Simple, Rapid Extraction of Collagen from Modern Bone

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# 1 Notes

## 1.1 References to include

* Botta et al. ([2012](#ref-botta2012)) - amide band positions
* Roche et al. ([2010](#ref-roche2010)) - preservation and carbonate content
* Grunenwald et al. ([2014](#ref-grunenwald2014)) - carbonate in enamel
* LeGeros ([1991](#ref-legeros1991)) - enamel IR bands
* Hankermeyer et al. ([2002](#ref-hankermeyer2002)) - dissolution rates

# 2 Introduction

Extracting bone collagen for stable isotope analysis is one of the most common methods for inferring ecological information from modern and fossil animals ([Koch et al., 1994](#ref-koch1994); [Clementz, 2012](#ref-clementz2012)). Bone is a composite material, made of both mineral and organic components. The mineral component is primarily bioapatite [Ca5(PO4)3OH] with carbonate (CO3) substitutions in the phosphate and hydroxyl sites ([Elliott, 2002](#ref-elliott2002)). Modern dry bone is about 20 - 30 wt% organic matter, while dentine is about 12 - 33 wt% organic ([Driessens and Verbeeck, 1990](#ref-driessens1990)), which in turn is about 89% collagen ([Ambrose, 1990](#ref-ambrose1990); [Driessens and Verbeeck, 1990](#ref-driessens1990); [Van Klinken, 1999](#ref-vanklinken1999)). Since collagen is relatively insoluble ([Schwarcz and Schoeninger, 1991](#ref-schwarcz1991)) it can persist in the fossil record for ~105 years ([Clementz, 2012](#ref-clementz2012)). Furthermore, since the amino acid composition and elemental content (~40% carbon, ~15% nitrogen) are well characterized, it is reasonably straightforward to identify and remove contaminants in collagen prior to analysis ([Tuross et al., 1988](#ref-tuross1988); [Ambrose, 1990](#ref-ambrose1990); [Tuross, 2002](#ref-tuross2002); [Szpak, 2011](#ref-szpak2011); [Fuller et al., 2015](#ref-fuller2015)). However, since bioapatite contains ~ 5% structural carbonate ([Elliott, 2002](#ref-elliott2002)) there are two main reservoirs of carbon in bone. Furthermore, the carbon isotope fractionation between diet and collagen differ significantly from the fractionation between diet and structural carbonate ([Passey et al., 2005](#ref-passey2005)). This means that collagen must be isolated from the mineral component prior to analysis.

Isolating collagen for stable isotope analysis is usually accomplished via reaction with hydrochloric acid (HCl) to dissolve the mineral component. HCl protonates the carbonate and phosphate groups of the hydroxylapatite structure to form carbonic and phosphoric acid ([Hankermeyer et al., 2002](#ref-hankermeyer2002)) which can then be decanted, leaving behind the residual collagen. This is sometimes followed by rinsing with sodium hydroxide (NaOH) to remove lipid and humic acid contaminants ([Longin, 1971](#ref-longin1971); [Brown et al., 1988](#ref-brown1988); [Ambrose, 1990](#ref-ambrose1990); [Pestle, 2010](#ref-pestle2010)). The rate of mineral dissolution strongly depends on the acid concentration, temperature, the particle size ([Hankermeyer et al., 2002](#ref-hankermeyer2002)) and change one of these variable will inevitably lead to faster or slower mineral removal. However, there is considerable variability in published methods for collagen extraction in reaction times, temperature, acid concentration, particle coarseness ([Ambrose, 1990](#ref-ambrose1990); [Pestle, 2010](#ref-pestle2010)) leading to ambiguity as to the best practices for mineral removal.

In this study we do not attempt to resolve the optimal conditions for collagen extraction *per-se*, instead, we focus only on evaluating one variable, the timing of mineral removal, while holding particle size, temperature, and acid concentration constant. Our results provide a simple, viable method for extracting collagen from well preserved modern and fossil bone. Furthermore, our methods provide a framework for evaluating the the efficacy of other collagen extraction methods at removing bone mineral under a variety of experimental conditions.

# 3 Background

## 3.1 Collagen Extraction

## 3.2 Fast Fourier Infrared Spectroscopy

Fast Fourier Infrared Spectroscopy (FTIR) irradiates a sample with a beam of infrared light. Photons of infrared light excite molecules into a higher energy state, and as a result, some wavelengths are absorbed while others are transmitted through the material unaffected. Since the molecular structure of a material determines which wavelengths are absorbed or transmitted, FTIR can be used to used to semi-quantitatively measure the chemical composition of a material and different absorbance bands can be assigned to specific chemical functional groups ([Stuart, 2004](#ref-stuart2004)). There has been considerable work using FTIR to investigate crystallographic and chemical changes to bioapatite and bone collagen during diagenesis ([Hassan et al., 1977](#ref-hassan1977); [Sønju Clasen and Ruyter, 1997](#ref-sonjuclasen1997); [Sponheimer and Lee-Thorp, 1999](#ref-sponheimer1999); [Roche et al., 2010](#ref-roche2010); e.g., [Grunenwald et al., 2014](#ref-grunenwald2014)), and as a result the band positions of the major chemical groups of these materials is well understood (Table 1).

Table 1: Nominal FTIR band positions of several relevant chemical groups.

| band position (cm-1) | Chemical Group | Reference |
| --- | --- | --- |
| 1630 | Amide I (CO–NH) | Botta et al. ([2012](#ref-botta2012)) |
| 1551 | Amide II (CO–NH) | Botta et al. ([2012](#ref-botta2012)) |
| 1545 | A-Type Carbonate (CO3) | ([LeGeros, 1991](#ref-legeros1991); [Rey et al., 1991](#ref-rey1991)) |
| 1415 | B-Type Carbonate (CO3) | ([LeGeros, 1991](#ref-legeros1991); [Rey et al., 1991](#ref-rey1991)) |
| 1231 | Amide III (CO–NH) | Botta et al. ([2012](#ref-botta2012)) |
| 1020 | Phosphate (ν3PO4) |  |
| 880 | Carbonate (CO3) |  |
| 565 | Phosphate (ν2PO4) |  |

# 4 Methods

![Figure 1: Diagram of methods.](data:application/pdf;base64,)

Figure 1: Diagram of methods.

## 4.1 Sample Collection

We selected three materials for analysis; dentine from a modern domestic goat (*Capra hricus*), bone from a white tailed deer (*Odocoileus viginianus*), and dentine from a blue shark (*Prionace glauca*). The goat tooth and deer bone were fortuitously collected while the blue shark teeth were collected as part of a previous study (**citation from Sora?**). We cleaned each sample of surficial contamination using a toothbrush grinding into a fine powder. The deer and blue shark samples were ground into a fine powder using a *SPEX 8000M Mixer Mill* for 20 minutes. For shark teeth we homogenized several teeth from the same individual in order to obtain enough material for our time-step experiments. The goat dentine was hand milled form in between enamel loops and did not require further grinding. The resulting powders were further homogenized via repeated stirring using vortexer to ensure that ontogenetic difference in isotope composition were minimized.

## 4.2 Demineralization

We demineralized aliquots of each bioapatite (deer bone, goat and shark dentine) using 0.1M hydrochloric acid at 4°C. We demineralized in 5 minutes increments from 5 to 60 minutes and in 10 minute increments from 70 to 120 minutes. For each time step we weighed 2.5 - 3 mg of powdered bioapatite into 1.7 mL micro-centrifuge tubes and added 1 mL of 0.1M HCl. The bioapatite was then placed in a 4°C refrigerator for the specified amount of time. Five minutes before the specified amount of time, the samples were centrifuged for 5 minutes at 10,000 RPM and promptly rinsed using demonized water. for example, for the 60 minute time step, the samples spent 55 minutes in the refrigerator and the 5 minutes in the centrifuge for a total of 60 minutes in acid. After rising the samples were frozen and lyophilized prior to FTIR and stable isotope analysis.

## 4.3 FTIR

We collected infrared absorbance spectra from 400 to 4000 cm-1 for 32 scans at a resolution of 4 cm-1 from each aliquot to characterize changes to bulk chemistry. In particular,

We collected infrared absorbance spectra from 4000 to 400 cm-1 for 32 scans at a resolution of 4 cm-1 from each aliquot to characterize changes to bulk chemistry, and in particular, the loss of phosphate and carbonate functional groups over time. We subtracted the background of each spectra by fitting a smoothed spline to several baseline points and smoothed each spectra slightly ([Stuart, 2004](#ref-stuart2004)) using a custom R script ([R Core Team, 2021](#ref-rcoreteam2021)). Several indices have been proposed proposed to characterize the chemical-crystal properties of bone apatite and collagen (Table 2). However most of these indices focus on characterizing the

We collected FTIR absorbance spectra from an aliquot at each time step to characterize changes to bulk chemistry. We used Bruker Vertex 70 Far-Infrared FTIR housed in the Nuclear Magnetic Resonance Facility at the University of California, Merced. We collected infrared absorbance spectra from 4000 cm-1 to 400 cm-1 for 32 scans at a resolution of 1 cm-1. We subtracted the background of each spectra by fitting a smoothed spline to several baseline points using a custom R script ([R Core Team, 2021](#ref-rcoreteam2021)), which is included in the supplementary material. A variety indices have been proposed to characterize the crystal-chemical properties of bone apatite and collagen (Table 2). Sponheimer and Lee-Thorp ([1999](#ref-sponheimer1999)) used

We used the ratio of the amide-I indices related to total carbonate and phosphate content as well as organic (collagen) content.

Table 2: FTIR indices used in this study. *B* indicates the absorbance band height at the specified wavenumber, while *V* indicates the depth of the “valley”. Since the maximum height of a particular absorbance band may not occur at exactly the reported wavenumber, we instead calculated the indices above using the local maxima or minima within ±15 cm-1.

| Index | Formula | Reference |
| --- | --- | --- |
| PCI |  | Sponheimer and Lee-Thorp ([1999](#ref-sponheimer1999)) |
| BPI |  | LeGeros ([1991](#ref-legeros1991)) |
| API |  | Sponheimer and Lee-Thorp ([1999](#ref-sponheimer1999)) |
| WAMPI |  | Roche et al. ([2010](#ref-roche2010)) |
|  |  | Lebon et al. ([2016](#ref-lebon2016)) |

## 4.4 Stable Isotope Analysis

the δ13C and δ15N values and 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 housed in the Stable Isotope Ecosystem Laboratory at the University of California, Merced. 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. Mean carbon and nitrogen isotope compositions were XXXXXX XXXXXX, XXXXX, XXXXX for USGS 40 and 41a, respectively. We also analyzed several aliquots of a homogenized squid tissue as an in house reference which returned δ13C and δ15N of YYYY and YYYY which are indistinguishable from the long-term average (ZZZZ).

# 5 Results

![Figure 2: FTIR spectra for Capra hircus dentine and dental collagen. EVENTUALLY THIS FIGURE WILL HAVE SPECTRA FOR ALL 3 MATERIALS](data:application/pdf;base64,)

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![Figure 3: Amide I - ν2PO4 ratios for all FTIR spectra.](data:application/pdf;base64,)

Figure 3: Amide I - ν2PO4 ratios for all FTIR spectra.

# 6 Discussion

# 7 Supplementary Information

All infrared spectra, stable isotope data, analysis code, and a draft version of this manuscript are available on at [github.com/robintrayler/collagen\_demineralization](https://github.com/robintrayler/collagen_demineralization)

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