Appendix A. Supplementary material

First successful detection of oestrogen, progesterone and testosterone in multiple human hard tissues, and their use as potential biomarkers of pregnancy.

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Sample Preparation

Whole tooth samples were abraded with a rose-head dental burr to remove any adhered material on the enamel and root surfaces. All calculus, teeth and bone fragments were submerged in isopropyl alcohol and gently mixed for 5 minutes. This wash procedure was repeated twice, and samples were allowed to air dry at room temperature in a clean fume cupboard.

Second and third molars were bisected with a rotary saw to a standard suitable for further histological or isotope analysis. The extraction of enamel, dentine and root was focused on one half of each tooth, retaining the antimere for future study. A novel methodology for extracting dentine and enamel was developed. Teeth were held, and dentine was drilled with a rose-head burr at a slow rotation to minimise the risk of burning the sample and to achieve maximal milling potential (Zhao et al., 2023). Dentine powder was collected in a clean tin foil boat. Once the correct sample weight was obtained, the remaining crown dentine was milled, weighed and stored. Dentine was removed from the enamel with either a round burr and/or a diamond burr.

The enamel shell was removed from the root at the cementum enamel junction by applying pressure and drilling through the junction. Any residual dentine or root was removed from the enamel fragments with a dental burr. Finally, the enamel fragments were washed twice in an isotonic bath to ensure no residual powdered dentine remained. The circumpulpal dentine and remaining cementum were abraded from the tooth roots.

Each sample of calculus, bone, enamel and root was ground separately to a fine powder using a stainless steel pestle and mortar; all tools were cleaned with acetone between samples. Dental burrs were cleaned additionally in an isotonic bath with distilled water to reduce cross-contamination of samples. All samples were stored in new sample bags or sterile Eppendorf or centrifuge tubes at each stage of the study to reduce external and cross-contamination. Protective gloves with an American National Standards Institute cutting standard of A8 were worn throughout tooth preparation. The intention was to provide high-cut protection to minimise hormone contamination from sweat and blood serum in the case of any potential puncture or laceration injuries obtained during sample preparation. Additional personal protective equipment included a Portwest bizflame overall, safety glasses, a head visor and chemical gauntlets.

Data validation

The experimental protocol enabled differentiation of negative results, positive results and false positives. Negative results were obtained when hormones were absent or present at levels below the detection sensitivity of the ELISA kit. The sensitivities and Limit of Detection of the EIA kits, determined by the manufacturer, are as follows:

- Arbor Assays multispecies DetectX® progesterone (K025-H1) sensitivity 47.9 pg/mL, Limit of Detection 52.9 pg/mL
- Arbor Assays multispecies DetectX® sensitivity 17β-Estradiol (oestrogen) (K030-H1) 39.6 pg/mL, Limit of Detection 26.5 pg/mL
- Arbor Assays multispecies DetectX® testosterone (K032-H1) sensitivity 9.92 pg/mL, Limit of Detection 30.6 pg/mL

Competitive multi-species ELISA test kits were used in this study, for which non-specific binding wells (NSB) and B0 wells are used to identify false positive readings. NSB and B0 wells contain neither samples nor standards. NSB wells detect conjugate non-specifically bound to the well, in addition to the background signal of the substrate (Arbor Assays, 2021). B0 wells, known as maximum binding wells, detect the maximum signal generated from the enzyme bound to the hormone antibody (Arbor Assays, 2021). Thus, NSB and B0 wells act as controls and are considered when calculating results. Another control measure was implemented on reading the optical density of each well, that being the addition of a reference wavelength of 540nm; this was used to compensate for the wavelength of the plate without its contents. During raw data calculation by the plate reader, the reference wavelength is deducted from the measurement wavelengths. Whilst using a reference wavelength was not essential according to the manufacturer's protocols, we considered it best practice to include it in the data interpretation.

The raw data generated from the optical density reading was interpreted via reduction of the data using a 4th order polynomial curve (4PLC). A commercially available cross-platform software, MyAssays (MyAssays, 2023) was used to calculate the 4PLC for hormone standards and individual sample readings. This software corrects samples by the mean of the NSB measurements. A 4PLC is fitted by plotting the hormonal standard concentrations versus the B0 measurements on semi-log axes. The software compares the raw data of the unknown samples to their fit within the 4PLC.

In the event of detected hormonal concentrations outside the standard measurement range or generated curve on 4PLC, the software is able to identify and highlight these readings. While most of these extrapolated readings provide a numerical value, these results are not statistically significant when assay controls are considered and thus are interpreted as false positives and omitted from the analysis.

References

Arbor Assays, 2021. In an EIA / ELISA or CLIA, what are NSB, B0, and Zero Standard? [WWW Document]. Arbor Assays. URL https://www.arborassays.com/questions/in-an-eia-elisa-or-clia-what-are-nsb-b0-and-zero-standard/ (accessed 11.22.23).

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