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BRIEF COMMUNICATION

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Particle size matters: The effect of particle size on carbon and oxygen isotope composition of bone hydroxyapatite

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Abstract

Objectives: Stable isotope studies often focus on hydroxyapatite (bioapatite) to answer questions of paleodiet, paleomobility, and paeloenvironment. This study seeks to determine the effect that sample particle size (in particular SA:V, or surface area to volume ratios) has on measured carbon and oxygen stable isotope values ($\delta^{13}C$ and $\delta^{18}O$) in bone hydroxyapatite.

Materials and methods: Previously ground *Homo sapiens sapiens* cortical bone samples were subdivided using geological screens to obtain three separate sub-samples, differing only in their particle size. These aliquots (n = 60) were then treated using established protocols to remove any exogenous organic material (2.5% NaOH) and adsorbed carbonates (0.1 M CH_3COOH), and analyzed for $\delta^{13}C$ and $\delta^{18}O$ using a Kiel-IV Carbonate Device coupled to a Thermo-Finnigan DeltaPlus IRMS.

Results: Data obtained indicate that decreased particle size leads to increases in both $\delta^{13}C$ and $\delta^{18}O$, with oxygen isotope values being more dramatically affected. Specifically, it is possible to produce isotopic shifts of as much as 1.0‰ and 4.0‰ for $\delta^{13}C$ and $\delta^{18}O$, respectively, solely by analyzing different sized particles from the same individual, bone, and sample.

Discussion: Based upon the variability seen between different size fractions from the same sample, it is clear that particle size has a meaningful impact on carbon and oxygen isotope composition. We attribute these shifts to the differential adsorption or precipitation of environmental carbon and oxygen during pretreatment. We recommend that particle size be added to the list of potential variables affecting isotope composition, alongside other factors including diagenesis, reagent concentration, and treatment time. We would also note that while most individuals exhibit consistent changes, some do not, and thus further investigation into these phenomena is warranted.

KEYWORDS

bone, hydroxyapatite, isotopes

1 | INTRODUCTION

Over the past three decades, stable isotope analysis has become an important tool for assessing questions from paleodiet (Hoppe, Stuska, & Amundson, 2005; Wang & Cerling, 1994), to paleomobility (Chazin, Gordon, & Knudson, 2019), to paleoclimate (Joachimski et al., 2009; Kohn & Law, 2006). In many instances, this analysis is conducted using hydroxyapatite, the most abundant mineral in bones and teeth, which preserves incredibly well, particularly in dental enamel, over both archaeological and geological time spans (Lee-Thorp, 2008).

As teeth and bone can be the only surviving vestiges of ancient peoples and animals, hydroxyapatite is an extremely useful substance for answering numerous archaeological, paleoanthropological and paleobiological questions.

As with any analysis, however, great care must be taken that the methods by which isotopic data are obtained do not skew results or create analytical "artifacts." Therefore, it is important for isotopic researchers to know the various factors that can alter results, particularly when comparing results from different studies conducted by different labs using different methods. In the present study, we focus on the isotopic analysis of hydroxyapatite (or bioapatite), examining, in particular, the effects that sample particle size has on $\delta^{13}C_{ap}$ and $\delta^{18}O_{ap}$ values.

Hydroxyapatite contains abundant biogenic carbon and oxygen, making it a useful substrate for analysis of the stable isotopes of both elements. As stable isotope analysis has grown in importance within archaeology and related fields, there have been efforts made to identify problems with pre-treatment methods and to suggest certain forms of best practice. In the case of hydroxyapatite, Garvie-Lok, Varney, and Katzenberg (2004) analyzed the effects that both treatment time and acid concentration have on hydroxyapatite samples, showing that a more dilute acid and shorter treatment times decreased recrystallization and sample dissolution, which Koch, Tuross, and Fogel (1997) previously had noted in their argument in favor of using lower concentrations of acid during pretreatment. However, Nielsen-Marsh and Hedges (2000) acknowledge that even low concentrations of acetic acid treatment can cause some recrystallization, although they argue that the effect is relatively minor and unlikely to cause diagenetic carbon to become reabsorbed into the sample. Pestle, Crowley, and Weirauch (2014) showed the potential for immense inter-laboratory variability, especially with hydroxyapatite, as a consequence of both differences in pretreatment and analytical instrumentation. Crucially, this study found an extreme difference between labs in hydroxyapatite oxygen isotope composition. Two recent studies (Pellegrini & Snoeck, 2016; Snoeck & Pellegrini, 2015) have examined in detail the relationship between various combinations of organic removal/oxidizing agents and acetic acid treatments for removal of secondary carbonates, and have found issues with many of the most commonly employed methods. Indeed, those authors have gone so far as to argue that, "(i)n order to preserve the original isotope composition of bioapatite, it may, therefore, be better to avoid any chemical pre-treatment," (Pellegrini & Snoeck, 2016, p. 95). Of particular relevance to the present work, Pellegrini and Snoeck have argued that the concentration of NaOCl used in organic removal is strongly correlated with observed shifts in both $\delta^{13}C$ and $\delta^{18}O$.

It is evident from the research already done on this topic that there are multiple variables for which researchers must account when treating bone samples for isotope analysis so as to avoid engendering isotopic shifts. While the research conducted to date has assessed the differences between labs, reagents, and instruments, no study has evaluated the impact of sample particle size on resulting isotopic values. While it is generally accepted that decreasing particle size (and thus increasing surface area-volume ratios) will increase reactivity/the rate of chemical reactions, this general principle has not been tested in the case of hydroxyapatite carbon and oxygen isotopic composition.

It is known that for most reactions involving solids, the rate of reaction increases as surface area relative to volume increases. Given that changes in particle size will lead to changes in surface area relative to volume (SA:V), and that SA:V has significant impact on the pace of chemical reactions, there is the potential for the production of isotopic shifts during pretreatment of hydroxyapatite as a function of sample particle size. As such, it is important to determine whether, and to what degree, particle size might impact resulting isotope ratios. Our working hypothesis is that smaller particle size (and higher SA:V) will have the same effect (directionally) as the use of higher concentration reagents (NaOCl and acetic acid) or longer treatment times. In this pilot study, we attempt to answer this question such that researchers can take this factor into account, particularly when comparing samples prepared (by different laboratories for instance) using different starting size fractions.

2 | MATERIALS AND METHODS

Twenty previously ground *Homo sapiens sapiens* cortical bone samples were selected at random from the collection held at the Archaeological Stable Isotope Lab at the University of Miami. These samples represent a range of localities from across the Atacama Desert of northern Chile, a region characterized by exceptional preservation of archaeological bone, and date to between 500–3,000 years before present. Given that the sites from which these samples were collected receive just 0.1–10 mm/yr of rain, the possibility that diagenetic dissolution/recrystallization has occurred is exceedingly low. Although different individuals were sampled at different locations and/or skeletal elements, all comparisons in this study are between sub-samples (aliquots) of the same sample (taken from the same place on the same bone of the same individual), and therefore issues of different remodeling rates are not relevant.

Samples were passed through geological screens to obtain at least 0.1 g each of 0.25–0.5 mm, 0.125–0.25 mm, and 0.063–0.125 mm size fractions, resulting in a total of 60 sub-samples or aliquots (three per individual). Sterile centrifuge tubes were labeled and weighed for each of these 60 aliquots, and approximately 0.1 g of ground bone was then added to each tube. Each tube was weighed after having the bone added to determine starting weight. The 60 aliquots were then separated into 5 batches of 12, ensuring that all size fractions or subsamples of the same sample (individual) were kept in the same batch.

All samples were treated using the protocol established in Lee-Thorp (1989) and Krueger (1991) and modified by Pestle (2010). Treatment began with the addition of 30 ml of 50% bleach (2.5% NaOCl) to each tube, and the tubes then were agitated briefly with a vortexer, covered loosely with a sheet of aluminum foil, and left to stand open overnight in a fume hood. On the second day, each sample was centrifuged for 5 min, decanted, and then a fresh 30 ml of 50% bleach was added. The samples were again left to stand open

overnight in the fume hood, still covered loosely with aluminum foil. On the third day, samples were centrifuged for 5 min and the bleach was then decanted. We then added 30 ml of distilled water to the samples, which were vortexed and then centrifuged for 5 min. This rinse process was repeated three more times, for a total of four rinses, or until the rinse water had a neutral pH. We then added 30 ml of 0.1 M acetic acid to each sample. Samples were then vortexed briefly and left to stand open for 2 hr. After 2 hr, air was slowly evacuated from the tubes using a vacuum manifold, until the samples achieved a low boil for 5 min. The samples were then vortexed and left to stand open for an additional 2 hr. Samples were then centrifuged for 5 min, and the acetic acid was decanted. We then added 30 ml of distilled water to each sample tube, and the tubes were then vortexed, centrifuged for 5 min, and decanted. This rinse step was repeated three additional times, for a total of four rinses, or until the rinse water had a neutral pH. Tubes were then placed open in an oven at 60°C overnight. The tubes containing samples were weighed again after treatment to determine end weight and calculate apatite yield. All reagents used in pretreatment on all 60 samples were drawn from the same batch, and all pretreatments took place over a total of 7 days.

Collagen content of the analyzed samples was also determined because of the potential influence of collagen not removed by hydroxyapatite pretreatment on the measured isotopic composition of that hydroxyapatite. Collagen extraction followed the method of Longin (1971), as modified by Pestle (2010). Start and end weights were recorded and used to calculate collagen yield (wt%) for each sample.

Isotopic analysis was performed in the Marine Geology and Geophysics Department's Stable Isotope Laboratory at the University of Miami's Rosenstiel School of Marine and Atmospheric Science. Hydroxyapatite samples were analyzed using a Kiel-IV Carbonate Device coupled to a Thermo-Finnigan DeltaPlus IRMS. Precision, as determined by replicate analysis of select samples, was better than 0.1‰ for $\delta^{13}C$ and 0.2‰ for $\delta^{18}O$. All isotope results were calibrated using an in-house carbonate standard calibrated to NBS-19, and results are reported relative to V-PDB. Bracketed standards were included in every analytical run. In order to minimize any effect of instrumental drift, the three size fractions of each sample (individual) were analyzed sequentially. As the number of samples necessitated two separate instrumental runs, we chose six samples to be analyzed in both runs. The average difference between replicates in the two instrumental runs was 0.05‰ for $\delta^{13}C$ and 0.2‰ for $\delta^{18}O$.

3 | RESULTS

For clarity of communication, we use the large particle size (0.25–0.5 mm) as the baseline from which we discuss all of the results. One sample, J-55, which produced outlier isotopic values, was excluded from all statistical analysis, resulting in a sample size of 19 individuals, or 57 total aliquots. As stated above, all comparisons referenced below are between or among sub-samples derived from the same location on the same skeletal element of the same individual. As the samples are identical, except in regard to particle size, and as they all received the identical pre-treatment, the only driver of difference should be difference in particle size and SA:V ratio.

As seen in Table 1, hydroxyapatite yields decreased significantly as particle size decreased. Put simply, smaller particle size resulted in lower yields (greater sample loss) post-treatment. Large particle size samples averaged 53 ± 11‰, medium samples 43 ± 12‰, and small samples 29 ± 12‰ (Figure 1). Wilcoxon signed–rank tests (the non-parametric equivalent of paired-sample t-tests) indicate that all of the pairings (large–medium, medium–small, large–small) were significantly different in terms of their hydroxyapatite yields ($Z = -3.8$, $n = 19$, $p < .01$).

As seen in Table 1, there are significant negative correlations between collagen yield and hydroxyapatite yield for every particle size (Spearman's P of −0.79, −0.81, and −0.64, for large, medium, and small particle sizes, respectively, $p < .01$ for all). This is not a surprising result because collagen and hydroxyapatite are the two main constituents of bone, and as one yield goes up, the other must go down. However, there were no significant correlations between collagen yield and the *difference* in hydroxyapatite yield between particle size fractions. For large–medium Spearman's P was 0.23, $p = 0.34$, for medium–small $P = -0.34$, $p = 0.15$, for large–small $P = -0.2$, $p = 0.4$. It would thus appear that the lower yields seen in smaller particle size

TABLE 1 Collagen yield and hydroxyapatite yields for large (0.25–0.5 mm), medium (0.125–0.25 mm), and small (0.063–0.125 mm) particle size fractions. Sample J-55 was not included in statistical analyses due to outlying isotope values

	Hydroxyapatite yield (wt%)			
Sample ID	Large	Medium	Small	Collagen yield (wt%)
H-96	39.0	30.0	29.0	19.3
J-41	64.0	57.0	29.0	0.1
J-44	61.0	52.0	33.0	3.6
J-48	53.0	47.0	38.0	15.8
J-49	77.0	66.0	54.0	0.0
J-51	75.0	71.0	47.0	3.2
J-54	66.0	49.0	43.0	7.1
J-55*	65.0	52.0	34.0	7.9
J-60	54.0	47.0	30.0	14.7
L-55	48.0	35.0	21.0	18.3
L-57	37.0	25.0	13.0	23.2
L-59	45.0	27.0	10.0	18.8
L-62	56.0	40.0	30.0	5.6
L-108	50.0	36.0	24.0	5.6
L-109	49.0	34.0	26.0	18.5
L-110	48.0	35.0	17.0	11.8
L-112	45.0	36.0	16.0	9.6
L-114	47.0	38.0	16.0	17.7
L-134	49.0	42.0	36.0	5.7
L-139	53.0	47.0	29.0	12.6

aliquots were not a consequence of the removal of more organic material (collagen) through faster/more complete bleach oxidation. Given that Snoeck and Pellegrini (2015) have found that NaOCl is most effective in removing organics from bone samples, it could simply be that the bleach treatment is effective in removing all, or nearly all, organics from these samples regardless of SA:V.

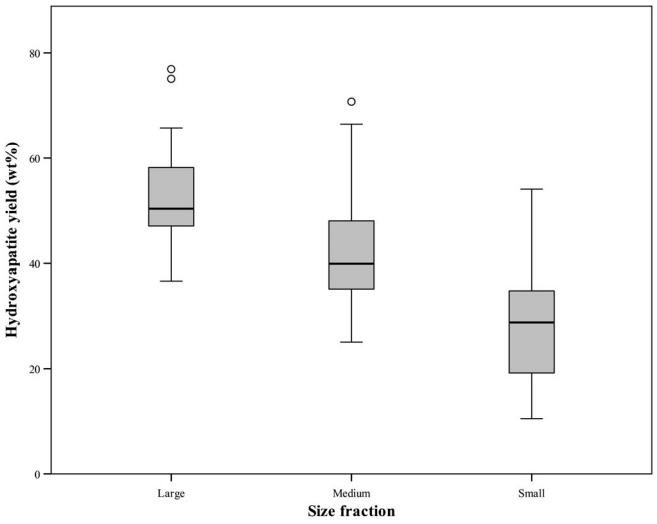

FIGURE 1 Hydroxyapatite yields for large (0.25–0.5 mm), medium (0.125–0.25 mm), and small (0.063–0.125 mm) particle size fractions

Turning next to $\delta^{13}C$ (Table 2), the average carbon isotope value for large particle size was −9.3 ± 1.2‰, for medium size was −9.1 ± 1.1‰, and for small particles, −9.1 ± 1.2‰. While the average difference between large and medium particle size $\delta^{13}C_{ap}$ was an increase of only 0.3 ± 0.3‰, this is nonetheless a statistically significant difference (Wilcoxon signed–rank, $Z = 2.8$, $n = 19$, $p < .01$). The average difference between medium and small particle size $\delta^{13}C_{ap}$ was −0.03 ± 0.3‰, a non-significant difference (Wilcoxon signed–rank, $Z = -0.4$, $N = 19$, $p = .71$). Finally, the average difference between large and small particle size $\delta^{13}C_{ap}$ values was 0.2 ± 0.4‰, a statistically significant difference (Wilcoxon signed–rank, $Z = 2.5$, $N = 19$, $p = .01$).

Considering $\delta^{18}O$ (Table 2), the average $\delta^{18}O_{ap}$ for large particle size was −4.3 ± 1.5‰, for medium-sized particles the average was −3.9 ± 1.7‰, and for small particles, the average was −3.0 ± 1.9. The average difference from large to medium particles was 0.3 ± 1.1, which is not statistically significant (Wilcoxon signed–rank, $Z = 1.2$, $N = 19$, $p = .22$). The average difference between medium and small particle $\delta^{18}O_{ap}$ was 1.0 ± 1.3, which is significant (Wilcoxon signed–rank, $Z = 2.7$, $N = 19$, $p < .01$). Finally, the average difference between large and small particle oxygen isotope ratios was 1.3 ± 1.0, which is statistically significant (Wilcoxon signed–rank, $Z = 3.8$, $N = 19$, $p < .01$).

TABLE 2 Stable isotope values for large (0.25–0.5 mm), medium (0.125–0.25 mm), and small (0.063–0.125 mm) particle size fractions. Sample J-55 was not included in statistical analyses due to outlying isotope values

	$\delta^{13}C_{ap\text{-}VPDB}$ (‰)			$\delta^{18}O_{ap\text{-}VPDB}$ (‰)		
Sample ID	Large	Medium	Small	Large	Medium	Small
H96	−11.7	−11.1	−11.0	−4.2	−2.0	−2.6
J41	−9.6	−8.6	−9.2	−2.8	−2.2	−0.9
J44	−9.5	−9.2	−9.2	−3.2	−3.2	−1.0
J48	−9.7	−9.6	−9.2	−2.3	−2.7	0.7
J49	−8.5	−8.9	−9.3	−2.4	−2.0	−1.5
J51	−8.7	−8.7	−9.0	−1.6	−2.3	−1.7
J54	−8.7	−8.6	−8.7	−2.6	−2.6	−0.9
J55*	−4.1	−8.4	−8.8	−1.1	−1.8	−1.8
J60	−8.8	−8.8	−8.9	−2.6	−1.5	−1.7
L55	−7.1	−6.8	−6.5	−5.3	−5.9	−4.2
L57	−9.6	−9.3	−9.5	−5.7	−4.0	−4.2
L59	−10.6	−10.2	−9.8	−5.4	−3.9	−1.4
L62	−9.8	−9.3	−9.7	−5.3	−4.0	−5.0
L108	−9.5	−9.7	−9.1	−4.4	−5.7	−3.5
L109	−7.5	−7.1	−6.9	−5.0	−5.2	−3.6
L110	−7.7	−7.5	−7.4	−4.7	−5.4	−4.3
L112	−8.6	−8.4	−8.2	−5.0	−5.9	−4.3
L114	−10.4	−9.9	−10.3	−6.1	−4.6	−5.0
L134	−10.7	−10.2	−10.6	−6.0	−4.1	−5.5
L139	−10.1	−10.1	−10.0	−6.3	−7.5	−6.0

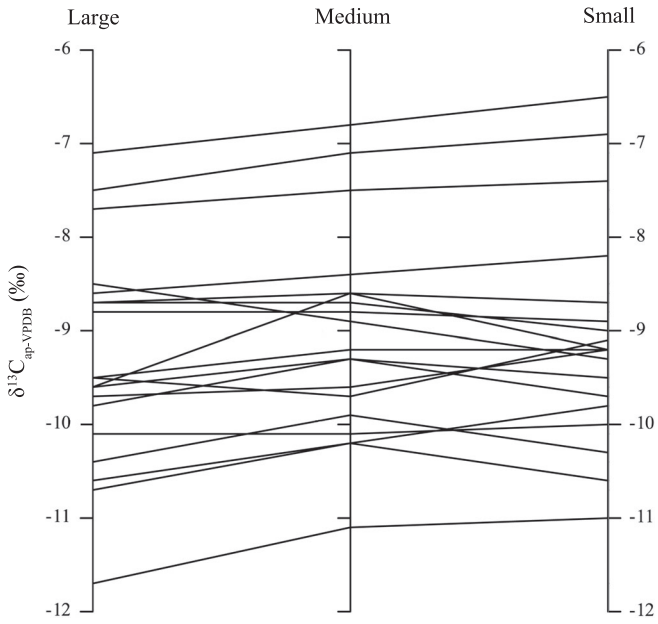

FIGURE 2 Carbon isotope signatures of large (0.25–0.5 mm), medium (0.125–0.25 mm), and small (0.063–0.125 mm) particle size fractions

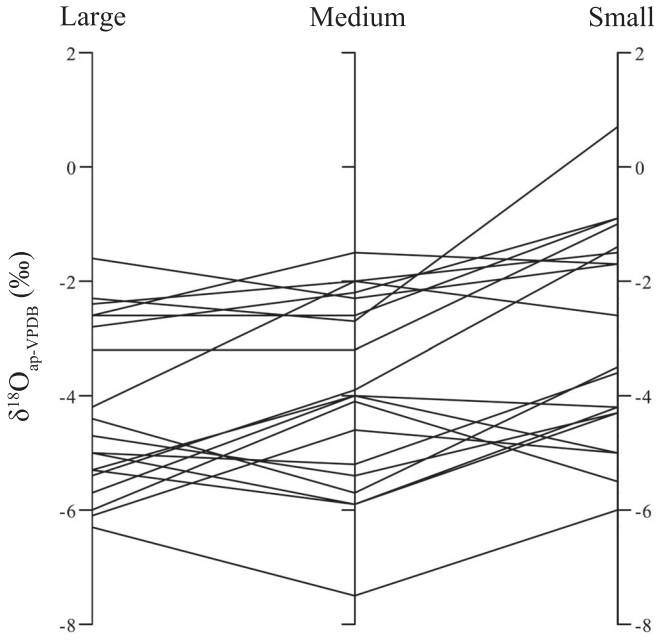

FIGURE 3 Oxygen isotope signatures of large (0.25–0.5 mm), medium (0.125–0.25 mm), and small (0.063–0.125 mm) particle size fractions

While there are differences (sometimes significant) in the average isotope values in the various size fractions, offsets among aliquots of given individuals may be more meaningful for thinking about the potential ramifications of size-induced fractionation.

Beginning first with carbon (Figure 2), although the overall trend is one of moderate ^{13}C enrichment as particle size is reduced, variation between particle size aliquots of the same individual can vary substantially. The range of differences seen between large and medium particle sizes extended from −0.4–1‰, between medium and small from −0.6–0.6‰, and between large and small from −0.8–0.8‰. Overall, then, particle size difference could engender variation, in the same individual, ranging from a decrease of 0.8‰ to an increase of 1.0‰ in $\delta^{13}C$. While the variation among size fractions of any one sample might be small (≤1‰) it is significantly larger than instrumental precision and could cause interpretative errors.

For oxygen (Figure 3), these individual differences are even greater. As with carbon isotopes, smaller particle size generally produces higher $\delta^{18}O_{ap}$ values, but with a greater range of variation. Values for differences in $\delta^{18}O_{ap}$ between large and medium particle size ranged from −1.3–2.2‰, between medium and small particle size ranged from −1.4–3.4‰, and between large and small particles from −0.1–4.0‰. Overall, then, particle size difference could engender variation in the same individual, ranging from a decrease of 1.4‰ to an increase of 4‰ in $\delta^{18}O$. As with $\delta^{13}C_{ap}$, these ranges far exceed instrumental error, resulting in a substantial increase of isotope values as particle size decreases; they are of such a magnitude (as much as a 5.4‰ difference) as to cause major interpretive errors if not considered correctly.

4 | DISCUSSION

These data make clear that differences in particle size used for hydroxyapatite isotope analysis can engender large isotopic differences, particularly for the oxygen isotope system. Indeed, it would appear that changes in particle size can produce effects of similar magnitude to better-understood changes in acid concentration and treatment time. Smaller particle size would appear to bring about increases in both $\delta^{13}C$ and $\delta^{18}O$, which does not match identically the results obtained by Garvie-Lok et al. (2004). In that study, $\delta^{18}O$ was found to consistently increase with increased acid treatment time, which is the same effect we observed with smaller particle size. However, whereas $\delta^{13}C$ was found to decrease with increased acid treatment time in that study, we observed an increase with smaller particle size (Garvie-Lok et al., 2004, p. 769). Thus, in our experiment, $\delta^{18}O$ follows the same trend as observed by Garvie-Lok et al. (2004), but $\delta^{13}C$ does not. We would also note that while most individuals exhibit consistent changes between $\delta^{13}C$ and $\delta^{18}O$, some individuals do not, and as a result further investigation into these phenomena appear warranted.

Our results are, however, in general agreement with those found by Pellegrini and Snoeck, who observed higher $\delta^{13}C$ subsequent to NaOCl treatment (an effect that was mitigated somewhat after acetic acid treatment), a shift that they attribute to the precipitation of exogenous carbonates derived from atmospheric CO_2 (Pellegrini & Snoeck, 2016, p. 94). The authors argue that the high pH of NaOCl causes atmospheric CO_2 to dissolve and convert, in solution, into carbonic acid then bicarbonate and carbonate ions, which readily precipitate as secondary calcium carbonate on the hydroxyapatite crystals (Pellegrini & Snoeck, 2016, p. 94). Because atmospheric CO_2 has $\delta^{13}C$

values higher than typical archaeological hydroxyapatite, such precipitation will lead to higher sample $\delta^{13}C$ values. Those authors found more variable results when it came to $\delta^{18}O$, although they observed uniform increases following combined NaOCl and acetic acid treatment (Pellegrini & Snoeck, 2016, p. 92), which also conforms with our findings. Precisely how the shift in oxygen isotope composition is occurring is unclear given the general similarity between sample and atmospheric $\delta^{18}O$ (Pellegrini & Snoeck, 2016, p. 94).

Although the ultimate cause of the particle size/SA:V related shifts observed in our data cannot be determined categorically, we can propose more and less likely drivers. Given that there were no consistent correlations between sample collagen yield and the isotopic shifts seen between particles of different size, differential removal of organic materials seems an unsatisfactory explanation. If the observed effects were a consequence of more complete removal of organics in higher SA:V particles, we would expect to find larger shifts in samples that had higher organic content to begin with. As such, dissolution/recrystallization and/or precipitation and incorporation of atmospheric carbon and oxygen, the effect of which is exacerbated in particles with greater SA:V, seems the most likely culprits. We posit that the hydroxyapatite crystals on the smaller-sized particles tend to dissolve and recrystallize more quickly or more completely (absolutely or proportionally), or that they simply present more surface area for the precipitation of adsorbed carbonates, thereby incorporating more exogenous (atmospheric or reagent-derived) carbon and oxygen and producing the observed isotopic shifts. Recrystallization has previously been judged to be responsible for observed isotope differences related to acid strength and treatment time (Garvie-Lok et al., 2004; Nielsen-Marsh & Hedges, 2000), and precipitation of atmospheric carbon (and possibly oxygen) was suggested as the mechanism driving isotopic shifts after more concentrated NaOCl treatments. We suggest that it is possible that a similar outcome is produced by an increase of the surface area to volume ratio. Put simply, starting with smaller particles (having greater SA:V) has a similar effect to the use of more concentrated reagents or the use of longer treatment times, both of which have been shown to engender isotopic shifts.

The magnitude of the differences observed in the present case are startling. Indeed, they are large enough, particularly in the case of oxygen, to have meaningful interpretive implications. As an illustration, the observed differences between small and large particles from the same individual for $\delta^{18}O$ were greater than 4‰. This large a difference could lead one to geolocate or source an individual far from her/his actual place of origin. Indeed, a 4‰ difference in the $\delta^{18}O$ of consumed water is equivalent to the difference between surface waters in Southern Florida and Northern Illinois, points some 2,000 km distant from one-another (Kendall & Coplen, 2001).

It is clear that more work needs to be done on this topic. However, it is also clear that particle size is one of a number of variables that can cause large differences in the isotopic signatures of identical samples, a finding that has significant implications for the comparability and accuracy of a variety of studies utilizing stable isotope analysis.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

All pertinent data are presented in the manuscript.

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