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Optimizing Pretreatments of Bioapatite for Forensic Analyses: Some Caveats

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Abstract:
The accuracy and precision of pretreatments that remove undesired materials from bioapatite are important in forensic science, environmental science, biology, medicine, and archaeology, especially when bioapatite's stable isotopes are measured. Because there may be diagenesis or contamination, many pretreatments have been tested in the attempt to obtain reproducible results. In this study a fractional experimental design is used to examine the relative efficacy of combinations of six common pretreatments: ultrasonication, bleach, hydrogen peroxide, hydrazine, acetic acid, acetate buffer, and ethanol using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and thermogravimetric analysis (TGA). Pretreatments can alter particle size and composition, impacting subsequent analyses. While pretreatments' temperature and concentration effects on organics and exogenous carbonates have been studied, particle size properties have largely been missed in recent pretreatment optimizations. A discussion of their ubiquitous impact is provided.

Keywords: *ATR-FTIR, Bioapatite, Mule Deer, Particle Size, Standardization, TGA*

1. INTRODUCTION

To reach the full potential for geolocating human remains based on isotopic analyses of bioapatite from tooth and bone, more consistent results between laboratories are crucial [1, 2]. A wider range of reference materials for validation and quantification of sources of error is needed for useful $\delta^{13}C$ and $\delta^{18}O$ databases [3]. Bioapatite phosphates and carbonates use different methods and variations in pretreatments arise as none of them remove all contaminants without altering the composition. Thus, researchers are still optimizing methods for forensic analyses [4, 5].

Ultrasonic treatment is used to remove surface dirt, including some exogenous carbonates [6, 7, 8]. Acids such as acetic acid and acetate buffer are used to dissolve unwanted carbonates, but structural carbonates may also be attacked [7]. Bleach (NaOCl) and hydrogen peroxide (H_2O_2) at various concentrations and temperatures are often used to oxidize and remove organic matter, both structural proteins and organic contaminants. However, these have been shown to alter the carbonates, so hydrazine (N_2H_4) has been suggested as a more selective agent for destroying the amide bonds in the structural proteins [9].

Seven pretreatments were applied to a composite of mule deer tooth enamel used as a proxy for human teeth. The robustness and efficacy at removing exogenous carbonates and organic matter were monitored using thermogravimetric analysis (TGA) [9, 10, 11, 12] and infrared attenuated total reflectance (ATR-FTIR) [7, 9]. The latter is most useful for monitoring alterations of the type A (OH replacing) and type B (PO_4 replacing) structural carbonates, affecting the composition of the hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) in the enamel. Data was collected using an efficient fractional experimental design that applied each pretreatment to molar enamel in various combinations to better characterize sources of potential error.

2. MATERIALS AND METHODS

Molars and premolars from three 2- to 5-year-old mule deer (*Odocoileus h. hemionus*) skeletonized over the course of a year were cleaned of dentine and surface dirt (humus and sandy silt) using a WC burr. About 4 g of enamel pieces were pulverized to < 250 µm in a Spex ShatterBox with a WC puck mill. The powder composition was characterized using XRF (PANalytical Epsilon4), combustion analysis (CE NC2100), ATR-FTIR (Bruker Alpha, 0.4 mm^2 diamond), XRD (Bruker D6 Phaser), and particle size distributions were measured using ImageJ ver. 1.52a freeware on photomicrographs from a Leitz polarizing light microscope.

A two-level fractional factorial design (Minitab DOE software) with 26 samples of the powder was used to measure the efficacy of combinations of seven pretreatments: (1) 10 min of ultrasonication, left in DI water (110 W, 40 kHz Branson 2800 M); (2) 30% H_2O_2; (3) 7.5% NaOCl; (4) 98% N_2H_4 at 20°C and at 55°C [13]; (5) 0.1 M acetic acid; (6) 1 M sodium acetate buffer (pH 4.5); and (7) 95% ethanol. All pretreatments were at 20°C for 24 hours unless noted otherwise. The seven individual pretreatments, four untreated samples, and 15 pretreatment combinations in random order comprised the 26 samples.

After each pretreatment step, the solution and three washes with deionized (DI) water were removed with a pipet after centrifugation (tabletop DLAB D1008 mini, 7000 rpm, 45 mm radius; or a floor model Beckman J2-HS centrifuge with JA-20 rotor, 4500 rpm, 108 mm, both with a relative centrifugal field of 2450 *g* at their r_{max}). The treated and untreated samples were left to air dry (ca. 40% relative humidity, RH) for 24 hours.

The ATR-FTIR analyses were conducted using 16 scans at a resolution of 4 cm^{-1} followed by the instrument's multipoint, "rubber band" baseline correction and normalization of the absorbances to the largest phosphate peak (1018 cm^{-1}) [14]. (The listed peak or band wavenumbers are empirical averages from the samples.) TGA (TA Instruments) was conducted using inert N_2 gas with a ramp rate of 10°C/min from 20°C to 1000°C. The 55°C hydrazine-treated sample was analyzed using combustion analysis in addition to the ATR-FTIR and TGA analyses in order to measure its residual nitrogen.

Statistical analyses of the data were conducted using multiple linear regression with backward elimination of insignificant factors ($P < 0.1$) in a commercial software package (Minitab, ver. 20.4).

3. RESULTS

Figures 1 and 2 show the ATR-FTIR and XRD spectra obtained for the untreated deer enamel powder and some comparison data. The untreated enamel's TGA curve is shown in Figure 3. Table 1 gives the composition from the XRF and combustion analyses. Table 2 summarizes wavenumber data used for preservation indices of alteration commonly calculated from IR data. Table 3 summarizes the TGA mass percentage losses for the temperature ranges associated with adsorbed water, organic, carbonates (overlaps organic char), and structural water components for the samples used to evaluate the pretreatments' efficacies [11, 12].

Figure 4 gives measured particle size distributions for untreated enamel before and after 12 water washes. Figure 5 shows the theoretical mean distances that different-sized spherical particles with a density of 2.9 g/cm^3 will travel in water with a typical RCF of 2450 *g* in a centrifuge. The most IR-absorbing 5–50 µm size range is truncated by inadequate centrifugation, impacting the composition [15], sampling statistics, and correspondence of instrumental methods.

Table 1. *Elemental composition (%) of the untreated deer enamel*

C	6.25
N	1.91
O	30.93[a]
Mg	0.76
Si	0.27
P	18.49
Cl	0.29
K	0.83
Ca	39.50
Fe	0.37
Sr	0.14
Ba	0.13

[a] *Obtained by difference*

Table 2. *ATR-FTIR absorbances for various pretreatments, ratioed to the 1018 peak* [a]

Pretreatments	1644	1544	1453	1411	601	590	560
Ultrasonication (Ult)	0.127	0.113	0.212	0.217	0.541	0.446	0.900
Hydrazine (Hyd)	0.131	0.110	0.192	0.207	0.564	0.430	0.902
7.5% Bleach (Ble)	0.068	0.066	0.212	0.212	0.541	0.438	0.861
30% H_2O_2 (Per)	0.120	0.086	0.181	0.191	0.564	0.430	0.911
95% Ethanol (Eth)	0.087	0.073	0.136	0.148	0.576	0.424	0.980
0.1 M HOAc (Ace)	0.141	0.109	0.190	0.184	0.547	0.415	0.930
1 M Buffer (Buf)	0.127	0.102	0.192	0.203	0.550	0.429	0.905
Ble-Eth-Ult-Hyd	0.129	0.108	0.196	0.207	0.561	0.437	0.905
Per-Ble-Buf-Ult	0.050	0.039	0.073	0.083	0.610	0.359	1.021
Ult-Eth-Buf	0.089	0.264	0.144	0.253	0.577	0.405	0.904
Ace-Eth-Ult	0.071	0.056	0.124	0.142	0.611	0.440	1.006
Hyd-Per-Buf-Eth	0.022	0.024	0.075	0.064	0.574	0.359	0.990
Per-Ble-Eth	0.075	0.116	0.184	0.206	0.549	0.432	0.866
Ace-Eth-Per	0.032	0.029	0.087	0.083	0.497	0.344	0.884
Ace-Ble-Ult	0.014	0.022	0.071	0.070	0.683	0.433	1.147
Hyd-Ble-Buf	0.017	0.029	0.079	0.086	0.664	0.487	1.146
Ble-Eth-Ace-Buf	0.022	0.023	0.078	0.077	0.716	0.447	1.160
Buf-Per-Ace	0.112	0.088	0.147	0.152	0.537	0.392	0.920
Ult-Ble-Eth	0.019	0.043	0.130	0.148	0.649	0.509	1.051
Buf-Per-Ult	0.047	0.050	0.128	0.128	0.573	0.439	0.985
Eth-Per-Ult	0.103	0.085	0.163	0.172	0.553	0.424	0.928
Ult-Hyd 55ºC-Eth	0.046	0.051	0.147	0.155	0.498	0.404	0.853
Untreated-1	0.073	0.064	0.135	0.138	0.560	0.414	0.945
Untreated-2	0.060	0.057	0.132	0.134	0.558	0.406	0.928
Untreated-3	0.055	0.056	0.123	0.121	0.588	0.405	0.940
Untreated-4	0.096	0.080	0.161	0.164	0.563	0.430	0.935

[a] *(Note: all absorbances were normed to* $A_{1018} = 1$*). C/C = Type (A+B)/B =* A_{1453}/A_{1411}*. C/P =* A_{1411}/A_{1018}*. IRSF =* $(A_{601} + A_{560})/A_{590}$*. Crystallite size ≈ (IRSF – 0.822)/0.048 = 59 nm for the untreated enamel. Compare this to 40 nm from the XRD FWHM measured at* 25.89^o*.*

Table 3. *TGA percentage of total mass lost with various pretreatments and mg of sample used*

Pretreatments	Water %	Volatile %	Char %	Carbonate %	Sample mg
Ultrasonication (Ult)	3.319	5.747	6.533	1.717	7.918
Hydrazine (Hyd)	3.105	5.904	5.209	1.690	8.590
7.5% Bleach (Ble)	3.475	3.767	3.959	1.953	14.153
30% H_2O_2 (Per)	3.449	5.625	5.179	1.784	15.438
95% Ethanol (Eth)	2.505	6.982	5.267	2.132	9.911
0.1 M HOAc (Ace)	2.608	7.526	5.517	1.736	4.982
1 M Buffer (Buf)	3.636	6.530	3.914	3.797	14.028
Ble-Eth-Ult-Hyd	3.738	1.112	1.353	3.293	8.114
Per-Ble-Buf-Ult	2.084	7.725	5.920	1.478	3.551
Ult-Eth-Buf	2.968	11.690	5.448	3.555	8.263
Ace-Eth-Ult	3.247	7.642	6.507	1.691	6.083
Hyd-Per-Buf-Eth	2.048	6.238	4.088	3.045	5.595
Per-Ble-Eth	2.599	6.179	5.283	1.732	5.942
Ace-Eth-Per	2.486	3.783	3.283	1.811	7.249
Ace-Ble-Ult	1.910	2.500	1.339	2.537	5.782
Hyd-Ble-Buf	1.391	3.180	1.808	4.199	4.387
Ble-Eth-Ace-Buf	2.015	3.747	3.272	1.368	2.740
Buf-Per-Ace	2.385	6.564	5.135	2.041	5.236
Ult-Ble-Eth	3.339	1.071	1.131	2.083	5.442
Buf-Per-Ult	3.248	8.318	7.193	1.871	7.573
Eth-Per-Ult	2.720	7.125	5.213	1.100	1.076
Ult-Hyd 55ºC-Eth	3.210	2.343	1.613	1.718	4.670
Untreated-1	4.239	7.969	4.920	1.640	11.212
Untreated-2	3.349	7.787	4.751	1.602	11.624
Untreated-3	3.644	7.181	5.321	1.713	11.166

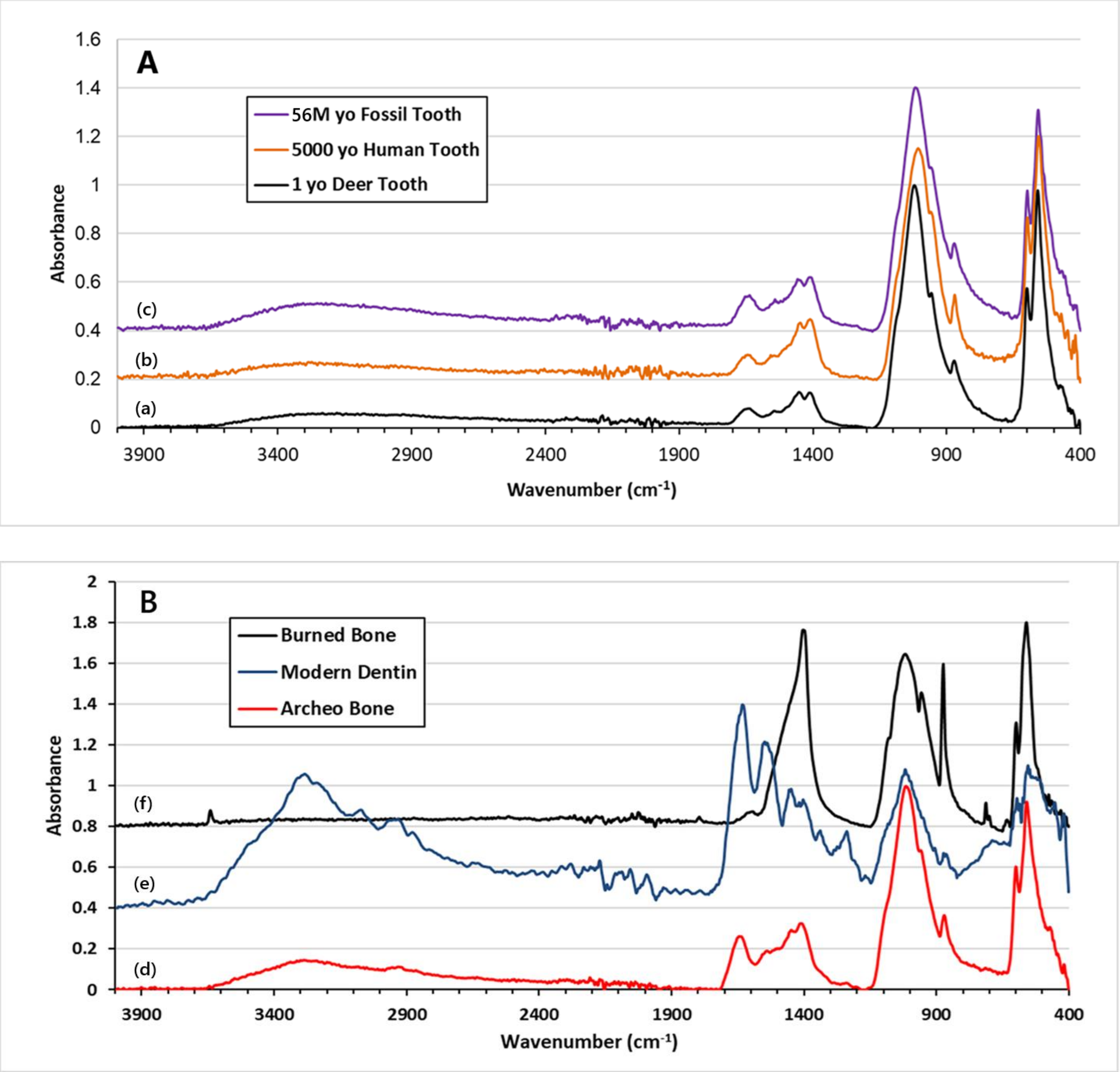

Figure 1. *(A) Baselined and normed ATR-FTIR spectra of untreated enamels (0.2 A offsets for clarity) from (a) a 1-year-old deer, (b) a 5000-year-old human, and (c) a 56-million-year-old fossil gar, illustrating the similarity and stability of enamel bioapatite between species over time.*

(B) Same conditions (0.4 A offsets): (d) untreated 130-year-old femur, (e) modern human dentin with its protein matrix [8], and (f) burned (500°C) femur with secondary carbonate peaks. These illustrate the major sources of interferences that are ameliorated with pretreatments.

4. DISCUSSION

The XRF and combustion analysis data (Table 1) and the XRD and ATR-FTIR spectra and TGA plot (Figures 1–3) mimic bioapatite from human enamel. The observed Ca/P is 2.14 compared to the expected 2.16 from stoichiometry, and the C/N is 3.265 compared to collagen's 3.243. Finally (Table 2), the C/P and IRSF indices (0.11 and 3.64) are within ± 1*s* of human values, implying deer enamel is a useful proxy for testing how pretreatments impact human enamel [16].

ATR-FTIR results should be treated as semiquantitative, as natural bioapatite has many spectral overlaps of its organic and mineral components. Peak heights, areas, and wavenumbers are also dependent on the particle size [17]. Moreover, the small volume of interaction with sample particles can limit the precision of analyses unless the sample is reloaded many times. The ATR window used here is 0.36 mm^2 and the penetration depth for the 1411 cm^{-1} carbonate peak is 0.5 µm in bioapatite, giving a sampling volume of 1.8 x 10^5 µm^3. This is equivalent to a single 100 µm diameter particle. For particles near 10 µm in diameter, this volume limits precision (%RSD) to 5% to 10%. (Particles < 1 µm are too small to interact in the mid-IR region [15].) Using many scans of the same sample placement without reloading does not fix this sampling limitation.

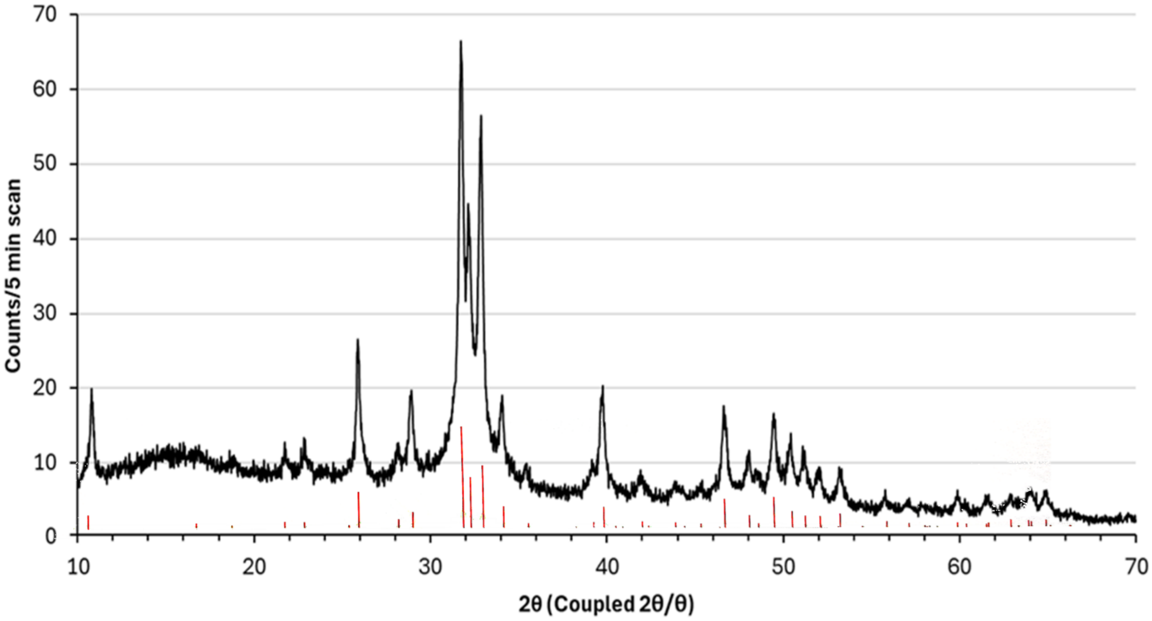

Figure 2. *XRD spectrum of untreated deer tooth enamel. Search-match of the 40,000 minerals in the spectral library best matched* $Ca_5H_{1.44}O_{13}P_{2.928}$ *(hydroxyapatite with partial substitution by F,* CO_3*, etc.; red bars).*

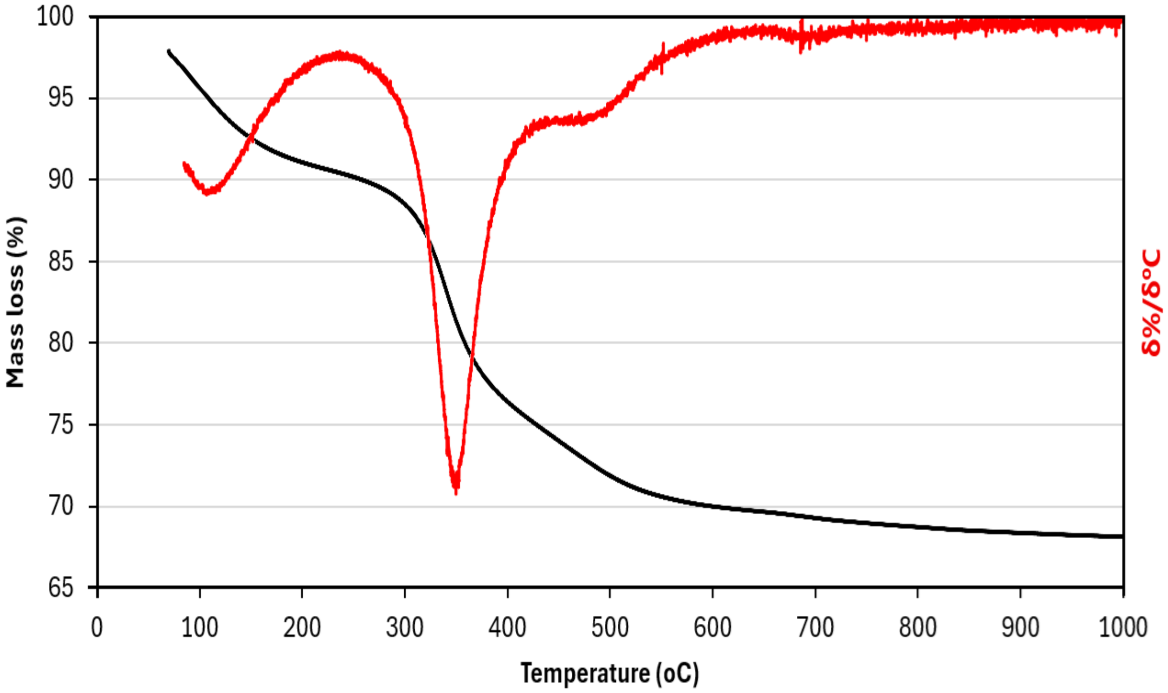

Figure 3. *Typical TGA curve and its derivative (in red) for untreated deer enamel. Note derivative minima at 106°C (adsorbed water), 340°C (volatile organics), 460°C ("char"), and 690°C (strongly bound water and carbonates).*

The sampling issue is exacerbated by the impact of pretreatment washes on particle sizes (Figures 4 and 5). Cavitation during ultrasonication and attack by pretreatments preferentially creates small particles of friable materials that are subsequently lost and not included in analyses. The 1644 peak is shifted by up to 6 cm^{-1} between untreated and treated samples ($P = 0.037$), likely due to changes in composition rather than particle size effects. There was also evidence that treatments like bleach may affect the phosphate crystallinity as the 601/560 ratio decreased.

Thus, ratios of absorbances used to monitor levels of alteration can follow the effects of pretreatments and of diagenesis over time, but they are primarily qualitative measures.

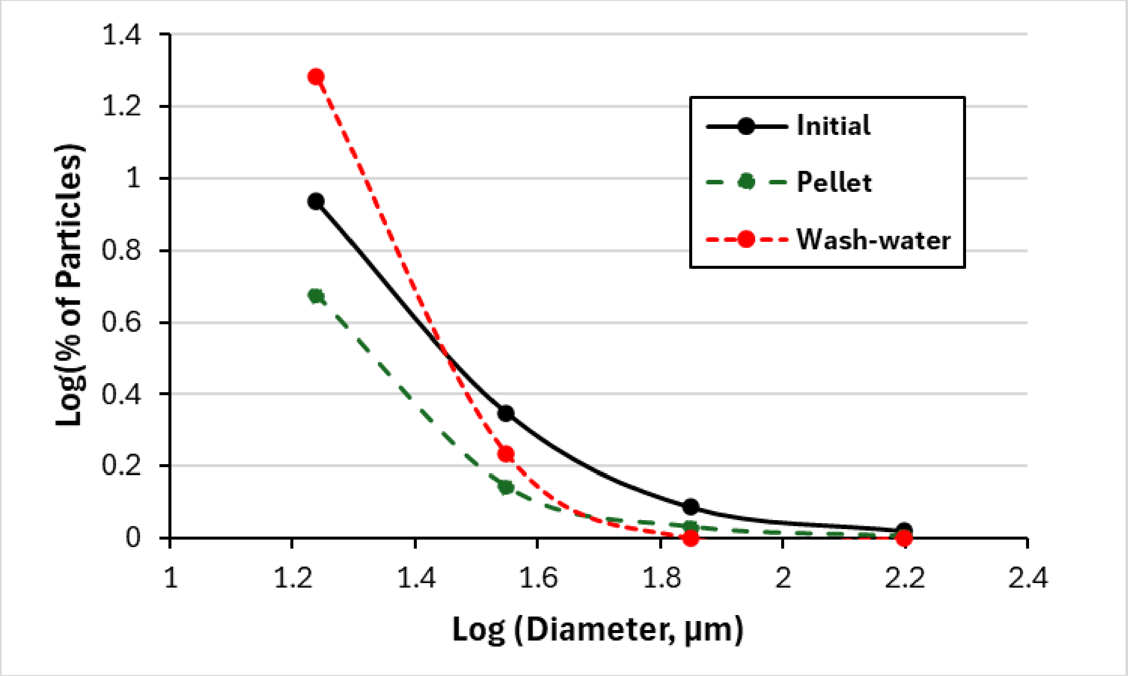

Figure 4. *Particle size distributions for the untreated deer enamel powder before and after washing with water 12 times, corresponding to three washes after each of four treatments. The pellet contains the retained particles used for analyses (e.g., ATR, TGA, stable isotopes). The mean aspect ratio (length/width = 5) of suspended particles was twice that found in the pellet.*

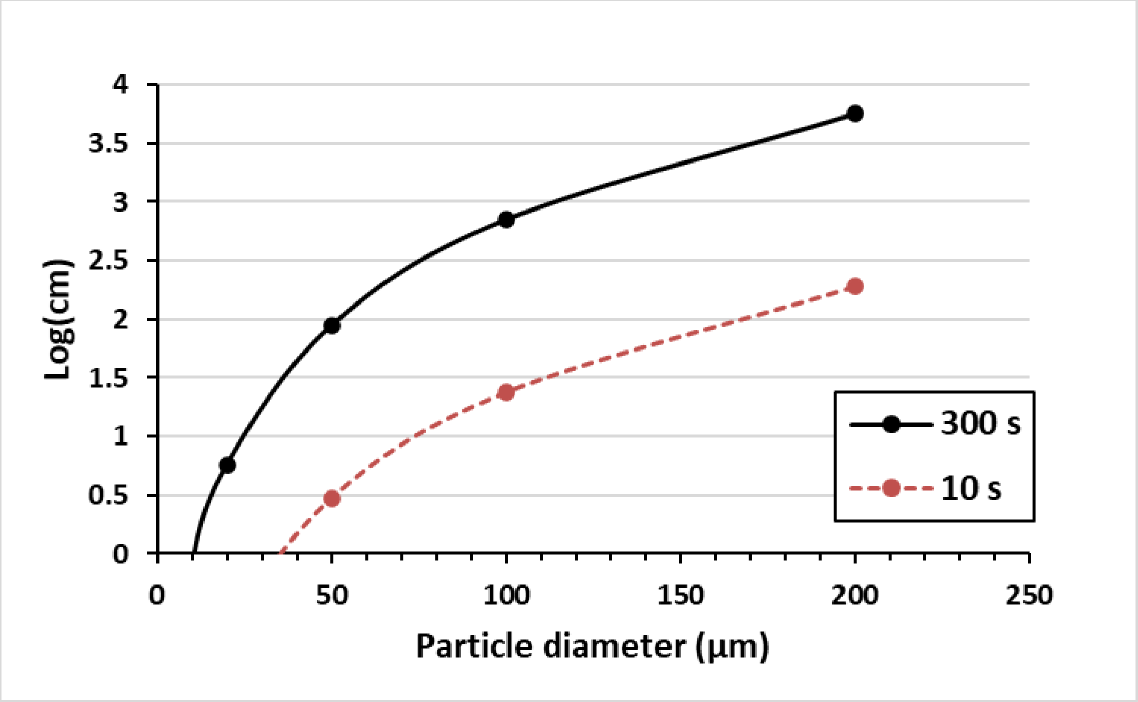

Figure 5. *Theoretical sedimentation distances for two centrifuge times at 2450 g for different spherical particle sizes. Many particles below log(cm) = 0 are suspended and lost when pretreatment reagents are removed with multiple water washes.*

Given spectral overlaps and particle size exclusions via ATR, TGA's use of larger sample sizes might be expected to give more accurate and precise analyses. However, TGA also suffers from overlaps. For example, as temperature increases, loss of water adsorbed on the surface is followed by water from the decomposition of organic compounds, followed by water bound in the bioapatite structure. These different water sources may not have distinct cutoff temperatures, and they overlap with the temperatures at which organics and carbonates are decomposed. Nevertheless, based on differences in the mass losses in Table 3, the impact of the pretreatments on the surface water, volatile organics, "char" (from organics and carbonates), and strongly bound carbonate and water can be obtained.

Based on the statistical analysis of the ATR-FTIR data in Table 2, bleach was found to be the most effective at removing proteins represented by the 1644 cm^{-1} peak (P = 0.038). It also lowered the 1453/1411 (C/C) ratio (P = 0.07) by removing an organic spectral overlap or by increasing type B carbonates. Increasing type B carbonates is counter to what is expected or desired [7], as they strain the crystal structure more, which should make them easier to remove. However, oxidation of organics can add carbonates, as was noted with H_2O_2 [7].

The buffer was as effective as bleach at lowering the 2950 cm^{-1} CH peak and was more effective at lowering the broad 3250 cm^{-1} OH peak (P = 0.076). The buffer was also the most effective at removing type B carbonates at 1411 cm^{-1} (P = 0.027). Finally, the buffer and acetic acid were the most effective at lowering the 1411/1018 (C/P) ratio (P = 0.027 and 0.089, respectively) and at raising the IRSF value (P = 0.012 and 0.045, respectively). H_2O_2 and N_2H_4 treatment at 20ºC for 24 hours slightly lowered the organic matter, but not significantly ($P > 0.10$).

The statistical analysis of the TGA data in Table 3 showed TGA is more quantitative than ATR-FTIR and better able to discern the magnitude of the pretreatment effects, though it is not as useful for discerning specific alteration effects. Using Bonferroni or Holm's test [18] for multiple comparisons from the multiple linear regression, backwards elimination found the amount of surface water measured between 70ºC and 230ºC was correlated with the mass of sample used ($P < 0.001$). This is not surprising, but it underscores the need for pre-drying samples at some specified standard RH, such as in a desiccator with a well-characterized drying agent.

As expected, the percentage of volatile organics lost between 230ºC and 400ºC was inversely related to the bleach ($P < 0.001$) and hydrazine (P = 0.003) pretreatments. While the 20ºC, 24-hour hydrazine was only 15% complete, the 55ºC, 24-hour method was 83% complete, based on %N from the combustion analyzer. However, the acetate buffer increased the volatile organic fraction, so it was not completely washed out even with three rinses with DI water (P = 0.009).

The mass loss between 400ºC and 600ºC in an inert atmosphere is primarily due to carbonates but also to refractory organic matter. The loss in this temperature range is highly correlated with the volatile organic fraction ($P < 0.001$). In air, more of the organic matter is lost between 230ºC and 400ºC, but some of the measured carbonates are secondary carbonates formed by the action of the pretreatments on organics in the sample. Bleach is the only pretreatment that was significant after applying the Holm's test (P = 0.006) [18]. However, H_2O_2 may increase the measured carbonates (P = 0.027), while hydrazine and ethanol may lower the carbonates (P = 0.042 and 0.050, respectively), possibly via their added washing cycles.

Finally, the mass loss between 600ºC and 900ºC is very small and probably contains a mix of tightly bound water and carbonate. None of the pretreatments had a significant effect after applying Holm's test, though backwards elimination suggested 55ºC hydrazine may increase the mass loss (P = 0.030). (Bonferroni correction implies $P > 0.007$ might not be significant.)

Overall, TGA verified the ATR-FTIR results and provided higher statistical significance due to the larger sample sizes it uses compared to the volume sampled by the Bruker ATR-FTIR in this study. However, TGA, sometimes used to assess burned remains, is totally destructive and time consuming compared to ATR-FTIR. Even the small ATR window and short treatment times used in this study proved adequate for detecting unwanted organic matter and exogenous carbonate contamination. Thus, ATR-FTIR is preferable for monitoring the impact of pretreatments, even those applied for only 24 hours in an efficient fractional factorial experimental design.

5. CONCLUSION

The results obtained in this study are in good agreement with work elsewhere [7, 16]. Extra care with washing is needed when using acetate buffers as they can contaminate the C and O content. Surface water can also add error if a consistent RH is not obtained. Physical and chemical pretreatments and washing protocols not only affect the composition, but also the particle size used for analysis, something not always considered in optimization studies. This can impact the comparability of geolocation results from different laboratories. Alterations [15] and IR absorbances used to monitor alteration are affected by particle size and homogeneity that varies with tooth maturation. While diagenetically altered bones and dentin are improved with pretreatments that remove exogenous carbonates and organic matter [16], this study underscores the importance of testing tooth samples before applying treatments. Often, pretreating enamel powder is unnecessary, as even physical cleaning (e.g., ultrasonication) and washing with water can fractionate and affect particle size, shape (aspect ratio), and composition, potentially impacting and limiting interlaboratory comparability [2, 3, 5].

6. DECLARATION OF COMPETING INTEREST

The authors declare no competing or conflicting interests.

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