

# Studies on Bone-Derived Calcium Phosphate Materials

Agnieszka Sobczak-Kupiec<sup>1</sup>, Klaudia Pluta<sup>1</sup>, Dagmara Malina<sup>1\*</sup> and Bożena Tyliszczak<sup>2</sup>

<sup>1</sup>*Institute of Inorganic Chemistry and Technology, Cracow University of Technology, 24 Warszawska St., 31-155 Cracow, Poland*

<sup>2</sup>*Department of Chemistry and Technology of Polymers, Cracow University of Technology, 24 Warszawska St., 31-155 Cracow, Poland*

Received October 12, 2016; Accepted February 02, 2017

**ABSTRACT:** In recent years, the development of composite biomaterials has been the subject of very intensive research. The elaboration of technology for manufacturing new biomaterials will allow their practical implementation and adaptation to changing market needs. One of the key components in the developed composite materials will be natural origin hydroxyapatite (HAp) and tricalcium phosphate (TCP) obtained from bone products. In this study, preparation and detailed characterization of bone-derived calcium phosphates as a component of biomaterial composites is proposed. This novel method of obtaining hydroxyapatite for biomedical applications allows the obtainment of a material with expected parameters. In this study, pork bones from meat cutting were subjected to a three-stage treatment: acid hydrolysis, initial calcination and proper calcination. In order to investigate the effect of lactic acid on the properties of the obtained materials, the preparation of a series of hydrolysis reactions with an increasing content of the hydrolysis reagent was assumed. Moreover, the third step of material preparation—proper calcination—was carried out at various temperature and time parameters. Subsequently, several experimental techniques were employed to investigate the expedient physicochemical properties of all calcium phosphate powders.

**KEYWORDS:** Bone-derived calcium phosphates, hydroxyapatite, tricalcium phosphate, biomaterials

## 1 INTRODUCTION

Biomaterial engineering is a rapidly developing branch of science. The evident destiny of biomaterials is to reduce disabilities and eliminate congenital or acquired defects; however, recently attention is also being paid to another important aspect. As civilization progresses, the number of people over the age of 65 years is systematically growing and societal aging is rapidly becoming a global phenomenon. Japan currently leads the world with nearly one-quarter of its population ages 65 and older, followed closely by Italy and Germany, where the share of the population ages 65 and older exceeds 20% [1]. Scientists in the U.S. predict that by the year 2050 the number of people of retirement age will have doubled [2]. What does that mean? It means that there will be a rising demand for implant materials as a consequence of the necessity to improve the health conditions of the elderly, who will need organ replacements to function properly.

The most important properties of a material intended as implant material are: 1) biocompatibility,

i.e., the material's ability to induce an acceptable response of the host, the interplay of the biomaterial with the medium of the living organism; 2) nontoxicity and minimal effects on the immune system, which means that the biomaterial must be chemically inert and does not cause inflammatory reactions and excessive tissue response and 3) bioactivity, meaning the ability of the implant's surface to adhere directly to soft or hard tissue without forming an intermediate layer of modified tissue [3–8]. Nevertheless, to properly fulfill an implant's role, the biomaterial should harmonize with the patient's body through the use of its own repair mechanisms, with adequate response stimulation on the level of individual tissues and cells.

From among all synthetic materials applied in medicine and dentistry, apatite bioceramics exhibit the biggest similarity to bone in crystallographic structure and chemical composition. Hydroxyapatite (HAp) and tricalcium phosphate (TCP) are composed of ions (mainly  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$ ) that naturally occur in tissue and, thus, they do not irritate the surrounding tissue, do not cause acute or chronic inflammation and stimulate bone repair processes, which enables the creation of a chemical bond at the implant-bone interface. They support intense bone ingrowth into the pores and provide the biological stability of the reconstructed tissue.

\*Corresponding author: dagmaramalina@chemia.pk.edu.pl

Additionally, apatite ceramics easily combine with other materials to make new composite materials with desired features [9, 10]. Currently, apatite bioceramics play a significant role in bone loss filling, paradentosis treatment, broken bone consolidation and stabilization, spinal column surgery and otolaryngology. They are also widely used in dental implants, as scaffolds for tissue engineering and coatings covering the surface of implants made of other biomaterials [11].

For the manufacture of ceramic implants, mainly hydroxyapatite and tricalcium phosphate (whitlockite) of natural or synthetic origin, or biphasic calcium phosphate bioceramics (BCP) containing both mentioned calcium phosphates, are commonly used [5, 12–14]. Methods for the obtainment of apatite powders can be divided into wet, dry, and others, including preparation from natural raw materials such as animal bones, egg shells and skeletons of corals [13–15]. These methods allow the obtainment of materials that vary in crystallinity, grain morphology and other physicochemical properties.

In the last decade, research concerning calcium phosphates (CPs) has focused on their preparation using natural resources such as beef or pork bones [16–21]. Studies on the structure and chemical composition of biological apatites as bone builder were exactly based on the development of works on synthetic apatites. After appropriate physicochemical treatment, resulting in the removal of protein and fat, followed by a pyrolysis process in temperatures about 1000 °C, this raw material may become an attractive material for further processing useful for certain medical applications. In order to obtain chemically pure apatites from bones, organic parts should be removed in the first step. Acid or alkaline hydrolysis method is mostly used [18, 22]. Commonly, for deproteinization and degreasing of the material, bones are treated with aqueous hydroxide solutions, suitable sodium, potassium or lithium at a concentration from 0.4–7.0 mol/dm<sup>3</sup> at temperature in the range of 20–300 °C [17, 23]. Then, the obtained bone slurry is a substrate in a two-stage calcination process [15, 20–22]. The calcination can be carried out in an atmosphere of air, oxygen, nitrogen, carbon dioxide or dry saturated steam [24–26]. Calcination of animal bones without chemical pretreatment is also used; however, such a method requires heat treatment at high temperatures [16, 27].

In this article, pork bones from meat cutting were subjected to the three-stage treatment: acid hydrolysis, initial calcination and proper calcination. In order to investigate the effect of lactic acid on the properties of the obtained materials, the preparation of a series of hydrolysis reactions with an increasing content of the hydrolysis reagent was assumed. The third step of material preparation was carried out at different

temperatures with the material kept at different times at a set temperature. The interesting expected results of these preliminary studies suggest that our team focused on the proper methodology of research, which suggests that the research may have a significant impact on the development of materials science and medicine.

## 2 MATERIALS AND METHODS

In this study, pork bones from meat cutting were used. After initial mechanical separation from organic residues and grinding, the bone pulp was boiled in a mixture of 80% lactic acid (POCH Poland S.A. Company) and deionized water. The stages of CP preparation are as follows:

**Stage 1 – Hydrolysis:** 100 g of bone pulp was placed in a round bottom flask and filled with 200 cm<sup>3</sup> deionized water (W). Afterwards, different volume of 80% lactic acid (LA) was added. The volumetric ratio of W/LA depended on the experiment: hydrolysis 1 – W:LA = 2:0.5, hydrolysis 2 – W:LA = 2:0.75 and hydrolysis 3 – W:LA = 2:1. The time of the process was 3 h and the duration of stage was 3 h.

**Stage 2 – Preliminary calcination:** Obtained bone sludge was separated from the remaining liquid fraction and preliminary calcination was done in a kiln chamber with electric heating in air atmosphere. The material was kept at 650 °C for 3 h; temperature was selected on the basis of thermogram analysis and previously published data of our team.

**Stage 3 – Proper calcination:** To investigate the effect of proper calcination parameters on the physicochemical properties of the final products, the material from initial calcination was ground in a porcelain mortar and then was heated in the same kiln chamber at temperature ranging from 750–1050 °C with a hundred-degree intervals for 2 or 3 h.

The final products were characterized by following analytical or instrumental methods. Phosphorus content was determined spectrophotometrically with an Evolution 220 UV-Visible spectrophotometer (Thermo Fisher Scientific Corp.) after former sample mineralization in the mixture of concentrated hydrochloric and nitric acids. Calcium was determined by complexometric titration method using disodium versenate in the presence of a mixed indicator (calcein and thymolphthalein). Contents of selected microelements and heavy metals such as As, Cd, Co, Cr, Cu, Ni and Pb were measured by the ICP with an iCAP 6500 DUO spectrometer from Thermo Fisher Scientific, total Hg content was determined using an AMA-254 atomic absorption spectrometer (Advanced Mercury Analyzer, Altec

Inc.). Phase composition was analyzed using X-ray diffraction (XRD) method with the use of a Philips X'Pert diffractometer with graphite monochromator, Cu K $\alpha$ , ( $\lambda = 0.1518$  nm), and Ni filter. Phase identification was based on XRD JCPDS-ICCD data for inorganic powder materials. The FTIR investigations were carried out with a Scimitar Series FTS 2000 Digilab spectrophotometer in the range of middle infrared of 400–4000  $\text{cm}^{-1}$ . Test samples were prepared using KBr pellet method. Thermal analysis of materials was performed using an EXSTAR SII TG/DTA 7300 thermogravimetric/differential thermal analyzer from SII NanoTechnology Inc. Analysis was conducted in a porcelain crucible in air at a temperature range of 20–1000  $^{\circ}\text{C}$  and the increase in temperature of 20  $^{\circ}\text{C}/\text{min}$ . The microstructure of samples was examined using a JEOL JSM-5510LV scanning electron microscope at voltage 20 kV and 15.0 mA. Specific surface area and pore parameters of powders were evaluated by BET method using Micromeritics ASAP 2405 accelerated surface area and porosimetry analyzer equipment.

### 3 RESULTS AND DISCUSSION

#### 3.1 Thermal Analysis of Bone Pulp

Major research on calcium phosphates preparation was preceded by thermal analysis of bone pulp in order to

determine the course of calcination process (Figure 1). Thermal decomposition of the analyzed material is multi-stage. The first stage is an endothermic process, which occurs at temperatures from 70–170  $^{\circ}\text{C}$ . It corresponds to water desorption from the surface of the sample with weight loss of approximately 8%. The next weight loss, in the range of 200–600  $^{\circ}\text{C}$ , is associated with exothermic decomposition of organic matter; the protein and fat residues already present in bone pulp after boiling in diluted lactic acid. The last weight losses at a temperature of ~650–800  $^{\circ}\text{C}$  are attributed to dehydroxylation processes, the thermal decomposition of hydroxyapatite and the loss of carbonate groups present in the natural bone [15].

Based on the derivatogram analysis we decided to conduct a two-stage calcination process of previously hydrolyzed bone material. The first step, pre-calcination at a temperature of 650  $^{\circ}\text{C}$  for 3 h of exposure, is essential to burn out organic compounds present in the material. Then it is assumed that lead calcining of the right milled material under varying conditions of temperature and time is needed in order to investigate the effect of various parameters on the physicochemical properties of the resulting product. Then, the obtained material, shredded in a ceramic mortar, was calcined in the same kiln chamber at a temperature ranging from 750–1050  $^{\circ}\text{C}$  with 100 degree intervals for 2 or 3 h of exposure.

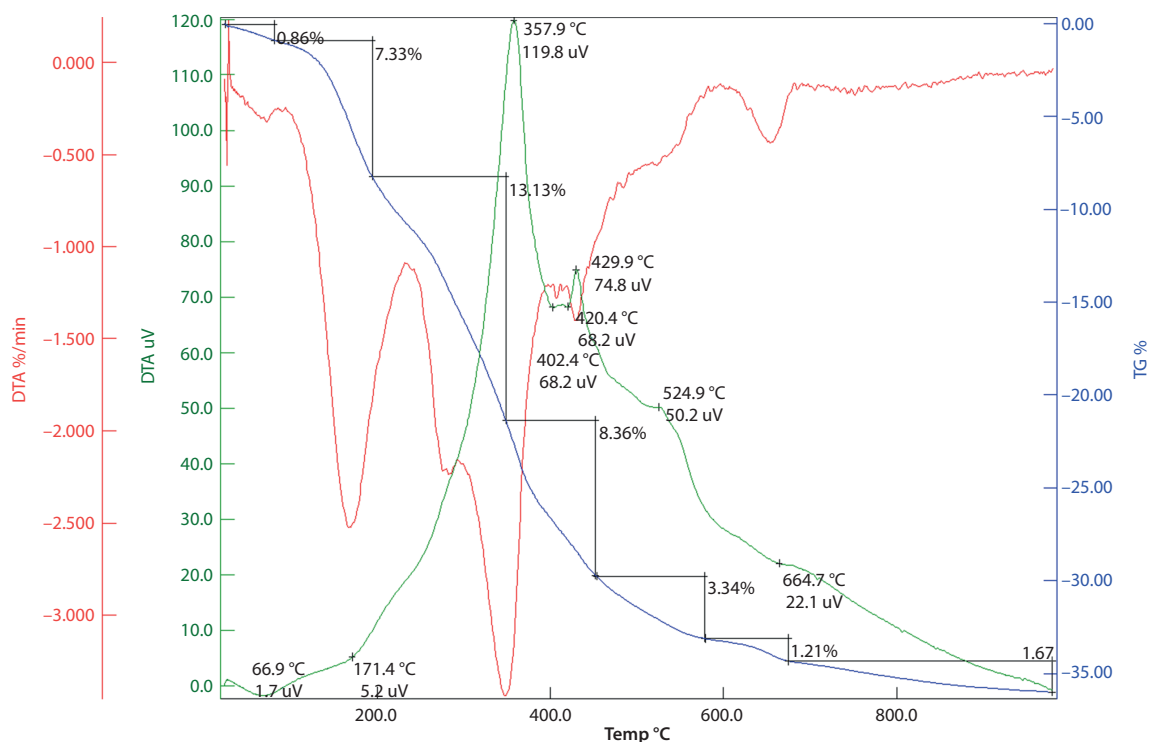


Figure 1 Thermal analysis of bone pulp.

## 3.2 Physicochemical Characterization of Final CP Products

### 3.2.1 XRD Phase Identification

Figure 2 presents X-ray diffraction patterns of calcium phosphate products obtained in the three-stage treatment. The proper calcination process carried out at a temperature range from 750–950 °C (for three conducted hydrolyses) failed to obtain a pure, white CP material. Only at 1050 °C were unburned compounds not present in the material. Based on the position of the Bragg reflections on the diffraction patterns, it was found that there are two crystalline phases: hydroxyapatite (marked as HAp) and tricalcium phosphate (marked as TCP) in CP products as a result of hydrolysis 1, regardless of the temperature and time of the proper calcination. However, it is worth mentioning that with an increase in temperature, all CP powders exhibit a higher intensity of the reflections, which is typical for materials with a high degree of crystallinity. The XRD analysis of the calcined products obtained after hydrolysis 2 of bone sludge again indicates the presence of HAp and TCP; however, an additional compound is also present in the material—calcium diphosphate (marked as  $C_2P$ ,  $Ca_2P_2O_7$ ).

However, this phase, with low intensity of reflections, is noticeable only in the materials calcined at 750–950 °C. Calcium diphosphate, because of its low water solubility and chemical inertness, is used as an abrasive for  $Sn^{2+}$  and  $F^-$  in toothpaste. In the highest

calcination temperature at 2 or 3 h of keeping material at a set temperature, only two phases are identified, TCP and HAp, and the crystallinity of the product increased significantly. Diffraction patterns of powders obtained using hydrolysis 3 (volumetric ratio of water to 80% lactic acid was 2:1) indicate the presence of calcium hydroxide in addition to the HAp, TCP and  $C_2P$  compounds. The increase of lactic acid share promotes the formation of new phases in the material.  $Ca(OH)_2$  occurs in the powders calcined in the temperature from 750–950 °C, which means that under the tested hydrolysis conditions the calcium ions are able to form different compounds, not only CPs. Differences in the structure and phase composition of the obtained materials caused changes in the contents of calcium and phosphorus, which we will discuss later. Summarizing, on the basis of the foregoing considerations it can be stated that in all products the significant influence of proper calcination temperature on the intensity of Bragg reflections is observed. With increased heat treatment the degree of crystallinity and phase purity of products also increased.

### 3.2.2 FTIR Spectroscopy

The FTIR spectra in Figure 3 demonstrate the presence of characteristic bands of CPs corresponding mainly to  $PO_4^{3-}$  and  $OH^-$  groups in all the tested CP products regardless of the W:LA volumetric ratio in hydrolysis. A band at a wavenumber of 603  $cm^{-1}$  corresponds to the triply degenerate asymmetric

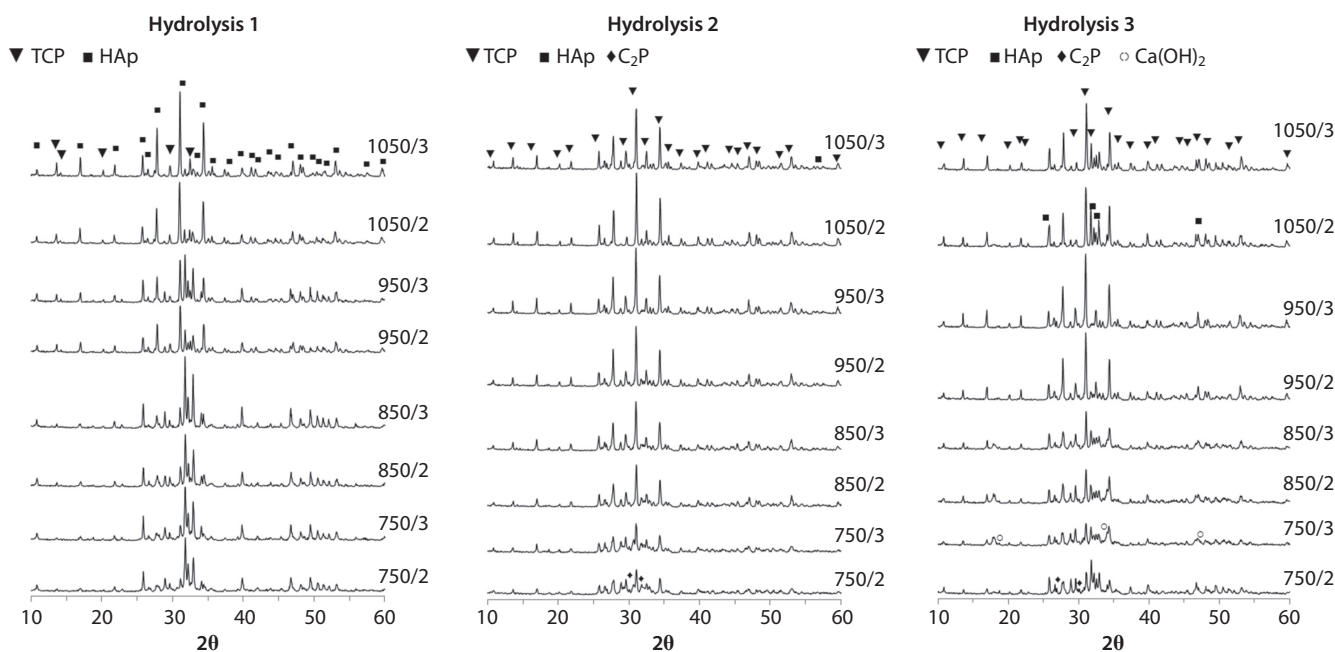
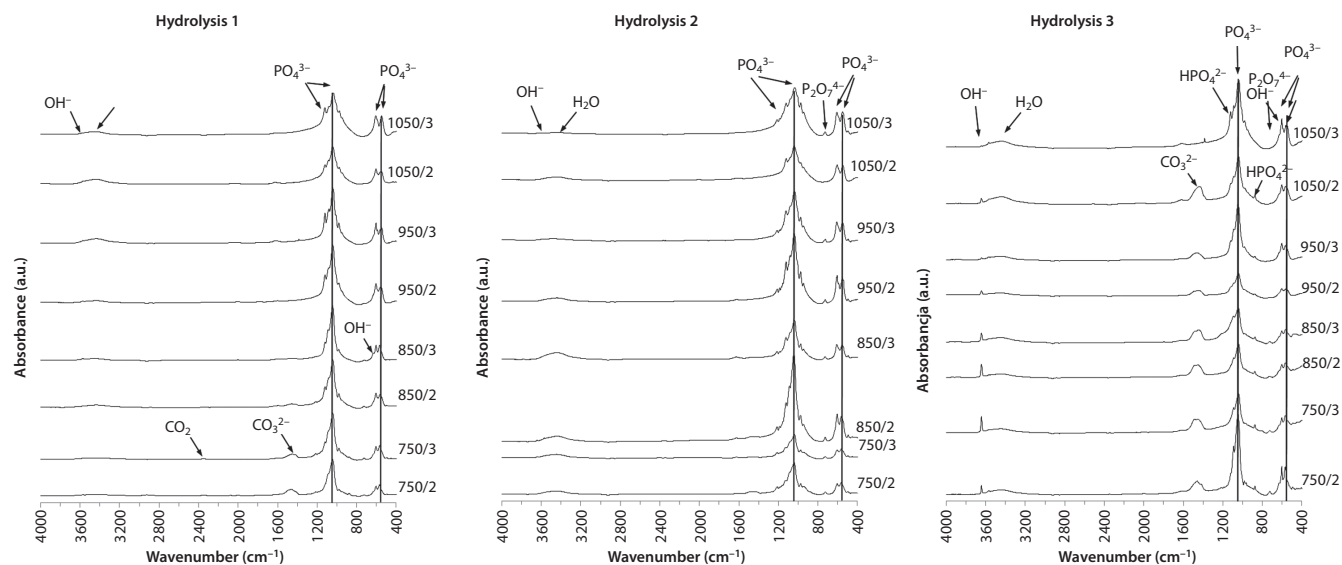


Figure 2 Diffractograms of calcination products obtained at different treatment parameters.



**Figure 3** FTIR spectra of calcination products obtained at different treatment parameters.

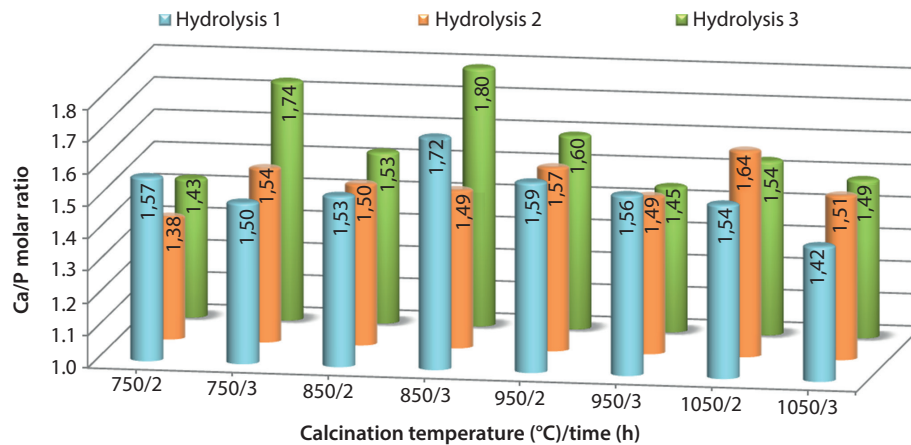
stretching vibration O-P-O of the central  $\text{PO}_4$  tetrahedron. Bands of low intensity within the wavenumber range of  $560\text{--}550\text{ cm}^{-1}$  correspond to the asymmetric vibrations of the  $\text{PO}_4^{3-}$  group from  $\alpha$ -TCP. Also, typical phosphate group bands of the highest intensity within the wavenumber range of  $1120\text{--}1043\text{ cm}^{-1}$  confirmed the presence of vibrational asymmetric stretching mode. A weak band at  $1462\text{ cm}^{-1}$  can be assigned to C-O vibration in the  $\text{CO}_3^{2-}$  group replacing some phosphate groups in the CP crystal lattice. This band is characteristic of CPs derived from animal bones. Typically, CPs stretching vibrations of  $\text{OH}^-$  group have been recorded as a weak band at wavenumber of  $640\text{ cm}^{-1}$  and band within the wavenumber range of  $3670\text{--}3570\text{ cm}^{-1}$ , which corresponds to the adsorbed water [15, 28].

The FTIR analysis of CP products hydrolyzed the second way also confirm the presence of CPs' characteristic bands, corresponding mainly to  $\text{PO}_4^{3-}$  and  $\text{OH}^-$  groups. Furthermore, the visible band with the maximum absorption at  $727\text{ cm}^{-1}$  corresponds to the pyrophosphate ion ( $\text{P}_2\text{O}_7^{4-}$ ) derived from calcium pyrophosphate shown in the diffraction patterns. The FTIR spectra of powders obtained as a result of calcination products of hydrolysis 3 also confirmed the presence of characteristic phosphate and hydroxyl groups. However, in this case, the peaks attributed to the oscillation of the hydroxyl groups at  $\sim 3600\text{ cm}^{-1}$  have a greater intensity than the samples obtained under other preparation conditions. Moreover, it appears that the conditions of hydrolysis 3 are not sufficient to completely replace the carbonate groups with phosphates. Thus the B-type hydroxyapatite is obtained, in which carbonate groups are incorporated

in place of the  $\text{PO}_4^{3-}$ . However, it could be noted that the use of the third method of hydrolysis under conditions of  $1050\text{ }^\circ\text{C}$  temperature of proper calcination process for 3 h of exposure, resulted in product with a lower content of carbonates in comparison with the other final powders obtained in this series.

### 3.2.3 Ca/P Molar Ratio

Figure 4 shows the Ca/P molar ratio of all obtained CP materials depending on the type of hydrolysis and parameters of proper calcination. For the first test series (hydrolysis 1), Ca/P ratio is set in the range from 1.42–1.72, wherein there is no temperature dependence. The low ratio confirmed the presence of TCP phase with Ca/P = 1.5, while the increase of this parameter may be caused by predominance of phase HAp over TCP. The second test series (hydrolysis 2 – W:LA = 2:0.75) also shows no significant changes in the value of the Ca/P ratio related to varied CP treatment conditions. Due to the presence of calcium phosphate phases, such as  $\text{Ca}_2\text{P}_2\text{O}_7$ , with a low Ca/P molar ratio ( $\text{Ca}_2\text{P}_2\text{O}_7 = 1.0$ ), the values for this test series is in the range between 1.38 and 1.64. These differences can be explained by variables in the contents of different phases in final materials. The variety of phases in CPs, as a result of hydrolysis 3 in the first stage of preparation, affects the contents of calcium and phosphorus as well. In products containing calcium, the higher value of Ca/P ratio is determined. In other CPs, due to the presence of TCP or/and  $\text{C}_2\text{P}$ , the Ca/P ratio is less than the expected stoichiometry ratio of pure hydroxyapatite (1.67). The content of calcium and phosphorus of the hydroxyapatite ceramic shall be determined



**Figure 4** Ca/P molar ratio of calcination products obtained at different treatment parameters.

**Table 1** Microelements and heavy metal contents of selected CPs powders obtained in varied proper calcination parameters at 1050 °C, 2 or 3 h.

Metal	Metal content [mg/kg]						Maximum limit [mg/kg]
	Hydrolysis 1		Hydrolysis 2		Hydrolysis 3		
	1050/2	1050/3	1050/2	1050/3	1050/2	1050/2	
As	<3						3
Cd	<0,5						5
Co	<1						–
Cr	1	<1	1	1	2	1	–
Cu	437	73	127	147	319	214	–
Hg	0.024	0.014	0.045	0.051	0.027	0.019	5
Ni	<1						–
Pb	21	4	7	9	16	11	30

in accordance with ISO standard 13779–3:2000(E) [29]. The calcium-to-phosphorus ratio, Ca/P, shall have value of 1,65–1,82 for the Ca/P ratio. Most of the obtained CP products met the ISO requirements, which qualifies the material for further analyses with regard to biomedical applications.

### 3.2.4 Content of Microelements and Heavy Metals

No undesirable metal contaminations are a key parameter in the assessment of material for biomedical applications. The limits of specific trace elements for ceramic apatites are given in Table 1. The contents of all tested elements in CP powders obtained as a result of different treatment conditions met the established standards (ISO standard 13779–1:2000(E)) [30].

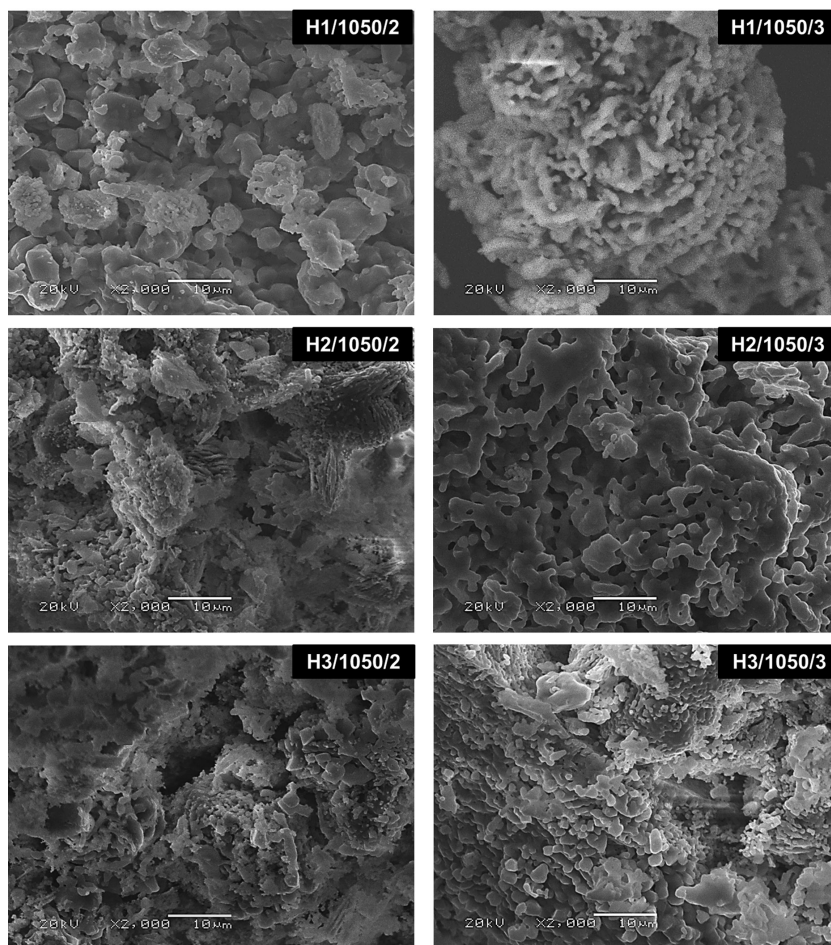
## 3.3 Structural Parameters of CP Products

### 3.3.1 SEM Images

The morphology analysis (Figure 5) of the powders showed that the crystallites of all materials are generally uniform in size and shape. The boundary between the individual grains is not evident and indicates a high degree of aggregation due to sintering of grains in all materials finally calcined in 1050 °C.

### 3.3.2 Surface Area and Pore Parameters

Table 2 summarizes the structural features of CP powders; surface parameters of three selected powders obtained under different hydrolysis conditions, and then calcined at 1050 °C for 3 h, are compared. Surface area analysis confirmed the theoretical assumptions



**Figure 5** SEM microphotography of selected CP powders obtained at varied proper calcination parameters at 1050 °C, 2 or 3 h (x 2000).

**Table 2** Surface area and pore parameters of selected calcination products.

Parameter	H1/1050/3	H2/1050/3	H2/1050/3
BET surface area [m <sup>2</sup> /g]	0,54	0,09	0,25
Total adsorption surface area of pores [m <sup>2</sup> /g]	0,165	0,029	0,157
Total adsorption volume of pores [cm <sup>3</sup> /g]	0,001398	0,000609	0,001861
Average pore diameter [nm]	10,28	25,85	30,03
Porosity [%]	30,33	30,79	63,36

that bone-derived HAp has small specific surface area not exceeding more than 1 m<sup>2</sup>/g, which is a characteristic of high-temperature calcined products. Natural bone minerals, not heat-treated, have surface areas of 87 and 100 m<sup>2</sup>/g, which is of the same order of magnitude as the surface area of synthetic unsintered hydroxyapatite for which surface areas of 17–82 m<sup>2</sup>/g were reported [31]. The process of calcination at temperatures above 700–800 °C results in a decrease of the order of magnitude in the surface area (up to 0.1 m<sup>2</sup>/g)

due to the grain melting and reduction of intergranular space in HAp particle. It has been observed that with the smallest share of lactic acid, the surface area is the highest in comparison with products with other tested CP products. Alike pore parameters determine application of these types of materials in implantology. Comparing the porosity of final CP products it can be noted that the material with the highest share of lactic acid has the highest porosity of over 63%—twice that of other materials obtained under hydrolysis 2

and 3 conditions. However, we must be aware that the total adsorption volume of pores of all tested powders ranges within 0,0006–0,001 cm<sup>3</sup>/g; therefore it can be concluded that the calcined CPs' bone origin is a solid material, not porous, since the pore volume in the material is negligible. Considering the low total volume and nanometric size of the pores (Table 2), the potential use of the obtained materials as a main component of bone scaffolds should be excluded. It has been proven that the minimum diameter of the pores allowing penetration of a living tissue should be 40–500 μm in size depending on the type of cell ingrowth into the spaces in bones. The porosity should be placed in the range from 20–60%; greater porosity causes a drastic decrease in the mechanical properties of CP materials [32].

## 4 CONCLUSION

Based on the results of these investigations, it can be stated that the transformation of pork bones into pure calcium phosphate materials for use in medicine is an attractive, viable and economical alternative to traditional methods of biomaterial production. Research issues presented in this study were selected in view of their cognitive significance and possible development in chemical technology for eventual use in medicine and dentistry. In the near future, our research team plans to develop a method for the preparation of a composite material containing the calcium phosphates obtained in this study to achieve the advisable bioactive materials with controlled microstructural and physicochemical properties. In the case of expected experimental results, these materials can become a strong base for the development of new implant materials for use in medicine.

## CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

## ACKNOWLEDGMENTS

This work was financed by the National Centre for Research and Development under the Lider project contract no. 037/481/L-5/13/NCBR/2014.

## REFERENCES

1. L.A. Jacobsen, M. Kent, M. Lee, and M. Mather, America's aging population. *Popul. Bul.* **66**, 1–20 (2011).

2. G.K. Vincent and V.A. Velkoff, The next four decades the older population in the United States: 2010 to 2050. Population estimates and projections. Current Population Reports, U.S. Department of Commerce Economics and Statistics Administration, U.S. Census Bureau (2010).
3. A. Szymański, *Biomaterialization and biomaterials*, Państwowe Wydawnictwo Naukowe, Warszawa (1991).
4. Z. Święcicki, *Bioceramic for orthopedics*, Instytut Podstawowych Problemów Techniki PAN, Wydawnictwo Spółdzielcze sp. z o. o., Warszawa (1992).
5. J. Marciniak, *Biomateriały*, pp. 68–90, Wydawnictwo Politechniki Śląskiej, Gliwice (2002).
6. B.D. Ratner, A.S. Hoffman, F.J. Schoen, and J.E. Lemons, *Biomaterials Science: An Introduction to Materials in Medicine*, pp. 1491–1493, Academic Press, San Diego (2004).
7. M. Navarro, O. Michiardi, and J.A. Planell, Biomaterials in orthopaedics. *J. R. Soc. Interface* **5**, 1137–1158 (2008).
8. N.R. Patel and P.P. Gohil, A review on biomaterials: Scope, application human anatomy significance human anatomy significance. *IJETAE* **2**, 91–101 (2012).
9. A. Sionkowska and J. Kozłowska, Characterization of collagen/hydroxyapatite composite sponges as a potential bone substitute. *Int. J. Biol. Macromol.* **47**, 483–487 (2010).
10. S. Jebahi, H. Oudadesse, H. el Feki, T. Rebai, H. Keskes, P. Pellen, and A. el Feki, Antioxidative/oxidative effects of strontium-doped bioactive glass as bone graft. *In vivo assays in ovariectomised rats. J. Appl. Biomed.* **10**, 195–209 (2012).
11. D. Malina, A. Sobczak-Kupiec, and Z. Wzorek, Biomaterials – the materials used in medicine. *Dental Engineering – Biomaterials* **11**, 14–17 (2014).
12. D.L. Wise, D.J. Trantolo, D.E. Altobelli, M.J. Yaszemski, J.D. Gresser, and E.R. Schwartz. *Encyclopedic Handbook of Biomaterials and Bioengineering. Part A: Materials*, Marcel Dekker Inc., New York (1995).
13. S. Błażewicz and L. Stoch, *Biocybernetics and biomedical engineering 2000. Tome 4: Biomaterials*, pp. 99–149, Akademicka Oficyna Wydawnicza EXIT, Warszawa (2003).
14. S.M. Best, A.E. Porter, E.S. Thian, and J. Huang, Bioceramics: Past, present and for the future. *J. Eur. Ceram. Soc.* **28**, 1319–1327 (2008).
15. A. Sobczak-Kupiec and Z. Wzorek, The influence of calcination parameters on free calcium oxide content in natural hydroxyapatite. *Ceram. Int.* **38**, 641–647 (2012).
16. G. Goller, F.N. Oktar, S. Agathopoulos, D.U. Tulyaganov, J.M.F. Ferreira, E.S. Kayali, and J. Peker, Effect of sintering of temperature on mechanical and microstructural properties of bovine hydroxyapatite (BHA). *J. Sol-Gel Sci. Techn.* **37**, 111–115 (2006).
17. K. Haberko, M. Bucko, J. Brzezinska-Miecznik, M. Haberko, W. Mozgawa, T. Panz, A. Pyda, and J. Zarebski. Natural hydroxyapatite: Its behaviour during heat treatment. *J. Eur. Ceram. Soc.* **26**, 537–542 (2006).
18. N.A.M. Barakat, M.S. Khila, A.M. Orman, F.A. Sheikh, and H. Y. Kim, Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods. *J. Mater. Process. Technol.* **209**, 3408–3415 (2009).



19. M. Younesi, S. Javadpour, and J. Bahrololoom, Effect of heat treatment temperature on chemical compositions of extracted hydroxyapatite from bovine bone ash. *Mater. Eng. Perform.* **20**, 1484–1490 (2010).
20. A. Sobczak, D. Malina, Z. Kowalski, and Z. Wzorek, Physicochemical properties of nanosilver suspension assigned to modification of hydroxyapatite sorbents. Preliminary investigations. *Przem. Chem.* **89**, 540–542 (2010).
21. A. Sobczak-Kupiec, Z. Wzorek, R. Kijkowska, and Z. Kowalski, Effect of calcination conditions of pork bone sludge on behaviour of hydroxyapatite in simulated body fluid. *Bull. Mater. Sci.* **36**, 755–764 (2013).
22. A. Sobczak, Z. Kowalski, and Z. Wzorek, Preparation of hydroxyapatite from animal bones. *Acta Bioeng. Biomech.* **11**, 23–28 (2009).
23. A.M. Janus, M. Faryna, K. Haberko, A. Rakowska, and T. Panz, Chemical and microstructural characterization of natural hydroxyapatite derived from pig bones. *Microchim. Acta* **161**, 349–353 (2008).
24. F. Zorn, F. Weber, A. Almeida, I. Taubert, R. Wagenknecht, and W. Eberle, Process for the production of spongiosa bone ceramic having low calcium oxide content, US Patent 5776843, assigned to Merck Patent Gesellschaft Mit Beschränkter Haftung (July 7, 1998).
25. J. Brzezińska-Miecznik, E. Kasprzyk, K. Haberko, A. Pyda, M. Bućko, W. Mozgawa, Ł. Zuch, B. Trybalska, and W. Reczyński, Sintering of natural origin hydroxyapatite in air and carbon dioxide atmospheres. *Materiały Ceramiczne* **60**, 274–277 (2008).
26. K. Haberko, M. Bućko, W. Mozgawa, A. Pyda, J. Brzezińska-Miecznik, and J. Carpentier. Behaviour of bone origin hydroxyapatite at elevated temperatures and in O<sub>2</sub> and CO<sub>2</sub> atmospheres. *Ceram. Int.* **35**, 2537–2540 (2009).
27. C.Y Ooi, M. Hamdi, and S. Ramesh, Properties of hydroxyapatite produced by annealing of bovine bone. *Ceram. Int.* **33**, 1171–1177 (2007).
28. A. Sobczak-Kupiec, R. Kijkowska, and D. Malina, Infusion of Ag-ion from aqueous solution into solid calcium phosphate of hydroxyapatite (HA) crystal structure. *J. Am. Ceram. Soc.* **99**(9), 3129–3135 (2016). DOI: [10.1111/jace.14317](https://doi.org/10.1111/jace.14317)
29. Standard specifications, Implants for surgery – Hydroxyapatite – Part 3: Chemical analysis and characterization of crystallinity and phase purity, ISO standard 13779–3:2000(E).
30. Standard specifications, Implants for surgery – Hydroxyapatite – Part 1: Ceramic hydroxyapatite, ISO standard 13779–1:2000(E).
31. S. Joschek, B. Nies, R. Krotz, and A. Göpferich, Chemical and physicochemical characterization of porous hydroxyapatite ceramics made of natural bone. *Biomaterials* **21**, 1645–1658 (2000).
32. J.H. Shepherd, R.J. Friederichs, and S.M Best, Synthetic hydroxyapatite for tissue engineering applications, in *Hydroxyapatite (HAp) for Biomedical Applications*, M. Mucalo (Ed.), pp. 235–298, Woodhead Publishing Series in Biomaterials, Number 95, Elsevier Ltd., London (2015).