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Effects of decalcification on bulk and compound-specific nitrogen and carbon isotope analyses of dentin

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RATIONALE: For bulk carbon and nitrogen isotope analysis of dentin, samples are typically decalcified. Since the non-protein carbon in dentin is low, whole sample analysis may produce reliable data. Compound-specific isotope analysis (CSIA) of bone and tooth dentin protein is a powerful tool for reconstructing the flow of carbon and nitrogen in modern and past food webs. Decalcification has also been used to prepare bone and dentin samples for CSIA, but the effects of this process on bulk dentin, amino acid composition, and their specific isotope values are not known.
METHODS: The bulk isotope values of raw and decalcified dentin from a sperm whale tooth were measured to determine the effects of decalcification and the accuracy of untreated dentin results. CSIA was also performed on decalcified and raw dentin to examine differences in the amino acid isotope values and molar composition between these two approaches.
RESULTS: Analysis of raw dentin yields precise and accurate bulk isotope measurements for this animal. The isotopic values of decalcified samples and raw dentin for individual amino acids were similar, but the average of the isotope value offsets between the two sample types was significant. The presence of inorganic material complicated raw sample processing for individual amino acid isotope values, and may have contributed to the isotopic differences between decalcified and raw samples.
CONCLUSIONS: Decalcification is not needed to measure bulk isotope values in dentin from this modern odontocete, probably because the lipid and carbonate concentrations are low and the carbon isotope values of dentin protein and carbonate are similar. This method should not be applied in some cases (e.g., with fossil dentin and modern bone). Decalcification should still be used prior to CSIA since significant matrix issues occur with raw dentin processing and decalcification does not alter the amino acid molar composition or isotopic values of dentin. Copyright © 2014 John Wiley & Sons, Ltd.

Carbon and nitrogen isotope ($\delta^{13}C$ and $\delta^{15}N$ values) analysis is a powerful tool for determining an animal's diet and the flow of carbon and nitrogen in a food web.[1–3] Bone or tooth collagen is often well preserved over long periods of time (from centuries to tens of millennia),[4] and is essential for reconstructions of modern and prehistoric diets via $\delta^{13}C$ and $\delta^{15}N$ analysis.[5–11] Yet, bulk collagen $\delta^{13}C$ and $\delta^{15}N$ values are influenced not just by diet composition, but also by biochemical cycling processes that influence the isotopic values at the base of the food web, which may shift over time and space.[12,13] Compound-specific isotope analysis (CSIA) of amino acids (AA) is now a valuable technique in ecological and biogeochemical studies for teasing apart diet versus baseline effects on a consumer's $\delta^{13}C$ and $\delta^{15}N$ values,[14,15] and it has also been widely used to determine trophic level for many taxa.[12,14,16] For instance, Germain *et al.* produced accurate trophic position estimates for harbor seals via a multi-trophic enrichment factor calculation.[17] However, it is possible that conventional decalcification prior to bulk or CSIA could alter the bulk protein, and the amino acid molar or isotopic compositions of the bone or tooth collagen. If so, trophic position and other data derived from bulk isotope analysis and/or CSIA of amino acids could be significantly compromised. It is therefore crucial to investigate the potential effects of this key chemical treatment on the stable isotope composition of collagen.

Collagen comprises 20 to 30% of the tissue by dry weight in both dentin and bone. Lipids comprise a significant fraction of the dry weight of bone, whereas, in dentin, they occur at such a low concentration that they are not a concern (20 mg total lipid/100 g bovine dentin).[18] To remove biogenic apatite, sedimentary carbonates, and fulvic acids, samples are typically demineralized in acid – often HCl – or a chelating agent, the supernatant containing the contaminants is discarded, and the residue containing the protein is rinsed to neutrality; the resulting material is considered to be collagen.[2,7] If humic acids are present, they are removed by a subsequent NaOH treatment.[2,7]
Lipids are typically removed from bone samples by treatment with an organic solvent (e.g., chloroform/methanol solution, petroleum ether).[19] These procedures remove non-protein components, including endogenous materials such as carbonate-

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bearing biological apatite (a phosphate mineral) and lipids, as well as exogenous contaminants (e.g., sedimentary carbonates, humic and fulvic acids), since they may distort isotopic measurements.

Decalcification methods could alter the isotopic composition of proteins in at least three different ways. First, water-soluble proteins other than collagen may be lost when the supernatant is discarded.[20,21] If the discarded proteins have a different isotopic composition from the remaining proteins, the bulk isotope results from treated samples may not represent the original material, although they would offer a cleaner value for bone or dentin collagen. Second, if demineralization partially hydrolyzes protein in a manner that causes a preferential loss of certain amino acids, decalcification would change the amino acid composition of the residue, which might in turn alter the bulk isotope values. Third, if demineralization breaks amide bonds, this would be expected to preferentially affect amide bonds containing low mass isotopes, such that the isotopic composition of the remaining collagen would be shifted to higher $\delta^{13}C$ and $\delta^{15}N$ values, as suggested by Bada *et al.* and Silfer *et al*.[22,23] The last process could change both bulk isotope values and the data generated by CSIA.

To avoid these potential effects of demineralization, an alternative option for isotopic analysis of dentin would be to directly analyze raw material from modern animals. This approach has, to our knowledge, never been carefully explored, but at least one group has taken this approach after reporting that experimental results (which were not presented in detail) showed that decalcified and raw samples yielded similar results.[24] This approach should not entail the potential consequences of altering the amino acid or isotopic composition of bone or dentin protein, which may occur in decalcification due to protein loss. Direct isotope analysis on raw bone or teeth is never recommended for fossil material because these sample types contain exogenous contaminants.[7] Yet even for modern samples, isotopically distinct non-protein components (e.g., lipid, carbonate) and abundant non-collagenous proteins might make analysis of raw material problematic, if the ultimate goal is to measure collagen isotope composition. Furthermore, components of the inorganic matrix might interfere with the chemical processing required for chromatography and CSIA.

In this study, we evaluate the effects of decalcification on both bulk and compound-specific amino acid stable isotope values of dentin using homogenized material from the tooth of a sperm whale (*Physeter macrocephalus*) collected in recent decades. We first examine the potential for analyzing dentin powder for bulk nitrogen and carbon isotope ratio values of modern samples. Next, we compare the results of individual amino acid $\delta^{13}C$ and $\delta^{15}N$ analysis and also amino acid molar percentage analysis, between hydrolysates of conventionally decalcified samples and raw tooth dentin. This study has four main components. First, we assess the necessity of treating dentin with a conventional decalcification method for bulk isotope analysis by directly comparing results from analysis of raw and decalcified dentin. Second, CSIA results are compared between decalcified and raw samples to assess if CSIA yields significantly different values. Third, the mole percentages of the amino acids in decalcified and raw samples are compared to determine if decalcification leads to any significant selective loss of some amino acids. Finally, we compare bulk raw dentin isotope values with corresponding $\delta^{15}N$ and $\delta^{13}C$ values for total hydrolyzable protein, derived from the CSIA of amino acids to assess the importance of inorganic matrix effects in raw dentin.

EXPERIMENTAL

Sample pretreatment

Dentin powder (approximately 100 mg) was collected across most of the growth layer bands deposited in the whale tooth using a hand-drill (Urawa Corporation, Kukshi, Japan; model no. UP201). This sample was homogenized manually and, together, encompasses the juvenile, maturing and adult stages of the whale (neonatal growth bands were excluded). Two sets of dentin powder were used as follows: one set for the decalcification treatment ('conventional decalcification') and the second set for raw dentin experiments (i.e., the control for bulk isotope analysis, and direct hydrolysis of raw dentin for CSIA). For each set, a total of ten samples were analyzed for bulk $\delta^{13}C$ and $\delta^{15}N$ values, and three samples for compound-specific $\delta^{13}C$ and $\delta^{15}N$ values.

For the decalcified treatment set, ~1–2 mg of powder dentin were acidified by adding 1 mL of 0.5 M HCl and then stored at 4°C for 22 h. The samples were then centrifuged at 10,000 rpm for 5 min, HCl solution was removed via aspiration, and the expected purified collagen was rinsed five times with Milli-Q water (Millipore, Billerica, MA, USA). Samples were stored at –20°C until they were freeze-dried in a Lyph Lock 4.5 freeze dry system (Labconco, Kansas City, MO, USA), and stored soon after in a desiccator until they were prepared for bulk and CSIA.

Bulk isotope analysis

Both raw dentin samples and extracted protein (mainly collagen) were weighed into tin cups (3 × 5 mm; Costech, Valencia, CA, USA) for elemental analyzer/isotope ratio mass spectrometry (EA/IRMS). These samples were analyzed for bulk $\delta^{13}C$ and $\delta^{15}N$ values in the Stable Isotope Lab at University of California at Santa Cruz on a EA 1108 elemental analyzer (Carlo Erba, Milan, Italy) coupled to a Thermo Finnigan Delta^{Plus} XP isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). The $\delta^{13}C$ values were referenced to a V-PDB standard, while $\delta^{15}N$ values were referenced to an AIR standard. Mass and drift corrections were performed by the analysis of gelatin standard replicates during each instrument session. Comparisons of standards resulted in standard deviations that were <0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$ values (7 standards analyzed at the start of each session, and a standard analyzed after every 8 samples during the session). We also report atomic C:N ratios (See Results and Discussion section).

Apatite in whale tooth dentin was extracted in order to measure the percentage carbonate. To extract apatite, hydrogen peroxide (30% H_2O_2) was added to raw dentin samples. After ~24 h, the liquid was removed by aspiration and the samples were rinsed five times with Milli-Q water. 1.0 M acetic acid (pH ~5) was then added to the samples. After ~24 h in the refrigerator (4°C), the acetic acid was also removed by aspiration, and the dentin samples were rinsed again five times with Milli-Q water and then freeze-dried.

The percentage carbon (converted into percentage carbonate) was measured on a model CM5012 carbon coulometer with a CM5130 acidification module (UIC, Inc., Joliet, IL, USA). The percentage carbonate was measured on two samples of whale dentin powder.

Compound-specific amino acid analysis

Prior to compound-specific isotope analysis via gas chromatography/isotope ratio mass spectrometry (GC/IRMS), all the samples were hydrolyzed and derivatized using methods described in McCarthy *et al.*[25,26] Briefly, hydrolysis was conducted under standard conditions (6 N HCl for 20 h at 110°C), and trifluoroacetic anhydride (TFAA) derivatives were prepared. For raw samples, acid hydrolysis dissolves the apatite mineral, such that calcium, phosphate and other inorganic constituents are present in solutions in subsequent preparation steps; these inorganic constituents are absent in samples that have been conventionally decalcified. The samples were stored in a −20°C freezer in a 1:3 TFAA/DCM (methylene chloride) mixture until the day on which they were analyzed. Immediately prior to analysis, the TFAA/DCM mixture was evaporated under a flow of N_2 and the samples were diluted in ethyl acetate.

The samples were analyzed for amino acid carbon and nitrogen isotope composition on a Thermo Trace gas chromatograph coupled to a Thermo Finnigan Delta^{Plus} XP isotope ratio mass spectrometer (oxidation furnace at 980°C (N) or 940°C (C) and reduction furnace at 650°C (N) or 630°C (C)). For $\delta^{13}C$ analyses, a DB-5 column (50 m × 0.32 mm, 0.52 μm film thickness; Agilent Technologies, Santa Clara, CA, USA) was used. For $\delta^{15}N$ analyses, a BPX5 column (60 m × 0.32 mm, 1 μm film thickness; SGE Analytical Science, Trajan, Austin, TX, USA) was used. The thicker film allowed the higher amino acid loadings required for measuring CSIA $\delta^{15}N$ values. The injector temperature was 250°C with a split He flow rate of 2 mL/min. The GC temperature program for carbon isotope analysis was: initial temp = 75°C hold for 2 min; ramp 1 = 4°C/min to 90°C, hold for 4 min; ramp 2 = 4°C/min to 185°C, hold for 5 min; ramp 3 = 10°C/min to 250°C, hold for 2 min; ramp 4 = 20°C/min to 300°C, hold for 5 min. The GC temperature program for nitrogen isotope analysis was: initial temp = 70°C hold for 1 min; ramp 1 = 10°C/min to 185°C, hold for 2 min; ramp 2 = 2°C/min to 200°C, hold for 10 min; ramp 3 = 30°C/min to 300°C, hold for 6 min. The amino acid $\delta^{13}C$ values were determined from the measured values of the amino acid derivatives following the approach of Silfer *et al.*[27] with corrections based on an amino acid mixture standard for which isotopic values had been independently determined by offline elemental analyzer analysis. The directly measured amino acid $\delta^{15}N$ values were also corrected based on bracketing external standards, as described in McCarthy *et al.*[26]

Using this approach, the $\delta^{13}C$ values of 13 amino acids could be reproducibly quantified in sperm whale dentin. These were: alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), hydroxyproline (Hpro), aspartic acid + asparagine (Asp), glutamic acid + glutamine (Glu), phenylalanine (Phe), and lysine (Lys). For analysis of $\delta^{15}N$ values, the same amino acids were measured, except that with the thicker film column Hpro and Pro typically co-eluted; therefore, for calculation of $\delta^{15}N$ values and molar percentages (see below), data from these peaks were combined and designated as Pro + .

The amino acid molar percentages (Mol%) were determined from the CSIA peak areas using an external standard approach and based on the amino acid standard versus sample peak areas. Because the $\delta^{15}N$ CSIA chromatography was both cleaner and more reproducible in the samples, we used these peak areas for our primary Mol% determinations.

Data analysis

All statistical tests were carried out in R statistical software.[28] Since most isotopic values failed the normality tests, a Box-Cox transformation was performed on all data.[29] For both $\delta^{13}C$ and $\delta^{15}N$ values, Student's *t*-tests were conducted for each amino acid to determine whether differences between the decalcification and raw methods were statistically significant. The differences between the decalcification and raw isotope values for each amino acid were calculated, as well as the average difference for all amino acids. To determine whether the average difference was significantly different from zero, we used a one-sample Student's *t*-test for a mean (null hypothesis: mean offset = 0). Student's *t*-tests were also performed on the Box-Cox-transformed Mol% values for each amino acid (for decalcified and raw samples) to identify significant differences in amino acid composition between the methods.

The isotopic results for total hydrolyzable amino acids (THAA), proxy values of total protein,[26,30] were calculated by taking mole percentage weighted averages of the carbon or nitrogen isotope values (the THAA $\delta^{15}N$ and $\delta^{13}C$ values are designated as $\delta^{15}N_{THAA}$ and $\delta^{13}C_{THAA}$, respectively). The isotopic values of Pro and Hpro were integrated together (as noted above, designated in results as Pro+), since these two amino acids co-eluted during the analysis. In total, seven protein amino acids that have been reported in collagen – arginine, histidine, methionine, tyrosine, hydroxylysine, tryptophan, and cysteine – were not detected in our analysis, either because of low concentration or because they are not measurable by our derivatization system. Therefore, any amounts that might be present in sperm whale dentin collagen are missing from the THAA calculations. However, the lack of data from these amino acids does not invalidate the THAA calculations because they only account for ~8% of the total amino acid pool in dentin collagen; see tables from Piez and Eastoe.[31,32] In particular, tryptophan and cysteine are consistently reported as being absent or at trace levels (<0.5% of the total amino acids) in dentin from mammals.[31–34] Student's *t*-tests for a mean were used to determine statistically significant differences between the THAA and bulk isotope values for both $\delta^{13}C$ and $\delta^{15}N$, and for each treatment (conventional decalcification and raw).

Since some protein is lost during conventional decalcification, the sample recovery was also determined for decalcified samples using the nitrogen amino acid mole percentage data. A yield for each sample (in moles) was calculated based on the yield of every amino acid in the sample (in nanomoles) from the mole percentage calculation, as well as the known GC/IRMS injection amount, dilution, and initial weight of the sample. The amino acid recovery for conventionally decalcified samples was the

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comparison of the yields (in moles) of the amino acids for decalcified samples and the corresponding amino acid yields for raw samples.

RESULTS AND DISCUSSION

Is decalcification necessary prior to bulk isotope analysis of dentin?

The bulk $\delta^{15}N$ and $\delta^{13}C$ values of the raw and conventionally decalcified samples were compared to assess whether decalcification is necessary to obtain accurate bulk isotope data from sperm whale dentin. The $\delta^{15}N$ and $\delta^{13}C$ values of raw and decalcified dentin were not significantly different (Fig. 1); however, the mean atomic C:N ratio of raw dentin (3.4 ± 0.02, $n = 10$) was significantly higher than that of decalcified dentin (3.2 ± 0.03, $n = 9$) ($p < 0.001$, data not shown).

The lack of any statistically significant difference in $\delta^{15}N$ values between raw and decalcified dentin samples suggests that if water-soluble proteins are lost during decalcification, they have roughly the same bulk $\delta^{15}N$ values as collagen (the protein that is not water soluble), and if any differential loss of particular amino acids occurs during treatment, this loss is too small to bias the bulk isotope results. This same reasoning applies to the lack of a significant difference in $\delta^{13}C$ values between raw and decalcified samples.

However, in the case of carbon, the raw samples contain a non-protein carbon source: the carbonate in biological apatite. The slightly higher C:N ratio in raw samples than in conventionally decalcified samples is therefore consistent with apatite carbonate containing a few weight percentage carbonate (Supplementary Table S1, Supporting Information). The average collagen yield following decalcification of our samples for bulk analysis ($n = 10$) was $24.0 \pm 1.8\%$ (a slight underestimate given unavoidable sample loss during the removal of acid and rinsing) and the average concentration of carbonate in biological apatite was 3.7% ($n = 2$). The average $\delta^{13}C$ value of dentin carbonate was -10.9 ± 0.1‰ ($n = 3$), thus approximately 2‰ enriched in ^{13}C relative to dentin collagen (-12.9 ± 0.1‰, $n = 9$), which is in line with expectations from studies of $\delta^{13}C_{apatite\text{-}collagen}$ values (equal to $\delta^{13}C_{bioapatite\ carbonate} - \delta^{13}C_{collagen}$) in other carnivorous marine mammals.[35] Isotope mass balance considerations suggest that, given this $\delta^{13}C_{apatite\text{-}collagen}$ value and these protein and carbonate concentrations, the raw samples should be no more than 0.1‰ ^{13}C-enriched relative to the collagen derived from conventional decalcification (Supplementary Table S1, Supporting Information). Our observation that the bulk $\delta^{13}C$ values of raw samples were not significantly different from the $\delta^{13}C$ values of collagen samples is consistent with this calculation (Fig. 1(b)); given that the EA/IRMS analytical error is of a similar magnitude to the theoretical $\delta^{13}C$ offsets, the differences due to apatite carbonate should be too small to be reliably observed.

The potential effect of apatite carbonate on the bulk $\delta^{13}C$ values of raw dentin depends on both its relative concentration and its isotopic ratio value. These same mass balance calculations indicate that even if the collagen concentration were as low as 20% and the apatite carbonate concentration as high as 6%, if the $\delta^{13}C_{apatite\text{-}collagen}$ value is 2‰, the raw samples will be no more than 0.2‰ ^{13}C-enriched relative to collagen. However, if the $\delta^{13}C_{apatite\text{-}collagen}$ value is higher, the divergence between the raw values and those of collagen may increase outside an acceptable range in samples where the collagen concentration is low and the carbonate concentration is high. Clementz *et al.* reported that terrestrial carnivores have $\delta^{13}C_{apatite\text{-}collagen}$ values of 4–6‰, and the values for terrestrial and marine herbivores may be higher still (6–9‰).[35] As a consequence, we strongly recommend measuring collagen and carbonate concentrations and isotope compositions on a test suite of samples for a new taxon before proceeding without decalcification to measure collagen $\delta^{13}C$ values in dentin. To reiterate a point made above, the analysis of raw bone or fossil material is not advised because of the potential presence of lipids and post-mortem contaminants.

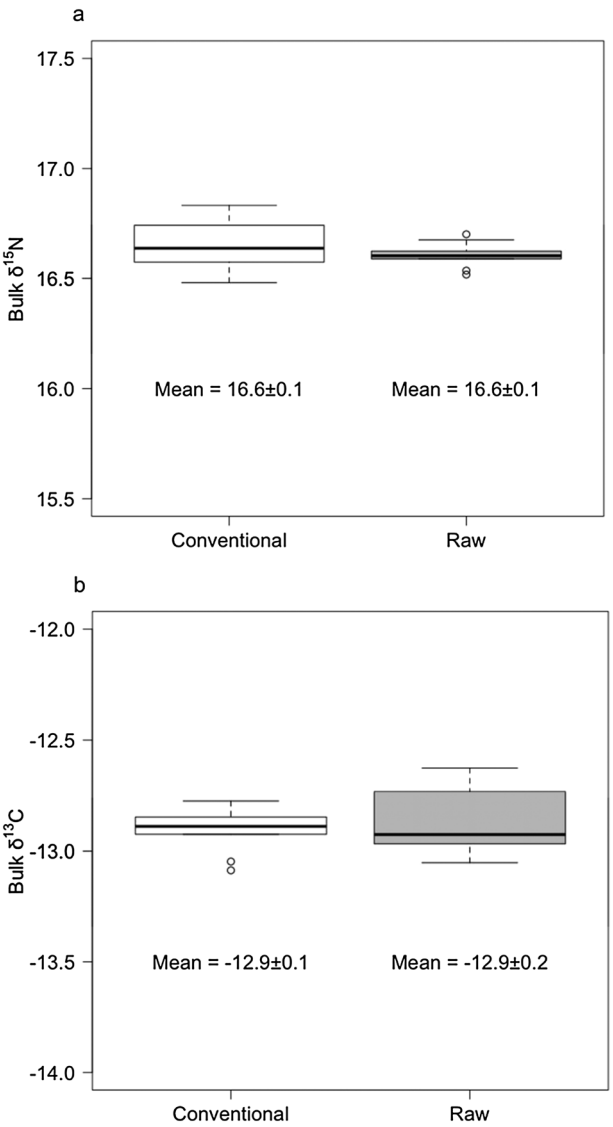

Figure 1. Bulk isotope values of conventionally decalcified ('Conventional') and raw dentin. Whisker plots show comparisons for replicate analyses ($n = 10$) for (a) bulk $\delta^{15}N$ values and (b) bulk $\delta^{13}C$ values. No significant differences were found between raw (grey) and decalcified (white) bulk dentin $\delta^{15}N$ and $\delta^{13}C$ values. The bold line within the box indicates the median, the upper edge of the box is the 75th percentile, the lower edge of the box is the 25th percentile, the upper whisker is the maximum value excluding outliers, and the lower whisker is the minimum value excluding outliers.

Does decalcification alter compound-specific isotope values?

Individual amino acid isotope values and precision

For both raw dentin and conventionally decalcified dentin, three replicate samples were prepared and then subjected to compound-specific isotope analysis. The average standard deviation for replicate determinations of $\delta^{15}N$ values for all amino acids (excluding Thr, see comment below) was low: 1.0‰ and 0.5‰ for decalcified and raw dentin, respectively. Consistent with observations for raw and decalcified bulk isotope values, the relative $\delta^{15}N$ values for decalcified and raw samples across all amino acids were also very similar (Fig. 2(a)). The general pattern of $\delta^{15}N$ values for the amino acids measured corresponds closely with expectations for heterotrophs,[12,14] with the trophic amino acid (AA) group (Glu, Asp, Ala, Ile, Leu, Pro+, and Val) having substantially higher $\delta^{15}N$ values than the source AA group (Gly, Ser, Lys, Phe). We note that Thr, now often classified as 'metabolic', does not fall into either category; it has extremely low $\delta^{15}N$ values (Supplementary Fig. S1, Supporting Information), as has also been previously reported for other marine mammals.[17] However, the standard deviation for Thr was also unusually high due to chromatographic issues (e.g., peak tailing and poor peak resolution in these runs). Therefore, the Thr $\delta^{15}N$ values were excluded in subsequent discussion, as well as from comparisons of the average $\delta^{15}N$ offset across all amino acids between decalcified and raw treatments. For all other amino acids except Lys, amino acids derived from decalcification had slightly lower $\delta^{15}N$ values than those from hydrolysates of raw dentin. Yet, none of the $\delta^{15}N$ differences were statistically significant at the individual amino acid level.

Phe and Glu are particularly important in the analysis of food web structure.[12,14] As noted above, the $\delta^{15}N$ values were not statistically different for either amino acid after decalcification, but Glu did have consistently better analytical precision than Phe. After decalcification, Glu had low $\delta^{15}N$ variation across the replicates (1 sd = 0.4‰), while the variability was higher for Phe (1 sd = 1.9‰). In comparison, the standard deviations for $\delta^{15}N$ values for raw dentin samples were 0.6‰ and 1.1‰ for Glu and Phe, respectively. These latter values are similar to the analytical precision typical of other natural sample types such as plankton,[25] mussels,[36] and seals.[17] It is possible that the relatively low molar abundance of Phe in dentin, coupled with chromatographic separation of Phe in decalcified sample runs, contributed to higher variability in the Phe $\delta^{15}N$ values. Overall, the observation that the standard deviation of $\delta^{15}N$ measurements across all amino acids except Thr was ≤1‰ for both treatments (decalcification and raw) indicates that the precision of CSIA for dentin protein is comparable with that expected for other biological samples. The lack of any statistically significant differences in $\delta^{15}N$ values for any amino acid further indicates that conventional decalcification does not alter the amino acid $\delta^{15}N$ values in any appreciable way.

As with the $\delta^{15}N$ values, the $\delta^{13}C$ values for the decalcified and raw dentin samples were very similar across all amino acids (Fig. 2(b)). At the same time, for most amino acids (except Val, Lys and Ala), the $\delta^{13}C$ values following decalcification were slightly lower than those from raw dentin. However, as with the $\delta^{15}N$ values, these differences were not statistically significant. The single exception was Ser, which had a significantly higher $\delta^{13}C$ value in raw dentin (p = 0.014, Fig. 2(b)). We believe this exception was due to the poor chromatography for Ser in these samples (see Experimental section). The analytical variability for Ser and Lys was anomalously high for both decalcified (1 sd = 3.3‰ and 3.9‰, respectively) and raw (1 sd = 2.8‰ and 4.0‰, respectively) samples, due to peak tailing and relatively poor resolution. Omitting Ser and Lys, the average $\delta^{13}C$ standard deviation for replicate analyses of decalcified samples was 2.1‰, while it was 1.1‰ for raw samples.

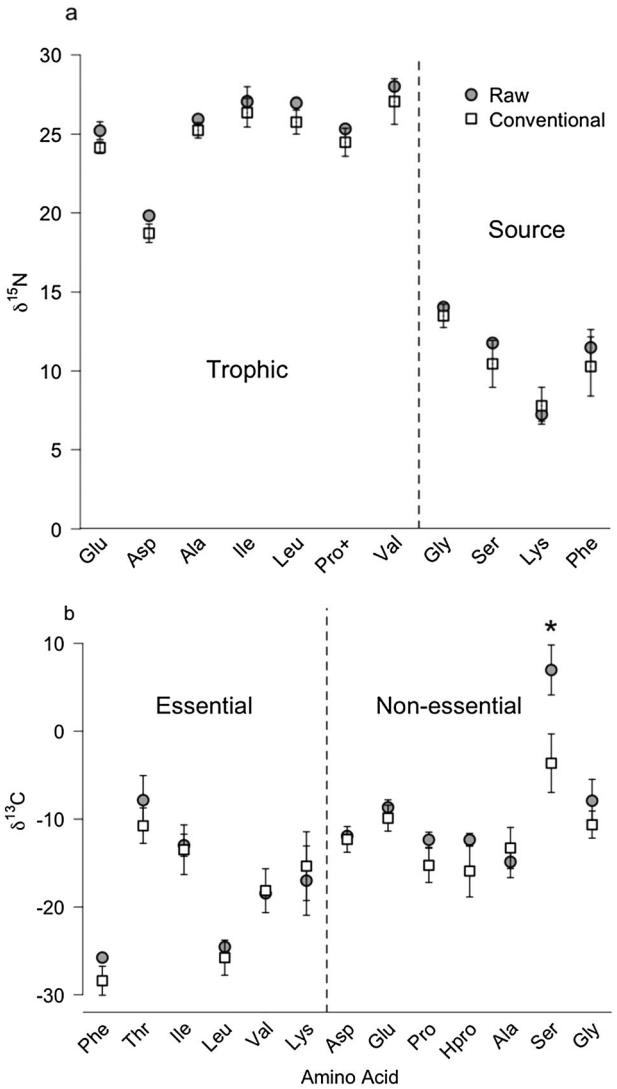

Figure 2. Amino acid isotope values for conventionally decalcified and raw dentin. Comparison of average amino acid isotope values from hydrolysates of conventionally decalcified dentin (white squares) versus raw dentin (filled circles). n = 3 for each treatment; symbols represent the mean and errors bars are standard deviations. (a) $\delta^{15}N$ values; there are no significant $\delta^{15}N$ differences for any amino acid (Student's t-test). As noted in the text, Thr $\delta^{15}N$ values are not shown (see Supporting Information). (b) $\delta^{13}C$ values; a statistically significant difference was observed for just one amino acid (Ser; p = 0.014, Student's t-test), but, as noted in the text, Ser had substantial chromatographic issues.

Overall, comparison of both the $\delta^{15}N$ and $\delta^{13}C$ values indicates that decalcification does not significantly alter the stable isotope values of amino acids in dentin. The analytical precision obtained from decalcified and raw sample types was generally similar to and consistent with that expected

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from other biological samples. Together, these observations suggest that decalcification should not appreciably alter the accuracy of individual amino acid isotope values, or parameters (such as trophic position estimates) derived from them.

What factors may have contributed to the pattern of amino acids in conventionally decalcified dentin being depleted in ^{15}N and ^{13}C relative to those in raw dentin?

Despite the absence of any statistically significant differences in isotopic values for individual amino acids, a consistent *pattern* in isotopic offset was observed for both $\delta^{13}C$ and $\delta^{15}N$ values in decalcified versus raw samples. The mean isotope offsets for all amino acids averaged together (decalcified dentin-raw dentin) were -0.7 ± 0.8‰ for $\delta^{15}N$ values (Fig. 3(a)) and -1.9 ± 3.1‰ for $\delta^{13}C$ values (Fig. 3(b)). Both these mean offsets were significantly different from zero ($p = 0.007$ and $p = 0.045$, respectively). Thus, on average, the amino acids were depleted in *both* ^{13}C and ^{15}N after decalcification compared with those derived from the raw dentin. Although the isotopic offsets are relatively small, the patterns are also consistent, and, therefore, could indicate an analytical issue with one or both of the sample types.

Several mechanistic explanations might underlie these observations. First, as noted above, an unavoidable effect of conventional decalcification is a loss of water-soluble proteins. If these proteins were compositionally or isotopically distinct, the amino acid compositions – and their corresponding isotope ratios – of isolated protein would be altered. In addition, partial hydrolysis of dentin proteins could result in the selective loss of some specific amino acids, and possibly an associated fractionation that might affect the isotopic composition of the residual (non-hydrolyzed) material. Finally, it is possible that the inorganic matrix of the raw dentin affects different aspects of the analysis, from chemical derivatization to instrumental analysis, with the potential to affect both molar and isotopic compositions. Therefore, it is important to stress that the isotopic values derived from raw dentin cannot *a priori* be assumed to be more accurate than those derived from decalcification.

To assess the mechanisms related to the loss of isotopically distinct water-soluble proteins, we compared amino acid distributions between raw and conventionally decalcified dentin. Because collagen has a unique amino acid composition (with high Mol% Gly and Pro+), it is unlikely that a substantial amount of some other protein could be lost in decalcified samples without changing the amino acid composition between raw and decalcified samples. In fact, the Mol% distributions were consistent for both raw and decalcified dentin (Fig. 4). For most amino acids there was no significant difference in Mol% values, with the exception of Ala, Val, and Glu ($p = 0.038$, 0.014, 0.029, respectively). An alternative Mol% calculation approach, based on peak areas from $\delta^{13}C$ analysis, gave similar results (Supplementary Fig. S2, Supporting Information). The few amino acids having significant differences in Mol% between raw and decalcified samples (Ala, Val, and Glu) also do not suggest a consistent pattern of amino acid loss for either procedure. For example, Ala and Val have higher Mol% values in the raw dentin, whereas Glu has a higher Mol% in the decalcified samples. However, because Mol% calculations are by definition internally normalized (i.e., expressed as a percentage of the total amino acids recovered), a change in recovery of any single amino acid will affect the Mol% of all others. It is typically quite difficult, therefore, to reliably interpret variable offsets in just a few amino acids. Overall, the broad conclusion from the Mol% comparison (Fig. 4) is that decalcification does not alter dentin amino acid composition in any substantial way. Thus, it is unlikely that a significant amount of water-soluble protein (with potentially different isotope values for individual amino acids) was selectively lost during conventional decalcification.

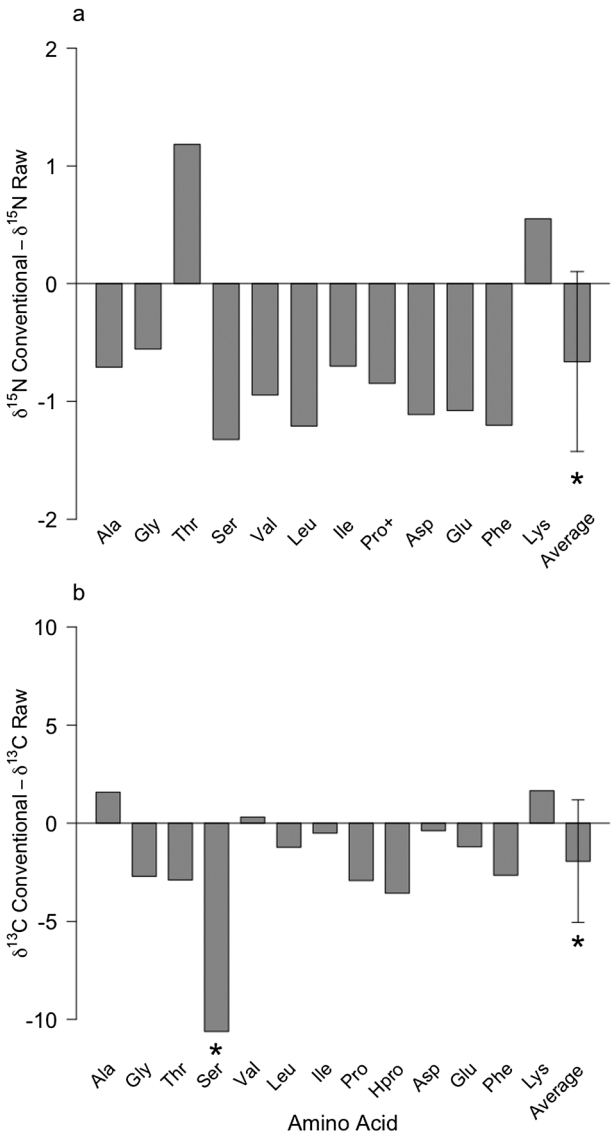

Figure 3. Offsets between amino acid isotope values of conventionally decalcified and raw dentin. Bars indicate difference in isotopic values (conventional decalcification – raw dentin) for each amino acid. 'Average' indicates the mean offset averaged across all amino acids. (a) $\delta^{15}N$ value offsets; average offset was statistically different from zero ($p = 0.007$, one-sample Student's *t*-test for a mean). (b) $\delta^{13}C$ offsets; average offset was also significantly different from zero ($p = 0.045$, one-sample Student's *t*-test for a mean). Asterisks indicate significant difference from zero. As also noted in the text, there were no significant differences in any offset for individual amino acids, except for Ser $\delta^{13}C$ values.

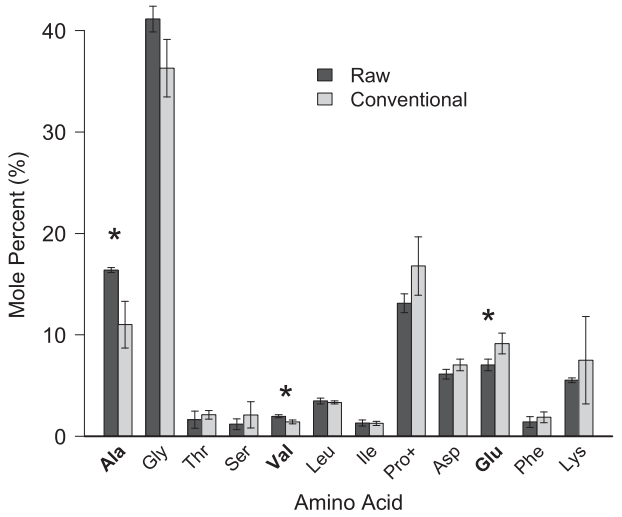

Figure 4. Amino acid molar composition of conventionally decalcified and raw dentin. Molar percentages (Mol%) are indicated for each amino acid for conventional decalcification (light grey) and raw dentin (dark grey) samples. No significant differences were found for most amino acids. Exceptions were Ala, Val, and Glu (p = 0.038, 0.014, 0.029 correspondingly, Student's t-tests).

The consistent isotope offsets that we observed between decalcified and raw samples (Fig. 3) imply a mechanism that affects essentially all amino acids in a similar manner, and for both elements. Partial hydrolysis of peptide bonds represents one such possibility. Both the theoretical and the experimental evidence suggest that acid hydrolysis of proteins might lead to an isotopic difference between the soluble and insoluble fractions.[22,23] Peptide bonds involving light isotopes (^{12}C and ^{14}N) are weaker than those involving heavy isotopes (^{13}C and ^{15}N). Therefore, if only partial hydrolysis occurs, bonds involving ^{12}C or ^{14}N should be cleaved preferentially. As a consequence, partial hydrolysis would be predicted to lead to a soluble fraction (amino acids and polypeptide fragments) enriched in the *light isotopes*, leaving behind a proteinaceous residue enriched in ^{13}C and ^{15}N relative to the original protein.[22,23] This process clearly cannot explain our results because the decalcified samples (i.e., the residue after acid treatment) show the opposite pattern: the proteinaceous residue after acid treatment has uniformly *lower* (not higher) stable isotope values for both carbon and nitrogen. We note that this result is also consistent with the basic assumption that only very limited (if any) peptide bond hydrolysis should occur at the weakly acidic, refrigerated conditions employed for conventional decalcification.

A final possibility is related to chemical matrix effects, i.e., that the abundant inorganic compounds in the raw dentin hydrolysates may have influenced the measured isotopic values. Without decalcification, the raw dentin hydrolysate solutions retain abundant calcium and phosphate from the mineral matrix. With the added Cl, they may then form a variety of inorganic complexes, including calcium chloride and phosphate compounds.[37,38] Indeed, we observed that the raw dentin sample solutions changed dramatically during processing. Immediately after derivatization, these solutions appeared similar to those of other protein derivatives. However, the final TFAA solutions became viscous after several days, and eventually fully gelatinous, and therefore progressively more difficult to inject onto the GC/IRMS instrument. While we did not attempt to analyze the structure or composition of these gels, they may have been polyphosphates and, in any case, could have interacted with the amino acid derivatives in these solutions in unpredictable ways. For freshly prepared samples (i.e., those run immediately after derivatization, before the solution became too gelatinous), the analytical precision of the raw dentin solutions was comparable with that of decalcified samples (Supplementary Fig. S4, Supporting Information). Within a few days, however, further processing became impossible. We note that similar sample problems (as well as problems even with EA data) were encountered in preliminary experiments testing the hydrolysis of decalcified samples for which supernatant (containing inorganic material) was not removed (Supplementary Fig. S3, Supporting Information).

Beyond the large practical issues that the raw samples presented for CSIA, the formation of gelatinous solutions from the raw dentin hydrolysates strongly supports the idea that the inorganic matrix might also affect the final measured molar or isotopic values of the organic portion (collagen and other proteins). Because it is not possible to conclude *a priori* that either raw or decalcified CSIA data should be more accurate, the possible effects of the inorganic matrix are difficult to directly evaluate. One approach would be to compare the isotopic values of all the proteinaceous material (as measured via GC/IRMS) with the measured bulk EA δ^{13}C and δ^{15}N values. As mentioned above, $\delta^{15}N_{THAA}$ and $\delta^{13}C_{THAA}$ represent proxies for the isotopic values of total hydrolyzable protein, derived from Mol%-weighted CSIA data. Because the bulk (EA-derived) isotope ratio values by definition represent all the amino acids present, the $\delta^{15}N_{THAA}$ and $\delta^{13}C_{THAA}$ values and bulk isotope values should be equal, *if* the CSIA results are representative of the amino acids in the original material (recalling that only ~8% of amino acids are not represented in the THAA calculation).[31,32] In contrast, a significant difference between these values could indicate fractionation in a given sample protocol.

The bulk and $\delta^{15}N_{THAA}$ and $\delta^{13}C_{THAA}$ isotope values for both decalcified and raw samples are presented in Fig. 5. Following conventional decalcification, there were offsets between the bulk isotope values and both the $\delta^{15}N_{THAA}$ and the $\delta^{13}C_{THAA}$ values, but they were in opposite directions and neither was statistically significant. This indicates that for conventional decalcification, the δ^{13}C and δ^{15}N values of the protein in the dentin (as measured by EA/IRMS) were the same, within error, as the δ^{13}C and δ^{15}N values of the total protein measured by CSIA. For raw samples, there was no offset between the bulk and the THAA δ^{13}C values, but there was a significant offset for the δ^{15}N values (p = 0.042). This result indicates that at least for nitrogen, total amino acids measured after processing of raw samples for CSIA *do not* correspond isotopically to those intact amino acids in the original dentin. This strongly suggests that isotopic alteration associated with the inorganic matrix occurred. The direction and magnitude of the offset between the $\delta^{15}N_{bulk}$ and $\delta^{15}N_{THAA}$ values are also consistent with the pattern observed for the isotope ratio values of conventionally decalcified and raw amino acids. Although unusual, the isotopically lighter amino acids are seemingly being sequestered from solution and condensing on the inorganic

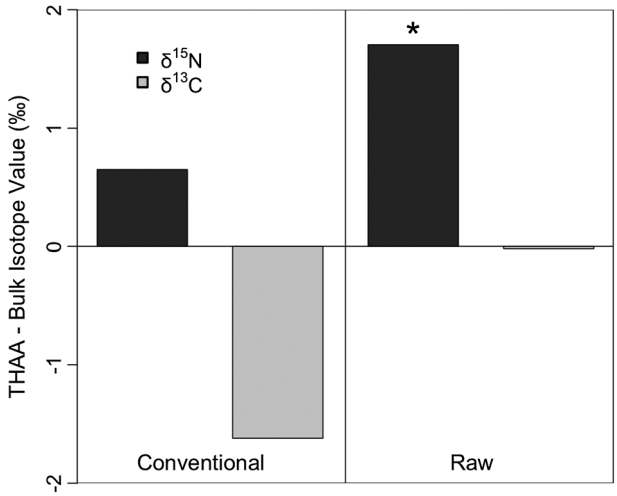

Figure 5. Offsets between total hydrolyzable amino acid isotope values ($\delta^{13}C_{THAA}$ and $\delta^{15}N_{THAA}$) and bulk isotope values for conventionally decalcified and raw dentin. Differences in isotopic values (THAA – bulk) are shown for $\delta^{15}N$ (dark grey) and $\delta^{13}C$ (light grey) values. As defined in the text the $\delta^{13}C_{THAA}$ and $\delta^{15}N_{THAA}$ values represent proxies for $\delta^{13}C$ and $\delta^{15}N$ values, respectively, of total hydrolyzable protein. Differences between the THAA and bulk isotope values indicate if the recovered amino acids are isotopically representative of the total N and C in each sample type. For conventionally decalcified samples, no significant differences were found between THAA and bulk isotope values (left panel). For raw dentin, the $\delta^{15}N_{THAA}$ value was significantly different from the raw bulk value (p = 0.042, one-sample Student's t-test for a mean, right panel).

matrix. Alternatively, the inorganic matrix could be interfering with the derivatization in a way that favors isotopically heavier amino acids.

However, clearly the THAA data are not fully consistent, since if lighter isotopes are lost by condensing inorganic material, the same offset would be expected for both $\delta^{15}N_{THAA}$ and $\delta^{13}C_{THAA}$ values. While we therefore cannot definitively conclude whether conventionally decalcified or raw isotope values are closer to the true isotope values for intact amino acids in dentin, the observed significant difference between the $\delta^{15}N_{bulk}$ and $\delta^{15}N_{THAA}$ values, together with substantial practical problems in processing raw material, suggests that CSIA values from conventionally decalcified (and more purified protein) dentin are closer to the natural amino acid isotope composition. However, it is important to re-emphasize that observed offsets in the average values of all amino acids between decalcified and raw samples were relatively small, and no significant differences in either C or N isotopic values were observed at the individual amino acid level.

SUMMARY AND CONCLUSIONS

This study evaluated the effectiveness of two methods for preparing dentin samples for bulk and compound-specific amino acid isotope analysis. For bulk carbon and nitrogen isotope analysis of tooth dentin from a sperm whale, we showed that direct analysis of raw dentin produces equivalent results, identical within error for both $\delta^{15}N$ and $\delta^{13}C$ values, to values obtained for conventionally isolated collagen. We conclude that conventional decalcification and lipid extraction are not necessary when measuring bulk $\delta^{15}N$ and $\delta^{13}C$ values in the dentin of sperm whales and possibly other odontocetes and marine carnivores, such as pinnipeds. However, we caution that this result will probably not extend to lipid-rich bone or fossils that may contain exogenous contaminants. In addition, decalcification may still be required for the analysis of herbivore dentin because the difference in $\delta^{13}C$ values between collagen and bioapatite is expected to be greater.

Our findings also show that conventional decalcification does not appreciably alter either the amino acid molar composition, or the compound-specific individual amino acid isotope values of the residual dentin proteins. Although we observed a small, but consistent, offset in the mean isotope values for amino acids between raw and decalcified dentin, our data suggests that the most likely cause is related to the highly concentrated inorganic matrix, which remained in the raw dentin sample solutions. Together with the practical analytical issues associated with raw dentin prepared for CSIA, we hypothesize that CSIA on raw dentin also produces less accurate results than CSIA on purified protein, probably because of the inorganic matrix interfering with amino acid recovery or with derivatization. Therefore, we recommend decalcification of dentin as a standard pretreatment prior to CSIA analysis.

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