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Investigation of different bone matrices by vibrational spectroscopy

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To study bones of various origins (human bone and bones from different animal species) the complementary spectral methods, infrared and Raman spectroscopy, were used. The main vibrational bands characteristic to major constituents of bones, that is collagen and calcium phosphate, were identified. Mature deer antler has the same constituents. The relative content of the organics in different bones was evaluated from Raman spectra as an integration ratio between the ν(CH) bands at 2825-3080 cm^{-1} and the $\nu_1[PO_4]^{3-}$ band centered at 960 cm^{-1}. This ratio decreases in the order: mature deer antler > human skull > fish > pig. The thermal behaviour of bones was investigated by infrared spectroscopy. Heating of powdered bones produces at 200 ^oC the diminishing of the organic component, marked by the reduced intensity of the amide III band observed at 1239 cm^{-1}. The spectra of samples heated at 900 ^oC put in evidence the removal of organic components and the presence of a variety of hydroxyapatite with an improved cristallinity degree, especially in the case of fish bone.

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1. Introduction

The use of biomaterials has shown a large evolution in the last years, especially as concerns the bone implants biologically similar to natural bone. Today a new generation of biomaterials, resorbable and bioactive that, once implanted, will help the body heal itself by tissue regeneration is developing.

The bone can be considered a composite material consisting of a fibrous protein, collagen, stiffened by an extremely dense filling and surrounding of calcium phosphate crystals. Other constituents are water, non-collagenous proteins and polysaccharides and, in many types of bone, living cells and blood vessels. The amount of water present in bone is an important determinant of its mechanical behaviour [1,2]. Collagen comprises about 85 to 90% of the protein in bone. The bone mineral is a variety of calcium phosphate, hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$.

Deer antlers have a very special property. They grow around 1-2 cm in length per day and reach full maturity in 12-14 weeks. Velvet antlers are removed after approximately 8 weeks of growth, although they are still immature and have not yet been fully calcified. The deer looses the antlers every year.

Vibrational infrared and Raman spectra of bones of different animal species were studied to compare the major components of them and the effect of the thermal treatment. In the perspective of using the mature antler of Romanian deer to obtain a new biomaterial with applications in bone reconstruction, a special attention was paid to its characterization by these spectral techniques.

2. Experimental

Fresh bone samples were cleaned and the excess of soft tissue was removed from the surface using a scalpel. The bone marrow was excised prior to the grinding of the bone into a powder that was degreased by repeated washing with acetone. The effect of this degreasing was the disappearance of the 1740 cm^{-1} band and the decrease of the intensity of ν(CH) bands from the 2800–3000 cm^{-1} domain of the infrared spectra. The studied samples were of different zoological origins: mature deer antler, human skull, pig and fish. For an easier identification of the vibrational bands, infrared and Raman spectra of bovine collagen and commercial hydroxyapatite samples were also studied.

Infrared spectra of powdered samples compressed in KBr discs were recorded using a Fourier transform infrared spectrometer JASCO 610. Raman spectra of bone powders were recorded on a BRUKER Raman spectrometer FRA 106/9 using 1064 nm excitation laser source.

3. Results and discussion

Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy are recognized as suitable methods for the investigation of the bone, a matrix of protein (about 90% type I collagen) and a calcium phosphate mineral, hydroxyapatite. In IR and Raman spectra the presence of the major components of bones might be identified relatively easy [3-6]. Raman spectroscopy has the advantage of narrower bands and a less sensitivity to water.

In Fig. 1 are presented infrared and Raman spectra of mature deer antler and in Table 1 is given the assignment of the most intense bands observed in these spectra. One can remark the high intensity of ν(CH) bands,

characteristic to vibrations of methyl and methylene groups, in the 2800-3000 cm^{-1} domain of Raman spectrum. Instead, in infrared spectrum, the bands characteristic to amide groups are more intense and could be used to study the cross linking of collagen in bones [7].

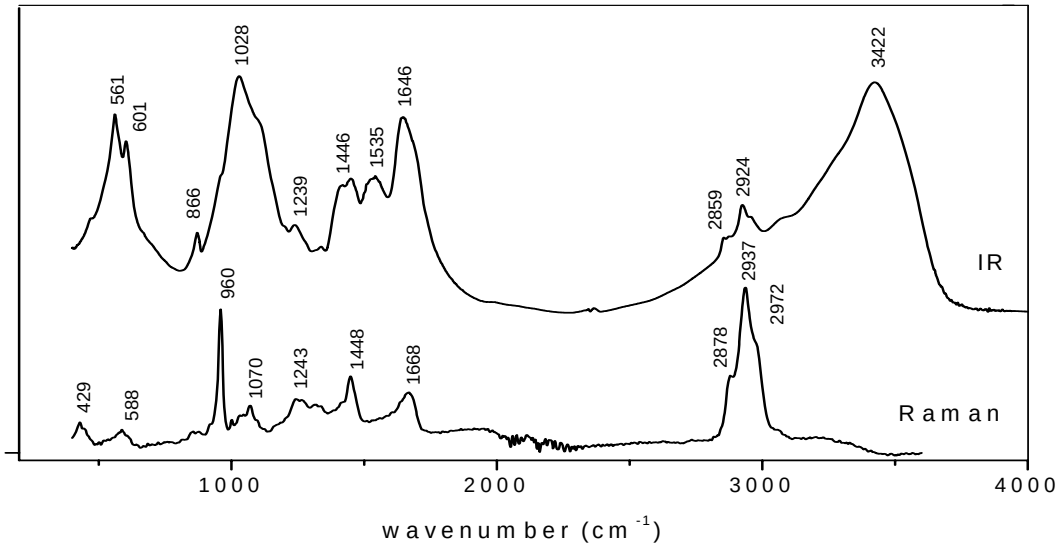

Fig. 1. Infrared spectrum (in absorbance) and Raman spectrum (Raman intensity) of mature deer antler.

Table 1. Assignment of the main bands from the infrared and Raman spectra of mature deer antler.

Organic matrix			Inorganic matrix		
Peak assignment	Peak position (cm^{-1}) in spectrum		Peak assignment	Peak position (cm^{-1}) in spectrum	
	IR	Raman		IR	Raman
$\nu_{asym}(CH_3)$	2956 sh	2975 sh	ν(OH) hydrogen bonded	3422 vs	
$\nu_{asym}(CH_2)$	2924 m	2937 vs	$\nu_3[CO_3]^{2-}$	*1446 m *1416 m	*1448 m
$\nu_{sym}(CH_2)$, $\nu_{sym}(CH_3)$	2859 m	2878 s	$\nu_1[CO_3]^{2-}$ and $\nu_3[PO_4]^{3-}$		1070 m-w
ν(C=O), CONH amide I	1646 s	1668 m	$\nu_3[PO_4]^{3-}$	1103 s 1028 vs	
δ(NH) + ν(CN), CONH amide II	1535 m		$\nu_1[PO_4]^{3-}$		960 s
$\delta(CH_3)$, $\delta(CH_2)$	*1446 m *1416 m	*1448 m	$\nu_2[CO_3]^{2-}$	*866 m-w	
ν(CN) + δ(NH), CONH amide III	1239 w	1243 m	$\nu_4[PO_4]^{3-}$	601 s 561 s	588 w
ν(C-C)	*866 m-w		$\nu_2[PO_4]^{3-}$		429 w

* Bands that can be assigned to both matrices

Relative intensity of bands: vs=very strong, s=strong, m=medium, w=weak, sh=shoulder

As concerns the bands assigned to phosphate group vibrations, these are well resolved in Raman spectrum and the most intense is the narrow band observed at 960 cm^{-1}. In isolated $[PO_4]^{3-}$ tetrahedra this band is assigned to the total symmetric ν_1(P-O) stretching vibration. The antisymmetric ν_3(P-O) stretching mode that is triply degenerate in the phosphate free ion generates in the infrared spectrum of bones a band with a complex contour between 900-1200 cm^{-1} (a maximum at 1028 cm^{-1} and a shoulder at ~ 1103 cm^{-1}). The triply degenerate ν_4 antisymmetric (P-O) bending vibrational mode is well resolved into at least two well defined peaks at 601 and

561 cm^{-1} in infrared spectrum of mature deer antler (see Table 1) and of synthetic hydroxyapatite [8]. One can see that mature deer antler has the major components of a bone and its vibrational spectra are similar to those of bones of another origin (Figs. 2 and 3). A higher water content in deer antler than in other bones is evidenced in infrared spectrum by the very intense band at ~ 3420 cm^{-1}.

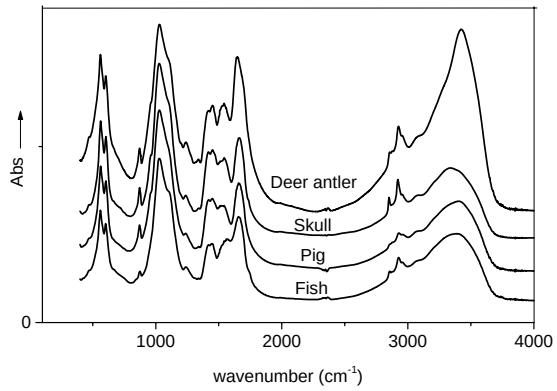

Fig. 2. FTIR spectra of bones of different origins: mature deer antler, human skull, pig and fish.

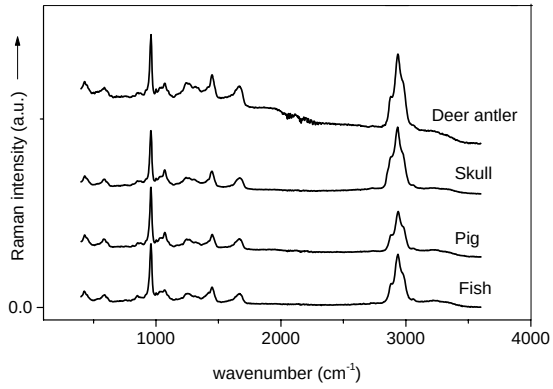

Fig. 3. Raman spectra of bones of different origins: mature deer antler, human skull, pig and fish.

Bones of different origins are distinguished especially by the organic/inorganic components ratio. In infrared spectra this is marked by the variable intensity of amide I or amide II bands (observed at ~1650 respectively 1535 cm^{-1}) comparatively to the intensity of the complex band from the 900–1200 cm^{-1} domain assigned to calcium phosphate. A quantitative evaluation of the relative content of the organics in different bones was done from Raman spectra in which there are isolated bands due to the organic and inorganic matrices. The ratio between area of the ν(CH) bands at 2825-3080 cm^{-1} and $\nu_1[PO_4]^{3-}$ band centered at 960 cm^{-1} decreases in the order: mature deer antler > human skull > fish > pig.

The effect of thermal treatment at 200 and 900 ^oC on powdered bones was investigated by infrared spectroscopy. Heating of bones at 200 ^oC produces the diminishing of the organic component well marked by the reduced intensity of the amide III band observed at ~1239 cm^{-1}. This process is illustrated for deer antler and fish bone in Figs. 4 and 5.

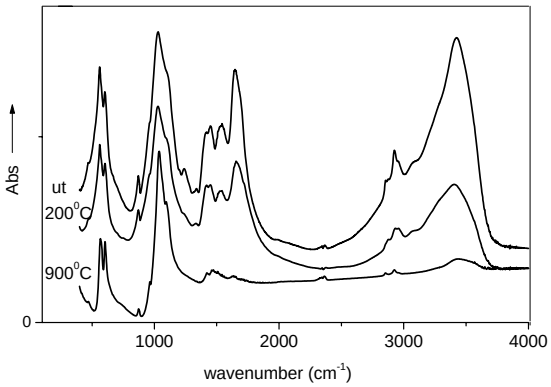

Fig. 4. FTIR spectra of mature deer antler untreated (ut) and thermal treated at 200 and 900^oC.

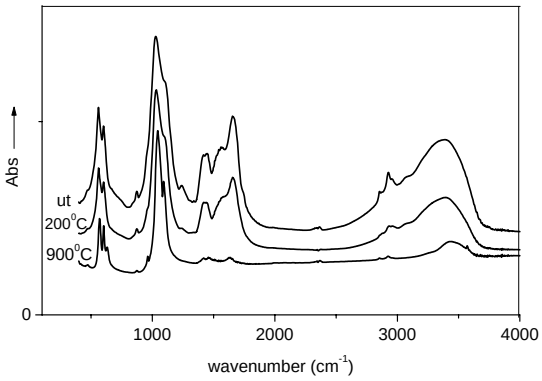

Fig. 5. FTIR spectra of fish bone untreated (ut) and thermal treated at 200 and 900 ^oC.

For the samples treated at 900 ^oC, the examination of spectra shows the expected complete removal of organic components and the modification of inorganic matrix, in fact an improvement of the cristallinity degree of hydroxyapatite. Presence of free OH groups is indicated by the new bands at ~3570 cm^{-1} [ν(OH)] and 630 cm^{-1} (OH libration). The antisymmetric stretching $\nu_3[PO_4]^{3-}$ band changed in a more narrow one, with well resolved three maxima at ~1100 (shoulder in spectra of untreated samples), 1040 and 960 cm^{-1}. Following the study of Pleshko [9], the modification of ν_3 $[PO_4]^{3-}$ pattern of untreated samples might be correlated with the size change of hydroxyapatite crystals from bones. The evolution of poorly crystalline hydroxyapatite from studied bones under thermal treatment was monitored using the method based on the splitting the phosphate ν_4 band observed in the 500-700 cm^{-1} region of infrared spectrum [10,11]. The crystallinity index of hydroxyapatite from bones varies

around 2.7 (except deer antler characterized by an index of 2.56) in untreated samples and slowly increases in samples treated at 200 °C (no more than 0.2 units). For samples treated at 900 °C the following values have been calculated: 3.40 for deer antler, 3.96 for human skull, 4.90 for pig and 5 for fish. We mention that this index was of 5.71 for the commercial hydroxyapatite heated at 1200 °C. The improvement with temperature of the crystallinity of hydroxyapatite from bones of different origins might be easily appreciated pursuing the evolution of OH libration band at ~630 cm^{-1} (Fig. 6). The most intense effect was evidenced in spectrum of fish bone and the most reduced in those of the deer antler and human skull.

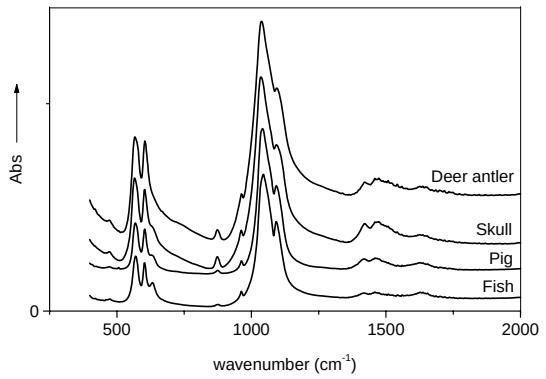

Fig. 6. FTIR spectra of bones of different origins: mature deer antler, human skull, pig and fish after thermal treatment at 900 ^0C.

Raman spectra of samples treated at 900 °C are not resolved, probably due to the fluorescence of samples, phenomenon partially observed also for deer antler powder treated at 200 °C.

4. Conclusions

Bones of different origins (mature deer antler, human skull, pig, fish) studied by infrared and Raman spectroscopy reveal the same major constituents. The characteristic vibrational bands of the organic and inorganic components were identified and assigned for mature deer antler. Spectra show that the collagen and hydroxyapatite content is dependent of the bone nature.

According to the evaluation based on Raman spectra the relative higher content of organics was noticed for mature deer antler.

The effect of thermal treatment at 900 °C determines the modification of crystallinity degree of hydroxyapatite. In the untreated bones, the hydroxyapatite is relatively poorly crystallized, and it can be characterized by an index evaluated from infrared spectra varying between 2.56 for mature deer antler and 2.72 for fish. This index increased to 3.40 for deer antler and 5 for fish, indicating that the crystallization of hydroxyapatite from mature deer antler needs higher heating temperature.

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