JIPK. Volume 14 No 2. November 2022
Sinta 1 (Decree No: 158/E/KPT/2021)
e-ISSN:2528-0759; p-ISSN:2085-5842
DOI=10.20473/jipk.v14i2.36261
Available online at https://e-journal.unair.ac.id/JIPK/index

JIPK
(JURNAL ILMIAH PERIKANAN DAN KELAUTAN)
Scientific Journal of Fisheries and Marine

Research Article

Autoclaving and Alkaline Hydrolysis Effects on the Particle Size and Solubility of Grouper (*Epinephelus* sp.) Nano-calcium Powder in *In Vitro* Gastrointestinal Tract Simulation

Pipin Kusumawati[1,2], Priyanto Triwitono[1], Sri Anggrahini[1], and Yudi Pranoto[1*]

[1]Program Study of Food Science, Department of Food and Agriculture Product Technology, Faculty of Agriculture Technology, Universitas Gadjah Mada, Bulaksumur, Yogyakarta, 55281. Indonesia
[2]Ambarrukmo Tourism Institute, Banguntapan, Bantul, Yogyakarta, 55198. Indonesia

OPEN ACCESS

ARTICLE INFO

Received: May 31, 2022
Accepted: July 26, 2022
Published: August 06, 2022
Available online: August 30, 2022

*) Corresponding author:
E-mail: pranoto@ugm.ac.id

Keywords:
Calcium Solvability
Fish Bone
Natural Calcium Source
Organic Content
Recycling Bone Waste

Abstract

Fish bone nano-calcium production may solve two challenges, providing calcium for lactose-intolerant people and recycling bone waste. Fish bone autoclaving prior to extraction reduces fat, denatures collagen, and softens bones but only few researches have compared autoclaving duration with nano-calcium product quality, particle size, and its solubility in *in vitro* testing. This study studied the influence of autoclaving duration followed by alkaline hydrolysis on nano-calcium characteristics to enhance calcium solubility in *in vitro* gastrointestinal simulation experiments. The dried grouper (*Epinephelus* sp.) bone was divided into four groups: 0A (no autoclaving), 3A (3 h autoclaving), 2x3A (double cycle for 3 h autoclaving), and 3x3A (triple cycle for 3 h autoclaving). Each group was followed by alkaline hydrolysis, designated as 0AH, 3AH, 2x3AH, and 3x3AH. The results showed that autoclaving for 3x3 hours followed by alkaline hydrolysis resulted in lowest nano-calcium particle size of 47.47 nm consisting of 30.73% calcium and 18.37% phosphorous. 3x3AH sample created the best calcium solubility (26.14%) in comparison to synthesized $CaCO_3$ (14.34%). In contrast to synthetic $CaCO_3$, grouper nano-calcium powder includes trace quantities of organic contents, such as protein and fat, which enhance calcium solubility. *In vivo* research should be established to study the bioavailability and influence of grouper nano-calcium powder on bone density.

Cite this as: Kusumawati, P., Triwitono, P., Anggrahini, S., & Pranoto, Y. (2022). Autoclaving and Alkaline Hydrolysis Effects on the Particle Size and Solubility of Grouper (*Epinephelus* sp.) Nano-calcium Powder in *In Vitro* Gastrointestinal Tract Simulation. *Jurnal Ilmiah Perikanan dan Kelautan*, 14(2):176-202. http://doi.org/10.20473/jipk.v14i2.36261

1. Introduction

Dietary calcium is one of the most essential elements for the human body and may be obtained by consuming a variety of dairy products (Acar *et al*., 2017). Calcium intake in Asian people is just 500 mg per day, less than half of the 1000-1300 mg per day recommended by the FDA (Cormick and Belizán, 2019). Many Asian populations, including Indonesian people reject dairy products due to high prevalence of lactose intolerance. Lactose intolerance is a disorder in which the body is unable to digest lactose found in milk due to a lack of lactase, a milk-digesting enzyme that obstructs the digestive process (Dewiasty *et al*., 2021; Hodges *et al*., 2019). Osteopenia, osteomalacia, and osteoporosis are just a few of the disorders that might endanger those who suffer calcium deficiency for an extended period of time (Acar *et al*., 2017; Cormick and Belizán, 2019; Hodges *et al*., 2019). Several cases of osteoporosis in the elderly population require surgery to replace porous or broken bones, thus requiring a durable and effective bio-cement nanomaterial for bone replacement (Al-Timimi and Tammemi, 2022). One way to overcome lactose sensitivity is to synthesize natural calcium from other sources, such as animal bones. Calcium sources derived from cow or pig bones often conflict with religious or health restrictions (Jung *et al*., 2007). The greatest option is to get natural calcium from fish bone sources. Indonesia is one of the world's greatest maritime nations, producing around 8.09 million tons of catch fisheries in 2021 (KKP, 2021). Fish bone waste accounts for around 9-15 % of the total weight of the fish (Malde *et al*., 2010; Bas *et al*., 2020). Calcium production from fish bone waste may address two issues simultaneously, to provide a supply of calcium for people who are lactose intolerant, and also the opportunity to recycle fishery waste that has the potential to damage the environment, create unpleasant odors, and affect human health.

There are two primary methods for producing nano-calcium: (1) the up-down approach, which involves heating the bone to a minimum of 600ºC (calcination method) to remove all non-mineral material and then grinding the bone to a nano size; and (2) the bottom-up approach, which is the inverse of the up-down approach. In the bottom-up approach, nano-calcium particles are formed from the basic material (atoms and molecules) that are united into nano-sized. This is often accomplished by extracting bone meal with acid/alkali, which degrades organic matter in the bone and increases the mineral content significantly (Abid *et al*., 2022).

Benjakul *et al*. (2017) evaluated the natural calcium content of tuna bones obtained by calcination and alkaline extraction. Calcium powder generated via calcination is brighter in color and contains more calcium but has a lower *in vitro* bioavailability than calcium powder produced through alkaline extraction. Thus, it is recommended to use an alkaline extraction procedure to get edible calcium with a high bioavailability.

Several researchers extracted fish bones using varying percentages of alkali, including Amitha *et al*. (2019) used 2% NaOH on grouper, emperor, and snapper bones, Nemati *et al*. (2017) used 2% NaOH on yellowfin tuna bones, Anggraeni (2019) used 1 N NaOH on tilapia bone, Kusumaningrum *et al*. (2016) used 1.5 N NaOH on belida bone, Prinaldi *et al*. (2018) used 1.5 N NaOH on yellowfin tuna, Sumarto *et al*. (2021) used 1.5 N NaOH on several species of *Clarias* sp.

The bone autoclaving pre-treatment before the extraction process is carried out to denature bone collagen protein and soften bones (Husna *et al*., 2020) has also been carried out in numerous studies, including those conducted by Yin *et al*. (2015) silver carp bone autoclaving for 1 hour, Ratnawati *et al*. (2020) autoclaving catfish bones for 1 hour, Talib and Zailani (2017) autoclaving yellowfin tuna bones for 2 hours, while Julianti (2017), Anggraeni (2019), and Husna *et al*. (2020). However, no studies have been conducted to compare the autoclave duration with the quality, particle size, and bioavailability of the nano-calcium in *in vitro* gastrointestinal simulation tests.

Kusumawati *et al*. (2022) performed an autoclaving pre-treatment of grouper bone for three hours followed by 1 N NaOH extraction, resulting in a calcium content of 23.24 % with a particle size of 281.4 nm. Thus, this research examined the effect of autoclave duration (3, 2x3 h, and 3x3 h) on the properties of the nano-calcium with the intention that nano-calcium particles would be finer in size and increase their bioavailability. In addition to lowering the size of nano-calcium particles, this research revealed that autoclaving followed by alkaline hydrolysis increased calcium and phosphorus levels. The presence of amino acids, saturated fatty acids (SFA), and unsaturated fatty acids (UFA) in nano-calcium powder in *in vitro* gastrointestinal test is found to have higher calcium bioavailability.

2. Materials and Methods

2.1 Material

The grouper (*Epinephelus* sp.) bone waste used in this study was the best species which contained highest calcium content and yield percentage among six

commercial fish bones according to Kusumawati *et al.* (2022) research. The bone was obtained from PT. Kelola Mina Laut Industrial Area Gresik, East Java Province. Bone samples were collected randomly, regardless of the fish's size, age, or sex. All fish bones were frozen and kept in the freezer until they were required. NaOH (Merck, 1310-73-2), HCl (Mallinckrodt, 7647-01-0), ultrapure water (Waterone, Onelab, PT. Jayamas Medica Industri), Pepsin porcine gastric mucosa (Sigma, 9001-75-6), Pankreatin from porcine pancreas - 4 x USP specifications (Sigma, 8049-47-6) and synthetic calcium carbonate ($CaCO_3$) (Lianyungang Shuren Kechuang Food Additive Co.) were used in this research.

2.2 Experimental Design

The grouper bone powder was prepared in the procedure outlined by Anggraeni (2019), with minor modifications. Under flowing water, the frozen fish bone was washed. The fish bone was then boiled for 1 hour, cleaned, and dried for 24 hours at 50°C in a cabinet drier.

The dried fish bone was then separated into four groups: 0A (no autoclaving treatment); 3A (3 h autoclaving treatment); 2x3A (double cycle for 3 h autoclaving treatment); and 3x3A (triple cycle for 3 h autoclaving treatment). The fish bone was then dried for 24 hours at 50°C in a cabinet dryer. The fish bone was crushed in a mortar and ground for 1 minute at 28,000 rpm using a multifunction disintegrator (IC-06B, Getra, Jakarta, Indonesia) into fish bone powder. A tiny sample of each fish bone powder group was obtained for further testing.

The manufacturing of nano-calcium powder was also carried out using the technique described by Anggraeni (2019) with minor modifications. Each group of grouper powder (0A-3x3A) was soaked in 1 N 1:5 HCl (Mallinckrodt, 7647-01-0) (w/v) for 1 hour while being agitated with a magnetic stirrer. It was then incubated for 24 hours at room temperature.

After discarding the HCl supernatant by centrifugation at 3000 rpm, the sediment was placed in a glass beaker and diluted with 1 N NaOH (Merck, 1310-73-2) solution in a 1:5 (w/v) ratio. The sediment was then hydrolyzed three times at 100°C for 60 min on a hotplate stirrer. Each round of hydrolysis was followed by centrifugation to separate the supernatant from the sediment.

The hydrolyzed sediment was added to the distilled water and neutralized with HCl 1 N (Mallinckrodt, 7647-01-0) until it achieved a pH of 6.9-7.1. It was then recentrifuged. The sediment was then moved to a ceramic tray and dried for 15-18 hours at 50°C in a cabinet drier. The dried sediment was refined for 1 min in a laboratory disc mill (Kawasaki T-100, Kobe, Japan), then sieved using a 203-mesh sieve (Haver & Boecker 59302 OELDE, Germany) and evaluated for further analysis.

There were eight samples in this study, with four autoclaved samples of varying durations and four autoclaved and alkaline hydrolysis samples. 0A is a sample that has not been autoclaved, 3A is a bone sample that has been autoclaved for 3 hours, 2x3A is a bone sample that has been autoclaved for 2 cycles of 3 hours, and 3x3 A is a bone sample that has been autoclaved for 3 cycles of 3 hours. 0AH stands for alkali hydrolyzed sample 0A, 3AH stands for alkaline hydrolyzed sample 3A, 2x3AH stands for alkaline hydrolyzed 2x3A, and 3x3AH stands for alkaline hydrolyzed 3x3A. The grouper bone powder is 0A-3x3A group sample, while the grouper nano-calcium powder is 0AH-3x3AH group sample.

2.3 Physical Test

2.3.1 Yield analysis

The yield analysis was determined by dividing the grouper bone sample or grouper nano-calcium powder net weight by the dried bone net weight. Due to the fact that the fish filleting procedure sometimes leaves a big quantity and also sometimes leaves a little amount of fish flesh, it was difficult to use the wet weight of fish bones as a baseline for calculating fish bone weight.

2.3.2 Color test

The color of the sample was determined using a colorimeter (Konica Minolta CR-400, Tokyo, Japan), with white standard values of L^* (lightness) = 95.50, a^* (greeness/redness) = -5.02, b^* (yellowness/blueness) = 9.31.

2.3.3 Particle size test

Yin *et al.* (2016) method was used for particle size measurement, with a little modification. 35 mg of the material was dissolved in 35 g (1:100 w/w) of ultrapure water. It was adjusted to a pH of 2.0 using 1 N HCl. The mixture was then homogenized for 15 min at 4000 rpm using a homogenizer equipment (Ika Ultra-Turrax 50T Basic, Selangor, Malaysia). Dynamic light scattering was used to determine the particle size distribution using the Malvern Zetasizer Nano ZS (Malvern, UK) equipment.

2.3.4 Scanning electron microscopy with energy dispersive x-ray spectroscopy (SEM-EDS)

SEM instrument (JSM-6510 LA, Jeol, Tokyo, Japan) with an electron-dispersive X-ray spectroscope (EDS) detector was also used to view samples, following the procedure of Benjakul *et al.* (2018) with a small modification. After coating the object with gold, a secondary electron accelerating voltage of 15 kV was utilized for observation.

2.4 Biochemical Test

2.4.1 Proximate analysis

The relative moisture content was determined using a moisture analyzer (MB-90, Ohaus, Parsippany, NJ, USA), the protein content was determined using Kjeldahl digestion in accordance with AOAC protocol no. 920.153, the fat content was determined using Soxhlet extraction in accordance with AOAC protocol no. 960.39, and the ash content was determined using gravimetry in accordance with AOAC protocol no. 928.08.

2.4.2 Fourier Transform Infrared Spectroscopy (FTIR) analysis

The chemical structure of the powder samples was determined using an FTIR instrument (Nicolet type iS10, Thermo Fisher Scientific, Massachusetts, USA). All samples were prepared using KBr pellets, and spectra were acquired in the 400-1400 cm^{-1} middle infrared range (Corrêa and Holanda, 2019).

2.4.3 X-Ray Diffraction (XRD) analysis

The crystallinity properties of grouper bone sample and grouper nano-calcium powder were determined using an X-Ray Diffraction equipment (Bruker AXS D8 Advance, Massachusetts, USA) with LYNXEYE XE-T detector. The sample was started at $2\theta = 8°$-$80°$ degree angle. The step scan data was 0.02 using Cu Kα radiation with a wavelength of $\lambda = 1.5$-4060. XRD generated a diffractogram that could be used to determine the mineral structural features of fish bone samples, including the composition phase of the chemicals contained inside, the lattice parameters, and crystal volume (Siddharthan *et al.*, 2009; Shi *et al.*, 2018). Phase identification matches measured peak locations to the Powder Diffraction File (PDF) for the relevant phase stored in the Qualx 2.0 (Italy) software's Crystallography Open Database. The percentage of crystallinity was calculated as the ratio of the area of the crystalline curve to the total of the areas of the crystalline and amorphous curves (Yusuf *et al.*, 2019).

2.4.4 Calcium solubility in an in vitro gastrointestinal tract system simulation

The purpose of this test was to measure the amount of calcium solubility in *in vitro* digestion simulations, using the method described by Benjakul *et al.* (2018) and by Pertiwi *et al.* (2020), with several modifications. 75 mg of sample was dissolved in 35 ml of 5 mM HCl-KCl (pH 1.5) and then added to 1 ml HCl-KCl 1 M buffer (pH 1.5) along with 1000 U/ml pepsin enzyme. The mixture in 50 ml conical tubes were then incubated for 60 min at 37°C in a water bath shaker. The mixture was then adjusted to pH 6.8 using 1M $NaHCO_3$ and added with the pancreatin enzyme 50 U/ml in 10mM tris-HCl pH 8.2. The mixture was then set to pH 7.5 by adding 1 M NaOH and incubated for another 3 h in a 37°C water bath shaker. After 3 h, the tube containing the mixture was immersed in boiling water for 10 min to bring the enzymatic process to an end. The volume of the solutions was added with ultrapure water to 50 ml then centrifuge at 7000 x g. The supernatant then transferred into a new 50 ml conical tubes and test for calcium content using the ICP-OES instrument (Agilent, type 720 detector DDC, Santa Clara California, USA).

2.4.5 Amino acid analysis

The 100 mg of dried material was mixed with 5 mL of 6 N HCl and drained for 22 hours in an oven at 110°C. After dilution, the material was filtered using a 0.45 m AABA and ultrapure water was added to the filtrate, followed by the addition of AccQ Fluor Borate, a fluorine reagent, and incubation at 55°C for 10 minutes following Ali *et al.* (2020) method. The High Performance Liquid Chromatography (HPLC) was performed using a Shimadzu CBM 20 A instrument (Kyoto, Japan). A Column Shim-pack VP ODS 5 μm 150 x 4.6 mm and a mobile phase Trisodium Citrate pH 3.25 were carried out using isocratic mobile phase at the flow rate 1 ml/min and 50 μl injection volume. The RF 20-A fluorescence detector was used at the wavelength of 450 nm. The analyzer computes the peak area proportionate to each amino acid's concentration.

2.4.6 Fatty acid analysis

The fatty acid analysis following Ali *et al.* (2020) methods. The 1 µL methylated sample (FAME) was injected into a Gas Chromatography (GC) instrument (Shimadzu GC-2010AF, Kyoto, Japan) with Column Restek FAMEWAX 30 m, ID 0.25 mm df 0.1 μm. As carrier gas, Helium (Pa 1 kg/cm^2) and Nitrogen (Pa 0.5 kg/cm^2) were utilized at a ratio of 1:50 with 1 μl injection volume. Hydrogen flow rate was 40 ml/min, whereas oxygen flow rate was 400 ml/min. The injector temperature was set to 250°C, and the MS (FID) detector temperature was also 250°C.

The temperature of the columns was initially fixed to 150°C and increased at a rate of 5°C/min to 240°C. Comparing the chromatogram peak to the fatty acid standard enabled the identification of the fatty acids.

2.5 Analysis Data

Yield analysis, color analysis, proximate analysis and calcium solubility in this research were carried out in triplicate. One-way analysis of variance (ANOVA) for statistical analysis was done using SPSS (IBM SPSS 26 Version, IL, USA), and means were analyzed using Duncan's multiple range test (DMRT) at $p < 0.05$.

3. Results and Discussion

3.1 Yield Analysis

The yield percentage is essential in economic viewpoint. The higher the yield rate of a product, the more profitable it is to produce. In this research, yield analysis was derived from the comparison of grouper bone powder or nano-calcium powder to dried bone weight.

According to the data, an autoclaving treatment 3 hours to 3x3 hours lowered the yield percentage by 10 to 30 % (Figure 1). After each autoclave cycle (1 cycle = 3 h), the fish bones were rinsed again while eliminating the collagen protein or blood vessels that had been displaced from the bone joints during the autoclaving process. This is done to decrease material other than bone so that it can enhance the proportion of calcium in the bone. The hydrolysis process was the most significant in reducing yield percentage, namely 43.37-52.37 %. The alkaline hydrolysis process will greatly reduce protein and fat levels due to protein denaturation reactions and saponification of free fatty acids that react with alkali. In the first cycle of centrifugation, three layers exist. The top white color soap layer will coagulate, the intermediate layer is a dark brown NaOH solution containing denatured protein that dissolves in NaOH, and the bottom layer is a light brown bone meal deposit containing calcium that will be hydrolyzed by alkali for two more cycles. In the second cycle, the soap layer started to dissipate, the color of the NaOH solution changed to a light brown hue, and the color of the bone meal changed to a creamy hue, indicating a decrease in organic matter content. In the third cycle, there were just two layers: a yellow layer of NaOH and a white layer of bone meal, indicating that the amount of organic matter had been drastically decreased. This reduction in organic matter content mostly affects the yield percentage at each step of extraction.

The yield percentage of Kusumawati *et al.* (2022) was between 21.75 and 50.25 % after 3 h of autoclaving treatment followed by 3 cycles of alkaline hydrolysis at 100°C. In the production of micro calcium powder, Ratnawati *et al.* (2020) achieved a yield of 11.19-51.58 % utilizing the agitation hydrolysis technique as opposed to the usual approach without hydrolysis. The conclusion that can be taken from this research is that the longer the stages of the manufacturing process, the finer the nano-calcium particle size and the purer the calcium; nevertheless, this is inversely related to the yield percentage. This was also proven in this study that the autoclaving process of 0 hours, 3 hours, 2x3 hours, 3x3 hours followed by alkaline hydrolysis 3 cycles of 100°C, the yield percentage decreased to 9%.

3.2 Color Analysis

The color analysis of white or as bright as possible is the most preferred color for nano-calcium powder. Natural nano-calcium color derived from natural materials, such as fish bones, mollusk shells, eggshells, and other animal bones, is challenging to match the brightness of artificial calcium color manufactured in factories (Hemung, 2013). Nano-calcium made from natural sources often includes organic substances (protein, fat, etc.) that may impart a brownish hue and degrade the product's brightness (Benjakul *et al.*, 2017).

Based on color analysis, the autoclaving duration is directly related to the brightness of the resultant fish bone nano-calcium powder (Table 1). As described in previous subchapter, each autoclave cycle involves the removal of organic substances, such as collagen or blood vessels, that have become loose during the autoclaving process. The decrease in organic matter minimizes the browning process in the product that may be induced by fat oxidation or the Maillard reaction of carbohydrates. However, after 2 cycles of 3 h (2x3A) and 3 cycles of 3 h (3x3A) of autoclaving, the product brightness did not rise significantly. Thus, the autoclaving technique for reducing organic matter content is limited to a maximum of 2 cycles of 3 h.

The alkaline hydrolysis process was more effective than the autoclaving duration treatment process for increasing the brightness value of fish bone nano-calcium powder. This increase in luminosity indicates that the alkaline hydrolysis treatment was more effective than the autoclave treatment at degrading organic substances (Table 2). In addition to decreasing organic matter by saponification reactions of free fatty acids, alkali hydrolysis treatment also denatures numerous proteins and proteolytic enzymes, such as proteases and lipases, which may cause quality and color degradation related to various enzymatic processes.

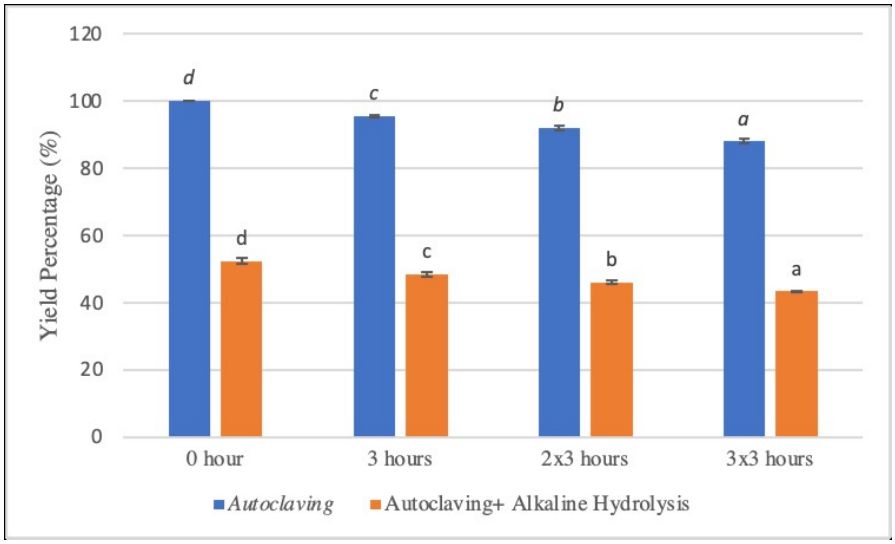


Figure 1. Yield Percentage of grouper bone powders and grouper nano-calcium powders. Different alphabets above the bar denoted significant differences using one-way ANOVA and Duncan's test ($p<0.05$).

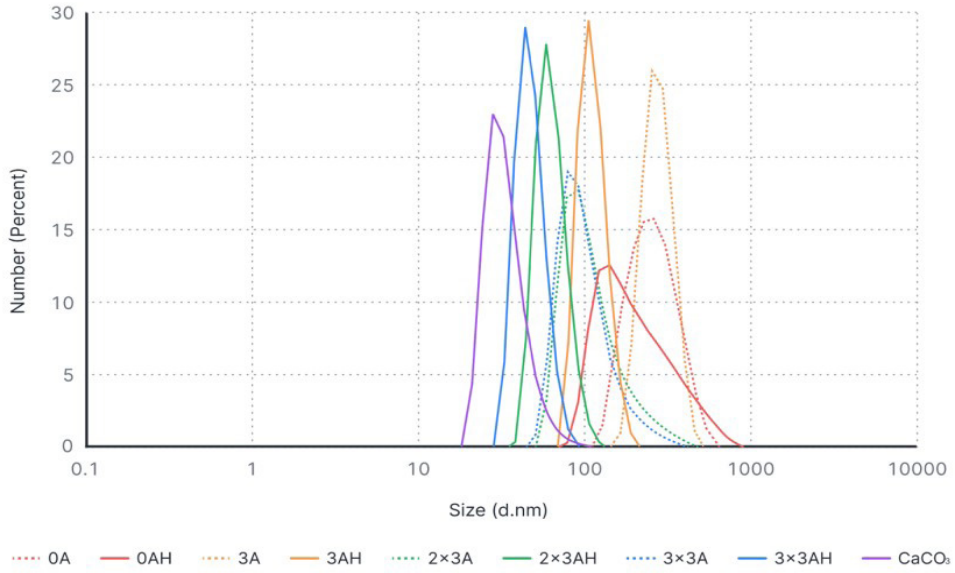


Figure 2. The distribution of particle size of grouper bone powders, grouper nano-calcium powders and synthetic $CaCO_3$

The nano-calcium brightness score produced in this study (88-89) is brighter than the catfish bone calcium from Ratnawati *et al.* (2020), which was 84.18, but less light than the biocalcium produced by Benjakul *et al.* (2017) with the brightness of 92-93 from tuna bone using the NaOH hydrolysis method and 95 by the sintering method. Meanwhile, Prinaldi *et al.* (2018) was able to hydrolyze tuna bones with HCl, resulting in a brightness of 92.61, slightly higher than synthetic $CaCO_3$ which is 92. To create nano-calcium with increased luminosity, hexane or other fat solvents may be applied to do a defatting pre-treatment prior to hydrolysis process.

According to Benjakul (2017), fat is the organic compound that has the most effect on color since it is readily oxidized and produces a brownish hue.

3.3 Particle Size Analysis

Nanoparticles consist of crystalline or amorphous solid particles with a mean diameter of 1 nm to 1,000 nm. There is a significant relationship between dietary component's size and its bioavailability. Nano-calcium is smaller than microscopic calcium, allowing calcium to penetrate into mineral receptor and be absorbed by the cells efficiently (Sumarto *et al.*, 2021).

Table 1. Color analysis of grouper bone powders, grouper nano-calcium powders and synthetic $CaCO_3$

Treatment	Duration	*L**	*a**	*b**	ΔE*
Autoclaving (A)	0 h (0A)	87.123±0.09^{b}	-3.13±0.01^{a}	10.09±0.12^{a}	8.62±0.1^{a}
	3 h (3A)	86.84±0.06^{a}	-2.34±0.04^{d}	11.41±0.26^{b}	9.31±0.01^{c}
	2x3 h (2x3A)	87.28±0.03^{c}	-2.46±0.05^{c}	11.27±0.15^{b}	8.82±0.02^{b}
	3x3 h (3x3A)	87.35±0.05^{c}	-2.63±0.06^{b}	12.10±0.23^{c}	8.94±0.07^{b}
Autoclaving + Alkaline Hydrolysis (AH)	0 h (0A)	89. 25±0.10^{b}	-3.71±0.23^{c}	10.83±0.37^{a}	7.46±0.02^{a}
	3 h (3A)	88.18±0.34^{a}	-3.88±0.21^{cb}	14.91±0.09^{bc}	9.28±0.35^{b}
	2x3 h (2x3A)	89.55±0.06^{b}	-4.09±0.63^{ab}	11.65±1.91^{ab}	6.58±1.38^{a}
	3x3 h (3x3A)	89.03±0.21^{b}	-4.17±0.08^{a}	13.40±1.21^{bc}	7.68±0.83^{a}
$CaCO_3$		93.87±0.01	-4.84±0.07	7.46±0.02	2.46±0.03

Description:

*L**: lightness ; *a**: redness/ greenness ; *b**: yellowness/blueness; ΔE*: total difference in color

Note: The values are expressed as mean standard deviation (n = 3). Using one-way ANOVA and Duncan's test, different alphabet superscripts within the same column demonstrate significant differences (p 0.05)

Table 2. Proximate analysis of grouper bone powders, grouper nano-calcium powders and synthetic $CaCO_3$

Treatment	Duration	Moisture %	Ash (%)*	Protein (%)*	Fat (%)*	Calcium (%)*	Phosphorous (%)*	Ca/P mole ratio
Autoclaving (A)	0 h (0A)	3.39±0.02^{c}	69.68±0.03^{a}	21.67±0.39^{c}	2.92±0.15^{a}	22.39±0.03^{a}	14.10±0.07^{b}	1.22
	3 h (3A)	2.33±0.12^{b}	71.14±0.23^{b}	21.75±0.03^{c}	3.76±0.04^{b}	23.12±0.47^{b}	14.89±0.05^{d}	1.19
	2x3 h (2x3A)	2.16±0.04^{b}	70.99±0.02^{b}	20.98±0.13^{b}	4.69±0.15^{c}	24.80±0.10^{c}	14.64±0.11^{c}	1.31
	3 x 3 h (3x3A)	1.93±0.16^{a}	72.17±0.06^{c}	19.78±0.08^{a}	4.76±0.01^{c}	24.47±0.02^{bc}	13.41±0.07^{a}	1.41
Autoclaving + Alkaline Hydrolysis (AH)	0 h (0AH)	2.32±0.45^{b}	90.68±1.16^{b}	0.86±0.58^{c}	0.12±0.02^{a}	27.55±0.01^{a}	17.73±0.52^{b}	1.2
	3 h (3AH)	1.53±0.00^{a}	90.68±0.40^{b}	0.74±0.04^{b}	0.74±0.04^{b}	30.18±0.34^{b}	16.83±0.07^{a}	1.32
	2x3 h (2x3AH)	1.73±0.27^{ab}	88.28±0.14^{a}	0.71±0.24^{b}	0.71±0.24^{b}	31.08±0.19^{c}	18.19±0.07^{c}	1.32
	3 x 3 h (3x3AH)	1.73±0.53^{ab}	87.73±0.04^{a}	0.63±0.04^{a}	0.63±0.04^{a}	30.73±0.32^{c}	18.37±0.32^{c}	1.29
$CaCO_3$		0.4±0.2	99.6±0.2	nd	nd	45.19±0.57	0.0195±0.00	1790.9

Note: * Dry weigh basis. The values are expressed as mean standard deviation (n = 3). Using one-way ANOVA and Duncan's test, different alphabet superscripts within the same column demonstrate significant differences (p 0.05); nd = not detected

This study produced nano-calcium with a particle size of 47.47-260.4 nm (Figure 2), which is significantly smaller than the calcium powder from catfish bones produced by Ratnawati *et al.* (2020) which were 3.52 -5.12 μm, Anggraeni (2019) who produced nano calcium from tilapia bone with a particle size of 500 nm, Prinaldi *et al.* (2018) research which produced nano-calcium from tuna bone with a particle size of 259-397 nm, and Benjakul *et al.* (2017) who produced biocalcium of 0.59-38.78 μm size.

In this experiment, the autoclave process followed by alkaline hydrolysis was able to lower the particle size of calcium, despite the fact that the percentage of size similarity remained below 30 %. The lowered size was related to the pressure of the autoclave, which shattered peptide connections and aided in the release of Ca particles (Nawaz *et al.*, 2020) the more the autoclave cycles the more calcium-binding chemical bonds were destroyed, releasing smaller calcium molecules. Yin *et al.* (2015) was able to produce nano-calcium with a nearly uniform size of 115 nm using a 6-hour wet milling procedure. To obtain finer and uniform nano-calcium particles, it can be done by adding milling treatment for some hours after the hydrolysis or powder drying process as was done by Nam *et al.* (2019) who succeeded in obtaining nano-calcium from Seabass with a uniform size of 50-70 nm. In this research, all grouper bone powder sample sizes evaluated by PSA were smaller than 1000 nm, hence all samples were classified as fish bone nano-calcium powders.

3.4 SEM-EDS Analysis

SEM Instruments can be used to observe the shape and size of micro and nano-sized particles with a magnification of 5-3,000,000x (Abdullah and Mohammed, 2019). Magnification of 1000x is sufficient for nano-calcium particles observation, whereas conventional microscopes are only capable of magnifications of 10-100x. The EDS detector is added to the SEM equipment to enhance quantitative investigation of the elemental types and quantities contained in a molecule or compound.

The grouper nano-calcium powders (0-3x3AH) morphology were hexagonal and spherical with non-uniform size while synthetic $CaCO_3$ particles had a comparatively finer and more uniform size, resembling tiny flakes (Figure 3). The spherical shape of the fish bone powder was also confirmed by Benjakul *et al.* (2017).

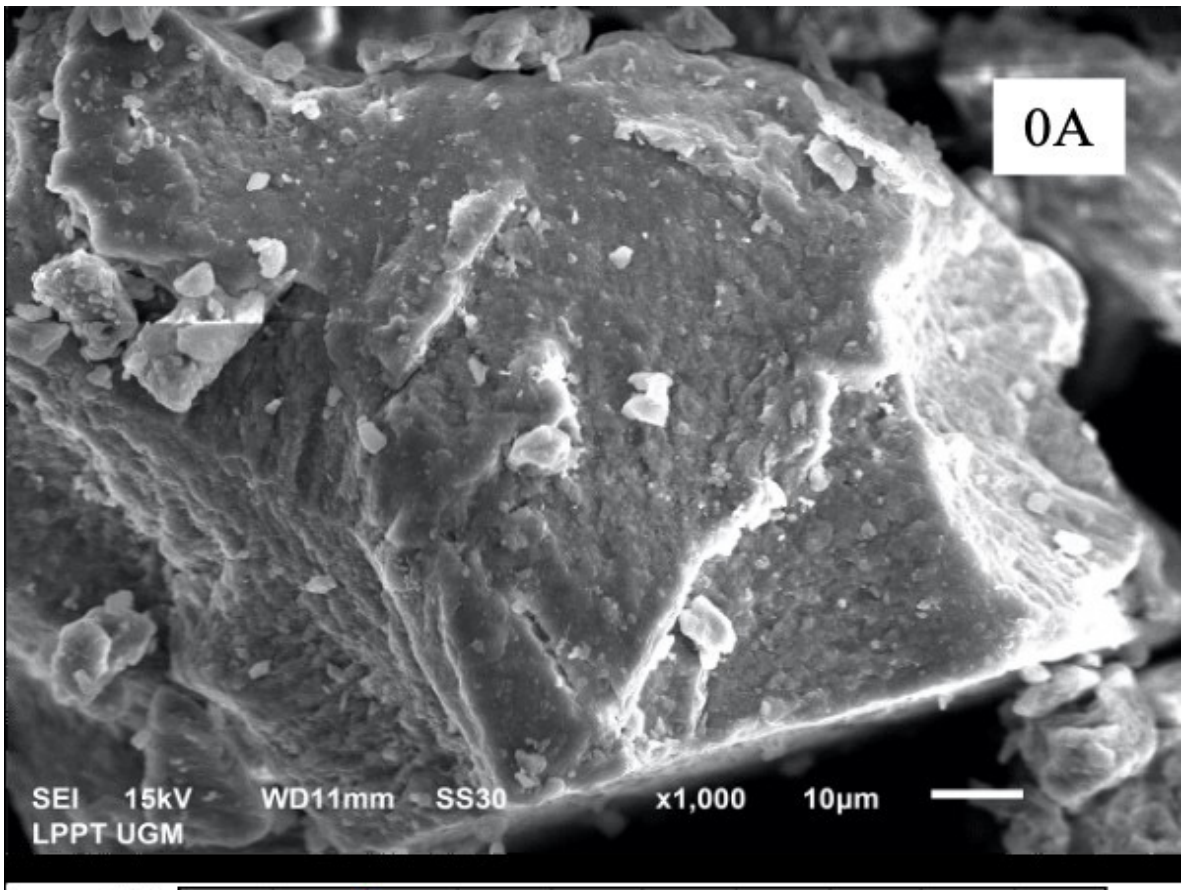
According to EDS detection, fish bone particles and $CaCO_3$ particles vary considerably. Fish bone particles contain organic elements in the form of protein, fat, and hydroxyapatite compounds with C (19.53-24.38 % in treatment A and 13.80-29.25 % in treatment AH) and O (33.54-39.07 % in treatment A and 32.98-41.80 % in the AH treatment) (Table 2) (Figures 4 and Figure 5). The organic constituents in $CaCO_3$ were C (11.61%) and O (46.66%), which form carbonate compounds (Figure 6).

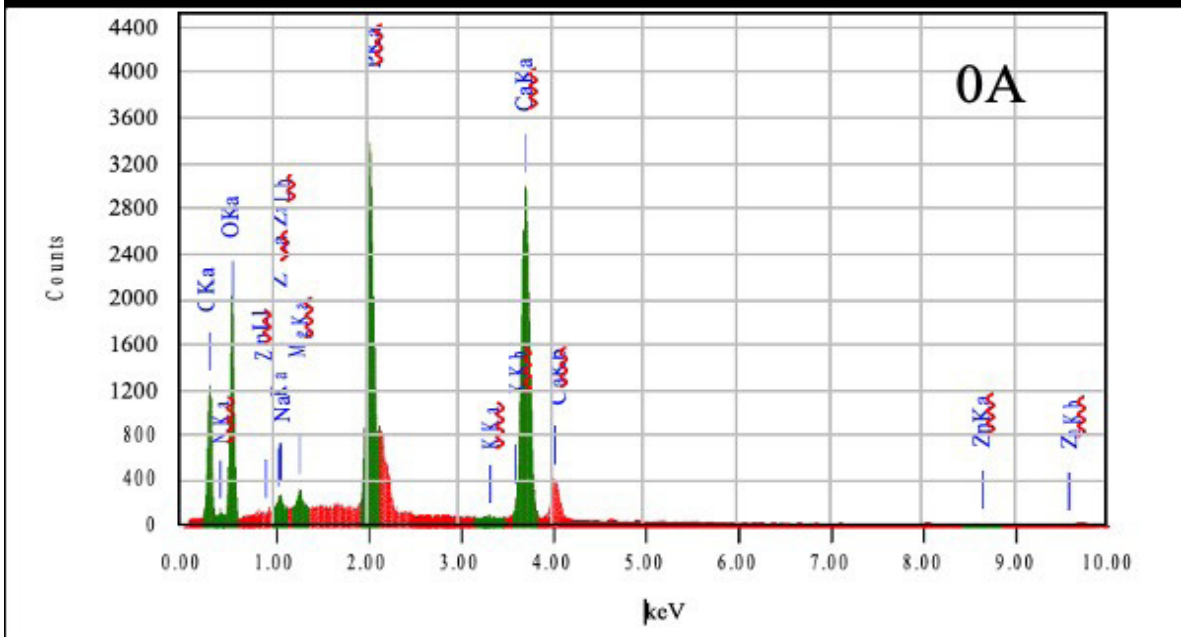
Another noteworthy difference between natural calcium powder (which in this research was derived from grouper bones) and manufactured $CaCO_3$ was the presence of naturally occurring minerals such as Na, Mg, K, Zn, and Cl in natural calcium powder. Natural calcium phosphate comprises of some trace minerals, including Na, Mg, K, and Zn, which makes it more similar to the chemical composition of human bone (Pu'ad *et al.*, 2019). According to Nam *et al.* (2019), the presence of trace minerals in bone extraction is natural since all of these elements have a role in bone metabolism in the body, for example Na and Mg, which substantially encourage the development of bones or teeth in organisms.

3.5 Proximate Analysis

Proximate analysis is a series of tests that estimate the content of the main nutrient components in a product, such as water, fat, protein, and ash content. There were significant differences in proximate analysis of grouper bone powder from autoclave treatment (0A-3x3A0) and grouper nano-calcium powder from autoclave and alkaline hydrolysis (0AH-3x3AH) (Table 2).

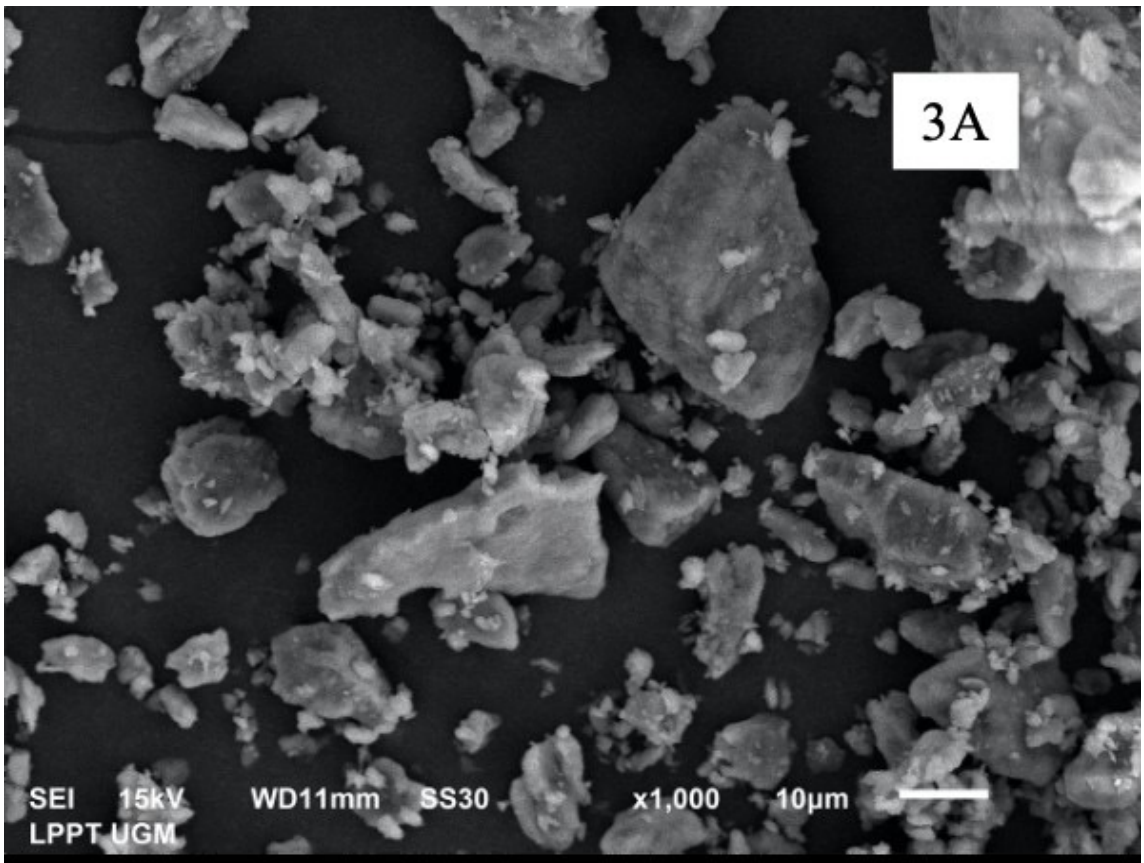
The significant difference between the autoclave (A) treatment and the autoclave followed by alkaline hydrolysis (AH) treatment was the protein and fat content (Table 2). The protein content in treatment A declining from 19.78 to 21.75 % to less than 1 % in treatment AH. Similarly, the fat level, which remained 2.91 % to 4.76 % in treatment A, may be reduced to less than 0.3% in treatment AH. Reducing the protein and fat content increased the ash/mineral content by around 18 %, as well as the calcium and phosphorus levels by 6 % and 3.8 %, respectively. The autoclave treatment followed by alkaline hydrolysis (AH) was highly successful in decreasing nano-calcium powder impurities.

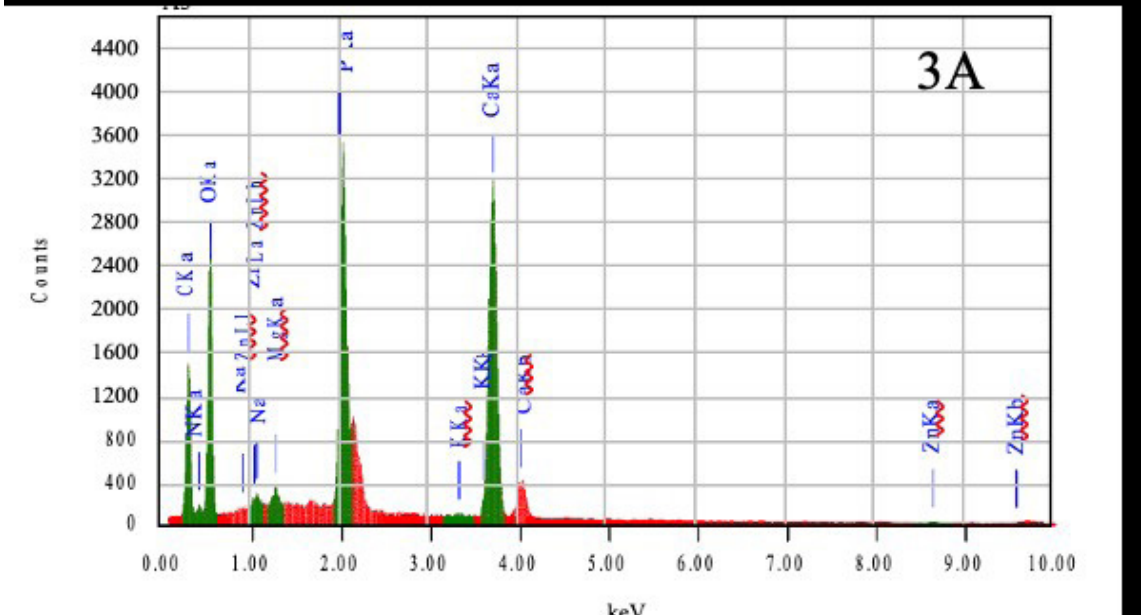




ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0537

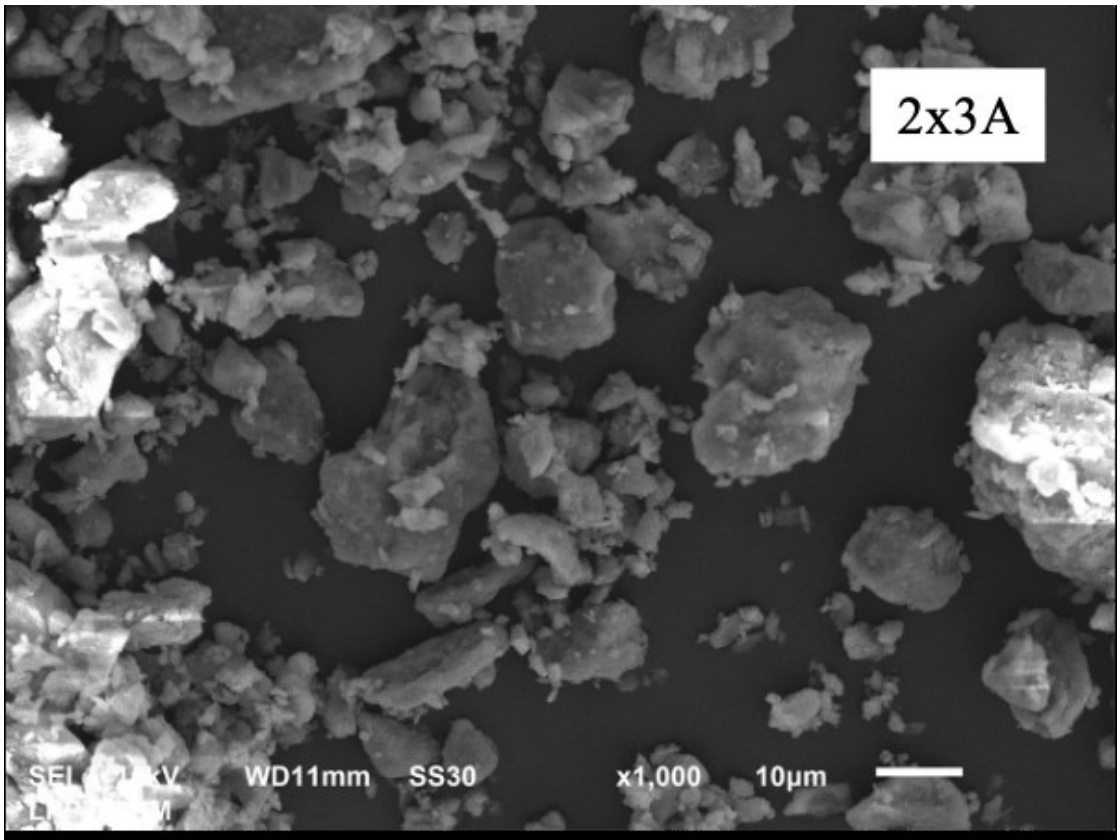
Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	19.53	150.91	28.85				10.1795
N K	0.392	10.55	481.64	13.37				8.6484
O K	0.525	37.89	702.04	42.02				27.9444
Na K	1.041	0.44	46.97	0.34				0.4889
Mg K	1.253	0.52	50.07	0.38				0.5569
P K	2.013	10.50	202.38	6.02				16.6006
K K*	3.312	0.09	22.47	0.04				0.1486
Ca K	3.690	20.01	368.62	8.86				34.8093
Zn K*	8.630	0.47	181.75	0.13				0.6235
Total		100.00		100.00				

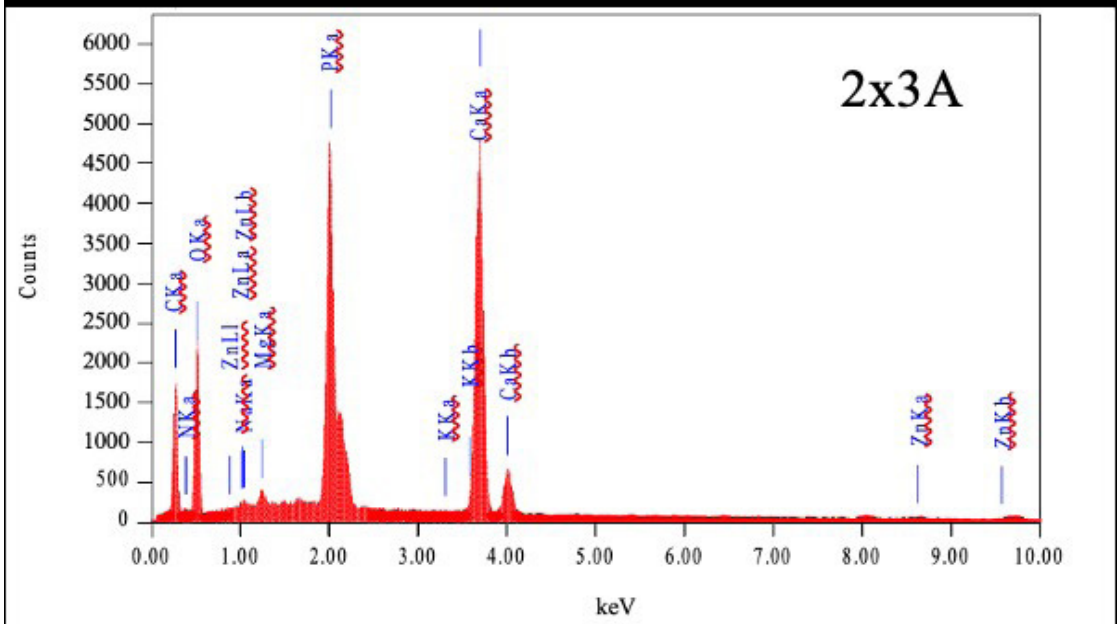




ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0527

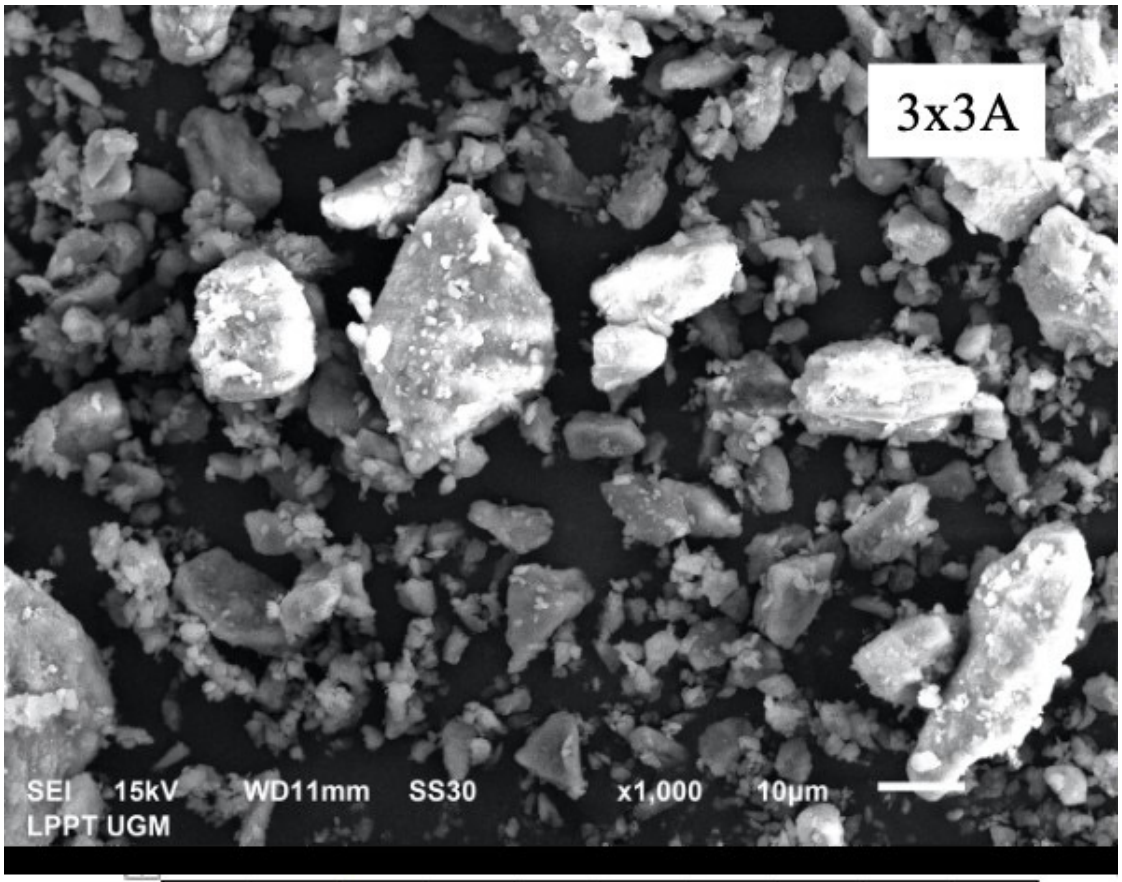
Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	20.00	0.10	28.99				10.9398
N K	0.392	11.51	0.26	14.30				9.9774
O K	0.525	39.07	0.29	42.52				29.6772
Na K	1.041	0.29	0.03	0.22				0.3234
Mg K	1.253	0.52	0.03	0.37				0.5634
P K	2.013	9.70	0.07	5.45				15.4770
K K*	3.312	0.04	0.02	0.02				0.0728
Ca K	3.690	18.43	0.12	8.01				32.3855
Zn K*	8.630	0.44	0.10	0.12				0.5834
Total		100.00		100.00				

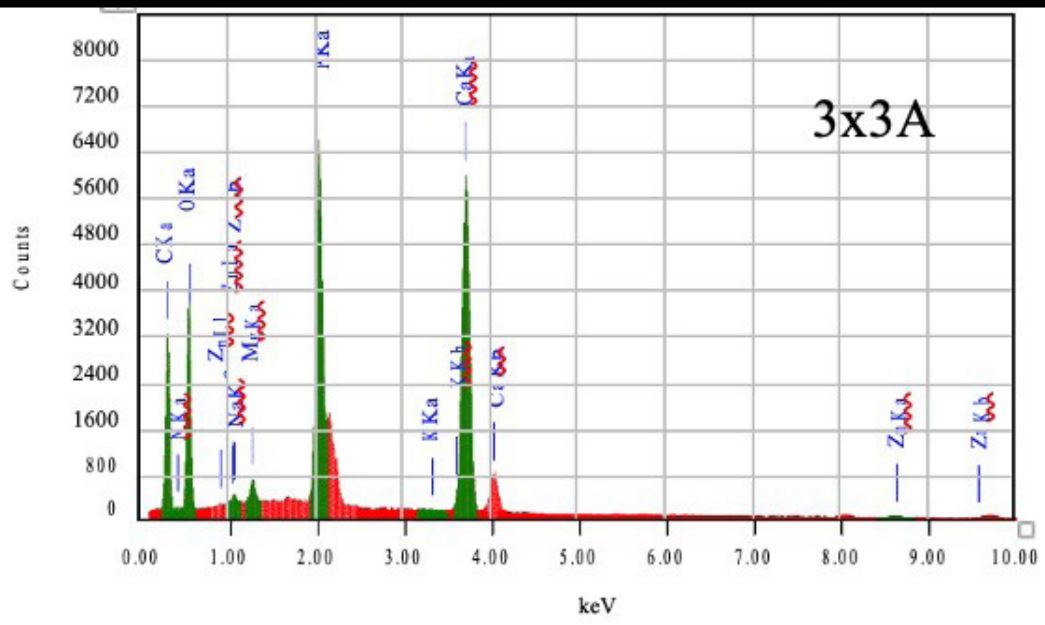




ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0527

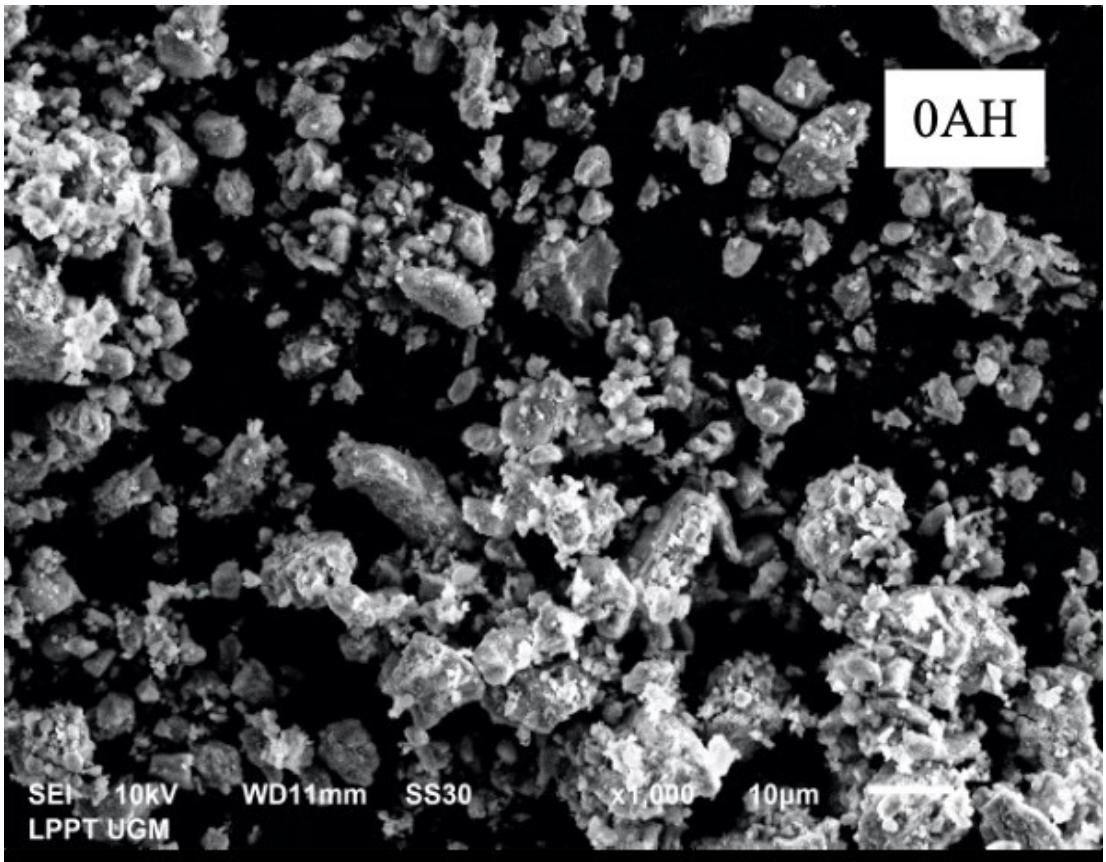
Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	20.74	0.02	31.28				10.6073
N K	0.392	10.14	0.11	13.12				7.4212
O K	0.525	33.54	0.10	37.99				22.7536
Na K	1.041	0.16	0.02	0.13				0.1760
Mg K	1.253	0.50	0.02	0.37				0.5421
P K	2.013	11.02	0.04	6.45				17.3934
K K*	3.312	0.03	0.01	0.01				0.0439
Ca K	3.690	23.08	0.07	10.43				40.0245
Zn K*	8.630	0.79	0.10	0.22				1.0379
Total		100.00		100.00				

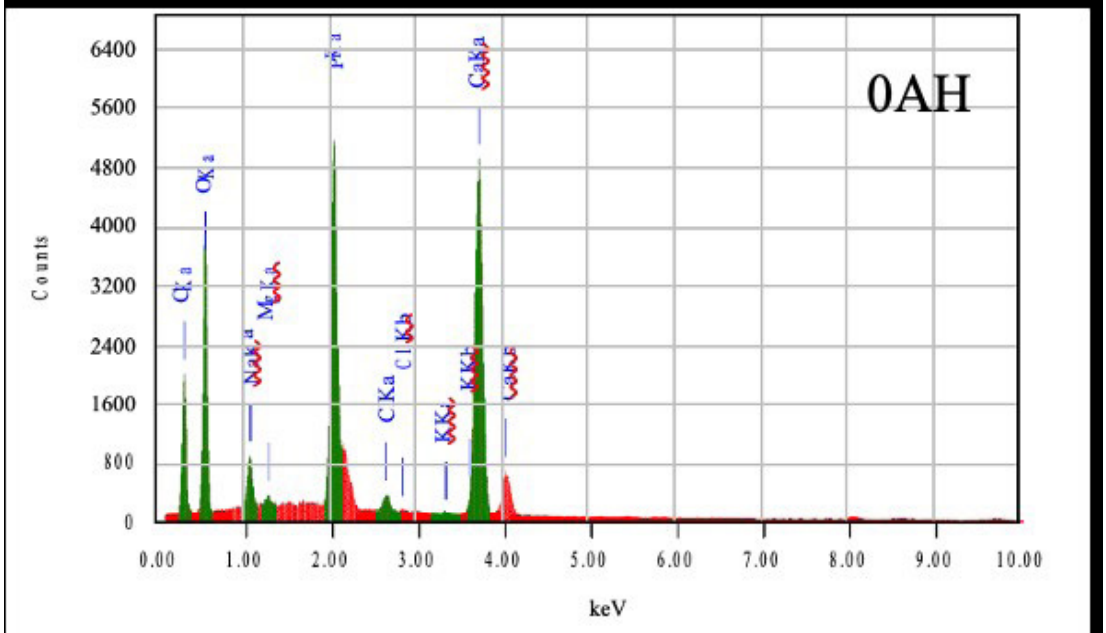




ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0473

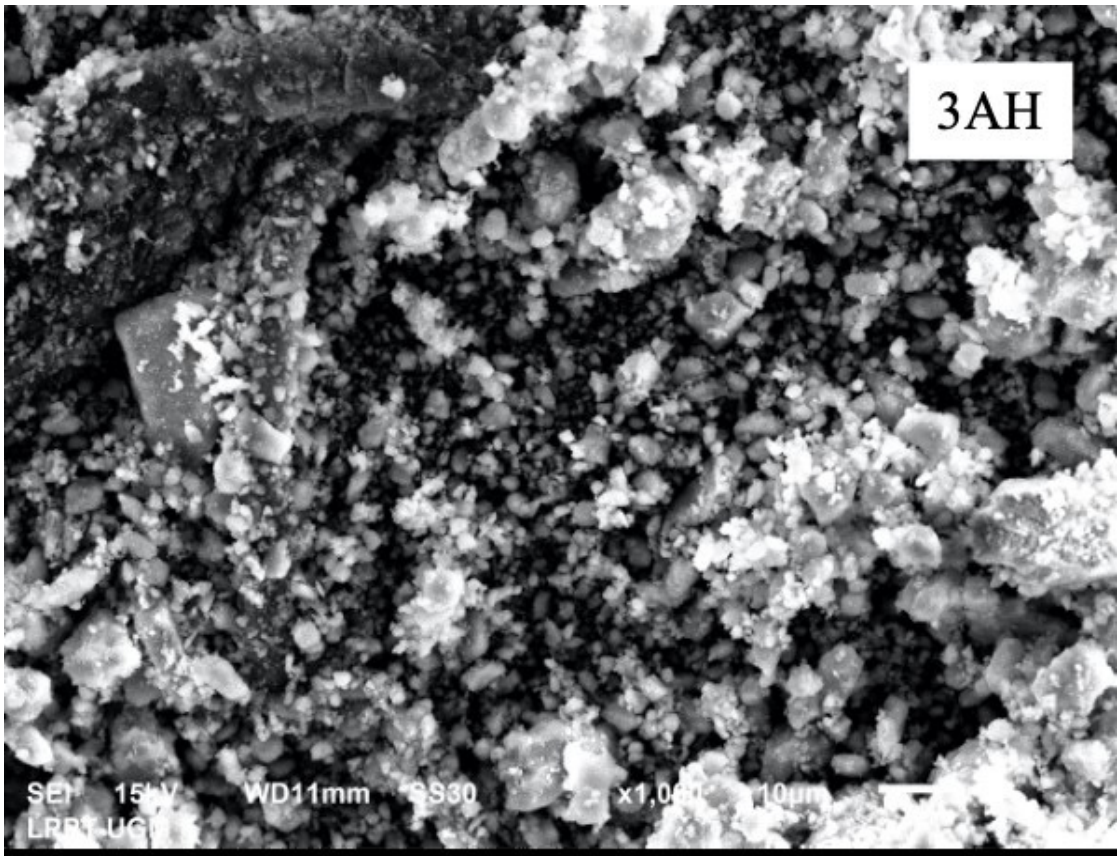
Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	24.38	0.08	35.31				13.4365
N K*	0.392	9.48	0.19	11.77				7.4442
O K	0.525	34.96	0.21	38.01				25.8975
Na K	1.041	0.24	0.02	0.18				0.2708
Mg K	1.253	0.69	0.02	0.50				0.7770
P K	2.013	10.01	0.05	5.62				16.3137
K K*	3.312	0.03	0.01	0.02				0.0607
Ca K	3.690	19.25	0.09	8.35				34.4948
Zn K	8.630	0.96	0.09	0.26				1.3047
Total		100.00		100.00				

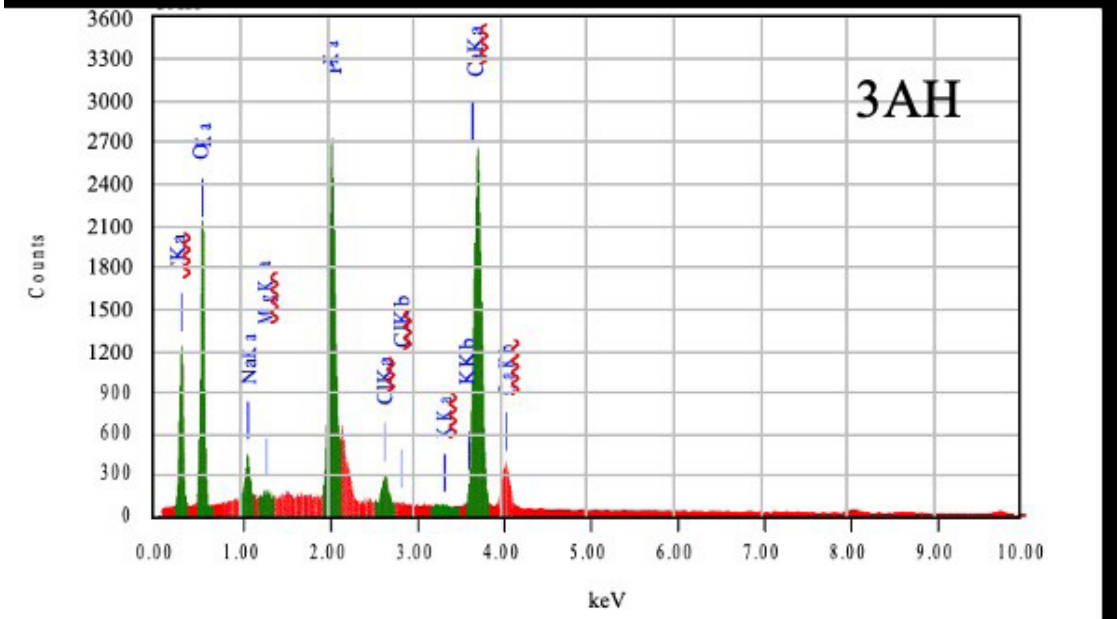




ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0432

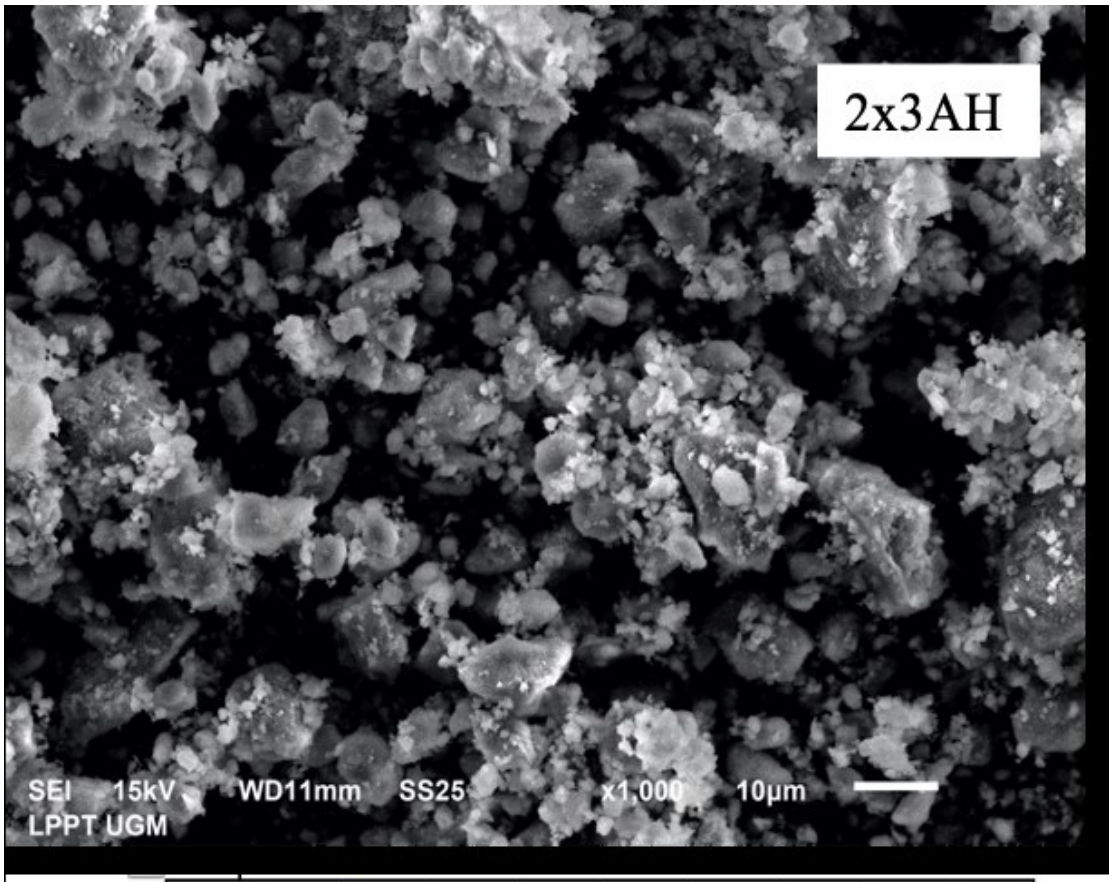
Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	22.06	0.02	33.55				9.9715
O K	0.525	41.75	0.09	47.66				32.8664
Na K	1.041	1.84	0.02	1.46				1.9578
Mg K	1.253	0.27	0.02	0.20				0.2753
P K	2.013	11.56	0.04	6.82				17.5225
Cl K	2.621	0.69	0.01	0.36				1.0835
K K*	3.312	0.05	0.01	0.02				0.0791
Ca K	3.690	21.78	0.06	9.93				36.2439
Total		100.00		100.00				

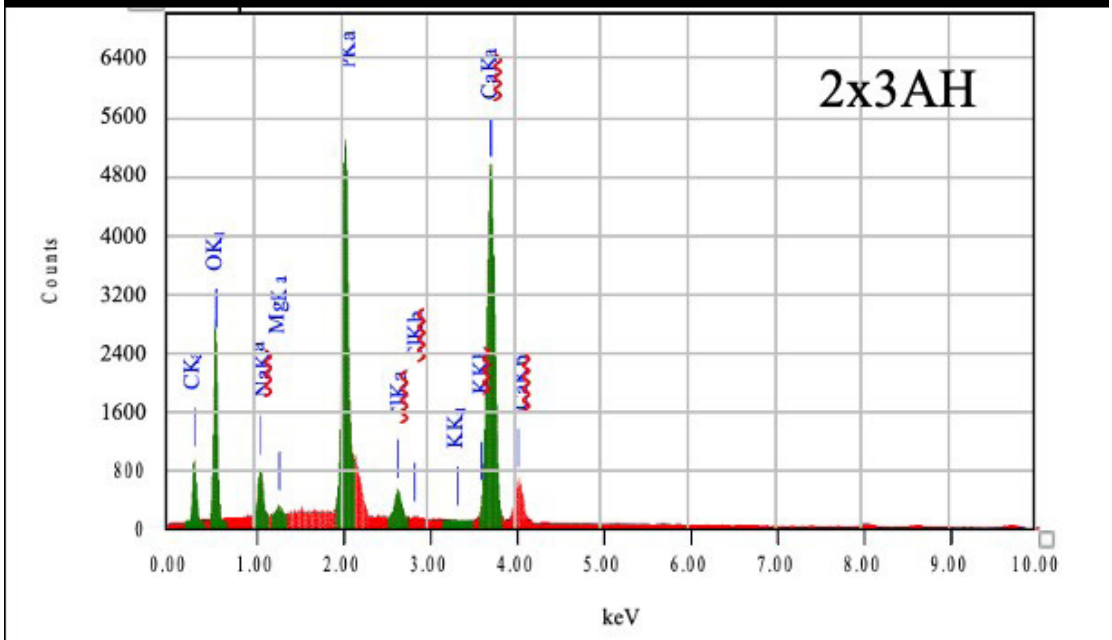




ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0541

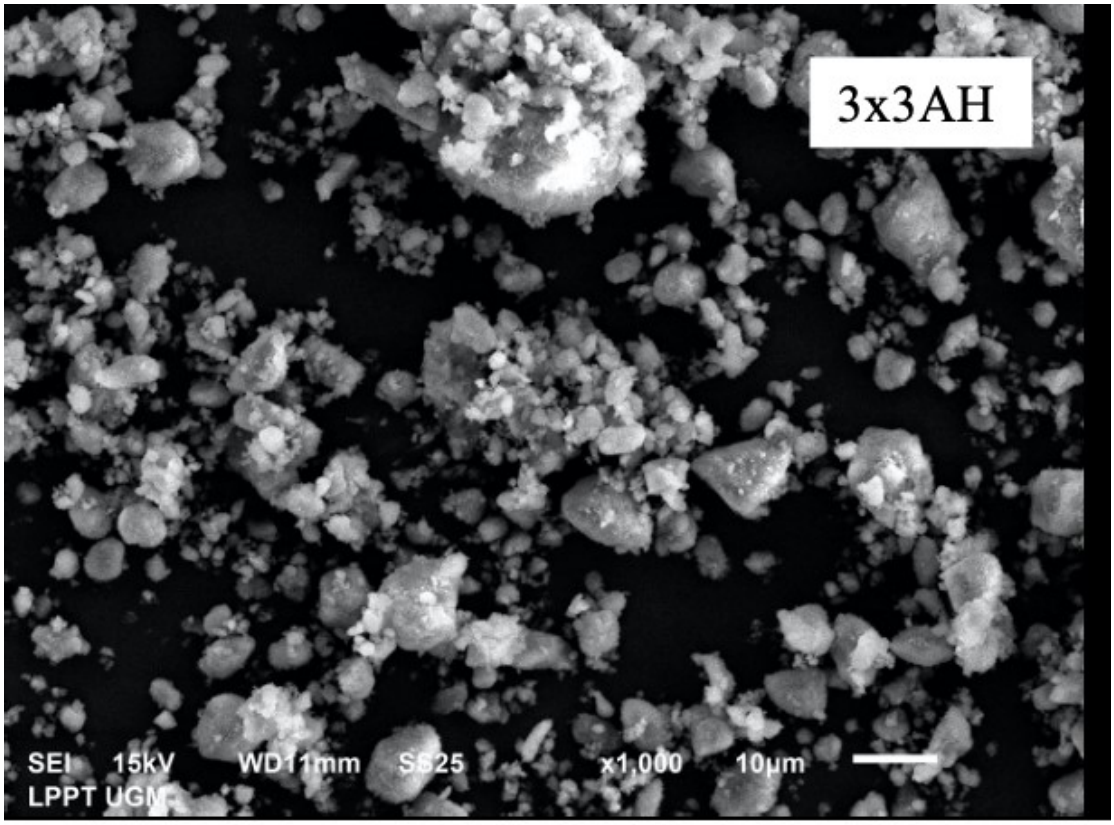
Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	23.77	0.14	35.59				11.3055
O K	0.525	41.80	0.33	46.99				33.1914
Na K	1.041	1.50	0.04	1.17				1.6116
Mg K*	1.253	0.16	0.02	0.12				0.1641
P K	2.013	10.43	0.09	6.06				16.0676
Cl K	2.621	1.02	0.03	0.52				1.6321
K K*	3.312	0.03	0.02	0.01				0.0434
Ca K	3.690	21.30	0.15	9.55				35.9843
Total		100.00		100.00				

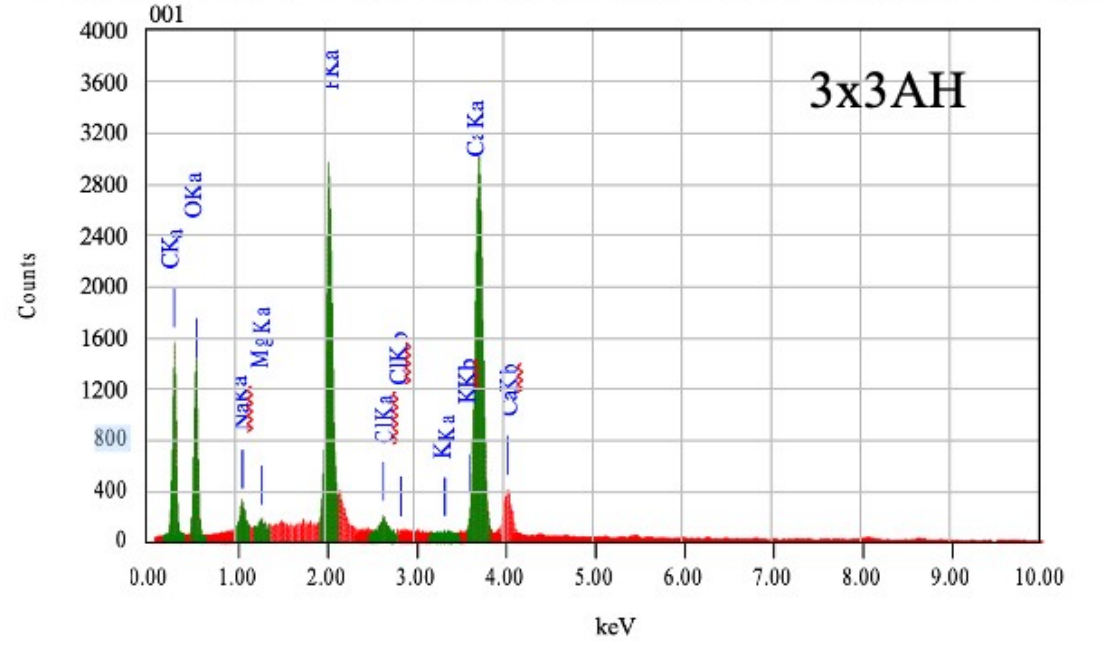




ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0398

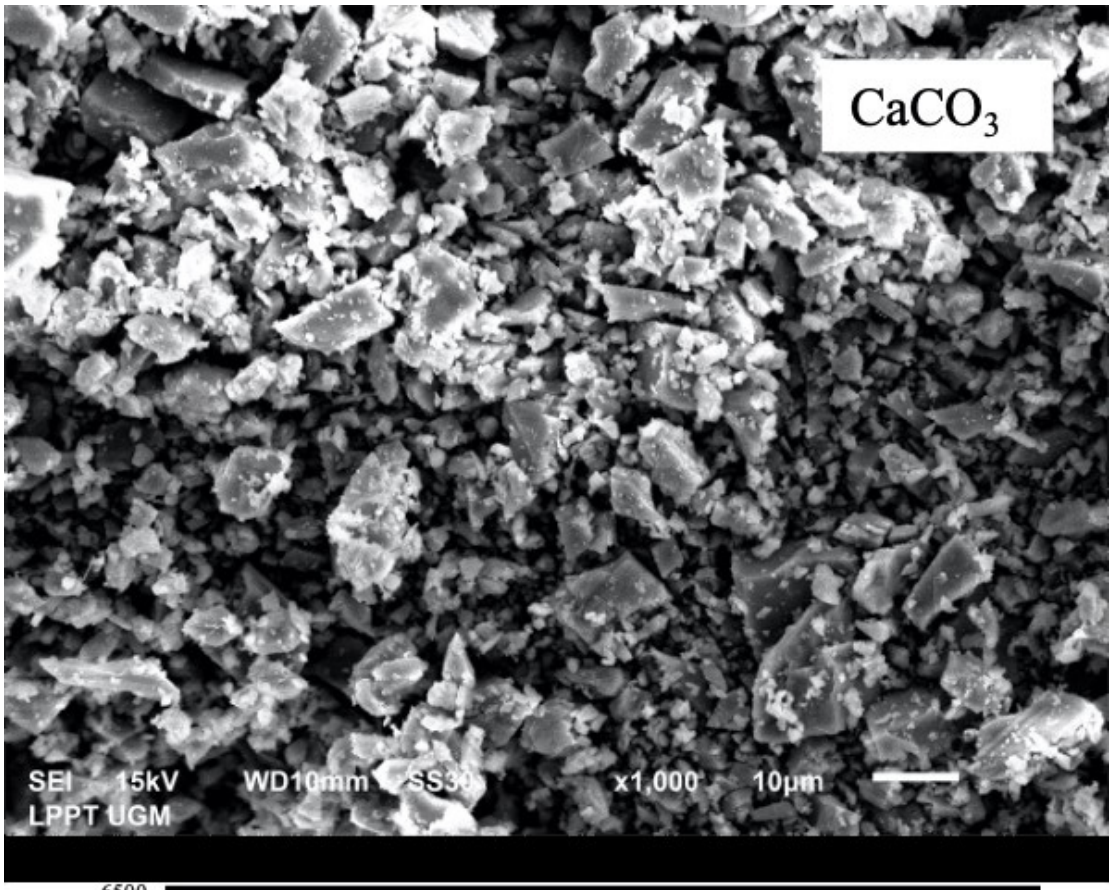
Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	13.80	0.02	23.18				4.8877
O K	0.525	39.94	0.10	50.38				27.4526
Na K	1.041	2.11	0.03	1.86				2.0789
Mg K	1.253	0.28	0.02	0.23				0.2650
P K	2.013	14.62	0.05	9.53				20.5224
Cl K	2.621	1.42	0.02	0.81				2.0404
K K*	3.312	0.03	0.02	0.02				0.0488
Ca K	3.690	27.80	0.08	14.00				42.7041
Total		100.00		100.00				

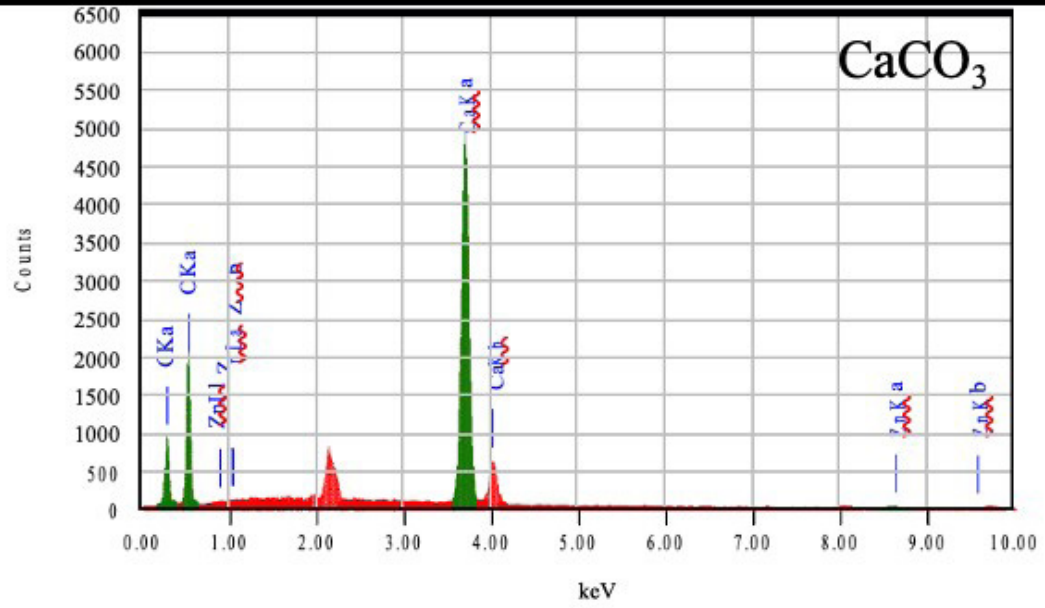




ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0352

Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	29.25	0.83	43.88				14.1590
O K	0.525	32.98	2.92	37.15				23.0932
Na K	1.041	0.94	0.29	0.74				1.0646
Mg K	1.253	0.25	0.15	0.19				0.2745
P K	2.013	11.86	0.93	6.90				18.8162
Cl K	2.621	0.56	0.22	0.29				0.9209
K K*	3.312	0.06	0.08	0.03				0.0945
Ca K	3.690	24.10	1.76	10.84				41.5772
Total		100.00		100.00				





ZAF Method Standardless Quantitative Analysis
Fitting Coefficient : 0.0721

Element	(keV)	Mass%	Sigma	Atom%	Compound	Mass%	Cation	K
C K	0.277	11.61	0.07	19.53				8.4332
O K	0.525	47.66	0.39	60.18				29.1160
Ca K	3.690	39.48	0.21	19.90				61.0068
Zn K*	8.630	1.25	0.17	0.39				1.4440
Total		100.00		100.00				

Figure 3. Scanning Electron Microscopy-Energy Dispersive X-Ray (SEM-EDS) results from grouper bone powders, grouper nano-calcium powders, and synthetic $CaCO_3$

Statistically, the autoclave duration treatment (A) decreased moisture content and protein but increased fat content and mineral content, including calcium and phosphorus. The treatment for autoclaving time followed by alkaline hydrolysis (AH) also showed little difference. Although the levels of organic compounds decreased with increasing autoclave time, there was no significant increase in the levels of minerals, calcium, and phosphorus between 3x2AH and 3x3AH.

Amitha *et al.* (2019) hydrolyzed grouper fish bone using alkali without autoclaving, achieving a nearly same ash level of 87.60 %, although having a greater water content of 5.96 % and protein content of 3.28 % more than in this study. Suntornsaratoon *et al.* (2018), which generated calcium supplement from tuna bone, had an ash content of 69.50 % consist of calcium 26.1 % and phosphorus 12.41 %, which was lower than this research, but greater amounts of impurities in the form of protein 24.06 %, fat 0.88 %. However, the findings of this research are still lower than those of Prinaldi *et al.* (2018) on tuna, which reported 98.66 % ash, 0.37 % water, 0.39 % protein, and 0.24 % fat.

3.6 FTIR Analysis

Fourier Transform Infrared (FTIR) is an instrument to analyze organic components (protein, lipid, water), the chemical bond, and organic structure. Based on these three spectra, it can be seen that the spectra produced from the grouper bone samples (Figure 4 and Figure 5) have more peaks than the spectra produced from the synthetic $CaCO_3$ sample (Figure 6), indicating that the chemical groups in the grouper bone samples are much more complex than those in the synthetic $CaCO_3$ sample.

All grouper bone powder samples (A and AH) showed a peak at 431 or 473 cm^{-1} which indicated a PO_4 compound (Prinaldi *et al.*, 2018), a peak of 603 cm^{-1} which indicated CH organic material (Nandiyanto *et al.*, 2019), a peak of 872 cm^{-1} as CO_3 ions (Yin *et al.*, 2016). According to Nandiyanto *et al.* (2019), peak 1236 cm^{-1} as amide III, peak 1456-1560 cm^{-1} as C-O-H (protein and collagen). The peaks of 1200-1600 were basically organic materials which can only be removed by sintering treatment. Furthermore, the peaks at 2854 and 2925 cm^{-1} were CH_2. The peak of 3691-3573 cm^{-1} was the hydroxyl stretching mode of hydroxyapatite (Prinaldi *et al.*, 2018; Biazar *et al.*, 2020).

The different peak between grouper bone powder (A) and grouper nano-calcium powder (AH) is in the AH group, all samples have a peak of 1638 cm^{-1} which indicates a CO_3 group and did not belong to group A, while the 1656 cm^{-1} peak indicates an amide group (Riaz *et al.*, 2018) and 1745 cm^{-1} which showed the ester/fat group (Nandiyanto *et al.*, 2019) only belonged to group A. It indicated that the alkaline hydrolysis process in the AH group succeeded in removing some of the protein and fat in group A until it was no longer detected on FTIR analysis.

Spectra on synthetic $CaCO_3$ only showed peaks of 473 cm^{-1} which indicated a phosphate group (Prinaldi *et al.*, 2018), 603 and 2925 cm^{-1} which indicated a CH group (Nandiyanto *et al.*, 2019) and 872 cm^{-1} which indicated carbonate ions (Yin *et al.*, 2016). The $CaCO_3$ spectra have also been confirmed to have a 99.995% similarity with Aldrich Calcium Carbonate CAS No. 472-34-1.

3.7 XRD Analysis

X-Ray diffraction (XRD) is a non-destructive technique used to determine the crystallinity structure of chemical substances. The phases of grouper bone powder (A), grouper nano-calcium powder (AH), and synthetic $CaCO_3$ were analyzed using XRD pattern diffraction. The crystal pattern was hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals, same for all grouper bone powder or nano-calcium powder samples with the maximum intensity at $2\theta = 31°$ (Figure 7a and Figure 7b). In this study, the dominant phase of the hydroxyapatite compound was the same as that described in several other studies (Biazar *et al.*, 2020; Nam *et al.*, 2019; Yusuf *et al.*, 2019).

Grouper bone powders (A) displayed slightly different diffraction patterns than grouper nano-calcium powders (AH). The XRD pattern of AH samples exhibited narrower and sharper diffraction peaks, indicating an increase in crystallinity. The 3 h autoclave treatment (3A) enhanced crystallinity while decreasing amorphousness in comparison to the treatment without autoclaving (0A) (Table 3). However, the autoclave's duration (3A-3x3A) had no effect on the crystallinity result. The highest increase in autoclave value occurred after alkaline hydrolysis (0AH-3x3AH).

The crystallinity of synthetic $CaCO_3$ samples was the highest, at 89.1 %, as shown by the sharp form of the diffraction pattern. In contrast to the HA sample, which had a diffraction peak at $2\theta = 31°$, the calcium carbonate sample had a peak at $2\theta = 29°$, with a phase analysis of Calcite 78%, Boron 12%, Cobalt 9.1%, and Chromium Iron Zirconium 0.5%.

Table 3. Crystallinity percentage of grouper bone powders, grouper nano-calcium powders and synthetic $CaCO_3$

Treatment	Duration	Crystallinity (%)	Amorphous (%)	Phase
Autoclaving (A)	0 h (0A)	59.3	40.7	Hydroxyapatite
	3 h (3A)	63.2	36.8	Hydroxyapatite
	2x3 h (2x3A)	63	37	Hydroxyapatite
	3x3 h (3x3A)	63.2	36.8	Hydroxyapatite
Autoclaving + Alkaline Hydrolysis (AH)	0 h (0A)	69.6	30.4	Hydroxyapatite
	3 h (3A)	71.9	28.1	Hydroxyapatite
	2x3 h (2x3A)	70	30	Hydroxyapatite
	3x3 h (3x3A)	71.6	28.4	Hydroxyapatite
$CaCO_3$		80.1	10.9	Calcite 78% Boron 12% Cobalt 9.1% Chromium Iron Zirconium 0.5%.

Table 4. Amino acid profile of grouper bone powders and grouper nano-calcium powders

Amino Acid Component (ppm)	Autoclaving				Autoclaving + Alkaline Extraction			
	0 h (0A)	3 h (3A)	2 x 3 h (2x3A)	3 x 3 h (3x3A)	0 h (0AH)	3 h (3AH)	2 x 3 h (2x3AH)	3 x 3 h (3x3AH)
Essential Amino Acid								
Histidine	93.44	143.75	167.3	208.23	93.63	157.45	173.52	189.35
Isoleucine	66.69	110.74	125.74	159.31	77.27	111.84	133.46	153.65
Leucine	103.09	151.92	177.85	218.03	102.54	157.94	182.51	189.01
Lysine	138.86	207.47	309.13	287.46	138.76	212.05	240.87	258.41
Methionine	42.04	66.67	72.86	94.86	41.65	66.29	77.04	79.98
Phenylalanine	59.11	82.31	87.74	104.39	51.11	84.6	94.02	96.34
Threonine	57.33	86.09	107.34	12.87	63.86	91.2	105.36	112.42
Tryptophan	20.33	27.82	35.13	49.37	26.7	31.69	40.11	38.34
Valine	124.24	193.63	229.81	280.11	137.25	206.84	236.42	244.15
Total	705.13	1070.4	1312.9	1414.6	732.77	1119.9	1283.31	1361.65
Non-essential As. Amino								
Alanine	79.46	112.46	135.14	157.6	82.96	114.83	133.14	139.39
Arginine	85.21	133.57	160.19	197.02	94.76	149.26	161.84	173.58
Asparagine	11.92	16.15	20.77	25.35	13.17	18.57	21.83	22.53
Aspartic acid	147.26	228.8	250.73	304.96	150.21	224.13	269.48	279.28
Cysteine	18.06	26.28	28.61	34.12	18.33	25.97	30.43	31.51
Glutamic acid	182.47	273.24	313.07	382.84	193.41	275.01	324.97	343.8
Glutamine	12.6	18.38	20.01	23.88	12.79	18.15	21.29	22.05
Glycine	61.15	85.63	101.166	121.81	65.79	92.55	104.39	114.38
Proline	52.08	74.55	86.95	97.73	50.26	73.06	87.47	90.16
Serine	41.74	60.68	82.43	93.58	46.82	69.51	81.38	109.47
Tyrosine	67.27	100.65	117.28	127.73	82.59	108.25	118.88	118.6
Total	759.22	1130.4	1316.35	1566.6	811.09	1169.3	1355.1	1444.75

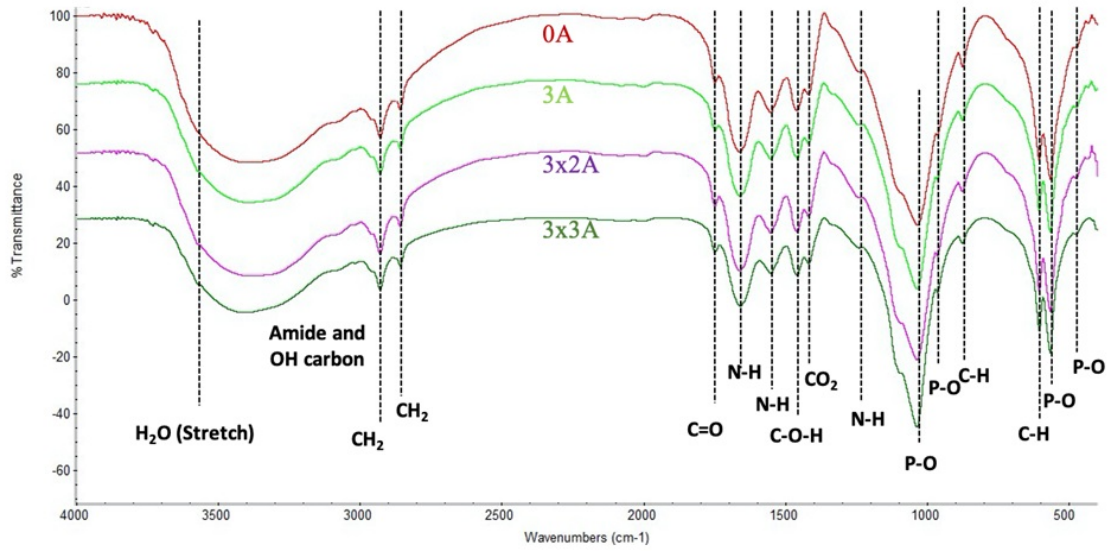


Figure 4. Fourier Transform Infra-Red (FTIR) analysis from grouper bone powders

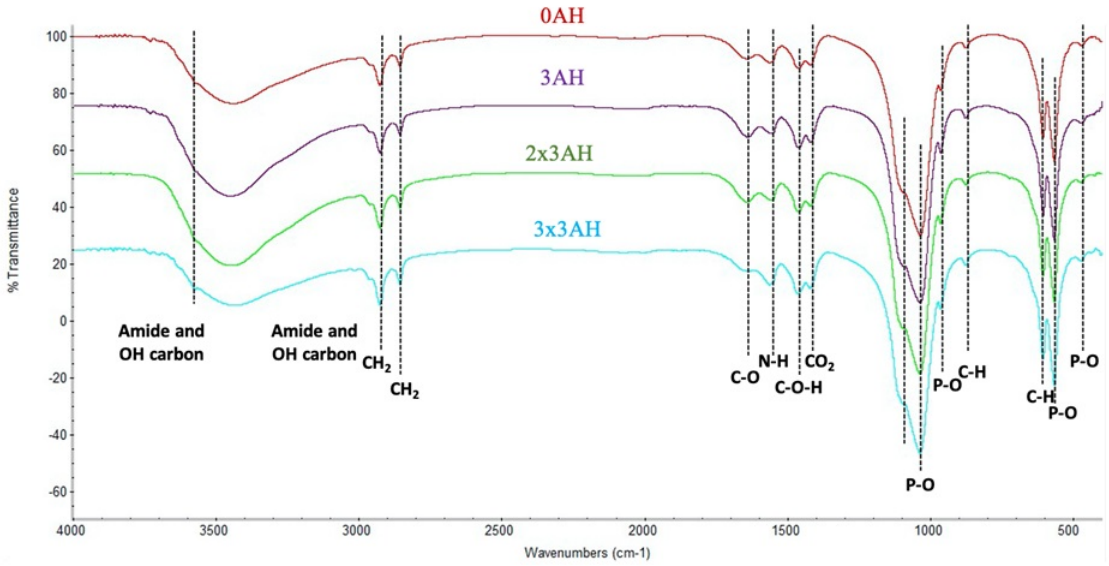


Figure 5. Fourier Transform Infra-Red (FTIR) analysis from grouper nano-calcium powders

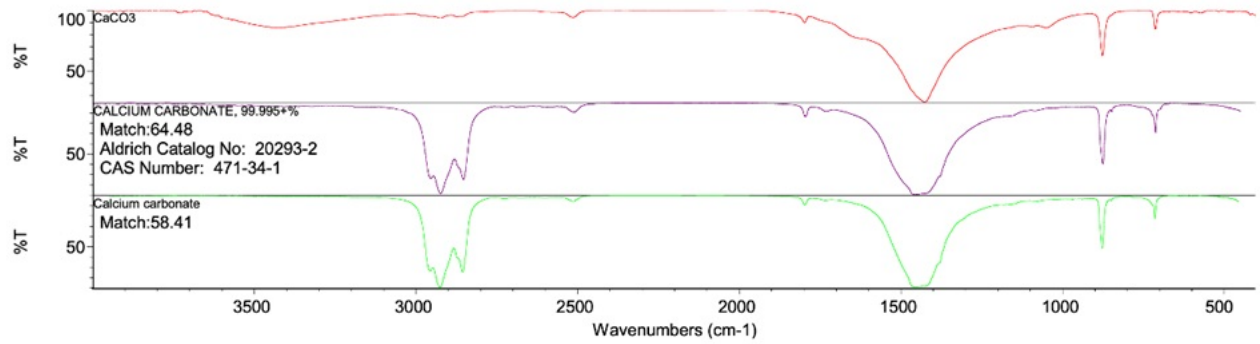


Figure 6. Fourier Transform Infra-Red (FTIR) analysis of synthetic $CaCO_3$

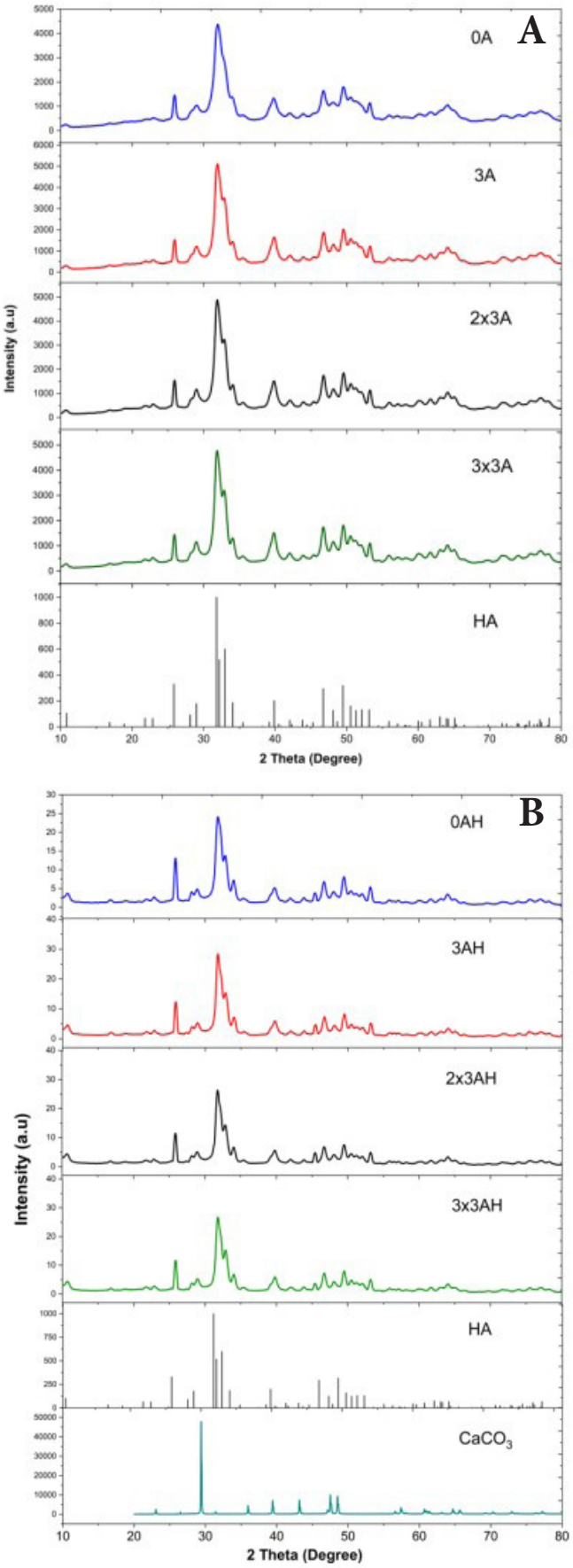


Figure 7. (a) XRD diffractogram of several autoclaving (A) treatments of grouper bone powders (HA: Hydroxyapatite); (b) XRD diffractogram of grouper nano-calcium powders and synthetic $CaCO_3$ (HA: Hydroxyapatite)

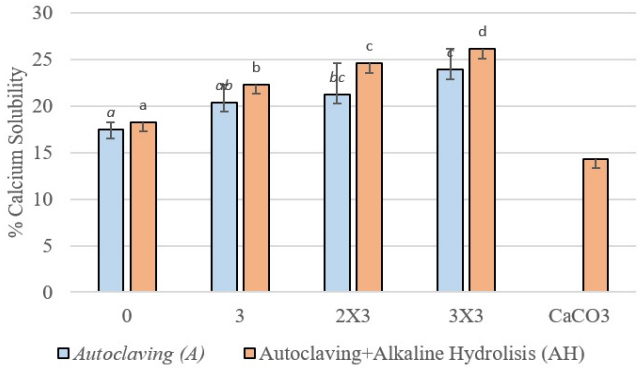


Figure 8. Calcium solubility of grouper bone powders, grouper nano-calcium powders, and synthetic CaCO3 samples in in vitro gastrointestinal simulation. Different alphabets above the bar denoted significant differences using one-way ANOVA and Duncan's test ($p<0.05$).

3.8 Calcium Solubility in In Vitro Gastrointestinal Simulation

In vitro digesting procedures are more ethical, quicker, and cheaper than *in vivo* approaches, which are time-consuming and pricey. As a technique for studying the digestibility or bioavailability of different meals, the gastrointestinal simulation is a digestion model that would yield very accurate findings in a short period (Lee *et al*., 2016).

Gastrointestinal tract simulation is a simple biochemical approach including at least two digestion processes (stomach and intestine) model whose products stay in a static bioreactor to simulate the gastrointestinal system (Alegría *et al*., 2015). In this study, the gastrointestinal tract simulation test was used as a model to determine the solubility of calcium after passing through the digestive tract of the stomach in an acidic environment and reacting with the enzyme pepsin and also passing through the intestinal tract in an alkaline environment and reacting with the enzyme pancreatin, so that the calcium that still disperse and dissolves is calcium, which can be absorbed by the microvilli in the intestinal epithelium.

The grouper bone powder sample (A) had a minimum solubility of 17.50 % in sample 0A and grouper nano-calcium powder (AH) had maximum solubility of 26.14 % in sample 3x3AH (Figure 8). Consequently, the autoclave time is exactly proportional to the increase in calcium solubility. Similarly, it was discovered that the time of the autoclave followed by alkaline hydrolysis increased the solubility of calcium.

Table 5. Fatty acid profile of grouper bone powders and grouper nano-calcium powders

Fatty Acid Component (ppm)	Autoclaving				Autoclaving + Alkaline Hydrolysis			
	0 h (0A)	3 h (3A)	2 x 3 h (2x3A)	3 x 3 h (3x3A)	0 h (0AH)	3 h (3AH)	2 x 3 h (2x3AH)	3 x 3 h (3x3AH)
Saturated Fatty Acid (SFAs)	1376.2	2143.85	3011.73	3375.9	1341.64	1893.43	2776.45	3147.18
C6:0 (Caproic acid)	19.84	29.41	41.85	45.9	20.53	26.59	36.02	43.46
C8:0 (Caprylic acid	111.86	173.66	243.53	272.86	108.96	153.5	224.74	255.82
C10:0 (Capric acid)	72.24	113.22	159.35	178.84	70.36	99.81	147.01	167.48
C12:0 (Lauric acid)	373.63	578.7	810.83	908.09	363.97	511.85	748.33	851.62
C14:0 (Myristic acid)	296.72	463.41	651.34	730.56	289	408.94	600.99	677.11
C16:0 (Palmitic acid)	339.46	527.73	740.47	829.84	330.65	466.29	683.31	769.59
C18:0 (Stearic acid)	148.24	237.34	336.67	379.26	144.31	208.02	310.43	352.89
C20:0 (Arachidic acid)	14.21	20.38	27.69	30.55	13.86	18.43	25.62	29.21
Unsaturated Fatty Acids (UFAs)	1353.7	2073.51	2944.12	3298.19	1318.64	1856.61	2717.04	3029.68
Monounsaturated Fatty Acids (MUFA)	1040.81	1580.76	2249.37	2517.79	1013.94	1422.77	2076.15	2297.55
C16:1 (Palmitoleic acid)	224.74	354.72	500.55	562.48	218.84	312.11	461.72	524.28
C18:1 (Oleic acid)	781.52	1199.16	1674.15	1871.73	761.44	1063.43	1545.51	1695.48
C20:1 (Gondoic acid)	30.15	20.38	65.73	73.66	29.37	41.41	60.66	66.07
C22:1 (Erucic acid)	4.4	6.5	8.94	9.92	4.29	5.82	8.26	11.71
Polyunsaturated Fatty Acids (PUFA)	312.89	492.75	694.75	780.4	304.7	433.84	640.89	732.13
C 18:2 (Linoleic acid)	166.43	264.5	374.21	421.03	162.02	232.29	345.12	397.7
C 18:3 (Linolenic acid)	73.15	112.64	157.48	176.18	71.27	99.8	145.36	167
C 18:4 (Steridonic acid)	30.9	49.63	70.49	79.45	30.08	43.46	64.99	74.97
C 20:4 (Arachidonic acid)	40.74	63.66	89.5	100.39	39.68	56.17	82.58	99.13
C 20:4 (Eicosatetraenoic acid)	25.08	39.68	56.04	63	24.42	34.89	51.69	53.01
C 20:5 (Eicosapentaenoic acid/ EPA)	96.68	153.51	217.1	244.22	94.13	134.85	200.22	222.44
C 22:5 (Docosapentaenoic acid)	10.46	16.83	23.91	26.96	10.18	14.73	22.05	26.58
C 22:6 (Docosahexaenoic acid)	35.88	56.8	80.23	90.2	34.94	49.94	74	89

The research of Yin *et al.* (2016) indicated that the smaller the calcium particles, the greater the solubility, with a maximum solubility of 19.27 %, which is still less than the solubility of the grouper nano-calcium powder created in this study.

If the solubility of calcium is determined solely by its particle size, then the sample with the highest solubility should be synthetic $CaCO_3$, which according to the PSA test had the smallest particle size among the grouper bone powder samples; however, the solubility of $CaCO_3$ is still lower (14.34 %) than that of grouper bone powder without autoclaving or alkaline hydrolysis (0A) which was 17.50 % (Figure 1). The primary distinction between synthetic $CaCO_3$ and grouper bone powder is that all grouper bone powder samples include organic material (protein and fat), while synthetic $CaCO_3$ does not. The existence of amino acids as components of proteins and the presence of different fatty acids as constituents of lipids are confirmed (Table 4 and Table 5).

Benjakul *et al.* (2017) hypothesizes that the relationship between the presence of protein and the increase in calcium solubility in vitro are (1) a special bond between calcium and protein that can increase calcium solubility or (2) protein acts as a buffer solution for gastrointestinal enzymes so that calcium solution reacts faster. Protein can neutralize the acidity of pepsin enzyme; hence it decreases the responding pancreatin base, thereby decreasing calcium deposits. In the Benjakul *et al.* (2017) investigation, the solubility of calcium was 3.78 to 4.65 % for sintered calcium and 10.12 to 10.80 % for calcium via alkaline hydrolysis, much lower than in this study.

The amount of fat in dietary calcium was substantially correlated with calcium solubility because fat was able to reduce the fluidity of lipid membranes, prolong the interaction of calcium with intestinal epithelial membranes, and enhance calcium absorption (Bandali *et al*., 2018).

3.9 Amino Acid Profile

The findings of the proximate test on the grouper nano-calcium powder sample suggest the existence of protein in a concentration of less than one percent (Table 2); thus, the amino acid profile was evaluated.

All samples included varying amounts of all kinds of amino acids, including essential and non-essential amino acids (Table 4). The dominant essential amino acids were lysine, valine, histidine, leucine, and isoleucine, and non-essential amino acids were aspartic acid, glutamic acid, arginine, alanine, glycine, and tyrosine. According to Mescher (2016), the three primary amino acids that construct type 1 collagen were proline, lysine, and glycine. The three polypeptides were aligned and formed into a stable triple helix owing to the presence of cysteine, which may bind procollagen strands through cysteine disulfide bonds.

Peptides that are naturally present in bone have been demonstrated to be able to improve calcium absorption in an *in vitro* test employing Caco-2 cells monolayers such as the findings of Liao *et al.* (2020) study. Three amino acid sequences Gly-Pro-Ala-Gly-Pro-His-Gly-Pro-Val-Gly, Phe-Asp-His-Ile-Val-Tyr and Tyr-Gln-Glu-Pro-Val-Ile-Ala-Pro-Lys-Leu has been found to work as a calcium chelating agent that shields calcium from digestive enzymes.

Tang and Skibsted (2016) also revealed that during passage from the stomach to the intestine, numerous kinds of charged amino acids were deprotonated, thereby having a favorable influence on calcium binding. The phenomena of increased calcium binding were also accompanied by binding of the binding sites of carboxylate binding to chelation by amino groups and carboxylate oxygen for leucine, aspartate, glutamate, alanine, and asparagine. Thus, the addition of different kinds of amino acids in the nano-calcium solution had been proven to improve the solubility of calcium via the gastrointestinal system so that it may subsequently be absorbed by intestinal epithelial cells.

3.10 Fatty Acid Profile

Fatty acids are molecules that construct the fat and are still identifiable in extremely low amounts in grouper nano-calcium powders. The hydrolysis procedure may lower the fat level by more than 4 %, but it cannot fully eradicate it. The primary sources of polyunsaturated fatty acids (PUFA) are known from fish and other aquatic foods and people acquire the majority of their eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) through ingesting fish, aquatic invertebrates, and algae (Taşbozan and Gökçe, 2017). The fatty acid profile in the grouper's bone powder in this study showed that fatty acid was also present in fish bone tissue, not only in flesh or fat tissue (Table 5).

In all samples, monounsaturated fatty acids (MUFA) predominated over polyunsaturated fatty acids (PUFA), but the percentage of unsaturated fatty acids (UFA) was near to that of saturated fatty acids (SFA). The autoclave duration raised the amounts of both saturated and unsaturated fatty acids. However, the

alkaline hydrolysis procedure reduced the quantities of fatty acids from 23-269 ppm.

According to the approximate statistics, autoclaving reduces the water content, consequently increasing the fat content and the amounts of saturated and unsaturated fatty acids (Table 2). The decrease in the levels of saturated fatty acids and unsaturated fatty acids after the alkaline hydrolysis process is due to the saponification reaction between alkali and free fatty acids; as a result, the soap layer is lost during the centrifugation process, resulting in a decrease in the levels of saturated fatty acids and unsaturated fatty acids. Alkali refine is a chemical lipid purification process that causes free fatty acids to react with alkali to form insoluble soap, thereby obtaining pure saturated and unsaturated fatty acids (Estiasih and Ahmadi, 2012).

As a source of calcium, certain kinds of fatty acids in fish bones are impurities in addition to reducing calcium levels, owing to the ease with which these fatty acids are oxidized during storage and form various aldehydes and ketones that induce alterations (Benjakul *et al*., 2017). In contrast, the inclusion of fatty acids in this product provided a number of benefits.

Young mice given n-3 PUFA diet with conjugated linoleic acid absorbed more calcium than those fed n-6 PUFA diet with conjugated linoleic acid (Kelly *et al*., 2003). Wang *et al*. (2016) showed *in vivo* that a MUFA-rich diet may enhance the thickness of the trabecular volume, but an SFA-rich diet has no effect on the trabecular volume. Meanwhile, Bandali *et al*. (2018) in *in vitro* study showed that administering SFA increased the bioaccessibility of calcium in the jejunum at normal pH. Depending on the kind of fatty acid, the presence of fat might therefore alter calcium absorption.

4. Conclusion

This research shown that 3x3 h of autoclaving followed by alkaline hydrolysis was able to shrink the size of nano-calcium particles and resulted in the highest calcium solubility compared to synthetic $CaCO_3$. In contrast to synthetic $CaCO_3$, grouper nano-calcium powders include trace quantities of organic substance, such as protein and fat. Proteins may act as proton donors, maintaining the pH stability and improving calcium solubility. Fat protects calcium from the alkaline pH in the intestine, preventing calcium precipitation. This research should be followed with *in vivo* study to demonstrate the bioavailability and impact of the grouper nano-calcium powder on bone density.

Acknowledgment

The authors are grateful Mr. Safrudin (PT. Kelola Mina Laut) for providing free samples of fish bones for this study. This study was conducted by Doctoral Dissertation Research funding from the Indonesian Government, the National Competitive Program-DIKTI in 2022 with grant number: 1939/UN1/DITLIT/PT.01.03/2022.

Author's Contributions

Everyone has contributed to the research. Each author contributed as follows, PK; gathered and analyzed the data and produced the paper. PT, SA, and YP; prepared and critiqued the article's key themes. All writers discussed the findings and contributed to the final draft.

Conflict of Interest

The authors state that no commercial or financial ties that may be considered as a possible conflict of interest existed during the conduct of the study.

Funding Information

This study was conducted by Doctoral Dissertation Research funding from the Indonesian Government, the National Competitive Program-DIKTI in 2022 with grant number: 1939/UN1/DITLIT/PT.01.03/2022.

References

Abdullah, A., & Mohammed, A. (2019). Scanning electron microscopy (SEM): A review. Paper presented at the Proceedings of 2018 International Conference on Hydraulics Pneumatics – HERVEX, Băile Govora, Romania.

Abid, N., Khan, A. M., Shujait, S., Chaudhary, K., Ikram, M., Imran, M., Haider, J., Khan, M., Khan, Q., & Maqbool, M. (2022). Synthesis of nanomaterials using various top-down and bottom-up approaches, influencing factors, advantages, and disadvantages: A review. *Advances in Colloid and Interface Science*, 300:102597.

Acar, S., Demir, K., & Shi, Y. (2017). Genetic causes of rickets. *Journal of Clinical Research in Pediatric Endocrinology*, 9(2):88-105.

Alegría, A., Garcia-Llatas, G., & Cilla, A. (2015). Static digestion models: General introduction. In K. Verhoeckx, P. Cotter, I. López-Expósito, C.

Kleiveland, T. Lea, & A. Mackie (Ed.), The impact of food bioactives on health: In vitro and ex vivo models. (pp. 3–12). New York: Springer International Publishing.

Al-Timimi, Z., & Tammemi, Z. J. (2022). Polymer Blends and nanocomposite materials based on Polymethyl Methacrylate (PMMA) for bone regeneration and repair. *Journal of Sustainable Materials Processing and Management*, 2(1):15-23.

Ali, M., Kusnadi, J., Aulanni'am, A., & Yunianta, Y. (2020). Amino acids, fatty acids and volatile compounds of Terasi Udang, an Indonesian shrimp paste, during fermentation. *AACL Bioflux*, 13(2):938-50.

Amitha, Raju, C. V., Lakshmisha, I. P., Kumar, P. A., Sarojini, A., Endra, G., & Pal, J. (2019). Nutritional composition of fish bone powder extracted from three different fish filleting waste boiling with water and an alkaline media. *International Journal of Current Microbiology and Applied Sciences*, 8(02):2942-2948.

Anggraeni, N. (2019). Bioavailabilitas nanokalsium hasil ekstraksi tulang ikan nila (*Oreochromis niloticus*) dengan variasi konsentasi pelarut basa dan lama ekstraksi. Thesis. Yogyakarta: Universitas Gadjah Mada.

Bandali, E., Wang, Y., Rogers, M., & Shapses, S. (2018). The influence of dietary fat and intestinal pH on calcium bioaccessibility: an *in vitro* study. *Food and Function*, 9(3):1809-1815.

Bas, M., Daglilar, S., Kuskonmaz, N., Kalkandelen, C., Erdemir, G., Kuruca, S. E., Tulyaganov, D., Yoshioka, T., Gunduz, O., Ficai, D., & Ficai, A. (2020). Mechanical and biocompatibility properties of calcium phosphate bioceramics derived from salmon fish bone wastes. *International Journal of Molecular Sciences*, 21(21):1-14.

Benjakul, S., Mad-Ali, S., Senphan, T., & Sookchoo, P. (2018). Characteristics of biocalcium from pre-cooked skipjack tuna bone as affected by different treatments. *Waste and Biomass Valorization*, 9(8):1369-1377.

Benjakul, S., Mad-Ali, S., & Sookchoo, P. (2017). Characteristics of biocalcium powders from pre-cooked tongol (*Thunnus tonggol*) and yellowfin (*Thunnus albacores*) tuna bones. *Food Biophysics*, 12(4):412-421.

Biazar, E., Joupari, M. D., Keshel, S. H., Amirhosein, D. N., Kamalvand, M., Sahebalzamani, M., Roniyan, R., Shabankhah, M., & Farajpour, F. (2020). Characterization and biocompatibility of hydroxyapatite nanoparticles extracted from fish bone. *Bioengineering Research*, 2(2):10-19.

Cormick, G., & Belizán, J. M. (2019). Calcium intake and health. *Nutrients*, 11(7):1-16.

Corrêa, T. H. A., & Holanda, J. N. F. (2019). Fish bone as a source of raw material for synthesis of calcium phosphate. *Materials Research*, 22:1-5.

Dewiasty, E., Setiati, S., Agustina, R., Roosheroe, A. G., Abdullah, M., Istanti, R., & de Groot, L. C. (2021). Prevalence of lactose intolerance and nutrients intake in an older population regarded as lactase non-persistent. *Clinical Nutrition ESPEN*, 43(1):317-321.

Estiasih, T., & Ahmadi, K. (2012). Pembuatan trigliserida kaya asam lemak ω-3 dari minyak hasil samping pengalengan ikan lemuru (*Sardinella longiceps*). *Jurnal Teknologi Pertanian*, 5(3):116-128.

Hemung, B. O. (2013). Properties of tilapia bone powder and its calcium bioavailability based on transglutaminase assay. *International Journal of Bioscience, Biochemistry and Bioinformatics*, 3(4):306-309.

Hodges, J. K., Cao, S., Cladis, D. P., & Weaver, C. M. (2019). Lactose intolerance and bone health: The challenge of ensuring adequate calcium intake. *Nutrients*, 11(718):1-17

Husna, A., Handayani, L., & Syahputra, F. (2020). Pemanfaatan tulang ikan kambing-kambing (*Abalistes stellaris*) sebagai sumber kalsium pada produk tepung tulang ikan. *Acta Aquatica: Aquatic Sciences Journal*, 7(1):13-20.

Julianti, S. R. (2017). Karakteristik fisikokimia dan bioavailabilitas nanokalsium hasil ekstraksi tulang ikan bandeng (*Chanos chanos*) menggunakan larutan basa. Thesis. Malang: Universitas Brawijaya.

Jung, W. K., Shahidi, F., & Kim, S. K. (2007). Calcium

from fish bone and other marine resources. In C. Borrow & F. Shahidi (Ed.), Marine nutraceuticals and functional foods. (pp. 419-429). Florida: CRC Press.

Kelly, O., Cusack, S., Jewell, C., & Cashman, K. D. (2003). The effect of polyunsaturated fatty acids, including conjugated linoleic acid, on calcium absorption and bone metabolism and composition in young growing rats. *British Journal of Nutrition*, 90(4):743-750.

KKP (Kementerian Perikanan dan Kelautan). (2021). Laporan Tahunan 2021. Jakarta: Ditjen Perikanan Tangkap.

Kusumaningrum, I., Sutono, D., & Pamungkas, B. F. (2016). Pemanfaatan tulang ikan belida sebagai tepung sumber kalsium dengan metode alkali. *Jurnal Pengolahan Hasil Perikanan Indonesia*, 19(2):148-155.

Kusumawati, P., Triwitono, P., Anggrahini, S., & Pranoto, Y. (2022). Nano-calcium powder properties from six commercial fish bone waste in Indonesia. S*qualen Bulletin Marine and Fisheries Postharvest Biotechnology*, 17(1):1-12.

Lee, S. J., Lee, S. Y., Chung, M. S., & Hur, S. J. (2016). Development of novel *in vitro* human digestion systems for screening the bioavailability and digestibility of foods. *Journal of Functional Foods*, 22:113-121.

Liao, W., Chen, H., Jin, W., Yang, Z., Cao, Y., & Miao, J. (2020). Three newly isolated calcium-chelating peptides from tilapia bone collagen hydrolysate enhance calcium absorption activity in intestinal caco-2 cells. *Journal of Agricultural and Food Chemistry*, 68(7):2091-2098.

Malde, M. K., Bügel, S., Kristensen, M., Malde, K., Graff, I. E., & Pedersen, J. I. (2010). Calcium from salmon and cod bone is well absorbed in young healthy men: A double-blinded randomised crossover design. *Nutrition and Metabolism*, 7(61):1-9.

Mescher, A. L. (2016). Junqueira's basic histology text & atlas (14th ed.) USA: McGraw Hill.

Nam, P. V., Van Hoa, N., & Trung, T. S. (2019). Properties of hydroxyapatites prepared from different fish bones: A comparative study. *Ceramics International*, 45(16):20141-20147.

Nandiyanto, A. B. D., Oktiani, R., & Ragadhita, R. (2019). How to read and interpret ftir spectroscope of organic material. *Indonesian Journal of Science and Technology*, 4(1):97-118.

Nemati, M., Huda, N., & Ariffin, F. (2017). Development of calcium supplement from fish bone wastes of yellowfin tuna (*Thunnus albacares*) and characterization of nutritional quality. *International Food Research Journal*, 24(6):2419-2426.

Nawaz, A., Li, E., Irshad, S., Hamad, H. H. M., Liu, J., Shahbaz, H. M., Ahmed, W., & Regenstein, J. M. (2020). Improved effect of autoclave processing on size reduction, chemical structure, nutritional, mechanical and in vitro digestibility properties of fish bone powder. *Advanced Powder Technology*, 31(6):2513-2520.

Pertiwi, M. G. P., Marsono, Y., & Indrati, R. (2020). In vitro gastrointestinal simulation of tempe prepared from koro kratok (*Phaseolus lunatus* L.) as an angiotensin-converting enzyme inhibitor. *Journal of Food Science and Technology*, 57(5):1847-1855.

Pu'ad, N. A. S. M., Koshy, P., Abdullah, H. Z., Idris, M. I., & Lee, T. C. (2019). Syntheses of hydroxyapatite from natural sources. *Heliyon*, 5(5):1-14.

Prinaldi, W. V., Suptijah, P., & Uju. (2018). Karakteristik Sifat fisikokimia nano-kalsium ekstrak tulang ikan tuna sirip kuning (*Thunnus albacares*). *Jurnal Pengolahan Hasil Perikanan Indonesia*, 21(3):385-395.

Ratnawati, S. E., Ekantari, N., & Ustadi. (2020). The utilization of catfish bone waste as microcalcium by different preparation methods. *E3S Web of Conferences*, 147(3):03031.

Riaz, T., Zeeshan, R., Zarif, F., Ilyas, K., Muhammad, N., Safi, S. Z., Rahim, A., Rizvi, S. A. A., & Rehman, I. U. (2018). FTIR analysis of natural and synthetic collagen. *Applied Spectroscopy Reviews*, 53(9):703-746.

Shi, P., Liu, M., Fan, F., Yu, C., Lu, W., & Du, M. (2018). Characterization of natural hydroxyapatite originated from fish bone and

its biocompatibility with osteoblasts. *Materials Science and Engineering: C*, 90:706-712.

Siddharthan, A., Sampath Kumar, T. S., & Seshadri, S. K. (2009). Synthesis and characterization of nanocrystalline apatites from eggshells at different Ca/P ratios. *Biomedical Materials*, 4(4):1-9.

Sumarto, Desmelati, Sari, N. I., Angraini, R. M., & Arieska, L. (2021). Characteristic of nano-calcium bone from a different species of catfish (*Pangasius hypophthalmus*, *Clarias batrachus*, *Hemibagrus nemurus* and *Paraplotosus albilabris*). IOP Conference Series: *Earth and Environmental Science*, 695(012055):1-8.

Suntornsaratoon, P., Charoenphandhu, N., & Krishnamra, N. (2018). Fortified tuna bone powder supplementation increases bone mineral density of lactating rats and their offspring. *Journal of the Science of Food and Agriculture*, 98(5):2027-2034.

Talib, A., & Zailani, K. (2017). Extraction and Purification of yellowfin tuna fishbone flour as an ingredient of future traditional medicine. *IOSR Journal of Pharmacy*, 7(11):8-14.

Tang, N., & Skibsted, L. H. (2016). Calcium binding to amino acids and small glycine peptides in aqueous solution: Toward peptide design for better calcium bioavailability. *Journal of Agricultural and Food Chemistry*, 64(21):4376-4389.

Taşbozan, O., & Gökçe, M. A. (2017). Fatty acids in fish. In A. Catala (Ed.), Fatty acids. (pp. 143-159). London: IntechOpen.

Wang, Y., Dellatore, P., Douard, V., Qin, L., Watford, M., Ferraris, R. P., Lin, T., & Shapses, S. A. (2016). High fat diet enriched with saturated, but not monounsaturated fatty acids adversely affects femur, and both diets increase calcium absorption in older female mice. *Nutrition Research*, 36(7):742-750.

Yin, T., Du, H., Zhang, J., & Xiong, S. (2016). Preparation and characterization of ultrafine fish bone powder. *Journal of Aquatic Food Product Technology*, 25(7):1045-1055.

Yin, T., Park, J. W., & Xiong, S. (2015). Physicochemical properties of nano fish bone prepared by wet media milling. *LWT - Food Science and Technology*, 64(1):367-373.

Yusuf, Y., Khasanah, D. U., Syafaat, F. Y., Pawarangan, I., Sari, M., Manuntu, V. J., & Rizkayanti, Y. (2019). Hidroksiapatit berbahan dasar biogenik (1st ed.). Yogyakarta: Gadjah Mada University Press.

