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

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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 μ 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, predominantly 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_{ox}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_{ox}IRPPY that would have substantiated a male assignment.

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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^{1–5} has been introduced as a powerful alternative to DNA analysis⁶ 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.^{3,7,8} 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_{ox}IRPPY peptide ($[M + 2H]^{2+}$ at m/z 440.22) from the Y-chromosomal amelogenin being then used for positive identification of biological males.^{8–10} 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.^{1,5} 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.^{11–13} 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_{ox}IRPPY (Y chromosome) and SIRPPYP (X chromosome).¹⁴ 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

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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 μL 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.³⁶ 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.¹⁴ 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 determination¹⁴ 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 ± 0.2 : m/z 376.19 (MH-SOCH₄), m/z 440.26 (b_4 -SOCH₄) 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 ± 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 ± 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 ± 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

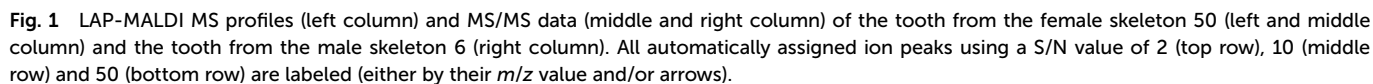
Five teeth from adult skeletons that were not analyzed before by the new LAP-MALDI workflow for biological sex estimation were prepared as described in Adair *et al.*¹⁴ and the Experimental section. The LAP-MALDI MS and MS/MS data were assessed for biological sex estimation using the classification rule 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 ± 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





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 predominately express the X-chromosomal version of amelogenin (100% for female; ~90% for male)³⁷ 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-

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 $\text{SM}_{\text{ox}}\text{IRPPY}$ fragment ions at m/z 376.19,

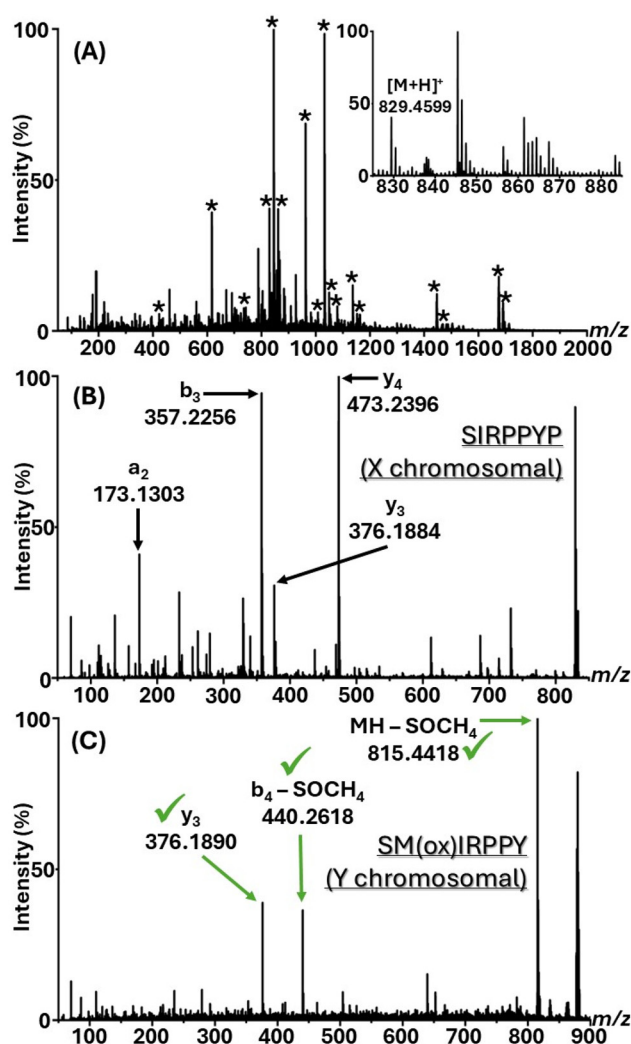


Fig. 2 LAP-MALDI MS and MS/MS spectra for the tooth of skeleton 136 (male; cf. Table 1). (A) LAP-MALDI MS profile with inset displaying the m/z region for the singly charged $SM_{ox}IRPPY$ (Y chromosome) and $SIRPPYP$ (X chromosome) peptides. Ion signals obtained from peptides extracted from the tooth 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 $SM_{ox}IRPPY$).

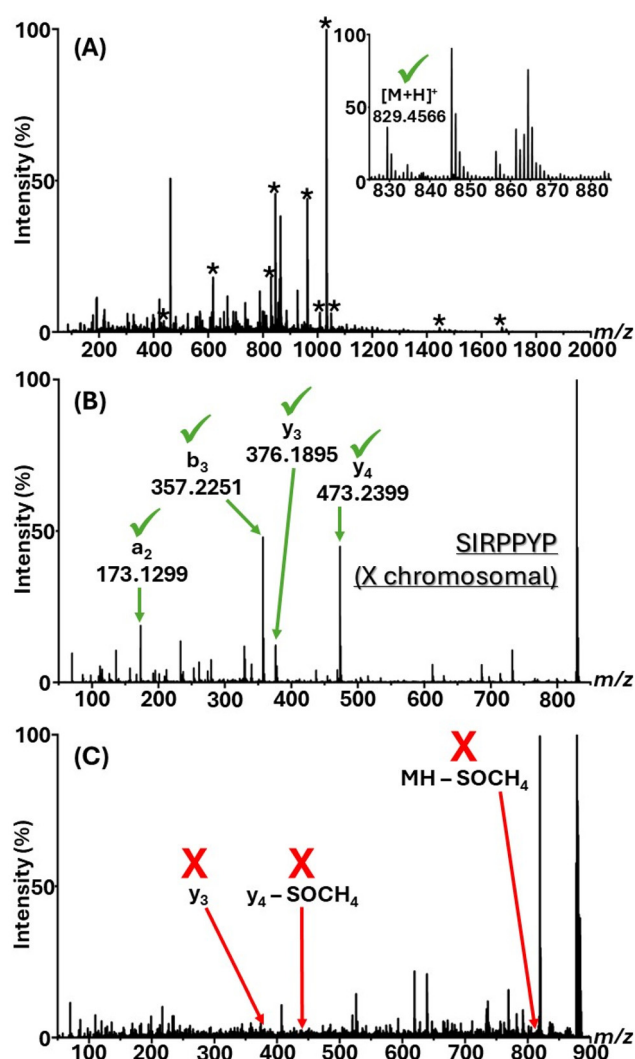


Fig. 3 LAP-MALDI MS and MS/MS spectra for the tooth of skeleton 106 (female; cf. Table 1). (A) LAP-MALDI MS profile with inset displaying the m/z region for the singly charged $SM_{ox}IRPPY$ (Y chromosome) and $SIRPPYP$ (X chromosome) peptides. Ion signals obtained from peptides extracted from the tooth 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 (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

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.^{2,3,10,13,38–41} For biological sex estimation, amelogenin peptides extracted from



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

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