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Fast methods based on mass spectrometry for peptide identification. Application to sex determination of human remains in tooth enamel

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ABSTRACT

Two new fast methods are proposed for the first time to assign biological sex to prehistoric human remains. Both methods are supported on sexually dimorphic amelogenin protein fragments (AMELX and AMELY) analysis in tooth enamel. The first one is based on flow injection analysis, electrospray ionization and high-resolution mass spectrometry (FIA-ESI-HRMS) with a run time of three minutes per sample. The second method is based on tandem mass spectrometry (FIA-ESI-MS/MS) with a low-resolution mass spectrometer. In this case, run time is one minute per sample.

When FIA-ESI-HRMS was used, two accurate mass-to-charge ratios, corresponding to the diprotonated ions of the molecular species $[M + 2H]^{2+}$ of both peptides, and 6 MS/MS transitions, 3 characteristics of peptide specific to the Y isoform of amelogenin and 3 to the X, were selected for identification purposes. In the FIA-ESI-MS/MS method, 6 MS/MS transitions, 3 characteristics of peptide specific to the Y isoform of amelogenin and 3 to the X, were measured. In both cases, no separation step is carried out once the sample is injected into the system.

The two methods were applied to a set of 16 tooth samples from prehistoric human remains. The results obtained in the sex determination with the rapid methods were confirmed using a liquid chromatographic based method (LC-HRMS). Results were in complete agreement among methods.

A very important increase of sample throughput was obtained with the new proposed methods. When the LC-HRMS method was used, time between sample injections was 101.4 min (run time, 100 min; time require for injection, 1.4 min). When the FIA-ESI-HRMS and FIA-ESI-MS/MS methods were used, in this time (101.4 min) it was possible to analyze 23 and 48 samples, respectively. Moreover, the possibility to assign sex using low-resolution mass spectrometers means that a greater number of laboratories could perform AMEL analysis because the cost of the instrumentation is reduced.

1. Introduction

Currently, there is an important increase in the development of non-separative methods based on mass spectrometry [1–5]. They have the advantage of providing results faster than the other techniques since separation of the analytes in the samples is not carried out. They have a high sample throughput, and a considerable amount of time and money is saved.

Teeth are a type of abundant fossil at archaeological sites. They contain life history traits such as sexual dimorphism, perinatal life characteristics, growth, etc. Dental enamel is the hardest tissue in vertebrates, and it is less porous than bone. Because of these features, it is the best-preserved tissue from remains which includes teeth. Mature enamel contains a slight amount of protein (<1%). Among it, amelogenin (AMEL), ameloblastin (AMBN), and enamelin (ENAM) are some examples of tooth specific proteins [6,7].

The assignment of biological sex has been carried out mainly by means of two methods: analysis of sexually dimorphic osteological features and detection of DNA markers [8–10]. The first approach is fast and non-destructive, but sex determination of human skeletal remains is more accurate after the individual reaches maturity and there is always a degree of uncertainty that depends on the method chosen, the material

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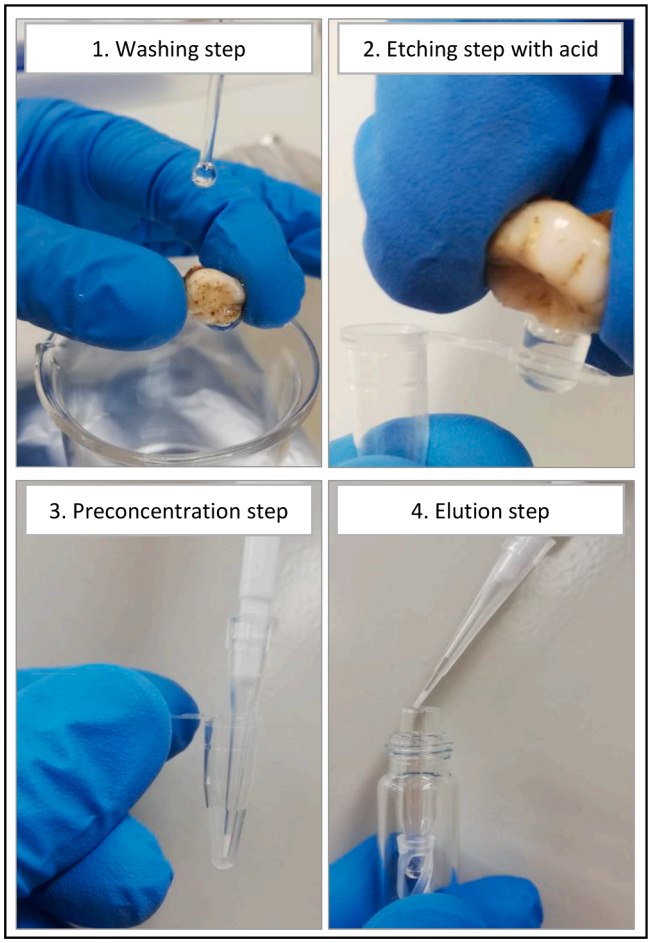

Fig. 1. Photographs of the main steps involved in the sample preparation.

Table 1
m/z ratios and PRM and MRM transitions used with the proposed non-separative methods.

HRMS		**Full MS**	**PRM**	**NCE**
	AMELX	540.2796	879.4343	20
			714.3920	
			366.1653	
	AMELY	440.2233	645.3707	20
			532.2870	
			279.1333	
MS/MS		**Precursor ion**	**Product ions**	**CE**
	AMELX	540.3	879.5	15
			714.4	
			366.2	
	AMELY	440.2	645.4	15
			532.3	
			279.1	

analyzed and even the experience of the observer. DNA analysis has some difficulties related to preservation, contamination, and expense. Another possibility to assign biological sex is to detect peptides specific to the X (AMELX) and Y (AMELY) chromosome-encoded gene products of amelogenin [6,7]. Protein is more robust than the relatively fragile DNA molecule, especially in the case of being associated with mineral surfaces such as enamel. In this sense, the development of mass spectrometry (MS) has made it possible to detect and identify a marker peptide specific to each amelogenin protein.

Sex determination from peptides in tooth enamel has recently been performed using different methods [6,7,10–17,18] based on liquid chromatography (LC) and high-resolution mass spectrometry (HRMS). Although excellent results were obtained, the times required for analysis are very high, with chromatographic run times of 60 min or more [6,7,11,12,16]. Moreover, the use of high-resolution mass spectrometers hinders the use of the proposed methods due to not all the laboratories have access to this kind of instrumentation because of its high cost.

Table 2
Characteristics of the studied samples.

No	Province	Location	Archaeological site	Chronology
1	Burgos	Villegas	Tres Chopos-Abarre	Bronze Age
2	León	Sahagún/Calzada del Coto	Canto Blanco	
3	Salamanca	Aldeanueva de la Frontera	Tordillos	
4	Salamanca	Aldeanueva de la Frontera	Tordillos	
5	Salamanca	Aldeanueva de la Frontera	Tordillos	
6	Segovia	Cuéllar	Barco de los Habares	
7	Valladolid	Alaejos	El Tablón	
8	Valladolid	Quintanilla de Onésimo	El Cementerio	
9	Valladolid	Tudela de Duero	Soto de Tovilla	
10	Zamora	Zamora	ConsejoConsultivo	
11	Soria	Caracena	Los Tolmos	
12	Zamora	Benavente	Cuestos de la Estación	
13	Valladolid	Tudela de Duero	Soto de Tovilla	Chalcolithic
14	Burgos	Villegas	Tres Chopos-Abarre	Age
15	Burgos	Villegas	Tres Chopos-Abarre	
16	Valladolid	Fuente Olmedo	Perro Alto	Bell Beaker period

Taking this into account, the aim of this work is to develop two different fast non-separative analytical methods based on MS for biological sex determination of human remains from peptides in tooth enamel. The first one is based on flow injection analysis, electrospray ionization and high-resolution mass spectrometry (FIA-ESI-HRMS). The second method is based on tandem mass spectrometry (FIA-ESI-MS/MS) using a low-resolution mass spectrometer to reduce instrumentation cost. As far as we know, this is the first contribution in this field using fast methods which do not include chromatographic separation. The results obtained with the two fast methodologies were confirmed using a liquid chromatography method (LC-ESI-HRMS).

Since the aim of this work is the development of different analytical methods and not a routine analysis of many samples, the number of prehistoric samples analyzed (16) is not high. However, on many occasions, archaeologists find human remains of hundreds of individuals and it is important to have methodologies in which the analysis time, the costs per sample and the expense of reagents are reduced compared to previous chromatographic analyses described in the literature. In addition, reliable results could be available in shorter periods of time, which facilitates work and decision-making.

2. Experimental section

2.1. Materials

Formic acid, trifluoracetic acid and acetonitrile were supplied by Sigma-Aldrich (Steinheim, Germany). HCl was obtained from Scharlau (Barcelona, Spain) and H_2O_2 was supplied by Panreac (Barcelona, Spain). The ultra-high quality (UHQ) water used was obtained with a Wasserlab Ultramatic purification system (Noain, Spain).

2.2. Sample preparation

Sample preparation has been adapted from prior publications [7,11] in which chromatographic methods are used. Tooth enamel was washed

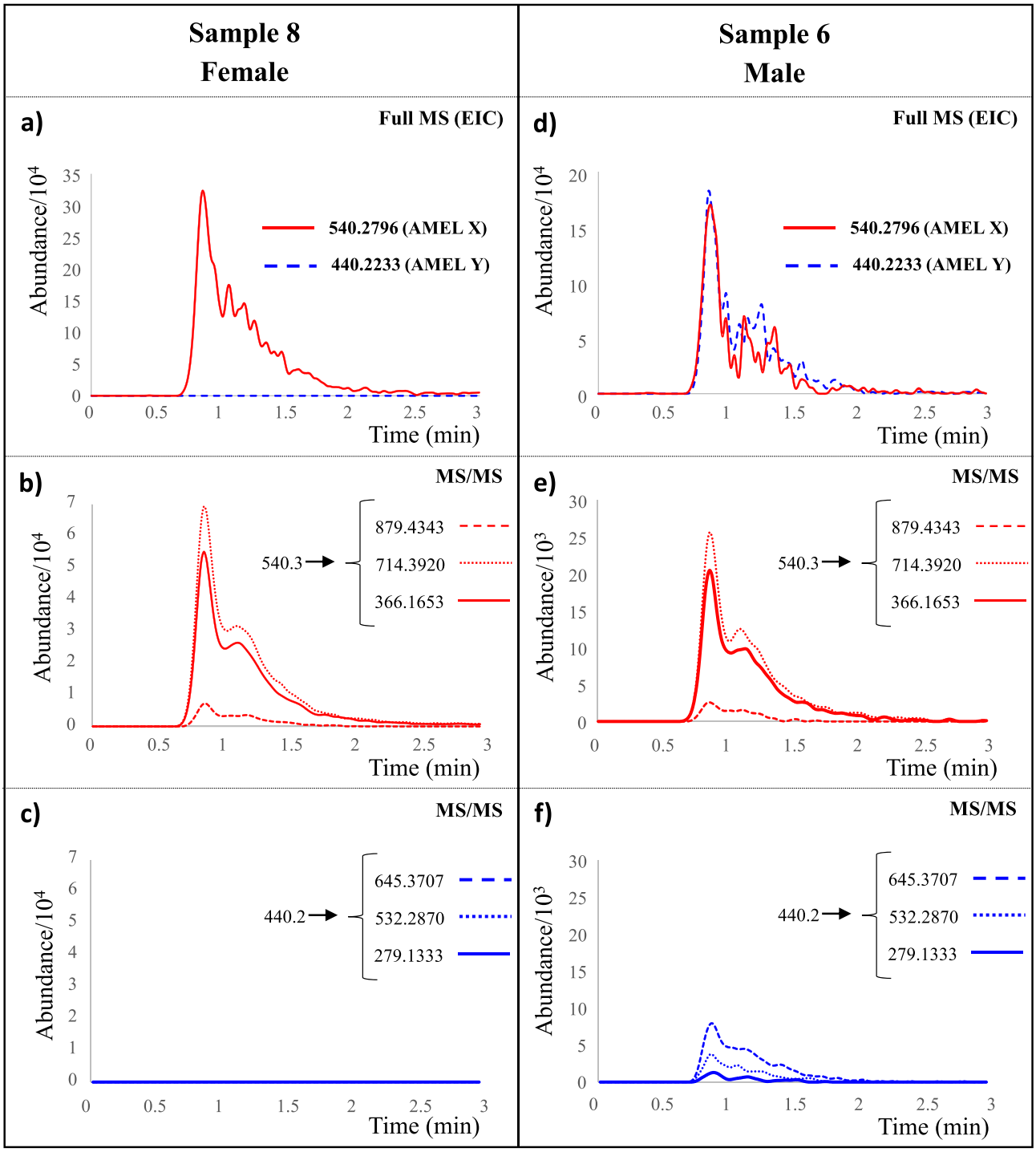

Fig. 2. FIA-ESI-HRMS method. Extracted ion chromatograms for the *m/z* ratios 440.2233 (AMEL Y) and 540.2796 (AMEL X) when using full MS analysis mode for sample 8 (a) and sample 6 (d). MS/MS signals for AMELX selected transitions for sample 8 (b) and sample 6 (e). MS/MS signals for AMELY selected transitions for sample 8 (c) and sample 6 (f).

with 3 % H_2O_2 for 30 s and then rinsed with ultrapure water. Then, 60 μL of 5 % (v/v) HCl was placed in the cap of a 0.2 mL Eppendorf tube and an initial etch was performed by lowering the tooth on the HCl and maintaining contact for 2 min. This first etch was discarded. A second 2-min etch was then performed and retained as the etch solution (sample solution).

A Zip-Tip® with 0.6 μL C18 resin (Merck Millipore, Ireland) was used for desalting and concentrating peptides. The sorbent was first conditioned with 3 cycles of 10 μL of acetonitrile, followed by 3 cycles of UHQ water with formic acid (10 μL) at a concentration of 0.1 % (v/v) (extract-discard mode). The sample was then extracted (10 cycles × 10 μL, draw-eject into the same vial). The Zip-Tip® was washed 6 times with 10 μL of UHQ water (0.1 % formic acid, v/v), with each wash discarded. Elution was performed using 4 μL of an acetonitrile/UHQ water/formic acid mixture (60/40/0.1 %, v/v). Extract was evaporated to dryness and reconstituted with 12 μL of UHQ water (0.1% trifluoroacetic acid, v/v) and analyzed. Each tooth could be analyzed several times, performing the sample treatment procedure at different points in the sample. Fig. 1 shows a scheme of the steps involved in the sample preparation.

Table 3
Results obtained in the determination of the sex of prehistoric human remains with the different methods proposed. Additionally, the sex estimated by anthropological methods is shown.

Samples	Sex			
	Proposed methods		Confirmation	Anthropological
	FIA-ESI-HRMS	FIA-ESI-MS/MS	LC-ESI-HRMS	
1	Female	Female	Female	Indeterminate
2	Male	Male	Male	Male
3	Male	Male	Male	Indeterminate[a] (6–8 years old)
4	Female	Female	Female	Female
5	Female	Female	Female	Female
6	Male	Male	Male	Female[b]
7	Male	Male	Male	Indeterminate[a] (5–6 years old)
8	Female	Female	Female	Indeterminate[a] (6–7 years old)
9	Male	Male	Male	Indeterminate[a](2–4 years old)
10	Female	Female	Female	Indeterminate[a] (7–11 months old)
11	Female	Female	Female	Indeterminate[a] (8–9 years old)
12	Female	Female	Female	Indeterminate[a] (6–8 years old)
13	Male	Male	Male	Male
14	–	–	–	Indeterminate
15	–	–	–	Indeterminate
16	Male	Male	Male	Male

[a] Subadult with estimated age indicated in parentheses.
[b] Estimation with greater uncertainty than the rest of the samples.

2.3. Instrumentation

When FIA-ESI-HRMS was used, extracts were injected into a Vanquish UHPLC system equipped with a Q Exactive Focus Orbitrap spectrometer detector (Thermo Fisher Scientific) without a chromatographic column. Carrier phase was formed by a 90:10 mixture (v/v) of UHQ water and acetonitrile with 0.1% formic acid. The carrier flow rate was 30 μL/min and the injection volume was set to 2 μL. Run time was 3.0 min. Electrospray ionization was done at + 3800 V, setting the capillary temperature to 320 °C and using nitrogen as desolvation gas. AMELX-(44–52) and AMELY-(58–64) peptides were detected in combined Full MS (250–650 *m/z*) and Parallel Reaction Monitoring (PRM) modes, using as precursor ions the molecular diprotonated cations $[M + 2H]^{2+}$of AMELX and AMELY (*m/z* 540.2796 and 440.2233, respectively), and a normalized collision energy (NCE) of 20%. Resolution was set at 70,000 at *m/z* 200. Information regarding MS parameters is shown in Table 1.

For FIA-ESI-MS/MS analysis, extracts were injected in an Acquity UPLC system from Waters (Milford, MA, USA) equipped with a Xevo TQ-S mass spectrometer detector. No analytical column was included in the instrumental configuration. The composition of the carrier phase is the same as described above. Flow rate and injection volume were set at 0.1 mL/min and 5 μL, respectively. Run time was 1.0 min. Electrospray ionization was carried out at +2500 V setting the source temperature to 150 °C. Desolvation was carried out by N_2 at 300 L/h and 400 °C. AMELX and AMELY were detected in MS/MS experiments based on the transition from the molecular diprotonated cations ($[M + 2H]^{2+}$) to different product ions (Table 1). Dwell time was set at 50 ms.

LC-ESI-HRMS method was adapted from previous publications [7,11]. Extracts were injected into the same instrument described for FIA-ESI-HRMS analysis and Full MS (400–1300 *m/z*) and PRM modes were used for MS detection. In this case, peptides were separated by means of a Discovery® BIO Wide pore C18 column (5 μm, 0.32 × 150 mm) with a mobile phase formed by a mixture of (A) UHQ water and (B) acetonitrile, both with 0.1 % formic acid (v/v). The mobile phase flow rate was 30 μL/min and the injection volume was set to 2 μL. Separation was achieved using a gradient starting at 1 % B, increased to 13.3 % B over 13 min, then to 25.6 % B from 13 to 28 min, to 45 % B from 28 to 38 min and to 90 % B from 38 to 41 min, held constant at 90 % B for 15 min, returned to 1 % B over 2 min and held conditions during 32 min. Run time was 100 min.

2.4. Prehistoric samples

Sixteen dental samples from eleven prehistoric sites in the Spanish North Sub-plateau were analysed (Table 2). Most of the samples (1 to 12) correspond to the Bronze Age (*ca.* 1850–1150 cal BC). Some older samples were also included from the Chalcolithic age (*ca.* 3000–2500 cal BC, samples 13–15) and the Bell Beaker period (*ca.* 2500–2000 cal BC, sample 16).

3. Results and discussion

3.1. FIA-ESI-HRMS

All the samples were analyzed using the FIA-ESI-HRMS based method. Fig. 2 shows, as an example, the signals corresponding to samples 8 and 6 (Table 2).

The upper part of the figure (Fig. 2a and 2d) shows the extracted ion signals for the *m/z* ratios 440.2233 and 540.2796, characteristics of AMELY and AMELX peptides, respectively, when using full MS analysis mode. In sample 8 (Fig. 2a), no signal was found for the *m/z* ratio 440.2233, while in sample 6 (Fig. 2d) both *m/z* ratios showed significant abundance. It is known that AMELY peptide is only present in the enamel dental of male individuals, while AMELX is present in both sexes [11].

Fig. 2b and c show the MS/MS signals (sample 8) for AMELX and AMELY selected transitions, respectively (Table 1). All transitions are specific to the corresponding AMEL. For the three transitions corresponding to AMELX peptide, signals were found, being the most abundant the transition 540.3 → 714.3920. For AMELY transitions no signals were found (Fig. 2c).

For sample 6, the transitions selected for both AMELX and AMELY peptides showed significant abundance (Fig. 2e and f). For AMELY, the most abundant transition was the one corresponding to *m/z* 440.2 → 645.3707. In view of these results, it can be stated that the FIA-ESI-HRMS methodology allows the differentiation of samples based on sex. Sample 8 corresponds to a female and sample 6 to a male.

The analysis of the remaining samples allowed, in a similar way, to assign the sex of all the individuals considering the presence or absence of the signals corresponding to the AMELY peptide. The results obtained are shown in Table 3. Assignment of sex was not possible for samples 14 and 15. Both samples did not show any remaining enamel: in the case of sample 14 due to problems related to its conservation and in the case of sample 15 due to a calcination process.

3.2. FIA-ESI-MS/MS

The set of samples previously analyzed using the FIA-ESI-HRMS methodology was analyzed with the FIA-ESI-MS/MS method to check its capacity in sex assignment. In this case, 3 transitions were selected for each AMEL peptide (Table 1).

The results obtained for samples 8 and 6 are shown in Fig. 3. The signal corresponding to sample 8 shows all the transitions of the AMELX peptide (Fig. 3a). However, no signals (Fig. 3b) different from that of the blank sample were found for AMELY peptide transitions.

In the case of sample 6, signal was found (Fig. 3c and d) for the transitions of both AMELX and AMELY peptides.

In view of these results, it can be stated that the FIA-ESI-MS/MS methodology allows the differentiation of samples based on sex using a low-resolution mass spectrometer. This represents an important

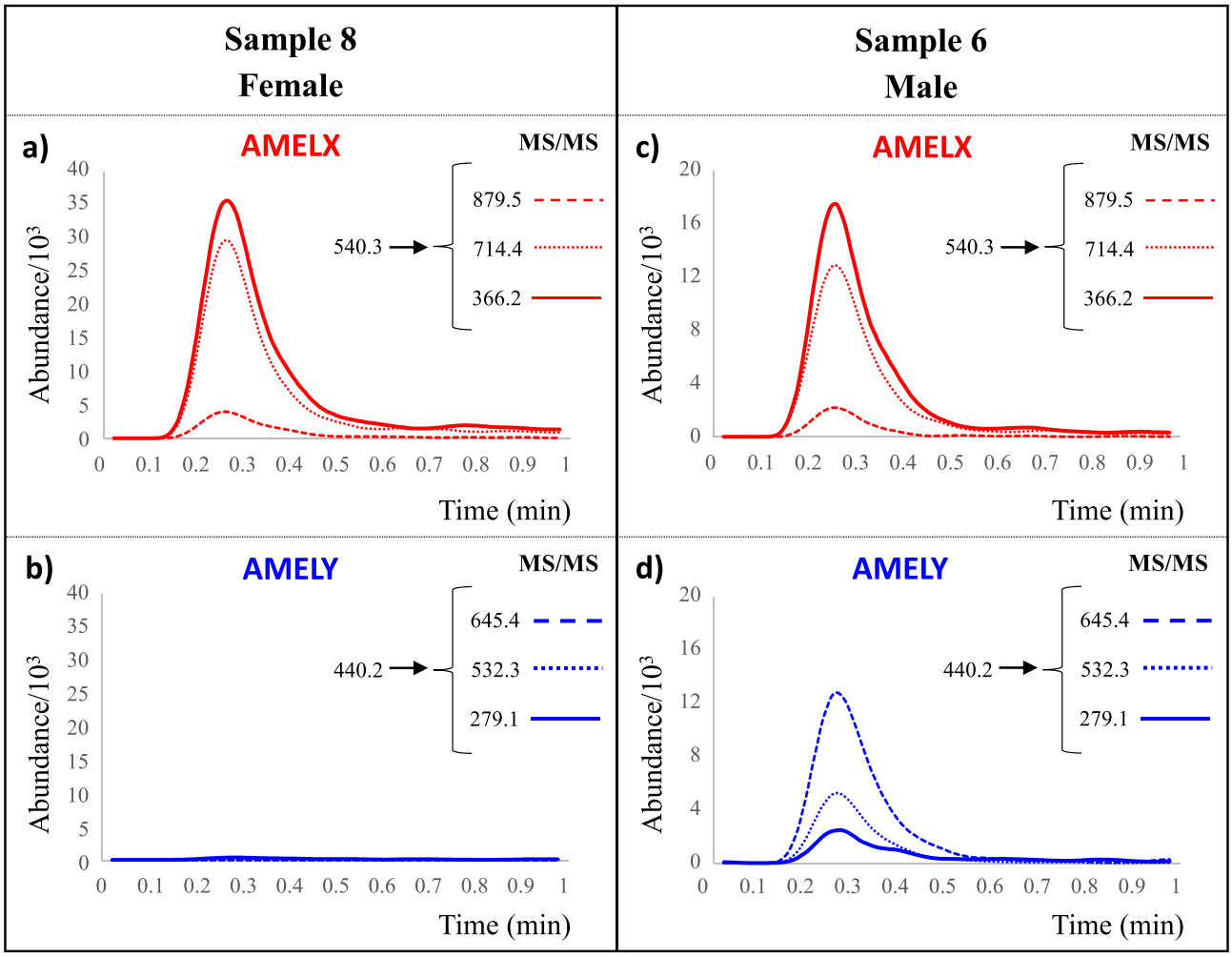

Fig. 3. FIA-ESI-MS/MS method. MS/MS signals for AMELX selected transitions for sample 8 (a) and sample 6 (c). MS/MS signals for AMELY selected transitions for sample 8 (b) and sample 6 (d).

advance since many laboratories could do these analyses since the price of the instrumentation decreases. Sample 8 corresponds to a female and sample 6 to a male. The analysis of the remaining samples allowed, in a similar way, to assign the sex of all the individuals considering the presence or absence of signal for the transitions of the AMELY peptide. The results obtained are shown in Table 3. The problems preventing sex assignment in samples 14 and 15 have been already described in the prior section (FIA-ESI-HRMS).

3.3. LC-ESI-HRMS

To confirm the results obtained with the non-separative methods (FIA-ESI-HRMS and FIA-ESI-MS/MS), the set of available samples was analyzed with the chromatographic method (LC-ESI-HRMS). The results obtained are shown in Table 3. In all cases, there was a good agreement between the two rapid methods and the chromatographic one.

Fig. 4 shows the results obtained for samples 8 and 6. The upper part of the figure shows the extracted ion chromatograms for the *m/z* ratios 540.2796 (AMELX, continuous line) and 440.2233 (AMELY, dotted line). In Fig. 4a, corresponding to sample 8, only the *m/z* ratio 540.2796 was found (t_R = 23.23 min). However, in Fig. 4d, both *m/z* ratios, 540.2796 and 440.2233, were found (t_R = 23.23 and 19.93 min, respectively). In view of these results, it can be stated that sample 8 is a female, and sample 6 is a male. Fig. 4b and e show the full MS spectra for AMELX and AMEL Y peptides, respectively. In the final part of the figure (Fig. 4c and 4f), the MS/MS spectra are shown when the ions of the molecular species were fragmented, using a NCE value of 20%. These results are in good agreement with previously published ones [7,11]. The transitions selected in the non-separative methods are among the most abundant in each case and they are specific to each peptide. The transition 440.2 → 408.2 was not selected for the FIA-ESI-MS/MS method despite being the most abundant in the case of AMELY peptide since it is also present in the female record. This fact indicates the presence of an isobaric interference with the same fragment ion.

3.4. Matrix effects

When stand-alone mass spectrometric methodologies are used, as is the case, ion suppression effects are a critical issue. To evaluate matrix effects, signal-to-noise ratios (S/N) obtained for the two peptides using the chromatographic (LC-ESI-HRMS) and the two non-separative methodologies (FIA-ESI-HRMS and FIA-ESI-MS/MS) were compared.

Matrix effect percentage (ME, %) was obtained according to the following equation:

$$Matrix\ Effect(\%) = \frac{A - B}{A} x100$$

Where A is the S/N ratio of the peptide when the chromatographic method is used and B in the S/N ratio of the peptide when the non-separative methodologies were used. S/N ratios were used instead of areas to compare the results obtained with two different instruments. When HRMS methods were compared, exact mass for both peptides was

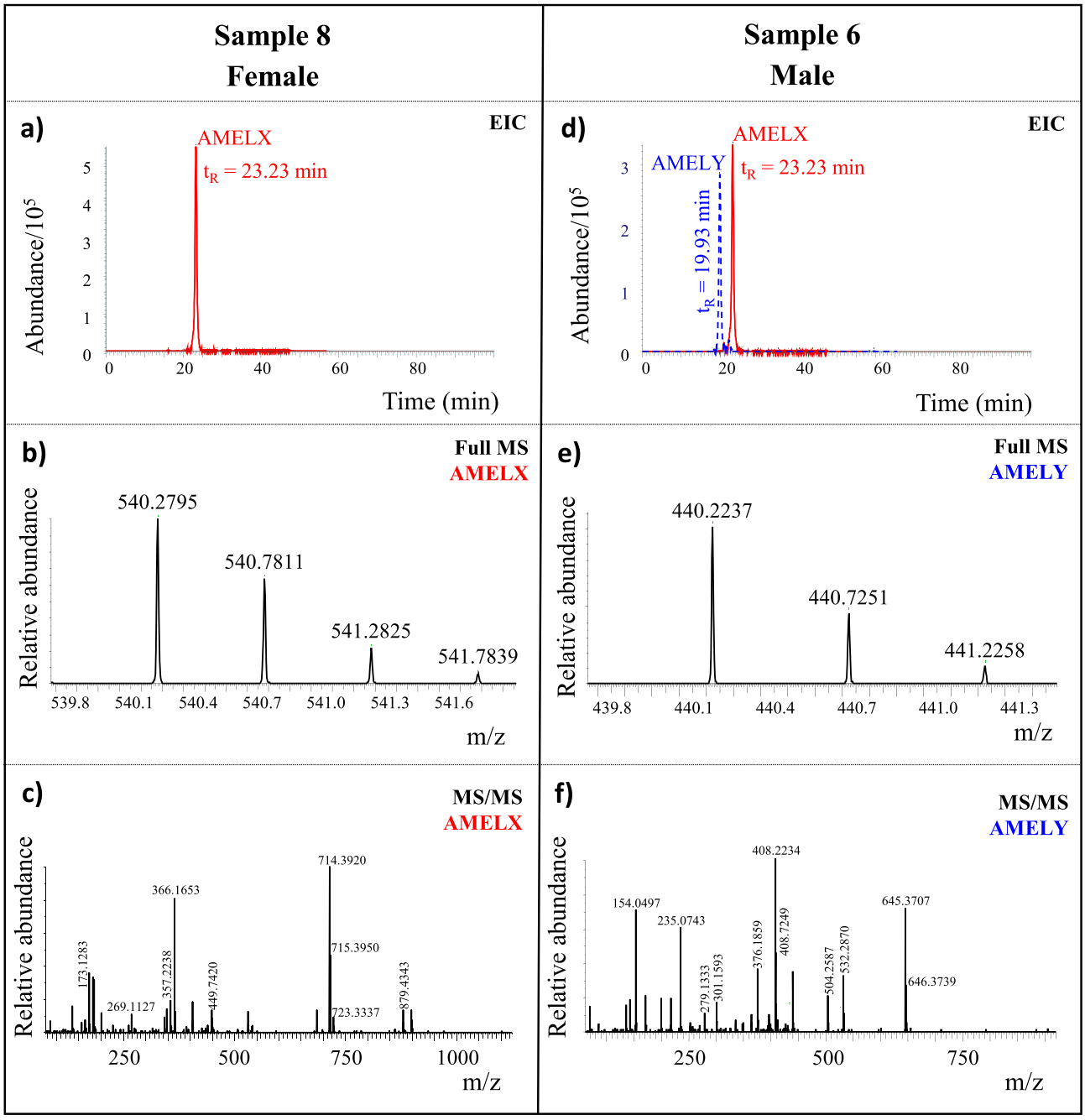

Fig. 4. LC-ESI-HRMS method. Extracted ion chromatograms for the *m/z* ratios 540.2796 (AMELX) and 440.2233 (AMELY) for samples 8 (a) and 6 (d). The full MS spectra for AMELX and AMELY peptides in the samples 8 (b) and 6 (e), respectively. PRM spectra for AMELX (precursor ion *m/z* 540.3) and AMELY (precursor ion *m/z* 440.2) peptides in the samples 8 (c) and 6 (f), respectively.

used. For MS/MS, the most intense transition for each peptide was compared against the exact mass of the chromatographic method.

Matrix effect (%) values are shown in Table 4. If ME ≈ 0, there is no matrix effects. If ME > 0%, an ion suppression occurs and, if ME < 0%, ion-enhancement occurs [19]. It was shown that ion suppression is present when non-separative methods are used, a common situation when this kind of methodologies are used [5]. Nevertheless, this was not detrimental to the methods proposed because only qualitative results are required, and sensitivity was enough to detect both peptides [20].

For the analyzed samples, the area ratio of AMELY to AMELX was between 0.4 and 1.3. These values are in good agreement with previous published results [11]. The sensitivity of the methods proposed, with the lowest AMELX S/N ratios for samples assigned as female (no AMELY detected) being 156 (HPLC-ESI-HRMS), 40 (FIA-ESI-HRMS) and 21 (FIA-ESI-MS/MS), ensures the correct sex assignment based on AMELY absence. In every case, S/N ratios are much higher than the S/N ratio corresponding to the limit of detection (S/N = 3). The sensitivity of the proposed method allows both peptides to be identified without any problem even in samples corresponding to few years old infants.

3.5. Time reduction

Fig. 5 shows a schematic diagram of the times required for the two proposed method. For comparative purposes, the chromatographic method has also been included.

As can be seen, a significant decrease on the run time was obtained

Table 4
Matrix effect (ME, %) values obtained for both peptides when both non-separative methods were used.

		FIA-ESI-HRMS ME (%)		FIA-ESI-MS/MS ME (%)	
Sample	Sex assignment	AMELX	AMELY	AMELX	AMELY
1	XX	81	—	87	—
2	XY	80	70	89	80
3	XY	53	58	78	67
4	XX	88	—	94	—
5	XX	72	—	86	—
6	XY	81	72	76	63
7	XY	83	58	61	47
8	XX	77	—	90	—
9	XY	67	43	90	61
10	XX	77	—	89	—
11	XY	83	52	89	64
12	——	——	——	——	——
13	XY	41	38	80	71
14	XX	68	—	82	—
15	XX	70	—	80	—
16	——	——	——		——

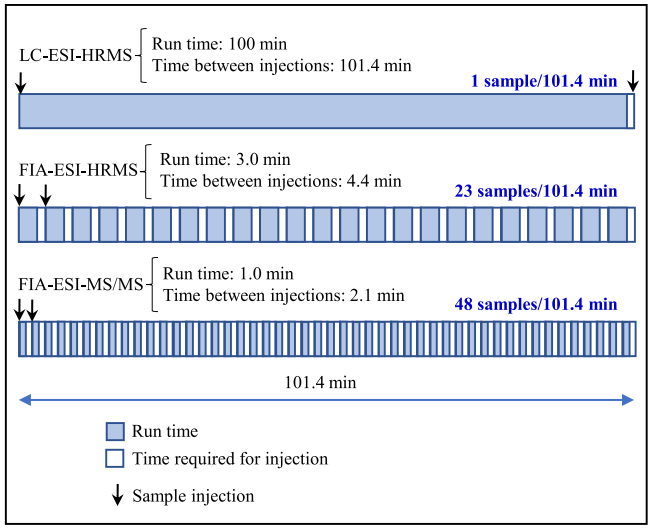

Fig. 5. Diagram of the times required, and number of samples analyzed with the two proposed methods and the confirmatory chromatographic one.

when the new non-separative methods were used. Times between injections for the FIA-ESI-HRMS and FIA-ESI-MS/MS methods were 4.4 and 2.1 min, respectively. When the LC-ESI-HRMS method was used, time between injections was 101.4 min. This involves a huge increment of sample throughput with the non-separative methods, being possible to analyze 23 (FIA-ESI-HRMS) and 48 samples (FIA-ESI-MS/MS) in the time required to analyze only one sample with the chromatographic method.

3.6. Anthropological assignment

Table 3 also shows the results obtained in the anthropological assignment of the samples. It is important to note that the anthropological method presents difficulties in estimating sex for individuals with younger ages. As shown in Table 3, most of the times that sex determination with the anthropological method was not possible it is due to this reason (samples 3, 7, 8, 9, 10, 11 and 12). The analytical methods developed in this work allowed the determination of sex in 14 of the samples studied. In the other two samples, there was no remaining enamel.

The only case in which the anthropological estimation of sex differs from the analytical methods proposed here correspond to sample 6. The state of conservation of the skeletal material forced the estimation to be based only on the observable characters in the skull, not being able to be compared with the pelvis, which generally offers greater precision [21]. The skull of this individual presented a gracile appearance [22], with morphological features that led to classification as a possible woman. This is a good example of the high uncertainty that should be accepted when anthropological assignment is based on observable qualitative criteria in the skeleton [23,24].

4. Conclusions

Two rapid methods have been developed for the assignment of sex in prehistoric human remains by analyzing peptides in tooth enamel. Both methods are based in non-separative analysis: they do not involve any chromatographic separation step prior to MS detection once the sample is introduced into the system. Results were confirmed using a LC method and, in all cases, the percentage of agreement between methods was 100%.

One of the greatest contributions of this work is the reduction of the time of analysis. Usually, the existing methods in the literature are based on chromatographic separation with run times of 60 min or more. With the proposed methods, the run time is considerably reduced: 1 min for FIA-ESI-MS/MS method and 3 min for FIA-ESI-HRMS. This time reduction is due two reasons: (1) no separation of the compounds is performed and (2) as there is not a chromatographic column, it is not necessary to re-equilibrate it before the next analysis. This reduction in analysis time is very significant.

Another outstanding contribution presented here is the ability to identify the sex of an individual without the need of high-resolution mass spectrometers (by using the FIA-ESI-MS/MS methodology). This allows many laboratories to perform this kind of analysis, as instrumentation expenses are critically reduced.

CRediT authorship contribution statement

Ana María Casas-Ferreira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Miguel del Nogal-Sánchez:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Ángel Esparza Arroyo:** Resources, Writing – review & editing. **Javier Velasco Vázquez:** Resources, Writing – review & editing. **Jose Luís Pérez-Pavón:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The 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.

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