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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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# 1 The effects of demineralisation and sampling point variability on the 2 measurement of glutamine deamidation in type I collagen extracted from 3 bone

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15

## 16 Abstract

17 *The level of glutamine (Gln) deamidation in bone collagen provides information on the diagenetic history of bone*  
18 *but, in order to accurately assess the extent of Gln deamidation, it is important to minimise the conditions that*  
19 *may induce deamidation during the sample preparation. Here we report the results of a preliminary investigation*  
20 *of the variability in glutamine deamidation levels in an archaeological bone due to: a) sampling location within a*  
21 *bone; b) localised diagenesis; and c) sample preparation methods. We then investigate the effects of pre-*  
22 *treatment on three bone samples: one modern, one Medieval and one Pleistocene. The treatment of bone with*  
23 *acidic solutions was found to both induce deamidation and break down the collagen fibril structure. This is*  
24 *particularly evident in the Pleistocene material (~80,000 years BP) considered in this study. We show that*  
25 *ethylenediaminetetraacetic acid (EDTA), when used as an alternative to hydrochloric acid (HCl) demineralisation,*  
26 *induces minimal levels of deamidation and maintains the collagen fibril structure. Areas of bone exhibiting*  
27 *localised degradation are shown to be correlated with an increase in the levels of Gln deamidation. This indicates*  
28 *that the extent of Gln deamidation may be a marker of diagenesis but that sampling is important, and that,*  
29 *whenever possible, subsamples should be taken from areas of the bone that are visually representative of the*  
30 *bone as a whole. Validation of our observations will require analysis of a larger sample set.*

31

32 **Keywords:** Bone, degradation, glutamine deamidation, collagen, mass spectrometry.

33

## 34            **Section 1        Introduction**

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

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

56        Collagen could be an ideal substrate for dating because it has extraordinary potential to be preserved in the  
57        fossil record. It was predicted that collagen could survive up to 500,000 years in optimal (i.e. cold) burial  
58        conditions (Collins *et al.*, 1995); it has since been found that, even in temperate environments (e.g. in Europe),  
59        collagen can survive for much longer than this, up to 1.5 million years (Buckley and Collins, 2011). However, the  
60        extent of degradation of collagen increases with thermal age (Dobberstein *et al.*, 2009; Smith *et al.*, 2003),  
61        which is defined as an estimate of the equivalent age based upon thermal history, assuming the sample had  
62        been held at constant temperature -10 °C ([www.thermal-age.eu](http://www.thermal-age.eu)). A relationship has been suggested between  
63        the thermal age and the level of glutamine deamidation (derived from composite estimates of deamidation in  
64        several peptides) observed in extracted bone collagen (van Doorn *et al.*, 2012; Wilson *et al.*, 2012). Given the  
65        difficulties of using amino acid racemization dating (AAR) to provide robust age information on collagen (Bada  
66        and Helfman 1975), such a link could provide the key to age estimation for bone samples beyond the range of  
67        <sup>14</sup>C dating. AAR and deamidation measurements in bone do both share some of the same issues, i.e. bone is  
68        ultimately an open system (Dobberstein, 2008; Grün, 2006; Pike *et al.*, 2002). However, one advantage of mass  
69        spectrometry is that, although some collagen may be leached/diffused out of the bone, we can be sure, using  
70        MS/MS analysis of the peptides, that what we are considering is indeed collagen, whereas AAR analyses  
71        incorporates amino acids from all remaining bone proteins, in addition to any contaminant amino acids. The

72 data reported by van Doorn *et al.*, (2012) showed high variability (ranging from 40% to 90%) in the levels of  
73 glutamine deamidation in peptides extracted and analysed from bones of the same age, obtained from the  
74 same site.

75

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

- 81 1) the location within the bone from which the sample was taken (section 3.1);
- 82 2) the visible preservation of the bone - comparing degraded and non-degraded sections (Section 3.2);
- 83 3) demineralisation method - comparing the effects of two demineralisation methods (using hydrochloric  
84 acid (HCl) and ethylenediaminetetraacetic acid (EDTA)) on the levels of deamidation (Sections 3.3 and  
85 3.4).

86

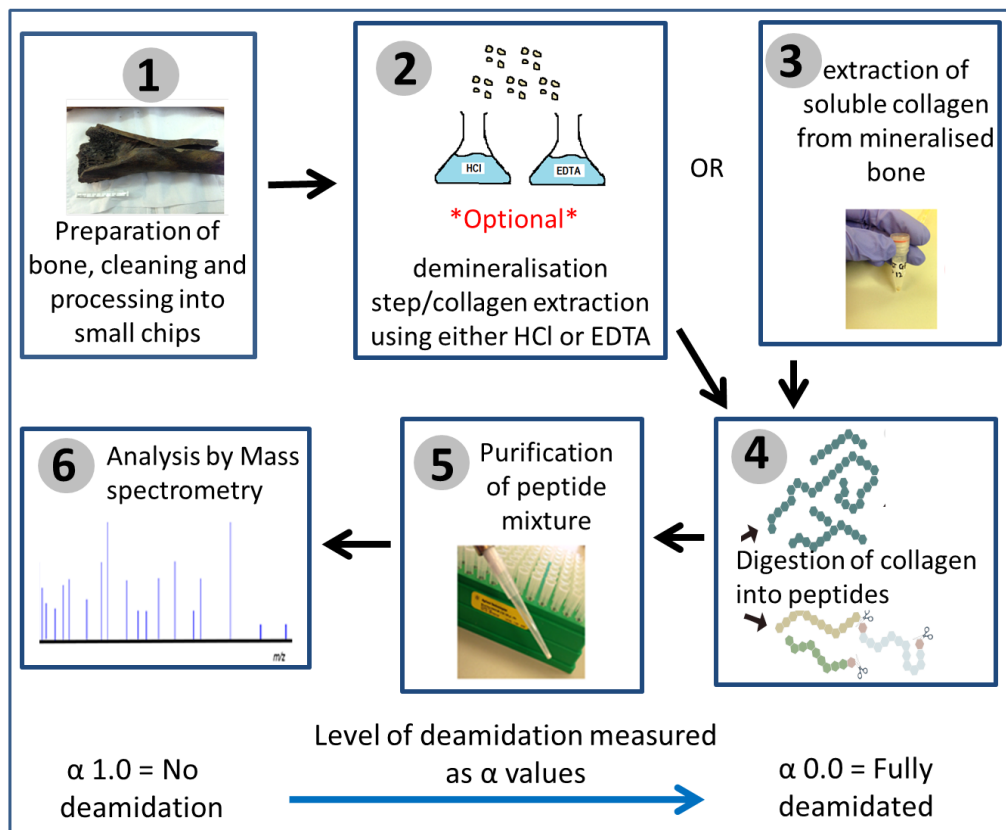
87 Second, we explore the preservation of collagen fibrils in samples of different ages, when demineralised using  
88 either HCl or EDTA. This was done using TEM to visualise three bones that differ considerably in age: modern,  
89 Medieval (bone used in previous sections), and Pleistocene (~80,000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

90

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

## 99 **Section 2 Methods**

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



102

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

112

## 113 Section 2.1 Preparation and cleaning of bone samples

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

### 117 Section 2.1.1 The Medieval bovine metatarsus.

118 The main sample used in this analysis was a bovine metatarsal bone (Figure 1) from the site Tanner Row (York,  
 119 UK), excavated by York Archaeological Trust. The bone is from an un-stratified context but is thought to date  
 120 between the 11th and mid-13th centuries. This bone was sub-sampled first by slicing into 17 cross sections;  
 121 some of these cross sections were then further sub-sampled by breaking parts of them into small chips. Because  
 122 deamidation may be induced thermally (van Doorn *et al.*, 2012), after cleaning (see Section 2.1), the bone was  
 123 cut into 17 slices (~ 3 mm in width) using a diamond-edged water-cooled band saw (Figure 2). The separate  
 124 slices were then cleaned in 50 mM ammonium bicarbonate solution and left to dry for one week in a fume hood

125 at room temperature. After slicing the bone, darker sections in the top centre of each of the slices were  
126 observed (Figure 2). These darker sections appeared macroscopically more degraded than the surrounding  
127 compact bone and were therefore removed using pliers before further analysis. The remaining pieces of each  
128 slice were immersed in liquid nitrogen for 60 seconds and then removed and broken into small chips using a  
129 small impacting hammer; the chips were then sieved through a 2 mm metal sieve and the retained chips (i.e.  
130 those of more than 2 mm) were rinsed in purified water and subjected to a range of different collagen  
131 extraction procedures (Figure 1; Sections 2.2, 2.3 and 2.4).

### 132 Section 2.1.2 Pleistocene bone

133 A fragment of bison metapodial bone excavated from a permafrost site in the Klondike region of Canada's  
134 Yukon Territory was investigated. This bone was AMS radiocarbon dated at the Center for Accelerator Mass  
135 Spectrometry, Lawrence Livermore National Laboratory, California USA, which provided in a non-age estimate  
136 ( $>50,300$   $^{14}\text{C}$  years BP; CAMS 157517). This sample was found in association with a volcanic ash (tephra) layer,  
137 Sheep Creek-K, that has been dated to  $\sim 80,000$  years old (Westgate *et al.*, 2008). As the exact age of this sample  
138 is unknown, we refer to this sample throughout this paper as Pleistocene in age. The bone piece was cleaned  
139 prior to all analyses as described in Section 2.1.

### 140 Section 2.1.3 Modern bone

141 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  
142 and defatted for 24 hours in acetone. The chunks were freezer-milled under liquid nitrogen.

## 144 Section 2.2 Extraction of collagen from mineralised bone using ammonium 145 bicarbonate

146 50 mM ammonium bicarbonate (pH 8) was added to each sample (approximately 100  $\mu\text{L}$  per 30 mg of bone).  
147 The sample was then warmed for one hour at 65  $^{\circ}\text{C}$  (adapting extraction procedures described in van Doorn *et al.*, 2011).  
148

## 149 Section 2.3 Hydrochloric acid demineralisation/collagen extraction

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

## 157 Section 2.4 EDTA demineralisation/collagen extraction

158 The EDTA demineralisation protocol of Koon *et al.* (2012) was adapted as follows. 0.5 M EDTA solution was  
159 prepared by dissolving 93.06 g of EDTA disodium salt in 500 mL of purified water, and the pH was then adjusted

160 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  
161 M EDTA (pH 7.4) added. The samples were stored at room temperature on an electric sample rocker, and the  
162 EDTA solution was replaced every three days. After 20 days the samples appeared to be visually demineralised,  
163 and the acid-insoluble fraction of collagen was gelatinised in 5 mL of pH 3.0 HCl at 80 °C for 24 hours, filtered  
164 through a 30 kDa centrifugal filter (Amicon) and freeze-dried. The resulting lyophilised collagen was then  
165 resuspended in 50 mM ammonium bicarbonate (pH 8.0) at a concentration of 2 mg/mL.

## 166 Section 2.5 MALDI-MS analysis

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

178

## 179 Section 2.6 Determining the level of deamidation in a peptide

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

194

## 195 Section 2.7 Analysis of collagen fibrils by transmission electron microscopy (TEM)

196 The modern, Medieval and Pleistocene bovid bone samples were prepared for TEM analysis following the  
197 protocol of Koon *et al.* (2012). Small bone chips around 60 mg in weight from each sample were treated either  
198 with 0.6 M HCl or 0.1 M EDTA. Once demineralisation was complete (approx. 2 weeks) the demineralisation  
199 solutions were discarded and the samples were prepared for TEM analysed following the protocol of Koon *et al.*  
200 (2012), An FEI Tecnai G2 transmission electron microscope fitted with a CCD camera was used for analysis. The  
201 typical optical settings used were as described in Koon *et al.* (2012) with a beam setting of 120 kV.

202

## 203 Section 3 Results

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

### 209 Section 3.1 Variation of Gln deamidation as a function of sampling location.

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

218

219 **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  
220 residue. Where possible the theoretical amino acid sequence of the peptides has been demonstrated by product ion analysis. For peptides  
221 where this was not possible, due to poor spectral quality, sequences were taken from published data (Wilson *et al.*, 2012) and assigned on  
222 the basis of the peptides’ accurate  $m/z$  values

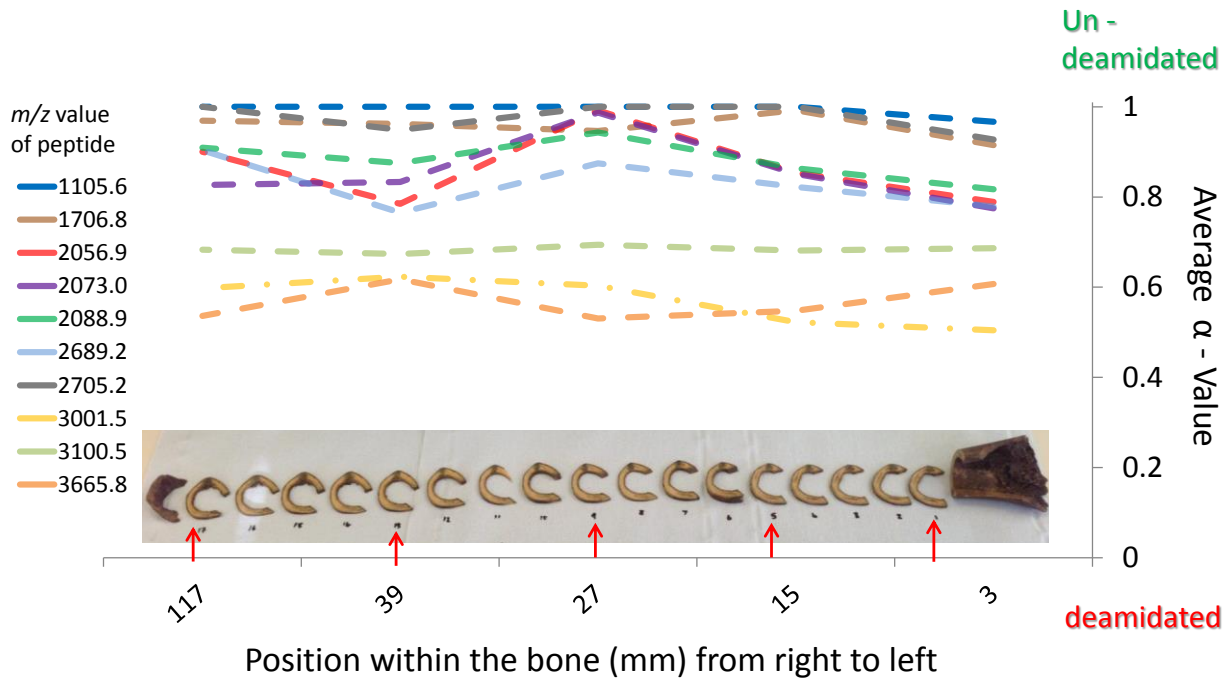
223 \* Assignment of sequence demonstrated using product ion spectrum.

[M + H] <sup>+</sup>	Peptide sequence	Collagen chain	Position in collagen chain
836.44	GPAGPQ <sup>*</sup> GPR <sup>*</sup>	COLL 1A1	[1084-1092]
1105.57	GVQ <sup>*</sup> GPPGPAGPR <sup>*</sup>	COLL 1A1	[685-696]
1690.77	DGEAGAQ <sup>*</sup> GPPGPAGPAGER	COLL 1A1	[612-630]
1706.77	DGEAGAQ <sup>*</sup> GPPGPAGPAGER	COLL 1A1	[612-630]

2056.98	TGPPGPAGQDGRPGPPGPPGAR*	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*	COLL 1A1	[1111-1140]
3001.50	GPSGEPGTAGPPGTPGPQGLLGAPGFLGLPGSR	COLL 1A2	[845-877]
3100.41	GLPGGPGAPGPQGFQGGPPGEPGEPGASGPMGPR*	COLL 1A1	[187-219]
3665.54	GSQGSQGPAGPPGPPGPPGPPGPSGGYEFGFDGDFYR*	COLL 1A2	[1079-1116]

224

225 Figure 2 shows the average  $\alpha$ -value for each peptide from the two chips from each slice. Some peptides  
 226 produce similar  $\alpha$ -values regardless of the sampling location (for example peptides with  $m/z$  values 3100.5,  
 227 1105.6, 1706.8, 2705.2), but other peptides (for example, peptides with  $m/z$  values 2056.9, 2073.0, 2689.1 and  
 228 3665.8 in particular) show greater variability with sampling location.



229

230 **Figure 2:**  $\alpha$ -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)  
 231 across the length of a Medieval bovine metatarsal bone. The average value for the two chips from each slice is plotted

232

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

$$236 \quad V_b = \frac{1}{(S-1)} \sum_{s=1}^S n_s (\bar{x}_s - \bar{\bar{x}})^2 \quad (1)$$

237 where  $S = 5$  is the number of slices,  $n_s$  is the number of  $\alpha$ -values from each slice (i.e. 2, here),  $\bar{x}_s$  is the mean  $\alpha$ -  
 238 value for slice  $s$  and  $\bar{x}$  is the grand mean, taken over all slices and the within-slice variance is given by equation  
 239 2:

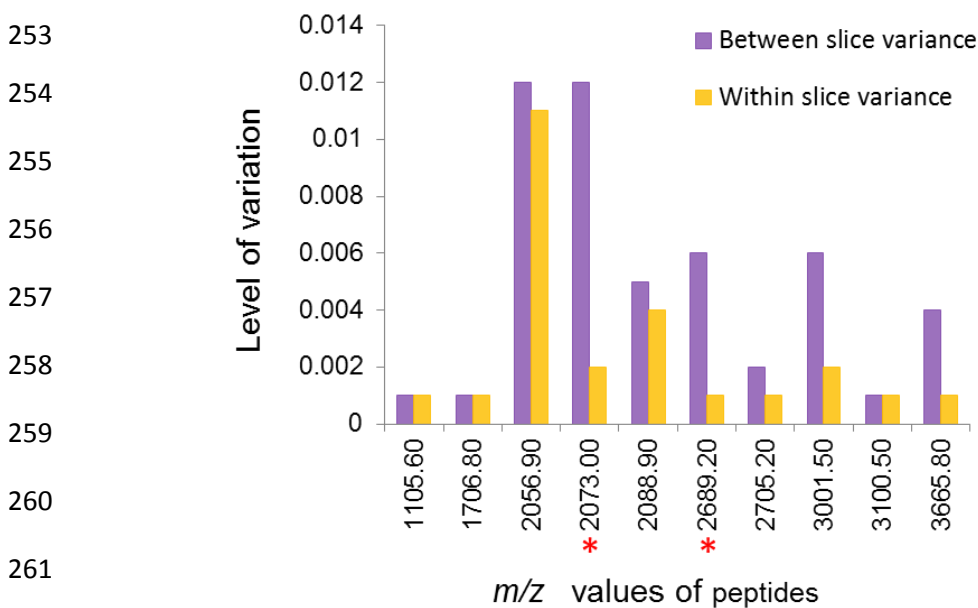
$$240 \quad V_w = \frac{1}{(N - S)} \sum_{s=1}^S \sum_{i=1}^{n_s} (x_{is} - \bar{x}_s)^2 \quad (2)$$

241 where  $N = 10$  is the total number of  $\alpha$ -values and  $x_{is}$  is the  $i$ th  $\alpha$ -value from slice  $s$ . Table 2 and Figure 3 show  
 242 the within-slice and between-slice variances for the ten peptides, together with the p-values obtained for F-  
 243 tests comparing the two variances. The variance between slices is shown to be significantly greater than the  
 244 variance within slices (at the 95% confidence level) for just two peptides, those with  $m/z$  values 2073.0 and  
 245 2689.2, although with a p-value of 0.038 for both the evidence against the null hypothesis is not strong. The  
 246 peptide with  $m/z$  value 2056.9 has the highest level of within-slice variation, but is of a similar level to the  
 247 variance between slices. The remaining peptides also show similar levels of variation within and between-slices.

248 **Table 2:** The variation in  $\alpha$ -values obtained from 10 peptides measured in tryptic digests of collagen, extracted from bone chips of different  
 249 slices compared with the variation obtained from replicate chips of the same slice. The p-values for F-tests show that, in general, the  
 250 between-slice variance is not significantly greater than the within-slice variance. \*denotes statistically significant values (at the 95%  
 251 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

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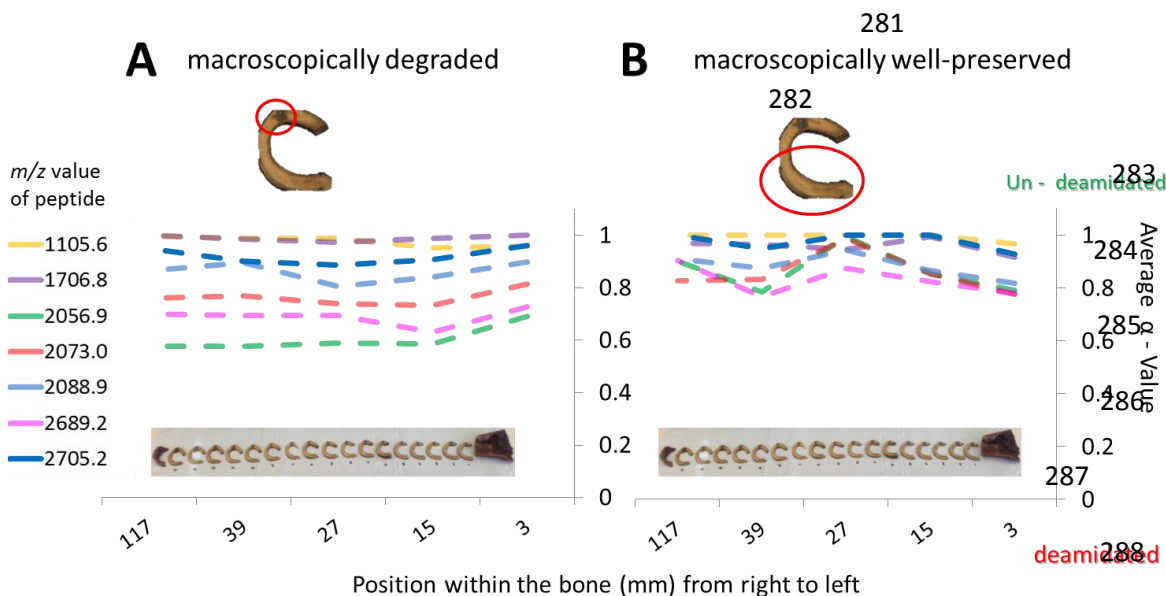


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**Figure 3:** The variation in  $\alpha$ -values obtained from 10 peptides measured in trypsin digests of collagen extracted from bone chips of different slices compared with the variation obtained from replicate chips of the same slice. The P-values 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)

### Section 3.2 Variation due to localised diagenesis.

In order to investigate the effect of localised diagenesis on  $\alpha$ -values, two chips were taken from the degraded sections of slices 1, 5, 9, 13, and 17 (Figure 4) and the  $\alpha$ -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  $\alpha$ -values obtained for macroscopically degraded sections were lower (i.e. the peptides were overall more deamidated) than those extracted from macroscopically well-preserved areas. Figure 4 shows the average  $\alpha$ -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  $\alpha$ -values of 0.99, 0.96, 0.88 and 0.98 respectively) also show little deamidation in the degraded chips (mean  $\alpha$ -values of 0.98, 0.99, 0.86 and 0.92 respectively).



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**Figure 4:** Comparison of  $\alpha$ -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  $\alpha$ -values are only plotted for the seven peptides which were observed in all five slices

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

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### 306 Section 3.3 The effects of acid demineralisation on deamidation.

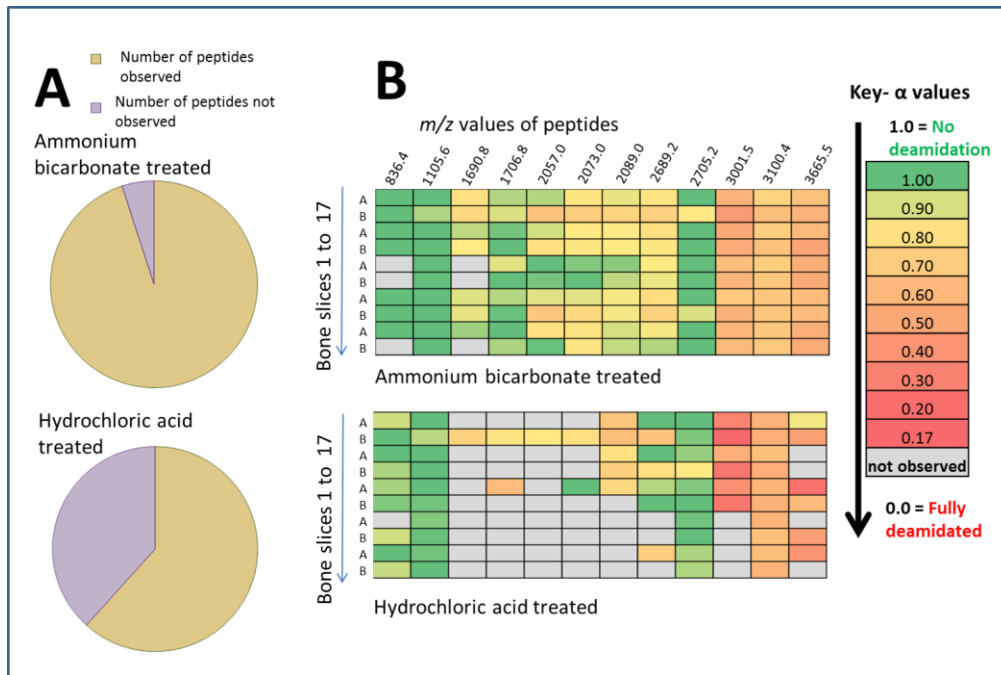
307 The removal of mineral using HCl is common in most bone preparation techniques, such as those for isotope  
308 analysis and radiocarbon dating (e.g. Brock *et al.*, 2007). An alternative to the use of HCl for the decalcification  
309 of bone is the use of EDTA as a chelating agent. EDTA decalcification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

311 HCl demineralisation was compared with the ammonium bicarbonate collagen extraction method developed by  
312 van Doorn *et al.* (2011), which does not involve the removal of mineral from the bone. We assessed the effects  
313 of HCl demineralisation on the overall deamidation using bone chips from macroscopically well-preserved areas  
314 of slices 1, 5, 9, 13 and 17. The  $\alpha$ -values of 12 peptides produced after HCl treatment (Table 2) were compared  
315 with those determined from chips from similarly well-preserved areas of the same slice, in which collagen was  
316 extracted using the ammonium bicarbonate extraction method. The 12 peptides were observed less frequently  
317 in spectra from samples treated with HCl than from those treated with only ammonium bicarbonate (Figure 5A).  
318 In the spectra obtained from the HCl-treated samples, only 74 (of a possible 120) observations of the peptides  
319 were recorded, compared with 114 in spectra from mineralised collagen extracted with ammonium bicarbonate  
320 (Figure 5A). This suggests the HCl treatment affects the peptides detected in the samples. Five of the twelve  
321 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  
322 HCl-treated samples. It should be noted that each of these peptides has an aspartic acid on the N-terminal side  
323 of glycine. The remaining peptides, observed in at least half of the HCl-treated samples, did not contain aspartic  
324 acid.

325 In observed peptides, the  $\alpha$ -values calculated for samples treated with HCl were generally lower than those  
326 from samples treated only with ammonium bicarbonate (Figure 5 (B)), indicating greater levels of deamidation  
327 in HCl-treated samples.

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

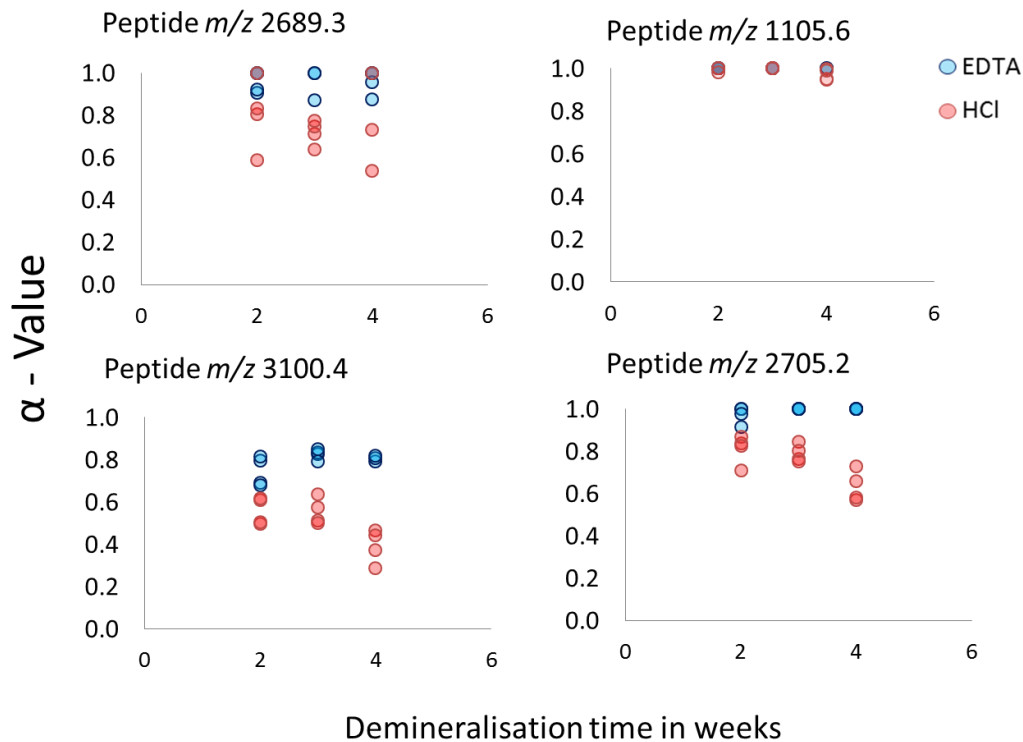
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### 343 Section 3.4 Effects of demineralisation time on $\alpha$ -values.

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

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

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361 **Figure 6:** Comparison of α-values obtained for four peptides after demineralisation in HCl or EDTA for 2, 3 or four weeks. Peptides with  
 362 smaller masses such as 1105 showed little deamidation regardless of the demineralisation method used. In samples pre-treated with HCl  
 363 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  
 364 not appear to induce deamidation over time

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

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

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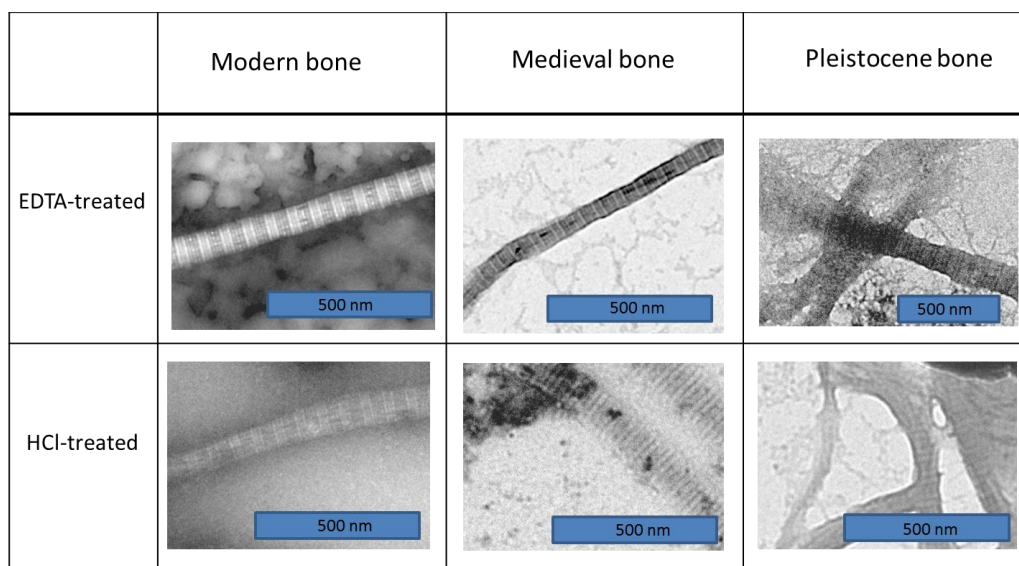
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**Table 3:** Average fibril width measurements from three samples of bone of different ages (modern, Medieval and Pleistocene). Fibril widths measured in all three samples were found to be statistically significantly different at the 95 % confidence 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

388

389 In the TEM observations, collagen fibrils are shown by the characteristic dark and light banding along the length  
 390 (Figure 8). This is due to the highly regulated structure and arrangement of the fibrils within the collagen  
 391 protein (Orgel *et al.*, 2001). However, the HCl-treated modern collagen resulted in fibrils with less uniform fibril  
 392 widths than those treated with EDTA, as well as regions of swelling along the fibril length (Figure 8). In contrast,  
 393 the collagen from the modern bone treated with EDTA resulted in a higher number of fibrils per square on the  
 394 grid than those treated with HCl, with less swelling and a more uniform fibril width (Figure 8). The effect of HCl  
 395 demineralisation was also evident in the Medieval bone. When treated with HCl, the extracted fibrils showed  
 396 less defined structure with areas of swelling and more disruption to the banding than those treated with EDTA  
 397 (Figure 8). The detrimental effect of HCl demineralisation on fibril structure was most evident in Pleistocene  
 398 bone, with very few collagen fibrils displaying the characteristic banding, whereas banding was still evident in  
 399 the majority of the fibrils in the EDTA-demineralised sample.



400

**Figure 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

## 402           Section 4     Discussion

403

### 404   Section 4.1   Spatial variation in deamidation levels within a sample

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

### 419   Section 4.2   Effects of sample pre-treatment and extraction methods on glutamine 420                   deamidation and the collagen fibril structure

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

429   Our results show that demineralisation treatment using HCl influences the extent of deamidation; HCl increases  
430   the level of glutamine deamidation and decreases the number of peptides detected in comparison with EDTA  
431   treatment. Both asparagine and glutamine deamidation have been studied in a range of sample types, from  
432   short synthetic peptides (Geiger and Clarke 1987; Li *et al.*, 2010; Robinson *et al.*, 1970; Robinson 2004; Stratton  
433   *et al.*, 2001) to proteins such as  $\alpha$ -crystallin of the eye lens (Takemoto and Boyle 1998), collagen (Hurtado and  
434   O’Connor 2012; van Doorn *et al.*, 2012; J. Wilson *et al.*, 2012), keratin (Araki and Moini 2011) and protein  
435   binders in paint (Leo *et al.* 2011). Asparagine is known to have two deamidation pathways: either via a cyclic  
436   succinimidyl (five membered ring) intermediate, or via direct side chain hydrolysis (Capasso *et al.*,  
437   1991; Radkiewicz *et al.*, 1996; Xie and Schowen 1999). The latter reaction has been found to be favoured at low  
438   or high pH (Robinson 2004). Glutamine can also deamidate via two pathways (Robinson 2004; Li *et al.*, 2010),

439 forming a cyclic glutimidyl (six membered ring) intermediate. The two residues have different rates of  
440 deamidation via cyclic intermediates, with glutamic acid forming at a slower rate than aspartic acid (Li *et al.*,  
441 2010). The most probable route of deamidation for both residues in a highly structured protein such as collagen  
442 is via direct side chain hydrolysis, due to the lack of flexibility necessary for the protein backbone to adopt the  
443 appropriate interatomic distance needed for the formation of the cyclic intermediates (van Duin and Collins  
444 1998). It is therefore likely that the two residues are equally stable in proteins such as collagen. However once  
445 in solution, gelatine (the soluble form of collagen) no longer has the same rigid structural constraints, and exists  
446 in the form of random coils.

447 We observe increased levels of glutamine in HCl (pH 1) treated samples. This is most likely due to an increase in  
448 direct side chain hydrolysis, which is less likely to occur during the ammonium bicarbonate or EDTA extractions,  
449 both carried out at around pH 8.0 (Robinson 2004). Low pH is known to induce peptide bond hydrolysis (Hill  
450 1965). However, in the experiments presented here the bone was treated in a fairly weak acid solution (0.6 M  
451 HCl) under refrigerated conditions (4-5 °C). It is therefore unlikely that these conditions would significantly  
452 hydrolyse the peptide bonds of the protein. Of the 12 Gln-containing peptides studied here, those that were not  
453 observed in spectra of HCl-treated samples all contained aspartic acid (peptide sequences in Table 1). The  
454 literature has shown that aspartic acid-proline bonds undergo hydrolysis at low pH under conditions where  
455 other aspartyl bonds are found to be stable (Pisskiewicz *et al.*, 1970). In the peptides measured here, the  
456 aspartyl is always to the N-terminal side of Gly; Radkiewicz *et al.* (2001) found that the degradation of aspartyl-  
457 glycine bonds can be promoted due to an increased rate of ring formation, with Asp-Gly having a short half-life  
458 compared to Asp bound to other amino acids (Ser, Ala, Cys and His). The half-life of Asp-Gly degradation at  
459 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  
460 that at low pH cyclisation at the aspartyl-glycine occurs, although currently not enough is known about how  
461 these bonds in collagen are affected over time, or at different pH. If the aspartyl-glycine bond is more prone to  
462 breakage than other Asp-amino acid bonds, this may explain the lack of Asp-Gly containing peptides in the  
463 spectra of HCl treated samples. However, from these experiments we have no direct supporting evidence of  
464 preferential breakage at the aspartyl-glycine bond.

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

## 471 **Section 5 Conclusions**

472 We have explored two potential causes of variation in Gln deamidation determined in bovid bone. This study  
473 found that for some peptides, levels of deamidation were reproducible across the length of areas of  
474 macroscopically well-preserved bone. Given that sample point variation was investigated in only one bone, the  
475 results obtained here are preliminary. In order to fully understand the possibility of sample point variation, a

476 wider study of multiple bone types would be necessary. Our results suggest that the level of glutamine  
477 deamidation is linked to the preservation state of collagen in bone, with macroscopically degraded sections  
478 resulting in increased levels of deamidation. Measurement of glutamine deamidation may therefore be a useful  
479 screening tool when selecting bone material for collagen-dependent analysis.

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

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

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

502

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