Correspondence
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Authors: RU Partan, Rachmat Hidayat, Eddy Mart Salim
Corresponding author: Rachmat Hidayat
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Optimizing *Anabas testudineus* Oil Processing by Oil Refinement Process on the Quality Parameters and Fatty Acid Composition of Fish Oil

Radiyati Umi Partan[1], Rachmat Hidayat[2*] , Eddy Mart Salim[1]

[1]Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, Palembang, Indonesia

[2]Department of Biology, Faculty of Medicine, Universitas Sriwijaya, Palembang, Indonesia

*Correspondence author

ABSTRACT

Anabas testiduneus, the climbing perch, is an endemic fish in Sumatra, Indonesia. This fish's uniqueness is that it contains much oil around its body. A large amount of oil in this fish can further explore the benefits, efficacy, and safety of its oil content. This study aims to assess the efficacy of the oil refinement process in optimizing extracted fish oil quality. About 5 kg of fresh fish were obtained from Palembang Auction Center. Fish oil extraction method consisted of purification process, which involves four main stages. They are degumming, neutralising, bleaching and deodorising. Total neutral lipids were determined using liquid chromatography. The acidity value was determined according AOCS official methods.Yield in after refinement was higher than crude oil methods ($p<0.05$). The levels of wax esters and free fatty acids were significantly lower after refinement than crude oil. Meanwhile, the level of triacylglycerides in crude oil is significantly lower than after refinement oil. In conclusion, oil refinement of A. *testudineus* fish oil extraction method is more effective compared to crude oil. It is not only in terms of oil quality, but also in quantity of fish oil.

Key words: fish oil, *Anabas testudineus*, oil purification, oil refinement.

Introduction

Fish is a food source that is rich in protein and very abundant in Indonesia. Fish can be processed into various foods that are rich in protein and have optimal nutritional content. South Sumatra is one of the provinces in Indonesia, where many rivers flow in the area. This causes this area to be very abundant in freshwater fish. The abundance of freshwater fish encourages many local foods in South Sumatra are made from freshwater fish, including *pempek* (fish cake), *pindang* (spicy fish soup) and *bekasam* (fish fermentation) (Partan et al., 2017).

Anabas testiduneus (Bloch, 1792), or locally known as Betok fish, is an endemic fish in Sumatra. Its shape is oval with a large and flathead. Its body is covered with green scales on the back and shiny white on the belly. *A. testiduneus* are found in rivers, lakes, swamps, puddles and can also eat small shrimp, small fish and other aquatic animals (Zalina et al., 2012). It has long dorsal fins, starting from the neck to the front of the base of the tail fin, 16-19 hard spiked fingers support the front: the back is shorter than the front; supported by 7 - 10 soft fingers. The anal fin is short; instead of the dorsal fin, the front is supported by 9 to 11 hard and sharp fingers, and 8 to 11 soft fingers support the back. The pectoral fins do not have stiff fingers, are supported by 14-16 soft fingers. It is located more on the body behind the gill covers. The pelvic fins are located in the front, below the pectoral fins, supported by hard pointed fingers and five soft fingers. The hard fingers of the pelvic fins may be displaced and can move around the dry mud surface. The bases of the pectoral, caudal, dorsal and anal fins are soft, all containing muscles and covered with small scales. These fish are generally fried or stewed. This fish's uniqueness is that it contains much oil around its body; even the water used to wash this fish will become very oily. A large amount of oil in this fish can further explore the benefits, efficacy, and safety of *A. testiduneus* oil content (Partan et al., 2017).

Previous research on *A. testudineus* oil has shown that *A. testiduneus* fish oil is rich in omega-3 (Partan et al., 2017). Omega-3 has the potential to inhibit cellular inflammatory processes, where inflammation is believed to play a role in the initiation of various degenerative health disorders, both cardiovascular (acute coronary syndrome) and endocrine (diabetes mellitus and dyslipidemia). Also, omega-3 can activate neuronal growth in the brain to enhance cognitive and intelligence improvements. Omega-3 is equally essential in regulating the immune system (body defence against various diseases). Omega-3 can regulate the immune system to increase the synthesis of immune cells when immunity decreases and reduces immune system activity when the immune system is overactive (Zalina et al., 2012; Partan et al., 2018).

The great potential of *A. testudineus* fish oil, of course, must be accompanied by efforts and exploration of the extraction method of fish oil. The better the technique and method of extraction of *A. testudineus* fish oil, the better fish oil production and omega-3 content will be. The fish oil extraction method using the oil refinement process is a method of extracting fish oil through degumming, neutralizing, bleaching and deodorization. The degumming stage is the stage

for separating the phospholipids; the neutralization stage is a step to reduce acidity, the bleaching stage is a step for removing coloured material, the deodorization stage is a step for eliminating unpleasant odours (Partan et al., 2019; Suseno et al., 2017; Bako et al., 2017).

This study aims to assess the efficacy of the oil refinement process in optimizing extracted fish oil quality. This research is the first research conducted to optimize the extraction method from *A. testudineus* fish oil. It is hoped that the optimal extraction method will be obtained in producing quality fish oil.

Material and Methods

Fish sampling and processing. About 5 kg of fish were obtained from the Palembang Fish Auction Center, South Sumatra, Indonesia in June 2020. *A. testudineus* fish are stored in a refrigerator -25^{o}C to minimise biochemical changes during fish transportation from the Auction Center to the Biotechnology Laboratory of the Faculty of Medicine, Universitas Sriwijaya, Indonesia. Furthermore, the whole of fishes was grinded and stored under -25^{0}C until used.

Fish oil extraction methods. The purification process, which involves four main stages: degumming (to separate phospholipids), neutralising (to reduce acidity), bleaching (to remove coloured matter) and deodorising (to remove unwanted odour compounds), described by Chakraborty and Joseph (2015) with a slight modification (Šimat et al., 2019) was used. 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 minutes. Samples after cooling were centrifuged (20 minutes, 4000 rpm) to remove degumming sap.

Neutralisation was carried out by slowly adding (drop by drop) NaOH solution (1 M) to the defatted oil. The samples were in combination with stirring and constant heating at 65◦C for 20 minutes. Neutralisation, the process was carried out until pH 7.0 was reached, and the sample was then heated to 70◦C for 20 minutes, cooled and centrifuged for 15 minutes at 4000 rpm. The obtained oil was washed by deionising water (3 times with 10 mL) with agitation (500 rpm) and heating at 50◦C in vacuum. The neutralised oil samples were separated by centrifugation (10 minutes, 2500 rpm).

After neutralisation, the oil obtained was carried out by beaching with an adsorbent (4 g/ 100 g oil) containing 1.13 g activated carbon. The oil sample was stirred using a magnetic stirrer

at 40^0C for 40 minutes under N2. After cooling, the oil samples were separated again by centrifugation (30 minutes, 3500 rpm). Deodorization of the oil sample bleach was carried out by distillation under vacuum. The oil mixture obtained after bleaching and deionised water (20 mL) was heated to 95-97^0C under vacuum for one hour, with continuous stirring. The refining process is repeated three times for each batch of oil.

Determination of yield. Yield was expressed as percentage of oil separated by the whole fish. 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 liquid chromatography on the HPLC system (Agilent 1200). Lipid separation was carried out at room temperature on the column (Lichrospher Diol 5 mm, 4x250 mm). The detection was carried out on an evaporative light scattering detector (Agilent 1200 series) at 45^0C and a pressure of 3.5 bar. The mobile phase consists 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 as follow: first, solvent A was flowing for 2 minutes, after that, solvent B was added in three steps, up to 10% in 10 minutes, to 44% in 15 minutes and 100% in 9 minute. The stationary phase was rinsed with solvent A during 6 min. The total solvent flow rate was kept constant at 1 mL/minute all along with the analysis. Calibration was carried out using standards of palmitil 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/minute, held at 200°C during 1 min, heated again to 220°C at 5°C/minute and finally held at 220°C for 20 minutes. 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.). Their quantification was made by relating the peak area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (2005). Calibration curves were made for each pair of internal standard and chromatographic standards to find the corresponding response factors [9].

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; Al-Mentafji, 2016).

Statistical analysis. Statistical analysis was performed using IBM SPSS 25 software. Data are presented as Mean ± SD (Standard Deviation). Furthermore, a bivariate analysis was carried out using the t-test, to assess the differences in mean levels and crude oil content and after the oil refinement process. Significance was set at $p<0.05$.

Results

Yield. The amount of obtained crude oil VS oil after the refinement process is shown in figure 1. Yield in after refinement was higher than crude oil methods, $p<0.05$.

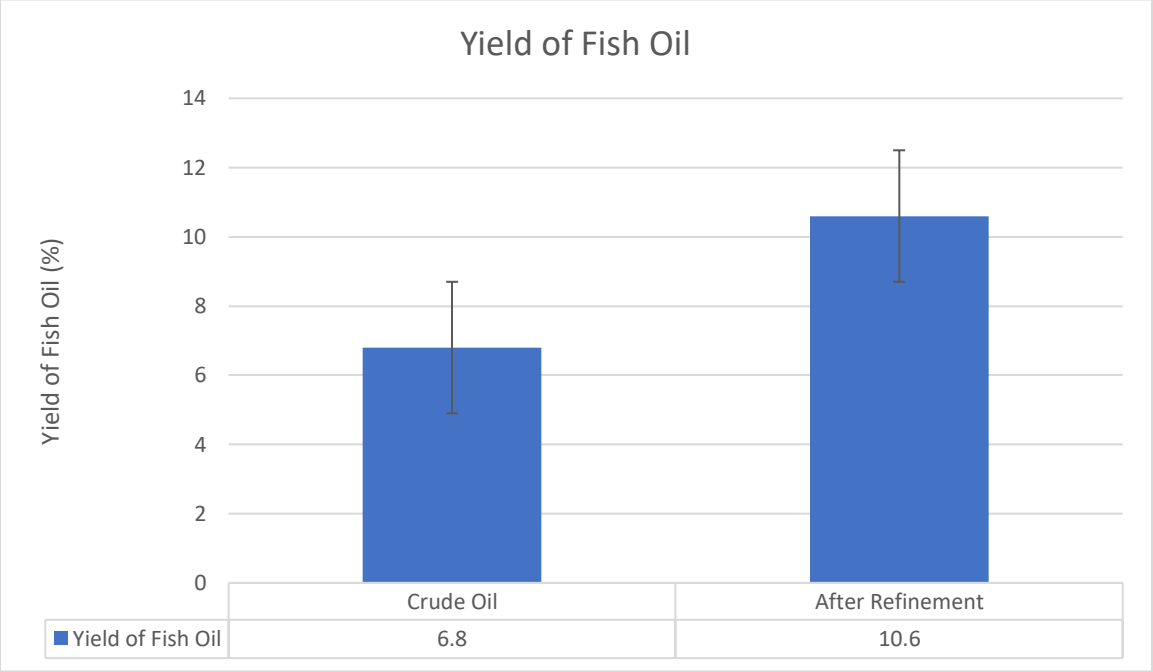

Figure 1. Yield (%) of obtained *A. testudineus* crude oil and after refinement process

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

Table 1. The levels of neutral lipids (%) in *A. testiduneus* oil in crude and after refinement process

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

*Paired t-test, $p < 0,05$ versus crude oil.

Free fatty acids profile. Analysis of the content of fatty acids showed no significant difference in the content of fatty acids of the two types of oil. However, the levels of EPA and DHA showed significant differences between the two extraction methods. EPA and DHA levels are higher in the after refinement process oil than crude oil.

Table 3. The fatty acids profile in *A. testudineus* crude oil dan oil after refinement process

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*

*Paired t-test, p< 0,05 versus crude oil

Determination of acid value. Figure 2 shows a significant difference in acid value between crude oil and oil after the refinement process. Acid value in the after refinement process is almost closer to the recommended rate than crude oil.

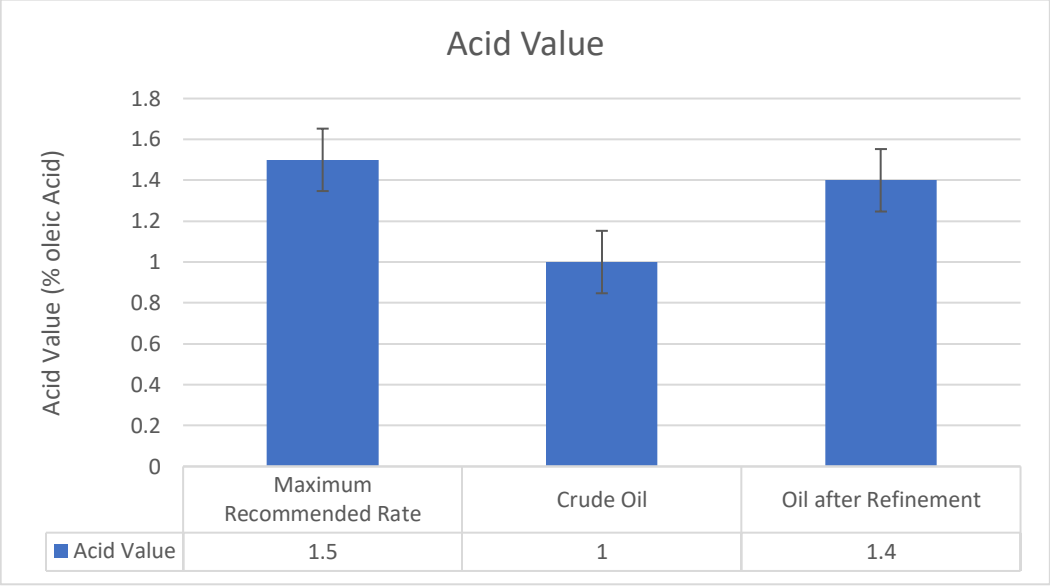

Figure 2. Acid Value Determination

Discussions

The fish oil production process is crucial 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 cells' metabolism to improve health. Essential fatty acids are the main precursors in producing various hormones and growth factors, especially sex hormones, such as testosterone, estrogen and androgens. EPA and DHA essential fatty acids play a role in maintaining cell membrane integrity to prevent damage to cells and increase cells' survival, predominantly neuronal cells. So that EPA and DHA are significant in increasing impulse transmission in nerve cells and impacting improving cognitive function. Given the enormous potential and benefits of fish oil, optimal efforts are needed to explore extraction methods, in order to obtain fish oil with optimal quantity and quality (Ramakhrihnan et al., 2013; Ivanovs et al., 2017; Deepika et al., 2014).

A. testudineus fish oil yield from the after refinement process shows a higher yield than crude oil. The refinement process separates the phospholipids, where the phospholipids are compounds that cause bonds between oil and tissue and fish meat. The opening and separation of the phospholipid result in the oil clumps and fat cells' opening, resulting in the oil's release and fluidity (Bonilla-Méndez et al., 2018; Wenwei et al., 2018; Sarker et al., 2012; Hajeb et al., 2015).

The neutral lipids analysis results in this study showed a significant difference in the wax esters (WE), tryacyl glyceride (TAG) and free fatty acid (FFA) levels in the oil extracted from *A. testudineus* fish in crude oil and after refinement (Latip et al., 2014; Baehaki et al., 2015). Low wax esters levels and high triacylglyceride levels can indicate low intracellular fat levels and high levels of extracellular fat bound to protein in meat (Anggraini et al., 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 in fish oil indicates high PUFAs in fish oil. 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 (Rachmawati et al., 2018; Ahmed et al., 2017; Nugraheni et al., 2017).

This study shows that oil refinement can obtain higher EPA and DHA content than crude oil. EPA and DHA is an unsaturated fatty acid with double bonds, where 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; Baehaki et al., 2015; Anggraini et al., 2015).

The acidity value is an essential parameter in oil quality, which is 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, which can reduce acidity. In this study, the TAG and FFA levels in oil refinement were higher than crude oil. Therefore, the higher acidity value in oil refinement methods is due to the role of FFA (Suseno et al., 2013).

Conclusion

Oil refinement of A. *testudineus* fish oil extraction method is more effective compared to crude oil. It is not only in terms of oil quality, but also in quantity of fish oil.

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Corresponding author:

Rachmat Hidayat, M.D., M.Sc.

Department of Biology, Faculty of Medicine, Universitas Sriwijaya, Indonesia

Address: Jl Dr. Moh Ali KM 3.5 Palembang, Indonesia, 30662

E-mail: dr.rachmat.hidayat@gmail.com

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Name of the authors: Radiyati Umi Partan, Rachmat Hidayat, Eddy Mart Salim

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Corresponding author

Name : Rachmat Hidayat

Signature: 

Date: January 5, 2021

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Radiyati Umi Partan[1], Rachmat Hidayat[2*] , Eddy Mart Salim[1]

[1]Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, Palembang, Indonesia

[2]Department of Biology, Faculty of Medicine, Universitas Sriwijaya, Palembang, Indonesia

*Correspondence author

ABSTRACT

Anabas testiduneus, the climbing perch, is an endemic fish in Sumatra, Indonesia. This fish's uniqueness is that it contains much oil around its body. A large amount of oil in this fish can further explore the benefits, efficacy, and safety of its oil content. This study aims to assess the efficacy of the oil refinement process in optimizing extracted fish oil quality. About 5 kg of fresh fish were obtained from Palembang Auction Center. Fish oil extraction method consisted of purification process, which involves four main stages. They are degumming, neutralising, bleaching and deodorising. Total neutral lipids were determined using liquid chromatography. The acidity value was determined according AOCS official methods.Yield in after refinement was higher than crude oil methods ($p<0.05$). The levels of wax esters and free fatty acids were significantly lower after refinement than crude oil. Meanwhile, the level of triacylglycerides in crude oil is significantly lower than after refinement oil. In conclusion, oil refinement of A. *testudineus* fish oil extraction method is more effective compared to crude oil. It is not only in terms of oil quality, but also in quantity of fish oil.

Key words: fish oil, *Anabas testudineus*, oil purification, oil refinement.

Introduction

Fish is a food source that is rich in protein and very abundant in Indonesia. Fish can be processed into various foods that are rich in protein and have optimal nutritional content. South Sumatra is one of the provinces in Indonesia, where many rivers flow in the area. This causes this area to be very abundant in freshwater fish. The abundance of freshwater fish encourages many local foods in South Sumatra are made from freshwater fish, including *pempek* (fish cake), *pindang* (spicy fish soup) and *bekasam* (fish fermentation) (Partan et al., 2017).

Anabas testiduneus (Bloch, 1792), or locally known as Betok fish, is an endemic fish in Sumatra. Its shape is oval with a large and flathead. Its body is covered with green scales on the back and shiny white on the belly. *A. testiduneus* are found in rivers, lakes, swamps, puddles and can also eat small shrimp, small fish and other aquatic animals (Zalina et al., 2012). It has long dorsal fins, starting from the neck to the front of the base of the tail fin, 16-19 hard spiked fingers support the front: the back is shorter than the front; supported by 7 - 10 soft fingers. The anal fin is short; instead of the dorsal fin, the front is supported by 9 to 11 hard and sharp fingers, and 8 to 11 soft fingers support the back. The pectoral fins do not have stiff fingers, are supported by 14-16 soft fingers. It is located more on the body behind the gill covers. The pelvic fins are located in the front, below the pectoral fins, supported by hard pointed fingers and five soft fingers. The hard fingers of the pelvic fins may be displaced and can move around the dry mud surface. The bases of the pectoral, caudal, dorsal and anal fins are soft, all containing muscles and covered with small scales. These fish are generally fried or stewed. This fish's uniqueness is that it contains much oil around its body; even the water used to wash this fish will become very oily. A large amount of oil in this fish can further explore the benefits, efficacy, and safety of *A. testiduneus* oil content (Partan et al., 2017).

Previous research on *A. testudineus* oil has shown that *A. testiduneus* fish oil is rich in omega-3 (Partan et al., 2017). Omega-3 has the potential to inhibit cellular inflammatory processes, where inflammation is believed to play a role in the initiation of various degenerative health disorders, both cardiovascular (acute coronary syndrome) and endocrine (diabetes mellitus and dyslipidemia). Also, omega-3 can activate neuronal growth in the brain to enhance cognitive and intelligence improvements. Omega-3 is equally essential in regulating the immune system (body defence against various diseases). Omega-3 can regulate the immune system to increase the synthesis of immune cells when immunity decreases and reduces immune system activity when the immune system is overactive (Zalina et al., 2012; Partan et al., 2018).

The great potential of *A. testudineus* fish oil, of course, must be accompanied by efforts and exploration of the extraction method of fish oil. The better the technique and method of extraction of *A. testudineus* fish oil, the better fish oil production and omega-3 content will be. The fish oil extraction method using the oil refinement process is a method of extracting fish oil through degumming, neutralizing, bleaching and deodorization. The degumming stage is the stage

for separating the phospholipids; the neutralization stage is a step to reduce acidity, the bleaching stage is a step for removing coloured material, the deodorization stage is a step for eliminating unpleasant odours (Partan et al., 2019; Suseno et al., 2017; Bako et al., 2017).

This study aims to assess the efficacy of the oil refinement process in optimizing extracted fish oil quality. This research is the first research conducted to optimize the extraction method from *A. testudineus* fish oil. It is hoped that the optimal extraction method will be obtained in producing quality fish oil.

Material and Methods

Fish sampling and processing. About 5 kg of fish were obtained from the Palembang Fish Auction Center, South Sumatra, Indonesia in June 2020. *A. testudineus* fish are stored in a refrigerator -25^{o}C to minimise biochemical changes during fish transportation from the Auction Center to the Biotechnology Laboratory of the Faculty of Medicine, Universitas Sriwijaya, Indonesia. Furthermore, the whole of fishes was grinded and stored under -25^{0}C until used.

Fish oil extraction methods. The purification process, which involves four main stages: degumming (to separate phospholipids), neutralising (to reduce acidity), bleaching (to remove coloured matter) and deodorising (to remove unwanted odour compounds), described by Chakraborty and Joseph (2015) with a slight modification (Šimat et al., 2019) was used. 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 minutes. Samples after cooling were centrifuged (20 minutes, 4000 rpm) to remove degumming sap.

Neutralisation was carried out by slowly adding (drop by drop) NaOH solution (1 M) to the defatted oil. The samples were in combination with stirring and constant heating at 65◦C for 20 minutes. Neutralisation, the process was carried out until pH 7.0 was reached, and the sample was then heated to 70◦C for 20 minutes, cooled and centrifuged for 15 minutes at 4000 rpm. The obtained oil was washed by deionising water (3 times with 10 mL) with agitation (500 rpm) and heating at 50◦C in vacuum. The neutralised oil samples were separated by centrifugation (10 minutes, 2500 rpm).

After neutralisation, the oil obtained was carried out by beaching with an adsorbent (4 g/ 100 g oil) containing 1.13 g activated carbon. The oil sample was stirred using a magnetic stirrer

at 40^0C for 40 minutes under N2. After cooling, the oil samples were separated again by centrifugation (30 minutes, 3500 rpm). Deodorization of the oil sample bleach was carried out by distillation under vacuum. The oil mixture obtained after bleaching and deionised water (20 mL) was heated to 95-97^0C under vacuum for one hour, with continuous stirring. The refining process is repeated three times for each batch of oil.

Determination of yield. Yield was expressed as percentage of oil separated by the whole fish. 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 liquid chromatography on the HPLC system (Agilent 1200). Lipid separation was carried out at room temperature on the column (Lichrospher Diol 5 mm, 4x250 mm). The detection was carried out on an evaporative light scattering detector (Agilent 1200 series) at 45^0C and a pressure of 3.5 bar. The mobile phase consists 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 as follow: first, solvent A was flowing for 2 minutes, after that, solvent B was added in three steps, up to 10% in 10 minutes, to 44% in 15 minutes and 100% in 9 minute. The stationary phase was rinsed with solvent A during 6 min. The total solvent flow rate was kept constant at 1 mL/minute all along with the analysis. Calibration was carried out using standards of palmitil 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/minute, held at 200°C during 1 min, heated again to 220°C at 5°C/minute and finally held at 220°C for 20 minutes. 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.). Their quantification was made by relating the peak area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (2005). Calibration curves were made for each pair of internal standard and chromatographic standards to find the corresponding response factors [9].

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; Al-Mentafji, 2016).

Statistical analysis. Statistical analysis was performed using IBM SPSS 25 software. Data are presented as Mean ± SD (Standard Deviation). Furthermore, a bivariate analysis was carried out using the t-test, to assess the differences in mean levels and crude oil content and after the oil refinement process. Significance was set at $p<0.05$.

Results

Yield. The amount of obtained crude oil VS oil after the refinement process is shown in figure 1. Yield in after refinement was higher than crude oil methods, $p<0.05$.

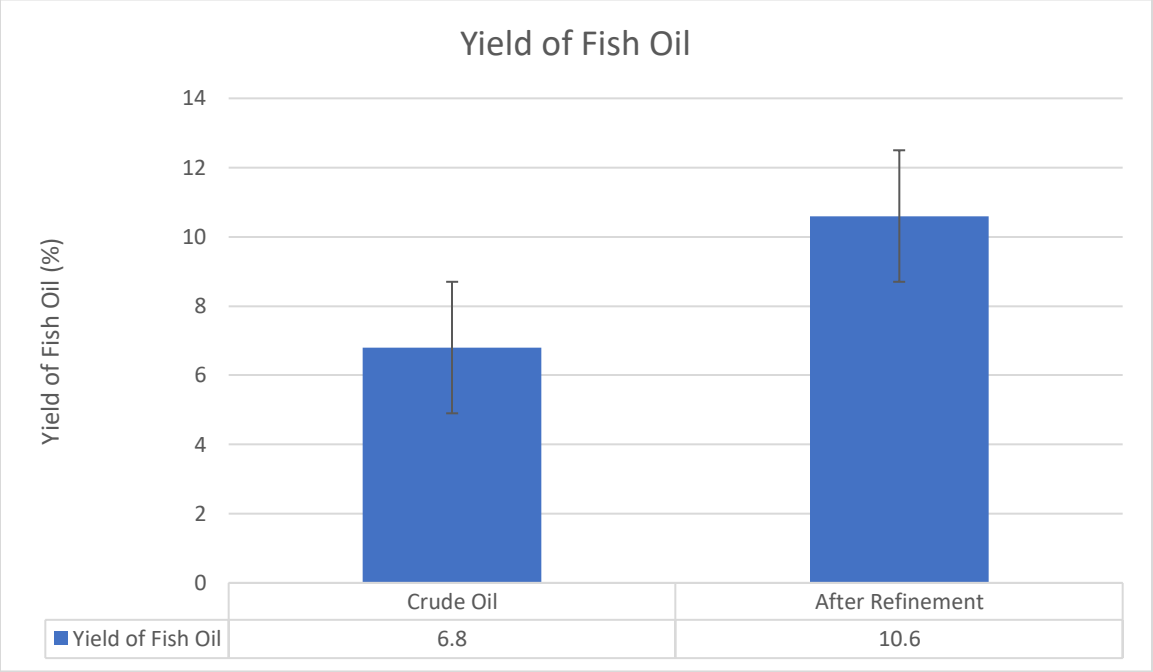

Figure 1. Yield (%) of obtained *A. testudineus* crude oil and after refinement process

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

Table 1. The levels of neutral lipids (%) in *A. testiduneus* oil in crude and after refinement process

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

*Paired t-test, $p < 0,05$ versus crude oil.

Free fatty acids profile. Analysis of the content of fatty acids showed no significant difference in the content of fatty acids of the two types of oil. However, the levels of EPA and DHA showed significant differences between the two extraction methods. EPA and DHA levels are higher in the after refinement process oil than crude oil.

Table 3. The fatty acids profile in *A. testudineus* crude oil dan oil after refinement process

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*

*Paired t-test, p< 0,05 versus crude oil

Determination of acid value. Figure 2 shows a significant difference in acid value between crude oil and oil after the refinement process. Acid value in the after refinement process is almost closer to the recommended rate than crude oil.

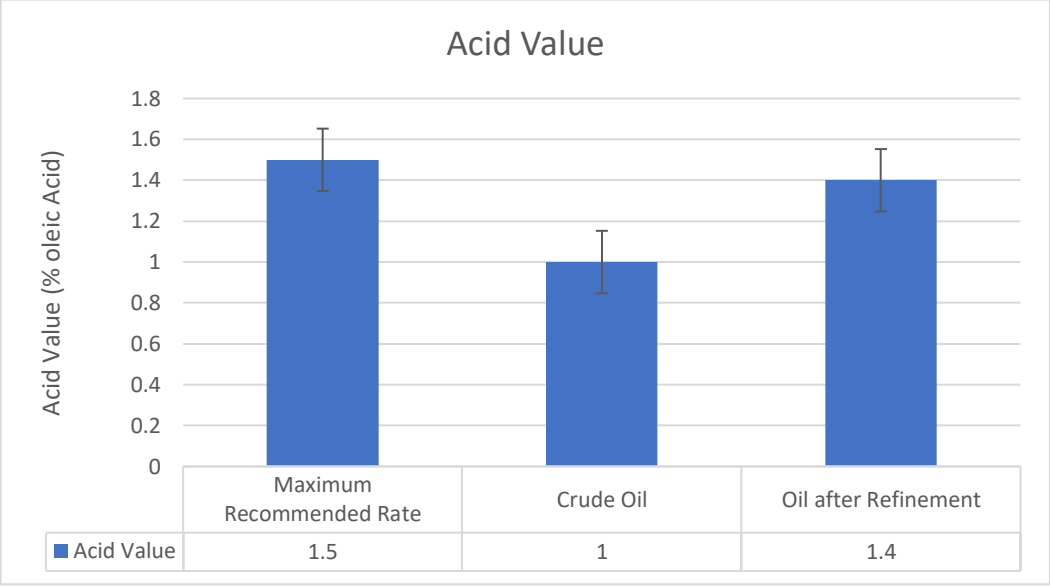

Figure 2. Acid Value Determination

Discussions

The fish oil production process is crucial 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 cells' metabolism to improve health. Essential fatty acids are the main precursors in producing various hormones and growth factors, especially sex hormones, such as testosterone, estrogen and androgens. EPA and DHA essential fatty acids play a role in maintaining cell membrane integrity to prevent damage to cells and increase cells' survival, predominantly neuronal cells. So that EPA and DHA are significant in increasing impulse transmission in nerve cells and impacting improving cognitive function. Given the enormous potential and benefits of fish oil, optimal efforts are needed to explore extraction methods, in order to obtain fish oil with optimal quantity and quality (Ramakhrihnan et al., 2013; Ivanovs et al., 2017; Deepika et al., 2014).

A. testudineus fish oil yield from the after refinement process shows a higher yield than crude oil. The refinement process separates the phospholipids, where the phospholipids are compounds that cause bonds between oil and tissue and fish meat. The opening and separation of the phospholipid result in the oil clumps and fat cells' opening, resulting in the oil's release and fluidity (Bonilla-Méndez et al., 2018; Wenwei et al., 2018; Sarker et al., 2012; Hajeb et al., 2015).

The neutral lipids analysis results in this study showed a significant difference in the wax esters (WE), tryacyl glyceride (TAG) and free fatty acid (FFA) levels in the oil extracted from *A. testudineus* fish in crude oil and after refinement (Latip et al., 2014; Baehaki et al., 2015). Low wax esters levels and high triacylglyceride levels can indicate low intracellular fat levels and high levels of extracellular fat bound to protein in meat (Anggraini et al., 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 in fish oil indicates high PUFAs in fish oil. 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 (Rachmawati et al., 2018; Ahmed et al., 2017; Nugraheni et al., 2017).

This study shows that oil refinement can obtain higher EPA and DHA content than crude oil. EPA and DHA is an unsaturated fatty acid with double bonds, where 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; Baehaki et al., 2015; Anggraini et al., 2015).

The acidity value is an essential parameter in oil quality, which is 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, which can reduce acidity. In this study, the TAG and FFA levels in oil refinement were higher than crude oil. Therefore, the higher acidity value in oil refinement methods is due to the role of FFA (Suseno et al., 2013).

Conclusion

Oil refinement of A. *testudineus* fish oil extraction method is more effective compared to crude oil. It is not only in terms of oil quality, but also in quantity of fish oil.

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Corresponding author:

Rachmat Hidayat, M.D., M.Sc.

Department of Biology, Faculty of Medicine, Universitas Sriwijaya, Indonesia

Address: Jl Dr. Moh Ali KM 3.5 Palembang, Indonesia, 30662

E-mail: dr.rachmat.hidayat@gmail.com

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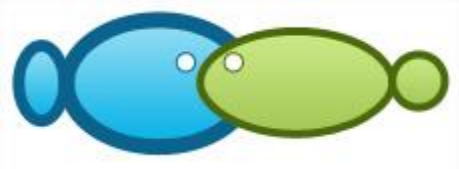

Optimizing *Anabas testudineus* oil processing by refinement process for quality parameters and fatty acid composition

[1]Radiyati U. Partan, [2]Rachmat Hidayat, [1]Eddy M. Salim

[1] Department of Internal Medicine, Faculty of Medicine, Sriwijaya University, Palembang, Indonesia; [2] Department of Biology, Faculty of Medicine, Sriwijaya University, Palembang, Indonesia. Correspondent author: R. Hidayat, dr.rachmat.hidayat@gmail.com

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 researchers to explore 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. testudineus* is better than the crude oil.
Key Words: *Anabas testudineus*, 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. testudineus* 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 et 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. testudineus* 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. testudineus* 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; Al-Mentafji 2016; 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 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; $p<0.05$).

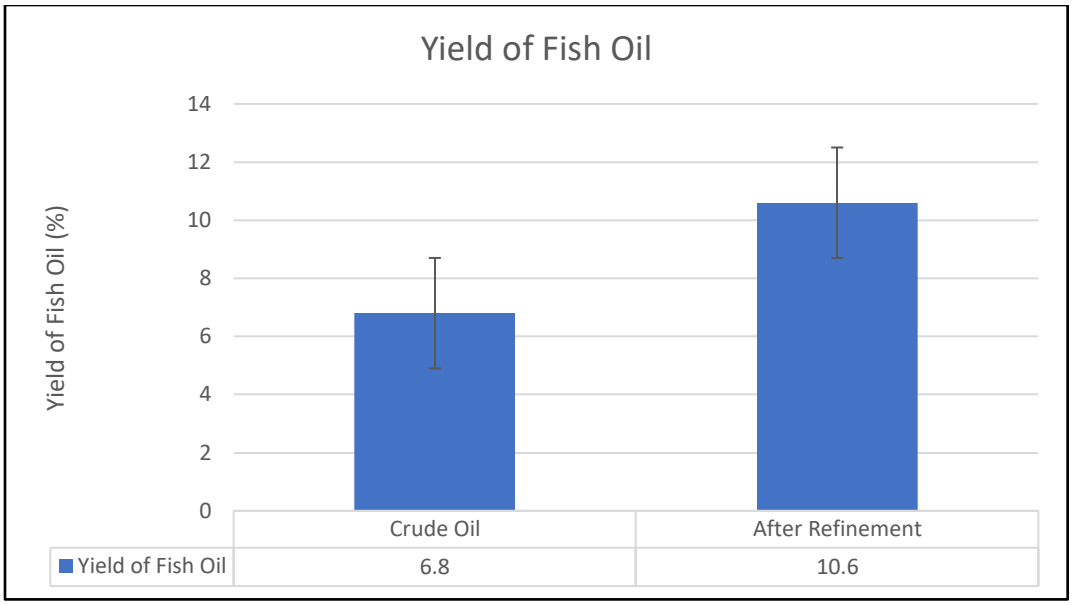

Figure 1. Yield (%) of *Anabas testudineus* crude and refined oil.

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.

Table 1

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 ($p<0.05$).

Free fatty acids profile. The analysis of the content of fatty acids showed no significant difference between the two types of oil. 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.

Table 2

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).

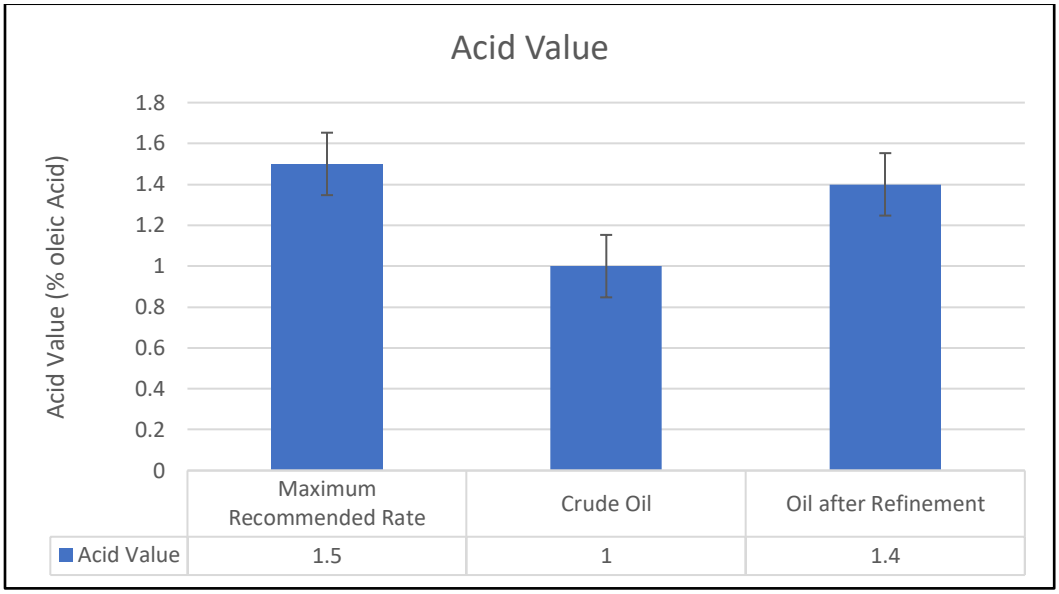

Figure 2. Acid value.

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 et al 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, resulting in the oil's release and fluidity (Sarker et al 2012; Hajeb et al 2015; Bonilla-Méndez et al 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* oil. Low wax esters levels and high triacylglyceride levels can indicate low intracellular fat levels and high levels of extracellular fat bound to proteins (Anggraini et al 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 et al 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 et al 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 oil refinement process of *A. testudineus* fish oil extraction method is more effective in elevated the quality of fish oil. The content of free fatty acid was increased 27,5% significantly after oil refinement process.

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

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Authors:
Radiyati Umi Partan, Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia, e-mail: andy.alfatih20@gmail.com
Rachmat Hidayat, Department of Biology, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia, e-mail: dr.rachmat.hidayat@gmail.com
Eddy Mart Salim, Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia, e-mail: mgs.irsan.saleh@gmail.com

Tudor Papuc <ptudor2008@yahoo.com>
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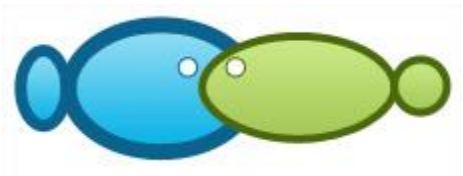

Optimizing *Anabas testudineus* Oil Processing by Oil Refinement Process on the Quality Parameters and Fatty Acid Composition of Fish Oil

[1]Radiyati U. Partan, [2]Rachmat Hidayat , [1]Eddy M. Salim

[1]Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia; [2]Department of Biology, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia. Correspondence author: Rachmat Hidayat, dr.rachmat.hidayat@gmail.com

Abstract. *Anabas testiduneus,* the climbing perch, is an endemic fish in Sumatra, Indonesia. This fish's uniqueness is that it contains much oil around its body. A large amount of oil in this fish can further explore its oil content's benefits, efficacy, and safety. This study aims to assess the efficacy of the oil refinement process in optimising extracted fish oil quality. About 5 kg of fresh fish were obtained from Palembang Auction Center. Fish oil extraction method consisted of a purification process, which involves four main stages. They are degumming, neutralising, bleaching and deodorising. 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 crude oil methods ($p<0.05$). The levels of wax esters and free fatty acids were significantly lower after refinement than crude oil. Meanwhile, the level of triacylglycerides in crude oil is significantly lower than after refinement oil. In conclusion, the oil refinement of A. testudineus fish oil extraction method is more effective than crude oil.

Key Words: fish oil, *Anabas testudineus*, oil purification, oil refinement.

Introduction. Fish is a food source that is rich in protein and very abundant in Indonesia. Fish can be processed into various foods that are rich in protein and have optimal nutritional content. South Sumatra is one of the provinces in Indonesia, where many rivers flow in the area. This causes this area to be very abundant in freshwater fish. The abundance of freshwater fish encourages many local foods in South Sumatra are made from it, including *pempek* (fish cake), *pindang* (spicy fish soup) and *bekasam* (fish fermentation) (Partan et al 2017).

Anabas testiduneus (*Anabantidae* family), or locally known as Betok fish, is a local fish in Sumatra. Its shape is oval with a large and flathead. Its body is covered with green scales on the back and shiny white on the belly. *A. testiduneus* are found in rivers, lakes, swamps, puddles and can also eat small shrimp, small fish and other aquatic animals (Zalina et al 2012). It has long dorsal fins, starting from the neck to the front of the base of the tail fin, 16-19 strong spiked fingers support the front: the back is shorter than the front; supported by 7 - 10 soft fingers. The anal fin is short; instead of the dorsal fin, the front is supported by 9 to 11 hard and sharp fingers, and 8 to 11 soft fingers support the back. The pectoral fins do not have stiff fingers, are supported by 14-16 soft fingers. It is located more on the body behind the gill covers. The pelvic fins are located in the front, below the pectoral fins, supported by strong pointed fingers and five soft fingers. The strong fingers of the pelvic fins may be displaced and can move around the dry mud surface. The bases of the pectoral, caudal, dorsal and anal fins are soft, all containing muscles and covered with small scales. These fish are generally fried or stewed. This fish's uniqueness is that it contains much oil around its body; even the water used to wash this fish will become very oily. A large amount

of oil in this fish can further explore the benefits, efficacy, and safety of *A. testiduneus* oil content (Partan et al 2017).

Previous research on *A. testudineus* oil has shown that its oil is rich in omega-3 (Partan et al 2017). Omega-3 has the potential to inhibit cellular inflammatory processes, where inflammation is believed to play a role in the initiation of various degenerative health disorders, both cardiovascular (acute coronary syndrome) and endocrine (diabetes mellitus and dyslipidemia). Also, omega-3 can activate neuronal growth in the brain to enhance cognitive and intelligence improvements. Omega-3 is equally essential in regulating the immune system (body defence against various diseases). Omega-3 can regulate the immune system to increase immune cells' synthesis when immunity decreases and reduces immune system activity when the immune system is overactive (Zalina et al 2012; Partan et al 2018).

The great potential of *A. testudineus* fish oil, of course, must be accompanied by efforts and exploration of the extraction method of fish oil. The better the technique and method of extraction of *A. testudineus* fish oil, the better fish oil production and omega-3 content will be. The fish oil extraction method using the oil refinement process extracts fish oil through degumming, neutralizing, bleaching and deodorization. The degumming stage is the stage for separating the phospholipids; the neutralization stage is a step to reduce acidity, the bleaching stage is a step for removing coloured material, the deodorization stage is a step for eliminating unpleasant odours (Partan et al 2019; Suseno et al 2017; Bako et al 2017).

This study aims to assess the efficacy of the oil refinement process in optimizing extracted fish oil quality. This research is the first research conducted to optimize the extraction method from *A. testudineus* fish oil. It is hoped that the optimal extraction method will be obtained in producing quality fish oil.

Material and Methods

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 fish are stored in a refrigerator -25oC to minimise biochemical changes during fish transportation from the Auction Center to the Biotechnology Laboratory of the Faculty of Medicine, Universitas Sriwijaya, Indonesia. Furthermore, the whole of fishes was grinded and stored under -250C until used.

Fish oil extraction methods. The purification process, which involves four main stages: degumming (to separate phospholipids), neutralising (to reduce acidity), bleaching (to remove coloured matter) and deodorising (to remove unwanted odour compounds), described by Chakraborty and Joseph (2015) with a slight modification (Šimat et al 2019) was used. 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 minutes. Samples after cooling were centrifuged (20 minutes, 4000 rpm) to remove degumming sap.

Neutralisation was carried out by slowly adding (drop by drop) NaOH solution (1 M) to the defatted oil. The samples were in combination with stirring and constant heating at 65◦C for 20 minutes. Neutralisation, the process was carried out until pH 7.0 was reached, and the sample was then heated to 70◦C for 20 minutes, cooled and centrifuged for 15 minutes at 4000 rpm. The obtained oil was washed by deionising water (3 times with 10 mL) with agitation (500 rpm) and heating at 50◦C in vacuum. The neutralised oil samples were separated by centrifugation (10 minutes, 2500 rpm).

After neutralisation, the oil obtained was carried out by beaching with an adsorbent (4 g/ 100 g oil) containing 1.13 g activated carbon. The oil sample was stirred using a magnetic stirrer at 40^0C for 40 minutes under N_2. After cooling, the oil samples were separated again

by centrifugation (30 minutes, 3500 rpm). Deodorization of the oil sample bleach was carried out by distillation under vacuum. The oil mixture obtained after bleaching and deionised water (20 mL) was heated to 95-97^{0}C under vacuum for one hour, with continuous stirring. The refining process is repeated three times for each batch of oil.

Determination of yield. The yield was expressed as a percentage of oil separated by the whole 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 liquid chromatography on the HPLC system (Agilent 1200). Lipid separation was carried out at room temperature on the column (Lichrospher Diol 5 mm, 4x250 mm). The detection was carried out on an evaporative light scattering detector (Agilent 1200 series) at 45^{0}C and a pressure of 3.5 bar. The mobile phase consists 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 as follow: first, solvent A was flowing for 2 minutes, after that, solvent B was added in three steps, up to 10% in 10 minutes, to 44% in 15 minutes and 100% in 9 minute. The stationary phase was rinsed with solvent A during 6 min. The total solvent flow rate was kept constant at 1 mL/minute all along with the analysis. 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 analysed by gas chromatography (GC) in a Hewlett Packard gas chromatograph (6890N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionisation 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/minute, held at 200°C during 1 min, heated again to 220°C at 5°C/minute and finally held at 220°C for 20 minutes. 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 (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; Al-Mentafji 2016).

Statistical analysis. Statistical analysis was performed using IBM SPSS 25 software. Data are presented as Mean ± SD (Standard Deviation). Furthermore, a bivariate analysis was carried out using the t-test, to assess the differences in mean levels and crude oil content and after the oil refinement process. Significance was set at $p<0.05$.

Results

Yield. The amount of obtained crude oil VS oil after the refinement process is shown in figure 1. Yield in after refinement was higher than crude oil methods, $p<0.05$.

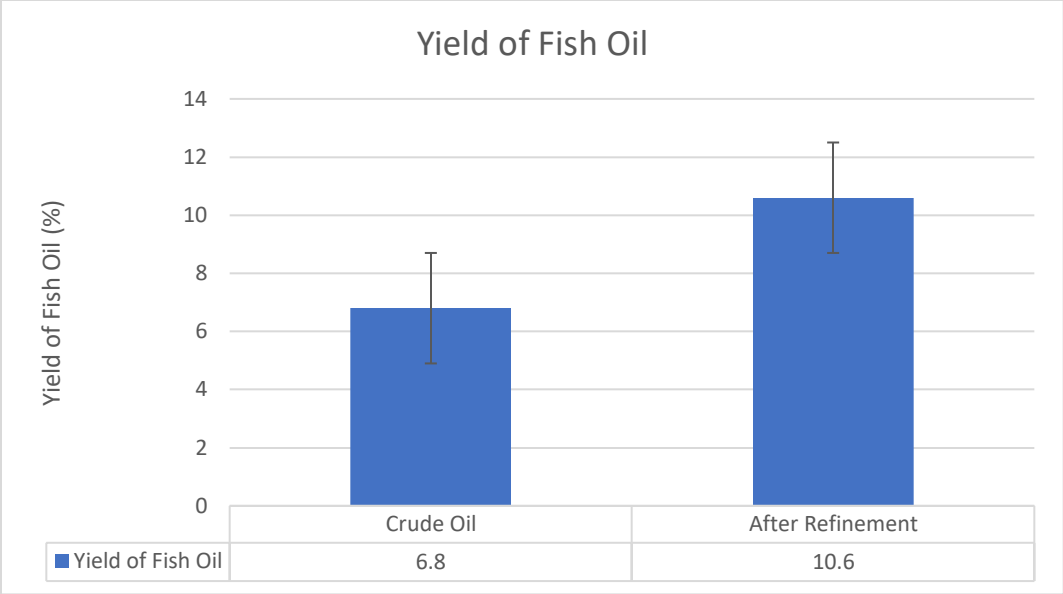

Figure 1. Yield (%) of obtained *A. testudineus* crude oil and after refinement process.

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

Table 1

The levels of neutral lipids (%) in *A. testiduneus* oil in crude and after refinement process

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

*Paired t-test, p< 0,05 versus crude oil.

Free fatty acids profile. Analysis of the content of fatty acids showed no significant difference in the content of fatty acids of the two types of oil. However, the levels of EPA and DHA showed significant differences between the two extraction methods. EPA and DHA levels are higher in the after refinement process oil than crude oil.

Table 3

The fatty acids profile in *A. testudineus* crude oil dan oil after refinement process

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*

*Paired t-test, p< 0,05 versus crude oil

Determination of acid value. Figure 2 shows a significant difference in acid value between crude oil and oil after the refinement process. Acid value in the after refinement process is almost closer to the recommended rate than crude oil.

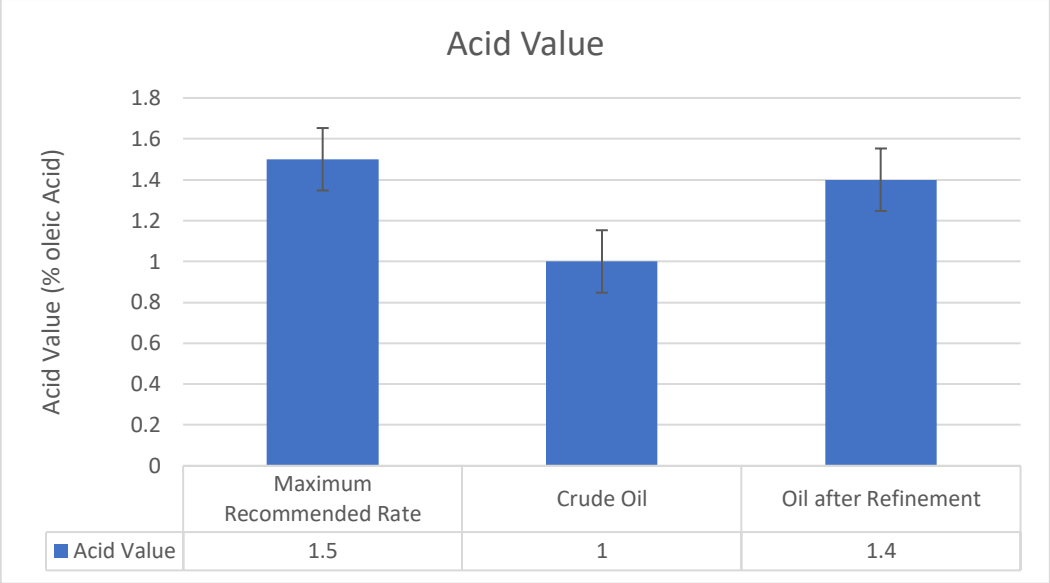

Figure 2. Acid Value Determination

Discussion. The fish oil production process is crucial 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 cells' metabolism to improve health. Essential fatty acids are the main precursors in producing various hormones and growth factors, especially sex hormones, such as testosterone, estrogen and androgens. EPA and DHA essential fatty acids play a role in maintaining cell membrane integrity to prevent cells' damage and increase cells' survival, predominantly neuronal cells. So that EPA and DHA are significant in increasing impulse transmission in nerve cells and impacting improving cognitive function. Given the enormous potential and benefits of fish oil, optimal efforts are needed to explore extraction methods to obtain fish oil with optimal quantity and quality (Ramakhrihnan et al 2013; Ivanovs et al 2017; Deepika et al 2014).

A. testudineus fish oil yield from the after refinement process shows a higher yield than crude oil. The refinement process separates the phospholipids, where the phospholipids are compounds that cause bonds between oil and tissue and fish meat. The opening and separation of the phospholipid result in the oil clumps and fat cells' opening, resulting in the oil's release and fluidity (Bonilla-Méndez et al 2018; Wenwei et al 2018; Sarker et al 2012; Hajeb et al 2015).

The neutral lipids analysis results in this study showed a significant difference in the wax esters (WE), tryacyl glyceride (TAG) and free fatty acid (FFA) levels in the oil extracted from *A. testudineus* fish in crude oil and after refinement (Latip et al 2014; Baehaki et al 2015). Low wax esters levels and high triacylglyceride levels can indicate low intracellular fat levels and high levels of extracellular fat bound to protein in meat (Anggraini et al 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 in fish oil indicates high PUFAs in fish oil. On the other hand, the hydrolysis of TAG leads to the formation of FFAs. Therefore, higher TAG and PUFA result in more FFA (Rachmawati et al 2018; Ahmed et al 2017; Nugraheni et al 2017).

This study shows that oil refinement can obtain higher EPA and DHA content than crude oil. EPA and DHA is an unsaturated fatty acid with double bonds, where 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; Baehaki et al 2015; Anggraini 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. In this study, the TAG and FFA levels in oil refinement were higher than crude oil. Therefore, the higher acidity value in oil refinement methods is due to the role of FFA (Suseno et al 2013).

Conclusion. Oil refinement of *A. testudineus* fish oil extraction method is more effective than crude oil.

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Authors:
Radiyati Umi Partan, Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia
Rachmat Hidayat, Department of Biology, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia, e-mail: dr.rachmat.hidayat@gmail.com
Eddy Mart Salim, Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia

Tudor Papuc <ptudor2008@yahoo.com>
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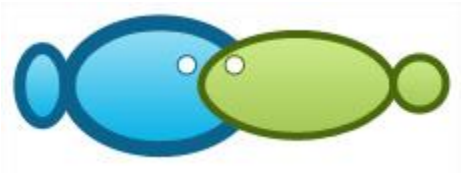

Optimizing *Anabas testudineus* oil processing by refinement process for quality parameters and fatty acid composition

[1]Radiyati U. Partan, [2]Rachmat Hidayat, [1]Eddy M. Salim

[1] Department of Internal Medicine, Faculty of Medicine, Sriwijaya University, Palembang, Indonesia; [2] Department of Biology, Faculty of Medicine, Sriwijaya University, Palembang, Indonesia. Correspondent author: R. Hidayat, dr.rachmat.hidayat@gmail.com

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. testudineus* is better than the crude oil.
Key Words: *Anabas testudineus*, 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. testudineus* 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. testudineus* 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. testudineus* 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.

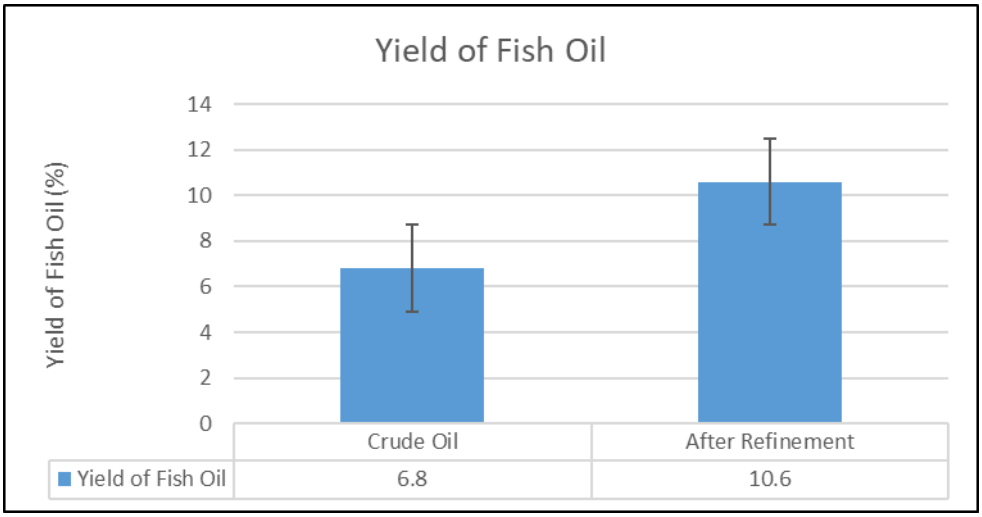

Figure 1. Yield (%) of *Anabas testudineus* crude and refined oil.

Table 1

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 ($p<0.05$).

Table 2

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).

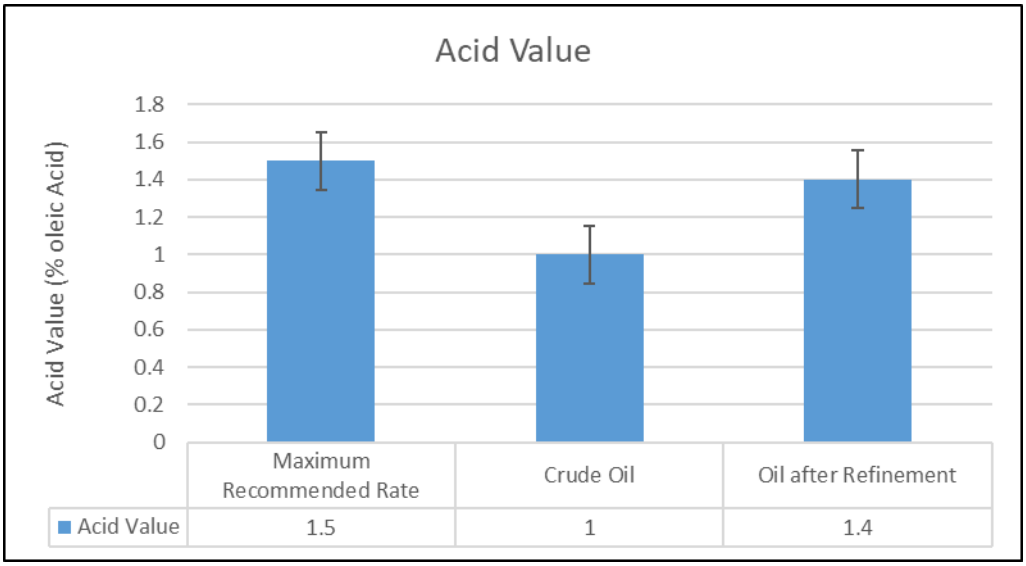

Figure 2. Acid value.

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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Authors:
Radiyati Umi Partan, Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia
Rachmat Hidayat, Department of Biology, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia, e-mail: dr.rachmat.hidayat@gmail.com
Eddy Mart Salim, Department of Internal Medicine, Faculty of Medicine, Universitas Sriwijaya, 30662 Palembang, Indonesia

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