Hydroxyproline extracted from *Edmontosaurus* fossil bone from the late Cretaceous

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INTRODUCTION

Research results from the last half century report organic molecules including proteins in fossil bones, in contrast with the common assumption that these would have been completely mineralized.[1] Sequencing by mass spectrometry of fossil proteins has given rise to the new discipline of paleoproteomics.[2] However, since biomolecules are labile, their identification in fossils is often contested. We present spectroscopic, microscopic, and mass spectrometric data from *Edmontosaurus annectens* (UOL GEO.1) bone from Upper Cretaceous Hell Creek Formation strata from South Dakota. We compare this with artificially decayed modern turkey (*Meleagris gallopavo*) bone. Hydroxyproline is most common in collagen. Its extraction and quantification using tandem LC-MS/MS has not yet been performed on acid-digested fossil and modern bones. Cross-polarized light micrographs of thin sections can corroborate endogenous bone collagen remnants.

- **Research question**: What technique(s) can detect collagenous remnants in fossil bone?

Bone Photography

Motion photogrammetry was used to 3D model the object prior to sampling (fig. 1). The model was reconstructed using 1061 images in Agisoft Metashape s/w version 1.7.2.

Further high-resolution photos were collected from a Keyence VHX-7000 4k digital microscope (fig. 2).

Fig. 1

Fig. 2

FTIR

FTIR in ATR mode experiments were performed using a Bruker Vertex 70©, 32 scans per spectrum, and a resolution of 2 cm^{-1}. Proprietary OPUS s/w was used. Thin cuts from *Edmontosaurus* (fig. 2) were ground to <50 µm particles with mortar & pestle.

The FTIR spectrum from the Edmontosaurus sample (fig. 3) shows a distinct PO_4 peak at wavenumber 1033 cm^{-1}, associated with bioapatite. Importantly, a small peak visible at 1652 cm^{-1} indicates the presence of carbonyl (C=O) groups,[3] possibly from endogenous proteinaceous remnants. The ratio of the peak intensities of the carbonyl and phosphate groups is 0.065 (0.011/0.168). Ratios can be related for age comparison. For thermally decayed modern turkey, this ratio is 0.455 (0.005/0.011).

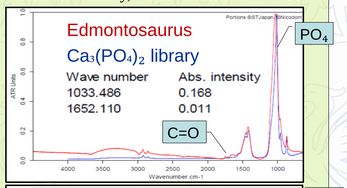


Fig. 3: *Edmontosaurus* bone ATR-FTIR spectrum

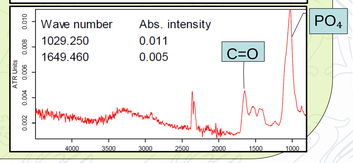


Fig. 4: Turkey bone ATR-FTIR spectrum

BOTTOM-UP PROTEOMICS

LC-MS/MS (Q/ToF) using a Waters™ Xevo G2-XS scanning in positive mode at 3.3 kV capillary voltage.

Trypsin digested *Edmontosaurus* and Modern turkey bone samples show congruence in detected peptides, with several collagen-derived peptides in common (fig. 5). The data are also consistent with bovine Achilles tendon results.[4]

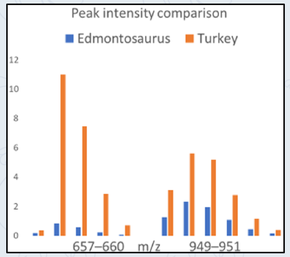
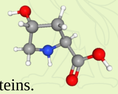


Fig. 5: LC-MS/MS
Aligned collagen fragmentation mass peaks for *Edmontosaurus* and turkey bone.

HYDROXYPROLINE



LC-MS/MS (LIT/Orbitrap) using a Thermo Scientific Orbitrap LTQ XL.

The ideal collagen amino acid

Hydroxyproline (Hyp) is found in few proteins. One of them is collagen, where its abundance is 7.8% in bovine bone. LC-MS/MS unambiguously identified Hyp in acid-digested, HCl-treated samples from two types of bone specimens. The formed parent ions (butyl esters of Hyp) at m/z 188 (MH^+) are further fragmented to ions of m/z 132 and 86. Ratios of the peak areas of these two transitions for authentic Hyp_{be} and the bone samples are compared and the results are the same (1.20 and 1.21 respectively). The chromatograms for both overlapped (fig. 8).

Table 1: Hyp content in *Edmontosaurus*.

Sample No.	nanomoles Hyp/ gram of bone	
1	41.7	(211 ppm)
2	19.1	(97 ppm)
3	6.7	(34 ppm)
4	8.9	(45 ppm)
5	15.4	(78 ppm)
6	8.6	(44 ppm)

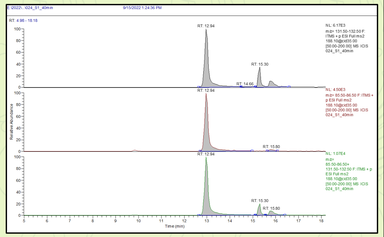


Fig. 8: Hyp chromatogram *Edmontosaurus*. Upper trace shows the transition 188-132, the second one 188-86, and the last one the sum of both transitions.

CONCLUSIONS

ATR-FTIR showed significant abundance of the carbonyl (C=O) moiety, indicating collagen presence in bone samples from modern *Meleagris* (turkey) bone and in *Edmontosaurus annectens* fossil sacrum bone.

Bottom-up proteomics provided congruence between collagen-derived peptides of the same, as well as with bovine results from the literature.

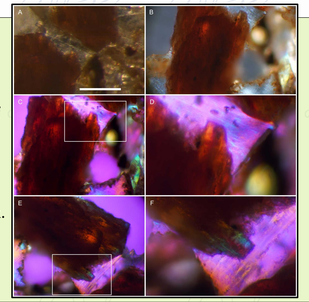
XPol microscopy of thin sections show evidence of bone collagen remaining in patches surrounded by regions without collagen, consistent with diagenetic decay.

LC tandem MS confirmed and quantified for the first time in fossil bone collagen hydroxyproline levels by comparison with modern bone.

Further research is needed to test whether these results would be replicated in other fossils. The combination of these techniques offers potential tools to characterize bone collagen decay patterns.

XPol

Fig. 6: Micrographs of *Edmontosaurus* (UOL GEO.1) thin sections show evidence of bone collagen.



(A) Stereomicrograph includes the region of interest (ROI) seen as a brown peninsula-shaped edge of a bone fragment left of center. Scale bar 200 µm, image collected at 100X.

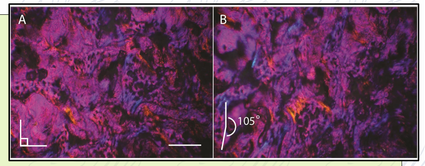
(B) Bone fragment under cross polars (Xpol) oriented to extinction. Thickness of opaque regions (at approx. 0.15mm) of tan-colored bone occlude light, but the thinner margins permit more light and appear gold.

(C) Same bone fragment in XPol with a 1st order red filter. Image 200X.

(D) Expanded view of inset shows the ROI with gold hue.

(E), (F) Same bone fragment after stage was rotated clockwise approx. 100 degrees reveals the gold areas turned blue, green, and lavender. This birefringence is consistent with intact bone collagen remnants.

Fig. 7: XPol micrographs of modern turkey thin sections show birefringence at right angles, consistent with bone collagen. Scale bar 200 µm.



Micrographs show XPol with 1st order red filter of artificially decayed *Meleagris* proximal epiphysis of femur. (A) Gold and blue regions suggest bone collagen remnants. Scale bar 200 µm. (B) Gold regions turn blue and blue regions turn gold after rotating the stage, showing birefringence consistent with bone collagen remnants. Lavender regions with little to no birefringence we interpret as collagen-depleted, similar to the patchy pattern of collagen that XPol reveals in fossil bone (fig. 6).

References

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Abbreviations key

ATR	= Attenuated Total Reflection
FTIR	= Fourier Transform InfraRed
LC-MS	= Liquid Chromatography Mass Spectrometry
XPol	= Cross-polarized light microscopy

Take a picture to contact Prof S. Taylor
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