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Utilization of Crude Palm Oil Waste to Produce Squalene

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Abstract: Indonesia is the world's largest producer of palm oil. The increasing demand of palm oil production generates a large amount of waste. Palm fatty acid distillate (PFAD) is one type of the wastes coming from crude palm oil (CPO) refinery process. Utilization of this waste from being discharged will reduce harmful into the environment. According to some studies, PFAD contains bioactive compounds including squalene. Squalene is a natural antioxidant extracted mostly from shark's liver. Due to the main source extinction, an alternative source of squalene has been studied and developed from edible vegetable oils including palm oil and PFAD. Therefore, a simple methodology to utilize PFAD waste as source of squalene was developed by saponification process continued with liquid-liquid extraction using different non-polar solvents: n-heptane, dichloromethane, and chloroform. The yield of crude squalene obtained from n-heptane, dichloromethane, and chloroform extracts were 81.86%, 75.35%, and 22.16% respectively while the squalene content of n-heptane, dichloromethane, and chloroform extracts were 0.97%, 4.55% and 0.67% respectively. The antioxidant activity of crude squalene extract was also carried out by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical-Scavenging Assay and dichloromethane extract showed the highest activity of 0.0073 mg DPPH/mg Extract. Therefore, dichloromethane solvent was chosen further to undergo another modified saponification process which resulted in the yield of crude squalene of 1.44% with squalene content of 73.52% and showed an increase antioxidant activity (0.2027 mg DPPH/mg Extract).

Keywords: *Palm Fatty Acid Distillate, Squalene, Antioxidant Activity, Saponification, Liquid Liquid Extraction*

1. Introduction

Crude palm oil (CPO) has been produced largely in Indonesia as the world's biggest palm oil producers with around 33.5 million tons production of CPO in 2014 (United States Department of Agriculture, 2014). Increased productivity of palm oil production and planted area expansion of the trees (Slette, 2014) cause more wastes to be generated. Due to the large waste production issues, some studies performed to minimize the waste produced. Fajri et al. (2014) investigated shikimic acid extraction contained in palm oil mill effluent and Sari et al. (2014) utilized palm empty fruit bunch for insulated sandwich panel core.

The CPO will be processed further to yield higher quality oil through oil refinery process. One of the wastes from oil refinery process is called Palm Fatty Acid Distillate (PFAD) which is also considered to be vegetable oil refinery distillate. Rather than being discharged, PFAD can be utilized to become a more valuable product. In general, PFAD waste is used in soap industry (Ping and Yusof, 2009) and as a raw material for synthesis of biodiesel (Catran et al., 2011). Despite of being used for soap and biodiesel, there is still a possibility of the waste to contain beneficial bioactive compounds such as vitamin E, squalene, and sterols, and for this reason, some studies have been conducted to extract these compounds. For instance, a research has been also carried out by Estiasih et al. (2013) to characterize the bioactive compounds of palm fatty acid distillate (PFAD) from several palm oil refineries, and furthermore a previous study about palm fatty acid distillate as a source of squalene was also conducted by Silangen et al. (2014). Since the presence of squalene in Indonesian palm oil refinery waste has been confirmed by previous study, the need to have an optimum extraction method to obtain pure squalene is important to be further investigated. From the economical perspective issue in the waste management system, a simple extraction method is more preferable to be developed.

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Squalene is known as a useful chemical that can be used as pharmaceutical ingredients and food supplement. The main richest source of squalene is found in shark's liver oil and therefore the price is relatively expensive (Günes, 2013). The world issues of marine life prevention including an extinction in the sharks populations which also confirmed by Baum and Myers (2003) causing a limit in the extraction of squalene from shark's liver. Another limitative reason to use this animal as the squalene source lies on the presence of different persistent organic pollutants (POPs) that can cause a harmful effect if consumed by human (Popa et al.,2014).

Recent interests in finding new alternative sources of squalene from the edible vegetable oil are being studied. Some researches have been conducted to identify squalene concentration from several plant oils. The first identification of squalene in olive oil had been conducted by Thorbjarnarson and Drummond (1935). The presence and concentration of squalene in several vegetable oil sources such as from soybean oil, grape seed oil, corn oil and palm oil have also been studied by Frega et al. (1992). However, recent technology reveals that extracting this valuable constituent directly from the vegetable oils is not economical to be performed since the concentration is too small. Popa et al. (2014) stated that the by-products obtained in the vegetable oil refinery process, such as the deodorizer distillate, contain 15 to 30% unsaponifiable fraction, with a concentration of up to 80% squalene, and depending on several factors, extraction of squalene from vegetable oil distillate has a chance to be conducted more economically. Therefore, PFAD, as considered to be by-product from vegetable oil refinery process, might be having a bigger chance to contain squalene.

2. Literature Review

2.1. Palm Oil Process

Palm oil contributes to 19% of worldwide vegetable oil and its fruit contains about 56% oil (Embrandiri et al., 2013). Generally, there are two main products of palm oil which are crude palm oil (CPO) and palm kernel oil (PKO). The crude palm oil is extracted from the flesh pulp of the fruit while the palm kernel oil is obtained by extracting from the seed of the fruit (Hassan et al., 2013). CPO is also used primarily for food products while the PKO is commonly used for oleo-chemical including cosmetics and soaps.A number of stages from the sterilization of the fresh fruit bunch (FFB) including the digestion, threshing, oil extraction and clarification of the oil are occupied in the process of crude palm oil production. A pressed cake after the oil extraction undergoes depericarping process where the whole of the pericarps or outer portion of the fruit is mechanically removed from the inner portion. Then the nut is cracked and the kernel oil is produced. For crude palm oil, it will undergo further refinery process to produce higher quality of oil. Two different refining process applied are physical and chemical refining. These techniques result in different final products. The difference lies on the addition of chemicals added (used in chemical refining) while the physical refining steps actually enhanced the physical characteristic in separating the impurities, such as particle size, boiling points, solubility, etc.

2.2. Palm Oil Waste and Palm Fatty Acid Distillate

In general,the wastes of palm oil production are divided into two types: solid wastes and liquid wastes and the wastes produced mainly are palm oil mill effluent (POME) and empty fruit bunches (EFB). It has been estimated that one tonne of crude palm oil production requires 5-7.5 tonnes of water in which about 50% ends up as POME (Embrandiri et al., 2013).Since palm oil industries produce large quantity of waste, some of these wastes are then utilized in many ways, for instance: Empty fruit bunch (EFB) can be used as fibre and particle boards, palm oil mill effluent (POME) is used for fertilizers, animal feed and phenolic antioxidants (Hassan et al., 2013).

In refining process of CPO, palm fatty acid distillate (PFAD) is a by-product of physical refining of crude palm oil (CPO) products. PFAD comprises about 4% in the waste. Ab Gapor (2010) also said that PFAD consists mainly of free fatty acids for about 81.7%, glycerides (14.4%), squalene (0.8%), vitamin E (0.5%), sterols (0.4%) and other substances (2.2%).

2.3. Squalene

Squalene is one of bioactive compounds that was discovered in 1906 by Mitsumaru Tsujimoto, a Japanese industrial engineer and is made up of 30 carbon polyprenyl compound including 6

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isoprenoids. The molecular formula is $C_{30}H_{50}$ (2,6,10,15,19,23-hexamethyl tetracosaheaxaene) can be seen in Figure 1. Squalene, which is structurally similar to beta-carotene, is known to be an intermediate product in cholesterol synthesis.

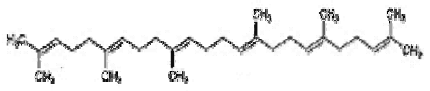

Figure 1. Chemical structure of squalene (Tjan, 2011).

Squalene is also categorized as triterpenes that carries oxygen independent of the hemoglobin and directly to cell membranes throughout the body reaching the regions having low oxygen supply. Squalene has been also accounted to have an inhibitory effect on cancer promotion and a high anti-tumor activity and is believed to enhance immune system and wound healing (Günes, 2013).

As a consequence of squalene's biochemical structure, it is extremely reactive to get into the oxidized form if consumed by human. The unsaturated carbons of squalene bind hydrogen ions from water and release 3 unbound oxygen molecules, providing the saturated form squalane, $C_{30}H_{62}$. Due to this reaction, the oxygen reaches the cells, the cellular metabolism is intensified, the function of certain organs, like liver and kidney, is enhanced (Kelly, 1999) and finally the vitality rises.

3. Research Method

The research began with saponification and then continued with liquid-liquid extraction using three different solvents including N-Heptane, Chloroform and Dichloromethane. Saponification was done by weighing 5 g of PFAD in 250 ml Erlenmeyer added with 0.25 g Ascorbic Acid, 2.5 ml of 2 M KOH and 44 ml Ethanol 96%. The mixture was heated 30 minutes in a water bath 70^{o}C. 100 ml Water and 75 ml non-polar solvents were added after saponification. Unsaponifiable fraction was separated. The modified saponification was done by weighing 5 g of PFAD in 250 ml Erlenmeyer added with 0.25 g Ascorbic Acid, 5 ml of 50% KOH (w/v) and 44 ml Ethanol 96%. The mixture was heated 1hour in a water bath 70^{o}C. 100 ml Water and 75 ml dichloromethane solvent were added and Unsaponifiable fraction was separated.

After extraction was done, the sample was evaporated and the total solid content was calculated. The dried extract was then analyzed by using Thin Layer Chromatography, UV-Vis Spectrophotometer, Fourier Transform Infrared (FTIR) Spectroscopy and Gas Chromatography Mass Spectrometry (GC-MS). The last step, all samples were checked for each antioxidant activity by using DPPH Radical-Scavenging Assay. All experiments were duplicated twice.

4. Results and Discussion

4.1. Saponification Process, Liquid-Liquid Extraction and GC-MS Result

Saponification was done to remove free fatty acids contained in PFAD, because the importance of doing saponification was to convert free fatty acids and triglycerides as much as possible into soaps and glycerol which led to increase the polarity, thus they were likely to be removed by polar solvent in the next process. This saponification step contributed to the success of the next liquid-liquid extraction which used a mixture of polar and non-polar solvents to further separate targeted bioactive compounds in the sapon mixture.

Subsequent to the saponification process was the liquid-liquid extraction process. Two immiscible solvents used were water and non-polar organic solvents (n-heptane, chloroform, and dichloromethane). Water was used to pull the polar sapon matters which were the impurities fatty acids that had already been converted into glycerol and soaps, while the non-polar solvents were used to extract the low-polarity constituent which was the squalene. However, there was a problem encountered during liquid-liquid extraction. Vigorous shaking in separating funnel during extraction resulted in an emulsion which caused by the soap formed during saponification that served as a bridge between aqueous phase (water) and organic phase (non-polar solvents). This emulsion influenced the activity of non-polar solvent to extract the squalene because the emulsion made the separation process of organic

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phase from mixture to become harder. It was possible that the squalene was trapped inside the emulsion and not yet transferred into the organic phase because when the separation took place, only the transparent organic phase was taken not the other else layer.

During experiment, the most emulsion formed occurred for chloroform solvent, this might be caused by the polarity it had which were the most polar among the other non-polar solvent. Therefore, those emulsions gave an impact to the yield from chloroform solvent which lowered volume of the chloroform layer collected during separation process.

Some analyses were done including TLC, UV-Vis Spectrophotometer, FTIR, and GC-MS. GC-MS confirmed that crude extract contained squalene and the best solvent extract that resulted in the highest content of squalene was dichloromethane extract with 4.55% squalene content and for the other squalene content from the other solvent extracts can be seen in Table 1.

Table 1: Squalene content of extracts based on GC-MS.

Extract	Squalene content based on GC-MS (%)
N-heptane	0.97
Dichloromethane	4.55
Chloroform	0.67

Table 2: Major constituents of extracts based on GC-MS.

N-Heptane Extract			Chloroform Extract			Dichloromethane Extract		
Retention Time (min)	Area (%)	Constituent	Retention Time (min)	Area (%)	Constituent	Retention Time (min)	Area (%)	Constituent
32.554	46.54	Palmitic Acid	32.464	62.79	Palmitic Acid	32.423	48.85	Palmitic Acid
-	-	Stearic Acid	-	-	Stearic Acid	32.533	17.40	Stearic Acid
33.595	48.24	Oleic Acid	33.512	34.44	Oleic Acid	33.485	26.18	Oleic Acid
37.250	0.97	Squalene	37.243	0.67	Squalene	37.243	4.55	Squalene

Table 2 shows the major constituents contained in the crude squalene extract. Mostly, the extract still contained palmitic acid, oleic acid and stearic acid. These acids were still categorized as free fatty acids that were not removed by saponification and liquid-liquid extraction process. Therefore, those free fatty acids were still found in the chromatogram. Those free fatty acids influenced the content of the squalene contained in the extract. The bigger the peak area of fatty acids means that the abundance amount of the free fatty acids towards squalene amount or in other words the free fatty acids are more concentrated than the squalene.By knowing the percentage of substance individual's area, it is concluded that squalene found is very small and categorized as minor components.

The abundance presence of free fatty acids indicated that the saponification process was still not optimum. If all free fatty acids were converted into soaps, the content of squalene obtained should be higher. The more fatty acids that were not turned into soaps influenced the extraction of squalene by non-polar solvents. The large amount of free fatty acids had also non-polar characteristic. Then, the addition of non-polar solvents could also have an interaction with the acids. This interaction influenced the interaction between non-polar solvent with squalene because there was a lesser space of squalene to be dissolved in the solvent and the rest of the space was occupied by free fatty acids. Every solvent had maximum capacity to hold and dissolve a matter, and since the GC chromatogram result showed that the extract still contained abundance acids amount, it pointed out that free fatty acids dominated the space for squalene in solvent extraction due to bigger chance to contact with the solvent.

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According to Table 1 which represents the squalene content of extract obtained by GC-MS, dichloromethane exhibits the highest content of squalene among the others. This result shows that dichloromethane had the least interaction with the free fatty acids. Therefore, it provided a larger space to be occupied with squalene. N-heptane had the second less interaction with free fatty acids and the most interaction occurred to chloroform.

4.2. Percentage of Yield

Some data had been collected during experiments and several solvents were used to extract squalene including n-heptane, dichloromethane, and chloroform. The summary of the data is shown in Figure 1 which represents the percentage yield of crude squalene extracts obtained from each 5 g of PFAD waste.

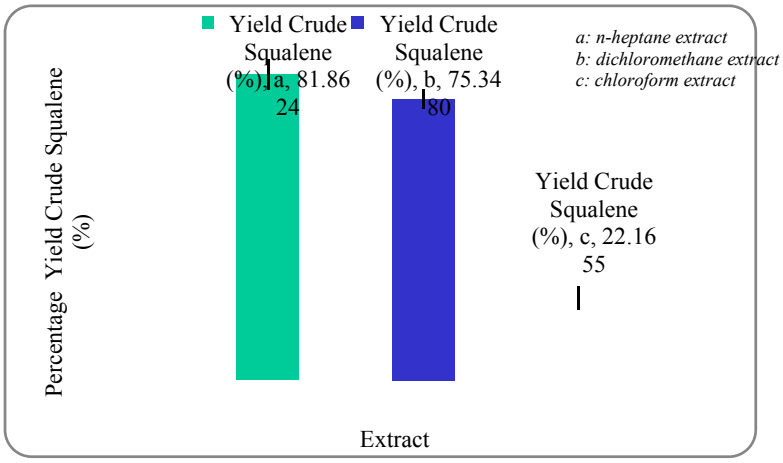

Figure 2. Percentage yield of crude squalene.

It can be seen in Figure 2 that N-heptane produced the highest yield for about ± 81% of crude squalene extract followed by dichloromethane ± 75% and chloroform ± 22%. This yield was related to the liquid-liquid extraction process. The yield of chloroform extract was the smallest because during extraction, the emulsion was the biggest to be formed. This emulsion occurred because of the sapon or soap that bridged the aqueous phase and organic phase. The more emulsion formed resulted in more lather which hardened separation of organic phase from the aqueous phase. Chloroform has the most polarity among the others. Thus, it was slightly to be more miscible with the aqueous polar phase.

The yield of heptane in contrast produced the highest yield that can be correlated with the polarity of heptane which was the least polar compared to dichloromethane and chloroform. This characteristic helped the separation process of organic phase from aqueous phase to be easier since both liquid was immiscible. Though the sapon was still formed in the n-heptane extraction, as time went by, when the liquid-liquid extraction was left for hours, the sapon went down back to the aqueous phase, leaving a clearer organic phase of n-heptane. Thus, the immiscible mark between organic phase and aqueous phase was easier to be observed and separated.

4.3. Antioxidant Activities Result

Antioxidant activities test from the crude squalene extract were also carried out to determine which solvent extract had the highest activities towards 2,2-dipehnyl-1-picrylhydrazyl (DPPH) radical. The result is presented in Figure 3.

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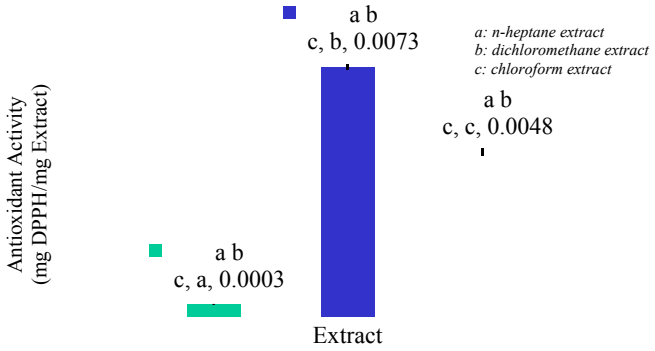

Figure 3. Antioxidant activities of crude squalene extracts.

The antioxidant activities were performed based on DPPH inhibition method which measured the amount of DPPH that was consumed by the antioxidant compound contained in the extract. The amount of DPPH consumed related to the decrease in the absorbance of extract and the calculation of DPPH consumed is based on the difference of absorbance control and absorbance extract. Then, the absorbance difference is converted into concentration of DPPH using DPPH calibration curve. The larger antioxidant agent contained in the extract, the higher the activities towards the DPPH radical.

The result of antioxidant activities is expressed in mg DPPH consumed per mg extract. As seen in Figure 3, the biggest activity was possessed by dichloromethane extracts with 0.0073 mg DPPH/mg Extract, followed by chloroform and n-heptane extracts. Referring back to the constituents' composition of extract it Table 2, there is an oleic acid contained in the extract. Cho et al. (2013) also stated that oleic acid had antioxidant activity again low-density lipoprotein (LDL) oxidation because it has double bond between carbons 9 and 10 (Relative to α-COOH group). Therefore, oleic acid is also known to be omega-9 and categorized as monounsaturated fatty acid (MUFA). In addition, consuming oleic acids also increased high-density lipoprotein (HDL) in the body (King, 2015). Thus, it can be pointed out that oleic acid might give contribution to the antioxidant activities of the crude extract. However, there was an anomaly occurred, n-heptane extract which had purity higher than chloroform extract should possess higher antioxidant activity but the impurities seemed to inhibit the antioxidant activity of n-heptane extract and this indicated that there was another compound cause this inhibition but was not investigated further.

4.4. Relation between Yield, Squalene Content and Antioxidant Activity of Crude Squalene Extract

Table 3: Relation between yield of crude squalene, squalene content and antioxidant activity of crude squalene extract

Solvent Extract	Solvent Polarity	Yield of crude Squalene	Squalene Content	Antioxidant Activity
N-Heptane	3	1	2	3
Dichloromethane	2	2	1	1
Chloroform	1	3	3	2

According to Table 3, the most polar solvent used as well as the best result of yield, squalene content and antioxidant activity are ranked with number 1. N-heptane extract has the highest yield of squalene and the yield is increasing proportionally with the decrease in the polarity of solvent used in extraction. This means the less polar solvents extract higher yields. For the squalene content, dichloromethane extract produced the highest squalene content followed by n-heptane extract and chloroform extract. It can be indicated that the squalene content did not correlate with the yield and polarity of the solvents used in extraction. The content of squalene is affected by the impurities contained in the extract and the influence of the solvent to the impurities. Solvent which produces the highest content of squalene has

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less interaction with the fatty acids impurities which means that dichloromethane has the least interaction with fatty acids while chloroform has the biggest interaction with fatty acids.

The antioxidant activities of crude squalene extract is affected by impurities contained in the extract. It is found that for dichloromethane and chloroform extract, the impurities enhanced the antioxidant activity which is predicted coming from the oleic acid group, a monounsaturated fatty acid that possesses an antioxidant activity. For n-heptane extract, it looks like that there is another impurity mechanism which inhibits the antioxidant activity which is not studied further in this study.

4.5. Modified Saponification

The first method of saponification yielded low squalene content due to large amount of free fatty acids content. Therefore a modification of saponification was done in order to increase the squalene content. Dichloromethane solvent was chosen as the solvent for further liquid-liquid extraction since it generated the highest content of squalene. This modified saponification managed to increase squalene content as shown in Figure 4. The increased content of squalene also produced a significant increase in the antioxidant activity as seen in Figure 5. However, in fact the free fatty acids resulted from saponification process are generally used in the soap industry as the raw material to make soaps because it produced lather. If the saponification was done optimally, then the other products besides fatty acids might contain a more concentrated squalene. Basically, the PFAD waste from CPO refinery can generate two products which are the raw material for making soaps and to produce squalene.

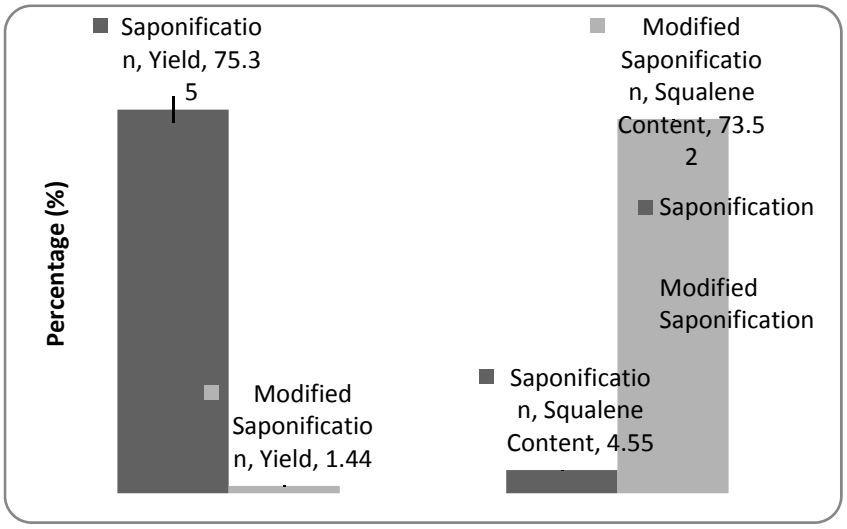

Figure 4. Yield and squalene contentresultcomparison of saponification and modified saponification.

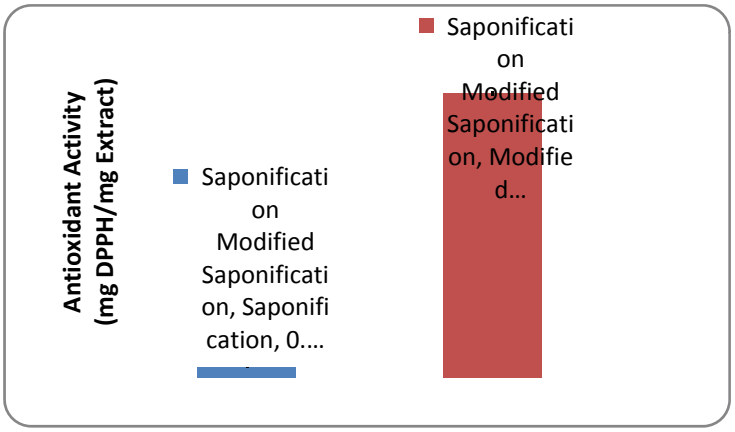

Figure 5. Antioxidant activity comparison of saponification and modified saponification.

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5. Conclusion

These experiments in the utilization of crude palm oil (CPO) waste to produce squalene resulted in several improvements that had been conducted. Based on the result, there are some conclusions that can be pointed out as follows: the waste from CPO can be an alternative source to produce squalene through saponification and liquid-liquid extraction using n-heptane, chloroform and dichloromethane solvents. The best solvent to produce the highest yield of crude squalene extract is N-heptane (81.86%). Meanwhile, the best solvent that resulted in the highest content of squalene is dichloromethane (4.55%) and the best solvent used to extract crude squalene with the highest antioxidant activity is dichloromethane (0.0073 mg DPPH/mg Extract). The modified saponification by adding more concentrated KOH and increased the process time resulted in higher squalene content which also increased the antioxidant activity of crude squalene. The free fatty acids can also be used for soap industry.

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