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Advances in Australasian Archaeometry

Proteomic Sex Estimation From Embedded Tooth Enamel

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ABSTRACT

Teeth hold insights into childhood diet, health and demography—including chromosomal sex. Proteomic analysis can detect peptide biomarkers from amelogenin genes encoded on X and Y chromosomes, allowing sex estimation. Embedding resins, necessary for thin sectioning teeth, can damage analytical systems, preventing proteomic analysis. We present a micro-dissection and chemical cleanup protocol for polymethylmethacrylate embedded teeth. Across iterative preparative and analytical phases, analyses of 15 enamel samples from eight individuals detected sex-specific amelogenin peptides. Of these, 13 samples matched known or previously estimated sexes. By refining embedded enamel preparation, sex can now be identified from teeth designated for semi-destructive techniques.

1 | Introduction

Estimation of sex from skeletal remains is an integral aspect of reconstructing identity and demography in archaeological and forensic investigations (Geller 2008; Gowland and Thompson 2013). Biological sex may play an important role in individual's health outcomes, morbidity and mortality (DeWitte 2010). Physiologically, sex is associated with differential innate and acquired immunity (Fish 2008; Klein and Flanagan 2016), risk of degenerative disease (Liu et al. 2003), costs of reproduction (Crump et al. 2024; Penn and Smith 2007) and even lifespan (DeWitte 2010; Zarulli et al. 2018). Despite sex being a biological variable, it has a complex interplay with individual and context-specific sociocultural norms manifesting in sex and gender-based differences, including migration patterns, community roles and occupation (Buonasera et al. 2025; Eerkens and Bartelink 2013; Gasparini et al. 2022; Zuckerman and Crandall 2019). Particularly in hegemonic cultures, sex (and by proxy gender) becomes a social determinant of health, with inequities shaping differential access to food and care (Ham 2025; Sen and Östlin 2009; Zuckerman and Crandall 2019). As children and infants are vulnerable to health insults (Silveira et al. 2007), identifying biological sex may provide a useful interpretive lens for investigating social inequities (Bethard and VanSickle 2020; Lewis 2018). The ability to estimate the sex of infants also facilitates nuanced insights into how differential maternal investment through breastfeeding may serve as a buffer for stressors (i.e., nutritional stress, disease) in prehistoric populations (e.g., Buonasera et al. 2022, 2020; Eerkens et al. 2022; Greenwald et al. 2023).

Traditional sex estimation methods for skeletal human remains use established anthropological and forensic standards that grade sexually dimorphic characteristics primarily using the pelvis and skull (Buikstra and Ubelaker 1994). This has well-documented limitations, particularly in archaeological populations where bone preservation is poor, or in cases where skeletal elements are comingled. Limitations include sexual dimorphism

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across different populations (size and robusticity), fragmentary or incomplete remains (i.e., only teeth) and juvenile mortality—as sexually dimorphic traits do not develop before puberty (Bethard and VanSickle 2020; Lamer et al. 2021; Stull et al. 2020). Until the recent development of proteomic approaches to estimate chromosomal sex (Parker et al. 2019; Stewart et al. 2017), difficulties in estimating sex for non-adults has ultimately hampered the investigation of possible sex-related differences in diet, care and exposure to stressors for children in the past (Adair et al. 2025; Buonasera et al. 2022; Gowland et al. 2021).

Proteomic sex estimation, like many DNA methods (Faerman et al. 1995; Hummel and Herrmann 1991; Inskip et al. 2019; Mannucci et al. 1994; Stone et al. 1996; Yokoi et al. 1989), relies on amelogenin—a protein involved in enamel formation that has two sexually dimorphic peptide isoforms coded on the X and Y chromosomes (AMEL-X and AMEL-Y respectively) (Parker et al. 2019; Snead et al. 1989; Stewart et al. 2016, 2017). Proteomic methods use shotgun mass spectrometry to assign sex based on the absence or presence of peptides originating from the AMEL-Y isoform (i.e., male = AMEL-X and AMEL-Y, female = AMEL-X only). There are two primary protocols for proteomic sex estimation: an enamel hydrochloric acid-etching method (Stewart et al. 2016, 2017) and a method using cut and milled enamel (Parker et al. 2019).

The Stewart et al. (2016) procedure extracts amelogenin peptides from surface enamel that is free from glues or embedding media and assigns sex using single biomarker peptides specific to AMEL-X and AMEL-Y respectively. This is described as a 'minimally destructive method,' yet micro-CT scans reveal enamel loss of 10% and dentine loss of 2% following acid-etching and scanning electron microscopy shows increased microporosity and deepened cracks and grooves (Brůžek et al. 2024). This method has been applied to archaeological remains (Gasparini et al. 2022; Rebay-Salisbury et al. 2022), including mineralising teeth (Adair et al. 2025; Gowland et al. 2021). It has been adapted to provide rapid analyses on both nano liquid chromatography–tandem mass spectrometry (nLC-MS/MS) and liquid atmospheric pressure matrix-assisted laser desorption/ionisation mass spectrometry LAP-MALDI MS systems (Adair et al. 2025; Casas-Ferreira et al. 2022; Cleland et al. 2024).

The Parker et al. (2019) method uses dissected ~20 mg enamel fragments subject to milling in acid and full solubilisation, followed by bioinformatics to identify and quantify all unambiguous AMEL-X and AMEL-Y peptide signals. Sex is estimated using a probabilistic function since samples containing AMEL-X alone may be either low-signal male samples or female sex. Unlike the acid-etch method, this approach has additional chemical steps including trypsin digestion and bioinformatic processing which increases proteomic signals and broadens detection and quantification of additional proteins, including immune proteins (Buonasera et al. 2024, 2025; Parker et al. 2019). It is a robust analytical technique in degraded contexts compared to osteological and genomic methods and has been successfully applied to archaeological teeth (Buonasera et al. 2022, 2024; Haas et al. 2020; Mays et al. 2023).

Teeth are common in the archaeological record due to their dominant mineral component (Forshaw 2014; Hillson 2014; Hollund et al. 2015). In ancient dental samples, there is a risk of chemical or physical change after deposition (diagenesis) (Hollund et al. 2015; Kendall et al. 2018). Dental tissues may become cracked, fragile, or chalk-like, rendering them vulnerable to fracture during cutting (Harmston et al. 2019). Many routinely-employed bioarchaeological techniques, such as dental histology, isotopic characterisation and trace element analysis are reliant on sectioned teeth (Esposito et al. 2024; Smith 2020). To reduce fracture risk, teeth are commonly embedded in slow-curing epoxy resins or the acrylic polymer polymethylmethacrylate (PMMA), physically stabilising tissues prior to sectioning (Bauer and Mahovlic 2003; Esposito et al. 2024; Hillson 2014; Smith 2020). In line with ethical considerations in archaeological specimen preservation (Squires et al. 2019, 2022; Stantis et al. 2025) and optimisation of protocols to minimise destructive analyses (Esposito et al. 2024), using previously embedded and sectioned teeth for sex estimation should be best practice. Unfortunately, proteomic analysis of embedded dental tissues presents a challenge for both the Parker et al. (2019) method and Stewart et al. (2017) protocol. Polymers and resins are highly viscous, interfere with reverse-phased chromatography and damage sensitive mass spectrometry instruments. Consequently, thousands of embedded teeth in museums and research collections worldwide have necessarily been excluded from proteomic sex estimation, limiting sex-based bioarchaeological interpretations for these individuals.

Embedded teeth pose two further challenges; physically sampling enamel encased in PMMA and removal of any PMMA that may have permeated enamel samples. Regions of enamel in contact with surrounding PMMA can be avoided using micromilling (Curtis et al. 2022; Sudron et al., forthcoming), facilitating targeted sampling. However, micro-dissected enamel may still contain PMMA contaminants, which must be removed prior to analysis. Here, we develop and test an enamel micro-dissection and chemical cleanup protocol for proteomic analysis. We assess whether organic solvents can sufficiently remove PMMA from previously embedded enamel without reducing proteomic signal. As PMMA is an organic polymer, it is soluble in ethyl acetate baths (Evchuk et al. 2005), however to our knowledge this approach has not been applied to embedded dental tissues. We test the effectiveness of pre-treatment protocols by comparing sex estimations to known sex for five living individuals and three archaeological individuals whose proteomic sex was previously estimated from unembedded teeth. Establishing the feasibility of an enamel micromill and chemical pre-treatment protocol will allow examination of proteomic sex and immunological information for numerous embedded teeth available in preexisting collections.

2 | Materials and Methods

2.1 | Dental Samples

Fifteen samples of 11 teeth from five modern and three archaeological individuals were analysed across three method development phases (Table 1). Clinically extracted, modern donated teeth were from individuals self-identified as genetically male or female. Ethics approval was not required from Griffith University because the university considers inclusion of

TABLE 1 | Known or previously estimated sex for each individual, experimental conditions and proteomic sex estimated in this study.

Phase one: modern samples							
Individual	**Tooth ID**	**Sample ID**	**Weight (mg)**	**Known sex**	**Embedded**	**Treatment**	**Proteomic sex**
Modern 1	SE1	SE1	11.9	Female	Yes	Chloroform/ methanol	Female
	SE3	SE3	11.9	Female	No	None	Male
		SE3u	34.5	Female	No	None	Female
		SE3t	36.5	Female	No	Ethyl acetate	Female
Modern 2	SE2	SE2	13.4	Male	No	None	Male
Modern 3	SE4	SE4	10.7	Male	No	None	Male
Phase two: modern samples							
Individual	**Tooth ID**	**Sample ID**	**Weight (mg)**	**Known sex**	**Embedded**	**Treatment**	**Proteomic sex**
Modern 4	T1	T1	20.3	Female	Yes	Ethyl acetate	Female
Modern 5	MS5	MS5	20.1	Male	Yes	Ethyl acetate	Male
	MS6	MS6	16.9	Male	No	Ethyl acetate	Male
Phase three: archaeological samples							
Individual	**Tooth ID**	**Sample ID**	**Weight (mg)**	**Previous sex ID[a]**	**Embedded**	**Treatment**	**Proteomic sex**
Burial 2	SMA71H_B2	SMA71H_B2	21.5	Male	Yes	Ethyl acetate	Male
Burial 11	SMA71H_ B11_LLM1	SMA71H_B11	21.9	Male	Yes	Ethyl acetate	Female
		SMA71H_B11t	19.8	Male	Yes	Ethyl acetate	Male
	SMA71H_ B11_RLM1	SMA71H_B11u	21.3	Male	No	None	Male
Burial 14	SMA71H_B14	SMA71H_B14a	12.5	Female	Yes	Ethyl acetate	Female
		SMA71H_B14b	15.0	Female	Yes	Ethyl acetate	Female

aProteomic sex estimation undertaken previously using unembedded teeth from the same individuals (Buonasera et al. 2024, 2025).

deidentified dental samples provided with full knowledge and consent to be outside of the scope of their ethics approval regulations. Archaeological enamel was sampled from three individuals from the *Asistencia San Pedro y San Pablo* coastal Mission Period Ohlone burial site, located in the San Pedro Valley, California, USA (SMA-71/H, 1787–1800 BP), where sex had previously been estimated proteomically (Buonasera et al. 2024, 2025; Eerkens et al. 2025). Permission to conduct this study was obtained from the state-assigned most likely descendant (CZ), the Amah Mutsun Tribal Band of Mission San Juan Bautista. Preparation and proteomic analysis of samples SE1, SE2, SE3, SE4, T1, MS5, MS6, SMA71H_B2, SMA71H_B11, SMA71H_B14a and SMA71H_B14a followed the protocols described here, with methods for SE3u, SE3t, SMA71H_B11u and SMA71H_B11t described in the Supporting Information.

Six teeth were soaked in methylmethacrylate monomer for several days at room temperature prior to embedding in PMMA at 48°C following established protocols (Smith 2020). As the teeth had been embedded and longitudinally sectioned to produce histological thin sections for ongoing and prior studies (e.g., Smith et al. 2024), mesial and distal tooth halves were available for enamel micromilling in the current study. Where necessary 2 mm thick longitudinal slices were taken to ensure the collection of sufficient enamel mass. For Burial 14, a marked developmental defect was visible in the enamel and dentine, underpinning a large demineralised occlusal pit (Figure 1). To obtain enough enamel from before and after the defect, enamel was sampled across three thick slices with a buffer region between the two sampling locations (Figure 2). Four unembedded intact teeth were longitudinally sectioned using an Allied Techcut four low-speed saw and 4-in. diamond-embedded blade, exposing internal enamel for sampling analogous to the embedded teeth. Bulk samples SMA71H_B11u and SMA71H_B11t were extracted at the University of Fairbank as described in the Supporting Information.

For all micromilled teeth, each longitudinal slice or tooth block was affixed with wax to a microscope slide, then secured to the Geomill326 (Izumo Web Ltd.) stage with tape to ensure sample stability for milling. Each tooth surface was spatially calibrated using Geomill326 software on a dedicated PC, coupled with a CMOS camera and Motic Microscope software to ensure sampling path accuracy (illustrated in Sudron et al., forthcoming: Figure 2). The tooth surface and drill bit were cleaned with ethanol following calibration. Images were taken of the region

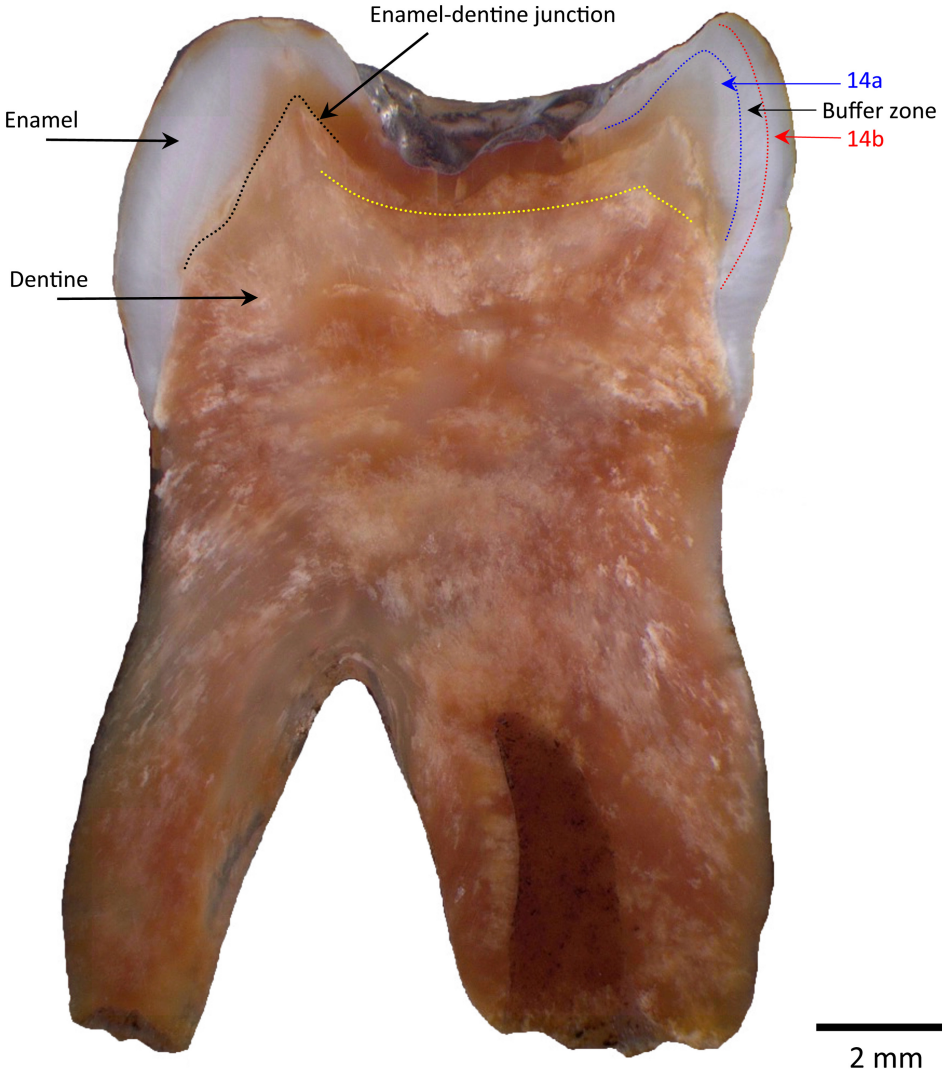


FIGURE 1 | Overview of embedded mesial half of the lower left first molar of Burial 14 prior to sampling. A developmental defect is shown in the dentine with a yellow dotted line. The enamel sampled pre-defect (SMA71H_14a) and post-defect (SMA71H_14b) is labelled in blue and red respectively, with the buffer zone between the two regions also indicated.

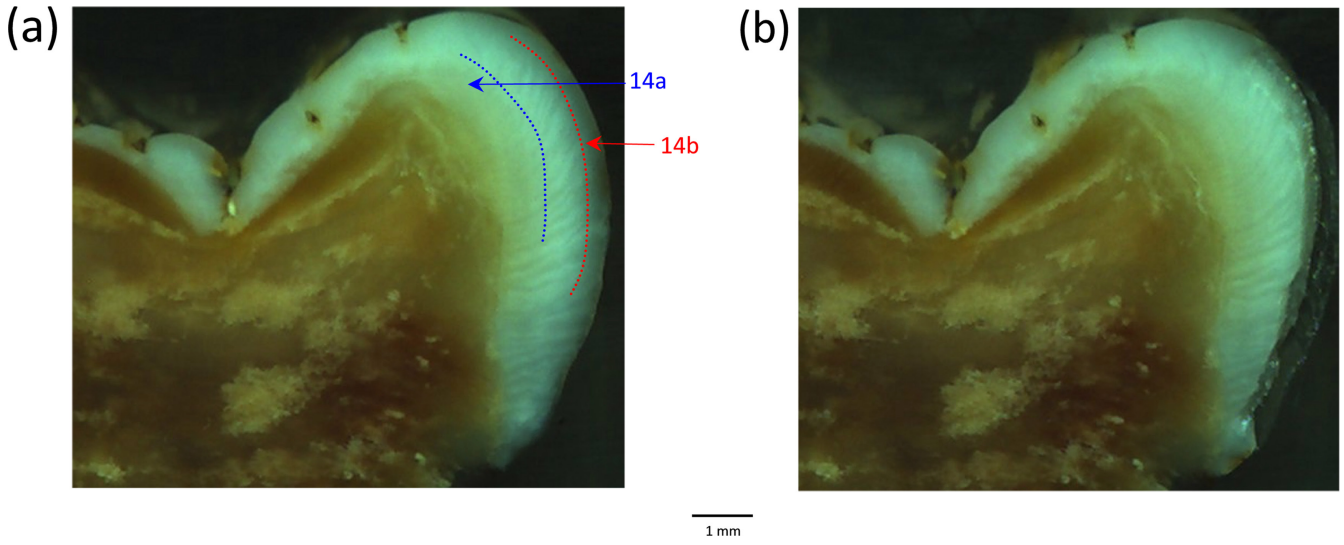


FIGURE 2 | Overview of embedded distal half of the lower left first molar of Burial 14 prior to enamel micro-dissection (a) and the same face after micromilling, (b) the post-defect region (SMA71H_14b).

targeted prior to sampling. Micromilling paths were manually programmed using live images of the tooth surface via the CMOS camera. Carious and diagenetically altered regions of enamel were avoided. Enamel was drilled at a maximum depth of 750 μm to ensure underlying dentine was not accidentally sampled. The tooth surface was then ground down and spatially recalibrated prior to continued sampling. Powdered enamel was removed at regular intervals using flat spatulas and transferred to a pre-weighed 2 mL screw-cap glass vial. Between individuals and between sub-samples for Burial 14, the tooth surface, drill bit and sampling tools were cleaned via ultrasonication and an ethanol rinse. Drilling was conducted at a low speed to minimise heat that could contribute to protein degradation (Bonicelli et al. 2022).

2.2 | Chemical Cleanup

Following enamel micro-dissection, enamel powder was subject to chemical pre-treatments to remove PMMA. Chemical treatment was conducted in two iterations to ensure feasibility. The first was tested in phase one, where 200 μL 50:50 chloroform/methanol was added to cover powdered enamel from the only embedded sample (SE1) in this phase. Chloroform/methanol solutions have been used to slowly extract dental remains from PMMA (Smith 2020), without consideration of impact on enamel peptides. After 1 h the supernatant was removed, with any remaining solvent evaporated overnight. Phase one testing also allowed established proteomic methods (Buonasera et al. 2020; Parker et al. 2019) to be piloted, ensuring they were reliable prior to their application to archaeological enamel.

A secondary, more stringent cleaning protocol was applied in phases two and three (Figure 3). Samples were cleaned by adding 200 μL of ethyl acetate prior to incubation (40°C, 1200 rpm, 60 min) on an Eppendorf Thermomixer 5350. Supernatant was removed into an aluminium weighboat and assessed for PMMA residue visually (residue appeared as an opaque film). Decanted ethyl acetate was replaced with fresh solvent, then incubated for a further hour. Incubation, residue assessment and replacement steps were repeated a further two times after PMMA residue was no longer visually detected. Remaining solvent was evaporated overnight. To ensure ethyl acetate treatment did not degrade enamel proteins, two unembedded pieces of enamel from individual Modern 1 were isolated, one was pre-treated with ethyl acetate (SE3t) as described below and one was not subject to pre-treatment (SE3u) (Supporting Information). Further comparative proteomic testing was undertaken on two first molars from an archaeological individual (Burial 11), one of which was unembedded and untreated (SMA71H_Burial 11_RLM1) and the other was embedded in PMMA and treated with the ethyl acetate protocol (SMA71H_Burial 11_LLM1; Supporting Information).

2.3 | Enamel Protein Extraction, Digestion and Cleanup

Following chemical treatment, enamel samples were transferred to 1.5 mL Eppendorf LoBind microcentrifuge tubes for protein extraction using established protocols (Parker et al. 2019; Buonasera et al. 2024). In general, the following protocol was used by our three different laboratories with slight variations (Supporting Information). To demineralise enamel, 200 μL of 1.2 M HCl was added to each sample. To reduce soluble proteins, samples were incubated and agitated (56°C, 1200 rpm, 60 min) on the Eppendorf Thermomixer 5350. After incubation, samples were spun down to consolidate liquid for 30 s using a desktop centrifuge. To adjust sample pH, 90 μL 2 M ammonium bicarbonate was initially added, then added in 10 μL increments as needed, aliquoting 2 μL of solution onto pH indicator paper until sample pH was between 7.5 and 8. To further solubilise proteins, 12 μL of 0.25% (w/v) protease max solution was added to each sample, followed by the addition of Trypsin (1 μL of 0.5 μg/μL, Thermo Pierce Inc.) and incubation in the dark at 37°C for 16–22 h. Samples were then centrifuged (15 min, 16,300*g*, 22°C) and the supernatant containing peptides (~200 μL) was transferred to 0.22 μm PTFE (or PDVF, Millipore MC-6V) centrifugal filter tubes. Where solution volume was more than ~700 μL, aliquots of the supernatant were filtered in batches. Samples

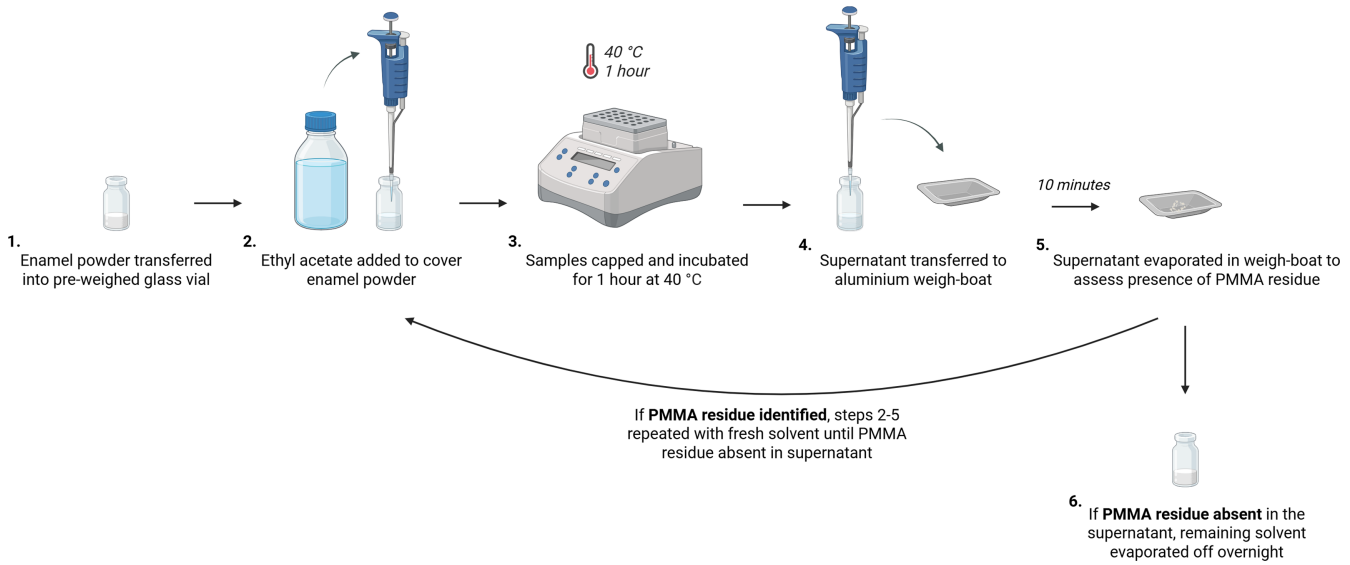


FIGURE 3 | Workflow illustrating ethyl acetate cleanup protocol. *Source:* Created in BioRender (https://BioRender.com).

were centrifuged in centrifugal filtration tubes (15 min, 16,300*g*, 22°C), with filtrates from each sample transferred to new 1.5 mL Eppendorf LoBind microcentrifuge tubes for sample cleanup. The filtrate was acidified by adding 10 μL of 10% trifluoracetic acid (TFA) until pH was adjusted to be between 1 and 2. Samples were vortexed for 5 s after adding TFA, before testing pH by aliquoting 2 μL onto a pH indicator paper. Samples were then spun on a desktop centrifuge for 30 s to separate the insoluble residue.

Peptide samples were cleaned and concentrated using C18 pipette tips (Pierce C18 pipette tips, 100 μL, Thermo Scientific). A fresh C18 tip was used for each sample. The reverse-phase column in the C18 tip was wetted by pipetting 50 μL of 50% acetonitrile (ACN) then discarding the solution. This was repeated three more times. The C18 tip was then equilibrated by pipetting 50 μL of 0.1% (v/v) TFA-ACN solution, discarding the solution and repeating three times. Samples were loaded and bound to the C18 tip by drawing 200 μL up and dispensing it back into the tube 20 times. The reverse-phase column was then washed by pipetting 50 μL of 0.1% (v/v) TFA-ACN solution from the washing tube and discarding. This step was repeated until no wash buffer remained (~10 times). Finally, the peptides were eluted by pipetting an elution buffer of 60% ACN, with 0.1% (v/v) TFA up and down in the elution tube 15 times, before expelling into the elution tube (1.5 mL Eppendorf LoBind microcentrifuge tube) for analysis. Samples were stored in a −20°C freezer until they were dried down prior to submission using a ThermoFisher SpeedVac for ~45 min.

2.4 | Proteomic Data Acquisition

Dried peptide samples were resuspended in 20 μL of 0.5% TFA at the Brisbane Translational Research Institute (TRI) or as described in the Supporting Information. Peptide concentration determination was attempted using microBCA, with no reading above background levels. As such, a 1:1:1:1 pooled sample mix of SE1–4 (2.5 μL of each sample, with 2.5 μL of 0.5% TFA to increase sample volume to enable multiple rounds of testing) was prepared to test detection using our MS method. A small fraction (1.2 μL) of this pooled sample was injected to test MS method length and evaluate content. Samples were applied to nLC-MS/MS on a Thermo Scientific Q Exactive Plus Orbitrap mass spectrometer in conjunction with an UltiMate 3000 HPLC nano-flow HPLC. The HPLC setup used a C-18 trap column and a 50 cm EasySpray C-18 analytical column (Thermo Fisher, catalogue 160454 and ES803A). Mobile phases were A: 0.1% formic acid and B: 80% acetonitrile with 0.1% formic acid. The mass spectrometer was run using an EasySpray source in positive ion mode and for this initial testing we used settings typical for detection of tryptic peptides by TopN DDA. Following MS method testing, 5 μL of each individual sample was run on a 100 min gradient. Samples were loaded in 3% B and eluted over a gradient from 3%–30% B over 64 min, followed by 30%–50% B over 10 min, with final washing and re-equilibration steps, all at 250 nL/min.

A MS survey scan was performed for a range of 300–1600 m/z at 70 k resolution. The MS/MS spectra were acquired at 17,500 k resolution using an inclusion list of 28 ions as described in Parker et al. (2019: Table A.1). The MS/MS was performed on 15 additional top ions acquired in the MS survey scan, if inclusion list ions were not found. Spectra were retained for all ions. For precursor ion selection an isolation mass window of 1.6 m/z was used, with an AGC target of 5e4, a minimum AGC target of 5e2 and a maximum inject time of 50 microseconds. Single charged ions and ions with greater than 4 + charge were excluded. The NCE was 27% and MS2 data was centroided. Peptide preferred match was set to 'blank'. The dynamic exclusion time used was 5 s. Sample-to-sample contamination was tested using a blank run of bovine serum albumin (BSA) standards (Table S3). Routinely 100 fmole BSA were run for quality control, which was at quantification level. For detection, not quantification, minimum amount run was as low as 1 fmole.

Mass spectrometry datasets were processed using PEAKS (version 10.6, build 20201221) (Zhang et al. 2012) peptide matching software (Bioinformatics Solutions Inc., Waterloo, ON) as described in Parker et al. (2019) and refined in Buonasera et al. (2020, 2024). AMEL-X and AMEL-Y peptides were quantified using the ion intensity of the primary precursor mass over charge ratio (m/z). Peptides composed of non-specific amelogenin amino acid sequences were filtered from results, with further probabilistic analyses detailed in Supporting Information.

3 | Results

Qualitative and quantitative proteomic sex estimates for all samples are listed in Tables 1 and 2, respectively. Of the six samples from phase one, five sex estimations matched known sex. Embedded enamel from individual Modern 1 (SE1) was estimated as female, matching known sex, with unembedded powdered enamel from the same individual (SE3) estimated as male. Further analysis of unembedded enamel from Modern 1 returned correct sex estimations for both untreated (SE3u) and treated (SE3t) enamel pieces. Proteomic sex estimations in phase two matched known sex for the modern individuals. Of the embedded teeth from modern individuals treated with ethyl acetate, proteomic sex of individual Modern 4 was estimated as female and Modern 5 (MS5) was estimated to be male (Table 2). Unembedded enamel from individual Modern 5 (MS6) was also estimated as male.

For the archaeological individuals included in phase three, sex estimations for Burial 2 and Burial 14 matched prior results from different unembedded teeth of these individuals, whereas an initial assessment of Burial 11 did not return the same sex estimate as Buonasera et al. (2024). Further post hoc analysis of unembedded (SMA71H_B11u) and embedded enamel (SMA71H_B11t) from Burial 11 was unambiguously estimated as male.

4 | Discussion

The present study aimed to test if sufficient proteomic signal could be detected in embedded enamel samples subjected to a cleanup protocol. Preliminary testing in phase one using enamel powder from predominately unembedded teeth was undertaken for the first time at the Brisbane Translational Research Institute (TRI). For individual Modern 1, sex

TABLE 2 | Proteomic sex estimation for modern individuals and archaeological individuals from the *Asistencia San Pedro y San Pablo*.

Phase one: modern samples									
Individual	**Tooth ID**	**Sample ID**	**Portion on column**	**AMEL-X (IC)**	**AMEL-Y (IC)**	**Pr(F)**	***n* pep X**	***n* pep Y**	**Sex**
Modern 1	SE1	SE1	5 μL/10 μL	5.77E+08	0.00E+00	0.71	45	0	F
	SE3	SE3	5 μL/10 μL	8.31E+08	9.47E+07	0.00	28	12	M
		SE3u[a]	8 μL/23 μL	1.73E+09	0.00E+00	0.77	347	0	F
		SE3t[a]	8 μL/23 μL	1.98E+09	0.00E+00	0.78	323	0	F
Modern 2	SE2	SE2	5 μL/10 μL	7.93E+08	1.34E+08	0.00	38	11	M
Modern 3	SE4	SE4	5 μL/10 μL	1.11E+09	1.60E+08	0.00	51	25	M
Phase two: modern samples									
Individual	**Tooth ID**	**Sample ID**	**Portion on column**	**AMEL-X (IC)**	**AMEL-Y (IC)**	**Pr(F)**	***n* pep X**	***n* pep Y**	**Sex**
Modern 4	T1	T1	5 μL/10 μL	1.10E+09	0.00E+00	0.73	19	0	F
Modern 5	MS5	MS5	5 μL/10 μL	9.02E+08	1.07E+08	0.00	25	19	M
	MS6	MS6	5 μL/10 μL	1.84E+07	2.43E+07	0.00	2	3	M
Phase three: archaeological samples									
Individual	**Tooth ID**	**Sample ID**	**Portion on column**	**AMEL-X (IC)**	**AMEL-Y (IC)**	**Pr(F)**	***n* pep X**	***n* pep Y**	**Sex**
Burial 2	SMA71H_B2	SMA71H_B2	5 μL/10 μL	3.17E+09	2.87E+08	0.00	16	6	M
Burial 11	SMA71H_B11_LLM1	SMA71H_B11	5 μL/10 μL	2.35E+09	0.00E+00	0.83	8	0	F
		SMA71H_B11t[b]	8.0 μL/20 μL	3.91E+08	3.74E+07	0.00	163	56	M
	SMA71H_B11_RLM1	SMA71H_B11u[b]	4.8 μL/20 μL	2.11E+09	1.50E+08	0.00	92	36	M
Burial 14	SMA71H_B14	SMA71H_B14a	5 μL/10 μL	3.08E+10	0.00E+00	0.97	105	0	F
		SMA71H_B14b	5 μL/10 μL	8.35E+09	0.00E+00	0.92	24	0	F

Note: Proportion on column = (a μL/b μL) = amount applied to column (μL a) of total extracted peptide raised in (μL b) of analysis solution (0.1% TFA, 2% ACN in MilliQ water).
Abbreviations: IC = combined peak intensities from mass spectrometry; Pr(F) = probability of female sex estimation; n pep = number of peptides unique to given amelogenin isoforms.
[a]Preparation and mass spectrometry undertaken at the University of Davis as detailed in the Supporting Information.
[b]Preparation and mass spectrometry undertaken at the University of Fairbanks as detailed in the Supporting Information.

estimations from embedded enamel (SE1) matched the known sex of female, however unembedded enamel from the same individual (SE3) was estimated as male. This may be due to contamination from modern male samples SE2 or SE4 that were prepared and run at the same time. Phase one also demonstrated that the chloroform/methanol chemical cleanup protocol applied to sample SE1 was likely insufficient to fully remove PMMA—with the residual undissolved polymer at low enough levels to not damage instrumentation. Polyethylene glycol (PEG) contamination was also detected across samples SE1, SE2, SE3 and SE4. PEG is one of the most common contaminants for nano-LC MS/MS workflows (Weaver and Riley 2006), particularly in samples with low peptide content as in our study. The polymer can be introduced via plastic pipette tips, microfuge tubes and solvents stored in plastic bottles. Unfortunately PEG contamination impacts proteomic data, resulting in ion suppression that may impact detection capability, precision, quantification and ultimately reliability of results (Annesley 2003; Furey et al. 2013), which was further amplified by detected keratin contamination. This contamination issue was rectified in phases two and three, as more stringent methods to eliminate potential PEG contamination were taken (i.e., sample collection in glass vials rather than Eppendorf tubes, passage of aqueous solutions through Sep-Pak C18 cartridges) and the more intensive chemical cleanup protocol was applied using organic carriers of PMMA (Evchuk et al. 2005).

Ethyl acetate cleaning provided correct proteomic sex estimations for two modern unembedded teeth from two donors (Modern 1 and Modern 5) and two embedded modern samples from two donors (Modern 4 and Modern 5). Unembedded enamel MS6, estimated as male, also matched a prior estimate obtained using the acid-etching method for the same tooth (Sudron et al. 2024). In phase three, proteomic sexes matched previous analyses from two of the three archaeological individuals (Burial 2 and Burial

14) buried at the *Asistencia San Pedro y San Pablo* archaeological site (Buonasera et al. 2024, 2025). The number of AMEL-Y and AMEL-X specific peptides characterised from the three archaeological individuals was, in some cases, two orders of magnitude lower than other archaeological samples from this assemblage (Buonasera et al. 2024, 2025) and immunological peptides were not identified. Previous studies employed unembedded teeth for proteomic analyses; our inter-laboratory comparison of the same individuals reveals unexpectedly shallow coverage for our samples. Although there was sufficient proteomic signal to estimate sex, they were low confidence assessments.

This low proteomic signal effect may be attributed to samples being treated as powder rather than pieces of enamel, the small volume (5 μL) of sample run on the column, fixative effects from PMMA embedding where biomolecules are bound to the PMMA and consequently discarded, or the use of a less sensitive mass spectrometer than employed in the earlier studies. The low proteomic signal is unlikely due to the effect of the solvent from the cleaning protocol, as unembedded enamel pieces from the Modern 1 individual had comparable AMEL-X specific peptides in untreated enamel and enamel treated with ethyl acetate rinses. The powdered nature of the archaeological samples is a likely explanation. Considering the viscosity of PMMA, when removing supernatant throughout the cleanup protocol, enamel may have been incidentally carried in the discarded solvent, resulting in additional sample loss. Consequently, enamel volumes may have been sub-optimal for nano-LC MS/MS analysis, impacting the quality of proteomic data produced.

The limited proteomic signal may also be due to burial effects and/or differences in laboratory analysis. Burial 11 was found to have an abnormally low overall number of peptides, including amelogenin, in both the present study and a previous study (Buonasera et al. 2024). Despite having a very low proteomic signal, AMEL-Y peptides were detected in the previous study, allowing this individual to be unambiguously estimated as male. AMEL-Y peptides were not identified for Burial 11 in the first sample analysed in the present study (SMA71H_B11) and AMEL-X IC was sufficient to estimate female sex for this individual (although the number of peptides was unusually low). Yet further post hoc analysis of embedded and non-embedded enamel samples from this individual, run on different instrumentation, returned good proteomic signal, revealed abundant Y peptides and were estimated to be unambiguously male, matching Buonasera et al. 2024. This variability in sex estimation could relate to spatial variability in diagenesis, but the comparatively lower number of detected peptides in the one sample could also be attributed to instrumental acquisition parameters, where peptides with low intensities, such as Y-chromosome linked AMEL-Y peptides, may be below the quantification threshold of the TRI instrumentation.

Comparison of unembedded, untreated enamel (SMA71H_B11u) with embedded and treated enamel (SMA71H_B11t) from Burial 11 both analysed under the same conditions at the University of Alaska (Supporting Information) provides insight into the effect of solvent treatment on proteomic signals. The removal of PMMA and subsequent proteomic sex estimation was successful for these samples, as they returned unambiguously male proteomic sex estimations. As measured by combined intensities of specific AMELX and AMELY peptides, prepared unembedded enamel from Burial 11 (SMA71H_B11u) contained a higher concentration of peptides per gram of extracted sample than enamel from the same tooth after it had been embedded in PMMA and had the PMMA removed through ethyl acetate cleanup (Table S1). Although the combined intensity of specific AMELX and AMELY peptides was greater for the unembedded sample, the number of specific peptides was greater for the previously embedded and solvent-extracted sample. On further analysis (Table S2), it appears that at least some of the increase in peptides can be attributed to a greater degree of post translational modification (PTM) in the previously embedded samples, as additional PTMs can create different versions of peptides. The difference in top peptides and the increased proportion of PTMs in SMA71H_B11t suggests that either the embedding process, the PMMA cleanup, or both, may affect the peptide signal for AMELX and AMELY. Additional experimental work can improve our understanding of whether and how much the peptide signal is degraded and if it is possible to further improve the PMMA removal process.

Proteomic analysis of unembedded teeth has recently revealed the preservation of immune proteins and cortisol when there is a strong proteomic signal (Buonasera et al. 2024; Wu et al. 2024). To detect low intensity immunological peptides from embedded teeth, enamel loss issues must be resolved. The introduction of a centrifuge step between cleanup rinses may ensure enamel powder is separated from the solvent prior to removal. Previously, Fourier-transform infrared (FTIR) imaging spectroscopy has been used to monitor PMMA dissolution in solvents (González-Benito and Koenig 2006; Kavda et al. 2023), potentially serving as a quantitative measure of PMMA removal for future applications of this protocol. Increasing the amount of protein introduced on the column would also increase the depth of peptide coverage. By further refining our method, future proteomic studies of embedded teeth may also be able to characterise hormonal and immunological signs of stress, allowing a deeper understanding of sex-based differences for archaeological populations.

5 | Conclusion

Embedding teeth allows for insight into ancient peoples' stress experiences, nursing histories, mobility and development (e.g., Lugli et al. 2022; Smith et al. 2021, 2024). Our ethyl acetate cleanup method facilitates proteomic sex estimation from embedded modern and archaeological teeth by successfully removing PMMA from embedded teeth, allowing the analysis of individuals previously precluded. Micromilling may be a useful complementary sampling tool for future proteomics work, particularly for teeth subject to extensive prior physical analyses (leaving little tissue remaining), or with carious lesions that limit pristine enamel. The narrow, shallow sampling paths and fine scale control facilitated by the small drillbit (Sudron et al., forthcoming) yielded sufficient volumes of enamel powder, ensuring masses were not inflated by accidental sampling of dentine or PMMA. The ability to estimate sex proteomically from teeth subject to invasive approaches decreases the unnecessary destruction of archaeological material (Esposito et al. 2024; Squires et al. 2019) and provides more intersectional

bioarchaeological interpretations. Proteomic sex estimations, particularly for infants and children, will supplement reconstructions of demography, disease and mortality, giving rich insight into sex-based differences in maternal investment, stress experiences, health, nursing histories and diet (Buonasera et al. 2022; Casas-Ferreira et al. 2022; Gowland et al. 2021; Greenwald et al. 2023; Ham 2025; Zhang et al. 2024).

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Data Availability Statement

The data that support the findings of this study are available in the text, tables and figures. Mass spectrometry proteomics data have been uploaded to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2022) with the dataset identifier PXD072257 (http://www.proteomexchange.org).

Peer Review

For transparency, the peer review documents associated with this article are available at https://doi.org/10.1111/arcm.70241.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Proteomic sex estimation for unembedded SMA71H_B11u (ME01) and previously embedded enamel SMA71H_B11t (ME02) from B11, processed at the UAF ADAPt Lab. **Table S2:** Post-translational modifications (PTMs) in sex-specific peptides for samples SMA71H_B11u (ME01) and SMA71H_B11t (ME02). **Table S3:** Sequence samples were loaded in, including blank injections, for each analytic run.