



Cite this: *Analyst*, 2025, **150**, 4051

## Rapid and accurate biological sex estimation by LAP-MALDI MS analysis of child teeth

Lily R. Adair,<sup>a</sup> Mary E. Lewis<sup>b</sup> and Rainer Cramer<sup>a</sup> 

We recently introduced a new rapid workflow for biological sex estimation of human skeletal remains from archaeological sites based on LAP-MALDI MS and MS/MS analysis of amelogenin peptides extracted from tooth enamel. With this workflow, a simple classification rule for biological sex estimation was established using panels of MS and MS/MS ion signals. Importantly, the employed LAP-MALDI analysis required only a single 1  $\mu$ L sample droplet and no further peptide separation. Here, we present the application of this rapid workflow and associated classification rule to a set of nine teeth from individuals of unknown biological sex dating to the medieval period (900–1540 CE), including teeth from four children, which were also assessed by conventional osteology. For the five teeth from adult skeletons, the recorded LAP-MALDI MS and MS/MS ion signal panels led to a 100% agreement in the assignment of the biological sex between osteology and LAP-MALDI. Such ion signal panels also provided biological sex estimation for the four child teeth, including three deciduous teeth. Two of these estimates agreed with the osteological assessment. The other two were not in agreement with the osteological assessment. In one case, predominately female osteological characteristics were obtained, while LAP-MALDI analysis clearly identified the individual as male owing to the unambiguous detection of the Y-chromosomal amelogenin peptide SM<sub>ox</sub>IRPPY. In the other case, male osteological characteristics were found, but the osteological assessment was more ambiguous and LAP-MALDI analysis could not detect any MS or MS/MS ion signals of SM<sub>ox</sub>IRPPY that would have substantiated a male assignment.

Received 11th June 2025,  
Accepted 4th August 2025

DOI: 10.1039/d5an00636h

rsc.li/analyst

### 1. Introduction

Biological sex estimation that is based on the differences between the X-chromosomal and Y-chromosomal amelogenin gene or its protein products has become an increasing area of interest in archaeology and the forensic sciences. Mass spectrometry (MS) analysis of amelogenin protein fragments extracted from tooth enamel<sup>1–5</sup> has been introduced as a powerful alternative to DNA analysis<sup>6</sup> as proteins such as collagens and amelogenin are often better preserved over time than DNA, in particular if they are embedded in a strong biomineral matrix like tooth enamel. It also provides minimal destructiveness, fast sample preparation/analysis and lower contamination risk, which is a common issue for DNA analysis.

MS analysis of amelogenin peptides is typically based on proteomic workflows that are applied to bottom-up proteomics with the exception that peptides are obtained by mild acid etching of the enamel rather than tryptic digestion.<sup>3,7,8</sup> Liquid chromatography (LC) separation and electrospray ionization

(ESI) are commonly employed for LC-ESI tandem mass spectrometry (MS/MS) analysis of these peptides with the doubly charged SM<sub>ox</sub>IRPPY peptide ( $[M + 2H]^{2+}$  at  $m/z$  440.22) from the Y-chromosomal amelogenin being then used for positive identification of biological males.<sup>8–10</sup> Proteomic LC-ESI MS/MS analysis can easily take more than one hour to analyze each sample, although three new methods with shorter sample preparation and analysis times have been published.<sup>1,5</sup> Nonetheless, these more recent methods still use ESI and an LC system, making the setup more complex and limiting the analysis times for subsequent sample analyses.

Until recently, matrix-assisted laser desorption/ionization (MALDI) MS and MS/MS analysis of peptides from enamel has been unsuccessful in detecting male-specific amelogenin peptides and has therefore not been employed for biological sex estimation.<sup>11–13</sup> However, a recent study with a hybrid Q-TOF mass analyzer demonstrated that liquid atmospheric pressure (LAP)-MALDI MS and MS/MS analysis can detect amelogenin peptides from both the Y and X chromosome that are chromosome-specific, *i.e.* SM<sub>ox</sub>IRPPY (Y chromosome) and SIRPPYP (X chromosome).<sup>14</sup> Thus, panels of precursor and fragment ions of these two peptides can be used for accurate biological sex estimation as the possible gene deletion of amelogenin on the Y chromosome, and therefore its lack of expression in

<sup>a</sup>Department of Chemistry, University of Reading, Whiteknights, Reading, RG6 6DX, UK. E-mail: r.k.cramer@reading.ac.uk; Tel: +44-118-378-4550

<sup>b</sup>Department of Archaeology, University of Reading, Whiteknights, Reading, RG6 6AB, UK



biological males, is extremely low in most populations, typically below 0.05%, apart from males from the Indian subcontinent.<sup>15</sup>

LAP-MALDI is a new ionization technique with elements of both ESI and MALDI. In particular, it allows the detection of multiply charged peptide ions with high ion yield stability,<sup>16</sup> while being a laser-based ionization technique with low sample consumption and the potential for high-throughput sample analyses.<sup>17–19</sup> So far it has been applied to research in microbial biotyping,<sup>20</sup> lipid analysis,<sup>20,21</sup> disease detection,<sup>22–24</sup> and can be employed in bottom-up proteomic workflows<sup>25</sup> as well as top-down proteomics analyses<sup>23,24,26</sup> on Q-TOF<sup>16,27</sup> and orbitrap<sup>28</sup> mass analyzers.

Here, we apply and extend the previously introduced workflow with its classification rule for biological sex determination based on panels of precursor and fragment ions from enamel-extracted amelogenin peptides using LAP-MALDI MS and MS/MS. It is shown for the first time that this exceptionally fast workflow provides high confidence in biological sex estimation for young children where osteology typically struggles owing to the absence of well-expressed osteological differences prior to puberty.

## 2. Experimental

### 2.1. Reagents and chemicals

Acetonitrile, water (LC-MS grade), trifluoroacetic acid (TFA) and aqueous 37% (v/v) hydrochloric acid (HCl), diluted to 10% with water, were bought from Fisher Scientific (Loughborough, UK). Ethylene glycol and  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) were bought from Merck (Gillingham, UK).

### 2.2. Samples and osteological analysis

All teeth were obtained from individuals excavated from a single site (St Ostwald's Priory, Gloucester, UK) and curated by the Department of Archaeology at the University of Reading. All sampling of the dental enamel was carried out according to the guidelines produced by the Advisory Panel on the Archaeology of Burials in England on destructive sampling of archaeological human remains (<https://historicengland.org.uk/content/docs/advice/science-and-the-dead-2nd-ed/>), and the British Association for Biological Anthropology and Osteoarchaeology (BABAO) Code for Biological Anthropology and Osteoarchaeology (BABAO) Code

of Ethics (<https://babao.org.uk/wp-content/uploads/2024/01/BABAO-Code-of-Ethics.pdf>). The metadata for all teeth investigated can be found in Table 1.

For the adults, age at death was estimated using standard osteological techniques based on degenerative changes to the pelvis and ribs.<sup>29</sup> The children were aged using dental and skeletal markers of growth, development and maturation. Biological sex determination for the adult individuals was based on assessments of the morphology of the pelvis, skull and humerus.<sup>29,30</sup> The accuracy of these methods depends on the experience of the osteologist and the age of the individual under study and is estimated to be between 95–100% accurate when the skeleton is well-preserved.<sup>31</sup> Determining biological sex for child skeletal remains based on the mandible,<sup>32</sup> pelvis<sup>33</sup> and humerus<sup>34</sup> is less reliable, often achieving accuracy rates of between 72–85%.<sup>35</sup> This low level of accuracy results from a lack of sexual differentiation in male and female skeletons before puberty, and the variability of trait expression during growth and development.<sup>35</sup>

### 2.3. Peptide extraction and purification

Peptides were extracted from tooth enamel as previously described.<sup>14</sup> In short, all teeth were individually cleaned in microcentrifuge tubes using ultrasonication for 30 seconds in just enough water to cover the tooth's crown. Subsequently, each tooth was incubated at room temperature (RT) in a small volume of 50  $\mu$ L of 10% HCl (v/v) for 1 minute and washed with water. After these initial cleaning steps, enamel peptides were extracted in another volume of 50  $\mu$ L of 10% HCl in the cap of a 1.5 mL microcentrifuge tube, by incubating the tooth's crown at RT in the 50  $\mu$ L droplet for 5 minutes. The entire droplet with the extracted peptides was then transferred to a new microcentrifuge tube. This droplet was then used to obtain a 10  $\mu$ L aliquot of the acid-etch extract that was cleaned up by C<sub>18</sub> Zip-Tips (ZTC18S096; EMD Millipore, Merck, Gillingham, UK) according to the manufacturer's protocol using an acetonitrile/water solution (50/50; v/v) for the final peptide elution step.

### 2.4. LAP-MALDI MS and MS/MS

The liquid MALDI matrix was prepared by dissolving CHCA in water : acetonitrile (3 : 7; v/v) to a concentration of 25 mg mL<sup>-1</sup>.

**Table 1** Metadata and biological sex estimation data of the teeth from individuals excavated from St Ostwald's Priory, Gloucester, UK, and analyzed in this study

Skeleton number	Period <sup>a</sup> (CE)	Sampled tooth	Age (osteology)	Sex (osteology)	Sex (Enamel MS analysis)
6	1120–1230	Left mandibular, 2 <sup>nd</sup> premolar (P)	26–35	Male	Male
7	1120–1230	Left mandibular, 2 <sup>nd</sup> premolar (P)	17–25	Female	Female
49	1120–1230	Right mandibular, 1 <sup>st</sup> premolar (P)	35–45	Male	Male
50	900–1120	Left maxillary, 1 <sup>st</sup> premolar (P)	17–25	Female	Female
63	1120–1230	Right mandibular, 2 <sup>nd</sup> premolar (P)	35–45	Male	Male
103	1250–1540	Left maxillary, 1 <sup>st</sup> incisor (D)	2.6–6.5	Male	Male
106	1250–1540	Left maxillary, 1 <sup>st</sup> incisor (D)	6.6–10.5	Male?	Female
136	1250–1540	Left mandibular, 1 <sup>st</sup> premolar (P)	2.6–6.5	Female	Male
332	1250–1540	Right mandibular, 1 <sup>st</sup> molar (D)	Child	Male	Male

<sup>a</sup> Time period in which the individual died. D = deciduous tooth; P = permanent tooth. '?' This sex assignment is based on a mixture of male and female characteristics with slightly more features indicating a male skeleton.



After thorough vortexing, until CHCA was fully dissolved, a volume of ethylene glycol (70% of the CHCA solution volume) was added. This mixture was also thoroughly vortexed to ensure the final liquid MALDI matrix was homogeneous.

LAP-MALDI sample spots were prepared by spotting first a volume of 0.5  $\mu$ L of the liquid MALDI matrix onto a stainless-steel MALDI sample plate, which was immediately followed by adding an equal volume of the analyte solution.

For MALDI MS and MS/MS analyses, freshly prepared liquid MALDI samples were run on a Synapt G2-Si (Waters Corp., Wilmslow, UK) with an in-house-developed LAP-MALDI source using a pulsed 343 nm laser (FlareNX 343-0.2-2; Coherent, Santa Clara, USA), which was focused on the centre of the liquid MALDI sample droplet. A detailed description of the LAP-MALDI source can be found in a previous publication.<sup>36</sup> The instrument settings and data acquisition parameters were kept the same as reported in the first LAP-MALDI MS and MS/MS study of amelogenin peptides.<sup>14</sup> For MS/MS analysis of the precursor ions at  $m/z$  829.5 and 879.4, the CID collision voltage was set to 40 V. All data acquisitions were in positive ion mode and used a scan time of 1 second per scan with the MS data acquisition and subsequent MS/MS data acquisitions lasting 1 minute each.

## 2.5. Data processing and analysis

Raw data files from MassLynx 4.2 (Waters) were processed with Mascot Distiller (Version 2.8.5, 64-bit; Matrix Science, London, England) for automated peak picking. For 'MS Peak Picking' as well as 'MS/MS Peak Picking', a correlation threshold (Rho) of 0.7 and a minimum signal-to-noise (S/N) of 2, 10 and 50 were selected. Baseline correction was applied with the isotope distribution fit method using 500 as the maximum peak iterations per scan. A minimum peak width of 0.005 Da, an expected peak width of 0.05 Da and a maximum peak width of 0.5 Da were selected. The peak list was evaluated according to the previously published classification rule for biological sex determination<sup>14</sup> but for all three different S/N values of 2, 10 and 50. In exact terms, male sex was only assigned when the peak list of the MS/MS analysis at  $m/z$  879.4 contained all of the following three fragment ions within an  $m/z$  tolerance range of  $\pm 0.2$ :  $m/z$  376.19 ( $MH-SOCH_4$ ),  $m/z$  440.26 ( $b_4-SOCH_4$ ) and  $m/z$  815.44 ( $y_3$ ). Female sex was only assigned when (i) the  $m/z$  829.46 precursor ion was detected in the MS analysis within an  $m/z$  tolerance range of  $\pm 0.1$ , (ii) the MS/MS analysis at  $m/z$  879.4 contained no fragment ions at  $m/z$  376.19,  $m/z$  440.26 and  $m/z$  815.44 within an  $m/z$  tolerance range of  $\pm 0.2$  and (iii) the peak list of the MS/MS analysis of  $m/z$  829.5 contained all of the following four fragment ions within an  $m/z$  tolerance range of  $\pm 0.2$ :  $m/z$  173.13 ( $a_2$ ),  $m/z$  357.22 ( $b_3$ ),  $m/z$  376.19 ( $y_3$ ) and  $m/z$  473.24 ( $y_4$ ).

## 3. Results and discussion

Prior to any LAP-MALDI MS and MS/MS analysis, all teeth were evaluated by standard osteological techniques without provid-

ing the results to the researcher who undertook the LAP-MALDI sample preparation and MS data acquisition, providing an appropriately blinded study for comparison.

### 3.1. LAP-MALDI MS and MS/MS for biological sex estimation

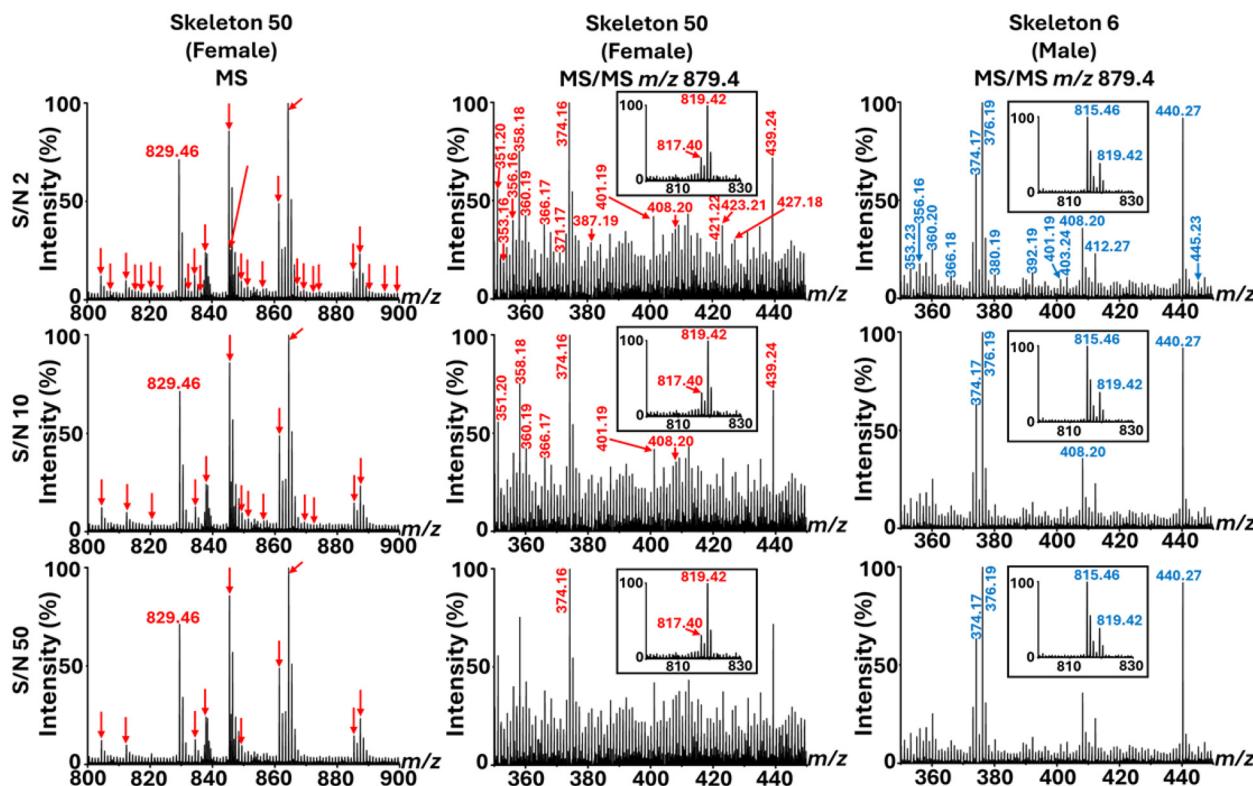
Five teeth from adult skeletons that were not analyzed before by the new LAP-MALDI workflow for biological sex estimation were prepared as described in Adair *et al.*<sup>14</sup> and the Experimental section. The LAP-MALDI MS and MS/MS data were assessed for biological sex estimation using the classification rule previously devised and further detailed with an accurate description of the applied  $m/z$  tolerances for peak detection (see 'Data processing and analysis' in the Experimental section).

As before the biological sex assignment for all teeth from adult skeletons showed 100% agreement between the osteological assessment and the LAP-MALDI MS and MS/MS analysis (see Table 1). It is noteworthy that even at a much lower S/N of 2 and a much higher S/N of 50 than the originally suggested S/N of 10, the sex classification rule using the LAP-MALDI MS and MS/MS data always arrived at the same classification, for all adult and child teeth. In this context, it should be noted that for all 19 teeth analyzed so far by LAP-MALDI and classified by this rule a classification result was obtained. No analysis led to an inconclusive sex assignment, although it only takes one missing ion peak out of three, for the male assignment to fail, and similarly only one missing ion peak out of five, for the female assignment to fail. Female sex assignment also fails if a sufficiently high noise peak appears at just one of three male-specific fragment ion peak position. The conclusive assignment to a single biological sex class in all cases further underlines the robustness of the presented workflow.

Fig. 1 shows the MS profiles and for  $m/z$  879.4 the MS/MS data for the tooth from skeleton 50 (female) for all three S/N values. For comparison, it also shows the MS/MS data for  $m/z$  879.4 for the tooth from skeleton 6 (male) for all three S/N value. In all spectra, only the peaks that were picked by Mascot Distiller according to the applied S/N value are labeled. All MS profiles in Fig. 1 clearly show the detection of a peak at  $m/z$  829.46, thus fulfilling the first requirement for female sex assignment. The MS/MS data of the female tooth reveals that none of the male-specific  $SM_{ox}IRPPY$  fragment ions at  $m/z$  376.19,  $m/z$  440.26 and  $m/z$  815.44 within an  $m/z$  tolerance range of  $\pm 0.2$  were detected for any S/N value, thus fulfilling the second requirement for female sex assignment. In contrast, the MS/MS data of the male tooth reveals that these three male-specific  $SM_{ox}IRPPY$  fragment ions were all detected for all S/N values, providing in all cases an unambiguous assignment as male.

LAP-MALDI analysis of this new set of 5 teeth from adult skeletons in a blinded experimental design, *i.e.* without prior knowledge of the osteological assignments before the LAP-MALDI assignments, further supports the validity of the LAP-MALDI-based workflow and data analysis for accurate biological sex estimation as a 100% agreement in the biological sex estimation was obtained. Confidence in the LAP-MALDI





**Fig. 1** LAP-MALDI MS profiles (left column) and MS/MS data (middle and right column) of the tooth from the female skeleton 50 (left and middle column) and the tooth from the male skeleton 6 (right column). All automatically assigned ion peaks using a S/N value of 2 (top row), 10 (middle row) and 50 (bottom row) are labeled (either by their  $m/z$  value and/or arrows).

assignments is further strengthened by the wide range of S/N values of 2–50 without a single case where the assignment deviated from that with an S/N value of 10. This wide range of applicable S/N values was obtained for all nine teeth despite the different types and levels of noise of the TOF MS and Q-TOF MS/MS data recorded on a Q-TOF mass spectrometer. The noise of TOF MS data is often dominated by chemical noise with ion signals at each nominal  $m/z$  value, which is virtually absent in MS/MS measurements. Thus, automated S/N determination for both data types is often inadequate using the same settings. Nonetheless, the automated peak detection based on analyzer-nonspecific S/N calculations of the data sets from both TOF MS and Q-TOF MS/MS measurements proved to be entirely adequate within this wide range of S/N values.

### 3.2. Biological sex estimation using child teeth

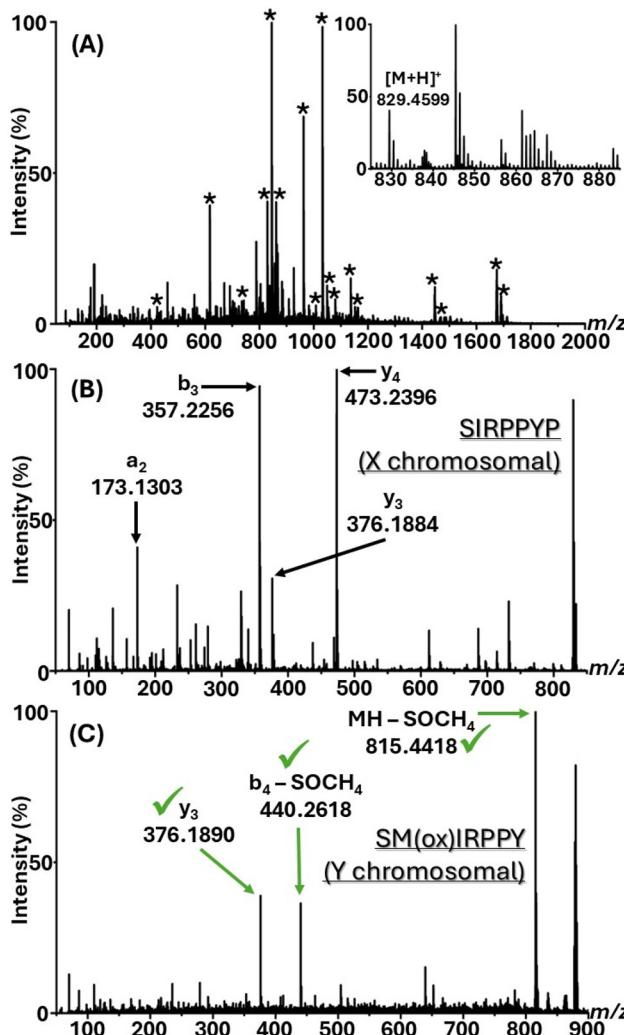
The analyses of the child teeth revealed some interesting results. While the more confident osteological biological sex estimates for skeletons 103 and 332 were confirmed by LAP-MALDI, the sex estimates for the other two skeletons provided different results. Skeleton 136 was estimated to be female as 9 of the 13 osteological features that could be observed on the mandible, humerus and pelvis were determined to have female characteristics. The MS/MS spectrum of the tooth from skeleton 136 for the precursor ions at  $m/z$  879.4 clearly shows all three  $SM_{ox}IRPPY$  fragment ions at  $m/z$  376.19,

$m/z$  440.26 and  $m/z$  815.44 (see Fig. 2) and is therefore unambiguous in assigning the biological sex as male.

Skeleton 106 was assigned a possible male estimate by osteology, as 5 of the 9 osteological features observed were determined to be male. However, for the tooth from skeleton 106, none of the three typical  $SM_{ox}IRPPY$  fragment ions were obtained by MS/MS analysis of the precursor ions at  $m/z$  879.4 (see Fig. 3(C)) at any S/N value investigated. However, at all investigated S/N values the  $m/z$  829.46 precursor ion was detected in the MS profile (see inset in Fig. 3(A)) and the peak list of the MS/MS analysis of  $m/z$  829.5 contained the fragment ions at  $m/z$  173.13 ( $a_2$ ),  $m/z$  357.22 ( $b_3$ ),  $m/z$  376.19 ( $y_3$ ) and  $m/z$  473.24 ( $y_4$ ) of the SIRPPYP peptide (see Fig. 3(B)). Thus, the MS and MS/MS data of the tooth from skeleton 106 fulfil all three requirements for the assignment as female.

Both biological sexes predominantly express the X-chromosomal version of amelogenin (100% for female; ~90% for male)<sup>37</sup> but only biological males express the Y-chromosomal amelogenin. Therefore, the detection of peptide ions that are specific to the Y-chromosomal amelogenin provides high confidence in the assignment of a biological male. Their absence, however, does not necessarily mean that the biological sex is female. Apart from a small fraction of biological males that do not express Y-chromosomal amelogenin, absence of MS or MS/MS ion signals could be a result of the loss of peptides or their low recovery during the extrac-

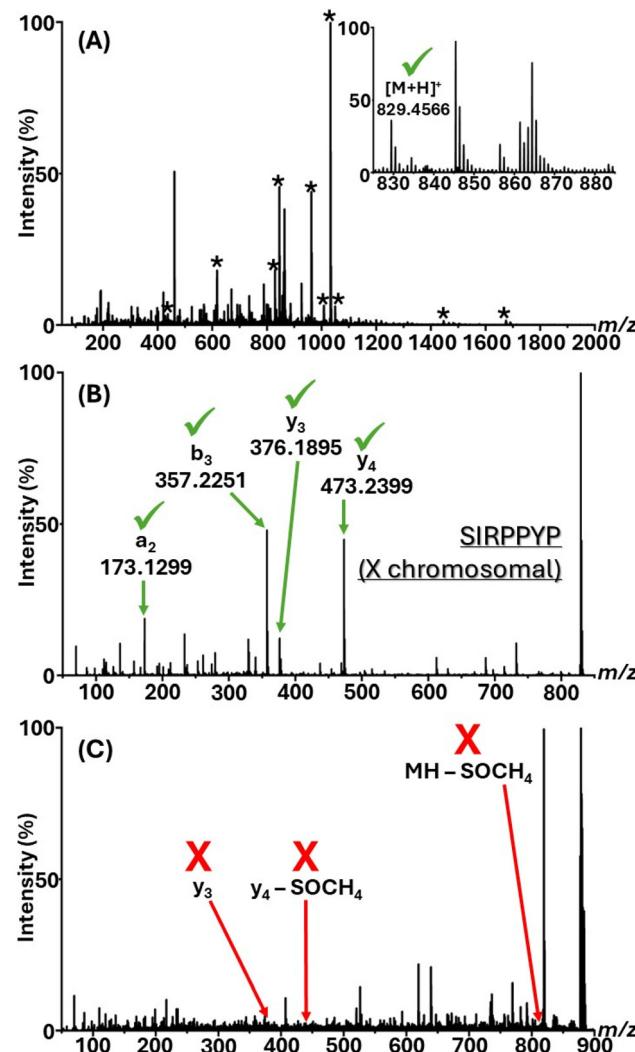




**Fig. 2** LAP-MALDI MS and MS/MS spectra for the tooth of skeleton 136 (male; cf. Table 1). (A) LAP-MALDI MS profile with inset displaying the  $m/z$  region for the singly charged  $SM_{ox}$ IRPPY (Y chromosome) and SIRPPYP (X chromosome) peptides. Ion signals obtained from peptides extracted from the tooth are labeled by asterisks. (B) LAP-MALDI MS/MS spectrum of the precursor ions at  $m/z$  829.5 (singly protonated SIRPPYP). (C) LAP-MALDI MS/MS spectrum of the precursor ions at  $m/z$  879.4 (singly protonated  $SM_{ox}$ IRPPY).

tion and further sample preparation stages. In addition, a high noise level could be a compounding factor, making it difficult to detect analyte ion signals at the required S/N level. For these reasons, a panel of MS and MS/MS ion signals that can be obtained from chromosome-specific peptides have been used for robust biological sex assessment. Both the absence of male-specific amelogenin peptide ions and sufficient ion signal intensities for the X-chromosomal peptide ions covering the same sequence region as the Y-chromosomal peptides are used to assign female sex, thus providing increased confidence compared to using the absence of male-specific ions only.

For the teeth where the osteology and LAP-MALDI sex assignments disagreed (from skeletons 106 and 136), replicate



**Fig. 3** LAP-MALDI MS and MS/MS spectra for the tooth of skeleton 106 (female; cf. Table 1). (A) LAP-MALDI MS profile with inset displaying the  $m/z$  region for the singly charged  $SM_{ox}$ IRPPY (Y chromosome) and SIRPPYP (X chromosome) peptides. Ion signals obtained from peptides extracted from the tooth are labeled by asterisks. (B) LAP-MALDI MS/MS spectrum of the precursor ions at  $m/z$  829.5 (singly protonated SIRPPYP). (C) LAP-MALDI MS/MS spectrum of the precursor ions at  $m/z$  879.4.

LAP-MALDI analyses using a new target plate and new LAP-MALDI sample preparation were undertaken. In both cases, the same sex assignments were obtained again, suggesting that no carry-over from other samples is accountable for the discrepancy, and at least for the male assignment the LAP-MALDI data is unambiguous.

#### 4. Conclusions

There has been an increasing interest in applying MS-based proteomic analysis to archaeological samples.<sup>2,3,10,13,38-41</sup> For biological sex estimation, amelogenin peptides extracted from

teeth allow for more (accurate) sex assignments than osteological techniques,<sup>42</sup> in particular in cases where bones are heavily degraded, absent or just not sufficiently informative due to the age of the individual. In general, MS-based peptide/protein identifications do not require any specific reagents as it is the case for primer-based DNA analysis. The latter would also require successful extraction of DNA from the tooth's pulp or dentin. However, so far all proteomic workflows published for biological sex estimation exploiting amelogenin peptides are still based on classic MS-based proteomic approaches with ESI and LC instrumentation as key components. The few attempts of utilizing conventional MALDI with a quick peptide extraction could not detect male-specific amelogenin peptides, thus failed for biological sex estimation.<sup>11–13</sup> The disadvantages in using ESI and LC instrumentation are the complexity of the workflow, the time needed to acquire the data and the cost per sample, even if no LC separation is applied and no additional proteolysis steps are included.

A recent sex estimation study on the largest set of teeth analyzed using ESI-based analysis of amelogenin peptides to date proposes a workflow that still requires a sample preparation time of 10 minutes or more and a turn-around time for data acquisition of 7.2 minutes.<sup>5</sup> The time for both steps together is only half of that when using the presented workflow with LAP-MALDI, currently with an overall sample preparation/analysis time of <10 minutes and the potential to reduce this further as the peptide extraction time of ~5 minutes and the data acquisition time of up to 3 minutes are rather generous. Furthermore, the cost per sample is mainly determined by the price of the tip-based SPE peptide purification of the tooth extract. As the tip-based SPE purification step is the main sample preparation step in the entire workflow, this workflow is also less complex than LC-based workflows and can be automated.

The success of LAP-MALDI in analysing tooth extracts for biological sex estimation where conventional MALDI has so far failed can be potentially explained by its hybrid nature, with characteristics of both ESI and MALDI in the ionization process and the instrumentation that can be utilized. The latter allows the use of high-performing instrumentation such as orbitraps, which has been recently exploited on an entirely commercial platform,<sup>28</sup> thus allowing the wider research community to employ LAP-MALDI. High ion signal stability is another contributing factor for a workflow that relies on ion signal intensities and their consistent detection. LAP-MALDI has been shown to be superior in recording stable ion signals compared to conventional MALDI.<sup>16</sup>

In summary, LAP-MALDI's latest application to the analysis of amelogenin peptides extracted from ancient teeth (including deciduous teeth) allows for rapid and accurate biological sex estimation, including its valuable application to child skeletal remains, where osteological techniques are less reliable, often leading to highly ambiguous results. The speed, low complexity and low-cost sample preparation of LAP-MALDI MS are additional major advantages that will help to increase the uptake of MS-based biological sex estimation using teeth. The

simplicity of this rapid and short workflow has also great potential in preparing amelogenin peptide extracts in remote location where no research laboratory or special expertise are available and by sending these extracts for immediate analysis to MS laboratories without sending the teeth. Finally, the use of an extremely simple but robust sex classification rule based on easily extractable information directly from the MS and MS/MS data allows accurate sex determination without the need for sophisticated bioinformatics and proprietary software or expert knowledge and therefore widens its applicability to research communities without the expertise in proteomics and mass spectrometry.

## Author contributions

Lily R. Adair: formal analysis, investigation, methodology, writing – review & editing. Mary E. Lewis: methodology, resources, validation, writing – review & editing. Rainer Cramer: conceptualization, formal analysis, funding acquisition, investigation, methodology, supervision, validation, writing – original draft, writing – review & editing.

## Conflicts of interest

Rainer Cramer has patent Method for Ion Production issued to University of Reading. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data supporting the results reported in this paper are openly available from the University of Reading Research Data Archive at <https://doi.org/10.17864/1947.001402>.

## Acknowledgements

This research was supported by the Engineering and Physical Sciences Research Council (EPSRC) through grant EP/V047485/1.

## References

- 1 A. M. Casas-Ferreira, M. del Nogal-Sanchez, A. E. Arroyo, J. V. Vazquez and J. L. Perez-Pavon, Fast methods based on mass spectrometry for peptide identification. Application to sex determination of human remains in tooth enamel, *Microchem. J.*, 2022, **181**, 107645, DOI: [10.1016/j.microc.2022.107645](https://doi.org/10.1016/j.microc.2022.107645).
- 2 J. A. Gamble, V. Spicer, M. Hunter, Y. Lao, R. D. Hoppa, D. D. Pedersen, J. A. Wilkins and R. P. Zahedi, Advancing sex estimation from amelogenin: Applications to archaeolo-



gical, deciduous, and fragmentary dental enamel, *J. Archaeol. Sci. Rep.*, 2024, **54**, 104430, DOI: [10.1016/j.jasrep.2024.104430](https://doi.org/10.1016/j.jasrep.2024.104430).

3 R. Gowland, N. A. Stewart, K. D. Crowder, C. Hodson, H. Shaw, K. J. Gron and J. Montgomery, Sex estimation of teeth at different developmental stages using dimorphic enamel peptide analysis, *Am. J. Phys. Anthropol.*, 2021, **174**(4), 859–869, DOI: [10.1002/ajpa.24231](https://doi.org/10.1002/ajpa.24231).

4 I. Mikšík, M. Morvan and J. Brůžek, Peptide analysis of tooth enamel - A sex estimation tool for archaeological, anthropological, or forensic research, *J. Sep. Sci.*, 2023, **46**, 2300183, DOI: [10.1002/jssc.202300183](https://doi.org/10.1002/jssc.202300183).

5 C. Koenig, P. Bortel, R. S. Paterson, B. Rendl, P. P. Madupe, G. B. Troche, N. V. Hermann, M. M. de Pinillos, M. Martinon-Torres, S. Mularczyk, *et al.*, Automated High-Throughput Biological Sex Identification from Archeological Human Dental Enamel Using Targeted Proteomics, *J. Proteome Res.*, 2024, **23**(11), 5107–5121, DOI: [10.1021/acs.jproteome.4c00557](https://doi.org/10.1021/acs.jproteome.4c00557).

6 Y. Nakahori, K. Hamano, M. Iwaya and Y. Nakagome, Sex Identification by Polymerase Chain-Reaction Using X-Y Homologous Primer, *Am. J. Med. Genet.*, 1991, **39**(4), 472–473, DOI: [10.1002/ajmg.1320390420](https://doi.org/10.1002/ajmg.1320390420).

7 E. Greco, A. M. Gennaro, D. Piombino-Mascali, D. Costanzo, S. Accardo, S. Licen, P. Barbieri, S. Fornasaro, S. Semeraro, E. Marin, *et al.*, Dental proteomic analyses and Raman spectroscopy for the estimation of the biological sex and age of human remains from the Greek cemetery of San Giorgio Extra, Reggio Calabria (Italy), *Microchem. J.*, 2023, **195**, 109472, DOI: [10.1016/j.microc.2023.109472](https://doi.org/10.1016/j.microc.2023.109472).

8 N. A. Stewart, R. F. Gerlach, R. L. Gowland, K. J. Gron and J. Montgomery, Sex determination of human remains from peptides in tooth enamel, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**(52), 13649–13654, DOI: [10.1073/pnas.1714926115](https://doi.org/10.1073/pnas.1714926115).

9 J. Bruzek, I. Mikšík, A. P. Kotterová, M. Morvan, S. D. Kaupová, F. Santos, A. Danielisová, E. Zazvornilová, B. Maureille and P. Velemínsky, Undertaking the biological sex assessment of human remains: The applicability of minimally-invasive methods for proteomic sex estimation from enamel peptides, *J. Cult. Herit.*, 2024, **66**, 204–214, DOI: [10.1016/j.culher.2023.11.021](https://doi.org/10.1016/j.culher.2023.11.021).

10 F. Lugli, G. Di Rocco, A. Vazzana, F. Genovese, D. Pinetti, E. Cilli, M. C. Carile, S. Silvestrini, G. Gabanini, S. Arrighi, *et al.*, Enamel peptides reveal the sex of the Late Antique 'Lovers of Modena', *Sci. Rep.*, 2019, **9**, 13130, DOI: [10.1038/s41598-019-49562-7](https://doi.org/10.1038/s41598-019-49562-7).

11 C. M. Nielsen-Marsh, C. Stegemann, R. Hoffmann, T. Smith, R. Feeney, M. Toussaint, K. Harvati, E. Panagopoulou, J. J. Hublin and M. P. Richards, Extraction and sequencing of human and Neanderthal mature enamel proteins using MALDI-TOF/TOF MS, *J. Archaeol. Sci.*, 2009, **36**(8), 1758–1763, DOI: [10.1016/j.jas.2009.04.004](https://doi.org/10.1016/j.jas.2009.04.004).

12 I. M. Porto, H. J. Laure, F. B. de Sousa, J. C. Rosa and R. F. Gerlach, New techniques for the recovery of small amounts of mature enamel proteins, *J. Archaeol. Sci.*, 2011, **38**(12), 3596–3604, DOI: [10.1016/j.jas.2011.08.030](https://doi.org/10.1016/j.jas.2011.08.030).

13 I. M. Porto, H. J. Laure, R. H. Tykot, F. B. de Sousa, J. C. Rosa and R. F. Gerlach, Recovery and identification of mature enamel proteins in ancient teeth, *Eur. J. Oral Sci.*, 2011, **119**, 83–87, DOI: [10.1111/j.1600-0722.2011.00885.x](https://doi.org/10.1111/j.1600-0722.2011.00885.x).

14 L. R. Adair, M. E. Lewis, M. J. Collins and R. Cramer, LAP-MALDI MS analysis of amelogenin from teeth for biological sex estimation, *J. Pharm. Biomed. Anal.*, 2024, **255**, 116599, DOI: [10.1016/j.jpba.2024.116599](https://doi.org/10.1016/j.jpba.2024.116599).

15 I. Stamfelj, Sex estimation based on the analysis of enamel peptides: False assignments due to AMELY deletion, *J. Archaeol. Sci.*, 2021, **130**, 105345, DOI: [10.1016/j.jas.2021.105345](https://doi.org/10.1016/j.jas.2021.105345).

16 R. Cramer, A. Pirk, F. Hillenkamp and K. Dreisewerd, Liquid AP-UV-MALDI Enables Stable Ion Yields of Multiply Charged Peptide and Protein Ions for Sensitive Analysis by Mass Spectrometry, *Angew. Chem., Int. Ed.*, 2013, **52**(8), 2364–2367, DOI: [10.1002/anie.201208628](https://doi.org/10.1002/anie.201208628).

17 B. Challen, M. Morris and R. Cramer, Ultra-High-Throughput and Low-Volume Analysis of Intact Proteins with LAP-MALDI MS, *J. Am. Soc. Mass Spectrom.*, 2023, **34**(6), 991–994, DOI: [10.1021/jasms.3c00068](https://doi.org/10.1021/jasms.3c00068).

18 H. Krenkel, J. Brown, M. Morris and R. Cramer, The use of salts, buffers and surfactants in LAP-MALDI MS, *Int. J. Mass Spectrom.*, 2023, **493**, 117134, DOI: [10.1016/j.ijms.2023.117134](https://doi.org/10.1016/j.ijms.2023.117134).

19 H. Krenkel, J. Brown, K. Richardson, E. Hoyes, M. Morris and R. Cramer, Ultrahigh-Throughput Sample Analysis Using Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry, *Anal. Chem.*, 2022, **94**(10), 4141–4145, DOI: [10.1021/acs.analchem.1c05614](https://doi.org/10.1021/acs.analchem.1c05614).

20 S. E. Lellman and R. Cramer, Bacterial identification by lipid profiling using liquid atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry, *Clin. Chem. Lab. Med.*, 2020, **58**(6), 930–938, DOI: [10.1515/cclm-2019-0908](https://doi.org/10.1515/cclm-2019-0908).

21 O. J. Hale and R. Cramer, Collision-induced dissociation of doubly-charged barium-cationized lipids generated from liquid samples by atmospheric pressure matrix-assisted laser desorption/ionization provides structurally diagnostic product ions, *Anal. Bioanal. Chem.*, 2018, **410**(5), 1435–1444, DOI: [10.1007/s00216-017-0788-6](https://doi.org/10.1007/s00216-017-0788-6).

22 S. E. Lellman, C. K. Reynolds, A. K. B. Jones, N. Taylor and R. Cramer, LAP-MALDI MS Profiling and Identification of Potential Biomarkers for the Detection of Bovine Tuberculosis, *J. Agric. Food Chem.*, 2023, **71**(37), 13899–13905, DOI: [10.1021/acs.jafc.3c01879](https://doi.org/10.1021/acs.jafc.3c01879).

23 C. Piras, O. J. Hale, C. K. Reynolds, A. K. Jones, N. Taylor, M. Morris and R. Cramer, LAP-MALDI MS coupled with machine learning: an ambient mass spectrometry approach for high-throughput diagnostics, *Chem. Sci.*, 2022, **13**(6), 1746–1758, DOI: [10.1039/d1sc05171g](https://doi.org/10.1039/d1sc05171g).

24 O. J. Hale, M. Morris, B. Jones, C. K. Reynolds and R. Cramer, Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Adds Enhanced Functionalities to MALDI MS Profiling for



Disease Diagnostics, *ACS Omega*, 2019, **4**(7), 12759–12765, DOI: [10.1021/acsomega.9b01476](https://doi.org/10.1021/acsomega.9b01476).

25 P. Ryumin, J. Brown, M. Morris and R. Cramer, Protein identification using a nanoUHPLC-AP-MALDI MS/MS workflow with CID of multiply charged proteolytic peptides, *Int. J. Mass Spectrom.*, 2017, **416**, 20–28, DOI: [10.1016/j.ijms.2016.12.006](https://doi.org/10.1016/j.ijms.2016.12.006).

26 L. R. Adair, I. Jones and R. Cramer, Utilizing Precursor Ion Connectivity of Different Charge States to Improve Peptide and Protein Identification in MS/MS Analysis, *Anal. Chem.*, 2024, **96**(3), 985–990, DOI: [10.1021/acs.analchem.3c03061](https://doi.org/10.1021/acs.analchem.3c03061).

27 O. J. Hale and R. Cramer, Atmospheric Pressure Ultraviolet Laser Desorption and Ionization from Liquid Samples for Native Mass Spectrometry, *Anal. Chem.*, 2019, **91**(22), 14192–14197, DOI: [10.1021/acs.analchem.9b03875](https://doi.org/10.1021/acs.analchem.9b03875).

28 B. Challen, L. R. Adair and R. Cramer, Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Using a Commercial Ion Source and Orbitrap Mass Analyzer, *Anal. Chem.*, 2024, **96**(40), 15865–15870, DOI: [10.1021/acs.analchem.4c04458](https://doi.org/10.1021/acs.analchem.4c04458).

29 T. D. White, M. T. Black and P. A. Folkens, *Human osteology*, Elsevier/AP, 2011, ch. 18, pp. 379–427.

30 C. G. Falys, H. Schutkowski and D. A. Weston, The distal humerus—a blind test of Rogers' sexing technique using a documented skeletal collection, *J. Forensic Sci.*, 2005, **50**(6), 1289–1293.

31 J. E. Buikstra and J. H. Mielke, Demography, diet, and health, in *Analysis of Prehistoric Diets*, ed. R. I. Gilbert and J. H. Mielke, Academic Press, 1985, pp. 359–422.

32 R. C. Sutter, Nonmetric subadult skeletal sexing traits: I. A blind test of the accuracy of eight previously proposed methods using prehistoric known-sex mummies from northern Chile, *J. Forensic Sci.*, 2003, **48**(5), 927–935.

33 L. A. Wilson, R. Ives, H. F. Cardoso and L. T. Humphrey, Shape, size, and maturity trajectories of the human ilium, *Am. J. Phys. Anthropol.*, 2015, **156**(1), 19–34, DOI: [10.1002/ajpa.22625](https://doi.org/10.1002/ajpa.22625).

34 T. L. Rogers, Sex determination of adolescent skeletons using the distal humerus, *Am. J. Phys. Anthropol.*, 2009, **140**(1), 143–148, DOI: [10.1002/ajpa.21060](https://doi.org/10.1002/ajpa.21060).

35 M. Lewis, Biological Anthropology of the Human Skeleton, in *Children in Bioarchaeology: Methods and Interpretation*, ed. A. Katzenberg and A. Grauer, John Wiley and Sons, 3rd edn, 2019, pp. 119–143.

36 P. Ryumin, J. Brown, M. Morris and R. Cramer, Investigation and optimization of parameters affecting the multiply charged ion yield in AP-MALDI MS, *Methods*, 2016, **104**, 11–20, DOI: [10.1016/j.ymeth.2016.01.015](https://doi.org/10.1016/j.ymeth.2016.01.015).

37 E. C. Salido, P. H. Yen, K. Koprivnikar, L. C. Yu and L. J. Shapiro, The Human Enamel Protein Gene Amelogenin Is Expressed from Both the X-Chromosomes and the Y-Chromosomes, *Am. J. Hum. Genet.*, 1992, **50**(2), 303–316.

38 E. Cappellini, F. Welker, L. Pandolfi, J. Ramos-Madrigal, D. Samodova, P. L. Rüther, A. K. Fotakis, D. Lyon, J. V. Moreno-Mayar, M. Bukhsianidze, *et al.*, Early Pleistocene enamel proteome from Dmanisi resolves *Stephanorhinus* phylogeny, *Nature*, 2019, **574**(7776), 103–107, DOI: [10.1038/s41586-019-1555-y](https://doi.org/10.1038/s41586-019-1555-y).

39 T. P. Cleland, S. A. McGuire, J. S. Beatrice, K. S. Moran and C. A. M. France, SPEED-E: A modified version of the sample preparation by Easy extraction and Digestion(-free) protocol for enamel-based sex estimation in archaeological remains, *J. Archaeol. Sci.*, 2024, **168**, 106006, DOI: [10.1016/j.jas.2024.106006](https://doi.org/10.1016/j.jas.2024.106006).

40 G. J. Parker, J. M. Yip, J. W. Eerkens, M. Salemi, B. Durbin-Johnson, C. Kiesow, R. Haas, J. E. Buikstra, H. Klaus, L. A. Regan, *et al.*, Sex estimation using sexually dimorphic amelogenin protein fragments in human enamel, *J. Archaeol. Sci.*, 2019, **101**, 169–180, DOI: [10.1016/j.jas.2018.08.011](https://doi.org/10.1016/j.jas.2018.08.011).

41 F. Welker, J. Ramos-Madrigal, M. Kuhlwilm, W. Liao, P. Gutenbrunner, M. de Manuel, D. Samodova, M. Mackie, M. E. Allentoft, A. M. Bacon, *et al.*, Enamel proteome shows that *Gigantopithecus* was an early diverging pongine, *Nature*, 2019, **576**(7786), 262–265, DOI: [10.1038/s41586-019-1728-8](https://doi.org/10.1038/s41586-019-1728-8).

42 T. Buonasera, J. Eerkens, A. de Flamingh, L. Engbring, J. Yip, H. J. Li, R. Haas, D. DiGiuseppe, D. Grant, M. Salemi, *et al.*, A comparison of proteomic, genomic, and osteological methods of archaeological sex estimation, *Sci. Rep.*, 2020, **10**, 11897, DOI: [10.1038/s41598-020-68550-w](https://doi.org/10.1038/s41598-020-68550-w).

