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REVIEW ARTICLE

Peptide analysis of tooth enamel – A sex estimation tool for archaeological, anthropological, or forensic research

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Proteomics has become an attractive method to study human and animal material, biological profile, and origin as an alternative to DNA analysis. It is limited by DNA amplification in ancient samples and its contamination, high cost, and limited preservation of nuclear DNA. Currently, three approaches are available to estimate sex–osteology, genomics, or proteomics, but little is known about the relative reliability of these methods in applied settings. Proteomics provides a new, seemingly simple, and relatively non-expensive way of sex estimation without the risk of contamination. Proteins can be preserved in hard teeth tissue (enamel) for tens of thousands of years. It uses two sexually distinct forms of the protein amelogenin in tooth enamel detectable by liquid chromatography-mass spectrometry; the protein amelogenin Y isoform is present in enamel dental tissue only in males, while amelogenin isoform X can be found in both sexes. From the point of view of archaeological, anthropological, and forensic research and applications, the reduced destruction of the methods used is essential, as well as the minimum requirements for sample size.

KEYWORDS
archeology, sex determination, tooth enamel

1 | INTRODUCTION

The estimation of sex is fundamental to many archaeological, anthropological, and forensic research. This determination has three possible approaches: osteology, genomics, or proteomics [1]. The first two approaches are traditional methods, but the proteomic one is a relatively new method with the increasing attention of scientists. Recently the use of proteomic methods in paleontology (so-called "Paleoproteomics") is rapidly growing, and it is expected that these methods can be helpful for many general applications and connect molecular biology, paleontology, archaeology, paleoecology, and history. Warinner et al. said about Paleoproteomics: "Growing from a handful of studies in the 1990s on individual highly abundant ancient proteins, paleoproteomics today is an expanding field with diverse applications ranging from the taxonomic identification of highly fragmented bones and shells and the phylogenetic resolution of extinct species to the exploration of past cuisines from dental calculus and pottery food crusts and the characterization of past diseases." [2]. From this point of view, the most frequently analyzed protein is collagen type I, which is a stable and rigid protein, as was demonstrated in a well-known study in 2007 by determining collagen type I at 68-million-year-old bones of *Tyrannosaurus rex* [3, 4]. However, at the last years, the ability to detect and identification of proteins in ancient (but not only) tissue was significantly increased and allows to use of protein not only for identification in tissue but also for various problems such as interaction with food,

Article Related Abbreviations: AMELX, amelogenin X isoform; AMELY, amelogenin Y isoform; DFA, discriminant function analysis; PCR, polymerase chain reaction.

interaction animal-human, environmental exchanges but also (from the view of this review) estimation of sex. Also, the significance of proteomic research has increased in archaeology [5]. It is important to note the differences in using the terms sex/gender. The terms sex and gender are often interchanged in conversation, documentation, and scientific literature, although they are not synonymous, and confusion in their usage is increasing. The biological concept of sex fundamentally differs from the social concept of gender, and the two terms are not interchangeable [6]. In this article, we will stick strictly to using the term sex only.

2 | OSTEOLOGICAL (MORPHOLOGICAL) ESTIMATION OF SEX

The existence of sexual dimorphism, that is, of differences in bone variables between males and females, is an inevitable prerequisite for any anthropological sex estimation method. According to the approach, the methods are divided into visual, which evaluates the development of characters according to categories, metric methods, which use the dimensions of bones and their size; and geometric-morphometric methods, which explore the interplay and differences between shape and sizes from a metric perspective [7]. Both genetic factors and the action of sex hormones influence the sexual dimorphism of the skeleton. Its final state is modified by external environmental factors (e.g., [8]). Before the development of secondary sexual characteristics, however, there was limited sexual dimorphism in skeletal features, rendering sexing methods unreliable it is not recommended to use them [9].

The full development of sexual dimorphism exists only in adulthood. In adults, the accuracy of the methods is different in every population and age group and depends on which bone is used. The dimensions of the skeleton show considerable variability. For these reasons, the methods must contain more variables that are not correlated with each other. In forensic anthropology and bioarchaeology, most methods for sex estimation rely on statistical models and tools generated through osteometric data collected from identified populations [10]. The most common method of sex estimation is discriminant function analysis (DFA). A discrimination score greater than the cut-off point between the sexes corresponding to a probability of 0.5 indicates a male and a score less than the cut-off point indicates a female. The accuracy of the methods is not absolute, and we always find a certain number of misclassified individuals who match the error. The skull and pelvis are still the preferred skeletal elements for sex estimation, with an accuracy that varies from 80% to 95% [11]. An overview of all possible anthropological methods for determining sex according to the skeleton is given in recent publications (e.g., [12–14]).

A common misunderstanding of DFA results is that the overall accuracy of sex classification can be applied to every individual in the sample. Every bone measurement and discriminant score shows an overlap between female and male distributions. The overlapping area represents the "zone of uncertainty", where the skeletal variables of females and males are similar and cannot reliably be distinguished from one another [15]. This "zone of uncertainty" varies depending on the size difference between the population where the method was proposed and the population in which we want to use it. We call this the population specificity of morphological methods, and ignoring it causes a dramatic decrease in accuracy and an increase in misclassification [16]. Exceptions are methods that use pelvic bone dimensions, which describe sexual dimorphism as a whole. Such methods have general validity and can be applied to all anatomically modern people with a high success rate and an error risk of less than 5% [17]. Unfortunately, in archaeological discoveries, the pelvis is often very damaged or completely missing [18]. For these reasons, the approach of primary and secondary sex diagnosis was proposed [19]. Primary sex diagnosis applies reliable methods in individuals with pelvic bone. In the sample obtained in this way, classification methods specific to the given population are proposed, which use extra-pelvic dimensions of the skeleton. These methods are applied to individuals with a missing pelvis [20]. Sex estimation in the "zone of uncertainty", that is, the overlapping region between sexes, should be avoided to reduce misclassification. Sex should be assigned only to those individuals with a posterior probability of being female or male higher than 0.95. Although such an approach limits the practical applicability of DFAs, because some portion of the individuals remains unclassified, it allows a high classification accuracy to be maintained at the individual level [15].

Another way to estimate the sex of a skeleton is to use visual methods and assess the degree of trait development using categories. These are highly subjective, and the variability of sexual dimorphism within and between populations is considerable. Only methods with a reference set from multiple populations and designed software for sex estimation can provide usable results [21–23].

3 | GENETIC ESTIMATION OF SEX

For a long time, hundreds of years, scientists searched for a system of sex-determining pathways. Aristotle (in 335 BCE) proposed that heat/cold could determine sex.

This environmental theory was popular until about 1900 – when sex chromosomes were discovered. However, Aristotle was partly right when in some reptiles, the temperature of the nest can determine the sex of the embryo [24]. The discovery of chromosomal sex determination is credited to Nettie M. Stevens in 1905 with the finding that in most animals' sex is determined chromosomally, with males producing two types of gametes (carrying a Y or X chromosome) and thus being heterogametic, while females produce only one type and they are therefore homogametic [25]. Despite the great variety of sex determination mechanisms in some lineages, there is a surprising consistency. Amongst eutherian mammals, birds and some insects rely on heteromorphic sex chromosomes, which differ from each other in size, morphology, and gene content, to determine the sex of the individual [26].

Molecular biology and genetics can use aDNA at the individual level to identify the biological sex of a skeleton, make phenotypic inferences from an individual's genotype, and identify specific pathogens within an infected individual [27]. Molecular biology techniques are increasingly used to identify the sex of skeletal remains when traditional anthropometric analyses do not successfully identify the sex of remains that are incomplete, fragmented, and/or refer to immature individuals [28–30]. As Raff [27] reports, aDNA research cannot be done in regular molecular biology laboratories. While next-generation methods have made it much easier to distinguish contamination from endogenous DNA based on DNA damage patterns, preventing contamination during the extraction process is still extremely difficult (e.g., [31, 32]). Contamination is such a pervasive problem that the work requires specialised facilities and workers trained in aDNA protocols. Sample decontamination is essential before beginning extraction. They will include one or more of the following steps: drilling or removing surface material, soaking or rinsing in bleach, and UV irradiation to cross-link surface DNA and extraction [33]. Laboratories investigating aDNA must be positively pressurised, with HEPA-filtered air, polymerase chain reaction (PCR) enclosures, separation from post-PCR or modern DNA laboratories, and strict access protocols. They should be staffed by researchers trained in the specialised laboratories and methods necessary for ancient biomolecules [27].

From the analytical point of view, we can also mention sex estimation by capillary gel electrophoresis with amelogenin locus as a marker from 1998 [34]. The analysis of ten male and ten female samples and double peaks verified the male sample, while one peak verified the female sample. As a result, two peaks were obtained—the first peak was for the X and the second for the Y locus.

The most common method of genetic sex estimation relies on differences in the amelogenin gene (e.g., [35, 36]). Using cloning techniques, the amelogenin gene was localized to the sex chromosomes. With the advent of PCR, researchers developed robust and relatively simple amplification techniques for determining the presence of X and Y chromosome versions of this gene. This technique exploited the fact that insertion/deletion polymorphisms between the X and Y chromosomes lead to differently sized amplicons, which can easily be visualised by size separation using gel electrophoresis. These methods were used in the first commercially available kits [37]. However, the amelogenin-based method can be problematic due to allelic dropout. It means that if only the copy present on the X chromosome is retained and not the copy on the Y chromosome, the individual will be falsely identified as female rather than male. Another problem is modern contamination from a male source, which could mislabel an ancient individual as male [27]. However, more recent profiling kits include assays for a separate insertion/deletion polymorphism and a short tandem repeat locus found only on the Y chromosome. Additionally, analysis of other short tandem repeats, other insertions, deletions, or single nucleotide polymorphisms on the X or Y chromosome can also be used to determine sex [37–40].

To overcome the before mentioned shortcomings, high-throughput shotgun sequencing has been proposed, which represents a more accurate method of sex assignment, as it simultaneously avoids the problem of allelic dropout by testing many more discriminating loci and allows the detection of contaminating fragments by assessing DNA damage patterns [41]. Skoglund et al. [42] evaluated the feasibility of this approach for applications in population-scale aDNA investigations. They found that approximately 100 000 reads are required for accurate sex estimation so that indexed genomes of up to 13 individuals could be pooled and simultaneously sequenced at low coverage at an estimated cost of < $300 per sample. While considerably more expensive than a simple amelogenin size-based analysis, it does have the advantage of being more accurate. It is likely that as shotgun sequencing costs continue to decrease, this approach will become much more routinely applied to archaeological populations [27]. However, this degree of preservation may be problematic for many archaeological remains, as Mittnik et al. [43] noted. To reduce the required number of mapped human sequences, Mittnik and colleagues proposed an alternative method of sex estimation using high-throughput shotgun-sequenced DNA. This method relies on the proportion of reads mapped to the human X chromosome compared to the proportion of reads mapped to each autosomal chromosome. By down-sampling reads from the same high-quality ancient DNA data sets used in Skoglund

et al. [41], the proposed method could give confident assignments with as few as 1000 human genomes reads [1].

However, both Skoglund's and Mittnik's approaches [42, 43] are limited and admit some risk of error when the confidence interval is within certain parameters [44]. To determine the genetic sex is compared the ratio for the number of Y-chromosomal 1240k positions with available data relative to the 1240k position on the X-chromosome. Individuals with a ratio greater than 0.35 were considered genetic males and individuals with a ratio less than 0.03 were considered genetic females [45]. The current rapid development of genomics will certainly enable even more sophisticated methods of genetic sex estimation. Current research is yielding very encouraging results in the field of archeology that were previously unavailable (i.e., [46–48]).

However, it cannot be neglected that the aDNA extraction process is invasive and generally involves the destruction of a small (<0.5 g) amount of bone or tissue. The material for extraction should not be used for osteological research, such as non-pathological ribs. Recently, a petrous bone from the inner ear has been an excellent source of well-preserved, high-quality aDNA and should therefore represent an optimal extraction target [49, 50]. However, even petrous bone is an essential element in anthropology that should not be sacrificed to obtain high-quality aDNA [51]. In addition, petrous also has several applications for scientific analysis beyond sex estimation from ancient DNA. There is a risk of potential bias in using the pars petrosa for ancient DNA analysis [52]. Ethical guidelines for petrous bone sampling need to be expanded further, as the demands for destructive sampling will only increase as the number of researchers and laboratories conducting aDNA research increases.

However, the genomic method looks like a standard and required method for the estimation of sex; there is also a developed proteomic method (see next section). One could ask the question: why? One of the answers is that genetic material is not so resistant to degradation, but what about the precision of sex estimation? An interesting comparison of the three methods (osteological, genomic, and proteomic) was made on 55 individuals between 2440 and 100 cal BP [1]. Agreement between all methods was excellent when DNA shotgun sequencing was about 100 000 total sequences. However, more than half samples were below this threshold, and the conflict of sex estimation increased. On the opposite proteomic signal was not decreasing so significantly. It was concluded that proteomic data could complement osteological and genomic results/data.

4 | PROTEOMIC ESTIMATION OF SEX

Nowadays, proteomic methods enable us to determine minor differences in protein composition (qualitative and quantitative) enabled by many influences such as diet, aging, breeding, and sex [53]. Of course, the gender approach is a popular modern society theme, as visualized by the European Commission: "Gendered Innovations. How Gender Analysis Contribute to Research", and thirty years of research have revealed that sex and gender biases are socially harmful and costly [54].

For archaeological, anthropological, or forensic research, it is necessary to consider tissues resistant to degradation caused by long-term effects, aggressive decomposition phenomena in the soil, and external physical and chemical damage. For these purposes, the ideal tissue is teeth enamel. Enamel is one of the most calcified tissues in mammalian organisms and can protect teeth for tens of thousands of years [55]. A nanocomposite bioceramic shields teeth against multiple chemical and physical (mechanical) efforts to disturb them. Protein amelogenins regulate crystallite formation during enamel development; however, they are specifically degraded during teeth maturation [56]. Amelogenin genes, in humans, are located on X and Y chromosomes (AMELX and AMELY, respectively). Proteins encoded by these genes have a different amino acid sequence (see Figure 1). The result of the presence of these two genes on the X and Y chromosomes is the sex-dependent presence of different proteins: AMELX for females and AMELX and AMELY for males [57]. During enamel maturation, proteins are degraded (proteolytic procedure), so mature tooth enamel is rich in various peptide fragments of constituted proteins [55]. We have to remind that amelogenin is a relatively small molecule highly concentrated in the extracellular matrix [58]. Because amelogenin is a major enamel protein, these peptides mainly originated from amelogenin. For this reason, free peptides from sex-dependent amelogenins (AMELX and AMELY) are a good choice for estimating sex. If we are looking for a universal sex determination pathway for all mammals, we have to pay attention: AMELX gene on the X chromosome X and AMELY on the Y chromosome are presented not in all mammalian families; it is presented in *Hominidae*, *Suidae*, and *Bovidae*, but rodent species have only one AMELX [58]. We must also mention that the tissue used (teeth enamel) is difficult to cross-contaminate.

In 1991 Fincham et al. [59] remark that a diagnosis of differences in human enamel proteins can permit the distinction of specimens according to the sex of the individual. Porto et al. [60, 61] discover/describe enamel proteins looking for sex-depending peptides. They used whole-crown

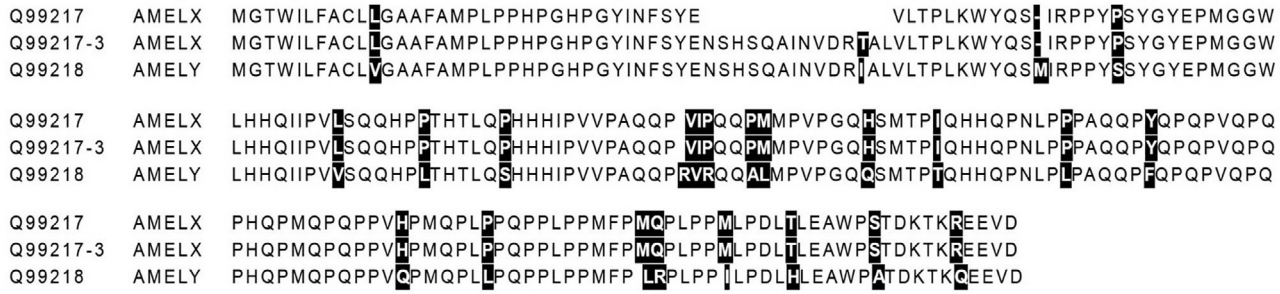

FIGURE 1 Structure of Amelogenins and their comparison. Data are from UniProtKB reviewed (Swiss-Prot): Q99217 Amelogenin, X isoform, Q99217-3 Amelogenin, X isoform, Rare isoform 3; Q99218 Amelogenin, Y isoform. Differences in amino acid sequence between X and Y amelogenins are indicated by white characters highlighted in black.

etching, enzymatic (trypsin) treatment, and analysis by MALDI-TOF/TOF. They found the amino-terminal amelogenin peptides in the ancient sample (Mummy—ad 800–1100). They also found a peptide (WYQSIRPPYP) specific for the amelogenin X-isoform but no peptide specific for Y-isoform. However, the authors did not specify the sex of the samples [60]. Although the MALDI-TOF/TOF method is suitable for protein and peptide analysis, nanoLC-MS/MS is a method of choice due to its higher sensitivity for peptide detection and identification. [62].

Another attempt to analyze human teeth enamel was made by Castiblanco et al. [63] in 2015. They investigated the protein composition of healthy contemporary pulverized teeth without the digestion of an enzyme (trypsin). After extraction and cleaning on C18 StageTips, they analyzed peptides/proteins on nLC/MS-MS using QExactive MS (Thermo Fisher Scientific). Seven proteins were identified when four were specific for enamel (AMELX/AMELY, enamelin, and ameloblastin). Although eight teeth were used, the sex was not mentioned, and the method was not used for its detection.

Stewart et al. [64] first published specific etching of single teeth to identify sex-sensitive amelogenins from enamel in 2016. This procedure (and with some slight modification) is now commonly used for peptide extraction from enamel (for the scheme, see Figure 2). The method consists of two parts [65]: etching and extraction. First teeth are mechanically treated (by dental burr) to clean them from all macroscopic impurities. Cleaning continued by water washing. Next, procedures were continued in the cap of a separate microcentrifuge tube (leaving a convex meniscus protruding above the lip when the tooth was lowering into the cap). The tooth's crown was washed with 3% H_2O_2 for 5 min, twice washed with H_2O, and etched with 10% (v/v) HCl for 2 min. All these solutions were discarded. A second 2-min etch was used for analysis. The next stage was extraction. C18 resin-loaded ZipTip (Millipore, MA, USA) was used for extraction. ZipTip was previously conditioned three times with 100% ACN and then three times with 0.1% (vol/vol) formic acid (each draw discarded). The peptides were bound to the ZipTip (by up and down pipetting by 10 times. The ZipTip was washed six times with 0.1% (vol/vol) formic acid (each wash discarded). Bounded peptides were eluted by 4-μl of 60% ACN/0.1% formic acid. This fraction was lyophilized and dissolved in 12 μl of 2% formic acid in water and analyzed by reversed-phase nanoLC/MS (with gradient similarly generally used for peptide separation, when used solvents were water and ACN with 0.1% TFA). The mass spectrometer was a hybrid linear ion trap orbitrap (Orbitrap XL, Thermo Scientific). They selected peptides AMELY-(58-64; SM(ox)IRPPY ($[M + 2H]^{2+}$, m/z 440.2233) peptide and AMELX-(44-52; SIRPPYPSY ($[M + 2H]^{2+}$, m/z 540.2796) (Figure 3).

Authors [64] also compared the influence of trypsin digestion on protein/peptide identification. However, trypsin increased the variety of peptides, but no significant differences were observed in "sex" proteins (enamel-specific proteins) when trypsin-treated and untreated samples were compared.

The same approach was used for sex estimation of the Late Antique (probably 4th–6th century) 'Lovers of Modena' [66]. The authors extracted peptides (after etching of teeth) by HyperSep SpinTips (Thermo Scientific) with C18 phase, and the analytical system was UHPLC-HRMS (Q Exactive MS from Thermo Scientific). Besides SM(ox)IRPPY (m/z 440.2233) peptide, they used at least two other peptides—SMIRPPY (m/z 432.2258) and M(ox)IRPPY (m/z 396.7073). All these peptides agreed in the distinction of specimens according to the sex, i.e. in the determination of AMELY. Surprisingly the 'Lovers of Modena' were males. The same method was successfully used to research sex-related morbidity and mortality for 30 non-adults from the Early Medieval Italian site from the 7th century AD [67]. The authors concluded that this method is used for archaeological (and forensic) research.

This method was also successfully used for sex estimation from deciduous and permanent teeth from non-adults

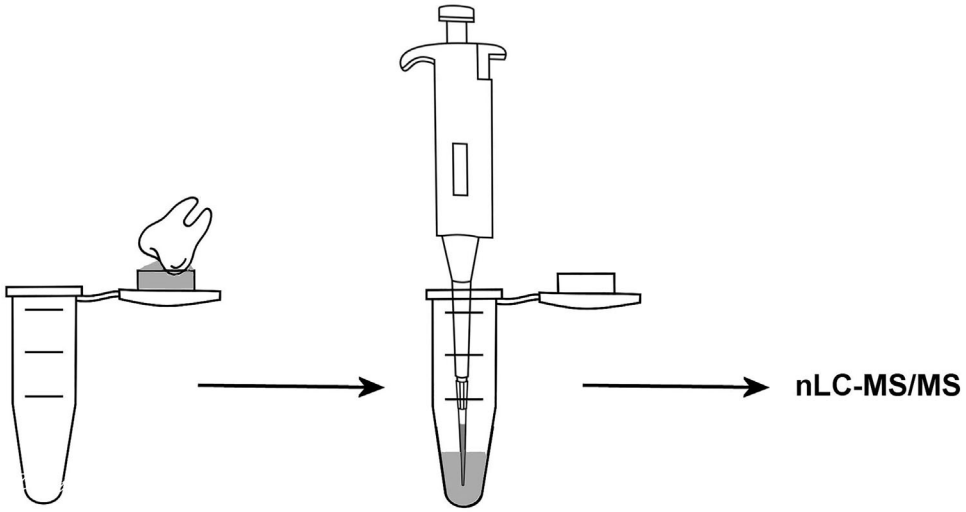

FIGURE 2 Workflow of the sample preparation (etching method) for amelogenin analysis.

(incl. perinatal subjects) from archaeological sites in England (1st–2nd centuries AD, and 18th–19th centuries) [68].

Again the same method [66, 67] was used for the sex estimation of the horsemen of the Early Middle age (7th century AD) [69]. Determination of sex by proteomic (enamel) method was in agreement with osteological and archaeological determination when in many cases, only proteomic analysis was the only acceptable method for sex estimation. Authors also used confident identification of AMELY by triple-peptide approach (*m/z* 396.7073 for M(ox)IRPPY, *m/z* 432.2258 for SMIRPY, and *m/z* 440.2233 for SM(ox)IRPPY).

The above-described etching techniques were studied for optimization using three treatment procedures by 1.5 M HCl: 3-sequential 4-min, 4 min only, and 10 min incubations [55]. Samples were desalted on SDB-RPS StageTips before analysis on nLC-MS/MS (using Q Exactive Plus Mass spectrometer) system. From the point of view of sex estimation, AMELX, and AMELY peptides authors recommend 10-min etching. The method was validated on a set of 23 archaeological teeth in comparison to two different methods of sex estimation: morphological and archaeological (based burial rite) methods. All three methods agreed except in one case (when the archaeological method was opposite to morphological and protein methods) [55]. Besides morphological methods, sex determination by this method was also described for sex estimation of prepubertal individuals from Roman Italy (1^{st}–4^{th} c. CE) and Late Roman Gaul (4^{th}–5^{th} c. CE) era [70].

Osteological, proteomic, and isotopic analyses were used to evaluate a 9000-year-old human burial from the Andean highland site. These analyses indicate that this early hunter was a young adult female. This challenge the man-the-hunter hypothesis [71]. For proteomic analysis, a small piece of enamel (20 mg) was cut from teeth, powdered, and demineralized by 1.2 M HCl. After reduction and alkylation, samples were treated with trypsin. The next step was extraction on SepPak C18. Peptides were analyzed by nLC-MS/MS (Thermo Scientific Q-Extractive Plus Orbitrap mass spectrometer). Multiple peptide detection was used for AMELX (and AMELY) identification [71].

Two fast methods (1-3 min) were also developed for sex estimation without the separation step [72]. Both methods are based on the FIA (flow injection analysis) using high-resolution mass spectrometry (Q Exactive Orbitrap MS from Thermo Scientific) or tandem MS (Xevo TQ-S from Waters, Milford). Analyzed peptides were again SM(ox)IRPPY and SIRPPYPSY when specific transition ions were selected. Sample preparation is the same as above. The advantages of these methods are rapid analysis (three, respectively 1 min per sample) and the possibility to use low-resolution mass spectrometers (MS/MS), i.e. relatively low-cost instruments, for sex estimation.

There are also described other amelogenin peptides as diagnostic peptides using a nanoLC system coupled to a Q Exactive orbitrap mass spectrometer [73, 74]. The sample preparation was, in principle, the same as in the method by Stewart et al. [65], but the AMELY peptide was SM(ox)IRPPYS (*m/z* 486.7393), and AMELX peptide was YEVLTPLKWYQSIRPPYP (*m/z* 750.7368) (Figure 4). This method was successfully applied for the estimation of the sex of a child (5–6-year-old boy) murdered in the Early Bronze Age from Schleinbach, Austria (c. 1950–1850 BCE) [73] as well as for successful sex classification of 70 (from 75) children under 12 years at death buried at the Early Bronze Age cemeteries in Franzhausen I, Austria (c. 2050–1680 BCE). The coincidence between archaeological and peptide-based results was very high

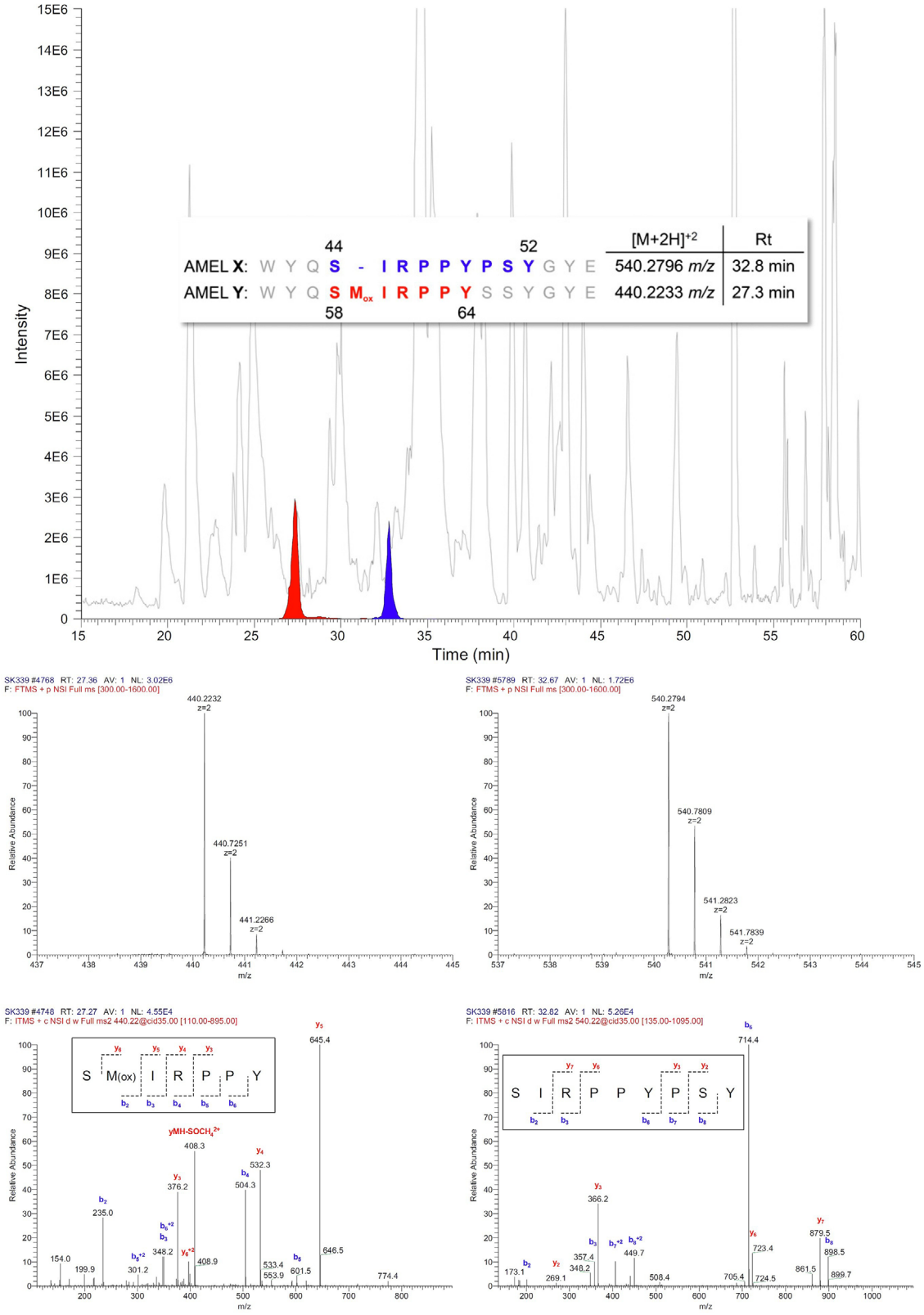

FIGURE 3 A base peak chromatogram (300–1600 *m/z*) with two marked peptides of amelogenin: AMELY-[58–64] and AMELX-[44–52]. The reconstructed ion chromatograms (to 4 ppm) for each are shown in red and blue; full-scan MS and corresponding MS/MS are shown below. Reprinted [65] with permission. Copyright 2017 National Academy of Sciences.

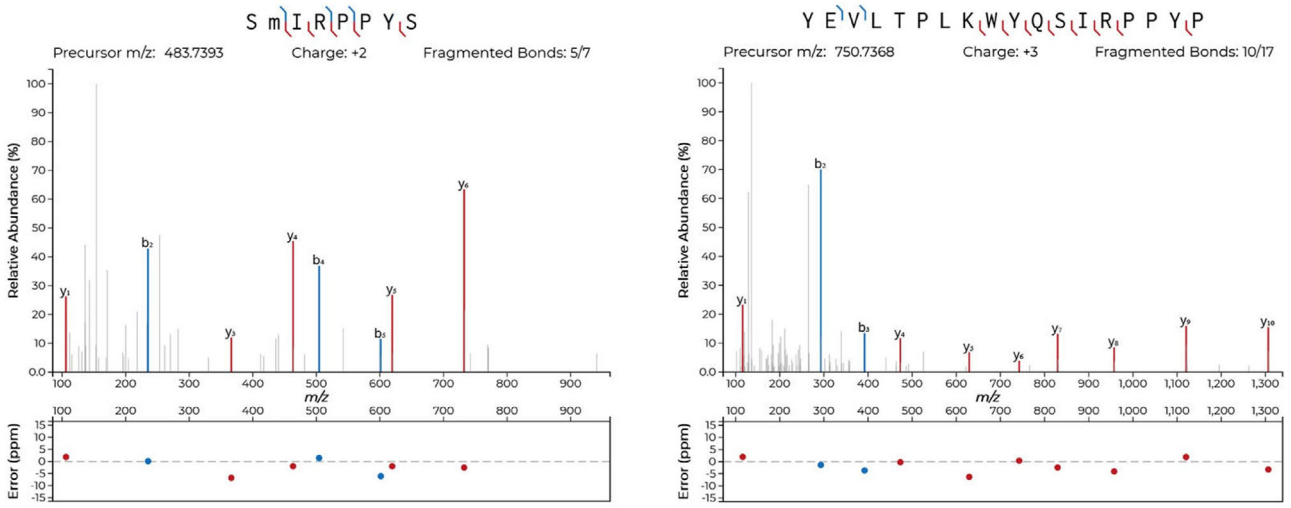

FIGURE 4 Left: MS/MS fragment spectrum of peptide SM(ox)IRPPYS (*m/z* 486.7393) (AMELY) and corresponding mass errors in ppm; Right: MS/MS fragment spectrum of peptide YEVLTPLKWYQSIRPPYP (*m/z* 750.7368).(AMELX) and corresponding mass errors in ppm. Reprinted [73] with permission. Copyright 2020 Springer Nature.

(62 of 63 individuals, 98.4%) when one was of the female sex, based on body position and orientation [74].

Another approach for analysis of archaeological samples was described for Iron Age individuals (ca. 2000–1000 years B.P.) from the tropical environment (northwest Thailand) [75]. It was possible to identify 212 proteins. Analyses were done at nLC-MS/MS system (OrbitrapVelos, Thermo Electron, Bremen, Germany) using the multiple reaction monitoring methods were used for the identification of two AMELX peptides (TALVLTPLK and WYQSIRPPYPSY(G)), and one AMELY peptide (IALVLTPLK). Regarding sample preparation, the tooth enamel/dentin was crushed, treated with 0.5 M HCl, reduced, alkylated, and cleaved by trypsin.

Another protein/peptide extraction was used by Froment et al. [76] to analyze 5000-year-old human teeth. In principle, they powdered whole teeth demineralized in EDTA, and proteins were denatured, lysed, washed, and alkylated. Finally, proteins were treated with trypsin. For analysis of peptides, nanoLC coupled to Orbitrap Fusion MS (Thermo Scientific) was used when using the targeted MS approach and parallel reaction monitoring (PRM). They demonstrated that the PRM method maximizes the sensitivity and reproducibility of "sex" peptides. The selected "sex" peptides were TALVLTPLK (*m/z* 478.3130) for AMELX and IALVLTPLK (*m/z* 474.3325) for AMELY (Figure 5).

A similar method for sample preparation, that is, demineralized (by 1.2 M HCl) milled teeth were alkylated and treated by trypsin, was used to analyze teeth aged up to 7300 years [77]. ZipTip C18 tips cleaned samples/peptides. These peptides were analyzed by nLC-MS/MS using Q Exactive Plus Orbitrap MS (Thermo Scientific). The authors used bioinformatic methods for the identification of AMELX and AMELY proteins. To elute false negative samples, that is, samples with low male signal, authors used a probability curve of female sex as a function of the logarithm of AMELX using logistic regression.

False female assignments were discussed in this context [77–79]. It is some probability that the method using AMELX and AMELY could produce inaccurate results due to the presence of low frequency of AMELY deletion variants in some populations [78]. However, after analysis of many genomic projects, it was concluded that the probability of false sex estimation is low, and AMELY deletion should not affect the routine estimation of the biomolecular sex [79].

The oldest dental proteome was probably studied in the Early and Middle Pleistocene hominin (*Homo antecessor*) and *Homo erectus* tooth [80]. For protein/peptide extraction, authors used three methods: 1) demineralization by HCl without alkylation and enzymatic digestion, 2) pellet after demineralization was reduced, alkylated, and digested by LysC and trypsin, and 3) demineralization by TFA without alkylation and enzymatic digestion. The first and third extraction gave more extensive peptide recovery than the second one. Peptides were analyzed by nLC-MS/MS using Q-Exactive HF or HF-X mass spectrometer (Thermo Fisher Scientific). It was described that an average peptide length decreased with the age of the enamel sample. Enamel-specific proteins were identified as enamelin, ameloblastin, MMP20, and amelogenins (both AMELX and AMELY). At the teeth of *Homo antecessor*, AMELY-specific peptide sequences (such as SM(ox)IRPPY) were found, and so it was concluded that he was male [80]. We must mention that *Homo antecessor* is an extinct archaic human species recorded in Spain that

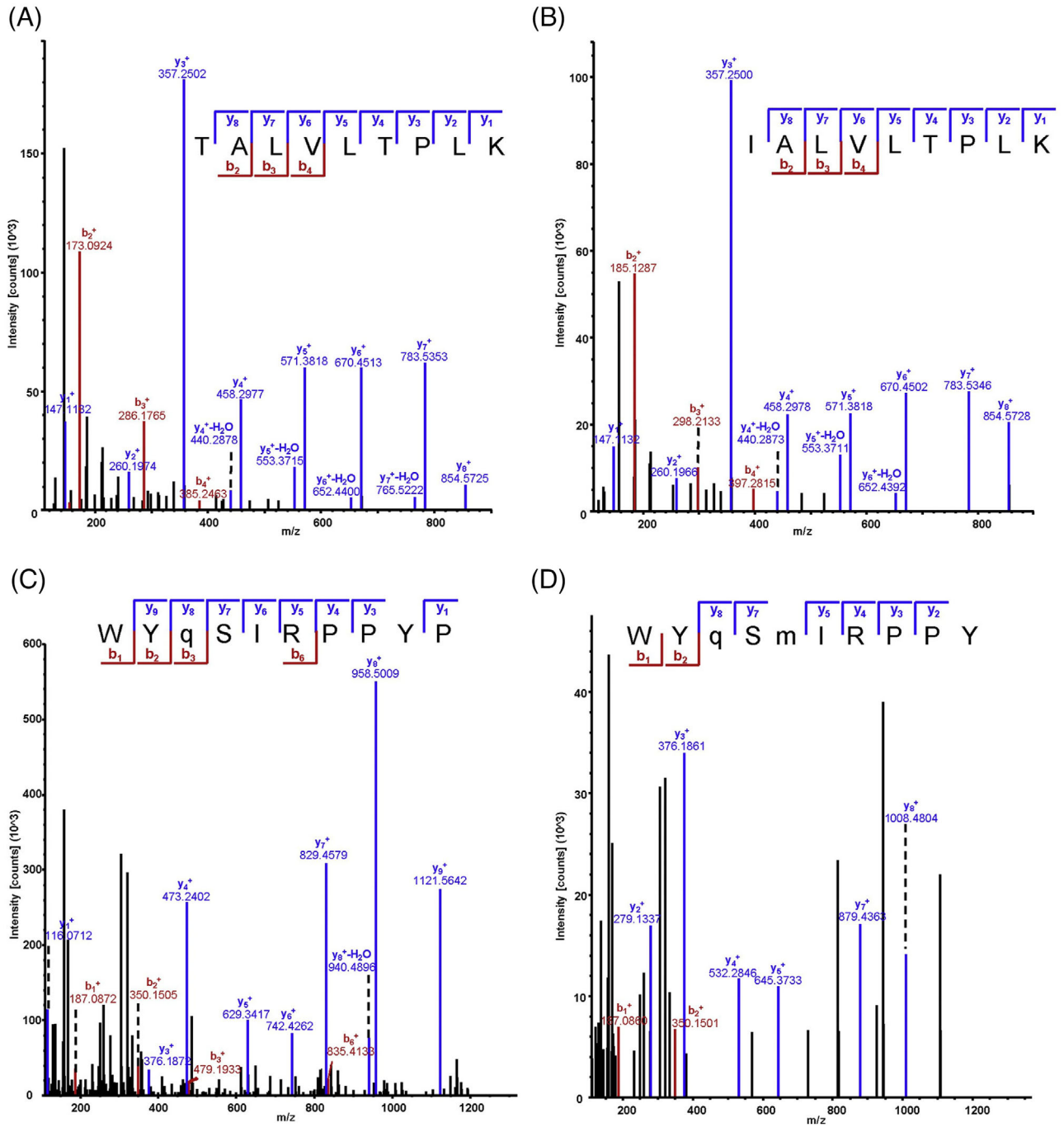

FIGURE 5 MS/MS spectra of specific peptides: (A) AMELX peptide, TALVLTPLK (precursor *m/z* 478.3130). (B) AMELY peptide, IALVLTPLK (precursor *m/z* 484.3325). (C) AMELX peptide, WYqSIRPPYP (precursor *m/z* 654.3259), and (D) AMELY peptide, WYqSmIRPPY (precursor *m/z* 679.3165). The series of y- and b-ions are highlighted in blue and red, respectively. q: deamidated glutamine residue; m: oxidized methionine residue. Reprinted [76] with permission. Copyright 2019 Elsevier B.V.

lived between 1.2 and 0.8 million years ago during the early Pleistocene.

In the end, we have to mention that acid etching of teeth is, mainly in comparison to genetic methods, a limited destructive method, but still is (limited) destructive and not quantitative (the signal cannot be normalized).

5 | CONCLUDING REMARKS

Sexual diagnosis is often crucial to archaeological, anthropological, and forensic research. Proteomic methods are relatively young, rapidly growing, and have applications in many scientific areas. Nowadays, there are used three

methods of sex estimation: osteology, genetics, and proteomics. In the sex estimation method, use protein amelogenin in X- and Y-form (AMELX and AMELY). It was established as a method that overcomes osteological and genomic methods as a more precious, sensitive, and relatively simple and rapid method. The crucial advantage is that this method uses teeth enamel. The enamel is one of the most calcified tissues in the mammalian organism. For this reason, it is relatively resistant to degradation caused by long-term effects, aggressive decomposition phenomena in the soil, and external physical and chemical damage. It was proven that proteins (peptides) in the enamel could be protected for tens or hundreds of thousands of years.

So it can be concluded that the proteomic method for sex estimation using isoforms of amelogenin be successfully used in archaeological, anthropological, and forensic research and overcome other previously used methods.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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