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Research Article

Effect of Different Papain Concentrations on Yield and Quality of Tuna Eye Oil

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Abstract

Docosahexaenoic acid (DHA), a crucial omega-3 fatty acid, plays a vital role in neurodevelopment and cardiovascular health. Indonesia relies heavily on imported fish oil, despite its significant potential in underutilized by-products like tuna eyes. This study investigates the optimization of papain enzyme concentration for enzymatic extraction of DHA-rich oil from tuna eyes, aiming to enhance yield and maintain quality. Using 1% papain at 55°C for 1 hour, the optimized process achieved an oil yield of 8.59 ± 0.69%, six times higher than cold extraction without enzymes. The extracted oil exhibited high oxidative stability with low Index of Atherogenicity (IA: 0.38–0.40) and Index of Thrombogenicity (IT: 0.20–0.21), while DHA content remained well-preserved at 27.82%. This method also demonstrated the capability to maintain oil quality even after prolonged storage during the COVID-19 pandemic. Compared to conventional methods, enzymatic extraction provides a sustainable and efficient alternative by reducing chemical solvent use, minimizing environmental impact, and maximizing the utilization of fishery by-products. These findings offer a scalable solution for producing high-value omega-3 oils, contributing to global dietary needs and promoting sustainability in the fishery industry.

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1. Introduction

Docosahexaenoic acid (DHA) is a critical omega-3 fatty acid comprising approximately 15% of the total fatty acids in the human frontal cortex, a region vital for signal transduction, synaptic transmission, and regulation of neurotransmitter pathways (Kuratko *et al*., 2013). As an essential component for fetal brain and retinal development, DHA plays an irreplaceable role in neurodevelopment and cognitive function. Its deficiency, particularly in fetuses, neonates, and young children, can result in neurodevelopmental delays and cognitive impairments (Coletta *et al*., 2010). To address these risks, the European Food Safety Authority (EFSA) (2016) recommends daily DHA intake of 100–200 mg for expectant and lactating women, which also mitigates age-related ailments such as cardiovascular diseases, cancer, inflammation, and Alzheimer's disease (Mason *et al*., 2020; Balakrishnan *et al*., 2021; Wei *et al*., 2021). Despite DHA's known benefits, Indonesia faces a high dependency on fish oil imports, totaling 9,124,579 kg in 2021 (MMAF, 2021). Concurrently, significant potential exists in utilizing underexplored by-products, such as tuna eyes, which are rich in DHA and EPA. Tuna, as one of Indonesia's flagship exports, generates considerable by-products, including heads, skins, and eyes, yet these remain largely untapped (Sayana and Sirajudheen, 2017; Trilaksani *et al*., 2023). This research focuses on optimizing enzymatic extraction of DHA-rich tuna eye oil using papain to enhance yield and reduce environmental impact, providing an innovative solution for sustainable fisheries by-product utilization.

Mass production of polyunsaturated fatty acids (PUFA) rich fish oil for human consumption is still lacking in Indonesia. The amount of imported fish oil rich in PUFA reached 9,124,579 kilograms in 2021 (MMAF, 2021). For the time being, tuna eye, a non-obligatory by-product of the tuna industry sector, exhibiting promise as a reservoir of PUFA, particularly DHA, has not been explored yet. PUFA's sources are reported to originate from tuna. Tuna (*Thunnus* sp.) is a flagship commodity of Indonesia with the second largest export value (960,339,781 USD) after shrimp (2,157,049,773 USD) (MMAF, 2022). Tuna is commonly processed into fillets, loins, sashimi, and canned products, which still leave by-products such as heads, skins, offal, and bones (Sayana and Sirajudheen, 2017). The respective proportion of fat in tuna eye is around 12-23% (Trilaksani *et al*., 2023) with concentrations of EPA and DHA are 7% and 35% (Renuka *et al*., 2016; Trilaksani *et al*., 2020). This circumstance provides an opportunity to aid the government in acquiring DHA and reducing the import volume.

Fish oil is typically extracted by high-temperature boiling and pressing. Exposure to high temperatures instigated oxidation and structural damage of omega-3 fatty acids (Chew and Nyam, 2019; Ferdosh *et al*., 2014). An alternative technique, including solvent extraction (Folch *et al*., 1957; Bligh and Dyer, 1959), is extremely complex and prone to causing environmental contamination (Ghaly, 2013; de Oliveira *et al*., 2017; Fernández-Lucas *et al*., 2017). The utilization of enzymatic oil extraction is a viable option due to its increased yield and safer operation (Qi-Yuan *et al*., 2016; Routray *et al*., 2018; Tacias-Pascacio *et al*., 2021). Salmon heads were extracted using 0.5% (w/w) bromelain enzyme, yielding 12.2 ± 1.1% (w/w) of oil (Mbatia *et al*., 2010). In a study conducted by Trilaksani *et al*., (2021), the integration of enzymatic extraction using papain 0.5% (w/w) with low-temperature centrifugation separation rose in yield that was three times higher (15.03 ± 2.43%) than that obtained by low-temperature centrifugation deserted (5.58% ± 0.01%). Enzymatic extraction improves both yield and quality of fish oil by selectively breaking down lipids and proteins, with papain effectively releasing oil from tissue matrices (Barrow and Shahidi, 2007; Ovissipour *et al*., 2012). Operating at low temperatures, this method preserves sensitive omega-3 fatty acids, such as EPA and DHA, ensuring better oxidative stability (Rubio-Rodriguez *et al*., 2010). Additionally, its solvent-free process enhances consumer safety and environmental sustainability by reducing chemical residues and pollution (Picot-Allain *et al*., 2021). This research is novel in applying an enhanced concentration of papain enzyme to tuna eye samples that have undergone prolonged storage during the COVID-19 pandemic. Extended storage can lead to oxidation and degradation of essential fatty acids, which reduces oil quality. Papain, known for its ability to break down protein and fat structures, can improve oil yield and oxidative stability (Barrow and Shahidi, 2007; Baptista *et al*., 2020). By optimizing papain concentration, this study examines the impact of storage on the yield and quality of omega-3 fatty acids, such as EPA and DHA (Wang *et al*., 2020). This approach offers a solution for the fish industry, aiming to enhance the quality and commercial value of stored fish by-products. While similar methodologies may have been explored in previous studies, this research is distinct in its focus on tuna eye samples. The primary aim of this study was to determine the optimal concentration of papain for the enzymatic extraction of tuna eye oil, with the goal of maximizing yield and enhancing the overall quality of the fish oil produced.

2. Materials and Methods

2.1 Materials

2.1.1 The equipments

The equipments used included an analytical balance (AND HR-250A), a Philips HR 211 blender, a camera, a centrifuge (Hitachi Model Part No. R12A6904357D0), a gas chromatograph (Shimadzu GC 2010 plus), a water bath shaker (Yamato, Japan), a UV-Vis 2500 spectrophotometer (Shimadzu), a viscometer (Toki Sangyo model TV-10), a magnetic stirrer, a CR-300 chroma meter (Konika Minolta).

2.1.2 The materials

The raw materials for this study included tuna (*Thunnus* sp.) eyes, each weighing approximately 200 g, sourced from the tuna loin industry in Bitung, North Sulawesi. Samples were transported under frozen conditions (-20°C) to preserve freshness and quality. Enzymatic extraction was performed using commercial papain enzyme (200,000 U/g), with 1% enzyme concentration optimized for maximum yield. For each extraction, 100 g of tuna eye paste was mixed with 500 mL of distilled water and incubated at 55°C for 1 hour in a water bath shaker at 150 rpm.

Analytical reagents used for quality assessment included Merck starch indicator, ethanol absolute, phenolphthalein (PP), isooctane, NaOH, glacial acetic acid (CH3COOH), KOH, anisidine, sodium thiosulfate (Na2S2O3), and potassium iodide (KI). Instrumentation included an analytical balance (AND HR-250A) for precise sample measurement, a centrifuge (Hitachi R12A6904357D0) for phase separation, and a UV-Vis spectrophotometer (Shimadzu UV-Vis 2500) for oil characterization.

The methodological framework was designed to optimize enzymatic extraction efficiency while maintaining the quality of DHA-rich oil. All procedures adhered to AOAC (2005) and Codex Alimentarius standards for fish oil analysis, ensuring reliability and reproducibility. This study builds on the protocol established by Rubio-Rodriguez *et al.* (2010), with modifications to suit tuna by-product characteristics.

2.1.3 Ethical approval

This study does not require ethical approval because it does not use experimental animals.

2.2 Methods

The sentence under the sub-chapter is written without distance from the title. Available mathematical equations, equations are made with equation editors and given numbers in the order they are.

2.2.1 Preparation and determination of tuna eye profile

Tuna (*Thunnus albacares*) eyes as a by-product of the tuna loining industry in Bitung City, North Sulawesi were frozen and transported with cargo to Jakarta and by car to THP Laboratory at -20°C. The tuna eyes were then thawed at a cooling refrigeration temperature for 12 hours. The process of visual determining tuna's eye profile using a camera (phone). The tuna eye was weighed using a scale, while the proportion comprised the components and the size of the eye (a minor scale corresponds to 0.1 cm). The assessment of tuna eye freshness encompassed the sensory evaluation of the eyeball, cornea, and pupil, in accordance to the Indonesian National Standard (SNI) No. 2729:2013, which specifies the eye appearance of fresh fish. A set of thirty tuna eye samples was used to determine the profile. The chemical constituent analysis of tuna eye included heavy metal analysis (SNI 2354-23 2021), water content (AOAC 2000 item 934.01 2000), ash content (AOAC 2005 item 938.08 2005), protein content (AOAC 2000 item 981.10 2000), lipid content (AOAC 2005), and carbohydrates (by difference) (AOAC 2000) were also analyzed.

2.2.2 Tuna eye oil extraction

The samples used were the flesh around an eye and eye muscle, which were then grounded into a paste. Comminuting was performed using a blender machine for approximately 3 minutes and held at refrigeration temperature for 15 seconds. The tuna eye mashed was centrifuged (11,200×g, 30 minutes, 4°C) and then the oil and other components (substrate) were separated. The oil generated from the first centrifugation was weighed and became the yield data for the first centrifugation. Components other than oil, such as liquids and solids, were remixed, followed by enzymatic extraction using the commercial type of papain enzyme. There were six treatments at this stage, consisting of A (0% enzyme), B (0.125% enzyme), C (0.25% enzyme), D (0.5% enzyme), and E (1% enzyme). The substrate was then incubated in a water bath shaker at 150 rpm,-55°C for 1 hour, followed by centrifugation (11,200×g, 4°C) for 30 minutes. The extracted tuna eye oil was collected with a pipette and became yield data 2. The oil from the first and second centrifugations was become total calculated yield. The oil was then stored in light-tight bottles at -20°C. Determination of the tuna eye oil profile included visual appearance, yield, and fatty acid profile (AOAC 2005 No. 969.33), index of atherogenicity (IA), and index of thrombogenicity (IT) as well (Trilaksani *et al.*, 2023).

Table 1. Effect of different concentration of papain enzyme on the values of L* (lightness), a* (redness), b* (yellowness) and whiteness of tuna eye oil.

Sample	L*	a*	b*	Whiteness (%)	Color
A	3.38^{cd}	-0.27^{a}	0.82^{a}	3.38±0.30^{ab}	Brown (*Raw Umber*)
B	2.88^{bc}	-0.42^{a}	-0.82^{a}	2.85±0.70^{bc}	Brown (*Wood Bark*)
C	2.34^{ab}	-0.25^{a}	0.62^{a}	2.34±0.09^{cd}	Brown (*Seal brown*)
D	1.97^{a}	-0.35^{a}	0.48^{a}	1.96±0.08^{d}	Brown (*Wood Bark*)
E	1.73^{a}	-0.28^{a}	0.50^{a}	1.73±0.13^{d}	Brown (*Cocoa Brown*)

Description: A papain enzyme 0%, B papain enzyme 0.125%, C papain enzyme 0.25%, D papain enzyme 0.5%, E papain enzyme 1%. Different superscripts in the same column shows that there are significant differences ($p < 0.05$).

2.2.3 Characteristics of Tuna Eye Oil

2.2.3.1 Tuna eye proportion

The proportion of tuna eyes was determined by measuring the weight of the vitreous humor, lens, and other parts compared to the total weight of the whole tuna eyes. The proportion was calculated using the following formula:

Tuna Eye Proportion (%) = Weight of tuna eye part (g) / Weight of whole tuna eyes (g) × 100%.

2.2.3.2 Chemical composition analysis of tuna eyes

The chemical composition of tuna eyes was analyzed using a modified AOAC method (2005). Moisture content was determined by method 925.45. Protein content was assessed using the Kjeldahl technique according to method 920.152. Ash content was measured by method 940.26, and fat content was determined using the Soxhlet extraction method 963.16. Carbohydrate content was calculated by difference.

2.2.3.3 Heavy metals analysis of tuna eyes

The heavy metals Cd, Pb (SNI 2354:5:2016), and Hg (SNI 2354:6:2016) were analysed using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Thermo Scientific 7900, Waltham, Massachusetts, USA). The measurement of As (SNI 2354:15:2017) was performed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Agilent Technologies 700, Santa Clara, United States).

2.2.3.4 The yield of virgin fish oil

The yield of fish oil extracted from tuna eyes was presented as a percentage. The analysis compared the amount of extracted fish oil with the total weight of the fish eyes used. The formula applied was as follows:

Yield (%) = Weight of fish oil (g)/Total weight of fish eyes (g) × 100%

Table 2. Oxidation parameters of tuna eye oil formed from enzymatic extraction

Treatment	FFA (%)	Acid value (mg-KOH/g)	Peroxide value (meq/kg)	Anisidin value (meq/kg)	TOTOX (meq/kg)
CXS 329-2017	-	< 3	< 5	< 20	< 26
A	1.14±0.04^{a}	0.99±0.08^{a}	127.93±2.76^{c}	30.01±3.85^{b}	285.87±3.85^{c}
B	1.42±0.17^{b}	1.34±0.29^{b}	137.92±1.26^{d}	31.79±7.89^{bc}	307.63±7.89^{d}
C	1.62±0.14^{bc}	1.51±0.20^{b}	141.40±2.63^{d}	34.42±1.63^{c}	317.21±1.63^{d}
D	1.66±0.13^{c}	1.61±0.23^{b}	115.27±1.98^{b}	40.14±0.75^{d}	270.67±0.75^{c}
E	2.00±0.25^{d}	2.09±0.49^{c}	88.85±2.76^{a}	62.23±1.41^{e}	239.93±1.41^{b}

Description: A papain enzyme 0%, B papain enzyme 0.125%, C papain enzyme 0.25%, D papain enzyme 0.5%, E papain enzyme 1%. Different superscripts in the same column shows that there are significant differences ($p < 0.05$).

Tabel 3. Fatty acid profile of tuna eye oil extracted enzymatically

Fatty Acid	A (%)	B (%)	C (%)	D (%)	E (%)
Lauric acid, C12:0	0.00±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Tridecanoic acid C13:0	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Myristic acid C14:0	2.67±0.00	2.67±0.09	2.71±0.00	2.74±0.01	2.74±0.01
Pentadecanoic acid C15:0	1.02±0.00	1.02±0.03	1.03±0.00	1.04±0.00	1.04±0.00
Palmitic acid C16:0	21.33±0.0	21.46±0.4	21.34±0.0	21.67±0.0	21.45±0.0
Heptadecanoic acid C17:0	1.64±0.00	1.65±0.02	1.65±0.00	1.68±0.00	1.68±0.00
Stearic acid C18:0	5.79±0.02	5.88±0.09	5.77±0.01	5.80±0.02	5.86±0.00
Arachidic acid C20:0	0.45±0.00	0.45±0.00	0.45±0.01	0.46±0.00	0.16±0.00
Heneicosanoic acid C21:0	0.13±0.00	0.15±0.00	0.15±0.00	0.16±0.00	0.46±0.00
Tricosanoic acid C23:0	0.17±0.00	0.16±0.00	0.19±0.00	0.19±0.00	0.19±0.00
Lignoceric acid C24:0	0.44±0.00	0.43±0.00	0.35±0.01	0.31±0.01	0.30±0.01
Total SFA (*Saturated Fatty Acid*)	33.68±0.04	33.89±0.45	33.68±0.02	34.08±0.01	33.90±0.02
Myristoleic acid C14:1	0.09±0.00	0.10±0.00	0.27±0.31	0.09±0.00	0.09±0.00
Pentadecenoic acid C15:1	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00
Palmitoleic acid C16:1	5.96±0.03	5.99±0.13	6.01±0.00	6.08±0.01	6.04±0.00
Heptadecenoic acid C17:1	1.19±0.01	1.20±0.01	1.22±0.00	1.23±0.00	1.22±0.00
Oleic acid C18:1n-9c	18.28±0.04	18.30±0.03	18.16±0.03	18.30±0.01	18.32±0.01
Eicosenoic acid C20:1	1.05±0.03	1.08±0.02	1.06±0.02	1.07±0.00	1.07±0.01
Total MUFA (*Monounsaturated Fatty Acid*)	26.83±0.03	26.92±0.11	26.97±0.31	27.03±0.01	26.99±0.00
Linoleic acid C18:2n-6c	1.37±0.03	1.38±0.01	1.36±0.00	1.34±0.00	1.36±0.00
α-Linolenic acid C18:3n-3	0.45±0.01	0.44±0.01	0.45±0.00	0.45±0.00	0.46±0.00
γ-Linolenic acid C18:3n-6	0.43±0.50	0.09±0.00	0.09±0.00	0.08±0.00	0.00±0.00
Eicosadienoic acid C20:2	0.33±0.00	0.33±0.00	0.32±0.00	0.32±0.00	0.32±0.00
Eicosatrienoic acid C20:3n-3	0.19±0.01	0.20±0.00	0.21±0.00	0.20±0.00	0.20±0.00
Arachidonic acid C20:4n-6	2.76±0.01	2.75±0.03	2.83±0.01	2.81±0.00	2.82±0.00
Eicosapentanoic acid(EPA), C20:5n-3	5.36±0.04	5.32±0.08	5.58±0.01	5.50±0.00	5.50±0.00
Docosahexaenoic acid (DHA), C22:6n-3	27.25±0.08^{a}	26.76±0.51^{a}	28.31±0.04^{b}	27.82±0.01^{b}	27.82±0.04^{b}
Total PUFA (*Polyunsaturated Fatty Acid*)	38.49±0.57^{ab}	37.57±0.63^{a}	39.33±0.02^{c}	38.68±0.01^{bc}	38.64±0.03^{bc}
n-3	33.25±0.11	32.72±0.59	34.55±0.03	33.97±0.01	33.97±0.04
n-6	4.36±0.04	4.38±0.04	4.43±0.00	4.39±0.00	4.34±0.00
n-9	18.42±0.03	18.44±0.02	18.30±0.03	18.44±0.01	18.46±0.00
Total Identified Fatty Acids	99.00±0.50	98.38±0.07	99.98±0.31	99.79±0.00	99.54±0.00

Description: A papain enzyme 0%, B papain enzyme 0.125%, C papain enzyme 0.25%, D papain enzyme 0.5%, E papain enzyme 1%. Different superscripts in the same column shows that there are significant differences ($p < 0.05$).

2.2.3.5 Oxidation analysis

The extracted fish oil was analyzed to determine its quality, including the peroxide value (AOAC 2005 Method Ca 5a-40), free fatty acids (AOCS, 1998), p-anisidine value (IUPAC 1987 No. 2504), and total oxidation (TOTOX) value (AOCS, 1997).

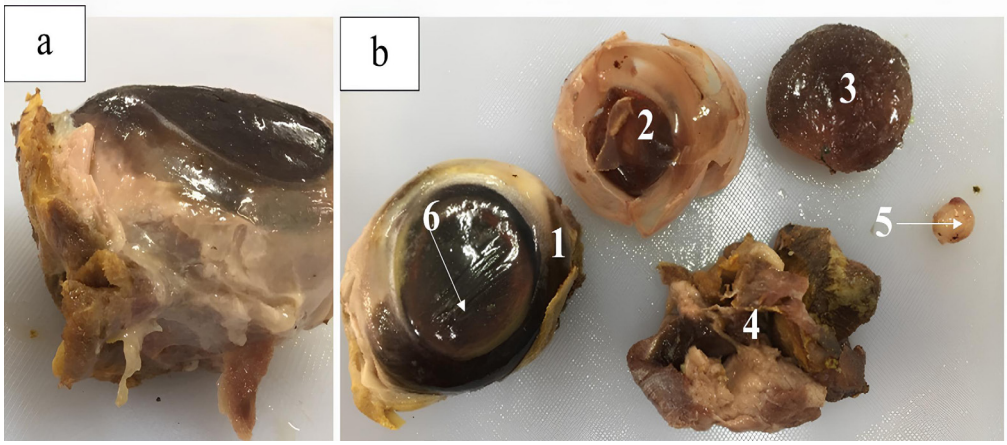

Figure 1. Visual appearance of tuna eyes. Description: a Visual appearance tuna eyes from beside, b part of tuna eye: b.1 tuna eye, b.2 sclera, b.3 vitreous humor, b.4 extraocular muscles, b.5 lens, b.6 cornea

2.2.3.6 Fatty acid profile

The fatty acid profile of tuna eye oil was evaluated using the AOAC 2005 No. 969.33 method. A 20 mg oil sample was placed in a Teflon-capped tube and mixed with 1 mL of 0.5 N NaOH in methanol. The mixture was heated in a water bath for 20 minutes, followed by the addition of 2 mL of a 20% BF3 solution and 5 mg/mL of the standard. After another 20 minutes of heating, the mixture was cooled and combined with 2 mL of saturated NaCl and 1 mL of isooctane, then vigorously shaken. The isooctane layer was pipetted off and transferred to a tube containing 0.1 g of anhydrous Na_2SO_4. After standing for 15 minutes, the separated liquid phase was extracted. The resultant oil phase was injected into the GC instrument (Shimadzu GC 2010 Plus, Kyoto, Japan) using a 1 µL injection of the Supelco 37 component fatty acid methyl ester mix. The GC instrument settings were a nitrogen (N_2) flow rate of 20 mL/minute, a water (H_2O) flow rate of 30 mL/minute, an injector temperature of 200°C, and a detector temperature of 230°C. The retention times and peaks were compared with standard retention times to identify the types and constituents of the fatty acids in the sample.

2.2.3.7 Atherogenicity index (ai) and thrombogenicity index (ti) of tuna eye oil

The atherogenic index (AI) and thrombogenic index (TI) are important factors for assessing the nutritional value of oil. These indices are calculated using the method developed by Ulbricht and Southgate (1991). The AI quantifies the relationship between primary saturated fatty acids and major classes of unsaturated fatty acids, calculated as follows:

$$AI = [(4xC14:0) + C16:0 + C18:0] / [\sum MUFA + \sum PUFA + n6 + \sum PUFA - n3]$$

The TI indicates the potential of the oil to form clots in blood vessels, calculated as follows:

$$TI = (C14:0 + C16:0 + C18:0) / (0.5\ MUFA + 0.5\ PUFA - n6 + 3\ PUFA - n3 + PUFA\text{-}n3/PUFA\text{-}n6)$$

2.3 Analysis Data

Data were expressed as the mean of three replicates and standard deviation (SD). Statistical analysis was done by ANOVA. The data obtained were processed using Microsoft Excel and the application of Statistical Product and Service Solution (SPSS) version 25 (IBM Inc.). Differences were considered to be significant at $p < 0.05$.

3. Results and Discussion

3.1 Results

3.1.1 Appearance and proportions of tuna eyes

The average weight and diameter of the tuna eyes utilized used in this research study were 85.24 ± 4.27 g and 5.65 ± 0.49 cm, respectively. This sample size was considered small size according to Trilaksani *et al.* (2023). The weights of the vitreous humor, lens, and sclera were 2.73 ± 0.53 g, 5.20 ± 0.68 g, and 23.73 ± 5.48 g, respectively (Figure 1).

3.1.2 Chemical composition of tuna eye

The chemical composition of tuna eye displayed that the highest proportion, 60.49 ± 0.36 percent, was water. The remaining components, protein, carbohydrate and ash contents, were 13.32 ± 0.33%, 15.53 ± 0.23% and 13.31 ± 0.01%, respectively. The analysis of heavy metals was performed in order to ensure food safety. Lead (Pb), mercury (Hg), arsenic

(As), and cadmium (Cd) were examined as heavy metal parameters. It seems that the heavy metal lead (Pb) was not detected. Cadmium and mercury were detected in concentrations of 0.06 ± 0.007 mg/kg and 0.11 ± 0.007 mg/kg respectively (meet the standard).

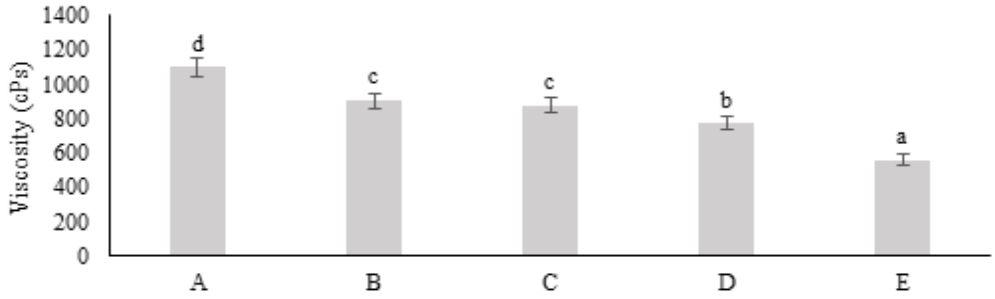

Figure 2. Viscosity of comminuted tuna eye flesh

Description: A papain enzyme 0%, B papain enzyme 0.125%, C papain enzyme 0.25%, D papain enzyme 0.5%, E papain enzyme 1%. Different superscripts in the same column shows that there are significant differences ($p < 0.05$).

3.1.3 Viscosity of tuna eye paste

Treatment A exhibited the highest viscosity value of 16613.33 cPs, which differed substantially ($p<0.05$) from the other treatments (Figure 2). Treatment F produced the least viscous solution (561.33 cPs) and differed substantially ($p<0.05$) from the other treatments. The variation in viscosity of tuna eye paste, as shown in Figure 2, can be attributed to enzymatic hydrolysis.

3.1.4 Appearance, brightness and color of tuna eye oil

Fish oil extracted from tuna eye paste by centrifugation consists of four components similar to the result of fish oil extracted from mackerels conducted by Ghaly (2013). Tuna eye oil, light lipid-protein, aqueous protein hydrolysate, sediment, and heavy lipid-protein comprise the first, second, third and fourth layer correspondingly (Figure 3). The layers are separated due to the different densities and molecule weight of the components. The denser lipid-protein layer settles at the bottom, while the less dense oil floats on top. The whiteness percentage of tuna eye oil was determined using a CR-300 chroma meter manufactured by Konika Minolta Japan. For all interventions, the observed differences were recorded using the b* parameter, which represents yellowness. As shown in Table 1, the yellowness values decreased as the concentration of the enzyme increased. The decline in yellowness means an alteration in the hue of the oil. Low values of whiteness are affected by the escalation of the papain enzyme concentration.

3.1.5 Yield of tuna eye oil

The extraction process consists of two phases. Centrifugal separation at a 4°C temperature constituted the first phase of extraction. The results of the oil extraction were weighed, after which the solid components were enzymatically extracted and subjected to a second round of centrifugation. The results obtained (Figure 5) indicated that the oil yield did not differ significantly between treatments ($p<0.05$). However, treatment A (0 papain) resulted in a significantly lower oil yield (8.55±0.69%) than treatment B (1% enzyme). The lack of significant variation in outcomes can be attributed to the comparatively limited range of enzyme concentrations across the different treatments. The enzyme concentration increased in direct proportion to the oil yield, as shown in Figure 5. The oil yields from the first extraction method (cold extraction) were comparable and did not differ significantly. However, the oil obtained from the second extraction varied because of the addition of different enzyme concentrations.

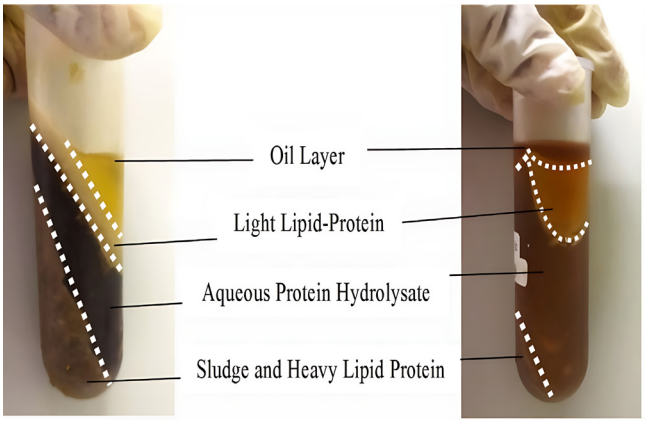

Figure 3. Visual appearance of tuna eye oil

3.1.6 Oxidation parameter of tuna eye oil

Tuna eye oil was analyzed and evaluated according to Codex Standard 329-2017, which is a specific standard for fish oil. The results showed that the parameters of free fatty acids (FFA) and acid number met the established criteria (Table 2). However, peroxide value, anisidine value, and total oxidation (TOTOX) exceeded the standards. Treatment E yielded the highest amounts of free fatty acid (FFA) and acid number (2.00±0.25% and 2.09±0.49 KOH/g, respectively). Treatment A conceded a peroxide value of 127.93±2.76 meq/kg, which subsequently rose and fell before reaching its minimum in treatment E (88.85±2.76 meq/kg). Thereafter, treatment E showed the highest anisidine value (62.23±1.41 meq/kg). Treatment C (0.25% enzyme treatment) gave the highest total oxidation (TOTOX) value of 317.21±1.63 meq/kg. The results indicated that enzymatic extraction had a higher oxidation value than cold extraction. The results indicated that enzymatic extraction had a higher oxidation value than cold extraction. The COVID-19 pandemic disrupted regular research timelines, often resulting in extended storage of biological samples. Such delays can impact data reliability and analysis, as prolonged storage, particularly in suboptimal conditions, may alter sample integrity and the outcomes of research studies (Tan *et al*., 2020; Smith *et al*., 2021). To maintain data accuracy in future studies, it is essential to address storage duration as a variable, especially under unforeseen circumstances (Jones and Lee, 2022).

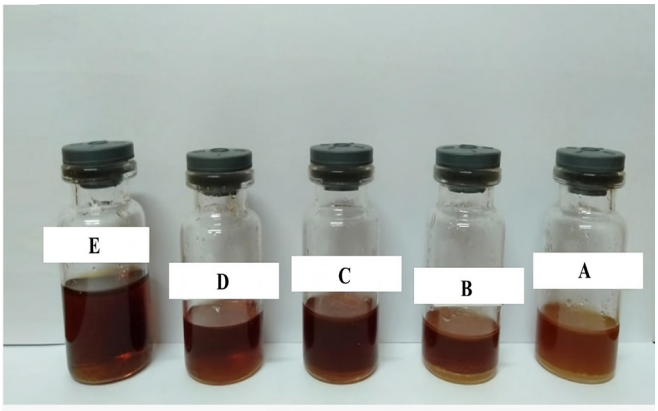

Figure 4. Visual appearance of tuna eye oil produced from enzymatic extraction. Description: A papain enzyme 0%, B papain enzyme 0.125%, C papain enzyme 0.25%, D papain enzyme 0.5%, E papain enzyme 1%.

3.1.7 Fatty acids of tuna eye oil

In tuna eye oil, polyunsaturated fatty acids (PUFA) were of great value, with details as follows: 37.34% total saturated fatty acids (SFA), 25.25% total monounsaturated fatty acids (MUFA), and 31.40% total polyunsaturated fatty acids (PUFA). Docosahexaenoic acid (DHA) comprised 26±31% of the fatty acid composition of tuna eye oil (expressively lower than DHA concentration extracted from fresh eye (Trilaksani *et al*., 2023), followed by palmitic acid (20±21%) and oleic acid (17±18%). A significance test ($p<0.05$) was performed on the fatty acids DHA, EPA, and palmitic to determine the effect of different enzyme concentrations on the fatty acid profile (Table 3). The predominant and essential fatty acids in the tuna eye were DHA and EPA. With the exception of palmitic acid, their levels were significantly different ($p<0.05$). DHA, EPA, and palmitic acid have been identified as the essential and predominant fatty acids in the tuna eye, were identified (Trilaksani *et al*., 2021). A higher enzyme concentration resulted in a decrease in the percentage of EPA and an increase in DHA.

3.1.8 Index of Atherogenicity (IA) and Index of Thrombogenicity (IT)

The Index of Atherogenicity (IA) ranges from 0.38 to 0.40, while the Index of Thrombogenicity (IT) ranges from 0.20 to 0.21 (Figure 6). There were no significant differences in IA and IT values between treatments. Both IA and IT values are low, indicated by values below 1.

3.2 Discussion

3.2.1 Appearance and proportions of tuna eyes

Organoleptic evaluation of tuna eyes was conducted corresponded to the Indonesian National Standard (SNI) No. 2729:2013; the score was 4.23 ± 1.57, with the cornea exhibiting a slight concavity and opacity, and the pupils predominantly gray in color and lacking gloss. The vitreous humor accounted for 26.74 ± 6.18 percent of the total, the sclera for 5.86 ± 0.77 percent, and the lens for 3.07 ± 0.60 percent. The appearance of the occular affects the amount of fat/oil produced. In this study. Species and size, storage time is a substantial determinant. When the eye is stored at frigid temperature, ice crystals will be formed, consequently when it is thawed, the meat losses moisture and shrink (Dawson *et al*., 2018).

3.2.2 Chemical composition of tuna eye

Different result was found in Sinulingga *et al.* (2024b) and La Dia *et al.* (2022) showed that more than 18% fat content was identified from tuna eye; it may possibly affected by size and freshness of raw material. In the contrary, arsenic content higher than the threshold (3.22 ± 0.007 mg/kg) specified in the Regulation of the National Agency of Drug and Food Control (BPOM) No. 9 of 2022 on Requirements

for Heavy Metal Contamination in Processed Foods (2.0 mg/kg). Arsenic (As) is a metalloid element that is abundant in aquatic ecosystems as a result of both natural and human activities. Arsenic can be classified into two distinct types: organic and inorganic. Arsenic, which is organic in nature, is frequently encountered in animal tissues. In trace amounts, it is converted to arsenobetaine (AsB) or other organic compounds (Boyle *et al.*, 2008). Arsenobetaine (AsB), a non-toxic compound, is expeditiously eliminated in its unaltered state by humans (Duker *et al.*, 2005). The presence of arsenic can infiltrate through various avenues, including seawater and sediment, food, and naturally occurring organic arsenic found in fat/fish oil (arsenolipid). Tuna fish are apex predators, which heightens the risk of arsenic exposure through consumed food. Organic liposoluble arsenic (arsenolipid) is commonly present in fish oil; for instance, certain species, such as tuna fillets, contain arsenolipid constituting 87% of the total arsenic (Taleshi *et al.*, 2010). Cod liver oil and herring fish have been found to contain arsenic at levels of 0.7-10 mg/kg (Lunde, 1967) and 5-6 mg/kg (Usydus *et al.*, 2009), respectively.

on the figure above, the variation in viscosity values of tuna eye paste is attributed to enzymatic hydrolysis. Peptide bonds can be fragmented into simpler forms via enzymatic hydrolysis (Figure 2). Proteins can have their physical and chemical properties altered through hydrolysis, while also gaining solubility, losing allergenicity, and acquiring nutritional benefits, such as enhanced digestibility (Amri and Mamboya, 2012). As the enzyme's hydrolysis increases with aggregating enzyme concentration, in consequence the tuna eye mashed viscosity declines (or becomes more dilute) (Bayu *et al.*, 2017).

3.2.4 Appearance, brightness and color of tuna eye oil

Low values of whiteness are affected by the escalation of the papain enzyme concentration. According to Zhang *et al.* (2021), enzymatic extraction of salmon head oil produced pigments of a reddish-brown hue that were darker than pigments obtained using alternative ultra-high-pressure extraction techniques. Prolonged storage of the sample induces an autoxidation reaction in the phospholipids, resulting in a dark hue. Autoxidation leads to the formation of dark-

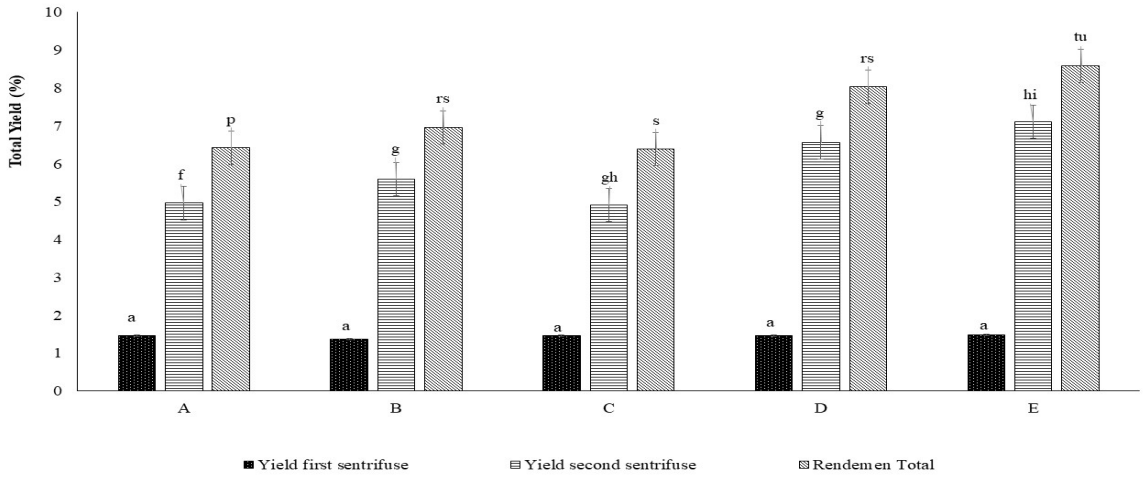

Figure 5. The total yield of tuna eye oil by enzymatic extraction, description: A papain enzyme 0%, B papain enzyme 0.125%, C papain enzyme 0.25%, D papain enzyme 0.5%, E papain enzyme 1%. Different superscripts in the same column shows that there are significant differences ($p < 0.05$).

3.2.3 Viscosity of tuna eye paste

Viscosity is a measure of the thickness or internal resistance of a fluid to flow. The degree of viscosity in a liquid is closely related to its resistance to movement. Liquids exhibit a range of flow characteristics some flow rapidly while others move more slowly. Substances such as water, alcohol, and gasoline, which flow quickly, have low viscosity, whereas substances like glycerin and honey, which flow more slowly, have high viscosity. The viscosity value directly influences the flow rate of a liquid (Firdausi *et al.*, 2008). Based

colored compounds, melanophosphatides, through the condensation reaction between phospholipid amino groups and aldehydes (Chew and Nyam, 2019). Apart from storage conditions, the increasingly dark brown color is also attributed to the higher concentration of enzymes used, which induce hydrolysis in the tuna eye muscle tissue, specifically myoglobin. Myoglobin is an essential heme protein (red in color) widely distributed in animal tissues, playing a crucial role in intracellular oxygen storage and diffusion (Sidell and O'Brien, 2006). During extraction and heating, myo-

globin and oxymyoglobin undergo changes that alter their color to yellowish-brown, resulting in metmyoglobin as a product of myoglobin denaturation (Grunwald and Richards, 2006). Table 1 illustrates the effect of different concentrations of papain enzyme on the b* (yellowness) values of tuna eye oil.

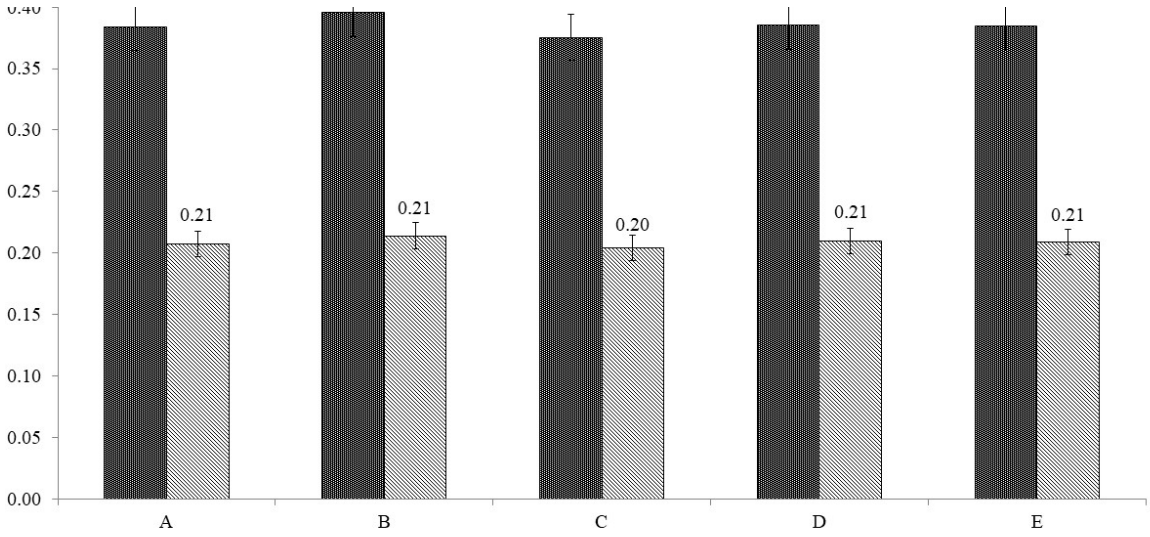

Figure 6. Index of Atherogenicity (IA) and Index of Thrombogenicity (IT) of tuna eye oil produced by enzymatic extraction. Description A papain enzyme 0%, B papain enzyme 0.125%, C papain enzyme 0.25%, D papain enzyme 0.5%, E papain enzyme 1%. Different superscripts in the same column shows that there are significant differences ($p < 0.05$).

3.2.5 Yield of tuna eye oil

Cold centrifugation is a straightforward technique for separating components in fish oil by employing centrifugal force (g-force) to distinguish them by molecular weight (Majekodunmi, 2021). Although this approach is generally straightforward and uncomplicated, a significant amount of unextractable oil remains in the muscle tissue; therefore, it has been suggested that the yield can be increased by employing protease enzymes such as papain, pepsin, bromelain, catalase to degrade the oil within the protein matrix has been suggested (Rubio-Rodríguez *et al*., 2012). The results indicated that a 1% enzyme concentration was the most effective treatment, yielding the highest extraction, though the differences among some treatment points were not statistically significant. This may be due to the narrow range between treatments, suggesting that future research should explore a wider range to achieve more optimal yields. Additionally, optimizing the temperature is recommended to determine the minimum extraction temperature to minimize fish oil degradation. Ghaly (2013), in his research on enzymatic extraction of mackerel using 0.5-2% enzyme concentration for 1-4 hours, observed hydrolysis of the tissue with a yield reduction at the second hour. This is likely due to excessive hydrolysis of lipid-protein interactions, suggesting that a one-hour hydrolysis time is optimal for oil extraction. Zhang *et al*. (2021) studied the enzymatic extraction of oil from yellowfin tuna (*Thunnus albacares*) by-products (skin, head, viscera) using enzyme concentrations of 0.5%, 1.0%, and 1.5% for 60, 90, and 120 minutes. They concluded that the optimum enzyme concentration of papain for the highest yield was 1% with a 60-minute hydrolysis time. The increased oil yield from hydrolyzed protein tissue is mainly due to changes in the structure and composition of the meat during hydrolysis. Protein hydrolysis alters the protein structure within the meat, breaking down the muscle network into peptides and amino acids, making the meat softer and more fragile. This condition facilitates the release of oil and fat within the meat. Hydrolysis also causes the breakdown of cell membranes, particularly lipid-containing membranes, releasing the trapped fats and oils (Verma, 2023).

3.2.6 Oxidation parameter of tuna eye oil

Similar results were reported by Trilaksani *et al*. (2021); the oxidation number of enzymatic extraction was twice that of cold centrifugation. The elevated oxidation number of fish oil may be attributed to several factors, such as the processing of the raw materials (using comparatively higher temperature during enzymatic extraction which leads oxidation) and the presence of high PUFA content. A tuna eye lubricant rich in PUFA is more susceptible to oxida-

tion (Damodaran and Parkin, 2017). Prevention can be achieved through an integrated approach that encompasses various stages, from upstream to downstream. Moreover, oxidized oil can be purified to remove contaminants that contribute to quality degradation. The high oxidation of tuna eye oil is also influenced by the duration of storage. During the COVID-19 pandemic, all laboratory activities were suspended, resulting in tuna eye samples being stored in a freezer (-20°C) for a period of two years. Following physical and enzymatic extraction, a significant change in oxidation value was observed compared to fresher tuna eye samples. Previous research indicates that the freshness of fish raw materials greatly affects the quality of omega-3 produced, particularly the composition of EPA and DHA. Trilaksani *et al*. (2023), La Dia *et al*. (2022), and Sinulingga *et al*. (2024a) found that highly fresh tuna eyes yield DHA concentrations exceeding 30%, approaching the value of omega-3 concentrates. The percentage of DHA produced in this study was only around 26-28%, likely due to the storage of samples during the pandemic (2 years). Rudy *et al*. (2016) stated in their study that storage duration affects the fatty acid profile of fish.

3.2.7 Fatty acids of tuna eye oil

Based on the results presented in Table 3, variations in extraction methods and enzyme concentrations notably influence the proportions of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Specifically, enzymatic extraction followed by heating led to a decrease in PUFA content, accompanied by increases in SFA and MUFA. This suggests that the heating process during enzymatic extraction may cause the breakage of double bonds, particularly in PUFA, thereby promoting the formation of more saturated fatty acids. PUFA emerges as the most susceptible to alteration compared to MUFA and SFA (Ivanovs and Blumberga, 2017; Chew and Nyam, 2019). Moreover, stove-heating generated free radicals, while subsequent microwave-reheating intensified the presence of stable free radicals. The stove-heating process was associated with the disruption of C=C and C=O bonds, potentially increasing the polarity of the fatty acids (Zhao *et al*., 2019). Fatty acids were categorized according to the initial position of the methyl (CH3) double bond on the fatty acid carbon chain, in addition to their degree of saturation. Classifications of omega-3 (double bond on the third carbon from the methyl group), omega-6 (double bond on the sixth carbon from the methyl group), and omega-9 (double bond on the ninth carbon from the methyl group) fatty acids have been established (Kimakova *et al*., 2018). Omega-9 fatty acids are classified as monounsaturated fatty acids (MUFA), whereas omega-3 and omega-6 fatty acids are classified as polyunsaturated fatty acids (PUFA). Heating and enzyme concentration had a significant ($p<0.05$) effect on the proportiondegres of omega-3, omega-6, and omega-9 fatty acids (Table 3). The results suggested that the primary determinant in the process of double bond cleavage in unsaturated fatty acids was the temperature (Ivanovs and Blumberga, 2017). However, it should be noted that the enzyme concentration also had a sinificant influence. The reduction, however, was not as significant as that of the thermal factor. Particularly in unsaturated fatty acids, which contain numerous double bonds and become more saturated when heated, fatty acid bonds were broken (Ivanovs and Blumberga, 2017; Chew and Nyam, 2019).

3.2.8 Index of Atherogenicity (IA) and Index of Thrombogenicity (IT)

Higher IA and IT values indicate a higher potential for the respective diet or food to increase the risk of atherosclerosis and heart disease. The Index of Atherogenicity (IA) is a measure used to assess the potential of a diet or food to increase the risk of coronary artery disease (atherosclerosis). IA refers to the ability of food or diet to increase LDL cholesterol (bad cholesterol) and decrease HDL cholesterol (good cholesterol), which are major risk factors for coronary artery disease. On the other hand, the Index of Thrombogenicity (IT) refers to the potential of a diet or food to increase blood clotting, which is a significant risk factor for heart disease and stroke. Foods with higher thrombogenic index tend to trigger more blood clot formation (Trilaksani *et al*., 2023). The Low IA and IT indicated that even though the oxidation value is high the ratio of unsaturated and saturated fatty acids remains stable.

4. Conclusion

The optimal treatment to achieve maximum yield in enzymatic extraction was 1% papain enzyme at 55°C for one hour. The maximum yield achieved was 8.59±0.69%, which was six times the yield of cold extraction (centrifugation) without enzymes (1.41±0.07%). In this study, adding enzyme concentration of up to 1% led to the following results: increased yield, decreased viscosity, and still keep remained well preserved PUFA, and improved oxidation quality. The primary factor influencing the reduction of PUFA and DHA during enzymatic hydrolysis was temperature, as opposed to an increase in enzyme concentration. Even though the oxidation value is higher during enzymatic extraction, the ratio of unsaturated and saturated fatty acids remains stable indicated by

the low IA and IT (below 1).

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Authors'Contributions

All authors contributed to the final manuscript. The specific contributions are as follows: Silva and Fahri collected the data and prepared the initial draft of the manuscript. Bambang created the figures, while Wini, Tati, and Joko developed the main conceptual ideas and conducted a critical review of the article. All authors engaged in discussing the results and made substantial contributions to the final version of the manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Declaration of Artificial Intelligence (AI)

The author(s) acknowledge the use of ChatGPT for language refinement and to find some information and we have check one by one the citation that we used in preparing this manuscript. All AI-generated content was rigorously reviewed, edited, and validated to ensure accuracy and originality. Full responsibility for the manuscript's final content rests with the author(s). To ensure transparency and support the review process, a comprehensive delineation of the tool's application is furnished in the "Introduction" or "Materials and Methods" section of this manuscript in compliance with the publisher's ethical guidelines.

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