RESEARCH ARTICLE

Nano-calcium Powder Properties from Six Commercial Fish Bone Waste in Indonesia

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Abstract

Low daily calcium intake in Asia, especially in Indonesia, is still a serious problem. The abundant fish bone waste from the fishery fillet industries in Indonesia, can be employed as an alternative source of calcium to meet daily calcium needs. This research aimed to determine which of the six fish species (tilapia, catfish, grouper, snapper, tuna, kingfish mackerel) produces the best quality and the most cost-effective nano-calcium powder for a recommendation to the stakeholders. The calcium was extracted using an alkali treatment. The properties of the produced nano-calcium powders were analyzed for: proximate composition, calcium and phosphorus levels, color brightness level, XRD, FTIR, particle size, and SEM-EDX image analysis. The tilapia bone had the finest particle size of calcium (87.37 nm), while the grouper bone had the biggest particle size (281.4 nm). The brightness of all yields varied from 83.83 (beige-kingfish mackerel) to 90.64 (white-tilapia). The average calcium content from EDX analysis varied from 21.51% (snapper) to 34.37% (grouper). The average phosphorus levels ranged from 10.73% (kingfish mackerel) to 15.99% (grouper). The EDX Ca/P molar ratio was 1.41-1.66 across all samples. The FTIR spectra showed that all samples contained PO, CH, CO, NH, and OH groups. The XRD spectra pattern determined that the two main components of the fish bone nano-calcium powder were >90% hydroxyapatite and halite. All fish bone samples have the potential to be used as raw material for nano-sized calcium. However, grouper bone with the highest calcium content and the highest nano-calcium yield was the best choice for further study.

Keywords: nano-calcium, fish bone, fishery industry waste

Introduction

Calcium is an essential mineral in human body. Almost 70% of a human's bone is calcium phosphate in hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) form, and 30% consist of organic materials such as glycoproteins and collagen (Biazar et al., 2020). Therefore, an inadequate calcium diet compared to body requirements resulted in various illnesses, i.e. including osteopenia and osteoporosis, which were prevalent in various countries (Bas et al., 2020). Regardless of the health risks, the calcium intake level in Asian people is negligibly low, less than 500 mg/day (Cormick & Belizán, 2019). The low level of calcium intake is profoundly related to the source of calcium. For instance, the calcium intake in Indonesia is low due to lactose intolerance (Dewiasty et al., 2021); inspite of dairy products is a preeminent source of calcium. Meanwhile, calcium derived from cow and pig bones was often associated with health issues and religious prohibitions (Jung et al., 2007) despite being another reliable source of calcium. Consequently, many calcium supplements were produced from marine products, fish bone, or mollusk shells.

Bioavailability level is concurrently crucial in calcium absorption containing from the source. For example, Calcium carbonate ($CaCO_3$) extracted from mollusk shells, has low bioavailability. Meanwhile, the calcium hydroxyapatite extracted from fish bones has better bioavailability than calcium carbonate (Suntornsaratoon et al., 2018).

Several previous studies have been conducted regarding calcium or hydroxyapatite extraction from fish bones of various species: tilapia (Abdullah et al., 2020), gourami (Handayani et al., 2019), yellowfin tuna (Nemati et al., 2017), skipjack tuna (Wardani et al., 2020), and Spanish mackerel (Anggresani et al., 2021). All of these studies, results cannot be categorized as nano-calcium because the particle size was bigger than 1 mm. Other studies have succeeded in making nano-sized calcium particles derived from salmon (Bas et

al., 2020), snakehead fish (Hariani et al., 2020), catfish (Sumarto et al., 2021), yellowfin tuna (Prinaldi et al., 2018), Sardinella (Hasan et al., 2020), *Caspian kutum* (Biazar et al., 2020), and triggerfish (Husna et al., 2020).

Indonesia is the largest archipelagic country globally, with a sea area of 3.25 million km^2 and 2.55 million km^2 of the Exclusive Economic Zone. It has enormous marine and fishery potential products, in which the fishery sector is one of the national development sector mainstays. Indonesia's capture fisheries production was approximately 8.02 million tons in 2020 (Pratama, 2020).

The massive fishery industries in Indonesia produce massive waste or by-products. Fish by-products such as bones, skins, fins, heads, scales, offals, and liquids can pollute the environment, causing foul odors and health risks. The fishery industry's potential for fish bone waste could reach 9-15% of its total weight (Bas et al., 2020). Accordingly, at least 800 thousand to 1.2 million of bone waste in Indonesia had been generated.

Thus, the production of nano-calcium derived from fish bone can address two main issues: it may reduce bone volume generated, and provide an alternate source of high bioavailability calcium for people with lactose intolerance cases.

To the best of our knowledge, there has been no previous studies on the characteristics of calcium powder extracted from fish bone by-products that is available abundantly as the results of commercial fish fillet processing industry. This information is vital for stakeholders in deciding which fish bone waste is the most prospective to be used as raw material for calcium production. Accordingly, the primary purpose of this research was to discover which of the six fish species produces nano-calcium powder with the highest calcium content, and the highest percent yield to be recommended to stakeholders for production.

Material and Methods

Preparation of Fish Bone

The raw material for nano-calcium powder production was fish bone by-products from six commercial fish species. These by-products were obtained from fish fillet processing industries in Indonesia. The marine fish bone of tuna (*Thunnus* sp.), kingfish mackerel (*Scomberomorus* sp.), snapper (*Lutjanus* sp.), and grouper (*Epinephelus* sp.) were obtained from PT Kelola Mina Laut (Industrial Area Gresik East Java). Whereas the freshwater fish bone of tilapia (*Oreochromis niloticus*) came from PT Aquafarm Semarang, Central Java, and catfish (*Pangasius* sp.) were acquired from PT. Sukis Seafood (Safe n Lock Eco-Industrial Park Sidoarjo, East Java). All fish bone waste was obtained with random internal factors (size, sex, or fish's age). All fish bones were frozen and stored in a freezer until used. Ultrapure water (Waterone, Onelab, PT. Jayamas Medica Industri), NaOH (Merck, 1310-73-2), and HCl (Mallinckrodt, 7647-01-0) were used in this research.

Coarse Bone Powder Production

The coarse bone powder was produced following Anggraeni (2019). Each species of frozen fish bone was rinsed under running water. The fish bone was then boiled for 1 h, cleaned, and dried in a cabinet dryer at 50 °C for 24 h. Fish bone was then put autoclaved at 121 °C for 3 h, and re-dried in a cabinet dryer at 50 °C for another 24 h. The fish bone was crushed in a mortar and ground with a multifunction disintegrator (IC-06B, Getra, Jakarta, Indonesia) at 52,591 *g* for 1 min. The obtained coarse calcium powder were then determined for their yield percentage and was prepared for further analysis.

Nano-Calcium Production

The production of nano-calcium powder was also carried out following Anggraeni (2019) with slight modification. The coarse calcium powder was soaked in 1 N HCl (with sample to solvent ratio of 1:5, w/v) while stirred for 1 h with a magnetic stirrer to make it homogeneous. Then, it was incubated at room temperature for 24 h.

Removing HCl from the coarse bone meal was done by centrifugation at 1,711 *g* for 15 min. The supernatant was discarded while the concentrated coarse bone meal pellet was put in a glass beaker, then was added with 1 N NaOH solution in a ratio of 1:5 (w/v). The pellet was then hydrolyzed on a hotplate stirrer at 100 °C for 60 min, repeated three times. Every hydrolysis repetition was carried out by centrifugation method to separate the supernatant from its sediment.

The distilled water was added to the hydrolyzed sediment, and was neutralized using 1 N HCl until it reached a neutral pH (6.9-7.1). Then, it was re-centrifuged. The sediment was then transferred into a ceramic tray and put in a cabinet dryer at 50 °C for 15-18 h. The dried sediment was refined using a laboratory disc mill (Kawasaki T-100, Kobe, Japan) for 1 min, then was sieved using a 200 mesh sieve. The yield was weighed and tested for further analysis.

Physiological Analysis

Yield percentage analysis

The fish bone obtained for this research were not uniform in size. The yield percentage analysis was calculated from nano-calcium net weight divided by the dried bone net weight and multiply by 100.

Color analysis

Colorimeter (Konica Minolta CR-400, Tokyo, Japan) was used to determine the sample's color, with the white standard measurement i.e. L^* = 92.87, a^* = -1.27, b^* = 3.33 and standard black measurement i.e. L^* = 21.30, a^* = -0.76, b^* = 1.27.

Particle size analysis

Particle size analysis followed the Yin et al. (2016) method and was slightly modified. As much as 50 mg sample was dissolved into 50 mL ultrapure water. It was adjusted to pH 2.0 using 1 N HCl. The mixture was then subjected to a homogenizer instrument (Ika Ultra-Turrax 50T Basic, Selangor, Malaysia) at 4,000 rpm for 15 min. The particle size distribution was determined by dynamic light scattering using the Malvern Zetasizer Nano ZS (Malvern, UK) instrument.

Scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDX)

Samples were also observed using scanning electron microscopy (JSM-6510 LA, Jeol, Tokyo, Japan) with an energy-dispersive X-ray spectroscope (EDX) detector, following Benjakul et al. (2018) method with a slight modification. Secondary electron 15 kV accelerating voltage was used in observation after the specimen was coated with gold.

Chemical Analysis

Proximate analysis

Proximate moisture content analysis was carried out using a moisture analyzer (MB-90, Ohaus, Parsippany, NJ, USA), protein content was measured using Kjeldahl digestion following AOAC protocol 920.153, fat content was determined using Soxhlet extraction following AOAC protocol 960.39, and ash content was calculated using gravimetry following AOAC protocol 928.08.

Determination of mineral level

Atomic Absorption Spectrophotometer (3110, Perkin Elmer, Massachusetts, USA) was used to determine Ca, Na, Mg, K, Zn, Fe, and Cu, following AOAC protocol 985.35 (Flame Atomic Absorption Spectroscopy method). UV-Vis Spectrophotometer (Aquamate 8100, Thermo Scientific, Germany) was used to determine phosphorus following AOAC protocol 958.01 (Spectrophotometric Molybdovanado-phosphate method).

Fourier transform infrared spectroscopy (FTIR) analysis

FTIR Instrument (Nicolet type iS10, Thermo Fisher Scientific, Massachusetts, USA) was used to determine the chemical structure of the powder samples. The spectra were recorded in the middle infrared regions of 400-1,400 cm^{-1}. All samples were prepared by KBr pellets methods (Corrêa et al., 2019).

X-Ray diffraction (XRD) analysis

X-Ray Diffraction instrument (Bruker D2 Phaser, Massachusetts, USA) was used to identify microstructural characteristics of fish bone samples. The sample was analyzed starting at an angle of 2q = 8-80° angle. The step scan data was 0.02 using Cu Kα radiation with a wavelength of λ1.54060. XRD produced a diffractogram that could determine the mineral structural characteristics, identifying the composition phase of the compounds contained in the sample, lattice parameters, and crystal volume of fish bone samples (Siddharthan et al., 2009; Shi et al., 2018). Phase identification compares the observed peak positions data to the Crystallography Open Database called the Powder Diffraction File (PDF) for the corresponding phase in the Qualx 2.0 software (Italy). The percentage of crystallinity represents a ratio of the crystalline curve area to the sum of the crystalline areas and amorphous curves (Yusuf et al., 2019).

Statistical Analysis

Experiments in this study were performed in triplicate. One-way analysis of variance (ANOVA) for statistical analysis was held using SPSS (IBM SPSS 26 Version, IL, USA), and means were compared using Duncan's multiple range test (DMRT) at $p<0.05$.

Results and Discussion

Yield Percentage Analysis

From an economics perspective, the yield percentage of a product is critical. The higher product's yield percentage, the more profitable it is to produce. The nano-calcium yield percentage calculation can be seen in Figure 1. Based on the results, the kingfish

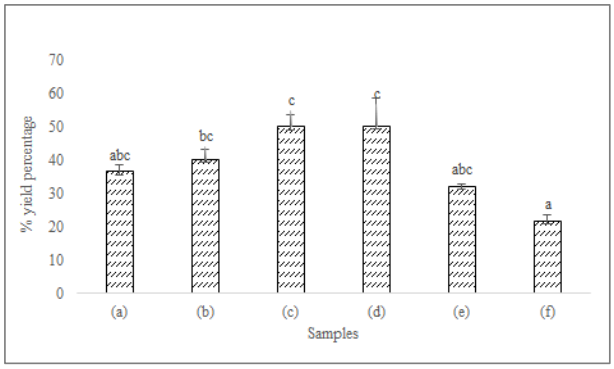

Figure 1. Nano-calcium powder yield percentage from different dried fish bones: a) tilapia, (b) catfish, (c) grouper, (d) snapper, (e) tuna, (f) kingfish mackerel. Different alphabet above the bar indicated significant differences using one-way ANOVA and Duncan's test (p<0.05).

mackerel and tuna bone had the lowest yield during the alkaline extraction process due to the high-fat content of the bone. Saponification occurred between fat and alkali during alkaline extraction, thereby increasing nano-calcium powder yield loss. The highest yield percentage of nano-calcium was derived from grouper and snapper fish bones; those species have large, dense, and heavy bone particles, making yields not easily dissipated during the washing and extraction process. In addition, the fat content in grouper and snapper bone was moderate, thus the alkaline extraction process did not significantly reduce the yield percentage.

According to the results, small fishes (tilapia and catfish) produced a lower percentage of nano-calcium powder than large fishes. Small fish bone had low-weighed particles that easily washed away and dissipated during the alkaline extraction. The yield percentage of nano-calcium powder from starry trigger (*Abalistes stellaris*) fish bone ranged from 7.55 to 12.94% (Husna et al., 2020), lower than our results. Meanwhile, Zainol et al. (2019) could produce a relatively high yield of 68% since the extraction process was only carried out once using 5 M NaOH at 100°C for 1 h. The frequency of the boiling cycle directly affects the quality of nano-calcium powder in terms of its purity. The organic residue would be lower but inversely proportional to the yield percentage of nano-calcium because of the lengthy production process.

Particle Size of Nano-calcium Powder

Nanoparticles are solid particles with a mean diameter of <1 µm or <1,000 µm, consisting of crystalline or amorphous particles (Uhlemann et al., 2021). All nano-calcium produced in this study were less than 1,000 nm in particle size. The particle size of food components are closely related to their bioavailability. Thus, measuring the particle size of the nano-calcium powder is essential. Nano-calcium is smaller than the microscopic size, enabling the calcium to rapidly enter the mineral receptor, and being absorbed by the cells (Sumarto et al., 2021). The particle size of nano-calcium powder was determined using a particle size analyzer (PSA) and the results are shown in Figure 2.

The average particle size of produced nano-calcium powder from the finest to the coarsest was tilapia (87.37 nm), kingfish mackerel (105.2 nm), tuna (150.2 nm), catfish (239.9 nm), snapper (254.7 nm), and grouper (281.4 nm). In this study, the particle size of nano-calcium derived from tilapia fish bone was smaller that reported by Anggraeni (2019), which was 500 nm. The particle size distribution of nano-calcium tuna observed in this research was finer than Prinaldi et al. (2018) and Benjakul et al. (2017). Their results were 259-397 nm and 590-38,780 nm, respectively.

According to PSA preparation, nano-calcium powder was soluble at low pH and agglomerated at neutral or alkaline pH. Nano-calcium should be dissolved in water with a pH of 2 to measure its size. According to Yin et al. (2015), the particle size and the pH of the solution influenced the solubility of calcium. The smaller the particle size means, the more degraded the collagen matrix and the wider the surface area of the particles with the solution, and the higher particle solubility.

Color Measurement

The image of fish, fish bones and nano-calcium powders can be seen in Figure 3. The color measurement results of nano-calcium powder from several fish species can be seen in Table 1.

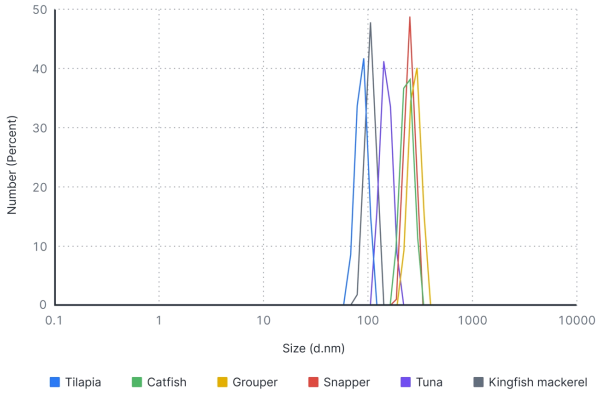

Figure 2. The particle size distribution of the nano-calcium powder from six fish species.

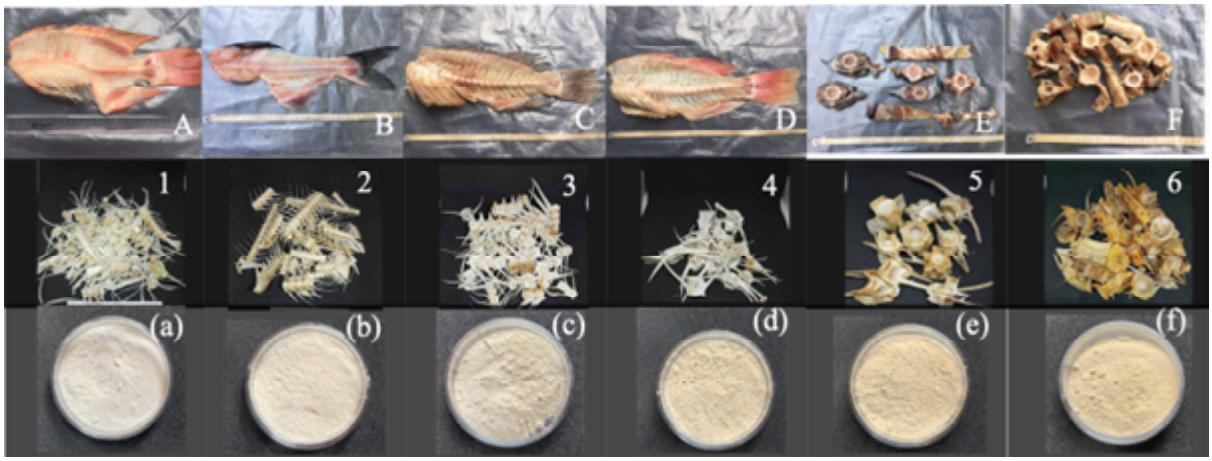

Figure 3. Upper: bone waste from fishery industries: A. tilapia, B. catfish, C. grouper, D. snapper, E. tuna, F. kingfish mackerel; Middle: dried fish bones: 1. tilapia, 2. catfish, 3. grouper, 4. snapper, 5. tuna, 6. kingfish mackerel Lower: nano-calcium powders: (a). tilapia, (b). catfish, (c). grouper, (d). snapper, (e). tuna, (f). kingfish mackerel.

Table 1. Color parameter of different fish bone powders

	Color Parameters	Fish Bone					
		(a)	(b)	(c)	(d)	(e)	(f)
Coarse Fish Bone Powder	*L* *	85.31±0.76^{a}	86.27±2.23^{a}	83.95±3.54^{a}	83.93±2.29^{a}	83.79±0.61^{a}	66.48±3.53^{b}
	a *	0.35±0.42^{a}	0.43±0.42^{a}	1.11±0.87^{a}	1.29±0.52^{a}	1.11±0.19^{a}	5.88±1.61^{b}
	b *	12.57±0.82^{a}	12.30±2.41^{a}	17.32±1.84^{b}	17.73±0.05^{b}	18.64±3.93^{b}	27.65±2.28^{c}
	DE *	81.57±0.33^{a}	82.20±1.96^{a}	80.60±3.15^{a}	80.63±2.24^{a}	80.72±1.32^{a}	66.59±2.45^{b}
Nano-calcium Powder	*L* *	90.64±0.90^{a}	90.53±0.33^{a}	87.53±0.38^{b}	86.33±0.33^{b}	85.55±1.18^{bc}	83.83±3.58^{c}
	a *	-0.29±0.65^{ab}	0.38±0.17^{a}	0.18±0.15^{bc}	0.75±0.07^{d}	0.51±0.19^{cd}	0.95±0.74^{d}
	b *	9.12±0.85^{a}	8.67±0.11^{a}	14.74±0.98^{b}	16.23±1.66^{b}	14.97±0.26^{c}	15.94±1.32^{bc}
	DE *	86.23±0.95^{c}	86.10±0.32^{c}	83.71±0.24^{b}	82.75±0.07^{b}	81.94±1.02^{ab}	83.49±3.31^{a}

Note: Values are expressed as mean ± SD (n = 3). Different superscript alphabets within the same row show significant differences using one-way ANOVA and Duncan's test (p<0.05) (a) tilapia, (b) catfish, (c) grouper, (d) snapper, (e) tuna, (f) kingfish mackerel

The most desirable color of the nano-calcium powder is white or as bright as possible, similar to hydroxyapatite crystal products (Zainol et al., 2019). The bone source's raw material and the organic material left in the fish bone, especially the protein and fat contents, influenced the nano-calcium powder's color, by causing darker shade (Benjakul et al., 2017).

In this study, the freshest raw material was freshwater fish. The fish were brought alive to the factory and directly filleted so that the rigor mortis phase did not occur. Marine fish, harvested from the sea and transported long distances to the plant, were exposed to a rigor mortis phase before being filleted, resulting in a significantly reduced quality compared to freshwater fish. According to Amos (2007), time and temperature tolerance are the most critical elements affecting the quality of fishery products. When a fish dies, its enzymatic activity continues, resulting in proteolytic alterations. Chemical spoiling processes are alterations that occur in the fish's lipid component. When lipids are oxidized to become peroxides, aldehydes, ketones, and lower aliphatic acids, they discolor to a brown or yellow tone. It can be seen that the color of the nano-calcium powder of freshwater fish in this research was much brighter than the color of marine fish's nano-calcium powders. According to the findings of the proximate analysis in Table 2, kingfish mackerel had the highest fat content of all samples, resulting to the darkest nano-calcium powder among other samples.

From brightest to darkest, the sorted nano-calcium powders were tilapia, catfish, snapper, grouper, catfish, tuna, and kingfish mackerel. The color of nano-calcium powders from tilapia, catfish, snapper, grouper, and

Table 2. Chemical composition of different fish bone powders

	Proximate Compositions	Fish Bone					
		(a)	(b)	(c)	(d)	(e)	(f)
Coarse Fish Bone Powder	Moisture (%)	1.66±0.39^{a}	2.16±0.74^{ab}	1.81±0.36^{a}	2.02±0.19^{ab}	2.47±0.33^{b}	1.73±0.23^{a}
	Ash (%)*	56.21±2.91^{a}	58.07±4.49^{a}	64.08±0.4^{b}	64.99±1.88^{b}	64.94±0.15^{b}	56.51±1.15^{a}
	Protein (%)*	24.91±0.13^{c}	23.15±3.42^{c}	24.17±0.35^{c}	23.02±0.28^{c}	18.26±0.70^{b}	15.55±2.18^{a}
	Fat (%)*	10.16±1.31^{d}	8.91±1.88^{c}	6.66±0.24^{b}	7.09±0.64^{b}	5.08±0.33^{a}	21.26±0.56^{e}
	Calcium (%)*	17.59±0.12^{b}	15.77±0.23^{a}	21.68±0.12^{e}	18.95±0.2^{c}	20.67±0.23^{d}	15.47±0.07^{a}
	Phosphorous (%)*	8.63±0.104^{c}	9.00±0.07^{d}	12.42±0.02^{f}	10.08±0.07^{e}	8.01^{b}	7.92±0.02^{a}
	Ca/P mole Ratio	1.58	1.35	1.35	1.45	1.99	1.51
Nano-calcium Powder	Moisture (%)	1.38±0.12^{a}	2.76±1.51^{b}	2.35±0.57^{ab}	1.98±0.38^{ab}	2.28±0.72^{ab}	1.66±0.30^{a}
	Ash (%)*	82.43±3.27^{b}	83.94±0.52^{bc}	86.76±1.77^{d}	85.89±0.36^{cd}	82.78±0.15^{b}	76.15±4.10^{a}
	Protein (%)*	0.83±0.42^{c}	1.01±0.54^{c}	1.08±0.73^{c}	1.00±0.39^{c}	0.67±0.40^{b}	1.18±0.61^{a}
	Fat (%)*	1.69±1.25^{a}	1.00±0.79^{a}	0.81±0.77^{a}	1.17±1.19^{a}	1.68±1.03^{a}	10.12±7.52^{b}
	Calcium (%)*	20.88±0.21^{a}	21.95±0.35^{b}	23.24±0.2^{c}	21.89±0.2^{b}	21.64±0.13^{b}	20.49±0.19^{a}
	Phosphorous (%)*	10.03±0.08^{b}	11.85±0.18^{c}	12.43±0.23^{c}	12.23±0.2^{d}	12.23±0.42^{cd}	9.33±0.16^{a}
	Ca/P mole Ratio	1.61	1.43	1.44	1.38	1.36	1.69

Note: *Dry weight basis. Values are seen as mean ± SD (n = 3). Different superscript alphabets within the same row show significant differences using one-way ANOVA and Duncan's test ($p < 0.05$); Fish bone: (a) tilapia, (b) catfish, (c) grouper, (d) snapper, (e) tuna, (f) kingfish mackerel.

catfish was white. In contrast, tuna nano-calcium powder was beige-white, and kingfish mackerel was the most common brownish-yellow color. The alkaline treatment in this study increased the brightness (L^*) of coarse fish bone powder. Extraction using alkaline treatment was sufficiently effective in removing various organic residues, especially fat content. Boiling coarse fish bone powder in a high-temperature alkaline solution could dissolve most organic materials such as fat and protein, thereby making a brighter color in the product. Prinaldi et al. (2018) extracted tuna bone powder with 1.5 N NaOH 1:3 at 100°C for 2 h in three cycles. The longer the solvent extraction procedure, the brighter the color of the produced nano-calcium, despite the risk of a reduced yield percentage.

At a minimum temperature of 700°C for 2 h, the calcination process is the most effective method to remove all organic residues, thus a brighter product would be produced. The calcination technique's disadvantage is a lower bioavailability than the chemically-based approaches (alkali treatment). The minimum quantity of protein remaining in the alkali-extracted of bio calcium powder boosted its solubility *in vitro* absorption tests, which would aid the absorption process *in vivo* (Benjakul et al., 2017).

Chemical Composition Analysis

The comparison of chemical composition in coarse fish bone and nano-calcium powder can be seen in Table 2. The water content of all types of fish bone meal, both coarse and nano-calcium powder, can be maintained by 1-2%. The increasing ash content in nano-calcium powder indicated that chemical extraction using alkali could reduce protein and fat content. Kingfish mackerel bone was high in fat, thus alkali chemical extraction could only reduce 52.4% fat. The increasing calcium and phosphorus content of coarse powder from the six types of nano-calcium powder was due to the high ash content after alkali extraction. The ideal Ca/P ratio of the calcium hydroxyapatite phase ($Ca_{10}(PO_4)6(OH)_2$) is 1.67 (Benjakul et al., 2017; Yusuf et al., 2019).

The perfect Ca/P ratio of 1.67 was difficult to achieve in calcium powder production because every calcium extraction process would significantly affect the Ca/P ratio (Yusuf et al., 2019). Benjakul et al. (2017) produced bio calcium powder using the alkaline versus the calcination method; the Ca/P ratios were 1.62-1.63, respectively. In comparison, Nam et al. (2019) produced hydroxyapatite with 1.69-1.82 Ca/P ratios from four different fish. Zairin & Phang (2018) reported 1.52-1.7 Ca/P ratios of hydroxyapatite from other calcination conditions. Natural hydroxyapatite such as nano-calcium from fish bone has a non-stoichiometric Ca/P ratio. The natural hydroxyapatite contains a few minerals, such as Na, Zn, Mg, K, Si, and CO_3, making it more comparative to the human bone's chemical composition (Pu'ad et al., 2019).

Table 3. The trace mineral element content of different fish bone powders

Mineral Element Content *(ppm)	Fish Bone					
	(a)	(b)	(c)	(d)	(e)	(f)
Na	34,653.70±147.26^{c}	29,465.11±433.35^{a}	3,5251.19±260.1^{c}	41,661.25±717.95^{e}	31,068.97±557.15^{b}	40,049.02±312.76^{d}
Mg	1,936.40±11.65^{a}	2,218.56±12.96^{c}	2,394.47±18.15^{e}	2,411.33±18.93^{e}	2,025.79±12.72^{b}	2353.23±12.37^{d}
K	380.53±6.00^{d}	276.57±11.85^{b}	284.70±4.6^{b}	285.5±4.8^{b}	137.14±4.92^{a}	355.03±3.13^{c}
Zn	100.88±4.58^{b}	134.82±1.41^{d}	133.54±1.97^{d}	93.36±1.56^{a}	118.97±2.12^{c}	197.96±2.81^{e}
Fe	40.37±12.59^{a}	166.28±21.39^{c}	24.40±6.81^{a}	16.157±0^{a}	50.12±46.67^{b}	92.15±41.26^{b}
Cu	4.61±0.32^{b}	5.08±0.53^{b}	4.47±0.53^{b}	2.72±0.3^{a}	4.51±0^{b}	2.83±5.43^{a}

Note: *Dry weight base. Values are seen as mean ± SD (n = 3). Different superscript alphabets within the same row show significant differences using one-way ANOVA and Duncan's test ($p < 0.05$); Fish bone: (a) tilapia, (b) catfish, (c) grouper, (d) snapper, (e) tuna, (f) kingfish mackerel.

The composition of trace minerals in nano-calcium (besides Ca and P) of the six fish species can be seen in Table 3. Based on these results, sodium was the most abundance mineral in fish bones, around 3-4%. Nano-calcium produced in this study has a ten-fold concentration of sodium and magnesium compared to hydroxyapatite extracted from different fish species using the calcination method (Nam et al., 2019). The trace minerals in the nano-calcium powder benefit natural calcium sources, while factory synthesized calcium sources often lack trace minerals. These trace minerals in the body will aid biochemical activities, particularly bone metabolism (Nam et al., 2019).

Talib & Zailani (2017) compared several chemical solvents to produce the highest percentage of calcium powder. Their research found that boiling the coarse bone powder in NaOH solvent was the most effective way to reduce organic material in the coarse bone powder. Suptijah et al. (2012) and Zainol et al. (2019) soaked coarse bone powder in HCl before alkaline extraction to accelerate protein hydrolysis. HCl could expand the matrix skeleton of fish bone, thus the solvent would be more easily absorbed and eventually release the calcium effectively. Kusumaningrum et al. (2016) found that the boiling frequency of coarse bone powder affected protein and fat contents and increased ash levels, especially calcium and phosphorus in the product.

Both freshwater and seawater fish did not show any significant differential chemical compositions in their bone (Table 2). However, kingfish mackerel bone showed significantly different values among other species. Since kingfish mackerel contains highly saturated and unsaturated fat, hexane may be utilized during the defatting step of kingfish mackerel bone to maximize mineral extraction.

FTIR Analysis

The FTIR spectra of fish bone fine meals from different fish species are shown in Figure 4. The peak spectra of the six fish species did not show any significant difference. The six spectra showed that the most prominent absorption of phosphate occurred in the 1,031 cm^{-1} region. Phosphate absorption band v4 (asymmetric bending vibration) is characterized by a split-shaped absorption band in the 563 and 603 cm^{-1} regions in all spectra, indicating the presence of hydroxyapatite crystals (HAP) (Lekahena et al., 2014). The FTIR spectra of the six nano-calcium products revealed carbonate, amine, hydrocarbon, and hydroxyl groups, indicating that minor organic materials in protein, fat, and water were still present. The similar spectra across all samples occured because the extraction process was the same, so the chemical composition was relatively similar. Previous researchers reported several spectral peaks at 564 cm^{-1} (Yin et al., 2015), 603, 700-725 (Cahyanto et al., 2017), and 1,033 cm^{-1} (Bonadio et al., 2013), indicating a high-intensity of a phosphate group. Peaks at 875 and 1,639 cm^{-1} showed a carbonate band (Boutinguiza et al., 2012). The peak band of 1,453 cm^{-1} suggested the presence of carbonate ion (Yin et al., 2015), while the peak of 1,533 cm^{-1} represented the amide group (Zainol et al., 2019). Peaks bands at 2,852 and 2,922 cm^{-1} indicated the presence of organic material in reasonably high intensity (Boutinguiza et al., 2012). The bands above 3,300 cm^{-1} displayed the water content in the sample with a sufficiently low intensity (Nam et al., 2019).

X-ray Diffraction (XRD) Pattern Analysis

XRD analysis was performed twice on coarse fish bone powder and fish bone nano-calcium powder to evaluate the initial phase of the coarse fish bone meal compared to nano-calcium powder after alkaline extraction. The XRD analysis of all six coarse fish bone powders determined the crystal pattern with phase formed as hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$), as shown in Figure 5(a). The highest intensity around the $2\theta = 31^{o}$ angles of hydroxyapatite crystals was identical for all samples and represented by the presence of peaks

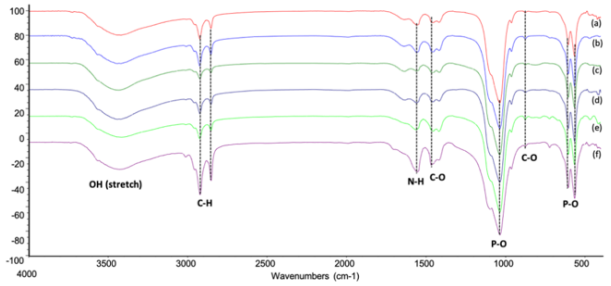

Figure 4. FT-IR spectra of different fish bone fine powder: (a) tilapia, (b) catfish, (c) grouper, (d) snapper, (e) tuna, and (f) mackerel by alkali treatment.

in (211), (112), and (300) hkl planes. The crystal system form was hexagonal. The six coarse fish bone powders showed the predominant phase of hydroxyapatite compounds as previously reported (Yusuf et al., 2019; Zainol et al., 2019). Hydroxyapatite is generally soluble in acids. The hydroxyapatite's solubility depends on tissue compatibility inside the body. Hydroxyapatite reacts actively with organic materials, especially fats and proteins (Yusuf et al., 2019). The XRD results of the tested nano-calcium powders can be seen in Figure 5 (b). The diffraction patterns of coarse fish bone powders were slightly different from those of fish bone nano-calcium powders. Table 4 shows that the XRD pattern analysis of fish bone nano-calcium powders revealed thinner and sharper diffraction peaks, suggesting an increase in the sample's crystallinity.

Table 4 concurrently shows the calculation of crystallinity in each species of coarse fish bone powders. The crystallinity percentage of six coarse fish bone powder species was calculated and found to be modest. The six coarse fish bone powders showed the predominant phase of hydroxyapatite compounds as previously reported (Yusuf et al., (2019); Zainol et al., (2019). There are two types of crystallinity in nano-calcium powders products. Nano-calcium from tilapia bone, catfish, and snapper bone have high crystallinity

Table 4. Compound composition of coarse fish bone and nano-calcium powders

Fish Species		Compound		Crystallinity (%)
		Hydroxyapatite/ HA (%)		
Coarse Fishbone Powder	Tilapia	100		51.353
	Catfish	100		66.134
	Grouper	100		62.494
	Snapper	100		63.369
	Tuna	100		55.297
	Kingfish Mackerel	100		62.962
		Hydroxyapatite/HA (%)	**Halite/ NaCl (%)**	**Crystallinity (%)**
Nano-calcium Powder	Tilapia	91.444	8.555	70.159
	Catfish	91.201	7.799	72.231
	Grouper	95.457	4.543	69.812
	Snapper	90.476	9.524	71.240
	Tuna	93.072	6.928	64.332
	Kingfish Mackerel	92.777	7.223	64.929

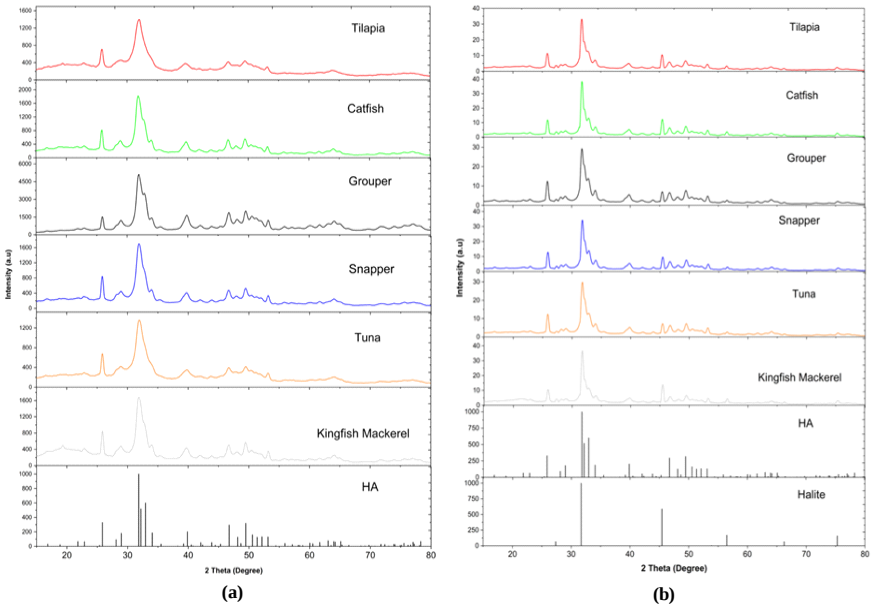

Figure 5. (a) XRD diffractogram of six coarse fish bone powders (HA: Hydroxyapatite) (b) XRD diffractogram of fish bone nano-calcium powders (HA: Hydroxyapatite).

(>70%). Whereas the grouper, tuna, and kingfish mackerel have intermediate crystallinity (30-70%). A crystallinity of 60-90% is required for biological applications of hydroxyapatite (Aminatun, 2015). As a result, these fish bone nano-calcium powder products may also be used in biomedical applications.

Further phase examination of the samples' by XRD diffraction pattern revealed that hydroxyapatite was the dominating phase, similar to the crystalline phase in fish bone coarse powders. The dominating phase demonstrates that the alkaline extraction method did not alter the crystalline phase but increasead the crystalline degree. The presence of the CO_3 group in the FTIR analysis revealed that the carbonate group was present in the sample in an amorphous state, thus undetectable by the XRD instrument.

Another feature that separates the XRD pattern of the nano-calcium powder from the coarse is a halite phase in the XRD patternin this method. Halite/NaCl compounds were found in trace amounts because, HCl was added after alkaline extraction until the sample reached a neutral pH. An acid solution added to an alkaline solution will neutralize the solution and produce salt. Another method for neutralizing the alkaline solution after the alkaline hydrolysis procedure is to rinse the sample with water repeatedly. However, this process is time-consuming and takes a large amount of water.

Scanning Electron Microscopy with Energy-Dispersive X-Ray Spectroscopy Image Analysis

SEM-EDX images of the nano-calcium powder from the samples are shown in Figure 6. The scanning electron microscopy scans revealed that nano-calcium powder of all fish species tended to agglomerate in the dry state, with an asymmetrical shape and a size of 100-1,000 nm. The outcomes of particle size analysis performed using PSA were varied from those obtained using SEM. The PSA tool's premise of operation is to determine the size of particles scattered in a liquid. The measured nano-calcium powder was dissolved in a solution and adjusted at a pH of 2 to charge its particles and produce electrostatic stability, which prevented the particles from colliding and even clumping together, allowing the PSA instrument to determine the size of each particle (Al-Gebory & Mengüç, 2018). In contrast, the SEM measurement represents the actual condition of the powder particles in a dried neutral pH state without any charge between them that inhibits the particles from adhering to one another, allowing the particles to agglomerate. The element profile obtained from the SEM-EDX analysis revealed that, in addition to Ca and P elements, the tested samples contained considerable levels of O and C elements and other natural trace elements such as Na, Cl, Mg, Fe, and Cu, and Zn.

Based on the calculation of the elemental composition on the surface of the nanoparticles, it was found that the Ca content ranged between 21.51% mass in snapper and 34.37% mass in grouper. Phosphorus content varied between 10.73% mass in kingfish mackerel and 15.99% in grouper. Tilapia, catfish, grouper, snapper, tuna and kingfish mackerel showed a Ca/P ratio of 1.49; 1.42; 1.66; 1.41; 1.43 and 1.57, respectively. The Ca/P mole ratio calculated in SEM-EDX was different from the stoichiometric value of hydroxyapatite, 1.67. Yusuf et al. (2019) called this calcium deficient hydroxyapatite (CDHA). As with the proximate test, the nano-calcium obtained from grouper showed the highest calcium content than those of the other samples. The Ca/P mole ratio of grouper nano-calcium was the nearest to the stoichiometric value of hydroxyapatite, 1.66.

The high ratio of carbon (C) and oxygen (O) components in the SEM-EDX test was also validated by FTIR analysis. In addition to the phosphate groups, these fish bone nano-calcium powders include a variety of organic substances, including lipids and proteins, which were also proven in the proximate results. Natural bone contains natural organic components composed of protein collagen fibrils in collagen type 1, which are bound to calcium phosphate in hydroxyapatite (Mescher, 2016). The inclusion of protein in fish bone calcium powder enhanced calcium absorption compared to pure synthetic calcium that contains no protein, according to Jung et al. (2006) and Suntornsaratoon et al. (2018) findings.

Conclusion

Each sample fish bones had a different chemical composition, physiological characteristics, and Ca/P ratio but had almost identical FTIR spectra and XRD diffraction patterns. The nano-calcium powders derived from the fish bones of six species were similar and contained PO, CO, CH, NH, and OH groups. The presence of organic compounds such as fat and protein affected the color of fish bone nano-calcium powder. The higher the protein or fat content in the nano-calcium powder, the darker the color. The XRD spectra pattern showed that the two main components of the fish bone nano-calcium powder were >90% hydroxyapatite, and the rest was halite. Six types of fish bone could be employed as raw material for nano-

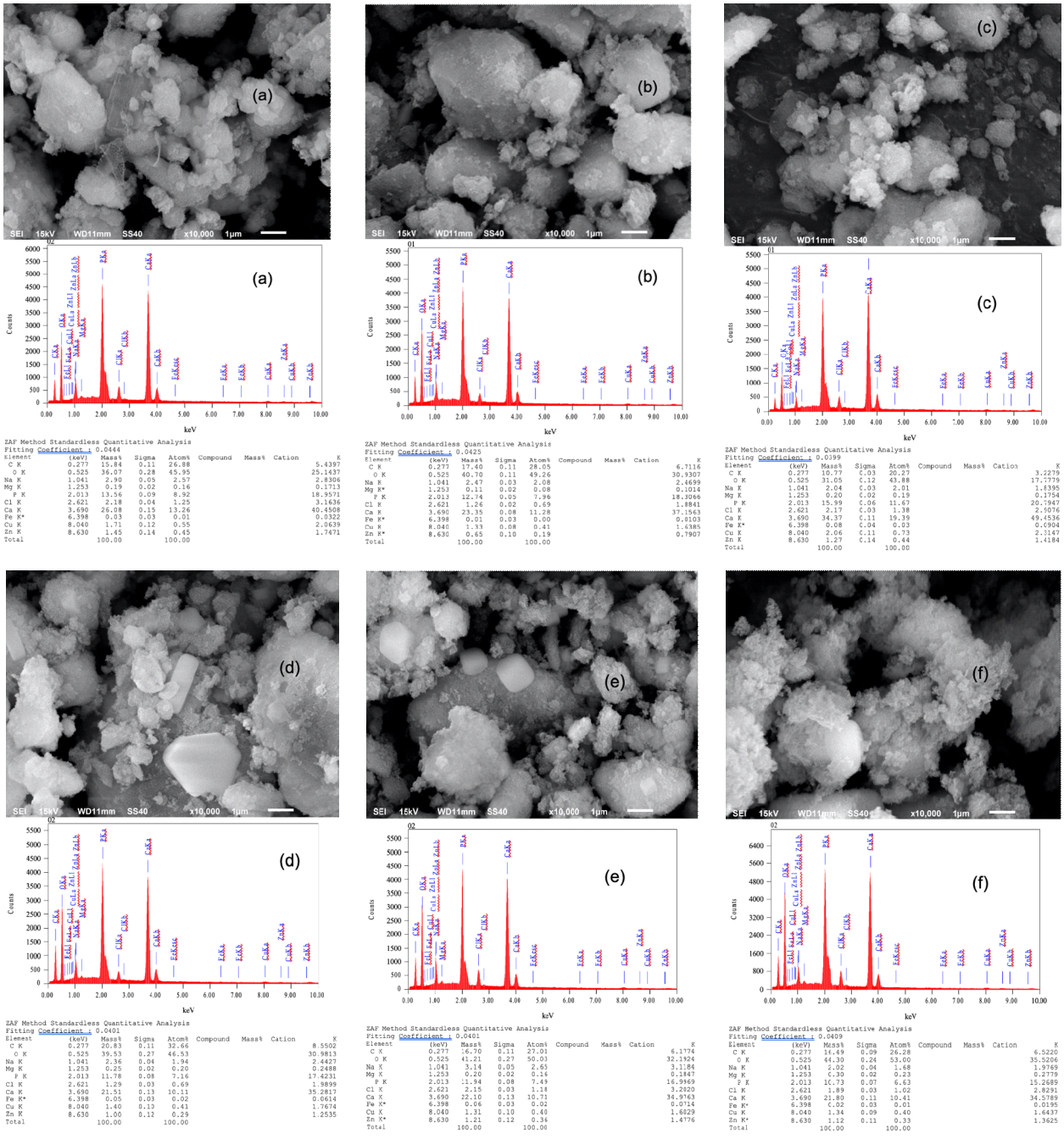

Figure 6. The appearance of nano-calcium powder particles from six different fish bones and their elemental compositions were determined by SEM-EDX: (a) tilapia, (b) catfish, (c) grouper, (d) snapper, (e) tuna, (f) kingfish mackerel.

sized calcium with 87.37-281.4 nm size ranges. However, the best choice for further study was grouper bone, with the highest calcium content and the highest nano-calcium yield percentage.

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Supplementary Material

Supplementary material is not available for this article.

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