# Eating the bone!
# An enzymatic protocol aiming at bone's organic matrix removal

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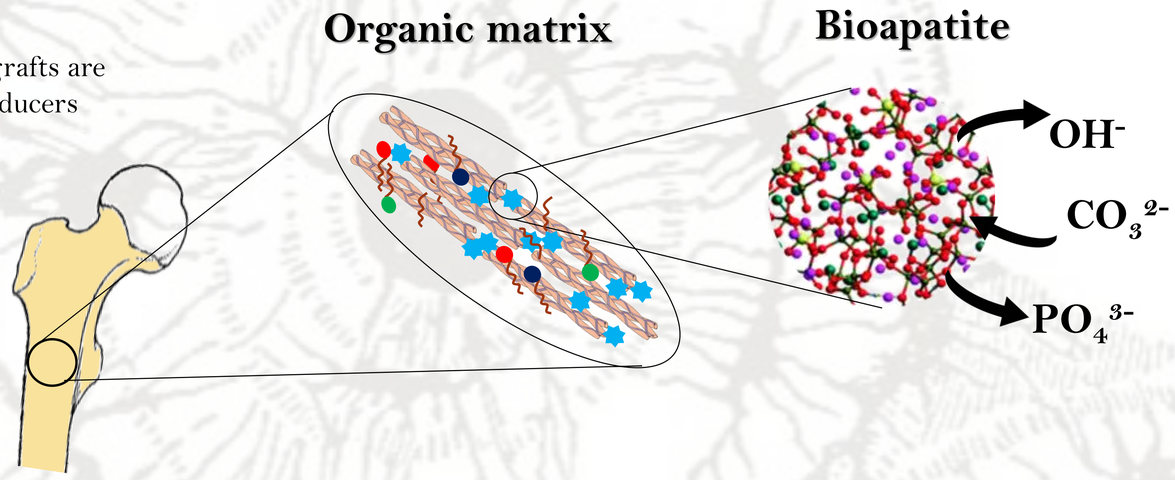
## INTRODUCTION

Human bone tissue is composed of bioapatite, a hydroxyapatite ($Ca_{10}(PO_4)_6OH_x$, HAp)) analogue substituted by carbonate at both phosphate and hydroxyl sites, and an organic matrix comprising several types of lipids and proteins (mainly type I collagen)

Bone tissue organic component removal is routinely performed in several fields of study (anthropology, forensics, archaeology, biochemistry, pharmacology and medicine)

In medicine, orthopaedic xenografts and allografts are used as implants or as bone regeneration inducers

The preparation of bone grafts includes its defatting and deproteination in order to obtain a biocompatible system with a suitable bioactivity and osteoconductivity, maintaining bone´s inherent structural and physical properties




The frequently used chemical treatments regarding the removal of lipids and proteins from the bone are associated to some negative effects on bioapatite lattice structure which may compromise its properties within a living system

The aim of the present study was to compare the efficiency and hypothetic negative effects of a new enzymatic defatting and deproteination protocol against the most widely used protocol, petroleum ether/hydrazine[1].

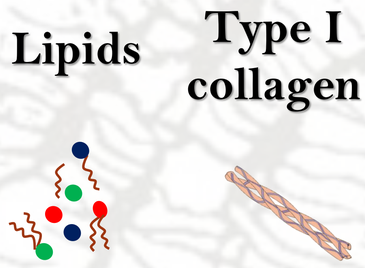
## EXPERIMENTAL

**Petroleum ether/Hydrazine method:**

First the bone was soaked in petroleum ether (80:20), in reflux, 10-12 h.
10 mL hydrazine (95%) *per* g of bone (constant stirring), 1 h at RT and 24 h at 55 ºC (renewing hydrazine at 15 h). The samples were washed with ethanol (80%), absolute ethanol and acetone (95%)
Samples were dried in a rotary evaporator under vacuum (at 60 ºC) and then under a dry air flux for *ca.* 3 h.

**Enzymatic method:**

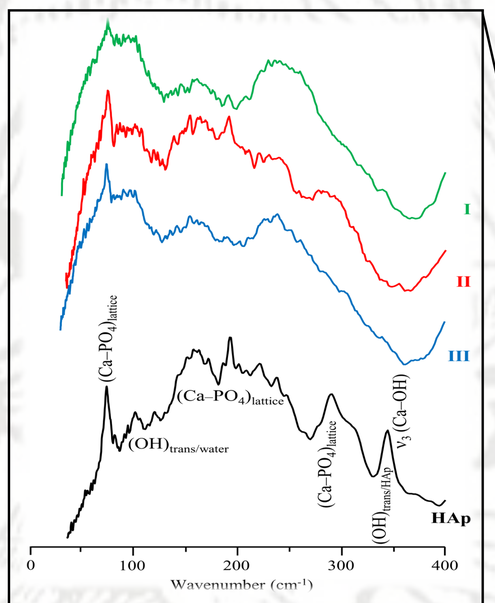
**Firstly performed:** Lipase from *Aspergillus niger* soaked in $Na_2CO_3/NaHCO_3$ buffer (pH = 9)
5% lipase ($w_{bone}/v$), 5 h at 40 ºC (constant stirring)




Type I collagenase from *Clostridium histolyticum* soaked in TESCA buffer (pH=7.4)
0.01% collagenase ($w_{bone}/v$) and 4 g $Ca^{2+}$ *per* mole of enzyme, 5 h at 37 ºC (constant stirring)

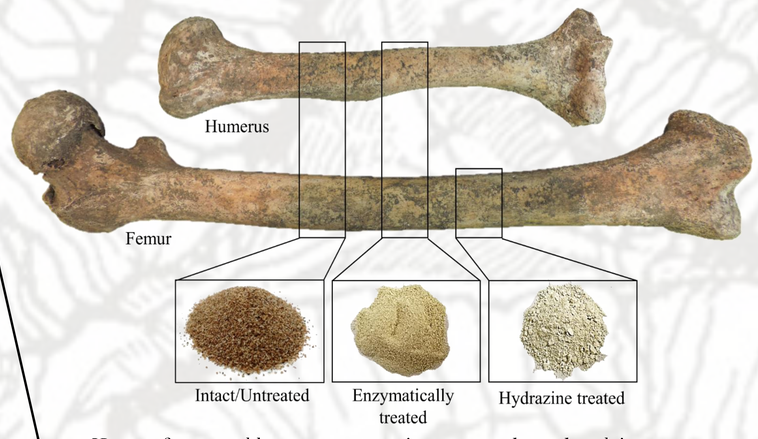
After both enzymatic digestions each enzyme was heat inactivated (70ºC, 15 min) and washed with sodium hypochlorite solution (3.25% *(w/v)*), 10 min, RT, followed by washing with deionised water. Samples were dried in an oven at 110 ºC for 5 h, and then under a dry air flux for *ca.* 24 h.

## RESULTS & DISCUSSION

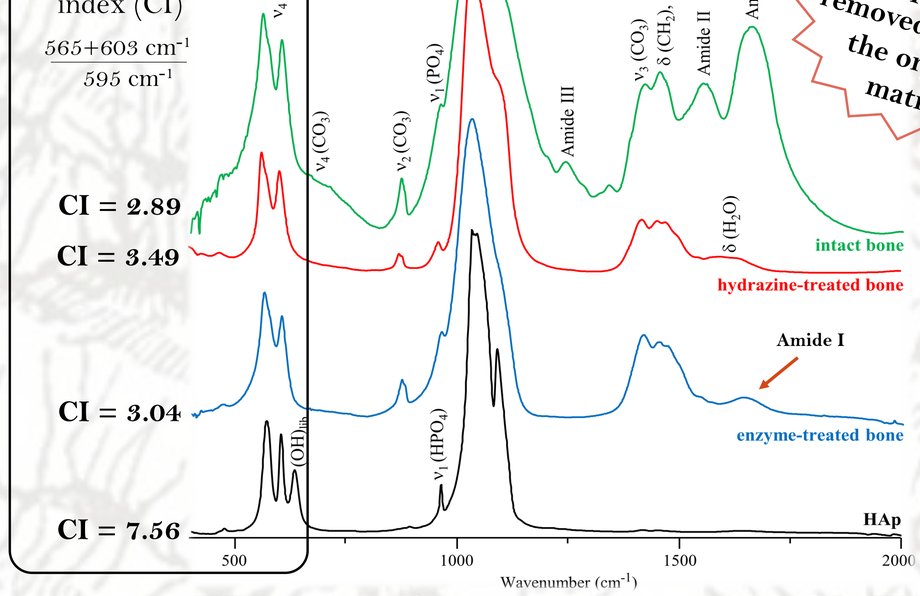



Low energy INS spectra of human femur, measured with the TOSCA spectrometer: intact (I), hydrazine-treated (II) and enzyme-treated (III). The spectrum of reference calcium hydroxyapatite (HAp, SMR2910b) is also shown, for comparison.

($Ca-PO_4$) signals were more affected by the hydrazine treatment than by enzymatic extraction,
**corroborating the CI values obtained through FTIR-ATR**




Human femur and humerus cut sections presently analysed: intact, enzymatically and hydrazine treated (after grinding).



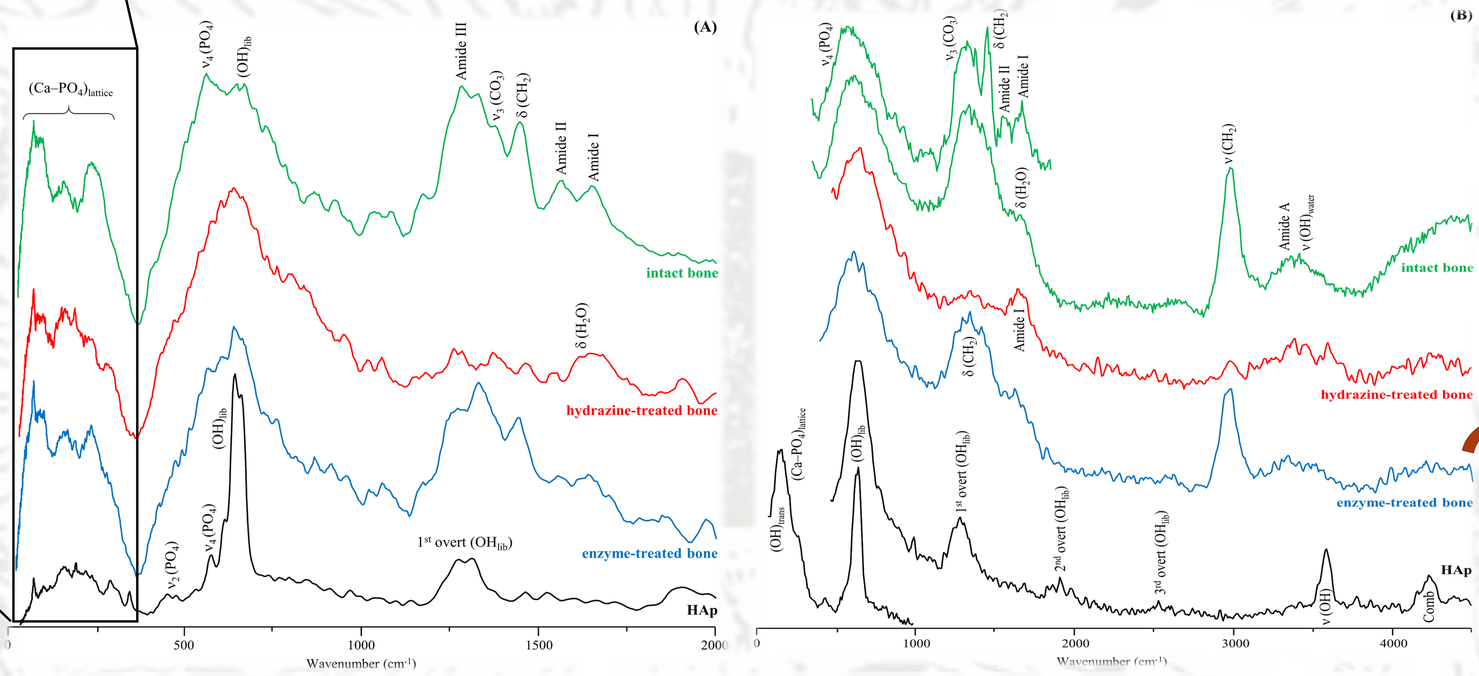

FTIR spectra of human femur – intact, hydrazine- and enzyme-treated. The FTIR spectrum of reference calcium hydroxyapatite (HAp, SMR2910b) is also shown, for comparison.

**Both protocols removed most of the organic matrix**

**No lipids detected**

**Traces of protein are present**

**Slight changes in crystallinity**




INS spectra of human femur – intact, hydrazine- and enzyme-treated – measured in two different spectrometers: (A) TOSCA and (B) MAPS (with 2024 and 5240 $cm^{-1}$ incident energies). The spectra of reference calcium hydroxyapatite (HAp, SMR2910b) is also shown, for comparison.

**Traces of protein - not detected through FTIR**
**No lipids left by petroleum ether**

**The presence of LIPIDS was clearly detected using the MAPS instrument**

## CONCLUSIONS

Petroleum ether extraction is a very efficient method for lipid elimination, on the other hand, hydrazine is not as successful for protein extraction: the amide I and amide A bands were still detected.

Petroleum ether/hydrazine method produced higher changes in bioapatite crystal lattice.

The high sensitivity of INS to H-associated vibrational modes unveils noticeable $\delta(CH_2)$ and $\upsilon(CH_2)$ signals reflecting the presence of lipids in the enzymatic treated samples.

Overall, the newly proposed enzymatic method for bone tissue defatting and deproteination was found to be quite promising, needing further optimisation.

**A mix of diferent lipases must be used in order to remove all the lipidic content**

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