QUANTITATIVE ANALYSIS OF BONE MINERAL USING FTIR

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ABSTRACT

Bone is a composite biomaterial mainly composed of packed collagen fibres and nano-sized hydroxylapatite (HA) crystals. Quantitative measurements of bone mineral composition can provide insights into the bone mineral formation in health and disease. Bone mineral characteristics such as crystallinity, carbonate and phosphate mineral content may vary as a function of age, sex, diet, bone location or pathological state of organisms. Fourier transform infrared spectroscopic is well suited to evaluate the variations in the chemical composition and mineralogy in bone.

INTRODUCTION

Bone is one of the most sophisticated biological structures developed by nature (Mann, 2001). The major constituent of bone is a calcium phosphate mineral that is similar in composition and structure to minerals within the apatite group, which form naturally in the Earth´s crust (Wopenka and Pasteris, 2005). Several biological processed are involved in bone development inducing significant differences in the chemistry and structure on this mineralized tissue.

Infrared (IR) spectroscopy has been used as one of the main techniques to detect and study synthetic and natural apatite (Boskey et al. 1998, Rey et al. 1990). This technique is also used to identify the presence of bone-like mineral in pathologic mineral deposits (Cassella et al. 1994, Lind et al. 2000). The aim of the present study is to give a more complete comprehension in the study of bone mineral using FTIR to investigate and determinate changes or variations of its composition.

MATERIALS AND METHODS

A series of homogenized bone samples from a species of painted turtle (*Chrysemys picta*) of various age were analyzed by FTIR. Clapper rails (*Rallus longirostris*) of the same age, newborn chicks, exposed to PCB´s and Hg in a contaminated marsh system in coastal of Georgia were also analyzed by X-ray powder diffraction and FTIR to study the possible alteration induced by exposure to toxicants on bone.

For the FTIR analyses, 3mg of bone samples was mixed with 200 mg of FTIR-grade KBr and pressed under vacuum at 9 metric tons of pressure for 20 min. Infrared spectra were obtained on a Magna 860 Nicolet FTIR instrument. For each sample, 1024 scans were collected at 2 cm^{-1} resolution. All curve fitting was performed and their integrated areas measured using curve fitting software (PeakFit®). Overlapping peaks were resolved using a second derivative methodology and fitted to a mixed derivative Gaussian and Lorentzian function. Locations of peaks in the main regions are shown in Figure 1. To minimize the effect of differences in sample size, peak areas were normalized to the area of 3800-2800 cm^{-1} band region associated with OH groups after removing C-H stretching peak area from this region. All data are expressed as intensities ratios.

The following parameters were used to describe bone composition and crystallinity from FTIR spectra analyses:

The degree of bone mineralization (*mineral*) was defined as the band intensity ratios of phosphate species in the bone mineral to organic matrix ratio (Pienkowski et al. 1997) and was estimated as follows:

$$mineral = A900\text{-}1200 / A1660 \quad [1]$$

where A900_1200 represent the amount of phosphate in bone and A1660 the amount of amide I groups (main band from bone organic matrix; Boskey 1999).

The crystallinity index (*CI*) was calculated as the ratio between peak areas at 1030 cm^{-1} (highly crystalline apatite) to 1010 cm^{-1} (poorly crystalline apatite) (Miller et al. 2001):

$$CI = A1030/ A1010 \quad [2]$$

The relative amount of carbonate in bone mineral (*minCO3*) was calculated as the ratio of the peak area at 1405 cm^{-1} (carbonate type B substitution, Rey et al. 1989) to phosphate band area (A900-1200):

$$minCO3 = A1405/A900\_1200 \quad [3]$$

All statistical analyses were performed using the software package SPSS 13.0 (SPSS Inc.).

Bone samples were also analyzed by X-ray diffraction (Philips PW1710) to confirm the mineralogy of the leg bone and its crystallinity. X-ray diffractograms of bone apatite confirm that the lattice structure is consistent with those of standard samples of synthetic and geologic hydroxylapatite, but the diffraction peaks are much

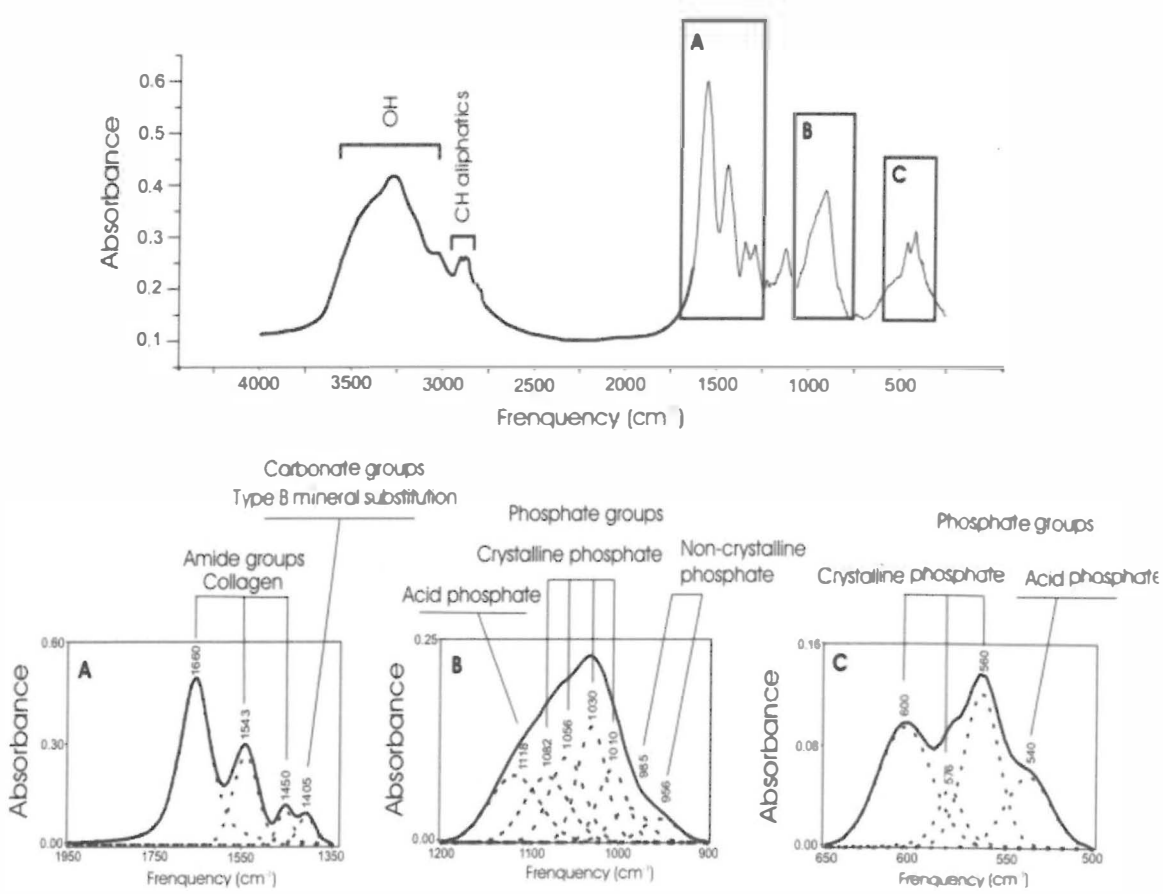

Figure 1: FTIR spectra of bone samples (400-4000 cm^{-1}) and overlapping peaks constituting the main band regions determined. Peaks position information was collected from the references: Paschalis et al. 1996, Boskey 1999, Bohic et al. 2000, Miller et al. 2001.

broader and less well resolved for bone (Figure 2). The width of an X-ray diffraction line represents a measure of the average bone crystallite size, perfection and ordering (i.e., crystallinity) in a particular diffraction plane (Cullity 1967), where narrower linewidths indicate increased crystallinity. The [002] reflection, which provides an index for *c-axis* crystal size, is probably the easiest way to determine the crystallinity of bone mineral (Miller 2001). The crystallinity parameter (CI) determined by FTIR analyses have been validated by comparison with X-Ray diffraction analysis of HA particles of known sizes (Pleshko et al. 1991).

RESULTS

We have applied this methodology to study changes in bone mineral composition caused by different processes including aging (in turtles) and environmental pollution (in birds). For instance, results obtained from deconvoluted FTIR spectra for *Chrysemys picta* provide representative information about the evolution of bone mineral composition as a function of age. The clearest change observed is an increase in bone mineral crystallinity with aging, indicating that crystals increase in size and/or perfection as bone maturation proceeds. This change is also correlated with an increase in the degree of mineralization on bone tissue, determined as the intensity ratio between phosphate and amide I bands.

In the case of birds (Clapper Rails) inhabiting highly polluted areas with organochlorines (PCB) and Hg, bone compositional parameters determined by FTIR revealed significant alteration of bone composition associated with toxicants. All observed variations and relationships between compositional parameters and toxicants levels indicated that contaminants have caused an alteration on the normal bone metabolism. Specifically, at the contaminated site, bone showed higher concentrations on carbonate and acid phosphate content that the Clapper rails from the unimpacted area. There were also notable changes in the crystallinity of bone mineral and the relative proportion of specific PO_4 groups in the different molecular environments of the tissue. Composition of bone minerals in specimens from the contaminated site the contaminated specimens is characteristic of more mature bone and revealed how toxicants have accelerated bone maturation.

DISCUSSION

The application of infrared spectroscopy to characterize bone properties and state of development as well as health state and disease is here described and applied to specific cases of study. For painted turtle bone samples the maturation states observed during aging of specimens probably signify that the apatite becomes more and more crystalline and stoichiometric.

Data obtained for Clapper rails bones showed a dose dependent change in the crystallinity of bone mineral which increasing toxicants levels. This could be due to a reduction or alteration in the rate of bone turnover caused by toxicants related to a modification on the normal bone metabolism. Similar toxicology studies has proved how many toxicants, like organochlorines and heavy metals, may cause skeletal defects and malformations inducing negatively alterations on bone formation and composition (Andrews 1989,Beard et al. 2000, Singh et al. 2000).

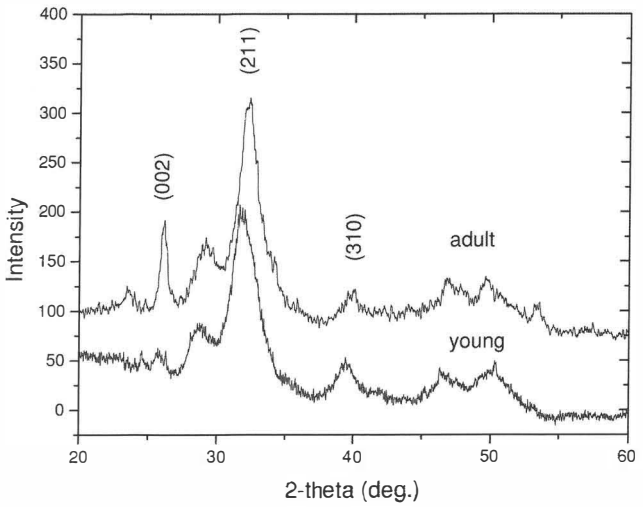

Figure 2: X-ray diffraction pattern from Clapper Rail leg bone characteristic of apatite with broad peaks typical of the nanocrystalline nature of bone mineral.

Complementary studies in laboratory models animals could determinate the toxicological effects involve on bone tissue formation under controlled conditions.

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REFERENCES

Andrews, J.E. (1989). Toxicology. 57, 83-96.

Beard, J., Marshall, S., Jong, K., Newton, R., Triplett-McBride, T., Humphries, B., Bronks, R. (2000) Arch. Of Environ Health. 55, 177-180.

Bohic, S., Rey, C., Legrand, A., Sfihi, H., Rohanzadeh, R., Martel, C., Barbier, A., Daculsi, G. (2000) Bone, 26, 341-348.

Boskey, A.L., Gadaleta, S., Gundberg, C., Doty, S.B., Ducy, P., Karsenty, G. (1998) Bone, 23, 187-196.

Boskey , A.L. (1999): In: «Dynamics of bone and cartilage metabolism», M.J. Seibel, S.P. Robins and J.P. Bilezikian, eds. Academic Press, San Diego, 23-50.

Cassella J.P., Pereira, R., Khillan, J.S., Prockop, D.J., Garrigton, N., Ali, S.Y. (1994) Bone, 15, 611-619.

Cullity, B.D. (1967): In «Elements of X-Ray Diffraction», Addison Wesley, Reading, MA, p.99.

Lind, P.M., Larsson, S., Oxlund, H., Hakansson, H., Nyberg, K., Eklund., T., Orberg, J. (2000) Toxicology 150, 41-51.

Mann, S. (2001): In «Biomeralization», R.G. Compton, S.G. Davies, J. Evans. Oxford University Press.

Miller, L. M., Vairavamurthy, V., Chance, M. R., Mendelsohn, R., Paschalis, E. P., Betts, F., Boskey, A. L. (2001) Biochimica et Biophysica Acta, 1527, 11-19.

Paschalis, E.P., Jacenko, O., Olsen, B., Menedelsohn, R., Boskey, A.L. (1996) Bone 19, 151-156.

Pienkowski, D., Doers, T.M., Monier-Faugere, M.C., Geng, Z., Camacho, N.P., Boskey A.L., Malluche, H.H. (1997). J Bone Miner Res, 12,1936–1943.

Pleshko, N., Boskey, A., Mendelsohn, R. (1991) Biophys. J. 60, 786-793.

Rey, C., Collins, B., Goehl, T., Dickson, I.R., Glimcher, M.J. (1989) Calcif. Tissue Int. 45, 157-164.

Rey, C., Shimizu, M., Collins, B., Glimcher, J. (1990) Calcif. Tissue Int. 46.384-394.

Singh, S.U.N., Casper, R.F., Fritz, P.C., Sukhu, B., Ganss, B., Girard, Jr. B, Sauvoret, J.F., Tenenbaum, H.C. (2000) J. of Endocrinol. 167, 183-195.

Wopenka, B., Pasteris, J.D. (2005). Materials Science and Engineering C, 25, 131-143.