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Undertaking the biological sex assessment of human remains: The applicability of minimally-invasive methods for proteomic sex estimation from enamel peptides

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Abstract: Being a part of the cultural heritage, skeletal human remains and grave objects are often the only evidence of people who lived many years, or even centuries or millennia, ago, and their preservation for future generations is thus of the utmost importance. The first task in analyzing skeletal remains is to build a biological profile of the individual, including in particular a sex estimation. Recently developed proteomic sex analysis, based on the detection of two sex-dependent forms of the amelogenin protein in tooth enamel, could offer a minimally-invasive and reliable approach applicable to both recent and past populations.

The aims of the present study are: 1) to validate the proteomic sex estimation approach with a delicate, minimally-destructive protocol using protein etching in recent and sub-recent identified samples of adult individuals; 2) for the first time, to evaluate the invasiveness of the extraction of amelogenin protein from teeth for proteomic analysis via scanning electron microscope (SEM) and microcomputed tomography (micro-CT); 3) to apply the method to an archaeological sample of unknown adult and juvenile individuals.

An assemblage of 60 teeth (32 males and 28 females) of recent and sub-recent origin was used to validate the approach. A sub-sample of 20 teeth (10 males and 10 females) was used to assess the invasiveness of the amelogenin extraction procedure. For the application of the method, samples of 15 adult and 32 juvenile teeth, both originating from medieval populations, were used.

Proteomic sex estimation achieved 100% accuracy in this sample. An SEM and micro-CT comparison of the dental surfaces before and after chemical treatment showed an approximately 10% loss of enamel and only 2% loss of dentine. The suitability and minimally-invasive character of the protocol for proteomic analysis in biological sex estimation was demonstrated, as was its applicability to archaeological samples.

Keywords: Human skeletal collections, cultural heritage, proteomics, sex estimation, sampling

1. Introduction

Human skeletal remains are an inseparable part of the cultural heritage, and are often the only information about people living in the past. They are preserved in museum and university collections, and are used for elaborating biological profiling methods [1]. Unfortunately, there are differences in legislation between countries [2–5] that affect the handling of skeletons, as well as differences in ethical standards e.g. [6,7]. The common denominators include some laws, such as the Valletta Treaty (Council of Europe, ETS No. 143, 1992), according to which all biological remains, artefacts, objects and any other remains of humankind from past epochs are subsumed under Cultural Heritage and are protected within the scope of a common European legacy. We must preserve for the future the status of archaeological human remains as part of the cultural heritage, and as indispensable empirical sources for the reconstruction of human population history through time and space [8].

The uniqueness of skeletal remains from any period leads conservators to minimize any interference with their integrity. Knowledge of the methods used and the degree of damage they cause are of enormous importance, but such damage is not always fully known [9]. Within the field of human osteology, the debate on ethical issues regarding the sampling, research and display of archaeological human and animal remains has been ongoing for decades, and has become more vigorous with the recent increase in ancient biomolecular studies [10,11]. Although the amount of sample needed for analysis can be just a few milligrams of bone or dental tissue, museums too are being inundated with destructive sampling requests. With respect to anthropological collections, we must consider the need to reduce or completely eliminate the mechanical destruction of skeletal remains deemed useful for scientific evaluation.

The preservation of organic molecules in teeth and bones has proven crucial for understanding the human past [12]. In recent years, proteomics has become an attractive method for studying human, animal, and biological profiles and origins. It is an alternative to DNA analysis, which is limited by the DNA amplification that is present in ancient samples, contamination, its high cost, and the limited preservation of nuclear DNA [13–15]. Currently, three approaches are available for estimating biological sex: osteology, genomics, and proteomics [16], but little is known about the relative reliability of these methods in applied settings [17].

Correct and reliable biological sex estimation of skeletal remains is important in various areas, from bioarchaeology to forensic science. The varying degree of preservation of skeletal remains limits the methods that can be used [18]. Morphological methods are based on the existence of sexual dimorphism in the skeleton, and are subject to population specificity which causes various error rates

[19,20]. The only part of the skeleton that allows a reliable estimate of sex is the pelvis [21]; in archaeological human skeletal assemblages, however, the pelvis is often heavily damaged or completely absent [22]. In addition, morphological methods have another major limiting factor: the inability to reliably or accurately estimate sex from immature elements with any degree of consistency [23,24]. According to Buonasera et al., biological sex estimation was possible for 100% of an archaeological sample by proteomics, for 91% by genomics, and for 51% by osteology; the agreement among the methods was high, but there were conflicts [17].

Proteomics provides a new, seemingly simple and cost-effective way to conduct sex estimation without the risk of contamination. It uses two sexually distinct forms of the amelogenin protein found in tooth enamel, detectable by liquid chromatography-tandem mass spectrometry (LC-MS/MS); the AMELY protein (amelogenin Y isoform) is present in enamel dental tissue only in males, while AMELX (isoform X) can be found in both sexes [13]. Enamel is the most mineralized part of the tooth, with mineral content making up about 97% of mature enamel; the rest is formed of proteins and other components, such as water. During enamel formation and maturation the matrix is removed almost completely through enzymatic degradation by proteases, resulting in its hardening and the extensive deposition of calcium-based minerals. Amelogenin is the predominant protein in the developing extra-cellular enamel matrix [25].

Although studies estimating biological sex by proteomic analysis of dental enamel use the same principle, they differ in their levels of sampling and pre-treatment. Some studies use more destructive sampling in the form of small enamel chunks extracted with a dentist's drill [26–30]; others offer a less invasive approach in the form of etching the enamel surface with hydrochloric acid [13,31,32].

1.1. Research aim

In this study, we aimed to (1) validate peptide-based enamel sex identification both in teeth from individuals of known sex from a recent population and in dissected individuals from the mid-20th century [33]; (2) assess the effect of protein extraction on the structural integrity of human dental enamel (damage to the enamel surface) via scanning electron microscope (SEM) and micro-computed tomography (micro-CT); and (3) to apply the method to an archaeological assemblage of unknown adult and immature individuals.

2. Material

To obtain and validate a predictor for biological sex based on enamel proteins, we used two different assemblages:

(1) a validation sample of teeth of individuals of known biological sex to verify the reliability of the method; a sub-sample of 20 teeth randomly selected from the validation sample served to verify the effect of protein etching on tooth enamel quality;

(2) an application sample of archaeological teeth from a museum collection, for which sex (in the case of adult individuals) was originally estimated by a morphological method.

2.1. Validation sample

This contains the identified teeth of adult individuals from two samples of the Czech population (Table 1). Group (1a) consists of 30 teeth of adult individuals of known age and sex (15 male and 15 female) from the recent Prague population, provided by dentists. Extractions were performed for medical reasons.

Group (1b) originates from the Pachner collection, housed in the Department of Anthropology and Human Genetics of the Faculty of Science, Charles University, Prague. It too comprises 30 identified teeth of individuals of known age and sex from the Prague population (17 male and 13 female), but who were autopsied in the 1940s. This collection dates to the 1930s and consists of individuals of known sex, age (date of birth and date of autopsy), and status (they represented the lower socio-economic classes of Bohemia) [33,34].

2.2. Application sample

This contains 15 adult and 32 non-adult teeth from the burial grounds of an Early Medieval population (9–11th century AD) at the Mikulčice agglomeration in Moravia [35], stored in the Department of Anthropology of the National Museum, Prague (Table 1). We selected adult individuals based on the conflicting results of morphological sex estimation by osteological methods between those published in the 1960s [36–38] and the revised sex estimates published in 2020 [39]. We also included non-adult individuals whose biological sex cannot be assessed by morphological methods [32,40]; they were selected to study the differences in diet between boys and girls among the early Slavs, which is the subject of another study [41]. This research was approved by the Institutional Review Board of Charles University.

Table 1. Composition of validation sample, sub-sample subjected to micro-CT and SEM and application sample.

	Validation sample of identified individuals (n=60)							Application sample (n=47)	
	Extraction medical reasons	M	F and M (n)	for Pachner's Collection	Identified (n)	M (n)	F and M (n)	Total (n)	Medieval population (9-11 th century AD)
	F (n)	M (n)	F and M (n)	F (n)	M (n)	F and M (n)	Total (n)	Adults	Non-adults
PSE	10	10	20	8	12	20	40	15	32
PSE + micro-CT and SEM	5	5	10	5	5	10	20	not performed	not performed

PSE = proteomic sex estimation; SEM = scanning electron microscope; micro-CT = microtomodensitometry

3. Methods

3.1. Biological sex estimation

Proteomic estimation of sex was done following the method of Stewart et al. [13], which is based on the analysis of two dimorphic peptides of amelogenin: AMELY-(58–64) peptide and AMELX-(44–52)

peptide from tooth enamel. The teeth of males contain both peptides (AMELY and AMELX), while female teeth contain only one (AMELX).

1 step – Sample preparation

The tooth surface was cleaned with water to remove obvious surface contaminants. The tooth crown was then placed in the cap of a 2-mL Eppendorf tube, washed with 3% H₂O₂ (200 µL) for 30 s and then rinsed with ultrapure water. It was then treated with 200 µL of 5% (v/v) HCl. An initial etch was performed by lowering the tooth onto the HCl and maintaining contact for 2 min. This first etch was discarded. A second 2-minute etch was collected for the analysis.

2 step – Protein etching

For peptide extraction, a C18 resin-loaded ZipTip (ZTC18S096; EMD Millipore) was used. This had previously been conditioned three times with acetonitrile (100%), and three times with formic acid 0.1% (v/v) (each draw discarded). Peptide solution was then pipetted 10 times to maximize binding to the C18 resin of the ZipTip material. Finally, the ZipTip was washed six times with formic acid 0.1% (v/v) (each wash discarded). Bonded peptides were eluted by 4-µL of acetonitrile/formic acid (60%/0.1%, v/v) and the resulting solution was collected into small vials. This fraction was lyophilized and dissolved in 20 µL of formic acid (2%) in ultrapure water, centrifuged on a benchtop centrifuge for 5 min to remove any particulate matter, and transferred (18 µL) to autosampler vials. This sample was injected for analysis by reversed-phase nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS).

3 step – Analysis method

The nanoLC apparatus was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a MaXis Q-TOF (quadrupole –time of flight) mass spectrometer with ultra-high resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. Five micro-liters of the peptide mixture were injected into a NS-AC-11 Bio-Sphere C18 column (particle size: 5 µm, pore size: 12 nm, length: 152 mm, inner diameter: 75 µm), with an NS-MP-10 BioSphere C18 pre-column (particle size: 5 µm, pore size: 12 nm, length: 20 mm, inner diameter: 100 µm), both obtained from NanoSeparations (Nieuwkoop, Netherlands).

Peptide separation was achieved via a linear gradient between mobile phase A (ultrapure water) and B (acetonitrile), both containing formic acid 0.1% (v/v). Separation was started with a gradient elution from 5% to 30% mobile phase B at 70 min. The next step was gradient elution to 50% B in 10 min, followed by a gradient to 100% B in 10 min. Finally, the column was eluted with 100% B for 30 min. Equilibration before the subsequent run was achieved by washing the column with 3 µL of 5% mobile phase B for 5 min. The flow rate was 0.25 µL.min⁻¹, and the column was held at ambient temperature (25 °C).

Online nano-electrospray ionization (easy nano-ESI) in the positive mode was used. The ESI voltage was set at + 4.5 kV, spectra rate 3 Hz. Operating conditions: drying gas (N₂), 4 L.min⁻¹; drying gas temperature, 180 °C; nebulizer pressure, 100 kPa. Experiments were performed by scanning from 420 to 550 m/z.

Proteomic determination of biological sex was done according to the peak area of EIC (extracted ion chromatogram) at a retention time of 35 min for m/z 440.22 (AMELY-peptide, marked as Y in the tables) and at a retention time of 43 min for m/z 540.28 (AMELX-peptide, marked as X in the tables).

3.2. Evaluation of the effect of protein etching on dental enamel

The effect of etching on enamel was performed in a subset of 20 teeth of individuals of known sex.

Scanning electron microscope (SEM)

Samples were scanned on a Hitachi S-3700 N Scanning Electron Microscope (SEM) at the National Museum in Prague. The teeth were documented overall, and then two areas on each were selected and scanned at several resolutions without tooth surface treatment (mechanical or ultrasonic cleaning). After the protein etching of the teeth, the same samples and the same areas on them were scanned again so that the changes that had occurred could be evaluated.

Microcomputed tomography (micro-CT)

Each tooth was scanned twice: before and after the application of the protocol of extraction for proteomic sex estimation with the same scanning parameters. The first scan took place under the same conditions as the SEM analysis of the surface (without mechanical or ultrasonic cleaning). Microtomodensitometric (micro-CT) data for these teeth were acquired using the micro-CT platform in the PACEA (Bordeaux). They were obtained with a “v|tome|x s 240” micro-CT scanner microfocus tube (GE Sensing and Inspection Technologies Phoenix X ray).

A total of 1750 radiographic projections (i.e. 1750 angular increments for 360 °rotation) were acquired with the following scan parameters: voltage 100 kV, current 160 µA, exposure 500 ms, voxel size 27.5 µm. The micro-CT data were reconstructed using Phoenix datos|x reconstruction 2 software and then exported as a 16-bit TIFF image stack. VG studio max software (Volume Graphics, release 2.2, Heidelberg, Germany) was used for the virtual slice visualization and three-dimensional rendering.

Using Avizo 9.5 (Thermo Fisher Scientific), a semi-automatic threshold-based segmentation was performed on the reconstructed images with subsequent manual corrections. After segmentation, volumetric reconstruction and visualisation of the micro-CT slices were performed using Avizo v. 9.5 software. Enamel thickness mapping before and after protein etching was performed with the Surface Distance module on the Avizo 9.5 software.

4. Results:

4.1. Biological sex estimation via peptides in dental enamel

A biological female determination of the sample is indicated by the detection of only one diagnostic peptide (AMELX); a biological male determination is indicated by the detection of both diagnostic peptides derived from each isoform (AMELX-(44–52), or AMELY-(58–64)) of amelogenin proteins. For the total 60 samples of individuals of known sex, the accuracy of sex estimation was absolute (Table 2).

Table 2. Amelogenin-sex estimation in a validation sample of adult teeth of known age and sex from a Czech population (30 males and 30 females).

Sample number	Sub-sample	Sex declared	Tooth sampled	Age (yrs)	Peak area		Proteomic sex
					X	Y	
Proteomic sex estimation							
8	A	F	3M	23	946,208	Not present	F

13	A	F	${}_2M$	39	1,896,641	Not present	F
15	A	F	3M	20	1,154,743	Not present	F
16	A	F	${}_3M$	21	1,527,914	Not present	F
17	A	F	${}_3M$	40	1,805,977	Not present	F
18	A	F	${}_3M$	25	874,56	Not present	F
19	A	F	P^2	64	3,008,422	Not present	F
20	A	F	M_3	21	1,325,183	Not present	F
21	A	F	M_3	55	3,032,252	Not present	F
22	A	F	M^3	24	673,982	Not present	F
1	A	M	${}_3M$	17	748,564	550,882	M
2	A	M	M^3	41	1,371,502	2,604,241	M
23	A	M	3M	28	973,924	1,296,839	M
24	A	M	${}_3M$	29	1,033,295	2,386,597	M
25	A	M	3M	55	2,330,862	3,339,066	M
26	A	M	${}_3M$	21	734,265	820,057	M
27	A	M	M_3	36	1,609,180	2,396,535	M
28	A	M	3M	35	1,295,788	2,389,316	M
29	A	M	3M	61	1,565,209	865,203	M
30	A	M	${}_3M$	30	1,490,656	2,013,183	M
37	P	F	P_2	34	2,323,905	Not present	F
40	P	F	2P	adult	929,849	Not present	F
43	P	F	M_3	51	4,721,920	Not present	F
50	P	F	${}_3M$	38	2,488,777	Not present	F
51	P	F	I^1	adult	258,116	Not present	F
52	P	F	M^1	70	4,448,210	Not present	F
55	P	F	3M	65	2,227,581	Not present	F
58	P	F	C	adult	2,259,169	Not present	F
33	P	M	C	adult	1,157,578	1,258,985	M
34	P	M	P^1	43	1,140,553	2,664,629	M

35	P	M	² P	38	1,967,799	3,615,260	M
36	P	M	² P	adult-	1,254,277	2,890,431	M
38	P	M	P ²	62	2,984,328	4,386,024	M
39	P	M	² P	32	2,070,259	4,178,386	M
41	P	M	M ¹	39	2,536,816	3,926,950	M
44	P	M	¹ P	adult	903,339	1,473,504	M
45	P	M	P ¹	32	4,197,536	7,638,730	M
56	P	M	¹ M	78	1,487,187	1,250,741	M
57	P	M	P ₂	61	3,344,463	4,761,168	M
60	P	M	² M	35	2,141,434	2,029,143	M
Proteomic sex estimation with micro-CT and SEM analysis							
9	A*	F	M ³	32	1,384,671	Not present	F
10	A*	F	₃ M	25	1,271,997	Not present	F
11	A*	F	M ₃	33	1,322,932	Not present	F
12	A*	F	M ₃	26	1,831,753	Not present	F
14	A*	F	M ₃	26	1,258,762	Not present	F
3	A*	M	₃ M	48	1,366,483	3,291,729	M
4	A*	M	M ₃	20	627,076	550,347	M
5	A*	M	₃ M	22	863,334	1,037,607	M
6	A*	M	³ M	31	611,035	1,317,945	M
7	A*	M	₃ M	24	551,819	1,299,878	M
31	P*	F	³ M	34	2,347,440	Not present	F
32	P*	F	₃ M	28	3,000,280	Not present	F
46	P*	F	M ³	adult	778,678	Not present	F
48	P*	F	² M	32	5,039,565	Not present	F
54	P*	F	M ¹	adult	1,210,069	Not present	F
42	P*	M	M ¹	73	1,092,764	876,029	M
47	P*	M	M ¹	33	2,718,667	3,445,208	M
49	P*	M	² M	adult	2,905,233	1,947,298	M

53	P*	M	¹ M	42	3,697,825	2,906,365	M
59	P*	M	² M	72	2,000,080	1,784,672	M

A = group 1a (teeth from extractions in dental clinics in 2019–2021), P = group 1b (teeth from Pachner's Identified collection from the first half of the 20th century), * = sub-sample for the effect of protein etching on tooth enamel quality. SEM = scanning electron microscope; micro-CT = microtomodensitometry. Tooth nomenclature: I = incisor, C = canine, P = premolar, M = molar, upper index = upper jaw, lower index = lower jaw, side of the index indicates side of the tooth (left or right).

After this validation of the proteomic sex estimation method, the method was applied to a sample of 15 teeth of adult individuals of archaeological provenance. For the needs of another study [41], we also estimated the sex of 32 permanent teeth of juvenile individuals from a medieval population (in which morphological sex estimation is impossible). Table 3 shows the results of the proteomic sex estimation of the application sample from the Early Medieval period. In the case of adults, 7 teeth showed both peptide signals and corresponded to males, while 8 teeth with only AMELX-peptide belonged to females. In the case of immature individuals, the nanoLC-MS/MS analysis showed that 16 teeth with only an AMELX-peptide signal belonged to female individuals; 16 individuals with the presence of both AMELX-peptide and AMELY-peptide signals corresponded to males.

Table 3. Amelogenin-sex estimation in an application sample of 15 adult teeth and a sample of 32 non-adult teeth, both from burial grounds of the Early Medieval (9–11th century AD) population from the Mikulčice agglomeration in Moravia.

Skeleton number	Age-at-death (yrs) (Stloukal 1963, 1964, 1967)	Tooth class	Peak area		Proteomic sex
			X	Y	
H87	40–50	C	3,496,055	3,991,318	M
H170	50–60	₂ P	3,745,990	4,076,427	M
H0171	40–59	C	3,209,222	Not present	F
H0187	50–60	P ₂	693,47	1,445,756	M
H0292	30–59	P ₂	835,227	Not present	F
H0314(bis)	adult	P ₂	4,902,831	Not present	F
H0314	30–40	P ²	2,952,606	3,665,371	M
H0324	30–40	I ₁	7,071,775	3,665,371	F
H0352	50–60	P ²	4,283,238	Not present	F
H0363	30–40	P ₁	1,986,341	2,610,787	M
H0406	40–50	² M	2,089,384	Not present	F

H0457	50–60	P ₁	4,678,292	Not present	F
H0647	40–50	₁ P	2,551,808	3,039,339	M
H0718	adult	P ²	5,232,203	Not present	F
H1088	adult	¹ P	2,893,400	3,510,927	M
H73-VI	5–6	M ¹	404,29	408,317	M
H143	6–7	¹ M	985,473	1,057,251	M
H160-VI	6–7	₁ M	806,03	Not present	F
H207	10–11	¹ M	713,327	1,009,648	M
H247	5–6	M ¹	799,429	Not present	F
H253	4	M ₁	1,254,321	Not present	F
H266	9–10	M ¹	187,41	446,308	M
H296	2	¹ M	770,229	1,102,988	M
H315	3–4	₁ M	1,211,179	Not present	F
H343	2–3	M ¹	1,260,716	1,923,015	M
H393	4–5	₁ M	1,186,517	Not present	F
H444	15–17	¹ M	799,936	1,221,249	M
H447	2–3	₁ M	776,379	644,6	M
H454	6–7	₁ M	949,155	Not present	F
H455	2–3	₁ M	533,605	Not present	F
H462	4–5	M ₁	1,498,764	Not present	F
H473	4–5	M ₁	556,056	702,444	M
H489	9	₁ M	759,236	1,023,086	M
H496	3–4	¹ M	560,303	Not present	F
H497	6–7	₁ M	999,491	694,996	M
H526	2–3	M ¹	348,887	593,259	M
H538	9	₁ M	297,597	Not present	F
H550	4–5	M ¹	566,669	Not present	F
H582	12–14	₁ M	733,286	1,023,541	M
H751	5–6	₁ M	388,359	Not present	F

H792	5–6	₁ M	1,006,139	1,233,096	M
H878	15–16	₁ M	915,958	825,651	M
H881	6	₁ M	949,087	Not present	F
H1041	6	M ₁	367,721	Not present	F
H1058	5	M ₁	465,283	Not present	F
H1154	5	M ₁	671,2	1,374,627	M
H1171	5–6	₁ M	640,652	Not present	F

Tooth nomenclature: I = incisor, C = canine, P = premolar, M = molar, upper index = upper jaw, lower index = lower jaw, side of the index indicates side of the tooth (left or right)

4.2. Tooth enamel changes due to protein etching

From a macroscopic point of view (visual evaluation), protein extraction does not affect the observed details of the tooth surface. It is only necessary to mention subtle colour changes and the reduction of the gloss of the enamel surface.

SEM analysis was used to examine and compare the surface morphology of each tooth from the validation group before protein etching and after etching. Figs. 1 and 2 show SEM images of the enamel surface of two examples: tooth 42 from the Pachner collection and tooth 6 from recent extractions in dental clinics. The collected SEM images show that all the samples showed variable and distinct surface changes. Because the teeth were not treated in any way before applying the etching protocol, after protein extraction from the enamel all traces of tartar and other substances present on the enamel surface disappeared.

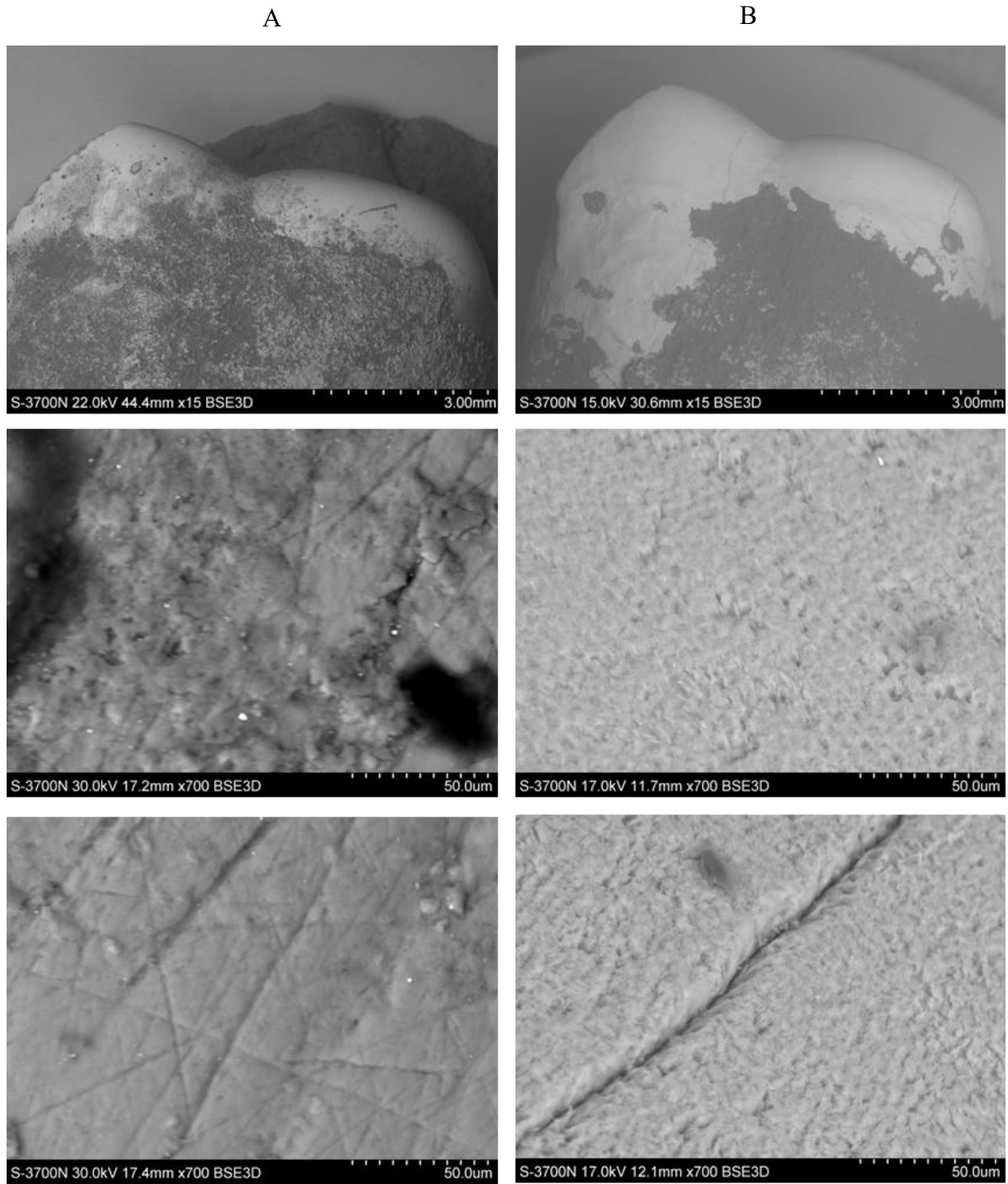


Fig. 1. Comparison of enamel surface alteration obtained by SEM before (A) and after (B) protein etching. Upper row – natural size; Middle row – area 1 at 500x magnification; Bottom row – area 2 at 500x magnification. Sample number 42 (from Pachner’s Identified collection, from the first half of the 20th century).

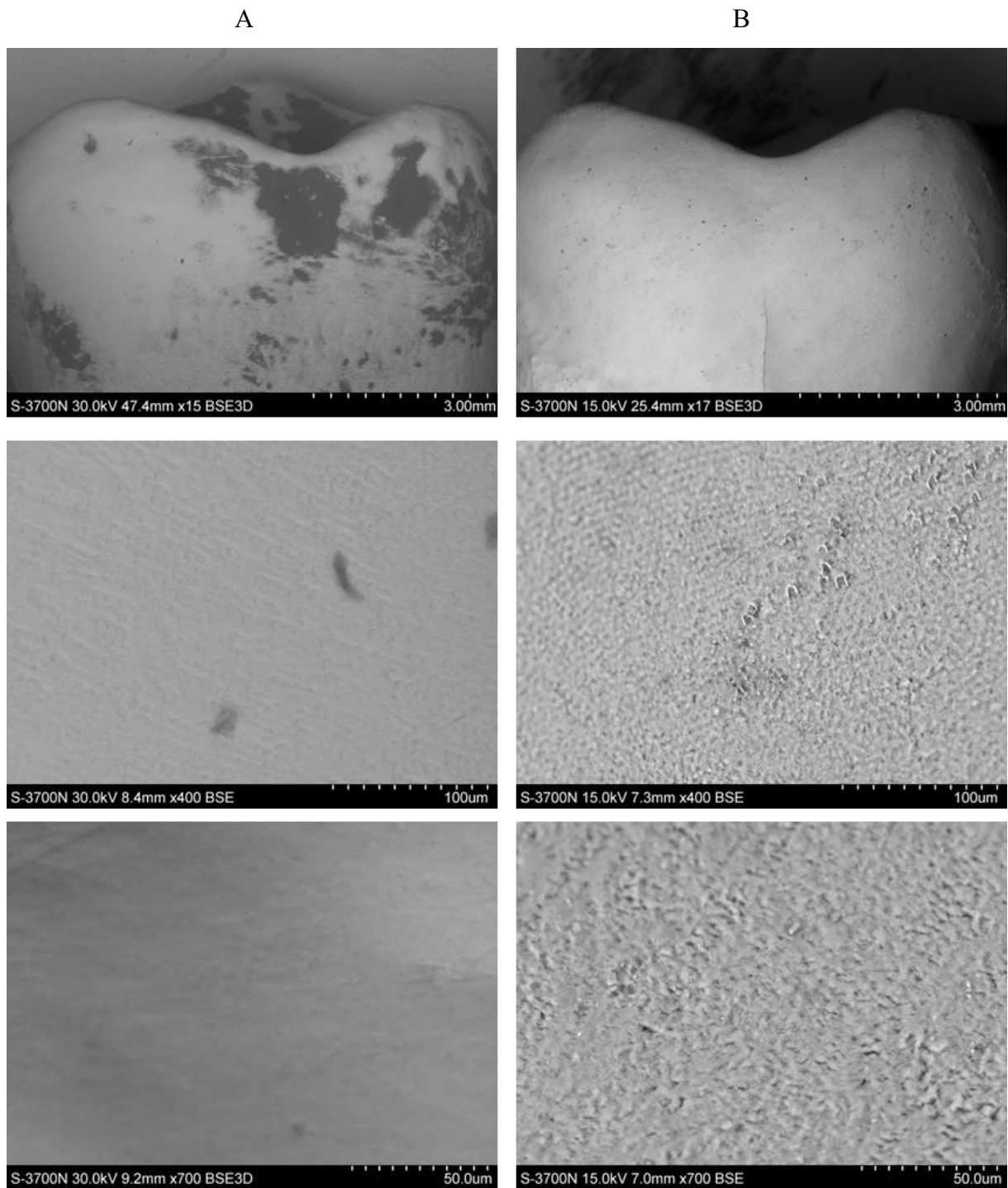


Fig. 2. Comparison of enamel surface alteration obtained by SEM before (A) and after (B) protein etching. Upper row – natural size; Middle row – area 1 at 400x magnification; Bottom row – area 1 at 700x magnification. Tooth sample number 6 (tooth from extraction in dental clinics in 2019–2021).

Additionally, most of the images from the SEM showed slight alterations of the surface with the presence of microporosity, possibly with deepening grooves and defects. The method of sample preparation for proteomic sex estimation appears to weaken the sample structure, which is also evident from the micro-CT analysis. We observed microcracks in some teeth before the analysis, which after protein etching were slightly wider (Fig. 3).

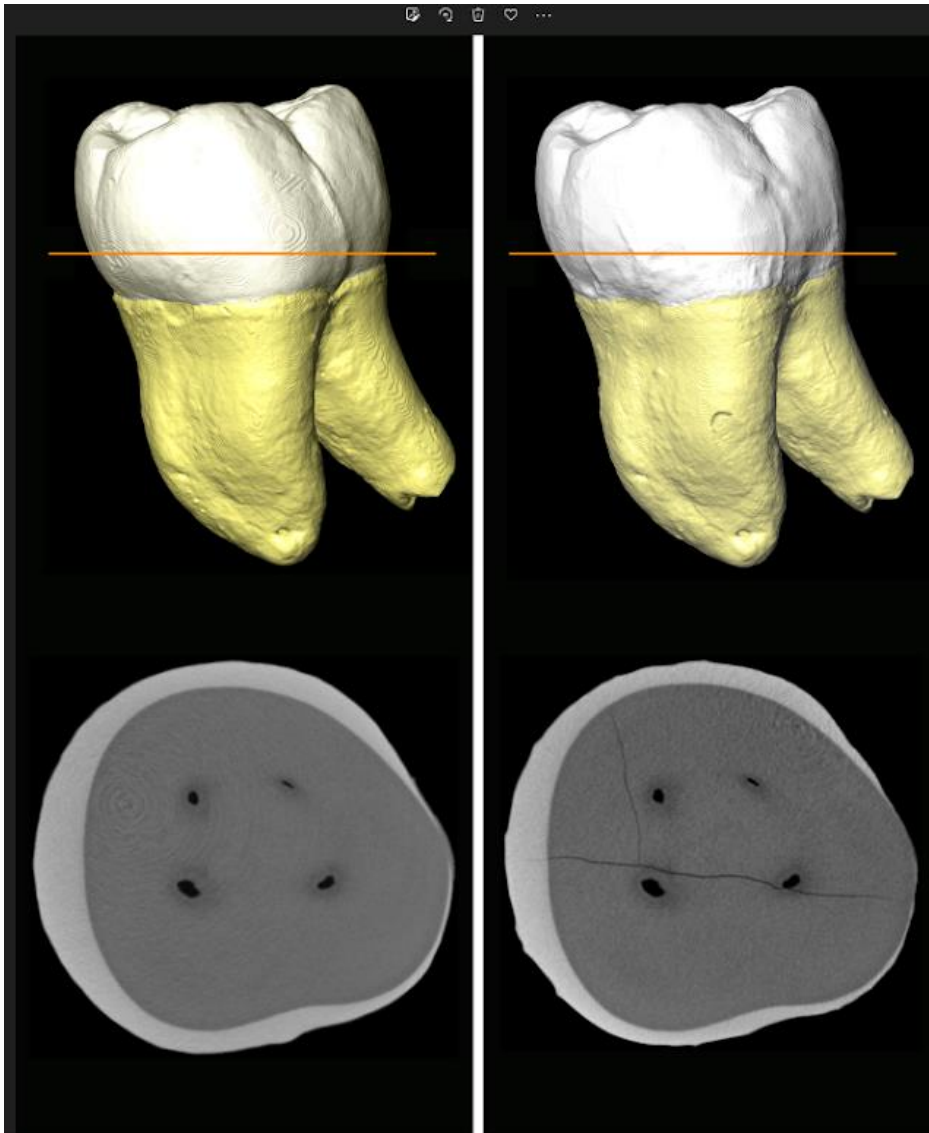


Fig. 3. Structural changes in tooth due to protein etching (Tooth sample number 3). Top left: 3D reconstruction of Tooth 3 before protocol application. Top right: after enamel etching according to the protocol to obtain protein for sex estimation. The horizontal line shows the plane of the section.

Quantitative changes in enamel thickness using colour maps are shown in Fig. 4. In the given example, the enamel surface on the occlusal plane shows visible enlargement of a cavity caused by tooth decay, which is caused by the action of the extraction solution. The enamel thickness is less, and the cavity after caries has increased due to the action of the extraction solution.

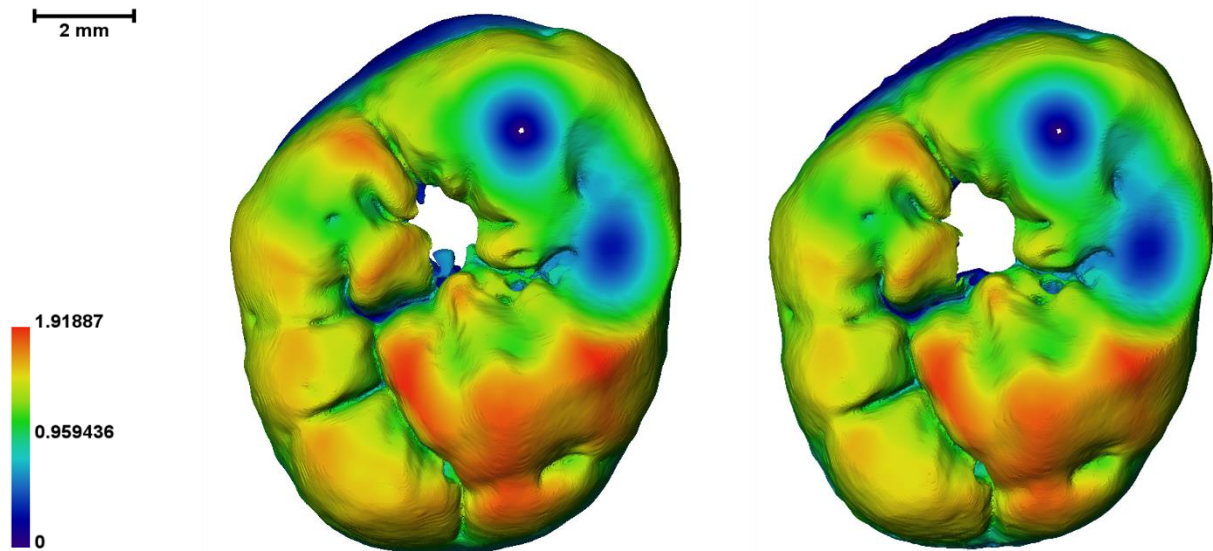


Fig. 4. Colour map of the enamel thickness on the molar occlusal surface. Red indicates areas of thicker enamel surface. The white area in the middle corresponds to an opening caused by dental caries. As a result of the extraction and the action of the acid, the opening became larger (left before etching, right after etching). Tooth sample number 46.

Another example of quantitative changes in enamel thickness using colour maps is shown in Fig. 5. After the application of the protein extraction protocol on the tooth enamel, there is a reduction in the thickness of the enamel, which is manifested by a change in colour from red to blue. This reduction in enamel thickness affects all of the teeth that have been treated. The colours are always less intense on the right colour map. Volume changes of both enamel and dentine, as well as of the entire tooth, are shown in Table 4. Due to the application of the extraction protocol, approximately 10% of the enamel is lost, but we also observed a 2% loss of dentine.

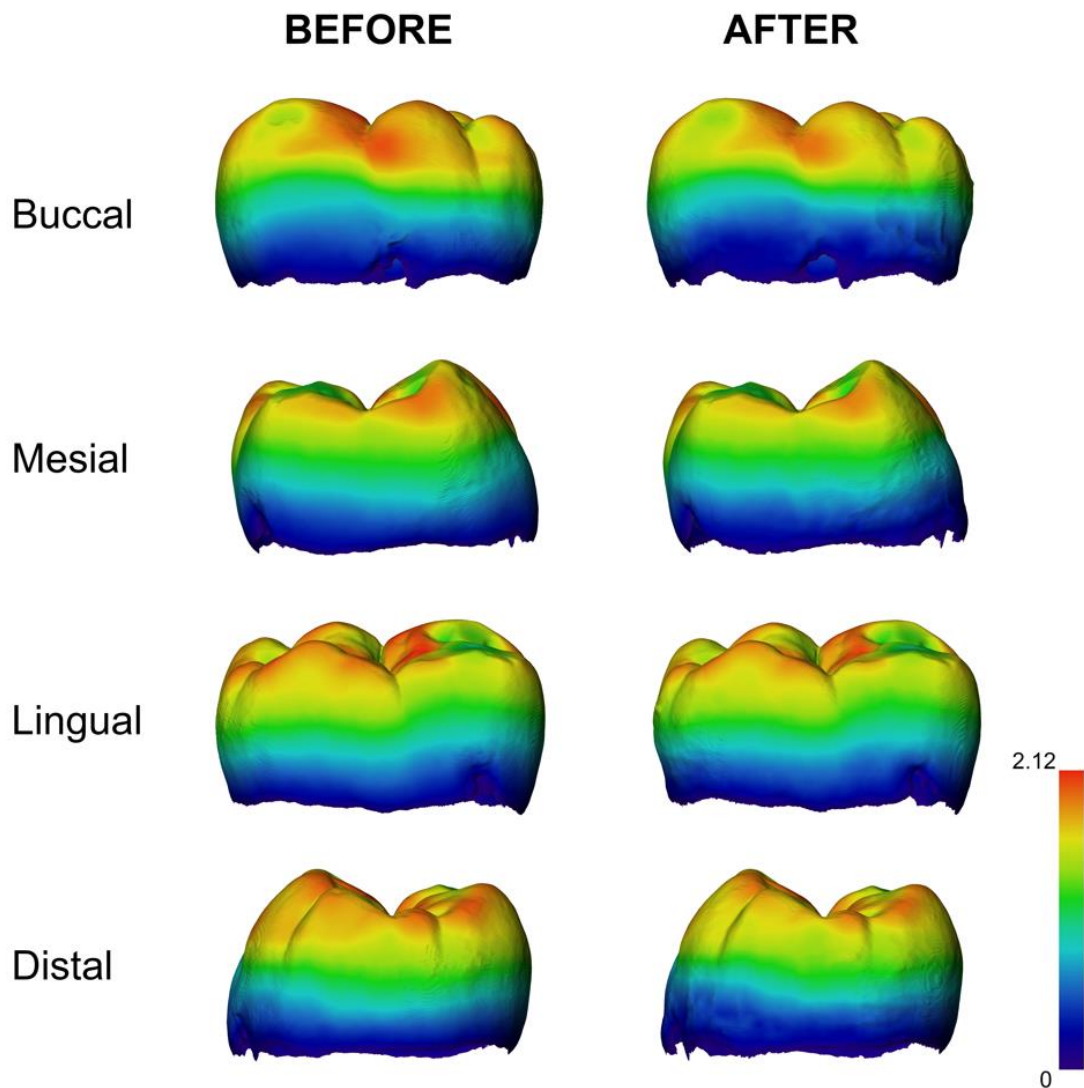


Fig. 5. Colour map of the tooth showing the enamel thickness before and after protein etching. Red indicates areas of thicker enamel surface. Tooth sample number 7.

Table 4. Micro-CT of selected teeth before and after protein etching. Absolute and relative values of tooth volume, enamel and dentine volume.

Sample number*	Tooth class	Enamel volume (mm ³)			Dentin volume (mm ³)			Total volume (mm ³)		
		Before	After	Difference (%)	Before	After	Difference (%)	Before	After	Difference (%)
3	₃ M	248.03	224.25	9.6	475.14	463.93	2.4	723.2	688.18	4.8
4	M ₃	285.38	264.53	7.3	632.08	623.11	1.4	917.5	887.64	3.3
5	₃ M	311.76	287.41	7.8	706.53	697.15	1.3	1018.3	984.56	3.3
6	³ M	191.38	167.6	12.4	499.1	485.5	2.7	690.5	653.1	5.4
7	₃ M	290.16	270.04	6.9	738.75	728.22	1.4	1028.9	998.26	3.0

9	M ³	151.3	131.57	13.0	403.34	393.32	2.5	554.6	524.89	5.4	
10	₃ M	205.22	178.91	12.8	390.81	383.48	1.9	596.0	562.39	5.6	
11	M ₃	191.6	166.52	13.1	620.17	603.85	2.6	811.8	770.37	5.1	
12	M ₃	182.37	157.88	13.4	465.99	453.56	2.7	648.4	611.44	5.7	
14	M ₃	162.68	139.6	14.2	389.2	372.79	4.2	551.9	512.39	7.2	
31	³ M	155.17	139.73	10.0	480.56	473.3	1.5	635.7	613.03	3.6	
32	₃ M	177.24	160	9.7	476.13	471.85	0.9	653.4	631.85	3.3	
42	M ¹	196.09	180.22	8.1	728.44	720.29	1.1	924.5	900.51	2.6	
46	M ³	155.55	141.07	9.3	531.76	516.31	2.9	687.3	657.38	4.4	
47	M ¹	277.28	260.76	6.0	936.66	928.53	0.9	1213.9	1189.29	2.0	
48	² M	237.48	220.53	7.1	553.83	547.77	1.1	791.3	768.3	2.9	
49	² M	185.94	166.71	10.3	688.07	675.21	1.9	874.0	841.92	3.7	
53	¹ M	171.39	148.34	13.4	798.43	786.98	1.4	969.8	935.32	3.6	
54	M ¹	100.7	91.35	9.3.	1005.0	984.73	2.0	1105.7	1076.08	2.7	
59	² M	237.44	218.2	8.1	1197.51	1178.42	1.6	1435.0	1396.62	2.7	
Mean				10.10				1.92	4.02		
Standard deviation				2.59				0.84	1.36		
95% confidence interval for the mean				[8.88, 11.30]				[1.53, 2.31]	[3.38, 4.65]		

*Numbers correspond to the sample number in Table 2

Tooth nomenclature: I = incisor, C = canine, P = premolar, M = molar, upper index = upper jaw, lower index = lower jaw, side of the index indicates side of the tooth (left or right)

As can be seen in Fig. 6, the percentage of total volume loss depends on the proportion of enamel in the total volume of the tooth (this proportion is calculated as enamel volume before etching / total volume before etching). This relationship is of moderate intensity ($r = 0.46$) and weakly significant ($p = 0.042$).

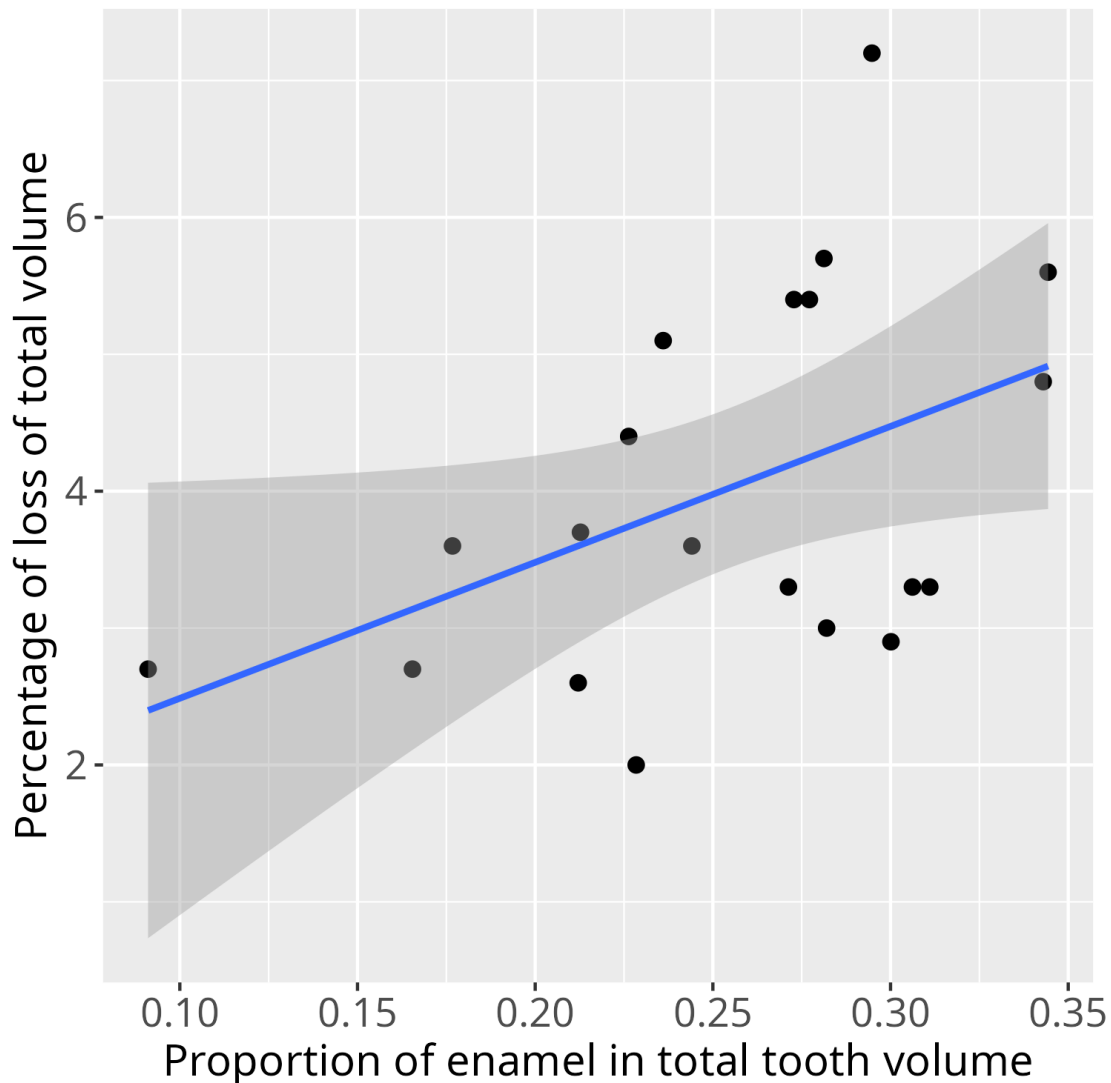


Fig. 6. Relationship between percentage of loss of total volume and the proportion of enamel in total tooth volume.

5. Discussion

The validity of proteomic sex estimation

Proteomics is an approach with a wide range of applications. The main strength of tandem mass spectrometry (MS/MS) is its ability to analyse complex protein mixtures [42]. The use of the resulting MS/MS spectra to determine the sequence of peptides is increasingly common not only in biological sex estimation [27,29,30,32], but also in art history [43–45] and the material analysis of historical textiles [46,47]. The use of proteomic methods in palaeontology is also increasing rapidly, and it is expected that these methods will be helpful for many general applications, connecting molecular biology, palaeontology, archaeology, palaeoecology, and history [42].

The advantage of proteomics in sex estimation is its high reliability and, compared to DNA analysis, the low risk of contamination and relative cost-effectiveness [17]; it is also less destructive. The absolute accuracy of the method used for the extraction and analysis of sex-specific proteins (Table 2) proved the suitability of the method for examining human skeletal remains, which, like cultural artefacts, form

an integral part of the cultural heritage. Reliable estimation of sex in sub-adults (which is impossible to assess directly from skeletal remains due to the undevelopment of sexually dimorphic traits), so far uncommon in bioarchaeological practice, significantly increases the explanatory potential of sub-adult skeletons and helps to avoid interpretation bias resulting from unknown sex ratios in specific age classes or socioeconomic groups [41]. Therefore, if sex estimation is needed, it is advisable to use protein extraction methods [13,32] without the need for tooth destruction [26,30]. Other reasons for its use include the low impact of manipulation on the archaeological skeletal material, which manifests itself in minimal invasiveness, as also proven in our results.

In addition, the technique employed allows the first two steps (sample preparation and protein etching) to be carried out in a laboratory with minimal instrumentation and equipment. Only the third and last stage, the protein analysis itself, must take place at a workplace with the appropriate liquid chromatography-tandem mass spectrometry equipment.

Invasiveness of the amelogenin protein etching protocol

The issue of the invasiveness of protein etching is a major topic in terms of cultural heritage preservation efforts. The influence of the various chemicals to which the hard dental tissue, in the case of proteomics the enamel, is exposed should be known. However, we are not aware of any previous study evaluating the extent of enamel loss when applying proteomics for sex estimation; to the best of our knowledge, the effect of protein extraction on the structural integrity of enamel assessed here for the first time. The protocol used in the present study uses low concentrations of H₂O₂ (3%) for 30 s for cleaning and demineralization of the tooth surface to remove calcium phosphate salts (calculus), and HCl (5%) etching for only 2 min [13] with immersion of only the dental crown. Such a concentration of H₂O₂ is even lower than the maximum concentration (up to 6% H₂O₂) in tooth whitening products, which a 2011 European directive considered to be cosmetic products according to Dias et al. [48]. With regard to HCl concentration and time to its exposition, the only possibility for comparison is offered by studies that deal with the effect of acids on teeth dissolution in a forensic context. The destructive effect of highly concentrated HCl (37%) on human dentition is indisputable. Several studies have established that highly concentrated HCl (depending, among other things, on the type of tooth) cause teeth to completely dissolve after a few hours [49–51]. Concerning the exposure time, Mazza et al. have reported no visible effect after 5 min of immersion in 37% HCl [49]. Gupta and Johnson observed morphological and radiographic changes to enamel after 30 mins–1 hour [52]. Jones et al. 2020 reported almost complete disintegration of enamel after 4 h and no enamel present after 12 h in HCl (37%) [51]. It has been confirmed that the acid concentrations as well as the length of exposure is important for the final morphological and chemical impact [49, 50], and left no traces behind. In our case, a much lower concentration and a much shorter exposure time were used, which leave no visible traces. We observed enamel loss of 10% and dentine loss of only 2% using micro-computed tomography (micro-CT) (see Table 4). However, it should be noted that this also involves the removal of surface dirt and dental calculus, which are included in the volume of dental tissue in the first scanning and are removed only before the protein etching itself.

Importance for sexing in archaeological assemblages

The application of proteomic sex estimation in an archaeological collection to an individual with questionable or contradictory results of biological sex estimation by anthropological methods allows the obtaining of correct information about biological sex, which can be used to study a whole range of issues such as demography, diet and burial rites [15,53]. In the present study, adult individuals, for whom the morphological sex assessments performed by two teams half a century apart showed differences, were selected for the application sample. Table 5 shows the results of proteomic sex

estimation in 15 adults from the Early Medieval period with biological sex estimate discrepancy. The proteomic analysis agrees with the first morphological sex estimate by Stloukal in approximately half of the cases [36–38], while the same ratio of morphological sex agreement occurs in the case of the second morphological sex estimate by Zazvonilová et al. [39]. Sexing by proteomic analysis was successful for all individuals, contrary to the morphological sex estimates where some individuals were not determined or where sex was estimated with uncertainty (in Table 5 indicated by question marks). Unlike morphological sex estimation, which provides a probability of the estimated sex, in proteomic analysis sex can be assigned.

Table 5. Contradictory morphological sex estimation in an application sample of 15 adult teeth performed by Stloukal (1963, 1964, 1967) and by Zazvonilová et al. (2020), in comparison with amelogenin-sex estimation. Concordance of the anthropological estimation with the proteomic sex estimation is indicated by an asterisk.

Skeleton number	Anthropological sex estimation		Proteomic sex estimation
	Stloukal (1963, 1964, 1967)	Zazvonilová et.al. (2020)	Present study
H87	F?	M*	M
H170	M*	F	M
H0171	M	F*	F
H0187	F?	M?*	M
H0292	M	F*	F
H0314(bis)	Not determined	M	F
H0314	Not determined	M*	M
H0324	M	F*	F
H0352	F*	M	F
H0363	M*	F	M
H0406	F*	M	F
H0457	F?*	M	F
H0647	M*	F	M
H0718	M?	F*	F
H1088	F?	M*	M

Regarding the non-adult individuals described in detail elsewhere [41], the current sample of sexed non-adults with known dietary history helped to reveal that there were no dietary differences between Great Moravian boys and girls during the first decades of their lives. Proteomics provides a new, relatively simple, and quite inexpensive method of sex estimation without the risk of contamination.

6. Conclusion

Our results demonstrated the suitability of a protocol that uses protein etching from an intact tooth and does not require its mechanical destruction, or that of parts of the crown. Results show the absolute accuracy of biological sex estimation in a sample of 60 individuals of known sex. Based on micro-CT analysis (n = 20), the etching protein process is minimally invasive and does not cause visible

changes to the dental enamel surface. Application to an archaeological sample of non-adult (n = 32) and adult (n = 15) individuals proved the suitability of proteomic biological sex estimation. Proteomics provides a convenient way to estimate sex in juveniles where anthropological methods cannot be applied. The status of such a procedure allows for wider dissemination of the method, which can thus become a routine technique in the analysis of archaeological skeletal material.

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