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Elimination of Elaidic Acid and Enrichment of Omega-3 Fatty Acid in Industrial By-Product of Fish Processing

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ABSTRACT. "Minyak Ala Muncar," abbreviated as MAM, is a by-product waste of a fish canning factory in Muncar Banyuwangi, Indonesia. According to GCMS, MAM contains essential fatty acids and trans Fatty Acids such as Elaidic Acid (EA), which could threaten the Indonesian population's health. Several purification methods have been applied to recycle the MAM. But, these methods did not concern the removal of EA from MAM. Even in other studies, the MAM was used as food fortification. Therefore, this study aimed to separate EA from the MAM and produced fractions of oil rich in n-3 fatty acids and free of EA. Three low-temperature crystallization methods, called winterization, namely crystallization with n-hexane, acetone, and urea, were conducted to remove the EA from MAM. The results showed that both crystallization with n-hexane and acetone produced ratio values 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 were 1.46 $\pm$ 0,05;1.36 $\pm$ 0.04; 0.73 $\pm$ 0.02; and 0.48 $\pm$ 0.04.

Keywords: Eicosapentaenoic acid, Elaidic acid, Low-temperature crystallization, Urea crystallization, Winterization

INTRODUCTION

Muncar Banyuwangi is one of Indonesia's most prominent centers of the fish canning industry. Both large and small factories of fish processing are operated in Muncar. One of the severe impacts resulting from this situation is oil waste. The oil was processed by local people and was circulated to fish, poultry, and animal breeders throughout Indonesia, used as animal feed supplements. This processed waste oil is called "Minyak Ala Muncar (MAM)." They treated the oil waste by heating it at high temperatures and adding caustic soda to separate the oil and residue.

GCMS assay showed that the MAM contained not only essential fatty acids such as linoleic acid (LA), α-linolenic acid (ALA), eicosa pentaenoic acid (EPA), docosa hexaenoic acid (DHA), but also it contained saturated fatty (SFA) acid and trans-Fatty acid (trans-FA) such as elaidic acid (EA) (Maulana et al., 2014). The presence of EA in MAM should be an essential concern, especially in health. The content of EA in oil poses a threat to the health of the Indonesian population. EA has been proven to correlate with the emergence of cancer risk factors (Ohmori et al., 2017), precipitating atherosclerosis (Menaa et al., 2013), and increasing hepatic lipogenesis as a hepatotoxic risk factor (Shao & Ford, 2013). EA could also diffuse into the mammary glands of nursing mothers, affecting breast milk quality (Daud et al., 2013). The preclinical trials showed that EA increased the metastasis of CT26 mouse Colorectal cancer (CRC) Cells by inducing the expression of stemness marker nucleostemin (NS) and epithelial-mesenchymal transition (Fujii et al., 2017). According to geometrical analysis, the trans fatty acid lipid structure was superior to the cis configuration, leading the lipid formation to a smaller area per lipid and a smaller diffusion coefficient (Tsai et al., 2015).

Several purification methods have been applied to purify the MAM, such as using adsorbent (Budiadnyani et al., 2020), neutralization with Alkali (RD et al., 2016), saponification and extraction with Aceton (Estiasih & Ahmadi, 2012), and Urea crystallization (Estiasih, 2010). But, these several methods did not notice about removal of EA from MAM. Other MAM research focused on the isolation of Lipid-Degrading bacteria (Sutrisno et al., 2016), and even the MAM was used as food fortification (Estiasih et al., 2017). EA was formed by heating at a high temperature during the processing of MAM. The application of high temperature during the processing of waste oil was known to affect the fatty acid content in the oil,

increase the oil toxicity (Okino-Delgado et al., 2017), and turn the cis-FA into trans-FA (Bockisch, 1998; Rani et al., 2010).

Three low-temperature crystallization methods, called winterization (Vázquez & Akoh, 2011) were applied to separate the EA and all trans-FA from MAM but keep the existence of EPA and DHA. According to Salimon et al., crystallization was able to separate PUFA from SFA and trans-FA (Salimon et al., 2012). The low-temperature crystallization methods applied in this process were winterization with n-hexane solvent, acetone solvent, and crystallization with urea. Saturated fatty acids and EA were known to have low solubility in acetone and n-hexane solvents, resulting in the best separation (Gunstone, 1996). The urea crystallization method was the most efficient, reproducible, fast, and environmentally friendly method (Haq et al., 2018). It could be applied to small and large amounts of waste (Patil & Nag, 2011). This study aimed to separate EA, a trans-FA, from the MAM and produced fractions of oil that are not only rich in n-3 fatty acids but also free of EA.

EXPERIMENTAL SECTION

MAM waste material was obtained from one of the producers in the Muncar Region, Banyuwangi, Indonesia. The FAME Mix 37 comp (SIGMA Aldrich) was used as a standard of fatty acid, TLC Plate (Merck).

Hydrolysis of Oil Became Free Fatty Acids

The MAM was hydrolyzed with a saponification technique using a solution of 15% Potassium Hydroxide in a mixture of water and methanol (1:1). The mixture was heated at 60 ^{O}C, accompanied by constant agitation (Guil-Guerrero & Belarbi, 2001). The soaped blends were then acidified using HCl until obtained pH 1. The *n*-hexane was added again, producing a two-layer, and the *n*-hexane layer was taken, then evaporated until the free fatty acids were obtained. These free fatty acids were ready to be purified.

Winterisation with Acetone and *n*-Hexane

Five grams of fatty acids were added with acetone 50 mL, then shaken until the fatty acids were completely dissolved. The mixtures were then stored in the freezer, with the temperature at -18 ^{O}C for 24 hours. Next, the combinations were removed from the freezer and filtered with a vacuum filter. Finally, the filtrate was evaporated to obtain a fatty acid fraction (Jala & Guo, 2012) , and we called it Acetone Crystallization Fraction (ACF). Next, the same procedure was carried out using *n*-hexane solvents until *n*-Hexane Crystallization Fraction (NCF) was produced.

Urea Crystallization

Five grams of fatty acids were added with 20 grams of urea (in 53.75 mL methanol). The mixtures were shaken until the fatty acids were completely dissolved. The mixture was then stored in the freezer (-18 ^{O}C) for 24 hours. Next, the mixture was removed from the freezer and filtered with a vacuum. The filtrate was kept in the freezer (-18 °C) for 3 hours and then filtrated. The filtrate was acidified with 6N HCl, then 20 mL of n-hexane was added. The *n*-hexane layer was separated from the mixture and then evaporated to produce a fatty acid fraction, and we named it Urea Crystallization Fraction (UCF).

Optimation of Time Duration of Fatty Acid Esterification

The Thin Layer Chromatographic was applied to determine the optimal length of esterification time. During the esterification process, the FAME formation was then monitored by thin-layer chromatographic (TLC) methods. One mL of fatty acids liquid was taken using a volume pipette at 30 minutes, 1 hour, 2 hours, and 3 hours after the esterification was running. Approximately 10 μL of the sample was then applied to the TLC plate. Elution was carried out using a combination of solvent systems; n-hexane-ethyl acetate-glacial acetic acid (95:5:1). The vapor of Iodine was then used as a reagent to observe a pigment of FAME.

Esterification of Fatty Acids

ACF, NCF, and UCF were then esterified using the method conducted by Guil-Guerrero and Belarbi (Guil-Guerrero & Belarbi, 2001). First, 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 completely dissolved. Next, the acetyl chloride was added as a catalyst to the mixture carefully, then refluxed at 70 °C with a paraffin bath under N_2 atmospheric conditions for 3 hours until FAMEs were obtained. Subsequently, FAMEs were separated from mixtures using n-hexane solvent and evaporated to obtain pure FAME.

FAME Observation In Fraction

Approximately 10 μL of each esterified sample was applied to the chromatographic plated, then eluted using a combination of the solvent as the same optimation procedure.

GCMS Analysis

The GCMS instruments used in FAME analysis were Variant-3900, GC/MS/MS Saturn 2000, CP-8400 autosampler, and GC (Shimazu, G-5000A) with a Hydrogen generator (Whatman) with a Bonded OV-1 column. The GC was conditioned to the ideal conditions of the analysis, in which the injector temperature was set at 240 °C. The detector temperature was set at 280 °C. The separation system in the GC thermostat oven was set with an initial temperature of 50 °C and held for 2 minutes. Then, the temperature was raised at a speed of 4 °C /minute until it reached 240 °C and was held for 2 minutes. So, the total duration of GC analysis for each sample was 51.5 minutes. ACF, NCF, UCF, and FAME standards were then injected into the GCMS system.

The area under the curve (AUC) produced from each top of the graph at the chromatogram was compared to the FAME standard with the same retention time parameters.

Fatty Acid Analysis

All information on fatty acids identified was further grouped based on the level of saturation into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Fatty acids were also classified according to the last position of the double bond, counting from the end of the methyl chain, or it was called the omega group.

Statistical Analysis

Data tables and figures were 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 set at $P < 0.05$ (Patil & Nag, 2011; Soleimanian et al., 2015). All experiments were carried out as three-time.

RESULT AND DISCUSSION

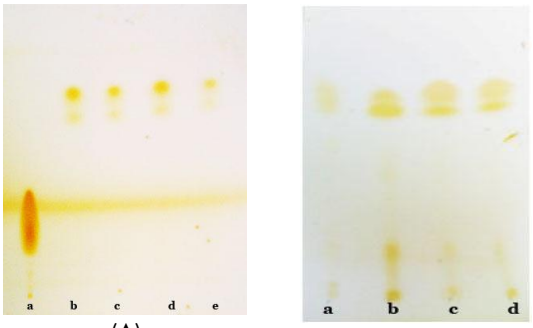
The esterification process of fatty acids into a methyl ester was intended to increase the level of volatility of fatty acids. This procedure was helpful once the time fatty acids were analyzed with the gas chromatography method. FAME was more volatile than the fatty acids form. The fatty acids that were esterified became FAME and were losing the hydroxyl group, which had a hydrogen bonding because the Hydrogen was replaced by the methyl group, which caused a decrease in the vapor point. Acetyl chloride was used as a catalyst because the material that would be esterified was an acid form, namely fatty acid. The esterification process of fatty acid would be more effective and faster when an acid catalyst is used. Guerrero (2001) also used acetyl chloride as a catalyst to esterify the UCF.

The chromatogram (**Figure 1**) shows that FAME is almost completely formed after 30 minutes of the esterification process. However, FAME began to form between 1 to 2 hours after the running process, so we take the 1,5 hours as the optimal duration of the esterification process.

Figure 1A showed that the fatty acid pigments have a lower rf than FAME. It is because fatty acids in the structure still have hydroxy groups, so they have a stronger affinity with the stationary phase, which was polar, while FAME was less polar than a fatty acid. After 1.5 hours of esterification, FAME from each fraction was successfully formed. This could be seen in **Figure 1B**, which shows that all bands were already above.

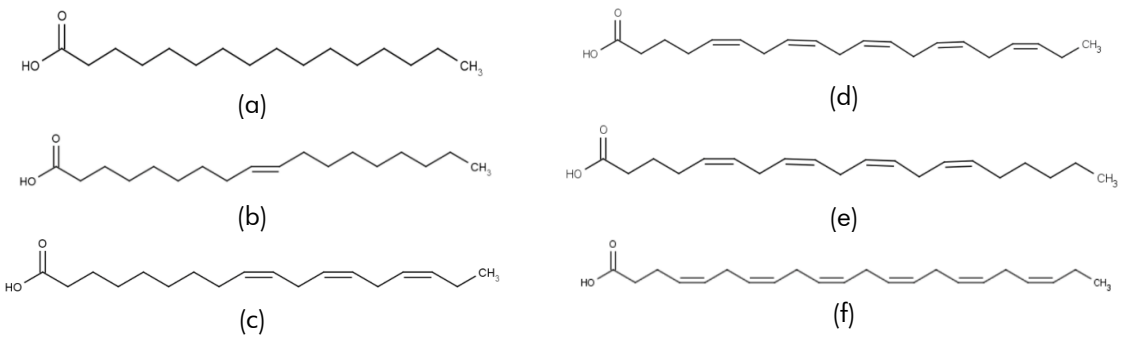
The Fatty Acid Content in MAM and Fraction

GCMS chromatograms of MAM, ACF, NCF, and UCF were displayed in **Table 1**. Palmitic acid was the most dominant fatty acid in the SFA group, and EPA was the most prevalent in the PUFA, whereas EA was the most predominant fatty acid in MAM. The structure of some fatty acids is shown in **Figure 2**.



(A) (B)

Figure 1. The Chromatogram of the observation of esterification process, (**A**) optimation of a duration of esterification process, a. 0; b. 0.5; c. 1; d. 2; e. 3 hours after the esterification process runs. (**B**) Observation of FAME formation in all fractions. a. FAME standard; b. UCF; c. ACF; d. NCF



(a) (d)

(b) (e)

(c) (f)

Figure 2. Structure of dominan fatty acid (**a**) Palmitic Acid (PA), (**b**) Elaidic Acid (EA), (**c**) α-Linolenic Acid, (**d**) Eicosapentaenoic Acid (EPA), (**d**) Arachidonic Acid (AA), (**d**) Docosahexanoic Acid (DHA)

Table 1. The Content of the fatty acids in "Minyak ala Muncar."

ΣC	n	MAM (w/v %)	ACF (w/v %)	NCF (w/v %)	UCF (w/v %)	SI
C12:0	-	0.10	0.31±0.10[a]	0.31±0.04[a]	0.25±0.04[a]	95
C14:0	-	1.93	4.77±0.35[a]	4.77±0.21[a]	0.61±0.05[b]	96
C15:0	-	-	0.19±0.01[a]	0.20±0.0 [a]	0.06±0.01[b]	94
C16:0	-	13.58	22.54±0.33[b]	21.29±0.08[a]	1.25±0.22[c]	94
C16:1	n-7	1.73	4.98±0.66[a]	4.77±0.26[a]	6.35±0.54[b]	96
C16:1	n-10	0.37	0.25±0.13[a]	0.36±0.07[a]	0.27±0.03[a]	92
C17:0	-	0.96	-	0.31±0.25	-	83
C17:0	-	0.27	-	-	-	83
C17:1	n-7	0.21	0.26±0.24[a]	0.29±0.23[a]	0.21±0.09[a]	91
C18:0	-	8.80	2.60±0.34[a]	3.67±0.34[a]	0.33±0.12[b]	96
C18:1	n-9	47.95	52.49±2.06[a]	52.29±0.62[a]	36.13±1.08[b]	95
C18:1	n-9	-	0.63±0.03[a]	0.68±0.08[a]	-	97
C18:2	n-6	-	-	-	3.48±0.30	93
C18:3	n-6	0.47	-	-	-	94
C18:3	n-3	-	0.82±0.16[a]	0.83±0.09[a]	4.95±0.12[b]	93
C18:4	n-3	1.40	-	-	-	90
C19:0	-	0.16	0.09±0.05[a]	0.12±0.05[a]	-	93
C20:0	-	1.54	0.29±0.02[a]	0.39±0.02[b]	-	93
C20:1	n-7	2.04	-	-	-	93
C20:3	n-7	0.55	-	-	-	93
C20:4	n-6	1.93	-	-	-	97
C20:4	n-3	0.81	-	-	0.29±0.07	92
C20:5	n-3	8.08	4.80±0.29[a]	4.79±0.18[a]	26.50±0.06[b]	96
C22:0	-	0.45	1.30±1.15[a]	0.94±0.76[a]	-	90
C22:1	n-9	0.95	0.29±0.07[a]	0.62±0.46[a]	-	91
C22:3	n-8	0.07	-	-	-	82
C22:5	n-6	0.44	-	-	-	94
C22:6	n-3	4.72	2.93±0.34[a]	3.22±0.27[a]	17.39±1.49[b]	95
C24:0	-	0.27	-	-	-	90
C24:1	n- 9	0.23	-	-	-	90

*w/v % in a pure fraction that was injected into GC.
Mean ± SD, n = 3

The content of EA in oil poses a threat to the health of the Indonesian population. EA have been proven to correlate with the emergence of cancer risk factors (Ohmori et al., 2017), precipitating atherosclerosis (Menaa et al., 2013), and increasing hepatic lipogenesis as a hepatotoxic risk factor (Shao & Ford, 2013). EA could also diffuse into the mammary glands of nursing mothers, affecting the quality of breast milk (Daud et al., 2013).

MUFA was the highest number of fatty acids in raw material (MAM), 53.48%, then SFA at 28.05%, and PUFA at 18.47%. Winterization with aseton decreased PUFA from 18.47% in MAM to 8.55% in ACF and cis-FA from 22.98% to 14.68% in ACF. However, the MUFA increased from 53.48% in MAM to 58.91% in ACF. Not only the MUFA but SFA and trans-FA also increased respectively, from 28.05% to 32.1%, and from 48.97% to 52.78% in ACF. **Table 1** shows that NCF produced by n-hexane winterization resulted in a similar fatty acid profile to ACF. However, only palmitic acid and arachidic acid had different values significantly (single-factor ANOVA with $p<0.05$).

Urea crystallization reduced SFA, MUFA, and trans-FAs to 2.51%, 42.97%, and 36.13% in UCF. Urea crystallization also increased the PUFA and cis-FAs to 52.61% and 59.45%, respectively. This result was in line with Salimon et al., where urea crystallization increased the composition of PUFA in oil by separating it from SFA and trans-FAs in oil (Salimon et al., 2012).

Urea crystals had a tetragonal geometry with a diameter of 5.67 Å. Therefore, the existence of aliphatic long-chain compounds could form a hexagonal structure with urea which convert the inner diameter became 8-12 Å (Wanasundara et al., 2005). However, more double bonds and the presence of cyclic in the compound would reduce the inclusion of urea crystals. It means that PUFA was more difficult than SFA to form inclusions with urea (Tang et al., 2018). In addition, urea complexation could interact not only with SFA in oil mixture but also with trans-FA such as EA. Therefore, SFA and trans-FAs would be interacted with urea to form the crystal complex, namely 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) (Salimon et al., 2012; Wanasundara et al., 2005).

According to Wanasundara et al., (Wanasundara, Shahidi and Wanasundara, 2005), eliminating SFA and trans-FAs with urea complexation may be difficult because some short-chain fatty acids do not interact to form complexes with urea crystals (Mu et al., 2016). Therefore, it was reasonable that the level of lauric acid in the UCF increased from 0.1 % in MAM to 0.25% in UCF. The ability of Urea crystallization to eliminate the SFA and trans-Fas was dependent on the ratio amount of urea and fatty acid, where an increase in the amount of urea would increase the number of fatty acids trapped in UCF (Mu et al., 2016).

The Fatty Acid Ratio Parameter In Fraction

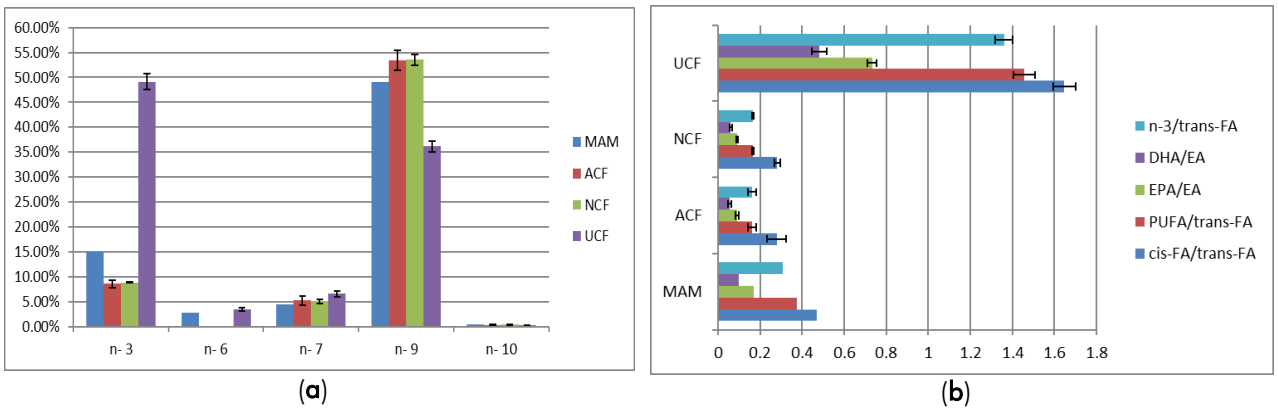
Omega (n) is a fatty acid-related naming system based on the position of the double bonds calculated from the end of the methyl at the fatty acid structure (Scrimgeour, 2005). n-3 means a double bond at the third position carbon from the methyl end in the fatty acid structure. EPA and DHA are an example of n-3 fatty acids, which most have health benefits, especially atherosclerosis. **Figure 2** displays that EPA has a similar structure to AA and only differs by one double bond at Carbon-17. Because of this similarity, both can interact with cyclooxygenase (COX) enzymes to produce prostaglandins (PG) which can interact with the same receptors. However, unlike AA, the prostaglandins produced by EPA are not pro-inflammatory agents (Calder, 2015; Dong et al., 2016). In addition, EPA is also highly correlated with an increase in the ratio of HDL to LDL, which can reduce risk factors for atherosclerosis (Abdelhamid et al., 2018). It allows EPA able to provide benefits against atherosclerosis and several other diseases. The status of fatty acid content based on omega classification can be seen in Figure 3a.

UCF was the fraction containing n-3 highest compared to ACF and NCF. The study has proved that The urea crystallization method could increase n-3 content from 15.01% in MAM to 49.13% in UCF. EPA was the highest of the n-3 group, which experienced an increased from 6.81% in MAM to 26.50% in UCF. DHA was in second, which increased from 4.72% to 17.39%. These phenomenons were in line with the study by Zhang et al. (Zhang et al., 2017), which stated that urea crystallization was the most efficient method for separating n-3 from SFA and MUFA.

The ratio between n-3 to trans-FA and EA (figure 3b) showed the quality of the fraction and the success parameter of the MAM recycling process. The crystallization method with acetone and n-hexane produced unsatisfactory fractions. The expectation of increasing PUFA/trans-FA and n-3/trans-FA ratios did not occur and instead decreased. ACF contained ratio value PUFA/trans-FA, n-3/trans-FA, EPA/EA, DHA/EA of 0.16 ± 0.02, 0.16 ± 0.01, 0.09 ± 0.01, and 0.06 ± 0.01 respectively. While NCF contained ratio value of 0.17 ± 0.01, 0.17 ± 0.01, 0.09 ± 0.01, and 0.06 ± 0.01 respectively. ACF and NCF did not show a significantly different ratio value based on the single-factor Anova analysis ($p<0.05$). It showed that EA highly dominated the ACF and NCF.

The UPF fraction obtained from the purification process was rich in n-3. The urea crystallization method increased the value ratio of PUFA/trans-FA, n-3/trans-FA, EPA/EA, and DHA/EA. The value ratio of each parameters namely 1.46 ± 0,05, 1.36 ± 0.04, 0.73 ± 0.02, 0.48 ± 0.04. Although EPA/EA and DHA/EA increased, these values were still lower than one point. It meant that there was still an amount of EA in the UCF. The urea crystallization method purified MAM more effectively and efficiently than acetone and n-hexane. Urea crystallization maintained the presence of PUFA n-3 groups, especially EPA and DHA, in the fraction (Crexi et al., 2012; Zhang et al., 2017).

Separating fatty acids with the winterization method using organic solvents depended on the ratio between fatty acids and solvents. It was closely related to the polarity of fatty acid and solvent and the cooling temperature(Vázquez & Akoh, 2011). Long-chain SFAs known have two molecular groups with different polarities. Carboxylic groups were a polar property, while long carbon chains were non-polar.



(**a**) (**b**)

Figure 3. (**a**) The omega group of fatty acids in all fractions (**b**) The ratio value in the fraction that results from each crystallization process.

The longer the carbon chain, the decreasing the polarity of fatty acids (Scrimgeour, 2005). That made the SFA content in acetone and n-hexane solvents increase.

The concentrations of palmitic acid as the most dominant SFA in both fractions increased from 13.58% in MAM to 22.54% in ACF and 21.29% in NCF. On the other hand, stearic acid decreased from 8.80% in MAM to 2.60% in ACF and 3.67% in NCF. Theoretically, stearic acid has a lower solubility in acetone if compared to palmitic acid, and its solubility decreases with decreasing temperature. So, the palmitic acid content in ACF and NCF should reduce (Wanasundara et al., 2005), but it did not happen. It was probably due to the diverse content of fatty acids in the oil, so it affected the behavior phase in the crystallization process, which became more complicated. In addition, it also caused a decrease in the nucleation velocity (Metin and Hartel 2005). Another cause was the presence of polymorphisms of fatty acid crystals so that saturated fatty acids did not crystallize (Soleimanian et al., 2015).

The crystallization method with acetone and n-hexane contained more n-9 than other omega groups, where the most dominant was EA. EA in ACF and NCF increased from 47.95% (MAM) to 52.49% (ACF) and 52.29% (NCF). While 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. This data was contradicted by Patil and Nag's research (Patil & Nag, 2011), where acetone was the most suitable solvent to separate SFA from PUFA, according to their result.

The decreasing of PUFA 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 (Wanasundara et al., 2005). At the crystallization method with a decrease in temperature, PUFA would be concentrated in the liquid fraction and separated from the crystals (Wanasundara et al., 2005). This data also contradicts other studies that state the concentration of PUFA increased sharply in the NCF fraction (Joseph & Chakraborty, 2017). The longer the carbon chain, the lower the polarity of the fatty acid. It makes fatty acids more soluble in acetone and hexane solvents. It may cause the high content of SFA in the fraction. Due to the high levels of SFA, the PUFA levels are seen to be lower.

CONCLUSIONS

The urea crystallization method significantly reduced the content EA (trans-FA) more effectively and efficiently compared to the acetone and n-hexane crystallization methods. Both crystallization with n-hexane and acetone produced ratio values of PUFA/trans-FA, n-3/trans-FA, EPA/EA, and DHA/EA below 0.3, respectively. While urea crystallization made the ratio value of PUFA/trans-FA, n-3/trans-FA, EPA/EA, and DHA/EA PUFA/trans-FA were more than one. The urea crystallization produced UCF, which is rich in PUFA, especially n-3, and less in SFA and EA. Only lauric acid from the SFA group increased in UFC because it was a short-chain fatty acid.

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