

Proteomic determination of sex followed the method of Stewart et al. (2017). This method is based on the analysis of two dimorphic peptides of amelogenin: the AMELY-(58-64) peptide and AMELX-(44-52) peptide from tooth enamel. Teeth of males contain both peptides (AMELY and AMELX) while female teeth contain only one (AMELX).

Sample preparation

The tooth surface was cleaned by water to remove obvious surface contaminants. At the next step tooth crown was placed at cap of a 2-mL Eppendorf tube and washed with 3% H₂O₂ (200 µl) for 30 s and then rinsed with ultrapure water. The next step treated by 200 µL of 5% (vol/vol) HCl. An initial etch was performed by lowering the tooth onto the HCl and maintaining contact for 2 min. This first etch was discarded. A second 2-min etch was then used for analysis. For peptide extraction from this solution was used a C18 resin-loaded ZipTip (ZTC18S096; EMD Millipore). It was previously conditioned three times with 100% acetonitrile, then three times with 0.1% (vol/vol) formic acid (each draw discarded). The peptides were bound to the ZipTip (by up and down pipetting by 10 times. The ZipTip was washed six times with 0.1% (vol/vol) formic acid (each wash discarded). Bounded peptides were eluted by 4-µL of 60% acetonitrile/0.1% formic acid. This fraction was lyophilized and dissolved in 20 µL of 2% formic acid in water, centrifuged on a benchtop centrifuge for 5 min to remove any particulate matter, and transferred (18 µL) to autosampler vials. Then this sample was injected for analysis by reversed-phase HPLC-MS.

Method of analysis

The nano-LC apparatus was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a MaXis Q-TOF (quadrupole – time of flight) mass spectrometer with ultra-high resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. Five microliters of the peptide mixture injected into a NS-AC-11 BioSphere C18 column (particle size: 5µm, pore size: 12 nm, length: 152 mm, inner diameter: 75 µm), with a NS-MP-10 BioSphere C18 pre-column (particle size: 5 µm, pore size: 12 nm, length: 20 mm, inner diameter: 100 µm), both obtained from NanoSeparations (Nieuwkoop, Netherlands).

The separation of peptides was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1% (v/v) formic acid. Separation was started with a gradient elution from 5% to 30% mobile phase B at 70 min. The next step was gradient elution to 50% B in 10 min, and then a gradient to 100% B in 10 min was used. Finally, the column was eluted with 100% B for 30 min. Equilibration before the subsequent run was achieved by washing the column with 3 µl of 5% mobile phase B for 5 min. The flow rate was 0.25 µl min⁻¹, and the column was held at ambient temperature (25°C).

Online nano-electrospray ionization (easy nano-ESI) in the positive mode was used. The ESI voltage was set at +4.5 kV, spectra rate 3 Hz. Operating conditions: drying gas (N₂), 4 l min⁻¹; drying gas temperature, 180°C; nebulizer pressure, 100 kPa. Experiments were performed by scanning from 420 to 500 m/z.

Results

Examples of MS (MS/MS) results are presented in Figs. S1-S3. The male sample gives the signal for two peptides (AMELY-peptide (440.22 m/z) and AMELX-peptide (540.28 m/z) but the female sample only for AMELX-peptide (540.28 m/z). The sex estimations for particular individuals included in the study are given in Table S1.

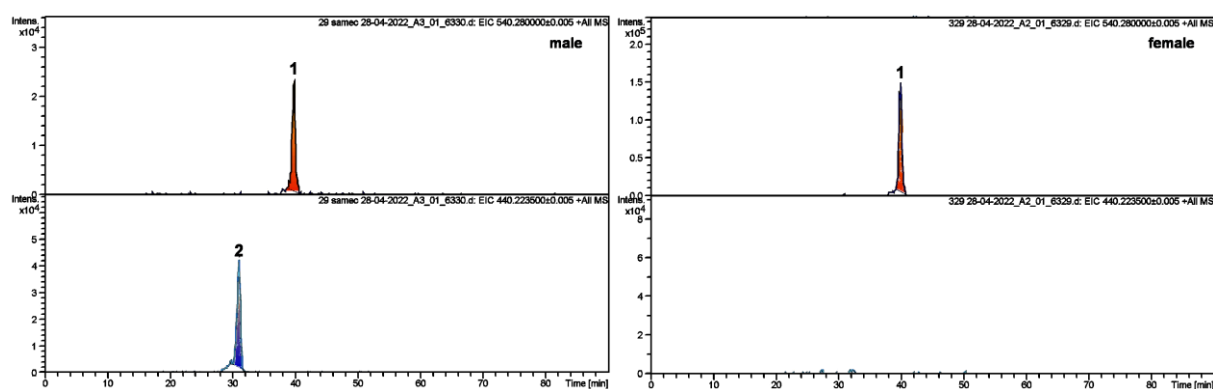


Figure S1 LC/MS analysis of male and female tooth 1 - AMELX-peptide – SIRPPYPSY, 2 - AMELY-peptide - SM(ox)IRPPY

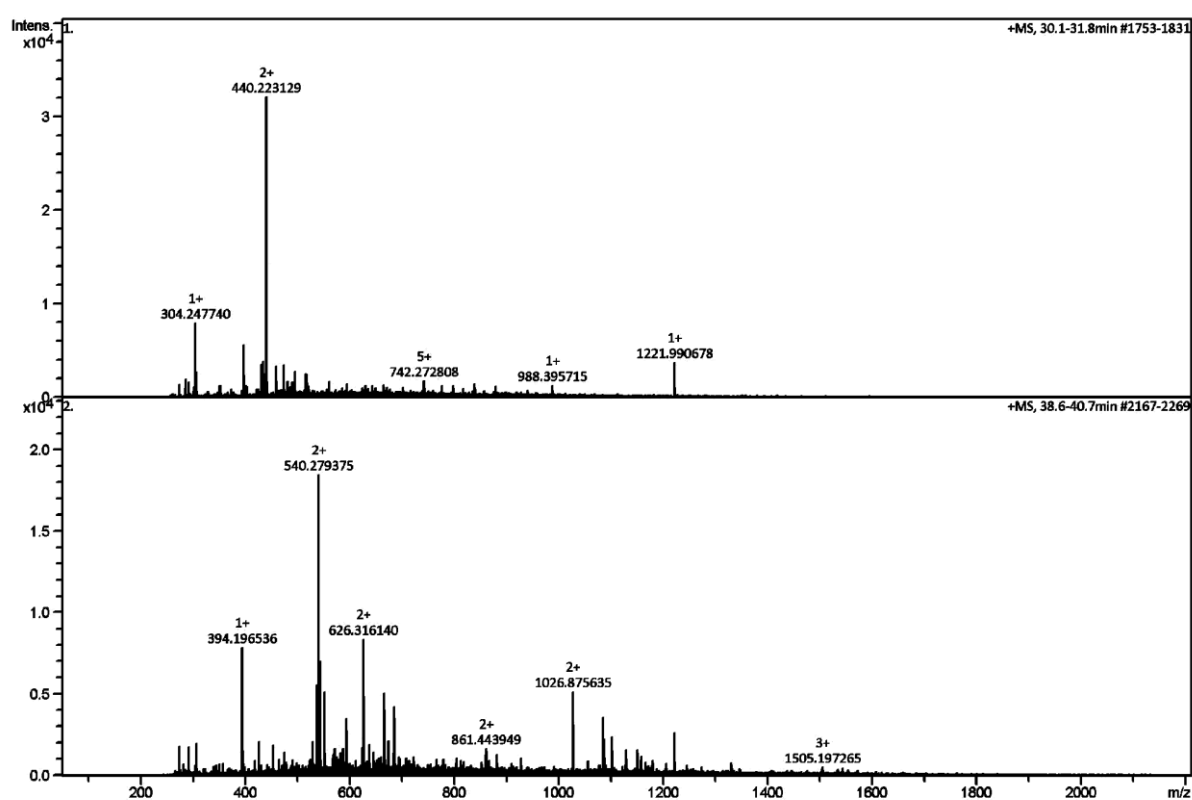


Figure S2 MS spectra of AMELY-peptide (SM(ox)IRPPY – 440.22 m/z) and AMELX-peptide (SIRPPYPSY – 540.28 m/z)

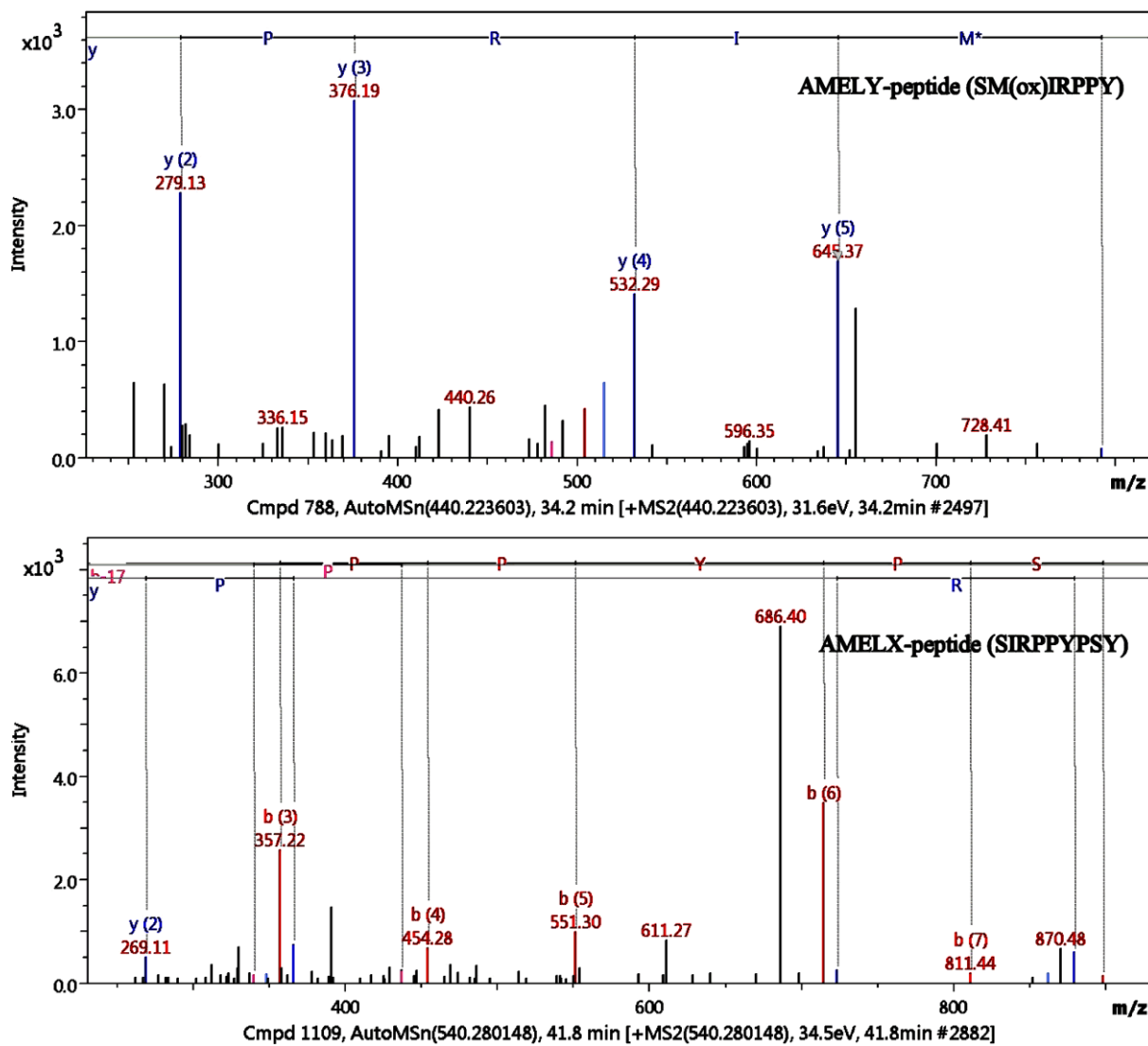


Figure S3 MS/MS spectra of AMELY-peptide (SM(ox)IRPPY – 440.22 m/z) and AMELX-peptide (SIRPPYPSY – 540.28 m/z)

Table S1

Sex estimations for subadult individuals included into the study

Grave No.	Sex	retention time/peak area	
		X	Y
H73-VI	M	404290	408317
H143	M	985473	1057251
H160-VI	F	806030	Not present
H207	M	713327	1009648
H247	F	799429	Not present
H253	F	1254321	Not present
H266	M	187410	446308
H296	M	770229	1102988
H315	F	1211179	Not present
H343	M	1260716	1923015
H393	F	1186517	Not present
H444	M	799936	1221249
H447	M	776379	644600
H454	F	949155	Not present
H455	F	533605	Not present
H462	F	1498764	Not present
H473	M	556056	702444
H489	M	759236	1023086
H496	F	560303	Not present
H497	M	999491	694996
H526	M	348887	593259
H538	F	297597	Not present
H550	F	566669	Not present
H582	M	733286	1023541
H751	F	388359	Not present
H792	M	1006139	1233096
H878	M	915958	825651
H881	F	949087	Not present
H1041	F	367721	Not present
H1058	F	465283	Not present
H1154	M	671200	1374627
H1171	F	640652	Not present

Reference

N. A. Stewart, R. F. Gerlach, R. L. Gowland, K. J. Gron, J. Montgomery, Sex determination of human remains from peptides in tooth enamel. *Proc. Natl. Acad. Sci. U.S.A.* 114, 13649–13654 (2017).