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RESEARCH ARTICLE

Comparing the performance of demineralization agents (HCl and EDTA) for stable isotope analysis of bone collagen with implications for quality control criteria and collagen yield

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Abstract

Stable isotope analysis of ancient bone collagen is a powerful technique for studying diet, migration, and ecology in archeological contexts. These analyses are, however, limited by collagen preservation and prohibitively low collagen yields. Harsh chemical demineralization is required to isolate the collagen from the mineral component of the bone, which in turn reduces the yield of material available for analysis. Demineralization is typically performed using hydrochloric acid (HCl), which reduces collagen yield via acid hydrolysis of peptide bonds. An alternative to a strong acid (HCl) treatment is the neutral chelating agent ethylenediaminetetraacetic acid (EDTA). To our knowledge, it has never been empirically tested whether EDTA treatment reduces collagen loss relative to HCl in samples that are known to be poorly preserved (i.e., low yields and/or collagen extracts failing other collagen quality control [QC] criteria). We tested the effect of the demineralization agent on collagen yield, stable isotope, and elemental composition of poorly preserved ancient bone samples. Collagen yield was significantly higher in EDTA-treated samples; however, this did not translate into a greater number of samples passing relevant quality control criteria. Stable isotope compositions (SIC) were also not significantly different between treatments. The atomic C:N ratio of samples treated with EDTA was significantly lower than equivalent samples treated with HCl, which is likely a product of increased deamidation of asparagine and glutamine residues when collagen is demineralized with HCl relative to EDTA. We conclude that although EDTA treatment may reduce collagen loss relative to HCl treatment, this does not necessarily increase the probability of producing reliable–stable isotope data from bone samples yielding low amounts of collagen. Based on our isotopic data, we suggest the following provisional collagen QC criteria for EDTA-demineralized samples: collagen yield > 2%, wt % C > 4%, wt% N > 2%, and atomic C:N ratio between 2.80 and 3.25.

KEYWORDS
bone chemistry, collagen yield, quality control, sample pre-treatment, stable isotope analysis

1 | INTRODUCTION

Stable isotope analysis of bone collagen can provide information on the diet and migration patterns of ancient humans and animals. Chemical pre-treatment methods are required to isolate the collagen from other bone components, the most important of which is the mineral fraction of the bone, bioapatite (Longin, 1971). HCl is commonly used for demineralization because of its low cost and ease of solution preparation. Additionally, there are established quality control (QC) criteria for both modern and ancient collagen treated with HCl that allow the recognition of collagen samples that have been altered in the burial environment and should therefore be excluded from future analyses (Ambrose, 1990; DeNiro, 1985; Guiry & Szpak, 2020, 2021; van Klinken, 1999). There is, however, a loss of protein material during strong acid demineralization via cleavage of peptide bonds that will reduce collagen yield (Procopio et al., 2021).

Treating samples with a neutral chelating agent such as EDTA has been proposed as an alternative to HCl to reduce collagen loss (Tuross, 2012). EDTA is regarded as a gentler demineralization agent that may be more suitable for poorly preserved samples, particularly because the demineralization process occurs more slowly than acidic agents (Collins & Galley, 1998; Tuross, 2012). If an insufficient amount of collagen is recovered from an ancient bone sample (i.e., an amount that will not yield a sufficient amount of gas upon combustion independent of % collagen yield), stable isotope analysis cannot be performed; therefore, maximizing collagen yield is an important consideration, especially for samples with low endogenous collagen content. Although it has been hypothesized that EDTA may be advantageous for optimizing collagen yield in ancient samples (Tuross, 2012), a robust comparison of collagen yield in poorly preserved samples treated with EDTA and HCl is lacking in the literature. If treatment with EDTA can produce samples with significantly higher collagen yields and reliable–stable isotope compositions, this would suggest that EDTA is a superior demineralization agent for poorly preserved samples.

We compared the collagen yield, stable isotope, and elemental composition of poorly preserved bone samples (i.e., those known to have low collagen yields based on previous analyses) treated with HCl and EDTA. Using these data, we assessed whether EDTA is a more suitable demineralization agent for obtaining higher yields of collagen from ancient samples. The stable isotope and elemental composition of samples treated with HCl and EDTA were compared to determine if any significant differences were present between samples treated with different demineralization agents. These data were used to assess the impact of applying quality control criteria developed specifically for samples treated with HCl to samples demineralized with EDTA.

2 | METHODS

2.1 | Materials

Poorly preserved camelid bones from archeological sites in Peru were used to compare the efficacy of pre-treatment agents for obtaining high collagen yields (Table 1). The bones ($n = 32$) have been identified as Camelidae, most likely llama (*Lama glama*), although possibly from alpaca (*L. pacos*), guanaco (*L. guanicoe*), or vicuña (*L. vicugna*). Previous analyses on the samples using HCl demineralization suggest that 24 of the samples have poorly preserved collagen (collagen yield <1% or not enough collagen for analysis), whereas eight of the samples have adequate collagen preservation (Szpak, 2013; Szpak et al., 2019). Adequate preservation is defined as collagen yield greater than 1% but not necessarily passing other relevant QC criteria (i.e., minima for %C and %N, C:N ratio within the typically accepted range).

2.2 | Bone preparation

Each bone was ground and sieved into fragments less than 2 mm in diameter using a mortar and pestle. The fragments were mixed via stirring and divided into two equal fractions for the different experimental treatments. The first fraction was demineralized in 0.5 M HCl for 30 min at room temperature (Longin, 1971). The samples were then rinsed four times using Type I water (resistivity > 18.2 MΩ·cm) and solubilized in 0.01 M HCl for 36 h at 75°C to isolate the collagenous portion of the bone into solution. Following solubilization, the collagen was lyophilized for 48 h. The second fraction of bone was demineralized in EDTA for 20 h. The samples were rinsed 15 times in Type I water (Tuross, 2012). Solubilization and lyophilization treatments for the EDTA-treated were conducted in the same manner as the HCl-treated samples.

2.3 | Stable isotope and elemental analysis

Collagen yield was calculated using the final mass of collagen divided by the mass of the initial bone sample. For each sample, 0.50–0.60 mg of collagen was weighed into a tin capsule. The samples were analyzed via a continuous flow isotope ratio mass spectrometer coupled to an elemental analyzer at the Trent Water Quality Center (Trent University, Ontario, Canada). Results were calibrated relative to ambient inhalable reservoir (AIR) for $\delta^{15}N$ using international reference standards USGS40 and USGS66 or USGS63 and Vienna Pee Dee Belemnite

Site	Location	Approximate age (calendar years BP)	*n* samples
Huaca de la Luna	Moche Valley	1250–1850	1
El Castillo	Santa Valley	1350–1650	5
Dos Cabezas	Jequetepeque Valley	1300–1650	1
Huaca El Pueblo	Zaña Valley	1250–1550	25

TABLE 1 Site and contextual information for bone samples used in the analyses.

(VPDB) for $\delta^{13}C$ using USGS40 and USGS66 (Qi et al., 2003; Schimmelmann et al., 2016) (Table S1). The analytical uncertainty for $\delta^{13}C$ measurements was ±0.10 ‰ and ±0.24 ‰ for $\delta^{15}N$ measurements using the equations presented by Szpak and Macdonald (2017). Internal reference materials were used to assess accuracy and precision throughout the analytical sessions (Table S2). Duplicate samples were used to test the repeatability of the analyses. An average difference of 0.05 ‰ between duplicate samples ($n = 6$) was observed for $\delta^{15}N$ and $\delta^{13}C$ values. In cases with insufficient collagen to analyze, the sample was omitted from all further testing (Tables 2 and 3).

2.4 | Statistical analyses

Wilcoxon tests for paired samples were used to assess differences in stable isotope and elemental composition between experimental treatments. Spearman's ρ values were calculated to compare the relationship between atomic C:N ratio and collagen yield for both treatments. Pearson's R values were generated to assess the linear relationship of elemental and isotopic compositions between treatments. These statistical analyses and data visualizations were performed using the PAST software (V4) and the RStudio package

TABLE 2 Experimental results for samples treated with HCl.

Bone ID	Sample ID	Site	Collagen yield	$\delta^{13}C_{VPDB}$/‰	$\delta^{15}N_{AIR}$/‰	Wt% C	Wt % N	C:N ratio	QC?	Feasibility of SIC?
1883	14495	Huaca El Pueblo	0.0						FAIL	
1885	14496	Huaca El Pueblo	0.4						FAIL	
1889	14497	Huaca El Pueblo	0.3						FAIL	
1891	14498	Huaca El Pueblo	0.0						FAIL	
1896	14499	Huaca El Pueblo	0.0						FAIL	
1904	14500	Huaca El Pueblo	1.7	−19.15	−1.32	0.8	0.2	5.11	FAIL	Not feasible
1911	14501	Huaca El Pueblo	0.0						FAIL	
1913	14502	Huaca El Pueblo	0.3	−14.42	10.66	4.2	0.9	5.20	FAIL	Feasible
1914	14503	Huaca El Pueblo	1.0	−18.69	4.05	2.7	0.8	3.91	FAIL	Borderline
1915	14504	Huaca El Pueblo	0.0	−19.28	2.68	2.6	0.6	4.71	FAIL	Flagged
1917	14505	Huaca El Pueblo	0.0	−15.57	4.85	8.7	2.2	4.59	FAIL	Borderline
1921	14506	Huaca El Pueblo	1.3	−17.57	7.44	2.8	0.7	4.73	FAIL	Feasible
1924	14507	Huaca El Pueblo	0.2	−14.70	12.86	4.8	1.0	5.45	FAIL	Borderline
1925	14508	Huaca El Pueblo	0.0						FAIL	
1928	14509	Huaca El Pueblo	2.5	−19.17	3.39	0.8	0.2	4.50	FAIL	Flagged
1933	14510	Huaca El Pueblo	16.2	−16.77	5.93	31.7	11.6	3.20	PASS	Feasible
1936	14511	Huaca El Pueblo	0.2	−16.46	7.90	2.9	0.6	5.66	FAIL	Feasible
1939	14512	Huaca El Pueblo	18.2	−11.62	5.68	37.1	13.6	3.19	PASS	Feasible
1942	14513	Huaca El Pueblo	1.5	−19.48	5.66	2.7	0.6	5.39	FAIL	Feasible
1944	14514	Huaca El Pueblo	13.2	−12.88	6.79	40.7	14.8	3.21	PASS	Feasible
1947	14515	Huaca El Pueblo	3.9	−15.21	6.94	23.4	8.4	3.26	PASS	Feasible
1958	14516	Huaca El Pueblo	0.0						FAIL	
1959	14517	Huaca El Pueblo	1.8	−15.94	6.46	17.6	6.1	3.39	PASS	Feasible
1960	14518	Huaca El Pueblo	0.0						FAIL	
1963	14519	Huaca El Pueblo	0.0						FAIL	
12449	13682	El Castillo	4.0	−15.66	12.70	10.7	2.9	4.33	FAIL	Borderline
1972	13683	Huaca de la Luna	1.0	−13.34	8.95	36.0	11.7	3.58	PASS	Feasible
12397	13684	El Castillo	1.0						FAIL	
2014	13685	Dos Cabezas	2.0	−15.80	6.61	39.3	13.8	3.33	PASS	Feasible
12405	13686	El Castillo	18.0	−14.11	8.19	40.7	15.1	3.14	PASS	Feasible
12446	13687	El Castillo	18.0	−13.32	10.04	40.8	14.2	3.36	PASS	Feasible
12444	13688	El Castillo	20.0	−14.34	5.95	38.8	14.3	3.17	PASS	Feasible

Note: A "PASS" in the QC column indicates that the sample passed all the collagen QC, as described in the text, whereas a "FAIL" in the QC column indicates that the sample failed at least one of these criteria. The "feasible SIC" column is based on the range of expected $\delta^{13}C$ and $\delta^{15}N$ values for Peruvian north coast camelids as described in the text. Samples highlighted in green have a C:N ratio that falls within the acceptable range.

ggplot2 (Hammer et al., 2001; Wickham, 2016). In our analysis, we considered acceptable collagen quality control criteria to be those that are most widely used (Ambrose, 1990; DeNiro, 1985): collagen yield > 1%, atomic C:N ratio between 2.9 and 3.6, wt% C > 13%, and wt% N > 4.5%.

To attempt to identify "unusual" isotopic compositions independent of whether a sample failed the QC criteria, we generated an expected range of $\delta^{13}C$ and $\delta^{15}N$ values for camelids from the northern coastal region of Peru (where the samples in our analyses are from) based on previously published archeological data. This process uses the same logic that DeNiro (1985) employed to develop his range of acceptable C:N ratios for ancient bone collagen. We included $\delta^{13}C$ and $\delta^{15}N$ values from bone collagen and hair keratin (from preserved hair and textiles made from camelid fiber) (Dufour et al., 2020; Santana-Sagredo et al., 2020; Szpak et al., 2014, 2016, 2018, 2019; Tomczyk et al., 2019). We adjusted the hair keratin $\delta^{13}C$ values by +0.6 ‰ and the $\delta^{15}N$ values by +1.5 ‰ to account for inter-tissue differences according to the collagen-hair pairings presented by Lehn et al. (2015). This resulted in 316 $\delta^{13}C$ and $\delta^{15}N$ values that passed quality control for bone collagen (Ambrose, 1990; DeNiro, 1985) and

TABLE 3 Experimental results for samples treated with EDTA.

Bone ID	Sample ID	Site	Collagen yield	$\delta^{13}C_{VPDB}$/‰	$\delta^{15}N_{AIR}$/‰	wt% C	wt% N	C:N ratio	QC?	SIC feasible?
1883	14261	Huaca El Pueblo	1.5	−18.90	−10.22	0.6	0.2	4.29	FAIL	Not feasible
1885	14262	Huaca El Pueblo	0.6						FAIL	
1889	14263	Huaca El Pueblo	2.1	−21.21	6.55	0.8	0.2	6.05	FAIL	Borderline
1891	14264	Huaca El Pueblo	1.6	−33.62	−0.48	1.4	0.3	4.97	FAIL	Not feasible
1896	14265	Huaca El Pueblo	1.4	−28.85	−17.58	0.4	0.1	4.81	FAIL	Not feasible
1904	14266	Huaca El Pueblo	1.4	−21.55	−1.79	0.4	0.1	4.72	FAIL	Not feasible
1911	14267	Huaca El Pueblo	1.6	−23.17	−10.05	0.6	0.1	5.52	FAIL	Not feasible
1913	14268	Huaca El Pueblo	0.3	−23.62	−4.40	0.6	0.1	4.90	FAIL	Not feasible
1914	14269	Huaca El Pueblo	1.9	18.94	−0.09	1.0	0.3	3.53	FAIL	Not feasible
1915	14270	Huaca El Pueblo	1.6	−23.57	−2.85	0.5	0.1	4.51	FAIL	Not feasible
1917	14271	Huaca El Pueblo	1.2						FAIL	
1921	14272	Huaca El Pueblo	1.5	−18.57	3.77	1.6	0.5	3.73	FAIL	Flagged
1924	14273	Huaca El Pueblo	2.0	−25.09	−3.26	0.5	0.1	4.91	FAIL	Not feasible
1925	14274	Huaca El Pueblo	1.6	−20.30	0.87	1.0	0.3	3.87	FAIL	Flagged
1928	14275	Huaca El Pueblo	1.2	−17.55	2.92	1.1	0.4	3.37	FAIL	Flagged
1933	14276	Huaca El Pueblo	3.7	−16.70	5.85	29.7	11.0	3.14	PASS	Feasible
1936	14277	Huaca El Pueblo	2.1	−21.09	−4.48	0.5	0.2	4.07	FAIL	Not feasible
1939	14278	Huaca El Pueblo	11.8	−11.53	5.70	39.1	14.5	3.15	PASS	Feasible
1942	14279	Huaca El Pueblo	1.3	−20.54	−2.10	0.9	0.3	3.58	FAIL	Not feasible
1944	14280	Huaca El Pueblo	7.9	−12.80	6.97	37.6	13.9	3.16	PASS	Feasible
1947	14281	Huaca El Pueblo	3.1	−15.17	6.79	30.8	11.4	3.15	PASS	Feasible
1958	14282	Huaca El Pueblo	0.2						FAIL	
1959	14283	Huaca El Pueblo	2.1	−15.50	6.29	19.6	7.3	3.13	PASS	Feasible
1960	14284	Huaca El Pueblo	1.8	−22.46	−9.15	0.5	0.1	4.19	FAIL	Not feasible
1963	14285	Huaca El Pueblo	1.8	−21.18	−0.04	0.5	0.2	4.04	FAIL	Not feasible
12449	13740	El Castillo	3.0	−17.11	7.24	12.8	4.9	3.03	PASS	Feasible
1972	13741	Huaca de la Luna	3.0	−14.00	5.93	5.0	1.9	3.07	FAIL	Feasible
12397	13742	El Castillo	3.0	−16.59	6.49	9.1	3.5	3.08	FAIL	Feasible
2014	13743	Dos Cabezas	4.0	−16.23	6.62	17.0	6.3	3.14	PASS	Feasible
12405	13744	El Castillo	19.0	−14.01	8.38	41.5	15.8	3.06	PASS	Feasible
12446	13745	El Castillo	19.0	−13.53	9.89	41.8	15.2	3.22	PASS	Feasible
12444	13746	El Castillo	20.0	−14.72	6.38	40.8	15.6	3.05	PASS	Feasible

Note: A "PASS" in the QC column indicates that the sample passed all the collagen QC, as described in the text, whereas a "FAIL" in the QC column indicates that the sample failed at least one of these criteria. The "feasible SIC" column is based on the range of expected $\delta^{13}C$ and $\delta^{15}N$ values for Peruvian north coast camelids as described in the text. Samples highlighted in green have a C:N ratio that falls within the acceptable range.

530 adjusted $\delta^{13}C$ and $\delta^{15}N$ values for hair keratin (Szpak & Valenzuela, 2020) (Tables S3 and S4). To assess the likelihood that the isotopic composition of the bone collagen that we analyzed had been altered in the burial environment, we defined two overlapping rectangles in bivariate ($\delta^{13}C$, $\delta^{15}N$) space. The first consists of a rectangle formed by the 2σ ranges around the mean $\delta^{13}C$ (−20.6 to −10.1 ‰) and $\delta^{15}N$ (+5.1 to +12.1 ‰) values for our published data (Figure S1). We considered collagen with an isotopic composition falling within this range to be "feasible." The second consists of a rectangle formed by the maximum ranges of the $\delta^{13}C$ (−21.9 to −8.3 ‰) and $\delta^{15}N$ (+4.1 to +14.8 ‰) for our published data (Figure S1). We considered collagen with an isotopic composition falling within this range but outside the "feasible" range to be "borderline." Finally, collagen with an isotopic composition falling outside of both ranges was "flagged," meaning that they are likely altered in the burial environment. We cannot consider any sample falling outside of our defined min–max ranges as "infeasible" strictly because it is possible that our surveyed published data do not adequately capture the full range of variation. Some samples, however, that produced isotopic compositions far outside the range observed for terrestrial vertebrates (e.g., $\delta^{13}C = -29$ ‰, $\delta^{15}N = -15$ ‰) can be confidently classified as "not feasible."

3 | RESULTS

3.1 | Collagen yield

Collagen yield was significantly lower in HCl than in EDTA-treated samples ($p = 0.03$). The average collagen yield was, however, very similar for both treatments (an average of 4.0% for both treatments). There was no significant difference in collagen yield for the samples that produced an atomic C:N ratio that was between 2.9 and 3.6 in both treatments ($p = 0.81$). For those samples that produced enough collagen to be analyzed (with either treatment) but were characterized by C:N ratios outside the range of 2.9 to 3.6, EDTA-treated samples had significantly higher yields than HCl-treated samples ($p = 0.001$). For the samples treated with HCl, 15/32 samples failed to yield enough collagen to pass quality control criteria (collagen yield >1%); however, some samples with less than 1% collagen still had enough material to combust in the EA-IRMS (Table 2). By contrast, the EDTA treatment produced only 3/32 samples with collagen yields below 1%. For HCl-treated samples, collagen yield was negatively correlated with atomic C:N ratio (Spearman's $\rho = -0.85$) (Figure 1). A weaker correlation was also observed for the same variables in EDTA-treated samples (Spearman's $\rho = -0.66$) (Figure 1). For a subset of the samples (ID: 14495-14499, all from the Huaca El Pueblo site), there was not enough collagen for analysis when HCl was used for demineralization, but there was enough when EDTA was used (Table 3). However, despite the fact that more of the samples treated with EDTA yielded enough collagen for analysis, they failed other quality control criteria (i.e., wt% C, wt% N, C:N), and therefore, they did not produce any additional useable data.

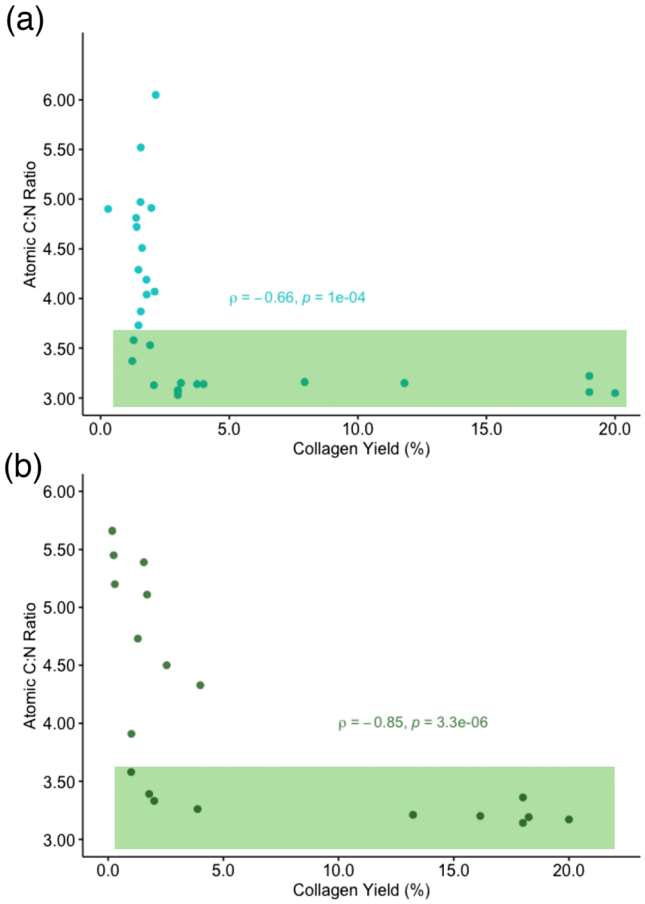

FIGURE 1 The plot of collagen yield against atomic C:N ratio for samples treated with (a) EDTA ($n = 29$), (b) HCl ($n = 19$). The ρ values from Spearman's correlation test are shown. The portion of the graph highlighted in green represents the range of atomic C:N ratios and collagen yield meeting quality control criteria established by DeNiro (1985) and Ambrose (1990). [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Stable isotope composition

The stable isotope compositions of the EDTA-treated samples were much more variable than those of the HCl-treated samples (Figure 2). A higher proportion of the EDTA samples produced isotopic compositions considered not feasible by our criteria (Tables 2 and 3), and many of these samples failed other traditionally utilized QC measures, especially atomic C:N ratio (Figure 2b) and minimum values for wt% C and wt% N (Figure 2d). The stable isotope composition of samples containing low amounts of collagen (<1%) was highly variable and often fell outside of the wide range of expected values that we generated for these camelids (Figure 2e,f, Tables 2 and 3). Of the 29 EDTA-treated samples that yielded enough collagen to produce isotopic data, 13 of the samples produced stable isotope compositions that were not feasible (e.g., $\delta^{15}N$ values below zero) (Table 3). Only one of the 21 HCl-treated samples produced similarly infeasible isotopic compositions (Table 2). Despite the EDTA treatment yielding more samples with sufficient collagen, the stable isotope compositions were not significantly different. There were

no significant differences in $\delta^{15}N$ or $\delta^{13}C$ between the EDTA and HCl-treated samples ($p = 0.77$ and $p = 0.86$, respectively, for all samples) (Table S5). The linear relationships between stable isotope composition of paired samples treated with either agent were strong for samples passing quality control criteria (Figure 3a,b) and weak for paired samples that did not pass QC (Figure 3c,d).

3.3 | Elemental composition

The atomic C:N ratios of samples treated with EDTA were significantly lower than equivalent samples treated with HCl ($p = <0.001$). On average, the atomic C:N ratio was 0.51 units lower in EDTA than in HCl-treated samples (Figure 4, Table S6). The C:N ratios were only

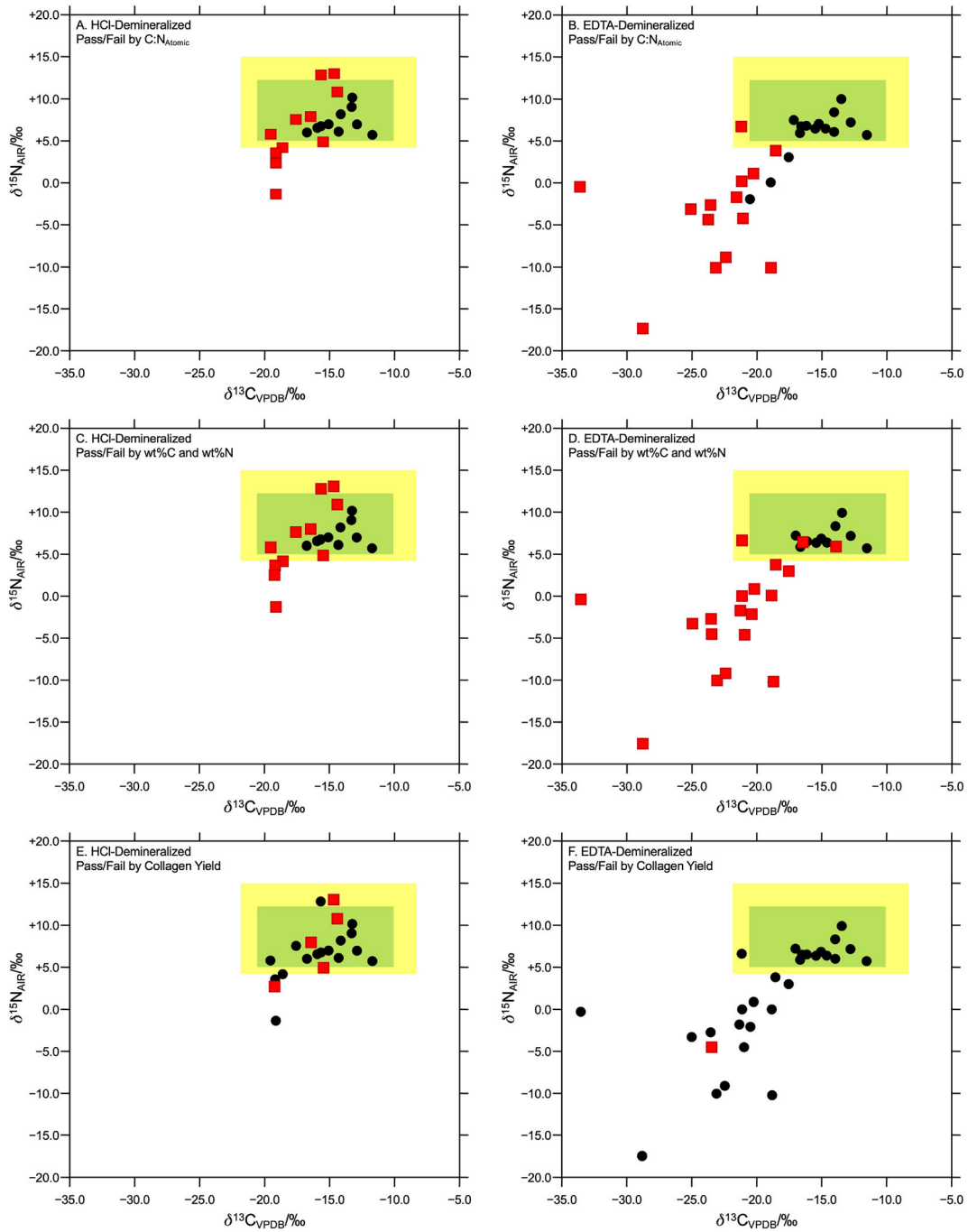

FIGURE 2 Stable carbon and nitrogen isotopic compositions for the HCl-treated (a, c, e) and EDTA-treated samples (b, d, f) compared with the expected ranges for camelids in the study region. The green box corresponds to the 2σ ranges around the mean $\delta^{13}C$ and $\delta^{15}N$ values, and the yellow box corresponds to the full range of $\delta^{13}C$ and $\delta^{15}N$ values for camelids from this region. Each panel depicts samples that passed or failed a particular collagen QC criterion: atomic C:N ratio (a, b), wt% C and wt% N (c, d), and collagen yield (e, f). Samples failing a particular QC criterion are shown as red squares, whereas samples passing that criterion are shown as black dots. [Colour figure can be viewed at wileyonlinelibrary.com]

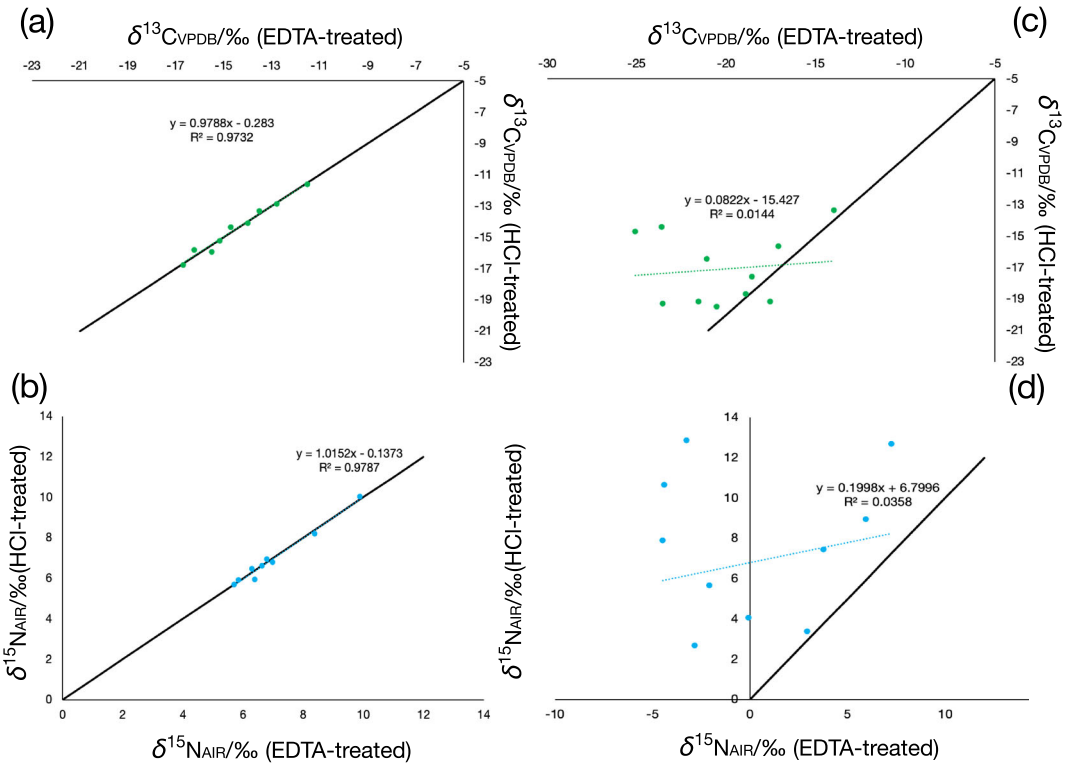

FIGURE 3 Linear regression plots of stable isotope composition compared between EDTA and HCl treatments. (a) $\delta^{13}C$ of samples passing QC criteria ($n = 10$), (b) $\delta^{15}N$ of samples passing QC ($n = 10$). (c) $\delta^{13}C$ of samples failing QC ($n = 11$), (d) $\delta^{15}N$ of samples failing QC ($n = 11$). The broken lines represent the regression lines for each comparison, and the solid black lines represent a 1:1 (i.e., x = y) line. [Colour figure can be viewed at wileyonlinelibrary.com]

0.12 units lower in EDTA (3.13 ± 0.05) relative to HCl-treated samples (3.25 ± 0.09) when the comparison was limited to only those samples that passed all the QC criteria. The wt% C and wt% N values were not significantly different between treatments ($p = 0.18$ and $p = 0.91$, respectively). Among the HCl-treated samples, 11 samples had atomic C:N ratios above 3.60, which is the most commonly used upper limit for quality control of ancient collagen samples (DeNiro, 1985; Guiry & Szpak, 2021). By contrast, only six of the same samples treated with EDTA had C:N ratios beyond this limit. A linear relationship was observed between atomic C:N ratios of equivalent samples treated with EDTA and HCl (Figure 4). Two samples treated with EDTA (14269 and 14275) had atomic C:N ratios that fell within the range that is traditionally associated with isotopically unaltered ancient bone collagen but had extremely low wt% C and N values and stable isotope compositions that are not reflective of the ecology of the animals from which these samples were obtained (Table 3).

4 | DISCUSSION

Obtaining sufficient collagen from poorly preserved bones is a major issue in stable isotope analyses of archeological and paleontological materials. We tested whether EDTA treatment reduces collagen loss relative to strong acid treatment in poorly preserved ancient samples. Collagen yield was significantly higher in samples treated with EDTA, but this increased yield did not translate into a greater number of reliable–stable isotope measurements. It has been suggested that foregoing strong acid demineralization may improve collagen yield in ancient samples (Tuross, 2012). Although this may be true, it does not necessarily result in improved stable isotope data, at least for the samples included in our study. Ancient samples that have low endogenous collagen content cannot be salvaged using a gentler chemical agent such as EDTA, despite this method's propensity to slightly increase the collagen yield.

The $\delta^{13}C$ and $\delta^{15}N$ values of paired samples treated with EDTA and HCl were not significantly different, despite different chemical agents being used for sample demineralization. This finding is consistent with other research that found no significant difference in the stable isotope composition of samples treated with EDTA and HCl (Tuross, 2012; Tuross et al., 1988; Wilson & Szpak, 2022). The atomic C:N ratios of EDTA-treated samples were, however, significantly lower than equivalent samples treated with HCl. The wt% C and N values were not significantly different between treatments, but this is likely due to the high variability in elemental composition across the poorly preserved samples (Tables 2 and 3). This difference in atomic C:N ratios between treatments can be explained by a chemical modification that occurs more frequently in HCl-treated samples, causing a loss of nitrogen but not carbon. Specifically, the deamidation of glutamine and asparagine residues, resulting in the loss of an NH_2 group, may explain these data (Procopio & Buckley, 2017; Simpson

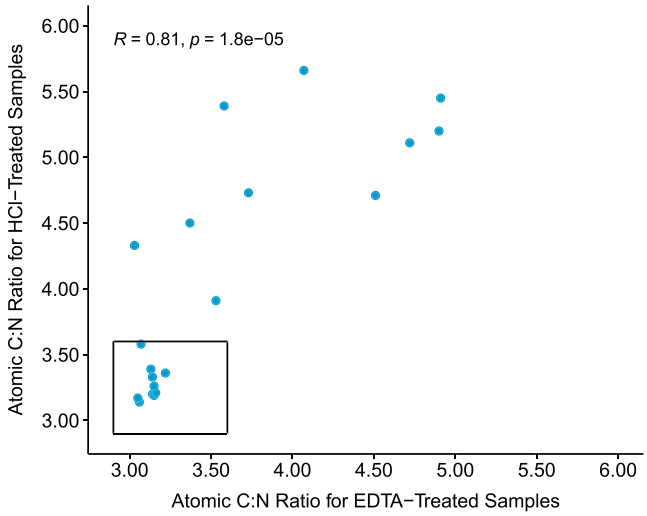

FIGURE 4 Scatterplot of atomic C:N ratio for EDTA-treated samples against HCl-treated samples. The portion of the graph demarcated by the black box represents the range of accepted atomic C:N ratios for bone collagen as defined by DeNiro (1985). Pearson's R is displayed on the graph. [Colour figure can be viewed at wileyonlinelibrary.com]

et al., 2016). An increased loss of nitrogen via deamidation in HCl-treated samples will result in a higher atomic C:N ratio, as we observed, but based on extensive modern experimental data, does not affect the stable isotope composition of the collagen (Wilson & Szpak, 2022). Therefore, a higher rate of deamidation in acid-treated samples may cause a difference in the elemental composition of HCl compared with EDTA-treated samples, as this mechanism is less likely to occur in the latter (Simpson et al., 2016). Although the stable isotope compositions of HCl and EDTA-treated samples were not significantly different, the difference in elemental composition for samples treated with these agents can still impact our interpretations of the data via the inclusion or exclusion of samples based on QC criteria.

Interpreting the reliability of stable isotope data from ancient samples depends on established quality control criteria (Ambrose, 1990; DeNiro, 1985; Guiry & Szpak, 2021). In our analyses, all samples treated with HCl that passed quality control criteria (C:N < 3.60) generated feasible stable isotope results (based on the known stable isotope compositions of camelids from this region). By contrast, multiple samples treated with EDTA would have passed the most commonly used quality control criteria (2.9 < C:N < 3.60, collagen yield > 1%), despite the fact that inaccurate stable isotope values were produced (e.g., $\delta^{15}N < 0$). This is significant because it demonstrates that samples treated with EDTA may pass the traditionally used QC criteria while still producing inaccurate results. Atomic C:N ratio is by far the most commonly reported QC criterion for ancient bone collagen in the archeological literature (79% of the time), followed by collagen yield (51% of the time) and wt% C and wt% N (45% of the time) (results according to the survey of Szpak & Macdonald, 2017). These results further highlight the importance of the wt% C and N QC criteria and that these criteria can, in some cases, better identify collagen samples that have undergone isotopic alteration in the burial environment than the atomic C:N ratio or collagen yield criteria.

In past and present studies using EDTA-treated samples, authors may have used unreliable stable isotope data in their interpretations because they assume, implicitly or explicitly, that QC criteria developed for HCl-treated samples apply equally well for samples treated with EDTA. The established quality control criteria do not take into consideration the variable rates of chemical mechanisms, such as deamidation between these two treatments, and therefore, should not be considered equally applicable to both EDTA and HCl-treated samples. Quality control criteria for bone collagen treated with EDTA should be specifically developed and validated for future studies of ancient samples using this chemical. The results of this study suggest that the existing collagen yield QC is too low for EDTA-treated samples as many of the EDTA-treated samples with collagen yield between 1% and 3% produced clearly unreliable stable isotope compositions and/or failed other QC criteria (Table 3). The commonly used upper limit for the C:N QC is also too high for EDTA-treated samples because of the effects of deamidation on HCl-treated samples (Wilson & Szpak, 2022). Evidence supporting this claim from our study comes from the fact that of the three EDTA-demineralized samples producing C:N ratios between 3.3 and 3.6, one produced an isotopic composition that was flagged, and two produced isotopic compositions that were not feasible; it is, therefore, probable that all three samples have altered isotopic compositions. Studies that have calculated C:N ratios based on amino acid composition data (e.g., Guiry & Szpak, 2020; Nehlich & Richards, 2009; Szpak, 2011) are also inappropriate for EDTA-treated samples. These studies assume that there is no post-translation modification of the collagen (no glutamine or asparagine present), and therefore, they present ranges of elemental compositions that would be obtained only from collagen that has been heavily deamidated as occurs in HCl-based demineralization.

Although our results do not demonstrate that demineralization with EDTA can produce collagen extracts that are superior to HCl from poorly preserved ancient samples, the scope of our study is by no means sufficiently broad to conclusively preclude this possibility. Our interpretations are limited by sample size and the fact that all the samples used in these comparisons originate from a similar burial environment. Increasing the sample size to include more poorly preserved samples from different burial environments and of a wider variety of geological ages is an important next step. There may be certain burial environments that yield bone samples with different chemical properties and patterns of diagenesis than those examined in this research that may benefit from a neutral demineralization agent over an acidic agent (Nielsen et al., 2018). Additionally, EDTA treatment may be beneficial for bones that have a low mineral content and would be destroyed by even a short demineralization in HCl. Because EDTA demineralization proceeds at a much slower rate than HCl demineralization, this agent might be well-suited for delicate samples that require greater attention to the completeness of demineralization

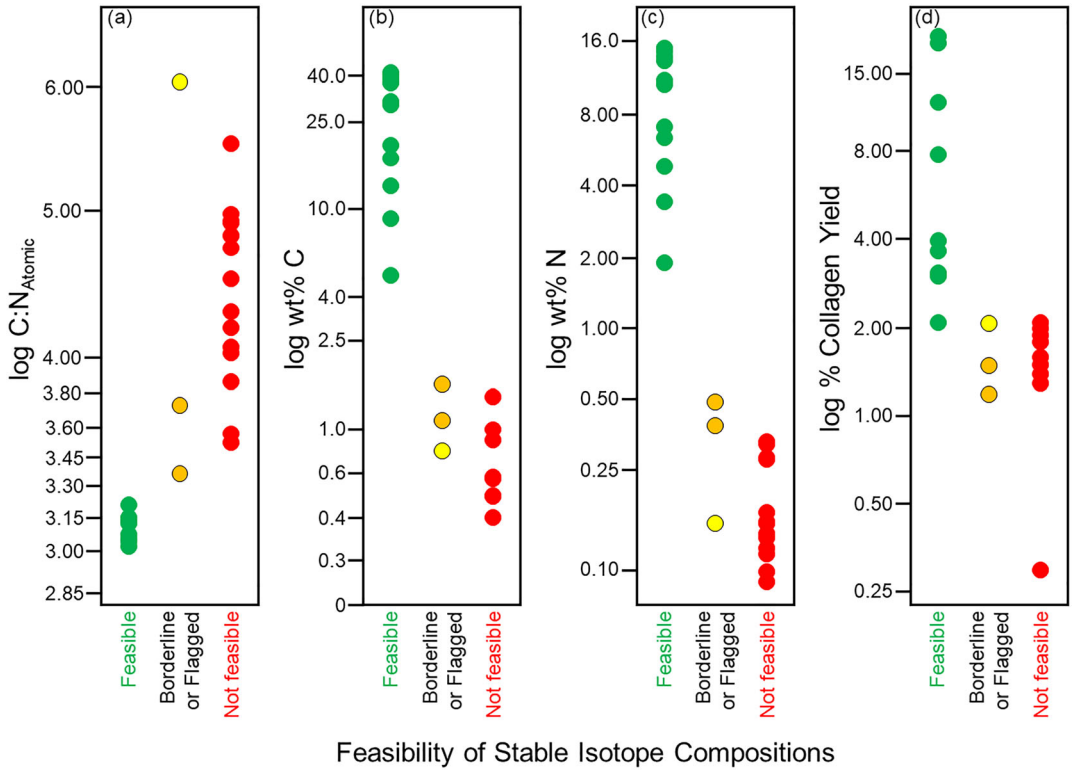

FIGURE 5 Distribution of collagen QC metrics for samples that had feasible, borderline (yellow-filled circles) or flagged (orange-filled circles) and not feasible isotopic compositions. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Proposed quality control criteria for EDTA-demineralized bone collagen.

Criterion	Provisional reliable range	Questionable range	Provisional unreliable range
Atomic C:N ratio	2.80–3.25	3.25–3.45	>3.45
wt% C	>4	2–4	<2
wt% N	>2	0.5–2	<0.5
Collagen yield	>2%	–	<2%

(Collins & Galley, 1998), but our results do not provide empirical support to these assertions.

Collagen QC criteria that are specific to EDTA-demineralized samples must be developed via a robust systematic study covering a broad range of environmental and temporal contexts. Our study was on a relatively small scale, but the results are suggestive of some potential modifications to the existing criteria. We compared the collagen QC criteria for samples that were classified as having isotopic compositions that were determined to be "feasible," "borderline," "flagged," and "not feasible" based on our survey of published data, as discussed previously (Figure 5). For the sake of clarity, we presented these criteria on logarithmic scales. This approach mirrors the one employed by DeNiro (1985) to develop the widely-used acceptable range for collagen C:N ratios—noting under what circumstances an independent metric (e.g., C:N ratio) produced isotopic compositions that were consistent with the ecology of a given organism. For each of these four metrics, there were clear differences in the QC criteria for EDTA-demineralized collagen, producing feasible and not feasible isotopic compositions. The collagen that produced isotopic compositions that were considered not feasible had the following characteristics: collagen yield < 2%, atomic C:N ratio > 3.5, wt% C < 1%, and wt% N < 0.4%. The collagen that produced isotopic compositions that were considered feasible had the following characteristics: collagen yield >2%, atomic C:N ratio <3.25, wt% C > 5%, and wt % N > 1.9%. Based on these values, we have generated provisional QC criteria for EDTA-demineralized bone collagen (Table 4). We did not observe any atomic C:N ratios below 3.03 in this study, but lower values have been observed in modern and well-preserved archeological bone, and we have therefore suggested 2.80 to be the minimum end of this range (Wilson & Szpak, 2022). Based on practical experience, however, it is far more likely that a highly degraded or contaminated sample will either produce a C:N ratio that is too high (as with all the EDTA-demineralized samples producing infeasible isotopic compositions in this study), and if it is particularly low, it will fail one

or more of the other QC criteria. Therefore, the upper end of this range is the more important one to define accurately. (Guiry & Szpak, 2021). With respect to the upper range for the atomic C:N ratio, this value is far lower than the traditionally utilized value of 3.60. Wilson and Szpak (2022) did not observe any modern or well-preserved EDTA-demineralized bone collagen samples that produced atomic C:N ratios above 3.09 for modern bone and 3.15 for well-preserved archeological bone. Moreover, the two EDTA-demineralized bone collagen samples in this study producing C:N ratios of 3.53 and 3.58 produced isotopic compositions that were clearly altered in the burial environment (Table 3). Therefore, although the exact atomic C:N ratio whereby a sample is likely characterized by an unreliable isotopic composition is still unknown, we can confidently assert that for EDTA-demineralized collagen, this value will be less than 3.60 and quite possibly much less than 3.60. Based on the criteria listed in Table 4, we would suggest that to be considered as having reliable isotopic compositions, an EDTA-demineralized sample should pass all four of these criteria. We would also stress that additional work following a similar approach is needed to test the efficacy of these proposed ranges in a variety of burial environments.

5 | CONCLUSION

Pre-treatment using EDTA slightly improved collagen yields in our dataset of poorly preserved ancient samples relative to strong acid demineralization. The stable isotope composition of these EDTA-treated samples was not significantly different from that of HCl-treated samples, nor were a greater number of reliable–stable isotope measurements produced. Our results suggest that a chemical mechanism such as deamidation is occurring at higher rates in HCl relative to EDTA-treated samples causing a discrepancy in the elemental composition that has important implications for how collagen QC criteria are applied to samples treated with EDTA. Future studies that employ EDTA for demineralization should be mindful that QC criteria developed for HCl-treated samples do not apply equally well to EDTA-treated samples. Based on our isotopic data, we suggest the following provisional collagen QC criteria for EDTA-demineralized samples: collagen yield > 2%, wt% C > 4%, wt% N > 2%, and atomic C:N ratio between 2.80 and 3.25.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the main text and supporting information of this article.

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