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SHORT REPORT

Effects of Sodium Hydroxide Treatment and Ultrafiltration on the Removal of Humic Contaminants from Archaeological Bone

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ABSTRACT This study compared bone collagen extraction techniques that included treatment with sodium hydroxide and 30 kDa ultrafilters using a set of well-preserved, humic-contaminated archaeological marine mammal bones. Treatment with sodium hydroxide was effective at removing humic contaminants from archaeological bone, although yields were significantly decreased. Yields were also significantly decreased by ultrafiltration although this study produced no evidence that 30 kDa ultrafilters were effective at selectively removing humic contaminants from archaeological bone. The combination of sodium hydroxide treatment and ultrafiltration did not produce superior results to the treatment involving only sodium hydroxide. Archaeological samples exhibiting darker colouration indicative of humic contamination should be treated with sodium hydroxide to remove these contaminants. Copyright © 2017 John Wiley & Sons, Ltd.

Key words: bone collagen; contamination; diagenesis; humic acids; stable isotopes; ultrafiltration

Supporting information may be found in the online version of this article.

Introduction

The proteinaceous component of bone, frequently referred to simply as 'collagen', is an important analytical substrate for stable isotope analysis of archaeological remains. Archaeological collagen will have undergone some degree of modification via biotic and abiotic attack. Collagen may be modified through interactions with contaminants in the burial environment, altering its chemical composition and producing isotopic compositions that are not representative of the endogenous collagen (van Klinken, 1999). The most significant of these contaminants are humic substances.

Humic substances occur naturally and are dark-coloured, predominantly aromatic, acidic and hydrophilic chemical compounds found in the soil. They are the by-products of microbial metabolism and are ultimately derived from decaying organic matter in the soil (Sutton & Sposito, 2005); their molecular weight ranges from 10^2 to nearly 10^6 Daltons (Da) (Shin *et al.*, 1999). Relative to collagen, humic substances are carbon rich (c. 50–60 wt%) and nitrogen poor (c. 1–5 wt%) (Christl *et al.*, 2000; Lobartini *et al.*, 1997). In the burial environment, humic substances are able to quickly penetrate the bone matrix and interact with collagen molecules; although the precise chemical mechanism behind the linking of humics to collagen is not known (van Klinken & Hedges, 1995). Given their high carbon content and the fact that they are derived from decaying organic matter with low $\delta^{13}C$ values (primarily from C_3 plant biomass), humics have long been recognised as potential contaminants of collagen extracted for stable isotope analysis and radiocarbon dating.

In studies of soil humic fractions, sodium hydroxide is commonly used to isolate these compounds from fulvic acids (acid soluble) and other soil compounds (Lobartini *et al.*, 1997). Following the same principles,

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an alkali treatment step (typically from 0.1 to 0.125 M NaOH) has been used regularly in the extraction and purification of bone collagen for isotopic analysis and radiocarbon dating (Hedges & Van Klinken, 1992; Katzenberg, 1989). The use of filtered centrifuge tubes ('ultrafilters') with a membrane that retains only molecules above a certain molecular weight (typically 30 kDa) is a common practice in collagen extraction for Accelerator Mass Spectrometry (AMS) dating and stable isotope analysis. Numerous studies have examined the pros and cons of the use of ultrafilters for collagen extraction as they relate specifically to AMS dating (Brock *et al.*, 2007; Brown *et al.*, 1988; Hüls *et al.*, 2009). While the use of ultrafilters for collagen purification has been applied in isotopic analysis (although not as extensively as in AMS dating), little published research exists concerning the utility of ultrafiltration for improving the accuracy and precision of isotopic or elemental data through the removal of various contaminants (but see Fuller *et al.*, 2014, Guiry *et al.*, 2016, Jørkov *et al.*, 2007, Sealy *et al.*, 2014).

The primary objective of this study was to compare the efficacy of sodium hydroxide and 30 kDa ultrafilters (separately as well as in concert) in the removal of humic contaminants from well-preserved archaeological bone.

Materials and methods

Materials

The primary materials used in this analysis consist of 20 northern fur seal (*Callorhinus ursinus*, hereafter NFS) bones from the historic (from late 1780s to early 1800s AD) occupation of Zapadni on St. Paul Island in the Pribilof Islands, Alaska (Veltre & McCartney, 2002). These samples were chosen for several reasons. First, a high degree of organic preservation was apparent as soft tissues (skin or hair) still adhered to the bone in some cases. Second, the bones were generally dark brown in colour, suggesting that collagen had undergone some type of humification reaction in the burial environment (Figure S1). Third, given the isotopic compositions that have been recorded in NFS tissues (c. from −15 to −12‰; Burton *et al.*, 2001, Szpak *et al.*, 2009), we anticipated relatively high $\delta^{13}C$ values for the endogenous collagen. In an environment devoid of C_4 plants, the relatively high NFS collagen $\delta^{13}C$ values should be affected to a substantial degree by humic contamination; and the selective removal of these contaminants by physical or chemical treatments should be apparent. One sample from Zapadni produced a $\delta^{15}N$ value that was approximately 6‰ lower than all of the others, suggesting that it had been misidentified and was not a NFS. The $\delta^{13}C$ value was still comparable to the others, however, and it was therefore included in further analyses except where noted.

The NFS samples were supplemented by five samples that were also well preserved, but that appeared not to be contaminated with humics based on the colouration of the bone. The specimens were also marine mammals (two ringed seals [*Pusa hispida*], two walruses [*Odobenus rosmarus*], and one beluga [*Delphinapterus leucas*]) from Arvik (QjJx-1), a Late Dorset (c. 1000 ^{14}C years BP) site located on Little Cornwallis Island in the Central Canadian Arctic Archipelago (Darwent, 2001). As with the NFS, these marine mammals were expected to have relatively high $\delta^{13}C$ values based on previous analyses conducted at this site (Jaouen *et al.*, 2016).

Experimental design

Each bone sample was physically cleaned by abrading the exterior surface of the bone with an NSK Ultimate XL micromotor (Nakanishi, Shimohanata, Japan) equipped with a diamond-tipped cutting wheel (Brasseler Canada, Québec City, QC, Canada). Four aliquots of 170 ± 30 mg were cut from the bone for each of four treatments as described in Figure 1. For each of the treatments, the bone was demineralized in 0.5 M HCl at 4°C. In all treatments, after demineralization, the samples were rinsed to neutrality with Type I water. In treatments SH/UF and SH/0, the demineralized bone sample was then treated with approximately 10 mL of 0.1 M NaOH at room temperature for successive 20 min periods until no colour change was observed in the solution. This step more closely follows the alkali treatments used in collagen extraction in radiocarbon labs (e.g., Beaumont *et al.*, 2010) than the 20 h treatment with 0.125 M NaOH that is often used when preparing collagen for light stable isotope analysis following Ambrose (1990). After the alkali treatment (SH/UF and SH/0) samples were rinsed to neutrality with Type I water and were then refluxed in sealed glass tubes at 75°C in 10^{-3} M HCl for 48 h. In the 0/0 and 0/UF treatments, the refluxing step immediately followed the post-demineralization neutralisation. After refluxing, the soluble collagen was then filtered with 5–8 μm filters (Elkay Laboratory Products, Hampshire, United Kingdom) to remove any remaining particulate matter (all treatments) and directly freeze dried in treatments SH/0 and 0/0. Samples in treatments 0/UF and SH/UF were filtered

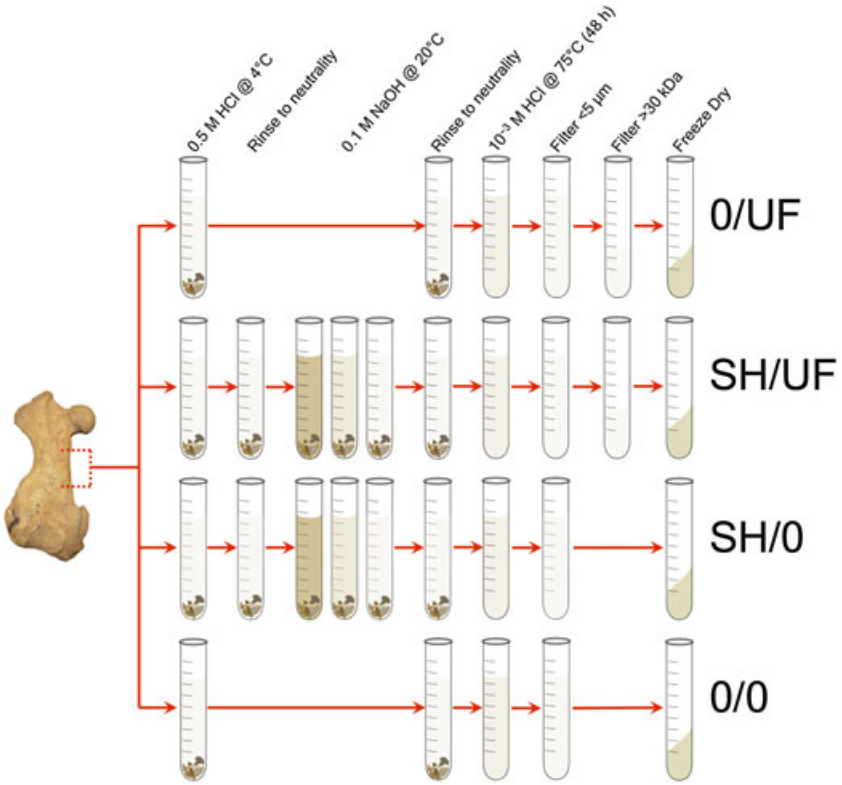

Figure 1. Schematic showing the steps involved in the four different collagen extraction protocols compared in this study. [Colour figure can be viewed at wileyonlinelibrary.com]

using a 30 kDa molecular weight cut-off ultrafilter (Pall Corporation, Fort Washington, NY) and subsequently freeze-dried. The dried collagen was then weighed into tin capsules for isotopic and elemental analysis.

Stable isotope and elemental analysis

Elemental and isotopic compositions were determined with an IsoPrime continuous flow isotope-ratio mass spectrometer coupled to a Vario Micro elemental analyser (Elementar, Hanau, Germany). Carbon and nitrogen isotopic compositions were calibrated relative to VPDB (Vienna Pee Dee Belemnite) and AIR (atmospheric nitrogen), respectively, using a two-point calibration anchored by USGS40 and USGS41. Analytical uncertainty was determined to be ± 0.26 for δ^{13}C and ± 0.29 for δ^{15}N (Szpak *et al.*, 2017); additional details on analytical accuracy and precision are provided in the supplementary material.

Data treatment

Comparisons for elemental and isotopic compositions among the treatments were performed using paired sample *t*-tests. Comparisons of within-sample homogeneity was performed using a one-way analysis of variance followed by a post-hoc Tukey's HSD test (equal variances) or Dunnett's T3 test (unequal variances); equality of variances was assessed using Levene's test.

Results and discussion

Collagen yield

The average collagen yields for the four treatments are presented in Table 1. Generally speaking, both the

Table 1. Results of the comparisons among treatments (paired t-tests) for collagen yield (p-values < 0.05 are in boldface)

	df		0/UF	SH/0	SH/UF
All samples	24	0/0	**0.001**	0.111	**0.004**
		0/UF	—	**0.019**	0.526
		SH/0	—	—	**0.012**
Zapadni	19	0/0	**0.005**	0.233	**0.026**
		0/UF	—	**0.025**	0.799
		SH/0	—	—	0.055
Arvik	4	0/0	**0.043**	**0.012**	**0.008**
		0/UF	—	0.539	0.068
		SH/0	—	—	0.051

NaOH and the ultrafiltration step decreased collagen yields, although these differences were not significant in all cases (Table 1). A decrease in yield that was of larger magnitude and greater statistical significance occurred with the ultrafiltration step than the NaOH treatment, for samples from both Zapadni (suspected to be contaminated with humics) and the Arvik (not suspected to be contaminated with humics). These findings are consistent with previous studies that have found decreased collagen yields associated with NaOH treatment (Boutton *et al.*, 1984; Minami *et al.*, 2004) and ultrafiltration (Guiry *et al.*, 2016).

Sample homogeneity

The homogeneity of samples was assessed on the basis of the standard deviation of replicate analyses of individual samples. The variable inclusion of contaminant material can influence sample heterogeneity and in turn overall analytical uncertainty (Guiry *et al.*, 2016). Results of a one-way analysis of variance demonstrated that treatment type had no significant effect on sample homogeneity for the following: $\delta^{13}C$ ($F_{[3,96]} = 0.544$, $p = 0.65$), $\delta^{15}N$ ($F_{[3,96]} = 0.917$, $p = 0.44$), wt% C ($F_{[3,96]} = 0.576$, $p = 0.63$), and wt% N ($F_{[3,96]} = 1.344$, $p = 0.27$).

Isotopic compositions

The average isotopic compositions for all samples are summarised in Table S6. There were significant differences in $\delta^{13}C$ values but not $\delta^{15}N$ values (Table 2) among the different treatments. For the humic-contaminated samples, the $\delta^{13}C$ values were lowest in the 0/0 and 0/UF treatments and highest in the SH/0 and SH/UF treatments (Table 2). The atomic C:N ratio of samples from the 0/0 treatment are plotted against the difference in $\delta^{13}C$ between each of the other treatments (SH/0, 0/UF, SH/UF) in Figure 2. For the Zapadni samples, which consisted of a single species and age class, there was a relationship between the atomic C:N ratio of the 0/0 treatment and the $\delta^{13}C$ value [Figure 2(d)] confirming that carbon-rich, nitrogen-poor humic contaminants were present in the samples and significantly influenced their $\delta^{13}C$ values.

Table 2. Results of the comparisons among treatments (paired t-tests) for $\delta^{13}C$ and $\delta^{15}N$ (p-values < 0.05 are in boldface)

	df	Variable		0/UF	SH/0	SH/UF
All samples	24	$\delta^{13}C$	0/0	0.226	**0.009**	**0.030**
			0/UF	—	**0.001**	**0.007**
			SH/0	—	—	0.054
		$\delta^{15}N$	0/0	0.772	0.276	0.275
			0/UF	—	0.936	0.857
			SH/0	—	—	0.808
Zapadni	19	$\delta^{13}C$	0/0	0.369	**0.010**	**0.011**
			0/UF	—	**0.002**	**0.008**
			SH/0	—	—	0.142
		$\delta^{15}N$	0/0	0.834	0.234	0.367
			0/UF	—	0.903	0.907
			SH/0	—	—	0.969
Arvik	4	$\delta^{13}C$	0/0	0.420	0.474	0.428
			0/UF	—	0.198	0.614
			SH/0	—	—	0.129
		$\delta^{15}N$	0/0	0.804	0.854	0.599
			0/UF	—	0.864	0.754
			SH/0	—	—	0.280

No differences among treatments for $\delta^{13}C$ were observed for the Arvik samples (not suspected to be contaminated) but they were for the Zapadni samples (appeared to be contaminated). The $\delta^{13}C$ values were significantly higher in treatments that used the NaOH step versus those that did not [Table 2; Figure 2(a-c)], suggesting that this step was effective at removing substances with $\delta^{13}C$ values that were lower than the endogenous collagen, most likely humic acids given their solubility in NaOH (Kipton *et al.*, 1992). Ultrafiltration had no discernable effect on the $\delta^{13}C$ values of humic-contaminated samples [Figure 2(a-c)] as there was no significant difference in the paired *t*-test comparisons for 0/0 versus 0/UF (0.369) or SH/0 versus SH/UF (0.142) (Table 2).

Unlike for $\delta^{13}C$, there was no relationship between the atomic C:N ratios of the 0/0 samples and their $\delta^{15}N$ values [Figure 3(d)]. The $\delta^{15}N$ values of both the Arvik and Zapadni samples were not consistently affected by either the NaOH treatment or ultrafiltration [Figure 3(a-c)], although the absolute difference in $\delta^{15}N$ between the treatment (SH/0, 0/UF, SH/UF) and the control (0/0) was greatest for 0/UF (0.43‰) relative to SH/0 (0.18‰) and SH/UF (0.21‰). The ultrafiltration treatment (0/UF) therefore changed the $\delta^{15}N$ values of the samples to a significantly greater extent than the NaOH treatment (SH/0) ($t = 2.35$, $df = 48$, $p = 0.02$), although the direction of this change was not consistent and samples with C:N ratios > 3.6 were not affected more than those with C:N ratios < 3.6 (Figure 3(a)). Because there was no difference in the change in the $\delta^{15}N$ values between the SH/0 and SH/UF treatments ($t = 0.60$, $df = 48$, $p = 0.55$), this suggests that this increased $\delta^{15}N$ variation is an artefact of the ultrafiltration process due to the retention of exogenous materials that are removed by the NaOH treatment.

Elemental compositions

Among treatments, there were significant differences in wt% C, but not wt% N (Table 3). Samples from

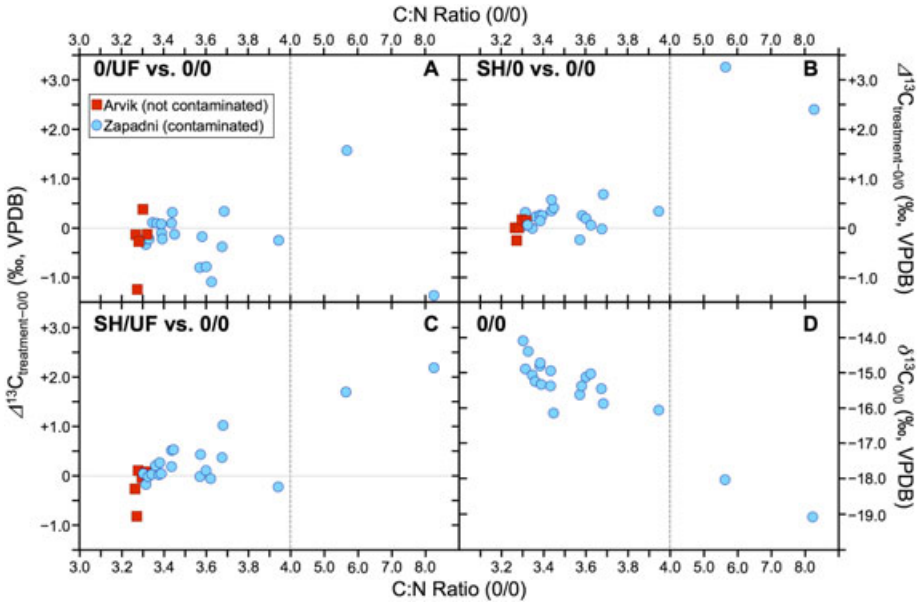

Figure 2. Comparison of the influence of treatment type on sample $\delta^{13}C$ values. Panel D (bottom right) plots the $\delta^{13}C$ values of the untreated Zapadni samples against their atomic C:N ratio. Panels A, B, and C plot the difference in $\delta^{13}C$ between each of the three treatments (0/UF, SH/0, SH/UF) and the control (0/0) on the y-axes ($\Delta^{13}C_{treatment\ -\ 0/0}$) and the C:N ratio of the untreated (0/0) samples on the x axes. Because two samples from Zapadni had extremely high atomic C:N ratios (in the 0/0 treatment), the scale on the x-axis is split with larger increments above than below 4.0; this division is marked by a broken line. Note that the Arvik (uncontaminated control samples) are excluded from Panel D because several species are represented and a comparison of their $\delta^{13}C$ values relative to their atomic C:N ratios would be meaningless. [Colour figure can be viewed at wileyonlinelibrary.com]

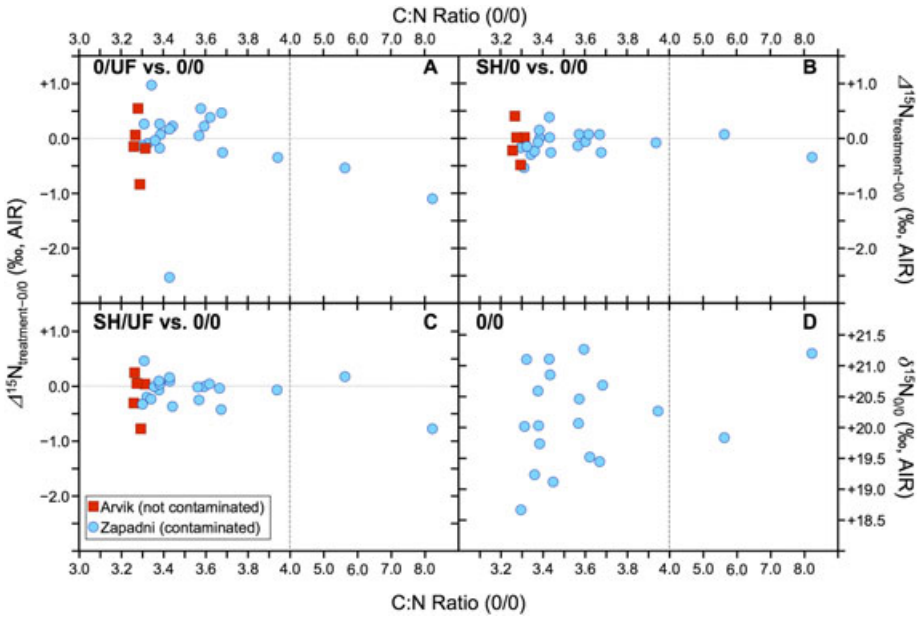

Figure 3. Comparison of the influence of treatment type on sample $\delta^{15}N$ values. Panel D (bottom right) plots the $\delta^{15}N$ values of the untreated Zapadni samples against their atomic C:N ratio. Panels A, B, and C plot the difference in $\delta^{15}N$ between each of the three treatments (0/UF, SH/0, SH/UF) and the control (0/0) on the y-axes ($\Delta^{15}N_{treatment\ -\ 0/0}$) and the C:N ratio of the untreated (0/0) samples on the x axes. Because two samples from Zapadni had extremely high atomic C:N ratios (in the 0/0 treatment), the scale on the x-axis is split with larger increments above than below 4.0; this division is marked by a broken line. Note that the Arvik (uncontaminated control samples) are excluded from Panel D because several species are represented and a comparison of their $\delta^{15}N$ values relative to their atomic C:N ratios would be meaningless. For the same reason, one sample from Zapadni (AZA 14107) was excluded from this plot because it produced an unusually low $\delta^{15}N$ value (+13.5‰) that suggested it was misidentified and is not a northern fur seal; this sample is included in the other three panels. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3. Results of the comparisons among treatments (paired t-tests) for all elemental compositions (p-values < 0.05 are in boldface)

	df	Variable		0/UF	SH/0	SH/UF
All samples	24	wt% C	0/0	**<0.001**	**0.004**	**<0.001**
			0/UF	—	**<0.001**	**0.011**
			SH/0	—	—	**<0.001**
		wt% N	0/0	0.838	0.631	0.081
			0/UF	—	0.711	0.133
			SH/0	—	—	0.092
		Atomic C:N	0/0	0.212	0.128	0.252
			0/UF	—	0.062	0.137
			SH/0	—	—	0.012
Zapadni	19	wt% C	0/0	**0.001**	**0.010**	**<0.001**
			0/UF	—	**<0.001**	0.054
			SH/0	—	—	**0.001**
		wt% N	0/0	0.903	0.466	0.095
			0/UF	—	0.404	0.088
			SH/0	—	—	0.248
		Atomic C:N	0/0	0.321	0.122	0.236
			0/UF	—	0.071	0.153
			SH/0	—	—	**0.013**
Arvik	4	wt% C	0/0	**0.038**	0.130	0.178
			0/UF	—	0.052	0.127
			SH/0	—	—	0.111
		wt% N	0/0	0.255	0.104	0.444
			0/UF	—	0.103	0.512
			SH/0	—	—	0.127
		Atomic C:N	0/0	0.069	0.130	0.171
			0/UF	—	0.066	0.135
			SH/0	—	—	0.389

Zapadni that were treated with NaOH were characterised by significantly lower wt% C than those that were not. Conversely, samples from Zapadni that were subjected to ultrafiltration were characterised by significantly higher wt% C values than those that were not. These elemental data were consistent with the isotopic data, suggesting that the NaOH treatment removed carbon rich compounds with low $\delta^{13}C$ values (humic acids) whereas the ultrafilters did not. Although wt% C differed significantly between treatments, atomic C:N ratios differed significantly only between the SH/0 and SH/UF treatments, with the SH/UF treatment producing higher atomic C:N ratios. With respect to the Zapadni sample, the SH/0 treatment produced the lowest atomic C:N ratios (3.47 ± 0.24), followed by SH/UF (3.58 ± 0.34), 0/0 (3.82 ± 1.14), and 0/UF (3.89 ± 1.20).

Discussion

As anticipated on the basis of a basic physical examination of the Zapadni material, the collagen was contaminated with carbon-rich, low $\delta^{13}C$ humic acids. These contaminants resulted in progressively lower $\delta^{13}C$ value with increasing sample wt% C [Figure 2 (d)]. Sodium hydroxide was effective at removing some of these contaminants, lowering the wt% C values and increasing the $\delta^{13}C$ values, while there was no evidence that ultrafiltration effectively removed any of these contaminants.

The fact that ultrafilters were not effective at removing humic contaminants from the samples, may be because of: (1) the majority of the humic acids having a molecular weight > 30 kDa; (2) the humic acids interacting with the collagen molecules in a manner that was not influenced by the initial acidification of the bone (with 0.5 M HCl) or the refluxing step (10^{-3} M HCl @ 75°C for 48 h); (3) a combination of (1) and (2). The specific interactions between humic substances and proteins are poorly understood in general (Arenella *et al.*, 2014; Tan *et al.*, 2008). It is, therefore, difficult to speculate on any specific chemical interaction that may have prevented the removal of the humic acids by 30 kDa ultrafiltration, but the suggestion that covalent linkages form between humics and collagen via Maillard-like reactions seems likely (van Klinken & Hedges, 1995). If this is the case, these humic substances will therefore not be removed through ultrafiltration even if their molecular weight is <30 kDa because they are bound to collagen fragments with molecular weights that are mostly >30 kDa.

The filters that were used in this study (and are commonly used in other studies) exclude material with a molecular weight <30 kDa. The vast majority of humic substances, however, have molecular weights that are >30 kDa. In a study of soil humic substances, Christl *et al*. (2000) found that the <30 kDa fraction of the humic component contained only 3 wt% C (of the entire humic component) whereas the >300 kDa fraction contained 52 wt% C (of the entire humic component). Moreover, there was a relationship between molecular weight and carbon content, with higher molecular weight fractions containing proportionally more carbon, 58.4 wt% in the >300 kDa fraction compared with 48.5 wt% in the <30 kDa fraction. This is not to suggest that the majority of humic substances that penetrate bone and interact with collagen are also predominantly >300 kDa, but that it is unlikely that most have very low molecular weights that would be effectively removed with ultrafiltration. The use of 30 kDa ultrafilters not only fails to remove the majority of humic substances on the basis of their molecular weight, but those that it does exclude are the least carbon rich, and therefore the least likely to confound

sample $\delta^{13}C$ values. The data from this study are consistent with this explanation as ultrafiltration did not result in higher $\delta^{13}C$ vales of the humic-contaminated samples and it also resulted in significantly higher wt% C in the sample, possibly because of the selective retention of particular compounds on the basis of the charge of the protein and filter membrane (Burns & Zydney, 1999) or the inclusion of predominantly degraded collagen molecules in the low molecular weight fragment and relatively few humic compounds. In order for humic substances to be removed by ultrafiltration, they would need to have a molecular weight of <30 kDa and interact with the bone collagen via hydrogen bonding or other weak intermolecular forces that are disrupted during the refluxing step. The results of this study suggest that at least one, if not both, of these conditions is not met.

Summary

Treatment with sodium hydroxide is effective at removing humic contaminants from archaeological bone, although yields are decreased. Yields are also decreased by ultrafiltration although this study produced no evidence that 30 kDa ultrafilters are effective at selectively removing humic contaminants from archaeological bone. When archaeological bone samples are suspected to be contaminated with humics (on the basis of their dark colouration), a sodium hydroxide treatment after demineralization is the most effective means of removing these contaminants.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Photos of three northern fur seal scapula samples used in this study showing the extent of apparent humic contamination. Sample numbers are indicated on the bottom of each panel.
Table S1. Standard reference materials used for calibration of $\delta^{13}C$ relative to VPDB and $\delta^{15}N$ relative to AIR.
Table S3. Standard reference materials used to monitor internal accuracy and precision.
Table S4. Mean and standard deviation of all check and calibration standards for all analytical sessions containing data presented in this paper. Note that means for calibration standards are not presented as they are pre-determined to be equal to the known value.
Table S5. Average standard deviations for sample replicates according to treatment.
Table S6. Stable carbon and nitrogen isotopic and elemental compositions for all samples analysed. Each row represents the mean of four replicate measurements ± one standard deviation.
Table S7. Summary statistics for stable isotopic and elemental compositions for each of the four treatments.