# THE APPLICATIONS AND LIMITATIONS OF A MINIMALLY DESTRUCTIVE APPROACH TO ARCHAEOLOGICAL PROTEOMICS

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# ABSTRACT

Although protein-based studies within archaeology have been applied for many years for bulk analyses, archaeological proteomics is a recent application. Not only can protein mass fingerprinting aid in identification of animal origin, it can reveal new information on the degradation process of proteins.

This study will focus on the MALDI-TOF-MS application ZooMS (Zooarchaeology by Mass Spectrometry) on collagen. Primarily collagen extracted from bone, although other collagenous materials were explored, notably leather. Although most bulk analyses protocols require decalcification prior to collagen extraction, and the first iterations of ZooMS analyses did also, this work presents a minimally destructive method to extract collagen from bone, involving extraction with ammonium bicarbonate buffer. The yield is consequentially lower than with a decalcified sample, however, the current generation of "soft"-ionization mass spectrometers is sufficiently sensitive for peptide fingerprinting these buffer extracts.

This allows us to investigate bone samples without loss of the protective mineral fraction. The contribution of this method is especially relevant to archaeological studies as it allows to (re-)investigate artefacts of worked bone without damaging the object and leaving microscopical features intact. The ability to re-analyze the exact same samples for DNA or other protein-based methods is just as relevant to studies of modern material, such as animal feed quality control. In addition, this study offers a currently unprecedented look at proteomics for animal origin detection in leather, treated with metal and organic tannages.

Aside from method development and exploration of the limits to buffer extraction approach, we investigate how deamidation of individual glutamine sites can give an indication of thermal damage in collagen. We present evidence that suggests that collagen retains its helical structure until nearly all collagen is lost, which offers further insight into the pattern of collagen degradation at a molecular level.

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| | |
|---|---|
| AAR | Aspartic (or amino) acid racemization |
| ACN | Acetonitrile |
| BG | Bulgaria |
| BSE | Bovine spongiform encephalopathy |
| °C | Degree Celsius |
| CHCA | α-cyano-4-hydroxycinnamic acid |
| C:N | Carbon to nitrogen (ratio) |
| CNBr | Cyanogen bromide |
| Da | Dalton |
| DAN | 1,5-diaminonapthalene |
| DHB | 2,5-dihydroxybenzoic acid |
| DNA | Deoxyribonucleic acid |
| aDNA | Ancient DNA |
| cDNA | Complementary DNA |
| mtDNA | Mitochondrial DNA |
| nuDNA | Nuclear DNA |
| DE | Germany |
| DSC | Differential scanning calorimetry |
| $E_a$ | Activation energy |
| EC | European commission |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ESI | Electrospray ionization |
| EU | European union |
| FI | Fluorescence index |
| FR | France |
| FT | Fourier transformation |
| FT-IR | Fourier transformation – infra red |
| g | Gram |
| GULO | L-gulonolactone oxidase |
| GuSCN | Guanidine thiocyanate |
| HMW | High molecular weight |
| ID | Identification |
| IT | Italy |

| | |
|---|---|
| JP | Japan |
| mm | Millimetre |
| *m/z* | Mass-to-charge (ratio) |
| MALDI | Matrix-assisted laser desorption/ionization |
| MAT | Microscopic analysis test |
| MBM | Meat and bone meal |
| MS | Mass spectrometry |
| MW | Molecular weight |
| nanoESI | Nanoscale electrospray eonization |
| nanoLC | Nanoscale liquid chromatography |
| NCP | Non-collagenous protein |
| NIR(S) | Near infra red (spectrometry) |
| NL | The Netherlands |
| NO | Norway |
| No. | Number |
| OHI | Oxford Histological index |
| PAP | Processed animal proteins |
| PCR | Polymerase chain reaction |
| RH | Relative humidity |
| RNA | Ribonucleic acid |
| RO | Romania |
| RU | Russia (Russian federation) |
| SAFEED | Safe FEED |
| SD | Sudan |
| SF | Splitting factor |
| SIMS | Secondary ion mass spectrometry |
| SP | Spain |
| SPE | Solid phase extraction |
| TEM | Transmission electron microscopy |
| TFA | Trifluoroacetic acid |
| TOF | Time-of-flight |
| TR | Turkey |
| UA | Ukraine |
| UK | United kingdom |
| UV | Ultraviolet |

| | |
|---|---|
| v/v | Volume/volume |
| wb | Whole bone |
| ZooMS | Zooarchaeology by Mass Spectrometry |

| | |
|---|---|
| Ala (A) | Alanine |
| Arg (R) | Arginine |
| Asn (N) | Asparagine |
| Asp (D) | Aspartic acid |
| Cys (C) | Cysteine |
| Gln (Q) | Glutamine |
| Glu (E) | Glutamic acid |
| Gly (G) | Glycine |
| His (H) | Histidine |
| Ile (I) | Isoleucine |
| Leu (L) | Leucine |
| Lys (K) | Lysine |
| Met (M) | Methionine |
| Phe (F) | Phenylalanine |
| Pro (P) | Proline |
| Ser (S) | Serine |
| Thr (T) | Threonine |
| Trp (W) | Tryptophan |
| Tyr (Y) | Tyrosine |
| Val (V) | Valine |

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And a big hug to all the LeCHEites (if that's now a word)! I've had a wonderful time and I'm glad to have met you all.

Finally, my family and friends overseas for dealing with me at such a distance for four years, yet making me feel at home whenever I decided to drop by. I'll never forget that those doors were always open and I can never thank you enough.

# DECLARATION

I hereby certify that the work described in this thesis is all my own work, except otherwise acknowledged, and has not been submitted previously for a degree at this or any other university.

Nienke Laura van Doorn (NLD)

## ARCHAEOLOGICAL PROTEOMICS

*Submitted for a textbook edited by Michael Richards, Max Planck Institute for Evolutionary Anthropology, to be published by Cambridge University Press*

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This chapter was initially co-written with Matthew Collins (MJC). Extensive edits were made by NLD.

## A NOVEL AND NON-DESTRUCTIVE APPROACH FOR ZOOMS ANALYSIS

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This chapter was written by NLD (except for the method section on FT-IR, written by Hege Hollund (HH)). All practical work (except FT-IR and SEM) and data analysis was performed by NLD. All FT-IR analyses were performed by HH.

## SITE SPECIFIC DEAMIDATION OF GLUTAMINE

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## ZOOMS: DETECTION OF ANIMAL ORIGIN OF MEAT AND BONE MEAL IN SINGLE PARTICLE ANALYSIS

*Submitted to Trends in Food Science & Technology*

This chapter was written by NLD with contributions from the co-authors. All practical work and data analysis was performed by NLD. Initial set-up and design of calibration through mMass was performed by MJC and Marc Wadsley.

# Chapter 1. GENERAL INTRODUCTION

In 2008, the Marie Curie funded LeCHE project (lactase persistence and the early cultural history of Europe) was launched to investigate a significant aspect in recent human evolution. The ability in adult humans to digest lactose, the sugar in milk, exists because lactase, the enzyme to digest lactose expression persists throughout human adulthood. Evidence suggests that mutations associated with lactase persistence originate from around 7500 years ago (Itan et al. 2009). Most Europeans today are able to digest milk and therefore the selective pressure for lactase persistence has been unique in its strength and speed. LeCHE brought together archaeologists, archaeozoologists, researchers of genetics, proteomics, stable isotopes and organic residues to examine the questions surrounding this phenomenon: when and where did this selection start and why is it so strong? Did the domestication of animals and especially the use of dairy products from domesticates contribute?

As a direct example of Use of animal products, primary (meat, hide, bone, horn, etc.) and secondary (which can be used repeatedly throughout the animal's life such as milk and traction), are commonly investigated by archaeozoologists by using age-at-slaughter distributions. Animals will have been slaughtered at different stages in their lives depending on the products needed and the patterns that emerge from the age of the bones reflect how people interacted with their domesticates and their main purpose for their culture. For age-at-slaughter profiles, determining the right species is important as the purpose of a certain animal to a culture can be vastly different (Balasse & Ambrose 2005). Especially sheep and goat are two species notoriously difficult to distinguish on morphology of bone alone and such mortality curves often group caprines together (Vigne & Helmer 2007). Therefore, the means to identify animal taxa through molecular analysis can aid tremendously to such research. Zooarchaeology by Mass Spectrometry (ZooMS, see also below and Chapter 2) provides the identification of taxa through collagen fingerprinting as one solution for archaeological questions within the LeCHE project where discrimination between animal genus is important for data interpretation.

In addition, stable isotope analysis, ancient DNA analysis and more approaches within the archaeological sciences are increasingly used to show — amongst others — changes in diet, migration and adaptation of human populations. Some of the most valuable specimen to archaeologists are bones and teeth from which important biomolecules and elements (DNA, protein, minerals) can be obtained. Not only do

bone and teeth persist in many environments over substantial periods of time, they are precious remains that can give direct information about the individuals of which the bones were found. Similarly, bone being the primary source of archaeological protein (collagen) makes it (currently) the tissue of choice for archaeological proteomic investigations.

Bone, despite being one of the most investigated and relevant materials in archaeological science, has had a reputation for being difficult material to obtain consistent results from due to its vascular nature, also being cited as behaving like an "open system" (Millard & Hedges 1996), prone to exogenous contamination and environmental influences (Hedges & van Klinken 1992). For example, aspartic acid racemisation in bone as a method for age determination in bone has been criticized (Hare 1980) due to some of the earliest studies containing several errors (reviewed in Johnson & Miller 1997), but has been revisited over recent years, largely to estimate age-at-death in forensic material (overview in Ritz-Timme & Collins 2002), while a direct relationship between aspartic acid racemisation in archaeological collagen and DNA amplification success has been contested (Collins et al. 2009; Fernández et al. 2009).

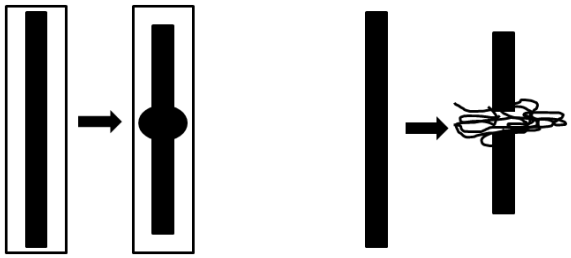

**Figure 1-1. Schematic illustration of the "polymer-in-a-box" principle in a cross-section of a collagen fibril. The illustration on the left represents a collagen molecule in a fiber with the outer box representing the (mineral) lattice of adjacent molecules. The image on the right represents a collagen molecule in solution. After exposure to heat, the molecule in solution is allowed in conformation to shrink and the collagen structure to collapse. Within the constraints of the lattice, the adjacent molecules do not allow the fibril conformational freedom to have the helix structure collapse (Miles & Ghelashvili 1999). In bone, the box is represented by both adjacent collagen fibrils and hydroxyapatite. In leather, the box can be similarly represented by chromium III complexes (Covington et al. 2008).**

Collagen, the main constituent in bone and its survival as the protein with arguably the greatest longevity through the archaeological and fossil record, has received similar refreshed attention. Whereas old ideas of collagen degradation had been based on a random scission pattern (Bowes & Cater 1965), not unlike decay of DNA (Kaiser et al. 2008), they have now shifted towards more sophisticated conceptions such as the "polymer-in-a-box" model (Miles & Ghelashvili 1999) or the "link-lock" principle (Covington et al. 2008) that suggest an intricate construction between collagen fibrils and bone mineral hydroxyapatite or otherwise surrounding lattice (Figure 1-1), leading to the observation that collagen is generally preserved in its helical form until too much collagen is lost, the structure collapses onto itself unable to retain its highly stable formation. In the link-lock principle it is explained that tannins may interact with collagen in two distinctive ways by covalently linking it in between the helices.

Collagen type I consists of three α-chains of which two are identical. The primary structure of collagen contains a repeating structure of Gly-Xxx-Yyy, which facilitates its unique triple helix structure. Xxx and Yyy can be any other amino acid residue, but are often proline or hydroxyproline. The triple helix is flanked by short telopeptides which do not contain glycine as every third residue and thus are not part of the stable helix, but are still essential towards the packing of the complete collagen fibril (Kadler et al. 1996).

These triple helices are found highly ordered within bone, as collagen is stacked in a staggered manner into a fibril (Figure 1-2). Because the staggered stacking of the triple helices within the fibril are not laid end-to-end, a periodic gap is created throughout the fibril, microscopically visible as a less denser region and creating a striated appearance, diagnostic of intact collagen. In turn, hydroxyapatite, the mineral fraction of bone is aggregated with the fibril in staggered platelets (Jäger & Fratzl 2000) and may nucleate in these less dense and less organized "gap" regions (Orgel et al. 2006) as opposed to the more highly structured "overlap" regions.

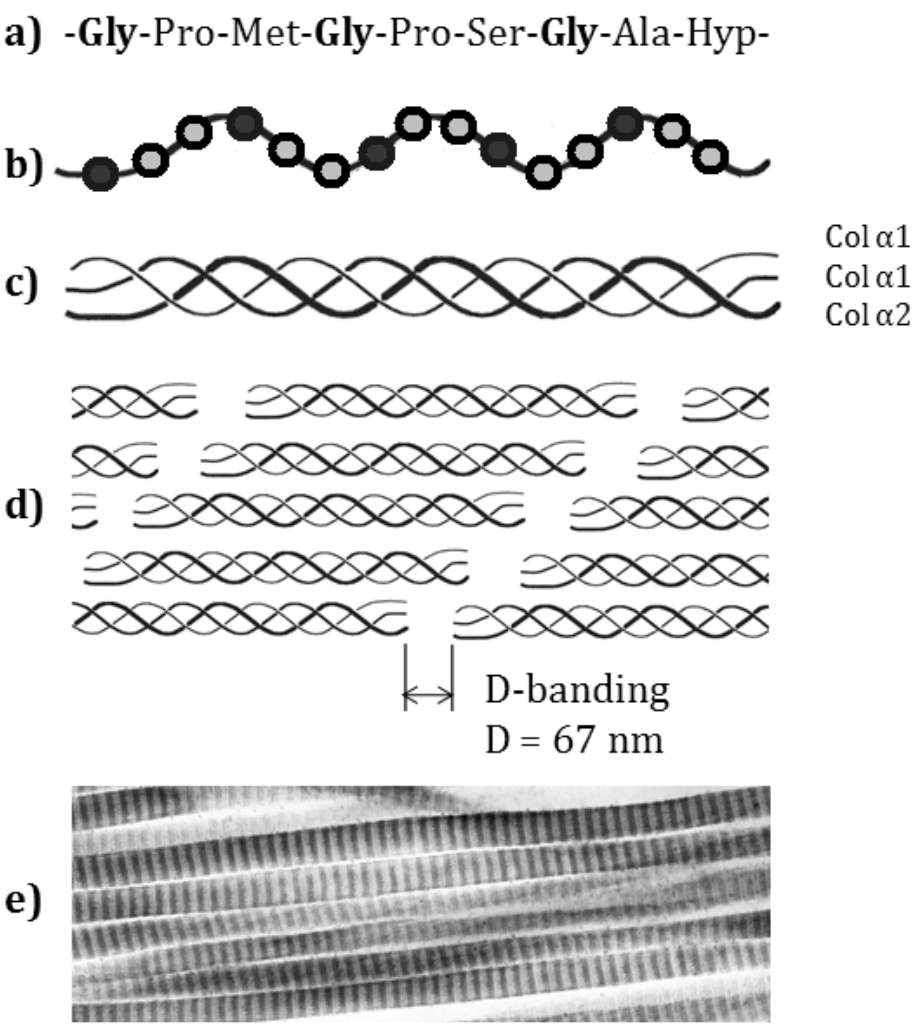


**Figure 1-2. Representation of the structural levels of collagen. From above to below: the primary structure of collagen (a), which, due to its repeated motif with a glycine (Gly ) residue at every third position, forms an α-helix (b). Three α-helices assemble into a triple helix or tropocollagen, flanked by non-helix telopeptides (c). The tropocollagen stacks in a staggered configuration, leaving a gap between end-to-end collagen molecules (d). This periodic gap is visible in microscopic images of collagen and gives it the characteristic banding pattern (e) (TEM image: Rob Young, Wellcome Images).**

Recent discoveries appear to re-emphasise that we are beginning to "unravel" the collagen molecule and are perhaps only at the precipice of understanding how the collagen molecule truly acts (and interacts) during inhumation or extended natural exposure to microbiological activity. Previously, it has been found that the carbon-to-nitrogen ratio in archaeological bone does not differ from that of modern bone until

97% of original collagen content has been lost (Ambrose 1991). Supposedly opposing finds suggest that our understanding of collagen degradation isn't yet complete (Dobberstein et al. 2009; Harbeck & Grupe 2009). Dobberstein et al observe that the amino acid racemisation (AAR) in archaeological bone collagen (as opposed to modern artificially degraded bone) is remarkably stable until almost no collagen remains, whereas Harbeck and Grupe observe bones with large amounts of low-quality collagen or collagen with altered isotopic distributions. Harbeck & Grupe (2009) also posit that degradation in archaeological and artificially degraded modern material are not equivalent. The observation that aspartic acid racemisation seems to be accelerated in degraded collagen only reinforces the idea of the collagen triple helix collapsing once it is beyond a certain point in decay (Collins et al. 1999), which Dobberstein and colleagues (2009) determined at approximately 99% of original collagen lost.

Another recent emergence, most notably in the field of DNA, is a focus on the development of non-destructive extraction methods of archaeological (bone) samples. This is challenging for collagen (and similarly DNA), as investigation of these (entrapped) molecules requires extraction from the sample — analysis cannot be achieved by scanning or other strictly non-invasive techniques. A non-destructive extraction of DNA from museum specimens has been explored through partial digestion in ancient insects (Thomsen et al. 2009). Successful mtDNA extraction was reported by Rohland and colleagues (2004), using a guanidine thiocyanate (GuSCN)/silica buffer on whole chimpanzee teeth. This was followed up with a protocol that uses a simple EDTA buffer prior to GuSCN/silica extraction (Rohland & Hofreiter 2007), the authors noting that simpler or gentler protocols seem to be equally or more efficient than more elaborate ones. Similar results have been found by Caputo et al (2012) on archaeological collagen extraction with acetic acid-based buffers and a similar EDTA buffer to that described above was used to extract and amplify nuDNA from whole teeth and skeletal material (Bolnick et al. 2012). Campos et al (2011) have argued, since methods which discard the EDTA fraction after the removal of hydroxyapatite are less efficient than those that include it, that DNA is strongly associated with hydroxyapatite and therefore will be prevalent in the EDTA fraction. The benefits of a non-destructive extraction include access to rare or otherwise valuable (due to ancestral history, for example) artefacts from museum collections, and (arguably more importantly) the potential for repeated investigation — either to screen, resample or to perform multiple analyses on the same materials.

Nowadays, multi-disciplinary approaches are increasingly common in archaeological sciences and comparisons between parameters from different analyses become more common. The aforementioned studies on non-destructive extractions report no visible alteration of the extracted source material. However, the use of acetic acid, proteinase K and/or EDTA in nearly all methods mentioned above, suggests that partial digestion of either mineral or protein has taken place. Considering that complete decalcification of bone is a slow process (days for HCl, weeks for EDTA; Callis & Sterchi 1998), it is likely that after decalcification treatments the artefact can still be recovered. However, microscopic features such as delicate skeletal characteristics or surface marks such as scratches and pits may be damaged in the process.

ZooMS, or Zooarchaeology by Mass Spectrometry, was first published in Buckley et al (2008; 2009) as a means for identifying an animal species by collagen peptide fingerprinting. Though sample requirement is minimal, it is destructive as a sizable fraction of bone mineral is removed during decalcification using HCl (see Chapter 3). Were we to omit this step, what would remain is an extraction in ammonium bicarbonate, which has no demonstrable decalcifying potency as it is neither an acid nor a chelating agent and does not actively digest protein. Ammonium bicarbonate is also directly compatible with enzyme digestions (e.g. trypsin) commonly used in proteomics.

Even in a calcified state, collagen denaturation increases gradually at temperatures below 110 °C (Li et al. 2003), producing gelatin (i.e. soluble collagen) without HCl treatment. If collagen is indeed retained in bone mostly as an intact helix, then with a whole bone extraction using the original ZooMS approach including demineralization *or* the proposed alternative we shouldn't expect any different results between extraction methods or even repeated extractions using only buffer and heating as more collagen would be released into solution.

The questions this study aims to answer are:

- Can ZooMS be performed without decalcification and obtain reliable and repeatable results in genus identification?
- Is this a "non-destructive" method?
- If peptides can be extracted for identification without decalcification, then what are the method's limits in terms of material and degradation state?

- Can ZooMS be used to identify animal origin for collagenous materials other than bone?
- Extraction of the extent of glutamine deamidation from MALDI data is possible (Dasari et al. 2009; Zhu et al. 2011); can this be used to predict a bone sample's age or chance of successful DNA amplification?
- What information does deamidation give us about collagen degradation?

# Chapter 2. ARCHAEOLOGICAL PROTEOMICS

Parts of this chapter have been published in: van Doorn, N.L. (No) Struggle for Survival. (2012) *May Contain Traces of Milk* by LeCHE. University of York, pp 143-158.

Authors: **Nienke L. van Doorn & Matthew J. Collins**

## 2.1. ABSTRACT

"Soft" ionization mass spectrometry (MS) methods have opened up a new area for the effective characterization of proteins. The term "soft" is used to indicate that energy used to ionise the peptide is not high enough to degrade the entire molecule, which allows for fingerprinting of enzymatic cleavage patterns through methods like Matrix Assisted Laser Desorption/Ionization (MALDI) and Electrospray Ionization (ESI).

In addition, current generations of instruments used for "soft" ionization MS are sensitive enough to detect as little as femtomoles of proteinaceous material. This sensitivity, combined with other advantages of MS such as available resolution and efficiency, makes these techniques an attractive source of data acquisition for archaeologists. Fingerprinting of ancient proteins can complement phylogenetic information acquired from ancient DNA, especially when considered that proteins display greater longevity through the archaeological and palaeontological record.

The sensitivity of current MS methods allows minimally invasive sampling and provides a greater chance of preservation of rare specimen or museum artefacts. More importantly, it creates options for revisiting a sample and including an estimate of the extent of biomolecule preservation for scaled screening prior to more expensive or elaborate investigations, sample selection and justification for more further analyses on the same material..

This chapter intends to provide an overview of the main "soft" ionization techniques, shed light on their applications to ancient proteins (Table 2-1) and the survival of various archaeological proteins illustrating the versatility of proteomics within the field of archaeology.

## 2.2. INTRODUCTION TO ARCHAEOLOGICAL PROTEOMICS

Archaeological studies of proteins are set to be transformed by analytical methods of soft-ionization mass spectrometry. The purpose of this chapter is to consider the basic

principles of soft-ionization mass spectrometry, the current state-of-the-art and the most promising molecules for future study[1].

The original investigations of archaeological proteins focused upon the recovery of amino acids (Abelson 1957), then microscopic detail (Doberenz & Wykoff 1967; Towe & Urbanek 1972) and immunological cross-reactivity (de Jong et al. 1974; Lowenstein 1980).Early attempts to sequence archaeological proteins directly met with almost no success, the one notable exception being Edman sequencing of the first 16 residues of the bone protein osteocalcin in New Zealand moa (Huq et al. 1990).

Amino acid and immunological investigations have remained common, the latter leading to an interest in the persistence of protein residues (Lowenstein 1981; Cattaneo et al. 1992; Loy & Hardy 1992; Eisele et al. 1995; Tuross et al. 1996). Despite the controversy surrounding a number of these approaches, there has been little doubt that intact sequences of peptides persist over archaeologically-relevant time scales. However, protein-based analysis has made little headway within archaeology beyond bulk analysis (for $^{14}C$ and stable isotopes) and more recently the use of amino acid racemization as a dating tool (Penkman et al. 2011).

The presence of intact protein sequences in archaeological bone was established even prior to Edman sequencing, following the work of Armstrong et al (1983) who successfully extracted peptides from collagen fossil bone following enzyme digests followed by chromatography. However the situation changed when Ostrom et al (2000) reported the successful detection of osteocalcin in fossil bones using soft-ionization mass spectrometry. This was only the second report of direct evidence of intact peptide sequences in archaeological substrates, and unlike the first report based on Edman sequencing which would prove to be overall slower and more expensive (Gooley et al. 1997), the work of Ostrom and colleagues (2000) soon led to

[1] The chapter is only a partial review of proteomics techniques; it does not consider two dimensional gel electrophoresis, a very important tool in proteomics research — albeit now one that is increasingly replaced by multi-dimensional chromatography. However in the case of the former technique it is worth mentioning the remarkable data obtained by Schmidt-Schultz and Schultz (2007) who were able to generate excellent one and two dimensional gel data from archaeological bone ranging up to 9,000 years in age. At present no other archaeological groups are using the 2D gel approach, and more research in this area would be valuable.

more publications. Soft-ionization mass spectrometry appeared to be particularly well suited to the analysis of ancient proteins compared to older techniques. Indeed the terms *archaeological proteomics* and *palaeoproteomics* are now being used to describe the use of technologies developed for proteomics applications to archaeological and fossil samples respectively. The *proteome* is "the complete set of proteins that result from the genome" (Wasinger et al. 1995; Wilkins et al. 1995). Proteomics, originally a term more strongly associated with 2-D gel electrophoresis (Jungblut & Wittmann-Liebold 1995), can also be defined as "*the qualitative and quantitative comparison of proteomes under different conditions to further unravel biological processes*" (definition from ExPASy 2007) and if so, only few archaeological applications are '*proteomics'* in the strictest sense. Much of classical proteomics research attempts to explore the link between expression of proteins and the functioning of the living biological system under investigation. Archaeological and palaeontological studies may explore the pattern of distribution of proteins but their goals are usually limited to identification of organisms, proteins or diseases.

## 2.3. INTRODUCTION TO PROTEIN MASS SPECTROMETRY

Mass spectrometry is a long-established technique, first developed in the late 19th century. The basic setup of a mass spectrometer consists of a means of ionisation, separation of analyte ions by mass-to-charge (*m/z*), followed by detection of ions to obtain a mass spectrum (Figure 2-1).

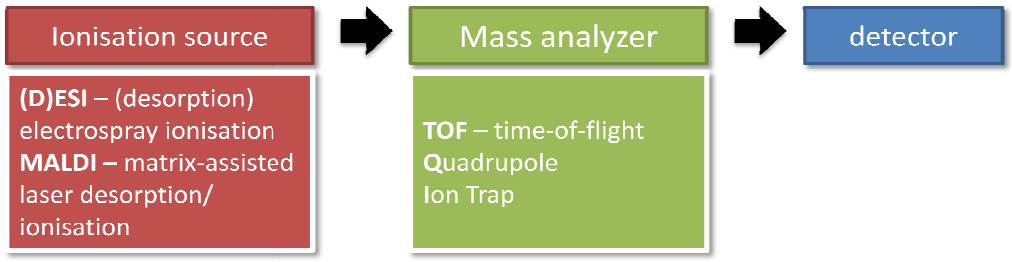


**Figure 2-1. Schematic illustration of three components of a mass spectrometer and examples as mentioned in Table 2-1.**

Mass spectrometers require charged gaseous analytes. Lipids and small molecules such as amino acids and nucleotides, must be made sufficiently volatile to be separated and ionised in the gas phase at temperatures sufficiently low to prevent them decomposing. However, most biomolecules (such as carbohydrates, proteins and DNA) are both large and polar, which means that they are not easily transferred into the gas phase and ionised. When investigating proteins by mass spectrometry, it

is common to digest the protein into peptides by using a sequence-specific protease. Analysing peptides instead of whole, mature (and possibly modified) proteins (of which the mass can be difficult to predict compared to peptides) makes the interpretation of mass spectra simpler. In the case of protein identification, mass spectrometry is most efficient in obtaining primary sequences from fragments up to circa twenty amino acids long.

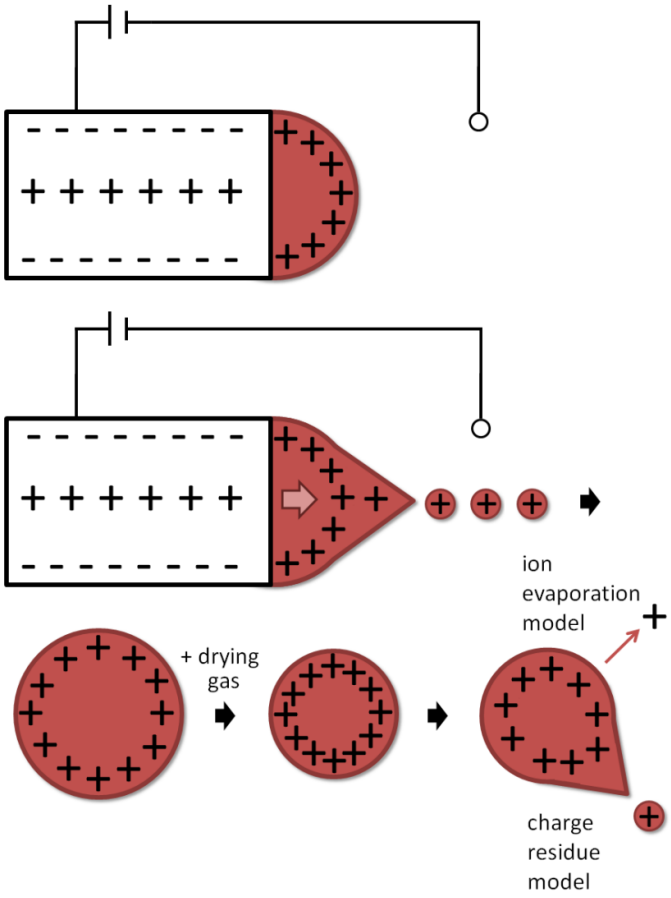


**Figure 2-2. Schematic illustration of electrospray ionisation. A droplet is forced through a hypodermic needle under high voltage, which causes same-charge ions to accumulate and electrostatic repulsion becomes stronger than the tension of the liquid surface. This results into a spray of droplets, which are passed through a drying gas. Evaporation of the solvent causes again an accumulation of charge, after which either ions can be desorbed from the surface or a new spray of droplets can be formed until all solvent is evaporated and charge is transferred to the analyte.**

Two types of ionisation strategies have contributed the most towards mass spectrometry of biomolecules: **electrospray ionisation** (ESI) (Yamashita & Fenn 1984) and **matrix-assisted laser desorption/ionisation** (MALDI) (Karas et al. 1985). These two methods are both "soft" ionisation methods, meaning that sufficient energy is provided to ionise the analyte but not enough to cause fragmentation of the formed ions, however the ionisation approaches are distinctly different.

### *2.3.1. IONISATION SOURCES*

With electrospray a (volatile) solvent in which the peptides are contained is pumped through a capillary tube at high voltage and under atmospheric pressure. A potential difference is applied between the capillary and a counter electrode. The field introduced by this potential induces a charge accumulation at the liquid surface at the end of the capillary. The drop that forms at the tip of the capillary while the voltage is increased, changes from spherical to a "Taylor cone"; the electrostatic repulsion from same-charge ions gathered in the liquid is higher than the liquid surface tension and small droplets are released from the tip of the cone (Figure 2-2). The droplets divide and decompose into a spray, which is passed through an inert "drying" gas.

The solvent in the droplets evaporates and the charge per volume unit increases. Under the influence of the strong electric field the droplets deform and Taylor cone formation can occur again to release even smaller droplets. The precursor droplet can produce several "generations" of nano-droplets as long as the solvent keeps evaporating and this is named the charge residue model. The nano-droplets continue to lose solvent and when the electric field on the surface becomes large enough, desorption of ions from the surface can occur, especially for small molecules (Wilm 2011). The latter is called the ion evaporation model.

Molecules below 1 kDa often contain one charge. Large molecules (intact proteins, large peptides) are stripped of the solvent by evaporation, and charge from the droplets will remain at the ionisable (polar) sites and will often result in multiply charged ions. As mass spectrometers measure mass-to-charge ratios, these larger ions can still be measured within the range of conventional mass spectrometers. Electrospray is well suited to interfacing with liquid chromatography as the components of the sample can be analysed by MS in real time, making it an ideal approach for complex samples. However, it is not possible to revisit the analyte.

MALDI uses a digested sample co-crystallised with an excess of highly-light-absorbing matrix on a target plate. Hundreds of samples can be deposited (spotted) as 1 µL

drops on the same plate and dried. A laser irradiates each spot sequentially and the energy absorbed by the matrix is used as heat to sublimate both matrix and analyte into the gas phase in excited state (Figure 2-3). The (often acidic) matrix donates a charge in the form of a proton to the analyte. The analyte now is charged and in the gas phase as ions, as is required for the mass analyzer. Singly-charged ions are formed, which makes MALDI spectra overall easier to interpret compared to ESI spectra. However, abundant peptides can mask less abundant ones. Because MALDI uses a solid substrate, samples can be analysed multiple times, stored and re-analysed. Hundreds of spots can be placed on a single MALDI target plate, which makes the set-up ideal for high-throughput analyses.

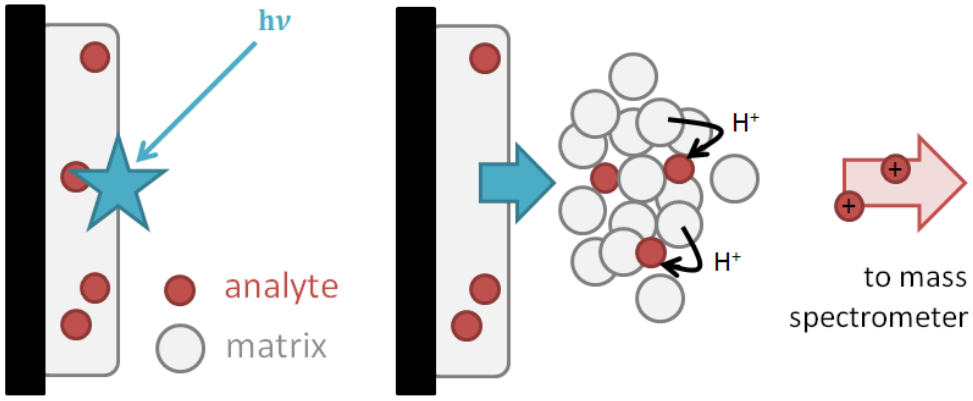


**Figure 2-3. Schematic illustration of matrix-assisted laser desorption ionization (MALDI). Analyte is co-crystallised with an excess of light-absorbing matrix, which is irradiated by a laser. Both analyte and matrix molecules are vaporized and the matrix ions in excited state donate a proton to the analyte, which can then be measured by the mass analyzer.**

### *2.3.2. MASS ANALYZERS*

Different instruments use a variety of different strategies to separate analyte ions, but all mass spectrometers measure the *m/z* of ions. Conceptually the simplest way of doing this is with a **time-of-flight analyzer** (TOF). Every ion is given the same amount of kinetic energy as any other ion with the same charge by the application of a constant voltage. Ions with lower *m/z* will have a higher velocity after their acceleration. Then the time for an ion to reach the detector is therefore determined by its *m/z*, i.e. the smaller the mass, the faster the ion travels. MALDI-TOF is able to perform the rapid analysis of proteins with a limit of detection of femtomoles (Vorm et al. 1994).

Another type of mass analyzer is the **quadrupole**. The quadrupole is in essence a mass filter, consisting of four metal rods that have an alternating potential applied to them as well as a fixed DC potential (Figure 2-4), so that the net charge of the rods inverts for a fraction of the time. For positive ions, the positive rods of the quadrupole function as a low-mass filter, whereas the negative rods filter for high-masses. A short interval of inversion of the potential over the positive rods can be enough to divert the direction of low mass ions to have them discharge. The inertia of high-mass positive ions causes them to discharge on negatively charged rods when the alternating current requires too many changes of direction to remain on a focused trajectory. Several alternations of the potential are needed for the quadrupole to function. This oscillation of potential lets only ions with a certain *m/z* pass through, creating instable trajectories for other *m/z*. The amplitude of the alternating current is adjusted so that the quadrupole passes analytes across the selected mass range to the detector and the collection of *m/z* creates the mass spectrum.

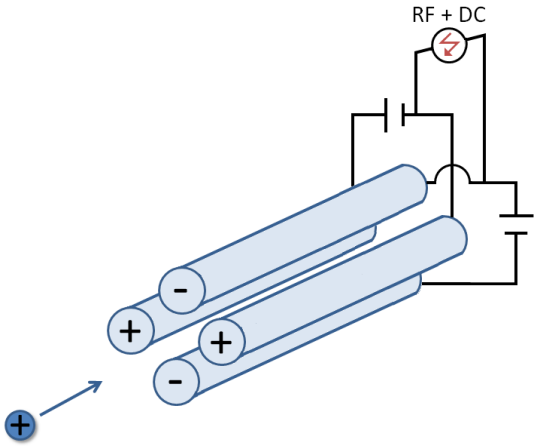


**Figure 2-4. Schematic illustration of a quadrupole configuration. An alternating (RF, radio frequency) current as well as a direct current are applied to four parallel rods.**

Another way to separate analyte ions is to trap them in an electric field after which ions with one *m/z* are ejected from the ion trap by an additional electric field. Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry also works with the trapping principle, but with a combination of electric fields and very strong magnetic fields. FT-ICR can lead to a very high resolution.

### *2.3.3. IDENTIFICATION OF PEPTIDES*

#### 2.3.3.1. Peptide mass fingerprinting

Identification of a protein is usually achieved by pattern matching of an experimentally obtained mass spectrum against a theoretical spectrum. In the case of an organism for which the complete genome is known, pattern matching is a powerful

approach. Even if there are multiple splice variants of a protein there should be a number of clearly recognisable peptides.

Proteins are digested prior to mass spectrometry with a specific protease with predictable cleavage patterns, usually trypsin. Trypsin will hydrolyse peptide bonds after each lysine and arginine residue. The pattern of tryptic cleavage is dependent on the primary structure of the protein, and is as such specific for each protein, forming a peptide mass fingerprint (PMF)(Pappin et al. 1993). For protein identification, the digested peptides are compared to *in silico* digests of known protein sequences stored in databases. The operator can select additional post-translational modifications that will alter the mass of the peptides, and any missed cleavages of the protease. The more of these additional options are included, the larger the amount of *in silico* peptides which may match the peptides, the slower the search and the greater the chance of false-positive matches.

Peptide mass fingerprinting can be very useful for the identification of relatively pure proteins, but become more complicated with the analysis of a mixture. PMF is a rapid and simple system to compare between samples, for example the same tissue changing over time or between individuals, or the same protein compared between species. In the case of bone it seems to be an ideal method to assess animal taxa from extracted collagen and it is the approach we have adopted for ZooMS (see below). If no protein can be unambiguously identified through PMF, peptide fragmentation is necessary to obtain sequence information.

#### 2.3.3.2. Tandem mass spectrometry

To identify an unexpected fragment or ambiguously identified protein, individual peptides can be further fragmented and the fragments analysed in a second mass spectrometer — hence tandem mass spectrometry or MS/MS. A parent ion of a particular *m/z* can be selected and fragmented, which is usually done by collision with neutral gas molecules (**collision-induced dissociation** or CID). After fragmentation, the resulting ions are measured through a second mass spectrometer. Combinations as MALDI TOF-TOF (Medzihradsky et al. 2000), in which the two time-of-flight mass analyzers are separated by a collision cell, and MALDI QqTOF (Loboda et al. 2000), in which a collision cell is placed between a quadrupole and a time-of-flight mass analyzer, are fairly recent combinations of mass analyzers which have become quite popular due to their high sensitivity, mass accuracy and comprehensive fragmentation through CID in comparison to their predecessor where fragmentation was achieved through post source decay (PSD) (Spengler et al. 1992).

During fragmentation of a peptide, the fragment ions are most common formed by disruption at the amide bond between two amino acids. If the charge is retained on the N-terminal part of the peptide, the fragment ions are called **b-ions**, whereas the fragment ions are called **y-ions** if the charge is retained at the C terminal part (Figure 2-5). The b-ions and y-ions form a series depending on which amide bond in the peptide is fragmented. Because each fragment ion in a series differs one amino acid from the next, their difference in mass corresponds with the mass of that amino acid in the sequence. Therefore, if all fragment ions could be detected, the primary sequence of the peptide could be reconstructed. Unfortunately, not all fragment ions are always detected and internal cleavage products or interfering peaks that were not generated from the intended parent ion (but from a parent ion with similar *m/z*, i.e. from an overlapping peak in MS1) can complicate the spectrum and its interpretation.

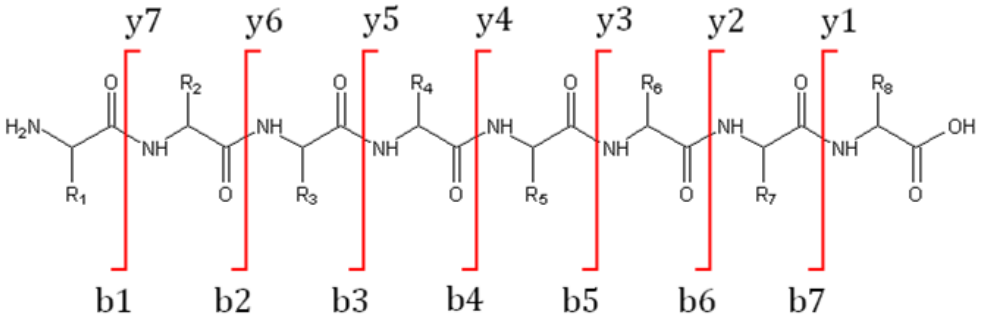


**Figure 2-5. Representation of b-ion and y-ion series from a peptide during fragmentation. Peptide fragmentation is usually induced by collisions with neutral gas and cleavage of the amide bonds leads to b-ions when the charge of the parent ion is retained by the N-terminal fragment or y-ions when it is retained by the C-terminal fragment.**

The amino acid sequence of the parent ion can be derived from mass spectrometry data of fragmentation data alone, which is called *de novo* sequencing. While *de novo* sequencing can be time consuming and complicated by incomplete ion series, it can still be useful when the peptide investigated is from a species of which the genome has not yet been sequenced (and therefore not all its peptides will be known). However, for species of which the genome has been sequenced, database matching of MS/MS spectra can be useful even if the full primary sequence cannot be determined through *de novo* sequencing.

There are different algorithms available for database searching. The most common two search engines are Mascot (Perkins et al. 1999) and Sequest (Eng et al. 1994). The Mascot search algorithm uses probability based matching between theoretical and experimental fragments. The chance that the number of matches is random is expressed as an identification score. Sequest uses a signal-processing algorithm called autocorrelation which calculates the overlap between the experimental spectrum and a theoretical spectrum, which is expressed in a score.

### *2.3.4. ZooMS*

The application of proteomics used in this thesis throughout is termed ZooMS (short for zooarchaeology by mass spectrometry), which is an identification method that uses a peptide mass fingerprinting approach (Figure 2-6). Although the primary sequence of collagen is overall strongly conserved, minimal differences give rise to mass differences in peptides measured through MS able to act as peptide markers, sufficient to distinguish most animal genera (Buckley et al. 2009) and, in some cases, species. The discrimination between genera and species is not dependent on identification of each peptide sequence. Some markers may be lost through degradation or rendering at high temperatures, however, it may still be possible to distinguish ruminants and omnivores, for example.

ZooMS is only one specific example of a number of fingerprinting approaches used in archaeology. A similar approach was used by Hollemeyer et al (2008) to identify sheep and cattle hair from Otzi's clothing using a library of keratin sequences from more than 300 species (Hollemeyer et al. 2002).

Although the method is of limited value in complex mixtures of proteins as numerous peptides may have similar masses, ZooMS exploits the fact that bone protein is predominately collagen. A bone extract is essentially a purified collagen extract, especially from archaeological remains (see section on protein degradation and preservation below). Collagen has a unique mechanism of preservation, so much so that collagen persists past many other biomolecules, especially over non-collagenous proteins in bone. In Chapter 3 it is shown that decalcification prior to gelatinization is not necessary and allows for minimally destructive analysis. In Chapter 6 and 7, we introduce a way of matching peptides to a collagen database using a Mascot algorithm.

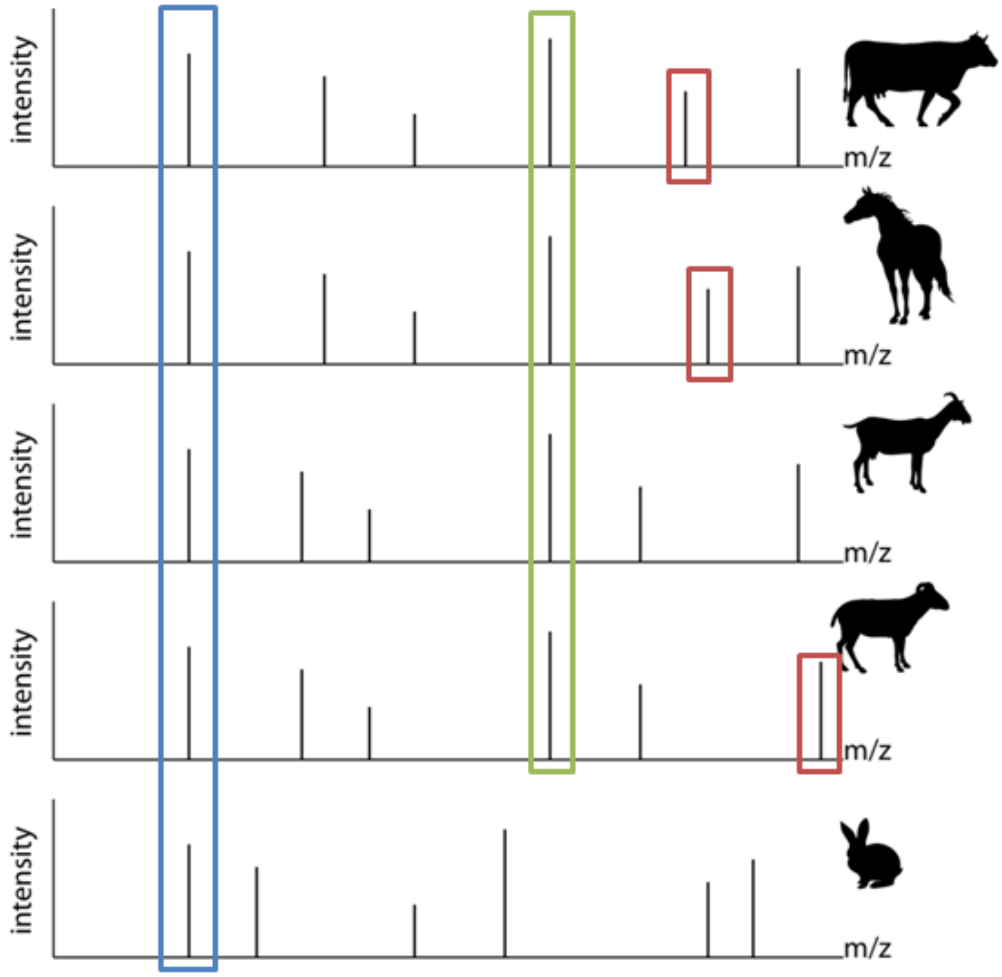


**Figure 2-6. A schematic illustration of ZooMS. Peptide mass fingerprints reflect differences between primary collagen sequences among taxa. High conservation in collagen sequences may lead to many peptides to not be indicative of species (blue), but some analogue peptides differ in primary sequence and consequentially mass, so that they can be diagnostic for a genus or species. In the illustrated example the peptide in green is likely a marker for ruminants and the peaks marked in red could potentially be species specific or at least help in the discrimination between two very closely related taxa, such as sheep from goat.**

## 2.4. Degradation and preservation mechanisms of ancient proteins

Non-enzymatic degradation of proteins mainly encompasses processes such as deamidation, hydrolysis and glycation. The latter does not as much degrade, as modify the protein.

Internal amide bonds of proteins easily hydrolyse if they are not otherwise protected, resulting in eventually the reduction of a protein to fragments and free amino acids (Bada 1991; Collins et al. 1999). Peptide bond hydrolysis occurs so rapidly and easily (Callis 1995) that especially in temperate climates (Bada 1991) this is a concern for archaeological samples.

Deamidation, the loss of an amide functional group as ammonia, is arguably the most common post-translational modification in proteins and the most rapid on the geological timescale (Bada & Miller 1968). In terms of mass spectrometric detection it results in a mass increase of +0.934 Dalton. Asparagine and glutamine are the amino acids affected at their sidechain, and are more readily affected than the peptide bond aminogroups in the protein backbone. Asparagine is more sensitive to deamidation than glutamine due to stable reaction intermediates (Daniel et al. 1996), but the rate of the reaction can be enhanced by several factors, such as having glycine or serine as an adjacent residue (Stephenson & Clarke 1989). For asparagine the rate of deamidation can be up to ten times faster than for glutamine (Terwilliger & Clarke 1981; Daniel et al. 1996).

One of the most commonly known and studied, but at the same time most complex reaction is non-enzymatic glycation of proteins, quite famously known as the Maillard reaction, named after Louis Camille Maillard who conducted research on reactions between amino acids and sugars (Maillard 1912). The Maillard reaction is used to describe the non-enzymatic and spontaneous "browning" reaction between proteins and carbohydrates. It is mostly known for its relevance in food processing and cooking, but also occurs in aging mammalian cells and collagen in pathologies such as diabetes (Avery & Bailey 2006), and glycation products normally accumulate in long-lived tissues (Bada et al. 1999). The intensity of browning is not the same for each amino acid and/or carbohydrate involved (Ashoor & Zent 1984). Characterizing heteropolymeric substances that remain as endproducts of the Maillard reaction has been a controversial matter for some time, as more characteristic volatile compounds diffuse out of organic matter (Evershed et al. 1997), but it is safe to say that organic matter in a buried environment is exposed to the Maillard reaction when protein and carbohydrate are present. Glycation tends to occur on the basic residues Arg and Lys, the same residues as those targeted by trypsin, so potentially these reactions could influence tryptic digestion. Therefore, alternative enzymes to trypsin (e.g. chymotrypsin), may be appropriate and may yield more peptides in samples known to

be prone to Maillard formation—e.g. those from dry, carbohydrate-rich environments.Warm climates, water flow and microbial activity promote protein degradation. Circumstances for excellent recovery of organics are similar to conditions for food preservation such as freezing (permafrost) and drying, which protects proteins from microbial attack. For similar reasons, anoxic conditions result in a better preservation of proteins (e.g. (Tuross & Dillehay 1995) and stagnant water results in better bone preservation in general as influx of fresh oxygenated water does increase the rate of diagenesis. However, anaerobic microbiota can increase carbon turnover and dissolve mineral by production of acids as a byproduct of the anaerobic pathway.

Beside environmental influences, there are mechanisms that preserve proteins at a molecular level. Biopolymers can benefit from a shielding effect in the sense that proteins often demand specific enzymatic breakdown, as opposed to more ubiquitous and non-specific reagents that degrade nucleic acids. A common mechanism that has been demonstrated to yield proteins persisting through geological timeframes, is mineralised tissue. The presence of mineral can prevent microbial attack and minimise enzyme access (Gernaey et al. 2001). Substances that interact with protein can preserve biomolecules in basically five different mechanisms: encapsulation, sorption, "micells", mineralised tissue or by a construction referred to as a "polymer-in-a-box": a remarkable and unique mechanism that is based on entropy of mineral lattices in combination with collagen fibrils.

### *2.4.1. ENCAPSULATION*

Encapsulation of proteins is a somewhat broader mechanism of protein preservation. Proteins can be enveloped in mineralised tissue, although this does not necessarily mean the proteins will not degrade further (Walton 1998). Any mineral based substance can have the capacity to reduce the degradation of organic matter. This protection may not be absolute or permanent (Baldock & Skjemstad 2000). Proteins do not necessarily receive protection only from mineralised substances. Fragments can be physically encapsulated in humic matter, preserving the proteinaceous material from both chemical hydrolysis and microbial degradation (Zang et al. 2000). If covalently linked to organics, proteins will be taken out of the active pool used for plant nutrition (Knicker & Hatcher 1997). This occurs by rearrangement of the peptide chains and can protect proteins in soil and sediments against microbial degradation up for 4000 years (Knicker et al. 1996). Also, non-covalent peptide linking through hydrogen bonds and hydrophobic interactions have proven to

preserve proteinaceous material in environments with low mineral content (Nguyen & Harvey 2001).

### *2.4.2. SORPTION*

Investigations have looked into survival of proteins on ceramics, as lipids have proven to survive very well on ceramic surfaces (Heron & Evershed 1993), and the protection mechanism may be similar on lithics. The detection of proteinaceous material on pottery has proven to be especially complicated (Evershed & Tuross 1996; Craig & Collins 2000; Craig & Collins 2002; Quiquampoix et al. 2006). Clay minerals, as an analogue to ceramic surfaces, adsorb proteins and peptides strongly and rapidly, and desorption is reported to not readily occur (Ding & Henrichs 2002). While independent of molecular size, it appears that isoelectric point and the nature of the residues are contributing factors, indicating that electrostatic interactions are the main determinant (Ding & Henrichs 2002). This is in concert with the observation that acidic residues persist due to the strong interactions with mineral particles (Brandt et al. 2002). Proteins directly attached to the mineral surface were found to show no enzymatic activity (Schmidt et al. 1990). It is possible that the interaction between the protein and mineral changes protein conformation considerably, and thereby protecting the organic material from biological deterioration because of severe conformational changes that do not correlate to proteins in a regular "biological" state (similar to theory in (Geigl 2002); (Collins et al. 2002)).

### *2.4.3. "MICELLS"*

It was discovered that organic molecules combine towards a multi-layered construction in a compact and distinct arrangement not unlike the formation of micelles, when placed in an aqueous environment with mineral particles or a mineral-based surface (Schmidt et al. 1990; Kleber et al. 2006). The polar functional groups of the abundant organic compounds will face either the mineral surface or the surrounding solution, thereby shielding any hydrophobic groups from direct contact with water, forming a highly organised layered system. Exchange rates of the layer directly attached to the mineral surface are much slower that the layer that is in direct contact with the surrounding environment.

The zonal model or multi-layered model does explain previous observations; amongst others it explains why organic molecules form in multi-layered clusters over a mineral base, rather than a mono-layer over the entire surface (Mayer 1999), thereby leaving siliceous surfaces largely uncovered (> 85%). This observation also presents an

explanation to why lipids are able to extensively cover more of the siliceous surface and receive excellent preservation by direct adsorption.

### *2.4.4. MINERALISED TISSUE*

Mineralised tissues are known to protect proteins from degradation, especially in bone where both collagen and non-collagenous proteins (NCPs) are found in ancient samples (Tuross et al. 1980; Schmidt-Schultz & Schultz 2004; Ostrom et al. 2006). Collagen is such a unique case in interacting with the hydroxylapatite mineral phase in bone that it will be discussed elsewhere in this section. Bone does contain more proteins than collagen alone, and these NCPs have often been found to persist, due to high affinity to and close interactions with hydroxylapatite (Masters 1987).

Osteocalcin is the most abundant NCP in bone, also known as bone Gla-protein, referring to the γ-carboxylated glutamate residue catalysed by vitamin K (Hauschka & Carr 1982). This residue adaptation causes conformational changes, and stabilises the portion of the protein that forms a α-helix. Strong interactions have been found with hydroxylapatite (Gernaey et al. 2001), the α-helix may also enhance the affinity for hydroxyapatite through calcium bridges (Lee et al. 2000), and osteocalcin also interacts strongly with collagen (Prigodich & Vesely 1997). Osteocalcin is very unstable *in vitro* (Lee et al. 2000), and only persists in archaeological or fossil samples when it can retain its interaction with the mineral phase. Any extreme changes in the mineral phase of bone affects osteocalcin survival (Smith et al. 2005). However, mtDNA survives better than osteocalcin and detected in archaeological samples in which osteocalcin is depleted (Buckley et al. 2008). The acidic Gla region may persist even beyond collagen survival. However, when collagen is lost, osteocalcin is unlikely to be found intact (Smith et al. 2005).

Albumin is another abundant protein in bone that exhibits a high affinity for calcium phosphate supports (Tuross 1993), and is not effectively released *in vitro* until the mineral support is decalcified. Albumin has been found to survive in a number of cases, not only reactively in ancient samples up to 4000 years old (Cattaneo et al. 1992; Cattaneo et al. 1995) or interestingly in fossil samples of 1.6 Myr old (Borja et al. 1997), but also in cremated bone (Cattaneo et al. 1994). However, even though albumin and osteocalcin are shown to interact with the hydroxyapatite crystals, the whole and intact proteins have not yet been convincingly detected in these ancient samples. Immunological methods could detect only the small reactive epitopes that

are left in intimate contact with mineral surfaces, as would be the case with the persistent acidic α-helix region of osteocalcin.

### *2.4.5. "POLYMER-IN-A-BOX"*

Collagen is a unique case in the story of protein survival. It has been recovered from bone when other biomolecules such as mtDNA and osteocalcin could not be detected (Buckley et al. 2008) and in fossilized remains from the Miocene (Wyckoff et al. 1963) and possible even older (Schweitzer et al. 2009). It has been known for a very long time that collagen has strong interactions with the hydroxylapatite crystals in mineralised tissues (Gernaey et al. 2001). Collagen in an unmineralised context is more readily hydrolysed and degraded by the enzyme collagenase, than in its mineralised form (Lees 1989; Collins et al. 1995). Demineralised collagen does not maintain its triple helical conformation in physiological solution, but attains a random coil at temperatures close to body temperature (Leikina et al. 2002).

To properly stabilise collagen and maximise its strength and elasticity needed to maintain the physiological functions of skin and bone, the fibrils must be sufficiently stabilized, yet retain a degree of conformational freedom for collagen turnover (Kadler et al. 1996). Collagen in bone is framed within hydroxyapatite crystals and adjacent collagen fibrils. Therefore, more energy is needed to "melt" the collagen fibre. This indicates that the fibres are stabilized because of the construction in which they are embedded in bone mineral (Miles & Ghelashvili 1999).

The major factors that influence collagen stability are interfibral water content and the interaction with mineral. Cross-linking has been mentioned as a factor (Collins et al. 1995; Trębacz & Wójtowicz 2005), and has been found to correlate with higher thermal stability of collagen fibrils (Koon et al. 2008), although it has also been found that cross-linking in itself does not add to a higher thermal stability of collagen (Miles et al. 2005). Crosslinking closes collagen fibrils in tighter, reducing intrafibrular space for water resulting in dehydration. Miles & Gelashvili (2005) (Miles et al. 2005) have shown that it is this water content that influences collagen stability effectively, and Trebacz & Wójtowicz (2005) (Trębacz & Wójtowicz 2005) have shown that dehydrated bone collagen is highly thermally stable in native and demineralised form. Presence of mineral does add to thermal stability. Similar results have been found for collagen in less mineralised dentine(Armstrong et al. 2006). The close interaction between collagen chains in the helix, as fibrils and dehydration are essential for its remarkable stability, protects collagen against microbial decay. But mineral is needed

to form and hold the fibrils of collagen in a physiological state to begin with (Kadler et al. 1996; Jäger & Fratzl 2000; Leikina et al. 2002). In the end, bone collagen survival depends on the intricate structure of the collagen fibrils, combined with the small hydroxyapatite crystals.

Collagen has been a favoured protein of choice for analysis. Mainly because it is the most abundant protein in bone and dentin, but even more so because it has been found present in a number of bones found from both the archaeological and fossil record of remarkable age (Wyckoff et al. 1963; Tuross et al. 1980; Asara et al. 2007).

Proteins such as osteocalcin, albumin and several immunoglobins have been investigated as well, in the hope that these less abundant proteins would be better markers or survivors of the tooth of time. Although osteocalcin and albumin have shown to exhibit interactions with mineral surfaces and especially hydroxyapatite, the results have been less positive (Gernaey et al. 2001; Dobberstein et al. 2009) than those involving collagen preservation.

## 2.5. PROTEOMICS STUDIES ON ARCHAEOLOGICAL MATERIALS

### *2.5.1. PROTEINS ON SURFACES*

#### 2.5.1.1. Pottery and Lithics

Detection of proteinaceous material from pottery has proven to be particularly complicated (Evershed & Tuross 1996; Craig & Collins 2000; Craig & Collins 2002; Quiquampoix et al. 2006). Clay minerals adsorb proteins and peptides strongly and rapidly, analogue to ceramic surfaces and desorption is reported not to occur readily (Ding & Henrichs 2002). With mass spectrometry, using a soft extraction method (Solazzo et al. 2008), or applied directly to the artefact surface (Heaton et al. 2009), the first identified sequences of proteins on archaeological pottery samples were obtained. With Desorption Electrospray Ionisation (DESI ) it was possible to identify protein digests and intact proteins of known standards applied to flint and glass surfaces. In addition, myoglobin could be identified from an archaeological potshard (Heaton et al. 2009).The ESI method sprays charged aqueous droplets (typically less than 5 μm and travelling at velocities in excess of 100 m/s) onto a sample surface positioned within millimetres of the spray source. The sample surface is wetted, producing a thin film of sprayed solvent on surface-bound analyte, with subsequent spray droplets colliding with this mixture, desorbing and transporting dissolved peptides into the mass spectrometer. The advantage of this method is that it requires

no sample preparation and is therefore well suited to the analysis of residues (e.g. lithics, pottery, art materials).

#### 2.5.1.2. Binding material

Proteinaceous material was commonly added to ancient mortars and binding material to initiate the drying process. The amount added, however, is small and the application of mass spectrometry has thus made it possible to identify proteins from this material (Kuckova et al. 2005; Kuckova et al. 2009). The sensitivity of MALDI-TOF MS is so high, that it was reported impossible to distinguish conclusively between the presence of only egg yolk or whole egg in paint, as even of trace amounts of proteins from egg white would show in the spectra (Kuckova et al. 2007). Furthermore, especially in the case of paintings or other painted artefacts, mass spectrometry hands the solution towards using minimal sample size (Tokarski et al. 2006), and proteomics could contribute greatly towards investigations on biodeterioration and preservation of cultural heritage (Tokarski et al. 2003). More recent studies explore the potential of deamidation to assess age and damage in binding material and found that deamidation in casein (commonly present in paint binders) allows deamidated peptides to function as markers to characterize aging molecular effects of historical artworks with(Leo et al. 2011). Interestingly, Leo et al concluded that collagen would not be as useful as casein due to the highly occurring presence of hydroxylations in the collagen molecule, thereby containing an increased heterogeneity of fragments compared to casein, even though they note the presence of hydroxylated peptides allows for a more confident protein identification. While this may be true for proteinaceous binding material in paints, for archaeological bone where almost exclusively collagen is present, the (potential) use of deamidation in bone collagen is discussed in Chapter 4.

#### 2.5.1.3. Metal

A notable approach is mentioned in the paper on blood recovered from metal surfaces (Mazel et al. 2007), using an indirect method to demonstrate the presence of haemoglobin through identification of protein and haeme or, alternatively, iron not associated with the mineral phase, with separate and independent methods. This approach was used to indicate the use of artefacts in sacrificial rituals and contexts. The presence of blood is shown through three steps: detection of the presence of proteins through time-of-flight secondary ion mass spectrometry (TOF-SIMS) allowing analysis without protein extraction, the presence of haeme by TOF-SIMS and

the detection of iron linked with proteins. This allows noninvasive detection of blood even when no intact bloodcells can be found.

### *2.5.2. PROTEINS IN EXCEPTIONALLY PRESERVED MATERIALS*

#### 2.5.2.1. Hair and textile

Natural hair fibers (i.e. wool) consist mainly of keratin, an insoluble and sulfur-rich protein. Because of the insoluble nature of the proteins, the extraction used for the proteins is more complicated than, for example, collagen, and non-destructive sampling is difficult. On the other hand, keratins may be more useful in species determination as sequences of keratins are more variable than those in the strongly conserved collagen. In hair analysis, proteomics has a high rate of success, where DNA amplification may fail or structural details may be lacking for microscopic analysis (Hollemeyer et al. 2008).

#### 2.5.2.2. Seeds

In 1977, Derbyshire and colleagues, investigating 700-year-old maize kernels, made a prescient comment that archaeological seed proteins may be useful in studies of domestication and plant evolution (Derbyshire et al. 1977). In the early 1980s, Shewry and colleagues used standard extraction and analytical protocols to compare the protein profiles of barley grains from ancient Egypt and historical samples from Rothamstead (Shewry et al. 1982). They were able, using a combination of sequential extraction and gel electrophoreis to demonstrate the presence of a number of seed proteins, but others, such as hordein, were not detected. Analysis by gel electrophoresis provides an obvious reason why: large smears were seen in the gels, indicating that proteins were degraded. Shewry and colleagues (1982) point out that seeds are under strong selective pressure and therefore the phenotypic analysis of seed proteins could be a valuable new source of information for archaeobotanical research. More recently, Cappellini et al (2010) have completed the first proteomics based studies using early and late Medieval grape seeds. The study reveals that seed storage proteins undergo hydrolysis resulting in peptides with progressive loss from one terminus. Cappellini was unable to precipitate proteins in early medieval seeds from Southern Italy, and DNA gave both greater recoverable information and greater persistence than proteins.

#### 2.5.2.3. Leather and parchment

Leather consists of collagen, but unlike bone is not mineralized, and the material is strengthened through crosslinking and dehydrating by tanning. Typically, leather is made from the dermis, the middle layer of animal skin, enforced with networks of

both helical collagen types I and III. The species origin of leather could be identified using ZooMS (see Chapter 6). The process of tanning raises the denaturation temperature of collagen, but does not prevent microbial activity. However, if burial conditions retard biodegradation then leather can persist. There are currently no known proteomics studies on leather, as far as we know, but Choudhury et al (2006) have performed 2D-gel electrophoresis followed by MALDI-TOF MS to identify proteins in pickled (acid hydrolysed) skin that may predict the quality of the leather to be made.

Parchment is not tanned, but is often kept beautifully and painstakingly preserved in archives. It is more fragile than leather, which is made to be more robust and resist heat and wetness. Parchment manuscripts act as sources of information in collagen from the archaeological record, and like bone, they would benefit from proteomics investigations as it looks at their molecular basis. Parchment has received recent interest from DNA researchers (Burger et al. 2000; Poulakakis et al. 2007; Campana et al. 2010), but DNA does not persist in leather due to the rigorous process of tanning. Here is where proteomics specifically can aid in identification (see Chapter 6).

### *2.5.3. PROTEINS IN BONES*

#### 2.5.3.1. Bone and other mineralized collagenous materials

Bones represent the most common source of archaeological proteins, and they are widely used in stable isotope analysis and radiocarbon dating. Following the first applications of soft-ionisation mass spectrometry to bones (Ostrom et al. 2000) more analysis has been conducted on this tissue than any other.

Ostrom and colleagues have surveyed preservation of ancient proteins in a range of fossils and were the first to detect a specific hydrolysis site: the cleavage of Asp-Pro. This finding explained the observation of (Craig & Collins 2000) based upon monoclonal antibody work that the N-terminal epitope was lost before the C-terminal epitope.

The ZooMS method, as discussed above, can potentially be applied to any collagenous tissue, and is therefore mentioned with regard to bone (Buckley et al. 2008; Buckley et al. 2009) and leather (see Chapter 6). The distinction between animal species can help in the analysis of archaeological assemblies, as recently a method has been established to differentiate between sheep and goat (Buckley et al. 2010), and even though fish collagen has until recently been absent from the protein databases, recent

investigations reveal the possibility to distinguish between fish species with a high rate of confidence (Richter et al. 2011).

#### 2.5.3.2. Fossils

A series of papers from a team led by Mary Schweitzer suggests that collagen may persist in palaeolontological bones, with reports of collagen peptides from both *Tyrannosaurus rex* (~68Ma) (Asara et al. 2007) and Hadrosaur (~80Ma) (Schweitzer et al. 2009). These results have proved controversial (Buckley et al. 2008; Pevzner et al. 2008) but have received some support (Bern et al. 2009) and a follow-up study (San Antonio et al. 2011). The ability of collagen to persist over such long time scales in bones from uncompromising burial environments would be significant, as it would imply that there may also be collagen present in bones from early hominid fossils (albeit at very low levels).

#### 2.5.3.3. Disease

The discovery of hydroxylation differences by mass spectrometry in osteocalcin revealed information on dietary development between species of humans and primates (Nielsen-Marsh et al. 2005). In a similar fashion, hydroxylation patterns in collagen could indicate certain pathologies. These differences lead to metabolic disease that fails to produce the expected sequence of healthy collagen. The most obvious example is scurvy. Vitamin C (L-ascorbic acid) is an essential co-factor for collagen hydroxylases (prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase; Myllyharju 2005), required for the decarboxylation of α-ketoglutarate. Ascorbic acid is synthesized from glucose via L-gulonolactone oxidase (GULO) but activity has been lost in bats, some passerine birds, guinea pigs and some primates (including humans). These organisms suffer from scurvy unless they ingest sufficient dietary vitamin C.

Schultz et al (2007) have reported over 200 extracellular proteins from compact bone of a Scythian king buried in southern Siberia (~2.7 kyr bp). They were able to detect a prostate specific antigen, a 34 kD glycoprotein by Western blotting; as the molecular weight on the Western was at double the molecular weight, the authors concluded that it was complexed with alpha 1-antichymotrypsin.

The cell wall of mycobacteria, responsible for several human pathogens, contains high molecular weight mycolic acids which have been used as biomarkers for pathologies in archaeological bone. Biomolecular analyses are believed to be more reliable than osteological analysis alone. MALDI-TOF MS has been commonly used for the

identification of diagnostic mycolic acids patterns, but this has not always gone unchallenged (Mark et al. 2010; Minnikin et al. 2010; Mark et al. 2011); neither has DNA analyses for the same pathogens (Wilbur et al. 2009). Boros-Major (2011) reported the presence of several *Mycobacterium* proteins in bones of up to 1300 years old that tested DNA positive for *M. tuberculosis* DNA and mycolic acids, using MALDI-TOF/TOF MS. The study showed as a first what proteomics can bring to aid towards this archaeological question: species specific identification of several sub-proteome level ancient proteins with reduced problems with contamination and the possibility for independent and high-throughput confirmation of other techniques.

**Table 2-1. Overview of recent studies in proteomic pertaining to archaeological science.**

| Material | Age | Reported identified proteins | Method | Reference |
|---|---|---|---|---|
| Binding material/ Glue | 1300-1400 AD | Ovotransferrin<br>Ovalbumin<br>Ovamucoid<br>Phosvitin<br>Lipovitellin | MALDI-TOF nanoLC/ nanoESI/ Q-q-TOF | (Tokarski et al. 2003) (Tokarski et al. 2006) |
| | 1300-1600 AD | Gelatin<br>Albumin<br>Ovalbumin<br>Casein | ESI-MS | (Peris-Vicente et al. 2005) |
| | 1336-1341 AD<br><br>Modern replicas | Gelatin<br>Casein<br>Albumin<br>Lactoglobulin | MALDI-TOF | (Leo et al. 2011) |
| | 1490 AD | Casein<br>Ovalbumin<br>Ovotransferrin<br>Collagen<br>vitellogenin | MALDI-TOF | (van der Werf et al. 2012) |
| | 1540 AD | Gelatin | MALDI-TOF | (Chamberlain et al. 2011) |
| | 1700 AD | Gelatin | FT-ICR MS | (Dallongeville et al. 2011) |

| | | | | |
|---|---|---|---|---|
| | 1800 AD | Ovalbumin<br>Casein | MALDI-TOF | (Kuckova et al. 2007), (Kuckova et al. 2005) |
| | Modern replicas | Gelatin | MALDI-TOF | (Romero-Pastor et al. 2012) |
| Bone | 68 Ma, 160-600 ka | Collagen | LC-MS/MS<br>TOF-SIMS<br>Orbitrap | (Asara et al. 2007)<br>(Schweitzer et al. 2009) |
| | 7500 BC - recent | Various NCPs (non-collagenous proteins) | 2D-PAGE | (Schmidt-Schultz & Schultz 2007) |
| | 800 y—53 ka BP | Osteocalcin | MALDI-TOF | (Ostrom et al. 2000), (Nielsen-Marsh et al. 2005) |
| | 43 ka | Various proteins | nanoLC-ESI Orbitrap | (Cappellini et al. 2012) |
| | Modern and archaeological | Collagen telopeptides | MALDI-TOF | (Buckley et al. 2008) |
| Ceramics | 1200-1400 AD | Myoglobin | nanoLC<br>nanoESI<br>FT ICR<br>MALDI-TOF | (Solazzo et al. 2008) |
| | Modern, experimental | i.a. Albumin<br>Myoglobin<br>Collagen<br>Actin | LC-MS | (Barker et al. 2012) |
| Dental pulp | Modern<br>13-14th century<br>18th century<br>6,500 BC | Collagen | MALDI-TOF | (Tran et al. 2011) |
| Food residue | 400 BC cal | Casein | LC-MALDI-TOF | (Hong et al. 2012) |
| Hair/Textile | 1600 AD | Keratin | MALDI-TOF | (Araki & Moini 2011) |

| | | | | |
|---|---|---|---|---|
| | 19-20th century | Keratin | nanoLC-MALDI-TOF | (Solazzo et al. 2011) |
| | 5300 y | Keratin | MALDI-TOF | (Hollemeyer et al. 2008) |
| Lithics/ Ceramics/ Glass/Flint | Modern, artificial, experimental | Albumin Casein Myoglobin Ovalbumin (artificially applied) | DESI-MS | (Heaton et al. 2009) |
| Metal (Patina) | 1100-1300 AD, 1900 AD | Haemoglobin | TOF-SIMS | (Mazel et al. 2007) |
| Mortars | 1100 AD | Casein Haemoglobin | MALDI-TOF | (Kuckova et al. 2009) |
| Pickled skin | Modern | Collagen I & III | 2D-PAGE MALDI-TOF | (Choudhury et al. 2006) |
| Soil | 240–270 AD 1500–1100 B.C | Keratin | MALDI-TOF | (Oonk et al. 2012) |

## 2.6. Potential of Archaeological Proteomics

When investigating bone collections, whether obtained from museums or site assemblages, it can be fundamental to minimise destructive sampling. A number of methods require significant amounts of bone to obtain enough protein for analysis, such as radiocarbon analysis, stable isotope analysis, and amino acid racemisation, even though some of these methods have evolved over time and now require less sample material. One of the main advantages of proteomics is that mass spectrometry requires only minimal amounts of protein, in the order of picograms. The amount of information that is obtained, however, is not compromised by reducing sample size.

Posttranslational modifications can potentially be used as markers for diagenesis. In principle, any biochemical marker that denotes change of collagen associated with a physiological feature, could be used as an indicative proxy for biological changes. Sequencing through mass spectrometry can elucidate phylogenetic information from proteins. All this information could be locked up in a minimal specimen and current advantages in proteomics as a whole and the studies currently performed on

archaeological proteomics could be a major revolution up and coming into archaeology.

### *2.6.1. SCOPE OF THIS THESIS*

This thesis aims to show the development and use of a minimally destructive approach of ZooMS (Chapter 3), application of this method for a thermal damage proxy by measuring the amount of glutamine deamidation in bone collagen (Chapter 4), and limitations that have so far been encountered using the minimally destructive approach, illustrated with two problematic case studies with archaeological samples and one experimental burial (Chapter 5).

Some of the problems touched upon in Chapter 5 were believed to be caused by cross-linking. So, to confirm these suspicions, ZooMS was applied to leather, a collagenous tissue that is purposefully crosslinked and may benefit from the strengths that ZooMS offers for species identification as much as bone does for both leather scientists and archaeologists. In Chapter 6 the results of collagen extraction and species identification through ZooMS from tanned hides is discussed.

Chapter 7 aims to show the application of ZooMS for a single-particle approach to the screening and identification of bone fragments in meat and bone meal.

In Chapter 8 final thoughts are given with regard to further improvements on the introduced minimally destructive approach to ZooMS and future potential towards archaeological science.

# Chapter 3. A NOVEL AND NON-DESTRUCTIVE APPROACH FOR ZOOMS ANALYSIS: AMMONIUM BICARBONATE BUFFER EXTRACTION

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## 3.1. ABSTRACT

Bone collagen is found throughout most of the archaeological record. Under experimental conditions, collagen is apparently preserved as an intact molecule, with amino acid compositions and isotopic profiles only changing when almost all of the protein is lost. The ubiquity of collagen in archaeological bone has led to the development of the use of collagen peptide mass fingerprints for the identification of bone fragments — Zooarchaeology by Mass Spectrometry (ZooMS). We report a novel, but simple method for the partial extraction of collagen for ZooMS that uses ammonium bicarbonate buffer but avoids demineralisation. We compared conventional acid demineralisation with ammonium bicarbonate buffer extraction to test ZooMS in a range of modern and archaeological bone samples. The sensitivity of the current generation of mass spectrometers is high enough for the non-destructive buffer method to extract sufficient collagen for ZooMS. We envisage a particular advantage of this method is that it leaves worked bone artefacts effectively undamaged post-treatment, suitable for subsequent analysis or museum storage or

display. Furthermore, it may have potential as a screening tool to aid curators in the selection of material for further molecular analysis – such as DNA sequencing.

## 3.2. INTRODUCTION

### *3.2.1. COLLAGEN SURVIVAL*

Collagen, the most abundant protein in bone and dentine, is found widespread in the archaeological record (Tuross et al. 1980; Higham et al. 2006) and can persist in fossils up to at least 600 ka (Buckley et al. 2011), perhaps considerably longer (Schweitzer et al. 2009). Type I (bone) collagen is a triple helix composed of two alpha 1 (COL1A1) and one alpha 2 (COL1A2) chains. COL1A1 is more highly conserved between genera than COL1A2 (Buckley et al. 2009; Buckley et al. 2010). A third alpha chain, COL1A3, has been reported exclusively in fish (Piez 1965).

Unlike DNA, which is typically fragmented to below 70bp when recovered from bone (e.g. Krause et al. 2010), collagen is sometimes treated as if it remains an intact protein. Radiocarbon and stable isotope preparation methods typically isolate an acid insoluble fraction, gelatinise that fraction by heating in weak acid and subsequently retain a high molecular weight >30 kDa fraction. Covington et al (2008) argue that collagen is stabilised by physical compression of the collagen fibril by mineral or chrome tanning agents, in a manner similar to the role of dehydration considered by Miles and Ghelashvili (1999). These findings are believed to be responsible for the observations that during artificial degradation, there is no perceptible reduction in collagen quality until there was less than 1% of the levels of collagen found in modern bone (Dobberstein et al. 2009). However, archaeological bone finds can contain more than one per cent of its original collagen yet show a signal of degraded collagen (Harbeck & Grupe 2009) according to commonly used diagenetic indicators: collagen wt%, C%, N% and C/N molar ratio (van Klinken 1999). Collagen degradation, though more elucidated in the past decades, can still not be fully or straightforwardly explained. Of the biomolecules found in archaeological specimen of direct biological significance, collagen has one of the highest chances of surviving over substantial periods of ancient history and in a quality that is useful for various types of analyses, such as proteomics.

Degraded collagen that has been significantly altered as to be detrimental to stable isotope analysis (Harbeck & Grupe 2009), is less of an obstacle for ZooMS. In contrast to other methods such as stable isotope analysis, protein mass-spectrometry doesn't

rely solely on bulk collagen and is less influenced by collagen quality in data interpretation.

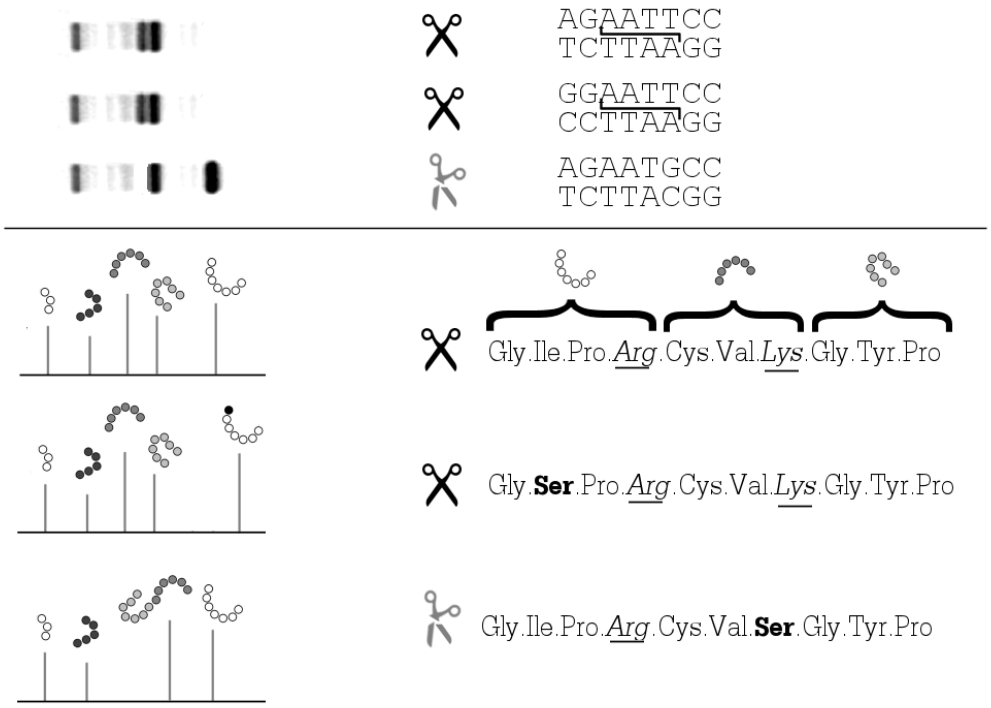


**Figure 3-1. Schematic illustration of DNA fingerprinting versus Protein Mass Fingerprinting (PMF). Both methods are based on the principle that the primary sequence holds motifs for restriction enzymes or proteases respectively and that resulting fragments after digestion differ in size and mass. A point mutation in a DNA sequence, when not interfering with the enzyme motif, will have no effect on the fragment length and therefore not be detected. In the case of PMF a non-synonymous point mutation will result in a change in the amino acid sequence, which is usually coupled to a mass change (excepting the isomeric residues Leu/Ile), even when the protease cleavage site (C-terminal to arginine and lysine residues in the case of trypsin) is unaltered.**

### *3.2.2. ZOOARCHAEOLOGY BY MASS SPECTROMETRY*

Ancient collagen integrity means that peptide mass can be used to identify the taxonomic source of archaeological bone samples in an approach termed ZooMS (Zooarchaeology by Mass Spectrometry; (Buckley et al. 2009)). ZooMS is a version of peptide mass fingerprinting (Pappin et al. 1993; Henzel & Watanabe 2003): an approach broadly analogous to DNA fingerprinting (Figure 3-1). A proteolytic enzyme (most commonly trypsin) is used to cleave proteins at specific residues, generating

peptide fragments of varying lengths, in a similar way to the action of restriction enzymes on DNA. Differences in primary sequence will result in a variation in masses of fragments, and these patterns are used as a fingerprint diagnostic for the original protein. In ZooMS, archaeological collagen peptide masses are matched to either known standards or masses predicted from collagen sequences (Buckley et al. 2009). Due to the slow rate of collagen evolution, the taxonomic resolution of ZooMS is typically no better than animal genus, much less precise than DNA based methods (c.f. Boyko et al. 2009). However, ZooMS does not allow template amplification and peptides are measured 'as is', which reduces contamination dominant signals.

### *3.2.3. MINIMALLY DESTRUCTIVE ANALYSIS*

The original ZooMS method (Buckley et al. 2009; Buckley et al. 2010) uses acid to demineralise bone prior to collagen extraction and mass spectrometric analysis. Despite small sample sizes (typically 1–5 milligrams of bone), the method is still destructive as samples are dissolved after acid digestion and gelatinisation. In the case of analysis of worked bone artefacts or a screening tool for subsequent analyses (e.g. DNA, stable isotope or radiocarbon analysis) a milder extraction process would be advantageous.

The current generation of mass spectrometers is capable of detecting femtomoles or attomoles of peptides (Vorm et al. 1994). We therefore speculated that we could leverage this instrumental sensitivity to analyse the small fraction of soluble protein that is reported to persist in ancient bone (Tuross et al. 1988; Collins et al. 2009) without the need for acid digestion. Here we report our findings in using a warm (65°C) ammonium bicarbonate buffer (pH 8.0) to leach collagen into solution for ZooMS analysis (Figure 3-2), enabling worked bone artefacts to be analysed without destructive sampling.

## 3.3. METHOD AND MATERIAL

### *3.3.1. MATERIALS*

All modern samples were from bovid bone meal autoclaved at 133°C for 20 minutes. Archaeological bone was powdered bovid bone from a Mesolithic-Neolithic site in Rosenhof (Germany) dated to 4900/4800 cal BC (Scheu et al. 2008) and whole bone fragments of bovid bone from mid-ninth to tenth century Coppergate, York (UK) (Ottoni et al. 2009), both well-preserved. For macroscopic comparison and Fourier transform infrared spectroscopy (FT-IR) investigation, fragments of bovid bone from Coppergate (UK) were used. Scanning electron microscopy was performed on early

medieval comb tines made from worked antler or bone (not established prior to investigation). See also Table 1.

**Table 3-1. Samples used in this study with abbreviations used in text for reference.**

| Sample description | Age | Site | Experiment | Reference | Abbreviation as used in text |
|---|---|---|---|---|---|
| Autoclaved bone meal | Modern | N/A | Method Comparison | Garrido-Varo et al. (2005) | M (modern) |
| Powdered bovid bone | 4900–4800 cal BC | Rosenhof (Germany) | Bradford Assay | Scheu et al. (2008) | P (prehistoric) |
| Bovid bone | 9–10th century AD | Coppergate, York (United Kingdom) | Bradford Assay / Macroscopic Comparison/ FT-IR | Ottoni et al. (2009) | H (historical) |
| Worked deer bone/antler comb tines | 6–9th century AD | Howe, Stromness (United Kingdom) | SEM | Ashby (2009) | W (worked) |

### *3.3.2. AMMONIUM BICARBONATE ($NH_4HCO_3$) BUFFER PROTOCOL*

Samples of bone (< 1 mg for modern bone; approximately 5 mg for archaeological bone) were incubated for 1 hour at 65°C in 50 mM ammonium bicarbonate [pH 8.0] in a polypropylene eppendorf tube or microplate. In pilot experiments a range of temperatures from 65 to 95°C, were tested; higher temperatures yielded poorer signals in mass spectrometric analysis with modern bone (results not shown). Samples were briefly centrifuged and the supernatant is (usually) discarded. The extraction was repeated, and this supernatant was collected for incubation with 1 µg/µL sequencing grade modified porcine trypsin (Promega, Southampton, UK) at 37°C for18 hours. See also figure 3-2. Due to hydrophobic substances interfering with spectra, the second hour extract obtains higher quality spectra and is therefore analysed, even though the first hour extract would contain more collagen and does (see table 3-2).

### 3.3.3. *COLD ACID PROTOCOL*

Samples (< 1 mg for modern bone; approximately 5 mg for archaeological bone) were incubated in 0.6 M HCl for 24 hours at 4°C. Demineralised samples were centrifuged and the supernatant discarded. The acid insoluble fraction was washed in ultrapure water to neutral pH, then incubated for 3 hours in 50 mM ammonium bicarbonate [pH 8.0] at 70°C. The supernatant was collected and incubated for 18 hours with 1 µg/µL sequencing grade modified porcine trypsin (Promega) at 37°C.

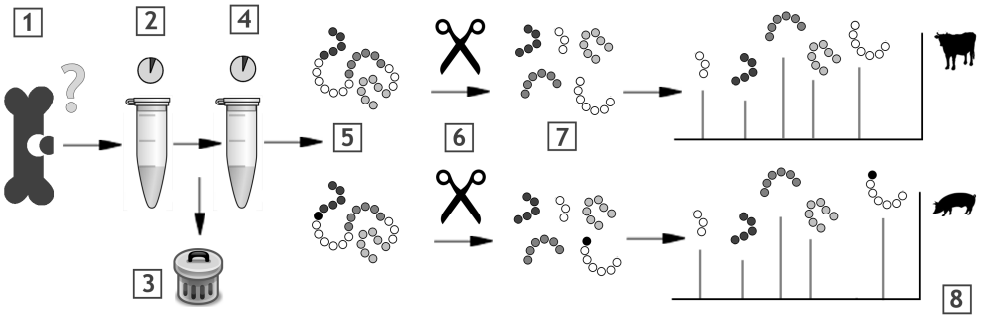


**Figure 3-2. Schematic overview of the buffer extraction method. [1] A small particle of bone is collected; [2] the sample is incubated for an hour at 65°C in ammonium bicarbonate buffer pH 8.0; [3] after incubation, the supernatant — containing protein and potentially interfering particles — is discarded; [4] the sample is incubated for another hour at 65°C in ammonium bicarbonate buffer; [5] the second extract containing collagen is collected; [6] trypsin is added to cleave the protein into peptides; [7] dependent on the primary sequence of collagen, peptides with varied masses are obtained; [8] these variations are visualised in the mass spectra and a species identity can be assigned, using ZooMS.**

### 3.3.4. *PEPTIDE PURIFICATION BY SOLID PHASE EXTRACTION*

The tryptic digest was purified over C18 resin (Millipore, Durham, UK; Porvair, Leatherhead, UK) to desalt and concentrate peptides by washing with 0.1% trifluoroacetic acid (TFA). Peptides were eluted in a final volume of 10 µL of 50% acetonitrile (ACN) / 0.1% TFA (v/v). 1 µL of eluate was mixed on a ground steel plate with 1 µL α-cyano-4-hydroxycinnamic acid matrix solution (1% in 50% ACN / 0.1% TFA (v/v/v)) and air dried.

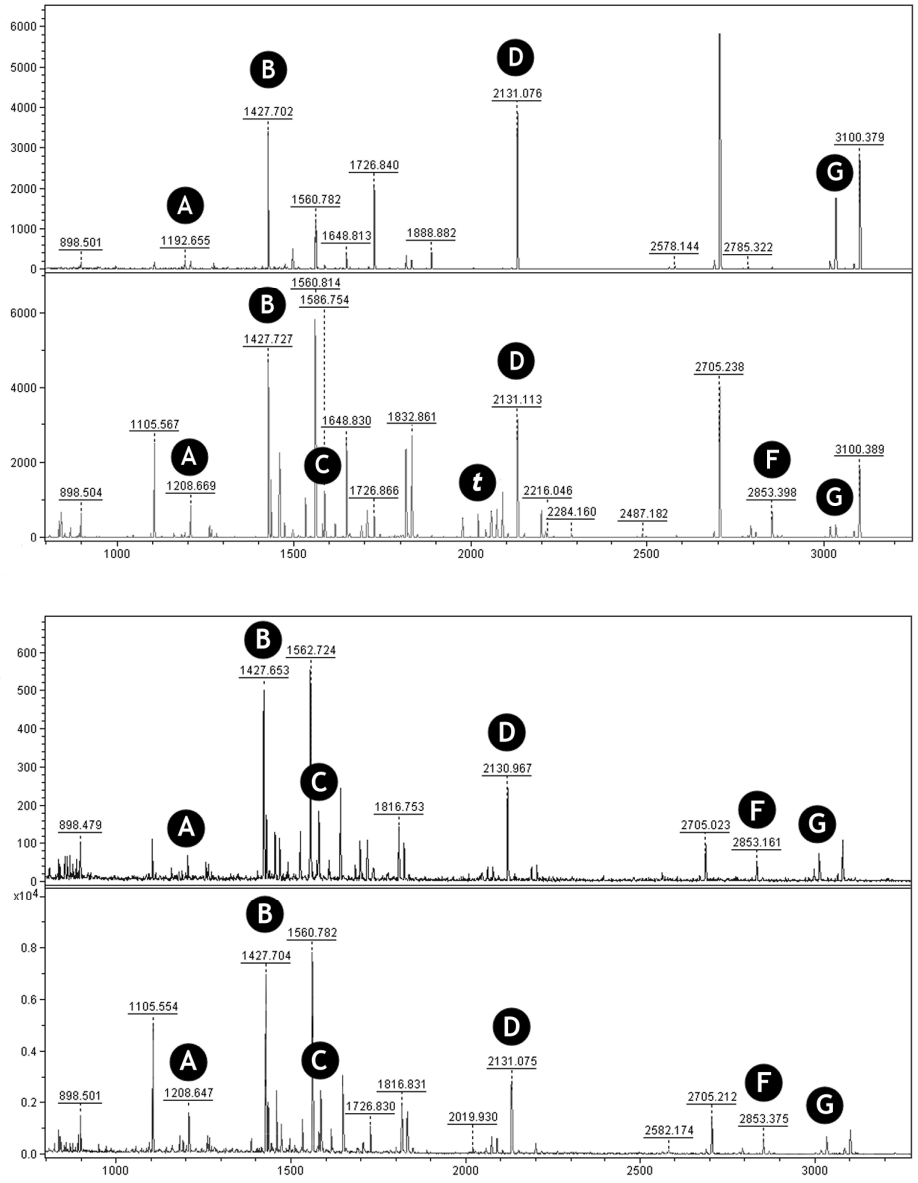


**Figure 3-3. MALDI-TOF spectra from bovid (*Bos taurus*) bone extracts. a) Modern bone (M, see Table 3-1), demineralised (above) non-demineralised (below); b) Archaeological bone (H, see Table 3-1), demineralised (above) non-demineralised (below). Assigned in capital letters A–G are *m/z* typical for cattle as designated by Buckley et al. (2009). *t* is used to indicate a trypsin autocleavage product.**

### *3.3.5. MASS SPECTROMETRIC ANALYSIS*

Each sample was analysed in reflector mode using a calibrated (to external peptide standards) Ultraflex III (Bruker Daltonics, Bremen, DE) MALDI-TOF instrument to measure mass-to-charge ratios (*m/z*) of tryptic fragments within a range of 800–4000

Da. Spectra were analyzed using flexAnalysis software v. 3.0 (Bruker Daltonics). Detected *m/z* with a signal-to-noise ratio above 3 were identified manually to *m/z* typical for cattle and deer (Buckley et al. 2009).

### *3.3.6. EXTRACTION EFFICIENCY MEASURED BY BRADFORD ASSAY*

Undigested material (M, H, P — abbreviations in Table 3-1) extracted by either cold acid or $NH_4HCO_3$ buffer method was collected and diluted 10 and 20 times in 50 mM ammonium bicarbonate [pH 8.0]. One volume of Coomassie Plus Protein Assay Reagent (Thermo Fischer Scientific, Loughborough, UK) was added to each sample and allowed to incubate 1 minute at room temperature. Samples were calibrated against standard range of 50 – 0.8 mg/ml BSA, and a 50 mM ammonium bicarbonate blank. All samples were measured in triplicate using a FLUOStar OPTIMA microplate reader (BMG Labtech, Offenburg, DE) in absorbance mode, with a BMG 0308A filter at a wavelength range of 570-585 nm.

### *3.3.7. MACROSCOPIC COMPARISON*

Two bone particles of approximately equal weight (approx. 70 mg) and size (10 mm diagonal) from the same archaeological bovine sample (H) were treated with either the cold acid method or the $NH_4HCO_3$ buffer method. After each incubation step, the samples were weighed, measured with a calliper and photographed. Concluding extraction as described in section 3.3.2, the samples were further exposed to three cycles of 12 hours at -20°C and 12 hours at room temperature in 50 mM ammonium bicarbonate buffer to examine the impact of freeze-thaw damage (i.e. repeated removal from a freezer).

### *3.3.8. SCANNING ELECTRON MICROSCOPY (SEM)*

Comb tines from a worked bone artefact (W) were treated with the $NH_4HCO_3$ buffer method. One of these was exposed to a freeze-thaw cycle due to storage. A third comb tine was left untreated as a control.

All three samples were examined by scanning electron microscopy (SEM). Samples were suspended in a copper shim and viewed uncoated at 5 kV on a JSM 6490LV microscope (JEOL, Tokyo, JP).

For (worked) bone surfaces, ultrasonic cleaning in the presence of a mild detergent is recommended (Rose 1983) to remove dirt from the surfaces. However, the $NH_4HCO_3$ buffer method involves a similar washing step, obviating the need for potentially destructive cavitation effects of ultrasound. Samples were cleaned with cotton wool,

but following extraction with $NH_4HCO_3$, macroscopic surface impurities were still visible.

### *3.3.9. FOURIER TRANSFORM INFRA RED SPECTROSCOPY (FT-IR)*

Bone apatite powder (2 mg) was ground with 200 mg of spectroscopic grade KBr. The powder was placed within a 12-mm disc and pressed into a pellet (3 mm window) using a hydraulic press at 10,000 psi. Pellets were scanned 16 times using a Perkin-Elmer FT-IR spectrometer. Spectra were recorded from 4,000 to 400 $cm^{-1}$ and baseline corrected.

Peak heights at wave-numbers 565 ($v_4$ $PO_4$), 605 ($v_4$ $PO_4$), the valley at ~590 $cm^{-1}$ between peaks 565 and 605 $cm^{-1}$ were measured to calculate the infra-red splitting factor (SF). The peak heights at wave-numbers 1,035 ($v_3$ $PO_4$), and 1,415 $cm^{-1}$ ($v_3$ $CO_3$) were measured for calculation of carbonate to phosphate ratio ($CO_3/PO_4$). These are two common semi-quantitative measurements used to evaluate diagenesis in apatite. Additionally, the collagen content can be estimated by calculating the ratio of intensities of the amide peak at 1,640 $cm^{-1}$ and the phosphate peak at 1,035 $cm^{-1}$ (Trueman et al. 2008).

The SF evaluates the crystalline structure of bioapatite. For modern bone, SF ranges between 2.5 and 2.9 (Wright & Schwarcz 1996). An increase in SF indicates re-crystallisation and an increase in crystal size and order. This is generally coupled with a decrease in $CO_3/PO_4$, i.e. a loss in carbonate (Sillen 1989). Measurements made on fresh cattle bone gave an amide-to-phosphate ratio ($Am/PO_4$) of 0.36. Any values below this would indicate a relative decrease in collagen content.

## 3.4. RESULTS

### *3.4.1. COMPARISON OF COLLAGEN EXTRACTION METHODS*

To assess the amount of protein extracted in the two protocols, first hour and second hour extracts from bone of three different ages (M, H, P; Table 3-1) was determined by Bradford Assay. The highest yield of protein with the Bradford Assay was always obtained in the first hour extract; concentrations decreased with subsequent extractions. However, MALDI-TOF-MS analysis of the first protein extract has consistently lower peak intensities and ion current than the following extract.

Autoclaved, modern animal bone extracted using either the acid and $NH_4HCO_3$ buffer methods produced sufficient collagen for MALDI–MS analysis and identification of peptides for speciation (Figure 3-3a). Similar results are obtained from archaeological

material (H in Table 3-1), showing that both methods of extraction can be used for ZooMS applications (Figure 3-3b).

Further repeated extractions still produced sufficient protein for identification by ZooMS, although below the detection limit of the Bradford Assay. There was no significant difference in yields of detectable protein with either the acid or ammonium bicarbonate methods. However, in archaeological samples, more protein is obtained using acid demineralisation, in both the first and any subsequent incubation. Estimated yields (Bradford Assay) are shown in Table 3-2 for each sample per milligram of original bone weight.

**Table 3-2. Bradford assay results of collagen extraction on well-preserved archaeological bone. Original sample size and weight are denoted and the protein yield as measured through photo-spectroscopic analysis. The results for extraction fractions, first hour and second hour, are shown. Yields are averaged over three sample replicates and technical triplicates. The measured protein concentration is normalised against original respective sample weight. Yields are denoted ± standard error.**

| Sample | Size (mm) | Weight (mg) | Yield cold acid (ug/mg) | | Yield $NH_4HCO_3$ (ug/mg) | |
|---|---|---|---|---|---|---|
| | | | 1h | 2h | 1h | 2h |
| Modern | 1-2 | 0.35 | 296.5 ± 67.4 | 62.50 ± 36.06 | 303.9 ± 74.8 | 106.6 ± 46.4 |
| Medieval | 5 | 9.8 | 20.54 ± 8.48 | 11.77 ± 3.65 | 0.84 ± 1.15 | 0.67 ± 0.29 |
| Neolithic | Powder | 6.3 | 27.20 ± 4.19 | 20.10 ± 2.32 | 7.52 ± 3.80 | 5.43 ± 2.47 |

### *3.4.2. MACROSCOPIC COMPARISON ON ARTEFACT PRESERVATION AFTER EXTRACTION*

Figure 3-4 shows the effect of each extraction step on bone material (H). Untreated, both samples A and B are of comparable size and weight. After acid treatment, sample A was decreased by 76% in weight (accounting for 63% by weight; Glimcher & Krane 1968). A transparent collagen ghost was left after air drying overnight. Ultimately, sample A lost approximately 90% of its original weight (74.3 mg) and decreased 50%

in size (11 mm). Sample B, extracted without decalcification, showed no significant decrease in either size (12 mm) or weight (59.9 mg). After multiple freeze-thaw cycles, no measurable changes were observed in either sample.

### *3.4.3. MICROSCOPIC AND MOLECULAR COMPARISON ON ARTEFACT PRESERVATION*

Scanning electron microscope images of untreated and treated artefacts (W) with the $NH_4HCO_3$ buffer method show no marked differences between bone surfaces (Figure 3-5). Both the untreated sample, and the treated samples (including one stored in a freezer) show surface damage, which may be caused by manufacture, use or taphonomy; the detail on these does not appear to change following extraction.

There is no evidence that the $NH_4HCO_3$ method measurably alters bone exposed to incubation. Acid extraction, unsurprisingly leads to changes in the mineral phase – specifically a lower IR-SF (i.e. a lower crystallinity), a higher proportion of carbonates and a high amide to phosphate ratio (indicating a relative increase in collagen over mineral).

The SF, $CO_3/PO_4$ and $Am/PO_4$ values of the $NH_4HCO_3$ buffer extracted sample are similar to the control values and both are within the typical range for modern bone. However, dissolution of apatite has reduced the crystallinity after cold acid treatment (Table 3-3). Low errors of SF values are also typical for unaltered bone.

## 3.5. DISCUSSION

A recent paper (Dobberstein et al. 2009) argued, on the basis of CNBr digestion and SDS-PAGE, that bone collagen remains largely intact in archaeological bone. Given the high temperature (> 150˚C) required to denature intact collagen (Kronick & Cooke 1996), the remarkably mild extraction procedure (2 x 1 hour @ 65°C) would be too low to fully denature undamaged collagen, yet yields repeatable and detectable results for ZooMS analysis in both modern and archaeological bone. A small, but significant soluble fraction is present in most bone through accumulated damage (Collins et al. 2009), and despite low yield, the coupling of concentration and purification by solid phase extraction and the sensitivity of MALDI-TOF-MS means that $NH_4HCO_3$ buffer extraction is sufficient for ZooMS analysis. We are currently exploring a larger body of samples, including material with varying thermal history, as the ability to release the minimal fraction of soluble collagen may be limited in some burial environments. The soluble fraction may have been leached out

completely prior to investigation if the inhumation period was exposed to an extensive water-influx.

**Table 3-3. FT-IR results on powdered bone for comparison between the cold acid method and the buffer method, and an untreated control. SF denotes the splitting factor, $CO_3/PO_4$ stands for the carbonate to phosphate ratio. $Am/PO_4$ is the amide to phosphate ratio. Results are noted ± standard deviation.**

| Sample | SF | $CO_3/PO_4$ | $Am/PO_4$ |
|---|---|---|---|
| Fresh bone | 2.75 ± 0.08 | 0.36 ± 0.08 | 0.36 ± 0.00 |
| Control (n = 3) | 2.76 ± 0.03 | 0.40 ± 0.008 | 0.38 ± 0.05 |
| Acid (n = 5) | 2.39 ± 0.35 | 0.44 ± 0.023 | 0.53 ± 0.02 |
| Water (n = 3) | 2.81 ± 0.10 | 0.39 ± 0.016 | 0.37 ± 0.06 |

Even with soluble collagen so easily flushed out, we consider the risk of cross-contamination during the inhumation period low. Due to the lack of amplification, there is little risk in a bone-preserving environment in which endogenous collagen would not exceed exogenous collagen by such a measure that contamination would show in mass spectra following this method. In addition, if any soluble exogenous collagen would be present, the first hour extract, which is discarded in our routine applications, would remove this.

### *3.5.1. SAMPLE INTERFERENCE*

Despite the fact that protein yields were highest in the first extract and decreased with each subsequent extraction (as estimated by Bradford Assay), the first extract invariably had lower peak intensity in mass spectra than the second extract. Subsequent extractions had progressively lower peak intensity, a pattern observed in both modern and archaeological bone.

It is unclear why the first extract has sub-optimal performance, but we suspect that this extract is enriched with compounds that co-crystallise with the matrix or otherwise prevent efficient ionisation of peptides more severely than with any subsequent extracts. The problem is more apparent in modern than archaeological extracts. The interfering component was probably moderately hydrophobic, given that the interference could be somewhat reduced (but not excluded) if extraction was

undertaken in polypropylene (which reportedly has a binding affinity for hydrophobic compounds) microplates rather than polystyrene (Sambrook & Russell 2001). One possible candidate is (phospho-)lipids; their rapid reduction in archaeological samples (Evershed et al. 1995; Millard 2001) would in part explain the lower interference in archaeological materials. However, we suspect a more complex range of compounds are responsible, as interference was also present in all archaeological samples, irrespective of age and burial environment.

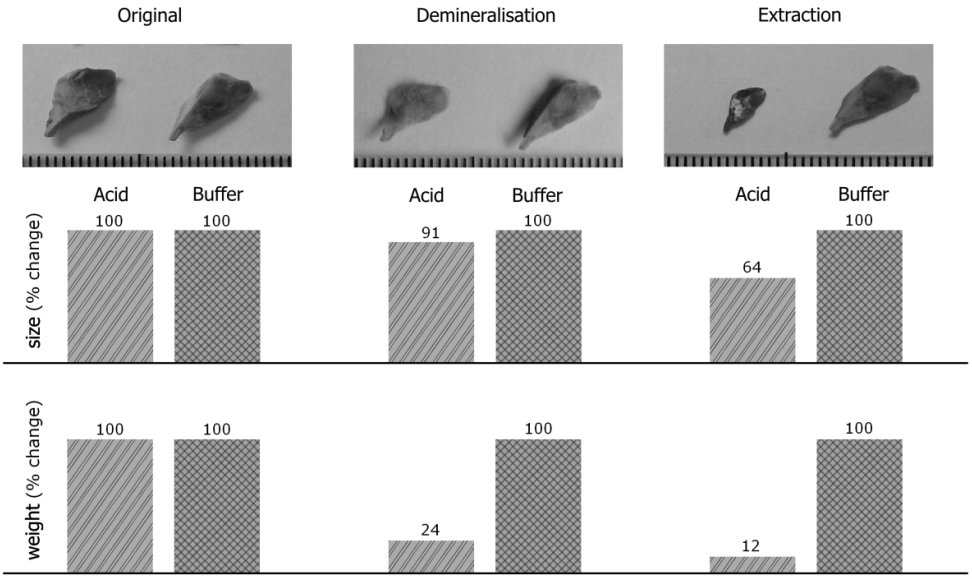


**Figure 3-4. Macroscopic comparison of the cold acid treatment versus the $NH_4HCO_3$ buffer method on well-preserved archaeological bovid bone (H, see Table 3-1). In the far left image (Original), both samples A (acid) and B (buffer) are untreated. After demineralisation, sample A has been incubated for 24 hours in 0.6 M HCl. Sample B is still untreated. In the last extraction sample A has incubated for 3 hours at 70°C in 50 mM $NH_4HCO_3$ buffer after decalcification. Sample B has been incubated twice for 1 hour at 65°C in $NH_4HCO_3$ buffer. Percentages of size and weight changes have been plotted below.**

### *3.5.2. ARTEFACT PRESERVATION AND METHOD POTENTIAL*

Our results show that in the case of well-preserved bone, the $NH_4HCO_3$ buffer extraction does not measurably alter the material. This allows worked artefacts to be investigated by ZooMS without destroying the sample and the artefact is only exposed twice to a weak (50 mM) ammonium bicarbonate buffer of pH 8.0 at 65°C for an hour (and this could be used as part of the cleaning process). It is unlikely that these

conditions will otherwise modify any object of mineralised tissue, as SF remained unaltered after treatment (Table 3-3). However, any major changes in crystallinity in bone prior to buffer extraction (i.e. acidic soil) may have an effect similar to cold acid extraction and potentially have a destructive effect.

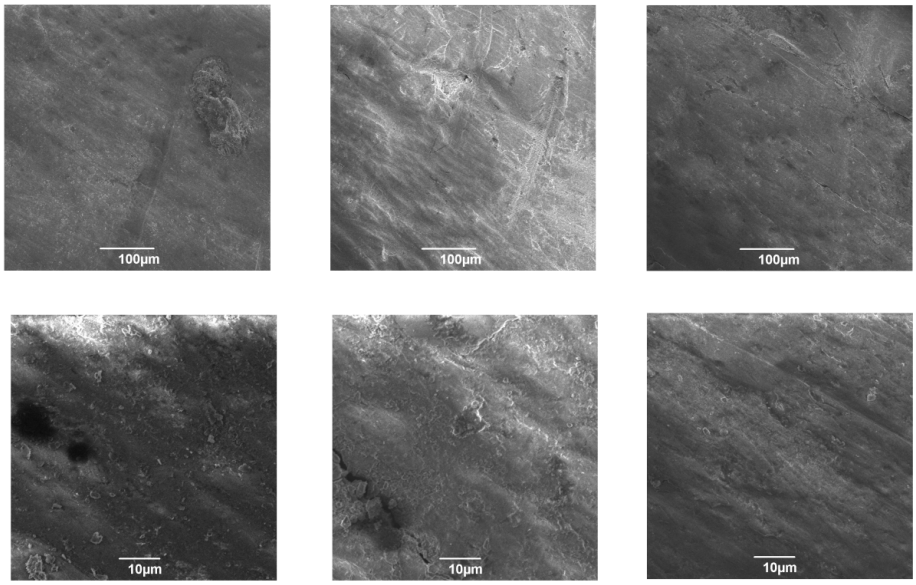


**Figure 3-5. SEM images of medieval comb tines (W, see Table 3-1). Left to right: untreated control; sample treated with $NH_4HCO_3$ buffer; sample treated with $NH_4HCO_3$ buffer and exposed to a freeze-thaw cycle. Above: magnification at 200x. Below: magnification at 1500x.**

With the $NH_4HCO_3$ buffer extraction the speed and sample throughput were vastly improved, while keeping overall costs down. Ultimately, extraction after cold acid treatment, by obtaining a higher protein yield, will have a higher efficiency and consequently a greater success rate. However, the partial leaching in the $NH_4HCO_3$ buffer extraction, allows the analyst to revisit the same specimen, and at the same time to investigate a higher number of samples at lower cost and time, as it obviates the need for a 24-hour decalcification step and neutralisation.

## 3.6. CONCLUSION

We have demonstrated the use of a mild $NH_4HCO_3$ buffer-based extraction method for extracting collagen from mineralised tissue. The $NH_4HCO_3$ buffer extraction yields sufficient collagen from well preserved mineralised tissue for mass spectrometric analysis and ZooMS identification of species. Potentially the method can be employed

to safely identify valuable artefacts or tiny fragments. It is also possible to repeat an analysis of the same material and furthermore the sample is not compromised if subsequent destructive analysis is required.

## 3.7. ACKNOWLEDGEMENTS

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# Chapter 4. SITE SPECIFIC DEAMIDATION OF GLUTAMINE: A NEW MARKER OF BONE COLLAGEN DETERIORATION

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Keywords: collagen, MALDI-TOF-MS, deamidation, glutamine, thermal age

## 4.1. ABSTRACT

**RATIONALE:** Non-enzymatic deamidation accumulates in aging tissues *in vivo* and has been proposed to be potentially useful as a molecular clock. The process continues post mortem, and here we explore the increase in levels of deamidation in archaeological collagen, as measured during Zooarchaeology by Mass Spectrometry (ZooMS) analysis.

**METHODS:** With the high sensitivity of current generation mass spectrometers, ZooMS provides a non-destructive and highly cost-effective method to characterise

collagen peptides. Deamidation can be detected by mass spectrometry as a +0.984 Da mass shift; therefore, aside from its original purpose, peptide mass-fingerprinting for bone identification, ZooMS concurrently yields a 'thermal indicator' of the samples.

**RESULTS:** By analysis of conventional ZooMS spectra, we determined the deamidation rate for glutamine residues in 911 bone collagen samples from 50 sites, with ages varying from medieval to Palaeolithic. The degree of deamidation was compared to diagenetic parameters and nearby sequence properties.

**CONCLUSION:** The extent of deamidation was found to be influenced more by burial conditions and thermal age than, for example, chronological age, the extent of bioerosion or crystallinity. The method lends itself mostly to screening heterogenic deposits of bone to identify outliers.

## 4.2. INTRODUCTION

### *4.2.1. PROTEIN DEAMIDATION*

Deamidation, a non-enzymatically occurring post-translational modification that asparagine (Asn or N) and glutamine (Gln or Q) residues undergo to aspartic acid (Asp or D) and glutamic acid (Glu or E) respectively, receives revived interest since it has been established that deamidation acts as a molecular clock in *in vivo* processes (Robinson & Robinson 2001; Takemoto et al. 2001; Robinson & Robinson 2004; Dasari et al. 2007). Recent studies of keratin, crystallin, casein and collagen (Haynes et al. 2002; Araki & Moini 2011; Leo et al. 2011) have demonstrated that sample age and damage show a correlation with deamidation during inhumation or above-ground storage. This study focuses on deamidation post mortem in archaeological samples.

The rate of deamidation is influenced by both steric hindrance and charged residues in the vicinity of deamidation sites, and therefore primary (Robinson et al. 1970; Kosky et al. 1999; Robinson & Robinson 2001), secondary (Xie & Schowen 1999) and tertiary (Kossiakoff 1988) structure, as well as temperature(Stratton et al. 2001) and pH (Hao et al. 2011). However, the situation is complicated by the fact that there are competing pathways for deamidation, either via condensation (to form a cyclic intermediate) or direct hydrolysis (Robinson et al. 2004). Condensation based mechanisms are arrested in structural proteins which prevent the reduction in inter-atomic distance required for the formation of the heterocycle, such as the highly ordered arrangement of collagen (van Duin & Collins 1998; Collins et al. 1999), arguably the 'ancient protein' with the most longevity. We therefore speculate that in

archaeological bone, the dominant pathway to deamidation of both Asn and Gln is direct side chain hydrolysis. Asparagine deamidation in collagen has recently been investigated with moderate success (Hurtado & O'Connor 2012). The process of glutamine deamidation is markedly slower and therefore less widely explored in studies on aging of tissues (Robinson & Robinson 2001). For archaeological samples, where the timespan for samples is greater, glutamine deserves more attention. In this paper we explore the potential of deamidation to function as a molecular clock in archaeological bone.

Collagen type I is the main organic constituent of bone and due to its characteristic mineral confinement (Miles & Ghelashvili 1999; Covington et al. 2008) can persist in temperate environments for more than 1 million years (Buckley & Collins 2011). Collagen varies slightly in primary sequence between species and can be used for genus identification (Buckley et al. 2009), using MALDI-TOF-MS (Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry) analysis with a method we term ZooMS (Zooarchaeology by Mass Spectrometry). The principle of ZooMS is based upon the sensitivity of the current generation of mass spectrometers, which allows high-throughput species identification (Richter et al. 2011) and the analysis of a partial bone extract containing only soluble collagen (van Doorn et al. 2011, also chapter 3).

Deamidation causes a mass shift of +0.984 Da and isotopic multiplets of both peptide variations will overlap in a MALDI-TOF MS spectrum. We can extract the degree of deamidation from ZooMS spectra with no modification to our existing protocols (Wilson et al. 2012), thereby not adding to the cost of analysis. Similar approaches indeed exist for MALDI-TOF-MS (Dasari et al. 2009; Araki & Moini 2011; Zhu et al. 2011). In the study by Leo et al (2011) it was argued that collagen contained too many hydroxylation variants to be of significant use and favoured casein for the study of damage in archaeological binding materials. Nevertheless, collagen being the main protein in bone, our method allows the use of deamidation in collagen peptides, including various hydroxylation variants, as an indication for single bone particles for a low cost. A further added value to this method is that this is performed using our high-throughput, non-destructive ZooMS protocol, which is minimally invasive and does not preclude subsequent DNA analysis on the same samples (von Holstein et al. submitted).

## 4.3. MATERIALS AND METHOD

### *4.3.1. SAMPLES*

All collected samples were bone fragments from a variety of species, mostly human (*Homo sapiens*) and cattle (*Bos taurus*), but also included reindeer (*Rangifer tarandus*), horse (*Equus caballus*), pig (*Sus scrofa*), sheep (*Ovis aries*), goat (*Capra aegagrus*), bison (*Bison bonasus*), dog (*Canis familiaris*), fish (halibut, *Hippoglossus hippoglossus*), and chicken (*Gallus gallus*). For an overview of all archaeological sites, see the map in Figure 4-1.

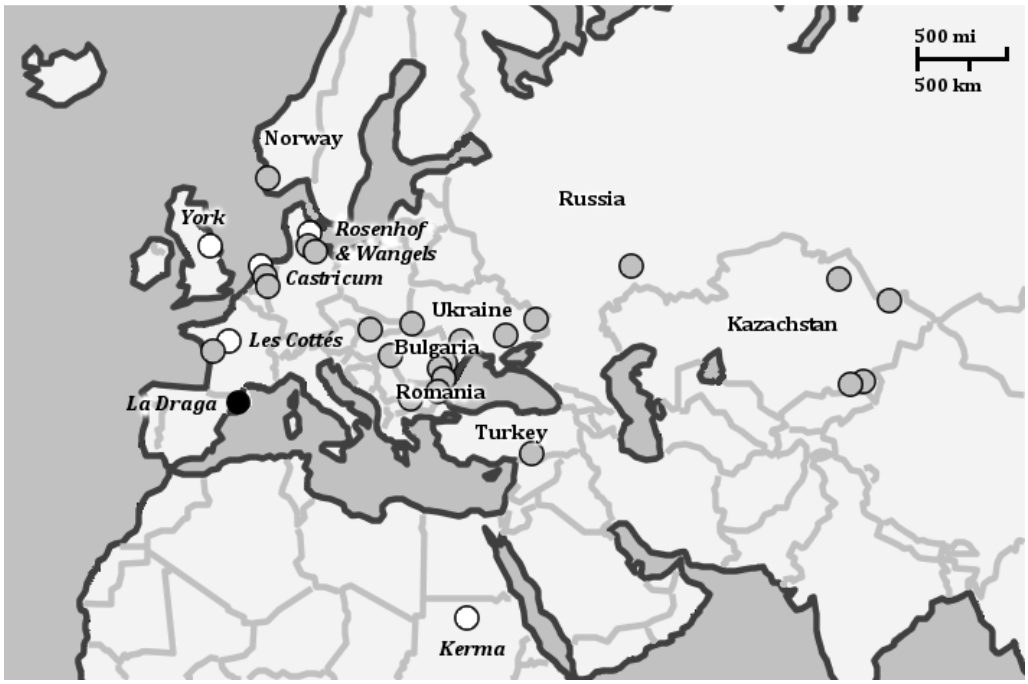



**Figure 4-1. All sites for samples investigated in this study. Indicated are the samples used in calibration of composite %Gln values (white), additional samples used for diagenetic comparisons (grey), and a sample site that did not yield substantial data for processing (black – La Draga). Site names from Table 4-2 are given in italics.**

### *4.3.2. PREPARATION OF SAMPLES*

#### 4.3.2.1. Non-destructive ZooMS

Samples of approximately 30 mg (5 mm) were collected in triplicate and transported in 96 well microplates. Upon receipt, samples were incubated for 1 hour at 65°C in 50 mM ammonium bicarbonate buffer (pH 8.0). The supernatant was discarded and the incubation repeated in 50 mM ammonium bicarbonate. Supernatant was collected and trypsinated overnight (≤18 h) at 37°C.

Trypsinated extract was used for Solid Phase Extraction (SPE) by use of a vacuum manifold (Porvair, Leatherhead, UK) and a C18 *BioVyon* 10 mg 96 well cartridge for desalting by washing with 0.1% trifluoroacetic acid (TFA) (similar to using C18 ZipTip). Samples were eluted in 50 µL 50% acetonitrile (ACN) / 0.1% TFA (v/v).

1 µL of eluate was spotted on a ground steel metal plate, mixed with 1 µL of α-cyano-4-hydroxycinnamic acid matrix (1% in 50% ACN / 0.1% TFA (v/v/v)) and allowed to air dry.

Each sample was analysed in reflector mode using a calibrated Ultraflex III (Bruker Daltonics, Bremen, DE) MALDI-TOF instrument to measure the *m/z* values (mass-to-charge values) of trypsinated fragments. Spectra were analyzed using flexAnalysis software v. 3.0 (Bruker Daltonics, Bremen, DE).

### *4.3.3. Calculation of Glutamine Deamidation*

A selection of deamidation markers was made, based on peptides that contain one or more Gln in known collagen sequences. Unlike in typical ZooMS analysis, preference was given to peptides that are commonly observed and are not necessarily species markers (see Table 4-1).

Deamidation causes a mass shift of +0.984 Da and therefore glutamine deamidation results in a shift of the isotope distribution. The extent of glutamine deamidation can be found by deconvolution of the two overlapping distributions (Wilson et al. 2012).The natural isotope distribution of a non-modified variant was calculated and the overlap of the deamidated and non-deamidated peptide multiplet is determined, expressed as %Gln. Using a composite value, percentages of original Gln from ten peptides were used to estimate the overall %Gln in each sample. A number of samples with varied thermal age were selected (Table 4-2) and the values of %Gln for each peptide were calculated as according to Wilson et al. (2012). To determine the extent of deamidation, a genetic algorithm was used to find the value of %Gln. After each generation, the fitness of each individual (representation of %Gln) is evaluated according to the sum of squares error between the observed peaks (Wilson et al. 2012).

The composite value for a whole sample was determined as follows: the relationship between %Gln of COL1A1-0510-1, one of the most stable and prevalent peptides, against the nine other peptides was determined (Figure 4-2; Appendix). All comparisons showed a logarithmic relationship between and provided $R^2$ values

ranging from 0.45 to 0.87. Predictive values for COL1A1-0510-1 were then averaged into a composite value for estimated overall %Gln of a sample, using weighted averages normalized against the total $R^2$:

**Equation 4-1.** $$\boldsymbol{Est\%Gln} = \frac{\sum y_n}{\boldsymbol{n}}$$

where: *Est%Gln* is our composite value; *n* is the number of peptides detected; *y* is the function as found from the peptide comparisons as shown in Figure 4-2 and the projected value of %Gln for COL1A1-0510-1 based on %Gln from other detected peptides; $R^2$ is the correlation coefficient found between COL1A1-0510-1 and peptide *n*.

### *4.3.4. Diagenetic parameters: Fourier Transform Infra Red Spectroscopy (FT-IR)*

A common and fairly simple method of assessing bone mineral integrity is by the crystallinity index, or splitting factor (SF), obtained by FTIR. FTIR can also be used to obtain several other preservation parameters, such as the carbonate to phosphate ratio ($CO_3/PO_4$), which also reflects changes in the crystal-structure since increased crystallinity leads to loss in carbonate. Furthermore, the ratio of the amide to phosphate peak ratio (Amide/$PO_4$) will reflect the relative amount of the organic phase of bone, the collagen, remaining in the bone (Trueman et al. 2008).

For the analysis, FTIR-ATR was used. This is an FTIR with an attenuated total reflection (ATR) unit, which operates by measuring the changes that occur in a totally internally reflected infrared beam when the beam comes into contact with a sample. The sample is placed directly, without any sample preparation, onto an optically dense crystal (Thompson et al. 2009). This method has been found to be advantageous in the analysis of bone preservation (Hollund et al. 2012).

Powders were drilled directly from a freshly cut cross-section using a hand-held drill with a tungsten-carbide drill-bit. A small amount of powder, enough to cover the prism window of the ATR stage (5x2.5 mm), was pressed to the diamond surface. Samples were scanned 32 times using FTIR-ATR (FTIR-8400S (Shimadzu, Tokyo, JP) with an ATR Golden Gate unit (Specac, Slough, UK)). Spectra were recorded from 4000 to 400 $cm^{-1}$ at 8 $cm^{-1}$ resolution and baseline corrected.

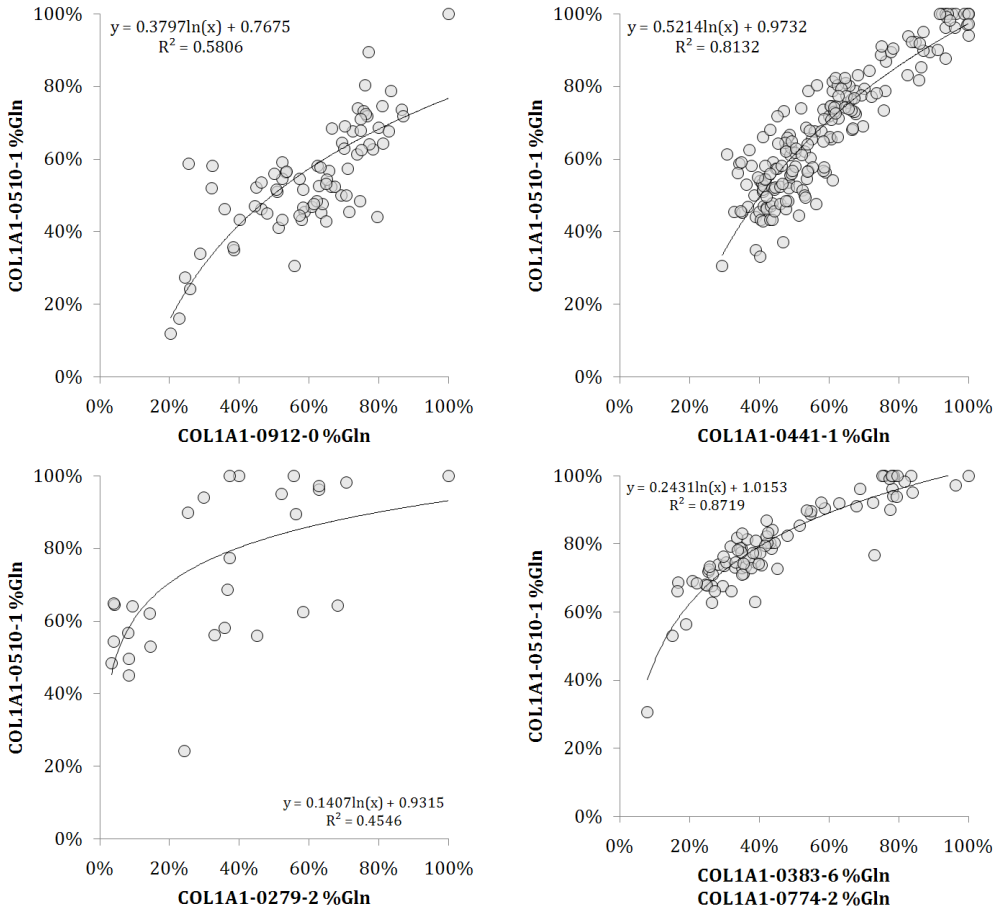



**Figure 4-2. %Gln for investigated peptides plotted against %Gln for COL1A1-0510-1. These relationships, established by an initial sample set, were used to predict equivalent %Gln for COL1A1-0510-1 based on the %Gln from other peptides. These values were then averaged to a composite value for an overall %Gln. A) COL1A1-0912-0, B) COL1A1-0441-1, C) COL1A1-0279-2, D) COL1A1-0383-6 & COL1A1-0774-2 (combined). The %Gln graphs for all peptides can be found in Appendix.**

Peak heights at wave-numbers 565 ($v_4\,PO_4$), 605 ($v_4\,PO_4$), the valley at ~590 $cm^{-1}$ between peaks 565 and 605 $cm^{-1}$ were measured to calculate the infra-red splitting factor (SF). The peak heights at wave-numbers 1035 ($v_3\,PO_4$), and 1415 $cm^{-1}$ ($v_3\,CO_3$) were measured for calculation of carbonate to phosphate ratio ($CO_3/PO_4$). The ratio of the intensities of the amide peak at 1640 $cm^{-1}$ and the phosphate peak at 1035 $cm^{-1}$ reflect collagen content (Trueman et al. 2008). The splitting factor (SF) evaluates the crystalline structure of bioapatite. For modern bone SF is reported to range between 2.5 and 2.9 (Wright & Schwarcz 1996). However, the SF value has been shown to be consistently higher when using FTIR-ATR compared to the method most commonly

used to analyse archaeological bone samples (Thompson et al. 2009). Measurements using FTIR-ATR on fresh cattle bone (8 replicates) gave an average of 3.17 (Hollund et al. 2012). An increase in SF indicates re-crystallisation and an increase in crystal size and order.

## 4.4. RESULTS

### *4.4.1. CALCULATION OF %GLN AND THERMAL EFFECTS*

A sample set was selected to represent varied thermal history, for which radiocarbon dates or established associated dates were available. Table 4-2 shows the %Gln for each of the peptides investigated. A value close to zero indicates that most Gln of the peptide are deamidated in solution. For some samples, no %Gln value could be calculated for certain peptides, especially in the case of samples from the La Draga site. This was either due to (i) the peptide being at a concentration below detection for MS, (ii) too low a signal to noise ratio or (iii) having a poor fit in the genetic algorithm used to evaluate the combined isotopic distribution (Wilson et al. 2012). Mass spectra for La Draga samples displayed very few high molecular weight peptides.

For each sample, thermal age was calculated for collagen gelatinization (activation energy $E_a$ = -173 kJ/mol) and is expressed in years equal to exposure at 10°C (Smith et al. 2003). Figure 4-3 shows composite %Gln values for all peptides combined plotted against thermal age, calculated as mentioned above. In this study we have compared %Gln to thermal age based on the activation energy for collagen gelatinization (we only extract a soluble fraction of collagen). A more useful comparison would be with the actual activation energy for glutamine deamidation in collagen, which has yet to be determined.

A trend is visible where thermally younger sample sets contain more peptides displaying a significant isotopic fit, as well as higher %Gln values, indicating that the level of deamidation is low. Particularly of note are the samples from Les Cottés and Kerma, regarding both their radiocarbon age and thermal age. As shown in Figure 4-3, deamidation in soluble collagen correlates more closely with the thermal age for collagen gelatinization than with chronological age. The samples from Kerma for example are approximately 5,000 years old, whereas samples from Les Cottés are approximately 38,000 years old. However, the Les Cottés samples are thermally much younger than the Kerma samples as they are dated to a period before the coldest part of the Last Glacial Maximum, and in part due to the difference in burial context as Les

Cottés is a karstic cave site. The pH and direct burial context are expected to lower thermal age considerably compared to burials from Sudan. Burial context is corrected for in thermal age estimations, however, there is no accounting available for pH at this moment. %Gln ratios show a relationship closer to thermal exposure rather than chronological age.

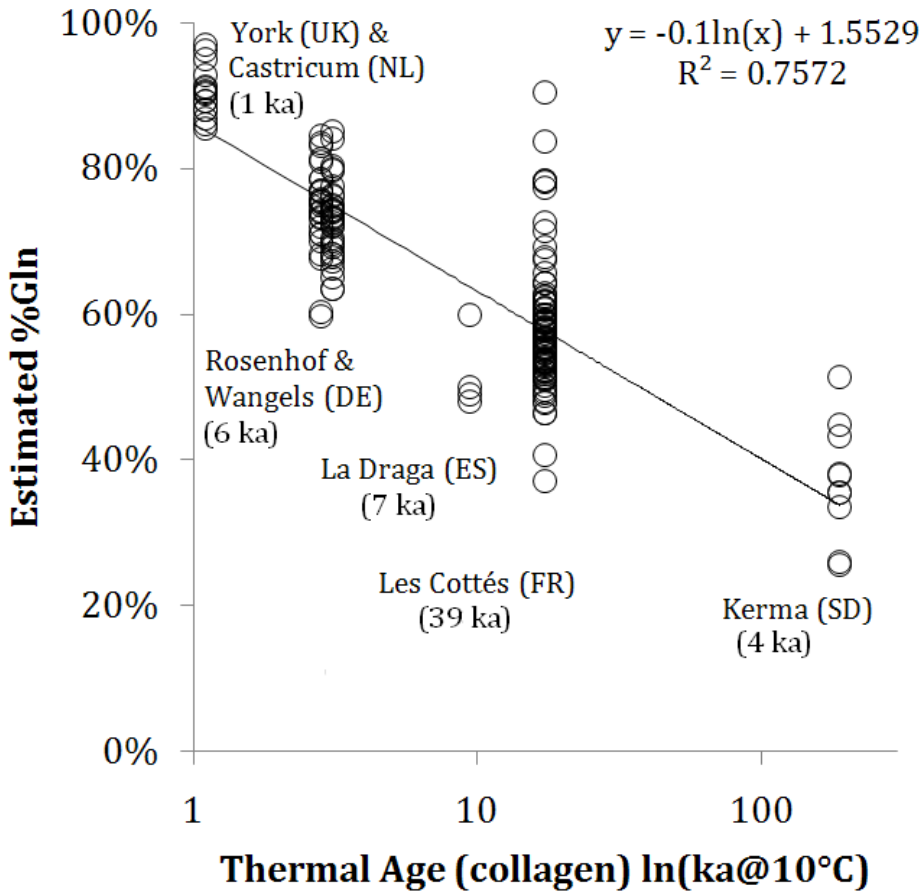



**Figure 4-3. Composite %Gln plotted against thermal age for collagen gelatinization (activation energy $E_a$ = -173 kJ/mol). Each marker represents a sample, averaged over three biological replicates. Approximate chronological age is denoted underneath the site names in thousands of years cal BP.**

### *4.4.2. Other Effects on Glutamine Deamidation*

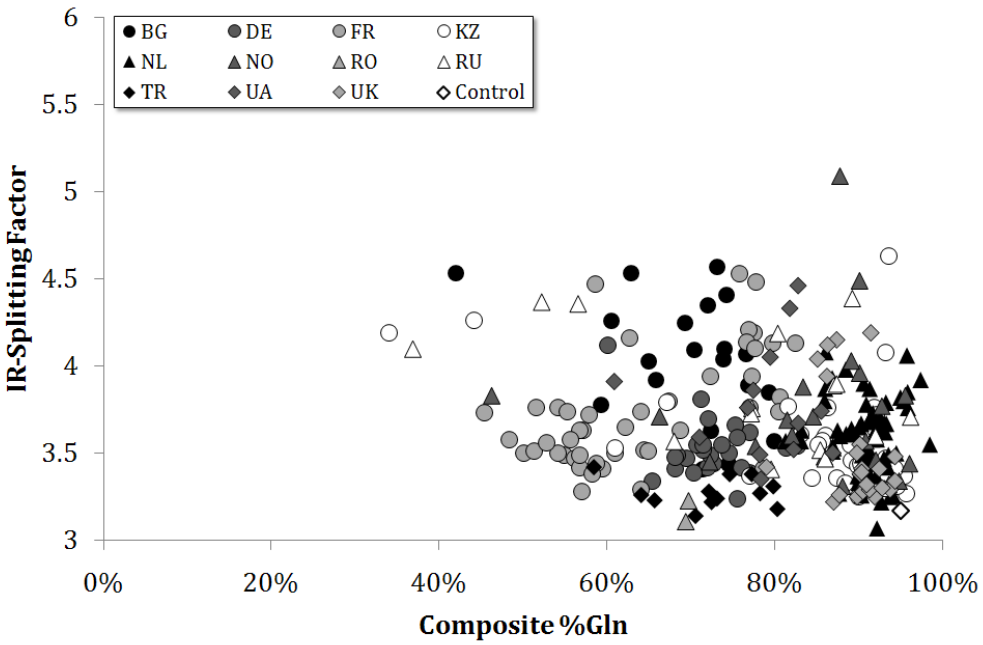
Although we see a relationship between %Gln and thermal age, we find little consistent data to indicate that motifs in the protein primary structure may have an influence. There is no consistency between deamidation rate and adjacent residues

(Table 4-1). For example, proline at the N-terminal position was found in both fast and slow deamidating peptides. There is no consistency between the calculated %Gln and the half-life of a glutamine with the same adjacent residues measured experimentally for pentapeptides. Whereas it has been argued that Gln at the Yyy position in the tripeptic motif of collagen (Gly-Xxx-Yyy) increases collagen stability (Silva et al. 2005), this motif was not found in any of the peptides with a high %Gln, indicating a slow deamidation rate. Increasingly hydroxylated variants of a peptide, however, tend to deamidate at a slower rate than the "original peptide".

High %Gln (low deamidation rates) seems to be more commonly associated with non-polar residues (A, I, P, V) in the peptides studied, whereas low %Gln (high rates) have polar residues adjacent (D, E, R) more often. However, it is possible that the effect of polarity on deamidation rates is related to higher structural levels rather than primary sequence.

The composite %Gln (Table 4-2) are compared to several diagenetic parameters for bone in Figure 4-3. Interestingly, most diagenetic parameters we've investigated do not seem to correlate with deamidation.

Specifically, crystallinity, $CO_3/PO_4$and amide/$PO_4$ ratios—common indicators of bone quality—and bioerosion (results not shown) do not establish a linear or otherwise progressive relationship with %Gln. Specifically, regarding the comparison with amide/$PO_4$ ratio, which reflects collagen content, a strong divide is visible, showing degradation of samples consistent with the theory that collagen (and by extension: bone) is either at the extremes of very well or very badly preserved (Dobberstein et al. 2009). Bone diagenesis does not appear to be a strong influence on glutamine deamidation.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NO
RO
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UK
Control
IR-Splitting Factor
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5
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0%
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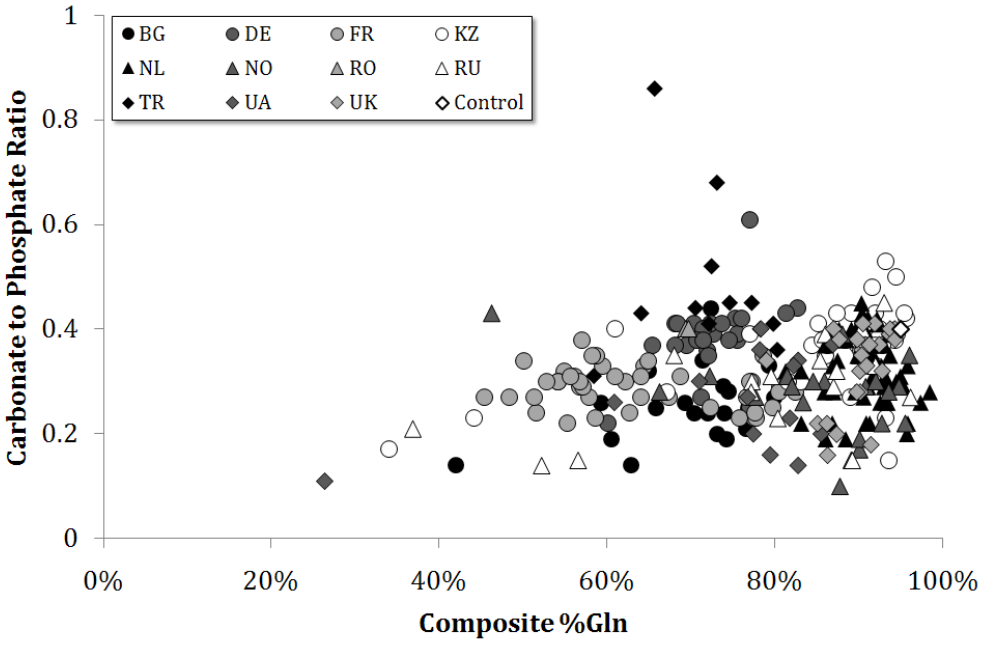
Composite %Gln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Carbonate to Phosphate Ratio
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20%
40%
60%
80%
100%
Composite %Gln

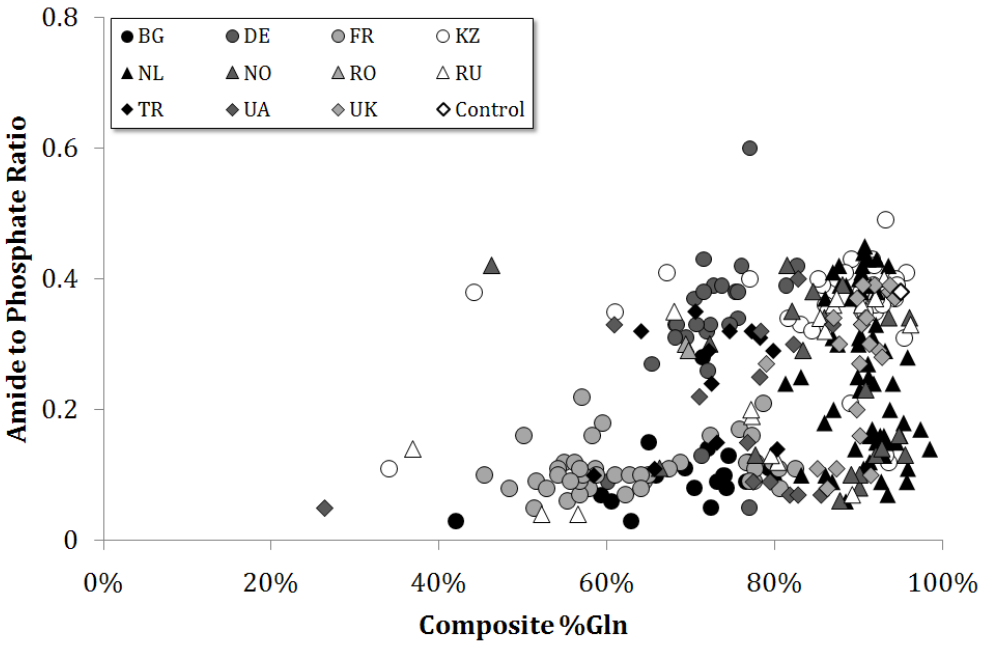


**Figure 4-4. Effects of diagenetic parameters on Gln deamidation in the soluble fraction. Above) Splitting Factor, Middle) $CO_3$ to $PO_4$ ratio, Below) Amide to $PO_4$ ratio. All charts show the lack of a significant relationship between deamidation and diagenetic parameters. Samples are grouped per geographical location (see also the map in Figure 4-1). BG = Bulgaria, DE = Germany, FR = France, KZ = Kazakhstan, NL = The Netherlands, NO = Norway, RO = Romania, RU = Russia, TR = Turkey, UA = Ukraine, UK = United Kingdom. The control is a well-preserved archaeological sample from a separate sample set (York, UK) with values similar to those found in modern bone.**

## 4.5. Discussion

### *4.5.1. Survival of Collagen*

Our method to detect deamidation involves the analysis of a collagen fraction extracted under denaturation conditions (2 x 1hr @ 65°C 50mM $NH_4HCO_3$ pH 8) comparable to the effect of exposure to room temperature (Li et al. 2003). Loss of soluble collagen from an active hydrological regime could explain why samples of intermediate thermal age from La Draga did not yield enough soluble collagen to calculate a %Gln for most of the peptides studied. Samples from Kerma (Sudan) of similar chronological age, but with a higher thermal age, yielded data for most target peptides (though with low %Gln), which can be attributed to the arid environment which generates, but does not remove, the soluble fraction. It can also be expected that in this stationary soluble fraction, glutamine deamidation can occur more freely

through the imide intermediate, as the helical structure would no longer be suppressing this pathway.

Demineralization with HCl to obtain total collagen for samples from La Draga, followed by gelatinization did not yield better data or higher %Gln. This could be because the primary mechanism of Gln deamidation in collagen is direct side chain hydrolysis, which is much less dependent upon higher order structure than Asn deamidation (Collins et al. 1999).

We do not yet know how the rate of glutamine deamidation varies between intact mineralized collagen and gelatin, but would expect the rate to be slower in the former. If this is the case, then differences in %Gln between a sample from an arid environment (Kerma) and one with an active hydrology (Rosenhof) would be expected. The former environment would (i) generate soluble gelatin more slowly but (ii) retain it for longer within the bone matrix than one with a significant flux of water. The range of variation will be further increased by the effective pH of the soil, with rates of direct hydrolysis increasing away from neutral pH. Alkalinity is likely to have a greater effect than acidity, as dissolution of bone apatite at low pH acts to buffer the collagen, until the point at which the protein is freed from its mineral cage and gelatinization accelerates. At alkaline pH, bone is an ineffective buffer and deamidation is catalysed by $OH^-$ ions. These differing factors may explain why samples with moderate levels of %Gln, from Northern Europe, do not correlate well with absolute age, infrared splitting factor or other diagenetic factors. For example, the samples from Les Cottés come from a carbonate rich environment, which may have had a buffering effect on collagen and therefore have a reasonable yield of peptides and %Gln considering their (thermal) age.

An additional explanation is the fact that diagenetic alterations caused by bioerosion will lead to localized changes in crystallinity, carbonate and collagen content whereas pockets of well preserved bone may remain. Furthermore, these diagenetic parameters reflect the global average while deamidation is measured on very small samples which may not represent the true overall state of the bone, which may account for the variation between biological replicates.

There has been recent discussion on what makes good collagen quality indicators and how useful they are. Whether an increasing splitting factor and overall quality of bone are related has been contested (Weiner & Bar-Yosef 1990; Nielsen-Marsh et al. 2000) although a general correlation has been noted (Nielsen-Marsh & Hedges 2000). Our

results suggest that changes in the mineral fraction of bone may not influence deamidation if the remaining collagen helical structure is intact.

In addition, as is the apparent case with samples from an active hydrological site, good collagen quality does not guarantee that we can obtain %Gln data from the soluble fraction. Our results show extremely well preserved (York) or extremely degraded (Kerma) samples will be observed as such—thermally intermediate samples show a wide range of %Gln (mainly those from Northern Europe—see the individual %Gln ranges in Table 4-2), indicating that burial environment will bear influence rather than chronological age. Repeated removal of the built up soluble fraction through leeching may give younger values because fresh gelatin is continuously generated. In such samples side-chain hydrolysis will be the dominant mechanism for deamidation, arguably displaying a younger glutamine or collagen age.

We do not yet know to what extent %Gln is useful to discriminate (i) older or (ii) more biochemically degraded material, but samples from a multi-levelled site where burial conditions are comparable, are likely where this method currently will be most successful to determine relative thermal age.

### *4.5.2. LIMITATIONS TO THE APPROACH*

A synthetic oligopeptide containing asparagines was shown to deamidate 70-80% during an overnight (~12 hour) trypsin digest (Krokhin et al. 2006), a standard step in our non-destructive protocol for MALDI-TOF-MS. In addition, our extraction steps are under similar conditions at 65°C, likely to allow accelerated deamidation. However, due to its lower susceptibility to non-enzymatic deamidation, we assume glutamine is not detectably affected by standard overnight trypsin digestions (Araki & Moini 2011; Leo et al. 2011). Initial kinetic experiments (results not shown) suggest that our extraction (2 hours @ 65°C) and trypsin digestion (≤ 24 hours @ 37°C) do not influence glutamine deamidation in collagen significantly, although we have yet to quantify to which extent it does at higher temperatures or longer incubations.

Further developments will include the determination of glutamine deamidation activation energy and modelling of the collagen molecule with these specific glutamines mapped in a 3D configuration. The importance of secondary and tertiary structure can therefore be more accurately shown, as we have shown that primary structure alone is not the main determinant of structural factors.

## 4.6. CONCLUSION

Deamidation has been used as an indicator for protein age and damage in a number of cases, yet not extensively for archaeological bone. Glutamine deamidation has because of its slower turnover rate been much less the focus of investigation, but covers the time span of the archaeological record and the detection of hydroxylated variants and their individual deamidation rates can add to our understanding of collagen degradation as a whole.

This method will not portray an optimised thermal history of each sample at present, but it can achieve repeatable results through a very inexpensive and fast protocol, simultaneously used for species identification. We acquire indications of relative damage, outliers within a group, and the primary indications and justification for future research to determine how structural factors add to the understanding of deamidation and the process of degradation as a whole within the collagen molecule.

Due to our high-throughput set-up we are also able to run multiple samples in a short amount of time to compare trends. The number of unique samples discussed in this paper is approximately one thousand (including biological replicates) for which the cumulative preparation and running time adds up to approximately 200 hours or about 20 days.

## 4.7. ACKNOWLEDGEMENTS

### *4.7.1. SAMPLE DONATIONS*

Rosenhof, Wangels, Neustadt and Seedorf: Ulrich Schmölke and Dr. Sönke Hartz (Stiftung Schleswig-Holsteinische Landesmuseen, Germany)

Castricum: Rob van Eerden, Archaeological service of the Province of Noord-Holland, The Netherlands.

Eindhoven: Nico Arts, the archaeological service of Eindhoven

Champs Durand: R. Joussaume, S. Corson and S. Braguier (L'Historial de la Vendée. Les Lucs-sur-Boulogne)

Kerma and La Draga: Roz Gillis (National Museum of Natural History (MNHN), France), Louis Chaix (Department of Genetics & Evolution, University of Geneva, Switzerland) and Jacqueline Studer (Museum of National History Geneva, Switzerland)

Les Cottés: Marie Soressi, Sahra Talamo (Max Planck Institute for Evolutionary Anthropology, Germany)

Kazachstan, Bulgaria, Ukraine, Russia: Sandra Wilde and Martina Unterländer (Johannes Gutenberg-Universität, Germany), Dr. Nadezhda Atanassova-Timeva, Dr. Stefan Alexandrov (Bulgaria samples), Alexander Khokhlov (Nikolaevka samples), Elke Kaiser (Frije Universitat, Berlin, DE)

Turkey: Christina Geörg (Johannes Gutenberg-Universität, Germany)

York: Terry O'Connor (University of York, United Kingdom)

### *4.7.2. FUNDING*

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**Table 4-1. Overview of the peptides used for thermal age calculation. Markers give the position of the first Gln appearing in the collagen sequence. Last numbers indicate for the number of hydroxylations. # Nomenclature of peptides: Collagen strand – position of active Gln from the beginning of the helix – # of hydroxylations; * Underlined proline indicates hydroxylation; § As the resolution of MALDI-TOF-MS is not sufficient to distinguish between the masses of these peptides, they may be observed as a mixture. ‡ As based on comparison of %Gln against thermal age of collagen gelatinization. †At pH 7.4, 37.0°C, 0.15 M Tris HCl in pentapeptide of the form GlyXxxGlnYyyGly (Robinson & Robinson 2004). • Peptides that contain two Gln commonly are observed as if only one Gln progressively deamidates (Wilson et al. 2012).**

| Marker # | Sequence* | *m/z* | Deamidation rate‡ | 1st order half-time (days)† |
|---|---|---|---|---|
| **COL1A1–0912–0** | GPAGP**Q**GPR | 835.4 | Slow | 630 |
| **COL1A1–0510–1** | GV**Q**GP<u>P</u>GPAGPR | 1104.6 | Slow | 640 |
| **COL1A1–0441–0** | DGEAGA**Q**GPPGPAGPAGER | 1689.8 | Medium | 610 |
| **COL1A1–0441–1** | DGEAGA**Q**GP<u>P</u>GPAGPAGER | 1705.8 | Slow | 610 |
| **COL1A1–0279–2** | GE<u>P</u>GPTGI**Q**GP<u>P</u>GPAGEEGK | 1862.9 | Fast | 620 |
| **COL1A1–0383–4§** | TGP<u>P</u>GPAG**Q**DGR<u>P</u>GP<u>P</u>GP<u>P</u>GAR | 2056.0 | Fast | 7600 |
| **COL1A1–0774–0§•** | GAPGADGPAGAPGTPGP**Q**GIAG**Q**R | 2056.0 | Fast | 630,7200 |
| **COL1A1–0383–5§** | TG<u>PP</u>GPAG**Q**DGR<u>P</u>GP<u>P</u>GP<u>P</u>GAR | 2072.0 | Fast | 7600 |
| **COL1A1–0774–1§•** | GAPGADGPAGA<u>P</u>GTPGP**Q**GIAG**Q**R | 2072.0 | Fast | 630,7200 |
| **COL1A1–0383–6§** | TG<u>PP</u>GPAG**Q**DGR<u>P</u>G<u>PP</u>GP<u>P</u>GAR | 2088.0 | Medium | 7600 |
| **COL1A1–0774–2§•** | GA<u>P</u>GADGPAGA<u>P</u>GTPGP**Q**GIAG**Q**R | 2088.0 | Medium | 630,7200 |
| **COL1A1–0383–7§** | TG<u>PP</u>GPAG**Q**DGR<u>P</u>G<u>PP</u>G<u>PP</u>GAR | 2104.0 | Medium | 7600 |
| **COL1A1–0774–3§•** | GA<u>P</u>GADGPAGA<u>P</u>GT<u>P</u>GP**Q**GIAG**Q**R | 2104.0 | Medium | 630,7200 |
| **COL1A1–0939–3•** | GFSGL**Q**GP<u>P</u>GP<u>P</u>GS<u>P</u>GE**Q**GPSGASGPAGPR | 2704.2 | Medium | 670,750 |

**Table 4-2. Overview of the sample sets chosen for the generation of a thermal age trend line. Samples were chosen for this baseline with a wide variance of thermal age (calculated for collagen gelatinization) and with knowledge of radiocarbon data for the actual age. The columns after the site show chronological age and predicted thermal age (expressed in damage equal to years of exposure at 10°C) respectively. %Gln (± standard deviation) is depicted both as a composite value and for each individual peptide. An asterisk (*) is denoted when no %Gln could be calculated. * As the resolution of MALDI-TOF-MS is not sufficient to distinguish between the *m/z* values of COL1A1-0383 and COL1A1-0774 (including their increasingly hydroxylated variants), they may be observed as a mixture.**

| Site | Age (ka Cal BP) | Thermal age (ka@10°C) | Est %Gln (composite) | COL1A1-0912-0 | COL1A1-0510-1 | COL1A1-0441-0 | COL1A1-0441-1 | COL1A1-0279-2 | COL1A1-0383-4* | COL1A1-0383-5* | COL1A1-0383-6* | COL1A1-0383-7* | COL1A1-0939-3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **York (N=9) (Ottoni et al. 2009)** | 1.050 | 0.6 | 95.00 (0.92) | 99.0 (2.5) | 99.9 (0.3) | 92.0 (6.0) | 95.9 (3.0) | 44.3 (10.0) | 64.0 (22.1) | 78.0 (3.2) | 78.0 (2.5) | 84.7 (5.0) | 83.4 (4.0) |
| **Castricum (N=18) (Hollund et al. 2011)** | 1.780 | 1.1 | 91.42 (3.63) | 60.5 (44.4) | 83.6 (30.6) | 65.2 (36.7) | 79.5 (29.8) | 16.9 (26.6) | 5.7 (14.4) | 24.6 (32.4) | 56.6 (32.8) | 63.6 (36.1) | 67.2 (31.3) |
| **Rosenhof (N=34) (Scheu et al. 2008)** | 6.600 | 3.1 | 72.94 (5.35) | 62.8 (32.8) | 70.4 (18.8) | 52.0 (23.1) | 55.3 (17.1) | * | 5.6 (9.5) | 29.6 (15.1) | 31.9 (11.7) | 25.3 (24.3) | 34.2 (24.2) |
| **Wangels (N=39) (Schmölcke 2001)** | 6.000 | 2.8 | 74.53 (6.27) | 32.1 (39.8) | 51.7 (35.6) | 37.3 (32.1) | 47.5 (32.8) | 1.4 (0) | 4.1 (8.9) | 23.2 (18.3) | 22.8 (19.9) | 16.9 (25.1) | 25.8 (26.1) |
| **La Draga (N=12) (Tarrús 2008)** | 7.200 | 9.4 | 51.75 (5.56) | * | 52.0 (5.0) | * | * | * | * | * | * | * | * |
| **Les Cottés (N=36) (Talamo et al. 2012)** | 38.700 | 17.3 | 57.54 (8.72) | 36.1 (33.1) | 55.1 (11.6) | 43.6 (22.1) | 46.6 (11.8) | 4.0 (12.3) | * | 0.9 (5.3) | 2.2 (9.2) | 3.3 (12.0) | 5.5 (17.9) |
| **Kerma (N=11) (Gillis et al. 2011)** | 4.000 | 188.4 | 37.26 (7.56) | 32.0 (16.1) | 24.2 (15.4) | * | 2.7 (0) | 3.0 (7.5) | 0.4 (0) | 7.3 (12.1) | 0.7 (0) | * | * |

# Chapter 5. EFFECTS OF BURIAL ENVIRONMENTS ON COLLAGEN EXTRACTION AND ANALYSIS: TWO CASE STUDIES

## 5.1. ABSTRACT

Zooarchaeology by Mass Spectrometry (ZooMS) is a tool available to archaeologists to examine bone material of minimal sample size to determine animal genus, thermal history and generally the extent of collagen preservation. The cost efficiency and speed of analysis make this an excellent screening tool and as the analyses can now be run without decalcification, it is especially appropriate for precious worked bone artefacts. However, we have encountered several complications during the analysis of bone samples with the non-destructive approach. Spectra could be variable, empty, weak, or be missing high molecular weight peptides (>2 kDa). Attempts to enhance yield by demineralization and longer gelatinization often improve matters, but not always. Several parameters were noted (waterflux in the burial environment, possible cross-linking) in which non-destructive ZooMS is less likely to return results, even though collagen is notably present in these problematic samples. We argue that different influences, especially linked to burial environment, may be the cause and that these options should be considered when using buffer extraction ZooMS for a selection of samples.

## 5.2. INTRODUCTION

Non-destructive ZooMS (ZooArchaeology by Mass Spectrometry) is a method available as a highly cost-effective method for bone sample screening (van Doorn et al. 2011). For this particular variation of ZooMS, a partial fraction of soluble collagen is extracted under mild conditions (65°C for 1 hour at pH 8.0). This leaves the bone sample, or at least the mineral matrix, intact and does not induce measurable weight or size changes, making it ideal for use on worked bone artefacts (see also Chapter 3).

However, with a milder application of ZooMS without removal of hydroxyapatite, a decline is observed in the overall success rate of samples run. It would be naturally expected that demineralisation would increase the success rate of ZooMS for positive identification. However, unsuccessful non-destructive extractions were found to be

not necessarily failure of analyses due to strictly a lower peptide concentration due to natural degradation, rather than possibly dependent on the nature of the (bone) samples investigated.

Bone has long been known as being vulnerable to exogenous contamination and inclusions: organic (humics) and inorganic (metals), soluble and insoluble, or physical intrusion (roots, bioerosion, Wedl tunneling) (Hedges & van Klinken 1992). Humics, for example, have been found to interact with bone and collagen specifically in a very fast reaction, reaching an average maximum uptake of humic substances (~25%) within a matter of hours (van Klinken & Hedges 1995). Humic substances are large (>100–1000 Da), dark-coloured, often aromatic molecules that target the amino acid side chains (lysine, hydroxylysine and to some extent arginine) and form Schiff's bases through the so-called Maillard reaction (Maillard 1912), mostly connected with visible browning in food (Thorpe & Baynes 2003). After the reversible formation of a Schiff's base, the compounds can rearrange into larger, polyphenolic substances named melanoidins (van Klinken & Hedges 1995). The interactions between these large melanoidins and collagen is quite similar to vegetable tanning in leather in which polyphenols form a crosslinking network between collagen fibrils and increase their resistance to temperature (Covington 2009). The presence of such humic substances has been linked to a delay in degradation by microbiota (Nicholson 1998; Covington 2009).

The non-destructive ZooMS protocol is meant to enable a screening method with possibilities to revisit and follow-up the same samples. While the non-destructive approach is beneficial on many aspects, a lower success rate of the non-destructive ZooMS protocol does pose problems. Low yields limit the ability to perform MS/MS analysis. A failure to generate a strong signal for high molecular weight peptides can restrict identification, for example for sheep-goat discrimination an important marker peptide is found above 3000 Da (Buckley et al. 2010). Furthermore, it would be expected that acid demineralisation, by removing all or part of the mineral component, would increase the success rate by increasing yields of gelatin, but strangely this is not always the case.

We will discuss several pitfalls for using the non-destructive approach that we have so far suspected or discovered. We have tried to identify these limitations in order to advise correctly and in advance anyone choosing to apply ZooMS to their samples.

## 5.3. MATERIALS AND METHODS

### *5.3.1. SAMPLE MATERIAL*

#### 5.3.1.1. Wangels, Germany

Cattle and ovacaprine samples from a Late Mesolithic-Early Neolithic marine site from North-East Germany were selected. The cattle samples have been previously studied for DNA analysis (Scheu et al. 2008) and more recently for various diagenetic proxies (Hollund et al. in prep) and glutamine deamidation (Chapter 5; van Doorn et al. 2012).

#### 5.3.1.2. La Draga, Catalonia

Ovacaprine samples from a Cardial Neolithic site (5300-5150 years cal BP) in Northern Spain (Catelonia) from marshland, a waterlogged burial context (Tarrús 2008) were selected. In Neolithic times, the village site was raised, but will probably have been exposed to regular floodings from Lake Banyoles.

#### 5.3.1.3. Kerma, Sudan

Ovacaprine samples from a Late Neolithic site dating from the end of the fourth millenium BC in a desert area (Chaix & Grant 1987). The samples were obtained from a necropolis 4 km East from Kerma (Gillis et al. 2011). The Kerma region opens onto the largest alluvial plain of northern Sudan and burial conditions were very hot and dry.

#### 5.3.1.4. Fiskerton, United Kingdom

Bone samples were obtained from an experimental set-up to determine the influence of re-watering on archaeological materials (Williams et al. 2006). Cooked and uncooked modern horse bone and archaeological cow bone was buried at varying levels up to 2 meters below ground. Of these levels the lowest was continually submerged in water, whereas the top level was above the water table. The two middle layers were exposed to a fluctuating water table (Figure 5-1) Bone samples remained buried up to thirty-six months. Unburied controls were included (modern and archaeological horse and archaeological cow).

Samples from Fiskerton were run in two separate stages: set A consisting of six samples that had been buried for 36 months (including two unburied controls, one modern and one archaeological); and set B consisting of two times four samples (two samples from each layer, one that had been buried for 36 months and one for six months) and in addition three unburied controls (one modern and two archaeological).

**Table 5-1. Overview of samples investigated using ZooMS.**

| Wangels | Bone element | Given ID | ZooMS ID |
|---|---|---|---|
| Wan-01 | Metatarsus | Bovine | Bovine |
| Wan-02 | 1st phalanx | Bovine | Bovine |
| Wan-04 | Metatarsus | Bovine | Bovine |
| Wan-05 | Metatarsus | Bovine | Bovine |
| Wan-07 | 2nd phalanx | Bovine | Bovine |
| Wan-09 | 2nd phalanx | Bovine | Bovine |
| Wan-10 | Talus | Bovine | Bovine |
| Wan-N | Tibia | Bovine | Bovine |
| Wan-11 | Humerus (R) | Sheep | Ruminant |
| Wan-12 | Radius (L) | Sheep | Ruminant |
| Wan-13 | Ulna (R) | Goat | Ruminant |
| Wan-14 | Horn (L) | Goat | No ID |
| Wan-15 | Radius (L) | Unknown ovacaprine | No ID |
| Wan-16 | Calcaneus (L) | Unknown ovacaprine | Ruminant |
| Wan-17 | Maxilla | Unknown ovacaprine | Ruminant |
| Wan-18 | Maxilla | Unknown ovacaprine | Ruminant |
| Wan-19 | Mandible (L) | Unknown ovacaprine | Ruminant |
| **La Draga** | **Bone element** | **Given ID** | **ZooMS ID** |
| LaDr-01 | Mandible | Sheep | No ID |
| LaDr-02 | Mandible | Sheep | No ID |
| LaDr-03 | Mandible | Sheep | No ID |
| LaDr-04 | Mandible | Sheep | No ID |
| LaDr-05 | Mandible | Sheep | No ID |
| LaDr-06 | Mandible | Sheep | No ID |
| LaDr-07 | Mandible | Unknown | No ID |
| LaDr-08 | Mandible | Goat | Ruminant |
| LaDr-09 | Mandible | Goat | No ID |
| LaDr-10 | Mandible | Goat | Ruminant |
| LaDr-11 | Mandible | Goat | No ID |
| LaDr-12 | Mandible | Goat | Goat |
| LaDr-13 | Mandible | Goat | Goat |
| LaDr-14 | Mandible | Goat | Goat |
| LaDr-15 | Mandible | Goat | No ID |
| LaDr-16 | Mandible | Unknown | No ID |
| LaDr-17* | Mandible | Goat | Goat |
| LaDr-18* | Mandible | Goat | Ruminant |
| LaDr-19* | Mandible | Goat | Ruminant |
| **Kerma** | **Bone element** | **Given ID** | **ZooMS ID** |

| | | | |
|---|---|---|---|
| Ker-01 | Mandible | Goat | Goat |
| Ker-02 | Mandible | Goat | No ID |
| Ker-03 | Mandible | Ovacaprine | Sheep |
| Ker-04 | Mandible | Sheep | Sheep |
| Ker-05 | Mandible | Sheep | Sheep |
| Ker-06 | Mandible | Sheep | Sheep |
| **Fiskerton** | **Bone element** | **Given ID** | **ZooMS ID** |
| Top layer | Radius | Horse | Horse |
| Upper-Middle layer | Radius | Horse | Horse |
| Lower-Middle layer | Radius | Horse | Horse |
| Bottom layer | Radius | Horse | Horse |
| Arch. Control 1 | Radius | Horse | Horse |
| Arch. Control 3 | Tibia | Bovine | Bovine |
| Modern Control | Radius | Horse | Horse |

* The other mandible of the same individual was sampled, corresponding with LaDr-08, LaDr-10 and LaDr-12 respectively.

### *5.3.2. PREPARATION OF SAMPLES*

#### 5.3.2.1. Acid Extraction ZooMS

Samples were incubated for >24 hours at 4°C in 0.6 M HCl. After demineralization, samples were rinsed with ultrapurified water to neutral pH. Samples were then incubated in 50 mM ammonium bicarbonate buffer (pH 8.0) for 3 hours at 65°C. The supernatant was collected and trypsinated overnight (≤18 h) at 37°C 1 µl of 1.0 µg/µl sequencing grade modified porcine trypsin (Promega, Southampton, UK).

#### 5.3.2.2. Non-destructive ZooMS

Samples of approximately 30 mg (5 mm) were collected in triplicate and transported in 96 well microplates. Upon receipt, samples were incubated for 1 hour at 65°C in 50 mM ammonium bicarbonate buffer (pH 8.0). The supernatant was discarded and the incubation repeated in 50 mM ammonium bicarbonate. Supernatant was collected and digested overnight (≤18 h) at 37°C with 1 µl of 0.4 µg/µl sequencing grade modified porcine trypsin (Promega, Southampton, UK).

Trypsinated extract was used for Solid Phase Extraction (SPE) by use of a vacuum manifold (Porvair) and a C18 *BioVyon* 10mg 96 well cartridge for desalting by washing with 0.1% trifluoroacetic acid (TFA) (similar to using C18 ZipTip). Samples were eluted in 50 µl 50% acetonitrile (ACN) / 0.1% TFA (v/v).

1 µl of eluate was spotted on a ground steel metal plate, mixed with 1 µl of α-cyano-4-hydroxycinnamic acid matrix (1% in 50% ACN / 0.1% TFA (v/v/v)) and allowed to air dry.

Each sample was analyzed in reflector mode using a calibrated Ultraflex III (Bruker Daltonics, Bremen, DE) MALDI-TOF instrument to measure mass-to-charge ratios (m/z) of trypsinated fragments. Spectra were analyzed using flexAnalysis software v. 3.0 (Bruker Daltonics, Bremen, DE).

#### 5.3.2.3. Calculation of Glutamine Deamidation

Ten peptides containing at least one glutamine residue were selected based on their consistent presence in MALDI mass spectra and conservation across animal collagen sequences. The difference between a glutamine and a glutamic acid residue is +0.984 Da, causing an overlap in the MALDI spectrum when both deamidated and non-deamidated versions of a peptide are present in the sample. The expected appearance based on normal isotopic distribution is used to calculate the relative amount of each peptide variant, which indicates the amount of glutamine deamidation. Glutamine deamidation correlates with thermal age (expressed in years exposed to 10°C) but weakly with bioerosion or bone quality. (See also Chapter 5; van Doorn et al. 2012; Wilson et al. 2012).

#### 5.3.2.4. Differential Scanning Calorimetry

Samples that were run wet were incubated in PBS, pH 7.4 until equilibrium was reached. The samples that were run dry were conditioned at 23°C at 65% RH (relative humidity) for 48 hours prior to analysis. Samples, (approximately 5–10 mg in weight), were then placed in an aluminium pan and sealed hermetically. Thermal analysis was undertaken in the temperature range -50 to 150°C at 5°C/minute under a nitrogen atmosphere using a DSC822e (Mettler-Toledo Inc, Columbus, OH, USA).

#### 5.3.2.5. Carbon/Nitrogen Analysis

Collagen was extracted from bone by an adapted protocol based on the original Longin (1971) extraction. Small bone fragments (0.1–0.5 g) were decalcified with 0.6 M HCl for 2 days. The acid was discarded and the residue gelatinized at 65°C for 1 day in 1 mM HCl, then ultra-filtered (30 kDa MW cut-off; Millipore, Durham, UK). Both residue and filtrate were lyophilized. Approximately 1 mg of collagen was folded into tin capsules and analyzed using an Elemental Combustion System CHNS-O analyser (Costech International Instruments, Cernusco sul Naviglio, IT).

Whole bone powder was analyzed using a CHN elemental analyzer (Flash EA1112 elemental analyzer from Thermo Scientific, Rodana, IT) for the weight percentages of nitrogen and carbon (%Nwb, %Cwb) and the carbon to nitrogen ratio (C:Nwb).

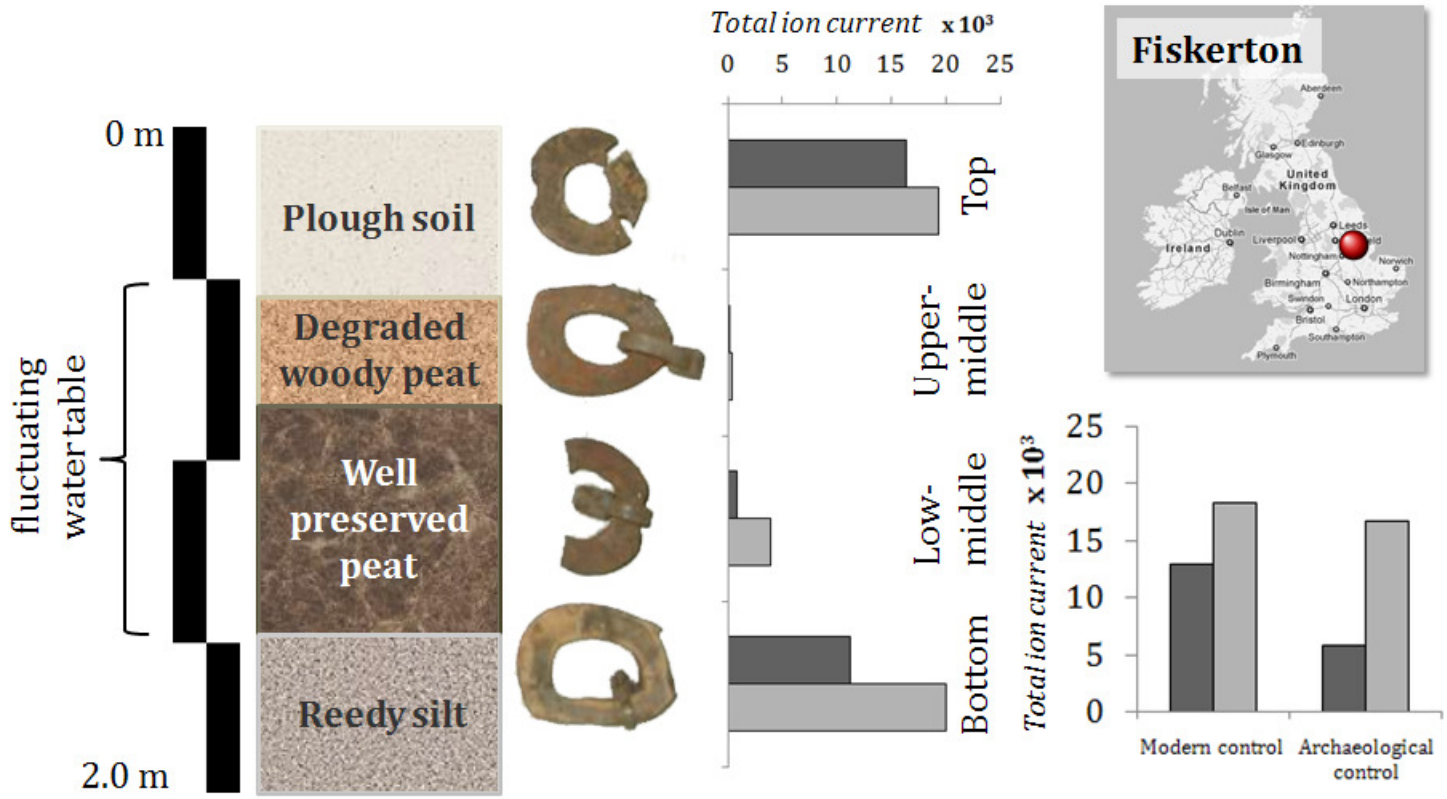


**Figure 5-1. Overview of results on the Fiskerton samples. The experimental set-up and results for set A. Depicted is the cumulative intensity for all m/z measured in a one hour extraction (dark grey) and a second one hour extraction (light grey) as compared to unburied controls.**

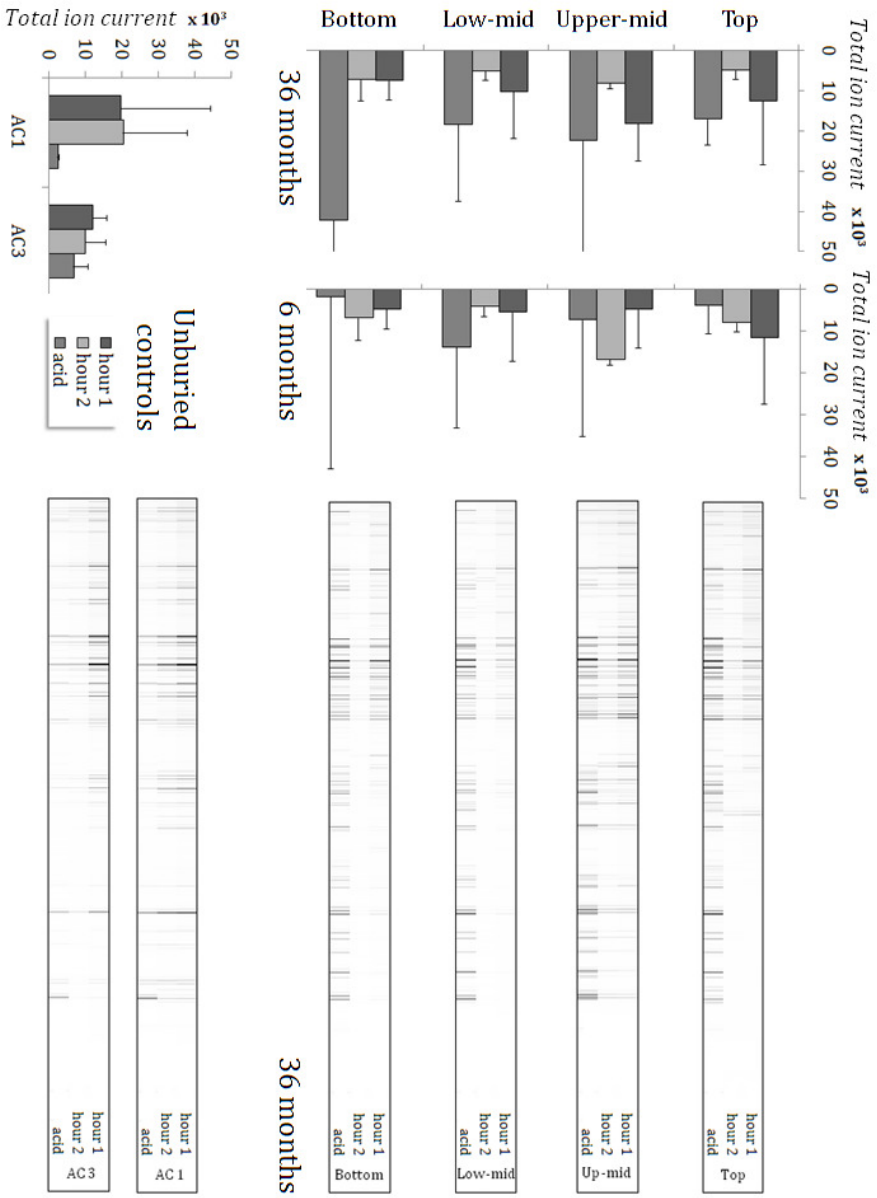


**Figure 5-2. Overview of results on the Fiskerton samples for set B. The same bone sets from the experimental burial as in set A were used, buried for 6 months and 36 months. Depicted is the cumulative intensity for all m/z measured in a one hour extraction (dark grey), a second one hour extraction (light grey) and a demineralised extract (middle grey) as compared to unburied controls. Also depicted are gel-like representations of spectra (technical replicates were combined). T bars indicate standard deviations. AC1 and AC3 are archaeological controls (animal bone).**

## 5.4. RESULTS

### *5.4.1. WANGELS: LACK OF MS SIGNAL IN COLLAGEN-CONTAINING BONE*

From Wangels, an Early Neolithic site in Northern Germany, we collected two sets of samples, originally for different research purposes. Cattle samples were collected to investigate diagenetic parameters (see also Chapter 4), whereas ovacaprine samples were collected originally for species identification studies to discriminate between sheep and goat (Table 5-1). The cattle samples were the only ones to have been sampled before, in this case for ancient DNA analysis and radiocarbon dating. Collagen analysis of **whole bone** powder from cattle samples (Table 5-2) appear as slightly below the strict norm of "well-preserved", but would not be considered "diagenetically altered" (Ambrose 1990). However, the low values for C:Nwb can also reflect contamination or decay. A high C:Nwb ratio (>5) can indicate exogenous carbon or extensive loss of nitrogen containing groups in collagen, whereas lower values can be due to loss of carbon from the mineral fraction (Tisnérat-Laborde et al. 2003).

Elemental (%C and %N) analysis of **extracted collagen** revealed yields slightly lower than would be acceptable for common radiocarbon or stable isotope analysis in the case of sheep bone (Table 5-3). The data suggests that collagen was still present in sufficient amounts (with the exception of sample Wan-14, a horn sample) for ZooMS analysis; %N was not unlike values reported for modern bone (Ambrose 1990).

**Table 5-2. Carbon and nitrogen concentration and atomic C:N for Wangels cattle samples for *whole bone* (wb). OHI = Oxford Histological Index, where a value of 5 indicates preservation on par with fresh bone and 0 indicates microscopic structure of bone is obliterated.**

| Sample | Excavation year | OHI | %C wb | %N wb | C/N wb |
|---|---|---|---|---|---|
| **Wan-01** | 1998 | 4 | 11.78 | 3.76 | 3.66 |
| **Wan-02** | 1997 | 3 | 12.25 | 3.9 | 3.66 |
| **Wan-04** | 1997 | 3 | 12.81 | 3.82 | 3.91 |
| **Wan-05** | 1998 | 5 | 14.1 | 4.56 | 3.61 |
| **Wan-07** | 1997 | 4 | 12.6 | 4.1 | 3.59 |
| **Wan-09** | 1997 | 4 | 12.31 | 3.84 | 3.74 |
| **Wan-10** | 1997 | 4 | 14.14 | 4.46 | 3.7 |
| **Wan-N** | 1997 | 3 | 11.63 | 3.73 | 3.64 |

**Table 5-3. Carbon and nitrogen concentration and atomic C:N for Wangels ovacaprine samples for *extracted collagen*. Collagen concentration is expressed as a weight percentage of whole bone.**

| Sample | Excavation year | Concentration (wt. % of bone) | C% | N% | C/N |
|---|---|---|---|---|---|
| **Wan-11** | 1999 | 11.66 | 41.13 | 14.22 | 2.89 |
| **Wan-12** | 1999 | 15.68 | 41.53 | 14.51 | 2.86 |
| **Wan-13** | 1999 | 12.09 | 42.51 | 14.96 | 2.84 |
| **Wan-14** | 1996 | 5.77 | - | - | - |
| **Wan-15** | 1996 | 9.89 | 38.05 | 12.90 | 2.95 |
| **Wan-16** | 1997 | 10.25 | 40.07 | 13.86 | 2.89 |
| **Wan-17** | 1999 | 17.07 | 41.29 | 14.16 | 2.92 |
| **Wan-18** | 1996 | 31.83 | 39.31 | 12.81 | 3.07 |
| **Wan-19** | 1996 | 8.99 | 41.53 | 14.54 | 2.86 |

Figure 5-3 shows the difference between cattle samples from Wangels and ovacaprine samples. The cattle samples showed sufficient peaks over the entire *m/z* range of the spectrum, allowing for identification by ZooMS as most taxa-specific peptides were detected. For the ovacaprine samples, however (despite being excavated around the same time as the cattle samples in the late 1990s), identification was complicated by a lack of peptides in the spectrum above 2 kDa. As shown in Figure 5-4, treating the ovacaprine samples with acid did not release more collagen peptides above 2 kDa or at least they were not visible in the sample as opposed to the lower molecular weight peptides.

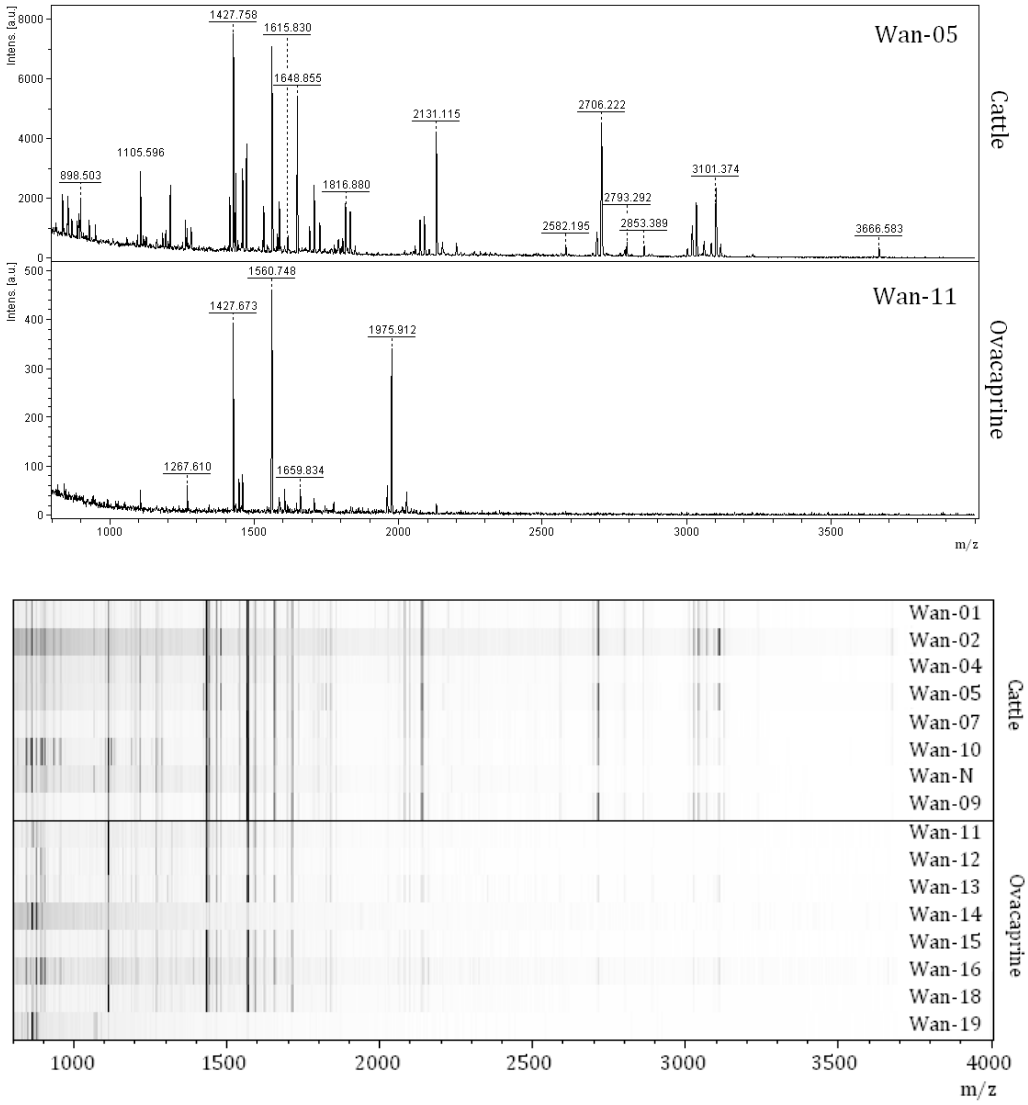


**Figure 5-3. Mass spectra and gel-view impression for samples from Wangels as mentioned in Table 5-1. The top eight samples are cattle, the lower eight are ovacaprine. Spectra as depicted in the gel-view were normalized against the signal (*m/z*) with the highest intensity (set to 100%).**

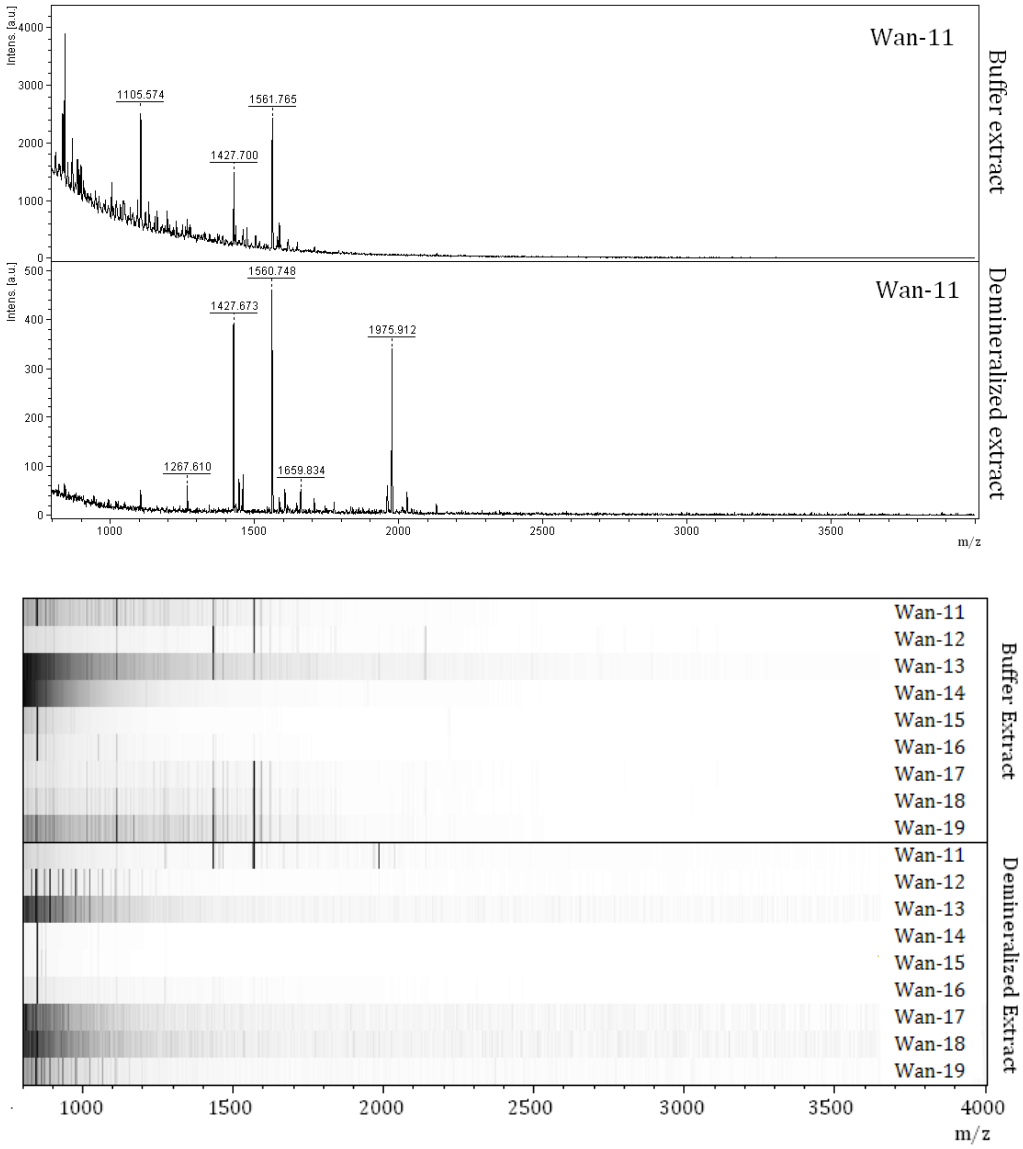


**Figure 5-4. Mass spectra and gel-view impression for ovacaprine samples from Wangels as mentioned in Table 5-1. The top eight samples are extracted with the non-destructive method, the lower eight are decalcified. Spectra as depicted in the gel-view were normalized against the signal (*m/z*) with the highest intensity (set to 100%).**

Figure 5-3 shows that ovacaprine samples show comparatively lower-intensity spectra. Interestingly, when these spectra are normalised to their relative intensities (highest peak = 100%), it becomes apparent that the ovacaprine samples have much lower signals for higher molecular weight peptides.

However, when deamidation rates are calculated for both sample sets, the ovacaprine samples do not have a significantly lower Q/E ratio than the cattle samples and this includes high molecular weight peptides (Figure 7-5).

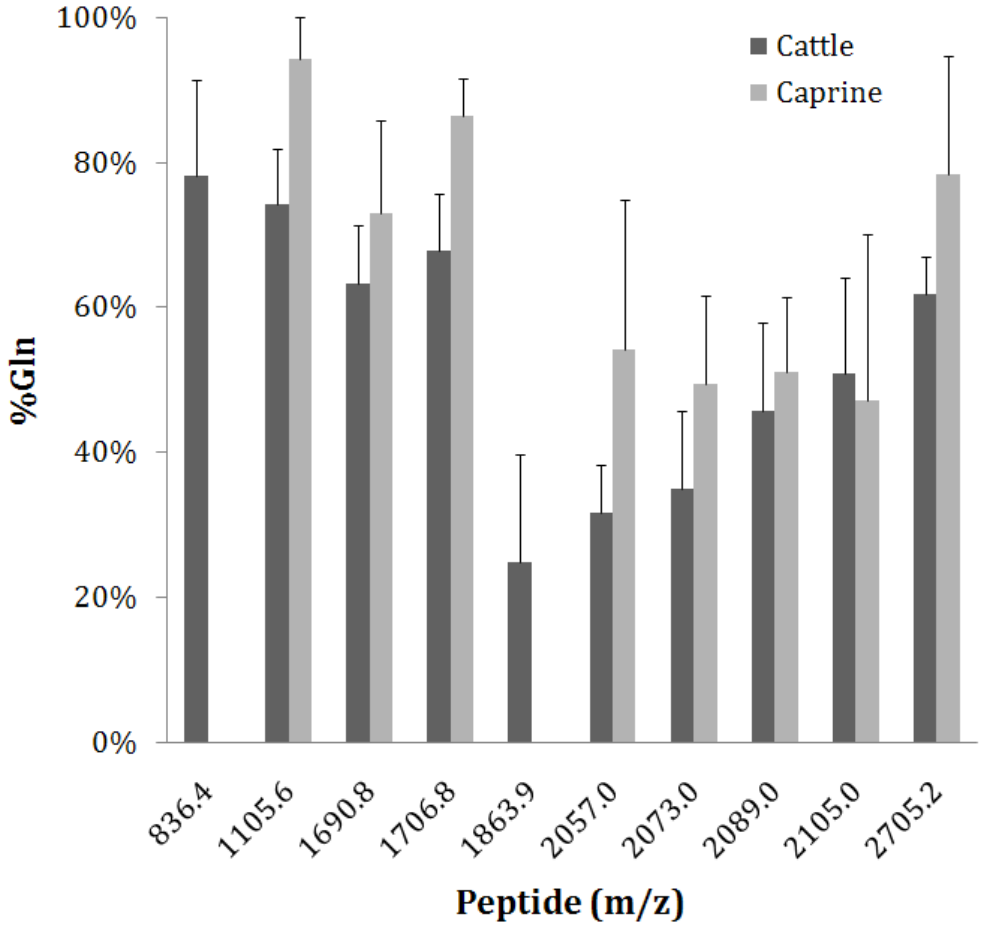


**Figure 5-5. Comparison of glutamine deamidation in collagen peptides found in samples from Wangels. %Gln represents the amount of original Gln is present in each peptide. T bars represent standard deviations. For peptides at m/z 836.4 and 1863.9 no data were found in caprine samples, possibly due to low fit, signal-to-noise ratio or peptide concentration.**

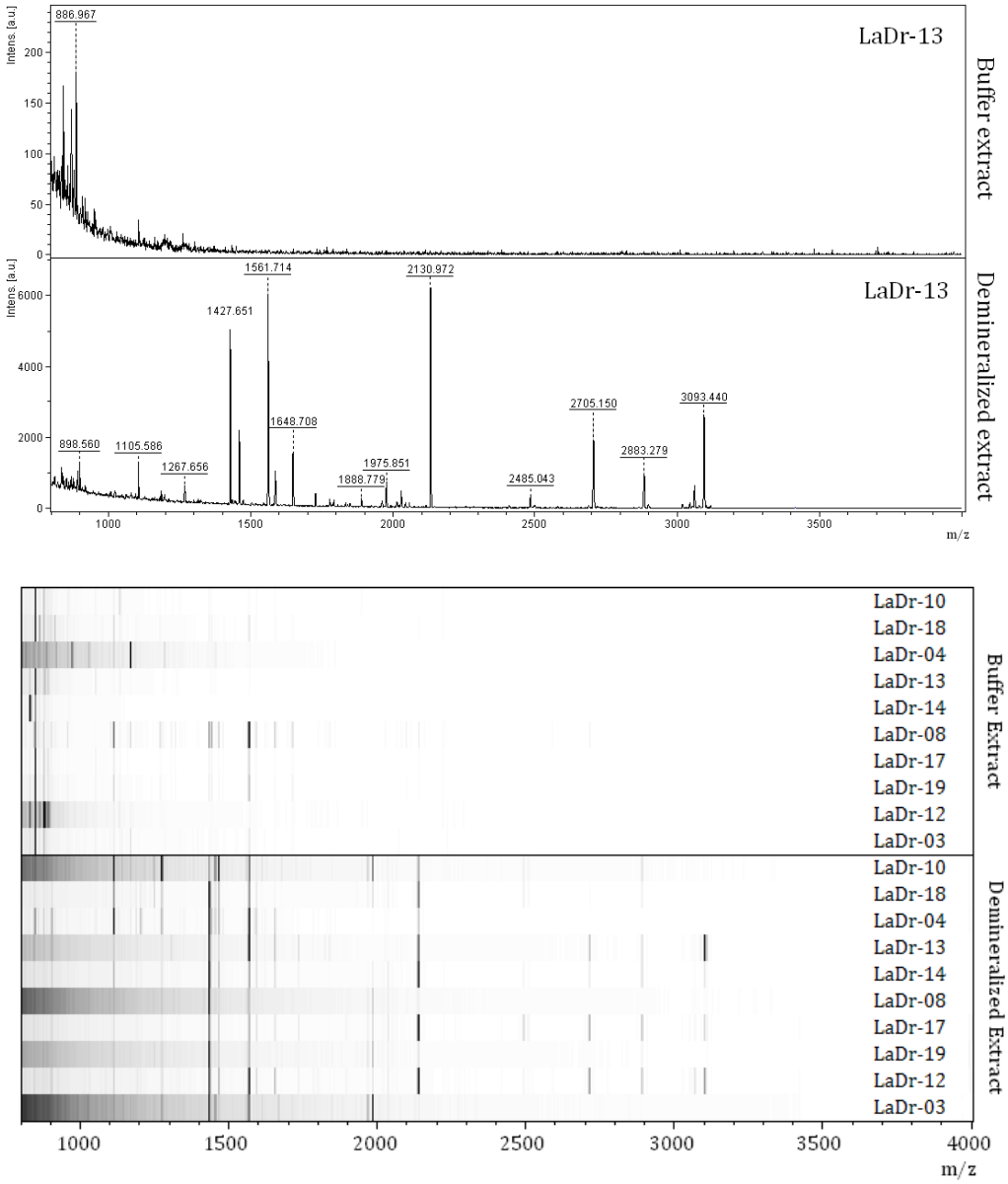


**Figure 5-6. Mass spectra and gel-view impression for samples from La Draga extracted by the non-destructive buffer method and repeated with demineralization. Spectra as depicted in the gel-view were normalized against the signal (*m/z*) with the highest intensity (set to 100%).**

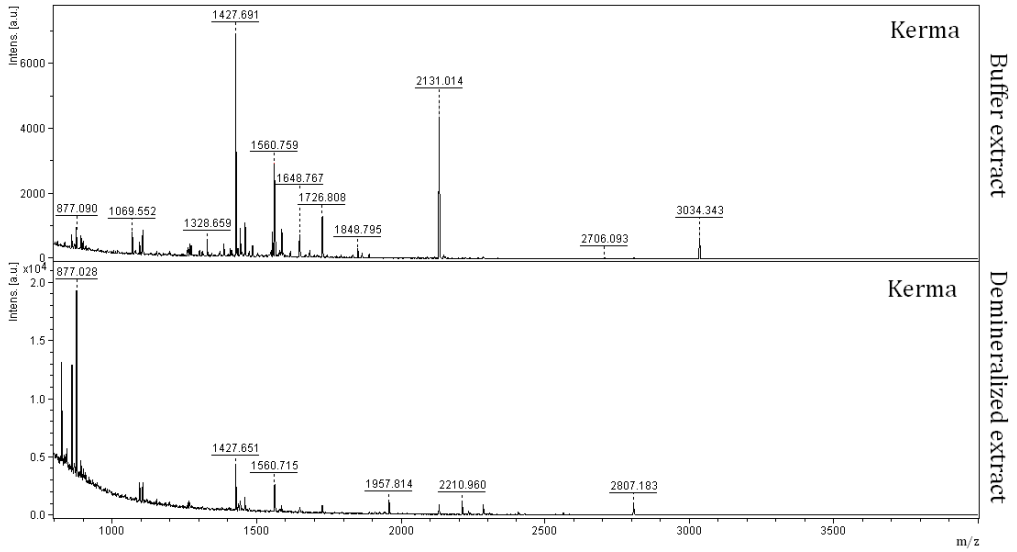


**Figure 5-7. Mass spectra for samples from Kerma extracted with the non-destructive buffer method and repeated with demineralization.**

### *5.4.2. La Draga and Fiskerton: Soluble Collagen loss in hydrologically active sites*

Samples from La Draga, a site with a fluctuating water level, displayed poor results using non-destructive ZooMS to the point that no identification of species could be given (Table 5-1). Spectra for samples from La Draga improved after decalcification (Figure 5-6). When investigated for glutamine deamidation (Chapter 4), only one of ten peptides was detected with sufficient isotopic fit to calculate %Gln.

In contrast to the samples from La Draga, samples from Kerma, an arid site in a desert area obtained excellent results with a buffer extraction. When the samples were decalcified, the spectra declined in quality (Figure 5-7).

We hypothesised that this was due to any soluble collagen generated to be leached away, leaving only a fraction behind that could be analysed by demineralising the bone. To affirm this theory, we investigated buried modern and archaeological bone material from a simulated hydrologically active site.

A monitoring project in Fiskerton, Lincolnshire, UK, was set up to investigate the effect of re-watering on the preservation of archaeological materials, including modern horse and well-preserved archaeological cow bone. Cross sections from long bones were placed at four different depths (top, upper middle, lower middle, bottom) within a plastic tube with bored holes to allow for water influx and were buried at a depth of 1.69 m without soil being packed against the samples (Figure 5-1). Samples

investigated in this study were removed from the experimental burial after 36 months (set A and set B) and 6 months (set B).

In set A the intensity of the spectra for samples obtained from the middle layers of the experimental burial (Figure 5-1), where water flux was expected to be greatest, was much lower compared to the top and bottom layer samples. Subsequent reanalysis displayed much less variation between layers, both for analysis of biological replicates of the same sample set (other fragments from the same bone sample) and other sample sets from the different levels at the Fiskerton site (Figure 5-2). One possible cause is that the samples were originally stored at -80°C before the experiments for set A were conducted. The biological replicates (for set B) were then stored cold (maximum temperature 4°C) until a resampling was undertaken, which was performed after 2 months and repeated 7 months later.

### *5.4.3. FISKERTON: DIAGENESIS*

The different extracts from the Fiskerton samples were also analysed for glutamine deamidation. Glutamine deamidation can be a useful indicator of collagen's thermal history (van Doorn et al. 2012).

Figure 5-8 shows the percentage of original glutamine content detected in selected peptides in a one hour ammonium buffer extract, a second hour buffer extract obtained from the same sample directly afterwards (both are non-destructive) and an extract after demineralisation and gelatinization of the same sample. Comparisons were made between the degradation rates of various peptides to reveal any differences between the extracts. Remarkably, the difference in deamidation between the soluble fraction (buffer extracts, notably the first hour) or insoluble fraction (acid extract) is limited, especially among the buried samples. Sometimes the insoluble fraction appears to be more deamidated, but other replicates from the same bone fragment show the opposite. The sequential buffer extracts display similar %Gln for most samples. Unburied controls show more consistency with the insoluble fraction having the lowest %Gln, rather than the opposite of what might be anticipated (assuming that deamidation is more rapid in the soluble fraction as collagen is less sterically restricted and will allow the faster imide-intermediate pathway for deamidation rather than side-chain hydrolysis ). The main difference is that more peptides are detected for the insoluble collagen fraction, with an accurate isotopic fit and signal-to-noise ratio.

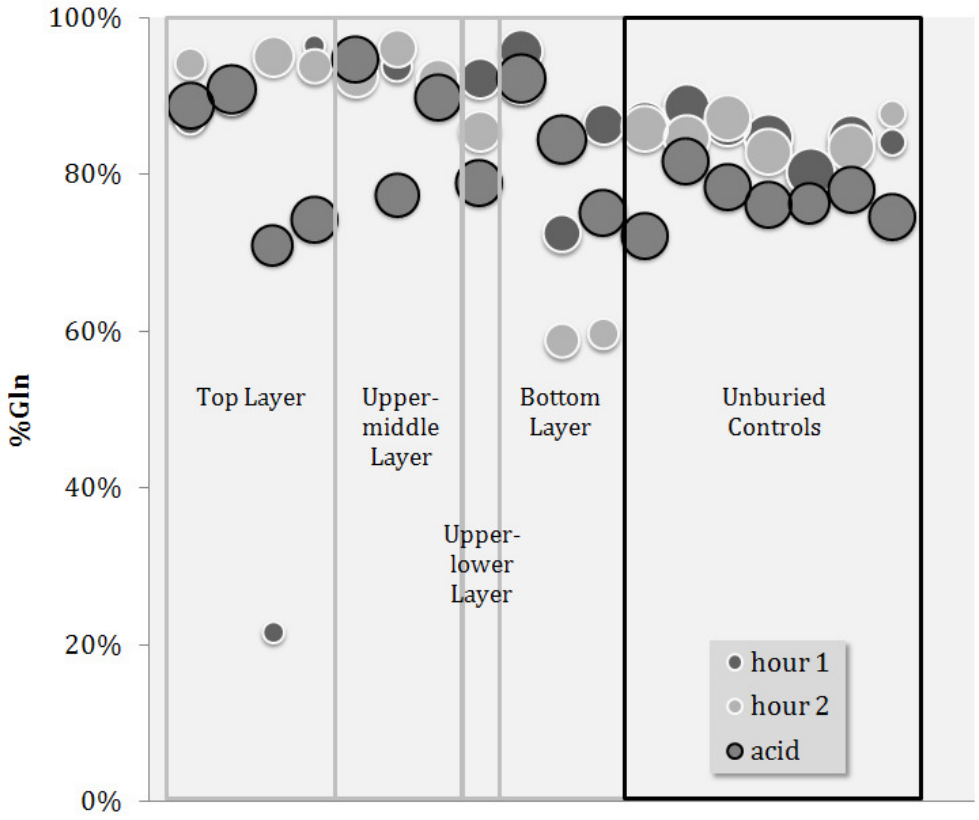


**Figure 5-8. Results of glutamine deamidation on extraction fractions from Fiskerton samples. Samples are lined along the x-axis and grouped per burial layer. The extractions investigated were a one hour buffer extract (hour 1), followed by a second one hour buffer extract (hour 2), followed by demineralization using acid and a three hour extraction in buffer (acid). These are the same bone samples as represented in Figure 5-2(36 months burial). %Gln represents the amount of original Gln present in each sample (van Doorn et al. 2012; Wilson et al. 2012). The size of the markers represents the number of peptides the calculation of %Gln is based upon.**

However, it can be argued, especially in the case of a hydrologically active environment that the less hydrophobic deamidated fragments are leached away and that this has led to a higher %Gln for the extracted fractions than would be expected from the buried samples. Two samples from the top layer (less hydrologically active environment) show a lower %Gln in the insoluble fraction. The samples have not been packed with soil, so it is possible that due to exposure in the top layer the samples have accumulated more damage than unburied controls would. It is also a lower %Gln

value than would be expected for samples "buried" in the UK for a maximum of 36 months. If this is a true effect that is shown in this small sample set, then burial environment should be taken into account for glutamine deamidation analysis.

## 5.5. DISCUSSION

### *5.5.1. CONDENSATION*

The overall yield of collagen does not appear to be a particular problem for ZooMS, because even in bones with < 1% collagen, intact collagen is still apparently present (Dobberstein et al. 2009). A more common and potentially more limiting issue is that samples can contain collagen, but may not yield good spectra, or alternatively only provide a spectrum for low molecular weight peptides, such as the ovacaprine samples from Wangels.

Our results showed that both cattle and ovacaprine samples from Wangels, excavated around the same time contain collagen in quality and amount acceptable for most bulk analyses. In addition, glutamine deamidation has occurred to a comparable extent in both sample sets, which makes sense as both the cattle and ovacaprine samples have been exposed to the same temperature over similar time frames, adding to their thermal age (the main determinant for glutamine deamidation) to be comparable (Smith et al. 2003; van Doorn et al. 2012). Hence, we may conclude that the lack of visible peptides in ovacaprine samples is not due to degradation or collagen loss (which according to C/N ratios and presence of low molecular peptides is at least comparable to the cattle samples).

Our working hypothesis is that crosslinking may be responsible for this pattern, as the larger the peptide the higher the probability that it contains a crosslink and is not cleaved by trypsin digestion. Lysines, with arginines, are both targets for non-enzymatic glycosylation and also for trypsin cleavage, explaining why some peptides may not appear consistently in all spectra, to the point that the observation of high molecular weight peptides is diminished. However, it should be considered that the higher molecular weight fraction is perhaps not targeted in specific and that crosslinking indiscriminately targets collagen lysine and arginine residues and affects peptides of all masses culminating into an overall lowered concentration of peptides, even though this is not what we observe.

An extraction at 65°C may have been too low a temperature for the ovacaprine samples from Wangels, as any "tanning" may have raised the melting temperature of

collagen. However, ovacaprine samples did not strongly differ in collagen quality from the cattle samples (Table 5-2 and 5-3). Attempts to assess the level of cross-linking using differential scanning calorimetry (DSC) failed to confirm hypothesis. Despite the fact that it was possible to isolate and gelatinise collagen for elemental carbon and nitrogen analysis, no transitions were observed by DSC suggesting a very broad temperature range for the collagen-gelatin transition, perhaps broadened by both hydrolysis and cross-linking. Furthermore, glutamine deamidation, a thermal rather than diagenetic affected parameter, did not show more damage in the ovacaprine bone material.

### *5.5.2. SOLUBLE COLLAGEN*

The strength of the non-destructive ZooMS approach is that we extract a soluble fraction of collagen from the sample. The inherent weakness is that if there is no readily available soluble collagen fraction, less protein can be extracted. This, however, should not necessarily exclude a sample from non-destructive ZooMS analysis, as intact collagen in the insoluble fraction can potentially be gelatinised in the extraction process, generating a fresh soluble collagen fraction, and analysed for species identification.

Our initial test on set A from the Fiskerton buried materials showed that the middle layers which were exposed to a fluctuating watertable, less proteinaceous material was observed after a buffer extraction. However, experiments on the same samples in set B did not show this pattern. The samples in the latter case were defrosted and stored at a temperature of 4°C for an extended period of time, potentially allowing for a new soluble fraction to be generated.

Samples from a hydrologically active site like La Draga displayed poor results from non-destructive ZooMS and spectra were improved after decalcification. Samples from the arid burial site in Kerma, Sudan, showed the reverse, indicating that most of the present collagen had gelatinized, but had not been leached out in the hot and dry environment. Consequentially, the acid extraction lost more of the present collagen than the buffer extraction did.

It seems that the loss of soluble collagen can obliterate mass spectra for samples from hydrologically active sites and that demineralization of bone is necessary to yield sufficient collagen for mass spectrometric analysis. This conclusion seems intuitive, yet could not be confirmed in the samples from the experimental burial in Fiskerton. The sensitivity of MALDI mass spectrometry may be the reason for this; not all

collagen needs to be leached out from the bone samples. If any collagen remains in helical form and is not otherwise retained within the bone sample (i.e. by tanning or the effect witnessed in the Wangels ovacaprine dataset), collagen can still be gelatinized and analysed.

## 5.6. CONCLUSION

We present here two case studies of archaeological sites — Wangels and La Draga — and one study of an experimental site (Fiskerton) that illustrate two different problems. Samples from the site Wangels repeatedly failed to generate >2 kDa peptides which complicated and prevented assignment of genus to ovacaprine samples. We argue that this is due to crosslinking, however, results did not indicate that the ovacaprine samples were richer in organics than the cattle samples from the same site as neither sample subset displayed a C/N ratio that would indicate for exogenous material. In addition, DSC failed to give any data apart from an expected increase in melting temperature.

Samples from the site of La Draga failed to provide peptides through buffer extraction (Chapter 4), but acid treatment did improve the spectra. This may be due to collagen being slowly leached out over time as the site is partially waterlogged and is exposed to a constant waterflux. However, we were unable to repeat this with simulated leached-out samples, i.e. the experimental burial at Fiskerton, presumably because the material from Fiskerton was not leached in a limited timeframe to the same extent as the La Draga samples and fresh gelatin was easily generated — which we observed in follow-up experiments.

This illustrates that the buffer extraction method does have its limitations and samples cannot simply be discarded for yielding insufficient data using MALDI MS when the buffer only method is used. Samples may contain collagen, even when the spectra may visibly lack peptides. This knowledge may eventually be of use for archaeologists while screening samples using ZooMS to troubleshoot problematic samples and hopefully may aid in finding solutions for increasing the efficiency of ZooMS.

## 5.7. ACKNOWLEDGEMENTS

We thank Paula Antunes (ICLT, University of Northampton, UK) for performing DSC analysis, Hege Hollund for sample collection (Wangels) and isotope analysis (Vrije Universiteit Amsterdam, NL), Debbie Coldwell and Sue Hartley for performing carbon/nitrogen elemental analysis (Univeristy of York, UK), Ulrich Schmölke and

Sönke Hartz for samples from Wangels, Roz Gillis for samples from La Draga, Hannah Koon for samples from Fiskerton.

# Chapter 6. SPECIES IDENTIFICATION OF VEGETABLE AND MINERAL TANNED LEATHER BY MALDI-TOF MASS SPECTROMETRY

## 6.1. ABSTRACT

Leather, the tanned skin or hide of animals, is today a $50 billion industry and has a long history, surviving deep into the archaeological record due to its increased hydrothermal stability and reduced bioavailability. However, lacking mineralization, it is more vulnerable to biodegradation than bone, the other common form of ancient collagenous tissue. Degradation can obliterate the characteristic grain structure of leather that allows identification of animal origin using microscopic methods. DNA survival may be compromised during leather production, where ZooMS may be useful for identifying the animal origin of leather in both archaeology and modern samples (e.g. counterfeiting or smuggling of exotica) as characterization is based upon collagen itself. We employ a slightly altered protocol of ZooMS, the non-destructive version using ammonium bicarbonate buffer to extract collagen from leather of three common tannages: hydrolysable and condensed vegetable tannage and chromium (III). Extraction at 95°C overall gained spectra from all three tannages and we were able to identify the genus of the leathers through MASCOT searches with an in-house database of collagen type I sequences. ZooMS is useful for most types of collagenous material. Non-tanned material, such as parchment, requires no different protocol than regular non-destructive ZooMS and provides excellent spectra. Tanned leathers require multiple steps to release peptides of varied molecular weight and concentrate the fraction for secure identification.

## 6.2. INTRODUCTION

The creation and use of animal hides dates back to at least prehistoric times and dated to at least 2000 BC (Reed 1972; Ryder 1984), and has persisted due to its versatility, endurance and resistance (Harlan & Feairheller 1977). Yet leather is sensitive to biodegradation (Strzelczyk et al. 1997), but can, when preserved under the right circumstances, survive through the archaeological record. Such finds can be artefacts originally made from leather, like clothing or adornments (Groenman-van Waateringe et al. 1999; Durrani 2012), or skins and hides preserved through naturally occurring "tanning" mechanisms (Painter 1991). Current hypotheses are that the presence of

*Sphagnum* moss degradation products in peat can aid in the preservation of skin and leather material in so called "bog bodies", through condensation of collagen with a unique pectin-like carbohydrate with bacterial retarding properties due to its weak acidity (Stalheim et al. 2009). A uronic acid known as 5-keto-D-mannuronic acid (5-KMA) was commonly referred to as "*sphagnan*" (Painter 1991; Painter 1998) but Ballance et al (2007) could not confirm the presence of 5-KMA in *Sphagnum* moss extracts. The interactions of *Sphagnum* moss degradation products with collagen have been partially characterized (Stankiewicz et al. 1997). In addition, leather can persist through the presence of corrosion products that retard biodegradation (Cameron 1991).

Leather is defined as the tanned intact hide of an animal skin of which the dermis (see inset of Figure 6-1), the stronger layer of animal skin, is used exclusively, unless it is intentionally kept fur-bearing with the epidermis intact (Reed 1972). In its preparation stages of processing to leather, raw skin is unhaired, defleshed, and soaked in alkaline baths (liming) to "open up" the fibrillar structure. For parchment and rawhide materials, materials are dried after deliming and finished. For leathers, the prepared hide is pickled (to optimize conditions for tanning) and finally tanned (see Figure 6-1, based on Reed (1972), Vuissoz et al (2007) and Covington (2009)). Two types of collagen are prevalent in skin: type I (a heterotrimer) and type III (homotrimer), which occur throughout the dermis in very consistent ratios, around 85:15 (Epstein Jr & Munderloh 1978).

Recently there has been an increased interest in DNA from archaeological parchment for palaeogenomic analysis (Burger et al. 2000; Poulakakis et al. 2007), but risks of transfer between parchments has been brought up (Campana et al. 2010). Leathers appear a less fruitful source of DNA as several steps in the beamhouse appear to remove most nDNA from leather (Reed 1972; Burger et al. 2000; Burger et al. 2001), although there have been reports of mtDNA surviving (Vuissoz et al. 2007). However, in theory, if there is any tanned material remaining, collagen should be present and if peptides can be extracted, we can employ ZooMS for animal origin identification.

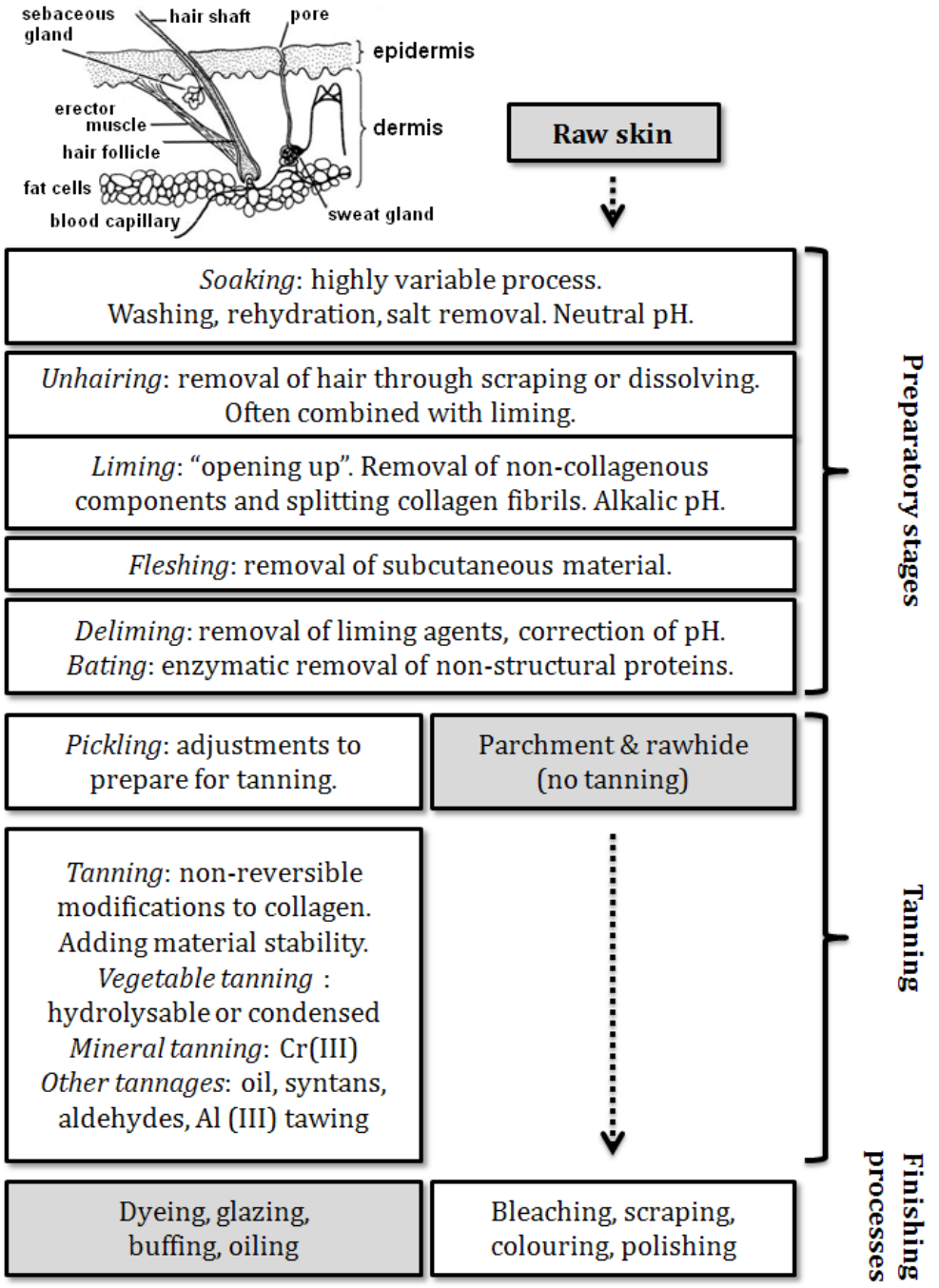


**Figure 6-1. Schematic overview of the process of leather making. Inset: cross-section of animal skin. ( Based on Reed 1972; Vuissoz et al. 2007; inset by Ruth Lawson Otago Polytechnic with amendments.; Covington 2009)**


**Figure 6-2. Close-up photographs of two leathers. Above: Ostrich leather; Below: "Imitation ostrich" leather, made from embossed bovine hide. In this particular example the difference in texture is macroscopically visible.**

Leather can be examined microscopically for specific grain patterns in the dermis. Even after unhairing, the follicles may still be visible, the elasticity and support of the

lower layers are strong enough that characteristic elements (the grain pattern) are preserved in the hide (Reed 1972). Unlike bone, leather lacks the protective mineral matrix that provides longevity to the collagen molecule by restricting both molecular mobility and enzyme accessibility (Collins et al. 2002). Therefore, protein analysis of archaeological leather can be useful if the grain of leather is too damaged or eroded to be identified by microscope. For contemporary use, this can potentially aid the identification of exotica: endangered species brought in through border security, or counterfeit material spiked with DNA or of which the grain pattern is altered through embossing (Figure 6-2).

Biodegradation does eventually occur in archaeological leather, although the stabilization obtained through tanning can aid protection (Covington 2009). Figure 6-3 shows the deliberate degradation of (tanned) hide powders by anaerobic microbiota into biogas, a mixture of carbon dioxide and methane. Untanned collagen is readily degraded, but the microbiota produce no more biogas above the level of a blank in the case of tanned hide powders. Figure 6-3 also shows that denaturation facilitates biodegradation and that after denaturation chromium tanned leather is vulnerable to biodegradation nearly as much as untanned hide. Hence, it should be possible to detect collagen peptides in degraded leather. However, leather tanned with mimosa, a condensed vegetable tannin, does not show an effect after denaturation, and the amount of biogas produced is still at the level of the negative control. Similar observations were made by Dhayalan et al (2007). This may indicate that without prior removal of strong vegetable tannins, tanning in leather may prevent us from seeing collagen during extraction (which involves denaturation), whereas chromium tanned leather may release collagen more easily.

Tanning is not necessarily synonymous to crosslinking, but the current requirements are that collagen needs to be irreversibly modified to obtain an increase in hydrothermal stability — the resistance to wet heat, commonly expressed as the "shrinkage temperature", $T_s$ (Covington 2009). Some tanning methods, such as aluminium (III) tawing, are reversible by washing out or heating, or do not significantly increase the hydrothermal stability of collagen and may therefore not fully be regarded as tanning, but are instead referred to as "leathering" (Covington 2009).

There are several different types of leather tannage, and in this chapter the most common three are discussed: "vegetable" tannage (most common in archaeological

leatherwork), mineral tannage such as chromium (III) (the most common contemporary tanning method) and aldehyde tannage (with glutaraldehyde as a common low-cost alternative).

The most complex method of tanning is with vegetable tannins, which comprise an entire array of different plant polyphenols, varying in weight and solubility and tanning efficiency, especially in archaeological material (van Driel-Murray 2002). Although different sizes of tannins are possible, those with chain lengths of $C_7$ to $C_{12}$ have a greater chance to increase thermal stability than longer or shorter chains (Scholnick et al. 1992), suggesting that these fit optimally within the microfibril structure of collagen, and keep the helices stabilised between themselves as opposed to binding within individual triple helices or binding collagen to surface material (Brown 1999). Hydrolysable vegetable tannins are highly astringent, exclusively associate with collagen through hydrogen bonding, and can be hydrolyzed within the leather, adding a buffering effect with carbolic acid (phenolic hydroxyl) residues (Covington 2009). Condensed tannins are typically less astringent. Similarly to hydrolysable tannins they interact with collagen through hydrogen bonds, but can also bind to collagen through covalent binding to lysine residues. For similar reasons, glutaraldehyde has been mentioned as a stable crosslinking agent for collagen fibrils specifically(Bowes & Cater 1968; Migneault et al. 2004).

The mechanisms on a molecular level for chromium tanning are still not fully understood (Covington et al. 2001), however, a high hydrothermal stability can be attained with a weight percentage of tanning agent almost ten times lower than most vegetable tannins, and does so much faster. The traditional view is that hydrated chromium (III) sulphate forms a binuclear complex between two ionized acidic residues with collagen (glutamic acid and aspartic acid) (Brown 1999) to cause an increase in hydrothermal stability, increasing the denaturation temperature of chromium tanned leather to resist temperatures up to 130°C. However, more recent X-ray studies have shown the active species is a linear tetra-chromium complex, itself crosslinked by counterions such as sulfate, but not necessarily crosslinking the collagen (Covington et al. 2001).

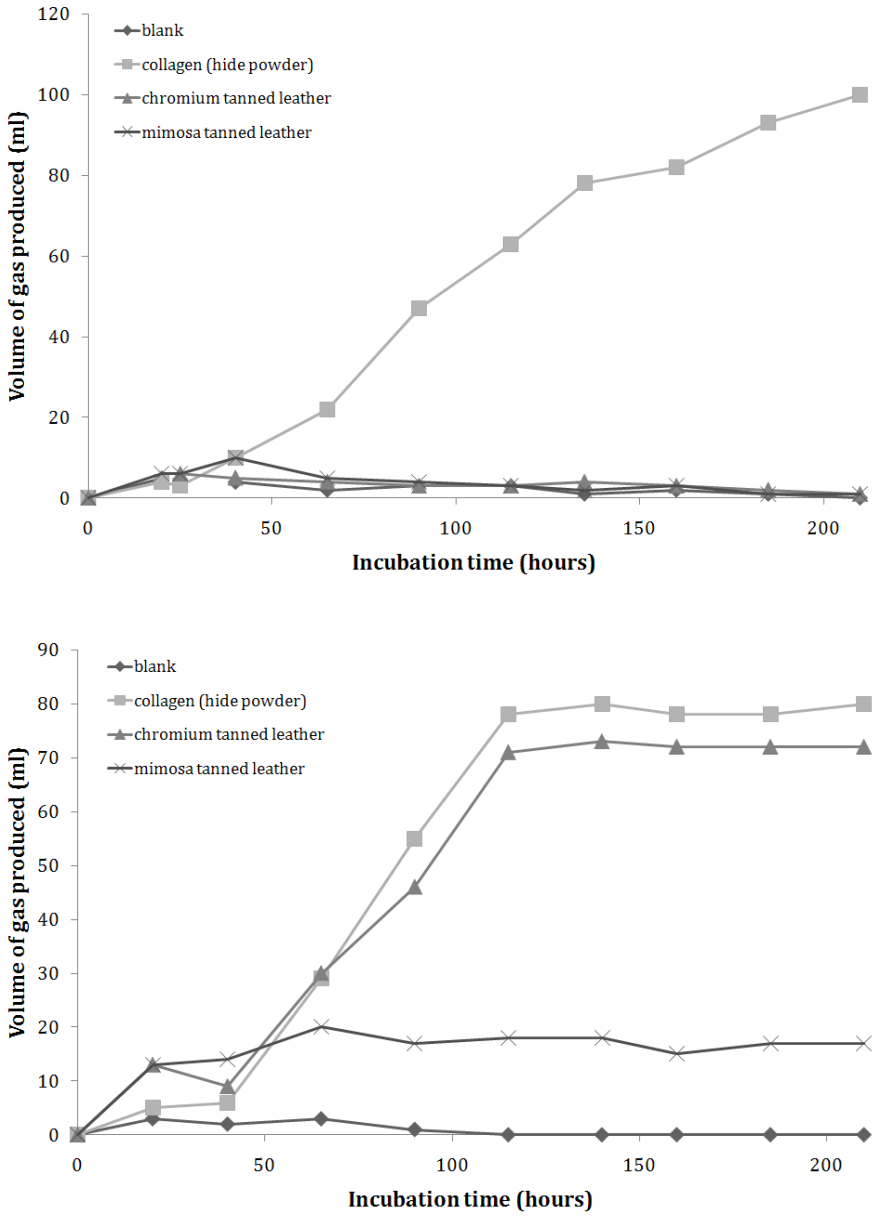


**Figure 6-3. Production of biogas from collagen by anaerobic bacteria (above) and after denaturation (below). With permission from: Covington (2009).**

Glutaraldehyde, similar to formaldehyde as a crosslinking agent, insofar as it does not react as a monomer, achieves comparable hydrothermal stability, is a small molecule that polymerizes prior to crosslinking through reactions of the aldehyde groups with the side-chain amino groups of basic residues (lysine and hydroxylysine) between collagen helices (Damink et al. 1995; Brown 1999). No complex networks of aldehyde oligomers are commonly formed, but as trypsin digestion (a staple of proteomics methods to cut a protein to peptides) targets the same residues, the enzyme digestion

is hypothesised to be inhibited and this may decrease the concentration of visible peptides.

Collagen can readily be dissolved from untanned hide (Bowes et al. 1955), but tanning is expected to complicate collagen extraction, due to an increased thermal stability imparted by the various tannages. Yet the possibility of running ZooMS on leather and other hide-derived material could be useful. It was suspected due to previous experiments with arguably strongly crosslinked material (Chapter 5), that leather would be equally complicated, especially in the case of vegetable tanning where arginine and lysine residues have reacted with the tannins. Trypsin targets these same residues during its enzymatic mechanism, ionic residues with narrow side-chains (Olsen et al. 2004), thus tannins bound to these residues may obstruct the enzymatic cleavage reaction. Steric limitations appear to dictate where crosslinking is initiated within the fibril. Potential binding sites with the collagen microfibrils were more prevalent in the more open gap region than the compacted overlap region of collagen (Brown 1999). Chromium tanned leather, with its raised hydrothermal stability may not produce peptides in the standard ZooMS protocol due to the relatively low temperature that is employed during extraction.

## 6.3. Materials and Methods

### *6.3.1. Samples*

All leather samples were donated by the Institute for Creative Leather Techologies (ICLT) University of Northampton, Northampton, UK. This included chromium (III) and modern vegetable tanned leathers from cow and calf, sheep, goat, deer, pig, buffalo, and ostrich. We also received bovine leather tanned with glutaraldehyde. Also included was leather made from cow-hide, embossed to appear like ostrich leather, referred to as "imitation ostrich" (Figure 6-2).

In addition, controls that were used in this study consisted of well-preserved medieval bone (for cattle) or heat-rendered modern bone (for pig and sheep) of known origin. For comparison of untanned hide, a parchment sample of sheepskin (Borthwick Institute Archives, University of York, UK) was taken into account as well. Comparative controls were extracted for 1 hour at 65°C (2 times), after which the ZooMS protocol as described below was followed.

### *6.3.2. Leather preparation*

1. Leather segments (300–800 mg) were autoclaved in ultrapure water for 3 hours at 120°C. Chromium tanned leathers were retrieved with relatively low

damage to the segments and with clear, but usually coloured, supernatant. Extracts of glutaraldehyde tanned leather was also retrieved with a clear supernatant. Vegetable tanned leathers were found to be blackened and fragmented, while the supernatant was typically "milky".

2. Leather segments (300–800 mg) were soaked in 50 mM ammonium bicarbonate (pH 8.0) over 48 hours at 65°C and the supernatant was refreshed every 6–12 hours to strain out colouring dyes that could potentially obscure MALDI signals. When the supernatant no longer diminished in colour, samples were incubated for 1 hour at 65°C and again for 1 hour at 95°C. Vegetable tanned leathers did not visibly fragment, blacken or produce a "milky" supernatant.

All supernatants were filtered using ultrafilters (> 30 kMW, Millipore, Durham, UK). 125 µl of residuate was added to 125 µl ammonium bicarbonate to pH 8.0 and digested for 18 hours at 37°C with 2 µg of trypsin.

### *6.3.3. ZooMS (non-destructive and traditional)*

Trypsinated extracts were desalted and concentrated over a C18 column (Porvair, Leatherhead, UK) and eluted with 50% ACN/0.1% TFA (v/v). 1 µl of eluate was mixed on a ground steel plate with 1 µl α-cyano-4-hydroxycinnamic acid matrix solution (1% in 50% ACN / 0.1% TFA (v/v/v)) and dried to air.

Each sample was analysed in reflector mode using a calibrated Ultraflex III (Bruker Daltonics, Bremen, DE) MALDI-TOF instrument to measure mass to charge ratios (m/z) of trypsinated fragments. Spectra were analyzed using flexAnalysis software v. 3.0 (Bruker Daltonics) and mMass (Strohalm et al. 2010). Peaks with a signal-to-noise ratio of below 5 were ignored before database matching. Search criteria in MASCOT allowed for 1 missed cleavage, variable deamidation of glutamine and asparagine, and hydroxylation of proline and lysine. Mass tolerance was set at 50 ppm. This in-house database contains collagen type I sequences from run standards and theoretical peptide fingerprints derived from translated cDNA sequences digested *in silico*.

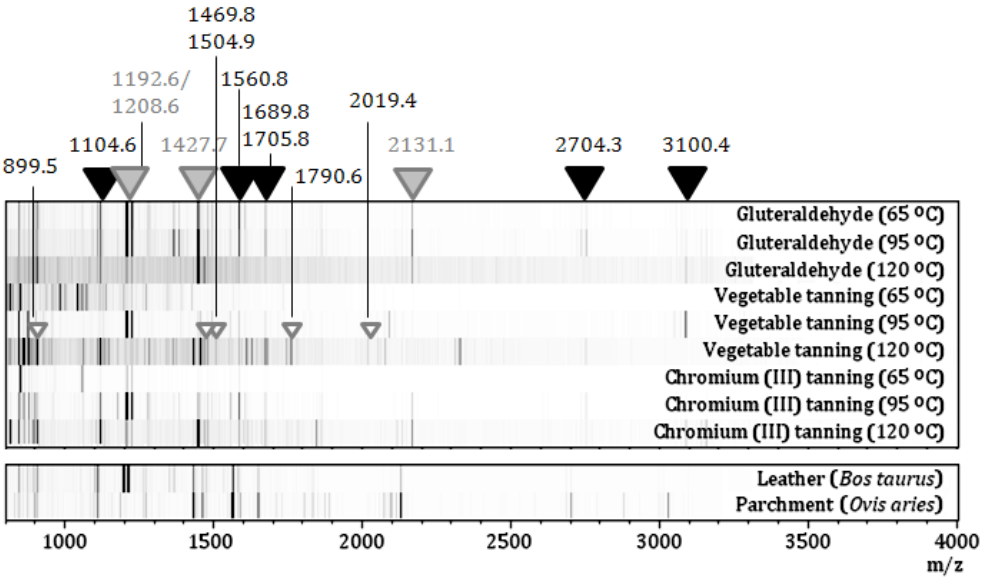


**Figure 6-4. Gel-view representation of MALDI-TOF spectra for the comparison of extraction temperatures on bovine leathers with different tannages. All samples are spectra from three technical replicates added together. The temperatures denoted refer to the extraction temperature. Above: mass spectra were normalized. Indicated are markers for collagen type I (black), collagen type I ruminant specific markers (grey), and peptides with the same mass as found by Choudhury (2006) to match collagen type III (white). Below: mass spectra for bovine leather (glutaraldehyde tanning) and medieval sheep-skin parchment.**

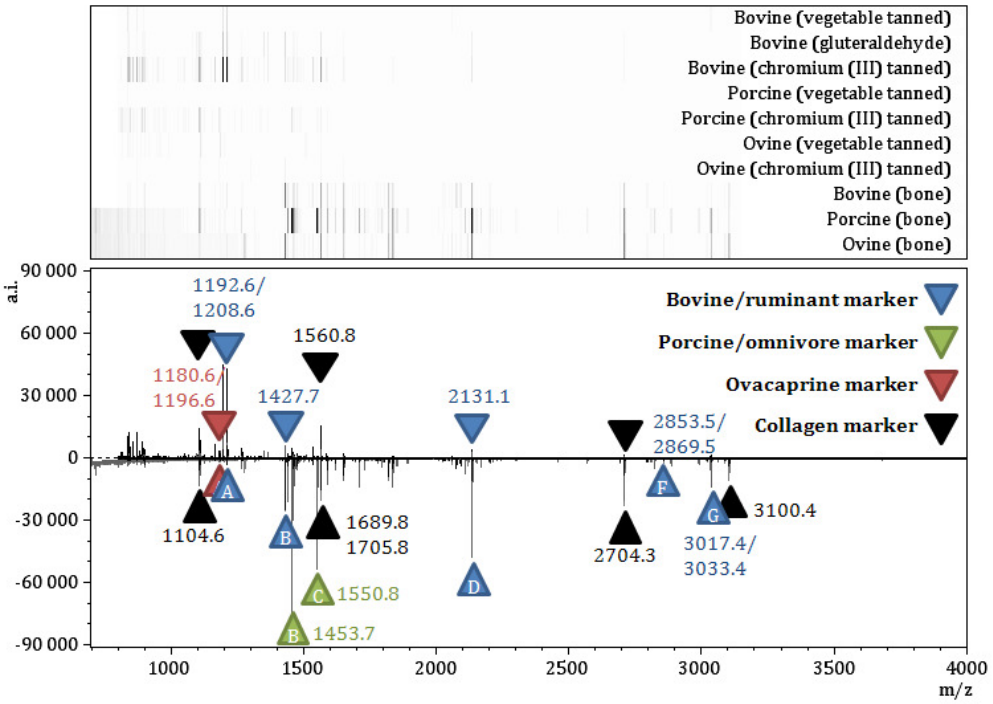


**Figure 6-5. Mass spectra and gel-view representation for three mammalian leathers with different tannages. Controls on modern bone extractions (65°C) are given on the flipside. All samples are cumulative spectra from three technical replicates. Indicated are markers for bovine/ruminant and specific markers for porcine (green) and ovacaprine (red) samples (Buckley et al. 2009), along with common (mammalian) collagen peptides in black.**

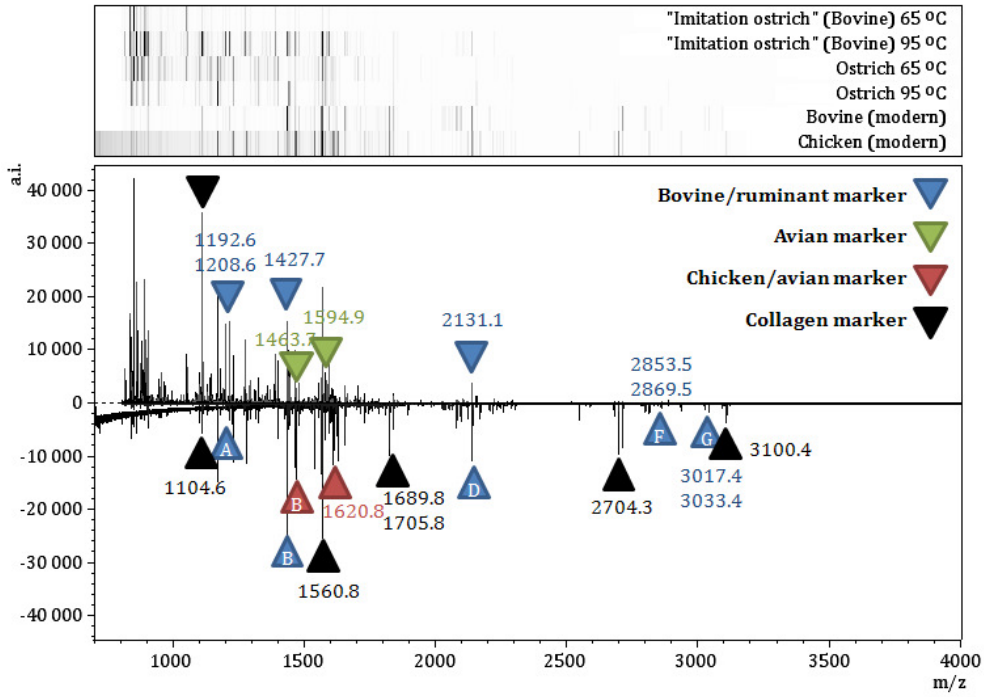


**Figure 6-6. Mass spectra and gel-view impression for two different leathers: original ostrich leather and bovine leather as "imitation ostrich". References of modern bone material are depicted on the flipside. All samples are cumulative spectra from three technical replicates. Indicated are bovine/ruminant markers (Buckley et al. 2009) in blue, known avian markers for the ostrich (green) and chicken (red) samples. Also denoted are common (mammalian) collagen markers in black.**

## 6.4. RESULTS

### *6.4.1. IDENTIFICATION*

Collagen in skin consists predominantly of type I (Epstein Jr & Munderloh 1978). As shown in Figures 6-4 and 6-5, as well as Tables 6-1 and 6-2 below, it was often possible to find markers for collagen type I as we commonly find in bone, as well as several markers for genus or family. However, not all markers that are observed consistently in bone samples were present in leather extracts. In five bovid and cervid samples, the ruminant marker 1427.7 (GIPGEFGLPGPAGAR) was not visible whereas other peptides regularly found in ruminant samples were present or even dominant in the spectra (GLPGVAGSVGEPGPLGIAGPPGAR; IGQPGAVGPAGIR for bovine; TGQPGAVGPAGIR for sheep, goat and pig).

In most cases, the identity determined by ZooMS matched with those given (Tables 1–3). One particular exception was chromium tanned sheepskin which carried a goat-specific marker: 3093.5 (GPSGEPGTAGPPGTPGPQGFLGPPGFLGLPGSR) (Buckley et al. 2010). The marker for sheep 3034.3 was not found in the spectrum for this sample and in the MASCOT search in our collagen database no match was found for 3093.5 for sheep.

Although we do not yet currently possess a complete collagen sequence for ostrich in our database, ostrich was compared to other avian material (Figure 6-6). The ostrich leather extract did not contain the peptide 1620.8 (GDPGPVGPVGPAGAFGPR), which is found in chicken. The ostrich sample did contain the peptide 1594.9 (GDPGPVGAVGPAGAFGPR) which is more common in avian samples, such as turkey and pheasant.

Compared to spectra obtained from well-preserved or modern bone, leather had overall poorer results. A reappearing trend is the lack of peptides over 2000 Da. When compared to either bone or parchment, the difference in distribution becomes apparent (Figures 6-4 and 6-5).

### *6.4.2. EXTRACTION TEMPERATURES*

As shown in Figure 6-4 and Figure 6-5 and summarised in Table 6-1 and 6-2, all treatments showed the potential to extract collagen type I. However, depending on the temperature or method of extraction used, the relative abundance of certain peptides changes. The 95°C extraction appears to have a selective preference for peptides compared to the other two temperatures. It should however be noted that at 120°C, the peptides were extracted in water, not ammonium bicarbonate buffer and run separately on the MALDI from the 65 and 95°C extracted samples.

Chromium tanned samples were better suited to treatments with higher temperature extractions, whereas overall, extraction at 95°C returned the best results, with the least unknown or unidentifiable peaks among the investigated samples. Glutaraldehyde tanning resulted in very clean spectra with the collagen markers being enhanced compared to the background or additional peaks present. However, this tanning method also consistently lacked the higher molecular weight fraction.

**Table 6-1. Overview of samples prepared as described in methods as preparation 1 at 120°C.**

| Sample | Tanning | MASCOT ID | Sup[a] | Other observations |
|---|---|---|---|---|
| Cattle | Chromium | Expect: 0.038<br>(*Bos primigenius*)<br>Coverage: 32% | C | |
| Deer | Chromium | Expect: 0.0097<br>(*Rangifer tardus*)<br>Coverage: 51%<br>Expect: 0.57<br>(*Dama dama*)<br>Coverage: 50% | C | |
| Goat | Chromium | Expect: 0.53<br>(*Capra hircus*)<br>Coverage: 15%<br>Expect: 5.6<br>(*Ovis aries*)<br>Coverage: 12% | C | |
| Pig | Chromium | Expect: 8.8<br>(*Sus scrofa*)<br>Coverage: 20% | C | PEG contamination |
| Sheep | Chromium | Expect: 0.82<br>(*Capra hircus*)<br>Coverage: 18%<br>Expect: 8.6<br>(*Ovis aries*)<br>Coverage: 15% | C | |
| Cattle | Glutaraldehyde | Expect: 17<br>(*Bos primigenius*)<br>Coverage: 6% | C | |
| Cattle | Vegetable | Expect: 13<br>(*Bos primigenius*)<br>Coverage: 20% | M | No/weak 1427.7 |
| Buffalo | Vegetable | | M | Strongest peaks are unknowns* |
| Calf | Vegetable | | M | Strongest peaks are unknowns* |
| Nigerian Goat | Vegetable | | C | Strongest peaks are unknowns* |
| "Imitation ostrich" (Cattle) | Vegetable | Expect: 0.4<br>(*Bos primigenius*)<br>Coverage: 39% | C | |
| Ostrich | Vegetable | Expect: 0.88 | C | |

| | | | | |
|---|---|---|---|---|
| | | (*Struthio camelus*) Coverage: 42% | | |
| Pig | Vegetable | Expect: 0.0092 (*Sus scrofa*) Coverage: 51% | C | Strongest peaks are unknowns* |
| Sheep | Vegetable | | C | Strongest peaks are unknowns* |

[a] Supernatant appearance: C = clear, M = "milky"

* Patterns are consistent with results from Choudhury et al. (2006) → collagen type III dominant

**Table 6-2. Overview of samples prepared as described in methods as preparation 2 at 65 and 95°C.**

| **Sample** | **Tanning** | **MASCOT ID (65°C)** | **MASCOT ID (95°C)** | **Other observations** |
|---|---|---|---|---|
| Cattle | Chromium | Expect: 2.7e-05 (*Bos primigenius*) Coverage: 60% | Expect: 2.4e-05 (*Bos primigenius*) Coverage: 17% | Poor spectrum (@ 95°C) |
| Cattle | Chromium | Expect: 2.1 (*Bos primigenius*) Coverage: 23% | Expect: 0.14 (*Bos primigenius*) Coverage: 54% | |
| Deer | Chromium | Expect: 0.17 (*Rangifer tardus*) Coverage: 31%<br>Expect: 2.1 (*Dama dama*) Coverage: 31% | Expect: 1.1 (*Capreolus capreolus*) Coverage: 11%<br>Other deer 11% | No 1427.7 (65°C) Poor spectrum (@ 95°C) |
| Goat | Chromium | No matches | No matches | PEG contamination |
| Pig | Chromium | Expect: 0.27 (*Sus scrofa*) Coverage: 19% | Expect: 2.7 (*Sus scrofa*) Coverage: 49% | |
| Sheep | Chromium | Expect: 4.6e-05 (*Capra hircus*) Coverage: 51% | Expect: 5e-10 (*Capra hircus*) Coverage: 42% | |
| Cattle | Glutaraldehyde | Expect: 0.099 (*Bos* | Expect: 3.1 (*Bos* | |

| | | | | |
|---|---|---|---|---|
| | | *primigenius*) Coverage: 44% | *primigenius*) Coverage: 44% | |
| Buffalo | Vegetable | No matches | Expect: 0.024 (*Bubalus buffalo*) Coverage: 41% | No/weak 1427.7 |
| Cattle | Vegetable | No matches | Expect: 0.038 (*Bos primigenius*) Coverage: 32% | No/weak 1427.7 |
| Cattle | Vegetable | No matches | Expect: 0.00027 (*Bos primigenius*) Coverage: 39% | No/weak 1427.7 |
| Nigerian Goat | Vegetable? | Expect: 93 (*Capra hircus*) Coverage: 11% | Expect: 0.95 (*Capra hircus*) Coverage: 17% | |
| "Imitation ostrich" (Cattle) | Vegetable | Expect: 0.031 (*Bos primigenius*) Coverage: 28% | Expect: 0.00057 (*Bos primigenius*) Coverage: 62% | |
| Ostrich | Vegetable | Expect: 4.5 (*Struthio camelus*) Coverage: 49% | Expect: 0.024 (*Struthio camelus*) Coverage: 41% | |
| Pig | Vegetable | Expect: 0.012 (*Sus scrofa*) Coverage: 30% | Expect: 0.014 (*Sus scrofa*) Coverage: 19% | |
| Sheep | Vegetable | No matches | No matches | PEG contamination |
| Cattle | None (bone) | Expect: 0.0016 (*Bos primigenius*) Coverage: 66% | N/A | Control |

**Table 6-3. Overview of results in leather identification by ZooMS with different extraction methods.**

| | 120°C | 95°C | 65°C |
|---|---|---|---|
| **Total ID** | **10** | **13** | **10** |
| *Agree* | *9* | *12* | *7* |
| *Disagree* | *1* | *1* | *1* |
| **No match** | **4** | | **3** |
| **Poor Spectrum** | | **2** | **2** |
| **Total** | **14** | **15** | **15** |

There were three samples which contained PEG, but the contamination was not reoccurring within the same sample in a different run and can be assumed to have been a randomly introduced laboratory contaminant, not present in the original leather sample.

There is a marked difference among several of the spectra of samples extracted at 120°C that were vegetable tanned, compared to chromium and glutaraldehyde tanned leathers. The latter spectra were overall similar to collagen type I dominant spectra as obtained from bone samples, whereas the vegetable tanned leathers showed a spectrum without any of the dominant collagen type I markers (Figure 6-4, Table 6-1).

### *6.4.3. PEPTIDE FRACTIONS*

One study of note should be mentioned. Choudhury et al (2006) investigated pickled sheep and lambskin by 2D-gel followed by MALDI-TOF MS on excised spots to distinguish characteristics that would contribute towards the quality of the leather. The hide used in this study is pickled, not tanned, yet some interesting overlap can be seen between Choudhury's results and this study. Their reported peptides do not correspond with the peptides we see in collagen type I dominant spectra as we have seen in chromium and glutaraldehyde tanned samples. This can possibly be attributed to the fact that they ran excised spots and not a whole sample, so there is a chance that those particular peptides are left out.

The peptides detected in the spots that Choudhury et al had matched to collagen type III are similar to those found in a characteristic vegetable tanned sheep-skin sample after extraction at 120°C (Figure 6-4 and table 6-5). Peptides that matched with the peptides Choudhury characterised as collagen type I were found to match with both the vegetable tanned sample and a chromium tanned sample.

**Table 6-4. Potential effect of visible peptides due to tanning.**

| | Chromium (III) | Glutaraldehyde | Vegetable | Bone (Control) |
|---|---|---|---|---|
| **Total peptides** | **155** | **133** | **82** | **176** |
| *N-terminal Arginine* | *101 (65%)* | *96 (72%)* | *55 (67%)* | *112 (64%)** |
| *N-terminal Lysine* | *54 (35%)* | *37 (28%)* | *27 (33%)* | *62 (36%)** |
| *C-terminal Arginine* | *114 (74 %)* | *106 (80%)* | *68 (83%)* | *120 (68%)* |
| *C-terminal Lysine* | *41 (26%)* | *27 (20%)* | *14 (17%)* | *56 (32%)* |
| **Miscleavages** | **56 (36%)** | **37 (28%)** | **31 (38%)** | **84 (48%)** |
| *Arginine* | *34 (61%)* | *22 (60%)* | *14 (45%)* | *52 (62%)* |
| *Lysine* | *22 (39%)* | *15 (40%)* | *17 (55%)* | *32 (38%)* |
| **Seq. Coverage** | **60%** | **44%** | **39%** | **66%** |

* Two peptides had no N-terminal amino acid as they belonged to the start of the helix.

## 6.5. DISCUSSION

In Chapter 5 some particularly difficult samples were discussed and our theory was that cross-linking of collagen may have been the main contributor to the absence of collagen peptides in the MALDI spectra of bone samples that were found to contain sufficient collagen for ZooMS analysis. However, we were able to detect several clear collagen (I) peptides from tanned leather by $NH_4HCO_3$ buffer extraction ZooMS.

Leather is made to be resilient and purposefully chemically resistant. Although there is a consistent pattern of higher molecular weight peptides missing from the MALDI spectra, peptides were visible and resulted in positive identification if heated to above the shrinkage temperature. In our experiments 95°C was optimal for vegetable tanned leather, 120°C for chromium. Vegetable tanned leathers could be successfully identified after extraction at 95°C. Chromium tanned leathers could be identified after extraction at each temperature investigated here. However, extraction at 120°C yielded good quality spectra overall, whereas extraction at lower temperatures sometimes led to missing markers (Table 6-2).

Surprisingly, extraction at 120°C was worse for vegetable-tannins, perhaps because it stimulated further crosslinking reactions with remaining active ingredients in the tannage, or extracts increasingly interfered with MALDI. Interestingly, we have found that these markedly different vegetable tanned samples extracted at 120°C generated

masses which correspond to those Choudhury et al (2006) have identified as collagen type III, meaning we may have a differential protein extraction due to the amount of tanning. Extraction at 65°C, the shrinkage temperature of raw skin collagen, is suitable for parchment (untanned hide) but yields overall poor results for leather, most likely due to its increased thermal stability through tanning.

**Table 6-5. Overview of *m/z* in vegetable and chromium tanned sheep leather found in common with Choudhury et al (2006). The top row indicates the spots excised from a 2D-gel and the identification of the proteins from those spots by MALDI-TOF.**

| | Spot 1: Col (III) | Spot 2: Col (III) | Spot 3: Col (III) | Spot 4: Col (I) |
|---|---|---|---|---|
| **Vegetable tanned** | 825.55<br>1469.86<br>1790.59 | 1834.61 | 825.55<br>899.50<br>1504.89 | 1504.90<br>1514.86<br>1650.76<br>2019.35<br>2934.50 |
| **Chromium tanned** | | | | 1183.62<br>1502.73<br>1514.76<br>1562.81<br>1652.84<br>2019.98 |

Additionally, as shown in Table 6-4 and Figure 6-7, it does seem that both vegetable tanning and glutaraldehyde tanning inhibit trypsin cleavage at collagen lysine residues. Vegetable tanned collagen produces markedly more lysine miscleavages than the other tannages. Glutaraldehyde and vegetable tanning also produce the least peptides that have an N-terminal lysine, implying that trypsin cleavage was inhibited. A similar pattern is visible for C-terminal lysines, however, it must be noted that the more pronounced reduction of lysine residues at the C-terminus may be due to the bias of MALDI to arginine-truncated peptides (Krause et al. 1999). To further suggest that trypsination may have been problematic can be judged from the fact that sequence coverage in chromium tanned samples is nearly as good as that in bone, whereas vegetable and glutaraldehyde tanned hide have markedly lower coverage at their corresponding optimal extraction temperatures.

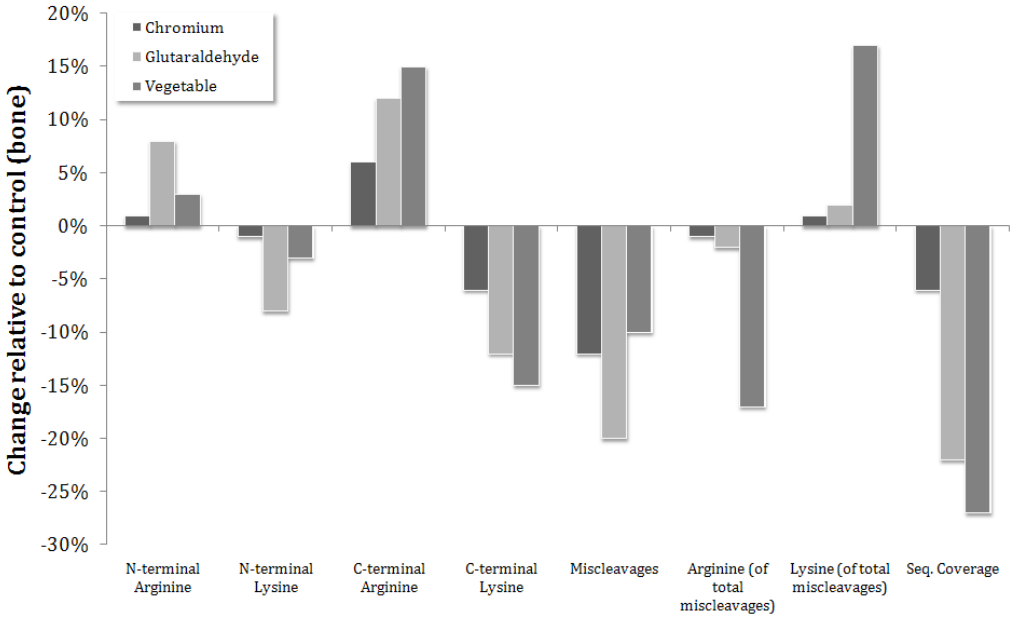


**Figure 6-7. The influence of tanning on the prevalence of lysine in observed peaks in MALDI spectra. Shown above is the difference between three types of tanned leather against well-preserved bone as a control. All samples are cattle (*Bos taurus*). Arginine and lysine content from miscleavages show the percentage of total miscleavages, not of the total of peptides. See also Table 6-4.**

This does not explain the consistent lack of HMW peptides, which is not exclusive to vegetable tannages. The competition of trypsin with tannins that crosslink at the same basic residues should not hold true for chromium tanned material as chromium complexes with acidic moieties. With the above explanation, we would expect more high molecular weight peptides to be visible, unless chromium would selectively crosslink at specific places throughout collagen fibrils. However, this does not seem to be the case as the crosslinking with chromium is rather unpredictable (Covington et al. 2001). In addition, many of the chromium tanned leathers were dyed. Although the colouring was removed as much as possible prior to extraction and part would be removed during SPE, it's possible that the remaining dyes may have interfered with the matrix absorbance efficiency, diminishing the overall visibility of peptides.

As a direct comparison: parchment has an even distribution across the MALDI spectrum and shows multiple *m/z* over 2 kDa. This would indicate that the difference lies in the tanning process as parchment (including medieval parchment) commonly was exposed to the same initial preparing stages as leather, like unhairing and liming (Reed 1972; Burger et al. 2000).

Vegetable tannins (which can account for 30–50% of the total volume of the finished leather) may accumulate in the gap region, the more "open" bands in the collagen fibril, rather than the overlap region of collagen (Brown 1999), due to less steric hindrance (Damink et al. 1995). Studies with catechin, a "model" vegetable tannin, show the atomic density around lysine residues is lower in the gap region of collagen and allow catechin to more freely interact (Brown 1999; Madhan et al. 2005). However, consider Figure 6-8, where our most commonly observed peptides in MALDI spectra are mapped on a collagen "unit cell", along with all theoretical tryptic peptides, cut after arginine or lysine. It appears that larger and smaller peptides are spread over this unit cell: it doesn't appear to be the case that larger peptides are found in the gap region as opposed to smaller peptides and therefore lacking in our spectra, neither is there any pattern in prevalence of lysine-terminated peptides that would suggest longer peptides are specifically targeted by tannins. The same can be said for both commonly observed and theoretical peptides. For example, whereas we have seen the arginine-truncated 1427.7 peptide missing from several spectra for vegetable tanned samples when other collagen (I) markers are present, it appears that this peptide lies within the overlap region. In addition, we seem to be losing collagen type I specifically when vegetable tanned leathers are exposed to 120°C for extraction.

These complications emphasise that future work is needed, mostly to look into more aggressive tannin and dye removal to understand what happens in leather and arguably other collagenous materials, such as organic-rich bone. We have shown that we can extract sufficient collagen type I peptides to give an indication of animal origin, and cleaner spectra would improve on this, especially for archaeological samples, as those will consist of vegetable tanned material and detection tests for vegetable tannins are well-established (Reed 1972; van Driel-Murray 2002). Aged leather has been noted to change in thermal behaviour and arguably decrease in stability (Budrugeac et al. 2003) which should facilitate identification of older material.

Deamidation is a key step in the processing skins for leather, as it increases the number of acid sites (at Asn and Gln residues) for the uptake of metal salts. Previous studies of deamidation have targeted the molar yield of nitrogen or the increase in the extent of racemization (Menderes et al. 1999). With no mineral fraction to protect the collagen helices, deamidation by side-chain hydrolysis during liming may be influenced differently as opposed to in bone collagen where deamidation proved to be correlated throughout the collagen molecule, even if the probability of deamidation

was different for each glutamine position. However, if it is successful it can potentially give an indication of how the leather was worked, which could be of value both to archaeologists and leather technologists.

## 6.6. CONCLUSION

Although there has been plenty of research on leather using DSC and even aDNA analysis, there is very little proteomics work done on tanned hide. However, the advantage of being able to investigate the collagen itself both for species identification and potentially an indication of the processing through deamidation makes proteomics an interesting venture for investigating materials made of hide.

1. ZooMS can potentially be used for identification of leather, as with most collagenous materials.
2. A temperature of 95°C for extraction proved ideal for extraction of the three tanning methods we investigated.
3. Cross-linking appears to inhibit collagen detection through MALDI-MS and further investigation will be needed for ZooMS to be most effective for leather identification.
4. Future work will include more stringent methods to remove tannins and dyes and generally improve the method for tanned materials and archaeological leathers specifically.

## 6.7. ACKNOWLEDGEMENTS

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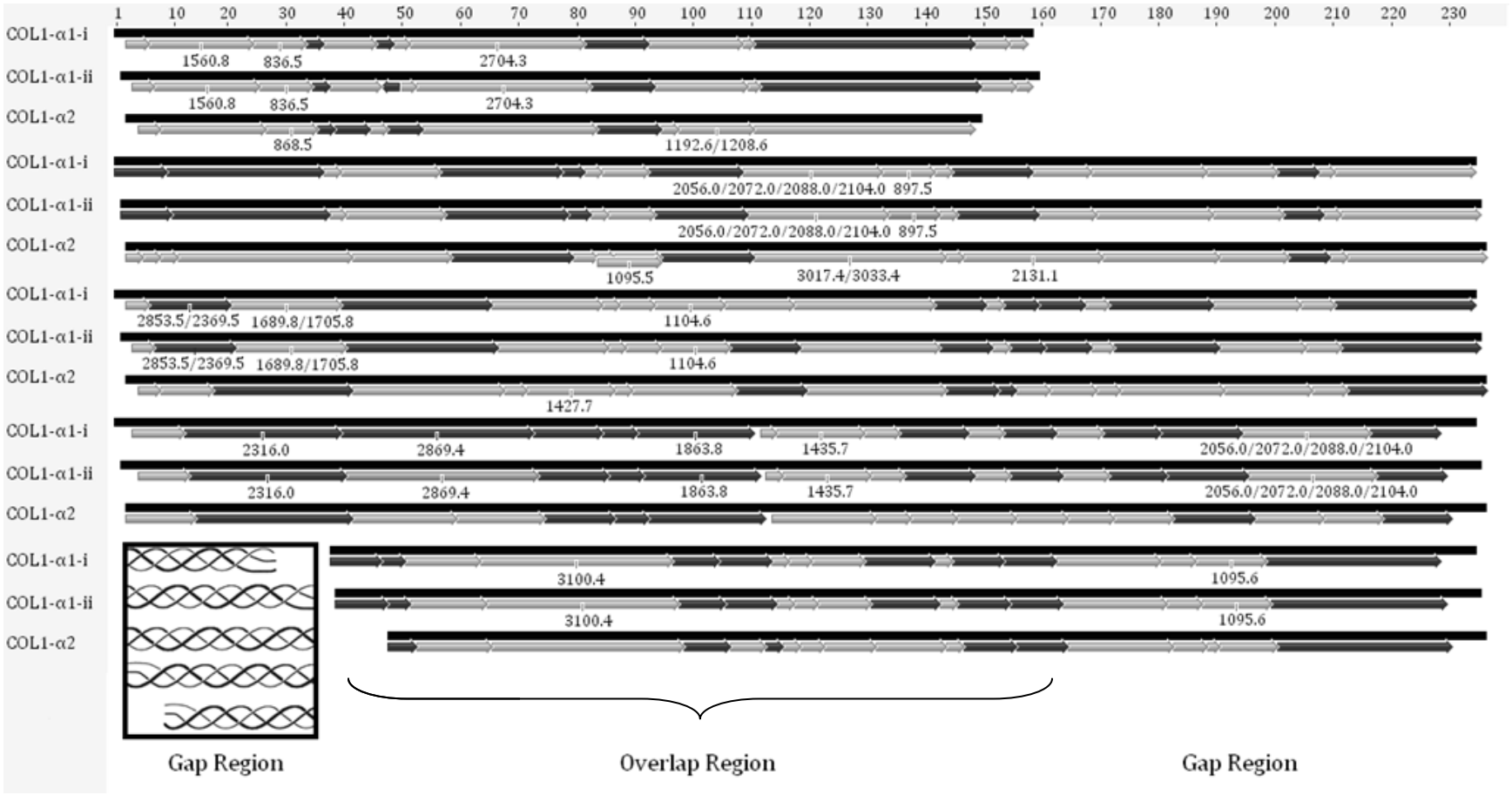


**Figure 6-8. A representation of a collagen "unit cell", the molecular building block of a collagen microfibril. Depicted are the staggered arrangement of five helices, each represented by the three subunits of two COL1-α1 strands and one COL1-α2 strand, showing the position of the gap region and the overlap region. The unit is shown so that no part of the original collagen sequence repeats other than in the COL1-α1 strands. The above unit is separated to show all theoretical trypsin cleavages (arrows: dark = lysine-truncated; light = arginine-truncated) and our most commonly observed peptides in cow (*Bos taurus*). The x-axis denotes the number of residues, the y-axis denotes the collagen subunits. Inset: schematic illustration of the "unit cell".**

# Chapter 7. ZOOMS: DETECTION OF ANIMAL ORIGIN OF MEAT AND BONE MEAL IN SINGLE PARTICLE ANALYSIS

Submitted to *Trends in Food Science & Technology*

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Keywords: MALDI-TOF-MS, Collagen, Gelatine, Meat and Bone Meal, single particle analysis, species identification

## 7.1. ABSTRACT

Identification methods of the animal origin of meat and bone meal are important as they potentially support the relaxation of the Extended Feed Ban which has been in place since the first confirmed case of BSE in Europe in 1986.

The high-resolution assessment method we propose builds directly upon the EU approved method of optical microscopy by subsequent decontamination and identification of individual bone fragments.

i. **Identification to genus** by examination of sequence variation in collagen, the protein which approximates 50% of the total volume of each bone fragment; due to the range of fish, birds and mammal collagen already sequenced, identifications do *not* require use of specific probes unlike both immunological and PCR methods,
ii. **Non-destructive analysis**, achieved by extraction of soluble collagen polypeptides using a mild buffer which leaves the particles intact, thus

rendering the particles suitable for subsequent analyses, e.g. DNA, amino acid or mineral analysis.

iii. **Low-cost high throughput** enabling 1,000s of particles to be analysed per day at a cost of <€15 per particle.

iv. **Robust detection** after rendering feeds at temperatures beyond the maximum limit set by regulatory authorities. We can detect animal origin of gelatin and of peptides which persist in amino acid hydrolysates.

v. **Detection of rendered material** by mapping of chemical damage on extracted peptides, enabling the discrimination of rendered and non-rendered material e.g. rodent bone in root vegetables, mammal bone in fish meal and high-temperature ruminant and porcine bone rendering.

## 7.2. INTRODUCTION

Current legislation (EC Regulation 999/2001) and amending regulations (EC Regulation 1292/2005, "extended feed ban") prevent certain use of meat and bone meal (MBM) in animal feeds due to risks of crosscontamination, notably following the outbreak of Bovine spongiform encephalopathy (BSE) in the UK in 1986, and it is further forbidden to feed proteins back to the same species (EC Regulation 1774/2002).

Thus far, the only method which has met with regulatory approval is microscopic analysis of bone particles (Commission Regulation (EC), 152/2009). Particles can be readily sedimented from meat and bone meal (MBM), and the method is sufficiently sensitive to police the 0.1% limit set by the EU authorities (EFSA Panel on Biological Hazards (BIOHAZ) 2011). Microscopy can discriminate terrestrial from non-terrestrial bone (i.e. fish) (van Raamsdonk et al. 2004; Fumière et al. 2009; Pinotti 2009), and has enabled some lifting of restrictions, such as the re-admittance of fish meal as a milk replacer for juvenile ruminants (EC Regulation 956/2008).

A wide range of alternative methods has been proposed. Dipstick immunological methods have been developed which can detect the presence of ruminant protein (von Holst et al. 2006; Karanam 2011). However, these cannot be used to assess the level of contamination. Tests that detect the animal origin of particles have been explored, including PCR on single granules (Fumière et al. 2010), Near Infrared (NIR) Spectroscopy (de la Haba et al. 2007), and protein mass spectrometric detection of osteocalcin (Balizs et al. 2011); for a review see van Raamsdonk et al (2007). Some of

these methods are compromised if the material has been rendered at a high temperature.

The processing of MBM, which must legally be treated for a minimum of 20 minutes at 133 ˚C and 300 kPa, works *against* most methods of detection (with the exception of microscopy methods), which are refined variants of techniques developed to identify untreated animal tissues and requires sufficiently sensitive analysis (DNA, immunology) or sufficiently heat stable targets (immunology) to capture a signal. Treatment at temperatures higher than 133 ˚C can destroy these targets (Frezza et al. 2003). Furthermore, these methods are not designed to recognise species accidently incorporated into MBM (e.g. vermin or marine mammals), as primers and antibodies need preparation in advance.

Our approach stems from techniques designed to detect ancient proteins, specifically a non-destructive method suitable for precious archaeological artefacts, which involves soaking in a mild buffer (van Doorn et al. 2011) and which extends a 96 well plate format of ZooMS that we developed to rapidly identify the genus of small fragmentary archaeological fish remains (Richter et al. 2011).

We make no selection as to target, but rather try to build collagen libraries of the vertebrate kingdom, and exploit the heat treatment to generate targets (soluble peptides) of the main organic constituent of bone (type I collagen). Bone collagen does not completely gelatinise at temperatures below 155˚C (Kronick & Cooke 1996), but the proportion of denatured collagen increases with heat

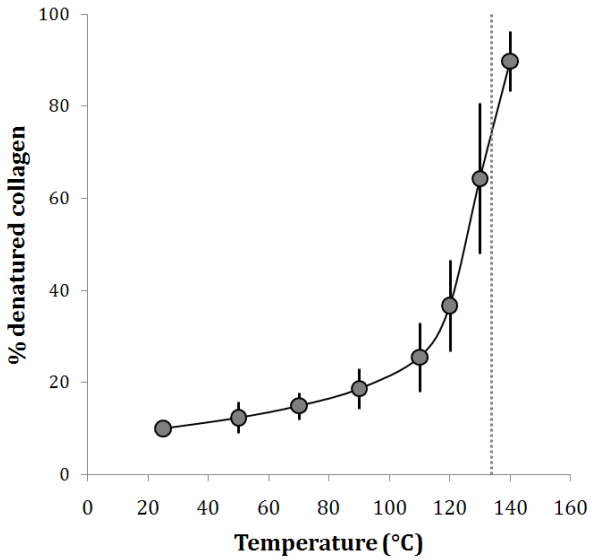


**Figure 7-1. Effect of heating (for 4 hours) on the generation of denatured collagen as detected by sensitivity to chymotrypsin (Li et al. 2003). The legally required treatment temperature of 133 ˚C is indicated (dotted line).**

treatment (Li et al. 2003). Thus the higher the temperature of MBM processing, the more soluble collagen is detected.

The increased release of soluble collagen with increased temperature is consistent with the polymer-in-a-box model of collagen survival (Miles & Ghelashvili 1999), but with a number of different "boxes" reflecting different degrees of hydration and mineral constraint. Heat treatment therefore generates soluble collagen, which we extract by a combination of heating at 65 °C and leaching.

Without an *a priori* selection of targets (as would be done in current PCR protocols for detection) the method is able to detect any bone fragment by extraction of soluble collagen, thus the range of species is wide (Buckley et al. 2009) and getting wider (Richter et al. 2011).

The method is specifically developed to work on small bone fragments and scaled for high-throughput analysis. We are developing sequence libraries to cover all commercial species (at present our databases includes partial collagen (type I) sequences from 54 mammals, 43 birds, 19 fish, 4 amphibians and 2 reptiles) and even if a species is not present in the database, the nearest member can be identified through the peptides present, identification to the level of phylogenetic family is possible.

## 7.3. Materials and Methods

All test material was prepared by VLA Luddington (UK).

### *7.3.1. Introduction to the Sample Sets*

As part of the EU STRATFEED project, a protocol was drawn up to ensure the quality, origin, and processing details of animal feeds would be fully documented and supported by European Fat Processors and Renderers Association (EFPRA). MBM samples, so-called "Set B-EFPRA" (Garrido-Varo et al. 2005), were produced in a pilot plant owned by Prosper de Mulder (UK) where batches of cattle, sheep, pig and chicken meals were ground through a 50 mm plate grinder and heated in an oven at 100°C. The cooker was vented for 10 min and the temperature raised to one of the focus test temperatures (133°C, 137°C, 141°C or 145°C), under 3 bar pressure. After 20 minutes, the pressure is released, and when the moisture level of 5-10% was reached, the MBM was discharged from the cooker, pressed and ground. Temperatures were raised during grinding but these temperatures were not monitored; on one occasion, a sample (of avian MBM) caught fire in the drying shed

after grinding. This resulted in the creation of a set of 16 standard samples (MBM of four species rendered at four temperatures) called Set B-EFPRA (Garrido-Varo et al. 2005), which have recently been characterized (Buckley et al. 2012).

### *7.3.2. MEAT AND BONE MEAL STANDARDS (UNIFORM SAMPLES)*

Negative feed matrices made from plant material (free from animal material, confirmed by PCR and ELISA) were contaminated with defined concentrations of MBM. The spiked feeds were then sedimented, sieved (>250 μm) and the bone fragment were picked out and sent for testing. In total, thirty-one (31) batches, each containing single species material (or protein-free material similar to bone) were analysed by ZooMS.

### *7.3.3. SIMULATED SAMPLES OF MIXED FEEDS*

Six sediments from animal feed samples (either petfood or compound feeds) were also analysed. Animal origin was determined by microscopic analysis test (MAT), enzyme-linked immunosorbent assay (ELISA) and PCR (see detailed contents in Table 7-1), prior to ZooMS analysis.

The results are based on either ELISA data (for samples dating before 2006) or PCR (for samples post-2006). ELISA could only identify "ruminant" and "porcine" categories, whereas PCR can identify bovine, ovine, porcine and avian species. Fish samples were determined using microscopy.

**Table 7-1. Details of submitted feed samples used in the study**

| No. | Sample | Detailed contents (ELISA, MAT and PCR) | Particles received |
|---|---|---|---|
| **A** | Petfood sample | Ruminant & Avian | 8 |
| **B** | Field sample | Bovine and fish | 10 |
| **C** | Petfood sample | Avian | 6 |
| **D** | Petfood sample | Ruminant & Porcine | 7 |
| **E** | Field sample | Bovine and Avian | 12 |
| **F** | Petfood sample | Ruminant (bovine?) | 7 |

All samples were processed into pellets or dried kibble and are representative of submitted animal feeds. The field samples are collected from mills in the UK and represent materials that would be sent to a lab for quality control. Using field samples exposes the test to a wider range of materials and processing conditions than if samples were made from composite materials. Each sample was processed similar to

the spiked feeds described above, i.e. pellets were ground, sedimented and sieved to obtain large (>250um) fragments for analysis.

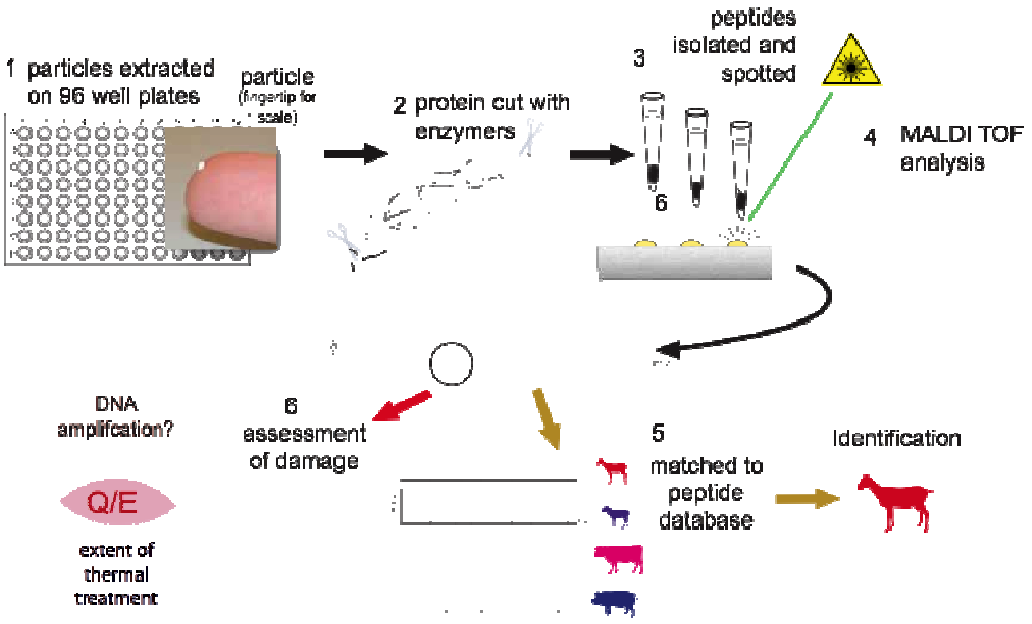


**Figure 7-2. Schematic overview of the ZooMS method. Samples are (1) extracted by heating at 65 °C in an ammonium bicarbonate buffer, pH 8.0; the extract is (2) cut into peptides which are (3) spotted with a matrix onto a target plate. The masses of the peptides are measured following desorption/ionisation of the sample using laser energy using MALDI-TOF-MS (4), the peptide masses detected by time-of-flight (TOF). The detection of specific peptides (5) are used to identify the animal, whereas degradation of individual peptides (6) may potentially be used to estimate thermal treatment and (ideally) the likelihood of DNA amplification.**

### *7.3.4. EXTRACTION AND ANALYSIS*

#### 7.3.4.1. Ammonium bicarbonate buffer extraction

Individual bone fragments were incubated twice each for an hour in 50 mM ammonium bicarbonate buffer (pH 8.0) at 65°C in a 96-well microplate format. The second incubation extract was digested overnight at 37°C with 0.4 µg sequencing grade modified porcine trypsin (Promega,Southampton, UK).

#### 7.3.4.2. Peptide purification by solid phase extraction

The tryptic digest was purified over a multiwell C18 column (Porvair, Leatherhead, UK) to desalt and concentrate peptides by washing with 0.1% trifluoroacetic acid (TFA). Peptides were eluted in a volume of 10 µL of 50% acetonitrile (ACN) / 0.1%

TFA (v/v). 1 µL of eluate was mixed on a ground steel plate with 1 µL α-cyano-4-hydroxycinnamic acid matrix solution (1% in 50% ACN / 0.1% TFA (v/v/v)) and air dried.

#### 7.3.4.3. Mass spectrometric analysis

Each sample was analysed in reflector mode using a calibrated Ultraflex III (Bruker Daltonics, Bremen, DE) MALDI-TOF instrument to measure mass to charge ratios (*m/z*) of trypsinated fragments. Spectra were analyzed using flexAnalysis software v. 3.0 (Bruker Daltonics) and mMass (Strohalm et al. 2010).

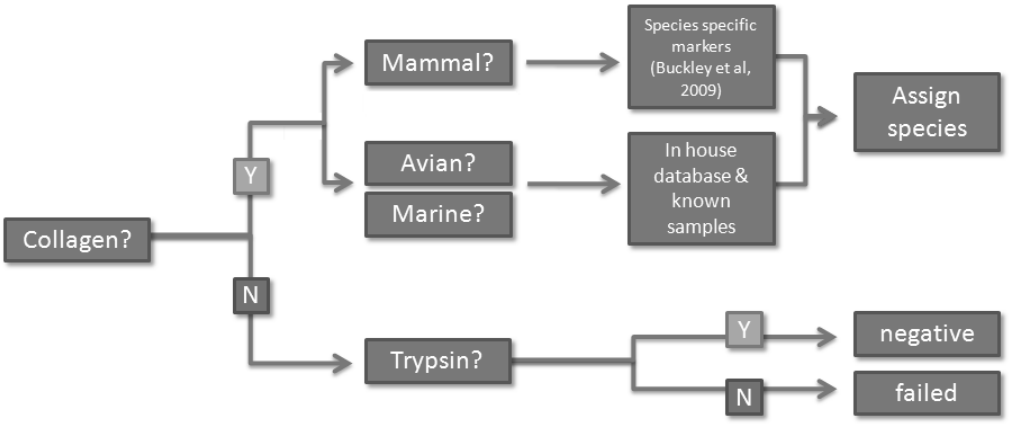


**Figure 7-3. Flowchart for determining species by ZooMS. Currently the method is only equipped to identify animal origin if collagen type I is present, hence if the dominant signal is not collagen or trypsin, it will be regarded as unsuitable for identification through ZooMS.**

### *7.3.5. IDENTIFICATION OF SAMPLES*

Manual assignment was performed according to the flowchart in Figure 7-3. The presence of collagen was determined by the presence of m/z values that correspond with peptides conserved throughout species. If collagen markers were not detectable, presence of m/z values typical of trypsin could indicate that the level of collagen would be below that of trypsin (8 pg per spot) and therefore classified as negative for collagen. If neither assessment could be made, the sample was considered "failed" and included in a rerun.

If collagen markers were present, taxon-specific markers were investigated. Characteristic peptides were identified manually from straight MS compared to those published in Buckley *et al* (2009) for mammalian taxa and comparisons to *in silico* digests of translated cDNA sequences when available (avian and fish peptides).

It should be noted that the icefish samples were not identified through a first designation as these collagen sequences were not part of our database or in our reference collection. However, the spectra for this species were found to be quite distinct and these were relatively easy to identify.

In addition, a method was devised for automatic assignment of spectra through recognition of peptides compared in an in-house database; internally calibrated spectra were then matched using the MASCOT search engine to retrieve the most likely hit in our database through probability-based scoring. Most sequences in our database are based on (modified) genomic sequences, translated into protein sequences and digested *in silico*.

Peaks with a signal-to-noise ratio of below 5 were ignored before database matching. Search criteria in MASCOT allowed for 1 missed cleavage, variable deamidation of glutamine and asparagine, and hydroxylation of proline and lysine. Mass tolerance was set at 50 ppm. This in-house database contains collagen type I sequences from run standards and theoretical peptide fingerprints derived from translated cDNA sequences digested *in silico*.

## 7.4. RESULTS

### *7.4.1. IDENTIFICATION OF BONE FRAGMENTS FROM UNIFORM SAMPLES*

We received thirty-one uniform sedimented MBM samples. These were all analysed per particle using our non-destructive protocol. Results for the identification of the individual bone fragments are shown in Table 7-2. **Expectation value** is "the number of matches with equal or better scores that are expected to occur by chance alone" (Matrix Science Ltd. 2012) and hence a lower value represents a more reliable hit. Assignment of "ruminant" to either ovine or bovine samples was considered high enough resolution for the purpose of this study. Often it was observed that several ruminant matches (cattle, sheep, elk, deer) were significant hits, although rarely *unique* significant hits.

Most samples could be identified both manually and through the database search and were in agreement with the given identification by the supplier. Interestingly, bovine material processed at 133 °C was positively identified and with less resolution at higher temperatures, whereas the reverse seems true for the porcine samples investigated here, which were rendered at 141 °C and positively identified.

**Table 7-2. Results of individual bone fragment identification analysis. The composition of each of these samples was known, and the results of manual designation (performed blind) followed by the designation by using our in-house database and a mascot search algorithm on the same spectra. Some samples were fully rerun (i.e. different particles) because poor spectra were obtained. Several positive samples were re-run as comparison.**

| No. | Composition | Manual[a] (*Rerun*) | MASCOT Search ID | Expectation value |
|---|---|---|---|---|
| 4 | Avian (141°C) | Avian | Turkey (*Meleagris gallopavo*) | Expect: 0.56 |
| | | | Chicken (*Gallus gallus*) | Expect: 1.2 |
| 3 | Bovine (133°C) | Bovine | Bovine (*Bos primiginius*) | Expect: 4.1e-05 |
| 15 | Bovine (133°C) | Ruminant (*Bovine*) | Bovine (*Bos primiginius*) | Expect: 0.00054 |
| 27 | Bovine (133°C) | Bovine | Bovine (*Bos primiginius*) | Expect: 5.6e-08 |
| 28 | Bovine (133°C) | Bovine (*Bovine*) | Bovine (*Bos primiginius*) | Expect: 2.6e-06 |
| 34 | Bovine (133°C) | Bovine | Bovine (*Bos primiginius*) | Expect: 5.4e-07 |
| 47 | Bovine (133°C) | Bovine | Bovine (*Bos primiginius*) | Expect: 5.6e-09 |
| 50 | Bovine (133°C) | Bovine (*Bovine*) | Bovine (*Bos primiginius*) | Expect: 1.4e-07 |
| 10 | Bovine (141°C) | Ruminant (bovine?) | Bovine (*Bos primiginius*) | Expect: 0.00024 |
| 44 | Bovine (141°C) | Ruminant | Elephant (*Loxodonta africana*) | Expect: 0.017 |
| 5 | Common dolphin | Porcine? | Clawed frog (*Xenopus*) | Expect: 26 |
| 6 | Ice fish | Fish | Fallow deer (*Dama dama*) | Expect: 0.28 |

| | | | | |
|---|---|---|---|---|
| 17 | Ice fish | Fish | Rainbow trout (*Oncorhynchus mykiss*) | Expect: 0.25 |
| 20 | Ice fish | Fish | Flounder (*Paralichthys olivaceus*) | Expect: 3.5 |
| 36 | Ice fish | Fish | Pufferfish (*Tetradon*) | Expect: 1.1 |
| 49 | Ice fish | Fish | Flounder (*Paralichthys olivaceus*) | Expect: 2.4 |
| 25 | Mineral (calcium carbonate) | Bovine | Sika (*Cervus nippon*) | Expect: 0.011 |
| 2 | Ovine (133°C) | No identification; haemoglobin | No identification | - |
| 11 | Ovine rib | Ruminant (bovine?) | Fallow deer (*Dama dama*) | Expect: 9.4e-05 |
| 12 | Ovine rib | Ovine + porcine | Ovine (*Ovis aries*) | Expect: 8.9e-08 |
| 13 | Ovine rib | Porcine? | Dolphin (*Tursiops truncatus*) | Expect: 1.3e-05 |
| | | | Ovine (*Ovis aries*) | Expect: 0.0001 |
| 14 | Ovine rib | Ruminant | Bovine (*Bos primiginius*) | Expect: 2.6e-05 |
| | | | Fallow deer (*Dama dama*) | Expect: 3.8e-05 |
| 40 | Ovine rib | Ruminant? | Irish elk (*Megaloceros giganteus*) | Expect: 1.9e-06 |
| | | | Ovine (*Ovis aries*) | Expect: 6.9e-06 |
| 7 | Porcine (133°C) | Bovine (*Porcine*) | Bovine (*Bos primiginius*) | Expect: 3e-06 |
| 39 | Porcine (133°C) | - (*Porcine*?) | No identification | - |
| 8 | Porcine (141°C) | Porcine | Porcine (*Sus scrofa*) | Expect: 0.0011 |

| | | | | |
|---|---|---|---|---|
| 32 | Porcine (141°C) | Porcine | Porcine (*Sus scrofa*) | Expect: 0.00047 |
| 9 | Sea lion | Porcine? | Ferret (*Mustela putoriusfuro*) | Expect: 2.8e-09 |
| 29 | Sea lion | Porcine? | Ferret (*Mustela putoriusfuro*) | Expect: 3.8e-06 |
| 1 | Shell | - (*Ruminant?*) | No identification | - |
| 48 | Shell | - (*Trypsin*) | No identification | - |

[a] Dark grey — all correct, Light grey — correct but unsure, Black — incorrect, White — no identification or no database matches. (*Italics*) — resampled and analysed in follow-up. * — significant. (n.s.) — not significant.

Sea mammals appear difficult to recognise. This is not only due to an overall poor signal from these samples, but also a lack of standards in our database. The sealion, was identified as a mustelid, the nearest carnivore to the pinnipeds in our database. With knowledge that the sample had been obtained from fishmeal we would have inferred a pinniped, but without seal and sealion sequences we could not improve upon this identification. Curiously, dolphin was matched with *Xenopus* (80% pairwise identity) despite the presence of a genomic sequence of common dolphin (*Tursiops truncates*) in our database.

Several samples were re-run with new particles from the same batch (1, 2, 7, 14, 15, 28, 39, 48 and 50) after manual designation was performed, based on poor results, which in some cases proved to be an improvement (sample 7 did not contain porcine identifiable peaks in the first run). In most this established the exact same result as the original analysis. However, the original signal of sample 7 appeared ruminant. The re-run on another particle was identified as porcine. This is an example in which we were most likely able to detect single particle contamination (marked with an asterisk in Table 7-3). Arguably, a similar statement can be made for sample 13 and a mixed signal was observed in sample 12.

Aside from samples that appeared to contain crosscontamination (7, 12 and 13), background signals may have dominated over what should have been a trypsin dominant (or blank) spectrum (1 and 25). Sample 2 showed degradation products of haemoglobin, indicating that the sample may have been too far degraded to pick up

any meaningful peptides or have established a pattern that does not typically match with those in our database.

**Table 7-3. Descriptive summary of individual bone fragment identification analysis. Presented are the percentage and number of samples (n, with the total number of samples in brackets) which were identified in agreement with the provided animal origin by the supplier, which were in disagreement and which did not yield an identification against the results given based on PCR data. Compared are manual identification versus identification through the database algorithm.**

| | Manual | | Automatic | |
|---|---|---|---|---|
| | % | n | % | n |
| **Agreement** | **71.0** | **22-24 (31)** | **74.2** | **23 (31)** |
| Terrestrial material | **75** | **15-17 (20)** | **75** | **15 (20)** |
| ***Ruminant*** | 80 | 12 (15) | 80 | 12 (15) |
| ***Porcine*** | 50-100 | 2-4 (4) | 50 | 2 (4) |
| ***Avian*** | 100 | 1 (1) | 100 | 1 (1) |
| Marine material | **62.5** | **5 (8)** | **75** | **6 (8)** |
| ***Fish*** | 100 | 5 (5) | 80 | 4 (5) |
| ***Sea mammal*** | 0 | 0 (3) | 66.7 | 2 (3) |
| Other | **66.7** | **2 (3)** | **66.7** | **2 (3)** |
| **Disagreement** | **19.4** | **6 (31)** | **19.4** | **6 (31)** |
| Terrestrial material | **10** | **2 (20)** | **15** | **3 (20)** |
| ***Ruminant*** | 6.7 | 1 (15) | 13.3 | 2 (15) |
| ***Porcine*** | 25 | 1* (4) | 25 | 1* (4) |
| ***Avian*** | 0 | 0 (1) | 0 | 0 (1) |
| Marine material | **37.5** | **3 (8)** | **25** | **2 (8)** |
| ***Fish*** | 0 | 0 (5) | 20 | 1 (5) |
| ***Sea mammal*** | 100 | 3 (3) | 33.3 | 1 (3) |
| Other | **33.3** | **1 (3)** | **33.3** | **1 (3)** |
| **Unable to detect** | **9.7** | **3 (31)** | **6.5** | **2 (31)** |

* Although this particle was officially described as originating from a porcine sample, the presence of clear bovine peptides suggests that this sample was either mislabelled or contained bovine contamination and was therefore misclassified.

Table 7-3 gives an overview of the species designation as given in Table 7-2. Percentages represent how many samples we could positively identify, those which were identified incorrectly and those which we were unable to detect due to poor spectrum quality (note that this differs in manual from automatic — signals that may be suitable for manual designation can be too low in intensity or contain masking degradation products for the search algorithm to pick up).

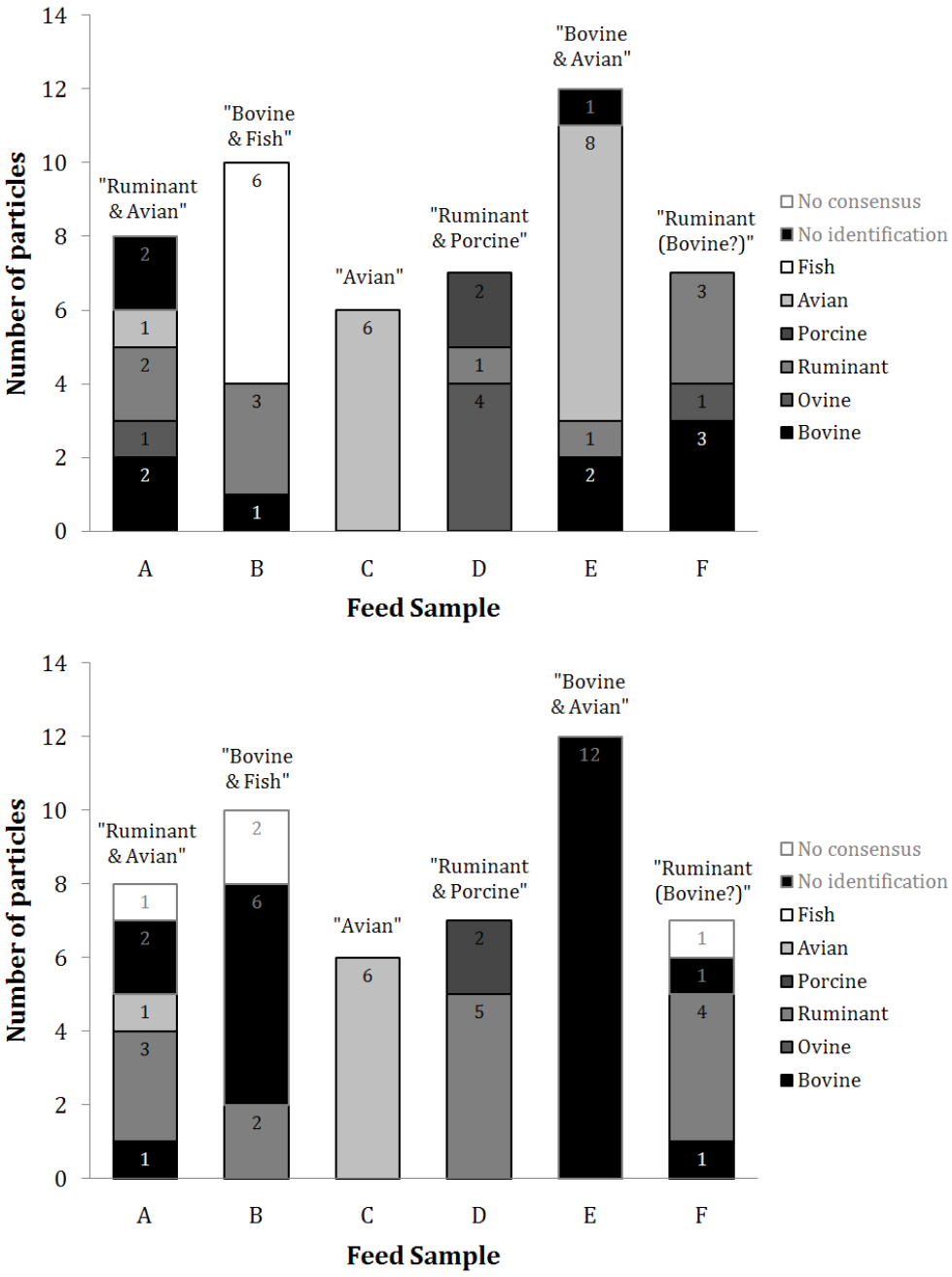


**Figure 7-4. ZooMS single-particle analysis of animal feeds (with the manufacturer's identification revealed above bars). Manual (above); automatic (below). Note that the ZooMS single-particle analysis samples B, C and E agree with assignments, but in the case of samples A, D and F additional information is provided about the source of the ruminant protein than is supplied by combined information provided by immunology, PCR and the manufacturers. "Ruminant" granules indicate that in these particles we failed to detect peptides that could be used to discriminate sheep from cow specifically with reasonable**

**certainty (see Buckley et al (2009)). "No identification" was assigned when identification could not be made or the database could not find a confident fit. "No consensus" indicates samples where three technical replicates contradict in their results. Positive identifications are indicated with a black outline and black/white lettering; "no identification" and "no consensus" are indicated with a half-tone outline and lettering.**

Assignments were made primarily to be species specific, although "ruminant" for either bovine or ovine samples was considered acceptable, "avian" for chicken and turkey, as was any type of fish for the designation "fish" even if the exact genus could not be determined or asserted.

### *7.4.2. IDENTIFICATION OF BONE FRAGMENTS FROM MIXED FEEDS*

We analyzed six representative samples of animal feeds blind and identifications were made on single particles from each feed sample, again approached with manual and digitized identification.

Although the exact composition of the samples was unknown in these cases, all results are consistent with the source material. Again, "ruminant" was considered an acceptable answer if ovine or bovine specific markers could not be detected, as was "avian" for turkey or chicken matches and "fish" for any fish species.

In the case of automatic designation, samples from feed B and E consistently failed to give any result. It is possible that the specific rendering of these feeds (both are field samples retrieved from operative mills in the UK) produced masking peptides and has prevented identification. Collagen peaks were, however, not absent, allowing for manual assignment. This rendering may also be the cause of why no fish material could be assigned, even though matches were found in the individual samples (Table 7-2).

## 7.5. DISCUSSION

Like microscopy and NIR, ZooMS allows for single-particle sample analysis. ZooMS has been designed specifically around the properties of bone collagen and the sensitivity of this generation's mass spectrometers, and works well on the sedimented fraction of PAP feeds.

ZooMS can accomplish a high resolution in species identification without loss of sample integrity. Terrestrial animal collagen signals are easy to distinguish from fish. Porcine and avian material can be distinguished from ruminant and in many cases

bovine signals can be distinguished from ovine. Even if this resolution is not always required, it does add to the certainty of the identification and if not all markers are visible, some distinction can usually be made (i.e. mammalian and ruminant collagen markers may be available even when species specific markers are not).

The ability to conduct single-particle analysis as a direct follow on from microscopic detection (unimpaired by the reagents used in sedimentation processes) means that the method can readily match the required 0.1% detection limit set by the EU authorities. In our hands, the ability to rapidly measure each particle combined with a 95% success rate (all samples, including resampling) enables us to dissect feed samples to a very high accuracy.

One main advantage of ZooMS is that by extraction of collagen from bone, the mass spectrometer measures peptides that are actual constituents of the bone sediment. This material is measured "as is" and does not require amplification, reducing the risk of contamination or masking signals. Mixed signals can be detected (for example the case of a porcine particle with bovine peptides).

The procedure is fast and allows for high-throughput of samples. The move away from user to MASCOT identification means that the method is becoming both more automated and less biased. There is no primer design or antibody preparation required in anticipation of what samples are expected, so samples can be approached blind without additional rounds or experiments to determine the animal genus. The *same* samples can be revisited afterwards with DNA primers for confirmation if so required, as the method is non-destructive and has been shown on archaeological material to not remove DNA to sub-amplifiable levels (von Holstein et al. submitted).

Measuring collagen directly has added potential. We have made initial investigations that indicate that glutamine deamidation can be used to investigate thermal history for archaeological bone (explored in Chapter 4). As of now, we do not have consistent results that show that the same applies for artificially heat-degraded material, but this may be an option in the future so that rendering at high temperatures may be detected and the likelihood of DNA survival can be further estimated.

As we build our databases, confidence in specific identifications will increase. For example, an archaeological focus on deer sequences means that these are overrepresented in the current database. This explains why ruminant samples with not enough diagnostic peptides present may be identified as deer (e.g. top-hits for sika

and fallow deer in this study). Examination of the output means that all ruminants in the database score strongly. However in such cases, the output would highlight the broad identity (ruminant), and a more experienced user could then investigate the spectra to assess which (if any) of the specific peptides used to discriminate the domestic ruminants (goat, sheep or cow) were present.

A more prevalent contamination issue surfaces when regarding the sensitivity of the mass spectrometer which may give rise to false positives. Trace contamination of collagen in non-collagenous material may be detected as a positive identification; note the combined signal in sample 12 and the limestone (calcium carbonate) sample (25) that should be protein free. Intensity of signal is not necessarily an indicator for material prevalence either, as there are multiple factors that can suppress overall signal intensity (see Chapter 5). However, collagen contamination does not occur as freely as with nucleic acids. Therefore, it is surmised that this is not a high-potential risk, but further research with larger sample sizes could give a clearer indication and cut-off threshold for what could be considered an actual false positive, i.e. when is detected and identified collagen above the set legal threshold of 0.1% (ruminant) MBM in feeds. MALDI-TOF-MS does not readily allow for absolute quantification (there are approaches for relative quantification using internal standards) and positive identifications of ruminant material above the threshold may have legal repercussions for the manufacturer.

With database matching, we showed that our methods overall are able to assign the correct category to each sample that has a positive identification, and ruminant material can be separated from others. The confidence of the assignment is largely based on the size and variety of the database. However, the database is being actively expanded with all samples that are run and in addition to possible damage markers through glutamine deamidation values, any surplus of generated peptides of markedly rendered material can also be added to the database, thereby enhancing the ways to detect feed treatment.

## 7.6. CONCLUSIONS

As we have shown, ZooMS provides an easily implemented approach that can identify a wide range of samples to a (required) genus level and often higher, and can be used to augment conventional microscopy methods for identifying the species provenance of bone fragments in animal feeds. We were able to positively identify 70–75% of all individual bone fragments. In addition, *all* positively identified material from the

submitted animal feed samples was in agreement with what the contents were reported to be by the official feed preparation laboratory. The database that is currently used for genus assignment is in perpetual construction, but by expansion will be able to relieve the bias that currently exists in both manual and automatic assignment.

We suggest that ZooMS is a valid method to augment conventional microscopy in the inspection of animal feeds containing meat and bone meal. ZooMS is cost-effective and allows for a fast high-throughput approach and also for the same samples to be revisited with alternative methods such as PCR.

## 7.7. ACKNOWLEDGEMENTS

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# Chapter 8. FINAL CONSIDERATIONS

## 8.1. SYNTHESIS OF FINDINGS

The first and main goal of this study was to develop a method that could extract collagen for MALDI-TOF based ZooMS analysis in as few stages as possible, with the intent of scaling the method towards a high-throughput format rather than handling and extracting each sample differently. Surprisingly, one of the first and simplest attempts succeeded in doing so, yielding collagen type I peptides in mass spectra after extraction in ammonium bicarbonate buffer (Chapter 3). This enabled a simplification of the ZooMS protocol, modified to a 96-well format for most steps (Richter et al. 2011). This format will be further developed and eventually automated.

As the demand for the buffer-only method increased, it was questioned whether this new approach worked equally well on all bone, even strongly deteriorated bone material, or even on other collagenous materials. To date, extractions from modern bone (Chapter 7), well-preserved archaeological bone from a wide range of sites (Chapter 4), leather and parchment (Chapter 6) and antler (von Holstein et al. submitted) have been successful.

This study initiated the examination of the potential of mass spectra as diagenesis proxies, gaining the most information from a single spectrum without a need to modify the protocol. We succeeded in assessing glutamine deamidation in bone samples and have found it to be consistent and a possible indicator of thermal damage, even if little functional correlation with DNA, histological and collagen quality criteria was found. Rather than linked to other degradation processes in bone, intact collagen appears to "record" thermal damage through the deamidation of glutamine.

## 8.2. CONCLUSIONS

This study has clearly illustrated that ZooMS can be very efficiently performed on modern and archaeological bone without decalcification. As Rohland and Hofreiter (2007) have observed for DNA, the simpler method appears to function well for collagen, at least for well-preserved mineralized tissue (bone, dentine, antler).

The ammonium bicarbonate buffer method is minimally destructive: it alters neither the mineral phase, nor the microscopic surfaces of worked bone artefacts measurably. However, the method could "damage" archaeological bone material that has been

recovered from acidic burial environments from which the protective mineral fraction has been leached away and the collagen is exposed to gelatinization.

There are only minimal differences between samples extracted with or without decalcification, although decalcified samples tend to yield more peptides overall. The buffer extraction has its limits and, curiously, does not always perform evenly even on samples from the same site. The case study from Wangels as shown in Chapter 5 may be an exception where site variability within the burial environment and reactive humic substances in the soil may have altered the bone collagen so that denaturation at 65°C yields insufficient peptides for analysis. Although, failure of the samples from Wangels may be because other components do not allow peptides or the matrix to properly co-crystallize or ionize and thus inhibit MALDI spectra.

In extension of that observation, though peptide mass fingerprinting of leathers was reasonably successful, vegetable tannages are problematic to collagen detection and may even target collagen type I specifically. The absence of peptides larger than 2 kDa (approximately 20 residues) has been observed in a variety of samples, from leather to (possibly degraded) archaeological bone. One explanation is that the likelihood of incorporation of cross-links increases for longer peptides, which would prevent them being released by trypsin digestion. However, if this were true we would expect to see the loss of >2kDa peptides more clearly in vegetable tanned than in chromium tanned leathers (vegetable tannins should target the lysine side-chain). We see little difference in the loss of >2kDa peptides, suggesting that the loss of longer peptides may require an alternative explanation.

We retrieve taxa identification from leather, parchment, sedimented bone particles in meat and bone meal, archaeological bone and antler with very similar protocols of extraction using an ammonium bicarbonate buffer without any prior treatments such as decalcification or removal of tannins.

Glutamine deamidation in nearly one thousand individual bone samples (including biological replicates; ~300 unique samples) has shown that there is a clear correlation with burial temperature (hence *thermal* age). However, it became equally apparent that several common indicators of quality for bone and collagen don't show a similar strong relationship.

With the exception of thermal age, relationships between %Gln content and established diagenesis proxies are similar: %Gln is variable for well-preserved

samples, and only very degraded samples have very low (<30) %Gln. These observations would be consistent with our proposed idea in Chapter 5 that Gln deamidation in helical collagen occurs by side-chain hydrolysis and its main determinant is temperature. In degraded bone, loss of collagen packing enables imide-mediated deamidation to also take place, accelerating the fall in %Gln.

## 8.3. FUTURE WORK AND CONSIDERATIONS

### *8.3.1. IMPROVEMENTS TO THE EXTRACTION*

There are options for improving the ammonium bicarbonate buffer extraction and although none are presented in this study for the sake of comparison between sample extractions, some of these have been explored. In Chapter 3 we introduced the method using two heating periods of both 65 °C. This was initially based on our observed results that extraction at 65 °C gained overall higher intensities of peptides investigated than at 80 or 95 °C. Using a Bradford Assay, a spectroscopic method to monitor the amount of protein, it was noted that the supernatant from the first hour extraction would contain more proteinaceous material than the second extract (Chapter 3). This is not observed in our mass spectra, where the intensities of second hour extracts are much stronger and we have theorised that this is because other substances interfere with peptide ionization and are filtered out through the extraction process to a lower level that allows peptides to ionize to an acceptable level.

The first extraction would therefore only need to remove these hydrophobic substances, which might occur at lower temperatures at which the protein is not yet extracted. Washing with organic solvents is an option, but would complicate the simple set-up of the buffer only method to obtain a result similar to soaking a bone sample for an additional hour in ammonium bicarbonate buffer. However, for individual samples expected to contain a high amount of hydrophobic molecules, adding an extraction step (using dicholoromethane for example) can substantially aid cleaning prior to protein extraction.

In pilot tests it was found that a 24 hour soak at 4 °C followed by a 1 hour extraction at 65 °C had stronger spectra than the studied protocol of 2 hours at 65 °C. Whereas the temperature difference should not carry a strong influence on, for example, our glutamine deamidation data as the energy transferred in the extraction process might still be too low (see Chapter 6; also Araki & Moini 2011; Leo et al. 2011)), it may be more beneficial to follow-up studies (especially those concerning DNA) to expose

samples to temperatures as low as possible. Although it should be noted that soaking for 24 hours may compromise the chance of retrieving DNA after ZooMS analysis as it can disperse into the supernatant of bone extractions (Campos et al. 2011). That said, the method allows adjustments regarding the intent of applying ZooMS (genus identification or glutamine deamidation) and the intent of the sample afterwards (DNA analysis, stable isotope analysis, conservation) if the same sample is to be used for such a follow-up analysis.

### *8.3.2. PROCESSING OF MS DATA*

The ammonium bicarbonate buffer extraction enables ZooMS analysis to be performed much faster than before, easily allowing close to a hundred samples to be run from extraction to data acquirement in no more than two days. However, this leads to a problem, we now also acquire many more spectra (especially as we routinely run each sample in triplicate). Analysing each spectrum by hand is slow; hence our description of the use of an in-house database for collagen sequences (Chapter 4) that will allow us to exploit a MASCOT-based search algorithm.

The study also highlights the limitations of the current database which is skewed towards certain clades, leading to a potential bias for species assignation. Chapter 7 showed that the database could use improvement, though the initial results were encouraging. The database could also be extended to include collagen type III for identification of hides, parchments and leathers (which comprise some 15% of collagen in these samples. Chapter 6 showed that we were unable to identify the samples that showed the typical spectra that Choudhury et al (2006) had identified as collagen type III.

### *8.3.3. LEATHER*

Originally, the tests of the ammonium bicarbonate buffer extraction on leather were performed to test the idea that crosslinks were the reason that longer (>2kDa) peptides were absent in the samples from Wangels (Chapter 7). It was possible to extract collagen peptides from tanned leathers with a simple protocol, but identification of animal origin in leathers may still improve if tannins (and dyes) are removed. These components may be interfering with peptide detection either by crosslinking or by suppressing peptide ionization. A similar removal of tannage may help future investigations of archaeological leathers, not only because archaeological leather is vegetable-tanned, but because the residual leather may comprise the collagen with the highest levels of tannage and may be difficult to remove after changes incurred over time by their environment (Reed 1972). Reports on the

thermal behaviour of aged leather suggests that extraction should be easier than of modern leather, because the resistance of collagen against heat declines over time (Budrugeac et al. 2003). However, we also observed that vegetable-tanned leather extracted at high temperatures (120 °C) lost its collagen (I) signal almost entirely (Chapter 6). Hence, removing the tannins prior to collagen extraction may be crucial.

In Chapter 6 it was also seen that several vegetable tanned leathers behaved differently when extracted at 120 °C (see table 6-1). Their extracts were clear, whilst most yielded a "milky" substrate. Nevertheless some of the "milky" extracts did yield collagen (I) peptides. The difference could be due to the type of vegetable tannins. As condensed tannins covalently link with collagen and hydrolysable tannins bind to collagen through hydrogen bonds (Covington 2009), it is possible that "milky" extracts were from hydrolysable tannins, while the clear extracts were from condensed tannins which did remain bound to collagen (I) similar to glutaraldehyde (which also has a clear supernatant) and therefore did not interfere with detection. Hydrolysable tannins usually consist of polyphenols, which are hydrophobic substances, and may therefore precipitate in an aqueous extract. Hydrolysable tannins cause a lower shrinkage temperature (<85°C) in collagen and the "tanning" reaction can be reversed (Covington 2009). In order to develop ZooMS analysis for archaeological leathers, a comparative study of different tannages would give better insight into what happens to collagen (I) prior to and during extraction.

### *8.3.4. DNA SURVIVAL AND DEAMIDATION*

DNA is known to deteriorate and fragment starting almost immediately after cellular death (Gilbert et al. 2003; Willerslev & Cooper 2005; Kaiser et al. 2008) unless extreme environmental conditions help to preserve it. DNA fragmentation can initially occur by lytic enzymes, released during autolysis of postmortem cells (Kaiser et al. 2008), and is mainly degraded by depurination, which then incurs damage to the DNA chain by disruption of the glycosyl bonds (Lindahl 1993; Hofreiter et al. 2001). Processes of deamination and hydrolysis further destabilize the DNA structure, leaving it markedly unstable and resulting in further fragmentation (Hofreiter et al. 2001). DNA also has a thermal age limit, unlikely to survive in hot and humid environments, whereas permafrost and arid conditions are more likely to yield any DNA of reliable quality.

A number of studies have drawn similarities between DNA and protein degradation (Bada et al. 1999; Poinar & Stankiewicz 1999), with the purpose to predict DNA

damage by the degree of protein degradation and amino acid racemization, as ancient DNA analysis is tricky and expensive. The chemistries of degradation of these biomolecules is similar; both involving hydrolytic cleavage of covalent bonds, after loss of residues that destabilize the backbone of either DNA or protein.

An often posed question is if ZooMS and — specifically our method of glutamine deamidation — can be used as a proxy for the level of DNA damage, DNA content or amplification success in ancient bone. It was questioned whether the chemistries of deamidation in collagen and deamination in DNA were perhaps similar enough to respond similarly to the environmental effects they are exposed to (Figure 8-1). However, the two biomolecules are in fact very different, as is their structure and their preservation within bone. Where the helical structure of collagen is a main determinant in why collagen survives as long as it does (and arguably delays glutamine deamidation; see Chapter 4), the helical structure of DNA reportedly does not add much protection (Lindahl 1993).

For a large selection of our dataset of European samples used for glutamine deamidation (described in Chapter 5) DNA information was available. Due to differences between laboratories in reporting their results, this data was reduced to whether there was or was not successful amplification of mtDNA, nuDNA or any DNA at all (either mtDNA or nuDNA). The range of glutamine deamidation (expressed as Est%Gln ("original glutamine content"), see also Chapter 4) for successful and unsuccessful DNA amplification is shown in Figure 8-2. The spread of deamidation values is too large to convey significant differences between successful or unsuccessful DNA amplification. However, it could be said that for the presence of amplifiable nuDNA, it appears that a loss of 40% or more of original glutamine content corresponds with the failure to detect the amplicon. However, a high proportion of glutamine is not a guarantee for DNA amplification success. This cut-off value does not seem to apply for mtDNA and glutamine deamidation is not an appropriate proxy. It should be noted that contamination with modern DNA could have been detected as that can never be fully eliminated. Also, amplification of aDNA is based on the survival of large fragments of DNA, so failure of amplification does not necessarily mean that there is no aDNA present in the samples (Cooper & Poinar 2000). The data presented in Figure 8-2 presents an indication of what the relationship between DNA survival and glutamine deamidation may have been, but the experiments and available data for this were not exhaustive or strictly comparable

as amplification was not obtained in the same way or with identical primers for the different subsets of the data from samples mentioned in Chapter 4.

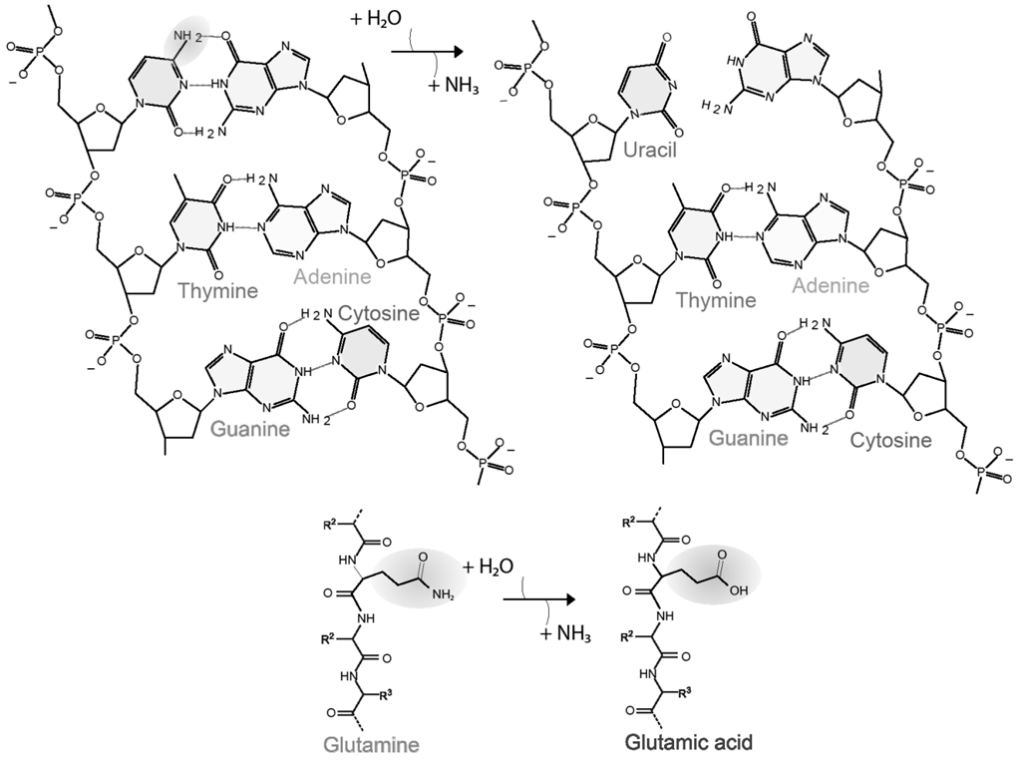


**Figure 8-1. Illustration of deamination in DNA (above: cytosine → uracil) and deamidation in collagen (below: glutamine → glutamic acid). Both reactions occur non-enzymatically and involve hydrolysis with the removal of an amide group of their side-chain.**

The problem with establishing a relationship between DNA preservation and glutamine deamidation is mostly due to choosing the right proxy and obtaining consistent results. In other words, in order to find out if there is a link between glutamine deamidation and DNA survival (rather than amplification) or C to T damage (the most common miscoding lesion in post mortem DNA (Vives et al. 2008)) caused by deamination (Figure 8-1). Such research would require a larger study, preferably performed in one laboratory or several with the same protocol, quality standards and reported in a consistent manner, although this is very difficult to accomplish.

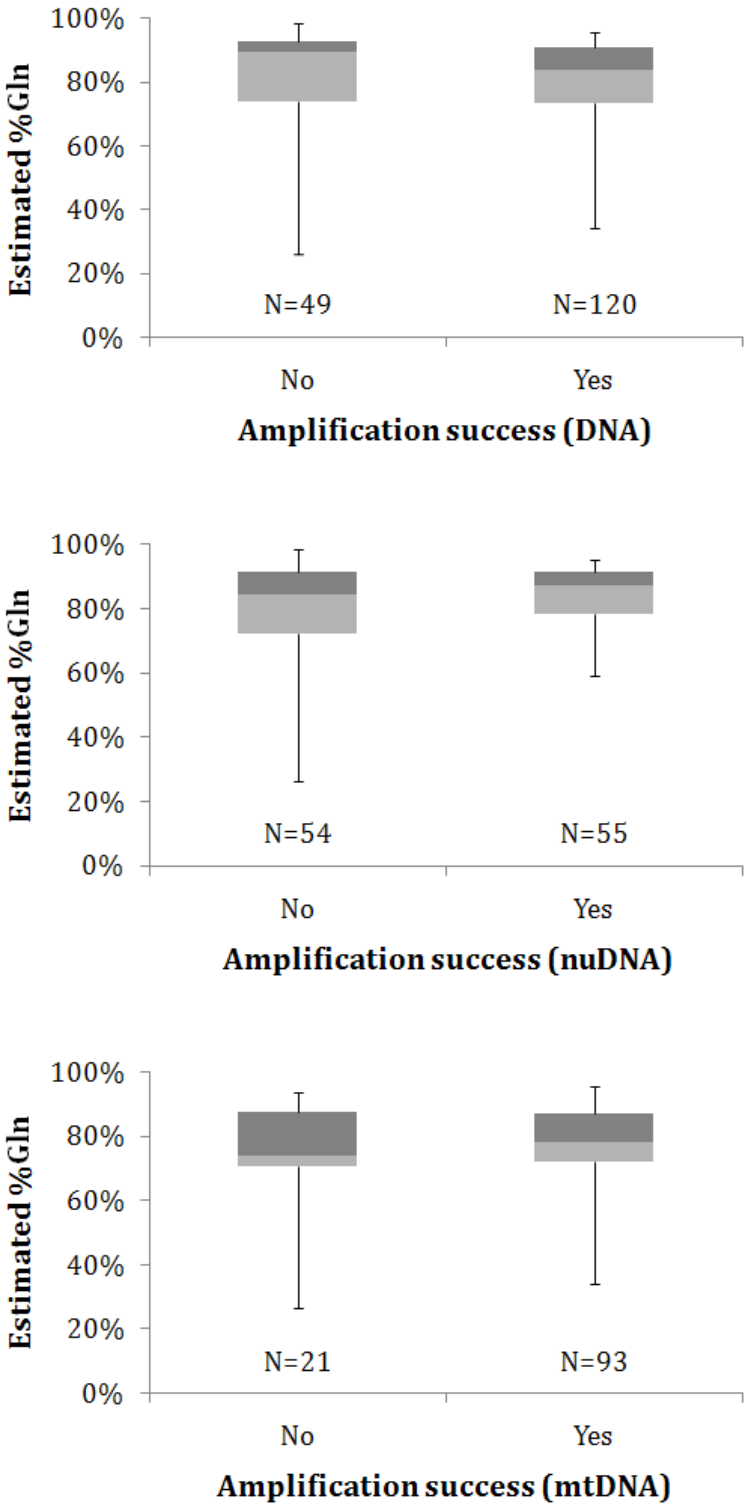


**Figure 8-2. Boxplots for the amount of "original glutamine remaining" (higher glutamine content means less deamidation has taken place) or Est%Gln (see Chapter 5) where DNA amplification had been successful or not successful. Each**

**segment of the boxplot (T-bars and colour-filled bars) represents a quartile of each sample, i.e. the range of 25% of the samples. The intersection of the two coloured bars is equal to the median value of Est%Gln for each category. Above: total DNA (combination of nuDNA and mtDNA subsets); Middle: nuDNA; Below: mtDNA. Samples are from the same dataset as described in Chapter 5 (more in Hollund et al. in prep).**

Bone cross-sections autofluoresce under UV-light and although it is uncertain what exactly causes this fluorescence (suggested are certain crosslinks and aromatic amino acid residues), different colours of fluorescence are observed, ranging from blue for well-preserved bone to brownish colours for degraded material (Hoke et al. 2011). Fluorescence has been found to correlate well with bone histology (bioerosion), as well as to an extent with collagen quality and positively with DNA amplification, similar to their correlation with bone histology (Hoke et al. 2011). UV fluorescence as a proxy for bone preservation shows a large spread for glutamine deamidation values when less than 5% of intact bone is observed, covering a range between both extremes of no estimated deamidation to full deamidation (Figure 8-3). Yet, in samples where a percentage of intact bone higher than 5% was observed, original glutamine content was not found below 40%. However, no meaningful relationship was found between glutamine deamidation and this Fluorescence Index, either overall or within the lowest increments (0–50% of intact bone; FI 0–2).

Correlation between glutamine deamidation and bioerosion was found to be inconsistent. The histological state of bone does correlate with the survival of amplifiable DNA (Colson et al. 1997; Haynes et al. 2002), stronger than glutamine deamidation. Multiple studies indicate that there is some inverse link between the diagenesis of hydroxyapatite and DNA survival as DNA does gain stability from the crystalline matrix (Götherström et al. 2002; Campos et al. 2011), and other studies have pointed out collagen or otherwise proteinaceous content of bone possesses at least a weak relationship with DNA survival (Poinar & Stankiewicz 1999). However, the relationship between glutamine deamidation and most diagenetic proxies (Chapter 5) are weak and only indicative when collagen is strongly degraded (Figures 5-4). Some factors that influence DNA survival do not predict a higher occurrence of glutamine deamidation, with the exception of thermal age.

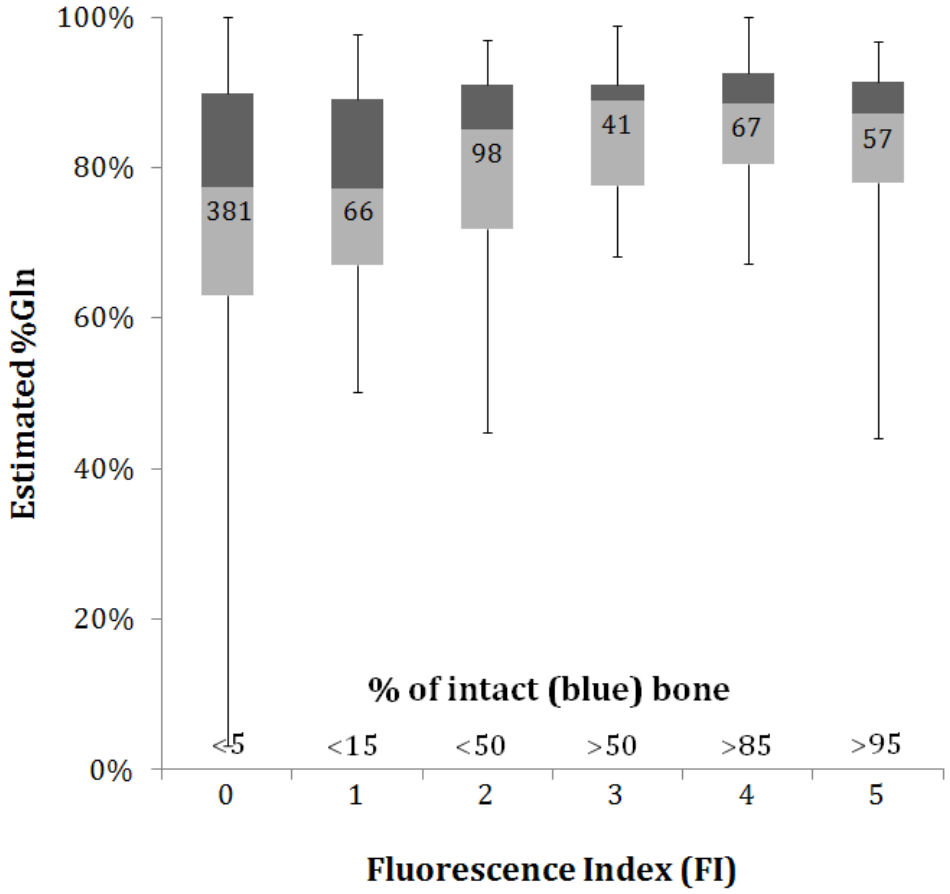


**Figure 8-3. Boxplots for "original glutamine remaining" (or Est%Gln; see chapter 5) for samples corresponding with the phases in the Fluorescence Index (FI), designed similar to the Oxford Histological Index (Hollund et al. submitted), and the percentage of intact bone present based on UV autofluorescence (Hoke et al. 2011). Each segment of the boxplot (T-bars and colour-filled bars) represents a quartile of each sample, i.e. the range of 25% of the samples. The intersection of the two coloured bars is equal to the median value of Est%Gln for each FI phase. Samples are from the same set as presented in Chapter 5 (Number of samples N denoted per boxplot). Note that the lowest %Gln calculated for FI=0 (<5% intact bone) was derived from at least three detected peptides. The most negative results (i.e. no peptides were detected and no Est%Gln could be determined) were present in FI=0 and 5 (respectively 4.5% and 5.3%).**

It would seem that for either estimation of DNA amplification success or a dating method, glutamine deamidation alone is not yet sufficient. Due to the contrasting

mechanisms of decay, glutamine content may be a proxy for DNA survival to exclude samples that are most likely DNA negative for amplification, but can currently not assertively predict DNA survival. The weak correlation with chronological age of samples and the much stronger correlation of deamidation with thermal age, suggests that rather than a method to determine age, it is a "molecular recording" as such with the thermal damage recorded in the amount of deamidation. In the end, what contributes to the deamidation assessment are the advantages of the buffer extraction: it is faster, more cost-efficient and less invasive or destructive than most other methods currently used. The method lends itself well to screening of a large collection, valuable specimen or multi-levelled sites to eliminate the most strongly deamidated samples, without compromising sample material and obtain a relative indication of thermal damage acquired, alongside species identification, if so desired, in the same run.

## 8.4. DISSEMINATION OF RESEARCH

### *8.4.1. PUBLISHED*

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### *8.4.2. UPCOMING*

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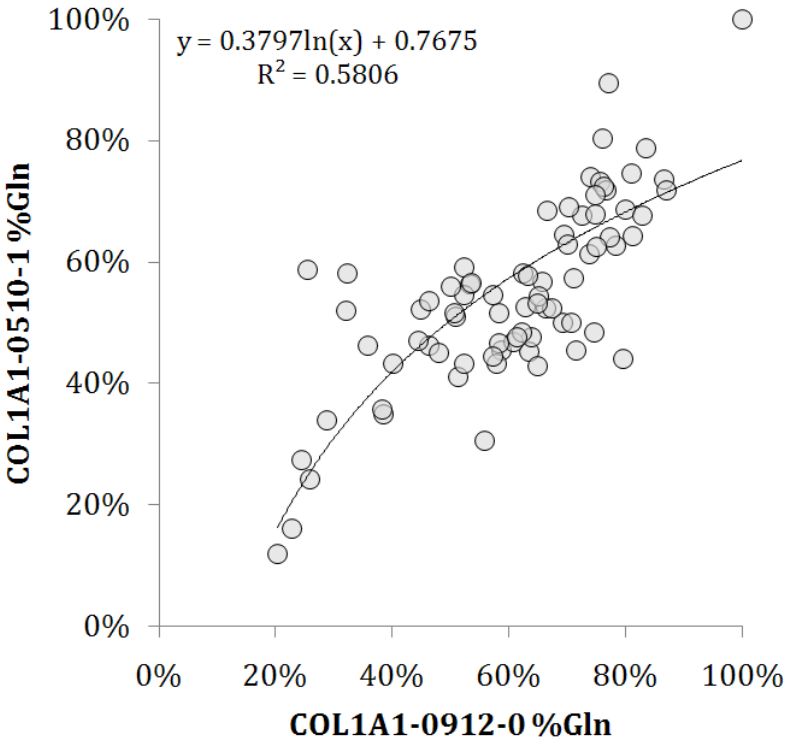
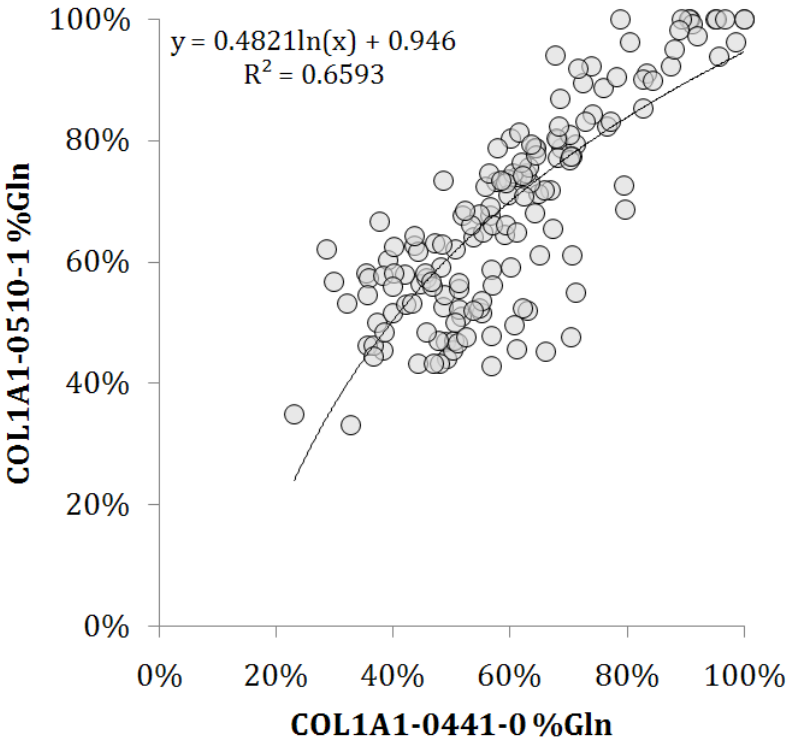
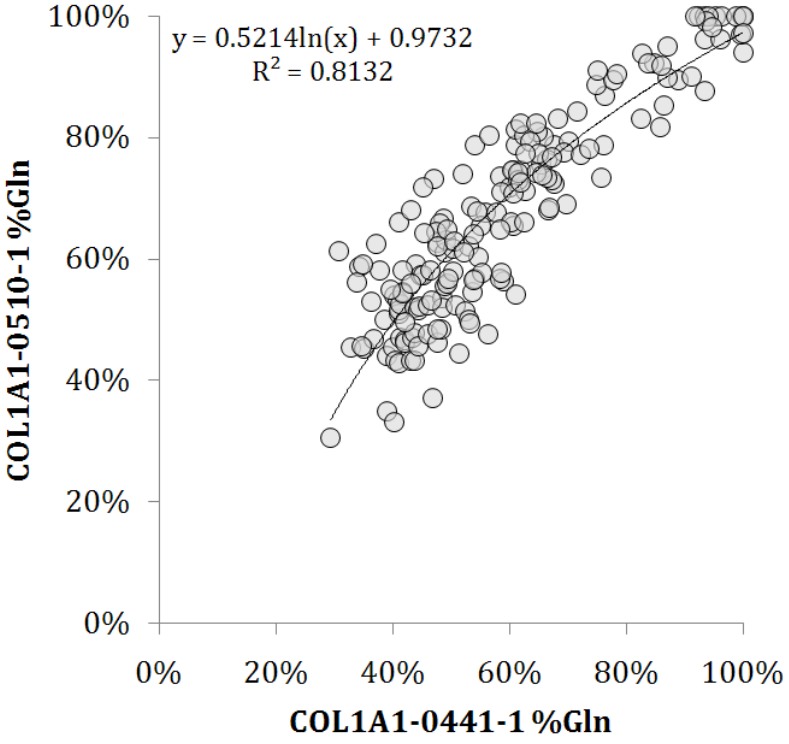
# APPENDIX


y = 0.3797ln(x) + 0.7675
R² = 0.5806
100%
80%
60%
40%
20%
0%
0%
20%
40%
60%
80%
100%
COL1A1-0510-1 %Gln
COL1A1-0912-0 %Gln


y = 0.4821ln(x) + 0.946
R² = 0.6593
100%
80%
60%
40%
20%
0%
0%
20%
40%
60%
80%
100%
COL1A1-0510-1 %Gln
COL1A1-0441-0 %Gln


y = 0.5214ln(x) + 0.9732
R² = 0.8132
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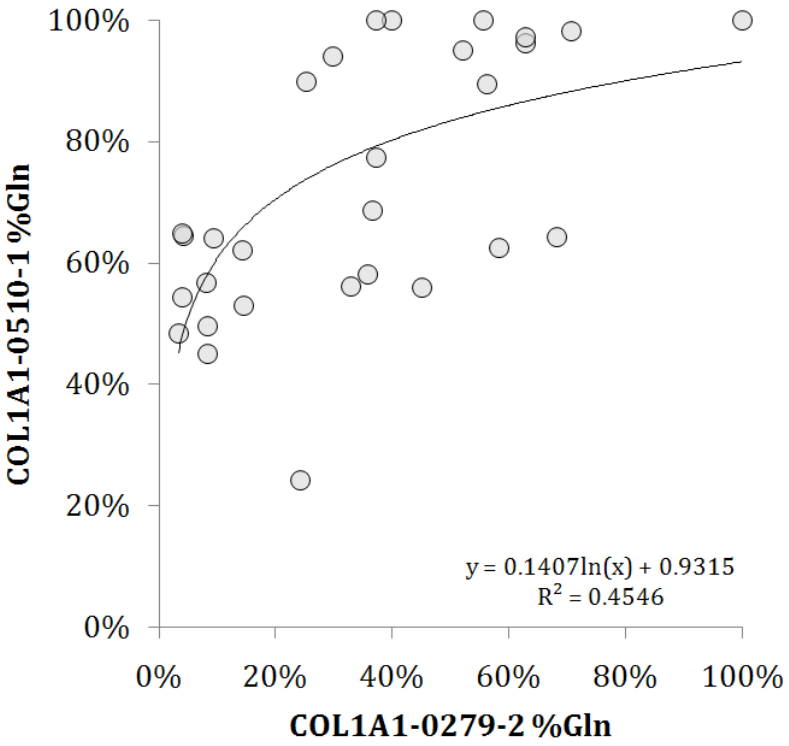
80%
60%
40%
20%
0%
0%
20%
40%
60%
80%
100%
COL1A1-0510-1 %Gln
COL1A1-0441-1 %Gln


y = 0.1407ln(x) + 0.9315
R² = 0.4546
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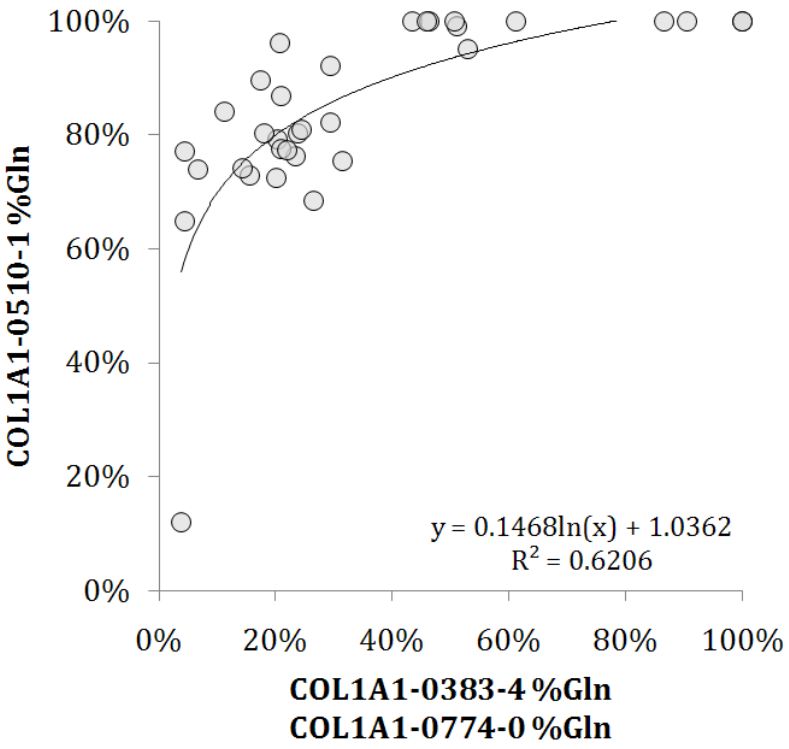
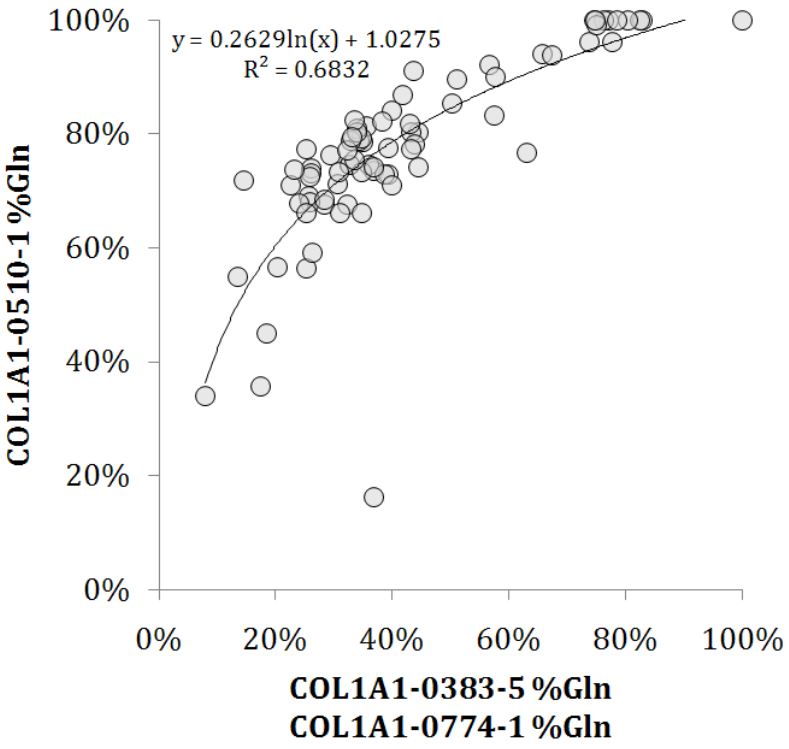
80%
60%
40%
20%
0%
0%
20%
40%
60%
80%
100%
COL1A1-0510-1 %Gln
COL1A1-0279-2 %Gln


100%
80%
60%
40%
20%
0%
COL1A1-0510-1 %Gln
y = 0.1468ln(x) + 1.0362
R² = 0.6206
0%
20%
40%
60%
80%
100%
COL1A1-0383-4 %Gln
COL1A1-0774-0 %Gln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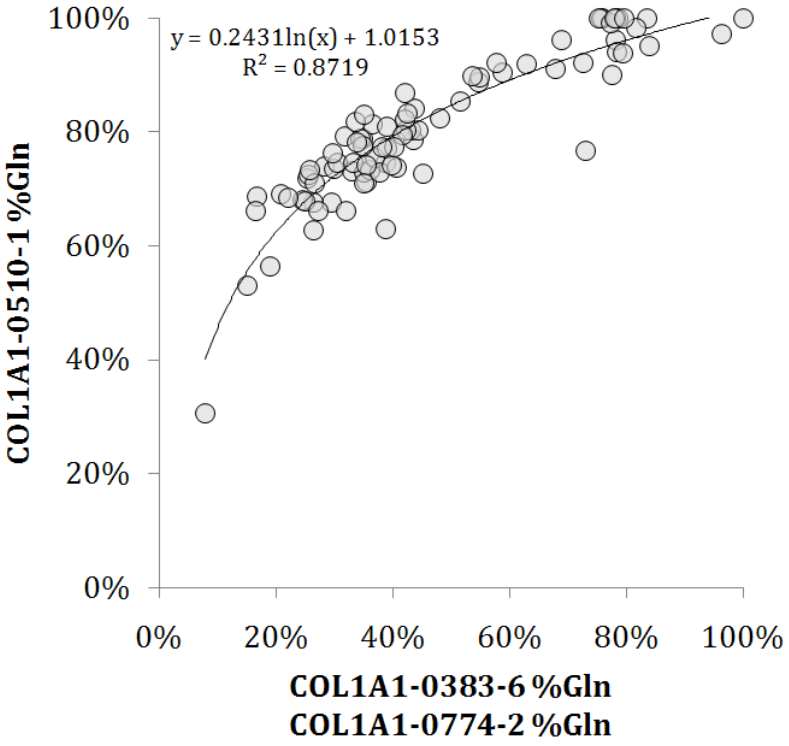
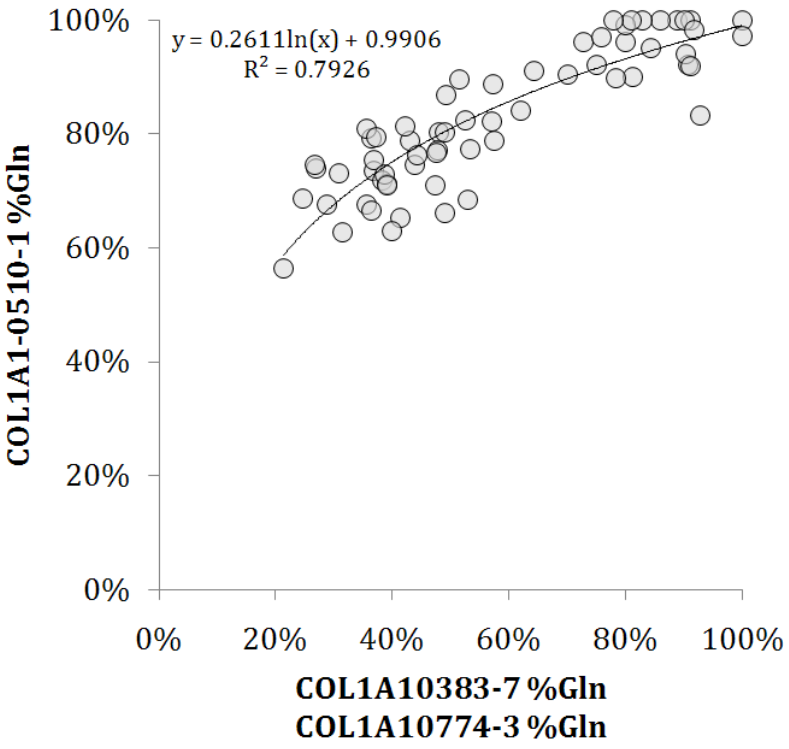
80%
60%
40%
20%
0%
COL1A1-0510-1 %Gln
y = 0.2629ln(x) + 1.0275
R² = 0.6832
0%
20%
40%
60%
80%
100%
COL1A1-0383-5 %Gln
COL1A1-0774-1 %Gln


y = 0.2431ln(x) + 1.0153
R² = 0.8719
100%
80%
60%
40%
20%
0%
0%
20%
40%
60%
80%
100%
COL1A1-0510-1 %Gln
COL1A1-0383-6 %Gln
COL1A1-0774-2 %Gln


y = 0.2611ln(x) + 0.9906
R² = 0.7926
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80%
60%
40%
20%
0%
0%
20%
40%
60%
80%
100%
COL1A1-0510-1 %Gln
COL1A10383-7 %Gln
COL1A10774-3 %Gln

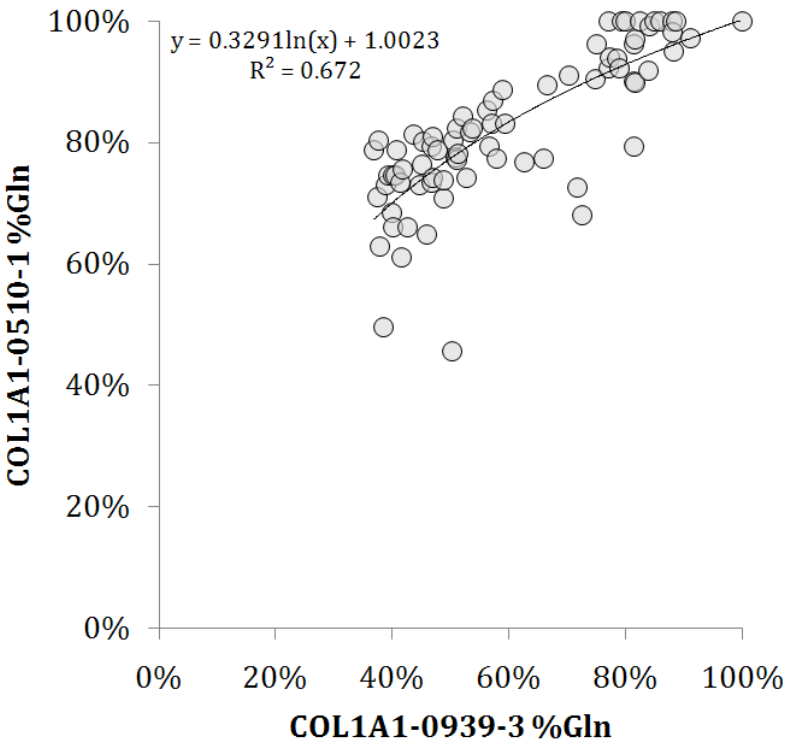


**Figure A-1. %Gln for investigated peptides plotted against %Gln for COL1A1-0510-1. These relationships, established by an initial sample set, were used to predict equivalent %Gln for COL1A1-0510-1 based on the %Gln from other peptides. These values were then averaged to a composite value for an overall %Gln. (See also Table 4-1).**