atlantis™ column (19 × 100 mm, particle size 5 μm, Waters Inc., Milford, MA, USA)) and the second separated the non-essential amino acids (NEAAs) on a mixed-mode chromatographic column (Primesep A column (22 × 250 mm, particle size 5 μm; SIELC)).

Different sets of chromatography conditions were tested in order to isolate enough hydroxyproline for AMS measurement and in a single injection. The best separation was observed using a gradient of MilliQ™ deionised water (eluent A) and 0.3% by volume phosphoric acid diluted with MilliQ™ deionised water (eluent B), as described in Table 3, at a total flow rate of 18 $mL.min^{-1}$. Both eluents selected are carbon free which is crucial for this method as any trace of organic solvent could significantly affect the radiocarbon date. We also noticed a variation of the retention time due to the temperature variation in the laboratory. We therefore added an oven to the system to keep the column at 30˚C. This new chromatographic procedure is faster than the previous methods. It allows for the separation of the amino acids present in collagen in a single injection on the Primesep A column in 290 min (Figure 2). With Method 1, each sample required 3 injections and separations over 200 min each. For Method 2, each sample had to pass through two different columns and to be concentrated/evaporated twice using the Genevac system. Between each change of column, the system had to be re-equilibrated and purged, which increased significantly the time required for each sample. With the opening of the chromatographic system to swap the 2 columns and additional handling of the sample in the laboratory, there was also a higher risk of lab-based contamination.

With the new method, the hydroxyproline is collected between 15.5 and 17.5 min representing a fraction of 36 mL. After the elution of the last amino acid, the system is flushed for another 30 min with 100% of Eluent B. The system is then flushed with 100% of MilliQ™ deionized water for a minimum of 2 hours before the next injection (not included in Table 3). The collected fraction containing the hydroxyproline in MilliQ™ deionised water is then concentrated using a Genevac EZ-2 Plus vacuum evaporator. Once transferred into an HPLC vial and totally dried, the amino acid hydroxyproline appears as a white residue which is then reconstituted in 25 μL of MilliQ™ deionised water and loaded by glass pipette onto 12 mg of Chromosorb™ in cleaned tin capsules (Figure 1). It would be difficult to transfer

the dried hydroxyproline into the tin capsules without losing material. It is therefore easier to re-solubilise it into MilliQ™ deionised water and adsorb it into Chromosorb™ already loaded in the tin capsule. The Chromosorb™ also helps to keep the "HYP solution" in the tin capsules while it is being transferred for combustion.

3.4 Quantifying the amount of hydroxyproline collected

In order to estimate the amount of hydroxyproline isolated during the chromatography process, we established a 6-point calibration curve using the peak area. This was prepared by injecting the hydroxyproline standard at known concentrations (Table 4). The results show a very good relationship between the amount of hydroxyproline injected and the response of the detector at 205 nm, with a R^2>0.999 (Figure 3). Based on this calibration, we established that the peak area needs to be at least ~5 million absorption units to obtain enough carbon for a large AMS target (~1.7 mg C) and ~2,500,000 units for a small AMS target (~0.8 mg C). If the peak area is lower than the latter value, a second injection is required (if enough material is available). If not, the sample is not analysed further for AMS dating.

3.5 Combustion and graphitisation

Samples, prepared as described above, were then analysed using a continuous flow isotope ratio mass spectrometry (CF-IRMS) system consisting of a combustion elemental analyser coupled to a gas source isotope ratio mass spectrometer (Sercon Limited, Crewe, UK). The samples were first oxidised to CO_2 and NO_X which was subsequently reduced to N_2, water was removed via a chemical trap and the two gases separated on a GC column packed with a Carbosieve™ (Supelco G60/80 mesh; Bellefonte, PA, USA) stationary phase. Sample gases were introduced to the mass spectrometer via a 50:1 splitter valve and the remaining CO_2 was collected cryogenically in a liquid nitrogen trap. Carbon dioxide collected in this manner was transferred to 10-mL rigs loaded with 2.0-2.5 mg of iron powder catalyst (<10 μm, 99.9+%, Sigma-Aldrich) that had been outgassed in the presence of 500 mbar of hydrogen (450°C, 1 hr). The carbon dioxide was then graphitised in the presence of hydrogen in a ratio of ~2.2 H_2:CO_2 (560°C, 6 hr). During the process, the carbon and nitrogen contents, atomic C/N ratio and stable isotopic ratios were recorded.

3.6 Correction of dates to include carbon contribution related to the HPLC procedure

All the radiocarbon determinations produced at the ORAU were corrected for trace carbon picked up during routine procedures such as pre-treatment chemistry, combustion and

graphitisation. For the HYP dates it is necessary to also include a correction for the extraneous dead (fM=0) and modern (fM=100) carbon added during the chromatographic separation. In order to calculate this, we regularly run background samples (which should not contain any ^{14}C) and modern samples of known age (see Table 2). The dating of the background standards did show that there was some modern carbon contamination, which needed to be taken in consideration (Table 5). The weighted mean of the Mary Rose dates was 318 ± 13 BP which is very close to the expected value (311 BP or 1545 AD). The correction for a dead carbon contribution was therefore negligible, although it was accounted for in our previous procedure, which used 2 columns [20]. The two new formulae applied to correct the AMS ages and account for the uncertainty on the age are reported below (Formula 1 and 2):

$$F^{14}C_{Hyp} = \frac{(AMS - MF_{Mod})}{MF_{Hyp}}$$

Formula 1: Formula for correcting AMS single amino acid ages where AMS is the measured $F^{14}C$; MF_{Mod} is the mass fraction of modern contamination ≈ (AMS_{Std} x C_{Std}) / C_T; MF_{HYP} is the mass fraction of sample hydroxyproline ≈ 1 – MF_{Mod}; AMS_{Std} is the measured $F^{14}C$ of the background standards; C_{Std} is the mass of carbon in the background standards measured on the mass spectrometer; C_T is the mass spectrometer measured mass of carbon in the sample.

$$\sigma F^{14}C_{Hyp} = \sqrt{\left(\left(\frac{1}{MF_{Hyp}} * \Delta AMS\right)^2 + \left(\frac{1}{MF_{Hyp}} * \Delta MF_{Mod}\right)^2 + \left(\frac{AMS - MF_{Mod}}{(MF_{Hyp})^2} * \Delta MF_{Hyp}\right)^2\right)}$$

Formula 2: Formula for calculating the corrected uncertainties on the AMS ages. See Formula 1 for details.

Experimentation at the ORAU over the last few years has led to the implementation of a number of improvements to the compound-specific radiocarbon dating of bone by the isolation of the amino acid hydroxyproline using semi-preparative chromatography. We have incorporated these improvements into the updated protocol described here and demonstrated the performance using a series of background and modern standards. Furthermore, we have successfully applied this method to date archaeological bone samples that have exhibited contamination effects that could not be removed with the routine procedures. These include material from several Palaeolithic sites in Eurasia such as Sunghir and Kostenki (Russia) [22, 23] and Vindija cave (Croatia) [24]. At Vindija, the direct dating of HYP resulted in a substantial change in the date of some of the bones from ~28,000 BP to 43,000 BP [24-26]. This site was initially considered to be a refugium for Neanderthal groups, but the new results

show that the late dates previously obtained were due to incomplete decontamination of the samples prior to the AMS measurement [24].

4 Conclusions

The necessity of applying compound-specific approaches for radiocarbon dating is already well established in the scientific community [27, 28]. The main limitation preventing this procedure being routinely used to date bones has been the time required to purify each sample. In order to make this method a more routine procedure we made significant modifications to the hardware and to the method itself, as described here. The use of a single column has reduced significantly the time required for each sample and the addition of the oven has improved the reproducibility of the separation. The use of a single column has also reduced the dead carbon contamination. This has simplified the correction of the dates compared with the previous approach without affecting measurement precision. It is now possible to prepare up to 10 samples per week and we expect this number to increase in the future. We also expect to see more radiocarbon facilities implementing this approach for bone dating, as well as for other molecules in different geo-archaeological substrates, such as lipids in sediments and ceramic sherds.

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Table 1: Carbon contribution for each amino acid in human bone collagen [17]

Amino acid	Abbrev.	EAA/ NEAA	Formula	MW	Carbon contribution in collagen (%)	HPLC elution order on Primesep A
Glycine	Gly	NEAA	$C_2H_5NO_2$	75.1	16.5	6
Proline	Pro	NEAA	$C_5H_9NO_2$	115.1	15.9	7
Hydroxyproline	Hyp	NEAA	$C_5H_9NO_3$	131.1	12.9	2
Glutamic Acid	Glu	NEAA	$C_5H_9NO_4$	147.1	9.3	3
Alanine	Ala	NEAA	$C_3H_7NO_2$	89.1	8.8	8
Arginine	Arg	NEAA	$C_6H_{14}N_4O_2$	174.2	7.3	17
Aspartic Acid	Asp	NEAA	$C_4H_7NO_4$	133.1	4.8	1
Lysine	Lys	EAA	$C_6H_{14}N_2O_2$	146.2	4.3	16
Leucine	Leu	EAA	$C_6H_{13}NO_2$	131.2	3.9	12
Phenylalanine	Phe	EAA	$C_9H_{11}NO_2$	165.2	3.2	14
Valine	Val	EAA	$C_5H_{11}NO_2$	117.1	3.0	9
Serine	Ser	NEAA	$C_3H_7NO_3$	105.1	2.8	4
Isoleucine	Ile	EAA	$C_6H_{13}NO_2$	131.2	2.1	11
Threonine	Thr	EAA	$C_4H_9NO_3$	119.1	1.9	5
Tyrosine	Tyr	NEAA	$C_9H_{11}NO_3$	181.2	1.0	13
Histidine	His	EAA	$C_6H_9N_3O_2$	155.2	0.9	15
Methionine	Met	EAA	$C_5H_{11}NO_2S$	149.2	0.7	10
Hydroxylysine	Hyl	NEAA	$C_6H_{14}N_2O_3$	162.2	0.5	

Table 2: Details of the reference samples dated after isolation of hydroxyproline using Prep-HPLC with their expected ages.

Lab reference	**Description**	**Expected ages**
P38675	Pig bone from the Mary Rose shipwreck	311 ± 8 BP
P19651	Bison bone from Alaskan permafrost	~60-80 ka BP
P18801 & P40856	Bison bone from Alaskan permafrost	~60-80 ka BP

Table 3: HPLC gradient for the separation of underivatised amino acids on Primesep A column with MilliQ™ deionised water as eluent A and 0.3% by volume phosphoric acid diluted with MilliQ™ deionised water as eluent B.

Time (min)	**Eluent A DI Water**	**Eluent B 0.3M H_3PO_4**
0 - 55	100%	0%
55 - 56	Linear gradient starting with 100% Eluent A and ending with 100% Eluent B	
56 - 290	0%	100%

Table 4: Details of the 6 injections of the hydroxyproline standard to establish the calibration curve.

Mass of HYP (in µg)	**Peak area**
4000	5718497
3000	4166798
2000	2778888
1000	1340366
500	660917
100	81528

Table 5: AMS results of the background and modern standards run on the HPLC to evaluate extraneous dead and modern carbon added during the chromatographic separation. P Code HYP refers to pretreatment based on the extraction of hydroxyproline from hydrolysed bone collagen [4, 9]. C_{Std} is the mass of carbon in the standards measured on the mass spectrometer. CRA is the conventional radiocarbon age, expressed in years BP [21]. AMS $F^{14}C$ corresponds to the fraction modern carbon as measured on the AMS instrument. Details of the independent ages for the background and modern standards are provided in Brock et al. [4]

P Number	Sample	P Code	C_{Std}	CRA	±	AMS $F^{14}C$	±
P18802.138	Fairbanks, Alaska bison	HYP	1.85	49200		0.00103	0.00057
P18802.152	Fairbanks, Alaska bison	HYP	1.35	45200	1800	0.00360	0.00082
P18802.153	Fairbanks, Alaska bison	HYP	1.76	48300	2100	0.00245	0.00064
P19651.141	Ash Bend bison	HYP	1.67	50400		0.00063	0.00063
P19651.163	Ash Bend bison	HYP	1.86	50500		0.00073	0.00057
P19651.148	Ash Bend bison	HYP	2.13	54400		0.00006	0.00054
P40854.2	Mary Rose	HYP	2.14	337	24	0.95889	0.00292
P40854.2	Mary Rose	HYP	2.12	330	24	0.95972	0.00290
P40854.2	Mary Rose	HYP	1.98	303	26	0.96294	0.00314
P39840.31	Mary Rose	HYP	0.90	291	28	0.96442	0.00342

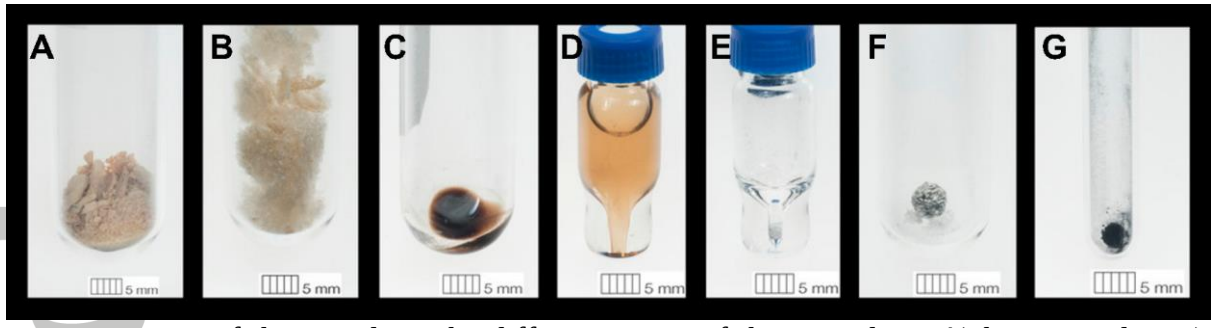

Figure 1: View of the sample at the different stages of the procedure: A) bone powder, B) bone collagen, C) hydrolysed collagen after evaporation of the acid, D) amino acids re-dissolved in NaOH for prep-HPLC, E) hydroxyproline fraction after evaporation of the water, F) hydroxyproline loaded on Chromasorb™ in tin capsule for combustion, G) graphite obtained after combustion of the hydroxyproline. (© Ian Cartwright, Institute of Archaeology, University of Oxford)

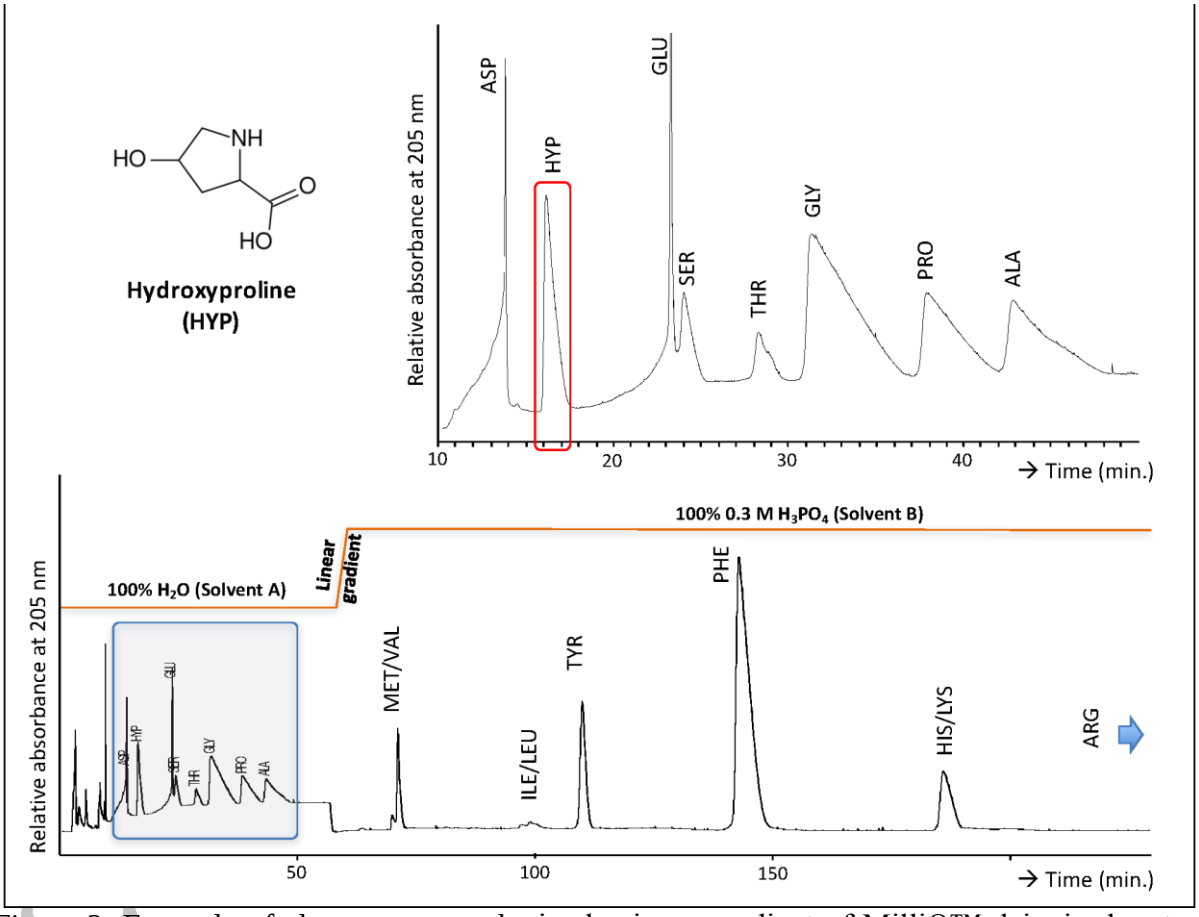

Figure 2: Example of chromatogram obtained using a gradient of MilliQ™ deionised water (eluent A) and 0.3% by volume phosphoric acid diluted with MilliQ™ deionised water (eluent B) as described in Table 3. Arginine elutes around 240-290 min.

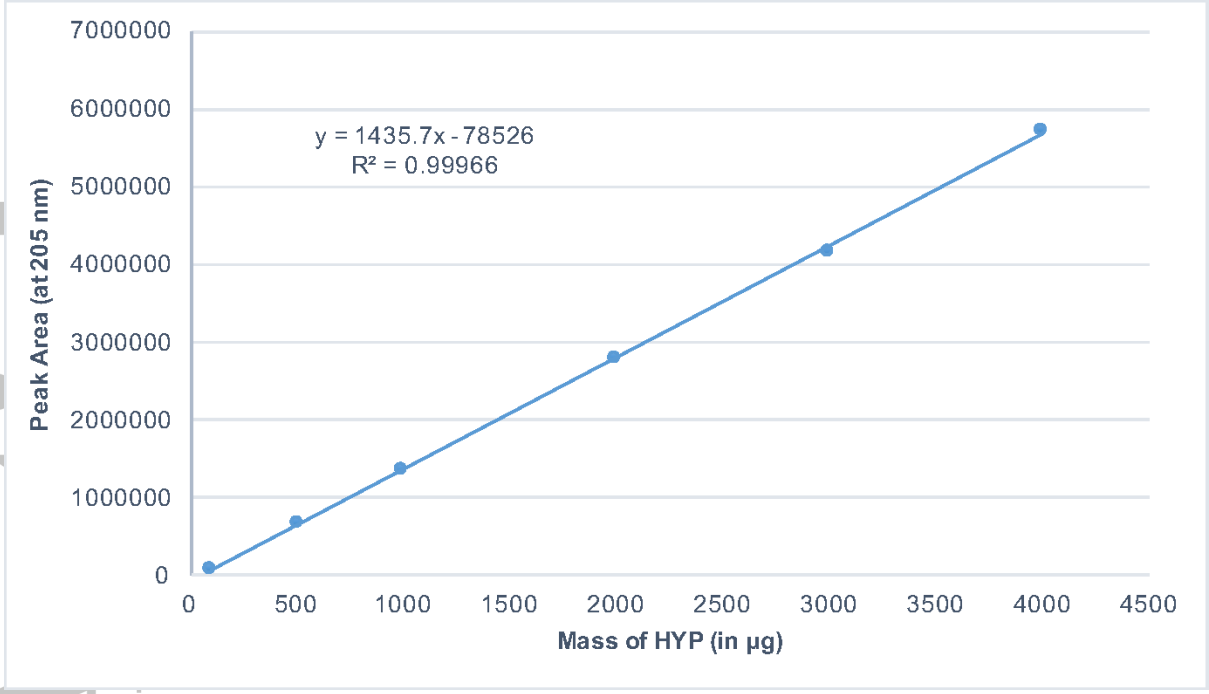

Figure 3: Calibration curve for the hydroxyproline standard (see Table 4 for data).