

Influence of the Calcination Temperature on the Crystallographic, Compositional and Morphological Properties of Natural Hydroxyapatite Obtained from Sheep Bones

Influencia de la temperatura de calcinación en las propiedades cristalográficas, composicionales y morfológicas de la hidroxiapatita natural obtenida de huesos de oveja

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Artículo de investigación científica y tecnológica

Abstract— In this article, the study of the influence of the calcination temperature on the crystallographic, compositional, and morphological properties of the natural hydroxyapatite taken from sheep bones was carried out. The obtained samples were characterized by thermogravimetric analysis (TGA), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR) and scanning electron microscopy, with elemental analysis (SEM-EDS) in order to obtain structural information, compositional and morphological properties. In the thermogravimetric curves, the decomposition temperatures of the organic phases present in the sheep bones powder were identified. In the X-ray diffraction analyzes, it was found that the diffraction patterns presented characteristic peaks of hydroxyapatite and, it was observed that the increase in temperature favors the growth crystallite size. Likewise, in the morphological analysis, changes in the samples were verified, finding greater agglomeration of the particles, while the EDS analyzes revealed the different Ca/P relationships for the calcination temperatures used. In the FTIR spectra, the

characteristic vibrational bands of the phosphate and hydroxyl functional groups present in the samples were observed. Moreover, due to the temperature changes, the vibrational bands corresponding to water and proteins disappeared, and a decrease in the carbonate bands was also identified.

Index Terms— Calcination temperature, FT-IR, hydroxyapatite, scanning electron microscopy, sheep bone, thermogravimetry, X-ray diffraction.

Resumen—En este artículo se estudió la influencia de la temperatura de calcinación en las propiedades cristalográficas, composicionales y morfológicas de la hidroxiapatita natural obtenida a partir del hueso de ovino. Las muestras fueron caracterizadas mediante análisis termogravimétrico (TGA), difracción de rayos X (DRX), espectroscopia infrarroja por transformada de Fourier (FT-IR) y microscopia electrónica de barrido con análisis elemental (SEM-EDS) con el propósito de obtener información estructural, composicional y morfológica. En la curva termogravimétrica se identificaron las temperaturas de descomposición de las fases orgánicas presentes en el polvo de hueso ovino. En los análisis de difracción de rayos X se encontró que los patrones de difracción presentan picos característicos de la hidroxiapatita y que el incremento de la temperatura favorece el crecimiento del cristal y su tamaño. Así mismo, en el análisis morfológico se comprobó dichos cambios en las muestras, encontrando mayor aglomeración de las partículas, mientras que los análisis EDS revelaron las diferentes relaciones Ca/P para las temperaturas de calcinación empleadas. En los espectros FTIR, se observaron las bandas vibracionales características de los grupos funcionales fosfato e hidroxilo presentes en las muestras. Además, debido al cambio en las temperaturas, desaparecen las bandas vibracionales correspondientes al agua y las proteínas y, disminución en las bandas de los carbonatos

Palabras claves— Difracción de rayos X, FTIR, hidroxiapatita; hueso de oveja, microscopio electrónico de barrido, temperatura de calcinación, termogravimetría.

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I. INTRODUCTION

IN the last 50 years, the diversity of advances and innovation in materials used in medicine and biotechnology, are testimony to the important scientific and technological advances, which respond to the needs of human beings caused mainly by the aging of the population and by the appearance of bone diseases [1, 2]. One of the materials studied to interact with biological systems is hydroxyapatite (HAp), whose chemical formula is $\text{Ca}_5(\text{OH})(\text{PO}_4)_3$ [3] and presents a monoclinic structure with the space group P63/m [3, 4]. It has been demonstrated, through experiments that the HAp has bioactive, biocompatible, biostable and osteoconductive characteristics [4-6]. In addition, it has ventured into biomedicine as a restorative material, used in ophthalmology, dentistry, and orthopedics [7-9]. In the latter, favorable results have been found for bio-hydroxyapatite, whose precursor is natural, and is present in bovine, porcine and human bones, among others [4], [10, 11].

In the same way, the HAp is found in mineral rocks, and can be obtained by synthesizing certain chemical components [12], using methods such as: sol-gel, hydrothermal, sonochemical, and the use of precursors from eggshells and corals [13]. One of the main advantages of bio hydroxyapatite is that it can be synthesized in large quantities from bio-waste produced by cattle bones, which makes this source a more ecological method in relation to others. However, some types of synthesis may have disadvantages due to the absence of osteoconductive and mechanical properties [14].

Within the synthesis processes of bio-hydroxyapatite are degreasing, alkaline treatment and calcination [15]. Calcination is of particular importance because it eliminates organic matter that has not been removed in other processes; for this reason, analyzes have been made of the influence of temperature on the structural, morphological and vibrational properties of the HAp obtained from mammalian bones, where an increase in the degree of crystallinity and crystal size has been found with temperature, contrary to what occurs with porosity. Likewise, it has been found with differential calorimetric scanning and thermogravimetric analysis that for $T > 700^\circ\text{C}$ there are no organic components in the matrix and that for $T > 800^\circ\text{C}$, an order-disorder transition occurs [16, 17]. Similarly, comparative studies of the quality of biohydroxyapatite obtained from bovine bone with that of commercial origin have been carried out [15], as well as the growth of HAp crystals during the calcination process due to the coalescence phenomenon for temperatures above 700°C , and the way in which the Ca/P molar ratio decreases for samples that are calcined at temperatures above 900°C [18]. Along the same lines, narrowing has been found in the X-ray diffraction peaks due to crystalline growth at calcination temperatures of approximately 700°C , confirming that crystalline growth is a process that begins when the sample has loss of organic matter and the change of bio-hydroxyapatite from nano to microscale [19].

Consequently, the instrumental techniques and methods used for the characterization of biomaterials and bone tissues are vital for their study. These can be divided according to the property to be studied, for example, crystallinity, particle size, chemical and structural analysis, and morphology with

techniques in each of these classifications [20], all relevant to make an analysis of the obtained HAp. Moreover, thermogravimetric analysis and differential calorimetric scanning provide important information on the properties and transition phases when a sample is exposed to heating [21].

It is important to recognize the components of the natural sources of HAp to identify what biomedical applications in prosthesis or partial bone replacement could be made [22–24]. For this reason, some studies have analyzed the stability of human bones and the extraction of type I collagen, finding that the mineralization produces a change in the chemical and mechanical properties of collagen in bone tissue, thereby reducing its elasticity and resistance [8], [25].

In the bibliographic review few studies have been found with ovine sources to produce hydroxyapatite [26, 27], in relation to those of other animal sources. Therefore, the intention of the article is to study the compositional, morphological, and crystallographic properties of HAp obtained from sheep bones, when varying the calcination temperature, and to analyze how these modifications intervene in the Ca/P molar ratio. The bone source was extracted from a slaughterhouse located in Marulanda, Caldas, Colombia. This was intended to transmit knowledge to the people of this community about the preparation of this source of bio-HAp and to take advantage of the organic waste from this place.

To carry out this study, the sheep femur bone powder was synthesized from a male specimen approximately two years old and characterized by means of thermogravimetric analysis (TGA), scanning electron microscopy with elemental analysis (SEM-EDS), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). It was found characteristic peaks of HAp in the XRD analysis, and vibrational bands inherent to the phosphate and hydroxyl functional groups with FTIR.

This paper is structured as follows: first, the methodology used for the synthesis of the ovine bones powder is described. Then, the analysis of the characterization of the bone powder samples is presented once they have been subjected to different temperatures. Finally, the most relevant conclusions of the study are reported.

II. METHODOLOGY

To carry out the synthesis of hydroxyapatite, the tibia and femur bones of a male sheep of approximately 2 years were used. Initially, the bone underwent a deproteinization phase, which consists of carrying out a hydrothermal process, where the bone marrow (diaphysis), patella, lipid membrane, and soft tissues are first extracted. Subsequently, the bones are placed in a pot with water, where they are cooked for approximately 30 minutes. Next, the tissues that could not be removed are extracted and the bones are split into chips with a size of about 3 cm. Then, they are taken to a microwave oven at a power of 700W in a solution of water and detergent, for 30 minutes four times, renewing the solution each time. Next, the bone chips were washed with tap water, and later, the pieces were dried

by placing them in the microwave for 30 min without the presence of liquid [5].

Then, the mechanical grinding of the bone pieces was carried out. For this, a mill with stainless steel balls 304 was used at 100 rpm. They were ground for approximately two days at intervals of time, until a powder of about 38 μm was obtained. The samples were calcined in a muffle furnace Acequilabs model MF-2003. The calcination temperatures of 600, 800 and 1000°C were used, with a ramp up of 5°C/min. N2 was used as carrier gas, and once the desired temperature was reached, the samples were left for 24 hours.

To characterize the samples, thermogravimetric analysis techniques were used with a Q50 V20.13 Build 39 equipment, at a ramp of 5°C/min from room temperature to 950°C. Also, scanning electron microscopy with elemental analysis and X-ray energy dispersion on a brand ZEISS Sigma 300 instrument. X-ray diffraction was performed on a D8 Bruker AXS equipment with parallel beam geometry, using a Cu-Kα source. Finally, Fast Fourier Transform Infrared Transmission Spectroscopy (FTIR) with BRUKER equipment, platinum Diamond 1 TTR accessory, and 4 cm-1 resolution was used. Fig. 1 is a step-by-step summary of the synthesis and characterization method of the obtained samples. In Fig. 2 (a) the tibia and femur of the specimen are shown. Fig. 2 (b) shows the pitcher used together with the heating rack and, in Fig. 2 (c) the bone chips.

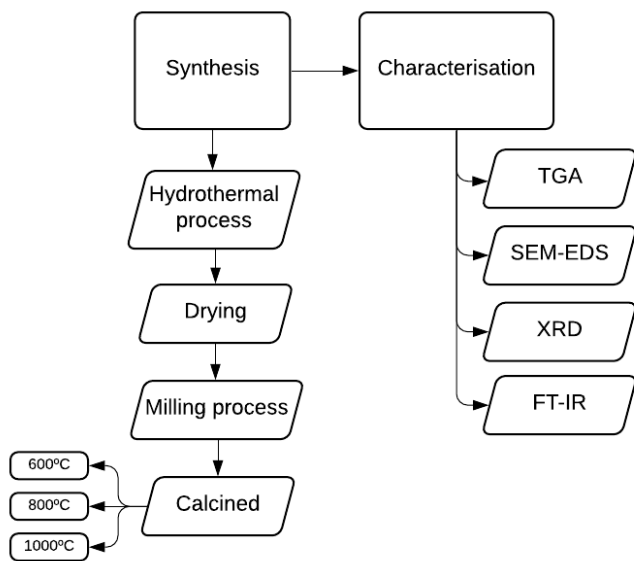


Fig 1. Block diagram for obtaining and studying HAp.

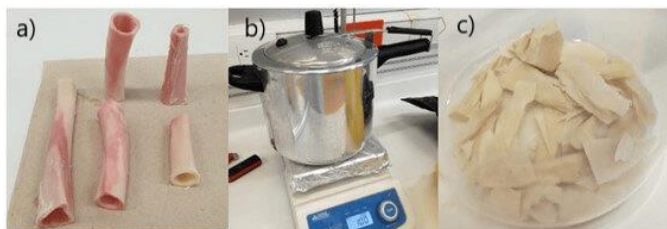


Fig 2. (a) sheep bones, (b) pressure cooker and, (c) bone chips.

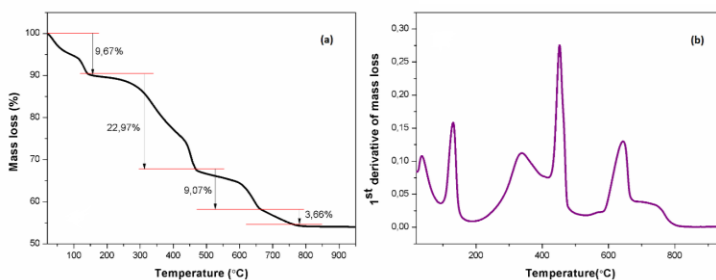
III. RESULTS AND DISCUSSION

A. TGA and the first derivative

In Fig. 3, the thermogravimetric curve and the first derivative of the TGA analysis of sheep bone powder sample can be observed, showing the changes suffered when subjected to a range from room temperature to 950°C. Thus, there is a minimum mass loss between 400°C and 500°C (see Fig. 3(a)). This denotes the decomposition of calcium hydroxide and carbonates into water, and carbon dioxide [28].

Fig 3. (a) TGA sample curve of the sheep bones powder (b) first derivative when samples are subjected to a range of temperatures from room temperature to 950 °C with a rate of 5°C/min.

The changes produced at lower temperatures of around 670°C correspond to the organic decomposition of fats and proteins



[17], [29], and for temperatures higher than this value the thermal changes are related to the physicochemical transformation of HAp [17].

The differences, due to the loss of mass between 200°C and 500°C correspond to the degradation and combustion of collagen. When making the comparison between the TGA curve with that of the first derivative (see Fig. 3 (b)), the expected correspondence is found, with a peak that shows considerable degradation at 644°C, due to the last protein transformation.

According to TGA, a change of 9.67% occurs up to 200°C due to the loss of water absorbed chemically and physically, and to the decomposition of part of the organic matter. Therefore, a difference of 32.04% is noted, and a considerable peak in the curve of the first derivative, corresponding to the organic decomposition and deneutralization of fats, which occurs between 200 and 650°C, and a variation of 3.66% from 650°C to 760°C due to the decomposition of the loss of carbonate from the organic matrix and part of the carbonates. For temperatures higher than 760°C and up to the limit of 950°C a variation of 0.38%, which is normally due to the formation of oxyapatite and oxide [17]. These last two percentages of losses, when dealing with the decarboxylation of the sample, gives way to the release of CO².

B. Morphological Analysis

In order to evaluate the morphological changes of the sheep bones powder when subjected to calcination processes, the SEM technique was used. Fig. 4 (a) shows the sheep bones powder without going through the calcination process, in which the granulation process is not yet very evident. In Figs 4 (b) and 4 (c) samples, that were subjected to 600°C, 800°C and 1000°C taken at 1000X are presented. According to the TGA analyzes, mentioned above, the considerable loss of mass up to 600 °C is not significant for the physical transformations of the sample in Fig. 4 (a) with respect to 4 (b), this is because the thermal changes between these are found associated with organic decomposition. Also, in both are agglomerated grains forming cauliflower-like distributions. On the other hand, in Figs 4 (c) and 4 (d), a growth of the intergranular space can be observed, which can be associated with the loss of the organic phase. The morphology presents changes with the increase in temperature, developing a spherical morphology. The formation of granules increases and agglomeration in the samples decreases with increasing temperature.

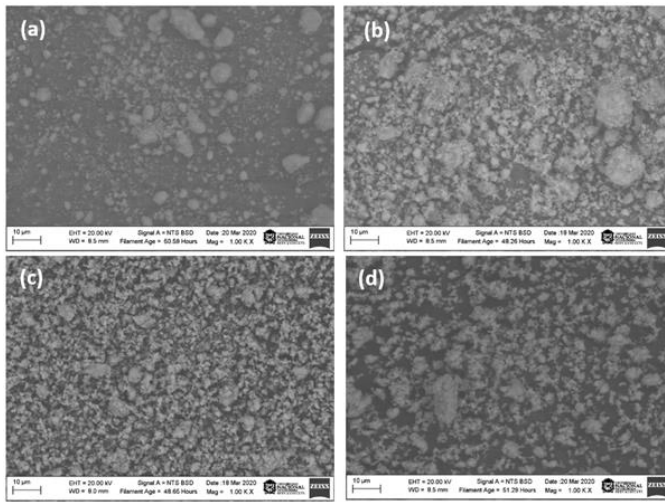


Fig 4. SEM photomicrographs (1000X). From (a) to (d) the uncalcined sample, and those calcined at 600 °C, 800 °C and 1000 °C, respectively.

The X-ray energy dispersion spectrometry (EDX) technique was used with the intention of finding the percentages of the elements present in the studied samples. The results of the chemical composition of the samples (uncalcined, 600 °C, 800 °C, and 1000 °C) show the presence of Ca, P, O and C, mainly in the 600 °C. In the 800 °C samples the presence is less which can be associated with the decarbonization process that begins before 800 °C as is shown in the TGA measurements. For the sample at 1000 °C, the presence of C is not seen because at this temperature this process has already finished Table I shows the Ca/P ratio for sheep bone powder samples subjected to different temperatures. The sample without being

subjected to a calcination temperature shows a ratio of 2.22, which may possibly indicate that there is a tetracalcium phosphate at this point [30]. For the treatment at 600 °C it is evidenced that the energy supplied, although it is sufficient to promote the loss of organic matter, is not sufficient to achieve the formation of stoichiometric hydroxyapatite. In addition, the relationship is maintained in the apatite biological range.

TABLE I.
CA/P MOLAR RATIO OF THE SHEEP BONE POWDER (SBP) STUDIED SAMPLES.

Sample	Ca/P ratio
SBP	2.22
SBP at 600 °C	1.88
SBP at 800 °C	1.76
SBP at 1000 °C	2.22

Now, for a sample subjected to 800 °C, the hydroxyapatite is close to the stoichiometric type. At 1000 °C, the sample becomes destabilized, causing an increase in the percentage of calcium in relation to that of phosphorus, which indicates a considerable substitution of phosphate ions for carbonate ions [31]. Similarly, the calcium richness of the samples subjected to 800 °C and 1000 °C can be associated with the CaO impurity [32], which can be confirmed with the peaks of the diffraction pattern.

C. X-Ray diffraction analysis

Fig. 5 represents typical patterns of HAp powder XRD, synthesized at temperatures of 600°C, 800°C, and 1000°C, respectively. They reveal the presence of HAp phases consistent with the database found in Crystallography Open Database with card 1521038 [33]. The main crystallographic (hkl) planes for HAp: (002), (210), (211), (112), (022), (310), (222) and (213) are identified in Fig. 3, showing as the most intense the (211) plane. The percentages of crystallinity (1) of the samples were 42.48, 89.94, and 89.77% for the samples at 600 °C, 800 °C and 1000 °C, respectively. This was calculated using diffractograms, where the highest intensity peak and a cautious near minimum were considered [34].

$$X_c = \left[\frac{I_{max} - I_{min}}{I_{max}} \right] * 100\% \quad (1)$$

Where X_c is the percentage of crystallinity, I_{max} is the highest intensity peak (211), and I_{min} is the minimum intensity between the previous plane and the (112) plane. It is notorious that from 600 to 800 °C there is a considerable increase in crystallinity, which is why at 600 °C the sample is considered amorphous. At this temperature, the organic matter has not been eliminated from the bone powder [5].

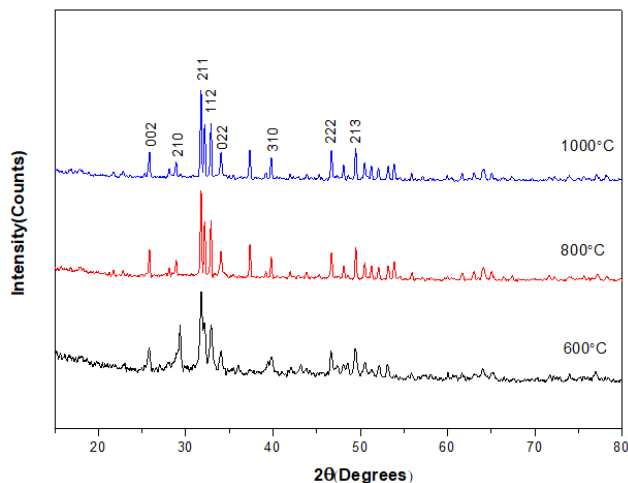


Fig 5. Diffraction patterns of the calcined samples at different temperatures.

Additionally, the size of the crystallite associated with the samples was found using the full width at half maximum (FWHM), which is related to the Scherrer equation. The crystallite sizes of 26.36 ± 7.80 nm, 54.42 ± 7.16 nm and 51.48 ± 5.47 nm were obtained for 600, 800 and 1000 °C, respectively.

The changes in the mineral phase of bones due to the loss of organic matter led to an increase in the size of the crystallite, this is because the platelet habit of the bone mineral is lost [35]. Thus, as the FWHM has been reduced, it is assumed that the formation of hydroxyapatite crystals begins, giving way to growth in crystallite sizes. At temperatures of 800 and 1000 °C it is found that there is stabilization of crystallite growth, likewise, in the samples relative to these temperatures a peak was found at 37.3° (2θ degrees) which is normally associated with CaO and constitutes an undesirable impurity in HAp. This is due to contact with water molecules, that results in breaking and even some disintegration into individual particles and alkalinity in the implant environment [32].

In addition, the lattice parameters of the hexagonal unit cell were found $a=b=9.39\pm 0.06$ Å and $c=6.89\pm 0.01$ Å, which are very close to those presented in the literature [19], [31], [36]. Moreover, it is observed that the size of the cell of the structure is not affected by the change in temperature. This stability can be associated with the presence of impurities such as Mg, Na and CO_3^{2-} [5].

D. FT-IR analysis

In Fig. 6, it is possible to observe the infrared spectra of the samples subjected to different temperatures. The vibration bands corresponding to the characteristic functional groups for hydroxyapatite such as phosphates PO_4^{3-} , and the hydroxyls OH^- can be appreciated. However, the spectral lines also show the presence of the carbonate group vibration CO_3^{2-} . These bands placed at 1415 and 875 cm^{-1} are associated with symmetric n3 and asymmetric n2 stretching, respectively [36]. This corresponds to the substitution of CO_3^{2-} by PO_4^{3-} in the structure of HAp and it gives way to carbonate hydroxyapatite. This substitution is typical in human bones, promoting excellent biocompatibility and osteoconductivity [37].

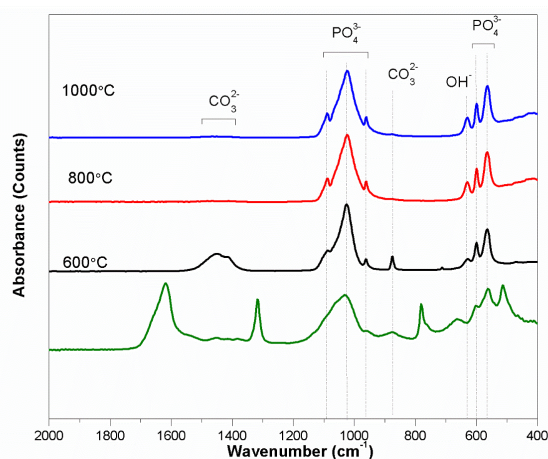


Fig 6. Infrared spectra of sheep bones powder without being subjected to temperature and, subjected to 600, 800 and 1000 °C.

Additionally, the peaks close to 1620 , 1320 and 782 cm^{-1} in the green color graph (not subjected to calcination), can be attributed to the presence of water and organic matter in the sample before being subjected to temperature [38]. Likewise, changes in the band close to 1000 cm^{-1} are associated with the decrease in carbonates, which is related to the increase in crystallinity [37]. It should be noted that the presence of carbonate groups has origin in the natural source of obtaining HAp.

IV. CONCLUSION

The results obtained show that the calcination temperature influences the changes in the properties of the sheep bone powder samples. With increasing temperature, forms closer to stoichiometric hydroxyapatite, higher degrees of crystallinity, and larger crystallite sizes are found. Also, for temperatures of 600 °C, a total loss of water and organic matter from the sample is observed. There is loss of carbonates at higher temperatures. This is evident in the FT-IR spectra, which added to the TGA results leads to suggest that 760 °C is an

appropriated temperature to obtain protein-free samples. In addition, the presence of CaO was detected in the diffractograms, which confirms that the samples obtained at 800 °C and 1000 °C are rich in Ca. The samples Ca/P ratio is higher than pure HAp, being the closest relation to this the 800 °C sample with a value of 1.76. Likewise, the reduction in the FWHM of the peaks in XRD represents an increase in the formation of crystals and in their size. It is found that this occurs as the calcination temperature increases as shown in the samples of SEM. This increment rises the formation of grains due to the phenomenon of coalescence, i.e., this temperature supplies energy for the formation of the crystallites. It should be noted that the increase in the FWHM is not necessarily linked to the quality of the crystallites. However, the lattice parameters of the crystal structure do not present major variations with temperature, it is feasible to associate it with the presence of impurities such as carbonates because the study material comes from a biogenic source. On the other hand, it should be noted that there is a need of samples calcination when they come from animal sources. As was mentioned earlier, at temperatures below 600 °C the sample is considered amorphous. For future studies, it is recommended to take temperature values between 600 and 800 °C, and 800 and 1000 °C, since for the Ca/P ratio considerable changes occur between these temperature range. Finally, according to the obtained results, the samples calcined at 800 °C are considered promising for biocompatibility studies.

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