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RESEARCH ARTICLE
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In Situ Bioavailability of Nano-calcium from Red Snapper (*Lutjanus malabaricus*) Bone Extract with Varying Extraction Duration

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ABSTRACT

Calcium is one of the essential macro minerals the body needs, and deficiencies in calcium can cause bone health problems. One of the abundant sources of calcium is red snapper fish bone waste; in addition, optimal calcium absorption occurs when calcium is in small particle sizes or nanoparticle form. This study obtained red snapper fish bones from PT Kelola Laut Nusantara, Pati. Initially, the fish bones were washed thoroughly with running water, boiled to remove attached meat, dried at 50°C for 6 hours, and reduced in size with a hammer mill. After extracting snapper fish bone powder in the second stage (60, 90, 120min), a sample: NaOH ratio of 1:4 was used to extract nano-sized bones. The next stage, nano-calcium with the smallest particle size was then tested for bioavailability in situ using mice, and compared with fish bone meal. The novelty of this study was to find the effect of extraction time on particle size, yield, water content, and nanocalcium nano-calcium produced. In addition, it can also determine the efficiency of nano-calcium nanocalcium and characterization of the use of base solvent concentration and selected extraction time variations based on particle size in the in-situ bioavailability process using mice as experimental animals. This study concludes that the best nano-calcium is nano-calcium with an extraction time of 90min, with a yield of 7.80%, particle size of 440.41nm, water content of 2.63%, ash content of 87.08%, protein content of 0.82%, fat content of 1.65%, calcium content of 21.84%, and phosphorus content of 12.76%. Total calcium absorption is 55.29%, with in situ bioavailability with experimental animals of 2.07%, and its morphology is uniformly consistent.

Keywords: Fish Bones; Calcium; Nano Calcium; Bioavailability; In Situ.

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INTRODUCTION

Nutritional issues are closely linked to food security, knowledge aspects, and the behaviour of not adopting a healthy lifestyle (Anggraeni et al., 2024). The deficiency of micro and macronutrients is a primary nutritional challenge in Indonesia. Macro-nutrient deficiency constitutes a health disorder resulting from the imbalance between needs, energy intake, and protein. Micro-nutrient deficiency occurs due to the inadequate intake of vitamins and minerals, including vitamin A, iron, and iodine (Darmanto et al., 2022). The lack of calcium, a crucial mineral, significantly impacts the required calcium intake, especially for premenstrual calcium needs in adolescents and pregnant women, and stunting prevention in children. Insufficient calcium intake leads to a 3.41 times higher likelihood of experiencing premenstrual syndrome compared to female students with adequate calcium intake (Muijah & Safitri, 2019). Fetal health disorders may result from nutritional deficiencies such as calcium and iron in mothers during pregnancy and lactation, potentially causing stunting in children (Media & Elfemi, 2021).

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Calcium is one of the essential micro-minerals that play a crucial role in the body (Upadhyay, 2017; Ismail, 2022). Furthermore, it is an essential macromineral required by the body. Calcium in bodily fluids plays a role in muscle contraction and relaxation, nerve impulse transmission, blood clotting, regulation of hormone secretion, and acts as a cofactor for several enzymes. Calcium also serves as a structural strengthener for bones and acts as a calcium reservoir, drawing reserves from bones when blood calcium levels decrease (Noprisanti et al., 2018). Inadequate calcium intake can lead to bone health disorders, with osteoporosis being one of the consequences of calcium deficiency. Based on the 2009 Department of Health data, the Indonesian population's calcium intake ranges from 270-300mg/day for adults and 318-380mg/day for pregnant women. The recommended calcium intake according to international standards is 1,000-1,200mg/day for adults and 1,200-1,500mg/day for pregnant women. This information indicates an imbalance between the calcium intake and requirements of the Indonesian population, making them susceptible to bone-related diseases (Husna et al., 2020).

The primary source of calcium commonly consumed today is dairy products and their derivatives. A drawback of dairy products and their derivatives is that individuals with lactose intolerance cannot consume them due to their tendency to cause diarrhea. Therefore, the need for alternative calcium sources everyone can consume is essential to address daily calcium intake. One potential alternative calcium source is the waste from the bones of red snapper fish (Anggraeni & Handayani, 2022). The utilization of fish bone waste is underutilized, making the extraction of nano calcium from red snapper fish bones highly promising as an alternative calcium source. Red snapper fish hold significant potential in supporting food needs (Jampilek et al., 2019). This is attributed to the continuous increase in red snapper fish production, reaching 4.32% and totaling 312,945 tons in 2021, with predictions indicating a continued rise. The production of red snapper fish fillets and surimi produce waste, including heads, tails, skin, and bones. These red snapper fish waste products currently need to be utilized more. However, the waste bones from red snapper fish have the potential to become a calcium source that can be transformed into nano calcium (Anggraeni & Handayani, 2022).

Nano-calcium is calcium with a tiny size (1-1000nm) produced through nanotechnology. The nano-sized particles are expected to enhance the optimal bioavailability of calcium in the body, originating from fish bones. Bioavailability is the absorption, transport, and utilization of nutrients. Bioavailability can also be defined as the amount of calcium absorbed by the body, equal to the calcium intake minus the amount excreted through the intestines and faeces (Anggraeni et al., 2016). The following sentence is incomplete, and there is missing information about why the transition to nanoparticles needs to be explained. One of the methods used to obtain nano calcium is extraction using NaOH, which aims to eliminate proteins and fats in the bones, thereby extending the shelf life and increasing the whiteness of bone meal.

This process also causes calcium to precipitate in the bones (Kusumaningrum et al., 2016). The determinant of the size of nanoparticle nano calcium derived from red snapper fish bones includes the extraction duration, which can be a significant factor. The extraction time affects the contact between the solvent and the material in the breakdown process, resulting in nano calcium being used as nanoparticles. Therefore, this research aims to determine the optimal extraction time for producing nano calcium from red snapper fish bones based on water content, particle size, yield, and bioavailability parameters.

MATERIALS & METHODS

This research was conducted from July to December 2023 at the Food Technology Laboratory, National Karangturi University, Indonesia, and the Research and Development Laboratory, Gadjah Mada University, Indonesia. Red snapper fish bones were obtained from PT Kelola Laut Nusantara, Pati, Indonesia.

Preparation and Production of Red Snapper Fish Bone Powder

The preparation of red snapper fish bones refers to the study conducted by Anggraeni et al. (2016). Red snapper fish bones were obtained from PT Kelola Laut Nusantara, Pati. The fish bones were thoroughly washed with running water. The cleaned bones underwent a boiling process to remove any remaining attached flesh. The boiled bones were then dried at 50°C for 6 hours. The dried bones were subsequently reduced in size using a hammer mill. The purpose of repeating the preparation process is to clean the fish bones and remove the remaining fat attached to the snapper bones to facilitate the nano-calcium extraction process.

Extraction of Red Snapper Fish Bone Nano Calcium

The nano-calcium extraction method was based on the method of Anggraeni et al. (2016). Coarse powder of red snapper fish bones was extracted using a 1N NaOH (Merck) solution with a sample solvent ratio of 1:4 at a temperature of 100°C for A (30), B (60), and C (90) minutes. The use of NaOH solution aimed to soften the red snapper fish bones. The extraction results were filtered using filter paper of Whatman No. 1 and neutralized to achieve a neutral pH. The sample solution was then dried at 50°C for 12 hours until the water content reached <8%, then milled using a ball mill and dried again at 50°C for 3 hours and sieved with a 200-mesh sieve.

Particle Size Analysis

A Particle Size Analyzer (PSA) was employed to determine the size of a sample. Particles were dispersed in a liquid medium, and the measured particle size refers to the size of individual particles. The particle size measurement using the Vasco-PSA brand, Arago DL 135 refractometer, Cordouan, utilized the Low Angle Laser Light Scattering (LALLS) method. The measurement involved applying light scattering, which is highly useful in conjunction with size-exclusion chromatography

techniques. The SEC column eluent passes through an index bias detector (which provides concentration size over time) and a laser scattering cell. The scattering intensity was measured as a function of time at a slight angle to the laser beam. Data from low-angle light scattering can be analyzed by assuming that low-angle data was equivalent to zero-angle scattering, which can be used for particle sizes ranging from 0.1 to 3,000µm with a He-Ne gas laser at an intensity of (λ=0.63µm).

Proximate Analysis and Minerals Analysis

Proximate analysis determined moisture content, ash, crude protein, and fat following the method described by Wijayanti et al. (2021b). Calcium analysis employs Atomic Absorption Spectrometry (AAS) (Perkin 100 type flame) with a wet digestion method at λ=422nm (Anggraeni et al., 2020), while phosphorus analysis utilizes UV-visible spectrophotometry at λ=660nm (Yang et al., 2023).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) samples were also observed using scanning electron microscopy (JSM-6510 LA, Jeol, Tokyo, Japan), following the Benjakul and Karnjanapratum (2018) method with a slight modification. After the specimen was coated with gold, a secondary electron 15 kV accelerating voltage was used in observation.

Calcium Solubility

The testing of calcium solubility refers to Wijayanti et al. (2021a). This test involved adding 0.5g of the flour sample to 40mL of ion-free distilled water. Subsequently, the sample solution was conditioned to pH levels of 2, 3, 4, 5, 6, 7, 8, 9, and 10 using 1N HCl and 1N NaOH. The sample was stirred for 20min at room temperature with a speed of 500rpm. The sample was then incubated in a water bath at 37°C for 2 hours. Afterwards, the sample was centrifuged for 10min at 1000rpm, and then the filtered sample was filtered using Whatman 42 filter paper. The calcium content in the filtrate was measured using Atomic Absorption Spectrometry (AAS) with a wavelength of 420nm. The calcium solubility was then calculated using the formula.

$$\text{Solubility (\%)} = \frac{\text{Dissolved calcium}}{\text{Total calcium level}} \times 100\%$$

In Vitro Digestion

Before conducting the in situ testing, the samples must undergo prior digestion. This testing refers to Abo El-Maaty et al. (2021). The digestion process involved adding 80mL of distilled water to each sample, adjusting the pH to 2 using 6M HCl, adding water to reach a total volume of 100mL, and supplementing with 3mL of pepsin solution (16 grams of pepsin in 100mL of 0.1 M HCl). The solution was incubated in a shaking water bath at 37ºC for 2 hours. The resulting digestion was centrifuged at 3500 rpm for 20 minutes. A 10mL portion of the digestion product (supernatant) was taken for calcium content testing using AAS at a wavelength of 420nm. In contrast, the remaining supernatant was stored in a refrigerator for the in situ testing the following day.

In Situ Perfusion

The protocol described and executed in the present study was with the permission of the Bioethics Committee for Medical / Health Research of The Faculty of Medicine, Sultan Agung Islamic University Semarang (Protocol No. 193/V/2023/Komisi Bioetik). The in situ bioavailability testing of nano calcium will be conducted based on the method outlined in the study by Liu et al. (2023). Wister rats (150–180g) were housed in groups of 30 animals in cages and maintained under standard conditions (12-hour light/dark cycle, 24ºC, and 35–60% humidity), with free access to a pelleted diet and purified drinking water. Subsequently, the rats were intraperitoneally anaesthetized with a dose of 100mg/kg body weight of ketamine. Once anaesthetized (approximately 45-50min), the rats were placed on a surgical table with their four legs tied. The abdominal surgery was performed longitudinally. A cannula was inserted and tied from the duodenum's upper part to the ileum's lower part. Then, the interior of the intestinal tract was flushed by flowing 0.9% NaCl until all feces were expelled. Afterwards, the injection process of the sample solution was carried out at a speed of 0.6-0.65mL/min. Samples exiting the intestines were collected every 15 minutes, and the perfusion lasts 120min. Calcium levels were determined on samples before and after the perfusion process.

$$\text{Amount of Ca absorbed} = \frac{\text{Ca levels (before} - \text{after) perfusion}}{\text{Ca levels before perfusion}} \times 100\%$$

Statistical Analysis

The experimental design employed a completely randomized design (CRD) with a single factor, namely the duration of nano-calcium milling. The data were analyzed utilizing the one-way Analysis of Variance (ANOVA) method, with significant data ($P<0.05$) being identified using the post hoc Tukey's Honest Significant Difference (HSD) test. Kruskal-Wallis analysis was performed on non-parametric data. The data was processed using the SPSS version 16 program.

RESULTS & DISCUSSION

The nano-calcium yield was the percentage of the primary raw material (fish bones) processed into the final product (nano-calcium). The variation in extraction time significantly impacted the nano calcium yield from red snapper fish bones. The longer the extraction time, the higher the nano-calcium yield produced. The water content of nano-calcium from red snapper fish bones at different extraction times indicated that the highest yield was observed in the 90-minute extraction treatment, which is 7.80±0.24%, while the lowest yield was in the 30-minute extraction treatment, namely 4.78±0.08%. The research results indicated that the nano-calcium yield varied significantly for each treatment. The yield in this study was lower than the nano calcium from red snapper fish bone extraction reported by Han et al. (2023) at 5.91% for base extraction and 4.41% for acid extraction. The duration of nano-calcium extraction from fish bones can influence the yield or quantity of nano-calcium obtained from the raw material. The relationship between extraction time and

yield can be influenced by several factors, including the extraction method used, temperature, pressure, solvent used, and raw material characteristics.

As the extraction time increased, more time was provided for the desired substance (in this case, nano calcium) to be released from the raw material (fish bones) and dissolved in the solvent or extraction medium. However, there are practical limitations related to extraction time. Longer extraction times require more energy and resources, and there is a point where the quantity that can be extracted reaches a saturation point. Yin and Park (2015) reported that the neutralization process can reduce nano-calcium yield due to losses during the process. Chandran et al. (2019) reported that longer extraction times result in higher yields due to increased contact opportunities between the raw material and the solvent. However, excessively long extraction times can lead to lower yields as the solution reaches saturation.

The research results showed that the particle size of nano-calcium from red snapper fish bones was lowest in the 90min treatment at 450.4±1.23nm and highest in the 30min treatment at 793.4±1.04nm (Table 1). In this study, nano-calcium fell into the nano category as its particle size was below 1,000nm. This aligns with Mehmood et al. (2022) statement that nanoparticle sizes range between 1-1,000nm. Arooj et al. (2014) reported that nanoparticles have 200-400nm particle sizes. Longer extraction times result in smaller particle sizes due to particle size reduction during the extraction process. Smaller particle sizes lead to higher bioavailability in the body (Anggraeni et al., 2016), suggesting that the 90min extraction in this study can produce nano-calcium from tilapia fish bones with higher bioavailability.

Table 1: Chemical and physical characteristics of nano-calcium

Parameters	Extraction Time		
	A	B	C
	30 minutes	60 minutes	90 minutes
Yield (%)	4.78±0.08^{a}	5.00±0.67^{ab}	7.80±0.24^{b}
Particle size (nm)	793.4±1.04^{c}	578.6±0.12^{b}	450.4±1.23^{a}
Moisture content (%)	2.67±0.09^{a}	2.66±0.76^{a}	2.63±0.65^{a}
Ash content (%)	87.03±0.76^{a}	86.08±0.56^{a}	87.08±0.27^{b}
Protein content (%)	0.76±1.13^{a}	0.78±1.78^{a}	0.82±0.67^{b}
Fat content (%)	1.70±1.15^{a}	1.68±1.04^{a}	1.65±0.07^{a}
Calcium content (%)	21.76±0.56^{a}	21.80±0.86^{a}	21.84±0.06^{a}
Phosphorus content (%)	12.45±0.08^{a}	12.67±0.46^{a}	12.76±0.05^{a}

Values (mean+SD) bearing different alphabets in a row differ significantly ($P<0.05$).

Particle size analysis proves that the calcium extraction method using precipitation produces nano-sized calcium. Extraction with 1N NaOH can dissolve minerals in the raw material until mineral nucleation occurs, forming nano-sized particles. The nano calcium in this study had larger particle sizes than the study by Benjakul et al. (2017), which reported a value of 20.29nm for tuna fish bone calcium. This difference may be attributed to different types of fish bones and milling speeds after the extraction process.

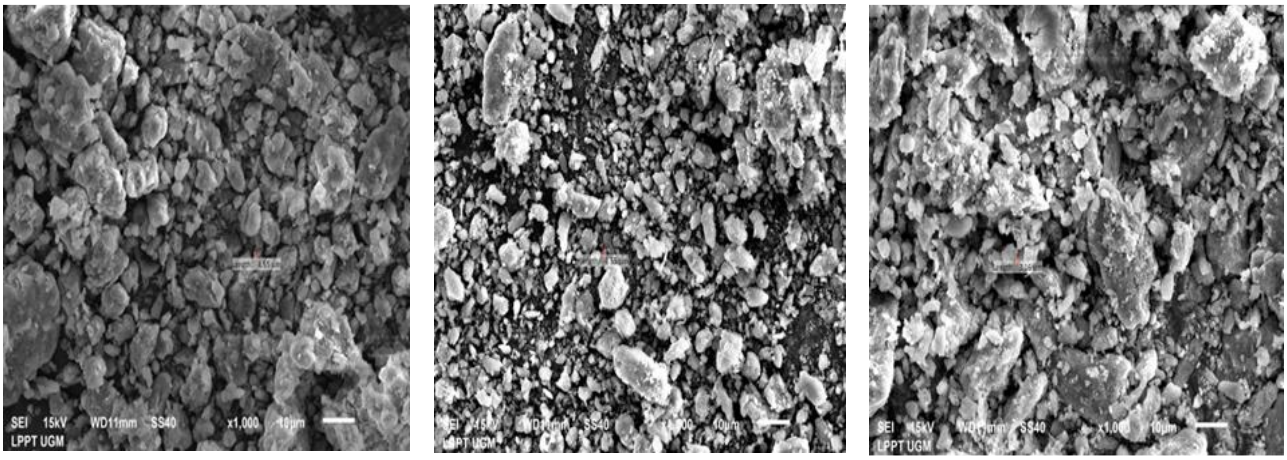
The variation in extraction time did not significantly affect the water content of nano-calcium from red snapper fish bones. Water content at different extraction times showed the highest level in the 30-minute extraction treatment at 2.67±0.09% and the lowest in the 90-minute extraction treatment at 2.63±0.65%. Lekahena et al. (2014) reported a water content of 4.67% for nano-calcium from tilapia fish bones with base extraction. Benjakul and Karnjanapratum (2018) reported a water content of 7.35% for calcium from fish bones. Differences in water content can be influenced by drying time and temperature after nano-calcium precipitation. Other factors affecting water content include the grinding process using a ball mill; the nano-calcium from red snapper fish bones was also dried in an oven at 50°C due to remaining moisture in the sample, causing clumping of nano-calcium on iron balls, and the ball mill wall, disrupting the grinding process. Therefore, the milling process also reduced the remaining water content during drying. Water content also decreased during sieving as air evaporated the remaining water. The evaporation process occurred faster due to the tiny particle size of nano-calcium, which was 200 mesh or equivalent to 75µm. One of the factors influencing reaction rate was surface area.

The research results indicated that the ash content of nano-calcium did not significantly differ between treatments. The highest ash content was in the 90min treatment at 87.08±0.27%, and the lowest was in the 30min treatment at 87.03±0.76%. The high ash content was due to the mineral content in the fishbone meal. The ash content of red snapper fish bone meal in the study by Wijayanti et al. (2021a) was 74.37%, much higher than in this study. This is attributed to the higher mineral content in their research. Aenglong et al. (2023) reported that the amount of minerals present influences the ash content in food materials.

The highest calcium mineral content was found in nano-calcium with a 90-minute extraction treatment at 21.84±0.06%, while the phosphorus content was 12.76±0.05%. Longer extraction times resulted in higher mineral content due to compound breakdown, enhancing the purity of nano-calcium. The calcium content in this study has met the Indonesian National Standard (SNI 01-3158-1992) for fish bone meal, with Quality I set at 30% wb and Quality II at 20% wb. The calcium content of red snapper fish bone meal in this study fell into Quality I. Nemati et al. (2017) stated that different fish types affect the calcium content in fish bone meal. The boiling process also affects calcium value, producing calcium.

The research results indicated that the fat content did not significantly differ between treatments. The highest fat content was in the 30-minute treatment at 1.70±1.15%, which fell into the low category. Husna et al. (2020) stated that low-fat content makes the quality relatively stable and less prone to spoilage. High fat content can cause the meal to taste fishy and result in oxidative rancidity due to fat oxidation, making the fish bone meal taste rancid and appear brownish.

The results of the morphology analysis of nano-calcium particles can be seen in Fig. 1. The analysis indicated that a longer extraction time will result in nano-calcium with a more uniform size. Based on the SEM test results, nano-calcium appeared very distinct, consisting of homogeneously shaped and sized particles with crystal forms resembling vaterite and some aragonite. Calcium crystals have different phases, namely calcite, aragonite, and vaterite. Calcite has a solid cubic shape, aragonite



(a) (b) (c)

Fig. 1: Morphology of red snapper fish bone nano calcium. (a)30 minutes; (b) 60 minutes; (c) 90 minutes.

resembles clustered needles, and vaterite resembles a flower (Li et al., 2023). Stepankova et al. (2023) stated that calcite has rhombohedral, scalenohedral cubic, and prismatic crystal forms. Aragonite forms clusters and discrete needle-like structures, while vaterite is spherical. The morphology of the nano-calcium produced differs from the research conducted by Khoerunnisa (2011), where nano-calcium from local clam shells had a more orderly needle-like structure resembling aragonite. Hasan et al. (2021) reported that nano-calcium from crab shells had a flower-like morphology or vaterite crystal type. These differences are attributed to variations in the nano-calcium production method. According to EL-Sokkary et al. (2012), calcite crystals strongly adhere to surfaces (most stable), aragonite easily detaches from walls, while vaterite is unstable and can transform into calcite mediated by the solvent.

The physicochemical results of red snapper bone nano-calcium showed that sample C, with an extraction time of 90min, was the best treatment. This was then carried out in situ bioavailability testing. The calcium solubility test uses pH levels ranging from 2 to 10. The calcium sources used were fish bone flour and the best-treated nano-calcium. The percentage of calcium solubility in the utilized calcium sources increased as the pH value decreased. The calcium source used exhibited the highest solubility at pH 2. This is because acid facilitates calcium release from the calcium source, turning it into Ca2+ ions more easily absorbed in the intestines (Yang et al., 2023). The acidity level (pH) influences calcium solubility and absorption. According to (Liu et al., 2023), the low pH of the stomach can dissolve calcium salts, which are then absorbed into the small intestine. The higher the calcium solubility, the greater the chance the body absorbs calcium. The calcium solubility graph at different pH levels can be seen in Fig. 2.

In situ bioavailability testing on the utilized calcium source was conducted to determine the calcium the body can utilize, simulated using the small intestine of rats. Before the in situ testing, the calcium source underwent digestion with pepsin enzymes to break down proteins, allowing calcium associated with proteins to be broken down into Ca2+ ions.

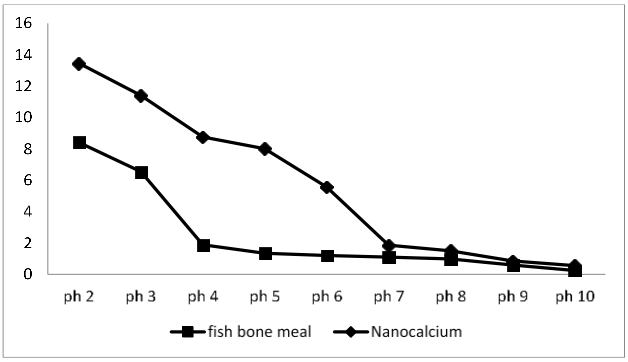


Fig. 2: Calcium solubility graph at different pH levels

The results of calcium solubility before and after digestion can be seen in Table 2. Based on the analysis results, a significant difference was observed between the solubility of fish bone flour and the selected nano-calcium with the smallest size, namely C. The dissolved calcium after digestion had a higher percentage of calcium content than calcium solubility before digestion. This is attributed to the digestion process using pepsin enzymes. The enzyme functions to break down proteins into peptides. Proteins entering the digestive system usually exist in complex molecular arrangements, so calcium bound to proteins must be broken down into Ca2+ ions. This can increase the calcium content in the dissolved calcium after digestion, while the calcium solubility before digestion (pH 2) has a low percentage. This is because the calcium source used is dissolved in an acidic environment (pH 2) only without digestion, so calcium bound to proteins cannot be broken down.

Table 2: Calcium solubility before digestion and dissolved calcium after digestion

Sample	Solubility of calcium before digestion (%)	Calcium solubility after digestion (%)	Difference (%)
Fish bone meal	9.11±0.14	10.76±1.08	1.65±0.02^{a}
Nano calcium	14.65±0.56	18.10±0.45	3.45±0.07^{b}

Values (mean+SD) bearing different alphabets in a column differ significantly (P<0.05).

The average calcium absorption can be seen in Fig. 3. The graph shows fluctuations in calcium absorption. The calcium absorption increased from 25 to 50. According to

(Li et al., 2023), higher calcium needs and lower calcium availability in the body will result in efficient calcium absorption, increasing calcium absorption in the small intestine. When calcium absorption from the used calcium source decreases, it is due to the body's adjustment to the blood calcium needs. If the blood calcium level rises, the calcium absorbed by the small intestine decreases. In contrast, if the blood calcium level is still low, the calcium absorbed by the small intestine will increase.

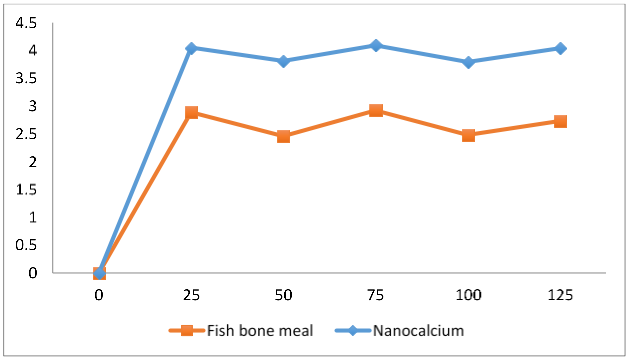


Fig. 3: Calcium Absorption time graph.

The total calcium absorption for each calcium source is presented in Table 3. The difference in calcium sources indicates a significantly different total calcium absorption, namely 37.01% for fish bone flour and 51.22% for nano-calcium. The highest percentage was found in nano-calcium, which is attributed to the higher solubility of calcium before digestion in nano-calcium compared to fish bone flour. Minerals will be absorbed when dissolved (Xiang et al., 2022). Additionally, particle size also influences calcium absorption.

Table 3: Total absorption and bioavailability of calcium

Sample	Sample calcium (mgCa/L)	Digested calcium (mgCa/L)	Calcium is absorbed (mgCa/L)	Total calcium absorption (%)	Calcium bioavailability (%)
Fish bone meal	7755.57	239.93±3.69	90.33±2.78	37.01±6.06	1.08
Nano calcium	18392.30	337.21±18.31	198.16±7.51	55.29±13.44	2.07

Table 4: Total absorption and bioavailability of calcium

Sample	Sample calcium (mgCa/L)	Digested calcium (mgCa/L)	Calcium is absorbed (mgCa/L)	Total calcium absorption (%)	Calcium bioavailability (%)
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Nano calcium	18392.30	337.21±18.31	198.16±7.51	55.29±13.44	2.07

Conclusion

Differences in extraction time significantly affected yield, protein content, and particle size but did not impact water content, fat, calcium, and phosphorus parameters of red snapper fish bone nano-calcium. The 90-minute extraction treatment was the most effective in producing nano calcium with a yield of 7.80%, particle size of 440.41nm, water content of 2.63%, ash content of 87.08%, protein content of 0.82%, fat content of 1.65%, calcium content of 21.84%, phosphorus content of 12.76%; total calcium absorption of 55.29% and bioavailability of 2.07%, along with a uniform morphological appearance.

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