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Evaluating bone collagen extraction methods for stable isotope analysis in dietary studies

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Abstract

The specific purpose of this study was to compare three different collagen extraction methods commonly used in isotope laboratories conducting dietary studies. We evaluated their resultant differences in $\delta^{13}C$ and $\delta^{15}N$, collagen quality and collagen yield. Our study was based on well-preserved skeletal material from the medieval period in Denmark. Our study shows that there is a systematic significant difference in the yield and the $\delta^{13}C$ values between the three methods. Using the method of DeNiro and Epstein [DeNiro, M.J., Epstein, S., 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochimica et Cosmochimica Acta 45, 341–351] with NaOH as cleaning agent, will, according to our study, give $\delta^{13}C$ values that are on average $\pm 0.32‰$ more positive than using the ultra-filtration method [Brown, T.A., Nelson, D.E., Vogel, J.S., Southon, J.R., 1988. Improved collagen extraction by modified Longin method. Radiocarbon 30 (2), 171–177, modified in Richards, M.P., Hedges R.E.M., 1999. Stable isotope evidence for similarities in the types of marine foods used by late Mesolithic humans at sites along the Atlantic coast of Europe. Journal of Archaeological Science 26, 717–722]. The third method, which is a modified version of the second method, excluded the ultra-filtration step. This method seems to give $\delta^{13}C$ values that lie in between the other methods. Our study did not show any significant difference in $\delta^{15}N$ values. Although the differences between the methods are very small, we conclude that the use of stable isotope analysis in food determination studies requires adherence to routine methods for preparing and measuring samples.

Keywords: Collagen extraction methods; Palaeodiet; Carbon; Nitrogen

1. Introduction

Stable isotope analyses of $\delta^{13}C$ and $\delta^{15}N$ measured in bone collagen are routinely used for the reconstruction of ancient diets and subsistence patterns (e.g., Ambrose, 1993; Bocherens et al., 2006; Honch et al., 2006; Jay and Richards, 2006; Katzenberg, 2000; Richards et al., 1998). Several techniques have been developed to prepare bone samples for isotope analysis. Most of these consider and adjust for factors such as humic acids and lipids that might influence the reproducibility of the measurements (Bronk Ramsey et al., 2004; Brown et al., 1988; Collins and Galley, 1998; Garvie-Lok et al., 2004; Lidén et al., 1995; Nielsen-Marsh and Hedges, 2000; Semal and Orban, 1995).

Generally, following Longin (1971), the extraction methods involve dissolving the mineral matrix in a HCl solution, subsequent solubilisation of collagen at elevated temperature in a weak HCl solution, followed by lyophilisation of the remaining collagen. However, there are various modifications including the addition of a treatment step with NaOH to remove humic acids before solubilisation of the collagen (DeNiro and Epstein, 1981), or the use of ultra-filtration to purify the

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solubilised collagen (Brown et al., 1988). These recommendations are commonly applied as cleaning steps in order to measure the original collagen used in dietary studies.

There are diverse chemical approaches in sample preparation used by different stable isotope laboratories. However, whatever the steps, the laboratories use each others results as references and comparison in dietary studies (e.g. Bayliss et al., 2004; Jørkov, 2002; Keegan, 1989). Previous studies of these extraction methods have examined the effects of ultra filtration in radiocarbon-dating and the contribution of lipids on the stable carbon isotope values (e.g. Bronk Ramsey et al., 2004; Lidén et al., 1995). Results have shown that ultra-filtration may still leave larger contaminating particles and lipids may alter the carbon signals. We therefore thought it necessary to investigate whether the extraction methods and cleaning steps may influence the isotopic result and hence potentially the interpretation of dietary variation.

The specific purpose of this study was to compare three sample preparation methods on well-preserved skeletal material by evaluating the resultant differences in $\delta^{13}C$ and $\delta^{15}N$, collagen quality and collagen yield. The first method (A) includes the treatment with NaOH, the second method (B) includes both ultra-filtration and filtration with Ezee filter separators (5–8 μm) (Elkay Laboratory Product) before lyophilisation as described in Richards and Hedges (1999) and Müldner and Richards (2005). The third method (C) is a modified version of method B, by excluding the ultra-filtration step. This modified version of method B is also used in laboratories conducting stable isotope analysis in dietary studies (Honch et al., 2006).

2. Materials and methods

The bone samples selected for this study were chosen from a large well documented skeletal collection from the medieval cemetery Ahlgade 15–17 in Holbæk, Denmark (Asmussen, 1997). The cemetery was used between ca. 1100–1573 AD, and contained more than 700 skeletons buried in clay soil. The material was chosen because of its excellent state of preservation (the bones were macroscopically intact with a hard structure and feel (i.e. non-flaky)). Eight bone samples from five individuals were each treated with the three extraction methods. Details on the samples, including age and sex of the individuals are given in Table 1.

Table 1
Sample details

Individual	Sample no.	Sex	Age (years)	Bone element	Bone structure
EG141	1	Male	45+	Femur (R)	ps
EG155	2	?	9	Femur (R)	s
EG160	3	Female	45+	Femur (R)	p
	4			Humerus (R)	p
EG161	5	Female	18–25	Femur (R)	p
EG295	6	Male	18–25	Femur (R)	cp
	7			Humerus (L)	cp
	8			Rib	cp

(R), right; (L), left. Bone structure when drilled: p, powder; ps, powder and shavings; s, shavings; cp, coarse powder.

Cortical bone samples were taken from the posterior side of the midshaft of femora and humeri. The surface of the bone samples was cleaned with a round milling cutter. Bone powder was then drilled out using a low speed Proxxon MICROMOT 40E drill (drill diameter: 2 mm). The resultant bone samples varied between consisting of fine bone powder (grains with an approximate diameter of 0.01 mm) (p), a mix of bone powder and small shavings with a size of 0.1–0.5 mm, to shavings with a size of 0.9–1.0 mm (ps). A further rib fragment and shards of a femur and humerus (sample nos. 6, 7 and 8) were ground manually in a mortar into coarse powder of 0.04–0.9 mm (cp). The samples from each bone were subdivided into three portions, and the three extraction methods were then applied.

2.1. Extraction method A

This extraction method followed the protocol of DeNiro and Epstein (1981). Bone samples were demineralised in 1 M HCl at 4 °C for 1 h, being stirred every 5 min. They were then rinsed to neutral pH with de-ionised water. 0.2 M NaOH was added to remove contaminating humic acids. Samples were rinsed again to neutral pH with de-ionised H_2O. HCl was added the sample tubes, obtaining a pH of 2.5. The samples were then covered and gelatinised in this weak acid solution at 70 °C for 16 h in order to concentrate the protein components. After removing insoluble residues by centrifuging the samples at 2500 rpm for 10 min, the remaining supernatant solution was evaporated at 100 °C for 6 h until reaching ca. 3 ml. The solution was then freeze-dried for 24 h.

2.2. Extraction method B

Collagen was extracted using the standard procedures by Brown et al. (1988), modified in Richards and Hedges (1999) by the use of Millipore Amicon Ultra-4 centrifugal filter (30,000 NMWL) prior to lyophilisation, so that molecules larger than 30 kDa were retained. As with method A, the samples were demineralised in 1 M HCl, at 4 °C for 1.5–10 h, until the release of CO_2 could no longer be observed. The use of 1 M HCl is a slight modification to 0.5 M HCl, which is commonly used (Richards and Hedges, 1999). The samples were then rinsed to neutral pH with de-ionised water. Weak HCl solution was added to obtain a pH of 2.5. Samples were then gelatinised at 70 °C for 24 h. After collagen solubilisation any insoluble residues were removed with a 5–8 μm Ezee mesh filter (Elkay Laboratory Products). The remaining solution was concentrated on the ultra-filters by centrifugation at 2500 rpm. The supernatant of purified "collagen" (>30 kDa), was then freeze-dried for 48 h.

2.3. Extraction method C

Method C followed the same protocol as method B, but the ultra-filtration step was left out.

The collagen extractions were carried out in our laboratory. All isotopic measurements were performed in at least

duplicate with a GV Instruments Isoprime stable isotope mass spectrometer combined with a Eurovector elemental analyzer (continuous flow) at the AMS Laboratory at the Institute of Physics and Astronomy, University of Aarhus, Denmark. Bulk collagen from each sample was weighed in duplicates to between 215–250 μg. To examine the accuracy and precision of analytical methods, a working standard gelatine material and an AMS standard reference (a whale bone produced with the preparation method A) (Heinemeier, 2005, personal communication) with known $\delta^{13}C$ and $\delta^{15}N$ were analysed in tandem with samples of bone collagen. These secondary standards are calibrated against approximately ten internationally recognised isotopic standards. The maximum analytical error (1σ) for $\delta^{13}C$ and $\delta^{15}N$ were 0.15‰ and 0.3‰ respectively.

Comparative statistical analyses following the procedures outlined by Bland and Altman (1986) were used to assess the three preparation methods. Friedman tests (FTS) and paired *t*-tests were applied to test for difference in C:N atomic ratio, $\delta^{13}C$ and $\delta^{15}N$ and % collagen yield between the methods (Altman, 1999).

3. Results

The results of the stable isotope measurements and comparative analysis are summarised in Tables 2–4 and Figs. 1 and 2.

3.1. Collagen preservation

On the basis of the quality indicators for bone collagen extracts (Ambrose, 1990, 1993; Schoeninger et al., 1989; van Klinken, 1999), the collagen yield, C:N atomic ratio and %C and %N were considered comparable for each of the three methods although the collagen yields did vary greatly between the methods (Table 2).

The lowest percentage collagen yield, ranging from 1.2% to 5.7%, was obtained using method B. Method A resulted in yields ranging from 3.5% to 16.7%, and method C resulted in yields ranging from 4.2% to 20.6%. However, %C by weight of the collagen samples was fairly consistent (Table 2): 41.1–44.7% (mean: 43.1%) for method A, 44.3–47.5% (mean: 45.8%) for method B, and 39.5–45.5% (mean: 42.8%) for method C. This was also the case for the %N: 15.0–16.6% (mean: 15.7%) for method A, 15.5–16.8% (mean: 15.9%) for method B, and 14.0–16.5% (mean: 15.4%) for method C.

3.2. $\delta^{13}C$ values

The results of the $\delta^{13}C$ measurements show that samples prepared following method A have values that are consistently less negative than samples prepared by method B, with method C having values that lie between the two (Table 2, Fig. 1). The differences are statistically significant (FTS = 14.250, $p = 0.001$). A comparative analysis of the differences between the methods showed that there is a statistically significant difference between all three methods (Table 3), albeit this

Table 2
Isotopic results of samples extracted by method A, B and C

Sample no.	$\delta^{13}C$			%C			$\delta^{15}N$			%N			C:N			% Yield		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	−18.99	−19.25	−19.28	44.4	46.6	44.0	12.43	12.77	12.40	15.7	16.0	15.8	3.3	3.4	3.2	13.50	2.79	18.43
2	−19.02	−19.38	−19.26	44.7	47.5	44.5	12.04	12.51	12.20	15.8	16.8	16.5	3.3	3.3	3.1	16.70	5.70	20.63
3	−20.25	−20.41	−20.40	41.1	45.9	39.5	10.92	11.26	11.18	15.0	16.4	14.0	3.2	3.3	3.3	6.60	1.80	6.30
4	−20.23	−20.54	−20.36	42.5	46.2	45.5	10.63	11.25	11.30	16.0	15.9	16.2	3.1	3.4	3.3	7.60	2.48	7.30
5	−18.82	−19.20	−19.13	43.0	46.2	42.3	12.26	12.47	12.28	15.9	16.2	14.9	3.2	3.3	3.3	4.47	1.20	4.50
6	−18.48	−18.86	−18.59	42.1	45.5	42.4	13.39	13.47	13.47	15.1	15.5	14.9	3.3	3.4	3.3	3.50	1.32	4.30
7	−18.57	−19.04	−18.67	43.3	44.5	42.4	13.17	13.36	12.79	16.6	15.7	15.9	3.1	3.3	3.1	10.20	2.10	4.20
8	−18.60	−18.99	−18.65	43.3	44.3	42.1	13.30	12.79	14.11	15.7	15.5	15.0	3.2	3.3	3.3	15.60	3.50	4.20

$\delta^{13}C$ and $\delta^{15}N$ measured in duplicate at the AMS ^{14}C Dating Centre, Department of Physics and Astronomy, University of Aarhus, Denmark. The maximum analytical error (1σ) for $\delta^{13}C$ and $\delta^{15}N$ were 0.15‰ and 0.3‰ respectively.

Table 3
Comparative analysis of the difference in $\delta^{13}C$ values between the methods

$\delta^{13}C$	Mean difference	2σ	t	p
Method A vs. B	0.32‰	0.22‰	8.195	<0.001
Method A vs. C	0.15‰	0.20‰	4.311	0.004
Method B vs. C	−0.17‰	0.30‰	3.140	0.016

difference is numerically small: the biggest difference is between Methods A and B, with a mean difference of 0.32 ± 0.22‰ (2σ).

3.3. $\delta^{15}N$ values

The analysis of the $\delta^{15}N$ measurements between the methods and of their difference was completed in a similar manner. There was no statistically significant difference in $\delta^{15}N$ values between any of the methods (Table 4, Fig. 2).

4. Discussion

When working with archaeological bone collagen, it is essential to be familiar with the mechanisms that can alter the isotopic signal as well as with the quality indicators that are available to assess collagen preservation. The sources of contaminants that can act to decompose the bone mineral (i.e. microbial attack, pH, groundwater activity and temperature etc.) are depended on the burial environment and vary both geographically and temporally (Hedges, 2002; Nielsen-Marsh and Hedges, 2000). Before applying the preferred preparation method, one should therefore consider the possible sources of diagenesis and contaminants from the burial environment that may have altered the original collagen isotopic signal. Today most isotopic studies (including dietary studies) follow the same criteria suggested by Ambrose (1990, 1993) and van Klinken (1999) for what is considered well preserved collagen. Methods B and C are widely used in radiocarbon laboratories and laboratories conducting stable isotope analysis in dietary studies (Bronk Ramsey et al., 2004; Honch et al., 2006; Richards et al., 2006; Richards and Hedges, 1999). Since several extraction protocols exist, it is important that in the end the protocols yield comparable results. The quality of the collagen should therefore also be compared as it may influence the result. The % collagen yield is one criteria, but not in itself the dominating factor. Of course, the ideal extraction method should maximise collagen yield while minimising the degradation of the extracted protein remnants and removing contaminants. By using method B, the yield of larger

Table 4
Comparative analysis of the difference in $\delta^{15}N$ values between the methods

$\delta^{15}N$	Mean difference	2σ	t	p
Method A vs. B	−0.22‰	0.68‰	−1.814	0.112
Method A vs. C	−0.20‰	0.77‰	−1.462	0.187
Method B vs. C	0.019‰	1.16‰	0.092	0.930

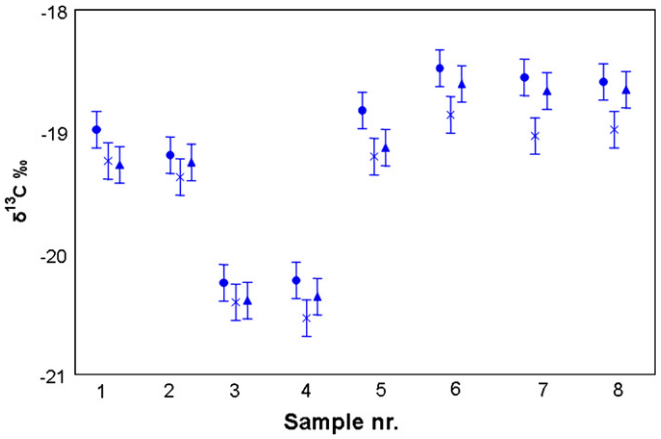

Fig. 1. $\delta^{13}C$ ‰ of the samples prepared by method A = ●, B = × and C = ▲. All samples have marked maximum analytical error of $1\sigma = \pm 0.15$‰.

peptides should be improved as contaminants are expected to be of lower molecular weight.

It was anticipated that collagen yields would be somewhat lower for the samples extracted with method B, since the use of ultra-filters lowers the overall yield by removing molecules that are smaller than 30 kDa. We performed an analysis of the collagen loss when ultra-filtrating. The analysis showed that the collagen yield is reduced by up to 86% (data not shown). The lowest collagen yield was obtained from bone sample no. 5, with a collagen yield of 1.2% using method B and 4.47% using method A (Table 2). This sample was taken from a femur, and produced fine powder during drilling of the bone sample. The powdery nature of the drilled sample could indicate that the collagen was more poorly preserved and the low yield would seem to confirm this. In contrast, bone sample no. 2 produced shavings when drilling. In our experience the quality of bone shavings seem to be an indication of well preserved collagen, although this would be difficult to quantify between different sites. This assumption was confirmed by the high collagen yield for all three methods. The method that gave the lowest yield of the

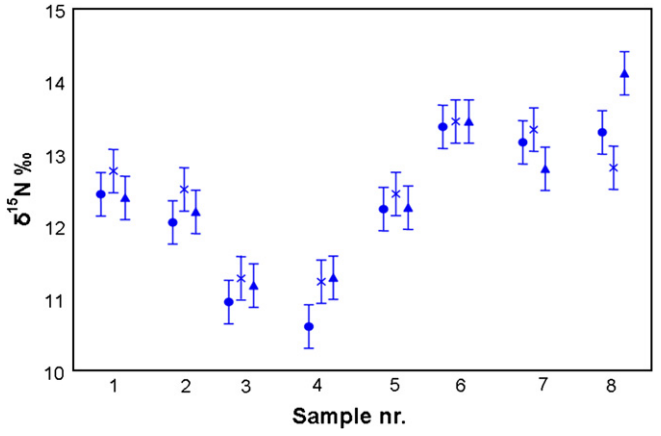

Fig. 2. $\delta^{15}N$ ‰ of the samples prepared by method A = ●, B = × and C = ▲. All samples have marked maximum analytical error of $1\sigma = \pm 0.30$‰.

three for this bone sample was method B. Again, this was likely to be due to the loss of collagen through the ultra-filter. However, powder (''p'') is not the only indication of poor collagen preservation in bone. Bone samples prepared from coarse powder (''cp'') have just as low a collagen yield. Schoeninger et al. (1989) reported that they achieved a much higher collagen yield if they avoided powdering, but they also stated that the superficial appearance, as well as the collagen yield or the C:N atomic ratio can not be used to predict how well collagen is preserved. However, in samples of poor preservation, preparation of chunks rather than powder should be achieved, as it has a greater chance of producing original intact collagen because the collagen fibre structure is preserved. Collins and Galley (1998) also showed that grinding of bone could damage the collagen. The significance should be less in collagen which is already damaged, as many of the long rigid collagen fibres have already been cut up. The high %C and %N values measured in the large molecules of method B could indicate two things: Either the original bone collagen is still contaminated, or the original collagen has molecular sizes which are smaller than 30 kDa and what is left is contamination.

The ultra-filters were pre-rinsed with 0.1 N NaOH and centrifuged twice with de-ionised water as suggested by the manufacturer. Contaminants that could interfere should be removed at this stage. Studies at the Oxford Radiocarbon Accelerator Unit (Bronk Ramsey et al., 2004) have shown that this pre-rinsing may not be sufficient to remove contaminants and that the suggested cleaning protocol by manufacturer might cause the C:N atomic ratios to be higher than the original collagen. A C:N atomic ratio within the range of 2.9–3.6 is an indicator of good collagen preservation (Ambrose, 1990). As with our C:N results (Table 2), the original C:N atomic ratio for each of the cleaning methods were all within the acceptable limits because the absolute proportion of contamination was very small. It is not all laboratories which are able to conduct such thorough cleaning protocol of the ultra-filters since it is both time consuming and costly.

The samples treated with NaOH (A) had C:N atomic ratios of 3.1–3.3 and the ultra-filtrated samples had slightly higher C:N atomic ratios between 3.3–3.4. The largest difference between the two methods was for sample no. 4 with C:N ratio of 3.1 and 3.4 for A and B, respectively.

Why samples extracted using method C have lower %C and %N values when the samples have only been Ezee filtered cannot be explained at this point. According to the Oxford ^{14}C database (van Klinken, 1999) the %C and %N values of intact collagen should be around 34.8 ± 8.8 and 11–16 wt%, respectively. Higher values might indicate addition of organic carbon with small amounts of inorganic matter can be expected in the extracts (Ambrose, 1990). And since the rest of the quality markers suggest good collagen, the collagen should be not rejected for analysis. It seems as if the use of NaOH in the A method removes the non protein contaminants as the samples have %C and %N values within the accepted range of 41.1–44.7% and 14–16%, respectively.

There is a systematic and significant difference in the δ^{13}C values between the methods although it was small. Using method A with NaOH as cleaning agent will according to our study give δ^{13}C values that are on average 0.32‰ more positive than using the ultra-filtration method (method B). Our study did not show any significant difference in δ^{15}N values. The variation in δ^{15}N between the methods of bone sample no. 8 is, however, noteworthy. This sample was taken from a rib (i.e. trabecular bone). The other seven samples were from the cortical part of long bones (i.e. compact bone). Since trabecular bone is more easily contaminated than compact bone, the variation in δ^{15}N between the methods might perhaps indicate that method C does not remove certain contaminants as well as methods A and B, which have more similar δ^{15}N values for this bone sample.

Samples extracted using method B should have had unwanted non-protein contaminants removed in the ultra filtration step. In our study, the treatment with NaOH seems to remove the non protein contaminants (base-soluble contaminants such as humic acids) better than the ultra-filter on material that has molecules less than 30 kDa. It produces a residue that is mostly derived from collagen, but can also contain extraneous organic and inorganic matter. The effect of those seems insignificant on skeletal material which is well preserved.

The δ^{13}C results could be biased by lipids as they are known to have δ^{13}C values more negative from those of protein (Lidén et al., 1995). However, the degree of interference is depended upon the amount of lipid preserved in bone. In this case we are analysing well preserved archaeological bone material from the medieval period and the chances of having lipids present is not so high as in modern bone samples, although cholesterol is known to be quite robust in archaeological time scales (Stott and Evershed, 1996). According to Lidén et al. (1995), the δ^{13}C in non-lipid-extracted collagen may be as high as 1.8‰ more negative than in samples of lipid extracted collagen. In order to avoid variability they therefore suggest removing lipids entirely in the extraction process by incorporating a methanol-chloroform solvent wash step.

Method B selects the high molecular weight material. As lipid molecules have a smaller weight than 30 kDa, the Ultra-filtration should remove any lipids that may have been present in the sample. Method A only removes the humic acids. Lidén et al. (1995) show that treatment with NaOH results in a decrease in collagen yield, contrary to the results of this study.

5. Conclusion

From this study we conclude that the use of stable isotope analysis in palaeodietary studies requires adherence to routine methods for preparing and measuring samples. How far apart measurements can be without causing variation in the dietary interpretation is in part a question of judgement. According to Lovell et al. (1986) a normal variation within a population is up to 0.3‰. Any difference in δ^{13}C larger than 0.3‰ may cause the interpreter to suggest a difference in subsistence. DeNiro and Schoeninger (1983) showed an intra-individual variation of up to 2‰; however, those studies were conducted on

animals raised on controlled diets and are not a satisfactory way to estimate baseline variability in human isotope ratios. Although a difference in collagen quality using the different methods was seen in this study and were in fact statistically significant, we believe it would have no influence on the overall interpretation of the isotope results. They do, however, indicate a need for consistency in the preparation method used and an awareness of the difference that they may give. Naturally, if laboratories make their own modifications to the extraction procedures, these should be duly noted.

We are aware that our study comprised only eight samples of well preserved bone from one Medieval cemetery in Denmark. We therefore think that a future study would benefit by including poorly preserved bone as well as material from different sites and time periods, to see how effective the three methods are for excluding contaminants.

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