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The effects of PVAc treatment and organic solvent removal on $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{18}\text{O}$ values of collagen and hydroxyapatite in a modern bone

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ABSTRACT

The stable isotopic analysis of archaeological and paleontological bones has become a common method to examine questions of ecology, climate, and physiology. As researchers addressing such questions incorporate museum collections in their studies, it is necessary to understand the isotopic effects of common preservation techniques utilized in such collections to ensure the preservation of original isotopic values. This study examines the effects of PVAc glue (polyvinyl acetate) applied in acetone solution and the subsequent removal of PVAc using various organic solvents on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of extracted bone collagen, the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of carbonate in bone hydroxyapatite, and the $\delta^{18}\text{O}$ values of phosphate in hydroxyapatite. The data demonstrate that isotopic values in the collagen and phosphate are unaffected by any combination of PVAc treatment and solvent application. The carbonates show little variation in $\delta^{13}\text{C}$ values, but exhibit variable $\delta^{18}\text{O}$ values upon exposure to the PVAc solution. It is here suggested that $\delta^{18}\text{O}$ values from carbonates in PVAc-treated bones do not retain an original isotopic value and should not be included in future studies.

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1. Introduction

Stable isotopes are a powerful tool for the study of natural systems. The use of stable isotopes from archaeological and fossil vertebrate remains to examine diet and trophic level (see reviews by the following: Ambrose, 1993; Kelly, 2000; Peterson and Fry, 1987), paleotemperature and climatic signals (Ambrose, 1991; Cormie and Schwarz, 1996; Gröcke et al., 1997; Heaton et al., 1986; Koch, 1998 and references therein, Levin et al., 2006; Sealy et al., 1987; Zazzo et al., 2002), and animal physiology (Ambrose, 1991; Fizet et al., 1995; Kohn et al., 1996) has become common practice. Necessarily this method relies upon the preservation of original isotopic values within the bone. Numerous studies have examined the natural degradation of bones and teeth and the inherent isotopic values with time (Ayliffe et al., 1994; DeNiro, 1985; Koch et al., 1997; Lecuyer et al., 1999; McNulty et al., 2002; Nelson et al., 1986; Tuross et al., 1989). Methodologies for determining the diagenetic state of a bone after exposure to natural elements are well established (Ambrose, 1990; Ayliffe et al., 1994; Harbeck and Grupe, 2009; Hedges et al., 1995; Iacumin et al., 1996; Person et al., 1995, van Klinken, 1999).

Less understood is the effect of treatments applied to specimens in the field and in collections for the purposes of preserving and

stabilizing specimens. Glues of various types are often used as adhesives and consolidants to physically stabilize fragile or fissile bones. As researchers look towards older collections in museums to provide comprehensive sample suites for analyses, it is essential to understand the possible effects of such treatments on the isotopic values of fossil bones. It is also critical to understand the effects of solvents used to remove said treatments when preparing bones for analyses.

Polyvinyl acetate (PVAc) and its derivatives within the polyvinyl ester family are one of the most commonly used consolidants for fossil bone preservation. This polymer (formula $(\text{C}_4\text{H}_6\text{O}_2)_n$) readily dissolves in organic solvents such as acetone and quickly solidifies upon drying making it a convenient field and laboratory method for stabilizing fractured specimens. Due to its highly soluble nature in organic solvents, it is often removed from specimens using a simple dissolution in acetone, methanol, or a similar solvent. These solvents are highly volatile and present a possible source of contamination or chemical interaction with the bones' organic proteins and inorganic mineral compounds that may alter the bone carbon and oxygen isotopic values. The PVAc itself in solution could also present a similar source of contamination or interaction.

Previous research has examined the effect of organic solvents on isotopic values of organic specimens. Treatment of tissue and blood samples with solvents such as formalin, ether and dimethyl sulfide often result in altered carbon and nitrogen isotopic values (Bosley and Wainright, 1999; Edwards et al., 2002; Hobson et al.,

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1997a). Moore et al. (1989) determined that carbon and nitrogen isotopes in bone collagen are unaltered by the application of an acetone solvent to remove Alvar, a vinyl acetal polymer related to PVAc, contingent upon the collagen extraction method incorporating steps to remove residual solvent and glue. Research is however lacking regarding the direct effect of common solvents besides acetone on bone protein and the inorganic mineral matrix, or the effect of PVAc itself on the stable isotopic values of bones.

This study involves a more thorough approach to test the effect of PVAc treatment, multiple solvents, and solvent removal methods on the stable isotopic values of a modern bone specimen. The effects on both the organic portion and the inorganic mineral fraction of the bone were examined to provide a more complete understanding of potential alterations to carbon, nitrogen, and oxygen isotopes in these various components. The organic portion of bone is composed primarily of the protein collagen which can be purified and analyzed for its carbon and nitrogen isotopic values. The inorganic mineral fraction of bone, hydroxyapatite [general formula $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$], contains several sites for oxygen which can be analyzed isotopically including phosphate ions, structural carbonates ($-\text{CO}_3$) substituted in the $-\text{PO}_4$ and $-\text{OH}$ sites, and carbonates present in labile sites of a more ambiguous configuration. The substituted carbonates can also be examined for stable carbon isotopic values.

2. Materials and methods

2.1. Sample material and preparation

Bone samples consisted of a modern whale rib which was sectioned into multiple disks. The sampling strategy incorporated both the outer compact bone and the more porous inner cancellous bone. The porosity of the bone overall was high, thus presenting a 'worst case scenario' whereby infiltration of the PVAc solution would be at a maximum when PVAc is used as whole-bone consolidant.

Treatments were organized into a complete block design. Half of the bone disks were treated with a 10% PVAc solution (PVAc dissolved in acetone) while the remaining half were left untreated. Disks treated with PVAc were completely submerged for 30 min in the PVAc solution, removed, and allowed to dry completely for 48 h during which time the acetone evaporated and the PVAc solidified. This treatment resulted in approximately 20% of the final disk weight consisting of PVAc. Both treated and untreated disks were divided into wedges with each wedge receiving a different combination of solvent exposure and drying methods. Solvents included 100% acetone, 100% chloroform, 50% chloroform:50% methanol, or 50% chloroform:50% ether. These solvents were chosen based on the common knowledge methods for removing PVAc from bones, and the suggested methods for removing lipids from bone (Burton et al., 2001; Hilderbrand et al., 1996; Hobson et al., 1994, 1997b; Post, 2002; Roth and Hobson, 2000) in an attempt to combine two solvent processes into one streamlined effort. The solvent-treated bone wedges were soaked for 48 h in a given solvent with the solvent removed and replaced after 24 h (~10 mL/g sample). The wedges were then rinsed three times in that solvent and exposed to one of the drying methods. Drying methods included air drying at room temperature (~23 °C) for 48 h, oven drying at 80 °C for 24 h, and vacuum drying for 24 h.

The combinations of treatments, solvent exposure, and drying methods were run in duplicate. One dry bone wedge from each combination was ground to a fine powder, a portion of which was used for a gas chromatography-mass spectrometric (GC-MS) analysis of solvent presence/absence, Fourier transform infrared spectroscopic (FTIR) analysis of PVAc presence/absence, extraction

of phosphate from bone hydroxyapatite for isotopic analysis, and extraction of structural bone carbonate for isotopic analysis. The remaining intact wedge was used for extraction of bone collagen for isotopic analysis. A sample of the PVAc stock pellets from which the PVAc solution was mixed was also available for isotopic analysis.

The phosphate present in bone hydroxyapatite was extracted according to the method of Dettman et al. (2001). Approximately 10–20 mg of powdered sample was reacted overnight in 2 M hydrofluoric acid solution to liberate the phosphate ions. The resulting supernatant was diluted and buffered with 20% ammonium hydroxide. A 2 M silver nitrate solution was added and the resulting silver phosphate (Ag_3PO_4) precipitate was separated by centrifuge, rinsed copiously with ultra-pure water, and dried at 60 °C. The Ag_3PO_4 solids were then analyzed for oxygen isotopes.

The structural carbonate in the bone hydroxyapatite was extracted according to modified methods of Bryant et al. (1996). Approximately 20–30 mg of powdered sample was soaked overnight in a 2–3% sodium hypochlorite solution to remove organic phases. The remaining solid was rinsed copiously with ultra-pure water and soaked in a 1 M acetic acid solution buffered with 1 M calcium acetate (pH ~ 4.5) for 4 h to remove any labile carbonates in the hydroxyapatite structure and secondary carbonate phases. The remaining solid material was rinsed, dried at 60 °C, and analyzed for carbon and oxygen isotopes.

Bone collagen was extracted according to modified methods of Longin (1971), DeNiro and Epstein (1978), and Bocherens et al. (1991). Approximately 200 mg of whole-bone sample was first sonicated in ultra-pure water to remove physical residue. Bones were then decalcified in 0.6 M hydrochloric acid at 4 °C for 24 h increments, replacing the acid each day until reaction ceased; average reaction time was 3–5 days. The remaining crude protein gelatin was rinsed to neutrality and dried at 60 °C. Samples were then soaked in 0.125 M sodium hydroxide. This step is typically incorporated to remove residual humic and fulvic acids resulting from organic degradation of fossil bones. Although these samples are modern and do not contain humic and fulvic acids, this step was included in an attempt to accurately replicate the chemical processes to which fossil and archaeological bones are likely to be exposed. The remaining solid residue was rinsed and soaked in 0.03 M hydrochloric acid at 95 °C for 18 h to separate the hot water soluble and insoluble phases. The supernatant containing the extracted gelatin was freeze dried to produce a solid collagen extract which was then analyzed for carbon and nitrogen isotopes.

2.2. Stable isotope mass spectrometry

All stable isotopic analyses were run on a Thermo Delta V Advantage mass spectrometer coupled to a ConFlo IV in continuous flow mode. Phosphate samples were introduced via a Thermo temperature conversion elemental analyzer (TCEA). Approximately 0.5 mg of Ag_3PO_4 was packed into silver cups and thermally decomposed at 1450 °C to form CO gas which was analyzed for oxygen isotopic values. Carbonate samples were introduced via a Gas Bench II system where the processed bone powders were acidified in concentrated phosphoric acid (~102%) at 25 °C to produce CO₂ gas which was analyzed for carbon and oxygen isotopes. Collagen samples were introduced via a Costech 4010 Elemental Analyzer (EA). Approximately 0.5 mg of purified collagen was packed into tin cups and combusted at 1050 °C to produce N₂ and CO₂ gases which were separated on a GC column and analyzed for carbon and nitrogen isotopes. The PVAc stock pellets were also analyzed for carbon isotopic values by combustion on the EA.

All isotopic values are reported in standard delta notation according to the following formula

$$\delta = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right] \times 1000$$

where δ represents the isotopic system of interest (i.e. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, or $\delta^{18}\text{O}$), R represents the ratio of the heavy to light isotope (i.e. $^{13}\text{C} : ^{12}\text{C}$, $^{15}\text{N} : ^{14}\text{N}$, or $^{18}\text{O} : ^{16}\text{O}$), units are in permil (‰), and the standards are V-PDB, atmospheric N_2 , and V-SMOW for carbon, nitrogen, and oxygen respectively. A set of internal standards is run with all samples and the raw values corrected using a 2-point linear calibration. All $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are reported with an error of $\pm 0.2\text{‰}$ (1σ). The $\delta^{18}\text{O}$ values from the carbonate fraction of the bone are reported with an error of $\pm 0.2\text{‰}$ (1σ). The $\delta^{18}\text{O}$ values from the phosphate fraction of the bone are reported with an error of $\pm 0.4\text{‰}$ (1σ).

2.3. Gas chromatography-mass spectrometry (GC–MS)

For each powdered sample, a known amount was sealed in a headspace vial and analyzed on an Agilent 6890 gas chromatograph with 5975 quadrupole mass spectrometer and an Agilent 7694E headspace analyzer (GC–MS). Reference samples of 0.2 μL of solvent were sealed in a headspace vial and analyzed on the GC–MS. Prior to headspace extraction vials were held at 50 °C for 5 min. The sample loop filled for 0.2 min at 55 °C and the transfer line to the GC was held at 60 °C. Helium carrier gas was used at a constant flow rate of 0.8 mL/min. A split splitless inlet was used in split mode at a temperature of 70 °C with a 20:1 split. Samples were separated on an Agilent J&W HP-5MS, 30 m \times 0.25 mm \times 0.50 μm column. The column was held at 25 °C for 5 min, then heated at 10°C/min to 150 °C and held for 5 min. The transfer line to the MS was held at a temperature of 200 °C. The mass spectrometer used electron impact, with the ion source at 230 °C and the quadrupole at 150 °C, measuring mass/charge ratios from 20 to 300 m/z . Peaks in the chromatogram were identified using both retention time and the corresponding mass spectrum.

Solvent peaks in the chromatograms were identified and integrated using Agilent ChemStation software. The solvent peak areas from the reference samples were used to determine approximately if the amount of remaining solvent was above or below 1 $\mu\text{L/g}$ powdered bone.

2.4. Fourier transform infrared spectroscopy (FTIR)

A small amount of powdered sample was analyzed using attenuated total reflectance (ATR) Fourier transform infrared spectroscopy using a Golden Gate ATR accessory in a Thermo Nicolet 6700 FTIR bench with a DTGS detector. Samples were placed on the diamond window of the ATR and spectra collected from 450 to 4000 cm^{-1} for 128 scans at a resolution of 4 cm^{-1} . Reference samples of PVAc, untreated bone, and the whale bone with known amounts of PVAc in the range of 4.0 to 0.1% were created and analyzed using FTIR. A partial least squares calibration was used to determine the amount of PVAc observed in the IR spectra of the samples. Concentrations above 0.5% PVAc could be quantified and showed a visible peak at 1734 cm^{-1} from the PVAc carbonyl group, distinct from the protein Amide I peak appearing at 1638 cm^{-1} .

2.5. Statistical considerations

All stable isotope data are analyzed using non-parametric statistical methods. The Wilcoxon signed-rank test was used to examine relationships between isotopic values of two paired groups (i.e. the presence/absence of PVAc). The Friedman test was

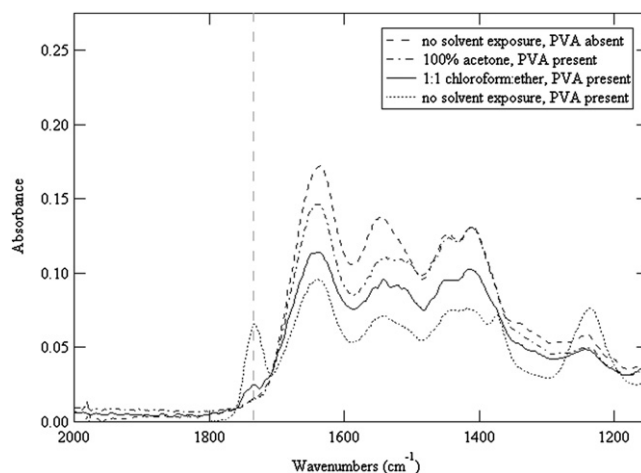


Fig. 1. Infrared spectra from bone exposed to four different treatment combinations: (1) untreated with no solvent exposure (long dashes), (2) treated with PVAc followed by soaking in 100% acetone to remove PVAc (dash-dot), (3) treated with PVAc followed by soaking in 50% chloroform:50% ether to remove PVAc (solid), and (4) treated with PVAc with no solvent exposure to remove PVAc (small dashes). Note the presence of PVAc indicated by a peak at 1734 cm^{-1} (gray dashed line). The minor peak at 1734 cm^{-1} in the chloroform:ether treated sample indicates a minimal amount of PVAc remaining and represents the maximum level of PVAc detected in any of the solvent-treated samples. Peaks at 1645, 1540, 1516, 1445, 1413 cm^{-1} are due to absorptions of the proteinaceous components of the bone. The peak at 1240 cm^{-1} is due to a combination of absorptions by both the bone and the PVAc.

used to examine the relationships between isotopic values of multiple dependant groups (i.e. solvent treatments).

3. Results

3.1. Removal of PVAc and solvents

GC–MS analysis indicated that traces of solvent were present in all samples. However even in samples with the largest amount of residual solvent, the amount of solvent remaining was less than 1 $\mu\text{L/g}$ sample. All drying methods are therefore considered successful and are grouped together for statistical purposes. The samples with the least amount of residual solvent were the samples treated with acetone or methanol and oven-dried. It is therefore recommended that PVAc be removed using acetone as a solvent followed by drying in an oven at 80 °C.

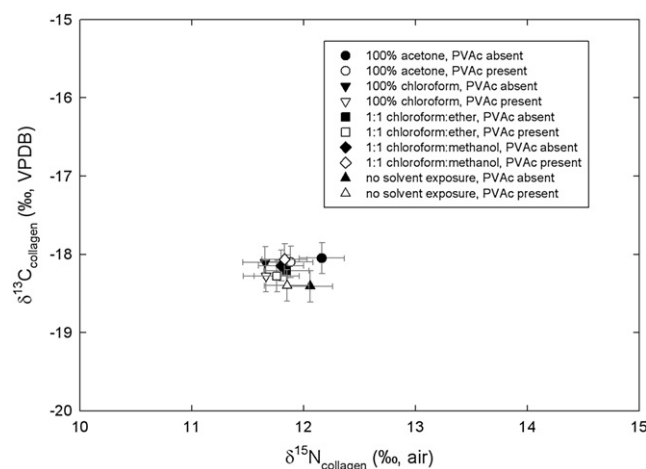


Fig. 2. Average carbon and nitrogen isotopic values of bone collagen for different treatment combinations. All errors are $\pm 0.2\text{‰}$ (1σ). Solid symbols indicate the absence of PVAc treatment, open symbols indicate the presence of PVAc treatment.

Table 1
Isotopic data.

PVAc treatment ^a	Solvent applied	Drying method ^b	Structural carbonate $\delta^{13}\text{C}$	Structural carbonate $\delta^{18}\text{O}$	Phosphate $\delta^{18}\text{O}$	Collagen $\delta^{15}\text{N}$	Collagen $\delta^{13}\text{C}$
Untreated	100% acetone	Air	-14.1	28.9	21.0	12.3	-18.0
Untreated	100% acetone	Air				12.4	-18.6
Untreated	100% acetone	Oven	-14.0	28.7	20.8	11.9	-18.0
Untreated	100% acetone	Vacuum	-14.3	29.0	21.2	12.1	-17.6
		<i>Average</i>	<i>-14.1</i>	<i>28.9</i>	<i>21.0</i>	<i>12.2</i>	<i>-18.1</i>
		<i>stdev</i>	<i>0.13</i>	<i>0.14</i>	<i>0.18</i>	<i>0.23</i>	<i>0.44</i>
Treated	100% acetone	Air	-14.6	30.1	20.6	12.1	-17.4
Treated	100% acetone	Air				11.8	-18.1
Treated	100% acetone	Oven	-14.9	32.3	20.7	12.1	-18.2
Treated	100% acetone	Vacuum	-14.1	28.3	21.2	11.8	-18.7
Treated	100% acetone	Vacuum				11.7	-18.1
		<i>Average</i>	<i>-14.5</i>	<i>30.2</i>	<i>20.8</i>	<i>11.9</i>	<i>-18.1</i>
		<i>stdev</i>	<i>0.42</i>	<i>2.0</i>	<i>0.31</i>	<i>0.20</i>	<i>0.47</i>
Untreated	100% chloroform	Air	-14.3	28.4	20.2	11.6	-17.9
Untreated	100% chloroform	Air				11.4	-18.0
Untreated	100% chloroform	Oven	-14.6	28.7	19.0	11.4	-18.4
Untreated	100% chloroform	Vacuum	-14.3	29.0	21.0	11.9	-18.1
Untreated	100% chloroform	Vacuum				11.8	-18.1
untreated	100% chloroform	Vacuum				11.9	-18.1
		<i>Average</i>	<i>-14.4</i>	<i>28.7</i>	<i>20.1</i>	<i>11.7</i>	<i>-18.1</i>
		<i>stdev</i>	<i>0.16</i>	<i>0.31</i>	<i>0.97</i>	<i>0.21</i>	<i>0.17</i>
Treated	100% chloroform	Air	-13.8	28.3	19.7	11.7	-18.0
Treated	100% chloroform	Air				11.7	-18.3
Treated	100% chloroform	Air				11.7	-18.1
Treated	100% chloroform	Oven	-15.5	30.5	21.0	11.7	-18.7
Treated	100% chloroform	Vacuum	-13.9	29.1	20.9	11.7	-18.1
Treated	100% chloroform	Vacuum				11.7	-18.2
Treated	100% chloroform	Vacuum				11.4	-18.6
		<i>Average</i>	<i>-14.4</i>	<i>29.3</i>	<i>20.5</i>	<i>11.7</i>	<i>-18.3</i>
		<i>stdev</i>	<i>0.96</i>	<i>1.1</i>	<i>0.76</i>	<i>0.11</i>	<i>0.27</i>
Untreated	50% chloroform:50% ether	Air	-13.9	28.6	20.6	11.7	-18.2
Untreated	50% chloroform:50% ether	Air				11.9	-18.2
Untreated	50% chloroform:50% ether	Air				11.7	-18.4
Untreated	50% chloroform:50% ether	Oven	-14.3	29.1	19.1	12.0	-17.9
Untreated	50% chloroform:50% ether	Vacuum	-14.0	29.6	21.1	11.9	-18.3
Untreated	50% chloroform:50% ether	Vacuum				11.8	-18.2
Untreated	50% chloroform:50% ether	Vacuum				11.9	-18.2
		<i>Average</i>	<i>-14.0</i>	<i>29.1</i>	<i>20.3</i>	<i>11.8</i>	<i>-18.2</i>
		<i>stdev</i>	<i>0.21</i>	<i>0.54</i>	<i>1.0</i>	<i>0.10</i>	<i>0.17</i>
Treated	50% chloroform:50% ether	Air	-13.9	29.0	20.7	11.8	-18.2
Treated	50% chloroform:50% ether	Air				12.0	-18.1
Treated	50% chloroform:50% ether	Air				11.9	-18.2
Treated	50% chloroform:50% ether	Oven	-14.6	30.0	20.7	11.9	-18.7
Treated	50% chloroform:50% ether	Vacuum	-14.6	29.9	20.5	11.9	-18.1
Treated	50% chloroform:50% ether	Vacuum				11.2	-18.2
Treated	50% chloroform:50% ether	Vacuum				11.7	-18.4
		<i>Average</i>	<i>-14.4</i>	<i>29.6</i>	<i>20.6</i>	<i>11.8</i>	<i>-18.3</i>
		<i>stdev</i>	<i>0.43</i>	<i>0.55</i>	<i>0.11</i>	<i>0.28</i>	<i>0.21</i>
Untreated	50% chloroform:50% methanol	Air	-14.1	28.9	20.7	12.0	-17.9
Untreated	50% chloroform:50% methanol	Air				11.8	-18.3
Untreated	50% chloroform:50% methanol	Air				11.8	-17.8
Untreated	50% chloroform:50% methanol	Oven	-15.0	29.9	20.6	11.5	-17.9
Untreated	50% chloroform:50% methanol	Vacuum	-14.0	28.5	21.0	12.0	-17.9
Untreated	50% chloroform:50% methanol	Vacuum				11.9	-18.2
Untreated	50% chloroform:50% methanol	Vacuum				11.7	-18.9
		<i>Average</i>	<i>-14.4</i>	<i>29.1</i>	<i>20.8</i>	<i>11.8</i>	<i>-18.1</i>
		<i>stdev</i>	<i>0.57</i>	<i>0.73</i>	<i>0.21</i>	<i>0.18</i>	<i>0.38</i>
Treated	50% chloroform:50% methanol	Air	-13.7	28.9	21.1	11.7	-18.0
Treated	50% chloroform:50% methanol	Air				11.7	-18.1
Treated	50% chloroform:50% methanol	Air				11.8	-18.5
Treated	50% chloroform:50% methanol	Oven	-15.3	29.4	20.9	12.0	-18.1

Table 1 (continued)

PVAc treatment ^a	Solvent applied	Drying method ^b	Structural carbonate $\delta^{13}\text{C}$	Structural carbonate $\delta^{18}\text{O}$	Phosphate $\delta^{18}\text{O}$	Collagen $\delta^{15}\text{N}$	Collagen $\delta^{13}\text{C}$
Treated	50% chloroform:50% methanol	Vacuum	-14.1	29.0	20.2	12.0	-17.8
Treated	50% chloroform:50% methanol	vacuum				11.8	-18.0
Treated	50% chloroform:50% methanol	Vacuum				11.9	-18.0
		Average	-14.4	29.1	20.7	11.8	-18.1
		stdev	0.82	0.23	0.46	0.12	0.23
Untreated	No solvent applied	N/A	-14.5	29.0	21.0	12.4	-18.4
Untreated	No solvent applied	N/A				12.3	-18.7
Untreated	No solvent applied	Oven	-14.3	29.5	20.8	11.5	-18.1
		Average	-14.4	29.2	20.9	12.1	-18.4
		stdev	0.13	0.38	0.17	0.50	0.34
Treated	No solvent applied	N/A	-14.3	29.0	19.9	11.9	-18.4
Treated	No solvent applied	Oven	-14.9	32.2	20.7		
		Average	-14.6	30.6	20.3		
		stdev	0.38	2.2	0.54		

Note all isotopic values are reported in ‰ notation referenced to V-PDB, V-SMOW, air for carbon, oxygen, nitrogen, respectively. Errors for all δ -values from collagen and structural carbonates are $\pm 0.2\text{‰}$ (1σ), errors for $\delta^{18}\text{O}$ from phosphates are $\pm 0.4\text{‰}$ (1σ).

^a Samples labeled 'treated' were exposed to a 10% PVAc solution in acetone.

^b Oven-dried samples were dried at 80 °C.

FTIR spectra from the majority of the samples do not show the presence of a peak from PVAc (1734 cm^{-1}) indicating that less than 0.5% PVAc remains in the treated samples. Spectra from the three chloroform:ether treated samples have a small peak at 1734 cm^{-1} indicating the samples contain 0.8–1.0% PVAc (Fig. 1). This suggests that the PVAc itself will not contribute to isotopic values as its own entity. Any isotopic effects from the PVAc will have occurred during chemical processing and before isotopic analysis.

3.2. Collagen isotopes

Average $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from isolated bone collagen showed no variation in isotopic values between different treatment groups (Fig. 2, Table 1). Values clustered tightly with a $\delta^{13}\text{C}$ range of -18.4‰ to -18.1‰ and a $\delta^{15}\text{N}$ range of $+11.7\text{‰}$ to $+12.2\text{‰} \pm 0.2\text{‰}$. No statistical differences were observed (all calculated p -values >0.05). It should be noted that the average $\delta^{13}\text{C}$ value for the pure PVAc stock pellets was -31.4‰ , clearly much more depleted than the bone material.

3.3. Structural carbonate isotopes

Average isotopic values from structural bone carbonates showed little variation in $\delta^{13}\text{C}$ values with a range of -14.0‰ to $-14.6\text{‰} \pm 0.2\text{‰}$ (Fig. 3, Table 1). The $\delta^{18}\text{O}$ values showed a much greater range of $+28.7\text{‰}$ to $+30.6\text{‰} \pm 0.2\text{‰}$ (Fig. 3, Table 1). While differences between values were not statistically significant (all calculated p -values >0.05), it should be noted that several of the $\delta^{18}\text{O}$ values are clearly variable outside the range of analytical error (i.e. $\pm 0.2\text{‰}$). Specifically, samples treated with PVAc tend to have more enriched $\delta^{18}\text{O}$ values. This discrepancy between observed trends and statistical tests is most likely a remnant of the non-parametric statistical methods which, while capable of accounting for small sample sizes and potentially non-Gaussian distributions, do tend to produce larger p -values and reduce the overall power of the statistical test.

3.4. Phosphate isotopes

Average oxygen isotopic values from the bone phosphate showed a range of $+20.1\text{‰}$ to $+21.0\text{‰} \pm 0.4\text{‰}$ with little variation in $\delta^{18}\text{O}$ between treatment groups (Fig. 4, Table 1). No statistical

differences were observed between treatments (all calculated p -values >0.05). Note that all $\delta^{18}\text{O}$ values from phosphates are plotted against the $\delta^{18}\text{O}$ from the structural carbonates in an effort to emphasize the difference between the oxygen results of the carbonate and phosphate portions of the inorganic bone material.

4. Discussion

The lack of variability in isotopic values of the bone collagen between different treatment groups is not unexpected. The PVAc compound contains no nitrogen so there is no opportunity for isotopic alteration of this element. Carbon is present in both the PVAc and the collagen, but the $\delta^{13}\text{C}$ of the former proved much more depleted than the latter, thereby providing a tracer for any contamination or chemical alteration of the collagen. The PVAc-treated samples did not appear significantly depleted compared to the untreated samples suggesting that no such contamination or chemical interaction occurred. Additionally, it is unlikely that the solvents themselves hydrolyzed the collagen or partially removed protein fragments and amino acids thereby altering $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$

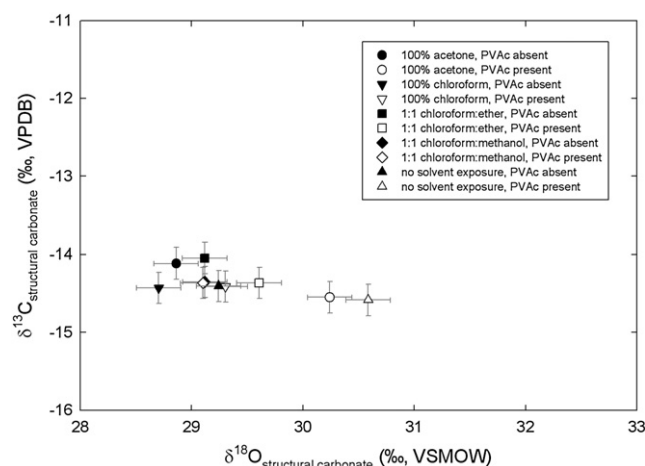


Fig. 3. Average carbon and oxygen isotopic values of structural bone carbonate for different treatment combinations. All errors are $\pm 0.2\text{‰}$ (1σ). Solid symbols indicate the absence of PVAc treatment, open symbols indicate the presence of PVAc treatment.

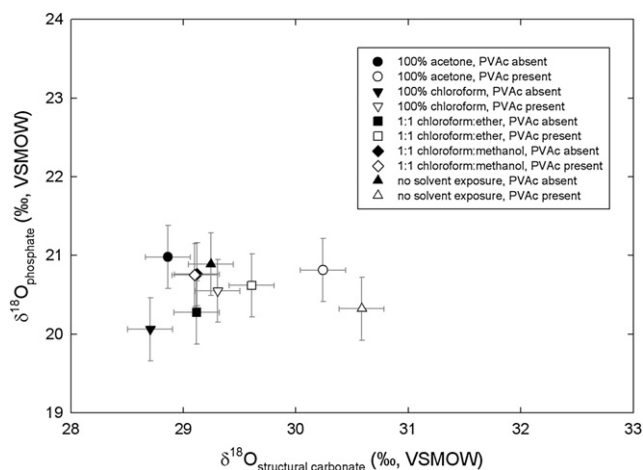


Fig. 4. Average oxygen isotopic values of bone phosphates for different treatment combinations plotted against average oxygen isotopic values of structural bone carbonate for comparison. All errors for $\delta^{18}\text{O}$ values from phosphate are $\pm 0.4\text{‰}$ (1σ); all errors for $\delta^{18}\text{O}$ values from carbonate are $\pm 0.2\text{‰}$ (1σ). Solid symbols indicate the absence of PVAc treatment, open symbols indicate the presence of PVAc treatment.

values. Collagen is a very stable and strong protein that requires acidic conditions and heat to denature. During the course of the collagen extraction, heat is used to hydrolyze the protein into soluble form which has the beneficial side-effect of volatilizing any remaining solvents and leaving behind any residual PVAc which could directly contribute to the isotopic values. This agrees with the previous research of Moore et al. (1989) who relied on a similar collagen extraction method to remove residual PVAc-like material and solvent.

The inorganic hydroxyapatite in the bone exhibited variation within the structural carbonate $\delta^{18}\text{O}$ values, but statistically insignificant variation in the structural carbonate $\delta^{13}\text{C}$ values and phosphate $\delta^{18}\text{O}$ values. The variation in structural carbonate $\delta^{18}\text{O}$ values is not, however, uniform as evidenced by some samples exhibiting greater enrichments than others (Fig. 3). It appears that the application of the PVAc treatment, as opposed to the solvent treatment for PVAc removal, has the greatest affect on the oxygen isotopic values in the structural carbonates. Since the FTIR analyses suggest that PVAc was removed from treated samples to a negligible level, it is unlikely that the PVAc itself is contributing directly to the isotopic values. It is more likely that the isotopic variation is due to chemical alteration during processing.

While the methods of this study do not directly examine the physical mechanisms of isotopic alteration in these structural carbonates, it is worthwhile to consider the potential processes that may be responsible. The PVAc was introduced in dissolved form in acetone solvent which presents several possible oxygen sites or oxygen-containing groups that could exchange with the bone material. The polyvinyl acetate contains both a singly-bonded and doubly-bonded oxygen from the ester group in the acetate side chain. When solubilized, polyvinyl acetate can hydrolyze to polyvinyl alcohol which contains a highly labile hydroxide ($-\text{OH}$) group (Lazár et al., 1989; Seymour and Carraher, 1988). Many manufacturers also include polyvinyl alcohol as a plasticizer in PVAc pellets which could be another source of labile $-\text{OH}$ groups (Horie, 2010).

These sources of oxygen from the PVAc solution could potentially exchange with oxygen in two different sites within the hydroxyapatite during the PVAc treatment and solvent removal processes. The phosphate site in the hydroxyapatite contains both carbonate ions and phosphate ions with covalently bonded oxygen atoms. The hydroxyapatite also contains an $-\text{OH}$ group which is

released in conjunction with CO_2 during acidification of carbonates in the mass spectrometry analysis. This $-\text{OH}$ group will exchange and equilibrate oxygen isotopic values with the released CO_2 during this process, thus transferring the isotopic signal obtained during the PVAc treatment and solvent removal processes to the final data. Of these possibilities, the oxygen atoms with the highest bond dissociation energies which present the least likely options for direct atomic exchange are those located in the covalently bonded sites of the carbonate and phosphate groups in the hydroxyapatite. The $-\text{OH}$ groups are much more labile and have relatively lower bond dissociation energies than the aforementioned oxygen atoms as well as oxygen atoms from the solvents used in the various treatments. As such, it is here suggested that direct exchange of $-\text{OH}$ groups between the polyvinyl alcohol in solution and the hydroxyapatite occurring during the PVAc treatment and possibly also the solvent removal process is the source of the observed isotopic variation in the bone structural carbonate analyses. Additional research into these potential pathways is needed to confirm this hypothesis.

This proposed mechanism of isotopic alteration would also explain the lack of variation in $\delta^{13}\text{C}$ values observed in these structural carbonates. Both the carbon atoms in the $-\text{CO}_3$ groups and the carbon atoms in the polymer chain of the PVAc or polyvinyl alcohol constitute the carbon backbone of a covalently bonded molecule, a bond which is less labile and more energetically difficult to break compared to the aforementioned $-\text{OH}$ groups. Thus direct exchange of carbon atoms is less likely. This new insight into the potential variations in isotope values of structural bone carbonates exposed to PVAc treatments suggests that $\delta^{13}\text{C}$ values will likely be unaffected. Caution should however be taken when utilizing $\delta^{18}\text{O}$ values from treated samples to analyze research questions, especially considering the variable and unpredictable effect of the treatment on the isotope values.

This proposed mechanism of direct $-\text{OH}$ exchange between PVAc solution and bone material could also explain the difference in $\delta^{18}\text{O}$ trends between the phosphates and carbonates. The phosphorus-oxygen bonds are relatively strong and require much greater energy to dissociate. The chemical processing and presence of PVAc in solution therefore has no apparent effect on stable isotopic values of bone phosphates. The chemical processing required to isolate phosphates eliminates all carbonate and hydroxyl groups from the sample material thus eliminating this isotopic contribution.

5. Conclusions

After exposing modern bone material to several different treatment combinations of PVAc and solvents, the following conclusions and recommendations can be drawn:

- (1) PVAc is most successfully removed from treated bones using acetone followed by drying at $80\text{ }^\circ\text{C}$ for at least 24 h.
- (2) The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of isolated bone collagen are unaffected by both PVAc treatment and solvent exposure provided the collagen extraction incorporates a heated acidic gelatinization step.
- (3) The $\delta^{18}\text{O}$ values from the phosphate in bone hydroxyapatite are unaffected by both PVAc treatment and solvent exposure.
- (4) The $\delta^{13}\text{C}$ values from the carbonate in bone hydroxyapatite are unaffected by both PVAc treatment and solvent exposure.
- (5) The $\delta^{18}\text{O}$ values from the carbonate in bone hydroxyapatite are altered by exposure to PVAc treatment in an unpredictable manner. These isotopic values are altered from their original state and care should be taken in utilizing such data for scientific interpretations.

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