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

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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 of other demineralization methods in use today including EDTA-demineralization and lipid removal.

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., $\delta^{13}C$, $\delta^{15}N$, 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 Middle 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) contains significant nitrogen contents, both the mineral and organic components contain carbon and stable isotope analysis of bulk bone or dentine will lead to $\delta^{13}C$ 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.

Collagen can be isolated from the mineral component of bone or dentine either by acid digestion of the mineral fraction using hydrochloric acid (HCl) or calcium binding via the chelating agent Ethylenediaminetetraacetic acid (EDTA) (Ambrose, 1990; Longin, 1971; Tuross et al., 1988). However, 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.

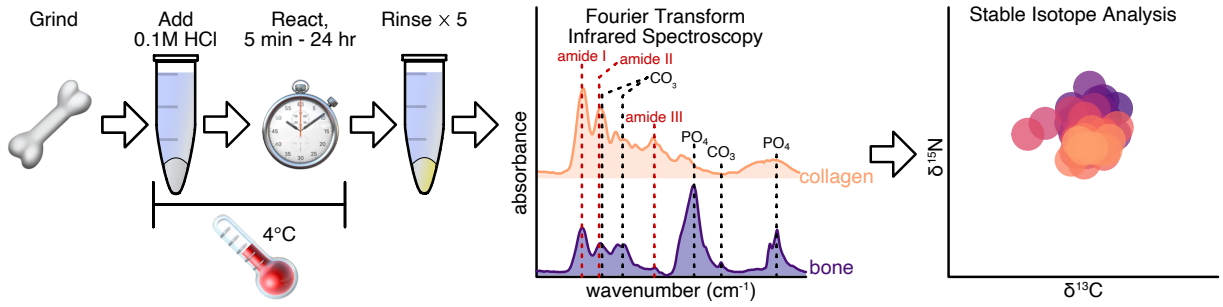

Figure 1: Diagram of methods.

Background

Elemental Composition of Bone

Bone and dentine are composite materials, with both mineral and organic components. Both phases are commonly analyzed for their stable isotope compositions (i.e., $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$) 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 $\delta^{15}N$ 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 $\delta^{13}C$ values due to different diet-tissue enrichment factors. The mineral phase is primarily bioapatite [$Ca_5(PO_4)_3OH$] with carbonate (CO_3) substitutions in the hydroxyl (type-A CO_3) and phosphate (type-B 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±9% carbon (Van Klinken, 1999); therefore, an average bone is about 9% collagen-bound-carbon by weight. Given these variation 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 (ε) differ for mineral and organic phases, bulk stable isotope analysis of bone without isolating either component will result in $\delta^{13}C$ 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 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 collegian is enriched by ~5‰ (Ambrose and Norr, 1993) bulk analysis (collagen plus carbonate) may differ by as much as 1‰, an ecologically meaningful amount.

Collagen Extraction

Isolating collagen is accomplished via reaction with either HCl or 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 bone mineral, and in well preserved specimens, HCl may be preferred due to its rapid reaction speed.

Hydrochloric acid (HCl) 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 sometimes followed by rising 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 which 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 with poorly preserved archaeological or fossil specimens (Beaumont et al., 2013; Sealy et al., 2014; Tuross et al., 1988). Particle size also varies considerably across methods, and furthermore, can strongly influence the rate of demineralization (Hankermeyer et al., 2002). While some authors suggest using bone "chunks", others use methods suggest first grinding samples into small pieces or fine powders (see review in: Sealy et al. (2014)). While collagen extraction methods vary significantly across published studies, inter-laboratory comparisons show that different methods produce comparable stable isotope compositions ($\delta^{13}C$, $\delta^{15}N$) (Pestle et al., 2014) and therefore, method development should focus on producing collagen of satisfactory quality.

The primary criteria used to assess collagen quality in modern and ancient specimens are col-

lagen yield, collagen carbon and nitrogen content, and the ratio of carbon to nitrogen content ($C:N_{atomic}$). 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 that 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 $C:N_{atomic}$ values. Furthermore, since lipids are relatively ^{13}C depleted, they can significantly change $\delta^{13}C$ values if not properly removed (Liden et al., 1995). This is especially true for fish and marine mammals which can have very high bone lipid contents. DeNiro (1985) proposed a $C:N_{atomic}$ 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 $C:N_{atomic}$ values that fall within the "acceptable" range. Other authors have suggested either narrower $C:N_{atomic}$ 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).

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 oth-

ers are transmitted through the material. Since the molecular structure of a material determines which wavelengths are absorbed or transmitted, FTIR can be use to semi-quantitatively, determine the chemical composition of a material ad assign different absorbance bands to specific chemical functional groups (Stuart, 2004).

There is considerable work using FTIR to investigate crystallographic and chemical changes to bioapatite and collagen during diagenesis and fossilization (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 (CO_3), phosphate (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 cm^{-1}.

band position (cm^{-1})	Functional Group
1650	Amide I (CO–NH)
1551	Amide II (CO–NH)
1545	A-Type Carbonate (CO_3)
1415	B-Type Carbonate (CO_3)
1231	Amide III (CO–NH)
1020	Phosphate (ν_3PO_4)
880	Carbonate (CO_3)
605	Phosphate (ν_2PO_4)

Materials and Methods

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

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 hours 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.

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 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_3 and B-CO_3 absorbance bands at 1545 cm^{-1} and 1415 cm^{-1}, respectively, but these bands partially overlap with the organic amide II and amide III bands. Therefore, quantifying CO_3 and PO_4 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_4 crystallography site and CO_3 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_3PO_4 absorbance bands $\left(\frac{B_{1650}}{B_{1020}}\right)$ to track the carbon and nitrogen content of archaeological bone samples. The ν_3PO_4 band is poorly resolved in demineralized collagen and hence, the 1020 cm^{-1} 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_2PO_4 absorbance bands $\left(\frac{B_{1650}}{B_{605}}\right)$ 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^{-1} for 32 scans at a resolution of 4 cm^{-1}. 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^{-1} by the height of the ν_2PO_4 band at 605 cm^{-1}. Since the band positions are approximate, we located the local maxima for each band with a window of ± 15 cm^{-1}. All corrections and calculations were performed using a custom R scripts (R Core Team, 2021), available in the supplementary material.

Stable Isotope Analysis

The $\delta^{13}C$ and $\delta^{15}N$ 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 $\pm 0.12‰$ for $\delta^{13}C$ and $\pm 0.15‰$ for $\delta^{15}N$ values. We also analyzed several aliquots of a homogenized squid tissue as an in-house reference which returned $\delta^{13}C$ and $\delta^{15}N$ values of $-18.8 \pm 0.1‰$ and $11.8 \pm 0.2‰$, respectively, which are indistinguishable from the long-term SIELO average.

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.

Collagen Yield and Quality

Our experiments successfully removed the mineral fraction from our samples and yielded collagen with in 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±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±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 $C{:}N_{atomic}$ values higher than 3.6, indicating the contribution of carbonate-bound carbon. Most acid treated sample $C{:}N_{atomic}$ 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 $C{:}N_{atomic}$ 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 $C{:}N_{atomic}$ values to residual lipids.

Taken together, our collagen yields and quality (C%, N%, $C{:}N_{atomic}$) 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.

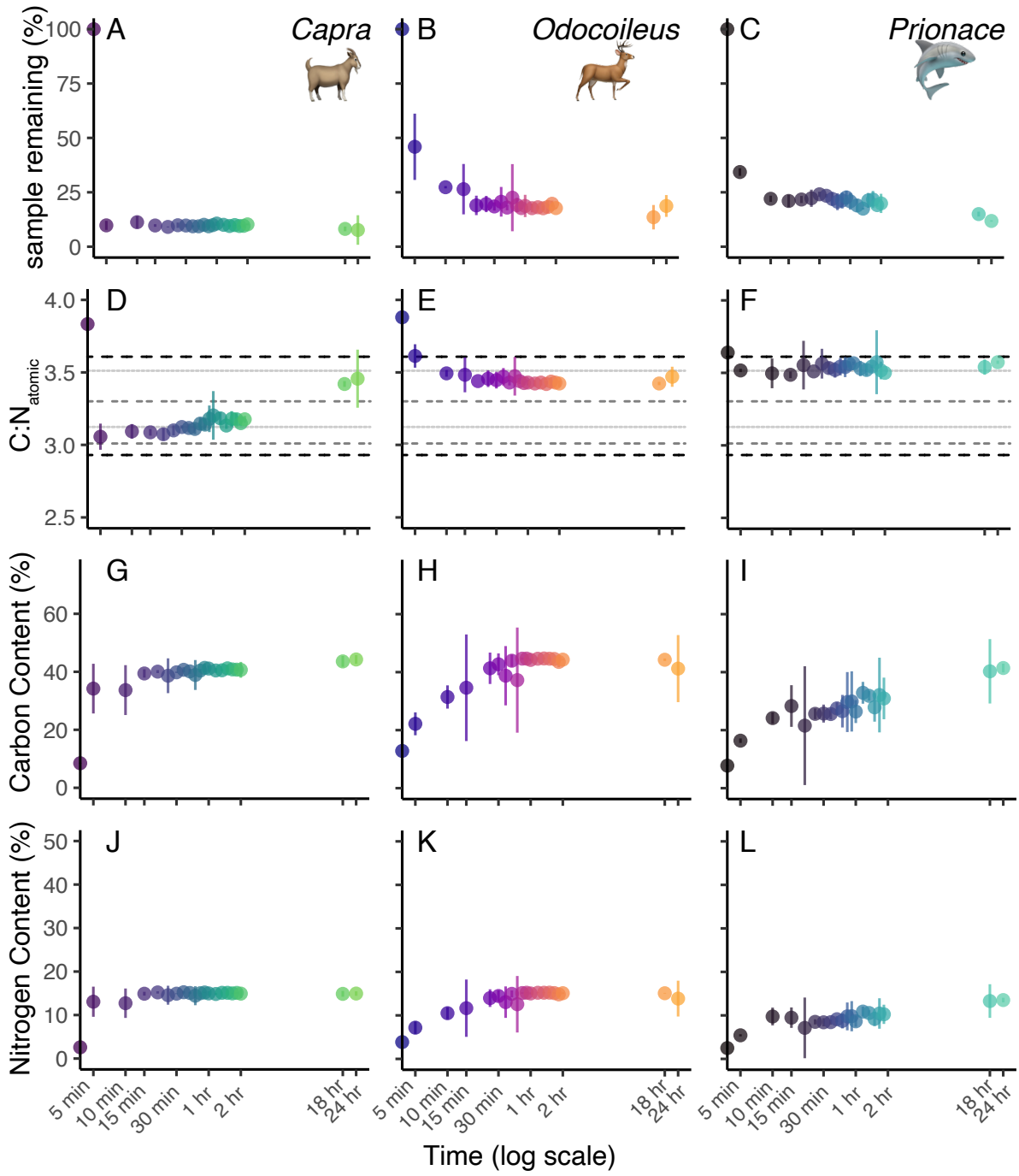

Figure 2: Fractional weight remaining (A-C), C:N_{atomic} ratios (D-F), carbon content (G-I), and nitrogen content (J-L) for all samples. The dashed lines in panels D-F indicates proposed acceptable ranges for well preserved collagen. Long dashes (black): is the 2.9–3.6 range of DeNiro (1985) and Ambrose (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).

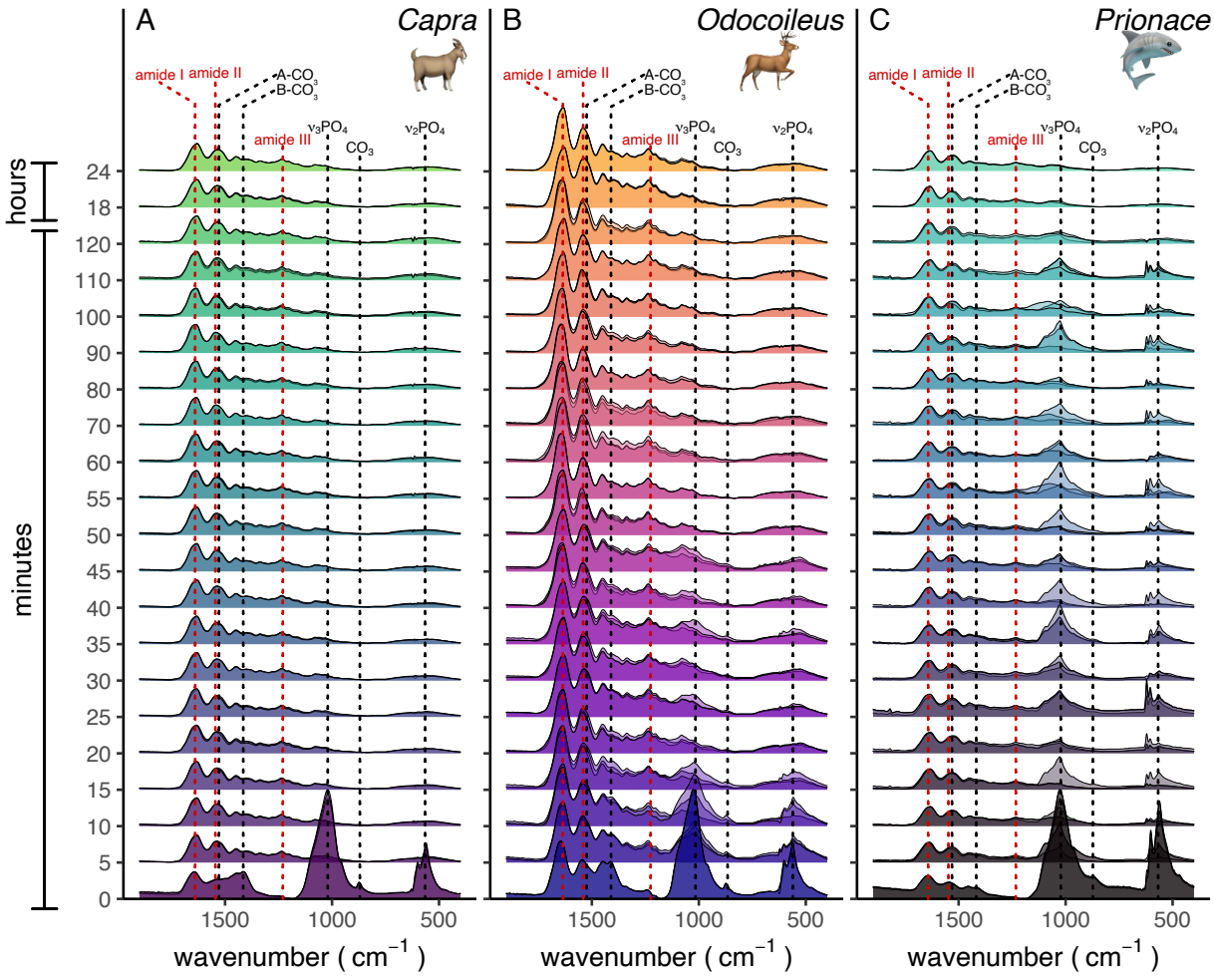

Figure 3: FTIR-ATR spectra for *Capra*, *Odocoileus*, and *Prionace* bioapatite and collagen.

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-bioapatite mixtures, with prominent absorbance bands for mineral (CO_3 = 880, 1415, 1545 cm^{-1}; PO_4 = 605, 1020 cm^{-1}) and organic (amide I = 1650 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±1.5 (±2σ; 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 CO_3 or 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 ; Figure 2). None of the 0 to 120 minute *Prionace* samples had WAMPI values within our acceptable range and 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.

Fidelity of Stable Isotope Compositions

The carbon isotope compositions of our samples were significantly affected by HCl acidification, likely resulting from the loss of mineral-bound carbonate (see Section). However, after the removal of carbonate, the $\delta^{13}C$ and $\delta^{15}N$ values showed only small changes across time steps, however none of these changes were larger than the variations expected from analytical uncertainty. 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,

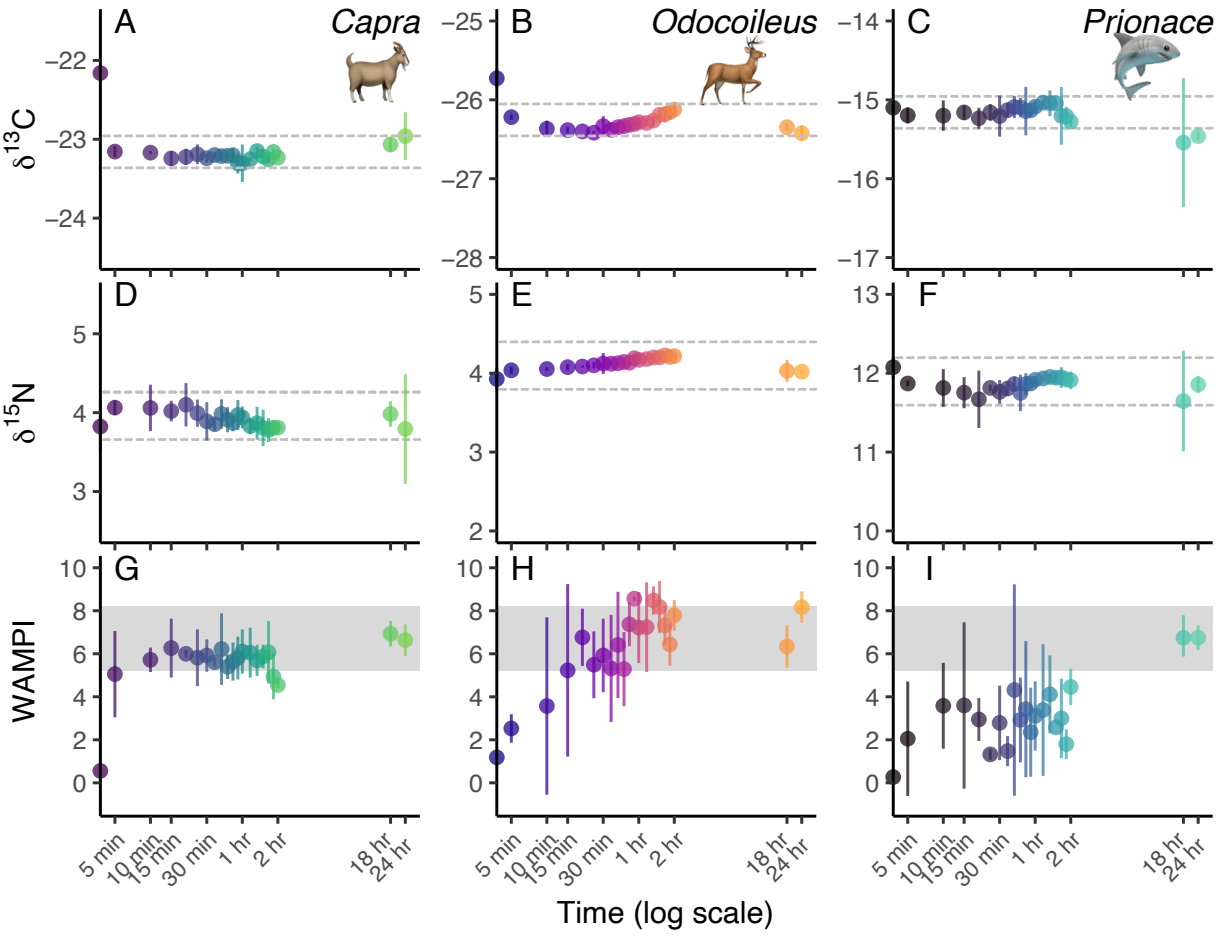

Figure 4: Stable isotope data (A-C: $\delta^{13}C$ values; D-F: $\delta^{15}N$ values) and FTIR index (G-I: WAMPI) for all samples. Colored points with error bars are the mean ±2 standard deviations for each time step. In many cases the error bars are smaller than the symbol. The dashed grey lines on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

suggesting a rapid removal of structural carbonate. After 5 minutes $\delta^{13}C$ values for these two materials show small variations in isotopic composition, although within analytical uncertainty (Figure 4 A-C). The $\delta^{13}C$ values of *Prionace* samples show very little change from 0 minutes to 2 hours, but the 18 and 24 hour time steps have lower $\delta^{13}C$ 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 CO_3 and collagen.

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 $\delta^{13}C$ and $\delta^{15}N$ 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 focus on marine invertebrates with example from vertebrate bone (Turner Tomaszewicz et al., 2015). For mammals, our results clearly contradict this recommendation and indicate removal of carbonate-bound carbon has significant, measurable, effects on $\delta^{13}C$ values, and little to no effect on $\delta^{15}N$ values. For reference, the *Capra* and *Odocoileus* samples show a 0.5‰ to 1‰ reduction in $\delta^{13}C$ values after carbonate removal, in line with our predictions (see: Section). 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.

In contrast to mammals, shark bioapatite is primarily fluorapatite [$Ca_5(PO_4)F$], has overall lower CO_3 contents, and is less soluble (Posner et al., 1984). Furthermore the diet-tissue carbon isotope enrichment factor for shark bioapatite are poorly understood and may vary as a function of both CO_3 content and via equilibration with dissolved seawater CO_3 (Vennemann et al., 2001). There

is no obvious influence of carbonate-bound carbon of $\delta^{13}C$ values from 0 minutes to 2 hours in our experiments, while the 18 and 24 hour time steps have slightly lower and more variable $\delta^{13}C$ values. Our results suggest that either there is not a large carbon isotope fractionation between carbonate and collagen or that CO_3 contents were low.

These isotopic differences, although small, can affect paleoenviromental and paleodietary interpretations. For example, a 1‰ shift in $\delta^{13}C$ 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.

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 and 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.

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 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 $\delta^{13}C$ values suggesting that structural carbonate strongly influenced 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 course 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.

Supplementary Information

All infrared spectra, stable isotope data, and analysis code, are available at github.com/robintrayler/collagen_demin

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