

# **ANALYSIS OF BONE COMPOSITION WITH RAMAN SPECTROSCOPY**

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## **ABSTRACT**

Bone is a composite material formed by the nucleation and growth of a mineral highly resembling calcium hydroxyapatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , within an organic matrix consisting mainly of type I collagen. Seventy percent of mature bone consists of the inorganic phase while the organic matrix and water fills the rest. Development of fast and reliable methodologies capable of analyzing chemically the major bone constituents is needed. In the present work, Raman spectroscopy, a non-destructive technique, was applied as such a tool on bovine cortical and trabecular parts of bone. Preliminary results show that their mineral to organic ratio differs at 30%.

## **INTRODUCTION**

Bone consists of two major components; an inorganic or mineral phase, which is a carbonated form of a finely crystalline calcium phosphate closely resembling hydroxyapatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , (bone apatite - BAP), and an organic phase which is made up mainly of type I collagen fibers (COL). Bone tissue also includes water and organic molecules, such as glycosaminoglycans, glycoproteins, lipids, peptides and enzymes. Ions such as sodium, magnesium, fluoride and citrate are also present, as well as hydrogenophosphate.

Traditional tools used in assessing the chemical composition of bone include electron microscopy, X-ray diffraction and wet chemical analysis. Use of these techniques has been proved time-consuming and cumbersome. Furthermore, during application the tissue is exposed to stresses that can alter its structure and/or its composition. The ideal analytical tool would be one in which minimal tissue preparation is required, while no loss of the amount and quality of information and sample is noted.

Fourier Transform Raman Spectroscopy is becoming applicable as analytical technique for biomedical applications because:

- (a) The need for tissue preparation is minimal, the measurement itself is non-destructive to the tissue and very small amounts of material can be successfully analyzed.
- (b) Molecular (ultra structural) level information is available, allowing investigation of functional groups, bonding types and molecular conformations [1-3].

This study investigates the composition of cortical and trabecular bovine bone tissue by comparing the mineral to organic material Raman signal ratio.

## EXPERIMENTAL

### *Materials and methods*

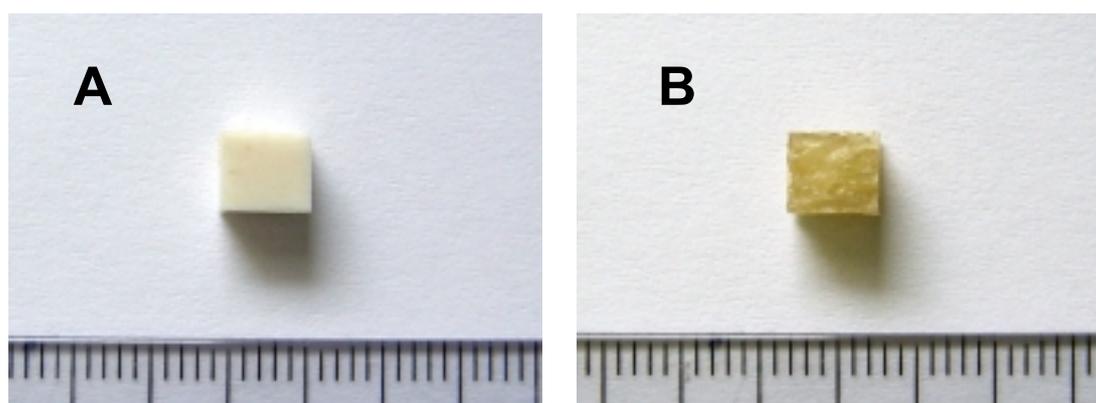
Cortical and trabecular bovine shinbones were used in this study. The bones were cleaned of soft tissue and stored in frozen state at  $-80^{\circ}\text{C}$ .

### *Preparation of bone samples*

Shinbones from six different animals were used. Four cubic specimen, measuring  $4 \times 4 \times 5$  mm, were extracted from the cortical and trabecular area of each bone (Figures 1A, 1B), using a small hacksaw and a diamond cutting wheel.

In order to minimize the influence of the presence of glycosaminoglycans, glycoproteins, lipids, peptides, enzymes and the other substances of bone marrow on Raman signal, a sequestration protocol was developed and applied. According to this, the specimen where immersed in a 1:1 methanol – chloroform solution for 2 hours and placed in an ultrasound bath for 1 hour. The solution was renewed and the procedure with alterations was repeated several times.

The efficiency of the protocol was tested on pure bovine collagen and synthetic HAP in which no dissolution of either substance was observed.



**Fig. 1.** Photos of cortical (A) and trabecular (B) bovine bone after the application of the cleaning protocol.

### *Instrumentation*

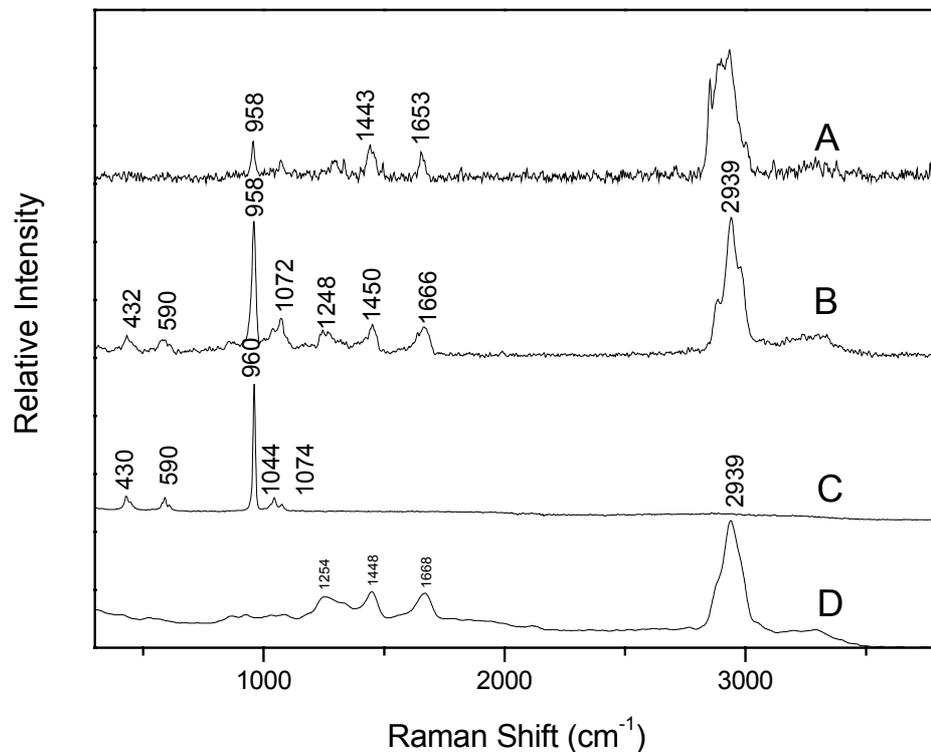
The Raman spectra were recorded using a FRA-106/S FT-Raman (Bruker) with the following characteristics: The laser excitation line used was the 1064 nm of a Nd:YAG laser. A secondary filter was used to remove the Rayleigh line. The scattered light was collected at an angle of  $180^{\circ}$ . The system was equipped with liquid  $\text{N}_2$  cooled Ge detector (D 418). The power of the incident laser beam was about 300 mW on the sample's surface. Typical spectra line width was  $0.5 \text{ cm}^{-1}$  while the recorded spectra were the average of 300 scans.

Three Raman spectra were recorded from different sides of each cubic sample.

## RESULTS AND DISCUSSION

### *Raman Spectra of Bone vs Synthetic Hydroxyapatite and Bovine Collagen*

The Raman spectrum of bone, as expected, resembles that of synthetic hydroxyapatite and bovine collagen (Fig. 2). The Raman bands associated with the hydroxyapatite at 430, 590, 961, 1044 and 1074  $\text{cm}^{-1}$ , and with the collagen at 1254, 1448, 1668 and 2939  $\text{cm}^{-1}$  can also be seen in the Raman spectra of the cortical and trabecular bovine bone [4-7]. The major Raman band assignments for bone (Table 1) include vibrational modes for phosphate, carbonate and the organic matrix bands of amide I and CH stretch. However, the positions, intensities and widths of  $\text{PO}_4^{3-}$  and  $\text{CO}_4^{3-}$  bands change in response to local perturbations in the crystal lattice caused by the local ion composition.



**Fig. 2.** Raman spectra of: A) untreated trabecular bovine bone; B) untreated cortical bovine bone; C) synthetically produced hydroxyapatite; D) bovine collagen.

Comparing the Raman spectrum of the trabecular bone before the application of the cleaning protocol (Fig. 2A) with the spectra of the synthetically produced hydroxyapatite (Fig. 2C) and the commercially available collagen (Fig. 2D) significant differences can be observed which can be attributed to the marrow components. The vibrations of the marrow components in the area of 2800 – 3050  $\text{cm}^{-1}$  overlap with the strong Raman band of the collagen at 2939  $\text{cm}^{-1}$ . Overlapping was also observed in the regions of amide I vibrations. Application of the cleaning protocol (experimental section) on the trabecular specimens proved to be sufficient to

eliminate the presence of the marrow components as can be seen by comparing Figs 2 and 3.

On the other hand, no significant differences were observed between the Raman spectrum of the untreated cortical bone (Fig. 2B) and the spectrum of the pure collagen (Fig. 2D). Application of the cleaning protocol on cortical bone did not affect its spectrum to a large extent. This is reasonable since the cortical bone is much more compact than the trabecular, and thus it contains smaller amounts of lipids and other substances of bone marrow.

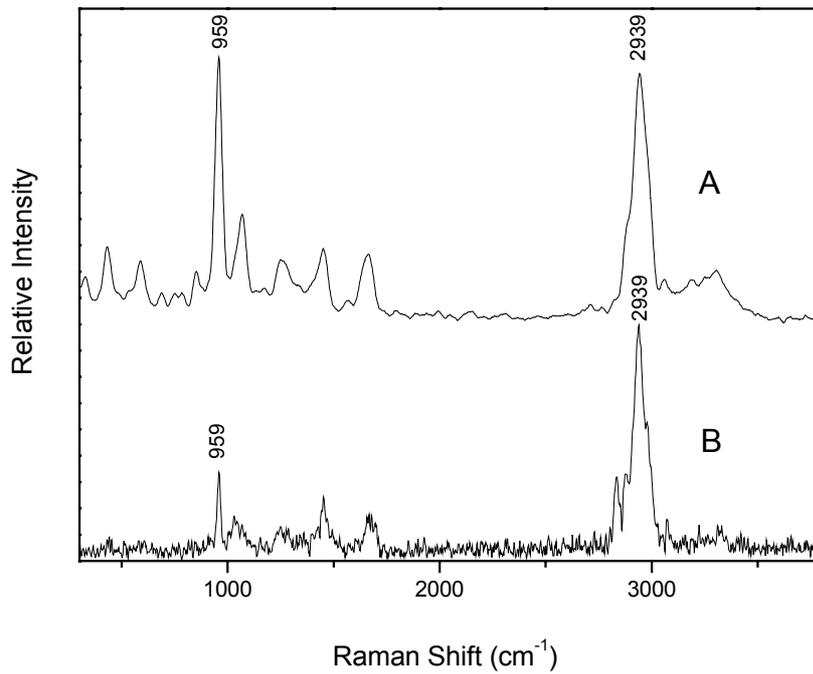
**Table 1.** Major Raman band assignments ( $\text{cm}^{-1}$ ) of trabecular and cortical bone, synthetic hydroxyapatite [8] and purchased bovine achilles tendon collagen.

Bands ( $\text{cm}^{-1}$ )	Trabecular bone tissue	Cortical bone tissue	Synthetic hydroxyapatite	Collagen
CH stretch	2804-3031 (s)	2939 (s)		2939 (s)
Amide I band	1653 (m)	1666 (m)		1668 (m)
CH <sub>2</sub> wag	1443 (m)	1450 (m)		1448 (m)
Amide III		1248 (m)		1254 (m)
CO <sub>4</sub> <sup>3-</sup> (stretch)		1072 (m)	1074 (m)	
CO <sub>4</sub> <sup>3-</sup> (bend)			1044 (m)	
PO <sub>4</sub> <sup>3-</sup> v <sub>1</sub> (symmetric stretch)	958 (m)	958 (s)	961 (s)	
PO <sub>4</sub> <sup>3-</sup> v <sub>4</sub> (bend) (asymmetric stretch)		590 (m)	590 (m)	
PO <sub>4</sub> <sup>3-</sup> v <sub>2</sub> (bend) (asymmetric stretch)		432 (m)	430 (m)	

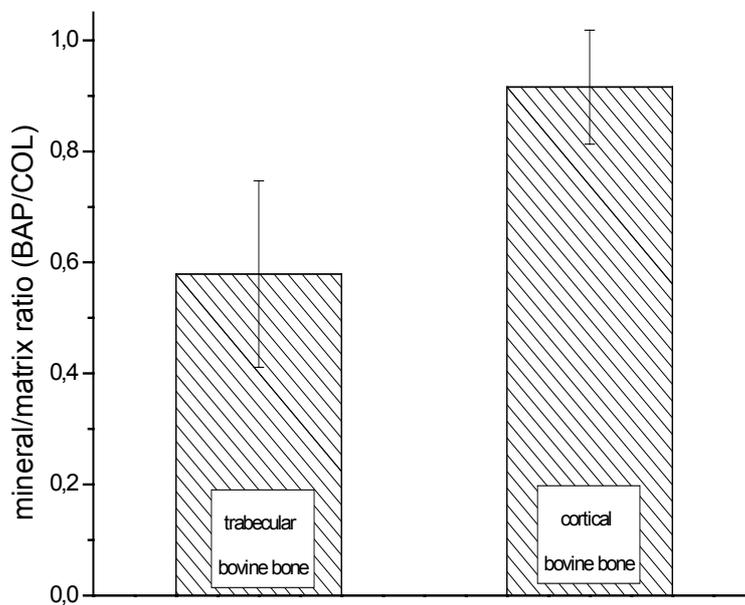
#### *Raman Spectra of Cortical vs Trabecular Bovine Bone*

The mineral-to-matrix ratio has been used in previous Raman studies on bone to examine the extent of mineralization. Markers of organic matrix included the amide I band at  $1666 \text{ cm}^{-1}$ , the area under the amide I envelope ( $1600 - 1720 \text{ cm}^{-1}$ ) and the CH<sub>2</sub> wag at  $1450 \text{ cm}^{-1}$  [9-11].

In this study, the CH stretch at  $2939 \text{ cm}^{-1}$  was used because it is a more robust measurement of matrix content and the  $959 \text{ cm}^{-1}$  band of the phosphoric group for the mineral part. The ratio of BAP/COL Raman intensities obtained from the specimens can be seen in Fig. 4. A difference of almost 30% was observed. Using the amide I vibration instead of the CH stretch band a similar difference was obtained (not shown). This result indicates that a cortical tissue is more mineralized than the trabecular counterpart.



**Fig. 3.** Raman spectra of: A) cortical bovine bone after the application of the cleaning protocol; B) trabecular bovine bone after the application of the cleaning protocol.



**Fig. 4.** The mineral-to-matrix ratio in cortical and trabecular bovine bone.

## CONCLUSIONS

In the present study, an attempt to study comparatively the chemical structure of the cortical and trabecular parts of bovine shinbone was made. Preliminary results indicate that cortical bone is more mineralized than the trabecular part. The difference appears to be as high as 30%. Raman spectroscopy was proved to be a useful tool for exploring the chemical microstructure of bone and may provide new insight into the structure-function relationship that determines the properties of the complex cortical-trabecular phase.

## ACKNOWLEDGMENTS

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