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Effects of glutamine deamidation on the oxygen isotope compositions of bone collagen

Curtis W. Russell, *The University of Western Ontario*

Supervisor: Dr. Fred J. Longstaffe, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Geology

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Abstract

The oxygen (O) isotope composition of mammalian collagen has the potential to provide information about the drinking water and hence geographic location and climate during the life of modern and ancient animals. An accurate and reproducible O-isotope analysis of collagen, however, has been elusive. Here, the O-isotope compositions of collagen from cortical bone of four megaherbivores were compared using two extraction procedures: the traditional Longin (HCl) method and the less commonly employed EDTA method. Preservation of O-isotope compositions was evaluated by measuring collagen peptide-chain masses, specifically the glutamine deamidation level (GDL). The EDTA method yielded collagen with a GDL of 1 – characteristic of unaltered collagen – and reproducible O-isotope results ($\pm 0.8\%$). Much lower GDL values (0.2-0.95) and poorer reproducibility ($\pm 1.6\%$) was obtained for the Longin method. This method uses strong acids to demineralize bioapatite releasing collagen, catalyzing reactions such as glutamine deamidation leading to O-isotope exchange with reagents, and should be avoided.

Keywords

stable isotopes, bone collagen, collagen extraction, Longin method, EDTA method, glutamine deamidation

Summary for Lay Audience

Bones can provide crucial information about the life of a mammal – the food they ate, the water they drank, and the region where they lived. These details can be determined from the proportion of heavy to light isotopes of carbon, nitrogen and oxygen captured and preserved in bones during life. Such measurements are obtained from bones using the harder mineral portion called bioapatite or the softer tissue called collagen. This study focused on measurement of oxygen isotopes in collagen, which should reflect the meteoric water where the animal lived. Typically, collagen is extracted from bone using strong acids such as HCl, which does not affect the original carbon and nitrogen isotope measurements that provide information about diet. Reactions during collagen extraction using HCl and heat, however, change the original oxygen isotope compositions, which otherwise would have carried information about drinking water origin. This study focused on limiting these changes by using a collagen extraction method that employed a weaker acid (EDTA) and avoided heating. The success of the new method was demonstrated by measuring the chain masses of peptides (smaller versions of proteins) that make up the collagen and by the acquisition of reproducible oxygen isotope data for the collagen. The results from this study can be applied to a variety of applications including paleoclimatic and forensic research, both of which can benefit from reproducible oxygen isotope compositions in bone collagen.

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Table of Contents

Abstract	ii
Keywords	ii
Summary for Lay Audience	iii
Acknowledgements	iv
Table of Contents	v
List of Figures	vi
List of Tables	vii
List of Appendices	vii
Chapter 1	
Introduction	
1.1 Thesis Overview and Objectives	1
1.2 Thesis Structure	2
1.3 Stable Isotopes	2
1.4 Bone Composition	3
1.4.1 Overview	3
1.4.2 Bioapatite	3
1.4.3 Collagen	4
1.4.4 Cortical Bone	4
1.4.5 Bone Degradation	5
1.5 Collagen Extraction	7
1.5.1 Methods	7
1.5.2 Measures of Collagen Quality	9
1.6 Controls on the Stable Isotope Compositions of Mammalian Bone ...	12
1.6.1 Carbon	12
1.6.2 Nitrogen	12
1.6.4 Oxygen	13

1.7 Research Design	14
Chapter 2	
Materials and Methods	
2.1 Bone Samples	16
2.1.1 Sample Preparation	16
2.2 Analytical Methods	18
2.2.1 Fourier Transform Infrared Spectroscopy	18
2.2.2 Powder X-ray Diffraction Analysis	19
2.2.3 Mass Spectrometry	19
Chapter 3	
Results	
3.1 Bone Physical and Structural Characterization	23
3.1.1 Colour	23
3.1.2 Fourier Transform Infrared Spectroscopy (FTIR)	25
3.2 Stable Carbon and Nitrogen Isotope Results for Collagen	26
3.3 Oxygen Isotope Results for Collagen	31
3.4 MALDI-TOF-MS Results for Collagen	37
Chapter 4	
Discussion	
4.1 Collagen Quality	46
4.2 Megaherbivore Sample Characterization	46
4.3 Oxygen Isotope Systematics in Bone Collagen	47
4.3.1 Oxygen isotope discrimination between collagen and meteoric water ($\Delta^{18}\text{O}_{\text{col-met}}$) in Megaherbivores.....	47
4.3.2 Glutamine Deamidation versus the Oxygen Isotope Composition of Bone Collagen	49
4.3.3 Previous Work	51

Chapter 5

Conclusion

5.1 Methods that Alter Original Isotopic Compositions	52
5.2 Collagen Preservation	52
5.3 Can Oxygen Isotope Compositions of Bone Collagen be Used to Infer Drinking Water Composition?	53
5.4 Future Work	53
References	55
Appendices	65
Curriculum Vitae	85

List of Figures

Figure 1.1 – Bone Cross Section	5
Figure 1.2 – Glutamine Deamidation Reaction	6
Figure 1.3 – Demineralization Time and Acid Strength	11
Figure 2.1 – Bone Samples	16
Figure 3.1 – <i>Bos taurus</i> Defleshing / Degreasing Effects	23
Figure 3.2 – Cortical Bone from <i>Bison priscus</i>	24
Figure 3.3 – Collagen from <i>Bos taurus</i> After Drying	24
Figure 3.4 – CI Values for Different Cortical Bone Positions	26
Figure 3.5 – Nitrogen-isotope values for each sample and extraction method used ..	30
Figure 3.6 – Carbon-isotope values for each sample and extraction method used ..	30
Figure 3.7 – Oxygen-isotope values for each sample and extraction method used ..	36
Figure 3.8 – Ideal versus Measured Peaks of Mass 836.43 for <i>B. taurus</i>	37
Figure 3.9 – Ideal versus Measured Peaks of Mass 1105.57 for <i>B. taurus</i>	38
Figure 3.10 – Ideal versus Measured Peaks of Mass 836.43 for <i>A. a americana</i> ..	39
Figure 3.11 – Ideal versus Measured Peaks of Mass 1105.57 for <i>A. a americana</i> ..	40

Figure 3.12 – Ideal versus Measured Peaks of Mass 836.43 for <i>B. priscus</i> ..	41
Figure 3.13 – Ideal versus Measured Peaks of Mass 1105.57 for <i>B. priscus</i> ..	42
Figure 3.14 – Alpha Values of Mass 836.43 and 1105.57 for the EDTA and Longin Methods of Collagen Extraction	43
Figure 4.1 – Carbon and Nitrogen Isotope Compositions of Megaherbivore Diets ..	47
Figure 4.2 – Comparison of collagen carbon and oxygen isotope results using the Longin (A) and EDTA (B) methods of collagen extraction	48
Figure 4.3 – Estimated Oxygen Isotope Megaherbivore Collagen-Meteoric Water Discrimination ($\Delta^{18}\text{O}_{\text{coll-met}}$) based on EDTA-extracted samples	49
Figure 4.4 – Glutamine Deamidation Versus $\Delta^{18}\text{O}_{\text{coll-met}}$ for Peptide Masses 836.43, 1105.57, and 2705.21	50

List of Tables

Table 1.1 – Collagen Extraction Method by Changes in Procedure	15
Table 2.1 – Sample Details	16
Table 2.2 – Peptide Chains and Their Respective Masses	22
Table 3.1 – Bioapatite Crystallinity Indices for Sampled Bones	25
Table 3.2 – Carbon and Nitrogen Isotope Compositions and Contents for Different Collagen Extraction Experiments	27
Table 3.3 – Longin versus EDTA Carbon- and Nitrogen- Isotope Average Compositions	29
Table 3.4 – Oxygen Isotope Compositions and Contents for Different Collagen Extraction Methods.	32
Table 3.5 – Experiment and Sample Variance of Oxygen Isotope Compositions ..	35
Table 3.6 – Alpha Values for Peptide Masses	44

List of Appendices

Appendix A – Bone Collagen Extraction Procedure Using EDTA	65
Appendix B – Bone Sample Catalogue	73
Appendix C – Trypsin Digest in Solution Protocol for Collagen Samples	78

Appendix D – MALDI-TOF-MS Program Code Guide	80
Appendix E – Glossary	83

I. INTRODUCTION

1.1 Thesis Overview and Objectives

The goal of this dissertation is to create a bone collagen extraction technique that eliminates or diminishes substantial changes in the original oxygen isotope composition that has plagued the traditionally employed hydrochloric acid (HCl)-based Longin method (Longin, 1971). Bone collagen extracted using the Longin method commonly does not preserve primary oxygen isotope compositions, which leads to faulty interpretation of these data. This study therefore explores in more detail the ethylenediaminetetraacetic acid (EDTA)-based collagen extraction method used in a few previous studies (e.g., Kirsanow and Tuross, 2011; Tuross et al., 2017; von Holstein et al., 2018) for acquiring samples that preserve original oxygen isotope compositions. Three of the samples used in this study are modern in age (*Bos taurus*, *Alces alces americana*, and *Ovibos moschatus*), and one is of Pleistocene age (*Bison priscus*).

Carbon, nitrogen, hydrogen, and oxygen are the key elements that make up the organic (bone collagen) and inorganic (bioapatite) components of bones. If preserved, the stable isotope compositions of these elements can serve as a proxy for animal diet patterns (i.e., drinking water source, food source) and climatic conditions (i.e., local precipitation, soil conditions) (Crowley, 2014; Mabee, 2019). While the Longin method allows for isolation of collagen that preserves original stable carbon and nitrogen isotope compositions, the oxygen isotope data have been less reliable (Tuross et al., 2017; von Holstein et al., 2018; Mabee, 2019).

This study therefore has the following objectives:

- 1) To examine the process(es) that cause alteration of the oxygen isotope compositions using the Longin method as opposed to the EDTA method, and through this comparison, describe a procedure for preparing bone collagen that eliminates, or limits to as a large degree as possible, laboratory-induced oxygen isotope alteration;
- 2) To test whether any difference in preservation quality between ancient and modern bone collagen affects the ability to obtain primary oxygen isotope compositions, and
- 3) To test whether collagen oxygen isotope compositions prepared by the EDTA method can be used to infer realistic drinking water oxygen isotope compositions.

These objectives are explored using bone samples from *Bos taurus* (Modern Cattle), *Bison priscus* (Steppe Bison), *Ovibos moschatus* (Muskox), and *Alces alces americana* (Eastern Moose).

1.2 Thesis Structure

This thesis is organized into five chapters. Chapter 1 briefly describes the purpose and approach to the study, along with some general background information. Chapter 2 describes the samples and methods used in the present study. Chapter 3 presents the isotopic results for the collagen, together with data concerning collagen alteration arising from the HCl versus EDTA collagen extraction techniques. Chapters 4 and 5 discuss these results and their implications, and summarize the major conclusions, respectively. Further details concerning samples and methodologies are provided in a series of Appendices.

1.3 Stable Isotopes

Stable isotopes are elements with the same number of protons and electrons, causing them to have nearly identical chemical properties, but differ in the number of neutrons, which causes them to have quite different isotopic properties. Stable isotopes differ from radioactive isotopes in a few important ways. Stable isotopes have nuclei that do not transform into a different element. Stable isotope compositions are measured as the ratio of the concentration of a given isotope of an element to other isotopes of that element, all calibrated to an accepted international standard. As stable isotopes of a given element are exchanged between phases, certain ‘light’ or ‘heavy’ isotopes may be exchanged in different ratios causing a ‘depletion’ or ‘enrichment’ in one or the other relative to the standard (Riebeek, 2005). The accepted value of most international standards for most stable isotopes is assigned a value of 0‰ (per mil). As such, positive values for a sample can be inferred as an enrichment of the isotope in question and negative values a depletion of that isotope relative to the standard.

Stable isotope ratios are reported using the following equation:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1], \quad (\text{Equation 1.1})$$

where X (in units of per mil, otherwise represented as ‰) represents the heavy isotope of interest, and R is the ratio of heavy to light isotope in both sample and standard material (Coplen, 2011). For oxygen in the current study, for example, X represents ^{18}O and R represents $^{18}\text{O}/^{16}\text{O}$. The standard for oxygen is Vienna Standard Mean Ocean Water (VSMOW) and is assigned a value of 0‰ exactly.

1.4 Bone Composition

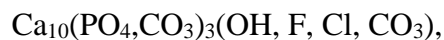
1.4.1 Overview

Modern mammalian bones consist of ~65% inorganic material, 25% organic material and 10% water, where bioapatite (biological apatite) represents the inorganic portion and collagen comprises the majority of the organic material (LeGeros, 1991; Mabee, 2019). The bone structure consists of an outer layer of cortical bone that surrounds an inner spongy layer called trabecular / cancellous bone. Collagen that resides in the cortical bone is generally the best-preserved *post-mortem* as this layer is thicker and structurally stronger than trabecular bone (Matsubayashi and Tayasu, 2019). During an organism's life, its bones are constantly remodelling and growing. Using a long bone as an example, bone growth starts mid-shaft and grows radially towards the two ends (Lyman, 1994). A bulk stable isotope composition of the bone would represent roughly the last 10-25 years of the organism's life (Ambrose and Norr, 1993; Manolagas, 2000), making it an adequate material to measure significant paleoenvironmental conditions during this time.

1.4.2 Bioapatite

Bioapatite is a calcium-phosphate mineral similar to hydroxyapatite (HAP) ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). Bioapatite's strength and rigidity allow the bone to absorb stresses that may be expected during the lifespan of the individual.

Bioapatite's chemical formula can be represented by:



whereby substitution of carbonate CO_3^{2-} into the PO_4^{3-} group is most common (LeGeros and LeGeros, 1984; Michel et al., 1995). These two groups are primary targets for oxygen isotope analysis (Stephen, 2000).

1.4.3 Collagen

There are as many as 28 different types of collagen (Miller & Gay, 1982; Ricard-Blum, 2010). The most mentioned types are fibrillar collagen (Type I), fibril-associated collagen (Type II), and sheet-forming collagen (Type III). Bone collagen, the subject of this research, is a subtype of Type I collagen. Collagen occurs in bones and in tooth dentin (Kuhn, 1987). Collagen is a tissue consisting of a polypeptide fibril structure (Shoulders and Raines, 2009). The polypeptide is composed of three peptide chains wound together. At its base, the chemical composition of a peptide depends on which amino acids are joined together to form the peptide (Bella, 1994). In general, collagen contains carbon, nitrogen, oxygen, and hydrogen (Bella, 1994).

1.4.4 Cortical Bone

As noted earlier, bone contains both trabecular and cortical bone (Matsubayashi and Tayasu, 2019; Suarez and Kohn, 2020) (Fig. 1.1). Trabecular, otherwise known as cancellous bone, is a spongy material where bone marrow is produced. Here, the main nutrient artery carries blood from the medullary cavity to the cortical portions of the bone. A combination of high porosity and high permeability of trabecular bone allows greater movement of blood through the cavity (Johnson et al., 2004). This feature also enables greater *post-mortem* interactions with water that can lead to alteration (Matsubayashi and Tayasu, 2019). Cortical bone is a compact, hard tissue that surrounds the trabecular bone, which is also composed of bioapatite and collagen. Cortical bone is typically less affected by *post-mortem* alteration, resulting in better preservation of *in-vivo* isotopic signals (Suarez and Kohn, 2020). In Suarez and Kohn's study, they note that portions of cortical bone closer to the periosteum have been potentially more exposed to the environment (Fig. 1.1) and hence may not be as well-preserved as the inner portion of the cortical bone. Cortical bone located closer to the center of this tissue is the best choice for unaltered material.

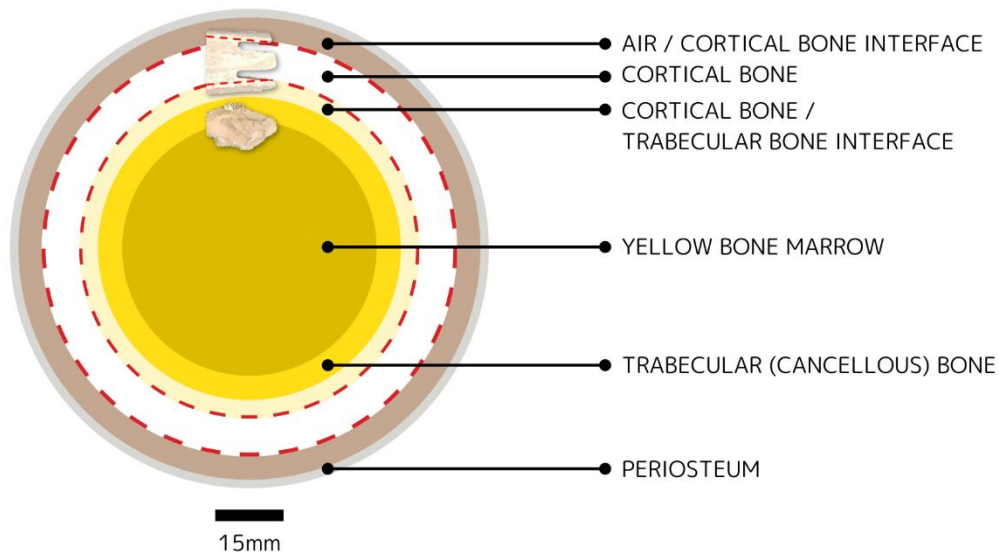


Figure 1.1 – Bone Cross-Section. Cartoon cross-section of a bone. The preferred sampling location for isotopic analysis lies within the red dashed lines. Figure created by Curtis Russell.

1.4.5 Bone Degradation

Bone subjected to *post-mortem* processes for any amount of time will have undergone alteration (Hoke et al., 2018; Nelson et al., 1986). ‘Degradation’ usually consists of two processes: chemical degradation (Collins et al., 2002; Berna et al., 2004) and microorganism / fungal degradation (Hackett, 1981; Bell et al., 1996; Nelson et al., 1986). Chemical degradation is generally a slower process that has a greater effect on bioapatite than collagen. Small hydroxyapatite (HAP) crystals comprising bioapatite in the bone structure are thermodynamically unstable, leading to changes in crystallinity during *post-mortem* processes. Crystallinity is a property that includes the crystal size and order as well as the number of defects in the bioapatite (Grypnas, 1976). Alteration typically results in recrystallization of HAP into larger crystals by a process called Ostwald Ripening (Trueman et al., 2004). Microbial degradation occurs mostly in the softer tissues of the body as bacteria and other organisms invade the decaying tissue. Considering collagen surrounds the rigid bioapatite structure, any notable dissolution/recrystallization of the bioapatite structure points to an additional possibility of collagen alteration. Collins et al. (1995) points out that collagen degradation is more of a concern due to hydrolysis reactions that can catalyze peptide breakage in the collagen fibrils.

Collins et al. (1995) modelled a degradation pathway for collagen that is catalyzed by hydrolysis of collagen and leads to loss of peptide fragments. Simpson et al. (2016) identified a specific chemical reaction – glutamine deamidation – in bone collagen that can be used to identify the extent of collagen degradation. Glutamine deamidation is a process, commonly catalyzed in the presence of acid, that converts an amide functional group from an amino acid (glutamine) into a carboxylic acid (glutamic acid) (Pal Chowdhury et al., 2019). This reaction involves addition of water to the glutamine structure, thereby potentially changing the original oxygen isotope composition to one that incorporates part of the oxygen isotope signal from the surrounding water (fig. 1.2).

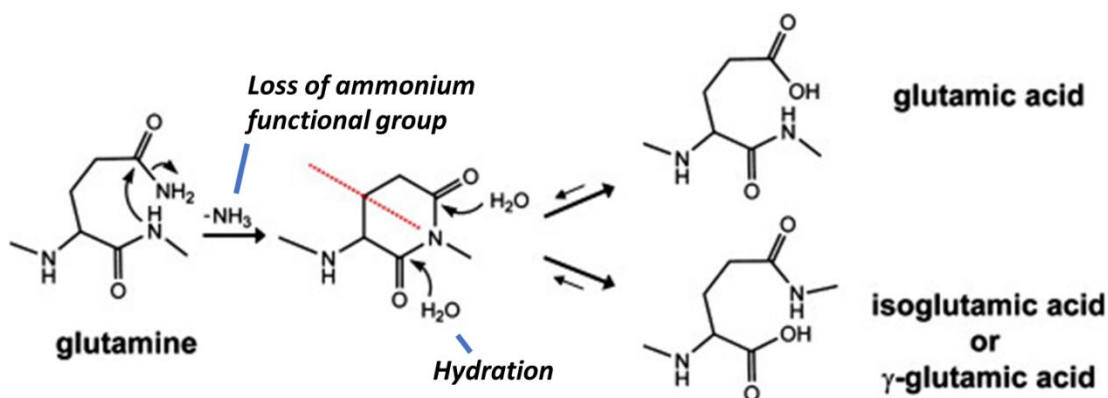


Figure 1.2 – Glutamine Deamidation Reaction. Two major chemical changes occur during glutamine deamidation: A) loss of ammonium functional group -NH₃ and B) Hydration of ammonium depleted glutamine. Adapted from Cournoyer (2008).

The oxygen exchange that occurs during hydration of the glutamine structure is the focus of this paper. Simpson et al., (2016) have suggested that glutamine deamidation is responsible for collagen degradation, but this paper aims to show how that degradation can be quantified with oxygen isotope variations.

During collagen extraction, bone is placed in contact with water for rinsing and acids for demineralization, making these optimal steps at which glutamine deamidation can occur. The extent of glutamine deamidation can be quantified for collagen peptide chains using Matrix assisted laser desorption / ionization time-of-flight mass spectrometer (MALDI-TOF-MS) analysis, as described by Wilson (2012).

Preservation. Good collagen preservation is required to ensure retention of stable isotope signatures acquired during life. Preservation depends largely on the environment of the bone after death. Hydrolysis takes place in the presence of water, implying that a wetter environment is less favourable for preservation quality. Plint et al. (2019), for example, used carbon and nitrogen isotope analysis to extract paleodietary information from bone collagen, in this case from an extinct Pleistocene beaver species. They noted that collagen preservation was commonly quite poor for this species. This result was attributed to the deposition of the bones in a semi-aquatic environment where the presence of water accelerated diagenetic processes. Animals that lived and died in terrestrial, rather than semi-aquatic, environments (i.e., grasslands) were therefore chosen for the present study to minimize such problems with the analysis of ancient (i.e., Pleistocene) samples.

Defleshing and Degreasing. Defleshing and degreasing are necessary to remove organic material from bone that could otherwise contaminate its collagen. Defleshing and degreasing, however, requires extended soaking in water-based solutions that can cause chemical degradation, depending on the procedure. Boiling a bone sample for longer than 16 hours, for example, can alter bone in a fashion similar to natural diagenesis (Roberts et al., 2002). Plint (2019) outlined a defleshing and degreasing method that limits exchange with water, which is described in more detail in Chapter 2.

1.5 Collagen Extraction

1.5.1 Methods

Extraction of bone collagen requires demineralization of bioapatite. As briefly noted earlier, the two commonly used acids for demineralization are HCl and EDTA. Use of HCl follows the Longin (1971) method whereas use of EDTA follows Koon et al. (2012) and Tuross et al. (1988). The original Longin (1971) method was modified by Brown (1998), Ambrose and Norr (1993), DeNiro and Epstein (1981) and Jarkov (2007); changes that were incorporated in this study. A full description of the two methods, as used in this study, is provided in Appendix A. Briefly, the procedures are as follows:

Longin Method. The Longin (1971) method is typically used when isolating collagen from bone for stable carbon and nitrogen isotope analysis. The Longin (1971) method uses HCl as the demineralizing agent. Most studies that use this method follow similar steps that include:

1. Crushing of bone samples to 0.18mm average particle diameter
2. Demineralization in 0.25-1M HCl (0.25M was used for this study) until collagen fragments are soft to touch
3. Rinsing with deionized water
4. Rinsing with NaOH to remove humic acids
5. pH adjustment to ~2-3
6. Gelatinisation in 0.001M HCl at 70-90°C for no more than 16 hours until evaporation of the solution is complete

The details of the Longin method can vary, depending on the preservation of the sample. Ultra-filtration of the remaining solution has been performed in several studies dealing with poorly preserved samples (Brown, 1988; Jørkov, 2007) which is often used for radiocarbon studies. This was omitted from this study as oxygen is the primary focus. Additionally, lower pH acid and warmer oven temperatures (80-100°C) are typically used for stable carbon and nitrogen isotope studies, whereas higher pH acids and cooler oven temperatures are used for additional analysis such as oxygen and hydrogen (Brown et al., 1988; Jørkov, 2007). Weaker concentrations of HCl were used in the present study to minimize collagen alteration as much as possible (Hoke et al., 2019).

EDTA Method. The EDTA method developed by Tuross et al. (1988), and furthered by Ambrose and Norr (1993) for collagen extraction is not as widely used due to the longer time required for demineralization and extra rinsing to desalt (Cleland et al., 2012). Briefly, this method is as follows:

1. Lyophilization (Freeze-drying) of samples
2. Lipid extraction to remove lipids from samples
3. Rinsing with DI water
4. Demineralization in 0.5M EDTA at room temperature, replacing the solution every 2-3 days
5. Rinsing with DI water

6. Rinsing with NaOH to remove humic acids
7. Rinsing with DI water
8. Lyophilization of samples

The use of EDTA versus HCl and lyophilization versus oven-drying of samples are the major differences between this method and the Longin method. As discussed in more detail later, these differences affect preservation of collagen fibrils, which can be key to minimizing alteration of original oxygen isotope compositions.

1.5.2 Measures of Collagen Quality

Carbon, Nitrogen and Oxygen Contents. The quality of collagen preservation following extraction from bone can also be evaluated using its carbon (wt%C) and nitrogen (wt%N) contents. Intact collagen should have %C of $34.8 \pm 8.8\text{wt}\%$ and %N of $13.5 \pm 2.5\text{wt}\%$ (van Klinken, 1999). In addition, collagen atomic C:N ratios should be in the range of 2.9-3.6 for both well-preserved ancient and modern collagen (Guiry and Szpak, 2020). Typically, modern collagen atomic C:N ratios average ~ 3.2 (Guiry and Szpak, 2020; van Klinken, 1999). Collagen preservation can also be evaluated using oxygen contents, as this study showed a strong correlation between oxygen contents (%O) ranging from 25-35wt% and collagen yields of >1% (See next paragraph).

Collagen Yield. Preservation of original carbon and nitrogen isotope compositions can also be evaluated by using the collagen yield following extraction (Equation 1.2):

$$\text{Collagen \% Yield} = \frac{(\text{Vial} + \text{Collagen Weight}) - (\text{Empty Vial Weight})}{\text{Dry Bone Weight}} \times 100 \quad (\text{Equation 1.2})$$

Bone collagen yields should be > 0.5% for preservation of primary carbon and nitrogen isotope compositions according to van Klinken (1999). Collagen yields should be >1% for preservation of oxygen isotope compositions (Ambrose, 1990; Szpak et al., 2017).

These measures, however, appear to be inadequate for evaluating whether primary oxygen isotope compositions have been preserved in collagen (Simpson et al., 2016; Wilson et al., 2012). For that purpose, glutamine deamidation levels in collagen should also be evaluated, as briefly described below (Chowdhury et al., 2019; Wilson et al., 2012).

Proteolysis and Glutamine Deamidation. Alteration of collagen depends on the reactions that affect its peptides (short strings of amino acids). Peptides form chains, typically called polypeptides, that are susceptible to chemical reactions specific to what peptides are contained within the chain. Peptide bond hydrolysis (proteolysis) is one such reaction. This reaction involves the cleaving of peptide chains by successive hydrolysis of peptide bonds. Substantial proteolysis can lead to glutamine deamidation, as is discussed more fully below (Shih, 1990). Glutamine is commonly the most abundant amino acid in animal collagen. After proteolysis occurs, glutamine is generated in the hydrolysate and can be joined with anions such as phosphate and bicarbonate to form glutamic acid (deamidation) (Gilbert et al., 1949). Shih (1990) notes that deamidation can be avoided by excluding anions that catalyze nonenzymatic deamidation. The Longin method, however, requires collagen samples to be left in 0.125M HCl at 90°C for no less than 16 hours. Complete protein deamidation would occur in HCl at 100°C in only 3 hours (Leach and Parkill, 1955), including the deamidation of glutamine. In addition to temperature, pH and the length of time spent in HCl would also induce deamidation, especially in the Longin method (Fig. 1.2). These two factors both increase the probability that proteolysis can occur. Other, earlier collagen extraction methods that use ammonium bicarbonate as a bone cleaning chemical are also considered problematic as bicarbonate anions accelerate non-enzymatic deamidation of glutamine (Gilbert et al., 1949). Any extraction method that promotes glutamine deamidation should be avoided as this can lead to oxygen isotope exchange as this is not favourable for preservation of original oxygen isotope compositions (Section 1.4.5), the EDTA method is preferred for collagen extraction (Simpson, 2016).

A central question addressed in this study, therefore, is whether a correlation can be made between increased levels of glutamine deamidation and changes to $\delta^{18}\text{O}_{\text{col}}$, and the extent to which this deamidation is extraction-method controlled. During collagen extraction (separation of collagen from bioapatite), water-based solutions interact with the sample (Longin, 1971; Matsubayashi & Tayasu, 2019). During these steps, oxygen in the collagen has the potential to exchange oxygen isotopes with water. For collagen extractions conducted using the Longin (1971) method, interaction with aqueous HCl solution is known to quickly degrade collagen (Brown, 1988). Identifying which steps in the collagen extraction cause the most isotopic exchange is key to producing collagen samples that retain their original oxygen isotope composition.

During collagen extraction, amino acids, which are the building blocks of proteinaceous tissues, interact with water and various acids. The interaction between these fluids and bone may introduce chemical degradation of the collagen in the form of deamidation. Deamidation occurs in peptides such as asparagine or glutamine (Robinson, 2004), and involves chemical transformation of an amide functional group into a carboxylic acid group (Lip Kwok and Chan, 2011). This process involves hydrolysis of the amide group, which can be catalyzed by acids such as HCl or Ethylenediaminetetraacetic Acid (EDTA) (Simpson, 2016). HCl is a strong acid (dissociation constant $K_a = 1.3 \times 10^6 \text{ mol/litre}$) that causes breakdown of collagen fibrils. By comparison, EDTA is a weak acid (dissociation constant $K_a \sim 3.085 \times 10^{-3} \text{ mol/litre}$), which generally does not degrade collagen fibrils. Figure 1.3 (from Simpson et. al, 2016) graphically demonstrates how the strength of acid (HCl versus EDTA) can lead to the conversion of glutamine into glutamic acid. Simpson's alpha value will be represented in this study as 'a'.

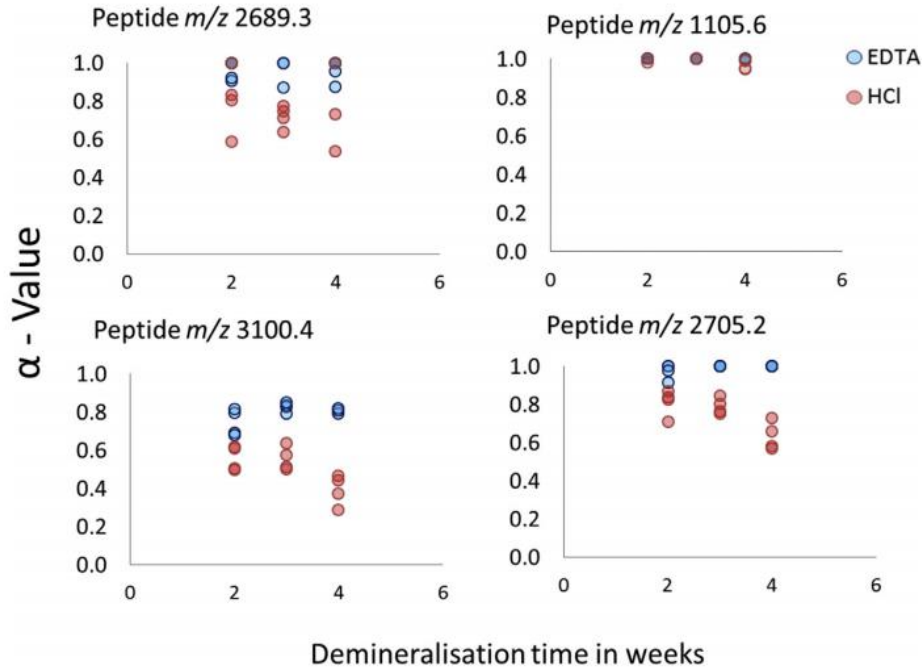


Figure 1.3 – Demineralization Time and Acid Strength. Glutamine deamidation is more susceptible when stronger acid (HCl) is used to demineralize collagen. Length of time spent in acid augments this effect. An alpha value of 1 means no deamidation, whereas 0 means complete deamidation.

The m/z values represent the mass over charge of the peptide chain being analyzed (Simpson, 2016). Copyright License #: 5135020108019.

1.6 Controls on the Stable Isotope Compositions of Mammalian Bone

Carbon, nitrogen, hydrogen, and oxygen, which largely come from food and water, are incorporated into bone when an animal feeds (Richards, 2015). As bone remodels during the lifespan of an animal, its isotopic composition can change, largely depending on variations in diet and drinking water consumed by the organism. Commonly, such differences reflect changing environmental conditions and/or animal migration (Crowley, 2014).

1.6.1 Carbon

The two stable isotopes of carbon (^{13}C and ^{12}C) are represented by the $^{13}\text{C}/^{12}\text{C}$ ratio. The international standard for reporting stable carbon isotope ratios as δ -values ($\delta^{13}\text{C}$) is Vienna Pee Dee Belemnite (VPDB). Stable carbon isotope ratios of bone collagen (denoted as $\delta^{13}\text{C}_{\text{coll}}$) are typically reflective of dietary proteins (Richards, 2015) which can be found in many C_3 and C_4 plants as well as other species in the food chain. C_3 plants differ from C_4 plants in that their photosynthetic pathway consists only of the Calvin cycle, where C_4 plants include production of phosphoenolpyruvate (PEP) prior to the Calvin cycle (Wang et al., 2012). Modern C_3 plants have an average $\delta^{13}\text{C}$ of $-28.1 \pm 2.5\text{‰}$ (O'Leary, 1981) and C_4 plants have an average $\delta^{13}\text{C}$ of $-12.7 \pm 1.4\text{‰}$ (Basu, 2015). Stable carbon isotope ratios measured for bone collagen from modern animals closer to -28.1‰ would suggest a C_3 diet, whereas ratios closer to -12.7‰ would reflect a C_4 diet (Jim, 2004). Ratios in between these values commonly suggest a mixed diet of either both types of plants or prey that fed on both plant types. There is an isotopic trophic enrichment effect with each step in the food chain. Trophic enrichment of $\delta^{13}\text{C}_{\text{col}}$ is commonly no more than $+2\text{‰}$ per trophic level and commonly less (Bocherens and Drucker, 2003).

1.6.2 Nitrogen

The two stable isotopes of nitrogen (^{15}N and ^{14}N) are represented as $^{15}\text{N}/^{14}\text{N}$ ratios. The international standard for reporting nitrogen isotope ratios as δ -values ($\delta^{15}\text{N}$) is atmospheric nitrogen (AIR), which is assigned a $\delta^{15}\text{N}$ of exactly 0‰ (Brand et al., 2014). Nitrogen isotope ratios in plants are commonly an indicator of soil conditions as nitrogen is fixed into bioavailable

compounds like NH_4^+ or NO_3^- in the soil by bacteria (Richards, 2015). Nitrogen can also be fixed from the atmosphere by plants with symbiotic nitrogen-fixing bacteria in their root nodules, especially leguminous plants (Richards, 2015). Non-legumes, in particular, commonly show a wide variation in $\delta^{15}\text{N}$ as a response to environmental factors that varies from study to study (Harper et al., 1989; Szpak et al., 2013; Tahmesabi et al., 2017). Values of $\delta^{15}\text{N}$ in animals are also indicative of trophic level, once the plant baseline is known (Fizet et al., 1995; Jaouen et al., 2019; Tahmesabi et al., 2017). Trophic enrichment for nitrogen isotopes typically range from +3-5‰ per trophic level (Bocherens and Drucker, 2003).

1.6.3 Oxygen

The oxygen isotope compositions of bone collagen ($\delta^{18}\text{O}_{\text{coll}}$) are the net of an animal's oxygen inputs and outputs, as modified by isotopic fractionations during tissue formation. Typical $\delta^{18}\text{O}_{\text{coll}}$ values reflect oxygen inputs from air, drinking water ($\delta^{18}\text{O}_{\text{dw}}$), food, and metabolic processes (Crowley, 2014). Values of $\delta^{18}\text{O}_{\text{dw}}$ generally reflect local freshwater ($\delta^{18}\text{O}_{\text{mw}}$), which represents precipitation plus any evaporative effects at the locality (Longinelli, 1984; Luz and Kolodny, 1985). Oxygen outputs include CO_2 exhalation and water loss via sweating and urination (Luz and Kolodny, 1985).

The combination of inputs and outputs can vary widely within and among species (Crowley et al., 2015). A key factor is the geographical location of an animal's home range. Higher latitude and altitude regions have systematically lower $\delta^{18}\text{O}_{\text{mw}}$ values than lower latitude and altitude localities (Dansgaard, 1964). Rayleigh Distillation (RD) is generally considered to be responsible for this difference. There is a preference for ^{18}O to concentrate in precipitation relative to water vapour, leaving the remaining air mass with a higher concentration of ^{16}O . As a result, the $\delta^{18}\text{O}$ of air masses systematically decrease as they move from equatorial to polar regions, causing the resulting precipitation also to have systematically lower $\delta^{18}\text{O}$. Temperature and humidity are also controls on oxygen isotope fractionation between liquid and vapour during RD, with the temperature effect being strongest near the poles (Hoefs, 2009). Outputs of oxygen can vary widely with temperature. In warmer regions, animals sustain greater water loss due to sweating and panting (Crowley et al., 2015). Arid regions can also effect oxygen output as animals would tend to retain water to combat the dry habitat.

The oxygen isotope ratios exhibited by an organism's tissues can vary throughout its life because of variations in oxygen sources and metabolic fluxes. Overall, the oxygen isotope compositions preserved in animal tissues, particularly megafauna (≥ 100 kg), can serve as a useful proxy for $\delta^{18}\text{O}_{\text{mw}}$ and hence an animal's location and migration across the landscape (Longinelli, 1984; Luz and Kolodny, 1985). Such studies generally focus on the oxygen isotope composition of bioapatite. Interpretation of $\delta^{18}\text{O}_{\text{col}}$ can be more complex. Nonetheless, whether seeking information on habitat type and location, migration patterns, dietary tendencies, or metabolic processes, these factors can sometimes be isolated using the $^{18}\text{O}/^{16}\text{O}$ ratios of bone collagen in combination with similar data for bioapatite (Clements et. al., 2009).

1.7 Research Design

Well-preserved bones of both modern and ancient (Pleistocene) megaherbivores have been analyzed, as both can represent different stages of *post-mortem* alteration, which may have the potential to affect preservation of original $\delta^{18}\text{O}_{\text{coll}}$ values. Traditional tests of crystallinity and preservation (bioapatite crystallinity by Fourier Transform InfraRed Spectroscopy (FTIR), collagen yield, chemical composition; see Chapter 2) were performed for all samples prior to collagen extraction. Each sample was then subdivided into three aliquots labelled L, E or M, to represent the collagen extraction procedure applied: Longin, EDTA, and Modified Longin or Modified EDTA, as summarized in Table 1.1.

Table 1.1. Collagen Extraction Method by Changes in Procedure.

The experiment design comprised 16 experiments conducted for 4 samples for a total of 64 samples + duplicates analyzed for $\delta^{18}\text{O}$.

Sample ID Tag	Method	Changed Parameter
L-1-01	Longin	Control
L-2-01 ^A	Longin	No Lipid Extraction
L-3-01	Longin	Oven Dried at 80°C
L-3-02	Longin	Oven Dried at 60°C
L-4-01 ^B	Longin	Sample crushed to 63 μ m
L-5-01	Longin	0.5M HCl used
L-6-01	Longin	Ultrasonic Pre-treatment
L-7-01 ^C	Longin	HCl diluted in $\delta^{18}\text{O} = +17.3\text{‰}$ H ₂ O
L-7-02 ^C	Longin	HCl diluted in $\delta^{18}\text{O} = -11.4\text{‰}$ H ₂ O
E-1-01	EDTA	Control
E-2-01 ^A	EDTA	No Lipid Extraction
E-3-01	EDTA	EDTA diluted in $\delta^{18}\text{O} = +17.3\text{‰}$ H ₂ O
E-3-02	EDTA	EDTA diluted in $\delta^{18}\text{O} = -11.4\text{‰}$ H ₂ O
E-4-01	EDTA	Sample crushed to 63 μ m
E-5-01	EDTA	Ultrasonic Pre-treatment
M-1-01	MODIFIED	Freeze-Dried Longin
M-2-01	MODIFIED	EDTA Method Duplicate

^A Used to determine if lipid extractions for modern or ancient bones was needed

^B Used to test whether isotopic exchange occurred during the crushing process as proposed by some studies (Simpson, 2016)

^C Used to test the extent of oxygen isotope exchange compared to +7.4‰ laboratory H₂O

2 MATERIALS & METHODS

2.1 Samples

Bone samples selected for this study are all from megaherbivores and are of sufficient mass (enough for distribution) to serve as potential standards for the oxygen isotope analysis of collagen at some point in the future (Table 2.1; Fig. 2.1). A full description of the samples and their origin is provided in Appendix B. The samples represent a spread of latitude locations, which offers a good opportunity to test for preservation in collagen of the expected differences in the oxygen isotope compositions ($\delta^{18}\text{O}_{\text{dw}}$) of drinking water. This sample choice also allows for a comparison of collagen oxygen isotope ($\delta^{18}\text{O}_{\text{coll}}$) behaviour in modern bone versus well-preserved ancient bone (in this case Pleistocene), which nonetheless may have undergone *post-mortem* / diagenetic alteration.

Table 2.1 - Sample Details.

Sample #	Animal Name	Bone Type	Location	Longitude	Latitude	Age	Dry Bone Vial Mass (11mL)
Bp-1	<i>Bison priscus</i>	Left Radius	Yukon Territory	135° W	67° N	Pleistocene	0.5g ¹
Bt-1	<i>Bos taurus</i>	Right Humerus	Petrolia, Ontario	82° W	43° N	Modern	0.5g
Aa-1	<i>Alces Alces americana</i>	Left Humerus	Sault St. Marie, Ontario	84° W	48° N	Modern	0.5g
Om-1	<i>Ovibos moschatus</i>	Fourth Cervical Vertebrae	Sachs Harbour, NWT	125° W	72° N	Modern	0.5g

¹This is the starting mass of each sample (Which includes both bioapatite and collagen) at the start of the extraction

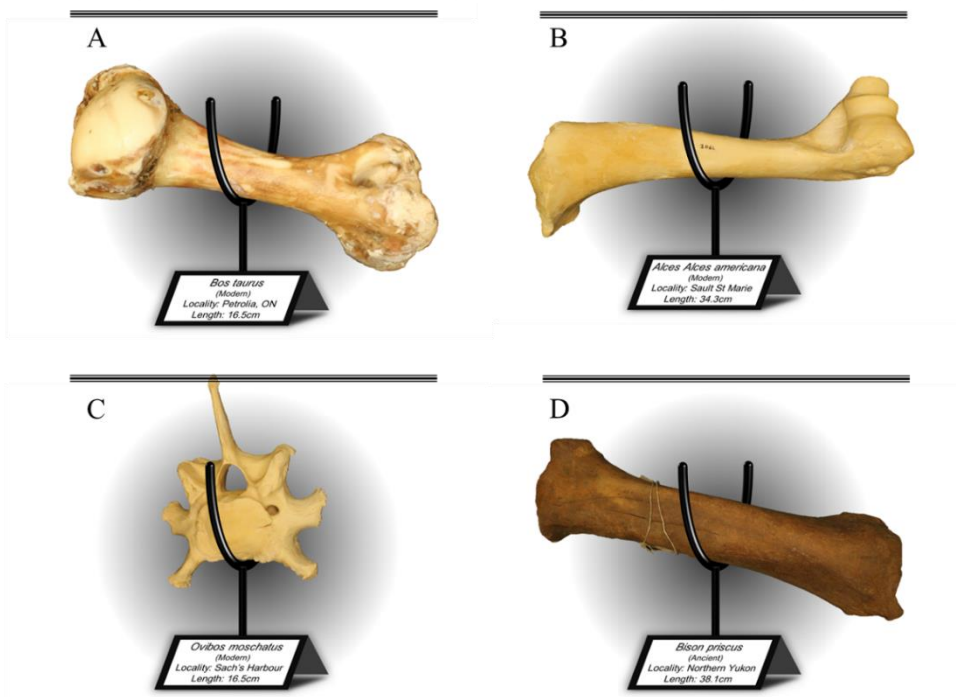


Figure 2.1 – Bone Samples. (A) *B. taurus* humerus; (B) *A. Alces americana* humerus; (C) *O. moschatus* c4 vertebrae; (D) *B. priscus* radius.

2.1.1 Sample Preparation

The sample preparation for the modern bone samples began with defleshing and degreasing. Plint (2019) outlined a method for cleaning the bone samples that limits alteration of bone surface – her “Method D”. The *B. taurus* humerus, which had not been defleshed earlier, was cleaned following this method:

- The bone sample was soaked in cold tap water to loosen and remove as much flesh by hand as possible.
- Remaining flesh was removed using a scalpel.
- The bone was rinsed again and placed in 30L of tap water and left covered for 3 days at 40°C.
- Sixty-three (63) mL of the protease enzyme (Neutrase®) was added to the water to accelerate defleshing, and the sample and solution left for an additional 16 hours at 40°C.
- The bone sample was removed from the solution and dried for several days at room temperature in a fume hood.

The other two modern samples (*Alces alces americana* and *Ovibos moschatus*) were degreased more aggressively by boiling them in water for more than 24 hours. The ancient bone sample (*B. priscus*) did not require defleshing or degreasing. Subsamples were then extracted from each bone using a diamond wheel on a Dremel® tool kit. To ensure that friction from the diamond wheel did not heat the bone, leading to alteration, brief pauses in cutting were accompanied by dampening of the diamond wheel with a KimWipe™ rinsed with acetone.

2.2 Analytical Methods

2.2.1 Fourier Transform Infrared Spectroscopy

Fourier Transform Infra-Red Spectroscopy (FTIR) was used to assess the state of preservation of bioapatite, and hence indirectly collagen, in the bone samples. The commonly used parameter for this assessment is the crystallinity index (CI). The CI indicates the degree of phosphate peak splitting in the PO₄ group of bioapatite and provides a measure of *post-mortem* recrystallization (Weiner and Bar-Yosef, 1990; Wright and Schwarcz, 1996). A CI greater than 4.3 suggests extensive hydroxyapatite recrystallization, which signifies poor preservation of bioapatite. FTIR crystallinity indices for unaltered samples of fresh bone are typically in the range of 2.8-3.0 but can be as high as ~3.5-4.3 for well-preserved archaeological samples (Webb et al., 2014).

The FTIR CI is calculated using equation 2.1:

$$\text{FTIR CI} = \frac{(A + B)}{C} \quad (\text{Equation 2.1})$$

where (A) and (B) refer to the height of v₄ PO₄ group peaks at 605 cm⁻¹ and 565 cm⁻¹, respectively, and (C) refers to the height of valley located at 595 cm⁻¹ between these peaks on a baseline-corrected spectrum (Munro et al., 2007; Webb et al., 2014; Weiner and Bar-Yosef, 1990; Wright and Schwarcz, 1996).

Sample pellets for the FTIR measurements were created by mixing ~2 mg of finely powdered bone (grain size 45-65 μm) with 200 mg potassium bromide (KBr) to compress with a hydraulic press at 10 tons for 10 minutes. Absorbance spectra were obtained at the Laboratory for Stable Isotope Science (LSIS) at the University of Western Ontario, London, Ontario, Canada using a Bruker Vector 22 FTIR Spectrometer, scanning 16 times from 800 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹. Two replicates were included to evaluate analytical error. Precision was ±0.47 across CI values, measured by sample duplicates. Accuracy (as measured to 3.2) was 0.0, indicating no shift in values from instrument error. For each of the four bone samples (*B. priscus*, *A. Alces americana*, *B. taurus*, *O. moschatus*), four subsamples were analyzed, taken from: the outer surface of the cortical bone, center of the cortical bone, cortical bone at the cortical

bone/trabecular bone interface, and trabecular bone, as discussed in section 1.4.4 (Fig. 1.1). The same cortical bone used in this analysis was used for the collagen extractions.

2.2.2 Powder X-ray Diffraction Analysis

Powder x-ray diffraction analysis (pXRD) was conducted for several key samples to measure the crystallinity of bone bioapatite. Powdered samples were gently front-packed into glass holders in preparation for the pXRD measurements. The pXRD data were collected using a Rigaku RU-200BVH rotating anode diffractometer, operating in step-scan mode and employing Co K α radiation ($\lambda = 0.1790210$ nm) at 45 kV and 160 mA. The measurements were collected from 28 to 44° 2 θ , with a 0.020° step size and a scan speed of 10°/min.

The pXRD CI was calculated following Person et al. (1995) and Bartsiokas and Middleton (1992) (Equation 2.2):

$$\text{XRD CI} = \{H[112] + H[300] + H[202]\}/H[211], \quad (\text{Equation 2.2})$$

where H = height above the local baseline (28–44° 2 θ) for each [hkl] line. The hkl values were assigned according to Joint Committee on Powder Diffraction Standards (JCPDS) card 9-432 for hydroxyapatite.

2.2.3 Mass Spectrometry

High Temperature Conversion Elemental Analyzer (TC/EA)-Continuous Flow (CF) Isotope Ratio Mass Spectrometer (IRMS). The oxygen isotope analysis of collagen was performed at LSIS. Aliquots of bone collagen for each sample were weighed into silver capsules and loaded into the Zeroblank[®] auto-sampler of the Thermo Scientific[™] High Temperature Conversion Elemental Analyzer (TC/EA). The carbon monoxide gas (CO) released from the glassy carbon reactor (1350°C) was purified on a GC column (held at 90°C) packed with a 5 Å molecular sieve and swept in a continuous flow of helium to a Thermo Scientific[™] Delta^{PLUS}XL[™] isotope-ratio mass spectrometer (IRMS) for oxygen isotope analysis (Matthews et al., 2021).

Collagen oxygen isotope compositions ($\delta^{18}\text{O}_{\text{col}}$) are reported relative to Vienna Standard Mean Ocean Water (VSMOW), as calibrated on a two-point scale using accepted values for international standards KHS (accepted $\delta^{18}\text{O} = +21.21\%$) and CBS (accepted $\delta^{18}\text{O} = +2.39\%$)

(Coplen T.B, 2019). The reproducibility (SD) of replicate analyses of these standard analyses ranged from 0.29‰ (CBS, n=16) to 0.52‰ (KHS, n=16). Keratin-Spectrum 1 was used to test the accuracy of the calibration; 20 analyses returned an average of $\delta^{18}\text{O} = +8.47 \pm 0.50\text{‰}$, which is currently the accepted value for this standard. Accuracy of the collagen measurements were measured by comparing both the Longin and EDTA method measurements to the average EDTA-extracted isotopic composition. Accuracy of the Longin method was $\pm 3.9\text{‰}$, whereas the accuracy of the EDTA method was $\pm 0.6\text{‰}$.

Elemental Analyzer (EA)- Continuous Flow (CF)-Isotope Ratio Mass Spectrometry (IRMS). Collagen stable carbon ($\delta^{13}\text{C}_{\text{col}}$) and nitrogen ($\delta^{15}\text{N}_{\text{col}}$) were measured using a Costech elemental analyzer (EA) combustion system attached to a Thermo Scientific™ Delta V IRMS operated in continuous flow mode with helium as the carrier gas. The combustion furnace was set at 1000°C. The stable carbon isotope data were calibrated to VPDB using a two-point scale anchored by USGS-40 (accepted $\delta^{13}\text{C} = -26.39\text{‰}$) (Qi et al., 2016) and USGS-41a (accepted $\delta^{13}\text{C} = +36.55\text{‰}$) (Qi et al. 2016). The reproducibility (SD) of replicate analyses of these standards ranged from 0.074‰ (USGS-40, n=16) to 0.404‰ (USGS-41a, n=18). The nitrogen isotope data were calibrated to AIR using a two-point scale anchored by USGS-40 (accepted $\delta^{15}\text{N} = -4.52\text{‰}$) (Qi et al. 2003) and USGS-41a (accepted $\delta^{15}\text{N} = +47.55\text{‰}$) (Qi et al. 2016). The reproducibility (SD) of replicate analyses of these standards ranged from 0.116‰ (USGS-40, n=16) to 0.214‰ (USGS-41a, n=17). These standards were also used to calibrate the carbon and nitrogen contents as well as the atomic C/N ratio of each sample.

Matrix-Assisted Laser Desorption/Ionization (MALDI) Time-of-Flight (TOF) Mass Spectrometry. This technique and instrumentation are used to measure microscale biological organic matter quantities, specifically peptide abundances (Tran et al., 2011). Glutamine deamidation can be observed through the analysis of peptides. Analysis of peptide masses requires that the targeted proteins be digested with sequencing grade lyophilized trypsin. If deamidation has occurred within a peptide chain, their masses should be different than the expected mass of that peptide chain. Deamidation of glutamine causes a +0.984 Da mass shift (van Doorn, 2012) that can be identified using MALDI-TOF-MS.

Collagen samples were digested according to the trypsin digest guide provided in Appendix C and then lyophilized to dry. No clean-up (see Appendix C) was performed prior to

analysis as there was no evidence of salts or buffers following the extensive rinsing of the samples post demineralization. Each sample was initially prepared as 1mg/mL in 0.1% formic acid (FA). This solution was then diluted to several concentrations (1, 0.5, and 0.25 mg/mL) to mix with the MALDI matrix, α -cyano-4-hydroxycinnamic acid (CHCA), which is prepared with 5.5 mg/mL in 50% acetonitrile, 0.1% Trifluoroacetic Acid (TFA) at 1:1 ratio. Each sample/matrix mix was spotted twice onto a MALDI plate. Data were acquired from each spot. The 0.25 mg/mL composition produced cleanest spectra (most peaks). Peaks for the EDTA samples were lower resolution than the Longin samples due to remnants of collagen still in the digest solution. The temperature regulator was not functioning and when the samples were removed it was observed that the Longin samples were fully digested whereas the EDTA samples still had some remnant collagen. Because of this problem, there were fewer targets available for analysis of the EDTA samples. Despite this, the EDTA samples still produced the cleanest spectra.

Mass spectrometry was performed using an AB Sciex 5800 TOF/TOF System, MALDI TOF TOF Series Explorer (AB Sciex) (Framingham, MA, USA) that is equipped with a 349nm Nd:YLF OptiBeam On-Axis laser. The laser pulse rate used was 400 Hz. Reflectron positive mode was used, as externally calibrated at 50ppm mass tolerance and internally at 10ppm using tryptic autolysis peaks. Each mass spectrum was collected as a sum of 500 shots.

The MALDI TOF data were interpreted using the R program of Julie Wilson (2012) (found here: <https://github.com/bioarch-sjh/q2e>). The peptides selected for matrix matching are listed in Table 2.2. The R program was compiled with this peptide list for each set of MALDI spectra. The program adds a hydroxylation factor on the ends of several chains. If a significant decrease in expected mass is identified in a peptide chain (i.e., >16 Da), a letter 'Z' is automatically added to the end of the chain to let the program know that the chain was influenced by other chemical reactions besides glutamine deamidation. This hydroxylation factor ensures that glutamine deamidation is the primary reaction that is being measured (Wilson et al., 2012).

Table 2.2 - Peptide Chains and their Respective Masses. The list of peptides analyzed in all six samples: Bt.L-1-01, Bt.E-1-01, Aa.L-1-01, Aa.E-1-01, Bp.L-1-01, Bp.E-1-01. M/z^+ represents mass (in Daltons) over charge (+1 for this study) of the peptide chain listed. Letters represent amino acids present in the chain, i.e. E = glutamic acid, Q = glutamine.

m / z+ (Da)	Peptide sequence
836.44	GPAGPQGPR
1105.57	GVQGPPGPAGPR
1690.77	DGEAGAQQPPGPAGPAGER
1706.77	DGEAGAQQPPGPAGPAGER
2056.98	TGPPGPAGQDGRPGPPGPPGAR
2073.01	GAPGADGPAGAPGTPGPQGIAGQR
2089.01	GAPGADGPAGAPGTPGPQGIAGQR
2689.26	GFSGLQGPPGPPGPSGEQGPSGASGPAGPR
2705.26	GFSGLQGPPGPPGPSGEQGPSGASGPAGPR
3001.5	GPSGEPGTAGPPGTPGPQGLLGAPGFLGLPGSR
3100.41	GLPGPPGAPGPQGFQGPPEPGEPEGASGPMGPR
3665.54	GSQGSQGPAGPPGPPGPPGPSGGGYEFGFDGDFYR

III. RESULTS

3.1 Bone Physical and Structural Characterization

Chemical reactions can be identified in organic matter through various qualitative properties including colour, structural integrity, and texture. These properties can serve as indicators of chemical changes. Section 3.1 outlines the physical changes observed during bone sample preparation and collagen extraction.

3.1.1 Colour

Bone colour changes were observed in association with several procedures. For example, the *Bos taurus* humerus underwent notable physical changes during defleshing and degreasing. This modern bone sample was obtained from a local butcher in a pristine *post-mortem* condition – still fleshy (Fig. 3.1A). Once the flesh was removed, the bone took on a glossy white appearance (Fig. 3.1B), and after the degreasing technique has been performed, the bone was ready for analysis (Fig. 3.1C). After drying at 20°C in air (in a fume hood) for three weeks, however, the bone surface became discoloured (Fig. 3.1D). A similar feature was observed for the Pleistocene *Bison priscus* sample.

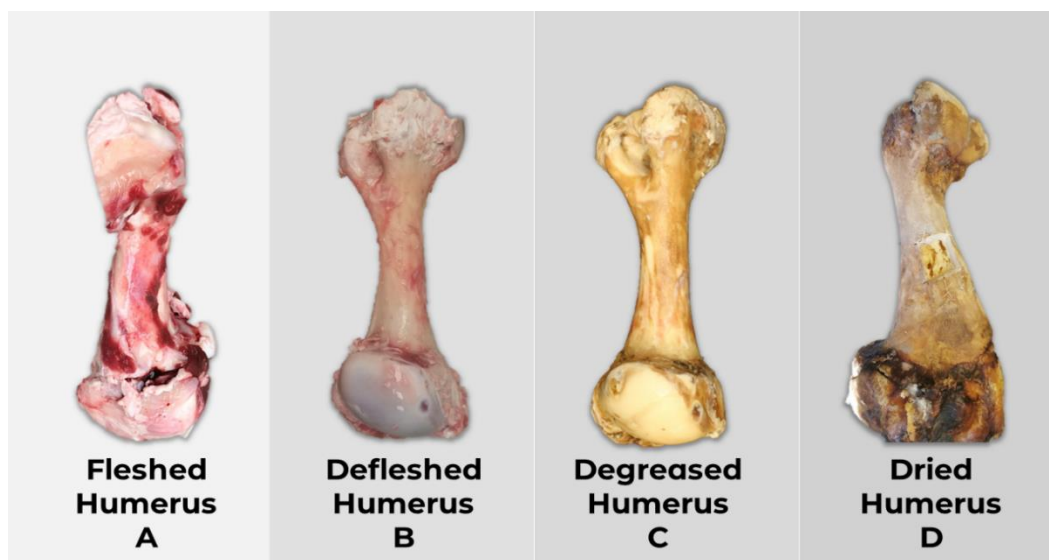


Figure 3.1 – *Bos taurus* Defleshing/Degreasing Effects. The humerus of a *Bos taurus* was cleaned using “Method D” of Plint (2019). (A) Fleshy humerus as obtained from the butcher; (B) Appearance after removing flesh/tissue, (C) Appearance after degreasing; (D) Appearance after 3 weeks of drying in 20°C.

Figure 3.2 illustrates the discolouration of 2 x 1cm sliced samples of cortical bone taken at the cortical bone / air and cortical bone / trabecular bone interface relative to the centre of the cortical bone in this sample.

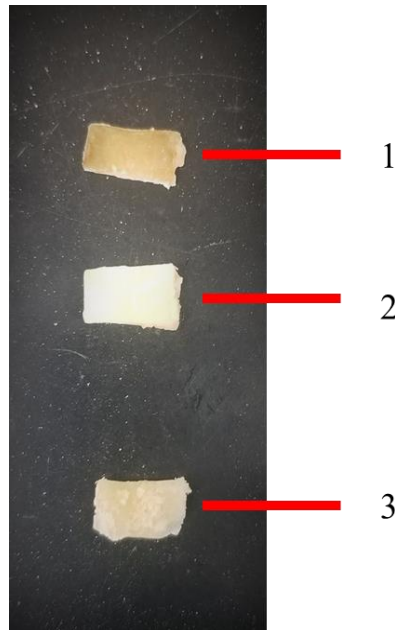


Figure 3.2 – Cortical bone from *Bison priscus*: (1) air / cortical bone interface, (2) centre of cortical bone, and (3) cortical / trabecular bone interface. Size of each piece is ~1cm across.

Third, following extraction from cortical bone, a colour change in the collagen from white to dark brown was noted after oven drying at 90°C (Fig. 3.3A). This colour change did not occur when the collagen was freeze-dried (Fig. 3.3B).

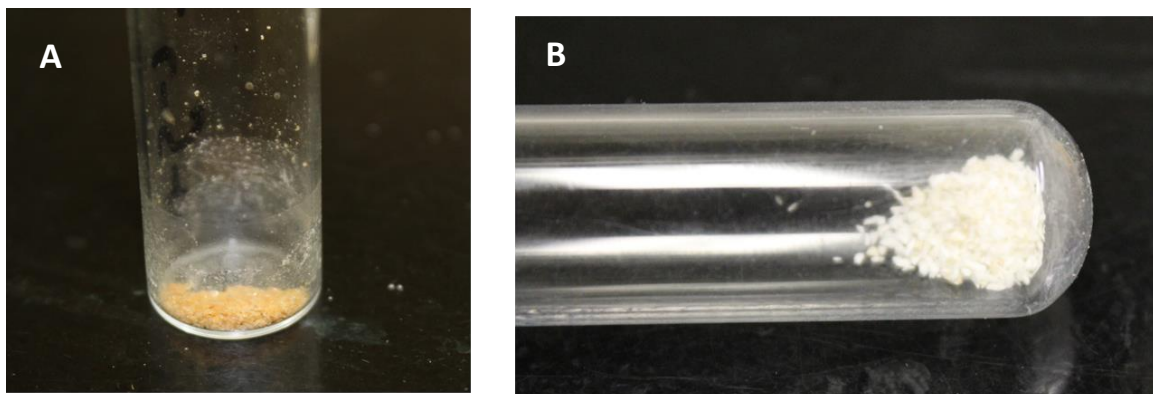


Figure 3.3 – Collagen from *Bos taurus* after drying: (A) Longin method (oven dried), and (B) EDTA method (freeze-dried).

3.1.2 Fourier Transform Infrared Spectroscopy (FTIR)

Crystallinity Index (CI) results for bioapatite from the bone samples are listed in Table 3.1. Most CI values for all modern samples and the ancient (*B. priscus*) bioapatite sample varied from 2.5-3.2, within the expected range for unaltered bone bioapatite. The first sample analyzed for *Bos taurus* (Bt-1-01) returned CIs above 3.8 (outside of the range for unaltered modern bone) (Webb et al., 2014). A new sample was cut from the same position and was more carefully shaved using the diamond wheel on the Dremel[®] kit to ensure the absence of residual organic material. Replicate analyses of this sample returned CI values within the acceptable range for unaltered bone (samples *Bos taurus*-2, *Bos taurus*-3, *Bos taurus*-4, Table 3.1). Bt.3-02 was used for collagen extraction.

Table 3.1 – Bioapatite Crystallinity Indices for Sampled Bones.

Measurements collected from the Fourier Transform Infrared Spectrometer analytical session

Sample #	Type	Height of v4 PO ₄	Height of v4 PO ₅	Height of Valley	CI
		Grs. @ 617cm-1	Grs. @ 563cm-1	@ 590cm-1	
Bp-1 ^A					
Bp-1-01	Cortical / Air Interface	0.09	0.08	0.06	3.26
Bp-1-02	Cortical Bone	0.09	0.10	0.07	2.98
Bp-1-03	Trabecular / Cortical Interface	0.09	0.08	0.05	3.03
Bp-1-04	Trabecular Bone	0.07	0.07	0.04	3.34
Bt-1					
Bt-1-01	Cortical / Air Interface	0.32	0.40	0.17	4.14
Bt-1-02	Cortical Bone	0.07	0.08	0.04	3.62
Bt-1-03	Trabecular / Cortical Interface	0.07	0.08	0.04	3.93
Bt-1-04	Trabecular Bone				
Bt-2-02	Cortical Bone	0.11	0.14	0.08	3.12
Bt-3-01	Cortical / Air Interface	0.03	0.04	0.03	2.61
Bt-3-02	Cortical Bone	0.03	0.04	0.03	2.50
Bt-3-03	Trabecular / Cortical Interface	0.03	0.04	0.03	2.52
Bt-3-04	Trabecular Bone	0.06	0.07	0.05	2.60
Bt-4-02	Cortical Bone	0.07	0.08	0.06	2.56
Aa-1					
Aa-1-01	Cortical / Air Interface	0.17	0.19	0.11	3.23
Aa-1-02	Cortical Bone	0.15	0.16	0.10	3.03
Aa-1-03	Trabecular / Cortical Interface	0.11	0.12	0.06	3.64
Aa-1-04	Trabecular Bone	NA	NA	NA	NA
Aa-2-02	Cortical Bone	0.03	0.04	0.02	3.02
Om-1					
Om-1-01	Cortical / Air Interface	0.14	0.16	0.11	2.85
Om-1-02	Cortical Bone	0.13	0.15	0.10	2.77
Om-1-03	Trabecular / Cortical Interface	0.09	0.08	0.05	3.33
Om-1-04	Trabecular Bone	0.09	0.09	0.06	2.91

^A Bt = *Bos taurus*; Bp = *Bison priscus*; Aa = *Alces alces americana*; Om = *Ovibos moschatus*

The average CI of all samples with position of sampling is illustrated in Figure 3.4. The least recrystallized (i.e., least altered) bioapatite occurs in the center of the cortical bone. The center rather than the edges of the cortical bone was used for collagen extractions (samples denoted by -02 as the suffix to the sample name).

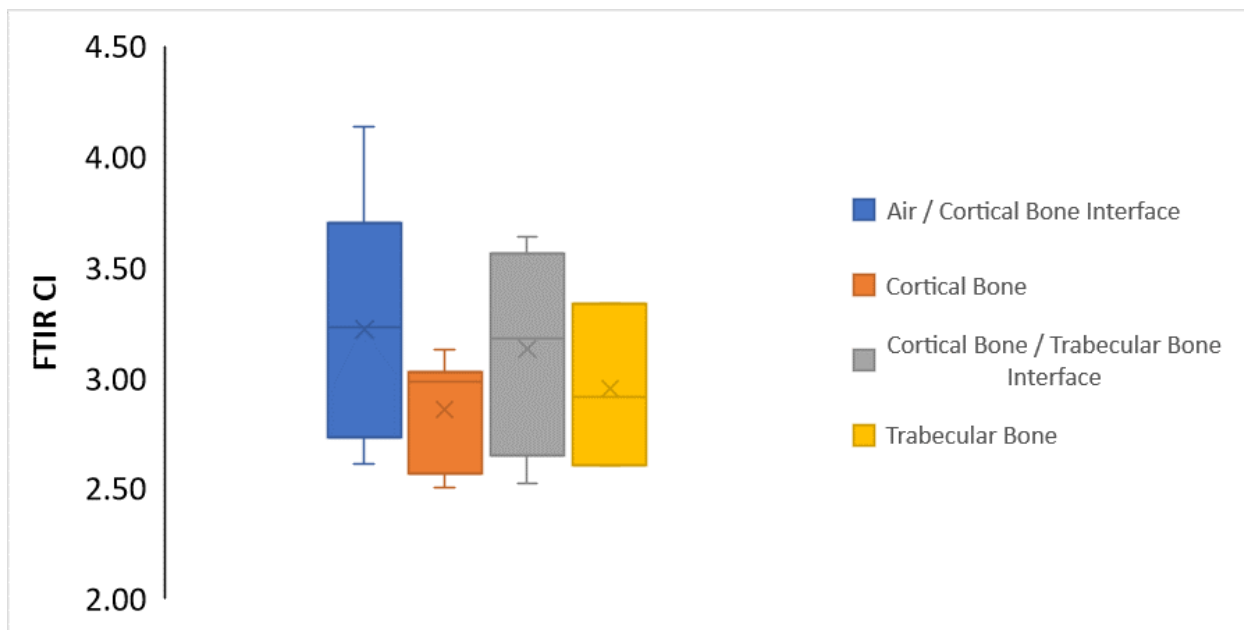


Figure 3.4 – CI Values for Different Cortical Bone Positions. The central portion of the cortical bone layer has the lowest CI average and smallest spread of values. ‘Whiskers’ on the plot for trabecular bone are coincident with the upper and lower boundaries of the box.

3.2 Stable Carbon and Nitrogen Isotope Results for Collagen

Table 3.2 summarizes stable carbon and nitrogen isotope results obtained for the collagen samples, as obtained after a variety of extraction protocols and treatments. The average atomic C:N ratio for the samples is 3.2 (2.7-3.6), which is acceptable for modern collagen (Guiry and Szpak, 2020; van Klinken, 1999). Samples that lay outside this range were not included in further analysis. The average carbon content is 43.2 wt% and the average nitrogen content is 15.5 wt%, both acceptable for well-preserved collagen (van Klinken, 1999).

Table 3.2 – Stable Carbon and Nitrogen Isotope Compositions and Contents for Different Collagen Extraction Methods.

Sample ID	Taxon	Region	Experiment	$\delta^{13}\text{C}$, ‰ (VPDB)	$\delta^{15}\text{N}$, ‰ (AIR)	wt. %C	wt. %N	Atomic C:N Ratio
Bt.L-1-01	Bos	Petrolia, ON	Longin Control	-10.7	5.2	45.8	16.6	3.2
Bt.L-2-01	Bos	Petrolia, ON	No Lipid Extraction	-10.9	5.2	43.8	16.1	3.2
Bt.L-3-01	Bos	Petrolia, ON	80C Oven Heating	-10.4	5.4	43.8	16.7	3.1
Bt.L-3-02	Bos	Petrolia, ON	60C Oven Heating	-10.7	5.5	45.3	16.9	3.1
Bt.L-4-01	Bos	Petrolia, ON	Smaller Sample Size	-10.5	5.4	46.2	17.1	3.1
Bt.L-5-01	Bos	Petrolia, ON	Stronger Acid	-10.3	5.4	45.4	17.1	3.1
Bt.L-6-01	Bos	Petrolia, ON	Ultrasound	-10.2	5.1	44.3	16.6	3.4
Bt.M-1-01	Bos	Petrolia, ON	Longin Freeze Dried	-9.7	4.8	42.0	15.1	3.2
Bt.M-1-01 Dup	Bos	Petrolia, ON	Sample Duplicate		5.2	42.5	15.6	3.2
Bt.M-1-01 Dup	Bos	Petrolia, ON	Sample Duplicate		4.9	41.8	15.3	3.2
Bt.M-2-01	Bos	Petrolia, ON	EDTA Method Duplicate	-9.6	4.9	44.4	16.0	3.2
Bt.E-1-01	Bos	Petrolia, ON	EDTA Control	-10.3	5.1	42.6	15.4	3.2
Bt.E-1-01b	Bos	Petrolia, ON	Aged Sample Duplicate	-11.9	5.5	44.4	15.3	3.4
Bt.E-1-01 ReX	Bos	Petrolia, ON	Re-extraction of Control	-11.4	5.6	44.0	16.8	3.1
Bt.E-2-01	Bos	Petrolia, ON	No Lipid Extraction (NLE)	-10.4	4.8	43.5	15.4	3.3
Bt.E-2-01 Dup	Bos	Petrolia, ON	Sample Duplicate	-10.4	4.9	43.3	15.5	3.3
Bt.E-2-01 ReX	Bos	Petrolia, ON	Re-extraction of NLE	-11.3	5.8	43.6	16.6	3.1
Bt.E-2-01 Dup ReX	Bos	Petrolia, ON	Re-extraction of NLE		6.2	43.4	16.5	3.1
Bt.E-3-01	Bos	Petrolia, ON	Heavy Water	-8.8	4.8	42.0	15.3	3.2
Bt.E-3-02	Bos	Petrolia, ON	Light Water	-10.7	5.2	40.7	14.8	3.1
Bt.E-4-01	Bos	Petrolia, ON	Smaller Sample Size <63um	-10.6	5.4	42.5	16.2	3.1
Bt.E-5-01	Bos	Petrolia, ON	Ultrasound	-9.7	5.0	42.6	15.5	3.4
Bp.L-1-01	Bison	Northern YT	Longin Control	-20.1	5.7	44.3	15.1	2.7
Bp.L-2-01	Bison	Northern YT	No Lipid Extraction	-20.1	5.7	44.0	15.9	2.8
Bp.L-3-01	Bison	Northern YT	80C Oven Heating	-20.1	5.8	41.9	15.8	3.1
Bp.L-3-02	Bison	Northern YT	60C Oven Heating	-20.0	5.8	44.3	16.8	3.1
Bp.L-4-01	Bison	Northern YT	Smaller Sample Size	-20.1	5.8	40.9	15.3	3.1
Bp.L-5-01	Bison	Northern YT	Stronger Acid	-20.1	5.9	44.8	16.8	3.1
Bp.L-6-01	Bison	Northern YT	Ultrasound	-20.0	5.7	36.2	13.3	2.7
Bp.M-1-01	Bison	Northern YT	Longin Freeze Dried	-20.5	5.7	33.7	12.3	3.2
Bp.M-2-01	Bison	Northern YT	EDTA Method Duplicate	-20.4	4.9	43.3	16.0	3.6
Bp.E-1-01	Bison	Northern YT	EDTA Control	-20.2	6.1	43.6	15.5	2.8
Bp.E-1-01b	Bison	Northern YT	Aged Sample Duplicate	-20.2	5.8	44.2	15.3	3.4
Bp.E-2-01	Bison	Northern YT	No Lipid Extraction (NLE)	-20.2	6.0	42.5	15.3	2.8
Bp.E-2-01 Dup	Bison	Northern YT	Sample Duplicate	-20.2	5.9	42.3	15.5	2.7
Bp.E-3-01	Bison	Northern YT	Heavy Water	-20.0	6.0	41.4	15.6	3.1
Bp.E-3-02	Bison	Northern YT	Light Water	-19.7	6.2	44.9	16.9	3.1
Bp.E-4-01	Bison	Northern YT	Smaller Sample Size <63um	-19.3	5.9	30.0	7.4	4.8
Bp.E-5-01	Bison	Northern YT	Ultrasound	-20.1	5.7	41.3	15.0	3.1

Aa.L-1-01	Alces	Sault St. Marie	Longin Control	-23.7	+0.03	43.5	15.6	2.8
Aa.L-2-01	Alces	Sault St. Marie	No Lipid Extraction	-24.1	+0.0	43.8	15.3	2.9
Aa.L-3-01	Alces	Sault St. Marie	80°C Oven Heating	-24.0	+0.4	43.8	15.0	3.4
Aa.L-3-02	Alces	Sault St. Marie	60°C Oven Heating	-23.7	+0.3	44.8	16.5	3.2
Aa.L-4-01	Alces	Sault St. Marie	Smaller Sample Size	-24.4	+0.3	47.3	16.0	3.4
Aa.L-5-01	Alces	Sault St. Marie	Stronger Acid	-24.0	+0.4	47.2	16.5	3.3
Aa.L-6-01	Alces	Sault St. Marie	Ultrasonic Cleaning	-23.7	+0.0	38.6	13.8	2.9
Aa.M-1-01	Alces	Sault St. Marie	Longin Freeze Dried	-24.8	+0.0	44.4	14.7	3.5
Aa.M-2-01	Alces	Sault St. Marie	EDTA Method Duplicate	-24.4	+0.4	47.6	13.5	4.1
Aa.E-1-01	Alces	Sault St. Marie	EDTA Control	-23.8	+0.9	43.5	15.2	2.9
Aa.E-1-01b	Alces	Sault St. Marie	Aged Sample Duplicate	-23.9	+0.3	44.4	15.0	3.5
Aa.E-1-01 ReX	Alces	Sault St. Marie	Re-extraction of Control	-23.8	-0.1	44.6	16.3	3.2
Aa.E-2-01	Alces	Sault St. Marie	No Lipid Extraction (NLE)	-24.2	-0.1	42.6	14.1	3.0
Aa.E-2-01 Dup	Alces	Sault St. Marie	Sample Duplicate	-24.5	+1.1	43.7	14.7	3.0
Aa.E-2-01 ReX	Alces	Sault St. Marie	Re-extraction of NLE	-24.3	+0.0	43.6	15.6	3.4
Aa.E-2-01 Dup ReX	Alces	Sault St. Marie	Re-extraction of NLE	-24.2	+0.5	43.4	16.2	3.3
Aa.E-3-01	Alces	Sault St. Marie	Heavy Water	-23.8	-0.3	48.4	16.7	3.4
Aa.E-3-02	Alces	Sault St. Marie	Light Water	-23.6	-0.2	47.1	16.8	3.3
Aa.E-4-01	Alces	Sault St. Marie	Smaller Sample Size <63um	-24.7	+0.2	45.5	15.0	3.5
Aa.E-5-01	Alces	Sault St. Marie	Ultrasound	-24.3	+0.3	42.7	14.8	3.2
Om.L-1-01	Ovibos	Sachs Habour, NWT	Longin Control	-23.0	+4.7	41.6	15.3	2.7
Om.L-2-01	Ovibos	Sachs Habour, NWT	No Lipid Extraction	-22.9	+4.8	43.6	16.1	2.7
Om.L-3-01	Ovibos	Sachs Habour, NWT	80°C Oven Heating	-23.0	+5.0	43.0	16.2	3.1
Om.L-3-02	Ovibos	Sachs Habour, NWT	60°C Oven Heating	-22.8	+5.1	43.9	16.6	3.1
Om.L-4-01	Ovibos	Sachs Habour, NWT	Smaller Sample Size	-22.8	+5.0	45.2	17.0	3.1
Om.L-5-01	Ovibos	Sachs Habour, NWT	Stronger Acid	-22.9	+4.9	43.7	16.5	3.1
Om.L-6-01	Ovibos	Sachs Habour, NWT	Ultrasonic Cleaning	-22.9	+4.8	42.2	15.7	3.7
Om.M-1-01	Ovibos	Sachs Habour, NWT	Longin Freeze Dried	-23.0	+4.7	41.9	15.7	3.1
Om.M-2-01	Ovibos	Sachs Habour, NWT	EDTA Method Duplicate	-22.9	+4.9	47.0	15.3	3.6
Om.E-1-01	Ovibos	Sachs Habour, NWT	EDTA Control	-22.9	+5.0	43.1	15.2	2.8
Om.E-1-01b	Ovibos	Sachs Habour, NWT	Aged Sample Duplicate	-23.1	+4.8	42.8	15.3	3.3
Om.E-2-01	Ovibos	Sachs Habour, NWT	No Lipid Extraction (NLE)	-22.9	+4.8	43.3	15.7	2.8
Om.E-2-01 Dup	Ovibos	Sachs Habour, NWT	Sample Duplicate	-23.0	+5.0	42.0	15.3	2.7
Om.E-3-01	Ovibos	Sachs Habour, NWT	Heavy Water	-22.6	+4.9	43.5	11.2	4.5
Om.E-3-02	Ovibos	Sachs Habour, NWT	Light Water	-22.7	+4.7	43.9	16.6	3.1
Om.E-4-01	Ovibos	Sachs Habour, NWT	Smaller Sample Size <63um	-23.0	+5.1	42.9	16.4	3.1
Om.E-5-01	Ovibos	Sachs Habour, NWT	Ultrasound	-22.9	+4.9	41.9	15.5	3.3

*Bt = *Bos taurus* ; Bp = *Bison priscus* ; Aa = *Alces alces americana* ; Om = *ovibos moschatus* .

Each experiment replaced one parameter from the control extraction protocol to test for variability between each change.

Pre-treatments include lipid extraction (tested in 2-01) and ultrasonic cleaning (tested in L-6-01, E-5-01).

Water isotopic composition was tested in several experiments (E-3-01,E-3-02). See text

Experiments with E in the sample name involved EDTA, L involved the Longin method, and M were a method check (duplicate and Longin freeze dried).

Table 3.3 provides the average values and standard deviations derived from Table 3.2 for each collagen extraction method used.

Table 3.3 – Longin versus EDTA Carbon and Nitrogen Isotopes Average Compositions.

Sample Type	Average $\delta^{13}\text{C}$ ‰ (VPBD)	Average $\delta^{15}\text{N}$ ‰ (AIR)	SD $\delta^{13}\text{C}$	SD $\delta^{15}\text{N}$
Bos taurus EDTA	-10.5	+5.3	0.9	0.4
Bos taurus Longin	-10.4	+5.2	0.4	0.2
Bison priscus EDTA	-20.0	+5.8	0.3	0.4
Bison priscus Longin	-20.1	+5.8	0.1	0.1
Ovibos moschatus EDTA	-22.9	+4.9	0.2	0.1
Ovibos moschatus Longin	-22.9	+4.9	0.1	0.1
Alces alces americana EDTA	-24.1	+0.2	0.3	0.4
Alces alces americana Longin	-24.0	+0.2	0.4	0.2

Figures 3.5 and 3.6 illustrate the spread in both carbon and nitrogen isotope compositions for each method and bone sample. EDTA-treated samples show a wider spread than the Longin method – particularly for nitrogen isotope compositions – but exhibit similar average isotopic compositions overall. The chemical formula of EDTA is $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$ consisting of both carbon and nitrogen, unlike the Hydrochloric Acid (HCl) of the Longin method. The inclusion of these elements in EDTA could be responsible for the larger variation in carbon and nitrogen isotope compositions as they would be a likely source of exchange.

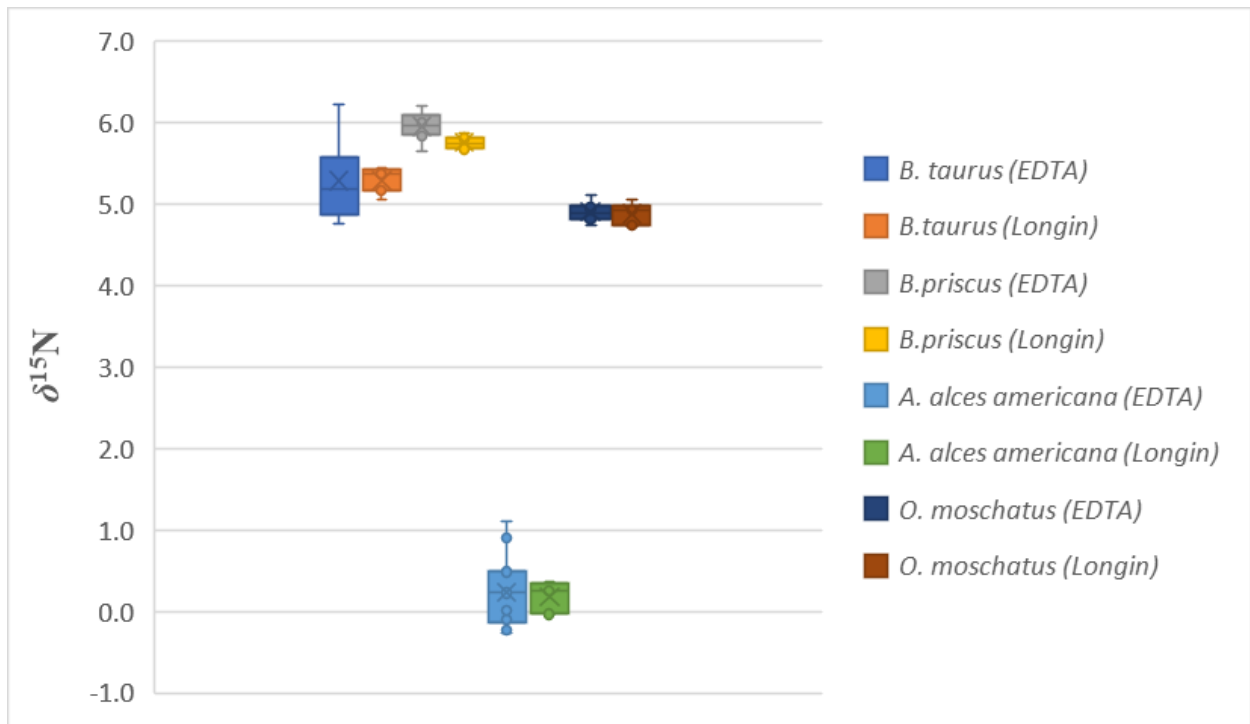


Figure 3.5 – Nitrogen isotope compositions obtained for each sample and extraction method used. EDTA-extracted samples show a greater spread than Longin-extracted samples.

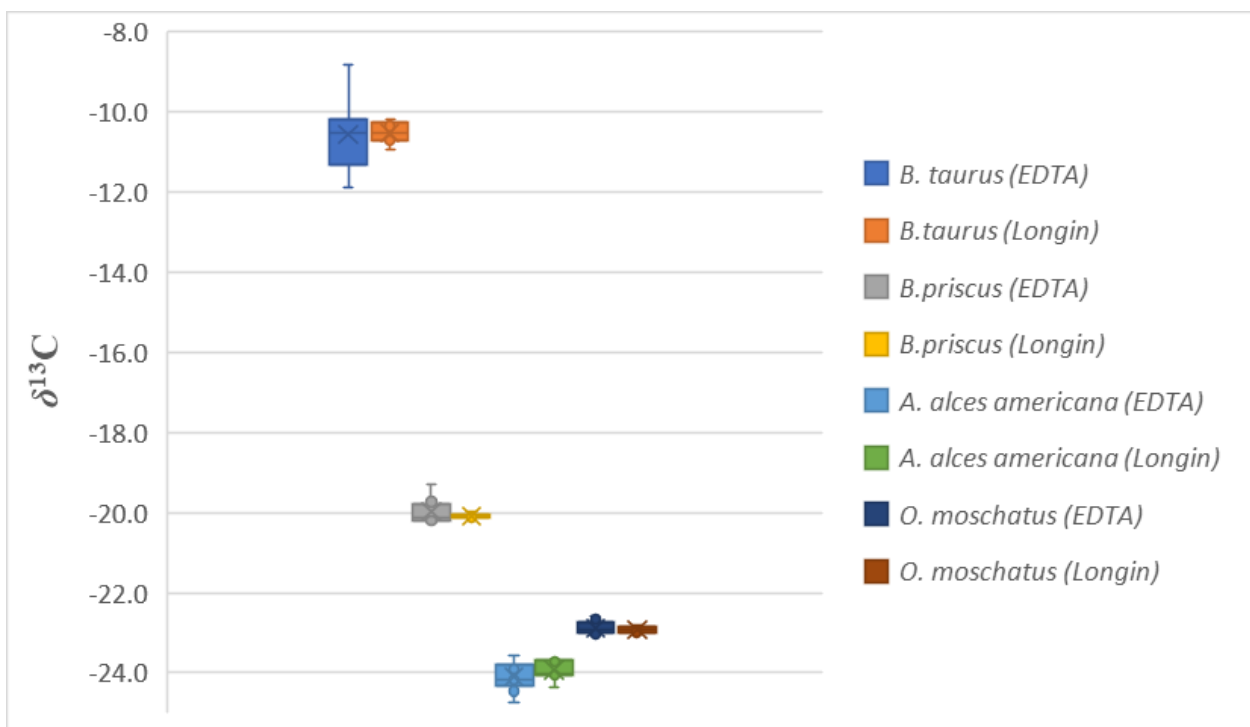


Figure 3.6 – Carbon isotope compositions for each sample and extraction method used. The EDTA extracted samples show a greater spread than the Longin extracted samples.

3.3 Oxygen Isotope Results for Collagen

Table 3.4 lists the oxygen contents and oxygen isotope compositions for all samples analyzed. The average oxygen content is 25 wt%, which lies within the typical range for collagen. Results with a 'L' in the sample name were obtained for collagen extracted using 0.25M HCl (Longin) demineralization. Those values of $\delta^{18}\text{O}_{\text{col}}$ lie in the range of +8.3 to +14.0‰ for samples from southern Canada and -0.3 to +13.1‰ for samples from northern Canada. Results with an 'E' in the sample name were obtained for collagen extracted using 0.5M EDTA demineralization. Those values of $\delta^{18}\text{O}_{\text{col}}$ lie in the range of +4.2 to +6.7‰ for samples from southern Canada and -4.0 to -1.8‰ for samples from northern Canada. Results with a 'M' in the sample name were obtained for collagen extracted using either EDTA or HCl depending on the experiment specified in Table 3.4. The accuracy of these measurements was calculated by comparing the standard deviation of the method measurements to the average $\delta^{18}\text{O}$ of the EDTA measurements. Collagen $\delta^{18}\text{O}$ is not known for megaherbivores, but the inclusion of glutamine deamidation levels in this study should assist in validating the results of the EDTA method. Accuracy of $\delta^{18}\text{O}$ for EDTA-extracted collagen was calculated to be $\pm 0.6\%$, whereas for Longin-extracted collagen it was $\pm 3.9\%$. A further test (M-1-01) was done for the Longin method using the freeze dryer instead of oven heating. Accuracy of $\delta^{18}\text{O}$ for Longin-extracted collagen that was freeze dried is $\pm 1.1\%$.

Table 3.4 – Oxygen Isotope Compositions and Contents for Different Collagen Extraction Methods.

Sample ID	Taxon	Region	Experiment	$\delta^{18}\text{O}$ ‰ (VSMOW)	wt.% O
Bt.L-1-01	Bos	Petrolia, ON	Longin Control	+14	27.8
Bt.L-2-01	Bos	Petrolia, ON	No Lipid Extraction	+13.3	27.7
Bt.L-3-01	Bos	Petrolia, ON	80°C Oven Heating	+10.1	23.0
Bt.L-3-02	Bos	Petrolia, ON	60°C Oven Heating	+12.1	23.8
Bt.L-4-01	Bos	Petrolia, ON	Smaller Sample Size	+10.5	24.2
Bt.L-5-01	Bos	Petrolia, ON	Stronger Acid	+10.6	25.1
Bt.L-6-01	Bos	Petrolia, ON	Ultrasound	+8.7	24.7
Bt.M-1-01	Bos	Petrolia, ON	Longin Freeze Dried	+7.3	23.7
Bt.M-1-01 Dup	Bos	Petrolia, ON	Sample Duplicate	+6.5	23.9
Bt.M-1-01 Dup	Bos	Petrolia, ON	Sample Duplicate	+6.4	14.5
Bt.M-2-01	Bos	Petrolia, ON	EDTA Method Duplicate	+4.4	24.4
Bt.E-1-01	Bos	Petrolia, ON	EDTA Control	+5.8	25.6
Bt.E-1-01b	Bos	Petrolia, ON	Aged Sample Duplicate	+6.0	24.0
Bt.E-1-01 ReX	Bos	Petrolia, ON	Re-extraction of Control	+6.7	23.2
Bt.E-2-01	Bos	Petrolia, ON	No Lipid Extraction (NLE)	+5.4	26.0
Bt.E-2-01 Dup	Bos	Petrolia, ON	Sample Duplicate	+5.5	26.3
Bt.E-2-01 ReX	Bos	Petrolia, ON	Re-extraction of NLE	+6.3	23.3
Bt.E-2-01 Dup ReX	Bos	Petrolia, ON	Re-extraction of NLE	+6.4	23.7
Bt.E-3-01	Bos	Petrolia, ON	Heavy Water	+5.4	25.1
Bt.E-3-02	Bos	Petrolia, ON	Light Water	+5.8	25.2
Bt.E-4-01	Bos	Petrolia, ON	Smaller Sample Size <63um	+6.0	23.6
Bt.E-5-01	Bos	Petrolia, ON	Ultrasound	+5.6	25.6

Bp.L-1-01	Bison	Northern YT	Longin Control	+12.3	27.9
Bp.L-2-01	Bison	Northern YT	No Lipid Extraction	+7.8	26.6
Bp.L-3-01	Bison	Northern YT	80°C Oven Heating	+13.1	23.9
Bp.L-3-02	Bison	Northern YT	60°C Oven Heating	+7.3	25.3
Bp.L-4-01	Bison	Northern YT	Smaller Sample Size	+8.7	24.9
Bp.L-5-01	Bison	Northern YT	Stronger Acid	+7.7	26.3
Bp.L-6-01	Bison	Northern YT	Ultrasonic Cleaning	+1.4	25.1
Bp.M-1-01	Bison	Northern YT	Longin Freeze Dried	-1.8	26.0
Bp.M-2-01	Bison	Northern YT	EDTA Method Duplicate	-1.1	26.0
Bp.E-1-01	Bison	Northern YT	EDTA Control	-2.5	27.2
Bp.E-1-01b	Bison	Northern YT	Aged Sample Duplicate	-2.3	23.0
Bp.E-2-01	Bison	Northern YT	No Lipid Extraction (NLE)	-2.9	24.8
Bp.E-2-01 Dup	Bison	Northern YT	Sample Duplicate	-2.6	27.5
Bp.E-3-01	Bison	Northern YT	Heavy Water	-2.9	24.1
Bp.E-3-02	Bison	Northern YT	Light Water	-4.3	26.1
Bp.E-4-01	Bison	Northern YT	Smaller Sample Size <63um	-2.1	22.1
Bp.E-5-01	Bison	Northern YT	Ultrasonic Cleaning	-3.1	25.0
Aa.L-1-01	Alces	Sault St. Marie	Longin Control	+10.1	26.2
Aa.L-2-01	Alces	Sault St. Marie	No Lipid Extraction	+12.4	27.1
Aa.L-3-01	Alces	Sault St. Marie	80°C Oven Heating	C (+35.9)	25.6
Aa.L-3-02	Alces	Sault St. Marie	60°C Oven Heating	+11.9	21.8
Aa.L-4-01	Alces	Sault St. Marie	Smaller Sample Size	+9.6	23.9
Aa.L-5-01	Alces	Sault St. Marie	Stronger Acid	+11.2	24.4
Aa.L-6-01	Alces	Sault St. Marie	Ultrasonic Cleaning	+8.3	25.1
Aa.M-1-01	Alces	Sault St. Marie	Longin Freeze Dried	+6.8	23.9
Aa.M-2-01	Alces	Sault St. Marie	EDTA Method Duplicate	+4.8	24.0
Aa.E-1-01	Alces	Sault St. Marie	EDTA Control	+5.1	25.6
Aa.E-1-01b	Alces	Sault St. Marie	Aged Sample Duplicate	+4.6	23.8
Aa.E-1-01 ReX	Alces	Sault St. Marie	Re-extraction of Control	+5.9	23.9
Aa.E-2-01	Alces	Sault St. Marie	No Lipid Extraction (NLE)	+4.6	26.0
Aa.E-2-01 Dup	Alces	Sault St. Marie	Sample Duplicate	+5.3	26.3
Aa.E-2-01 ReX	Alces	Sault St. Marie	Re-extraction of NLE	+6.2	23.4
Aa.E-2-01 Dup ReX	Alces	Sault St. Marie	Re-extraction of NLE	+5.5	23.1
Aa.E-3-01	Alces	Sault St. Marie	Heavy Water	+5.1	25.0
Aa.E-3-02	Alces	Sault St. Marie	Light Water	+3.6	24.9
Aa.E-4-01	Alces	Sault St. Marie	Smaller Sample Size <63um	+5.4	24.5
Aa.E-5-01	Alces	Sault St. Marie	Ultrasonic Cleaning	+4.4	25.1

Om.L-1-01	Ovibos	Sachs Habour, NWT	Control	+4.3	29.3
Om.L-2-01	Ovibos	Sachs Habour, NWT	No Lipid Extraction	+5.6	27.8
Om.L-3-01	Ovibos	Sachs Habour, NWT	Oven Dried at 80°C	+8.3	23.8
Om.L-3-02	Ovibos	Sachs Habour, NWT	Oven Dried at 60°C	+4.0	24.4
Om.L-4-01	Ovibos	Sachs Habour, NWT	Sample crushed to 63 μ m	+3.3	24.5
Om.L-5-01	Ovibos	Sachs Habour, NWT	0.5M HCl used	+11.6	25.2
Om.L-6-01	Ovibos	Sachs Habour, NWT	Ultrasonic Pre-treatment	-0.3	24.8
Om.M-1-01	Ovibos	Sachs Habour, NWT	Longin Freeze Dried	-1.0	24.5
Om.M-2-01	Ovibos	Sachs Habour, NWT	EDTA Method Duplicate	-1.5	25.4
Om.E-1-01	Ovibos	Sachs Habour, NWT	Control	-2.9	25.5
Om.E-1-01b	Ovibos	Sachs Habour, NWT	Aged Sample Duplicate	-4.0	24.5
Om.E-2-01	Ovibos	Sachs Habour, NWT	No Lipid Extraction (NLE)	-2.7	25.5
Om.E-2-01 Dup	Ovibos	Sachs Habour, NWT	Sample Duplicate	-2.6	25.8
Om.E-3-01	Ovibos	Sachs Habour, NWT	EDTA diluted in $\delta^{18}\text{O} = +17.3\text{‰}$ H ₂ O	-3.4	24.8
Om.E-3-02	Ovibos	Sachs Habour, NWT	EDTA diluted in $\delta^{18}\text{O} = -11.4\text{‰}$ H ₂ O	-3.3	25.2
Om.E-4-01	Ovibos	Sachs Habour, NWT	Sample crushed to 63 μ m	-1.8	21.6
Om.E-5-01	Ovibos	Sachs Habour, NWT	Ultrasonic Pre-treatment	-3.3	24.9

Each experiment replaced one parameter from the control extraction protocol to test for variability between each change.

¹ Bt = Bos taurus; Bp = Bison priscus; Aa = Alces alces americana; Om = Ovibos moschatus.

² This experiment tested the necessity of lipid extraction (this differs from sample to sample)

³ Dup = Sample duplicate; ReX = The collagen was re-extracted (no bioapatite present)

⁴ The water isotopic composition used during extraction was changed here to augment any oxygen exchange

Experiment variance was calculated for each sample to evaluate how much each oxygen isotope composition differed from the control result. The experimental variance was calculated using equation 3.1:

$$\omega_e = \sigma_x^2 \quad (\text{Equation 3.1})$$

where x represents the measurements from one sample with the same method used (either EDTA or Longin) and σ is the standard deviation for these measurements. Sample variance was calculated for each sample to observe the variance between methods used for that particular sample. Sample variance was calculated using equation 3.2:

$$\omega_s = \sigma_{X_i}^2 \quad (\text{Equation 3.2})$$

where X is the method used (either EDTA or Longin), X_i is the measurements from the same sample and extraction protocol used, and σ is the standard deviation for that sample.

Table 3.5 - Experiment and Sample Variance of Oxygen Isotope Compositions. EDTA results include all samples extracted using EDTA. Longin results include all samples extracted using HCl.

Sample Name	Average $\delta^{18}\text{O}$ ‰ (VSMOW)	SD $\delta^{18}\text{O}$ ‰	n value	Experiment Variance, ω_e ‰	Sample Variance, ω_s ‰
<i>Bos taurus</i> EDTA	+5.8	0.6	12	0.4	0.2
<i>Bos taurus</i> Longin	+10.0	2.7	7	7.3	0.2
<i>Bison priscus</i> EDTA	-2.6	0.9	8	0.8	0.1
<i>Bison priscus</i> Longin	+7.1	5.0	7	25.0	10.1
<i>Ovibos moschatus</i> EDTA	-2.8	0.8	8	0.6	0.6
<i>Ovibos moschatus</i> Longin	+4.5	4.2	7	17.6	0.9
<i>Alces alces americana</i> EDTA	+5.0	0.7	12	0.5	0.6
<i>Alces alces americana</i> Longin	10.0	2.0	7	4.0	1.6

The *Bos taurus* EDTA and Longin samples both share the same sample variance. This is misleading as it only signifies that each method shows similar variance compared with itself instead of a comparison between. A more accurate way to view the data would be to add the experiment variance with the sample variance to get total variance. For *Bos taurus* EDTA, the total variance would be 0.6‰ where the total variance for *Bos taurus* Longin would be 5.5‰. Fig. 3.6 illustrates the difference in spread among the oxygen isotope compositions of collagen extracted using each method. The EDTA method shows significantly less variability in $\delta^{18}\text{O}_{\text{col}}$ than the Longin method.

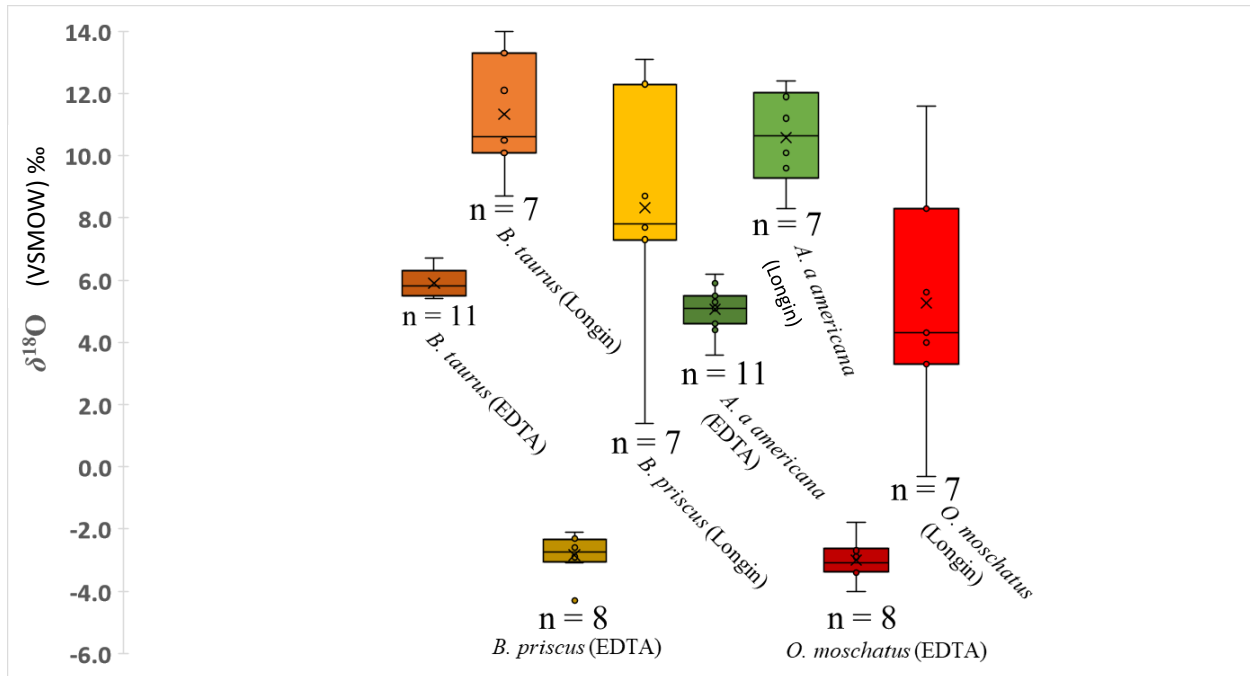


Figure 3.7 – Oxygen-isotope compositions for each sample by collagen extraction method used. The EDTA extracted samples (left for each species) show a lower spread than the Longin extracted samples (right for each species).

3.4 MALDI-TOF-MS Results for Collagen.

As described earlier, MALDI-TOF-MS analyses were used to evaluate the structural integrity of collagen fibrils after collagen extraction from six long bone sub-samples: Bt.L-1-01, Bt.E-1-01, Aa.L-1-01, Aa.E-1-01, Bp.L-1-01, Bp.E-1-01. The data obtained for these sub-samples are illustrated in Figures 3.8-3.13 and uploaded to this online directory:

<https://github.com/Crusse56/Maldi-Data>. The key importance of the following graphs is observing the mass shift of +0.984 Da caused by glutamine deamidation. This can be observed in the Longin extracted samples (L) where the blue peak is much higher than the ideal isotope distribution of that mass. The blue peak should be located at the mass peak to its left (lower than the ideal distribution). The EDTA extracted peaks (green) are not higher than the ideal isotope distribution and therefore show no signs of mass shifts.

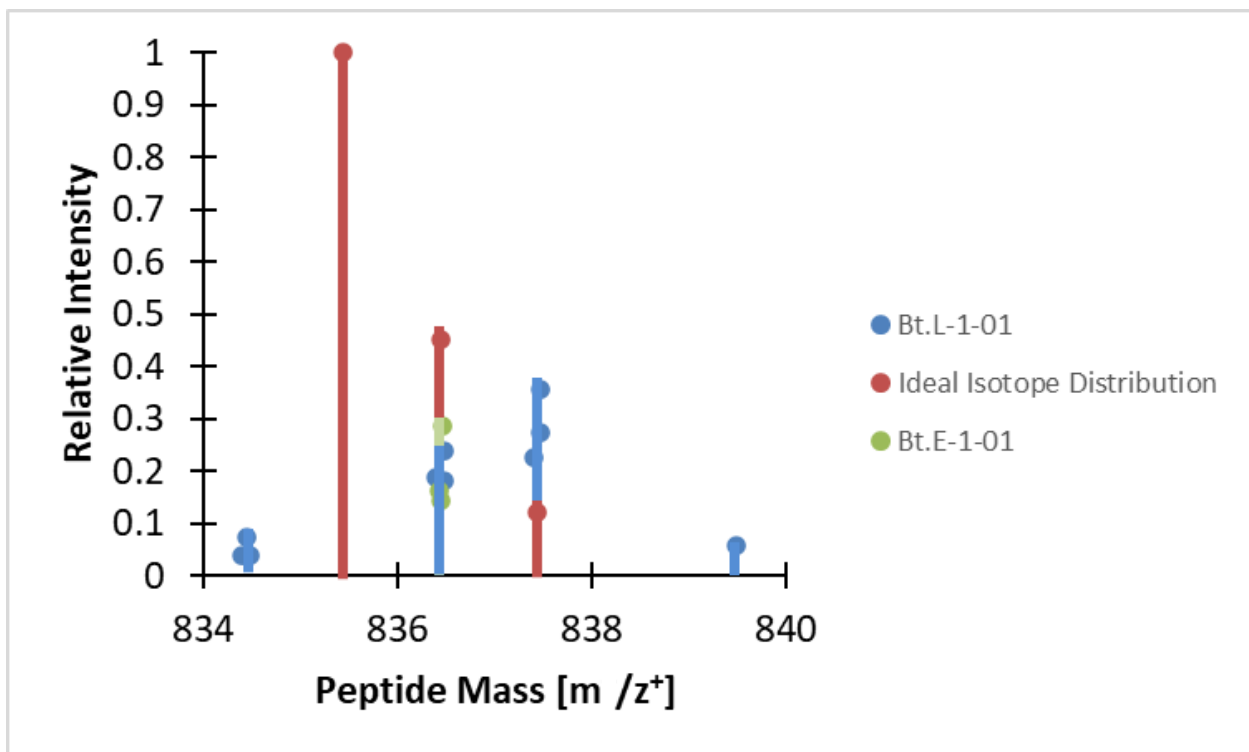


Figure 3.8 – Ideal Peaks versus Measured Peaks of Mass 836.43 for *B. taurus*. Peaks for the Longin method (blue) are shifted by $\sim +0.984$ Da from that expected for a glutamine residue (Blue peak higher than red peak) Peaks for the EDTA method (green) shows no evidence of being shifted.

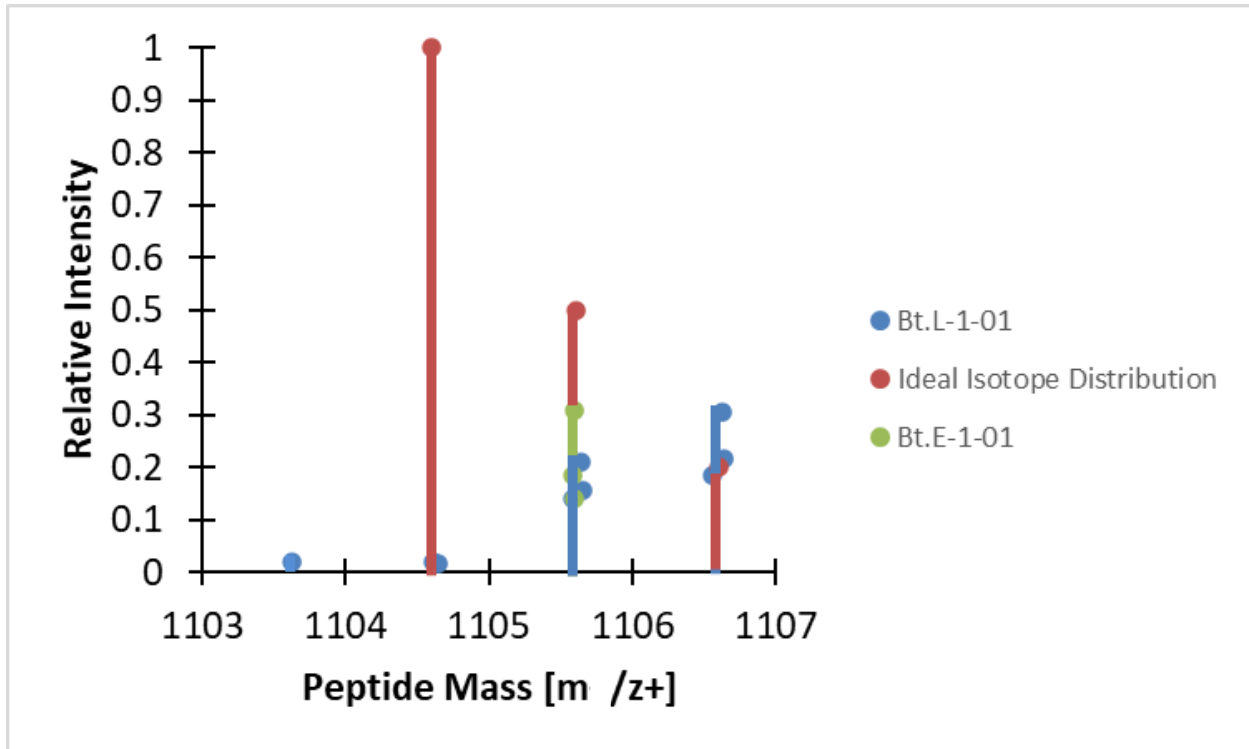


Figure 3.9 – Ideal Peaks versus Measured Peaks of Mass 1105.57 for *B. taurus*. Peaks for the Longin method (blue) are shifted by $\sim +0.984\text{Da}$ from that expected for a glutamine residue. Peaks for the EDTA method (green) shows no evidence of being shifted.

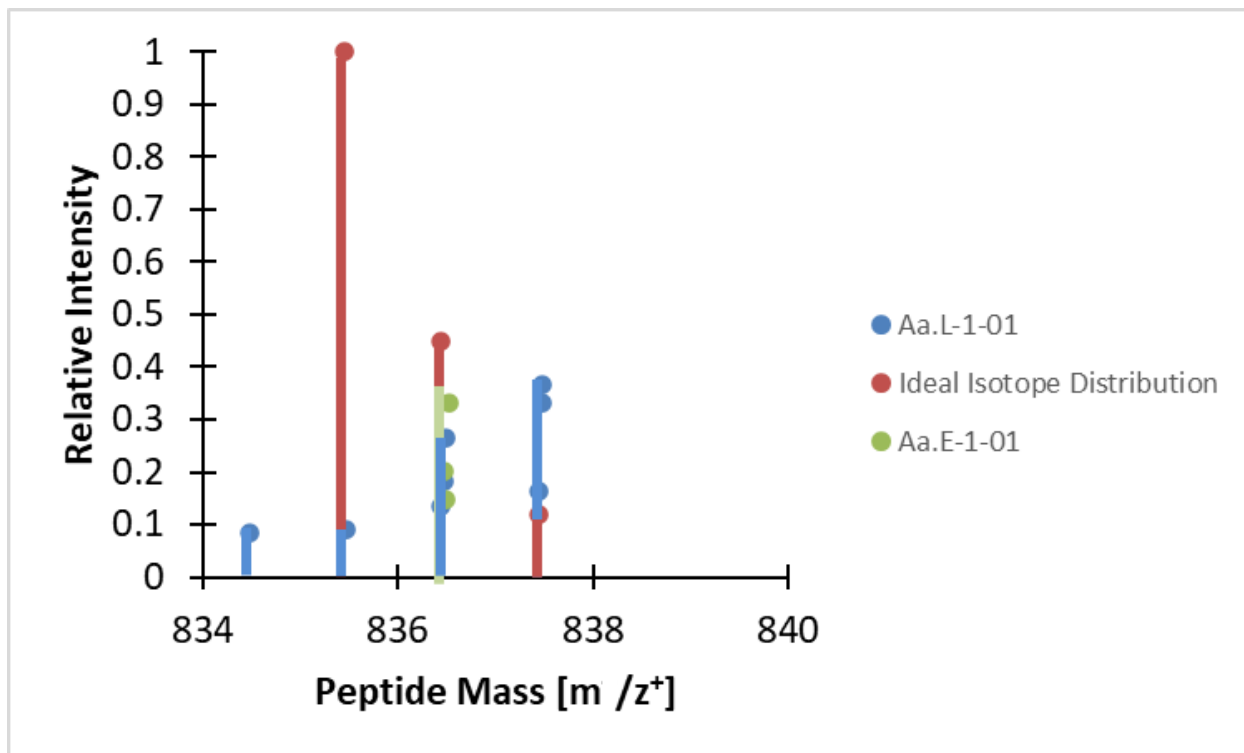


Figure 3.10 – Ideal versus Measured Peaks of Mass 836.43 for *A. a americana*. Peaks for the Longin method (blue) are shifted by $\sim +0.984\text{Da}$ from that expected for a glutamine residue. Peaks for the EDTA method (green) shows no evidence of being shifted.

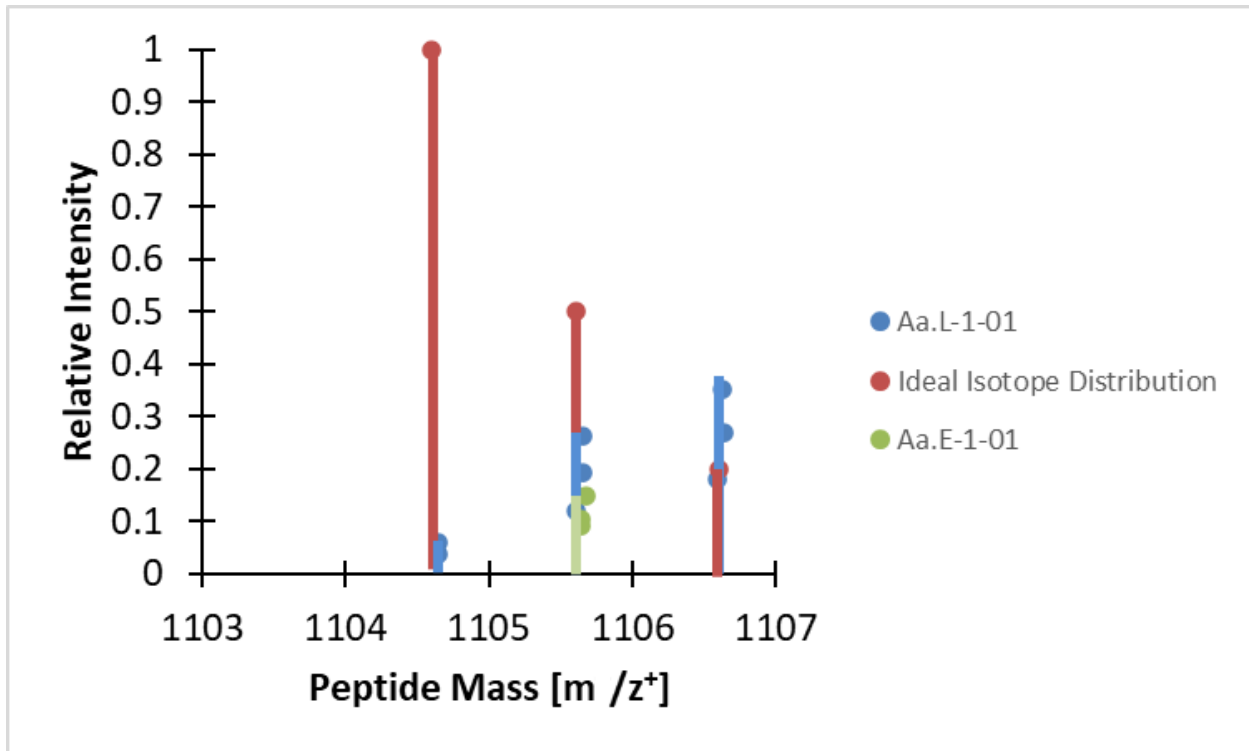


Figure 3.11 – Ideal versus Measured Peaks of Mass 1105.57 for *A. a americana*. Peaks for the Longin method (blue) are shifted by $\sim +0.984$ Da from that expected for a glutamine residue. Peaks for the EDTA method (green) shows no evidence of being shifted.

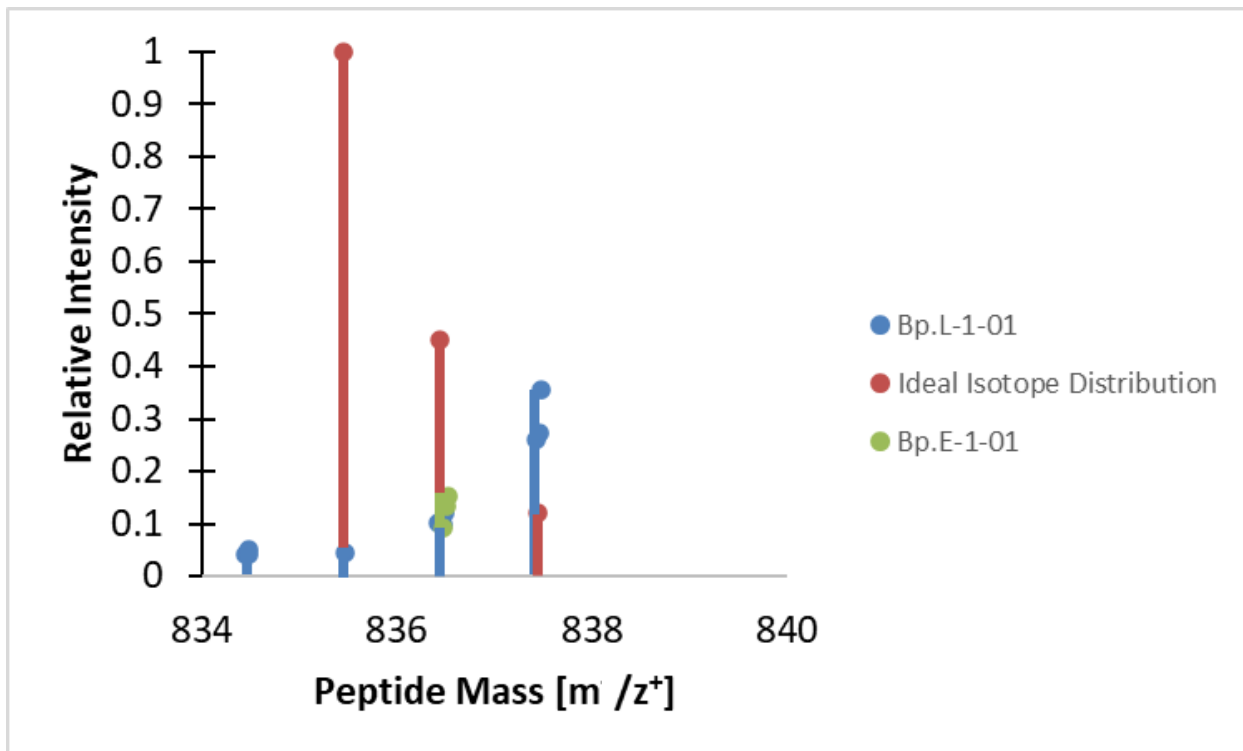


Figure 3.12 – Ideal versus Measured Peaks of Mass 836.43 for *B. priscus*. Peaks for the Longin method (blue) are shifted by $\sim +0.984$ Da from expected for a glutamine residue. Peaks for the EDTA method (green) shows no evidence of being shifted.

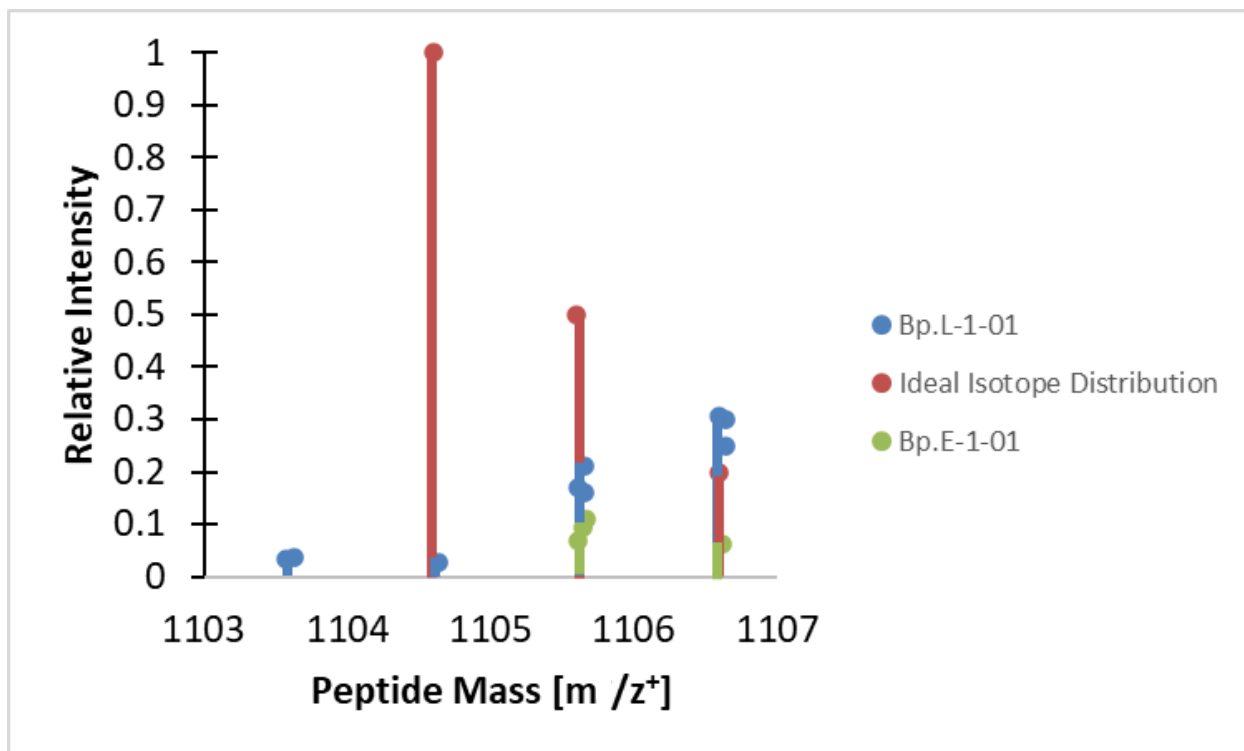
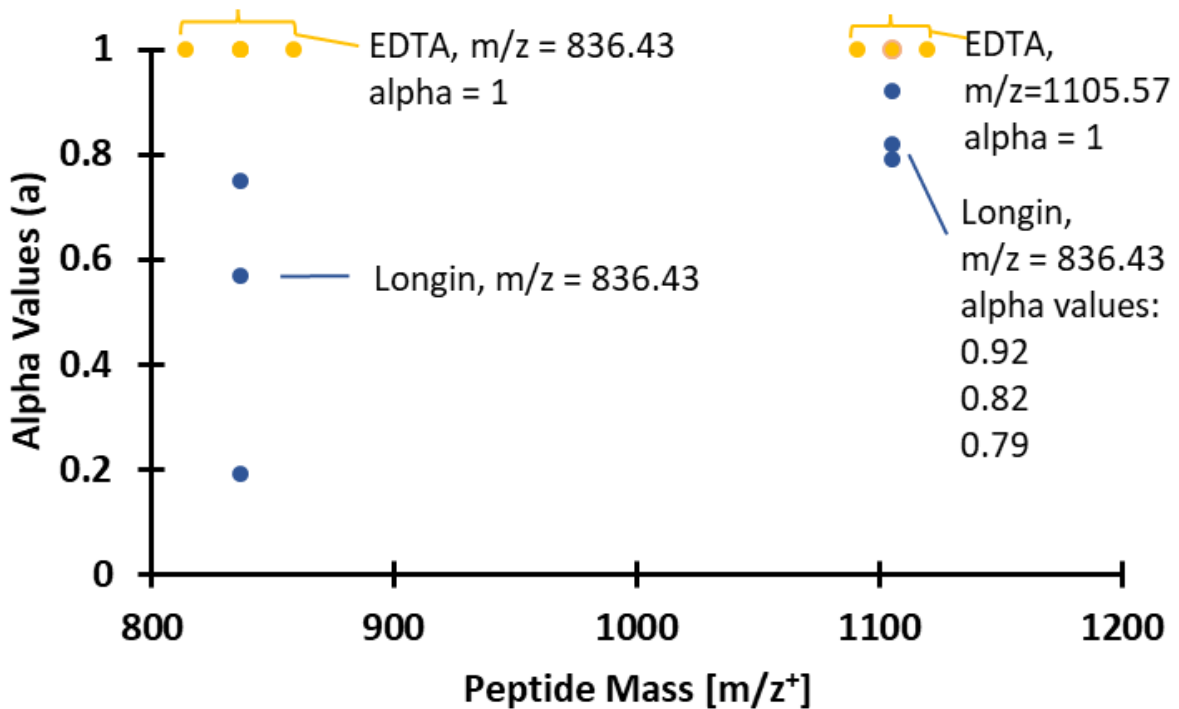


Figure 3.13 –Ideal versus Measured Peaks of Mass 1105.57 for *B. priscus*. Peaks for the Longin method (blue) are shifted by $\sim +0.984$ Da from expected for a glutamine residue. Peaks for the EDTA method (green) shows no evidence for being shifted. This is the only figure that shows a second peak for EDTA, which is still lower in intensity than the ideal distribution indicating it hasn't been mass shifted.

The primary objective of using MALDI-TOF-MS in this study was to identify glutamine deamidation. This is most likely not the only chemical change that occurred during collagen extraction, but it was the only chemical reaction that could be tested from the data obtained using the R-program developed by The University of York, UK. A description of this code and how it was used in this study is provided in Appendix D. Briefly, MALDI-TOF data for each sub-sample were added to a data folder (each file contains two columns: mass / charge and peak count), the program's available "PeptideList" file was edited to include only the peptides of interest, the program was compiled for each sample using program's 'make' function, and alpha values (α) were returned for each sub-sample and saved in a separate file. The relative intensity vs mass / charge (m/z) plots (e.g., Fig 3.14) were then compared to an ideal isotope distribution for each collagen peptide. The Longin method samples had a higher resolution of data which is why there are more peaks available on Figures 3.8-3.13.

Alpha values (α), which provide a measure of deamination for all collagen sub-samples, were returned consistently for MALDI-TOF-MS masses 836.43 and 1105.57. Ideally, α -values should be equal to 1 ± 0.1 , indicating no deamidation. The formulas used to calculate α can be found in Wilson et al. (2012). Consistent with the results of Simpson et al. (2016) and Wilson et al. (2012), all EDTA extracted collagen sub-samples all had α -values of 1 (Fig. 3.14). Longin method-extracted (HCl) collagen sub-samples had α -values of less than 1, indicating deamidation. Unlike our study, however, Simpson et al., (2016) did not find α -values below 0.7 for the peptide chains at 836.43 or 1105.57 demineralized with HCl, probably because they extracted the collagen at 2-8°C. Higher temperatures were used in the present study (room temperature, $\sim 22^\circ\text{C}$), which likely caused the HCl to react more extensively with the collagen,



leading to greater glutamine deamidation.

Figure 3.14 – Alpha Values of Mass 836.43 and 1105.57 for the EDTA and Longin Methods of Collagen Extraction. All EDTA results for the 836.43Da (three) and 1105.57Da (three) peaks have α -values of 1 and appear in the exact same spot (shown here by horizontal tie lines). The results for the Longin method have α -values lower than 1 indicating that glutamine deamidation has occurred.

Table 3.6 – Alpha Values for Peptide Masses.

This table contains numerical values for the data in fig 3.14

Sample ID	Peptide Mass (m/z⁺)	Alpha Values (a)
Bt.L-1-01	836.43	0.57
Bt.L-1-01	1105.57	0.79
Aa.L-1-01	836.43	0.75
Aa.L-1-01	1105.57	0.92
Bp.L-1-01	836.43	0.19
Bp.L-1-01	1105.57	0.82
Bp.L-1-01	2705.21 ¹	0.03
Bt.E-1-01	836.43	1
Bt.E-1-01	1105.57	1
Aa.E-1-01	836.43	1
Aa.E-1-01	1105.57	1
Bp.E-1-01	836.43	1
Bp.E-1-01	1105.57	1
Bp.E-1-01	2705.21	1

¹ Because 2705.21 was only visible for two of the samples, it is not shown in Figure 3.14

IV. DISCUSSION

4.1 Factors that Increase Oxygen Isotope Alteration

Three factors should be considered when preparing collagen samples for oxygen isotope analysis. First, sampling of altered bone should be avoided. The discoloured outer surface of fresh bone that commonly results from degreasing and drying can be removed with a Dremel[®]. Second, during collagen extraction, temperatures above 22°C should be avoided to reduce collagen alteration arising from the weakening of collagen fibrils. At lower temperatures, reaction times are slower, especially during demineralization. The slower reaction times would decrease the risk of over-soaking the sample in acid. Oven treatment should be avoided and replaced by freeze-drying, and fully demineralized samples should be stored in a refrigerator or freezer to avoid further interaction with ambient moisture. Third, the strength of acid should be considered. Both EDTA and HCl acid cause alteration of collagen, but EDTA has very little effect on the oxygen isotope composition due to its less acidic pH. A 0.5M solution of EDTA is adequate to cause demineralization, but not acidic enough to affect the collagen fibrils. It is suggested then, to use a 0.5M EDTA solution.

4.2 Inferred Diet for Modern and Ancient Bone

Collagen samples extracted using the Longin method produced more reproducible stable carbon and nitrogen isotope results than collagen extracted using EDTA. This outcome is likely due to the reagents used during extraction. A HCl solution, which is used during Longin extraction, contains no nitrogen or carbon. An EDTA solution, by comparison, contains both nitrogen and carbon, would could potentially exchange with or contaminate collagen. Accordingly, collagen extracted using the Longin method was used to assess the diets of these megaherbivores.

To obtain dietary information, the collagen isotopic results were first corrected using known isotopic discrimination factors between collagen and diet. There is a +5‰ increase for

carbon ($\Delta^{13}\text{C}_{\text{coll-diet}}$) and a +3‰ increase for nitrogen ($\Delta^{15}\text{N}_{\text{coll-diet}}$) from diet to collagen (Richards, 2015). For the *B. priscus* sample, a further correction for the Suess effect was made to account for the change in atmospheric $\delta^{13}\text{C}$ from -6.5 to -8.4‰ since the Pleistocene age due to industrialization (Bocherens, 2003). See equations 4.1-4.3:

$$\text{Modern: } \delta^{13}\text{C}_{\text{diet}} = \delta^{13}\text{C}_{\text{coll}} - \Delta^{13}\text{C}_{\text{coll-diet}} \quad (\text{Equation 4.1})$$

$$\text{Pleistocene: } \delta^{13}\text{C}_{\text{diet}} = \delta^{13}\text{C}_{\text{coll}} - \Delta^{13}\text{C}_{\text{coll-diet}} + \text{Suess effect} \quad (\text{Equation 4.2})$$

$$\delta^{15}\text{N}_{\text{diet}} = \delta^{15}\text{N}_{\text{coll}} - \Delta^{15}\text{N}_{\text{coll-diet}} \quad (\text{Equation 4.3})$$

All four samples used in this study were collected from megaherbivores. This being the case, the nitrogen isotope compositions are all consistent with those expected for terrestrial herbivores, (i.e., tropic level 1) (Schoeninger, 1984).

The modern *B. taurus* originated from a local cattle farm 5km east of Petrolia, ON Canada. It is typical for farms to feed their cattle with corn or corn silage, but this animal came from a farm that does not restrict ingestion of other plant matter. C3 plants have an average $\delta^{13}\text{C}$ of $-28.1 \pm 2.5\text{‰}$ (O'Leary, 1981) and C4 plants have an average $\delta^{13}\text{C}$ of $-12.7 \pm 1.4\text{‰}$ (Basu, 2015). The *B. taurus* diet $\delta^{13}\text{C}$ of $-16.0 \pm 0.9\text{‰}$ suggests a C3 influence on diet (which was likely grass from nearby farmland) (Fig. 4.1). It isn't known whether the C4 diet was corn, but a mixed diet of C3 grass and C4 corn would be consistent with the results obtained for this animal.

The Pleistocene *B. priscus* was a late Beringian Steppe megaherbivore who was primarily a grazer (Mabee, 2019). Its dietary $\delta^{13}\text{C}$ of $-27.0 \pm 0.3\text{‰}$ (corrected to modern atmospheric $\delta^{13}\text{C}_{\text{CO}_2}$) is typical of C3 plant matter (Fig. 4.1), likely grasses as would be expected for a grazer at this locality, which was devoid of C4 plants during the Pleistocene (Mabee, 2019).

The modern *A. a americana* is an eastern moose. Its dietary $\delta^{13}\text{C}$ of $-29.0 \pm 0.4\text{‰}$ also suggests a diet of C3 plant matter, particularly shrubs, which tend to have lower $\delta^{13}\text{C}$ among C3 plants (Schwartz-Narbonne et al., 2019) (Fig. 4.1). This result is consistent with the modern moose's preference of heavily wooded areas and marshland.

The modern *O. moschatus* is a muskox, who are primarily mixed feeders. Its dietary $\delta^{13}\text{C}$ of $-28.0 \pm 0.2\text{‰}$ is also consistent with a diet of C3 plant matter, including subshrubs (Fig. 4.1).

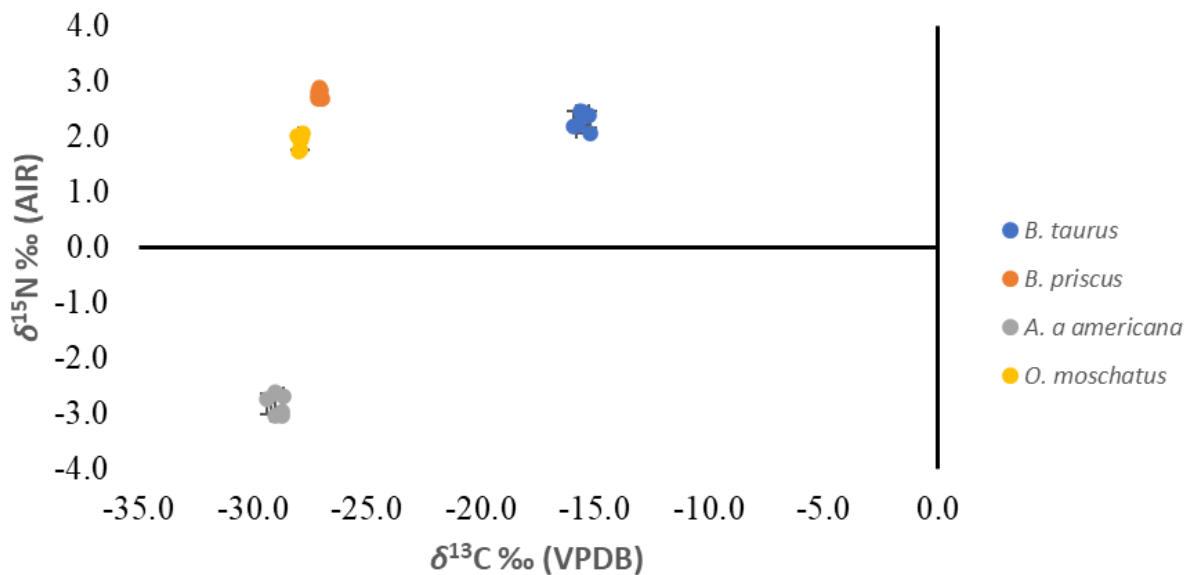


Figure 4.1 – Carbon and Nitrogen Isotope Compositions of Megaherbivore Diets, as inferred from collagen $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, after correction for collagen-diet isotopic discrimination factors and for *B. priscus*, the Suess effect correction.

4.3 Oxygen Isotope Systematics of Bone Collagen

4.3.1 Oxygen isotope discrimination between collagen and meteoric water ($\Delta^{18}\text{O}_{\text{coll-met}}$) in Megaherbivores

To further validate the accuracy of the isotope measurements, $\delta^{18}\text{O}_{\text{coll}}$ can be plotted alongside $\delta^{13}\text{C}_{\text{coll}}$ to illustrate the disparity between extraction methods. Figure 4.2 compares the carbon versus oxygen isotope compositions of collagen obtained using the Longin (Fig. 4.2A) versus EDTA (Fig. 4.2B) methods. While $\delta^{13}\text{C}_{\text{coll}}$ is similar for both methods, only the EDTA method produces distinctly different and reproducible values of $\delta^{18}\text{O}_{\text{coll}}$ for megaherbivores. Accordingly, only results obtained using the EDTA method should be used to determine the oxygen isotope mammalian discrimination between collagen (coll) and body water (bw) ($\Delta^{18}\text{O}_{\text{coll-bw}}$).

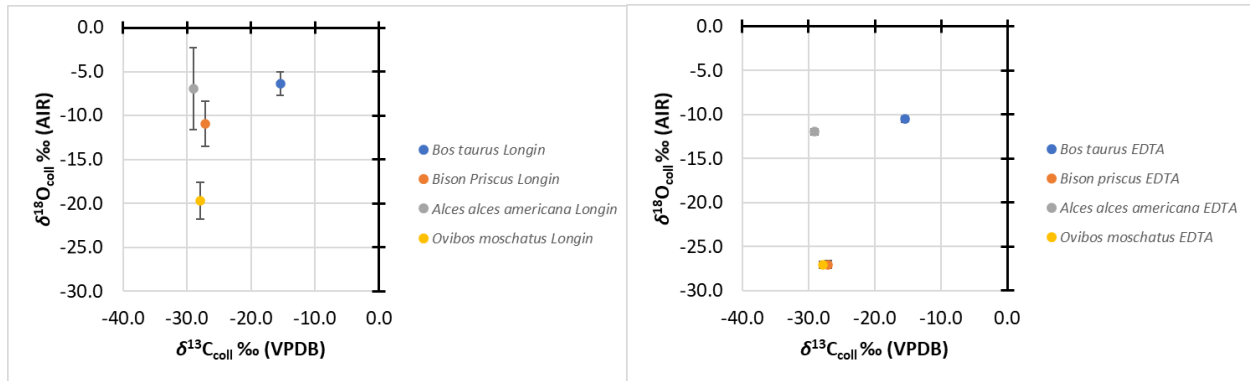


Figure 4.2 – Comparison of collagen carbon and oxygen isotope results using the Longin (A) and EDTA (B) methods of collagen extraction. Error bars for carbon and oxygen in B and carbon in A are smaller than the size of the data points.

Currently, the value of $\Delta^{18}\text{O}_{\text{coll-bw}}$ is not well known. We can approximate this value by assuming that body water has a similar isotopic composition to drinking water, and then by further assuming that drinking water has the same oxygen isotope composition of local meteoric water where the animal lived. It is recognized that these assumptions likely underestimate $\delta^{18}\text{O}_{\text{bw}}$ by a few per mil (Daux et al., 2008; France et al., 2018). That said, values of $\delta^{18}\text{O}_{\text{coll}}$ obtained using the EDTA method can be compared to the expected oxygen isotope composition of meteoric water ($\delta^{18}\text{O}_{\text{met}}$) for the regions from which the samples originated. For *B. taurus*, the expected average $\delta^{18}\text{O}_{\text{met}}$ is $\sim -10.2\text{‰}$ (Hamilton, 2015); for *A. a. americana*, $\sim -8.7\text{‰}$ (Bowen, 2007); and *O. moschatus*, $\sim -23.7\text{‰}$ (Bowen, 2017). For the Pleistocene *B. priscus*, a glacial paleoenvironment has been assumed in which the majority of water ingestion occurs during summer months on a dry mammoth steppe when $\delta^{18}\text{O}_{\text{met}}$ is expected to be higher ($\sim -18.5\text{‰}$) (Mabee, 2019; Metcalfe et al., 2014).

The estimated oxygen isotope discrimination for each sample is illustrated in Figure 4.3, as calculated using equation 4.4:

$$\Delta^{18}\text{O}_{\text{coll-met}} = \delta^{18}\text{O}_{\text{EDTAcoll}} - \delta^{18}\text{O}_{\text{met}} \quad (\text{Equation 4.4})$$

The average value of $\Delta^{18}\text{O}_{\text{coll-met}}$ for all samples is $16.6 \pm 3.0\text{‰}$. The value of $\Delta^{18}\text{O}_{\text{coll-bw}}$ can be expected to be ~ 1 to 2‰ smaller. Crowley et al. (2014) found that $\Delta^{18}\text{O}_{\text{coll-met}}$ for rodent collagen was 12.5-15‰; however, these values were obtained using Longin extracted collagen samples. A higher value might be expected due to the higher $\delta^{18}\text{O}_{\text{coll}}$ values typically resulting

from Longin extraction; however, the effect of a smaller physiology may significantly decrease the discrimination. Kirsanow and Tuross (2011) found that the $\Delta^{18}\text{O}_{\text{coll-met}}$ for enamel phosphate was 18‰ which was obtained using the EDTA method. Additionally, Bryant et al., (1996) produced a value of 17.6‰ for phosphate oxygen. This is more closely aligned with the 16.6‰ obtained here. Additional analysis on bone collagen can help make this value more precise.

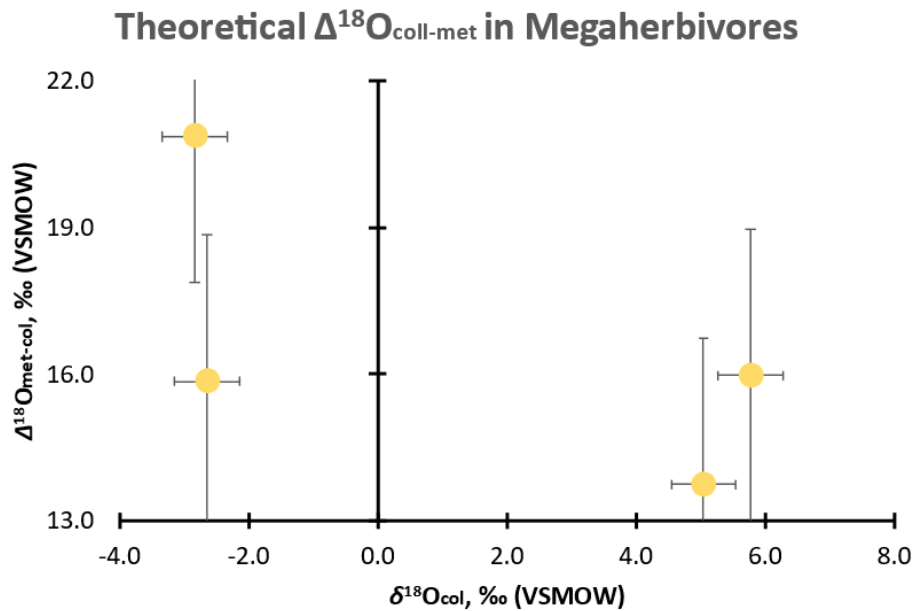


Figure 4.3 – Estimated Oxygen Isotope Megaherbivore Collagen-Meteoric Water Discrimination ($\Delta^{18}\text{O}_{\text{coll-met}}$) based on EDTA-extracted samples. Points on the right were obtained from southern Canadian latitudes, and points on the left were obtained from northern Canadian latitudes.

4.3.2 Glutamine Deamidation versus the Oxygen Isotope Composition of Bone Collagen

A key finding of this study is the relationship between the α -values that describe glutamine deamidation and the oxygen isotope compositions of bone collagen for peptide masses 836.43, 1105.57, and 2705.21. This behaviour is illustrated in Figure 4.4 and summarized in Table 4.1 in a comparison of $\Delta^{18}\text{O}_{\text{coll-met}}$ obtained for those samples (both HCl- and EDTA-extracted) for which α -values were obtained. There is an inverse relationship between $\Delta^{18}\text{O}_{\text{coll-met}}$ and α for all three peptide masses, which suggests that glutamine deamidation directly

affects the oxygen isotope composition of collagen. In the case of the samples analyzed in this study, deamidation has driven $\delta^{18}\text{O}_{\text{coll}}$ to higher values. This apparent enrichment in ^{18}O of collagen that has undergone deamidation likely results from exchange with the reagents used (including water). This would imply that the relationship between glutamine deamidation and oxygen isotope exchange depends on the laboratory procedures and isotopic compositions of the water used.

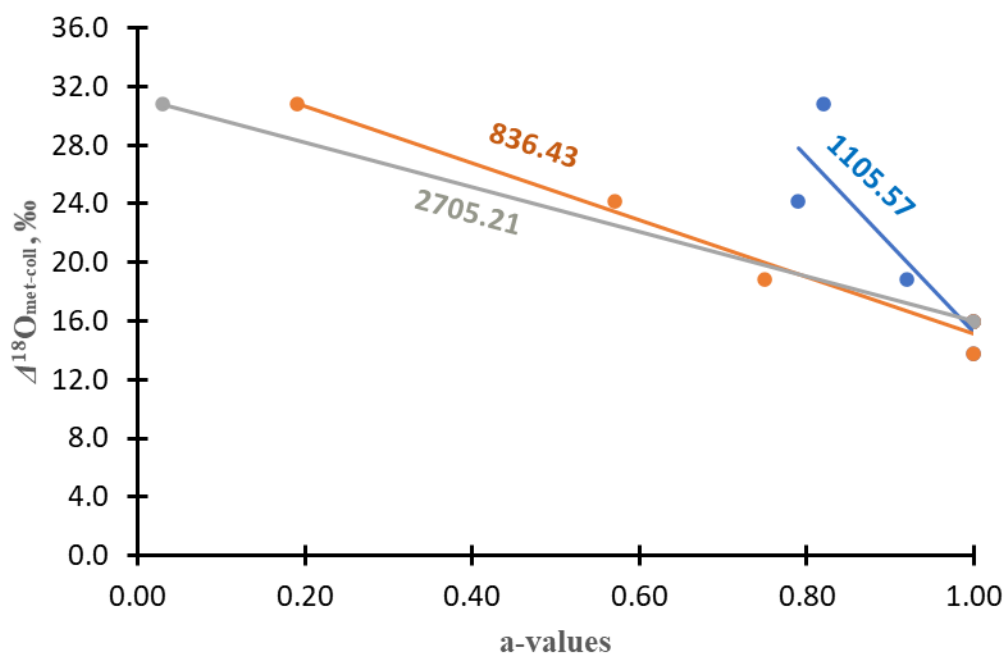


Figure 4.4 - Glutamine Deamidation Versus $\Delta^{18}\text{O}_{\text{coll-met}}$ for Peptide Masses 836.43, 1105.57, and 2705.21. See Table 4.4 and text.

Figure 4.4 illustrates the relationship between $\delta^{18}\text{O}_{\text{coll}}$ and a-values for glutamine deamidation for several peptide masses. It is a linear relationship showing that with increasing chemical alteration (i.e. glutamine deamidation), oxygen enrichment ($\Delta^{18}\text{O}_{\text{met-coll}}$) increases and becomes more variable. The EDTA samples represent the origin of the relationship and the Longin extracted samples represent the tails. If ultrafiltration was performed, it would be expected that this would be further top-left in the diagram. The exact values for Figure 4.4 can be found in Table 4.1.

Table 4.1 - Glutamine Deamidation Versus $\Delta^{18}\text{O}_{\text{coll-met}}$ for Peptide Masses 836.43, 1105.57, and 2705.21 Data Table.

Sample ID	Taxon	Region	Experiment	Peptide m/z	$\delta^{18}\text{O}$, ‰ (VSMOW)	BD	a-values
Bt.L-1-01	Bos	Petrolia, ON	Longin Control	836.43	+14.0	25.0	0.57
Bt.L-1-01	Bos	Petrolia, ON	Longin Control	1105.57	+14.0	25.0	0.79
Bt.E-1-01	Bos	Petrolia, ON	EDTA Control	836.43	+5.8	16.8	1.00
Bt.E-1-01	Bos	Petrolia, ON	EDTA Control	1105.57	+5.8	16.8	1.00
Bp.L-1-01	Bison	Northern YT	Longin Control	836.43	+12.3	30.8	0.19
Bp.L-1-01	Bison	Northern YT	Longin Control	1105.57	+12.3	30.8	0.82
Bp.E-1-01	Bison	Northern YT	EDTA Control	836.43	-2.5	16.0	1.00
Bp.E-1-01	Bison	Northern YT	EDTA Control	1105.57	-2.5	16.0	1.00
Bp.E-1-01	Bison	Northern YT	EDTA Control	2705.21	-2.5	16.0	1.00
Bp.E-1-01	Bison	Northern YT	Longin Control	2705.21	+12.3	30.8	0.03
Aa.L-1-01	Alces	Sault St Marie, ON	Longin Control	836.43	+10.1	22.1	0.75
Aa.L-1-01	Alces	Sault St Marie, ON	Longin Control	1105.57	+10.1	22.1	0.92
Aa.E-1-01	Alces	Sault St Marie, ON	EDTA Control	836.43	+5.1	17.1	1.00
Aa.E-1-01	Alces	Sault St Marie, ON	EDTA Control	1105.57	+5.1	17.1	1.00

* $\Delta^{18}\text{O}$ was calculated by subtracting the accepted meteoric water value (see text) from the $\delta^{18}\text{O}$

* $\delta^{18}\text{O}$ values were obtained from the designated experiment in column 1

4.3.3 Previous Work

Von Holstein et al. (2018) had examined how different collagen extraction conditions (i.e. temperature, water composition) affected the oxygen isotope exchange. They concluded that acidic solutions should not be used for collagen extraction. A similar conclusion was reached by Simpson et al. (2016) who showed that glutamine deamidation was the culprit, catalyzed by the acids. While it was suggested that variability between duplicate samples made it difficult to validate the results, no comparison was made between oxygen isotope composition and collagen degradation – an addition that the present study corrects (Fig. 4.4).

V. CONCLUSIONS

5.1 Methods that Alter Original Isotopic Compositions

This study focussed on the effects of acids (i.e. EDTA and HCl) and drying methods (i.e. oven-heated and freeze-dried) on the isotopic composition of collagen as a result of demineralization of bioapatite. The type of acid used had only a limited effect on the stable carbon and nitrogen isotope compositions of collagen and their reproducibility. The reproducibility of $\delta^{13}\text{C}$ for EDTA-extracted collagen averaged $\pm 0.4\text{‰}$ (SD), whereas for Longin-extracted collagen it was $\pm 0.3\text{‰}$. The reproducibility of $\delta^{15}\text{N}$ of EDTA-extracted collagen averaged $\pm 0.3\text{‰}$, whereas for Longin-extracted collagen it was $\pm 0.2\text{‰}$. The choice of acid, however, strongly affected the quality of the oxygen isotope results obtained for collagen. The reproducibility of $\delta^{18}\text{O}$ for EDTA-extracted collagen was $\pm 0.8\text{‰}$, whereas for Longin-extracted collagen it was $\pm 1.6\text{‰}$. Accuracy of $\delta^{18}\text{O}$ for EDTA-extracted collagen was $\pm 0.6\text{‰}$, whereas for Longin-extracted collagen it was $\pm 3.9\text{‰}$. A further test of drying methods proved that freeze drying brought the Longin method measurements closer in accuracy to the EDTA method. Accuracy of $\delta^{18}\text{O}$ for Longin-extracted collagen that was freeze dried is $\pm 1.1\text{‰}$. Accordingly, collagen samples intended for oxygen isotope analysis should be prepared from bone using an EDTA extraction method involving freeze-drying. The inaccurate and poorly reproducible oxygen isotope results obtained for collagen extracted using HCl (e.g., Longin method) results from glutamine deamidation of the collagen fibrils which was catalyzed by the very strong acidity and high oven temperatures.

5.2 Collagen Preservation

The quality of oxygen isotope data obtained for collagen can be assessed using a combination of collagen yield (wt.%), oxygen contents, and glutamine deamidation levels, as measured using α -values. Unaltered collagen should have a collagen yield $>1\text{wt.}\%$, and should have oxygen contents between 25-35wt%. The expected glutamine deamidation levels (α) for unaltered collagen should be 1. If all parameters are met, regardless of the age of sample, then the oxygen isotope value should be accepted assuming the analysis presented no other significant operation errors.

5.3 Can Oxygen Isotope Compositions of Bone Collagen be Used to Infer Drinking Water Composition?

The results from four Canadian megaherbivores analyzed in this study suggest that the oxygen isotope discrimination between collagen and local meteoric water ($\Delta^{18}\text{O}_{\text{coll-met}}$) is $\sim 16.6 \pm 3.0\text{‰}$ (section 4.3.1). Meteoric water represents the primary source of animal drinking water, but its relation to collagen oxygen involves a big assumption – meteoric water is the primary source of oxygen in collagen and all other sources are negligible. While this assumption enables $\delta^{18}\text{O}_{\text{coll}}$ to be related to $\delta^{18}\text{O}_{\text{mw}}$, it ignores the possible influence of other oxygen inputs (i.e. food water, metabolises) (Crowley, 2015).

5.4 Future Work

This study examined the glutamine deamidation levels of 6 collagen samples (3 EDTA-extracted, and 3 Longin-extracted). When compared to the modern *B. taurus* reference, all EDTA-extracted collagen samples showed no evidence of deamidation. Nonetheless, a larger dataset that compares EDTA- versus HCl-extracted collagen should be acquired to further test the limits of deamidation that can be accepted before significant alteration of collagen isotope compositions can be anticipated.

Given that oxygen isotope alteration can occur during collagen extraction from bone, a set of bone standards of known collagen oxygen isotope composition should be developed. Such standards will allow individual laboratories to assess the efficacy of their protocols for extracting collagen in which the primary oxygen isotope composition has been preserved.

Collagen oxygen isotope compositions, at least for large mammals, may be useful for determining the oxygen isotope composition of meteoric water, which differs in a systematic fashion with latitude and altitude (Mezga, 2014; Singh, 2017). Such data should have many applications, including identification of paleoclimatic conditions using well-dated fossil and subfossil bone, and in forensic investigations. In the former case, the oxygen isotope composition of collagen from the bones of ‘Ice Age’ megafauna may be as – or even more – useful as similar

data for bioapatite in determination of glacial-interglacial climatic variations. In the latter case, human remains are the core component of most homicide and missing-persons investigations. Bones are often the only record left of an individual's origin and identity. The ability to obtain accurate oxygen isotope compositions for collagen, which can then be related to geographic origin, could be key information in such investigations. Whether the oxygen isotope composition of human bone collagen, both in modern and ancient populations, is preserved and largely determined by drinking water, is worthy of further investigation.

References

- Ambrose, S.H. & Norr, L. (1993). Experimental evidence for the relationship of the carbon isotope ratios of whole diet and dietary protein to those of bone collagen and carbonate. In: Lambert JB and Grupe G (Editors), *Prehistoric Human Bone: Archaeology at the Molecular Level*. Springer-Verlag, Berlin, 1-37.
- Ambrose, S.H. (1990). Preparation and characterization of bone and tooth collagen for isotopic analysis. *Journal of Archaeological Science*, 17, 431–451., doi:10.1016/0305-4403(90)90007-r.
- Bartsiokas, A. & Middleton, A.P. (1992). Characterization and dating of recent and fossil bone by X-ray diffraction. *Journal of Archaeological Science* 19, 63–72.
- Basu, S. & Agrawal, S., Sanyal, P., Mahato, P., & Kumar, S. & Sarkar, A. (2015). Carbon isotopic ratios of modern C3–C4 plants from the Gangetic Plain, India and its implications to paleovegetational reconstruction. *Palaeogeography Palaeoclimatology Palaeoecology*. 440. 10.1016/j.palaeo.2015.08.012.
- Bella, J., Eaton, M., Brodsky, B. & Berman, H. (1994). Crystal and molecular structure of a collagen-like peptide at 1.9 Å resolution. *Science* 266, 75-81. doi: 10.1126/science.7695699
- Bell, L.S., Skinner, M.F. & Jones, S.J. (1996). The speed of post-mortem change to the human skeleton and its taphonomic significance. *Forensic Science International*, 82, 129-140.
- Berna, F., Matthews, A. & Weiner, S. (2004). Solubilities of bone mineral from archaeological sites: the recrystallization window. *Journal of Archaeological Science* 31, 867–882.
<https://doi.org/10.1016/j.jas.2003.12.003>
- Bocherens, H. & Drucker, D. (2003). Trophic level isotopic enrichment of carbon and nitrogen in bone collagen: case studies from recent and ancient terrestrial ecosystems. *International Journal of Osteoarchaeology* 13, 46-53. doi: 10.1002/oa.662
- Bowen, G.J., Ehleringer, J.R., Chesson, L.A., Stange, E. & Cerling, T.E. (2007). Stable isotope ratios of tap water in the contiguous United States. *Water Resources Research* 43, W03419.
<https://doi.org/10.1029/2006wr005186>

Bowen G.J. (2017). The Online Isotopes in Precipitation Calculator, Version 3.1. Available from: <http://www.waterisotopes.org>.

Brand, W.A., Coplen, T.B., Vogl, J., Rosner, M. & Prohaska, T. (2014). Assessment of international reference materials for isotope-ratio analysis (IUPAC Technical Report). *Pure and Applied Chemistry* 86, 425–467. <https://doi.org/10.1515/pac-2013-1023>

Brock, F., Wood, R., Higham, T., Ditchfield, P., Bayliss, A. & Ramsey, C. (2012). Reliability of nitrogen content (%N) and carbon:nitrogen atomic ratios (C:N) as indicators of collagen preservation suitable for radiocarbon dating. *Radiocarbon* 54, 879-886.
doi:10.1017/S0033822200047524

Brown, T.A., Nelson, D.E., Vogel, J.S. & Southon, J.R. (1988). Improved collagen extraction by modified Longin method. *Radiocarbon* 30, 171–177.
<https://doi.org/10.1017/s0033822200044118>

Bryant, J.D., Koch, P.L., Froelich, P.N., Showers, W.J. & Genna, B.J. (1996). Oxygen isotope partitioning between phosphate and carbonate in mammalian apatite. *Geochimica et Cosmochimica Acta* 60, 5145–5148. [https://doi.org/10.1016/s0016-7037\(96\)00308-0](https://doi.org/10.1016/s0016-7037(96)00308-0) *Tellus*, 16(4): 436-468.

Cleland, T.P., Voegelé, K. & Schweitzer, M. H. (2012). Empirical evaluation of bone extraction protocols. *PLoS ONE* 7, e31443. <https://doi.org/10.1371/journal.pone.0031443>

Clementz, M.T., Fox-Dobbs, K., Wheatley, P.V., Koch, P. & Doak, D.F. (2009). Revisiting old bones: Coupled carbon isotope analysis of bioapatite and collagen as an ecological and palaeoecological tool. *Geological Journal* 44, 605 - 620. <https://doi.org/10.1002/gj.1173>.

Collins, M.J., Riley, M.S., Child, A.M. & Turner-Walker, G. (1995). A basic mathematical simulation of the chemical degradation of ancient collagen. *Journal of Archaeological Science* 22, 175-183.

Collins, M.J., Nielsen-Marsh, C.M., Hiller, J., Smith, C.I., Roberts, J.P., Prigodich, R.V., Wess, T.J., Csapo, J., Millard, A.R. & Turner-Walker, G. (2002). The survival of organic matter in bone: a review. *Archaeometry* 44, 383-394.

- Coplen, T.B., Brand, W.A., Gehre, M., Gröning, M. & Meijer, H. (2006). New guidelines for $\delta^{13}\text{C}$ measurements. *Analytical Chemistry* 78, 2439-2441.
<http://pubs.acs.org/doi/abs/10.1021/ac052027c>.
- Coplen, T.B. (2011). Guidelines and recommended terms for expression of stable-isotope ratio and gas-ratio measurement results. *Rapid Communications in Mass Spectrometry* 25, 2538-2560.
- Coplen, T.B. (2019). Report of stable isotope compositions: reference material USGS40.
<https://isotopes.usgs.gov/lab/referencematerials/USGS40.html>
- Coplen, T.B. (2019). Report of stable isotope compositions: reference material USGS4a.
<https://isotopes.usgs.gov/lab/referencematerials/USGS41a.html>
- Cournoyer, J.J. & O'Connor, P.B. (2008). Chapter 16: analysis of deamidation in proteins. *Comprehensive Analytical Chemistry*, 375-410. [https://doi.org/10.1016/s0166-526x\(08\)00216-x](https://doi.org/10.1016/s0166-526x(08)00216-x)
- Crowley, B. (2014). Oxygen isotope values in bone carbonate and collagen are consistently offset for new world monkeys. *Biology Letters*, 10. <https://doi.org/10.1098/rsbl.2014.0759>
- Crowley, B.E., Melin, A.D., Yeakel, J.D., & Dominy, N.J. (2015). Do oxygen isotope values in collagen reflect the ecology and physiology of neotropical mammals? *Frontiers in Ecology and Evolution*, 3. <https://doi.org/10.3389/fevo.2015.00127>
- Dansgaard, W. (1964). Stable isotopes in precipitation. *Tellus* 16, 436–468. Stockholm University Press. <https://doi.org/10.1111/j.2153-3490.1964.tb00181.x>
- Daux, V., Lécuyer, C., Héran, M.-A., Amiot, R., Simon, L., Fourel, F., Martineau, F., Lynnerup, N., Reyhler, H. & Escarguel, G. (2008). Oxygen isotope fractionation between human phosphate and water revisited. *Journal of Human Evolution*. 55, 1138-1147.
<https://doi.org/10.1016/j.jhevol.2008.06.006>
- DeNiro, M.J. & Epstein, S. (1981). Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et Cosmochimica Acta* 45, 341-351. [https://doi.org/10.1016/0016-7037\(81\)90244-1](https://doi.org/10.1016/0016-7037(81)90244-1)
- Elorriaga-Verplancken, F., Auriolles-Gamboa, D., Newsome, S.D. & Martínez-Díaz, S.F. (2013). $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values in dental collagen as a proxy for age- and sex-related variation in foraging strategies of California sea lions. *Marine Biology* 160, 641-652.

Fizet, M., Mariotti, A., Bocherens, H., Lange-Badré, B., Vandermeersch, B., Borel, J. & Bellon, G. (1995). Effect of diet, physiology and climate on carbon and nitrogen stable isotopes of collagen in a late Pleistocene anthropic palaeoecosystem: Marillac, Charente, France. *Journal of Archaeological Science* 22, 67-79.

France, C.A.M., Qi, H. & Kavich, G.M. (2018). Combined influence of meteoric water and protein intake on hydrogen isotope values in archaeological human bone collagen. *Journal of Archaeological Science* 96, 33-44. <https://doi.org/10.1016/j.jas.2018.05.011>

Gilbert, J.B., Price, V.E. & Greenstein, J.P. (1949). Effect of anions on the non-enzymatic deamidation of glutamine. *Journal of Biological Chemistry* 180, 209.

Griffiths, P. (1983). Fourier transform infrared spectrometry. *Science* 222, 297-302.

Grypnas, M. (1976). The crystallinity of bone mineral. *Journal of Material Science* 11, 1691-1696

Guiry, E.J. & Szpak, P. (2020). Quality control for modern bone collagen stable carbon and nitrogen isotope measurements. *Methods in Ecology and Evolution* 11, 1049-1060. <https://doi.org/10.1111/2041-210x.13433>

Hamilton, S.M. (2015). Ambient groundwater geochemistry data for southern Ontario, 2007-2014; Ontario Geological Survey, Miscellaneous Release-Data 283-Revised.

Harper, L., Giddens, J., Langdale, G. & Sharpe, R. (1989). Environmental effects on nitrogen dynamics in soybean under conservation and clean tillage systems. *Agronomy Journal* 81, 623-631.

Hoefs J. (2009). *Stable Isotope Geochemistry* 6. Berlin: Springer-Verlag.

Hackett, C.J. (1981). Microscopical focal destruction (tunnels) – in exhumed human bones. *Medicine, Science and the Law* 21, 243-265.

Hoke, N., Rott, A., Johler, S., Reul, A., Beck, A. & Günther, A. et al. (2019). How bone degradation, age, and collagen extraction methods affect stable isotope analysis. *Archaeological and Anthropological Sciences* 11, 3357-3374.

- Jaouen, K., Richards, M., Le Cabec, A., Welker, F., Rendu, W. & Hublin, J. et al. (2019). Exceptionally high $\delta^{15}\text{N}$ values in collagen single amino acids confirm Neandertals as high-trophic level carnivores. *Proceedings of the National Academy of Sciences* 116, 4928-4933.
- Jim, S., Ambrose, S. & Evershed, R. (2004). Stable carbon isotopic evidence for differences in the dietary origin of bone cholesterol, collagen and apatite: implications for their use in palaeodietary reconstruction. *Geochimica et Cosmochimica Acta* 68, 61-72.
- Johnson, E.O., Soultanis, K. & Soucacos, P.N. (2004). Vascular anatomy and microcirculation of skeletal zones vulnerable to osteonecrosis: vascularization of the femoral head. *Orthopedic Clinics of North America* 35, 285–291.
- Jørkov, M.L.S., Heinemeier, J. & Lynnerup, N. (2007). Evaluating bone collagen extraction methods for stable isotope analysis in dietary studies. *Journal of Archaeological Science* 34, 1824-1829.
- Kadam, S., Tiwari, B., Álvarez, C. & O'Donnell, C. (2015). Ultrasound applications for the extraction, identification and delivery of food proteins and bioactive peptides. *Trends In Food Science & Technology* 46, 60-67.
- Kirsanow, K., Makarewicz, C. & Tuross, N. (2008). Stable oxygen ($\delta^{18}\text{O}$) and hydrogen (δD) isotopes in ovicaprid dentinal collagen record seasonal variation. *Journal of Archaeological Science* 35, 3159-3167.
- Koon, H.E.C. (2012). Identification of cooked bone using TEM imaging of bone collagen. *Forensic Microscopy for Skeletal Tissue* 915, 249-261. In: Bell, L. (Ed.) *Forensic Microscopy for Skeletal Tissues. Methods in Molecular Biology (Methods and Protocols)* 915. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-61779-977-8_15
- Leach, S.J. & Parkhill, E.M.J. (1955). Amide nitrogen in proteins. *Proceedings of the International Wool Textile Research Conference, Australia*, C, 92.
- LeGeros, R.Z. & LeGeros, J.P. (1984). Phosphate minerals in human tissues. In: Nriagu, J.D., Moore, P.B. (Eds.), *Phosphate Minerals*. Springer-Verlag, Berlin Heidelberg, Germany, 351-385.
- Lip Kwok, P. & Chan, H. (2011). Pulmonary delivery of peptides and proteins. *Peptide and Protein Delivery*, 23-46.

- Longinelli A. (1984). Oxygen isotopes in mammal bone phosphate: A new tool for paleohydrological and paleoclimatological research? *Geochimica et Cosmochimica Acta* 48, 385-390.
- Longin, R. (1971). New method of collagen extraction for radiocarbon dating. *Nature* 230, 241-242.
- Luz B. & Kolodny Y. (1985). Oxygen isotope variations in phosphate of biogenic apatites, IV. Mammal teeth and bone. *Earth and Planetary Science Letters* 75, 29- 36.
- Mabee, S. (2019) Using Stable Isotopes to Understand Survival versus Extinction of Late Pleistocene Muskoxen. PhD Dissertation, The University of Western Ontario, London, Ontario, Canada.
- Manolagas S.C. (2000). Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocrine Review* 21, 115-137.
- Matsubayashi, J. & Tayasu, I. (2019). Collagen turnover and isotopic records in cortical bone. *Journal of Archaeological Science* 106, 37-44.
- Matthews, C.J.D., Longstaffe, F.J., Lawson, J.W. & Ferguson, S.H. (2021). Distributions of Arctic and Northwest Atlantic killer whales inferred from oxygen isotopes. *Scientific Reports* 11. <https://doi.org/10.1038/s41598-021-86272-5>
- Metcalf, J.Z., Longstaffe, F.J. & Hodgins, G. (2013). Proboscideans and paleoenvironments of the Pleistocene Great Lakes: landscape, vegetation, and stable isotopes. *Quaternary Science Reviews* 76, 102-113. <https://doi.org/10.1016/j.quascirev.2013.07.004>
- Mezga, K., Urbanc, J. & Cerar, S. (2014). The isotope altitude effect reflected in groundwater: a case study from Slovenia. *Isotopes in Environmental and Health Studies* 50, 33-51. <https://doi.org/10.1080/10256016.2013.826213>
- Michel, V., Ildefonse, P. & Morin, G. (1995). Chemical and structural changes in *Cervus elaphus* tooth enamels during fossilization (Lazaret Cave): a combined IR and XRD Rietveld analysis. *Applied Geochemistry* 10, 145-159.
- Miller E.J. & Gay, S. (1982). Collagen: An overview. *Methods Enzymology* 82, 3-32.

- Munro, L., Longstaffe, F.J. & White, C.D. (2007). Burning and boiling of modern deer bone: Effects on crystallinity and oxygen isotope composition of bioapatite phosphate. *Palaeogeography, Palaeoclimatology, Palaeoecology* 249, 90-102.
- Nelson B.K., DeNiro M.J., Schoeninger M.J. & De Paolo D.J. (1986). Effects of diagenesis on strontium, carbon, nitrogen and oxygen concentration and isotopic composition of bone. *Geochimica et Cosmochimica Acta* 50, 1941-1949.
- O'Leary, M. (1981). Carbon isotope fractionation in plants. *Phytochemistry* 20, 553-567.
- Pal Chowdhury, M., Wogelius, R., Manning, P. L., Metz, L., Slimak, L. & Buckley, M. (2019). Collagen deamidation in archaeological bone as an assessment for relative decay rates. *Archaeometry* 61, 1382-1398. <https://doi.org/10.1111/arcm.12492>
- Person, A., Bocherens, H., Saliège, J-F., Paris, F., Zeitoun, V. & Gérard, M. (1995). Early diagenetic evolution of bone phosphate: an X-ray diffractometry analysis. *Journal of Archaeological Science* 22, 211-221.
- Plint, T., Longstaffe, F.J. & Zazula, G. (2019). Giant beaver palaeoecology inferred from stable isotopes. *Scientific Reports* 9. <https://doi.org/10.1038/s41598-019-43710-9>
- Qi, H., Coplen, T.B., Geilmann, H., Brand, W.A. & Böhlke, J.K. (2003). Two new organic reference materials for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements and a new value for the $\delta^{13}\text{C}$ of NBS 22 oil. *Rapid Communications in Mass Spectrometry* 17, 2483-2487. <https://doi.org/10.1002/rcm.1219>
- Qi, H., Coplen, T.B., Mroczkowski, S.J., Brand, W.A., Brandes, L., Geilmann, H. & Schimmelmann, A. (2016). A new organic reference material, L-glutamic acid, USGS41A, for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements – a replacement for USGS41. *Rapid Communications in Mass Spectrometry* 30, 859-866.
- Richards, M. (2015). Stable isotope analysis of bone and teeth as a means for reconstructing past human diets in Greece. *Hesperia Supplements* 49, 15-23.
- Roberts, S.J., Smith, C.I., Millard, A. & Collins, M. (2002). The taphonomy of cooked bone: Characterizing boiling and its physico-chemical effects. *Archaeometry* 44, 485-494. <https://doi.org/10.1111/1475-4754.t01-1-00080>.

- Robinson, N.E., Robinson, Z.W., Robinson, B., Robinson, A.L., Robinson, J.A., Robinson, M.L. & Robinson, A.B. (2004). Structure-dependent nonenzymatic deamidation of glutaminyl and asparaginyl pentapeptides. *Journal of Peptide Research* 63, 426-436.
<https://doi.org/10.1111/j.1399-3011.2004.00151.x>
- SHIH, F. F. (1990). Deamidation during treatment of soy protein with protease. *Journal of Food Science* 55, 127-129. <https://doi.org/10.1111/j.1365-2621.1990.tb06033.x>
- Schoeninger, M.J. & DeNiro, M.J. (1984). Nitrogen and carbon isotopic composition of bone collagen from marine and terrestrial animals. In *Geochimica et Cosmochimica Acta* 48, 625-639.
[https://doi.org/10.1016/0016-7037\(84\)90091-7](https://doi.org/10.1016/0016-7037(84)90091-7)
- Schwartz-Narbonne, R., Longstaffe, F.J., Kardynal, K.J., Druckenmiller, P., Hobson, K.A., Jass, C.N., Metcalfe, J.Z. & Zazula, G. (2019). Reframing the mammoth steppe: Insights from analysis of isotopic niches. *Quaternary Science Reviews* 215, 1-21.
<https://doi.org/10.1016/j.quascirev.2019.04.025>
- Shoulders, M. & Raines, R. (2009). Collagen structure and stability. *Annual Review of Biochemistry* 78, 929-958.
- Simpson, J.P., Penkman, K.E.H., Demarchi, B., Koon, H., Collins, M.J., Thomas-Oates, J., Shapiro, B., Stark, M. & Wilson, J. (2016). The effects of demineralisation and sampling point variability on the measurement of glutamine deamidation in type I collagen extracted from bone. *Journal of Archaeological Science* 69, 29-38. <https://doi.org/10.1016/j.jas.2016.02.002>
- Singh, B.P. (2017). Original isotopic composition of water in precipitation by different methods. *Applied Water Science* 7, 3385-3390. <https://doi.org/10.1007/s13201-016-0500-6>
- Suarez, C.A. & Kohn, M.J. (2020). Caught in the act: a case study on microscopic scale physicochemical effects of fossilization on stable isotopic composition of bone. *Geochimica et Cosmochimica Acta* 268, 277-295. <https://dx.doi.org/10.1016/j.gca.2019.10.008>
- Szpak, P., Orchard, T.J., McKechnie, I. & Gröcke, D.R. (2012). Historical ecology of late Holocene sea otters (*Enhydra lutris*) from northern British Columbia: Isotopic and zooarchaeological perspectives. *Journal of Archaeological Science* 39, 1553-1571.

Szpak, P., White, C.D., Longstaffe, F.J., Millaire, J-F & Vásquez Sánchez, V.F. (2013). Carbon and nitrogen isotopic survey of northern Peruvian Plants: baselines for paleodietary and paleoecological studies. *PLoS ONE* 8, e53763. <https://doi.org/10.1371/journal.pone.0053763>

Szpak, P., Metcalfe, J.Z., & Macdonald, R.A. (2017). Best practices for calibrating and reporting stable isotope measurements in archaeology. *Journal of Archaeological Science* 13, 609-616. <https://doi.org/10.1016/j.jasrep.2017.05.007>

Tahmasebi, F., Longstaffe, F.J. & Zazula, G. (2018) Nitrogen isotopes suggest a change in nitrogen dynamics between the Late Pleistocene and modern time in Yukon, Canada. *PLoS ONE* 13, e0192713. <https://doi.org/10.1371/journal.pone.0192713>

Tran, N.T.T., Wang, T.-H., Lin, C.-Y., Tsai, Y.-C., Lai, C.-H., Tai, Y. & Yung, B.Y.M. (2011). Direct synthesis of rev peptide-conjugated gold nanoparticles and their application in cancer therapeutics. *Bioconjugate Chemistry* 22, 1394-1401. <https://doi.org/10.1021/bc2001215>

Trueman, C.N.G., Behrensmeyer, A.K., Tuross, N. & Weiner, S. (2004). Mineralogical and compositional changes in bones exposed on soil surfaces in Amboseli National Park, Kenya: diagenetic mechanisms and the role of sediment pore fluids. *Journal of Archaeological Science* 31, 721-739.

Tuross, N., Fogel, M.L. & Hare, P.E. (1988). Variability in the preservation of the isotopic composition of collagen from fossil bone. *Geochimica et Cosmochimica Acta* 52, 929-935.

Tuross, N., Warinner, C., Kirsanow, K. & Kester, C. (2008). Organic oxygen and hydrogen isotopes in a porcine controlled dietary study. *Rapid Communications in Mass Spectrometry* 22, 1741-1745.

van Doorn, N.L., Wilson, J., Hollund, H., Soressi, M. & Collins, M.J. (2012). Site-specific deamidation of glutamine: a new marker of bone collagen deterioration. *Rapid Communications in Mass Spectrometry* 26, 2319-2327.

van Klinken, G.J. (1999). Bone collagen quality indicators for palaeodietary and radiocarbon measurements. *Journal of Archaeological Science* 26, 687-95.

Verburg, P. (2006). The need to correct for the Suess effect in the application of $\delta^{13}\text{C}$ in sediment of autotrophic Lake Tanganyika, as a productivity proxy in the Anthropocene. *Journal of*

Paleolimnology 37, 591–602. Springer Science and Business Media LLC.
<https://doi.org/10.1007/s10933-006-9056-z>

von Holstein, I.C.C., von Tersch, M., Coutu, A.N., Penkman, K.E.H., Makarewicz, C.A. & Collins, M.J. (2018). Collagen proteins exchange oxygen with demineralisation and gelatinisation reagents and also with atmospheric moisture. *Rapid Communications in Mass Spectrometry* 32, 523-534.

Wang, C., Guo, L., Li, Y. & Wang, Z. (2012). Systematic comparison of C3 and C4 plants based on metabolic network analysis. *BMC Systems Biology* 6, S9.

Wilson, J., van Doorn, N. L. & Collins, M. J. (2012). Assessing the extent of bone degradation using glutamine deamidation in collagen. *Analytical Chemistry* 84, 9041-9048.
<https://doi.org/10.1021/ac301333t>

Webb, E.C., White, C.D. & Longstaffe, F.J. (2014). Investigating inherent differences in isotopic composition between human bone and enamel bioapatite: implications for reconstructing residential histories. *Journal of Archaeological Science* 50, 97-107.
<https://doi.org/10.1016/j.jas.2014.07.001>

Weiner, S. & Bar-Yosef, O. (1990). States of preservation of bones from prehistoric sites in the Near East: a survey. *Journal of Archaeological Science* 17, 187-196.

Wright L.E. & Schwarcz, H.P. (1998). Stable carbon and oxygen isotopes in human tooth enamel: identifying breastfeeding and weaning in prehistory. *American Journal of Physical Anthropology* 106, 1-18.

APPENDIX A

Bone Collagen Extraction Procedure Using EDTA

L.S.I.S. Technical Memorandum #19-01

Version 2

Sawyer Rowe

Stephanie Mabee

Curtis Russell

OVERVIEW:

Collagen is extracted using methods modified from Tuross et. al (2008) and von Holstein et. al (2018). In summary, bones that have undergone lipid extraction are immersed in a weak EDTA solution buffered to near neutrality to dissolve the mineralized components. Once demineralization is complete, a collagen residue is left behind, which is then treated with NaOH to remove any humic and fulvic acids. The residue is rinsed and freeze-dried to remove any moisture. This technical memorandum outlines the required steps to extract the collagen from bone.

SAFTEY CONSIDERATIONS:

All standard laboratory safety protocols (safety glasses, closed-toed shoes, etc.) must be adhered to while conducting the steps described in this technical memorandum. Lab coats and gloves should be worn when handling solvents, acids and bases, or other caustic chemicals. All work involving solvents must be conducted under a fume hood.

APPARATUS:

Dremel rotary tool

Sieves (0.85mm/#80 Mesh and 0.18mm/#20 Mesh) and pan

Mortar and pestle

Weighing paper

Tweezers/scoopula

Balance

Centrifuge

11 mL 16x100mm glass collagen vials

150/250mL beakers

4mL glass vials with caps

1L Erlenmeyer flask with side arm and rubber stopper / hose

9" Pasteur pipette

Distilled water

0.5M EDTA buffered to near neutrality (pH 7.0-8.0)

0.1M NaOH

3:1 Chloroform: Methanol solution

I. Setup

- i. Wipe down the bench surface, Dremel, mortar and pestle, sieves and any other tools such as tweezers or scoopulas with acetone or isopropyl alcohol and Kimwipes. If you are analyzing phosphate or carbonate in addition to collagen, acetone or isopropyl will not remove very fine dust particles. Instead, wash all tools with warm water and Sparkleen in the sink, rinse 3 times with DI water and allow to dry. Excess water may be patted dry with paper towels. Do not press too hard on the mesh when washing the sieves or the mesh may separate from the frame and ruin them. Similarly, do not press scoopulas or tweezers into the sieve mesh to remove fragments as this may bend or break the mesh and ruin the sieve. Repeat this cleaning process between each sample. When cleaning the sieves between samples, blowing compressed air through the mesh before washing can help to clear some of the particles.
- ii. Select bone samples for analysis and remove any surface dirt and contaminants with a wire brush or Dremel wheel. Use the Dremel to remove any trabecular bone and remaining soft tissue until you have ~500-700mg of cortical bone for modern samples, and ~1g of cortical bone for archeological or poorly preserved bone samples. Starting with <50mg of cortical bone is not recommended and starting with a sample weight >50mg of cortical bone will help mitigate sample loss that occurs during crushing. Be careful not to allow the Dremel wheel to remain in one location too long or the bone may heat to the point isotopic exchange occurs because of collagen damage and inorganic matrix recrystallization. Leaving the Dremel wheel in one location too long can also result in loss of too much of the needed cortical bone for collagen extraction.

If needed, the sonicator may be used to remove adherent soil on the surface of bones, particularly for thin or fragile bones such as ribs where the cortical bone layer is thin and the Dremel may not be appropriate. To perform this, place the sample in a 50mL beaker and fill it halfway with DI water, then fill the sonicator tub with DI water to the fill line. Place the samples in the sonicator basket and set the time for 5 minutes. Be sure to check the sonicator tub's water temperature at the end of each 5-minute cycle as it will begin to warm. If the water becomes too warm, isotopic exchange may occur. Replace the sonicator tub's water if it becomes warm at the end of a cycle. As soil is dislodged from samples it will dirty the water in the 50mL beaker, pour off the water and replace with fresh DI water as needed until there is no further discoloration of the water. This indicates no further soil remains on the sample and the water in the 50mL beaker may be poured off and allow the samples to dry for ~24 hours.

II. Crushing and Weighing Bone

- i. In your lab book construct a table with the following columns: Sample ID and Dry Weight.

- ii. Stack your sieves with the 0.85mm sieve atop the 0.18mm, and a pan below the 0.18mm sieve. If you are conducting phosphate (<0.045mm), carbonate (<0.18mm) or FTIR (<0.063mm) analysis you may add the appropriate additional sieves.
- iii. Place your bone samples in a mortar and pestle and begin crushing. Padding may be placed below the mortar and pestle to minimize noise and vibration while crushing. It is important to periodically place the crushed material in the 0.85mm sieve and agitate the sieves by placing both hands on the sides and gently tapping and shaking from side to side to make the fragments “dance” to sort particle sizes periodically. Periodically sorting the particle sizes will help to minimize sample loss during crushing and prevent too wide a distribution of fragment sizes which can interfere with demineralization. Remove any material remaining in the 0.85mm mesh and replace it in the mortar for further crushing. Material which is of the appropriate size for collagen analysis will be resting on the 0.18mm mesh (appropriately sized powder for other analysis will also be resting on that procedure’s respective mesh size).
- iv. Once the sample has been crushed sufficiently that no more material remains in the 0.85mm mesh, remove the material from the 0.18mm mesh and place it on a folded weigh paper. If you are conducting carbonate, phosphate or FTIR analyses and have added sieves, material which has collected on those should similarly be removed to weigh papers at this time.
- v. Prepare the balance by brushing away any bone or dust fragments that may have accumulated and wipe down the surface with acetone or isopropyl and Kimwipes. Place a second piece of weigh paper on the balance and “tare” the scale so the screen shows 0.00000g. Fold the weigh paper with the crushed bone fragments in half and carefully transfer them to the paper on the balance and weigh out 0.500 ± 0.150 g (ensure the balance door is closed when recording weights). Record the sample ID and weight in your notebook.
- vi. Transfer the weighed bone fragments from the folded weigh paper into an 11mL collagen vial. Tear off strips of Kimwipes and fold the strips in half and secure them over the mouth of the vial with rubber bands and place the vials in a 150/250mL beaker. Repeat for all samples and place in the freeze dryer overnight.

Lipid Extraction step omitted as it was not used for the majority of the samples. It should be noted that if your samples have lipids in them (should be tested before all the experiments), then a lipid treatment should be done to remove lipids.

III. Demineralization

- i. Construct a demineralization checklist in your notebook, a template for which is given at the end of this technical memorandum.
- ii. Add ~8mL of 0.5M EDTA to the vials, once again using the pressure of the spray bottle to agitates the bone fragments. Check the pH of the liquid and record this in your notebook, along with the date you began demineralization.
- iii. Cap the vials but be sure to inspect the liner of the cap before doing so. If the liner is missing or loose, do not use the cap as glue residue on a cap missing a liner or from a cap liner which falls into the sample could cause contamination. Once capped be sure to agitate the vials, either by gently shaking/swirling them by hand, or by placing them on the vortex at a setting of 6-8 for ~10 seconds. After agitating, tip the sample rack on its side (propped up by something like a bottle cap or block to keep the acid away from the cap liner) in the fume hood, with the bone fragments spread along the side of the vial to speed the demineralization process.
- iv. After two days of soaking, wipe down a 9" pipette and a pair of tweezers or scoopula with acetone or isopropyl and Kimwipes and connect the pipette to the sidearm flask as noted in the "Lipid Extraction" section. Pipette off as much of the liquid as possible while being careful not to suck up any collagen fragments. Be sure to wipe down the pipette with acetone or isopropyl and Kimwipes before moving on to the next sample.
- v. Check the progress of the sample demineralization by pressing some collagen fragments into the side of the vial with tweezers or a scoopula. If demineralization is complete, the fragments will feel soft and have some "give" to them, similar to pressing on a piece of rubber. If demineralization is not complete the fragments will feel hard and may give a clicking sound when pressed on by metal instruments.

IV. Demineralization, Part II

- i. If demineralization hasn't completed, repeat steps ii to v making sure to note what day the EDTA solution was changed. Once the EDTA solution has been exchanged the samples may be left in the fume hood for 2-3 days at a time, but it is recommended the EDTA solution be exchanged every 2 days until demineralization is complete by repeating steps ii. and iv. to expedite the process.

Not all samples will demineralize at the same rate. Should one or more samples finish demineralizing before others, pipette off as much EDTA solution as possible as described above and store the capped vial in a 150/250mL beaker in the fridge until the remaining samples have finished demineralizing. Be sure to label the beaker with your name and project, as well as the date you put them in the fridge. Note the date each sample finished demineralizing in your notebook.

- ii. Once samples have finished demineralizing and as much EDTA solution has been pipetted off as possible, add ~8mL of DI water to the vials. Once again, the pressure of the squirt bottle should be used to agitate the fragments, and the vials should also be gently shaken/swirled by hand or vortexed to ensure the fragments are well agitated and as much surface area is rinsed by the DI water as possible.
- iii. After adding the DI water, place the collagen vials in the proper holder for the centrifuge and balance them. If the centrifuge cups are not of equal weight, empty collagen vials may be added to either side and filled with DI water as needed until balanced.
- iv. Place the balances samples in the centrifuge and spin them down at 2800rpm for 7 minutes; be sure to double check the centrifuge settings before each run.
- v. After centrifuging, pipette off as much DI water as possible and repeat steps vii to ix 6 more times for a total of 7 rinses. Be sure to note the completion of each rinse on the checklist so as not to lose track of which rinse you are on.

V. Humic and Folic Acid Removal

- i. Following the 7th DI water rinse and spin down, pipette off as much DI water as possible, and add ~8mL of 0.1M NaOH to the samples. Once again be sure to agitate the collagen fragments either by hand or vortex as outlined above, to ensure as much surface area is rinsed with NaOH as possible.
- ii. Tip the vials on their side with the sample fragments spread up the side of the vial to increase surface area as outlined in step iii. of the demineralization stage above. Allow the samples to sit for 20 minutes. Note if there is a color change during this step. Samples with large amounts of humic or folic acids will turn the NaOH solution a yellow-brown or even black color. Be sure to note the time you begin the NaOH rinse in your notebook.
- iii. After 20 minutes, balance and centrifuge the samples at 200rpm for 7 minutes and pipette off as much NaOH solution as possible.
- iv. Repeat steps i and ii until there is no color change in the NaOH solution after 20 minutes; you may wish to use something white like a sheet of paper to hold behind the collagen vials while inspecting the solution color to better observe if a small amount of color change has occurred. If analyzing modern samples there is likely to be very little, if any humic and folic acids present and a single NaOH rinse may be sufficient.
- v. Once the humic and folic acids have been extracted and the NaOH solution pipetted off, add ~8mL of DI water and agitate and centrifuge as described above before pipetting off the DI water. Repeat this process 6 more times for a total of 7 rinses. Be sure to note the completion of each rinse in your notebook so as not to lose track.

VI. Final Freeze Drying and Weighing

- i. After completing the final DI water rinse, tear off strips of Kimwipes and place them over the mouth of the vials and secure them with rubber bands and store them in a 150/250mL beaker. Do not cap the vials.
- ii. Place the vials in the freeze dryer overnight once more to remove any moisture.
- iii. Once freeze drying is completed, the collagen fragments may be transferred into 4mL glass vials, allowing the 11mL collagen vials to be cleaned and reused for other projects (important since collagen extraction is a common procedure in the LSIS).
- iv. Samples may be powdered by mortar and pestle using the cryomill as the collagen fragments will not powder at room temperature, but rather flatten into “oatmeal” flakes. Once powdered in the cryomill transfer the samples to a folded weigh paper and return it to the 4mL samples vial. It should be noted the use of the cryomill will cause condensation on the sample and will thus necessitate additional freeze drying.
- v. As described above, tear up Kimwipes and secure with rubber bands over the vial mouth and place the vials in a 150mL beaker to freeze dry overnight. Do not cap the vials.
- vi. Alternatively, you may skip powdering with the cryomill and weigh the sample into 4x3.2mm tin or silver weigh capsules using tweezers. After each fragment is placed into the capsule, the vial should be capped and shaken for ~3 seconds to randomize the fragments before another is selected.
- vii. To begin weighing, obtain the most recent EACF and TCEA lab books and obtain the current standards in use. A table with a standardized sequence of standards and samples as well as weights is posted by the microbalance, but if in doubt, consult the TCEA/EACF technical memoranda or the laboratory staff. On the next blank page in the TCEA or EACF book copy the template of the preceding run, as well as your surname, and last 2 digits of the year, followed by your run number, e.g., “Doe TCEA 19-02”. If using the EACF and analyzing only carbon or nitrogen isotope compositions, be sure to note so in the EACF book.
- viii. Wipe down the microbalance, tweezers, scoopula and bench with Kimwipes and acetone or isopropyl. Place a weigh capsule on the microbalance, close the door and “tare” the scale. Once the balance displays 0.0000mg, open remove the weigh capsule and begin to either scoop the powdered sample or sample collagen fragments into the capsules.
- ix. Weigh the capsule each time you add sample. When weighing for C or N, use tin capsules and be sure to check the preceding runs for the standard weights. When

weighing collagen samples, a good target weight 0.390 ± 0.01 mg. For oxygen a good target weight is 0.600 ± 0.01 mg. Record the sample weight in the EACF or TCEA book.

- x. Once the target weight is reached the capsule may be sealed by placing it upright on a weigh paper, and using tweezers or curved forceps, crimp the top of the capsule and rolling the top edge over like a paper bag. Using 2 sets of tweezers, compress the capsule into a ball or cube and place it into a 96-well Elisa tray.
- i. When finished weighing, secure the lid of the tray with a rubber band to prevent samples from jumping trays during transport or storage since this will almost certainly require you to re-weigh 2 or more samples. Label the tray and store in a desiccator for at least 48 hours for C or N isotope analysis and 72 hours for O isotope analysis.

VIII. References

Kirsanow, K., Makarewicz, C., & Tuross, N. (2008). Stable oxygen ($\delta^{18}\text{O}$) and hydrogen (δD) isotopes in ovicaprid dentinal collagen record seasonal variation. *Journal of Archaeological Science*, 35(12), 3159-3167.

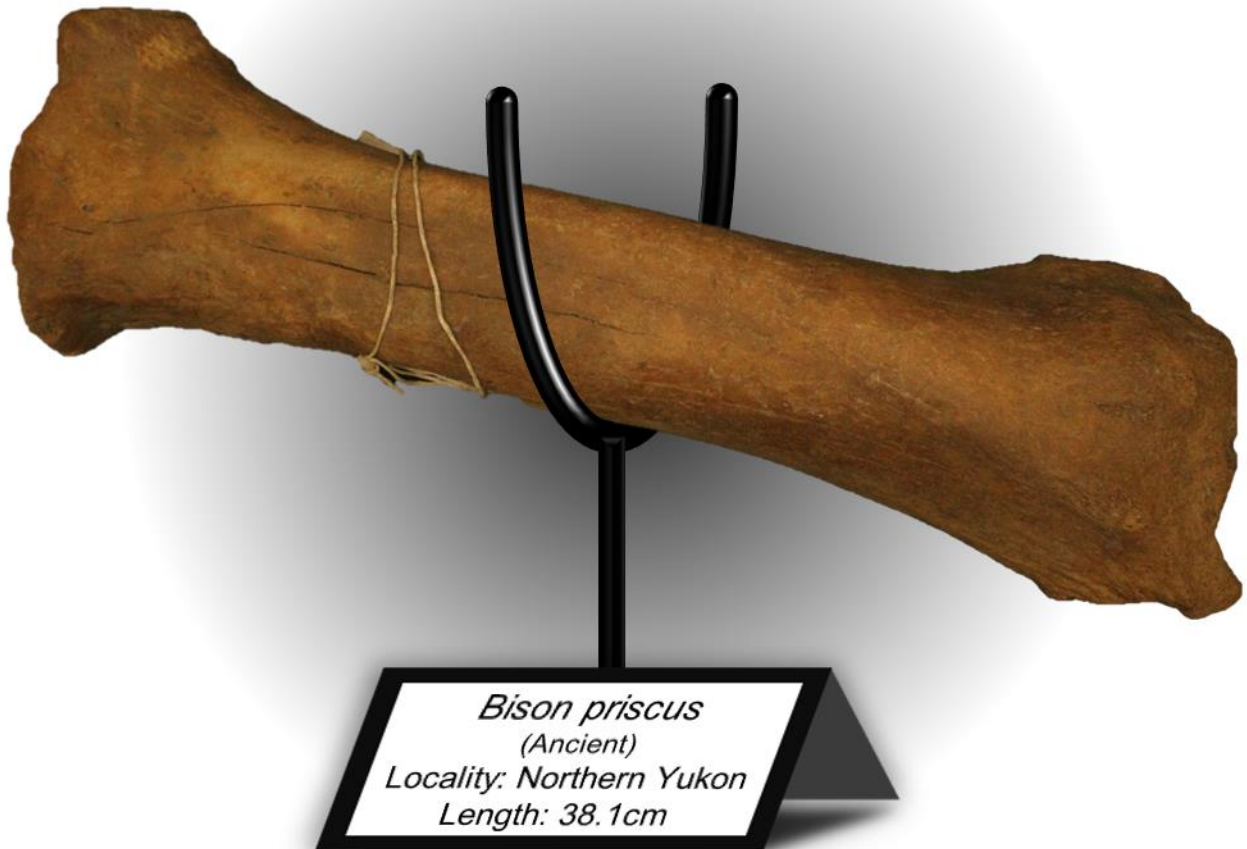
Tuross, N., Warinner, C., Kirsanow, K., & Kester, C. (2008). Organic oxygen and hydrogen isotopes in a porcine controlled dietary study. *Rapid Communications in Mass Spectrometry*, 22(11), 1741-1745. doi:10.1002/rcm.3556

von Holstein, I. C. C., von Tersch, M., Coutu, A. N., Penkman, K. E. H., Makarewicz, C. A., & Collins, M. J. (2018). Collagen proteins exchange oxygen with demineralisation and gelatinisation reagents and also with atmospheric moisture. *Rapid Communications in Mass Spectrometry*, 32(6), 523-534. doi:10.1002/rcm.8064

APPENDIX B
Bone Sample Catalogue

Images Collected by Curtis Russell

Bison priscus (Steppe Bison)



Description:

This is the left radius bone from a younger muskox (based on length and density of the bone). It has not been radiocarbon dated but based on the rate of decomposition and the visible discolouration, it is presumed to be of Pleistocene age. The bone was preserved in paleosol material, which was found both inside and outside of the bone. Dating this soil would provide an age minimum for the bone if dating of the bone proves difficult. There is a crack in the bone that permeates through its radius and propagates along the c-axis. The sample extracted from this bone was removed at least 2 cm away from the crack.

Ovibos moschatus (Muskox)



Description:

This is a modern C4 vertebrae from a Muskox. It has been aggressively degenerated in the laboratory, giving it a surface a chalky appearance. The internal bone shows mild osteoporosis suggesting an aged muskox.

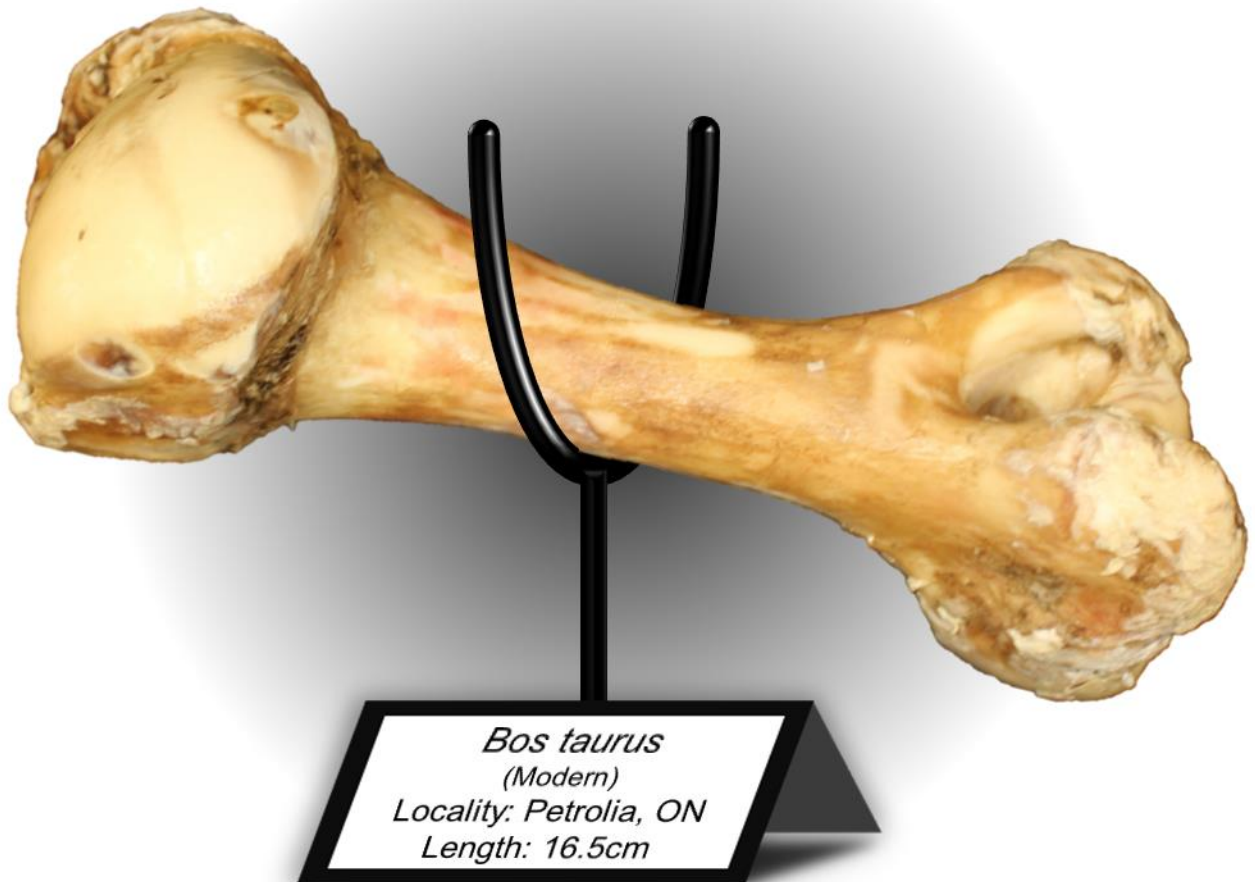
Alces Alces americana (Eastern Moose)



Description:

This is a modern left humerus from an Eastern Moose that was harvested on a side road in 2008. Age is estimated to be early - middle aged (suggested by the lack of bone porosity). It was donated to this research by Ed Estaugh from the Anthropology Department at Western University. The bone appearance exhibits chalkiness and dryness, presumably inherited from aggressive defleshing pre-treatment.

Bos taurus (Modern Cattle)



Description:

This is a modern right humerus from a 2-week-old calf (age determined from local butcher, and as evident from low crystallinity in bone). The bovine was killed on a farm 5 kilometres east of Petrolia on a farm that fed their animals with a combination of maize and grass. They were not fed grass directly but were instead permitted to eat the grass that was available to them. The sample was degreased following Plint's "Method D" (Plint, 2019; p 36), and has a greasy appearance as a result of light defleshing.

APPENDIX C

Trypsin Digest in Solution Protocol for Collagen Samples

MALDI-TOF-MS Technical Memorandum

Version 1.1

Curtis Russell

Kristina Jurcic

I. Sample Preparation

1. Calculate the final volume of your sample vials by multiplying the weight of the sample by your desired concentration of trypsin (Ideal is $1.2\mu\text{g}/\mu\text{L}$).
2. Bring samples to half the final volume in LysC water and then add the other half in NH_4HCO_3 (100mM).
3. Add $50\mu\text{L}$ of Trifluoroethanol (to a final of 5%).
4. In each Trypsin capsule, combine $100\mu\text{L}$ of NH_4HCO_3 with the trypsin for a total volume of $100\mu\text{L}$ of dissolved trypsin in each capsule.

II. Trypsin Digest

* For the trypsin to work properly, the concentration of detergents (*i.e.*, SDS) and other chaotropes (*i.e.*, Urea) needs to be at a compatible final concentration. When you add the correct amount of trypsin make sure you add it in a volume that will dilute your sample to the desired concentration of the limiting interfering agent. Alternatively, protein precipitation (or other appropriate clean-up / desalting) can be done at this time to remove interfering agents and excess IAA.

1. Quantify protein sample by BCA or Pierce 660nm assay.
2. Add the trypsin solution at a ratio of trypsin to sample of 1:50 – 1 mg of trypsin for every 50 mg of protein.
3. Gently vortex and spin the sample. To allow complete digestion, place the rack in the 37°C incubator (water bath or shaking thermal block @500-700 rpm) overnight, or for at least 18 hrs. Gentle or periodic mixing is optional.
4. Next morning add more trypsin at a (1:100) ratio and maintain at 37°C for another 4 hours with mixing at 1400 rpm if possible.
5. Acidify sample using 0.1% formic acid.

III. Sample Clean-Up

1. If detergents are present in the digest, they must be removed first. For non-ionic detergents (*i.e.*, Tween, NP-40, CHAPS, Triton-X), or anionic (*i.e.*, SDS), strong cation exchange (SCX) chromatography is the recommended removal method.

APPENDIX D

MALDI-TOF-MS Program Code Guide

Simon Hickinbotham

Julie Wilson

This information is also available on the README file of the program

Make sure you are in the directory (folder) where the code is; then the code can be compiled using the Makefile, *i.e.*, just type "make".

To run the code type:

```
./Q2E <filelist> <peptidelist> <parameterfile>
```

<filelist> should be replaced by the name of a file (*e.g.*, exampleFileList) containing the names (including the path) of the files to be processed. Note that the name of the file will be used in the names of the output files, *e.g.*, exampleFileListResults.

The format of this file should be the same as that given in the file exampleFileList, *i.e.*, the number of files to be processed and the number of replicates on the first line followed by each filename on a new line. The filename should include the path to where the files are.

Replicates must follow consecutively.

<peptideList> should be replaced by the name of a file containing the list of peptides with the number of peptides on the first line. Each following line should have the monoisotopic mass of the peptide, the number of Qs in the peptide (1 or 2) and the sequence of the peptide (with a Z added for each hydroxylated proline).

Output:

The programme calculates the amount of glutamine deamidation for each peptide in the file <peptidelist>.

If only one glutamine is being considered, the value of beta1 (between 0.0 and 1.0) shows the level of undeamidated glutamine, with 0.0 being totally deamidated and 1.0 being not deamidated at all. The value beta2 in this case is just 1.0-beta1 and shows the level deamidated glutamine, *i.e.* of glutamic acid.

If there were two glutamines being considered, then beta1 would show how much (of either) was undeamidated; beta2 would show how had one deamidated glutamine (either one), and beta3 would show how much had both glutamines deamidated (with $\beta_3 = 1.0 - \beta_1 - \beta_2$).

More than two glutamines can also be considered, but as the number of glutamines increases, the number of peaks (from the monoisotopic mass) being fitted, FITPEAKS, should also be increased.

Beta values are calculated for each set of replicates (if SNR level and fit in GA are ok so that value can be considered reliable). These are combined according to SNR levels, *i.e.*, better spectra have more weight.

The output to the screen shows why a spectrum has not had a value calculated for it (poor SNR or poor fit in GA). The results are also written to the file <filelist>Results.

The beta values are also output in a comma-separated file called <filelist>Betas.csv.

This has the number of sets of replicates on the first line (how many betas to expect for each peptide). Each following line contains the mass at which the peptide is found (monoisotopic mass + 1), and then the beta value for each replicate set. Where there are two Qs, the betas for a sample follow each other with the smallest value first.

Parameters that can be adjusted:

In the parameterFile you will find:

```
FITPEAKS 4
GALIM 0.02
SNRLIM 3.0
FIRSTMASS 800.0
LASTMASS 4000.0
CSV 0
```

FITPEAKS is the number of peaks in the isotope distribution to be included in the fit. The default is 4, but if there really are two Q's and both are deamidated to some extent then there may be 5 decent sized peaks and it would be worth changing this to 5, but there is no point in adding peaks that are just at noise level.

GALIM is related to the fitness criterion in the genetic algorithm. 0.02 is the default, but to get only the best fitted peptides, use 0.01.

SNRLIM is the SNR threshold to be used. The default value is 3.0.

FIRSTMASS is the first m/z value to be considered and LASTMASS is the last.

CSV determines the file type.

If CSV = 1, the code is set up to read comma separated files with intensities given as real numbers, as output by mMass after processing.

If CSV = 0, the code is set up to read space delimited files with counts given as integers, as output when exporting from Flex Analysis.

All of the above items can be changed.

Q2Einfo.docx

The Q2E service calculates the proportion glutamine (Q) that has degraded to glutamic acid (E) in peptides analyzed by MALDI-MS. For each chosen peptide, the theoretical distribution is calculated and the measured distribution for each sample compared with the theoretical distribution to determine the extent of glutamine deamidation.

See: Assessing the extent of bone degradation using glutamine deamidation in collagen. Julie Wilson, Nienke L van Doorn, Matthew J Collins. Anal. Chem. 84(21), 9041-9048. (2012). (<http://pubs.acs.org/doi/abs/10.1021/ac301333t?mi=z48nb4&af=R&pageSize=20&searchText=aging>)

The user supplies a file containing the names of the files to be processed together with the data files. The format of this file should be the same as that given in the file `exampleFileList`, *i.e.*, the number of files to be processed and the number of replicates on the first line followed by each filename on a new line. Replicates must follow consecutively.

Note that the name of the file will be used in the names of the output files, *e.g.*, `exampleFileListResults`.

A file containing a list of peptides is also supplied by the user. This file should have the number of peptides on the first line. Each following line should have the monoisotopic mass of the peptide, the number of Qs in the peptide (1 or 2) and the sequence of the peptide (with a Z added for each hydroxylated proline).

APPENDIX E

Glossary

Abbreviations and Definitions

Longin method – A method of extracting collagen from bone that involves using HCl acid to demineralize the bioapatite, freeing the collagen.

EDTA method – A method of extracting collagen from bone that involves using EDTA acid to demineralize the bioapatite, freeing the collagen.

post-mortem – A latin term that means post death

fibrillar collagen – Collagen that forms in bundles called ‘fibrils’

$\delta^{18}\text{O}_{\text{coll}}$ = oxygen isotope composition of organic collagen

$\delta^{18}\text{O}_{\text{dw}}$ = oxygen isotope composition of drinking water (typically from $\delta^{18}\text{O}_{\text{met}}$)

$\delta^{18}\text{O}_{\text{met}}$ = oxygen isotope compositions of meteoric water

$\Delta^{18}\text{O}_{\text{coll-met}}$ = discrimination (isotopic enrichment) from meteoric water to organic collagen

$\Delta^{18}\text{O}_{\text{coll-bw}}$ = discrimination (isotopic enrichment) from body water to organic collagen

Curriculum Vitae

Name : Curtis Russell

Post-Secondary Education and Degrees : The University of Western Ontario
London, ON, Canada
2019-2022 M.Sc.

The University of Western Ontario
London, ON, Canada
2015-2019 B.Sc.

Honours and Awards : Robert Hodder Award
2019

Brandon J. Papp Award
2019

Western Scholarship of Distinction
2015

Related Work Experience: Teaching Assistant
The University of Western Ontario
2019-2021