



COMPARING THE EFFECTS OF TWO WET PRECIPITATION METHODS ON THE YIELD OF CHICKEN EGGSHELL-DERIVED HYDROXYAPATITE

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Abstract

Research into better and more improved biomaterials is at the forefront of modern biomedicine. A calcium phosphate compound known as Hydroxyapatite is one of the most bioactive and biocompatible osteoconductive ceramic materials currently known to man. It occurs naturally in the bones, teeth, and shells of some animals and the leaves, and stalks of some plants. It can also be synthesized artificially. Eggshells are very prominent sources of Hydroxyapatite and currently constitute waste, and environmental pollution due to ineffective, wrong, and incorrect disposal. In this research, two modified variants of the wet chemical precipitation method were used to produce hydroxyapatite from chicken eggshells gotten from the same source. The obtained samples were then characterized using XRD and SEM. The results of the characterization were analyzed to determine the most efficient, cost-effective, and less tedious method of production. It was discovered that although the orthophosphoric acid-based wet precipitation method was more efficient in producing phase pure hydroxyapatite, the nitric acid-based wet chemical precipitation method was found to be better than the orthophosphoric-based wet chemical precipitation method in terms of particle size, agglomeration, elemental analysis, and other observed properties.

Keywords: Environmental impact, characterisation, hydroxyapatite, bio-based, biomaterials

Introduction

Human tissue regeneration and bone regrowth have been a major target of advanced research for at least 50 years (Agbeboh *et al.*, 2020). However, in recent years, researchers have made possible the purposeful regeneration and gradual regrowth of human hard tissue, including the manufacturing of artificial body parts, orthopedic implants, and prosthetics (Agbeboh *et al.*, 2020; Kusmanto *et al.*, 2008). Victims of disease and accidents, as well as the aged who suffer from various bone ailments, are now able to manage their conditions, heal the affected parts, and return to productive or comfortable living conditions. The currently available solutions to orthopedic growth and hard tissue regeneration issues are still far from perfect. The orthopedic implants still do not completely possess the most important physiognomies of human tissues; they cannot maintain an adequate blood supply, they do not self-repair and they cannot change their structure or evolve in response to their

environment (Jones, 2005). This means that there is still a dire need for prolific research to proffer better solutions and perfect the already existing ones.

Bone defects are varied and can cause mild to severe discomfort. They can be repaired or corrected using procedures of tissue engineering that rely on the use of different bioactive molecules, in combination with cells and scaffolds which are dependent on adequate blood supply for preservation and sustenance. The scaffolds must, therefore, possess angiogenic and osteogenic features to allow for improved blood flow and growth (Jones, 2005). Both features are greatly improved when scaffolds are combined with growth factors in the form of bioactive molecules and also when the release of protein is triggered by micro-environmental conditions (Kusmanto *et al.*, 2008). Calcium Hydroxyapatite is one of such bioactive molecules that has been combined with or used in the

manufacture of scaffolds by various researchers over the years in a bid to improve the properties of the scaffolds and improve blood flow (Agbeboh *et al.*, 2020). Calcium Hydroxyapatite or Hydroxyapatite (HAp) is a calcium phosphate (CaP) compound that possesses favourable biomedical characteristics due to its crystal structure, and its similarity to the mineral phase of bone and teeth (Ramesh *et al.*, 2007). It is a white solid with a hexagonal structure that has a strong affinity for heavy metal ions, this makes it very useful in environmental protection as an absorbent (Azis *et al.*, 2018). HAp became popular in the 1960s due to its excellent biocompatibility, haemocompatibility, non-degradability, osteoconduction, osteoinduction (due to the release of calcium and phosphate ions), nonimmunogenic, and noncarcinogenic reactions (Ige *et al.*, 2012). It is widely used as fillers within polymeric matrixes, photocatalyst, the coating on prosthetic devices, clinical bone and hard tissue replacement, etc.

Pure and thermally stable HAp can be obtained from different sources which may be synthetic or natural. Continuous research is being carried out to develop safer, cheaper, and more efficient methods of obtaining HAp from both natural and synthetic sources. Although natural sources are deemed safer because of their immunological properties and intra-body reaction, they are usually non-stoichiometric and also contain other beneficial elements in quantities that are suitable for biocompatibility (Agbeboh *et al.*, 2020). However, the synthetic sources are usually purer, stoichiometric, and can be tuned to have specific desirable properties and exact Ca/P ratios, consequently making them suitable for targeted applications in specific areas of the human body (Carrodeguas and Aza, 2011).

Giant strides in research into the extraction of HAp from different natural sources have led to rapid improvements in the quality of HAp obtained from each source. Some natural sources from which HAp has been successfully obtained are bovine and human bones, fish bones and shells, snail shells, eggshells, oyster shells, corals, leaves, and stalks of plants (e.g. mint, basil, Trifolium, and green tea) and algae (Oladele *et al.*, 2018; Akram *et al.*, 2014). Many of these natural resources are waste materials that are inexpensive and easily accessible (Olajide *et al.*, 2019). In recent years, research into the use of waste materials for the synthesis of hydroxyapatite and other biocompatible materials has increased drastically due to the advantage of converting the waste to usable products thereby eliminating harmful waste from the environment. Prominent among these materials are eggshells, especially chicken eggshells because they are rich in calcium (which is in form of calcium carbonate, an essential material for hydroxyapatite production) and a substantial source of waste from restaurants, houses, bakeries, etc. causing a serious

menace to the environment (Oladele *et al.*, 2019). The shell of an egg accounts for about 11% of the egg (Wu *et al.*, 2015). The eggshells consist of about 94% CaCO_3 , 1% magnesium carbonate, 1% calcium phosphate, 4% organic matter and insoluble proteins (Li-Chan and Kim, 2008). It has three structural layers, a ceramic-looking cuticle, a lamellar, and a spongy layer (Jojo *et al.*, 2015).

The synthesis of high purity, osteogenic, and angiogenic HAp of appropriate morphology and Ca/P ratios from natural sources can be laborious depending on the synthesis route or method employed (Núñez *et al.*, 2018). Various synthesis routes have been developed for the production of hydroxyapatite from eggshells biogenic waste materials, each having its intricacies and levels of difficulty (Sanosh *et al.*, 2009). Lee and Oh (2003) produced various calcium phosphate bioceramics including hydroxyapatite from eggshells reacted with phosphoric acid, Nayar and Guha (2009) carried out the in-situ synthesis of HAp by reacting eggshells with a solution of Diammonium Hydrogen Phosphate (DAHP) and hydrochloric acid but concluded that it was not suitable for biomedical purposes, this was due to the ethical issues posed as a result of the biomolecules introduced into the HAp, to control the sizes and shapes of the HAp particles produced. Microwave irradiation of eggshells in the presence of diammonium hydrogen phosphate was also used by Krishna *et al.* (2007) to produce non-cytotoxic nanosized HAp with increased osteoblast cell growth rate. Azis *et al.* (2018) used a sol-gel process involving precipitated calcium carbonate from eggshells to synthesize a calcium carbonate compound shown by XRD, FTIR, and SEM to be hexagonal crystals of calcium hydroxyapatite. These are just a few of the numerous techniques so far developed by researchers to furnish HAp of different sizes, porosity, cytotoxicity, and other physical and chemical properties. However, two simple and cost-effective routes have emerged as the most favourable, swift, and efficient synthesis routes for the production of HAp from eggshells. They are an orthophosphoric acid-based process that uses orthophosphoric acid as its phosphate source (Oladele *et al.*, 2019), and a wet chemical-based process that uses diammonium hydrogen phosphate as its phosphate source (Sopyan *et al.*, 2008). Each route produces HAp with different characteristics in varying amounts of time. This is because the properties and characteristics of the HAp produced are highly dependent on the source of the materials used in its production, the particle size of the eggshell powder, the concentration of the chemicals and the reaction methods (Oladele *et al.*, 2019). It is, therefore, necessary to develop a method that furnishes HAp from natural wastes efficiently using cheap

materials and equipment in a shorter time, and mass-producing to match the quality and volume obtainable from synthetic sources. This present research attempts to compare the HAp produced from eggshells from the same ecological source using the two selected wet chemical precipitation methods. The wet chemical precipitation process was selected because it is a cost-effective process.

Materials and Method

Materials

The eggshells used in this work were all gotten from the same source: chicken eggshells of the Leghorn breed of Nigerian chicken obtained from a poultry farm located in Ihumudumu town, Ekpoma, Esan region of Edo state, Nigeria. The Nitric acid ($\text{HNO}_3 \geq 99.0\%$ purity), Orthophosphoric acid ($\text{H}_3\text{PO}_4 \geq 85\%$ purity), Diammonium Hydrogen Phosphate ($(\text{NH}_4)_2\text{HPO}_4 \geq 99.0\%$ purity) and Distilled water were obtained from Pascal Scientific Ltd., Akure, Ondo State, Nigeria.

Preliminary Processing of Eggshells

The eggshells from the eggs of a local leghorn breed of white chickens (*Gallus gallus domesticus*) having an average weight of 600-700 g were collected, cleaned, boiled in distilled water for 10 min according to Oladele *et al.* (2019) to remove its membranous layer, then they were washed and dried for 24 h at 80 °C in an oven.

Thermal Treatment of Eggshells

The eggshells from the eggs of a local leghorn breed of white chickens (*Gallus gallus domesticus*) having an average weight of 600-700 g were collected, cleaned, boiled in distilled water for 10 min according to Oladele *et al.* (2019) to remove its membranous layer, then they were washed and dried for 24 h at 80 °C in an oven.

Thermal Treatment of Eggshells

The dried eggshells were pulverised into finely powdered particles, which were then sieved using a sieve shaker to determine the particle size, and particles below 25 µm were obtained. To ensure complete conversion, the eggshells were put in a crucible, placed in an electric muffle furnace and first heated from room temperature to 450 °C at a rate of 10 °C /min, the temperature was held constant for 1 h to ensure that all the organic material burns off and the smoke being emitted stops. Then secondly the shells were heated to 700 °C at a heating rate of 4 °C/min and held at this temperature for 2 h, to ensure all the proteins and organic elements were completely burnt off, and also to

begin the process of decarbonization and emission of CO_2 as shown via the chemical equation in Equation 1.



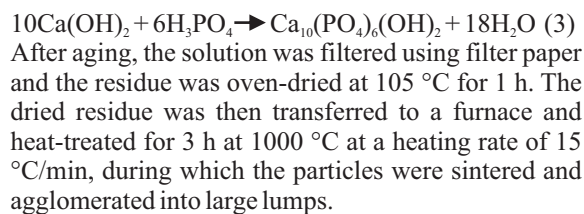
The third step was carried out by heating the powdered eggshell samples from 700 °C to 1000 °C at a heating rate of 4 °C/min and holding for 3 h to ensure complete decarbonization and transformation of the eggshells to calcium oxide (CaO). The calcium oxide was confirmed by X-Ray Diffraction (XRD) and then used in the synthesis of hydroxyapatite via the two different wet chemical precipitation methods which are further discussed in the next session.

Hydroxyapatite Synthesis using Orthophosphoric Acid

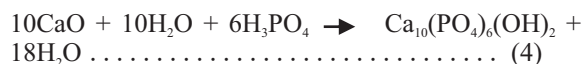
After calcination, the samples were allowed to cool to room temperature within the furnace and then 3 g of the CaO obtained was measured into 100 ml of distilled water in a beaker. The beaker was then placed on an electric heating plate maintained at 90 °C for 2 h. The expression was as shown in Equation 2.



The solution was then reacted with 20 ml of analytical grade orthophosphoric acid (H_3PO_4) while stirring at 120 rpm according to the processes outlined by Hui *et al.* (2010) and Oladele *et al.* (2019). A suspension of precipitates was initially observed, and upon further stirring, for about 30 min the precipitates dissolved into the solution. The pH of the solution was kept constantly below 2 by dropwise addition of sodium hydroxide solution when needed. After all the precipitates had dissolved, signifying completion of the reaction as shown in Equation 2, stirring was stopped, the beaker was removed from the heating medium and allowed to cool and age for 7 days, the aging process followed Equation 3.



The lumps were then pulverized and ground to powder via vigorous ball milling and grinding for 48 h to reduce the particle size. The powdered particles were subjected to sieve analysis after grinding to determine the particle size, and particles below 90 µm were obtained (Wu *et al.*, 2015). Due to the possibility of calcium hydroxide being formed from the reaction between CaO and water, the chemical reaction is adjusted as shown in Equation 4.

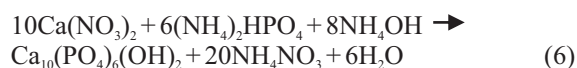
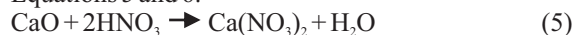


This is, therefore, the expected chemical reaction for the synthesis of hydroxyapatite using orthophosphoric acid-based wet chemical precipitation method

Hydroxyapatite Synthesis using Diammonium Hydrogen Phosphate and Nitric Acid

After the calcination of the eggshells to obtain CaO by the release of carbon dioxide, the CaO was reacted with nitric acid and diammonium hydrogen phosphate in stoichiometric amounts according to the steps outlined by Rujitanapanicha *et al.* (2014). At first 3 g of the CaO was reacted with 25 % Nitric acid at room temperature to obtain calcium nitrate $\text{Ca}(\text{NO}_3)_2$ while stirring constantly. After the heat emitting reaction, the solution was allowed to cool down to room temperature and 12.73 g of diammonium hydrogen phosphate $(\text{NH}_4)_2\text{HPO}_4$ dissolved in 30 ml distilled

water was added dropwise to the solution while maintaining the pH between 8-10 using ammonium hydroxide (NH_4OH) . All through the reaction, the solution was constantly stirred for 1 h to maintain a homogenous reaction according to the chemical Equations 5 and 6.



Upon completion of the reaction the solution was aged for 24 h and then the precipitates were filtered and oven-dried at 120 °C for 2 h. The dried precipitates were then calcined at a heating rate of 15 °C/min to 1000 °C in an electric muffle furnace, and held for 3 h to furnish HAp, and allow for sintering and agglomeration of the HAp particles. The powdered particles were subjected to sieve analysis to determine the particle size, and particles below 40 µm were obtained.

Table 1: X-ray Diffraction Peak Analysis Results for the Eggshell Powder

No.	ICDD - PDF Ref. Code	Mineral Name	Compound Name	Chemical Formula	Crystal System	d [Ang]	2Theta [deg]
1	01-074-1226	Lime	Calcium Oxide	CaO	Cubic	2.38900	37.621
2	01-089-2779	-	Calcium Hydroxide	Ca(OH) ₂	Hexagonal	1.79250	50.901

The phase analysis displayed in Table 1 shows that all the major peaks of CaO and some of $\text{Ca}(\text{OH})_2$ were present in the powder sample and comparison exposes a high correlation between the peaks, *d* spacing and

diffraction pattern of the sample and that of both CaO and $\text{Ca}(\text{OH})_2$ reference patterns (01-075-0264 and 01-089-2779 respectively) showing that the calcined eggshell powder are composed mainly of CaO and $\text{Ca}(\text{OH})_2$.

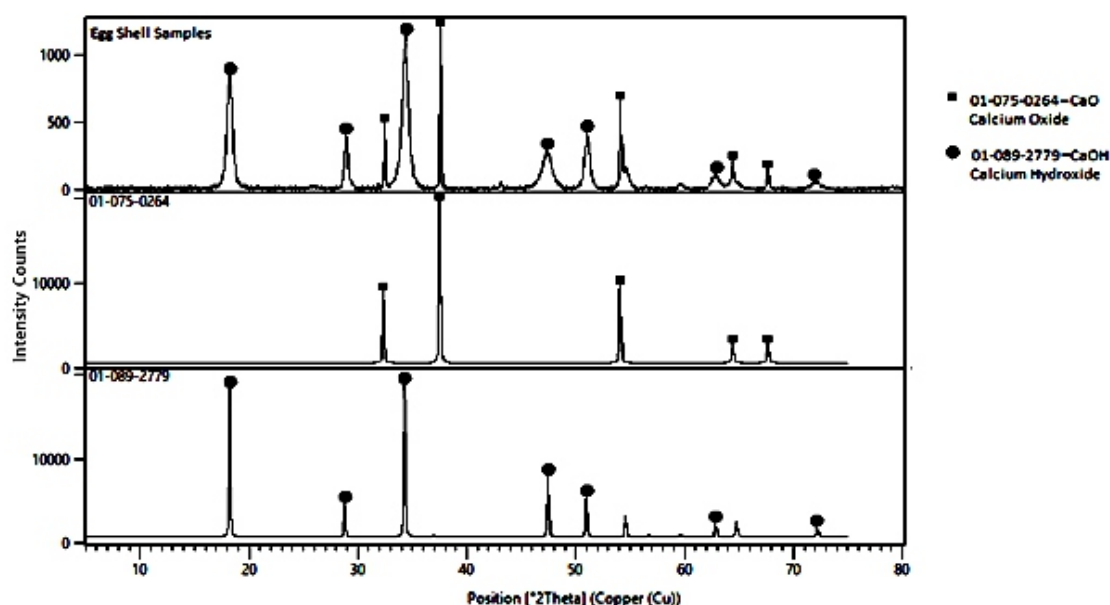


Figure 1: XRD Spectra of Calcined Eggshells

The SEM micrograph of the eggshell powder is shown in Figure 2a, and Figure 2b shows a histogram displaying the particle size distribution measured from the SEM micrographs with a mean size of 3.2 μm . The morphology can be observed from the micrographs, the white phases represent the eggshell particles which

are shown to have sintered and formed agglomerates due to calcination, these agglomerates and the spaces between them are caused by the combined action of heat which sinters the particles together and the expulsion of burnt organic matter in form of gases which leaves spaces between the sintered calcium compounds that remain.

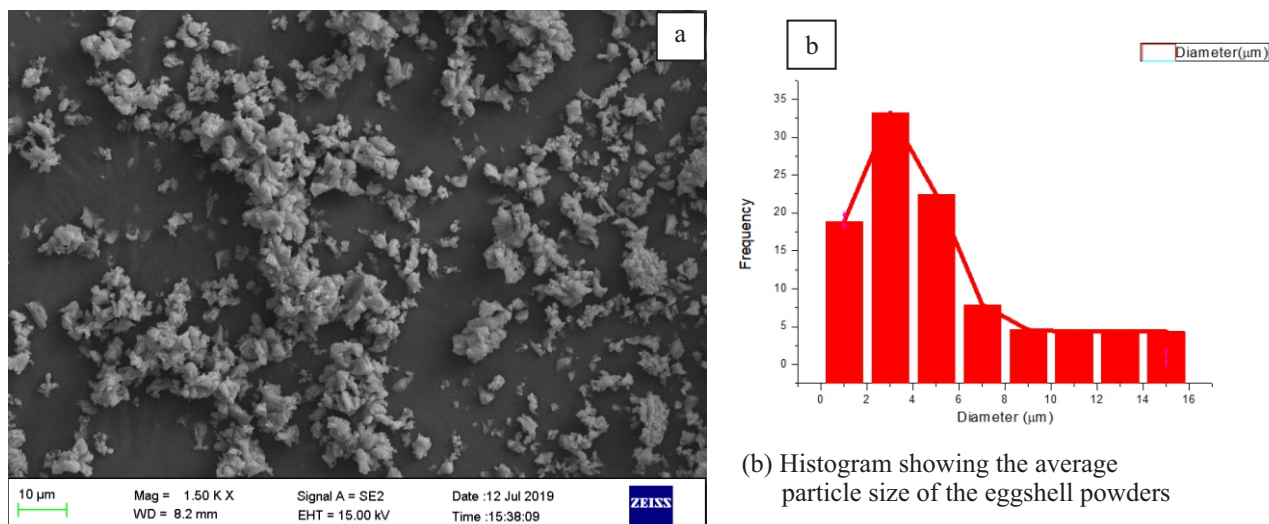


Figure 2: (a) SEM Micrographs of the eggshell powders and

Characterisation of Synthesised HAP Powders

The calcium oxide generated from the calcining process was used to synthesize HAP using two different wet precipitation methods. Both wet precipitation methods require the use of calcium as starting materials. The major difference between both methods being the chemicals reacted with the calcium to yield HAP. The XRD patterns of the HAP derived using orthophosphoric acid are shown in Figure 3, the peaks observed in the spectrum within the 2θ range of

20° – 90° matched the peaks of Calcium Phosphate with ICDD - PDF card no. 00-045-1061 for, and hydroxyapatite (HAP) with ICDD - PDF card no. 01-072-1243. Upon analysis, it was observed that the synthesized sample powders contained a few peaks for Calcium Phosphate and all the major peaks for HAP phases (Goloshchapov et al., 2013; Balazsi et al., 2007). Table 2 outlines the X-ray diffraction peak list for the eggshell and orthophosphoric acid-derived hydroxyapatite powders.

Table 2: X-ray Diffraction Peak Analysis Results for the Eggshell and Orthophosphoric Acid-derived Hydroxyapatite Powders

No	ICDD - PDF Ref. Code	Mineral Name	Compound Name	Chemical Formula	Crystal System	d [Ang]	2Theta [deg]
1	01-072-1243	Hydroxylapatite, syn	Calcium Phosphate Hydroxide	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Hexagonal	3.89005	22.842
2	00-045-1061	-	Calcium Phosphate	$\text{Ca}_2\text{P}_2\text{O}_7$	Unknown	3.78000	23.517

This shows that there was some untransformed calcium phosphate after the reaction had occurred and aging time completed. Therefore, extra reaction time and

aging time were required for a complete transformation to occur (Minh *et al.*, 2012).

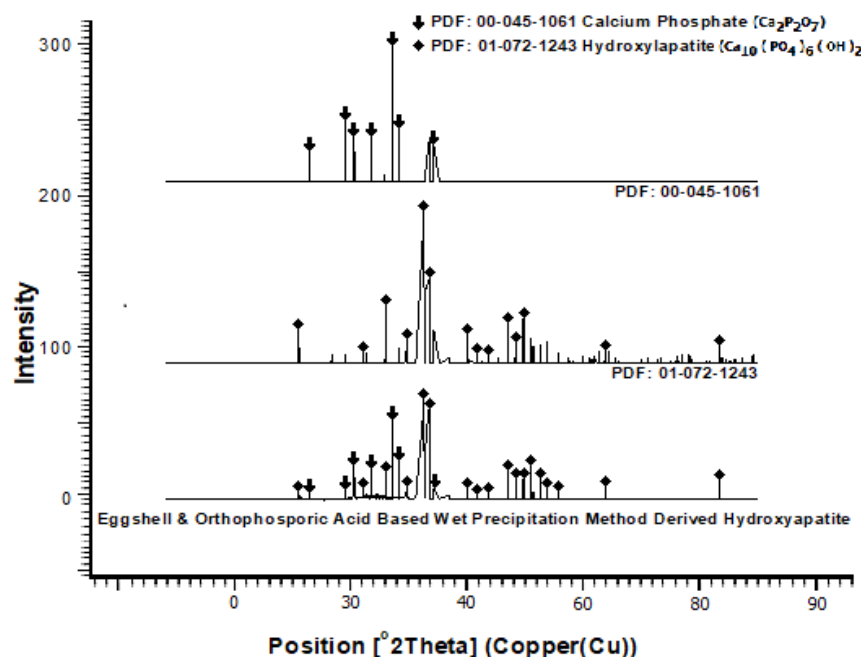


Figure 3: XRD spectrum of the eggshell and orthophosphoric acid-derived hydroxyapatite powders

Figure 4 shows the XRD spectrum of the HAp derived using nitric acid and diammonium hydrogen phosphate. From the spectrum, three phases are identified, based on the peaks observed in the spectrum within the 2θ range of $0-90^\circ$ which were compared with PDF card no. 01-086-2031 for Phosphorus Oxide,

PDF card no. 01-076-0694 for hydroxyapatite with a monoclinic crystal structure (M-HAp) and PDF card no. 01-074-0566 for hydroxyapatite with a normal hexagonal crystal structure (HAp) and the analysis revealed that the synthesized sample powders contained some of the peaks for Phosphorus Oxide all the major peaks for both M-HAp and normal HAp phases

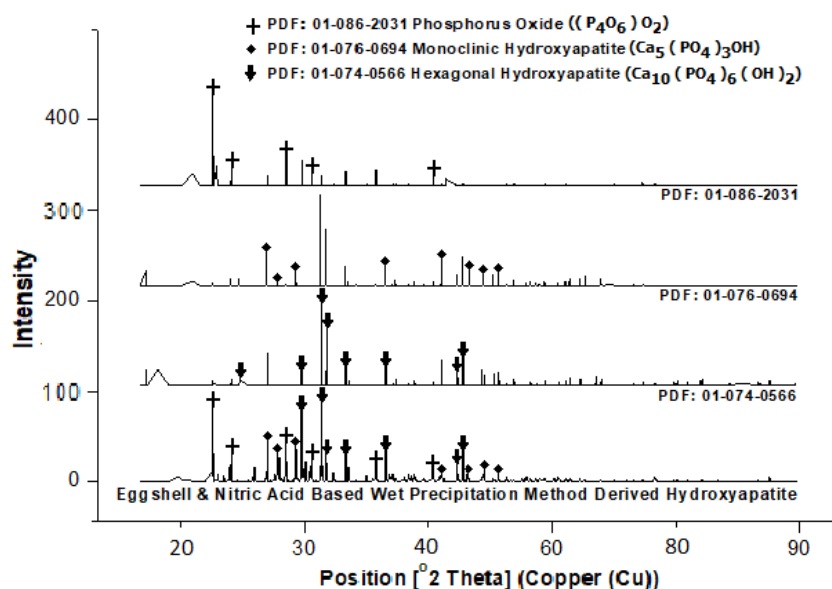


Figure 4: XRD spectrum of the eggshell and nitric acid-derived hydroxyapatite powders

The powder particle size depends to a large extent on the reaction temperature, reaction time, and the aging time of the reaction solution before calcination (Minh *et al.*, 2012). This implies that the reaction time was too

short for all phosphorus oxide phases to completely react with the nitric acid and diammonium hydrogen phosphate, thereby leaving behind some untransformed phosphorus oxide and resulting in the

different types of crystals formed. The X-ray diffraction peak list for the eggshell and nitric acid-derived hydroxyapatite powders evidently showing all the phases including the phosphorus oxide phases present, and the major phases matched are outlined in Table 3. The results confirm that the transformation of

several Calcium phases into phase-pure HAp and the size and type of HAp crystals formed is largely dependent on the aging time allowed. It could be inferred from literature that the aging time was insufficient for the monoclinic phases to transform completely into hexagonal HAp phases (Bakan et al., 2013).

Table 3: X-ray Diffraction Peak Analysis Results for the Eggshell and Nitric Acid-derived Hydroxyapatite Powders

No.	ICDD - PDF Ref. Code	Mineral Name	Compound Name	Chemical Formula	Crystal System	d [Ang]	2Theta [deg]
1	01-076-0694	Hydroxylapatite, syn	Calcium Phosphate Hydroxide	$\text{Ca}_5(\text{PO}_4)_3\text{OH}$	Monoclinic	3.08388	28.929
2	01-086-2031	-	Phosphorus Oxide	$(\text{P}_4\text{O}_6)\text{O}_2$	Monoclinic	3.01184	29.637
3.	01-074-0566	Hydroxylapatite	Calcium Hydroxide Phosphate	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Hexagonal	2.72047	32.897

SEM Micrographs of the HAp derived from the orthophosphoric acid wet precipitation method are shown in Figure 5 (a). The white parts of the micrographs represent the HAp particles which are shown to have sintered due to calcination, the mean particle size was determined from the SEM

micrographs to be 79.1 μm , a histogram showing the average particle size is shown in Figure 5 (b). The sizes, morphology, and shape of the particles were a direct result of the ball milling and grinding process in accordance with previous findings (Rujitanapanicha *et al.*, 2014).

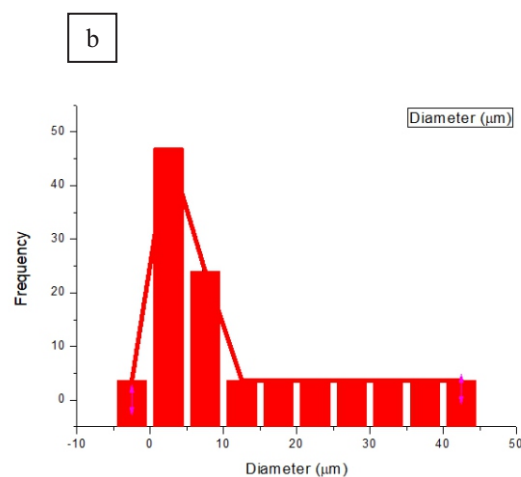
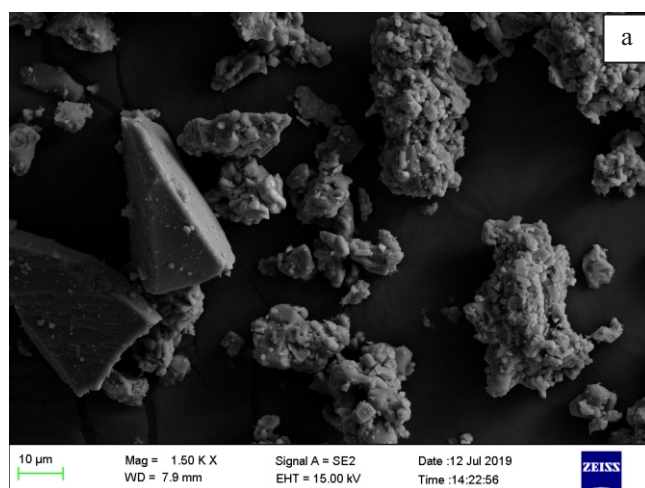


Figure 5: (a) SEM Micrographs of the eggshell and orthophosphoric acid-derived HAp powders and (b) Histogram showing the average particle size of the eggshell and orthophosphoric acid-derived HAp powders

The SEM Micrographs of the HAp derived from the nitric acid and diammonium hydrogen phosphate-based wet precipitation method are shown in Figure 6(a) while the histogram in Figure 6 (b) showed its

average particle size. The particles were observed to be significantly smaller as they were initially fine powders which were, however, shown to have agglomerated due to the high temperature of calcination and the longer holding time to form the lumps visible in the micrographs. The morphology of each particle of these agglomerates resembles somewhat needle-like fibres with a mean particle size determined from the SEM micrographs to be 4.4 μm in agreement to the findings of Sanosh *et al.* (2009).

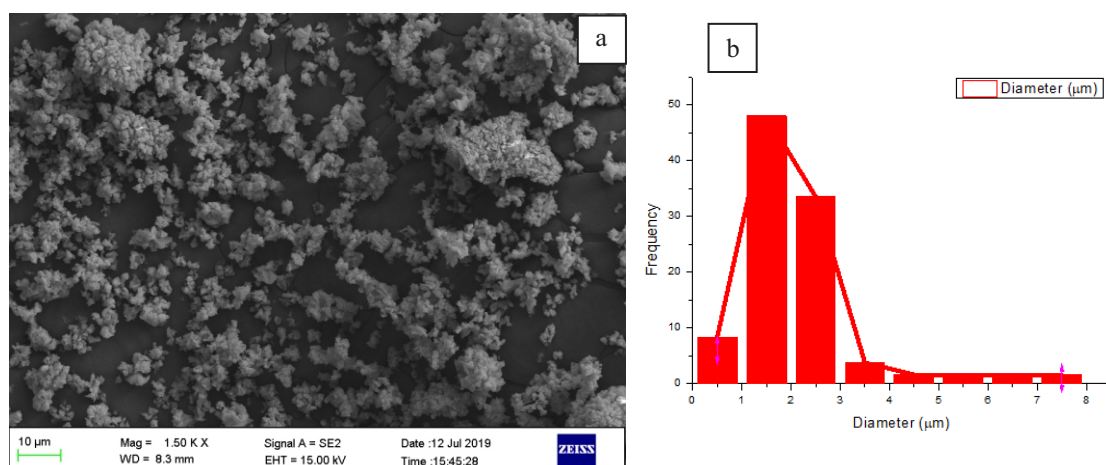


Figure. 6: (a) SEM Micrographs of the eggshell and nitric acid-derived hydroxyapatite powders and (b) Histogram showing the average particle size of the eggshell and nitric acid-derived hydroxyapatite powders.

Efficiency of Wet Precipitation Methods

From the results, over 5 g of samples were obtained for each method after the final calcination. Pure phase hydroxyapatite was achieved, although, there was an incomplete transformation of all the reactants into HAp during the reaction period. Even with this, the orthophosphoric acid wet precipitation method yielded only a calcium phosphate phase and the pure phase hydroxyapatite was formed after the reaction was completed. On the other hand, the nitric acid and diammonium hydrogen phosphate-based wet precipitation method gave the impression that more phases on the CaP spectrum could be obtained, which could be advantageous because the bones and teeth within the body are made up of different CaP phases including HAp (Kusmanto *et al.*, 2008).

The orthophosphoric acid wet precipitation method resulted in the formation of pure phase hydroxyapatite at exactly the Ca/P molar ratio of 1.67. The fact that the reaction was performed at high temperatures of about 90 °C for 2 hours aided the efficiency of the process (Rujitanapanicha *et al.*, 2014; Oladele *et al.*, 2019; Lee and Oh, 2003). Compared to the nitric acid and diammonium hydrogen phosphate-based wet precipitation method which was conducted at room temperature of about 25 °C.

The orthophosphoric acid wet precipitation method requires constant stirring at 90 °C close to the boiling temperature of water for 2 hours 30 mins before the full reaction is completed. While this improves the efficiency of the process, it is, however, time-consuming and energy-intensive, with a high risk of spillage which could result in injury during the process. The nitric acid and diammonium hydrogen phosphate-based wet precipitation method in

comparison requires a shorter constant stirring time and the whole process takes 1 hour. It also conserves energy as the reaction does not require external heating to proceed.

It is worthy of note that the orthophosphoric acid wet precipitation method is performed at a pH of 2 and is, therefore, considered an acidic process. The nitric acid and diammonium hydrogen phosphate-based wet precipitation method is maintained at a pH between 8 and 10 and is considered basic, acidic processes are considered inherently more dangerous and therefore, require more meticulous safety measures (Daramola *et al.*, 2019).

The orthophosphoric acid wet precipitation method requires a shorter time of just 1 hour and a lower temperature of 105 °C to oven-dry but a longer aging time of about 7 days, this is considerably more than the nitric acid and diammonium hydrogen phosphate-based wet precipitation method which requires only 24 hours aging time but 2 hours to oven-dry at a temperature of 120 °C.

At the end of the orthophosphoric acid wet precipitation method, the HAp particles are completely sintered due to the calcination at 1000 °C for 3 hours and require grinding to break the significantly stronger bonds formed as a result of sintering and reduce their particle size. On the other hand at the end of the nitric acid and diammonium hydrogen phosphate-based wet precipitation method the particles are already in fine powdered form, with particles weakly bonded and having individual sizes in micrometres.

Based on careful consideration of these reaction properties it is clear that for complete and efficient synthesis of pure phase HAp the orthophosphoric acid wet precipitation method is most efficient and more economical, since it requires just one major chemical for the synthesis of the product and less calcination time. But nitric acid and diammonium hydrogen phosphate-based wet precipitation method is safer, requires shorter time and is more environmentally friendly. It is recommended, that a method be selected in view of the respective advantages already stated. The

particle size, pH, and purity should also be considered to ensure an efficient and safe biomedical incorporation of the product. This should be done to ensure a perfect match between the developed material and its area of application.

Conclusion

This research methodically compares two wet precipitation processes carried out to produce hydroxyapatite, a very important biomaterial of exceptional biocompatibility from waste eggshells. This was done to determine the most efficient and environmentally acceptable process. The results revealed that the orthophosphoric acid wet precipitation method was the most efficient process and economical for HAp production based on a complete transformation of the starting materials to pure phase HAp while the nitric acid and diammonium hydrogen phosphate-based wet precipitation produced HAp of different crystalline properties. This method was safer and more environmentally friendly. HAp from these eggshells conforms to the acceptable biomaterials desirable for biomedical applications.

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