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TECHNICAL NOTE

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A New Approach to Amino Acid Racemization in Enamel: Testing of a Less Destructive Sampling Methodology*

ABSTRACT: Aspartic acid racemization has been found to be an accurate measure of age at death for recent forensic material. This paper examines the practicality of using acid etching of the tooth surface to extract amino acids from the enamel for racemization analysis. By serial etching of the tooth and contamination of the teeth with bovine serum albumin prior to etching, the ability of etching to remove contamination was assessed. The destructiveness of the method was visualized and quantified using micro-computed tomography (micro-CT). By bleaching the teeth and by deeper etching it was possible to obtain more consistent values. While etching had little effect on the enamel at the macroscale, it did have an impact at the microscale. The quantities of enamel removed varied depending upon the tooth morphology, but were not large. Acid etching of enamel thus appears to be a promising new method for extracting proteins for amino acid racemization age estimation noninvasively.

KEYWORDS: forensic science, forensic anthropology, amino acid racemization, age estimation, enamel, acid etching, contamination

In both forensic and archaeological case work, being able to determine the age at death of an individual is of critical importance. However, in skeletonized remains, our ability to estimate age has largely been hampered by the reliance of most age determination methodologies on degenerative processes, which are strongly influenced by lifestyle and health. As a result, there has been interest in the development of aging methods which utilize chemical processes which occur in the body during life. One such method that has received attention in forensic contexts is amino acid racemization in dentine. This method has been found to have higher accuracy than many other age estimation techniques when applied to unburnt remains (1). However, because the dentine is inaccessible without drilling or sectioning of the tooth, this method is highly destructive, and appears to be influenced by the postmortem history of the sample (2). The proposed application of forensic age estimation methods to living individuals (1) would also be more practical if sampling was less destructive.

Dental enamel may provide a suitable alternate substrate to dentine (3). Racemization in enamel is less well correlated with age than racemization in dentine (4,5) but the correlation is still adequate for forensic age estimation purposes. Existing methods for extracting amino acids from the enamel require sectioning, which results in the destruction of the majority of the tooth (e.g., 5). In this paper, we present the details of a new sampling method which uses acid etching to extract proteins from the surface enamel. As the tooth remains largely intact, it should be possible for the tooth to be used for other analyses, and to be returned to the body of the deceased after analysis. The amino acids extracted by this method have been found to show a good correlation between aspartic acid racemization and age of the tooth (6).

Materials and Methods

All modern teeth were provided by the Clockhouse Dental Surgery (York) and consisted of extracted teeth from individuals of unknown age. Long-term burial is likely to enhance surface contamination of enamel, therefore unprovenanced samples from the late Saxon/early Norman cemetery at Newcastle Blackgate were used in the serial etching experiment. The remains analyzed were buried directly in the ground, and thus had been in contact with the soil from their burial in the 8th–12th centuries AD until their excavation in 1990–1992. Since excavation, the teeth had been in storage, most recently at the University of Sheffield in the Department of Archaeology. Samples which were free from dental caries and severe dental wear were selected, specifically a maxillary third molar from one individual and a mandibular first premolar and mandibular third molar from a second individual. Both individuals were estimated to be over 30 years of age at the time of death from the level of wear of their teeth. However, the precise age at death of these individuals was not known.

Two separate experiments (contamination and serial etching experiments) were conducted to determine the ability of a combination of bleaching and acid etching to extract uncontaminated amino acids from dental enamel for racemization analysis. The serial etching experiment was intended to investigate the potential for the

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method to detect contamination within the tooth itself. The contamination experiment focused on the ability of this method to remove contaminants acquired during the burial period. A third experiment (micro-computed tomography [micro-CT]) was designed to both visualize and measure the destructiveness of the acid etching technique.

Serial Etching Experiment

For the serial etching experiment, the surfaces of each tooth were cleaned by applying one 0.2 mL PCR tube filled with 6 M HCl (total volume *c*. 230 μL) to each of the labial and lingual sides of each tooth for 1 min, then rinsed with high performance liquid chromatography (HPLC) grade methanol (Fisher Scientific UK Ltd, Loughborough, UK). On one side of the tooth, samples of enamel were serially etched by applying each of eight 0.2 mL PCR tubes filled with 6 M HCl (total volume *c*. 230 μL) for two consecutive 30-sec time intervals, followed by six consecutive intervals of 1 min (without intervening washing between etching steps). The tooth surfaces were then rinsed with ultrapure water and placed in 12% sodium hypochlorite. After 2 days, they were rinsed again in ultrapure water and HPLC grade methanol before serial sampling of the other side of the same tooth. As before, samples were taken by applying sixteen 0.2 mL PCR tubes filled with 6 M HCl (total volume *c*. 230 μL) for two consecutive 30-sec time intervals, followed by 14 consecutive intervals of 1 min. To establish the ease with which surface contamination can be acquired, a break of 3 weeks was included between the 8-min and 9-min sampling events. During this period, the tooth was first rinsed with ultrapure water, then wrapped in foil and stored at room temperature.

Contamination Experiment

One canine, two premolars, and two molars extracted from living individuals without caries or fillings were selected for the contamination experiment. No age information was available for these teeth.

The surfaces of each tooth were cleaned by applying a 0.2 mL PCR tube filled with 6 M HCl (total volume *c*. 230 μL) to each of the labial and lingual sides of each tooth for 1 min, then rinsed with HPLC grade methanol. The teeth were then placed in 12% wt/vol sodium hypochlorite for 2 days to remove contaminating proteins from handling of the teeth and then rinsed in ultrapure water and HPLC grade methanol. Samples were taken by applying two 0.2 mL PCR tubes filled with 6 M HCl (total volume of each tube *c*. 230 μL) to each of the labial and lingual surfaces for two consecutive time intervals of 1 min, with the second sample from each side retained for analysis. The teeth were then dipped in 20 mg/mL bovine serum albumin (BSA) for 2 min and baked at 88°C for 2 h. The teeth were allowed to cool and then sampled again by the method outlined above.

All the samples were hydrolyzed at 110°C for 6 h in a N_2 enriched atmosphere in sterile glass vials. Although racemization may be induced by the use of acid in the sampling methodology, the extent of induced racemization is expected to be minimal as all the samples analyzed were either hydrolyzed shortly after collection or stored in a freezer until they were hydrolyzed. In this study, a hydrolysis temperature of 110°C has been used, rather than the 100°C used in previous studies of enamel racemization (4,5). The use of a higher hydrolysis temperature will result in a larger amount of induced racemization during the hydrolysis step than was observed in previous research. However, as the same hydrolysis procedure was used for all the samples included in this study, the amount of induced racemization will be similar for all the samples. Hydrolysis for 6 h at 110°C was chosen as this protocol was recommended by Kaufman and Manley (7). The Asx (aspartic acid and asparagine) D/L values obtained in this study will thus be higher than was reported in previous research, but should bear a constant relationship to these values. Based upon the kinetics of racemization in enamel observed by Ohtani and Yamamoto (5), using a hydrolysis temperature of 110°C rather than 100°C should increase the offset by *c*. 0.0027 units on the $\ln[(1 + D/L)/(1-D/L)]$ scale.

After hydrolysis, the samples were dried on a rotary evaporator overnight. When dry, the samples were rehydrated in 50 μL of internal standard solution (L-homo-arginine, 0.01 mM, 0.003 M HCl, 0.77 mM sodium azide, with a pH of 2). The sample vials were vortexed to aid dissolution, centrifuged, and the supernatant collected and analyzed by HPLC (Agilent Technologies UK Ltd, Wokingham, UK). Samples were separated on a Hypersil BDS column (Hypersil, Runcorn, UK) by reverse phase HPLC using a modified version of the method of Kaufman and Manley (7). Absolute concentrations of L- and D- amino acids were calculated from the integrated area under each peak and normalized to the peak area of the internal standard, L-homo-arginine.

Destructiveness of the Technique

In addition to the investigation of the ability of acid etching and bleaching to obtain uncontaminated amino acids from dental enamel, the present study also sought to determine the extent of enamel loss resulting from the application of this technique. To this end, the volumes of two modern teeth before and after etching were measured using micro-CT. Whereas scanning electron microscopy would have allowed visualization of the impact of etching on the tooth surface at a higher resolution, the use of micro-CT made it possible to both visualize and quantify the loss of enamel from the tooth surface simultaneously. Both teeth used were modern extracted teeth from individuals of unknown age, with no evidence of caries or fillings.

The two teeth were initially sent to the Centre for Medical Engineering and Technology at the University of Hull, where they were scanned using micro-CT. The teeth were then sampled as described above, before being returned to the University of Hull for a further micro-CT scan.

Scanning was performed using an X-Tek HMX160 micro-CT system (X-Tek, Tring, UK), with each scan taking *c*. 3 h to complete. The X-ray source used a voltage of 78 KV and a current of 6 μA with an aperture setting of 10% and a 0.1 mm Cu filter. About 1000 projections were taken while rotating the specimen through 360°, taking 128 frames per projection that were averaged to reduce noise artifacts and produce the final result for each projection. Reconstruction took *c*. 2 h and was conducted using NGI CT Control software (X-Tek) applying a Butterworth filter; this created 500 slice images at a resolution of 1000 × 1000 pixels. The resulting scan resolutions were *c*. 14 μm × 14 μm × 28 μm and 17 μm × 17 μm × 33 μm for tooth 1 and tooth 2, respectively. Volumetric reconstructions were created in VGStudioMAX (Heidelberg, Germany) and two-dimensional 16-bit slice images (Tiff format) were exported into AMIRA image segmentation software (TGS Inc., Chelmsford, MA) for analysis. The teeth were mounted on a stand that could be fixed in the same position on the scanner turntable to ensure, as much as possible, that they were in the same position during the second scan. In addition, scan parameters such as object distance from the x-ray source and angular increments during scanning were kept constant, ensuring equal

resolutions for each tooth. However, inevitably there were some minor differences in position that were visible in the magnified three-dimensional reconstructions. To overlay the etched and unetched teeth exactly, some manual manipulation was performed followed by automatic registration in AMIRA, which computes an affine transformation using an iterative optimization algorithm. A hierarchical strategy is applied, starting at a coarse resampling of the dataset, proceeding to finer resolutions. All transformations were deemed rigid, thus no deformation took place during the re-orientation process.

Results

The method for amino acid analysis applied here is able to detect and separate successfully L-Asx, D-Asx, L-Glx (L-glutamic acid and L-glutamine), D-Glx, L-Ser, D-Ser, glycine (Gly), L-alanine (L-Ala), and L-homo-arginine (Fig. 1). The Asx D/L values for the bleached and unbleached sides of each tooth are shown in Fig. 2. In all three cases, the Asx D/L at the tooth surface is very high, but gradually decreases to comparatively consistent values in the deeper sections of the enamel. The Asx D/L value for the bleached side of the tooth tended to be lower than that of the unbleached side and reached a steady state much earlier.

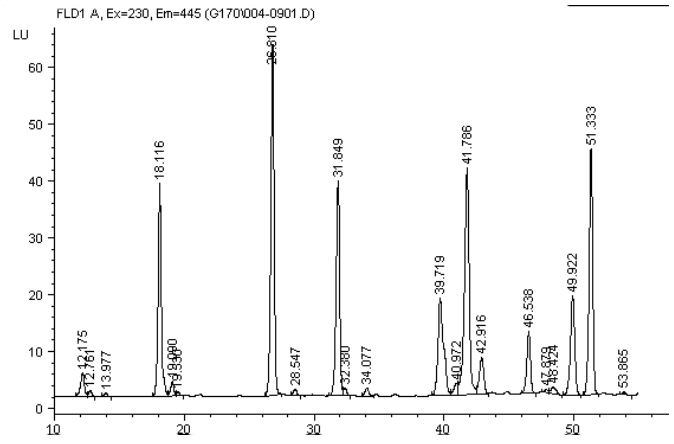

FIG. 1—*Chromatogram of a typical enamel sample.*

The values obtained for Glx D/L (Fig. 3) also tended to be high at the surface of the tooth. In teeth A and B, the Glx D/L values decreased as the samples were taken from deeper sections; the values obtained in the deeper parts of the enamel were significantly lower than those for Asx (Fig. 3). For tooth C, there is a high level of variability in the Glx D/L values and the depth of sampling appears to have little impact on the Glx D/L values obtained, although it is unclear why.

All the samples taken prior to contamination appear to resemble the amino acid composition of enamel previously reported (8–10), as demonstrated by Principal Components Analysis (PCA) of the amino acid compositions of the samples (Fig. 4, with the amino acid weightings of each principal component in Table 1; PCA conducted using MINITAB [Minitab Inc., State College, PA]). Even after contamination, the amino acid compositions of both sets of tooth samples resembled enamel rather than BSA, but concentrations somewhat surprisingly fell.

Destructiveness of the Technique

Little change to the tooth surface after etching was visible with the naked eye. However, examination using micro-CT showed that the areas that had been etched left a mottled texture on the enamel surface (Figs. 5 and 6) with some cracking of the enamel. The extent of the acid etching could also be detected in thinning of the enamel in the sampled regions of the tooth.

The impact of acid etching appeared to vary between the teeth; compare tooth 1 (Figs. 5 and 6; little change) with tooth 2 (Figs. 7 and 8; extensive surface alteration and enamel loss). (Note that in Figs. 6 and 8, the shaded regions are the surfaces from the original scan images, before etching, overlaid on the teeth after etching. The etched teeth images have been fractionally enlarged to show more clearly the etched region; however, this did not affect the numerical calculations.) The difference in the effect of the etching is reflected in the measured changes in tooth volume, with tooth 2 having lost twice as much enamel during etching as tooth 1. This is probably because of the different surface morphologies of the two teeth. The sampled surfaces of tooth 2 were flatter than those of tooth 1, allowing greater amounts of the enamel surface to come into contact with the acid. Despite the difference in absolute loss, because of the differences in size both teeth lost *c*. the same total volume (3%) as a result of the bleaching and etching process (Table 2).

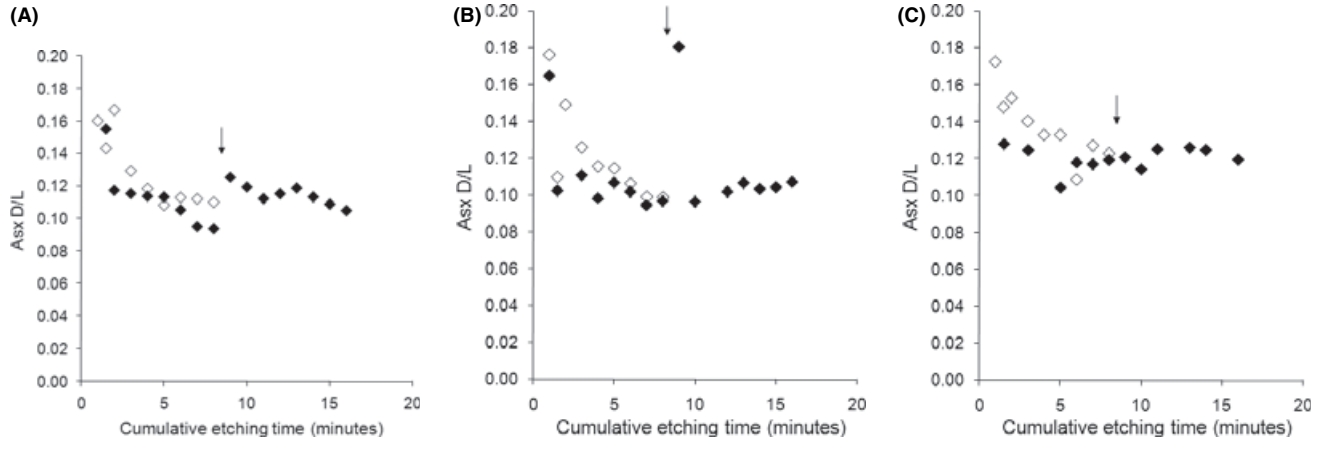

FIG. 2—*Asx D/L values for the unbleached (closed diamond) and bleached (open diamond) sides of teeth A, B, and C plotted against etching time. The gap in sampling of the teeth is marked by an arrow. Asx, aspartine.*

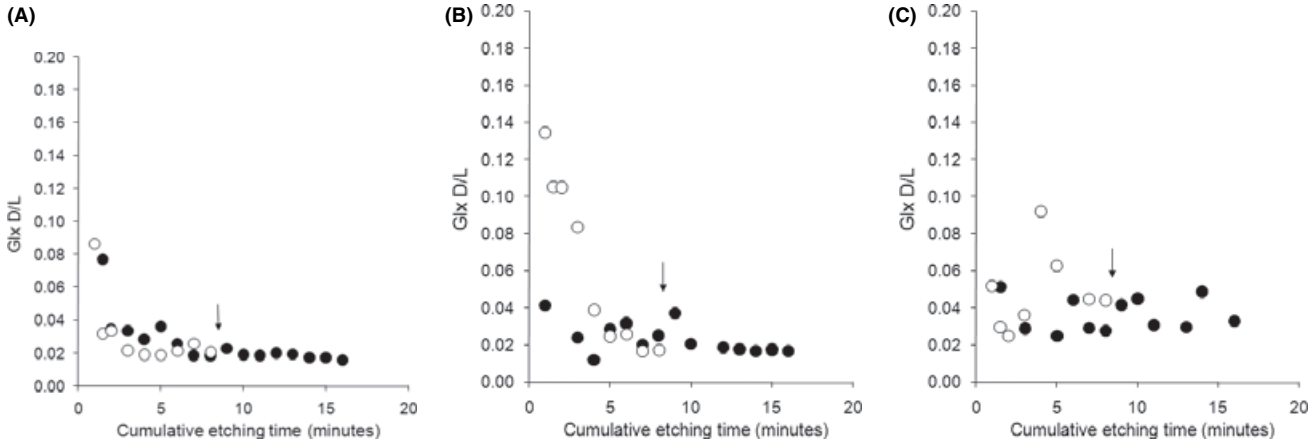

FIG. 3—*Glx D/L values for the unbleached (closed triangle) and bleached (open triangle) sides of teeth A, B and C plotted against etching time. The gap in sampling of the teeth is marked by an arrow.Glx, glutamine.*

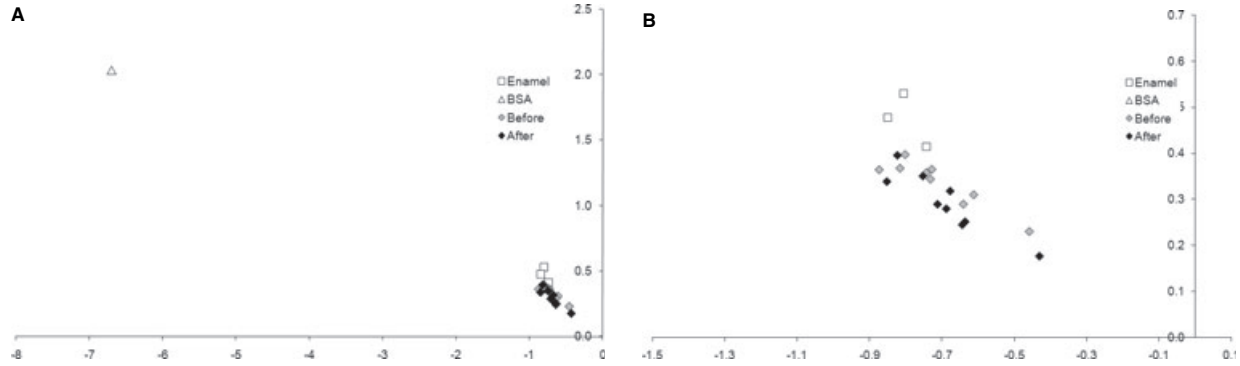

FIG. 4—*Separation of whole enamel proteins from bovine serum albumin by Principal Components Analysis of amino acid compositions (principal components 1 and 2 shown) before and after contamination, showing the full range of values (A) and the values of the samples taken before and after contamination (B). The values for enamel are from Weidmann and Eyre (8), Robinson 1975 (9), and Robinson 1977 (10). BSA, bovine serum albumin.*

TABLE 1—*Weightings of the amino acid concentrations in principal components 1 and 2.*

Amino Acid	Principal Component 1	Principal Component 2
Asx	−0.597	−0.329
Glx	−0.769	0.069
Ser	−0.214	0.836
Ala	−0.082	−0.434

Asx, asparagine; Glx, glutamine; Ser, serine; Ala, alanine.

Discussion

At present, aspartic acid racemization in dentine is apparently unable to provide reliable age estimates for individuals who have been buried for periods longer than two decades (1). This appears to be because of the combined effects of diagenesis of dentine proteins in the burial environment and contamination. This study has presented details of a new method for extracting amino acids from dental enamel, which arguably should be a more suitable substrate for analysis. However, one of the key problems with the use of surface sampling is the possibility of contamination of the amino acids extracted with foreign proteins. This paper has investigated the ability of the combined acid etching and bleaching method presented here to remove surface contamination and to extract uncontaminated amino acids for analysis.

Ability of the Method to Remove Contamination

The results of the contamination experiment indicate that limited acid etching alone can remove much of the contaminating protein on the tooth surface, but that even extensive etching cannot remove it all. Although the amino acid composition of the enamel seems to change after contamination, there does not appear to be any systematic variation in the direction of the change in composition for any particular tooth. Thus, it seems most likely that any changes in the enamel amino acid composition before and after contamination result from sampling variation rather than the contamination process. This idea is supported by the greater similarity of the amino acid composition of all the deliberately contaminated samples to enamel rather than to the contaminant (BSA) and the lack of an increase in amino acid concentration after contamination.

The results of the serial etching experiment clearly indicate that contamination of the enamel surface is potentially a major problem in the analysis of enamel for forensic age estimation. All the teeth analyzed have very high Asx and Glx D/L values at the surface of the tooth, relative to their age. It could be argued that the high level of Asx racemization observed at the tooth surface is because of chemical changes occurring in the burial environment. Asx is known to racemize rapidly even when peptide-bound via formation of a five-membered succinimide heterocycle (11). However, this

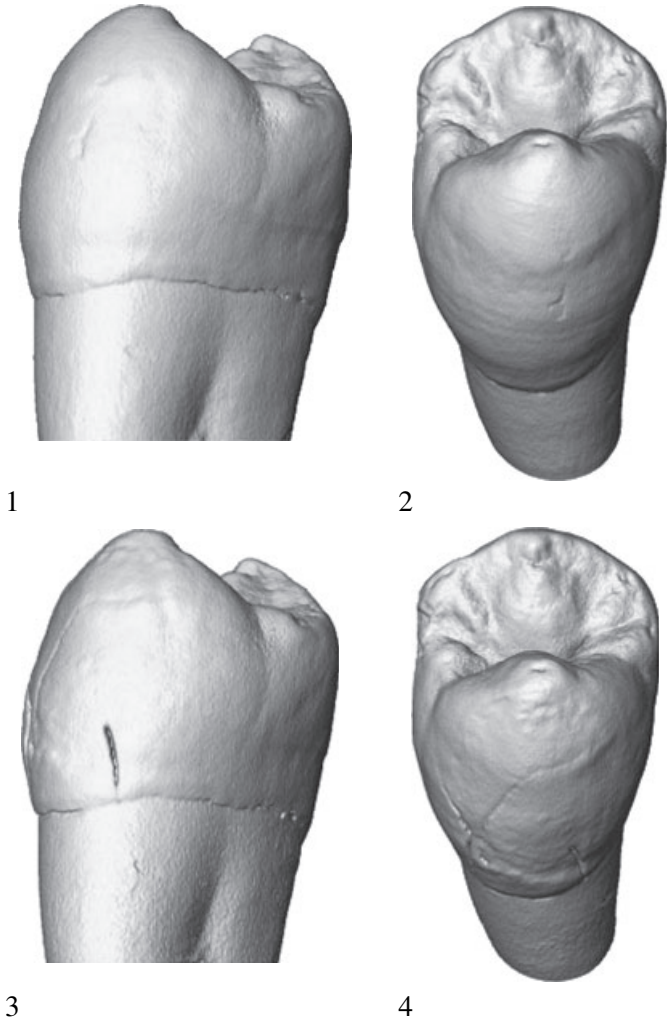

FIG. 5—*Enamel surface of tooth 1 before (1,2) and after (3,4) etching.*

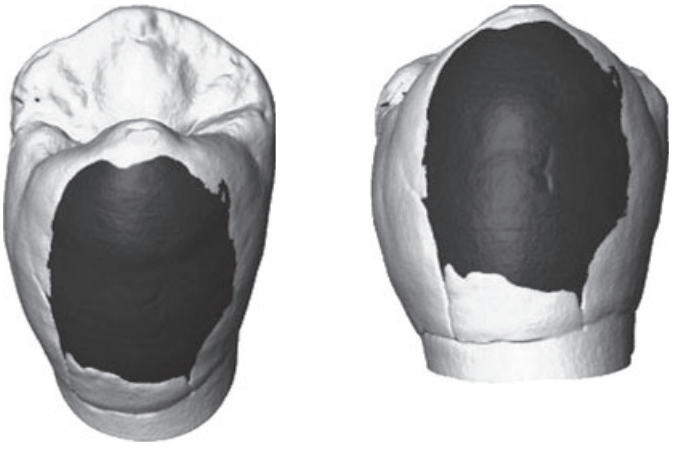

FIG. 6—*Area of enamel loss from tooth 1 because of etching, represented by the shaded area of the tooth surface.*

idea cannot explain the high Glx D/L values observed near the tooth surface in this experiment. Although Glx is similar to Asx chemically, it does not racemize rapidly when peptide-bound, as the six-membered heterocycle is energetically unfavorable (12).

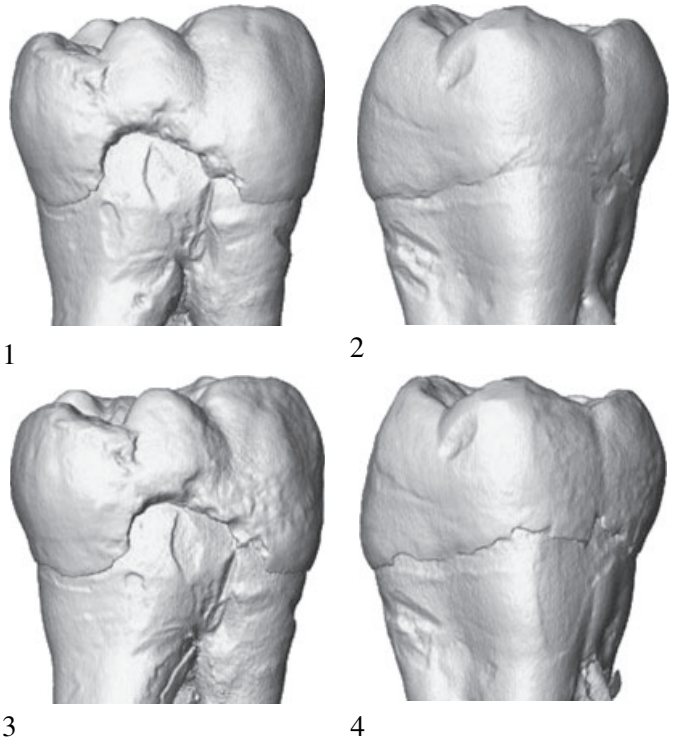

FIG. 7—*Enamel surface of tooth 2 before (1,2) and after (3,4) etching.*

The Asx D/L values for each sample are much lower than would be expected on the basis of the Glx D/L values, given relative rates of decomposition of Asx and Glx (13) coupled with low levels of free amino acids. Thus, the high Glx D/L values cannot be explained by a chemical process of decomposition.

A more likely explanation is contamination of the enamel with bacterial peptidoglycan. During life, the enamel surface is covered by a biofilm known as the pellicle, which is created by the adhesion of salivary particles to the enamel surface and has a substantial bacterial content. It is possible that some of the bacterial cell wall components (including peptidoglycan) may be retained at the tooth surface after its extraction or postmortem. For archaeological teeth, bacterial contamination could also occur in the burial environment. The bacterial cell wall contains microbial peptidoglycans, which are known to contain high levels of D-amino acids, including substantial amounts of D-glutamic acid (14–16). Bacteria also can contain racemases which catalyze the racemization reaction (17). In either case, the presence of bacteria would be expected to increase the amount of D-amino acids in the sampled region of the tooth.

The results obtained in the deeper parts of the enamel were much more consistent. The ability of bleach in combination with acid etching to depress the D/L values earlier in the etching sequence than was observed for unbleached teeth suggests that the proteins located at the enamel surface are not well protected by mineral. It seems that bleaching the tooth prior to sampling can greatly improve the quality of the age estimates obtained using this method.

The effect on Asx D/L and Glx D/L of the break in etching incorporated into this experiment on some of the teeth analyzed here suggests that contamination may be easily acquired. Although precautions were taken to minimize the risk of contamination (including postsampling rinsing, use of sterile glassware for analysis, and storage of the teeth in foil), the values of Asx D/L in the

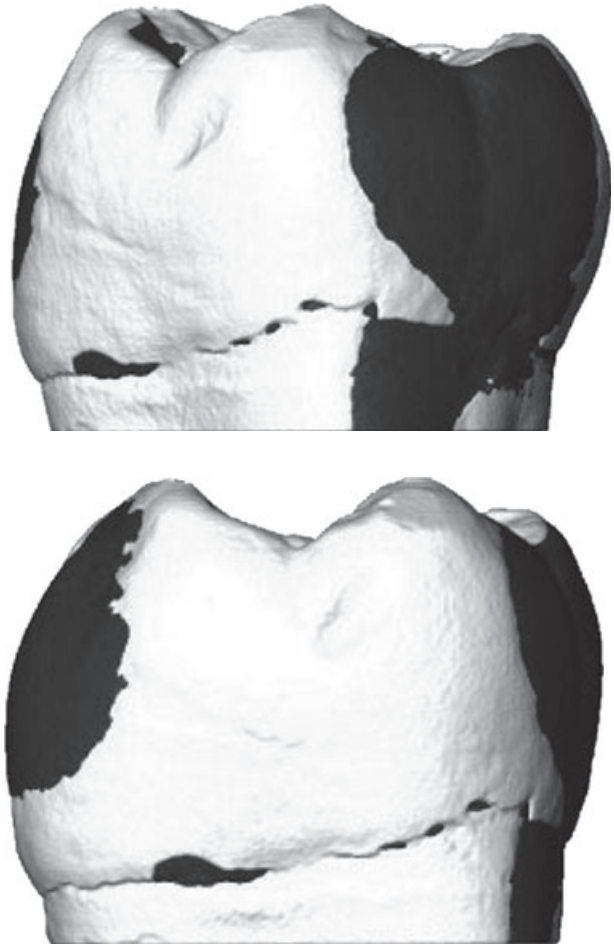

FIG. 8—*Area of enamel loss from tooth 2 because of etching, which is represented by the shaded area of the tooth surface.*

first etch after the break for teeth A and B are elevated over those from the previous etch, possibly reflecting the acquisition of contaminating amino acids. This suggests that there is a potential for detectable contamination of enamel protein samples within the laboratory environment. However, subsequent etches appear to be able to remove the majority of the acquired contamination, as the Asx D/L values decrease in later etches. The trends observed in the Asx D/L values for teeth A and B are poorly reflected in the Glx D/L values, and it is noteworthy that tooth C shows no evidence of contamination in its Asx D/L values. As a result, the increases observed here in Asx D/L for all three teeth are not statistically significant (*t*-test for paired samples; $t = -1.6$; $p = 0.25$). This along with the small sample size of this experiment means that it is not possible to determine conclusively that the increased Asx D/L values observed after the break are because of laboratory contamination.

The presence of high levels of contamination at the enamel surface suggests that samples taken from enamel near the tooth surface will produce an artificially high age estimate. The age estimates produced by the inner enamel are more reproducible, and it seems likely that these samples will better reflect the true age of the individual.

Destructiveness of the Technique

Acid etching appears to cause alteration of the appearance of the sampled area at the microscale, and to reduce the thickness of the enamel in the sampled region. The mottling of the enamel surface observed in our study is similar to that observed previously by dental researchers examining the impact of acid etching on the enamel surface and is caused by preferential etching of the interior or the periphery of the enamel prisms to produce a honeycomb structure (e.g., 18). The etching and bleaching process presented here appears to also create small cracks on the enamel surface. Although unobtrusive when viewed with the naked eye, these cracks could hamper the use of the method for age estimation, as they will increase the risk of contamination of samples with underlying dentine proteins. Cracking may be related to the age of the sample, as it was commonly observed in archaeological remains, but may be less problematic in forensic cases, where the length of the burial period is substantially shorter and the condition of the teeth correspondingly superior.

The amount of the enamel which is removed by etching varies between the teeth despite efforts to ensure homogeneity in sampling. This variability is consistent with previous research conducted by dental researchers on the rate of enamel loss during acid etching (19) and is thought to be because of the variable content of enamel in different individuals and in various parts of the tooth. The results obtained here indicate that some of the variability is determined by the contours of the analyzed surface, with flatter tooth surfaces losing more enamel than curved surfaces because of the larger amount of enamel in contact with the acid on a flat enamel surface. The nature and extent of the changes to the enamel surface as a result of acid etching should be considered when applying this method to the analysis of forensic or archaeological material. Furthermore, although acid etching appears to remove noticeable amounts of enamel and may create cracks on the enamel surface, the method presented here is still less destructive than existing methods for enamel analysis, where the teeth studied are sometimes completely destroyed.

For the purposes of forensic odontology, this work suggests that enamel could provide a useful substrate for aspartic acid racemization analyses, if sampling is conducted away from the surface of the enamel. This could also indicate that the potential for the application of this method in earlier samples, including those from archaeological contexts, may be greater than previously assumed. Further work is needed to clarify the extent to which enamel can be used for these analyses and to what extent diagenesis of the tooth in the burial environment could affect the results obtained.

TABLE 2—*Tooth volumes before and after etching for teeth 1 and 2.*

Tooth	Volume (mm^3)	Change in Volume (mm^3)	% Change in Volume	Approximate Mass Apatite Lost (mg)*
1 (before etching)	310.70	11.71	3.77	35
1 (after etching)	298.99			
2 (before etching)	690.98	23.94	3.46	72
2 (after etching)	667.04			

*Apatite density = 3.021 g/cm^3.

Acknowledgments

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