

# Evaluating the Efficacy of Collagen Isolation Using Stable Isotope Analysis and Infrared Spectroscopy

Robin B. Trayler      Pedro Valencia Landa      Sora L. Kim

## **Abstract**

Stable isotope analysis of bone and dental collagen is one of the most common methods to investigate the ecology of modern and extinct human and animal populations. However, since bone and dentine are composite materials with both organic and mineral components, the mineral component must be removed prior to analysis. In this study we investigated the timing and efficacy of mineral removal from bone and dentine. We performed a series of time-step experiments that show that mineral removal can be quantified over short periods of time using Fourier Transform Infrared Spectroscopy (FTIR), and collagen alteration can be tracked using a combination of stable isotope analysis and elemental analysis. We tested our methods on three modern materials: mammalian bone, mammalian dentine, and shark dentine. Our results show: 1) mineral removal is a necessary step, as structural carbonate has a strong influence on stable isotope compositions; 2) demineralization using weak acid (0.1M HCL) does not appear to alter the elemental and isotopic compositions of collagen. Our methods can be used as a framework to evaluate the need-for and efficacy of other demineralization methods in use today including EDTA-demineralization and lipid removal.

# 1 Introduction

Analyzing collagen via stable isotope analysis is one of the most common methods for inferring ecological information from the remains of modern, archaeological, and fossil animals (Clementz, 2012; Koch et al., 1994). Collagen has a slow turnover rate (Hedges et al., 2007), and analyzing its stable carbon and nitrogen isotope composition (i.e.,  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , respectively) provides time-averaged insights into diet and trophic dynamics (Ambrose, 1990; Clementz, 2012; DeNiro and Epstein, 1981, 1978; Koch, 2007; Koch et al., 1994). Collagen is relatively insoluble (Schwarcz and Schoeninger, 1991) and persists in the fossil record for tens of thousands of years, allowing the investigation of animal ecology from the Late Pleistocene through the Anthropocene (Clementz, 2012). Since the amino acid composition and elemental content (~35% carbon, ~11% nitrogen) are well characterized, it is relatively straightforward to quantify the purity of collagen (Ambrose, 1990; Fuller et al., 2015; Szpak, 2011; Tuross, 2002; Tuross et al., 1988). However, the isolation of pure collagen from biologic material such as teeth and bones is complicated as these are composite materials with both mineral and organic (i.e., collagen, lipids) components. While only the organic substrate (i.e., collagen) has a significant nitrogen content, both the mineral and organic components contain carbon and stable isotope analysis of bulk bone or dentine will lead to  $\delta^{13}\text{C}$  values that reflect a weighted average of both. It is therefore necessary to extract the organic collagen from the inorganic mineral prior to stable isotope analysis.

There is considerable variation in published (and practiced) demineralization methods. Some studies recommend higher acid concentrations but shorter reaction times (Ambrose, 1990; Pestle, 2010), while others use lower acid concentrations and long reaction times (Sealy et al., 2014), or forgo mineral removal all together (Brault et al., 2014; Jacob et al., 2005; Mateo et al., 2008; Turner Tomaszewicz et al., 2015). In this study, we do not attempt to resolve the optimal conditions (e.g., time, temperature, acid concentration, etc.)

for all collagen extraction methods. Instead, we focus on developing a framework for assessing the efficacy of a given mineral removal method, with set experimental conditions. We held acid concentration and temperature in our experiments constant and varied the acid-reaction time from 5 minutes to 24 hours (Figure 1). We used this experimental design to test the rate and efficacy mineral removal in three materials collected from modern animals - white tailed deer bone (*Odocoileus virginianus*), goat dentine (*Capra hircus*), and blue shark dentine (*Prionace glauca*). We assessed our methods using three metrics: 1) collagen yield and quality, 2) stable isotope compositions, and 3) the ratio of organic to inorganic material. Our results show that mineral removal is a necessary first step when analyzing the stable isotope composition of collagen. Furthermore, collagen isolation can be rapidly achieved using even low molarity acid, a beneficial quality when preparing large numbers of sample. Finally, we show that for a given method, the extent of mineral removal can be quantified using standard techniques available at many major research universities, and these metrics can be used as a framework to evaluate the preferred collagen extraction technique coupled with bioapatite of interest to any research group.

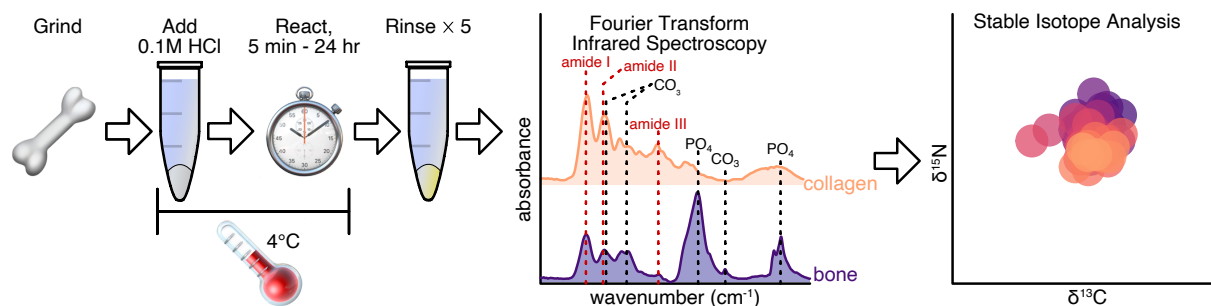


Figure 1: Diagram of methods, including sample preparation and analyses.

## 2 Background

### 2.1 Elemental Composition of Bone

Bone and dentine are composite materials and both mineral organic components are commonly analyzed for their stable isotope compositions (i.e.,  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ,  $\delta^{18}\text{O}$ ) to infer dietary sources, trophic position, and geographic provenance. Essentially, all nitrogen in bone is contained in the organic phase and some studies have analyzed dentine for  $\delta^{15}\text{N}$  values without mineral removal (i.e., Guiry et al., 2016). Most pertinent to this study, both the mineral and the organic components contain carbon sources, which have differing  $\delta^{13}\text{C}$  values due to different diet-tissue enrichment factors. The mineral phase is primarily bioapatite  $[\text{Ca}_5(\text{PO}_4)_3\text{OH}]$  with carbonate ( $\text{CO}_3$ ) substitutions in the hydroxyl (type-A  $\text{CO}_3$ ) and phosphate (type-B  $\text{CO}_3$ ) crystallographic sites (Driessens and Verbeeck, 1990; Elliott, 2002; Elliott et al., 1985). Bioapatite carbonate contents vary considerably, with reported values ranging from 3 to 13% (Driessens and Verbeeck, 1990). Since carbonate is empirically 20% carbon, assuming an average carbonate content of about 5% (Sydney-Zax et al., 1991; Zazzo et al., 2005) means that bone contains about 1% carbonate-bound carbon, by mass. Modern dry bone and dentine are 12 to 33% collagen by weight (Ambrose, 1990; Driessens and Verbeeck, 1990; Van Klinken, 1999). Collagen carbon contents can vary significantly based on preservation, but well-preserved samples are usually  $35\pm 9\%$  carbon (Van Klinken, 1999); therefore, an average bone is about 9% collagen-bound-carbon by weight. Given these variations in both collagen and mineral content, demineralization methods to isolate organic collagen prior to stable isotope analysis may vary among specimens.

Since the carbon isotope diet-to-tissue enrichment factors ( $\epsilon$ ) differ for mineral and organic phases, bulk stable isotope analysis of bone without isolating either component will result in  $\delta^{13}\text{C}$  values that represent a mixture of the two components. For example, bioapatite-carbonate in many mammalian herbivores is enriched relative to diet by 11-

14‰ depending on body size and gut physiology (Passey et al., 2005), while collagen is enriched relative to diet by about 5‰ (Ambrose and Norr, 1993). Given the average carbon contents of each phase (discussed above), a weighted average of bone collagen and bone carbonate should be enriched by about 6‰ albeit with considerable variability. Since purified collagen is enriched by ~5‰ (Ambrose and Norr, 1993) bulk analysis (collagen plus carbonate) may differ by as much as 1‰, an ecologically meaningful amount.

## 2.2 Collagen Extraction

Isolating collagen is accomplished either by acid digestion of the mineral fraction using hydrochloric acid (HCl) or calcium binding via the chelating agent Ethylenediaminetetraacetic acid (EDTA) (Ambrose, 1990; Guiry and Szpak, 2020; Longin, 1971; Tuross et al., 1988; Van Klinken, 1999). Demineralization via EDTA is usually recommended for archaeological or paleontological specimens where collagen may be poorly preserved. However, EDTA demineralization is often slow and can take weeks to months to fully remove mineral, and in well preserved specimens, HCl may be preferred due to its rapid reaction speed.

Hydrochloric acid protonates the carbonate and phosphate groups of bioapatite, to form carbonic and phosphoric acid (Hankermeyer et al., 2002) which can then be decanted, leaving behind the residual collagen. This procedure is routinely followed by rinsing with sodium hydroxide (NaOH) to remove lipid and humid contaminants (Ambrose, 1990; Brown et al., 1988; Longin, 1971; Pestle, 2010). The rate of mineral dissolution with HCl is strongly dependent on the acid concentration, temperature, reaction time, and particle size (Hankermeyer et al., 2002), and changes to one of these variables should lead to more or less mineral removal. However, HCl is a potent protein hydrolyzing agent (Rosenberg, 2013) and it can induce amino-acid loss in poorly preserved specimens that may alter stable isotope compositions (Pestle, 2010).

There is considerable variation in published methods for collagen extraction. While

some studies suggest rapid removal of mineral using higher acid concentrations (Pestle, 2010), others use lower HCl concentrations, in particular for poorly preserved archaeological or fossil specimens (Beaumont et al., 2013; Sealy et al., 2014; Tuross et al., 1988). Particle size also varies across methods, and influences the rate of demineralization (Hankermeyer et al., 2002). While some authors suggest using bone “chunks”, others grind samples into small pieces or fine powders (see review in: Sealy et al. (2014)). How to best determine when demineralization is complete is less clear. Most stable isotope and radiocarbon studies of collagen uses qualitative metrics to assess the degree of demineralization. These methods look for some combination of the cessation of bubbling from CO<sub>2</sub> release (Jørkov et al., 2007; Sealy et al., 2014), the softness of the of the bone “chunks”, and the visual appearance of a translucent collagen pseudomorph (Tuross et al., 1988; Wilson and Szpak, 2022). While collagen extraction methods vary significantly across published studies, inter-laboratory comparisons show that different methods produce comparable stable isotope compositions ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) in most cases (Pestle et al., 2014) and therefore, method development should be taxa or locality specific and focus on producing collagen of satisfactory quality.

The primary criteria used to assess collagen quality in modern and ancient specimens are collagen yield, collagen carbon and nitrogen content, and the ratio of carbon to nitrogen content ( $\text{C:N}_{\text{atomic}}$ ). These criteria are based on the conservative amino acid composition of collagen, which varies little across a wide variety of taxa (Szpak, 2011). Collagen content in fresh bone should be about 35±9% (Van Klinken, 1999), although these contents may be lower in dentine or degraded bone. Low collagen yields may indicate alteration due to degradation and preferential loss of amino acids (Van Klinken, 1999). Ambrose (1990) initially suggested that samples that yield less than 3.5% were suspect of degradation, though later work has lowered this threshold to 1% yield (Van Klinken, 1999). Similarly, total collagen carbon and nitrogen contents can indicate alteration, with proposed minimum contents of 13% and 4.5% for carbon and nitrogen, respectively (Guiry and Szpak, 2020).

The main sources of contamination in modern and fossil collagen are endogenous lipids, humic acids, and non-collagenous proteins (Guiry and Szpak, 2021). Both humic acids and lipids are carbon rich but nitrogen poor and lead to higher  $C:N_{\text{atomic}}$  values. Furthermore, since lipids are relatively  $^{13}\text{C}$  depleted, they can substantially change  $\delta^{13}\text{C}$  values if not properly removed (Liden et al., 1995). This effect is especially true for fish and marine mammals which can have very high bone lipid contents. DeNiro (1985) proposed a  $C:N_{\text{atomic}}$  range of 2.9–3.6 as indicative of well-preserved collagen, although this range has been suggested to be somewhat too liberal, where samples with carbon-bearing lipid or humid compounds may still have  $C:N_{\text{atomic}}$  values that fall within the “acceptable” range. Schwarcz and Nahal (2021) determined the theoretical midpoint of  $C:N_{\text{atomic}}$  as 3.243, and suggested values above this likely represent exogenous contaminants, while lower values indicate preferential loss of amino acid. Other authors have suggested either narrower  $C:N_{\text{atomic}}$  or taxon specific ranges. For example, Van Klinken (1999) suggests a range of 3.1–3.5 based on long term data collection in the Oxford radiocarbon facility, while others have suggested adjusting the upper limit of the DeNiro (1985) range (2.9–3.6) for fossil collagen and a slightly narrower range of 3.00–3.30 for modern specimens, depending on preservation characteristics, likelihood of contamination, and research tolerance for altered isotopic compositions (Guiry and Szpak, 2021, 2020).

## 2.3 Fast Fourier Infrared Spectroscopy

Fast Fourier Infrared Spectroscopy (FTIR) irradiates a sample with a beam of infrared light, which excite molecules into a higher energy state. As a result, some wavelengths are absorbed while others are transmitted through the material. Since the molecular structure of a material determines which wavelengths are absorbed or transmitted, FTIR can be used to semi-quantitatively determine the chemical composition of a material and assign different absorbance bands to specific chemical functional groups (Stuart, 2004).

There is considerable work using FTIR to investigate the crystallographic and chem-

ical properties of bioapatite and collagen (Grunenwald et al., 2014; Hassan et al., 1977; Roche et al., 2010; Sønju Clasen and Ruyter, 1997; Sponheimer and Lee-Thorp, 1999). As a result, the absorbance band position of the major chemical functional groups of both materials are well understood (Table 1). In particular, it is possible to distinguish the functional groups associated with the carbonate ( $\text{CO}_3$ ), phosphate ( $\text{PO}_4$ ), and organic amide groups, which produce distinct absorbance bands. The relative size of these bands is proportional to concentration (Grunenwald et al., 2014; Lebon et al., 2016), meaning that FTIR can be used to track changes in mineral/ collagen abundance and alteration (see review in: Olcott Marshall and Marshall (2015)).

Table 1: Nominal FTIR band positions of several relevant chemical groups. Actual band positions may be shifted by several  $\text{cm}^{-1}$ .

band position ( $\text{cm}^{-1}$ )	Functional Group
1650	Amide I ( $\text{CO-NH}$ )
1551	Amide II ( $\text{CO-NH}$ )
1545	A-Type Carbonate ( $\text{CO}_3$ )
1415	B-Type Carbonate ( $\text{CO}_3$ )
1231	Amide III ( $\text{CO-NH}$ )
1020	Phosphate ( $\nu_3\text{PO}_4$ )
880	Carbonate ( $\text{CO}_3$ )
605	Phosphate ( $\nu_2\text{PO}_4$ )

Much of this work has focused on detecting changes during diagenesis and fossilization (Roche et al., 2010; Sillen and Parkington, 1996; Sponheimer and Lee-Thorp, 1999). In bioapatite, type-A and type-B carbonate appear to be differentially susceptible to alteration and FTIR is used to track changes to the carbonate environment as a possible proxy for alteration of  $\text{CO}_3$  stable isotope compositions (Roche et al., 2010; Sponheimer and Lee-Thorp, 1999). Other work has developed indices to characterize the crystallinity



of bioapatite as a proxy for recrystallization during fossilization (Roche et al., 2010; Sponheimer and Lee-Thorp, 1999; Weiner and Bar-Yosef, 1990) or as an indicator of deliberate or accidental burning of bone (Ellingham et al., 2016; Snoeck et al., 2014; Thompson et al., 2013, 2009). Several studies also used FTIR to screen bone for the presence of organic collagen as a precursor to radiocarbon or DNA analysis (Chowdhury et al., 2021; Fredericks et al., 2012; Lebon et al., 2016; Leskovar et al., 2020; Olsen et al., 2008). Infrared spectra from isolated collagen are widely used to characterize the chemical structure of different collagen types, particularly for medical applications (see review of: Riaz et al. (2018)). More pertinent to this study, FTIR can be used to identify contaminants in collagen (e.g., humic acids, carbonates, lipids) prior to radiocarbon or other analysis (Chadefaux et al., 2009; D’Elia et al., 2007; Liden et al., 1995).

## 3 Materials and Methods

### 3.1 Sample Collection

We selected three modern materials for analysis: dentine from a modern domestic goat (*Capra hircus*) tooth, bone from a white-tailed deer (*Odocoileus virginianus*), and teeth from a blue shark (*Prionace glauca*). The goat and deer specimens were fortuitously surface collected, and the shark teeth were collected as part of a previous study (Kim et al., 2012). We cleaned each sample of surficial contamination using a toothbrush and deionized water before grinding into a fine powder using either an agate mortar and pestle or SPEX 8000M Mixer Mill. Since blue shark teeth are quite small, we homogenized several teeth from the same individual and in the functional position (i.e., outermost tooth series) to obtain enough material for our experiments. The resulting powders for all specimens were further homogenized via repeated stirring using a vortexer to minimize effects from isotopic zoning in mammal teeth (Kohn et al., 1998; Trayler and Kohn, 2017) and varia-

tions among multiple teeth from individual sharks (Shipley et al., 2021; Vennemann et al., 2001).

### 3.2 Demineralization

We demineralized our samples in 5-minute increments from 5 to 60 minutes, and 10-minute increments from 70 to 120 minutes as well as 18- and 24-hour time steps. All experiments were performed in triplicate. For each time step, we weighed 2.5 - 4.0 mg of powdered bone or dentine into 1.7 mL micro-centrifuge tubes and added 1 mL of cold (4°C) 0.1M HCl. Samples were briefly stirred using a vortexer and then placed in a 4°C refrigerator (Figure 1). Five minutes before the specified amount of time, samples were removed from the refrigerator and centrifuged at 10,000 RPM for five minutes, then promptly rinsed 5 times with deionized water. For example, the 5-minute sample was placed directly in the centrifuge after acidification, and the 60-minute time step samples spent 55 minutes in the refrigerator and 5 minutes in the centrifuge for a total of 60 minutes in HCl. After rinsing, the samples were frozen and lyophilized prior to FTIR and stable isotope analysis. Since we are primarily interested in quantifying mineral removal, we made no attempt to remove lipids from our samples nor did we gelatinize collagen prior to analysis. However, we do recognize that these steps are important for some materials that are expected to be lipid rich or contain non-collagenous proteins.

### 3.3 FTIR Indices

Several indices are proposed to assess the quality and preservation of the collagen and bioapatite in bone and dentine. However, many of these rely on absorbance regions with considerable overlap among the organic and inorganic functional group bands. As a result, some indices cannot be reliably calculated for both unprocessed bone and fully demineralized collagen, limiting their usefulness in this study. For example, quantifying carbonate

content or loss is complicated by the overlap among carbonate, phosphate, and amide bands. The A-carbonate-on-phosphate and B-carbonate-on-phosphate indices are commonly used to infer carbonate content in bone and tooth enamel (Grunenwald et al., 2014; Roche et al., 2010; Sponheimer and Lee-Thorp, 1999; Sydney-Zax et al., 1991). However, calculating these indices require reliable measures of the A-CO<sub>3</sub> and B-CO<sub>3</sub> absorbance bands at 1545 cm<sup>-1</sup> and 1415 cm<sup>-1</sup>, respectively, but these bands partially overlap with the organic amide II and amide III bands. Therefore, quantifying CO<sub>3</sub> and PO<sub>4</sub> loss is confounded when using these absorbance bands.

Instead of directly measuring carbonate loss, we quantify the reduction of phosphate relative to organic content as a proxy for mineral removal. Since most carbonate is bound in the PO<sub>4</sub> crystallography site and CO<sub>3</sub> content affects bioapatite solubility (Elliott, 2002; Sillen and LeGeros, 1991), phosphate removal should directly track carbonate loss. Lebon et al. (2016) used the ratio of the amide I and ν<sub>3</sub>PO<sub>4</sub> absorbance bands ( $\frac{B_{1650}}{B_{1020}}$ ) to track the carbon and nitrogen content of archaeological bone samples. The ν<sub>3</sub>PO<sub>4</sub> band is poorly resolved in demineralized collagen and hence, the 1020 cm<sup>-1</sup> region and is instead a broad slope, on the “shoulder” of the amide III region (see: Figure 1). Roche et al. (2010) proposed a similar index the Water-Amide-on-Phosphate-Index (WAMPI) as the ratio of the Amide I and ν<sub>2</sub>PO<sub>4</sub> absorbance bands ( $\frac{B_{1650}}{B_{605}}$ ) to track organic loss in fossil bone. We use the WAMPI as our proxy for ratio of organic and inorganic components of bone, where higher WAMPI values should correspond to mineral removal.

We collected ATR-FTIR spectra using a Bruker Vertex 70 Far-Infrared FTIR housed in the Nuclear Magnetic Resonance Facility at the University of California, Merced. Spectra were collected from 400 - 2000 cm<sup>-1</sup> for 32 scans at a resolution of 4 cm<sup>-1</sup>. We subtracted the background of each of each spectrum by fitting a smoothed spline to several baseline points (points expected to have an absorbance of 0) and smoothed each spectrum slightly (Stuart, 2004). We then calculated the WAMPI for each spectrum by dividing the height of the amide I band at 1650 cm<sup>-1</sup> by the height of the ν<sub>2</sub>PO<sub>4</sub> band at 605 cm<sup>-1</sup>. Since the

band positions are approximate, we located the local maxima for each band with a window of  $\pm 15 \text{ cm}^{-1}$ . All corrections and calculations were performed using custom R scripts (R Core Team, 2021), available in the supplementary material.

### 3.4 Stable Isotope Analysis

The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values as well as elemental carbon and nitrogen contents of all samples were measured using a Costech 4010 Elemental Analyzer coupled with a Delta V+ Isotope Ratio Mass Spectrometer with ConFlo IV housed in the Stable Isotope Ecosystem Laboratory of the University of California, Merced (SIELO). Carbon and nitrogen isotope compositions were corrected for instrument drift, mass linearity, and standardized to the international VPDB and AIR scales using the USGS 40 and USGS 41a standard reference materials. The average reproducibility for these reference materials was  $\pm 0.12\text{‰}$  for  $\delta^{13}\text{C}$  and  $\pm 0.15\text{‰}$  for  $\delta^{15}\text{N}$  values. We also analyzed several aliquots of a homogenized squid tissue as an in-house reference which returned  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of  $-18.8 \pm 0.1\text{‰}$  and  $11.8 \pm 0.2\text{‰}$ , respectively, which are indistinguishable from the long-term SIELO average.

## 4 Results and Discussion

All data (FTIR spectra, elemental content, and stable isotope compositions) and analysis code are available in the supplemental material and at [github.com/robintrayler/collagen\\_demineralization](https://github.com/robintrayler/collagen_demineralization)

### 4.1 Collagen Yield and Quality

Our experiments successfully removed the mineral fraction from our samples and yielded collagen within the acceptable range across all quality metrics (Figure 2). In general, all samples showed a rapid reduction in fractional weight after 5 minutes of acid treatment, followed by a prolonged period of weight loss until the fractional weight stabilized, which we

interpret as indicative of the final collagen yield. *Capra* dentine sample weights stabilized after 5 minutes for an average yield of  $9.5 \pm 1.1\%$ . The *Odocoileus* bone sample weights took longer to stabilize with a plateau after about 20 minutes, for a final yield of  $18.6 \pm 2.6\%$ . Finally, while the *Prionace* sample weights appear to plateau after about 30 minutes, the 18- and 24-hour time steps show a further reduction in sample weight suggesting that full mineral removal took between 2 and 18 hours.

The elemental content of our samples mirrors our fractional weight results (Figure 2 G-L). Both *Capra* carbon and nitrogen contents stabilized after about 10 minutes while *Odocoileus* contents plateaued after 30 minutes. *Prionace* elemental contents do not clearly plateau by two hours and instead gradually increased suggesting that the mineral component was not fully removed.

In all cases, untreated samples (0 minutes) had  $C:N_{\text{atomic}}$  values higher than 3.6, indicating the contribution of carbonate-bound carbon. Most acid treated sample  $C:N_{\text{atomic}}$  values fell within the broadest acceptable range for well-preserved collagen (2.9–3.6) (Ambrose, 1990; DeNiro, 1985), although not all samples fell within the more conservative ranges proposed by Guiry and Szpak (2020) and Van Klinken (1999). The  $C:N_{\text{atomic}}$  values for both *Odocoileus* and *Prionace* samples were systematically higher than 3.30. Given that we did not attempt to remove lipids from our samples prior to analysis, we attribute these slightly higher  $C:N_{\text{atomic}}$  values to residual lipids.

Taken together, our collagen yields and quality (i.e., C%, N%,  $C:N_{\text{atomic}}$ ) metrics are indicative of well-preserved collagen. While this is an expected result for our modern samples, establishing the fidelity of our extracted collagen is key for interpreting our infrared spectroscopy and stable isotope results.

## 4.2 FTIR and the Rate of Mineral Removal

Our results show mineral removal can be tracked and quantified using infrared spectroscopy. Untreated samples (0 minutes) show the expected spectra for collagen-

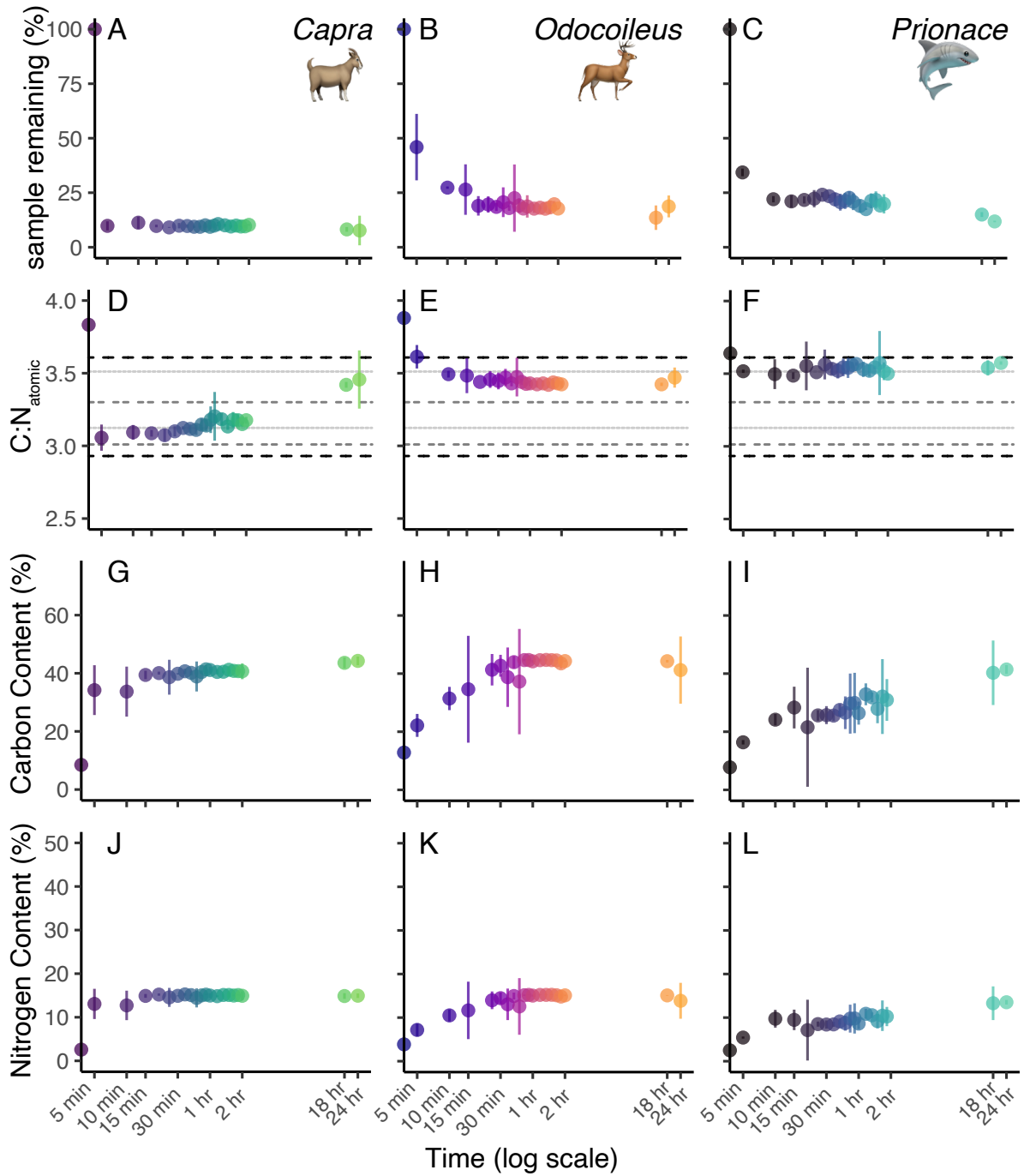


Figure 2: Fractional weight remaining (A-C), C:N<sub>atomic</sub> ratios (D-F), carbon content (G-I), and nitrogen content (J-L) for all samples. The dashed lines in panels D-F indicates proposed acceptable ranges for well-preserved collagen. Long dashes (black) is the 2.9–3.6 range of DeNiro (1985) and Ambrose (1990), paired-dash line (medium grey) is the more conservative range of 3.00–3.30 of Guiry and Szpak (2020) and short-dashed line (light grey) is 3.1–3.5 acceptable range of Van Klinken (1999).

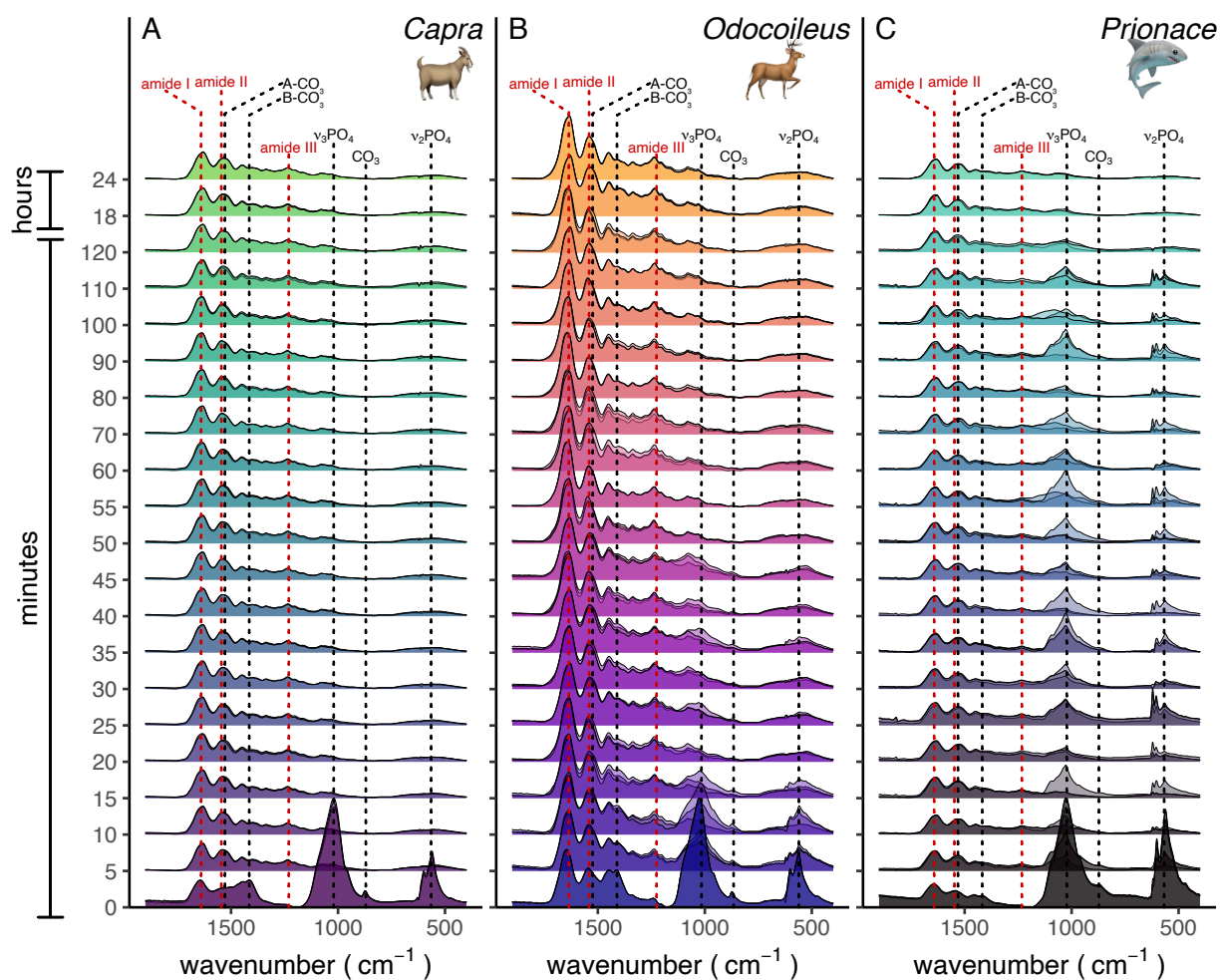


Figure 3: FTIR-ATR spectra for *Capra*, *Odocoileus*, and *Prionace* bioapatite and collagen. Undemineralized samples (time-0) have prominent PO<sub>4</sub> and CO<sub>3</sub> absorbance bands which are rapidly reduced after treatment with HCl.

bioapatite mixtures, with prominent absorbance bands for mineral ( $\text{CO}_3 = 880, 1415, 1545 \text{ cm}^{-1}$ ;  $\text{PO}_4 = 605, 1020 \text{ cm}^{-1}$ ) and organic (amide I =  $1650 \text{ cm}^{-1}$ ) phases (Figure 3). As acid treatment time increased, the absorbance bands associated with structural phosphate and carbonate became less prominent while the organic amide I, II, and III bands became more pronounced.

In all cases, the 18- and 24-hour treatment groups returned the expected spectrum for pure collagen, and we interpret the WAMPI values ( $6.7 \pm 1.5$  ( $\pm 2\sigma$ ; absorbance/absorbance) for these samples as indicative of complete mineral removal (Figure 4). With the exception of untreated dentine (0 minutes), nearly all of our *Capra* samples fell within this WAMPI range. The 110- and 120-minute treated *Capra* samples had slightly lower WAMPI, however the spectra for these samples do not show any signs of  $\text{CO}_3$  or  $\text{PO}_4$  absorbance bands. *Odocoileus* WAMPI values do not overlap the acceptable range until 20 minutes of acidification, which corresponds with our observation that the sample fractional weight reached a plateau at this time (see Section 4.1; Figure 2). None of the 0-to-120 minute *Prionace* samples had WAMPI values within our acceptable range and  $\text{PO}_4$  absorbance bands are still visible in FTIR spectra until 120 minutes (Figure 3). Again, this agrees well with our observation that *Prionace* sample fractional weights showed a prolonged gradual reduction over the entire period of our experiments. In contrast to mammals, shark bioapatite is primarily fluorapatite [ $\text{Ca}_5(\text{PO}_4)\text{F}$ ], has overall lower  $\text{CO}_3$  contents, and is less soluble (Posner et al., 1984). As a result of homogenizing whole teeth, the *Prionace* samples also have a higher mineral content, which likely explains the slower demineralization of these samples.

### 4.3 Fidelity of Stable Isotope Compositions

The carbon isotope compositions of our samples were substantially affected by HCl acidification, likely resulting from the loss of mineral-bound carbonate (see Section 2.1). However, after the removal of carbonate, the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values showed only small changes



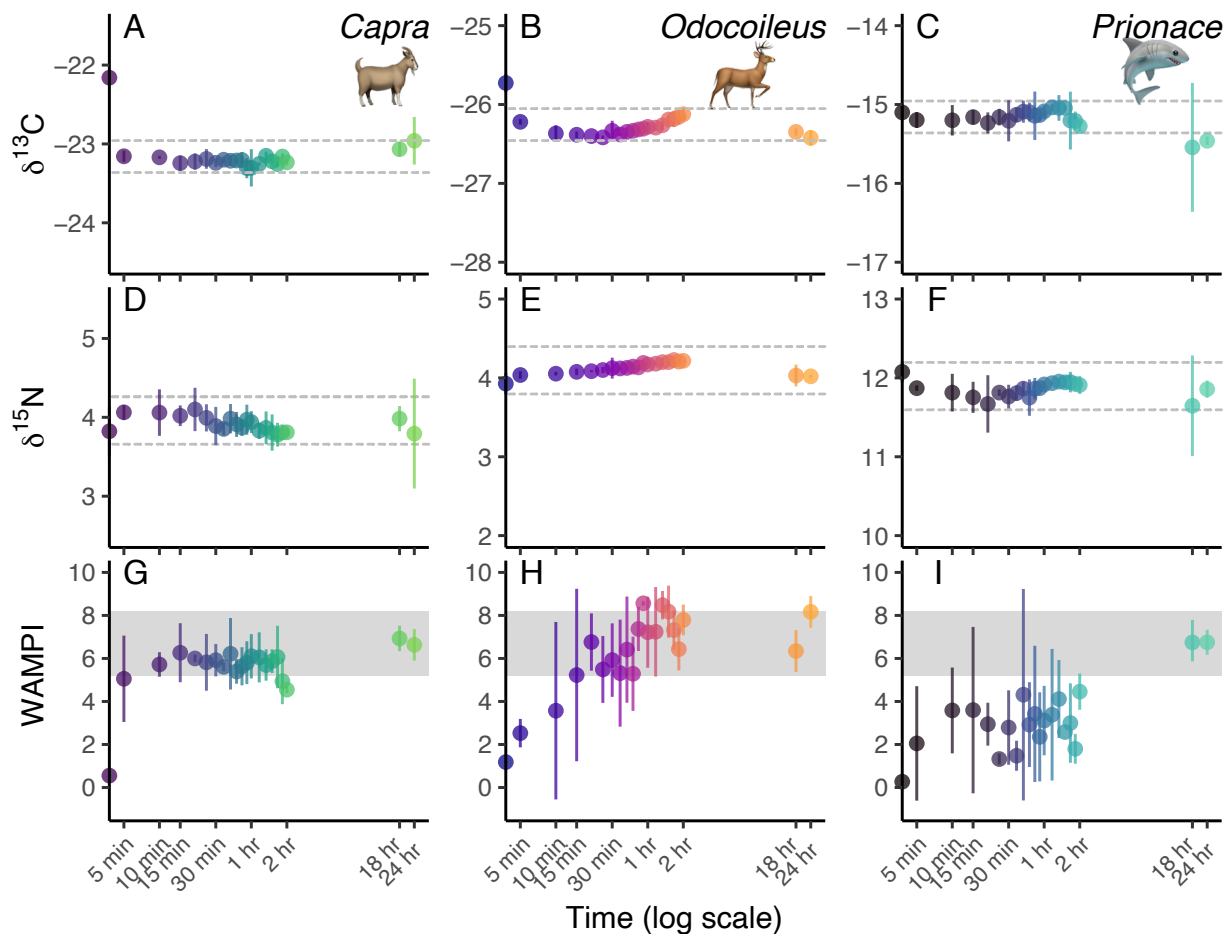


Figure 4: Stable isotope data (A-C:  $\delta^{13}\text{C}$  values; D-F:  $\delta^{15}\text{N}$  values) and FTIR index (G-I: WAMPI) for all samples. Colored points with error bars are the mean  $\pm 2$  standard deviations for each time step. In many cases the error bars are smaller than the symbol. The dashed grey lines on panels A - F indicate long-term  $\pm 2\sigma$  analytical reproducibility, centered on the data. Notice that the vertical-axis scaling differs among panels A - F. The shaded grey band on panels G - I indicates the mean  $\pm 2\sigma$  of WAMPI values for 18 and 24 hours samples, which we interpret as the WAMPI range for fully demineralized collagen.

across time steps, however none of these changes were larger than the variations expected from analytical uncertainty (i.e.,  $\pm 0.2\text{‰}$  for  $\delta^{13}\text{C}$  and  $\pm 0.4\text{‰}$  for  $\delta^{15}\text{N}$  values; ( $\pm 2\sigma$ )). This suggests that acid-treatment did not affect collagen stable isotope compositions (Figure 4 D-E).

Both *Capra* and *Odocoileus* carbon isotope compositions shifted after 5 minutes of acid treatment, suggesting a rapid removal of structural carbonate. After 5 minutes  $\delta^{13}\text{C}$  values for these two materials show small variations in isotopic composition, although within analytical uncertainty (Figure 4 A-C). The  $\delta^{13}\text{C}$  values of *Prionace* samples show very little change from 0 minutes to 2 hours, but the 18- and 24-hour time steps have lower  $\delta^{13}\text{C}$  values relative to the shorter time steps. Furthermore, this shift is larger than the analytical uncertainty range, suggesting material changes to isotope compositions in the 18- and 24-hour samples. Curiously, the untreated *Prionace* samples do not show carbon isotope offset associated with carbonate-bound carbon present in both the *Capra* and *Odocoileus* samples, suggesting either lower carbonate contents or a smaller isotopic spacing between  $\text{CO}_3$  and collagen (Karnes, 2022).

#### 4.4 The Need for Mineral Removal

Our results indicate that mineral removal is necessary, and the stable isotope composition of whole bone should not be used as an alternative to isolated collagen. Several studies have suggested that acid treatment of mineralized tissues is unwarranted because the procedure influences  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, and the influence of carbonate bound carbon is small (Jacob et al., 2005; Mateo et al., 2008; Turner Tomaszewicz et al., 2015). However, these studies have primarily focused on marine invertebrates with some examples from vertebrate bone (Turner Tomaszewicz et al., 2015). For mammals, our results clearly contradict this recommendation and indicate removal of carbonate-bound carbon has substantial, measurable effects on  $\delta^{13}\text{C}$  values, and little to no effect on  $\delta^{15}\text{N}$  values. For reference, the *Capra* and *Odocoileus* samples show a 0.5‰ to 1‰ reduction in  $\delta^{13}\text{C}$

values after carbonate removal, in line with our predictions (see: Section 2.1). Our results agree with recent work by Wilson and Szpak (2022), who also show that mineral fraction of bone must be removed prior to collagen analysis.

Fluorapatite shark teeth have lower carbonate contents and are less soluble than mammalian enamel. Furthermore, the diet-tissue carbon isotope enrichment factor for shark bioapatite is poorly understood and may vary as a function of both  $\text{CO}_3$  content and via equilibration with dissolved seawater  $\text{CO}_3$  (Karnes, 2022; Vennemann et al., 2001). There is no obvious influence of carbonate-bound carbon on  $\delta^{13}\text{C}$  values from 0-minutes to 2-hours in our experiments, while the 18- and 24-hour time steps have slightly lower and more variable  $\delta^{13}\text{C}$  values. Our results suggest that either there is not a large carbon isotope fractionation between carbonate and collagen or that  $\text{CO}_3$  contents were low.

These isotopic differences, although small, can affect paleoenvironmental and paleodietary interpretations. For example, a 1‰ shift in  $\delta^{13}\text{C}$  values would have a large impact on dietary modeling (e.g., Phillips, 2012) and paleoprecipitation calculations (Kohn, 2010). Since bone carbonate contents and diet-carbonate enrichment factors can vary significantly (Passey et al., 2005; Tejada-Lara et al., 2018), we feel the most straightforward method of accounting for the different isotopic fractionations among diet, carbonate, and collagen is to simply remove the carbonate-bearing mineral fraction.

## 4.5 Evaluating Demineralization Methods

Our methods provide a framework for evaluating mineral removal from bone and dentine (Figure 1). While most demineralization methods use similar techniques, the details can vary among laboratories which can lead to significant differences in stable isotope compositions (Pestle et al., 2014). However, there is not a clear best method, as bone or teeth with different preservation characteristics may require different treatments to successfully isolate collagen (Fuller et al., 2014; Pestle et al., 2014; Tuross et al., 1988). Our results show that a combination of stable isotope analysis, elemental analysis, and FTIR

can be used to track mineral removal and structural changes to bone and collagen over the course of hours. Varying the reaction temperature, acid concentration, particle size, and or reaction time of our experiments would likely produce different results, as mineral removal would likely be faster or slower. However, our study provides an approach to quantify the effects of changes to one of these variables while assessing method efficacy and the resulting collagen quality.

Finally, these methods can be extended to quantify the effects of other treatments. For example, while we did not remove lipids for our experiments, they can be a significant source of excess carbon in many materials and a variety of techniques are used to remove lipids prior to analysis (Post et al., 2007). Lipid functional groups produce distinct absorbance bands in infrared spectra (Liden et al., 1995) which can be used to quantify their presence and evaluate lipid removal. Other exogenous contaminants (e.g., humic acids, labile carbonates) are also visible using infrared spectroscopy (D'Elia et al., 2007) and our workflow could likewise be used to check for the removal of these compounds.

## 5 Conclusions

Removing the mineral component of bone and dentine prior to stable isotope analysis of organic collagen is a necessary step. While the method of mineral removal may not have a strong influence on stable isotope compositions (Pestle, 2010; Wilson and Szpak, 2022), the timing and efficacy of mineral removal should be quantified to ensure consistent results. Here, we presented methods for assessing the efficacy of a given demineralization method using a combination of elemental analysis, stable isotope analysis, and FTIR. Our results show that mineral loss can be tracked using infrared spectroscopy and that demineralization using weak acid (0.1M HCl) does not appear to alter the stable isotope or elemental composition of collagen. In contrast, whole, undemineralized bone and dentine have measurably different  $\delta^{13}\text{C}$  values suggesting that structural carbonate strongly influ-

enced these measurements. For finely powdered samples, demineralization can be quite rapid, with the majority of the mineral component removed in under two hours. However, using chunks or coarse powders will likely require longer reaction times, as will less soluble materials such as shark enameloid. Finally, this study offers an approach to the timing of mineral removal and isotopic effects are an effective framework for investigating the impact of other treatment methods (e.g., lipid and humic acid removal) and can be used to fine tune existing methods.

## 6 Supplementary Information

All infrared spectra, stable isotope data, and analysis code, are available at [github.com/robintrayler/collagen](https://github.com/robintrayler/collagen)

## 7 Acknowledgments

We thank Maya Morris for her help with time step experiments and Dr. David Rice for his assistance with FTIR data collection. We also thank Gina Palefsky for providing the *Odocoileus* bone and Matthew J. Kohn for providing the *Capra* tooth. This work was supported by NSF-EAR-1830480 (SLK), NSF-EAR-2138163 (RBT and SLK) and UC Merced startup funds to SLK.

## References

- Ambrose, S.H., 1990. Preparation And Characterization Of Bone And Tooth Collagen For Isotopic Analysis. *Journal of Archaeological Science* 17, 145.
- 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: *Prehistoric Human Bone*. Springer, pp. 1–37.
- Beaumont, J., Gledhill, A., Lee-Thorp, J., Montgomery, J., 2013. Childhood diet: A closer examination of the evidence from dental tissues using stable isotope analysis of incremental human dentine. *Archaeometry* 55, 277–295.
- Brault, E.K., Koch, P.L., Gier, E., Ruiz-Cooley, R., Zupcic, J., Gilbert, K.N., McCarthy, M.D., 2014. Effects of decalcification on bulk and compound-specific nitrogen and carbon isotope analyses of dentin. *Rapid Communications in Mass Spectrometry* 28, 2744–2752.
- Brown, T.A., Nelson, D.E., Vogel, J.S., Southon, J.R., 1988. Improved collagen extraction by modified Longin method. *Radiocarbon* 30, 171–177.
- Chadefaux, C., Le Hô, A.-S., Bellot-Gurlet, L., Reiche, I., 2009. Curve-fitting Micro-ATR-FTIR studies of the amide I and II bands of type I collagen in archaeological bone materials.
- Chowdhury, M.P., Choudhury, K.D., Bouchard, G.P., Riel-Salvatore, J., Negrino, F., Benazzi, S., Slimak, L., Frasier, B., Szabo, V., Harrison, R., 2021. Machine learning ATR-FTIR spectroscopy data for the screening of collagen for ZooMS analysis and mtDNA in archaeological bone. *Journal of Archaeological Science* 126, 105311.
- Clementz, M.T., 2012. New insight from old bones: Stable isotope analysis of fossil mammals. *Journal of Mammalogy* 93, 368–380.
- D’Elia, M., Gianfrate, G., Quarta, G., Giotta, L., Giancane, G., Calcagnile, L., 2007. Evaluation of possible contamination sources in the  $^{14}\text{C}$  analysis of bone samples by FTIR

spectroscopy. *Radiocarbon* 49, 201–210.

DeNiro, M.J., 1985. Postmortem preservation and alteration of in vivo bone collagen isotope ratios in relation to palaeodietary reconstruction. *Nature* 317, 806–809.

DeNiro, M.J., Epstein, S., 1981. Influence of Diet on the Distribution of Nitrogen Isotopes in Animals. *Geochimica et Cosmochimica Acta* 45, 341–351.

DeNiro, M.J., Epstein, S., 1978. Influence of diet on the distribution of carbon isotopes in animals. *Geochimica et Cosmochimica Acta* 42, 495–506.

Driessens, F.C., Verbeeck, R.K., 1990. *Biomaterials*. CRC Press, Boca Raton, FL.

Ellingham, S.T., Thompson, T.J., Islam, M., 2016. The effect of soft tissue on temperature estimation from burnt bone using Fourier transform infrared spectroscopy. *Journal of forensic sciences* 61, 153–159.

Elliott, J.C., 2002. Calcium Phosphate Biomaterials. *Reviews in Mineralogy and Geochemistry* 48, 427–453.

Elliott, J.C., Holcomb, D.W., Young, R.A., 1985. Infrared Determination Of The Degree Of Substitution Of Hydroxyl By Carbonate Ions In Human Dental Enamel. *Calcified tissue international* 37, 372–375.

Fredericks, J.D., Bennett, P., Williams, A., Rogers, K.D., 2012. FTIR spectroscopy: A new diagnostic tool to aid DNA analysis from heated bone. *Forensic science international: Genetics* 6, 375–380.

Fuller, B.T., Fahrni, S.M., Harris, J.M., Farrell, A.B., Coltrain, J.B., Gerhart, L.M., Ward, J.K., Taylor, R., Southon, J.R., 2014. Ultrafiltration for asphalt removal from bone collagen for radiocarbon dating and isotopic analysis of Pleistocene fauna at the tar pits of Rancho La Brea, Los Angeles, California. *Quaternary Geochronology* 22, 85–98.

Fuller, B.T., Harris, J.M., Farrell, A.B., Takeuchi, G., Southon, J.R., 2015. Sample preparation for radiocarbon dating and isotopic analysis of bone from Rancho La Brea. *La Brea and beyond: The paleontology of asphalt-preserved biotas*, ed. JM Harris. Natural History Museum of Los Angeles County, Science Series 151–167.

- Grunenwald, A., Keyser, C., Sautereau, A.-M., Crubézy, E., Ludes, B., Drouet, C., 2014. Revisiting carbonate quantification in apatite (bio) minerals: A validated FTIR methodology. *Journal of Archaeological Science* 49, 134–141.
- Guiry, E.J., Hepburn, J.C., Richards, M.P., 2016. High-resolution serial sampling for nitrogen stable isotope analysis of archaeological mammal teeth. *Journal of Archaeological Science* 69, 21–28.
- Guiry, E.J., Szpak, P., 2021. Improved quality control criteria for stable carbon and nitrogen isotope measurements of ancient bone collagen. *Journal of Archaeological Science* 132, 105416.
- 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.
- Hankermeyer, C.R., Ohashi, K.L., Delaney, D.C., Ross, J., Constantz, B.R., 2002. Dissolution rates of carbonated hydroxyapatite in hydrochloric acid. *Biomaterials* 23, 743–750.
- Hassan, A.A., Termine, J.D., Haynes, C.V., 1977. Mineralogical studies on bone apatite and their implications for radiocarbon dating. *Radiocarbon* 19, 364–374.
- Hedges, R.E.M., Clement, J.G., Thomas, C.D.L., O’connell, T.C., 2007. Collagen turnover in the adult femoral mid-shaft: Modeled from anthropogenic radiocarbon tracer measurements. *American Journal of Physical Anthropology* 133, 808–816.
- Jacob, U., Mintenbeck, K., Brey, T., Knust, R., Beyer, K., 2005. Stable isotope food web studies: A case for standardized sample treatment. *Marine Ecology Progress Series* 287, 251–253.
- 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.
- Karnes, M., 2022. Enigmatic Carbonate Isotope Values in Shark Teeth: Evidence for Environmental and Diet Controls (PhD thesis). University of California, Merced.



- Kim, S.L., Casper, D.R., Galván-Magaña, F., Ochoa-Díaz, R., Hernández-Aguilar, S.B., Koch, P.L., 2012. Carbon and nitrogen discrimination factors for elasmobranch soft tissues based on a long-term controlled feeding study. *Environmental Biology of Fishes* 95, 37–52.
- Koch, P.L., 2007. Isotopic study of the biology of modern and fossil vertebrates, in: Michener, R., Lajtha, K. (Eds.), *Stable Isotopes in Ecology And Environmental Science*. Blackwell Publishing, pp. 99–154.
- Koch, P.L., Fogel, M.L., Tuross, N., 1994. Tracing the diets of fossil animals using stable isotopes. *Stable Isotopes in Ecology and Environmental Science* 63–92.
- Kohn, M.J., 2010. Carbon Isotope Composition of Terrestrial C<sub>3</sub> Plants as Indicators of (Paleo)Ecology and (Paleo)Climate. *Proceedings of the National Academy of Sciences* 107.46.
- Kohn, M.J., Schoeninger, M.J., Valley, J.W., 1998. Variability in oxygen isotope compositions of herbivore teeth: Reflections of seasonality or developmental physiology? *Chemical Geology* 152, 97–112.
- Lebon, M., Reiche, I., Gallet, X., Bellot-Gurlet, L., Zazzo, A., 2016. Rapid quantification of bone collagen content by ATR-FTIR spectroscopy. *Radiocarbon* 58, 131–145.
- Leskovar, T., Pajnič, I.Z., Geršak, Ž.M., Jerman, I., Črešnar, M., 2020. ATR-FTIR spectroscopy combined with data manipulation as a pre-screening method to assess DNA preservation in skeletal remains. *Forensic Science International: Genetics* 44, 102196.
- Liden, K., Takahashi, C., Nelson, D.E., 1995. The effects of lipids in stable carbon isotope analysis and the effects of NaOH treatment on the composition of extracted bone collagen. *Journal of archaeological science* 22, 321–326.
- Longin, R., 1971. New method of collagen extraction for radiocarbon dating. *Nature* 230, 241–242.
- Mateo, M.A., Serrano, O., Serrano, L., Michener, R.H., 2008. Effects of sample preparation on stable isotope ratios of carbon and nitrogen in marine invertebrates: Implica-

- tions for food web studies using stable isotopes. *Oecologia* 157, 105–115.
- Olcott Marshall, A., Marshall, C.P., 2015. Vibrational spectroscopy of fossils. *Palaeontology* 58, 201–211.
- Olsen, J., Heinemeier, J., Bennike, P., Krause, C., Hornstrup, K.M., Thrane, H., 2008. Characterisation and blind testing of radiocarbon dating of cremated bone. *Journal of Archaeological Science* 35, 791–800.
- Passey, B.H., Robinson, T.F., Ayliffe, L.K., Cerling, T.E., Sponheimer, M., Dearing, M.D., Roeder, B.L., Ehleringer, J.R., 2005. Carbon isotope fractionation between diet, breath CO<sub>2</sub>, and bioapatite in different mammals. *Journal of Archaeological Science* 32, 1459–1470.
- Pestle, W.J., 2010. Chemical, elemental, and isotopic effects of acid concentration and treatment duration on ancient bone collagen: An exploratory study. *Journal of Archaeological Science* 37, 3124–3128.
- Pestle, W.J., Crowley, B.E., Weirauch, M.T., 2014. Quantifying inter-laboratory variability in stable isotope analysis of ancient skeletal remains. *PLoS one* 9, e102844.
- Phillips, D.L., 2012. Converting isotope values to diet composition: The use of mixing models. *Journal of Mammalogy* 93, 342–352. <https://doi.org/10.1644/11-mamm-s-158.1>
- Posner, A.S., Blumenthal, N.C., Betts, F., 1984. Chemistry and structure of precipitated hydroxyapatites, in: *Phosphate Minerals*. Springer, pp. 330–350.
- Post, D.M., Layman, C.A., Arrington, D.A., Takimoto, G., Quattrochi, J., Montana, C.G., 2007. Getting to the fat of the matter: Models, methods and assumptions for dealing with lipids in stable isotope analyses. *Oecologia* 152, 179–189.
- R Core Team, 2021. *R: A Language and Environment for Statistical Computing*.
- Riaz, T., Zeeshan, R., Zarif, F., Ilyas, K., Muhammad, N., Safi, S.Z., Rahim, A., Rizvi, S.A., Rehman, I.U., 2018. FTIR analysis of natural and synthetic collagen. *Applied Spectroscopy Reviews* 53, 703–746.

- Roche, D., Ségalen, L., Balan, E., Delattre, S., 2010. Preservation Assessment Of Miocene–Pliocene Tooth Enamel From Tugen Hills (Kenyan Rift Valley) Through FTIR, Chemical And Stable-Isotope Analyses. *Journal of Archaeological Science* 37, 1690–1699. <https://doi.org/10.1016/j.jas.2010.01.029>
- Rosenberg, I.M., 2013. *Protein analysis and purification: Benchtop techniques*. Springer Science & Business Media.
- Schwarcz, H.P., Nahal, H., 2021. Theoretical and observed C/N ratios in human bone collagen. *Journal of Archaeological Science* 131, 105396.
- Schwarcz, H.P., Schoeninger, M.J., 1991. Stable isotope analyses in human nutritional ecology. *American Journal of Physical Anthropology* 34, 283–321.
- Sealy, J., Johnson, M., Richards, M., Nehlich, O., 2014. Comparison of two methods of extracting bone collagen for stable carbon and nitrogen isotope analysis: Comparing whole bone demineralization with gelatinization and ultrafiltration. *Journal of Archaeological Science* 47, 64–69.
- Shiple, O.N., Henkes, G.A., Gelsleichter, J., Morgan, C.R., Schneider, E.V., Talwar, B.S., Frisk, M.G., 2021. Shark tooth collagen stable isotopes ( $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ ) as ecological proxies. *Journal of Animal Ecology* 90, 2188–2201.
- Sillen, A., LeGeros, R., 1991. Solubility profiles of synthetic apatites and of modern and fossil bones. *Journal of Archaeological Science* 18, 385–397. [https://doi.org/10.1016/0305-4403\(91\)90073-X](https://doi.org/10.1016/0305-4403(91)90073-X)
- Sillen, A., Parkington, J., 1996. Diagenesis of bones from Eland’s Bay Cave. *Journal of Archaeological Science* 23, 535–542.
- Snoeck, C., Lee-Thorp, J., Schulting, R., 2014. From bone to ash: Compositional and structural changes in burned modern and archaeological bone. *Palaeogeography, palaeoclimatology, palaeoecology* 416, 55–68.
- Sønju Clasen, A.B., Ruyter, I.E., 1997. Quantitative Determination Of Type A And Type B Carbonate In Human Deciduous And Permanent Enamel By Means Of Fourier Trans-

form Infrared Spectrometry. *Advances in dental research* 11, 523–527.

Sponheimer, M., Lee-Thorp, J.A., 1999. Alteration of enamel carbonate environments during fossilization. *Journal of Archaeological Science* 26, 143–150.

Stuart, B.H., 2004. *Infrared spectroscopy: Fundamentals and applications*. John Wiley & Sons.

Sydney-Zax, M., Mayer, I., Deutsch, D., 1991. Carbonate content in developing human and bovine enamel. *Journal of dental research* 70, 913–916.

Szpak, P., 2011. Fish bone chemistry and ultrastructure: Implications for taphonomy and stable isotope analysis. *Journal of Archaeological Science* 38, 3358–3372.

Tejada-Lara, J.V., MacFadden, B.J., Bermudez, L., Rojas, G., Salas-Gismondi, R., Flynn, J.J., 2018. Body mass predicts isotope enrichment in herbivorous mammals. *Proceedings of the Royal Society B* 285, 20181020.

Thompson, T., Gauthier, M., Islam, M., 2009. The application of a new method of Fourier Transform Infrared Spectroscopy to the analysis of burned bone. *Journal of Archaeological Science* 36, 910–914.

Thompson, T., Islam, M., Bonniere, M., 2013. A new statistical approach for determining the crystallinity of heat-altered bone mineral from FTIR spectra. *Journal of Archaeological Science* 40, 416–422.

Trayler, R.B., Kohn, M.J., 2017. Tooth enamel maturation reequilibrates oxygen isotope compositions and supports simple sampling methods. *Geochimica et Cosmochimica Acta* 198, 32–47. <https://doi.org/10.1016/j.gca.2016.10.023>

Turner Tomaszewicz, C.N., Seminoff, J.A., Ramirez, M.D., Kurle, C.M., 2015. Effects of demineralization on the stable isotope analysis of bone samples. *Rapid Communications in Mass Spectrometry* 29, 1879–1888.

Tuross, N., 2002. Alterations in fossil collagen. *Archaeometry* 44, 427–434.

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

- 610 Van Klinken, G.J., 1999. Bone collagen quality indicators for palaeodietary and radiocar-  
611 bon measurements. *Journal of Archaeological Science* 26, 687–695.
- 612 Vennemann, T., Hegner, E., Cliff, G., Benz, G., 2001. Isotopic composition of recent shark  
613 teeth as a proxy for environmental conditions. *Geochimica et Cosmochimica Acta* 65,  
614 1583–1599.
- 615 Weiner, S., Bar-Yosef, O., 1990. States of preservation of bones from prehistoric sites in  
616 the Near East: A survey. *Journal of Archaeological Science* 17, 187–196.
- 617 Wilson, T., Szpak, P., 2022. Acidification does not alter the stable isotope composition of  
618 bone collagen. *PeerJ* 10, e13593.
- 619 Zazzo, A., Balasse, M., Patterson, W.P., 2005. High-resolution  $\delta^{13}\text{C}$  intratooth profiles  
620 in bovine enamel: Implications for mineralization pattern and isotopic attenuation.  
621 *Geochimica et Cosmochimica Acta* 69, 3631–3642.