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Recycling of "Minyak Ala Muncar" by Three Crystallization Methods

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Abstract

"Minyak Ala Muncar" or abbreviated as MAM is a by-product waste of fish canning factory in Muncar Banyuwangi Indonesia. MAM was widely distributed throughout Indonesia to be used as animal feed supplements. MAM was very potential to be converted into oil rich in n-3 through the recycling process. The recycling process needs to be conducted because MAM contained trans-FA especially EA which could have a detrimental impact on health. Therefore, the aim of this research was to produce an oil fraction that rich in n-3 and low in trans-FA content. Three crystallization methods namely crystallization with n-hexane solvent, acetone solvent, and urea were chosen as the recycling method. The MAM Recycling research was carried out through several stages, namely oil hydrolysis, winterization with solvents (acetone, n-hexane), urea crystallization, fractions esterification, Fatty Acid content analysis. The results showed that both crystallization with n-hexane and acetone produced ratio value of PUFA/trans-FA, n-3/trans-FA, EPA/EA, and DHA/EA below 0.3 respectively. While urea crystallization was able to produce ratio numbers for each PUFA/trans-FA, n-3/trans-FA, EPA/EA, and DHA/EA PUFA/trans-FA 1.46±0.05, 1.36±0.04, 0.73±0.021, 0.48±0.035.

Abbreviations

AA Arachidonic Acid

ACR Aseton Cristallization fraction

ALA ∝-Linolenic Acid

Cis-FA cis-fatty acids

DHA Docosahexanoic acid (22:6n3)

DPA Docosapentaenoic acid (22:5n3)

EPA Eicosapentaenoic acid (20:5n3)

EA Elaidic Acid

FAME Fatty acid methyl ester(s)

GC Gas Chromatography

HDL High Density Lipoprotein

HCR Hexane Cristallization fraction

LA Linoleic Acid

LDL low density lipoprotein

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MAM Minyak Ala Muncar

MUFA Monounsaturated fatty acid(s)

n-3 omega-3

NUCF Non-urea complexing fraction

OA Oleic Acid

PUFA Polyunsaturated fatty acid(s)

SFA Saturated fatty acid(s)

SDA Stearidonic acid (18:4n3)

Trans-FA trans-fatty acids

UCR Urea Cristallization fraction

INTRODUCTION

Muncar Banyuwangi is one of the biggest fish canning industry centers in Indonesia. Both large industries and small fish processing factories operated in Muncar. One of the serious impacts resulted from this situation is oil waste that is circulated throughout Indonesia by the local community used as animal feed supplements for fish, chicken, goats, and cows. The local communities give the term waste oil as "Minyak Ala Muncar (MAM)" which means oil typically of Muncar. they treated the waste by heating at high temperatures accompanied by the addition of caustic soda so that the oil and residue are separated. This process is known as saponification in oil purification [1], [2].

GCMS assay showed that the MAM rich in a variety of fatty acids namely LA, AA, ALA, EPA, DHA, palmitic acid, stearic acid, arachidic acid, myristic acid, Palmitoleic acid, and EA [3]. EPA, DHA, ALA are a type of n-3 PUFA that have a good influence on the cardiovascular system [4] including preventing sudden death [5]. EPA and DHA also could accelerate the healing process of keloids [6], increased HDL levels, reduced LDL, reduced platelet aggregation [7], and reduced the growth of breast cancer cells [8]. The combination of n-3 and n-6 groups in oil also has an impact on health. The ratio between n-3 and n-6 in the body greatly affects somebody's health [9]. Greenland Eskimos have the lowest death rate from coronary heart disease compared to the other races. it is because of the ratio between n-3 against n-6 in Eskimo's dietary nutrition is highest compared to other races. This ratio greatly affects the increase or decrease in LDL in the body [10].

The presence of EA in MAM should be an important concern because not only its large amount reaching 26.8% in MAM [3] but also EA caused many health problems. EA appears in MAM was allegedly due to the high-temperature of heating during processing, where heating was known affecting of fatty acid content [11] and increasing of oil toxicity [12]. Under conditions of high heating temperatures repeatedly and continuously, cis-FA such as OA could turn into trans-FA such as EA [2], [13].

The recycling process that could be conducted on MAM was to separate the content of EA and all trans-FA from n-3. The most possible method to be applied to MAM recycling is crystallization. This method is easy to apply because it uses very simple equipment [14] and can be applied to samples in large quantities. Crystallization was known able to separate PUFA from SFA and trans-FA [15].

The crystallization methods applied in this recycled process were crystallization with n-hexane solvent, acetone solvent, and urea. Saturated fatty acids and EA were known to have low solubility in acetone and n-hexane solvents so that resulted in the best separation [1]. Urea crystallization method was the most efficient, reproducible, fast, and environmentally friendly method [16] and could be applied to waste [17] both to a large size or a little. The three crystallization methods are carried out at freezer temperature (under 0 ^{O}C). Crystallization at zero or below zero was an effective step in the process of fractionating

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fatty acids and their derivatives [18], because the solubility of both fatty acids also decreases along with the decrease in temperature [19].

In this research, three crystallization methods consisting of crystallization with n-hexane solvent, acetone solvent, and urea complexation were applied in MAM recycling which aims to produce oil fractions that rich in n-3 PUFA especially EPA and DHA and low in trans-FA especially EA.

Experimental Procedures

Materials

MAM waste material was obtained from one of the producers in the Muncar Region, Banyuwangi Indonesia. The MAM had been processed before by the producer with heating and adding caustic soda. The fatty acid standard used was the FAME Mix 37 comp (SIGMA).

Methods

Hydrolysis of oil became free fatty acids

MAM was firstly analyzed its fatty acid content by the GCMS method. The oil was then hydrolyzed with a saponification technique using a solution of 15% Potassium Hidroxide in a mixture of water and methanol (1:1). The mixture was then heated at 60 ^{O}C accompanied by constant agitation. The mixture was then separated from the compound that not soaped compound by using a n-hexane solvent so that it obtained a compound that soaped. The mixture was then acidified to pH 1 using HCl, then n-hexane was added. the n-hexane layer was taken and then evaporated so that it obtained free fatty acids [20].

Winterisation with acetone and n-hexane

5 grams of fatty acids were added to 50 mL acetone in an Erlenmeyer, then shaken until the fatty acids dissolve completely. The mixture was then stored in the freezer with the temperature set below 0^{O}C for 24 hours. The mixture was then filtered with a vacuum filter, then the resulting filtrate was evaporated until a fatty acid fraction was obtained [21]. The same procedure was also carried out using n-hexane solvents.

Urea crystallization

5 grams of fatty acids were added with 20 grams of urea (in methanol). The mixture was then shaken until the fatty acids dissolve completely. As with crystallization with acetone, the mixture was stored in the freezer for 24 hours. The mixture was then filtered with a vacuum filter, and the filtrate was put back in the freezer for 3 hours. The mixture was refiltrated and the filtrate obtained was acidified with 6N HCl, then 20 mL of n-hexane was added. the n-hexane layer was separated and then evaporated to produce a fatty acid fraction.

Esterification of fatty acids

Fatty acids obtained from the three crystallization methods were then esterified by the method carried out by Guil-Guerrero and Belarbi [20]. Fatty acids were put into a three-hole flask, then added absolute methanol at a ratio of 1:20 (w/v). the mixture was then stirred until it dissolved completely. The acetyl chloride catalyst was then added to the mixture carefully then the mixture was refluxed at 70^{O}C with a paraffin bath under N_2atmospheric conditions [20] for 3 hours until FAME was obtained. Subsequent FAME was taken using n-hexane solvent. The mixture was then evaporated to obtain pure FAME.

Fatty Acid Analysis

The GCMS instruments used were Variant-3900, GC/MS/MS Saturn 2000, CP-8400 autosampler and GC (Shimazu, G-5000A) was equipped with a Hydrogen generator (Whatman) with a Bonded OV-1 column. The GC instrument was firstly conditioned by setting the system according to the ideal conditions of the analysis, namely the injector temperature of 240 ^{O}C, detector temperature 280 ^{O}C, the separation system was set with an initial temperature of 50^{O}C, held for 2 minutes, then the temperature was raised at a speed of 4 ^{O}C/minute until reach temperatures of 240 ^{O}C. The temperature of 240^{O}C was then held for 2 minutes.

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So the total duration of analysis was 51.5 minutes for each sample. The standard FAME and the FAME sample crystallized by the three methods were then injected into the previously set GCMS system.

Statistical Analysis

Data tables and figures are presented in the format of mean +standard deviation (SD) and analyzed with one-way ANOVA using the Microsoft Excel program with the significance of the data difference was set at P <0.05 [17], [18]. All experiments were carried out as three-time.

Results and Disscussion

The fatty acid content in MAM

MAM waste hydrolysis was conducted to break the bonds between fatty acids and glycerol [1]. This process aims to facilitate the process of separation of fatty acids in the oil. Fatty acids naturally appear in the form of triacylglycerol, that glycerol which is esterified with three fatty acids [1], [22]. The crystallization method using acetone or n-hexane was known to be able to produce the best separation compared to other solvents [1].

GC analysis of MAM materials and fractions obtained from the three crystallization methods can be seen in table 1 and fig 1. GC data show that waste materials contain very diverse fatty acids. MUFA was the highest number of fatty acids, which was 53.48%, then SFA 28.05%, and PUFA 18.47%. EA was the most dominant fatty acid in MAM that was equal to 47.95% and contributes 89.65% of the total MUFA. Palmitic acid was the most dominant fatty acid in SFA group which was 13.58% while the most dominant in the PUFA group was EPA which was 8.08%. The presence of n-3 fatty acids in MAM beside a large amount of EA makes it as very potential material to be recycled into n-3 rich oils that beneficial to health. EA is a geometrical isomer of oleic acid and has a trans configuration. Therefore, EA is known as trans-FA [1].

The content of EA in oil poses a threat to the health of the Indonesian population. Although the oil is specialized as animal feed, trans-FA will diffuse to animal products so that their eggs and meat will be contaminated with trans-FA. trans-FA have been proven to correlate with the emergence of cancer risk factors [23], precipitating atherosclerosis [24], breast cancer [25], and increasing hepatic lipogenesis as a hepatotoxic risk factor [26]. trans-FA can also have side effects on fetal growth and development [27]. trans-FA can also diffuse into the mammary glands of nursing mothers so that it affects the quality of breast milk [28].

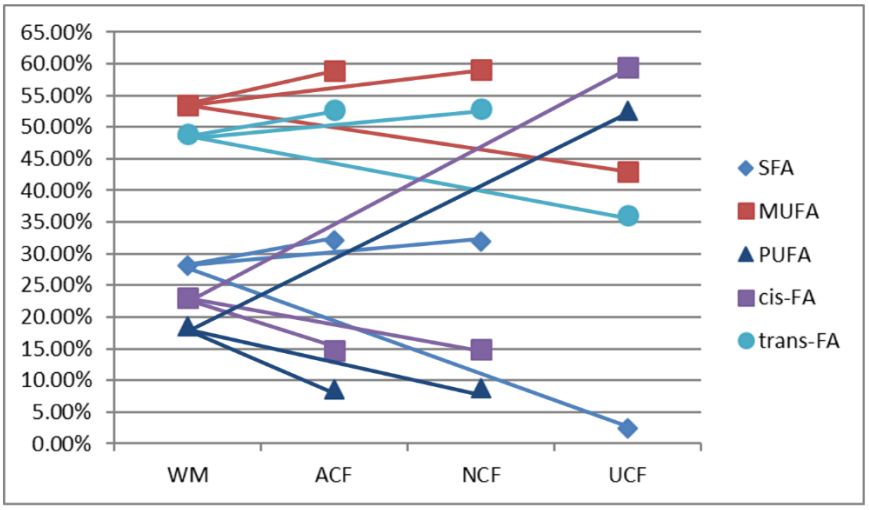

Fig. 1. Percent content (%) of the fatty acid group in "Minyak ala Muncar"

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There are several methods of separating fatty acids in the oil, namely freezing methods, enzymatic lipase reactions [29], [30], super critical fluid extraction [31], [32], Preparative HPLC with stationary phases modified using $AgNO_3$ reagents [32], [33], crystallization with ethanol solvents [31], n-hexane and ethyl acetate in cold temperature conditions [32], [33], and urea crystallization [16], [32]. However, crystallization methods were the best choice to be applied to recycle MAM. The results of the crystallization process can be seen in table 1.

Table 1. The Content of The Fatty Acids in "Minyak ala Muncar"

Σ"	**Trivial Name**	**n**	**WM (%)**	**ACF (%)**	**NCF (%)**	**UCF (%)**
C12:0	Lauric acid	-	0.10	0.31 ± 0.10^{a}	0.31 ± 0.04^{a}	0.25 ± 0.04^{a}
C14:0	Myristic acid	-	1.93	4.77 ± 0.35^{a}	4.77 ± 0.21^{a}	0.61 ± 0.05^{b}
C15:0	Pentadecanoic acid	-	-	0.19 ± 0.01^{a}	0.20 ± 0.0^{a}	0.06 ± 0.01^{b}
C16:0	Palmitic acid	-	13.58	22.54 ± 0.33^{b}	21.29 ± 0.08^{a}	1.25 ± 0.22^{c}
C16:1	palmitoleic acid	n-7	1.73	4.98 ± 0.66^{a}	4.77 ± 0.26^{a}	6.35 ± 0.54^{b}
C16:1	7-methyl-6c-palmitoleic acid	n-10	0.37	0.25 ± 0.13^{a}	0.36 ± 0.07^{a}	0.27 ± 0.03^{a}
C17:0	Margaric acid	-	0.96	-	0.31 ± 0.25	-
C17:0	14-methylpalmitic acid	-	0.27	-	-	-
C17:1	10c-Heptadecenoic acid	n-7	0.21	0.26 ± 0.24^{a}	0.29 ± 0.23^{a}	0.21 ± 0.09^{a}
C18:0	Stearic acid	-	8.80	2.60 ± 0.34^{a}	3.67 ± 0.34^{a}	0.33 ± 0.12^{b}
C18:1	Elaidic Acid (EA)	n-9	47.95	52.49 ± 2.06^{a}	52.29 ± 0.62^{a}	36.13 ± 1.08^{b}
C18:1	Oleic Acid (OA)	n-9	-	0.63 ± 0.03^{a}	0.68 ± 0.08^{a}	-
C18:2	Linoleic acid (LA)	n-6	-	-	-	3.48 ± 0.30
C18:3	-Linolenic acid (GLA)	n-6	0.47	-	-	-
C18:3	∝-Linolenic acid (ALA)	n-3	-	0.82 ± 0.16^{a}	0.83 ± 0.09^{a}	4.95 ± 0.12^{b}
C18:4	stearidonic acid	n-3	1.40	-	-	-
C19:0	Nonadecanoic acid	-	0.16	0.09 ± 0.05^{a}	0.12 ± 0.05^{a}	-
C20:0	Arachidic acid	-	1.54	0.29 ± 0.02^{a}	0.39 ± 0.02^{b}	-
C20:1	13c-Eicosenoic acid	n-7	2.04	-	-	-

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Σn	Trivial Name	n	WM (%)	ACF (%)	NCF (%)	UCF (%)
C20:3	Eicosatrienoic acid	n-7	0.55	-	-	-
C20:4	Arachidonic acid (AA)	n-6	1.93	-	-	-
C20:4	8c,11c,14c,17c-eicosatetraenoic acid (bishomostearidonic acid)	n-3	0.81	-	-	0.29±0.07
C20:5	Eicosapentaenoic acid (EPA)	n-3	8.08	4.80±0.29[a]	4.79±0.18[a]	26.50±0.06[b]
C22:0	Behenic acid	-	0.45	1.30±1.15[a]	0.94±0.76[a]	-
C22:1	13t-docosenoic acid	n-9	0.95	0.29±0.07[a]	0.62±0.46[a]	-
C22:3	8t,11t,14t-docosatrienoic acid	n-8	0.07	-	-	-
C22:5	Docosapentaenoic acid (DPA)	n-6	0.44	-	-	-
C22:6	Docosahexaenoic acid (DHA)	n-3	4.72	2.93±0.34[a]	3.22±0.27[a]	17.39±1.49[b]
C24:0	Lignoceric acid	-	0.27	-	-	-
C24:1	Nervonic acid	n- 9	0.23	-	-	-
-	Unidentified matter	-	-	0.75±0.39	0.45±0.06	2.16±1.74

There were fatty acid groups in Fraction resulted from Aceton crystallization (ACF) that have decreased and increased. Fatty acid groups that were decreased include PUFA which fell from 18.47% to 8.55% and cis-FA from 22.98% to 14.68%. The fatty acid groups that were increased include MUFA from 53.48% to 58.91%, SFA from 28.05% to 32.1%, and trans-FA from 48.97% to 52.78%. The crystallization method with the n-hexane solvent turned out to produce a fraction (NCF) with a fatty acid content profile that was similar to the acetone solvent (table 1). Only palmitic acid, stearic acid, and arachidic acid produced significantly different value (single-factor ANOVA with $p<0.05$).

The success of separating fatty acids with the winterization method using organic solvents is very dependent on the ratio between fatty acids and solvents. This is closely related to the polarity of fatty acid and solvent and the cooling temperature [34]. Long-chain SFA has two molecular groups with different polarities. Carboxylic groups have a polar property while long chains carbon are non-polar, the longer the decreasing polarity of fatty acids [22]. This makes the SFA content in acetone and n-hexane solvents were increased. The concentrations of Palmitic acid as the most dominant SFA in both fractions increased from 13.58% in WM to 22.54% in ACF and 21.29% in NCF. While stearic acid actually decreased from 8.8% in WM to 2.6% in ACF and 3.67% in NCF. Theoretically, stearic acid has a lower solubility in acetone if compared to palmitic acid and its solubility decreased with decreasing temperature. So, the palmitic acid content in ACF and NCF should decrease [19], but it did not happen. This was probably due to the diverse content of fatty acids in the oil so that it affected the behavior phase in the crystallization process which became more

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complicated. In addition, it also caused the decreasing in the nucleation velocity [35]. Another cause was the presence of polymorphisms of fatty acid crystals so that saturated fatty acids did not crystallize [18].

The phenomenon of the decreasing of PUFA content in both ACF and NCF was contrary to the theory in general, where the solubility of fatty acids in organic solvents tends to increase along with a large number of double bonds [19]. EPA as a PUFAs dominant component in ACF and NCF has decreased from 8.08% to 4.80% in ACF and 4.79% in NCF. At the crystallization method with a decrease in temperature, PUFA would be concentrated in the liquid fraction and separated from the crystals [19]. This data also contradicts other studies that state the concentration of PUFA increased sharply in the NCF fraction [33].

The Crystallization with urea produced a fraction (UCF) with a different profile of fatty acid groups compared to ACF and NCF. Urea crystallization was able to reduce SFA to 2.51%, MUFA to 42.97%, and trans-FAs to 36.13%. On the other hand, this method was also able to increase PUFA to 52.61% and cis-FAs to 59.45%. This result was in line with research by Guil-Guerrero and belarbi [20] where urea crystallization able to increase the composition of EPA. Urea crystallization was also known to be able to separate PUFA and cyclic fatty acids with SFA, MUFA, and trans-FAs in oil [15], so as to increase PUFA recovery [20].

The palmitic acid content in the urea crystallization fraction decreased to 1.25% and stearic acid to 0.33%. Urea crystals had a tetragonal geometry with a diameter of 5.67 Å. The existence of aliphatic long-chain compounds capable to make a binding to form a hexagonal structure with an inner diameter of 8-12 Å [19]. The more of double bonds and the presence of cyclic would reduce the inclusion with urea crystals. This means PUFA was difficult to form inclusions with urea [32]. In addition, the trans fatty acid structure was preferred to form urea complexes. Therefore, in the crystallization of urea, SFA and trans-FAs will be carried by urea to form the Urea Inclusion component (UIC). Whereas cis-FAs, PUFA, and cyclic fatty acids keep remaining in a solution known as non-Urea Complexing Fraction (NUCF) [15]. Even so, the urea crystallization fraction still contained SFA and EA. According to Wanasundara et al [19], totally eliminating SFA and trans-FAs with urea complexation may be difficult, because there some short-chain fatty acids did not interact to form complexes with urea crystals [36]. Therefore, it was very reasonable that the level of lauric acid in the urea fraction did not decrease at all. It might also be due to the limited capacity of urea so that it could not interact fully with all SFA and trans-FAs, where an increase in the amount of urea would increase the number of fatty acids trapped in UCF [36].

The content of fatty acids in MAM based on the omega (n) group

Omega (n) is a fatty acid-related naming system based on the position of the double bonds calculated from the end of the methyl at the fatty acid structure [22]. n-3 means that there is a double bond in the third position carbon calculated from the very end methyl group. ALA, EPA, DHA are an example of n-3 fatty acids which are most often used for health. Status of fatty acid content based on omega classification can be seen in Fig. 2.

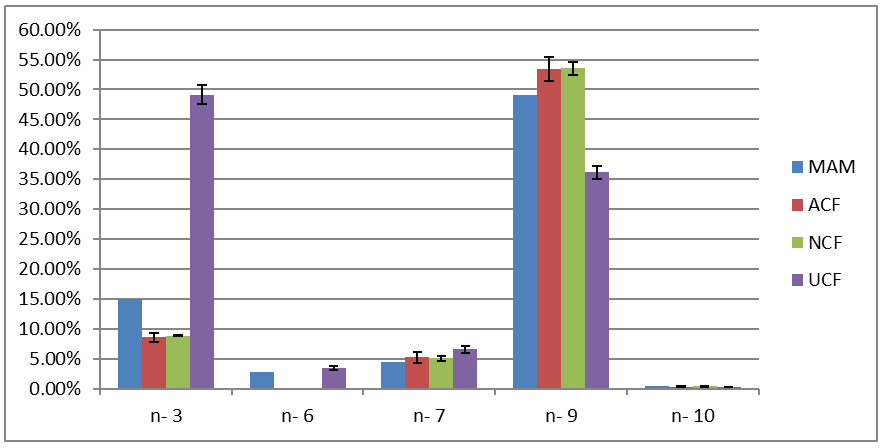

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Fig. 2. The fatty acid content in each fraction of based on the type of omega classification group

Fig 2 shows that the fraction of urea complexes containing n-3 was the highest compared to n-3 other omega groups. The urea crystallization method has been proven to increase n-3 content from 15.01% (MAM) to 49.13%. EPA was ranked on the top in the n-3 group which increased from 6.81% to 26.50%. DHA was in the second with an increase in content from 4.72% to 17.39%. This data conclusion is similar to the study conducted by Zhang et al [37] which also stated that urea crystallization was the most efficient method for separating n-3 from SFA and MUFA. The crystallization method with acetone and n-hexane contains more n-9 than other omega groups where the most dominant was EA. The content of EA in ACF and NCF increased from 47.95% (WM) to 52.49% (ACF) and 52.29% (NCF). While the n-3 group actually declined such as EPA from 8.08% to 4.80% (ACF) and 4.79% (NCF). This data was contradicted with Patil and Nag research [17], where acetone was the most suitable solvent to separate SFA from PUFA according to their result.

The crystallization method with acetone and n-hexane produced unsatisfactory fractions. the ratio value of PUFA/trans-FA and n-3/trans-FA that expected to increase precisely decreased compared to MAM. ACF contained ratio value PUFA/trans-FA, n-3/trans-FA, EPA/EA, and DHA/EA of 0.162 + 0.021, 0.162 + 0.011, 0.092 +0.009, and 0.056 + 0.009 respectively. While NCF contained ratio value of 0.167 + 0.003, 0.167 + 0.003, 0.092 +0.005, and 0.062 + 0.004 respectively. Both ACF and NCF did not show a significantly different ratio value based on single-factor Anova analysis ($p<0.05$). This showed that the ACF and NCF were highly dominated by EA. All value of the entire ratio can be seen in Fig. 3.

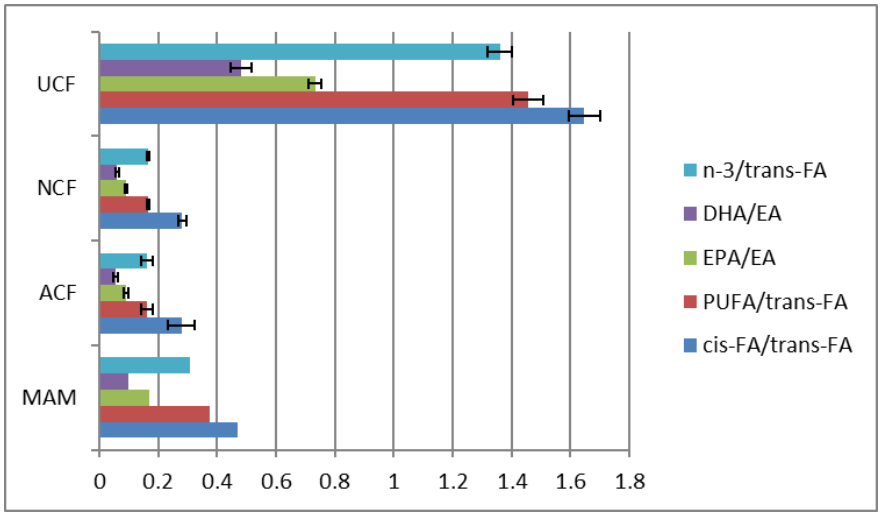

Fig. 3. The ratio value in the fraction that results from each crystallization process

Overall, the urea crystallization method was known to be able to provide more effective and efficient purification compared to the acetone and n-hexane crystallization methods. Urea crystallization was able to maintain the presence of PUFA n-3 groups, especially EPA and DHA in the fraction so that the levels increased [37]. Based on figures 2 and 3, the UPF fraction was very rich in n-3. UPF was able to increase the PUFA/trans-FA ratio from 0.37 to 1.45 and n-3/trans-FA from 0.3 to 1.36. The urea crystallization method proved to be able to produce fractions very rich in n-3 fatty acids. Even so, this method still needs to be further optimized because there was still an EA which has a higher amount compared to EPA and DHA. As shown in Figure 3, the ratio of EPA/EA and DHA/EA, although increasing, but still below one.

To eliminate EA completely, it is necessary to combine two purification methods such as urea crystallization method with molecular distillation which was able to separate EPA and DHA from sardine oil [38] or with Argentated Silica Gel Column Chromatography which was able to separate PUFA from tuna oil [36].

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Conclusion

The urea crystallization method was significantly able to reduce the content of SFA and EA (trans-FA), and be able to maintain n-3 PUFA especially EPA and DHA in the oil fraction. Its seem from the increase of ratio value of PUFA/trans-FA from 0.37 to 1.45 and n-3/trans-FA from 0.3 to 1.36. Even so, the crystallization of Urea has not completely eliminated EA from the oil fraction. The only SFA that survived in the three factions was lauric acid. Overall the Urea crystallization method was able to recycle MAM became n-3 rich fraction.

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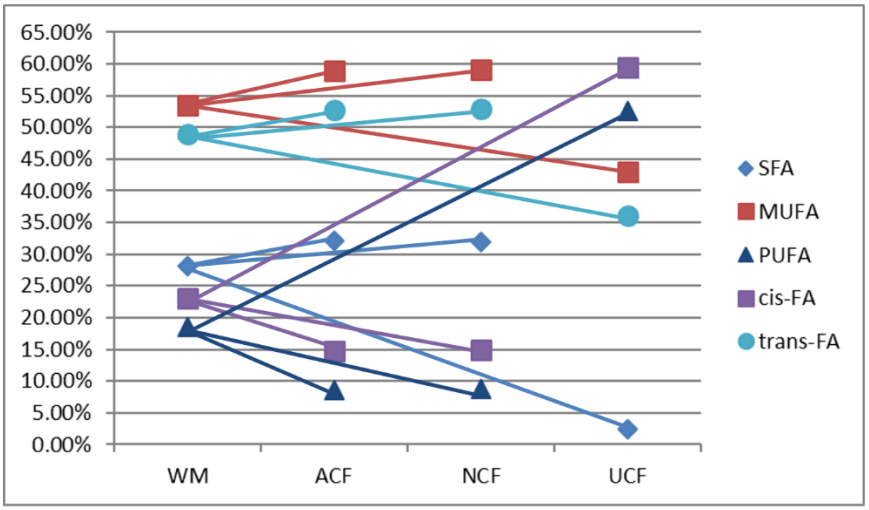

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