

Original article

In-situ digestion and micro-destructive sampling for dental proteomic sex determination analysis using polyvinylalcohol-based gel



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ARTICLE INFO

Article history:

Received 25 June 2025

Accepted 15 January 2026

Available online 31 January 2026

Keywords:

Non-invasive sampling

Teeth

Proteomics

Sex determination

Hydrogels

ABSTRACT

Background: Determining biological sex is pivotal for archaeological and forensic studies, providing insights into past societies, burial practices, and population demographics. Traditional methods, such as morphological analysis and DNA-based techniques, face limitations, including inconclusive results for juveniles, contamination, and genetic material degradation. Additionally, these approaches often involve significant destruction of artifacts, raising ethical concerns. Dental proteomics has emerged as a robust alternative, utilizing the stability of enamel-bound proteins, such as amelogenin isoforms (AMELx and AMELy). However, current sampling techniques are invasive, requiring substantial material removal and risking the integrity of culturally significant specimens.

Results: This study introduces a novel, minimally invasive technique for enamel protein sampling using a polyvinyl alcohol (PVA)-based highly viscous polymeric dispersion (HVPD). Acting as an elastic gel-like system, HVPD enables in-situ digestion and efficient extraction of proteins without compromising the tooth's structural integrity. The method was successfully applied to contemporary and archaeological samples, demonstrating effective recovery of amelogenin isoforms, reduced contamination risk, and compatibility with degraded specimens. This minimally invasive approach achieves reliable detection of all key amelogenin markers required for sex determination, matching the analytical outcome of conventional destructive protocols while preserving specimen integrity.

Significance: This methodology offers transformative potential for archaeology, anthropology, and forensic science, bridging scientific innovation with ethical stewardship. By preserving the physical and cultural integrity of artifacts, it addresses a critical need for sustainable research practices. Reliable biological sex determination in challenging contexts expands the scope of proteomic applications, providing new insights into past societies while safeguarding irreplaceable heritage for future generations.

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Introduction

The analysis of ancient human remains is a cornerstone of understanding historical civilizations, providing insights into their cultural practices, social structures, and biological traits. The determination of biological sex is especially crucial, as it forms the basis for demographic studies, interpretations of burial practices, and re-

constructions of past societies. Traditionally, this has been achieved through morphological assessments of skeletal features or through DNA-based analyses. However, both methods face significant limitations [1–3]. Morphological analyses are often inconclusive for juveniles or degraded skeletal remains, while DNA-based techniques are hindered by contamination and the degradation of genetic material over time [4–6]. Moreover, such techniques frequently require the destruction of precious materials, a practice often at odds with ethical considerations surrounding cultural heritage preservation [7].

Dental proteomics has emerged as a robust alternative [3,8], capitalizing on the remarkable stability of proteins embedded within the mineralized enamel matrix. Amelogenin has two isoforms encoded on the X and Y chromosomes (AMELx and AMELY), and their peptide fragments in enamel uniquely reflect an individual's biological sex: females (XX) express only AMELx, whereas males (XY) express both AMELx and AMELY. Amelogenin analysis offers a reliable means of determining biological sex even in challenging archaeological contexts. Unlike genetic material, enamel proteins are less susceptible to diagenesis, environmental degradation [9,10], and provide a complementary avenue for analysis when DNA is either unavailable or insufficiently preserved. In practice, confident detection of an AMELY-derived peptide conclusively identifies a male, while the absence of AMELY (with only AMELx peptides found) suggests a female, though caution is warranted, as poor preservation can cause false negatives for males, and some probability methods can be applied to estimate the correct female assignment [11–13]. Early studies established that this proteomic sexing is highly reliable even on very old or juvenile remains, achieving close agreement with osteological sex when applied to archaeological samples up to ~7300 years old and even to sub-adult (deciduous) teeth [12,13]. These findings demonstrated that enamel proteomics provides a robust sex indicator when sufficient peptide signals are obtained, and they greatly expanded the scope of sex estimation to remains (such as infants, juvenile or sub-adults) that lack diagnostic osteo-morphology [14]. Despite their advantages, the current methodologies for enamel sampling are destructive, requiring the removal of substantial material from the skull, usually an entire tooth. Such invasive techniques are often unsuitable for objects of cultural significance, leading to the pressing need for a non or minimally invasive alternative.

The analytical workflows to recover enamel proteins have varied in their invasiveness and practicality. Traditional methods typically require extracting a physical sample of enamel from the tooth and fully demineralizing it to release the peptides. For example, protocols adapted from paleoproteomics involve cutting a fragment of enamel (on the order of 10–30 mg) from the tooth, followed by complete dissolution of the mineral matrix in strong acid (e.g. trifluoroacetic acid or HCl) [11]. This approach yields a rich peptide mixture that can be analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), allowing the identification of multiple amelogenin peptides (including those unique to AMELx or AMELY). Such bulk dissolution methods have excellent analytical sensitivity – they typically detect numerous amelogenin peptides per sample and thereby enable unambiguous sex determination [3]. Indeed, recent studies using bulk enamel proteomics were able to monitor eight characteristic AMELx/AMELY peptides in each sample, achieving high accuracy in sex assignments based on the presence or absence of Y-chromosomal peptides [13]. These methods also proved to be effective on poorly preserved remains in respect to other techniques (DNA or osteo-morphology). From an analytical performance standpoint, therefore, full enamel dissolution maximizes peptide recovery and the likelihood of detecting the male-specific markers even in very ancient or degraded specimens. The drawback of such approaches is their destructiveness to the specimen. Removing a chunk of enamel inevitably causes per-

manent damage to the tooth, and the tooth is often extracted from the skeletal remains entirely for sampling. Dissolving ~15 mg of enamel effectively destroys that portion of a tooth [11].

In archaeological and forensic contexts, where human remains are regarded as irreplaceable cultural heritage or trial evidence, this practice conflicts with modern ethical guidelines [15] that demand researchers minimize destruction and preserve remains for future generations or to repeat the tests. Even the loss of a single tooth (or part of it) can be significant, as it alters the integrity of a skeleton or diminishes the material available for other analyses [16]. Guidelines in bioarchaeology now emphasize that skeletal sampling is “not a limitless resource” and must be conducted with utmost restraint [8]. Accordingly, we have sought to reduce the physical impact of proteomic sex determination methods.

A seminal example is the surface acid-etching method [14], which forgoes drilling out a chunk of enamel in favor of gently etching the tooth's surface with a mild acid to extract proteins. In this technique, a small drop of diluted acid (e.g. ~5 % HCl) is applied to a tooth's enamel for a brief period, dissolving only a thin layer of the surface and releasing trace amelogenin peptides, which are then analyzed by nanoLC-MS/MS. This method has been shown to reliably recover the diagnostic AMELx and AMELY peptides while leaving the tooth largely intact, provided it is detached from the original skull. Subsequent work has validated and refined such surface etching protocols on larger sample sets. For instance, Brůžek et al. (2024) [16] applied an enamel etching procedure to 60 documented teeth (32 male, 28 female) and achieved 100 % sexing accuracy, thereby confirming that proteomic sex estimation can be performed successfully without extensive destruction of the tooth. Crucially, they also used scanning electron microscopy (SEM) and micro-CT to quantify the physical impact of the acid treatment on teeth: the etching removed roughly 10 % of the enamel thickness and about 2 % of dentine, and it widened some pre-existing microcracks in the enamel surface. These findings illustrate that even ‘minimally invasive’ chemical extraction can produce measurable changes to tooth structure. Although the removal of a limited portion of enamel represents a substantial reduction in damage compared to traditional destructive sampling, it is still relevant from a conservation perspective, particularly in cases where only one or a few teeth remain, as is common in pre-historic graves. Importantly, performing an acid etch usually still requires the tooth to be out of its socket (to control the acid application and collect the dissolved sample), meaning the tooth often must be removed from the skull. Thus, while surface etching protocols reduce the extent of material sacrificed and align better with ethical standards, they do not entirely eliminate concerns about irreversible changes to teeth and skeletal remains. Modern bioarchaeological studies increasingly report these limitations; for example, researchers show that acid etching, although macroscopically subtle, produces measurable microscopic alterations, including the removal of at least ~15 mm³ of enamel, as documented in controlled assessments [16]. In summary, minimally destructive approaches mark a significant advance by drastically cutting down the required sample size and damage, thereby aligning better with ethical standards, but they still involve some degree of permanent modification to human remains.

Alongside efforts to minimize damage, recent proteomic methods have also targeted improvements in analytical efficiency and sensitivity. Traditional LC-MS workflows, while sensitive, are relatively slow and labor-intensive, which historically limited proteomic sexing to small sample batches [17]. To address this, researchers [12] accelerated the sample preparation and analysis steps. Parker's study introduced a faster digestion protocol and demonstrated that even very small enamel samples could yield identifiable amelogenin peptides, suggesting the potential to scale down the required sample without sacrificing detection. In that

study, a suite of amelogenin peptides was consistently recovered from each tooth, and the presence/absence method for sexing proved robust across diverse archaeological contexts. Several studies have advanced enamel-proteomics workflows through either targeted MS approaches or complementary analytical strategies [11], while additional work has demonstrated the broader applicability of enamel protein extraction and analysis [18] collectively contributing to the refinement of current methodologies.

Most recently, an automated high-throughput pipeline was described using parallel reaction monitoring (PRM) on an LC-MS platform to specifically monitor a handful of sex-indicative peptides with high sensitivity [11]. This targeted approach dramatically increases throughput by focusing the mass spectrometer on just the key amelogenin peptide ions, enabling dozens of samples to be processed in a single day without extensive fractionation or spectral library searching. Similarly, Adair et al. [19] reported a novel workflow that eliminates the time-consuming liquid chromatography step altogether: after a minimal acid extraction of enamel, they employ LAP-MALDI MS/MS (liquid atmospheric-pressure matrix-assisted laser desorption/ionization) to directly analyze the peptides. By forgoing LC separation, this method achieves a turnaround time of ≤ 10 minutes per sample from extraction to result. Only a tiny enamel area needs to be treated with 10 % HCl to release peptides, and the subsequent MALDI analysis rapidly detects the Y-specific amelogenin peptide (e.g. the diagnostic oxidized SMIRPPY sequence) among a total of 27 identified enamel peptides. The entire process is streamlined such that it can be performed in basic laboratory settings (or even portable labs) without sophisticated chromatography equipment. These developments in speed and sensitivity mean that proteomic sex determination is more feasible to apply on a larger scale, including screening many individuals or integrating into standard archaeological workflows. Nevertheless, it should be noted that even these improved pipelines have until now required some form of enamel sampling (drilling powder, taking a micro-flake, or dipping the tooth in acid solution). Thus, while the analytical footprint (time, cost, sample size) has been reduced, the physical footprint on the human remains has remained a concern.

To address these challenges, this study introduces an innovative in-situ sampling technique using PVA-based HVPD [20,21], which behaves as an elastic gel-like system after drying. This approach minimizes physical damage to the artifact while enabling the efficient extraction of enamel proteins. By providing a micro-destructive yet highly effective method for proteomic analysis, this technique offers a transformative solution for archaeological and anthropological research, ensuring that valuable specimens remain intact for future study. Furthermore, the micro-destructive nature of this methodology aligns with conservationist principles, fostering greater collaboration between scientific inquiry and the preservation of culturally and forensically significant human remains and materials.

Research aim

This study aims to develop and validate a micro-destructive, in situ method for proteomic analysis of dental enamel to improve biological sex determination in archaeological and forensic contexts. Specifically, we seek to: (i) introduce and test a novel polyvinyl alcohol-based hydrogel (HVPD) capable of extracting enamel peptides through surface digestion without visibly altering or removing material from the tooth; (ii) evaluate the impact of acidic HVPD treatment on enamel structure; and (iii) assess the applicability of this approach to highly degraded archaeological specimens. Unlike conventional proteomic protocols, which typically require invasive sampling, this method permits direct application to teeth in situ, including those embedded within skeletal remains, thereby mini-

mizing intervention and eliminating the need for tooth extraction or transport. By preserving both the structural and contextual integrity of human remains, the proposed technique aligns with current ethical standards in cultural heritage science and offers broad interdisciplinary relevance across analytical chemistry, bioarchaeology, and conservation science.

Materials and methods

Materials

Poly(vinyl alcohol) [PVA] (87–89 % hydrolyzed, Mw 85,000–124,000, Sigma-Aldrich), dipropylene glycol [DPG] (99 %, isomer mixture, Aldrich), 2-methyl-1,3-propanediol [MPD] (99 %, Aldrich), glycerol [GLY] (analytical grade, Merck), polyethylene glycol [PEG] (average Mn 300), and ethanol [EtOH] (purity ≥ 98 %, Fluka) were utilized in the preparation of HVPD. Hydrochloric acid [HCl] (37 %, Carlo Erba) was used to adjust the pH of HVPD. Hydroxyapatite (analytical grade, Thermo Scientific Chemicals) was used as received for mockup preparation. Water was purified using a Millipore MilliRO-6 Milli-Q gradient system (resistivity > 18 M Ω -cm).

Preparation of PVA-based HVPD

The preparation process was carried out following the protocol reported by Guaragnone et al. [20], incorporating minor adjustments to fit the specific requirements of enamel micro-sampling. A 20 wt % PVA dispersion was initially prepared by dissolving the polymer in Milli-Q water at 90 °C under continuous stirring for 2 h. The temperature was then reduced to 70 °C to facilitate the addition of plasticizers, specifically PEG (0.4 wt %), DPG (2.5 wt %), MPD (2.5 wt %), and GLY (0.6 wt %). Following approximately 30 min of homogenization, the temperature was further adjusted to 55 °C, at which point 17 wt % ethanol was introduced and mixed rigorously until a clear and uniform polymeric dispersion was achieved. The HVPD was subsequently acidified to a pH of 0 ± 0.5 using aqueous HCl, enabling the degradation of enamel hydroxyapatite and the release of entrapped proteins. The dispersion's pH was verified using indicator paper (MQuant with a pH range 0–6.0 by Merk) [20].

Preparation of hydroxyapatite mock-ups

To simulate a realistic tooth enamel surface, mock-up pellets were prepared by sintering pure hydroxyapatite powder under 10 tons pressure for 10 min using an FTIR table press. The resulting samples were used directly, without further modifications, to evaluate the effect of acidic HVPD on the crystalline matrix morphology.

Morphological study of the hydroxyapatite-HVPD interaction

Hydroxyapatite pellets were prepared as mock-ups to replicate tooth enamel and the HVPD was subsequently tested on these. Surface roughness of the pellets, both before and after treatment, as well as the step height between treated and untreated areas, were assessed using a high-resolution 3D digital microscope featuring a white-light confocal point scanning system (Hirox HRX-01 & NPS, Hirox Europe). This system offered an axial resolution of 76 nm, a lateral resolution of 1.3 μ m, and a measurement range of 150 μ m. The collected data were processed and analyzed using the MountainMap software suite (Digital Surf, France), which provided an in-depth analysis of surface roughness and allowed for accurate calculations of the Sa parameter across the measured area. Sa, defined as the arithmetic mean of absolute values of the Surface Heights,



Fig. 1. A drop of HVPD applied on sample 9.

is calculated by averaging the absolute deviations of the surface heights from a reference plane: higher Sa value indicates a rougher surface with greater height variations, whereas lower Sa value signifies a smoother surface [22]. Additionally, the step measurement was determined by calculating the difference between the mean height of the untreated and treated areas, based on three independent profiles taken along the boundary used for statistical analysis.

Application and removal

The HVPD was carefully applied to the enamel surface using a syringe to control the deposition, as shown in Fig. 1. Care was taken to avoid contact with the underlying dentin, which could introduce unwanted peptides and compromise the proteomic analysis. The polymeric dispersion was allowed to dry for four hours under controlled conditions to form a cohesive film. During this period, environmental contaminants were minimized by conducting the process in a clean air environment. The dried film was subsequently peeled from the enamel surface using precision tools, ensuring a clean removal without leaving residues or causing visible damage to the artifact. Photographic documentation before and after application was conducted to assess the preservation of the sample, further validating the micro-destructive nature of the technique. Iterative refinements to the process were implemented to balance protein yield with artifact preservation.

Peptide extraction and analysis

The peeled-off film was processed using a two-step purification protocol. Initially, peptides were enriched through C18 StageTips, followed by a secondary purification via strong cation exchange chromatography. This purification strategy reduces the contribution of residual hydrogel components to the chromatographic background, improving peptide detectability for the targeted amelogenin markers. Nevertheless, some PEG-derived signals remain present, and although they do not overlap with the monitored ions, their persistence indicates an area for future methodological refinement. Offline StageTip purification was performed essentially as described in Taverna et al. [23]. Briefly, 100 μL of 5 %

(w/w) trifluoroacetic acid (TFA) were added to the film already in Phosphate-Buffered Saline (PBS, 1 mL total). Approximately 1 mL of the resulting extract was loaded onto by C18 Stage Tips prepared in 200 μL pipette tips. After sample loading and washing with 0.1 % TFA, peptides were eluted in 20 μL of a 50 % (v/v) acetonitrile solution. The eluate was diluted with 80 μL of 80 % acetonitrile 0.5 % formic acid (Wash SCX) and loaded onto a SCX StageTip previously conditioned with 50 μL of Wash SCX. After sample loading and washing with Wash SCX solution (50 μL), peptides were eluted with 10 μL of 500 mM ammonium acetate, 20 % (v/v) acetonitrile. The eluate was evaporated to dryness and reconstituted in 12 μL of mobile phase A (see below). Four microliters were injected for nanoLC-MS/MS analysis.

NanoLC-MS/MS analysis was performed on an EasyLC 1200 instrument coupled to a Q-Exactive “classic” (Thermo Fisher Scientific) as described in Greco et al. [13]. Briefly, peptides were separated on a reversed-phase column (0.075 \times 130 mm id \times L) in-house packed with C18 silica particles (Dr. Maisch). Peptides were loaded directly on-column and eluted at 230 nL/min by a 20-min linear gradient (from 10 % B to 50 % B). Mobile phase A was 2 % acetonitrile, 0.1 % formic acid v/v, whereas mobile phase B was 80 % acetonitrile, 0.1 % formic acid v/v. Peptides were ionized by nanoelectrospray in positive ion mode. MS acquisition followed a mixed data-dependent acquisition/parallel reaction monitoring (DDA/PRM) strategy, consistent with previously published workflows by the authors [13]. PRM constituted the primary analytical mode for sex determination, while DDA scans were systematically acquired in parallel. The inclusion of DDA provides an untargeted spectral record that verifies peptide presence independently of PRM transitions and offers a long-term archival resource, allowing potential future identification of additional enamel peptides or isoforms of relevance. Nevertheless, in the authors’ experience, monitoring the ions subjected to PRM scans already provides all information needed for biological sex determination. A full MS1 spectrum (resolution 35,000, scan range 350–1000 m/z) was followed by MS2 scans on eight consecutive precursors at 17,500 resolution: 431.22, 423.22, 540.28, 525.30 (AMELx peptides), 483.74, 440.22, 396.71, 11,432.22 (AMELy peptides). Settings for PRM events were: 60 ms of max. injection time, 1.6 m/z if isolation window, normalized collision energy of 25.

Ion chromatograms were constructed using Xcalibur (Thermo Scientific) with a mass tolerance of 50 ppm. For comparative purposes, results obtained with the HVPD method were evaluated alongside those from the conventional acid-etching protocol previously described by Greco et al. [13]. This comparison was included to benchmark the performance of HVPD against an established technique commonly used in dental proteomics biological sex estimation. This dual-mode acquisition ensures maximal information recovery from micro-destructive samples and aligns with the ethical commitment to preserve and re-use spectral data beyond the immediate analytical objective.

To assign a probability to sex estimation, the methodology by Parker et al. was adopted [12]. Combined ion peak intensity signals of peptides belonging to for AMELX_HUMAN (AH) were calculated from extracted MS1 chromatograms and normalized per mg of extract. The probability of biological female sex ($\text{Pr}(F)$) was then calculated based on the logistic regression:

$$\ln \frac{\text{Pr}(F)}{1 - \text{Pr}(F)} = -12.8622 + 0.7496 \times \ln(\text{AH} + 1)$$

Sample selection

The study included a total of 10 samples: five contemporary (Samples no. 1, 2, 3, 9, 10) teeth from individuals with known biological sex and five archaeological specimens (Samples no. 4, 5, 6,

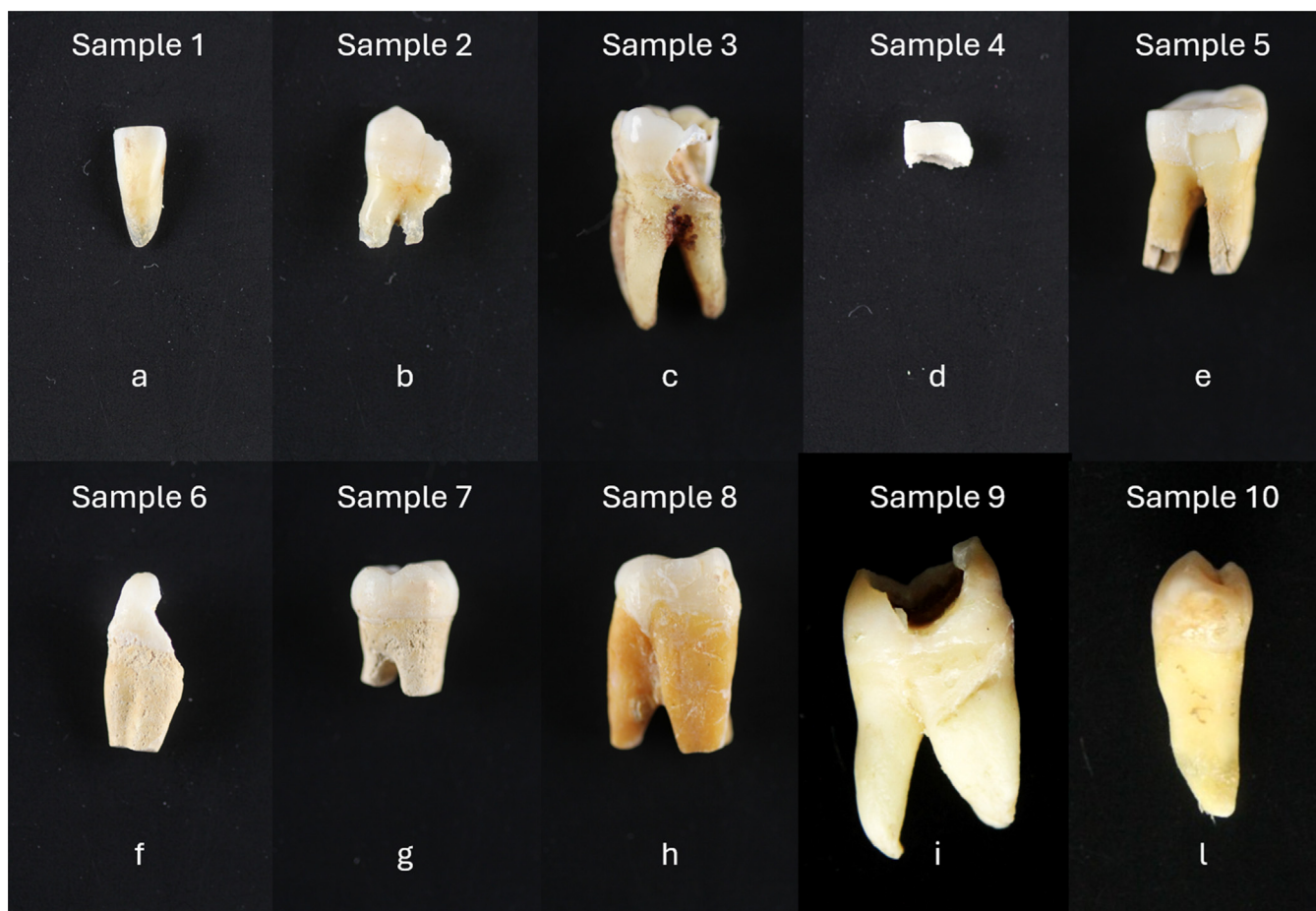


Fig. 2. Sample 1 (a), sample 2 (b), sample 3 (c), sample 4 (d), sample 5 (e), sample 6 (f), sample 7 (g), sample 8 (h), sample 9 (i), sample 10 (l).

7, 8) retrieved from the Neolithic site of Sultana, Romania (4700–4200 BCE), shown in Figs. 2(a-l) and table T1. The contemporary samples served as controls to validate the method's accuracy, while the archaeological samples tested its applicability to highly degraded materials. Particular attention was given to ensuring that the archaeological specimens exhibited varying degrees of preservation, enabling a robust assessment of the technique's versatility. The inclusion of both well-preserved and highly degraded samples provided insights into the limitations and adaptability of the hydrogel approach, establishing its efficacy across a spectrum of real-world scenarios.

Results and discussion

Effects of HVPD on the morphology of hydroxyapatite layer

The profiling of the hydroxyapatite mockup surfaces before and after exposure to the acidic HVPD provided valuable insights into the interaction between the HVPD and the enamel, as well as its ability to selectively erode the crystalline enamel matrix in a controlled and precise manner. Particularly notable was the reduction in the arithmetical mean height (S_a), which decreased by 37.5 %, from $1.855 \mu\text{m}$ to $1.162 \mu\text{m}$ (Figs. 3(a-b)–4(a-b)). This reduction highlights a microscopical change in the roughness of the surface of the enamel, indicative of the highly targeted and localized erosive action of the acidic HVPD on the crystalline structure. Additionally, a well-defined step of $3.3 \pm 0.5 \mu\text{m}$ was observed at the interface between the treated and untreated regions of the enamel, marking a clear transition zone where the surface morphology was

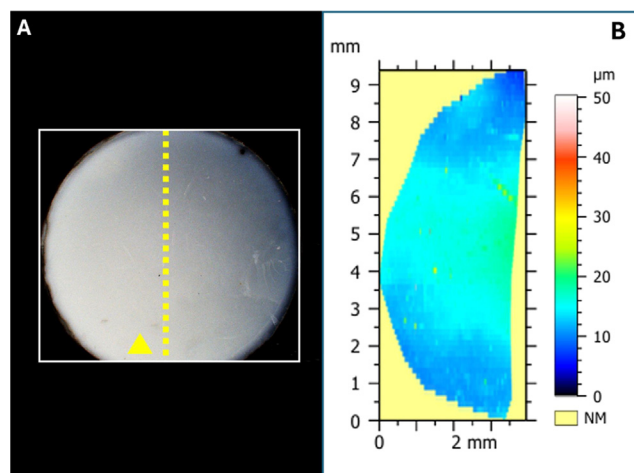


Fig. 3. Visible imaging of the hydroxyapatite pellet prior to interaction with acidic HVPD (A). Besides the visible image, the corresponding topographic false-color map, ranging from blue (low elevations) to red (high elevations), illustrates the surface profile of the left pellet area (triangle) before HVPD application (B). NM is for Not Mapped area. Surface roughness is reliably assessed by the S_a parameter.

altered due to HVPD's acidic exposure (Fig. 4 panel B). This step represents a sharp demarcation between areas subjected to the treatment and those that remained unaffected, further emphasizing the localized and targeted nature of the HVPD's effect. The four-hour application time of the HVPD represents a methodological trade-off: unlike acid-etching protocols, which typically oper-

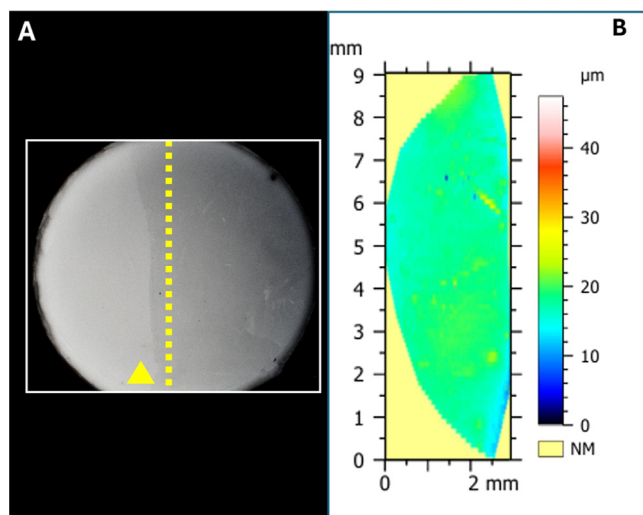


Fig. 4. Visible imaging of the hydroxyapatite pellet after the interaction with acidic HVPD (A). Besides the visible image, the corresponding topographic false-color map, ranging from blue (low elevations) to red (high elevations), illustrates the surface profile of the left pellet area (triangle) after HVPD application (B). NM is for Not mapped area. Surface roughness is reliably assessed by the Sa parameter.

ate over 20–50 min, HVPD requires extended contact to achieve controlled, micro-scale digestion. Because etching protocols rely on brief exposure, their damage profiles cannot be directly extrapolated to the longer HVPD contact time, and the two approaches should not be compared on the basis of duration alone. For this reason, our comparison between HVPD and conventional etching focuses on the nature of material removal rather than on duration: HVPD induces confined, micrometer-scale alteration, whereas etching protocols, despite their brevity, still produce measurable enamel loss as documented in prior controlled studies.

Sampling efficiency

The PVA-based HVPD demonstrated remarkable efficiency in extracting proteins from enamel while maintaining the physical integrity of the samples. Unlike traditional acid-based methods, which require the destruction of significant amounts of enamel, this technique achieved selective protein extraction with minimal material removal. The polymeric dispersion's viscoelastic properties allowed it to adhere uniformly to the enamel surface and form a cohesive film that could be cleanly removed. Comparability refers to the consistent recovery of the diagnostic AMELx and AMELy peptides required for sex estimation, rather than to absolute peptide abundance, which is inherently higher in destructive methods due to the much larger amount of enamel dissolved. This success underscores the transformative potential of this approach in reconciling scientific inquiry with the ethical preservation of culturally and historically significant human remains. Furthermore, detailed analysis revealed that the HVPD consistently enabled detection of the diagnostic AMELx peptides, and AMELy peptides when present, across specimens exhibiting different degrees of diagenetic alteration, confirming its robustness for applications in diverse archaeological contexts. Here, 'performance' refers strictly to successful recovery of peptides sufficient for sex determination.

Proteomic profiles and biological sex determination

The proteomic analysis yielded clear identification of eight amelogenin isoforms peptides (reported in Table 1) in all samples. Contemporary samples showed a consistent correlation between detected peptides and the known biological sex of the donors.

Male samples exhibited robust signals for AMELy peptides, while female samples exclusively displayed AMELx peptides, as reported in Figs. 5–6 and figures S1–S10. In the archaeological specimens, similar patterns were observed, with the presence or absence of AMELy peptides reliably indicating biological sex. Notably, even in samples with significant diagenetic alteration, the method proved effective, underscoring its suitability for degraded materials. Proteomic analysis was conducted in parallel on four specimens using a well-established acid-etching-based enamel-protein extraction method to serve as benchmark for evaluating the performance of the new method. The analysis was performed according to Greco et al. [13] and consisted in incubating a 10 mg enamel fragment in hydrochloric acid followed by C18 StageTip purification. All comparative assessments refer to this destructive benchmark, representing conventional proteomic sampling. Both the gel-based and the reference methods provided reliable identification across all samples. The reference method benefitted from a larger amount of starting material, which improved the detection of high levels of AMELx peptides and, consequently, the probability of correct female assignment as reported, together with peak area values for all detected peptides, in Table T2.

In conclusion, the PVA-based HVPD method enables accurate sex determination in contexts where traditional archaeological and osteological methods show limited reliability [13], thus significantly expanding the analytical toolkit available for archaeological studies.

Impact on sample preservation

Photographic documentation before (Fig. 2a-l) and after (Fig. 7a-d) sampling revealed negligible visual changes to the enamel surface. Unlike destructive bulk-dissolution protocols, which remove measurable portions of enamel, the hydrogel approach avoided macroscopic damage. However, because microscopic alterations on actual teeth were not measured in this study, the nature and extent of any sub-surface changes cannot be inferred from the hydroxyapatite mock-ups alone. This minimal impact is critical for the preservation of culturally, historically, and forensically significant samples, including human remains. Moreover, the controlled application and removal process ensured that the enamel's structural integrity remained intact, aligning with the ethical standards of conservation [7,24]. Extended trials on highly valuable Neolithic human tooth [25], including those from museum collections, further validated the methodology's applicability in high-stakes contexts where preservation is paramount [34].

The new proteomic method described in the present work introduces a paradigm shift by virtually eliminating damage to the tooth and the need to disturb the skeletal assemblage. This innovative approach uses a polyvinyl alcohol (PVA) hydrogel film to perform in situ acidic digestion and extraction of amelogenin peptides directly from the tooth's surface. In practice, a thin PVA gel (pre-loaded with a mild acid) is applied to the enamel in situ, for example, painted onto a tooth that is still set in the jaw of a skeleton, and left in place to gently solubilize and digest a superficial layer of the enamel proteins. The gel is then peeled off, now carrying the released amelogenin peptides, which can be analyzed to detect AMELx and AMELy isoforms. The advantages of this technique are major: first, it obviates any need to remove the tooth from the skull or to physically scrape/cut the enamel. The sampling is micro-destructive to the naked eye, the tooth remains in place and essentially unaltered, satisfying the highest standards of preservation and respecting the integrity of human remains. Second, because the PVA film confines the reagents to the tooth's surface, there is no uncontrolled exposure of the specimen to liquid chemicals; the underlying dentine and surrounding bone are not affected, and after the gel is removed the tooth retains its original

Table 1
Precursors monitored in PRM scans. Fragment ions used for producing extracted ion chromatograms (XICs) are also reported.

m/z precursor	Gene name	Peptide sequence	Fragments used for XICs
440.22	AMEly	SMoxIRPPY	154, 408.2, 645.4
396.71	AMEly	MoxIRPPY	364.7, 655.3
432.22	AMEly	SMIRPPY	143.1, 191.1, 645.1
483.74	AMEly	SMoxIRPPYS	451.7, 732.4
540.28	AMELx	SIRPPYPSY	366.1, 686.3, 714.4
525.30	AMELx	SYEVLTPLK	223.1, 357.2, 670.4, 799.5
423.33	AMELx	MPLPPHPG	104.1, 504.2, 714.4
431.22	AMELx	MoxPLPPHPG	504.2

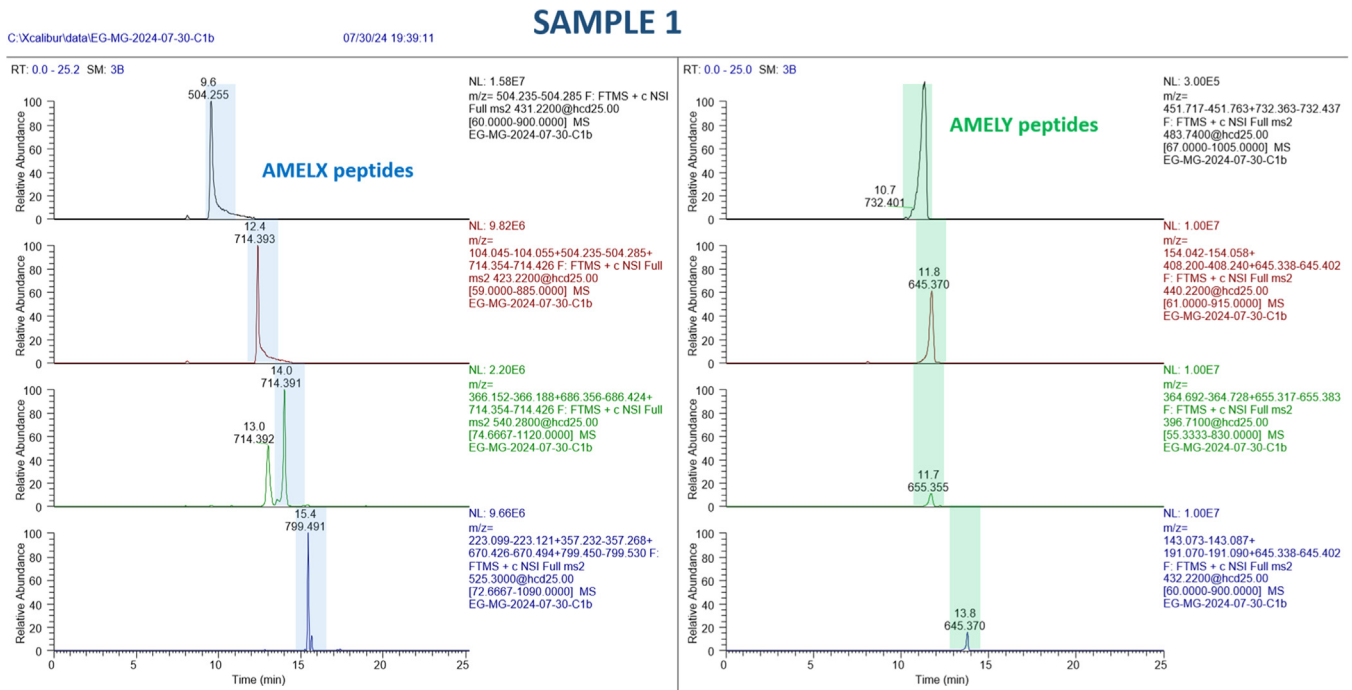


Fig. 5. Chromatogram of 4 AMELx and 4 AMELY peptides of Sample 1.

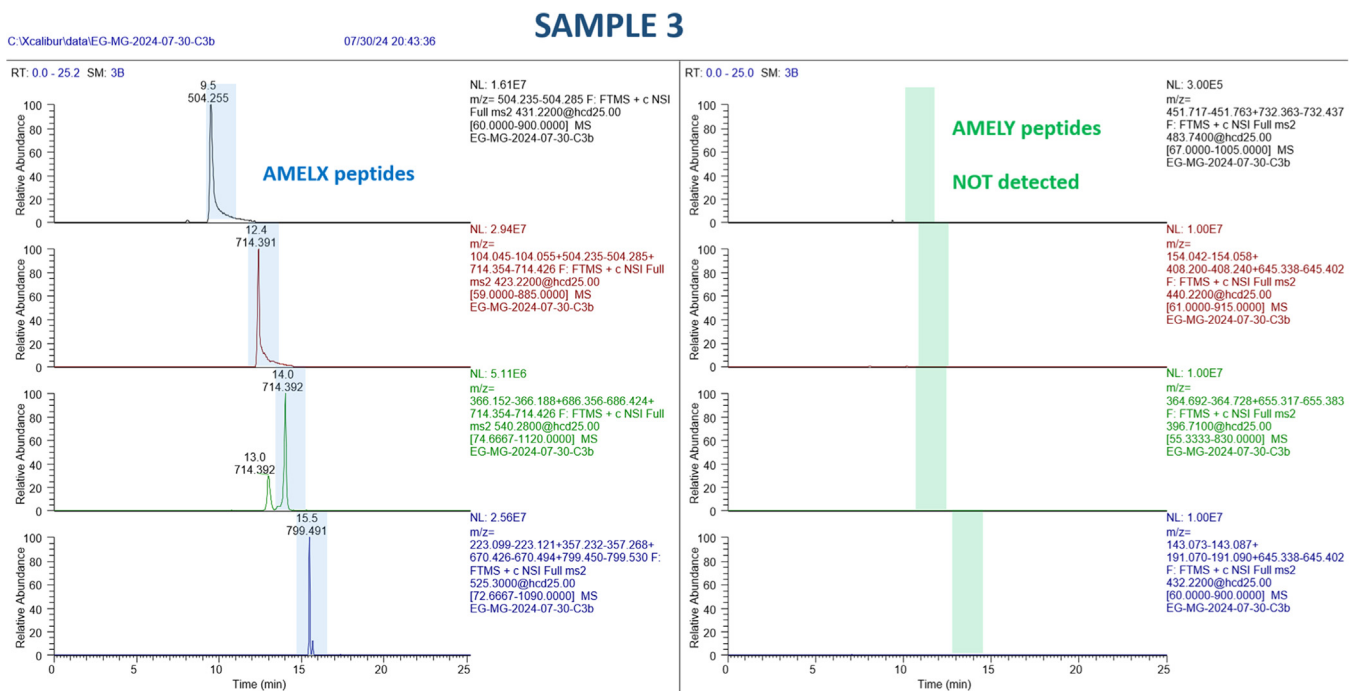


Fig. 6. Chromatogram of 4 AMELx and 4 AMELY peptides of Sample 3.

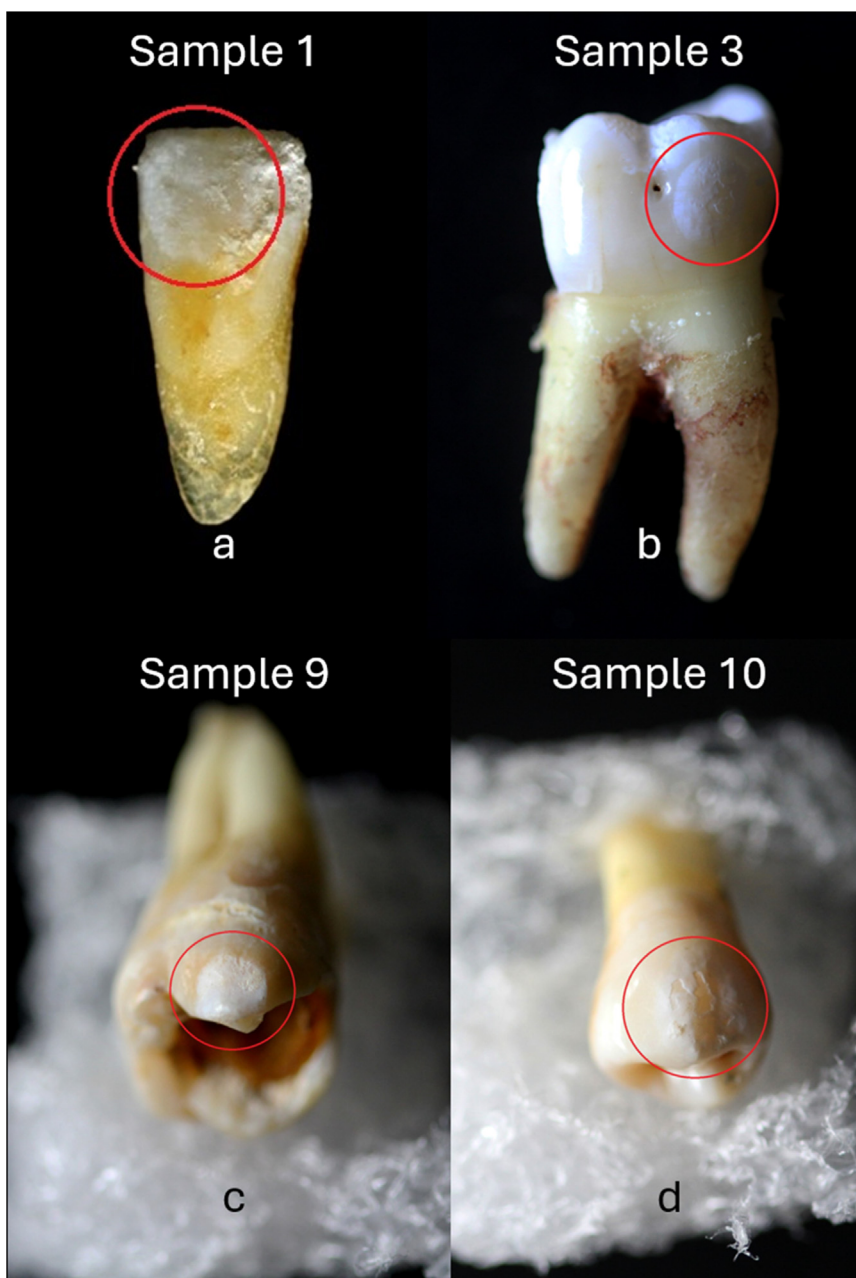


Fig. 7. Sample 1 (a), sample 3 (b), sample 9 (c), and sample 10 (d) after HVPD sampling. These areas show the only visual marks in the sample set.

appearance and structural stability. This means the method can be deployed on skeletal remains in the field or in museum collections without any transportation of the human remains themselves. Archaeometrists, archaeologists or curators can perform the gel application on-site (even on fragile or curated specimens), then simply send the used gel or resulting peptide extract to a lab for mass spectrometric analysis. This dramatically reduces logistical and ethical hurdles, since sensitive human remains no longer need to be relocated or subjected to destructive sampling. Finally, the PVA-gel method has been shown to recover sufficient amelogenin peptides to enable confident sex determination. The HVPD extracts yielded clear detection of all required AMELX and AMELY markers, achieving the same functional outcome as destructive acid-etching protocols, despite recovering peptides from a markedly smaller amount of enamel. In summary, the PVA hydrogel approach achieves what earlier methods strived for but never fully attained: a minimally invasive yet effective proteomic sex-determination technique that

preserves the physical integrity of teeth and skeletons. This breakthrough aligns perfectly with contemporary ethical guidelines in bioarchaeology, as it virtually eliminates harm to human remains while still harnessing the power of state-of-the-art proteomics. By enabling in situ sampling with no damage and no need to extract or transport remains, the PVA hydrogel method represents a significant advancement over prior techniques, opening the door for routine, on-site sex determination of archaeological skeletons without compromising their preservation.

Limitations and future directions

While the method represents a significant step forward in minimally-invasive proteomics, there are areas for further refinement. The PVA-based HVPD formulation could be refined to further improve its peptides extraction efficiency, particularly for specimens with severe diagenetic alterations, while reducing its already

limited invasiveness. Notably, the application of the polymeric dispersion on the selected teeth showed a not complete formation of the polymer film. The rate of drying can be estimated to be about 95 %, because the surface of the teeth appears only lightly wet. This means that some interactions between the polymeric dispersion and certain components of enamel may occur preventing them from completely drying. In addition, PEG-related background peaks were still detectable in the chromatograms. Although these did not interfere with the targeted amelogenin ions, reducing or eliminating this residual contribution will be a desirable improvement for future formulations. Future studies will investigate such interactions to identify the cause of this interference and optimize the polymeric dispersion's composition to address this challenge. The residue left can be easily removed by gentle rinsing with water by wiping. As clearly demonstrated above, the amount of proteins extracted was enough to get all the requested information

Additionally, expanding the proteomic database to include a broader range of amelogenin variants across diverse populations would improve the method's applicability in global anthropological research. Future studies could also explore the integration of advanced imaging techniques to monitor the sampling process in real-time, ensuring even greater precision. Collaborations with conservation scientists could lead to the development of multifunctional hydrogels that combine sampling capabilities with protective applications for long-term teeth preservation.

Importantly, enamel proteomics determines only chromosomally derived biological sex. It cannot detect intersex conditions, nor can it address gender identity or the culturally specific social roles that individuals may have embodied. These limitations must remain central to any interpretive framework, particularly when working with past populations whose social categories of personhood may not align with modern constructs. In our previous study [13], proteomic analysis corrected earlier sex attributions based solely on burial goods, illustrating both the utility of the method and the need for caution when linking biological sex to social identity. By explicitly acknowledging these boundaries, we aim to promote interpretations that remain scientifically rigorous while respectful of the individuals represented by the remains.

Ethical considerations

As researchers engaged in archaeological and anthropological studies involving human remains, we firmly uphold the ethical principles highlighted by Squires et al. [15]. Our research rigorously adheres to the core ethical standards of respecting human dignity, ensuring cultural sensitivity, and actively engaging in collaboration with relevant stakeholders.

The polyvinyl alcohol-based hydrogel (PVA-based) method presented in this study particularly embodies these ethical commitments, offering a completely minimally-invasive procedure that requires no physical alteration or extraction of teeth from human remains. This approach aligns impeccably with current ethical guidelines, safeguarding the integrity and dignity of human skeletal material, which must be viewed as subjects of respect rather than mere research objects.

Our method further respects the principles of scientific objectivity, data protection, and confidentiality. It facilitates responsible dissemination and education by enabling accurate biological assessments without compromising the preservation of cultural heritage materials. By providing an innovative, ethical, and respectful analytical tool, our PVA-based method helps foster confidence among descendant communities, heritage institutions, and the broader public in how human remains are studied and preserved, and contributes positively to the dialogue surrounding ethical practices in bioarchaeological research.

The use of modern teeth was conducted in full compliance with ethical standards: all donors provided written informed consent, and the study protocol was reviewed and approved by the Institutional Review Board, with samples fully anonymized prior to analysis.

Conclusions

This study introduces a transformative methodology for minimally-invasive enamel proteomics, combining analytical rigor with the preservation of culturally invaluable human remains and associated materials. The use of PVA-based HVPD enables efficient in-situ digestion and extraction of enamel-bound peptides while minimizing damage, offering a viable alternative to traditional destructive methods. By demonstrating its efficacy in both contemporary and archaeological specimens, this technique sets a new standard for proteomics in archaeology and anthropology. Its potential to expand our understanding of past societies while safeguarding Cultural Heritage underscores its significance as a tool for interdisciplinary research. Furthermore, the methodology exemplifies the synergy between scientific innovation and ethical stewardship, ensuring that the narratives embedded within ancient remains are preserved for future generations.

Data availability

All raw MS data were deposited in the ProteomeXchange repository under accession number PXD067050. Results of data-dependent acquisition scans, which were not discussed in this manuscript, were searched using MaxQuant v 2.03.01 using default parameters except for the following: (i) digestion unspecific; (ii) minimum peptide length 6 amino acids, peptide length between 6 and 12 amino acids; the FASTA file was downloaded from the Uniprot database after searching for "human enamel" proteins (238 sequences).

Author contributions

EG: Conceptualization, Project administration and Writing Original Draft, **EG, RG, MG:** Methodology and Supervision, **MB, AC, LB, CG:** Investigation, **EG, MG, EM:** Formal analysis, **AM, PP, CL:** Resources, **PB, EM:** Validation, **AC, LB:** Visualization

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Acknowledgments

RG and AC acknowledge CSGI for supporting this research. LB and RG also acknowledge PRIN2022 REAGENERA (MUR) for the support. EG acknowledges Dr. Yuri Gabbino, INSTM for the support by grant no. IND.23/8, and the USF's Mediterranean Diet Archaeology Project.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.culher.2026.01.012](https://doi.org/10.1016/j.culher.2026.01.012).

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