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The effects of demineralisation and sampling point variability on the measurement of glutamine deamidation in type I collagen extracted from bone

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ABSTRACT

The level of glutamine (Gln) deamidation in bone collagen provides information on the diagenetic history of bone but, in order to accurately assess the extent of Gln deamidation, it is important to minimise the conditions that may induce deamidation during the sample preparation. Here we report the results of a preliminary investigation of the variability in glutamine deamidation levels in an archaeological bone due to: a) sampling location within a bone; b) localised diagenesis; and c) sample preparation methods. We then investigate the effects of pre-treatment on three bone samples: one modern, one Medieval and one Pleistocene. The treatment of bone with acidic solutions was found to both induce deamidation and break down the collagen fibril structure. This is particularly evident in the Pleistocene material (~80,000 years BP) considered in this study. We show that ethylenediaminetetraacetic acid (EDTA), when used as an alternative to hydrochloric acid (HCl) demineralisation, induces minimal levels of deamidation and maintains the collagen fibril structure. Areas of bone exhibiting localised degradation are shown to be correlated with an increase in the levels of Gln deamidation. This indicates that the extent of Gln deamidation could provide a marker for diagenesis but that sampling is important, and that, whenever possible, subsamples should be taken from areas of the bone that are visually representative of the bone as a whole. Although validation of our observations will require analysis of a larger sample set, deamidation measurements could be a valuable screening tool to evaluate the suitability of bone for further destructive collagen analyses such as isotopic or DNA analysis, as well as assessing the overall preservation of bone material at a site. The measure of bone preservation may be useful to help conservators identify bones that may require special long-term storage conditions.

1. Introduction

Bone can survive in the burial environment for millions of years (Collins et al., 1995) and can provide *direct* information about an organism during its life and *post mortem*. Bone contains both organic (mainly proteins) and inorganic components, with the most abundant protein being type I collagen (Rich and Crick, 1961). This fibrous protein consists of three polypeptide chains of similar length (two α-1 chains and one α-2 chain) that form a tightly-wound triple helix (Rich and Crick, 1961; Shoulders and Raines, 2009; Viguet-Carrin et al., 2006; Whitford, 2008). The presence of the hydroxyapatite (mineral) crystals, which embed and protect the protein, contribute to the stability and preservation of bone over geological timescales (Turner-Walker, 2008; Covington et al., 2010).

The extraordinary preservation of collagen in bone has been exploited by archaeologists and palaeontologists seeking to address

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challenges such as species identification (Buckley et al., 2009; Welker et al. (2015)), diet (Ambrose and Norr, 1993) and radiocarbon age (Libby, 1960; Reimer et al., 2013). Recently, the radiocarbon dating of single amino acids such as hydroxyproline (Marom et al., 2012; McCullagh et al., 2010) and improved pre-treatment methods (Brock et al., 2007, 2010; Ramsey and Higham, 2007) have enabled radiocarbon dating to be applied to samples as old as ~50 ka BP (Van der Plicht and Palstra, 2014). However, bones recovered from Middle and Early Palaeolithic and palaeontological sites must be dated by association with other materials, which can be used as substrates for other absolute dating methods (e.g. luminescence or U-series). Therefore a method that could date bone material *directly* would be a valuable tool to archaeologists and palaeontologists. Deamidation measurements could also be used as a screening tool to evaluate the suitability of bone for further destructive collagen analyses such as isotopic or DNA analysis, as well as assessing the overall preservation of bone material at a site. The measure of bone preservation may be useful to help conservators identify bones which may require special long term storage conditions.

Collagen could be an ideal substrate for dating because it has extraordinary potential to be preserved in the fossil record. It was predicted that collagen could survive up to 500,000 years in optimal (i.e. cold) burial conditions (Collins et al., 1995); it has since been found that, even in temperate environments (e.g. in Europe), collagen can survive for much longer than this, up to 1.5 million years (Buckley and Collins, 2011). However, the extent of degradation of collagen increases with thermal age (Dobberstein et al., 2009; Smith et al., 2003), which is defined as an estimate of the equivalent age based upon thermal history, assuming the sample had been held at constant temperature −10 °C (www.thermal-age.eu). A relationship has been suggested between the thermal age and the level of glutamine deamidation (derived from composite estimates of deamidation in several peptides) observed in extracted bone collagen (Van Doorn et al., 2012; Wilson et al., 2012). Given the difficulties of using amino acid racemization dating (AAR) to provide robust age information on collagen (Bada and Helfman, 1975), such a link could provide the key to age estimation for bone samples beyond the range of ^{14}C dating. AAR and deamidation measurements in bone do both share some of the same issues, i.e. bone is ultimately an open system (Dobberstein, 2008; Grun, 2006; Pike et al., 2002). However, one advantage of mass spectrometry is that, although some collagen may be leached/diffused out of the bone, we can be sure, using MS/MS analysis of the peptides, that what we are considering is indeed collagen, whereas AAR analyses incorporates amino acids from all remaining bone proteins, in addition to any contaminant amino acids. The data reported by Van Doorn et al. (2012) showed high variability (ranging from 40% to 90%) in the levels of glutamine deamidation in peptides extracted and analysed from bones of the same age, obtained from the same site.

Here, we explore the potential causes of this variation, and we test two hypotheses: 1) that variation may occur due to natural variability within the biological tissue; and 2) that variation may be induced in the laboratory, during sample preparation. First, we perform a series of experiments that focus on preservation and decay of a single, well-preserved bovine metatarsus of Medieval age. From this bone we determine the variability of glutamine deamidation using mass spectrometry (MS) as a function of:

1) the location within the bone from which the sample was taken (section 3.1);
2) the visible preservation of the bone - comparing degraded and non-degraded sections (Section 3.2);
3) demineralisation method - comparing the effects of two demineralisation methods (using hydrochloric acid (HCl) and ethylenediaminetetraacetic acid (EDTA)) on the levels of deamidation (Sections 3.3 and 3.4).

Second, we explore the preservation of collagen fibrils in samples of different ages, when demineralised using either HCl or EDTA. This was done using TEM to visualise three bones that differ considerably in age: modern, Medieval (bone used in previous sections), and Pleistocene (~80,000 years old) (Section 3.5).

Our aim is to improve the understanding of the effects that sample location and pre-treatment methods may have on collagen preservation. This will allow not only more accurate determination of the extent of deamidation in bone collagen, but also may be useful for other analytical methods that require the removal of mineral, such as radiocarbon dating, isotopic analysis or species identification through collagen mass finger printing (ZooMS). The results presented here derive from a single bone, and therefore need to be further investigating using a range of bone types, preservation levels and ages. Nonetheless, our results provide data that are key to the appropriate interpretation and exploitation of the suggested relationship between deamidation levels and diagenetic history.

2. Methods

An overall schematic of the process we have used for the preparation, extraction and analysis of collagen by mass spectrometry is shown in Fig. 1.

2.1. Preparation and cleaning of bone samples

All three bone sample types (modern, Medieval and Pleistocene) were cleaned at room temperature (~22 °C) by soaking in 50 mM ammonium bicarbonate solution (pH 8.0, prepared in purified water, 18.0 MΩ) overnight. After cleaning, the bones were allowed to dry in a fume hood at room temperature.

2.1.1. The medieval bovine metatarsus

The main sample used in this analysis was a bovine metatarsal bone (Fig. 1) from the site Tanner Row (York, UK), excavated by York Archaeological Trust. The bone is from an un-stratified context but is thought to date between the 11th and mid-13th centuries. This bone was sub-sampled first by slicing into 17 cross sections; some of these cross sections were then further sub-sampled by breaking parts of them into small chips. Because deamidation may be induced thermally (Van Doorn et al., 2012), after cleaning (see Section 2.1), the bone was cut into 17 slices (~3 mm in width) using a diamond-edged water-cooled band saw (Fig. 2). The separate slices were then cleaned in 50 mM ammonium bicarbonate solution and left to dry for one week in a fume hood at room temperature. After slicing the bone, darker sections in the top centre of each of the slices were observed (Fig. 2). These darker sections appeared macroscopically more degraded than the surrounding compact bone and were therefore removed using pliers before further analysis. The remaining pieces of each slice were immersed in liquid nitrogen for 60 s and then removed and broken into small chips using a small impacting hammer; the chips were then sieved though a 2 mm metal sieve and the retained chips (i.e. those of more than 2 mm) were rinsed in purified water and subjected to a range of different collagen extraction procedures (Fig. 1; Sections 2.2, 2.3 and 2.4).

2.1.2. Pleistocene bone

A fragment of bison metapodial bone excavated from a

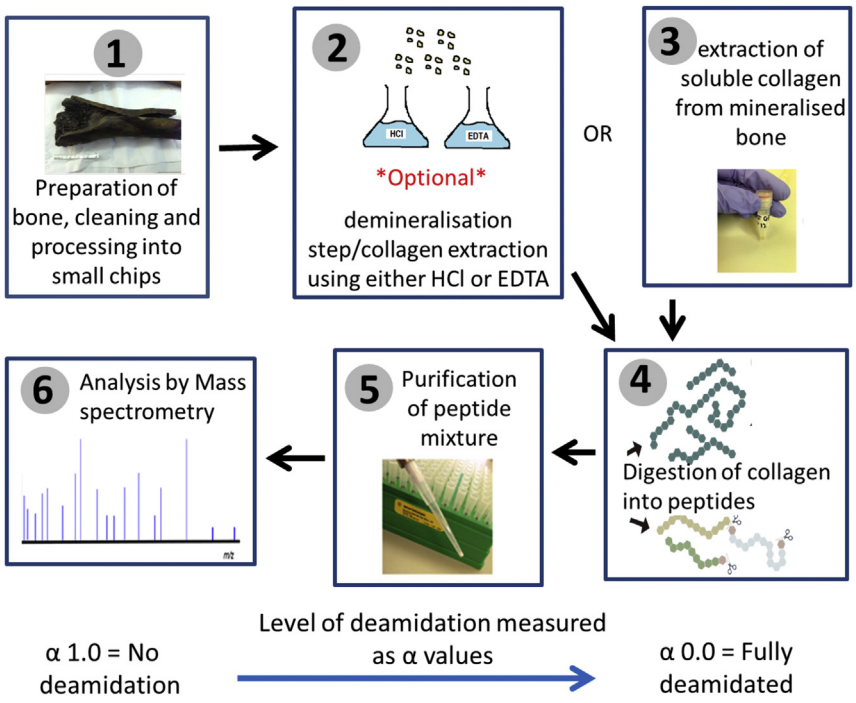

Fig. 1. A schematic of sample preparation protocols. (1) Samples are cleaned in 50 mM ammonium bicarbonate at room temperature overnight. The sample is then cut into small pieces as required; (2) For the demineralisation experiments, the bone is demineralised using either HCl or EDTA, gelatinised, ultrafiltered, freeze dried and the resulting lyophilised collagen is re-suspended in ammonium bicarbonate solution (3) If step two has not been performed then collagen is extracted directly from the mineralised bone by warming in ammonium bicarbonate solution (at 65 °C) for one hour; (4) A tryptic digestion of the extracted protein is carried out overnight in ammonium bicarbonate solution at 37 °C; (5) The resulting peptide mixture is purified using solid phase ZipTips; (6) the peptide mixture is analysed by MALDI-MS (section 2.5); the spectrum is used to estimate the level of deamidation occurring in specific peptides (section 2.6). The calculated glutamine deamidation level is given by the α-value, with a value of 1.0 representing no deamidation and 0.0 indicating complete deamidation of glutamine to glutamic acid.

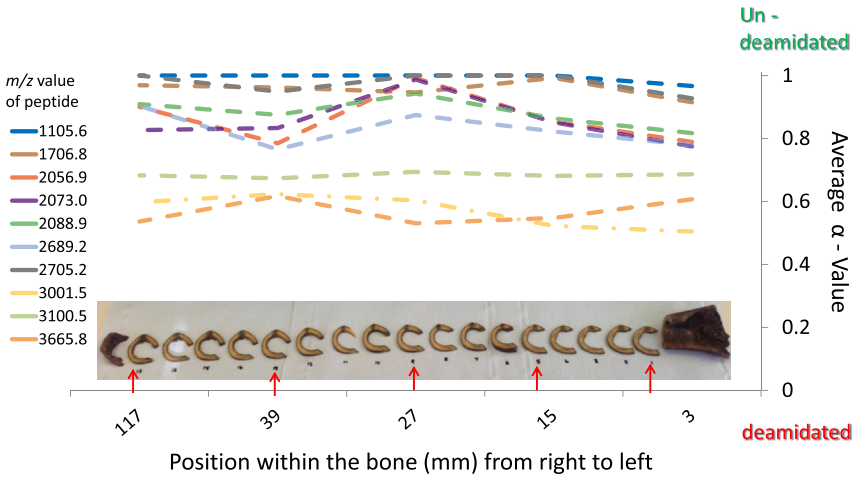

Fig. 2. α-values for 10 peptides, in 10 samples, obtained from two chips from each of the five different positions (slices 1, 5, 9, 13 and 17) across the length of a Medieval bovine metatarsal bone. The average value for the two chips from each slice is plotted.

permafrost site in the Klondike region of Canada's Yukon Territory was investigated. This bone was AMS radiocarbon dated at the Center for Accelerator Mass Spectrometry, Lawrence Livermore National Laboratory, California USA, which provided in a non-age estimate (>50,300 ^{14}C years BP; CAMS 157517). This sample was found in association with a volcanic ash (tephra) layer, Sheep Creek-K, that has been dated to ~80,000 years old (Westgate et al., 2008). As the exact age of this sample is unknown, we refer to this sample throughout this paper as Pleistocene in age. The bone piece was cleaned prior to all analyses as described in Section 2.1.

2.1.3. Modern bone

A piece of modern bovine tibia obtained from a local butcher (Newcastle) was prepared by Dr C. Smith (Smith et al., 2005): the periosteum and marrow were removed with a scalpel and the bone was then sawn into chunks and defatted for 24 h in acetone. The chunks were freezer-milled under liquid nitrogen.

2.2. Extraction of collagen from mineralised bone using ammonium bicarbonate

50 mM ammonium bicarbonate (pH 8) was added to each sample (approximately 100 μL per 30 mg of bone). The sample was then warmed for one hour at 65 °C (adapting extraction procedures described in Van Doorn et al., 2011).

2.3. Hydrochloric acid demineralisation/collagen extraction

For demineralisation in hydrochloric acid (HCl) the standard preparation protocol for stable isotope analyses of Ambrose (1990) was adapted: each chip was placed in a 15 mL polypropylene centrifuge tube and 5 mL of 0.6 M HCl (pH 1) added. The samples were stored at 2–8 °C and the HCl replaced every three days. After 10 days the samples appeared to be visually demineralised, and the acid-insoluble fraction of collagen was gelatinised in 5 mL of pH 3.0 HCl (purified water adjusted to pH 3.0 with 0.6 M HCl solution) at 80 °C for 24 h, filtered through a 30 kDa centrifugal filter (Amicon) and freeze-dried overnight. Prior to MS analysis the lyophilisate was resuspended in 50 mM ammonium bicarbonate (pH 8.0) at a concentration of 2 mg/mL.

2.4. EDTA Demineralisation/collagen extraction

The EDTA demineralisation protocol of Koon et al. (2012) was adapted as follows. 0.5 M EDTA solution was prepared by dissolving 93.06 g of EDTA disodium salt in 500 mL of purified water, and the pH was then adjusted to 7.4 using 0.5 M NaOH. Each bone chip was placed in a 15 mL polypropylene centrifuge tube and 5 mL of 0.5 M EDTA (pH 7.4) added. The samples were stored at room temperature on an electric sample rocker, and the EDTA solution was replaced every three days. After 20 days the samples appeared to be visually demineralised, and the acid-insoluble fraction of collagen was gelatinised in 5 mL of pH 3.0 HCl at 80 °C for 24 h, filtered through a 30 kDa centrifugal filter (Amicon) and freeze-dried. The resulting lyophilised collagen was then resuspended in 50 mM ammonium bicarbonate (pH 8.0) at a concentration of 2 mg/mL.

2.5. MALDI-MS Analysis

The collagen extracts suspended in ammonium bicarbonate solution (pH 8.0)) were digested with 1 μL of porcine trypsin solution (0.4 μg/μL 50 mM acetic acid) overnight at 37 °C. Digests were purified using 100 μL C18 solid-phase tips (Millipore ZipTips). After loading, the tips were washed with 0.1% trifluoroacetic acid (TFA) solution. Peptide mixtures were then eluted in 50 μL of 50:50 (v/v) acetonitrile: 0.1% TFA). The resulting peptide mixtures, consisting predominantly of tryptic peptides, were analysed using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). A volume of 1 μL of sample solution was spotted on a ground steel MALDI target plate, followed by 1 μL of α-cyano-4-hydroxycinnamic acid matrix solution (1% in 50% ACN/0.1% TFA (w/v/v)). The sample and the matrix solutions were mixed together on the plate and allowed to air-dry. Each sample was spotted on to the MALDI target plate in triplicate. Each spot was analysed in reflector mode using a calibrated ultraflex III (Bruker Daltonics, Bremen, Germany) MALDI-TOF instrument. Spectra were analysed using flex Analysis software version 3.0 (Bruker Daltonics).

2.6. Determining the level of deamidation in a peptide

The deamidation of glutamine results in an overall mass increase of 0.984 Da. One disadvantage of the TOF instrumentation used in this work is that due to the insufficient resolving power of the mass analyser, it was not possible to resolve the deamidated and undeamidated signals: the nth peak of the deamidated peptide signal (typically the mono-isotopic signal) overlaps the $(n+1)^{th}$ peak of the undeamidated form (typically the signal for the species containing one ^{13}C atom). The extent of deamidation of glutamine (Q), converting it to glutamic acid (E) can be estimated by deconvolution of the two overlapping distributions as described in Wilson et al. (2012). For a peptide containing just one glutamine residue, a value between zero and one (referred to as the α-value) denotes the proportion of glutamine that is deamidated, and is determined by optimizing the fit of overlapping theoretical distributions with the experimental distributions. An α-value of 1 indicates no deamidation, while a value of 0 results from complete deamidation. The method can be extended to peptides with more than one glutamine residue. Each sample was analysed in triplicate by MALDI-MS and the α-value obtained from a weighted average of the three spectra, where the weights reflect the signal to noise ratio (S/N) of each peptide. Full details are given in Wilson et al. (2012). The code used to calculate deamidation levels is available as an R package from GitHub (https://github.com/franticspider/q2e.git).

2.7. Analysis of collagen fibrils by transmission electron microscopy (TEM)

The modern, Medieval and Pleistocene bovid bone samples were prepared for TEM analysis following the protocol of Koon et al. (2012). Small bone chips around 60 mg in weight from each sample were treated either with 0.6 M HCl or 0.1 M EDTA. Once demineralisation was complete (approx. 2 weeks) the demineralisation solutions were discarded and the samples were prepared for TEM analysed following the protocol of Koon et al. (2012), An FEI Tecnai G2 transmission electron microscope fitted with a CCD camera was used for analysis. The typical optical settings used were as described in Koon et al. (2012) with a beam setting of 120 kV.

3. Results

The results obtained for the Medieval bone are described in terms of the variation in α-values calculated from the MALDI-MS data with respect to: a) the sub-sampling location (and localised areas displaying “macroscopic degradation” on the bone) and b) the collagen extraction protocol. These results are then linked to the structural properties observed in collagen extracted from modern, Medieval and Pleistocene bone, investigated by TEM (Section 3.5).

3.1. Variation of Gln deamidation as a function of sampling location

To investigate the variability in levels of glutamine deamidation (α-values) between different sampling locations within a bone, chips were sub-sampled from parts of macroscopically well-preserved sections of slices 1 (~3 mm from the right), 2 (at ~15 mm) 3 (at ~27 mm), 4 (at ~39 mm) and 5 (at ~117 mm) were sampled (Fig. 2). Two chips were taken from each slice, and extracts from each of these two chips were analysed in triplicate by MALDI-MS. Each triplicate analysis generated one α-value; the two α-values generated for each chip were then averaged, and the average α-values for each slice are what is represented on Fig. 2. Although, initially, twelve peptides were investigated (Table 1), α-values are only reported here for the ten collagen peptides that were observed in collagen extracts from all five slices (Fig. 2).

Fig. 2 shows the average α-value for each peptide from the two chips from each slice. Some peptides produce similar α-values regardless of the sampling location (for example peptides with *m/z* values 3100.5, 1105.6, 1706.8, 2705.2), but other peptides (for

Table 1
12 peptides that are observed in MALDI mass spectra of tryptic digests of bovine type I collagen and contain at least one glutamine residue. Where possible the theoretical amino acid sequence of the peptides has been demonstrated by product ion analysis. For peptides where this was not possible, due to poor spectral quality, sequences were taken from published data (Wilson et al., 2012) and assigned on the basis of the peptides' accurate m/z values.

$[M + H]^+$	Peptide sequence	Collagen chain	Position in collagen chain
836.44	GPAGPQGPR[a]	COLL 1A1	[1084–1092]
1105.57	GVQGPPGPAGPR[a]	COLL 1A1	[685–696]
1690.77	DGEAGAQGPPGPAGPAGER	COLL 1A1	[612–630]
1706.77	DGEAGAQGPPGPAGPAGER	COLL 1A1	[612–630]
2056.98	TGPPGPAGQDGRPGPPGPPGAR[a]	COLL 1A2	[552–573]
2073.01	GAPGADGPAGAPGTPGPQGIAGQR	COLL 1A1	[934–957]
2089.01	GAPGADGPAGAPGTPGPQGIAGQR	COLL 1A1	[934–957]
2689.26	GFSGLQGPPGPPGPSGEQGPSGASGPAGPR	COLL 1A1	[1111–1140]
2705.26	GFSGLQGPPGPPGPSGEQGPSGASGPAGPR[a]	COLL 1A1	[1111–1140]
3001.50	GPSGEPGTAGPPGTPGPQGLLGAPGFLGLPGSR	COLL 1A2	[845–877]
3100.41	GLPGPPGAPGPQGFQGPPGEPGEPGASGPMGPR[a]	COLL 1A1	[187–219]
3665.54	GSQGSQGPAGPPGPPGPPGPPGPSGGGYEFGFDGDFYR[a]	COLL 1A2	[1079–1116]

[a] Assignment of sequence demonstrated using product ion spectrum.

example, peptides with *m/z* values 2056.9, 2073.0, 2689.1 and 3665.8 in particular) show greater variability with sampling location.

Considering each slice as a group, the usual equations for within-group and between-group variance can be used to calculate the variances within and between slices for each peptide (Snedecor, 1934). Thus, the between-slice variance is given by equation (1):

$$V_b = \frac{1}{(S-1)} \sum_{s=1}^{S} n_s (\bar{x}_s - \bar{\bar{x}})^2 \quad (1)$$

where $S = 5$ is the number of slices, n_s is the number of α-values from each slice (i.e. 2, here), $\bar{x}_s$ is the mean α-value for slice s and $\bar{\bar{x}}$ is the grand mean, taken overall slices and the within-slice variance is given by equation (2):

$$V_w = \frac{1}{(N-S)} \sum_{s=1}^{S} \sum_{i=1}^{n_s} (x_{is} - \bar{x}_s)^2 \quad (2)$$

where $N = 10$ is the total number of α-values and x_{is} is the *i*th α-value from slice *s*. Table 2 and Fig. 3 show the within-slice and between-slice variances for the ten peptides, together with the p-values obtained for F-tests comparing the two variances. The variance between slices is shown to be significantly greater than the variance within slices (at the 95% confidence level) for just two peptides, those with *m/z* values 2073.0 and 2689.2, although with a p-value of 0.038 for both the evidence against the null hypothesis is not strong. The peptide with *m/z* value 2056.9 has the highest level of within-slice variation, but is of a similar level to the variance between slices. The remaining peptides also show similar levels of variation within and between-slices.

3.2. Variation due to localised diagenesis

In order to investigate the effect of localised diagenesis on α-values, two chips were taken from the degraded sections of slices 1, 5, 9, 13, and 17 (Fig. 4) and the α-values compared with those obtained from chips in macroscopically well-preserved areas of the same bone slice. The spectra obtained from chips from locally degraded regions contained fewer peaks than those from the macroscopically well-preserved chips, with the heavier peptides (*m/z* 3001.5, 3100.5 and 3665.8) absent in spectra of samples from degraded regions. In the spectra from visibly degraded chips, there were a total of 106 observations of these peptides in comparison to 114 observations in the spectra from well-preserved chips (out of a possible 120). In most cases, the average α-values obtained for macroscopically degraded sections were lower (i.e. the peptides were overall more deamidated) than those extracted from

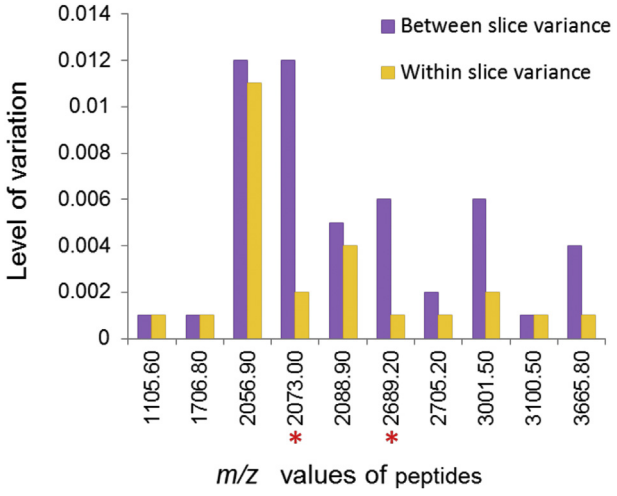

Fig. 3. The variation in α-values obtained from 10 peptides measured in trypsin digests of collagen extracted from bone chips of different slices compared with the variation obtained from replicate chips of the same slice. The P-values for F-tests show that, in general, the between-slice variance is not significantly greater than the within-slice variance. *denotes statistically significant values (at the 95% confidence level).

Table 2
The variation in α-values obtained from 10 peptides measured in tryptic digests of collagen, extracted from bone chips of different slices compared with the variation obtained from replicate chips of the same slice. The p-values for F-tests show that, in general, the between-slice variance is not significantly greater than the within-slice variance. *denotes statistically significant values (at the 95% confidence level).

m/z of peptide	1105.6	1706.8	2056.9	2073.0	2088.9	2689.2	2705.2	3001.5	3100.5	3665.8
Between-slice variance, V_b	0.001	0.001	0.012	0.012	0.005	0.006	0.002	0.006	0.001	0.004
Within-slice variance, V_w	0.001	0.001	0.011	0.002	0.004	0.001	0.001	0.002	0.001	0.001
p-value for F test	0.486	0.486	0.451	0.038*	0.398	0.038*	0.233	0.13	0.486	0.08

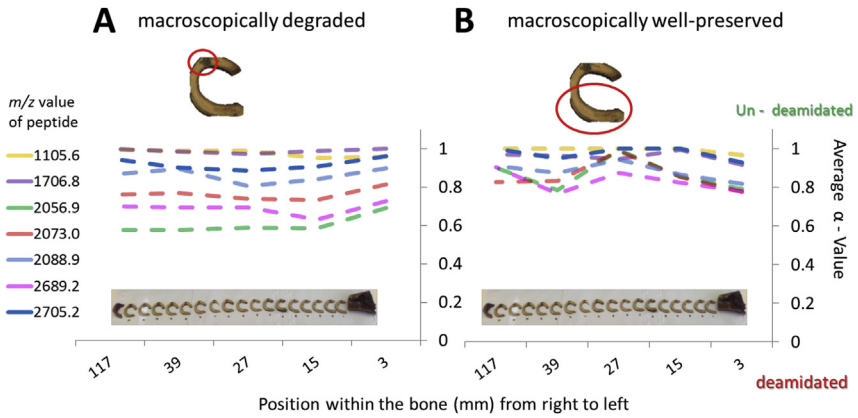

Fig. 4. Comparison of α-values obtained from peptides observed in tryptic digests of collagen extracted from macroscopically degraded sections of bone (A: left) with those from macroscopically well-preserved areas of the same slice (B: right). Here α-values are only plotted for the seven peptides which were observed in all five slices.

macroscopically well-preserved areas. Fig. 4 shows the average α-values for the two chips in each case. Interestingly, the four peptides that show least deamidation in well-preserved chips (*m/z* values 1105.6, 1706.7, 2088.9 and 2705.2, with mean α-values of 0.99, 0.96, 0.88 and 0.98 respectively) also show little deamidation in the degraded chips (mean α-values of 0.98, 0.99, 0.86 and 0.92 respectively).

Other peptides (*m/z* values 2056.9, 2073.0 and 2689.2) show greater changes between the visibly well-preserved (mean α-values of 0.86, 0.85 and 0.83 respectively) and degraded areas (mean α-values of 0.59, 0.76 and 0.69 respectively). Fig. 4 shows that the variation in deamidation levels along the length of the bone is slightly less for the degraded samples than the spread for the well preserved region-derived samples. This can also be seen in Supplementary Table S1, which gives the average difference between slices in comparison to the difference between chips from the same slice. Despite generally higher α-values in the well-preserved samples, the levels of deamidation along the length of the diaphysis is not consistent in some peptides. It is possible that the greater variation in α-alpha-values for sub-samples taken from the well-preserved slices may be due to the fact that the degraded sub-samples were taken from a smaller region of the bone. As we have seen degraded samples with much lower alpha-values than those presented in Fig. 4, we do not believe that the alpha-values for the degraded sub-samples represent an endpoint of deterioration.

3.3. The effects of acid demineralisation on deamidation

The removal of mineral using HCl is common in most bone preparation techniques, such as those for isotope analysis and radiocarbon dating (e.g. Brock et al., 2007). An alternative to the use of HCl for the decalcification of bone is the use of EDTA as a chelating agent. EDTA decalcification is often used when trying to minimise damage to the surface histology of bone (Jonsson et al., 1986; Tuross, 2012).

HCl demineralisation was compared with the ammonium bicarbonate collagen extraction method developed by Van Doorn et al. (2011), which does not involve the removal of mineral from the bone. We assessed the effects of HCl demineralisation on the overall deamidation using bone chips from macroscopically well-preserved areas of slices 1, 5, 9, 13 and 17. The α-values of 12 peptides produced after HCl treatment (Table 2) were compared with those determined from chips from similarly well-preserved areas of the same slice, in which collagen was extracted using the ammonium bicarbonate extraction method. The 12 peptides were observed less frequently in spectra from samples treated with HCl than from those treated with only ammonium bicarbonate (Fig. 5A). In the spectra obtained from the HCl-treated samples, only 74 (of a possible 120) observations of the peptides were recorded, compared with 114 in spectra from mineralised collagen extracted with ammonium bicarbonate (Fig. 5A). This suggests the HCl treatment affects the peptides detected in the samples. Five of the twelve peptides (*m/z* 1690.8, *m/z* 1706.8, *m/z* 2057.0, *m/z* 2073.0, *m/z* 2089.0) were observed in less than half of the HCl-treated samples. It should be noted that each of these peptides has an aspartic acid on the N-terminal side of glycine. The remaining peptides, observed in at least half of the HCl-treated samples, did not contain aspartic acid.

In observed peptides, the α-values calculated for samples treated with HCl were generally lower than those from samples treated only with ammonium bicarbonate (Fig. 5 (B)), indicating greater levels of deamidation in HCl-treated samples.

3.4. Effects of demineralisation time on α-values

In order to compare the effects of HCl (pH 1) and EDTA (pH 7.4) on glutamine deamidation, the remaining unanalysed chips from the macroscopically well preserved sections of the 17 slices of bovine metatarsal were mixed together. A total of 24 chips from this sample set were demineralised for up to four weeks in either HCl or EDTA (see sections 2.3 and 2.4). For each demineralisation method, four chips were removed from the solutions after 2, 3 or 4 weeks. The collagen was extracted as described in sections 2.3 and 2.4. The resulting collagen extracts were digested and purified as described in section 2.5 and analysed using mass spectrometry.

For each of the samples, levels of glutamine deamidation were calculated (section 2.6). The patterns observed can be split into three categories: 1) peptides (i.e. *m/z* 2689.3, 2705.2 and 3100.4) which showed lower α-values (i.e. more deamidation) with increased variability when treated with HCl than EDTA (Fig. 6). 2) peptides (i.e. *m/z* 2705.2 and 3100.4) which showed increased levels of deamidation on acid treatment over time, with α-values for *m/z* 2705.2 ranging from 0.57 to 0.87 in HCl-treated samples; this peptide shows little or no deamidation in samples treated with EDTA over the four week period, with values of EDTA treated samples producing α-values ranging from 0.92 to 1.00. 3) Some of the smaller peptides (*m/z* values 836.4 and 1105.6) showed little difference in deamidation levels regardless of the demineralisation procedure used, or the length of time they were treated. Examples

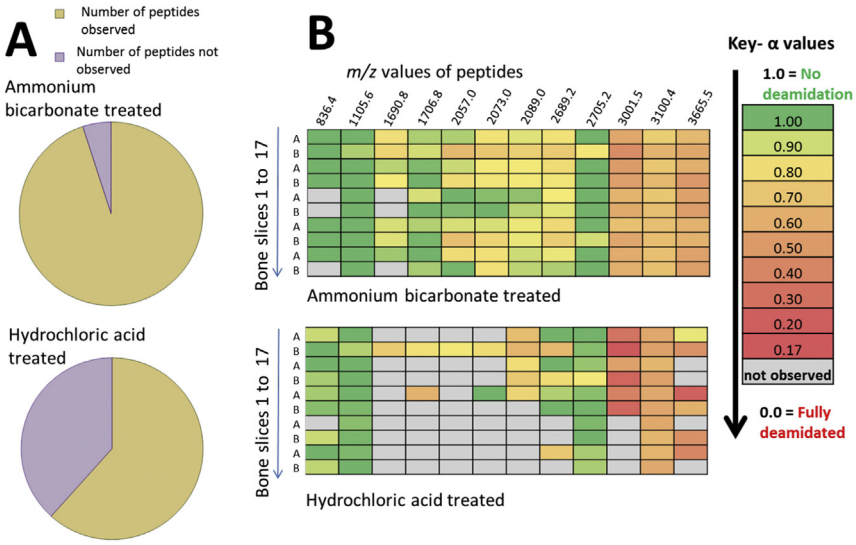

Fig. 5. (A) A comparison of the number of times the peptides in Table 1 were observed in spectra obtained from samples treated with HCl or ammonium bicarbonate solutions. (B) Comparison of α-values obtained for these 12 peptides in spectra from macroscopically well-preserved areas of the Medieval bone (2 each from slice: 1, 5, 9, 13, and 17) after treatment with ammonium bicarbonate (top) or HCl (bottom).

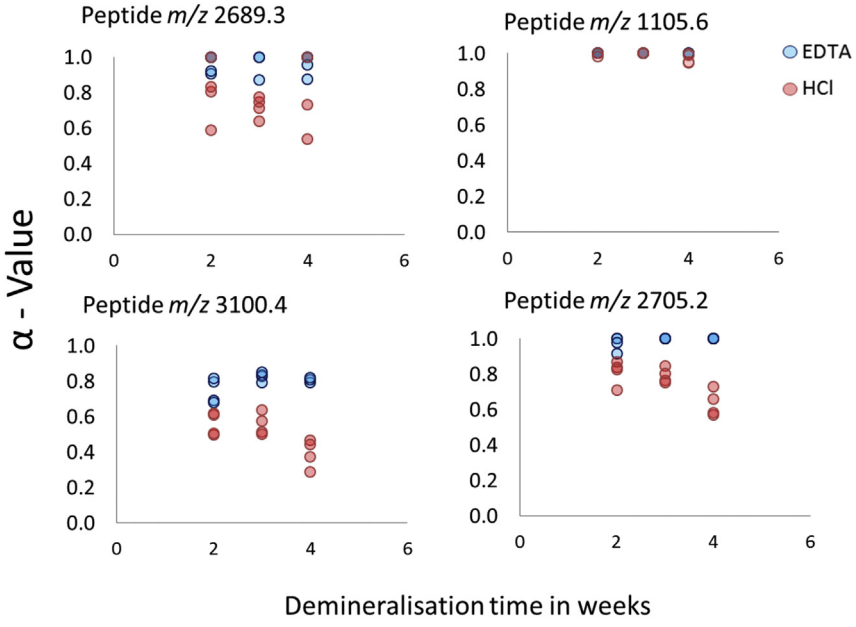

Fig. 6. Comparison of α-values obtained for four peptides after demineralisation in HCl or EDTA for 2, 3 or four weeks. Peptides with smaller masses such as 1105 showed little deamidation regardless of the demineralisation method used. In samples pre-treated with HCl three peptides (m/z 2689.3, 2705.2 and 3100.4) showed an increase in deamidation over time, in contrast to EDTA pre-treatment which did not appear to induce deamidation over time.

from the three categories are shown in Fig. 6.

3.5. Comparison of collagen fibril structure in modern, Medieval and Pleistocene bone demineralised with either EDTA or HCl using transmission electron microscopy (TEM)

To investigate the effect of different demineralisation methods on the structure of collagen fibrils, three bovid bones of different ages, modern, Medieval and Pleistocene were used. Bone chips from each sample type were sampled and the mineral from each sample was removed using either HCl or EDTA. The extracted collagen was visualised using TEM and the preservation state and average width of the collagen fibrils was investigated. Measurements of the width were taken at ten points along the length of 20 fibrils, resulting in a total of 200 measurements for each of the six samples. The distribution of measurements was assessed to be plausibly normal for each sample and the statistical significance of the difference in mean fibril width between HCl and EDTA treated samples was determined using a two-tailed, two sample t-test for unequal variances for each of the modern, Medieval and Pleistocene samples. In each case, the average fibril width was found to be significantly larger for HCl-treated samples than in EDTA-treated samples (Table 3).

In the TEM observations, collagen fibrils are shown by the characteristic dark and light banding along the length (Fig. 7). This is due to the highly regulated structure and arrangement of the

Table 3
Average fibril width measurements from three samples of bone of different ages (modern, Medieval and Pleistocene). Fibril widths measured in all three samples were found to be statistically significantly different at the 95% confidence level, when prepared using the two pre-treatment methods. In each case, the t-test shows the average fibril width is significantly greater in HCl-treated samples.

Sample	Mean fibril width (HCl treated)	SD	Mean fibril width (EDTA treated)	SD	p-value
Modern bone	90.63	14.63	76.88	13.31	1.99 E-20
Mediaeval bone	96.36	29.90	72.18	23.37	1.02 E-17
Pleistocene	96.77	33.68	69.11	15.91	5.2 E-22

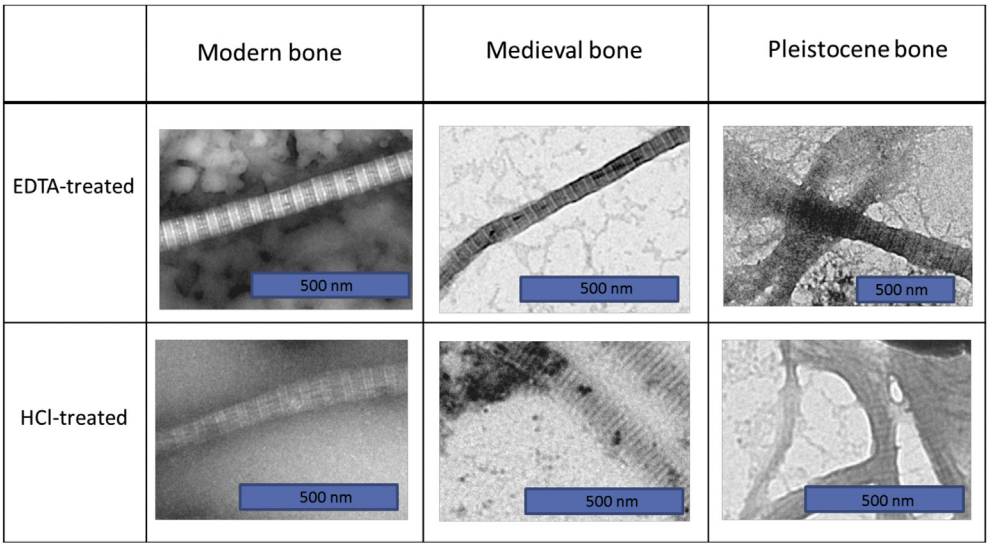

Fig. 7. Transmission electron micrographs of collagen extracted from modern, Medieval and Pleistocene bone treated with either 0.6 M HCl or 0.5 M EDTA.

fibrils within the collagen protein (Orgel et al., 2001). However, the HCl-treated modern collagen resulted in fibrils with less uniform fibril widths than those treated with EDTA, as well as regions of swelling along the fibril length (Fig. 7). In contrast, the collagen from the modern bone treated with EDTA resulted in a higher number of fibrils per square on the grid than those treated with HCl, with less swelling and a more uniform fibril width (Fig. 7). The effect of HCl demineralisation was also evident in the Medieval bone. When treated with HCl, the extracted fibrils showed less defined structure with areas of swelling and more disruption to the banding than those treated with EDTA (Fig. 7). The detrimental effect of HCl demineralisation on fibril structure was most evident in Pleistocene bone, with very few collagen fibrils displaying the characteristic banding, whereas banding was still evident in the majority of the fibrils in the EDTA-demineralised sample.

4. Discussion

4.1. Spatial variation in deamidation levels within a sample

Our findings show that, in the Medieval bovine metatarsal bone investigated here, the sampling location across areas of well-preserved compact bone does not generally contribute significantly to differences in the level of deamidation observed. This may be attributable to highly structured and repetitive nature of the protein and the dense packing of the surrounding mineral. Samples taken from areas of bone that displayed localised macroscopic diagenesis showed elevated levels of deamidation of some peptides. This may be due to localised differences in the bone structure in this “darkened” region; for example, bone is less compact and more porous at sites of muscle attachment than the surrounding bone (Hawkey and Merbs, 1995; Mann and Hunt, 2013) and therefore may be more susceptible to diagenetic processes. It should be noted that only one bone was used to investigate sampling point variability in this study and although the protein structure is conserved throughout different bone types (e.g. long or flat bones) the level of mineralisation or the effect of structural anatomical differences on levels of glutamine deamidation has not been investigated. The increased deamidation from areas of the bone that display localised, macroscopic diagenesis highlights the importance of sampling from areas that are representative of the overall preservation of the bone, i.e. by avoiding areas that are clearly and visibly compromised.

4.2. Effects of sample pre-treatment and extraction methods on glutamine deamidation and the collagen fibril structure

The gentle collagen extraction method developed by Van Doorn et al. (2011) has the advantages of being fast to perform and minimally destructive to the bone, as it does not require decalcification pre-treatment. However, we have found that this extraction does not always yield sufficient amounts of collagen for successful MS analysis. Extraction using only ammonium bicarbonate solution may result in partial collagen extraction for a number of reasons. For example, as the buffer-soluble collagen is easily extracted, it is possible that much of it may be lost due to leaching or exchange within the burial environment, especially in sites with fluctuating water tables (High et al., 2015). Also, the buffer-soluble fraction is likely to be gelatinised and therefore may not be truly representative of the general state of preservation of the majority of the mineralised bone collagen.

Our results show that demineralisation treatment using HCl influences the extent of deamidation; HCl increases the level of glutamine deamidation and decreases the number of peptides

detected in comparison with EDTA treatment. Both asparagine and glutamine deamidation have been studied in a range of sample types, from short synthetic peptides (Geiger and Clarke, 1987; Li et al., 2010; Robinson et al., 1970; Robinson, 2004; Stratton et al., 2001) to proteins such as α-crystallin of the eye lens (Takemoto and Boyle, 1998), collagen (Hurtado and O'Connor, 2012; Van Doorn et al., 2012; J. Wilson et al., 2012), keratin (Araki and Moini, 2011) and protein binders in paint (Leo et al. 2011). Asparagine is known to have two deamidation pathways: either via a cyclic succinimidyl (five membered ring) intermediate, or via direct side chain hydrolysis (Capasso et al., 1991; Radkiewicz et al., 1996; Xie and Schowen, 1999). The latter reaction has been found to be favoured at low or high pH (Robinson, 2004). Glutamine can also deamidate via two pathways (Robinson, 2004; Li et al., 2010), forming a cyclic glutimidyl (six membered ring) intermediate. The two residues have different rates of deamidation via cyclic intermediates, with glutamic acid forming at a slower rate than aspartic acid (Li et al., 2010). The most probable route of deamidation for both residues in a highly structured protein such as collagen is via direct side chain hydrolysis, due to the lack of flexibility necessary for the protein backbone to adopt the appropriate interatomic distance needed for the formation of the cyclic intermediates (Van Duin and Collins, 1998). It is therefore likely that the two residues are equally stable in proteins such as collagen. However once in solution, gelatine (the soluble form of collagen) no longer has the same rigid structural constraints, and exists in the form of random coils.

We observe increased levels of glutamine in HCl (pH 1) treated samples. This is most likely due to an increase in direct side chain hydrolysis, which is less likely to occur during the ammonium bicarbonate or EDTA extractions, both carried out at around pH 8.0 (Robinson, 2004). Low pH is known to induce peptide bond hydrolysis (Hill, 1965). However, in the experiments presented here the bone was treated in a fairly weak acid solution (0.6 M HCl) under refrigerated conditions (4–5 °C). It is therefore unlikely that these conditions would significantly hydrolyse the peptide bonds of the protein. Of the 12 Gln-containing peptides studied here, those that were not observed in spectra of HCl-treated samples all contained aspartic acid (peptide sequences in Table 1). The literature has shown that aspartic acid-proline bonds undergo hydrolysis at low pH under conditions where other aspartyl bonds are found to be stable (Pisskiewicz et al., 1970). In the peptides measured here, the aspartyl is always to the N-terminal side of Gly; Radkiewicz et al. (2001) found that the degradation of aspartyl-glycine bonds can be promoted due to an increased rate of ring formation, with Asp-Gly having a short half-life compared to Asp bound to other amino acids (Ser, Ala, Cys and His). The half-life of Asp-Gly degradation at 37 °C, pH 7.4 was found to be 41–71 days, in comparison with 266 days for Asp-His and Asp-Ala. It is possible that at low pH cyclisation at the aspartyl-glycine occurs, although currently not enough is known about how these bonds in collagen are affected over time, or at different pH. If the aspartyl-glycine bond is more prone to breakage than other Asp-amino acid bonds, this may explain the lack of Asp-Gly containing peptides in the spectra of HCl treated samples. However, from these experiments we have no direct supporting evidence of preferential breakage at the aspartyl-glycine bond.

Low pH has been found to emphasise areas of damage in cooked collagen, as it induces observable swelling at sites of damage (Koon et al., 2010). The TEM findings presented in this paper support the theory that HCl treatment of bone causes degradation of the collagen structure and that older bone may be more susceptible to pH-induced damage. Greater knowledge of the contribution of the 3D structure to the stability of residues at specific sites would help further understanding of the breakdown pathways of bone collagen, as well as of the observed differences in deamidation rates for different Gln-containing peptides.

5. Conclusions

We have explored two potential causes of variation in Gln deamidation determined in bovid bone. This study found that for some peptides, levels of deamidation were reproducible across the length of areas of macroscopically well-preserved bone. Given that sample point variation was investigated in only one bone, the results obtained here are preliminary. In order to fully understand the possibility of sample point variation, a wider study of multiple bone types would be necessary. Our results suggest that the level of glutamine deamidation is linked to the preservation state of collagen in bone, with macroscopically degraded sections resulting in increased levels of deamidation. Measurement of glutamine deamidation may therefore be a useful screening tool when selecting bone material for collagen-dependent analysis.

When looking to extract collagen, especially from old or poorly preserved bone, it appears that EDTA-treatment is preferable to HCl-treatment. We conclude that, although acid demineralisation has been shown to be suitable for other types of collagen analyses (e.g. for radiocarbon dating, or dietary studies (Sealy et al., 2014)), this pre-treatment method clearly disrupts the collagen structure and causes some damage to the protein structure. EDTA demineralisation is preferable for mass spectrometric analyses aimed at quantifying the extent of glutamine deamidation in samples where ammonium bicarbonate extraction is unsuccessful, or in particularly degraded or old samples.

In the 12 peptides considered here, some appeared to be more stable than others and underwent deamidation more slowly, similar to the observation of Van Doorn et al. (2012) and Wilson et al. (2012), who calculated different half-lives for glutamine in different peptides. We suggest that these stable peptides may be particularly useful when evaluating the preservation state of Pleistocene bone material. On the other hand, rapidly-deamidating peptides may be most suited to determination of the extent of diagenesis in younger (Holocene and/or Late Pleistocene) bones. In order to further investigate the relationship between thermal age and glutamine deamidation, a number of bones from dated sites are currently being analysed which should help answer this question.

This technique could be used as a low cost method to identify bones with good collagen preservation prior to subsequent destructive analyses, such as radiocarbon dating or DNA analysis. Using this technique to map preservation across a single bone could help clarify how protein in a bone degrades over time. Understanding the effects of bone pre-treatment methods on the collagen structure could aid the success of species identification by peptide mass fingerprinting, helping to optimise the recovery of species-specific collagen peptides. Finally we feel that measurements of glutamine deamidation may offer a new way of quantifying and visually mapping the preservation of protein within bone.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jas.2016.02.002.

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