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Thermal analysis study of human bone

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We have studied the thermal stability of human bone tissue and also its bone-extracted type-I collagen. We have used differential thermal analysis (DSC), infrared spectroscopy (FTIR) and gas chromatography. For type-I Collagen, variations of an exothermic maximum peak were observed between 500 and 530°C depending on the extraction method. These variations are related to high thermal stability of extracted collagen as opposed to thermal stability found in bone tissue, which maximum exothermic peak was found at ≈350°C. Total combustion enthalpy ΔH values are similar: -8.4 ± 0.11 kJ/g for bone tissue, and between -7.9 to -8.9 kJ/g in extracted collagen (depending on the extracting method). These findings, along with the results obtained by infrared spectroscopy and chromatographic techniques, demonstrate that the loss of thermal stability in type I collagen is due to its interactions with carbonate hydroxyapatite nanocrystals. The interactions cause a change in the molecular properties of collagen during mineralization, (specifically in its cross-links and other chemical interactions) which have an effect on fiber elasticity and on strength of bone tissue as a whole. We discuss the decomposition/combustion process and also how calorimetric measurement affects specific interactions between mineral and organic phases.

1. Introduction

Bone, teeth and shells as biological structures have been considered as some of the most exciting subjects in materials science, archaeology and medicine research. As *composite materials*, they contain both mineral and organic phases. In dentin in teeth and bone, we have hydroxyapatite carbonate $Ca_5(PO4, CO_3)_3OH$, (HA) and type I collagen. As for shells, they contain calcium carbonate (calcite or aragonite) along with unidentified biopolymers. Structural stability depends on the interactions between both phases. For the case of type I collagen and HA composite (in bone tissue and dentin in teeth) both phases have been studied and characterized applying chemical treatments in some cases, and extracting these phases from the hard tissue in other cases. Crystallinity, crystal size, texture [1, 2], chemical substitutions and solubility [3], are some of the issues involved in the characterization of HA. For type I collagen, the main focus of several studies has been the protein structure [4–6], while in other studies, some relevant parameters such as enthalpy and denaturation temperature (obtained by calorimetric measurements) have been related to the thermal stability of the molecules [7–10].

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Several studies of collagen deal with its structural functionality and abundance in living organisms. Collagen has a role in some human diseases, mainly hereditary, and in the development of collagen/HA-like materials. Collagen is stabilized by the action of strong intermolecular forces [11, 12]. Much research on collagen deals with thermal responses at temperatures where denaturation occurs. When the molecule is in a hydrated state, denaturation is associated with an endothermic peak with a maximum at approximately 60°C [13]. Some studies have been done to understand the changes in the thermal stability of type I collagen when it passes from the hydrated to the dehydrated state [14]. One is concerned here with the process of degradation but not with combustion, interpreting degradation as a partial breakage of the molecule into smaller fragments and oxidative reactions. In the last case the process produces oxide molecules such as NO_2, CO_2, SO_2, etc. Other studies deal with the thermal stability of collagen during denaturation under oxidative damage [15]. Kato and co-workers have suggested a possible path to collagen fragmentation by proline oxidation followed by cleavage of the GlyPro peptide bonds [16]. There are also other protein degradation models [17, 18]. The relationship between the thermal behavior and the collagen conformation is essential for the understanding of the molecular properties of collagen.

The oxidative damage of proteins as a consequence of degradation involves amino acid modification, fragmentation, an increased susceptibility to proteolysis, aggregation and crosslinking.

The aim of our work is the thermal characterization of bone by several methods and to connect the thermal properties to the nature and behavior of type I collagen in bone tissues. We initially study the combustion process of the protein within a controlled thermal treatment. In this way valuable information should be obtained allowing a better knowledge of the scheme of degradation in type I collagen, its effects in bone properties as the collagen structure changes by oxidation and finally its interaction with the apatite mineral phase.

2. Materials and methods

2.1. Sample preparation

Bone samples were supplied by the Anthropological Research Institute at National Autonomous University of Mexico. Bone pieces from a healthy young human skull (labeled as S-bone) and adult human radium (R-bone) were used; there were no visible signs of illness in the bone tissue.

Bone pieces were cut and then washed by softly shaking with distilled water in an agate mill for 1 h. Water was extracted and substituted for ethanol and again shaken for 5 days. During this procedure, the ethanol solution was substituted every 3 h. This method was useful to remove impurities and lipids from the collagen matrix. After washing, the samples were dried and powdered in an agate mill and sieved through a 325 mesh. The particle sizes obtained were less than 44 μm.

Type I collagen was obtained by using two methods applied on fragments from S-bone sample. In the first method, the protein content was extracted by boiling the powdered bone sample in distilled water for 1 h. Subsequently, the liquid phase was left decant and a solid phase was obtained by desiccation. The collagen sample obtained by this method was labeled as EXT-Collagen. In the second method, the procedure began by the use of EDTA solution (0.1 M, 7.4 pH) applied on a $2 \times 2 \times 2$ mm bone fragment for 2 days. The solution was replaced every 24 h. After treatment the sample was desiccated and labeled as EDTA-Collagen. Commercial type I collagen from Bovine Achilles tendon (Sigma-Aldrich C-9879) was included in the analysis as the reference sample (labeled as Sigma-Collagen).

The powdered samples S-bone, R-bone, EXT-Collagen, EDTA-Collagen and Sigma-Collagen were analyzed by using differential scanning calorimetry (DSC), thermogravimetry (TGA), gas chromatography and FT-IR Spectroscopy (Fourier transform infrared spectroscopy).

2.2. Thermal analysis, FT-IR spectroscopy and gas chromatography experiments

DSC and TGA measurements were carried out in a Thermal Analysis System 9900, Du Pont 910 (DSC module) and a Du Pont 951 (TGA module) respectively. For DSC and TGA measurements the heating rate was constant and equal to 10°C/min in an air atmosphere. The sample amount used for DSC experiments were 1.0 and 2.0 mg for collagen and bone samples respectively; and 20 mg for all cases in TGA experiments. Because there are no ΔH values reported associated for type I bone collagen combustion that were reproducible and corrected for the organic content (measured by TGA), the DSC measurements were taken for three specimens of the same labeled sample. Thus, information on thermal stability of the molecules in the presence of mineralized HA can be understood.

The FT-IR analysis was carried out by using a Nicolet 680 equipment. The collagen and bone samples were mixed with KBr powder (100:1 ratio) and compressed to form pellets. IR spectra for collagen and bone samples were obtained after isothermal treatments up to the temperatures of 20, 200, 300, 400, 500 and 600°C in a furnace with during 5 min each temperature.

Gas chromatography was performed only in the S-bone sample, with a Tremetrics Instrument in two steps. A specific column configuration was used in each step in order to identify the different gaseous products released during the thermal treatment. In the first step, the measurements were done by employing a Carboxen 1000 column capable to detect O_2, H_2O and N_2O using an atmosphere of 20% oxygen and 80% helium. The data were collected at 250, 300, 350, 400, 550, 650 and 700°C temperatures. In the second step, a Porapak N Porous Polymers column was employed, capable to detect N_2, NO, CO_2, O_2 and N_2O. The data collection was done by using the temperatures described in the first step.

2.3. Calorimetry results

DSC measurements show a similar thermal behavior for the S-Bone and R-Bone samples (Fig. 1). In both

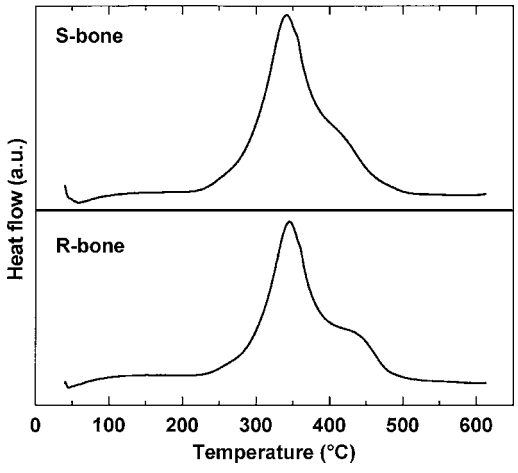

Figure 1 DSC plots of young human skull (S-bone) and adult human radium (R-bone) with maximum peaks at 340 and 345°C respectively. It can be seen a right shoulder at 425°C. The exothermic transition represents the degradation and combustion process from 220 to 530°C in both samples.

DSC plots there is first an endothermic peak (50–90°C) which represents the dehydration process (mostly water molecules removed from surfaces of the samples). An exothermic peak with maximum about 345°C appears with a right shoulder at 425°C. These signals comprising the range from 220 to 530°C represent a process in which the degradation and combustion of collagen occurs. They cannot be distinguished one from another at a specific temperature because both processes contribute simultaneously to the exothermic signal.

The DSC curves of EXT-Collagen, EDTA-Collagen and Sigma-Collagen are shown in Fig. 2. The EXT-Collagen shows three exothermic transitions, the first and smaller one at 340°C, the second and third peaks at 480 and 530°C respectively are clearly sharper. The EDTA-Collagen thermogram has only one exothermic transition about 500°C, while the Sigma-Collagen sample presents three transitions about 185, 300 and 490°C respectively.

The average enthalpy values, ΔH, for bone and collagen samples are listed in Table I. Note that ΔH values

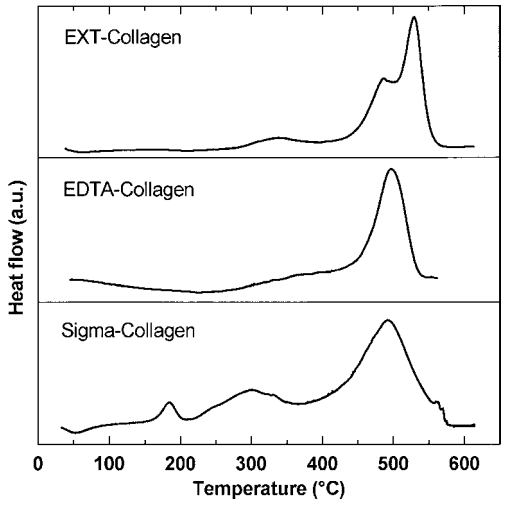

Figure 2 DSC thermograms of EXT-Collagen, EDTA-Collagen and Sigma-Collagen. All thermograms were measured with the same experimental conditions, 10°C/min heat rate and air atmosphere.

TABLE I Average enthalpy values of bone and collagen samples

Sample	Number of measurements	Average ΔH (kJ/g)
R-bone	3	-8.39 ± 0.15
S-bone	3	-8.42 ± 0.11
EXT-Collagen	3	-8.89 ± 0.07
EDTA-Collagen	3	-7.91 ± 1.01
Sigma-Collagen	3	-7.39 ± 1.39

corresponding to S-bone and R-bone appear so similar because both samples had not been exposed to any kind of protein degradation process before experiments; they were trended the same way.

From TGA-curves of bone and collagen samples (Figs 3 and 4 respectively) it can be observed that about 10% of the weight of the sample, being superficial water completely evaporates before 200°C. In the next step, the percentage of collagen mass lost by combustion was about 24 and 29% for R-bone and S-bone samples respectively. In cases of EXT-Collagen and Sigma-Collagen this percentage increase up to 80 and 90% respectively (Fig. 4). These values were used to estimate the mass of organic content for obtaining the

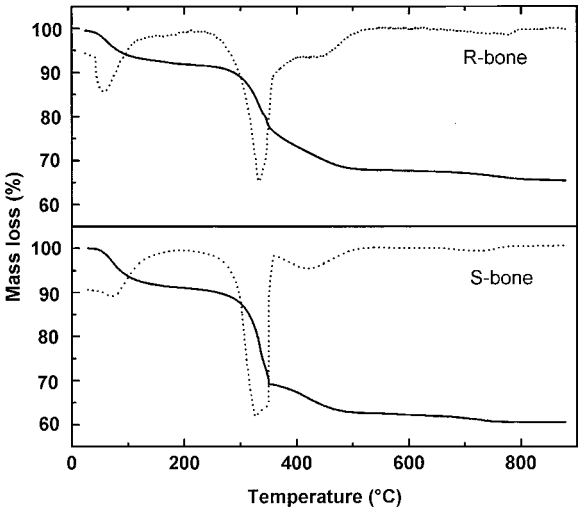

Figure 3 TGA curves of R-bone and S-bone (continuous lines) with their respective first derivatives (dot lines).

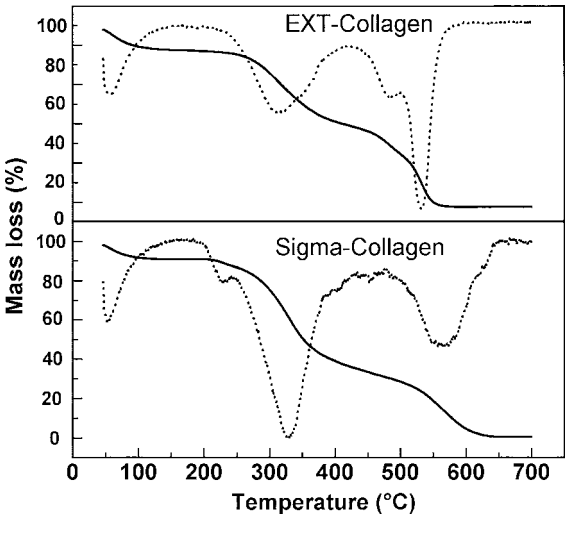

Figure 4 TGA curves of EXT-Collagen and Sigma-Collagen (continuous lines), with their respective first derivatives (dot lines).

ΔH (in kJ/g) corresponding to the protein combustion according to the collagen percentage in bone. The mass loss measured from 600 to 800°C was found to be 2% for both S-bone and R-bone. This value might correspond to the carbonate groups into the hydroxyapatite crystal structure. In the thermally extracted collagen 7.3% of mass is left after combustion which corresponds to hydroxyapatite content that was not removed during the extraction method. The TGA first derivative plots are represented in Figs 3 and 4 showing more accurate temperature values associated with the percentage of mass lost.

2.4. FT-IR and gas chromatography approach to degradation-combustion process

The FTIR spectra of S-bone and R-bone prepared in KBr pellets have similar characteristics, while the FTIR spectra of EXT-collagen has differences principally at the 1600–400 cm^{-1} range, because of the absence of mineral phase (Figs 5 and 6). At the wavenumber of 3400 cm^{-1} the OH signal decreased as the thermal treatment temperature increases. When the temperature reaches 400°C this band almost disappears. The same occurs for the C–H wavenumbers (2960 cm^{-1}) which clearly are weaker at 400°C. In both samples the events occurring between 20 and 200°C are mainly associated with the evaporation of superficial water and protein denaturation. From 200 to 400°C the main change is due to structural water that has a strong chemical interaction with the protein. When the degradation and combustion of collagen occur, the signal corresponding to amide I at 1650 cm^{-1} (the C=O stretch) clearly disappears from 300 to 400°C. For amide II at 1550 cm^{-1} and amide III at 1230–1240 cm^{-1} (C–N stretch and N–H vibration of C–N–H in plane bending modes) the signals attenuation seems to occur in the 200–300°C range. The ratio between C=O and C–N number of bonds is almost the same in type I collagen (although C–N bond is slightly more abundant). Both bonds participate in the peptide bond, and it seems that the breakage of almost all the C–N bonds happens before the transformation of the C=O group in CO_2. Therefore, the backbone of the protein starts to break into fragments between 200 and 300°C, perhaps after 250°C when the DSC exothermic peak begins to increase and when the mass loss slope in the TGA plot is larger. There are also many inorganic bands from hydroxyapatite carbonate: for P–O at 560 and 1030 cm^{-1}, and for carbonate group at 890, 1100, 1370, 1420 and 1470 cm^{-1}. These do not present any change during the thermal treatment range because hydroxyapatite has substantial changes up to 1200°C [19, 20] and are overlapping some organic bands that became visible in the collagen spectra.

By using a carboxen 1000 column it was found that at 400°C the area units corresponding to the evolution of O_2 (Fig. 7) had their minimum value and conversely the area units of CO_2/NO_2 had their maximum value. This column cannot distinguish between the evolution of both gases. In Fig. 8 we determine the CO_2 production with a Porapak N column. Two maxima at 350 (the larger one) and 500°C can be observed, being both products produced from the degradation and combustion of collagen. Since there are different nitrogen oxide gases produced in the process of collagen combustion,

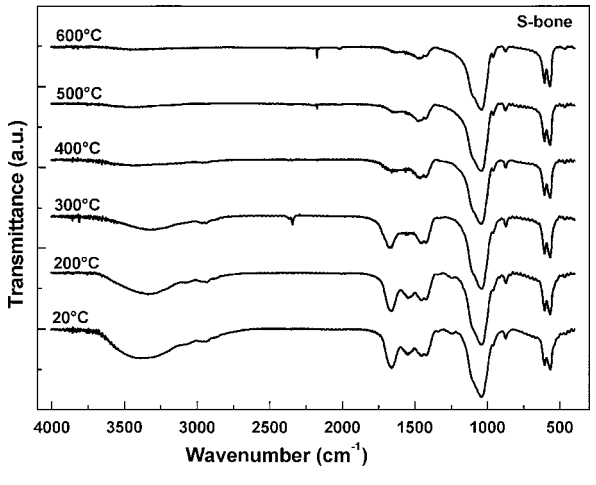

Figure 5 FTIR transmittance spectra of S-bone. The thermal treatments were at 20, 200, 300, 400, 500 and 600°C.

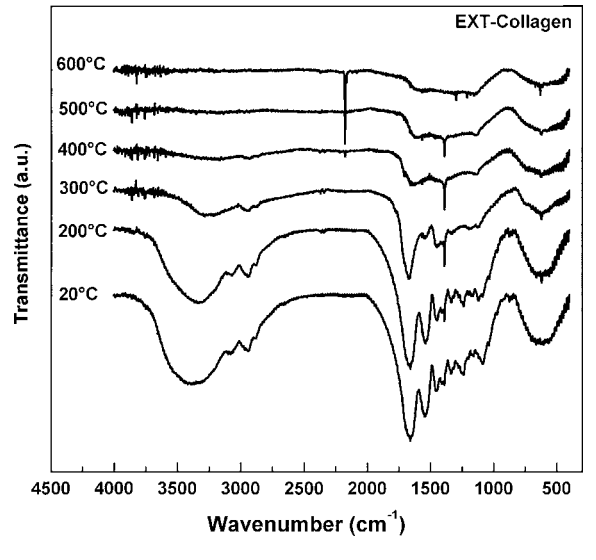

Figure 6 FTIR transmittance spectra of EXT-Collagen sample. The thermal treatments were at 20, 200, 300, 400, 500 and 600°C.

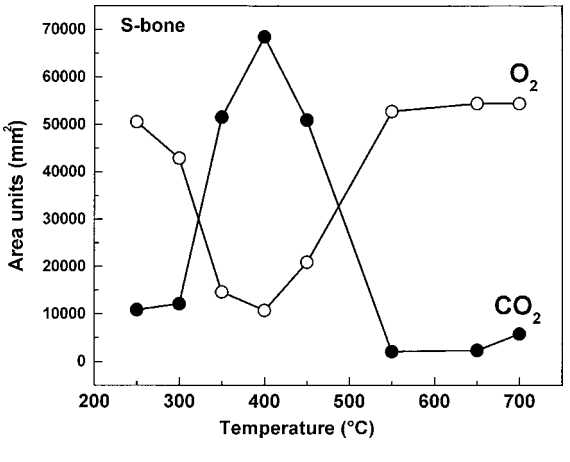

Figure 7 Gas chromatography plot employing a Carboxen 1000 column. The plot shows the gas detection (area units) at different selected temperatures.

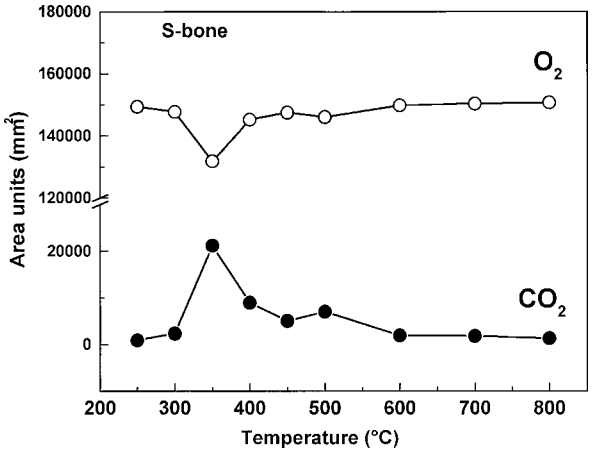

Figure 8 Gas chromatography plot employing a Porapak N Porous Polymers column. The plot shows the gas detection (area units) at different selected temperatures.

it was necessary to recognize which of them leaves during collagen combustion so as to know at which time there is no nitrogen in the protein structure. Nitrogen oxides such as N_2O, N_2O_3, N_2O_4 and N_2O_5 were not considered because of their chemical and physical properties. Therefore, only the NO and NO_2 production was considered as plausible possibility.

2.5. General discussion

From calorimetric experiments it is possible to establish that all the collagen samples (EXT-Collagen, EDTA-Collagen and Sigma-Collagen) are more thermally stable than collagen in bone. In the case of EXT-Collagen, the higher stability can be explained by the fact that the denatured protein does not transform to its original conformation after the treatment, but interlaces with other collagen molecules giving rise to a different structure. It is possible that the new arrangement contains larger number of crosslinks or secondary bonds due to closer interactions among molecules (the rigidity is higher so that the solid samples are brittle). In bone, these crosslinks or bonds would appear in the interfaces between the inorganic and organic phases (gap zone) in which Oxlund and coworkers report the existence of crosslinks formed specifically by lysyl and hydroxylysyl residues located in the non-helical telopeptide zone [21]. The crosslinks play a very important role in the interaction between the inorganic and organic phases. It is interesting to observe that, if gap density is proportional to the area/volume ratio, then the interfaces might affect the thermal stability of the protein.

For the EDTA-Collagen, the last explanation in terms of the crosslinks influence and secondary bonds is not useful, since there is no thermal treatment or mechanism that could originate protein crosslinks. Therefore, it is possible that the higher thermal stability, in contrast with bone, is diminished by the fact that hydroxyapatite crystals act as fracture centres. Then, the absence of these fracture centres (involving higher thermal stability) is characteristic of the non-mineralized collagen tissue as is shown in the Sigma-Collagen sample. The new arrangement showed by EXT-collagen visible at the sample surface (which seems brittle in contrast to EDTA-Collagen and Sigma-Collagen sample surfaces that have a rough topology) may not contribute to the stability but may have an effect on the enthalpy value.

Sakae *et al*. [22] stated that during collagen matrix mineralization, the number of crosslinks decreases as the hydroxyapatite crystals grow up into the fibril intraspaces. Thus it seems important to consider the role of the hydroxyapatite crystals as fracture centres, than as higher or lower number of collagen crosslinks, to explain the thermal stability.

Sakae *et al*. [22] analyzed dentin and obtained ΔH values for type I collagen with and without presence of a mineral phase. The reported ΔH values are -13.87 ± 0.52 kJ/g for human dentin and -15.12 ± 1.18 kJ/g for demineralized human dentin (using an EDTA solution). In Table I the average enthalpy values for the bone and collagen samples are listed. The higher values of ΔH in dentin tissue (as compared to bone) might be due to additional stability from higher mineralized tubular dentin structures which have lower organic content. The ΔH values show small changes when the collagen degradation and combustion occurs regardless of the presence of hydroxyapatite crystals in the bone tissue. The thermal stability of the extracted collagen seems not due to changes in the protein molecule, but rather a consequence of a relationship with the crystal phase (forming fracture centres).

We observe a continuous and uninterrupted degradation and combustion process of the collagen molecule structure with no bond breakage preference from the protein backbone or amino acids side chains. From the FTIR spectra it can be seen that the C–N bonds are broken before the C–H and C–C bonds. The C–N energy bond of 292 kJ/mol, is smaller than the C–C energy bond of 344 kJ/mol, according to Pauling [23], but from 300°C and up the molecule loses some of its bonds. We suspect that the NO_2 gas appears at lower temperatures, such as 250°C. At 300–350°C the degradation and combustion process acts over the molecule fragments (the newly formed aliphatic chains) which in the latest oxidized states possibly cause the left shoulder of the DSC curve of both bones. In the TGA curves and their respective first derivatives, the pronounced slope change at 350°C might also be related to the fraction of aliphatic chains and their different kinetic reaction combustion process.

The interpretation of the DSC curve must be related to the way in which the sample was prepared. It has been stated by Nielsen-Marsh *et al*. [24] that bone shows different thermal behavior if it is ground or studied in larger pieces and this affects the shape of the DSC curve, and thus the ΔH values. In order to obtain useful information about interactions between the mineral and organic phases, the way in which the bone is prepared should be taken into account.

In our analysis the particle sizes of the samples were homogeneous and less than 44 μm so that degradation and combustion processes happen as a single thermal transformation.

3. Conclusions

We infer that the presence of a mineral phase reduces the thermal stability of the protein; the small crystallites act as fracture centres. A possible physical model of fracture considering interactions between COO^- groups in the collagen molecule with some groups in the apatite crystallite can be used according to results of recent published work [25, 26].

Mineralization then changes the thermal and mechanical properties of collagen in bone which in turn reduces the structural interactions providing elasticity (when associated in fibrils like cartilage) and strength in the whole tissue.

Understanding in detail the thermal response of bone may provide valuable information about the change ocurred in it during different processes (i.e., collagen degradation in archaeological bones, implantation using biomaterials and biomimetic structures, etc.). Finally, regarding the analogy of the thermal analysis with those specific processes, the results may have relevance in research fields such as archaeology, material science and medicine.

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