Optimizing *Anabas testudineus* oil processing by refinement process for quality parameters and fatty acid composition

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Abstract. *Anabas testiduneus,* the climbing perch, is an endemic fish in Sumatra, Indonesia. Its uniqueness consists in the oil of its body. The abundant oil content in this fish allows the exploration of its health benefits, efficacy and safety. This study aims to assess the efficacy of the oil refinement process in optimizing the extracted fish oil quality. About 5 kg of fresh fish were obtained from Palembang Auction Center. The fish oil extraction method consisted of a purification process, which involved four main stages: degumming, neutralizing, bleaching and deodorizing. Total neutral lipids were determined using liquid chromatography, and the acidity value was assessed according to AOCS official methods. Yield after refinement was higher than in the crude oil (p<0.05). The levels of wax esters and free fatty acids were significantly lower after refinement than in the crude oil. The level of triacylglycerides in crude oil is significantly lower than after refinement. In conclusion, the refined oil of *A. testidineus* is better than the crude oil.
Key Words: *Anabas testidineus*, fish oil, oil purification, oil refinement.

Introduction. Fish is a food source rich in protein and abundant in Indonesia. Fish can be processed into various foods rich in protein and other nutrients. South Sumatra is a province in Indonesia with many rivers, being abundant in freshwater fish. The abundance of freshwater fish encourages the production of many local foods in South Sumatra, including "pempek" (fish cake), "pindang" (spicy fish soup) and "bekasam" (fermented fish) (Partan et al 2017).

Anabas testiduneus (Anabantidae family), locally known as "betok", is a local fish in Sumatra. Its shape is oval with a large and flat head. Its body is covered with green scales on the back, with a white color underside. *A. testiduneus* are found in rivers, lakes, swamps, puddles and can eat small shrimp, small fish and other aquatic animals (Zalina et al 2012). It has long dorsal fins. The anal fin is short. The pelvic fins are located in the front, below the pectoral fins. The hard rays of the pelvic fins may be displaced and can move outside the water. These fish are generally cooked fried or stewed. Its uniqueness consists in the large quantity of oil; even the water used to wash it will become oily. The abundant oil content in this fish allows researchers to explore its health benefits, efficacy and safety (Partan et al 2017).

Previous research on *A. testidineus* oil showed that it is rich in Omega-3 (Partan et al 2017). Omega-3 can inhibit cellular inflammatory processes, where inflammation could play a role in the initiation of some degenerative health disorders, both cardiovascular and endocrine (Calder 2013). Omega-3 can activate neuronal growth in the brain, enhancing cognitive and intelligence improvements (Healy-Stoffel & Levant al 2018). Omega-3 is essential in regulating the immune system, by increasing the synthesis of immune cells when immunity decreases and by reducing activity when the immune system is overactive (Zalina et al 2012; Partan et al 2018).

The great potential of *A. testidineus* fish oil must be accompanied by efforts and exploration of the extraction method of the oil. A better technique and method of extraction of *A. testidineus* oil will bring better fish oil production and Omega-3 content.

The fish oil extraction method using the oil refinement process extracts fish oil through degumming, neutralizing, bleaching and deodorization. The degumming separates the phospholipids; the neutralization reduces acidity; the bleaching removes colored material; the deodorization stage eliminates unpleasant odors (Bako et al 2017; Suseno et al 2017; Partan et al 2019).

This study aims to assess the efficacy of the oil refinement process in optimizing extracted fish oil quality. It is hoped that the optimal extraction method will be obtained for producing quality fish oil.

Material and Method

Fish sampling and processing. This study was conducted in Palembang, South Sumatra, Indonesia. About 5 kg of fish were obtained from the Palembang Fish Auction Center, Indonesia, in June 2020. *A. testudineus* were stored in a refrigerator at -25°C to minimize biochemical changes during fish transportation from the Auction Center to the Biotechnology Laboratory of the Faculty of Medicine, Sriwijaya University, Indonesia. The fish were grinded and stored at -25°C until use.

Fish oil extraction methods. The purification process involves 4 main stages: degumming (to separate phospholipids), neutralizing (to reduce acidity), bleaching (to remove colored matter) and deodorizing (to remove unwanted odor compounds). The method is described by Chakraborty & Joseph (2015), and it had a slight modification (Šimat et al 2019). The extracted crude oil (500 g) was mixed with phosphoric acid (5 mL). The mixture was stirred in a water bath heated to 70°C for 20 min. After cooling, samples were centrifuged (20 min at 4000 rpm) to remove degumming sap.

Neutralization was carried out by slowly adding (drop by drop) NaOH solution (1 M) to the defatted oil. The samples stirred and constantly heated at 65°C for 20 min. Neutralization was carried out until pH 7.0 was reached. The sample was then heated to 70°C for 20 min, cooled and centrifuged for 15 min at 4000 rpm. The obtained oil was washed with deionizing water (3 times with 10 mL), agitated (500 rpm) and heated at 50°C in vacuum. The neutralized oil samples were separated by centrifugation (10 min at 2500 rpm).

After neutralization, the oil obtained was bleached with an adsorbent (4 g per 100 g oil) containing 1.13 g activated carbon. The oil sample was stirred using a magnetic stirrer at 40°C for 40 min under N_2. After cooling, the oil samples were separated again by centrifugation (30 min at 3500 rpm). Deodorization of the bleached oil sample was carried out by distillation under vacuum. The oil mixture obtained after bleaching and washing with deionized water (20 mL) was heated to 95-97°C under vacuum for one hour, with continuous stirring. The refining process was repeated three times for each batch of oil.

Determination of yield. The yield was expressed as a percentage of oil separated from the fish. The yield was calculated as follows (Suseno et al 2013):

% yield = (weight of fish oil/weight of fish) x 100%

Neutral lipids evaluation. Total neutral lipids were determined using a HPLC system (Agilent 1200). Lipid separation was carried out at room temperature. The column used was a Lichrospher Diol 5 mm, 4x250 mm. The detection was carried out on an evaporative light scattering detector (Agilent 1200 series) at 45°C and a pressure of 3.5 bar. The mobile phase consisted of a mixture of solvents: (A) hexane/acetic acid (99.5/0.5 by volume) and (B) hexane/1-propanol/acetic acid/water (85/14.4/0.5/0.1 by volume).

The solvent gradient used was the following: first, solvent A flowed for 2 min; solvent B was added in three steps, up to 10% in 10 min, 44% in 15 min and 100% in 9 min. The stationary phase was rinsed with solvent A during 6 min. The total solvent flow rate was kept constant at 1 mL min^{-1}. Calibration was carried out using standards of

palmitic palmitate (99%), tripalmitin (>99%), dipalmitin (99%), monopalmitin (99%) and palmitic acid (99%) in hexane. The calibration curves showed a good correlation according to the exponential relationship described for an evaporative light scattering detector (Suseno et al 2013).

Determination of fatty acids. The determination of the fatty acid profile was carried out using the AOAC method. The fatty acid methyl esters were first prepared and then analyzed by gas chromatography (GC) in a Hewlett Packard gas chromatograph (6890N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionization detector (FID). The separation was carried out with helium (1.8 mL min^{-1}) as a carrier gas. A fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) was used. The column temperature was programmed starting at a constant temperature of 180°C during 20 min, heated to 200°C at 1°C min^{-1}, held at 200°C during 1 min, heated again to 220°C at 5°C min^{-1} and finally held at 220°C for 20 min. A split injector (50:1) at 250°C was used. The FID was also heated at 250°C. Most of the fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Co., Singapore). Their quantification was made by relating the peak area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (AOAC 2005). Calibration curves were made for each pair of internal standard and chromatographic standards to find the corresponding response factors.

Determination of acid value. The acidity value was determined according to the AOCS official methods Ca 5a-40 (AOCS 1990; Suseno et al 2013; Taati et al 2018).

Statistical analysis. The statistical analysis was performed using IBM SPSS 25 software. Data are presented as mean ±SD (standard deviation). Furthermore, a bivariate analysis was carried out to assess the differences using the t-test. Significance was set at $p < 0.05$.

Results and Discussion

Yield. The amount of obtained crude oil and oil after refinement is presented in Figure 1. The yield after refinement was higher than crude oil yield (after refinement: 10.6±1.22%; crude oil: 6.8±0.45%; $p < 0.05$).

Neutral lipids evaluation. Table 1 shows that the neutral lipids level has a significant difference between crude oil and refined oil. The levels of wax esters and free fatty acids were significantly lower after refinement than in crude oil. Meanwhile, the level of triacylglycerides in crude oil was significantly lower than in the refined oil. There is no significant difference between the two methods for cholesterol levels.

Free fatty acids profile. The analysis of the content of fatty acids showed no significant difference between the two types of oil (Table 2). However, the levels of EPA and DHA showed significant differences between the two extraction methods. EPA and DHA levels are higher in the refined oil than in crude oil.
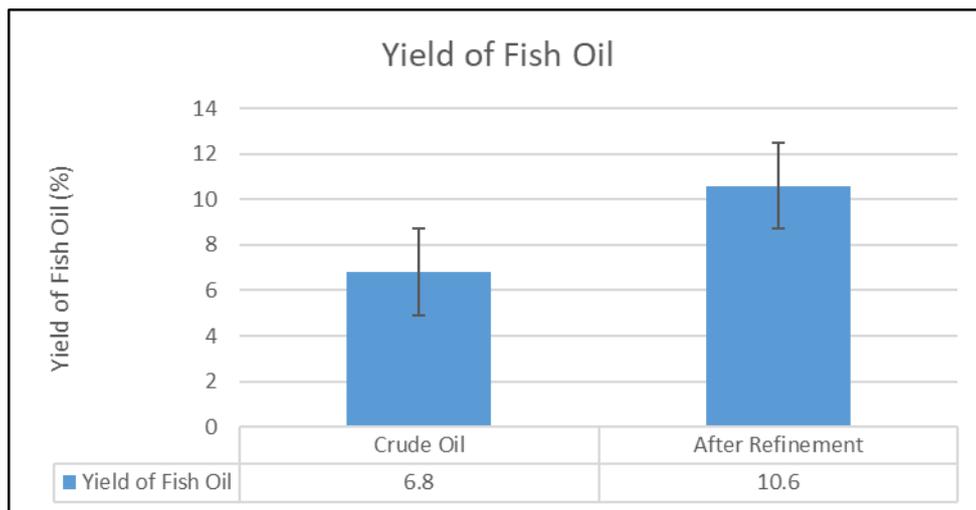
Figure 1. Yield (%) of *Anabas testudineus* crude and refined oil.

The levels of neutral lipids (%) of *Anabas testiduneus* in crude and refined oil

Components	Crude oil	Oil after refinement process
Wax esters (WE)	2.32±0.3	1.22±0.2*
Triacylglycerides (TAG)	94.21±3.02	89.32±4.34*
Free fatty acids (FFA)	3.56±0.32	4.54±1.12*
Cholesterol (CHOL)	2.21±2.01	2.34±2.02

Note: * - significant difference ($p < 0.05$).

The fatty acids (%) profile of *Anabas testudineus* crude oil and refined oil

Fatty acids	Crude oil	Oil after refinement
C12:0	0.09±0.01	0.05±0.03
C14:0	5.11±1.02	3.02±1.03
C15:0	0.87±0.2	0.89±0.35
C16:0	11.76±0.5	12.65±0.43
C16:1	6.31±2.4	6.45±3.32
C17:0	1.75±1.2	1.86±1.01
C18:0	5.19±2.54	3.89±2.94
C18:1c	16.23±4.56	16.89±5.54
C18:2c	1.29±1.54	1.82±1.54
C18:3 n6	1.09±1.14	1.59±1.21
C18:3 n3	1.86±0.04	2.19±0.02
C20:0	0.89±0.54	0.99±0.67
C20:1	0.59±0.44	0.79±0.43
C20:4 n3	1.85±0.89	1.89±0.87
C20:4 n6	1.95±0.98	1.96±0.77
C20:5 n3	4.19±2.99	4.29±3.54
C22:0	0.89±0.54	0.77±0.76
C22:5 n3	4.76±2.54	4.99±3.34
C22:6 n3	19.89±9.54	20.87±8.98
EPA and DHA	4.89±0.34	11.97±0.94*

Note: * - significant difference ($p < 0.05$).

Determination of acid value. Figure 2 shows a significant difference in acid value between crude oil and refined oil. The acid value in the refined oil is closer to the recommended rate than that of the crude oil (Deepika et al 2014).
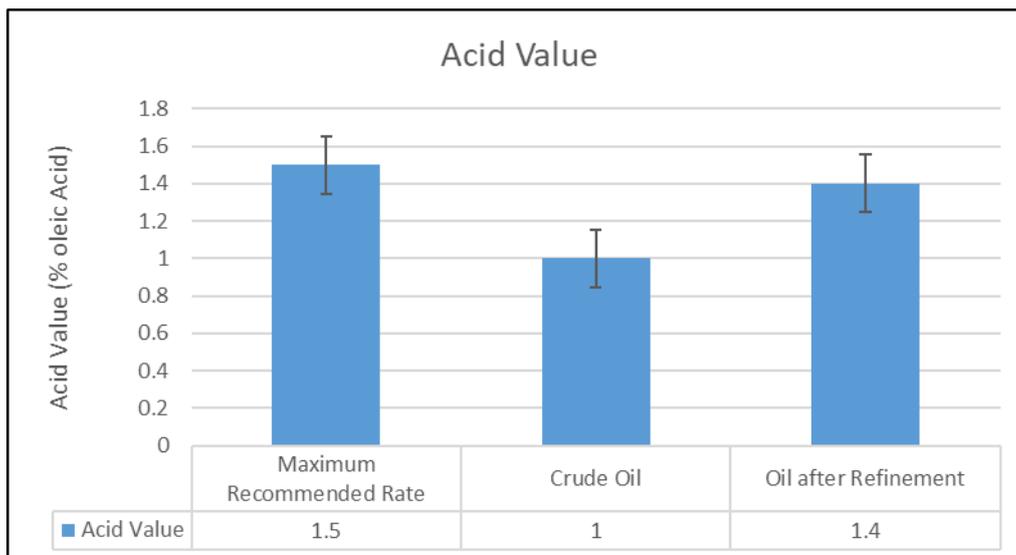
Figure 2. Acid value.

The fish oil production process is essential for exploration and optimization efforts, especially concerning the optimal fish oil extraction method. Fish oil is rich in essential fatty acids, which play a role in optimizing cell metabolism (Jannas-Vella et al 2017). Essential fatty acids are the main precursors in producing various hormones and growth factors, especially sex hormones, such as testosterone, estrogen and androgens (Collodel et al 2020; Komal et al 2020). EPA and DHA essential fatty acids play a role in maintaining cell membrane integrity to prevent cell damage and increase cell survival, predominantly for neuronal cells (Jannas-Vela et al 2017; Komal et al 2020). Thus, EPA and DHA are significant in increasing impulse transmission in nerve cells and in impacting improving cognitive function. Given the high potential and benefits of fish oil, efforts are needed to explore extraction methods to obtain fish oil of better quality and quantity (Ramakhrihnan et al 2013; Deepika et al 2014; Ivanovs & Blumberga 2017).

A. testudineus oil yield from the refining process is higher than that of crude oil. The refinement process separates the phospholipids, which are compounds that cause bonds between oil and tissue. The opening and separation of phospholipids results in opening of oil clumps and fat cells and in oil release and fluidity (Sarker et al 2012; Hajeb et al 2015; Bonilla-Méndez & Hoyos-Concha 2018; Wenwei et al 2018).

The neutral lipids analysis results in this study showed a significant difference in the wax esters (WE) (crude oil 2.32±0.3%; after refinement 1.22±0.2%), tryacyl glyceride (TAG) (crude oil 94.21±3.02%; after refinement 89.32±4.34%) and free fatty acid (FFA) levels (crude oil 3.56±0.32%; after refinement 4.54±1.12%) in the oil extracted from *A. testudineus*. Low wax esters levels and high triacylglyceride levels can indicate low intracellular fat levels and high levels of extracellular fat bound to proteins (Anggraini & Yunianta 2015). The weak binding of extracellular oil to protein causes an increase in fish oil extraction, followed by an increase in triacylglycerol and a decrease in WE. A high TAG level in oil indicates high levels of PUFA. On the other hand, the hydrolysis of TAG leads to the formation of FFAs. Therefore, higher levels of TAG and PUFA result in more FFA (Ahmed et al 2017; Nugraheni et al 2017; Rachmawati & Samidjan 2018).

This study shows that oil refinement can obtain higher EPA and DHA content than in crude oil. EPA and DHA are unsaturated fatty acids with double bonds. Crude oil tends to have many phospholipids, which cause a less optimal release of fish oil from fish tissue and muscle (Latip et al 2014; Anggraini & Yunianta 2015; Baehaki et al 2015).

The acidity value is an essential parameter in oil quality, influenced by FFA and other non-lipid acids, such as acetic acid. In general, oils containing high amounts of TAG and PUFA have high FFA levels, reducing acidity (Bruun et al 2021). In this study, the

TAG and FFA levels in the refined oil were higher than in crude oil. Therefore, the higher acidity value in the refined oil could occur due to the role of FFA (Suseno et al 2013).

Conclusions. It was concluded that the oil refinement process of *A. testudineus* fish oil in the extraction method is more effective, and elevates the quality of fish oil. The content of free fatty acids was increased significantly after the oil refinement process.

Conflict of Interest. The authors declare that there is no conflict of interest.

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