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Optimization of Essential Fatty Acids Via Esterification of the Native North Sumatera's Freshwater Fish

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ABSTRACT

Nutritionally balanced diets required animal proteins which are obtained in both marine fish and freshwater fish. Chemical constituents obtained in freshwater fish are especially high in protein and rich in healthy lipids. Polyunsaturated fatty acids (PUFA), the essential fatty acids, for example, are responsible for central nervous system function, play important roles in inflammatory responses and immune system, significant for structural components of cell membranes, and heredity. Indonesia is known as one of Asia's greatest freshwater fish producers, and North Sumatera, a part of Indonesia's mainland, is one of the biggest in the country. *Cyprinus carpio* and Indonesian snakehead fish (*Channa striata*) are common freshwater products consumed in daily life and in the traditional ceremonies of the biggest population called Bataknese. The results showed that for saturated fatty acid (SFA), palmitic acid (C16:0) was dominant for goldfish and snakehead fish, which are about 31.8% and 42.7%, respectively. In addition, oleic acid (C18:1) was the largest monounsaturated fatty acid (MUFA), more than 45% for goldfish and around 26% for snakehead fish. In contrast, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) of PUFA are found to be higher in snakehead fish oil than in goldfish, which indicates a rich amount of nutrients. Furthermore, the fatty acid distributions are more in the sn-2 position in both goldfish and snakehead fish. The fatty acids content and distribution in this study were determined by enzymatic hydrolysis of extracted fish oil followed by esterification. The individual percentage of fatty acid was quantified using Gas Chromatography-Flame Ionization Detector (GC-FID).

1. INTRODUCTION

Fish as food has been considered a good source of micronutrients, vitamins, and minerals. The nutritional value mainly focuses on polyunsaturated fatty acid (PUFA), which affects human health. n-3 PUFA, which cannot be synthesized in mammals, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have several health benefits, including enhancing brain function, arthritis, mental illness, reducing heart attack risk and inflammation and is recommended for pregnancy and breastfeeding for an infant [1-3]. Therefore, fatty acids become important for human health. In addition, fish processing and feeding habits significantly influence fatty acid contents, while the other minor nutrients seem less impacted [4]. Fatty acid works as a growth energy source, reproduction and metabolism for the fish itself.

According to the Food and Agriculture Organization of the United Nations (FAO), the total aquaculture and fisheries production accounts for 70 percent of the total produced by Asian countries. In 2020, the major producer was China, with a share of 35 percent, followed by India (8 percent), Indonesia (7 percent), Viet Nam (5 percent) and Peru (3 percent) [5]. However, the total fish consumption in Indonesia is far behind the other Asian countries despite the increasing demand for freshwater fish due to limited marine fish resources. It is native to Asia and was introduced to North America and Europe. To date, in Europe, freshwater fish is not only for consumption but also use as water quality control, recreational fisheries and ornamental fish.

Freshwater fish is a source of protein and valuable fatty acids and lipids. This may vary due to diet habits, phases of the life cycle, sexual maturity, and environmental factors among freshwater fish such as giant gourami (*Osphronemus goramy*), catfish (*Mystus atrifasciantus*), tilapia (*Oreochromis niloticus*), milkfish (*Chanos chanos*), etc. Indonesian

snakehead fish (*Channa striata*) and goldfish (*Cyprinus carpio*) have been important commodities in Indonesia. The previous report showed enhancement of common carp nutritious ingredients (larger share of total PUFAs and saturated fatty acid (SFA): PUFA ratio) in the human diet using algae for fishmeal [6-8]. *C. striata* was reported to have a good source of fatty acid and high protein content that makes it promising to prevent stunting. An interesting fatty acids profile shows that unsaturated fatty acids are more than saturated fatty acids [9, 10]. Currently, in India, contributions of freshwater fish to total protein were increased from 0.4 to 1.7% to fulfill the nutritional needs of people. This indicates the promising potential of freshwater fish as a source of healthy food [11]. Moreover, it is well-known as a therapeutic agent in traditional medicine (energy boosters during illness) and food industries that reveal its excellent nutritional content for health to prevent stunting.

This study presents the physicochemical characterizations of typical freshwater fish oil from North Sumatra, Indonesia. The study regarding meat quality of freshwater fish is still limited since the chemical composition of each fish is highly dependent on the area, we considered that the profile of this fish will be different from freshwater fish in other areas. Fatty acid content and distributions were determined before and after enzymatic hydrolysis, followed by esterification using gas chromatography (GC).

2. MATERIALS AND METHODS

2.1 Collection of fish meat, oil extraction and purification

Goldfish and snakehead fish were collected from a local fish market situated at Medan fish market, North Sumatera, Indonesia. The collected fish were segregated manually to obtain the meat. The fish fillets were cut into pieces and ground into fine particles. About 500g of fish was added with 1000mL of n-hexane and heated at 80℃ for 3h under a vacuum. Finally, centrifugation was performed for 60min at 80℃ to recover fish oil contents. A dark container was used to store fish oil after being dried. The following formula calculated the percentage fish oil yield [12, 13]:

$$\%\ \text{Oil yield} = \frac{Weight\ before\ extraction – Weight\ after\ extraction \times 100\%}{Weight\ of\ sample\ before\ extraction} \quad (1)$$

Further, the physicochemical characterizations of fish oil were determined by the physical properties such as cloud point and total solid, according to Regost et al. [14], Robin et al. [15], and Rørå et al. [16]. The chemical properties, i.e., peroxide value (PV) [17, 18], saponification number [19, 20], free fatty acid (FFA) content [21, 22], and iodine number [20, 23].

$$\text{PV (milliequivalents peroxide/1000g sample)} = \frac{(S-B)\times N\times 1000}{weight\ of\ sample, g} \quad (2)$$

where, B=volume of titrant (mL of blank); S=volume of titrant (mL of sample); N=normality of $Na_2S_2O_3$ solution.

$$SN = \Sigma \frac{560\times Ai}{MWi} \quad (3)$$

where, Ai is the percentage, and MWi is the molecular mass of each component.

FFA was determined by dissolving approximately 2.0g of fish oil in 25mL of ether-alcohol solution (2:1) and two drops of 1% phenolphthalein indicator according to Eq. (3).

$$\%\ FFA = \frac{(V.M.28.2)}{m} \quad (4)$$

where, % FFA represents the free fatty acid (%), V is the solvent volume, M is the molarity of the NaOH, and m is the mass of the fish oil sample.

$$W = \frac{12.69\times c\times (V1 - V2)}{m} \quad (5)$$

where, *W* corresponds to the iodine value of the sample, g (I_2)/100g, *c* is the concentration of $Na_2S_2O_3$ solution (mol/L), V_1 is the volume of the standard solution (mL), V_2 is the sample solution (mL), and *m* represents the mass of sample (g) according to Wijs method [24].

The fatty acid composition of the triacylglycerol (TAG) at the *sn*-1 or *sn*-3 position (*sn*-1+3) was calculated using Eq. (6). Further, the fatty acid composition of the TAG at the *sn*-1 or *sn*-3 position (*sn*-1+3) was calculated using Eq. (6). Further, the ratio of fatty acids at the *sn*-2 position was determined using Eq. (7) [25, 26].

$$[\text{sn-1+3}] = (3\times[\text{TAG}] - [\text{sn-2}])/2 \quad (6)$$

Abundance in TAG ratio at sn-2:

$$[\text{sn-2}]/(3\times[\text{TAG}]) \quad (7)$$

where, [*sn*-1+3], [*sn*-2], and [TAG] are fatty acids composition at *sn*-1+3, fatty acids composition at *sn*-2, and fatty acid composition in TAG, respectively.

2.2 Enzymatic hydrolysis of fish oil

Hydrolysis of fish oil was performed in an Erlenmeyer at room temperature in an atmosphere with a lipase enzyme as a catalyst. Fish oil was first extracted from a dried fish fillet in n-hexane as a solvent. Extraction was performed at 80℃ for 50 minutes followed by distillation at 70℃ for 1h. Next, 100mg of activated lipase (incubated at 37℃ for 10 h while shaking every hour for 10 min diluted in 50mL ethanol) was added into 6g of fish oil followed by adding 10mL of distilled water, 2.5mL of $CaCl_2$ 0.063M and 5mL of tris-HCl buffer solution. The mixture was transferred into a dropping funnel to separate the water/glycerol combination from the FFA. The top layer was then taken and evaporated to obtain pure FFA. Distillation of FFA was performed for further investigation.

As shown in Figure 1, FFAs are mainly obtain by hydrolyzing of TAGs in the presence of lipase. Lipases (known as triacylglycerol acylhydrolyase commonly used in the breakdown of acyl glycerol bond in the presence of water). The current mechanism leads to the formation of the mono-glycerides (MG), di-glycerides (DG), FFA and the glycerol as by-products [27].

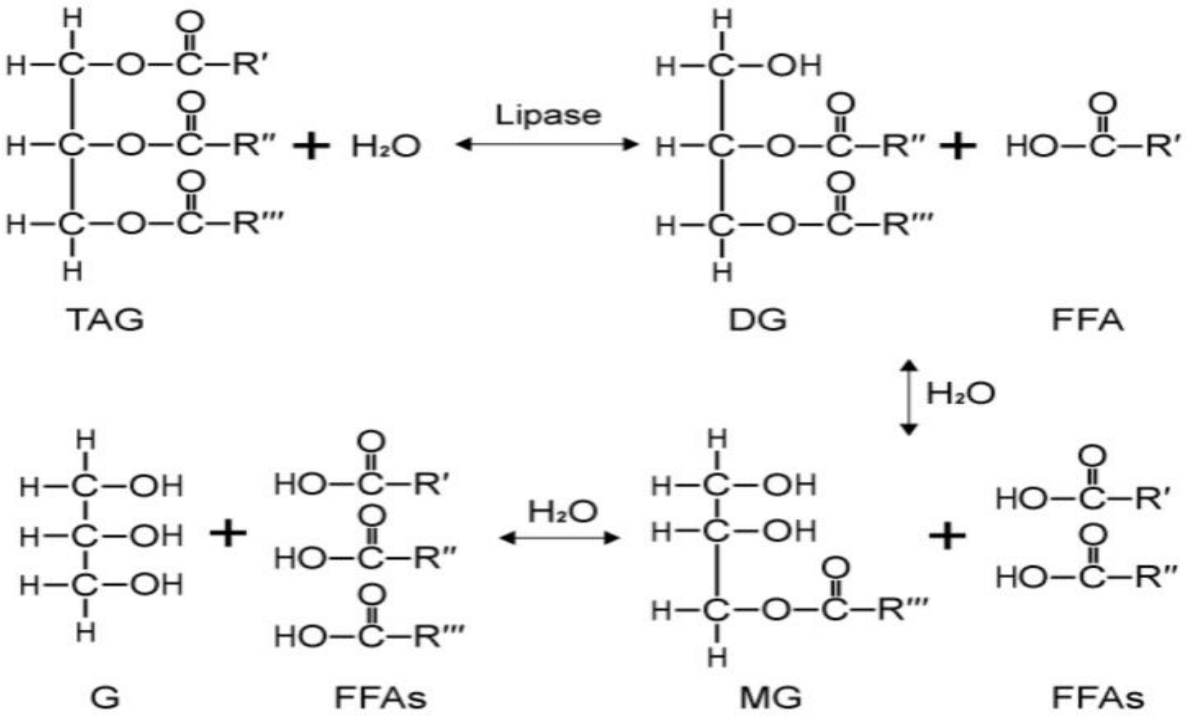

Figure 1. Enzymatic hydrolysis of TAG from fish oil into FFA
Monoacylglyceride (MG), Diacylglyceride (DG), Glycerol (G) [28].

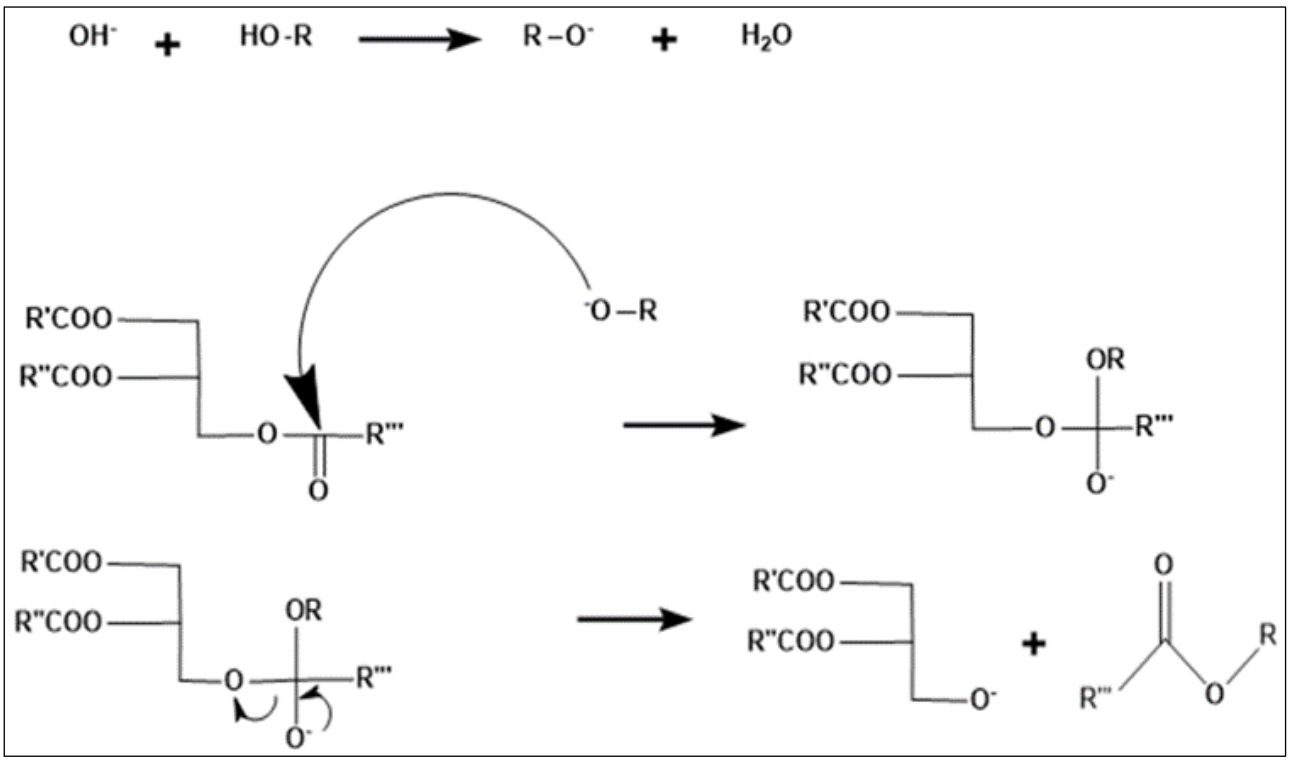

Figure 2. Esterification base-catalyzed mechanism [29]

2.3 Fatty acids esterification

Next, 25mg fatty acids were diluted in the 0.5 N NaOH in methanol while heating at 100℃ for 5 min, cooled to 30℃, and added 1mL of BF3 and heated at 100℃ for 5 min. The mixture was cooled to room temperature, and 1mL of n-hexane was added while shaking. 2mL of saturated NaCl was added to separate fatty acid from the mixture. The fatty acid mixture was obtained by adding 50mg Na_2SO_4 anhydrous to remove water and completely evaporate it. The fatty acid was analyzed using flame ionization detector (FID) gas chromatography (GC-FID) (Shimadzu QP 2010 ULTRA) and connected to the DB-23 column. All materials and chemicals were analytical grade and purchased from Merck (Darmstadt, Germany) without further purification. GC method was chosen since this method allows sensitivity and reproducibility of fatty acid analyses with a high-quality capillary column. Moreover, GC was widely adopted as an applicable tool in a

number of fatty acids research areas.

In this reaction, alkali metal (Na) was used as catalyst. Figure 2 shows the mechanism of homogeneous base-catalyzed reaction. In the first step, proton was accepted and transferred by alkali metal to the alcohol. The formation of alkoxides occurred in this step. Next, in the second step, basic alkoxides generate nucleophilic that reacts with carbonyl group. NaOH is dissolved in methanol to obtain methoxide anion ($–OCH_3$) then this anion attacks the ester compound to yield a new ester.

3. RESULT AND DISCUSSION

3.1 Characterization of fish oil

The oil content of *C. carpio* and *C. striata* were relatively high ~75.0% w/w and ~70.0% w/w, respectively, consistent with the established work [30]. Table 1 shows the result of physical properties such as cloud point and total solid, indicating high impurity for goldfish and snakehead fish. The peroxide number is lower than 5%, which meets the World Health Organization standard. However, the high saponification number and free fatty acid content correspond to the high volatile matter and moisture content. The iodine number of goldfish cultivated in ponds is higher than that of cages, indicating the higher amount of unsaturated fatty acids present there [31].

Fatty acid analysis of the original feedstocks by GC-FID results are presented in Table 2. The results show that both fish oils have large amounts of polyunsaturated fatty acids. The significant differences between the total fatty acid compositions of these two types of oils lie in the amount of oleic, linoleic, eicosapentaenoic and docosahexaenoic acids. For both oils, oleic acid was the most abundant fatty acid (around 46%), followed by linoleic acid 17.9% and 8.1% for goldfish and snakehead fish, respectively, supporting the previous report for typical freshwater fish [32]. Similarities in both TAG and fatty acids of fish oils are expected in the diet, spawning, season, and environment. A high amount of oleic, palmitoleic, and arachidonic acid generally characterizes freshwater fish. In the tropical region, fish show a high SFA and MUFA level while PUFA is low. Further, a good distribution of palmitic acid (SFA), oleic acid (MUFA), DHA and EPA (PUFA) in the fish body [33]. PUFA of freshwater fish is considered less than that of marine fish, with a great balanced amino acid content, which is good for health [34].

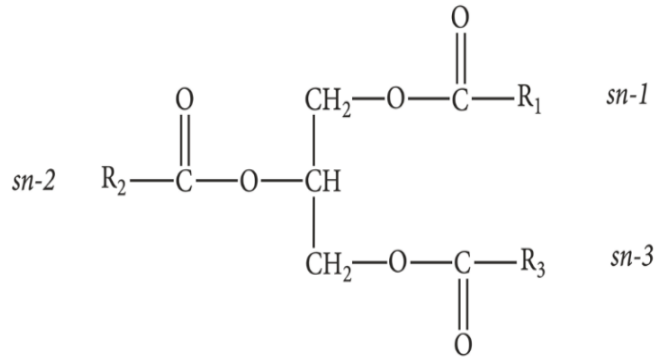

Figure 3. Triacylglycerol (TAG) molecule structure

The TAG molecule structure of fish oil shown in Figure 3. Table 3 shows the distribution of major SFAs, PUFAs and MUFAs in the *sn*-2 position of the TAGs. Generally, a higher EPA and DHA were observed for snakehead fish than for goldfish bound in the *sn*-2 position. However, amount of EPA and DHA of freshwater fish is lower compared to sea fish. In contrast, C18 PUFA was found higher in freshwater fish than those of marine fish. This might be due to the lesser enzyme activity, water temperature, salinity of the water, and nutrients while seasonal variations and geographic region will influence the lipid levels. On the other hand, sn-3 PUFA has been found in a significant proportion or even more in some freshwater fish than some sea fish as found in this study. This indicates that freshwater fish is promising for supplements and nutraceuticals industries for its similar characterizations found in marine fish [35]. In contrast, MUFAs C16:1 and C18:1 occurred in the terminal *sn*-1 and *sn*-3 positions for snakehead fish, while none was observed in goldfish. Furthermore, the abundance ratio of omega-3 to omega-6 of goldfish and snakehead fish before and after enzymatic hydrolysis is 1:1, which corresponds to the recommendation.

Table 1. Physicochemical characterization of fish oil

Characteristics	Goldfish		Snakehead Fish Cultivated in Ponds	Unit
	Cultivated in Cages	Cultivated in Ponds		
Cloud point	51.50	54.50	62.50	℃
Total solid	36.00	34.00	27.00	°Brix
Peroxide number	2.84	2.60	3.10	meq/kg
Saponification number	103.26	109.59	103.99	mg KOH/g
Free fatty acid	2.69	15.07	3.60	%
Iodine number	13.38	15.07	21.18	mg/100g

Table 2. Goldfish and snakehead fish fatty acids before and after enzymatic hydrolysis

Fatty Acid	Carbon Position	Name	Goldfish (%)				Snakehead Fish (%)	
			Cultivated in Cages		Cultivated in Ponds		Before	After
			Before	After	Before	After		
Saturated fatty acids	C:14-0	Myristic acid	1.800	1.690	2.165	1.465	1.672	1.568
	C:16-0	Palmitic acid	23.190	21.640	22.620	22.040	28.440	27.157
	C:18-0	Stearic acid	5.686	4.022	5.415	4.543	11.049	10.045
	C:20-0	Aracidic acid	-	-	-	-	1.135	1.001
	C:22-0	Behenic acid	-	-	-	-	0.494	0.387

	C:24-0	Lignoceric acid	1.143	0.943	1.155	1.009	-	-
		Σ SFA	31.819	27.295	31.355	29.057	42.790	40.158
	C:16-1	Palmitoleic acid	3.940	3.074	3.574	3.282	2.810	2.696
	C:17-1	Cis-10-Heptadecanoic acid	0.523	0.425	0.474	0.425	-	-
	C:18-1	Oleic acid $^{\omega-9}$	46.030	45.890	46.890	46.780	27.161	26.153
	C:20-1	Eicosenoic acid $^{\omega-9}$	1.472	1.279	1.310	1.232	0.866	0.759
	C:22-1	Erucic acid $^{\omega-9}$	-	-	-	-	0.450	0.410
		Σ MUFA	51.965	50.668	52.248	51.719	31.287	30.018
Unsaturated fatty acids	C:18-2	Linoleic acid $^{\omega-6}$	17.850	16.120	17.960	15.870	8.157	8.047
	C:18-3	γ-Linoleic acid $^{\omega-6}$	0.801	0.446	0.503	0.388	-	-
	C:18-3	Linolenic acid $^{\omega-3}$	1.188	1.057	1.155	1.120	1.216	1.199
	C:20-2	Eicosadienoic acid	0.786	0.780	0.724	0.518	-	-
	C:20-3	Eicosatrienoic acid $^{\omega-3}$	0.617	0.482	0.549	0.396	-	-
	C:20-5	Eicosapentaenoic acid $^{\omega-3}$	0.556	0.434	0.495	0.380	5.323	5.196
	C:22-6	Docosahexaenoic acid $^{\omega-3}$	0.767	0.465	0.553	0.376	4.313	4.208
		Σ PUFA	22.565	19.784	21.939	19.048	19.009	18.650
		Σ USFA	74.530	70.452	74.187	70.767	50.296	48.668

Table 3. Fatty acid distribution in triacylglycerol of freshwater fish

Fish Oil	Position	Fatty Acid Distribution (%)									
		16:1	18:1	18:2	18:3	γ-18:3	20:1	20:3	20:5	22:1	22:6
Goldfish:											
Cultivated in cages	1+3			9.691	11.026	44.319		21.880	21.942		39.374
	2			90.308	88.973	55.680		78.119	78.057		60.625
Cultivated in ponds	1+3			11.636	3.030	22.862		27.868	23.232		32.007
	2			88.363	96.969	77.137		72.131	76.767		67.992
Snakehead fish	1+3	4.057	3.711	1.349	1.398	-	12.356		2.386	8.889	2.435
	2	95.943	96.289	98.651	98.602	-	87.644		97.514	91.111	97.565

4. CONCLUSION

The fatty acids of native North Sumatera goldfish and snakehead fish characterizations and distributions are successfully determined. Palmitic acid and oleic acid are found to be large in SFA and MUFA in goldfish and snakehead fish, respectively. Fatty acids in sn-2 positions are highly distributed in both goldfish and snakehead fish. This information will aid to produce a promising source of natural rather than synthetic essential fatty acids at an affordable price for a healthier and balanced diet. The fatty acid composition data in this research can be a groundwork for research in fish scale chemistry in the future. This finding may lead to the growth of freshwater fish cultivation along with the optimizing fatty acids content in freshwater fish (flesh quality) especially in North Sumatera, Indonesia.

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