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IMPROVING METHODOLOGY OF SQUALENE EXTRACTION FROM PALM FATTY ACID DISTILLATE (PFAD) THROUGH ENHANCED PRE-TREATMENT PROCESS

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ABSTRACT

Indonesia has been accounted as one of the biggest palm oil producers in the world with annual production capacity reaching over 34 million tons. As a consequence, the amount of wastes resulting from this industry requires immense attention to be given. One of the wastes resulted is the Palm Fatty Acid Distillate (PFAD), which in previous researches has been proven to contain some beneficial bioactive compounds such as squalene. Squalene is known as one of the best natural emollients for pharmaceuticals and cosmetics, so that many researches have given the attempt to extract squalene from PFAD. Despite all attempts, large amount of impurities such as free fatty acids (FFA) were still to be found present in squalene extract. Therefore, in this research an effort to enhance the pre-treatment process of PFAD was done by combining saponification process and centrifugation, in order to remove FFA prior to extraction process. Three different pre-treatment scenarios in single stage liquid-liquid extraction (LLE) were compared in their effect on squalene content found in the extract using GC-MS analysis. The analysis showed that the squalene content increased from 5.370 to 9.320 % (w/w) when centrifugation was applied. Adding another round of saponification to this method has increased the content even further to 23.940 %. Furthermore, the application of multiple stage extraction could increase the squalene content to 37.450 %.

Keywords: *Liquid-liquid extraction; multiple stage extraction; Palm Fatty Acid Distillate; squalene.*

ABSTRAK

Indonesia merupakan salah satu negara penghasil minyak sawit terbesar di dunia, dengan kapasitas produksi melebihi 34 juta ton per tahun. Dengan besarnya volume produksi minyak sawit, jumlah limbah yang dihasilkan pun sangat besar dan memerlukan perhatian khusus. Salah satu limbah industri minyak sawit adalah Distilat Asam Lemak Minyak Sawit (PFAD), yang telah dibuktikan mengandung beberapa senyawa bioaktif, di antaranya adalah *squalene*. *Squalene* merupakan salah satu krim alami terbaik untuk pengobatan dan kosmetik, sehingga banyak upaya telah dilakukan untuk mengekstrak *squalene* dari PFAD melalui proses bertingkat tunggal dan jamak. Akan tetapi, beberapa pengotor seperti asam lemak bebas masih ditemukan dalam ekstrak *squalene* yang diperoleh. Oleh karena itu, dalam penelitian ini dilakukan upaya untuk mengoptimalkan proses perlakuan awal, dengan menggunakan teknik separasi untuk mengurangi jumlah asam lemak sebelum ekstraksi dilakukan. Dari percobaan ini, ekstraksi tingkat tunggal memperlihatkan hasil analisa GC-MS kandungan *squalene* yang meningkat dari 5,370 ke 9,320 % (b/b) jika dilakukan sentrifugasi. Penambahan proses penyabunan pada metode ini juga meningkatkan kandungan squalen lebih lanjut ke 23,940 %. Dengan penggunaan ekstraksi bertingkat jamak dapat meningkatkan kandungan squalen menjadi 37,450 %.

Kata kunci: *Distilat Asam Lemak Minyak Sawit (PFAD); ekstraksi bertingkat jamak; ekstraksi cair-cair; squalene.*

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INTRODUCTION

In recent years, palm oil has become one of the most potential resources of vegetable oils in the world. Indonesia has been one of the biggest countries that produces palm oil which yields over 30 million tons yearly where 80 % is exported. There are several waste products, which come from the production of palm oil such as empty fruit bunches, palm press fiber, palm oil mill effluent, palm kernel cake, palm kernel shell, and sludge cake (Prasertsan and Prasertsan, 1996). Due to the high production rate of palm oil in Indonesia, the high amount of palm oil waste offers high potency to be utilized. One of the wastes that is generated through the refinery process of crude palm oil is Palm Fatty Acid Distillate (PFAD), which can be used as feedstock for animal feed, soap industry, oleo chemical industry and combustion for local power generation (Zero & Rainforest Foundation Norway, 2015).

PFAD was also found to contain some beneficial bioactive compounds, among those is squalene (Yusuf, *et al.*, 2015), which can be used mainly for food supplements and pharmaceutical ingredients. In addition, it also can be considered as one of the best natural emollients in pharmaceuticals and cosmetics (Gapor, 2010). Moreover, squalene can be applied as a detoxification factor, as an eye and skin antioxidant. It has been widely known, that the demand for squalene is increasing from time to time, while there is limited availability from conventional sources such as shark liver oil. At the time being, the availability of shark liver oil is low due to many regulations prohibiting the killing of sharks. With this condition, the interest to get squalene from other sources has been arisen. Obtaining squalene from PFAD became very favorable to be explored, despite several obstacles faced related to its purity (Gapor, 2010).

Based on a previous study (Yusuf, *et al.*, 2015), squalene extract can be obtained by using organic solvents such as dichloromethane (DCM) through liquid-liquid extraction (LLE). Wandira, *et al.* (2017) has proposed an optimum condition for the saponification and extraction process to extract squalene from PFAD, resulting in an extract with a squalene content of around 24 %-w. In a separate

study, Sibuyo, *et al.* (2017) has applied multiple stage LLE to extract squalene from PFAD using DCM, and has proven that compared to a single stage extraction, this method could increase squalene content up to 1.33 times, depending on the ratio used between PFAD and DCM. However, in these researches, the content of FFA in the extract was still considerably high. It could be assumed, that the saponification process conducted prior to LLE, was not able to remove the majority of FFA contained in the PFAD. Hence, this research was aimed at enhancing the pre-treatment process, and to study the effect of incorporating different sequences of PFAD pre-treatment methods on the squalene content in the extract.

MATERIALS AND METHOD

Materials

The materials used in this research include PFAD sample, L-ascorbic acid (Merck, Germany), potassium hydroxide (Merck, Germany), ethanol 96 % (JT-Baker), dichloromethane (Mallincrodt, USA), iso-propanol (Mallincrodt, USA), toluene (Mallincrodt, USA), and distilled water. The sample of PFAD was taken from a palm oil refinery industry in Bekasi, Indonesia.

Research Methodology

In this experiment, several pre-treatment methods were applied (Figure 1). It comprised of three different research paths, which were then followed with acid base titration analysis. Research path 1, is principally the suggested squalene extraction method from previous researches (Sibuyo, *et al.*, 2017 & Wandira, *et al.*, 2017), whose results this research aimed to improve. In research path 1, PFAD would go under a saponification process prior to a liquid-liquid extraction, with the purpose to as much as possible reduce the amount of FFA, by converting it into glycerol and soap. After saponification, LLE process would proceed using dichloromethane (DCM) as a solvent, where afterwards, the extracted sample was analyzed by using gas chromatograph – mass spectroscopy (GC-MS) analysis for the determination of squalene content.

In the second and third research paths, a combination between saponification and centrifugation was applied, prior to LLE. Centrifugation is a process that can separate a mixture based on density differences by applying centrifugal force field. The product of saponification between PFAD and KOH are mainly glycerol and soap, with the rest of unreacted FFA and impurities contained in the mixture. Given that the density of glycerol was 1.26 g/cm³ and the density of soap is 0.932 g/cm³, centrifugation was

considered to be a practical method to separate the two phases. Free fatty acid, with a density of 0.961 g/cm³ is expected to be found in a bigger portion in the bottom layer together with glycerol, while squalene with a density of 0.858 g/cm³ should be found in the upper layer together with the soap. This way, it is expected that adding a centrifugation process after saponification will give favor to the subsequent process, which is the extraction of squalene.

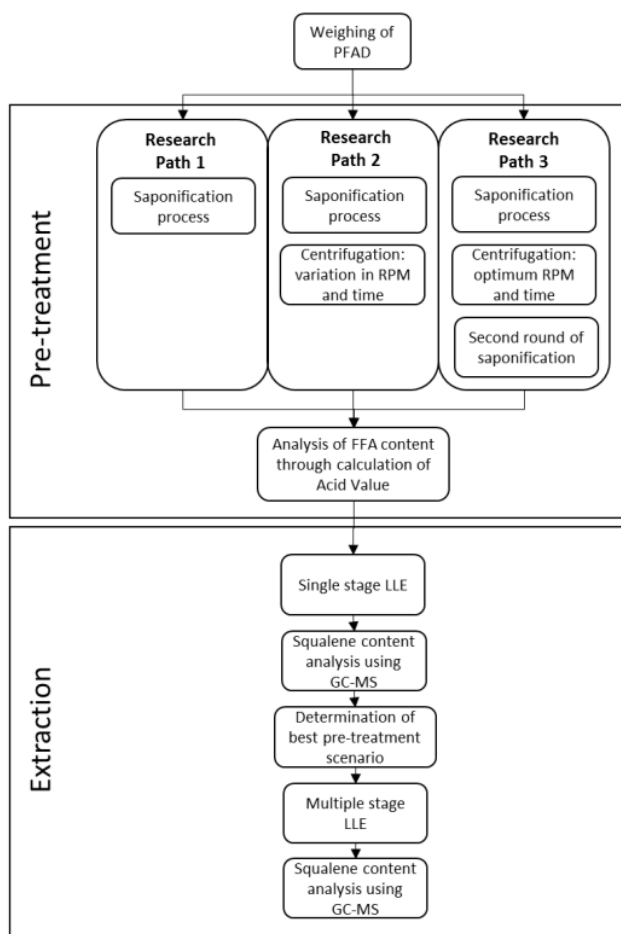


Figure 1. Experiment design

Multiple stage extraction process with 3 stages was applied in the latest part of the experiment in order to determine how much increase of squalene

content can be achieved by using a combination of the selected pre-treatment scenario and the multiple stage extraction process.

Pre-Treatment Process

The pre-treatment process will comprise saponification and centrifugation processes. As much as 10 g of PFAD sample was added to 0.500 g of ascorbic acid in the three necks round bottom flask. Then, 88 ml of 96 % ethanol was added into the mixture. Heating was applied to maintain the temperature at 70°C by using water bath while being continuously stirred using magnetic stirrer. The mixture was saponified with 10 ml of 50 % w/v concentration of potassium hydroxide (KOH) using reflux condenser in water bath for 60 minutes. Then, centrifugation of the mixture occurred twice at 3,000 rpm and 5,000 rpm for 10 and 30 minutes. In the research path 3, the saponification procedure was repeated after the centrifugation process.

Extraction of Sample

Single Stage Extraction

The sample and 100 ml of distilled water was poured into 500 ml separator funnel at ± 25°C and shook in vertical direction carefully. Following this step, 75 ml of dichloromethane was added into the mixture and left for approximately an hour until two separated layers were formed. The transparent

layer of the mixture was removed and the remaining liquid was mixed with another 75 ml of dichloromethane and left for an hour for extraction. The transparent layer was removed once again to be collected with the previous obtained extract. This repetition was conducted three times, and the total volume of the transparent layer was measured and labelled as Extract 1.

Multiple Stage Extraction

The multiple stage extraction was conducted following the schematic diagram shown in Figure 2. Each circle in this figure represents a single extraction process, with the exact procedure to be performed as explained previously. The numbers written in the circles show different separator funnels used in each step. The procedure for multiple stage extraction as shown above is a means to batch-wisely approximate a continuous multiple stage process. The first three stages shown in the diagram is the pre-conditioning stage, where stages 4 until 6 are considered to be the approximated real condition. Hence, the analysis of squalene content was done on extracts E9, E12 and E15, and additionally also on extract E1, so that a comparison between squalene content prior and subsequent to multiple stage extraction can be made.

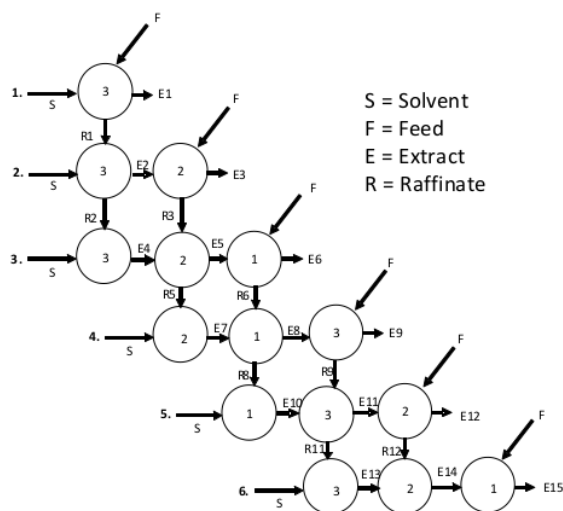


Figure 2. Multiple extraction process with 3 stages

Analysis Techniques

Titration Acid-Base Analysis

In order to determine the fatty acid percentage in oil, titration acid-base analysis based on ASTM D 974 (American Standard 2003) was conducted. This is the standard test method for acid and base number by color indicator titration. This method can be used to indicate the acidic or base constituents in petroleum products and lubricants that are soluble in mixtures of toluene and isopropanol. Titration was conducted in this study by the addition of 50 ml of iso-propanol and 50 ml of toluene into the sample in 250 ml conical flask. The addition of 20 drops of naphthol benzene indicator into the solution then followed, and this mixture was titrated with standard alkali solution (potassium hydroxide 0.087M), while being vigorously rotated until dark green color was observed. The volume of standard KOH was used to determine the free fatty acid value.

Gas Chromatograph – Mass Spectroscopy (GC-MS) Analysis

Gas Chromatograph – Mass Spectroscopy (GC-MS) analysis was used to analyse the squalene content of the resulting extracts. The column that was used was HP Ultra 2 Capillary Column Length

\times Internal Diameter \times Film Thickness = 30 m \times 0.25 mm \times 0.25 μ m. Helium (He) gas was used as the carrier gas. The initial temperature of oven was set at 70°C and held for 0 minute, then rising at 3°C/min to 150°C. The instrument was then injected with 1 μ l sample with the constant flow of 0.9 μ l/min. Thus, it was being on hold for 1 minute and finally rising at 20°C/min to 280°C and was held for 26 minutes. The temperature was set for injection port at 250°C, ion source at 230°C, interface at 280°C and quadruple at 140°C. The detector used was coupled to mass spectrometry.

RESULTS AND DISCUSSION

Optimization of pre-treatment process

The extraction method by Sibuyo, *et. al.* (2017) & Wandira, *et. al.* (2017), which only suggested a single saponification method as a pre-treatment prior to LLE, was to be improved through addition of centrifugation and another round of saponification in this research. In order to analyze whether there is a decrease in FFA content in the PFAD sample in each path, titration acid-base analysis was conducted. The volume of KOH added to change the titrated sample color into dark green was used to calculate the FFA content (%-w).

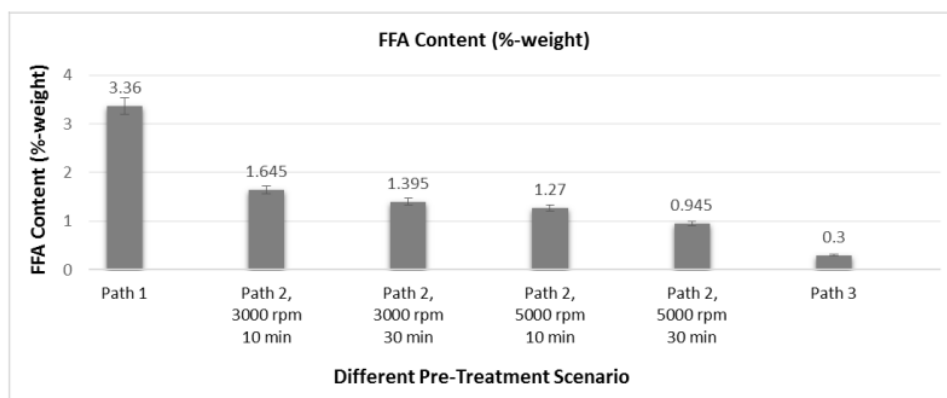


Figure 3. Free fatty acid content (%-weight) from different pre-treatment scenario

The result summarized in Figure 3 shows that by applying centrifugation after saponification process (research path 2), the FFA content can be reduced to less than half of its initial amount. The FFA content has decreased from $3.36 \pm 0.03\%$ to $0.945 \pm 0.135\%$ when a centrifugation at 5,000 rpm for 30 minutes took place subsequent to saponification process. Adding another round of saponification subsequent to the centrifugation process (research path 3) has shown further improvement in removing FFA. Research path 3, which applied a combination between centrifugation and double saponification, has shown results with lowest FFA content ($0.3 \pm 0.06\%$) and therefore is proven to be able to remove a large portion of FFA contained in PFAD.

In order to confirm that the application of research path 3 is not only going to remove FFA but will as well have an effect on the squalene content in the final extract, the observation was continued by performing single stage extraction subsequent to the pre-treatment. Squalene content analysis of the

resulting extracts was then conducted by means of GC-MS analysis.

All extract samples resulting from the single LLE process and the squalene standard were injected into GC-MS to obtain the chromatograms and mass spectrums. The result of GC-MS was observed qualitatively by chromatogram, including the quantitative analysis by the area under each peak which was shown in the chromatogram. Based on the chromatogram of squalene standard as shown in Figure 4 below, the retention time of squalene was shown at 26.107 min. Therefore, there should be a peak with similar retention time in the chromatogram if the extract does contain squalene. An example of GC-MS analysis result on one extract is shown in Figure 5, where a similar peak to the squalene standard could be observed at an approximately same retention time. This indicates qualitatively that this extract indeed contains squalene. Afterwards, a quantification of the amount of squalene present in the extract was done by determining the area below the peak.

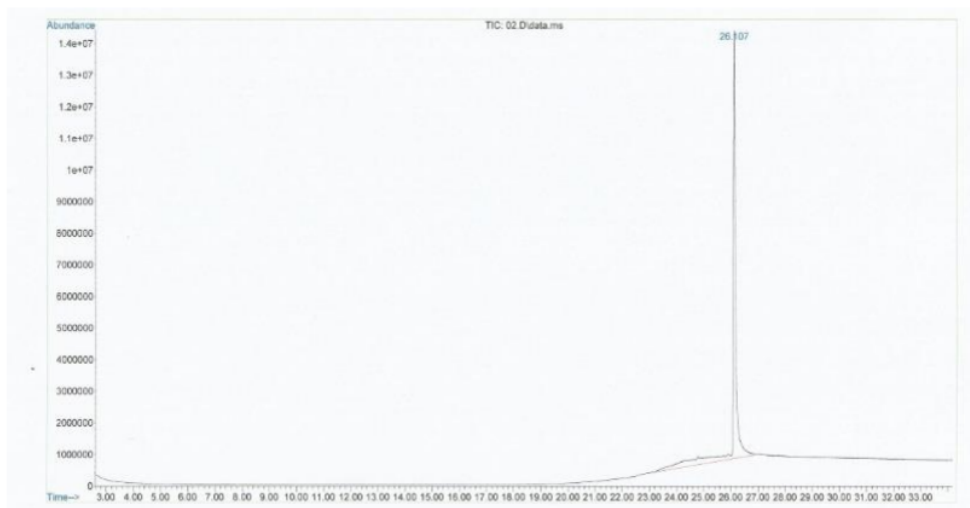


Figure 4. Chromatogram of squalene standard at retention time 26.107 min

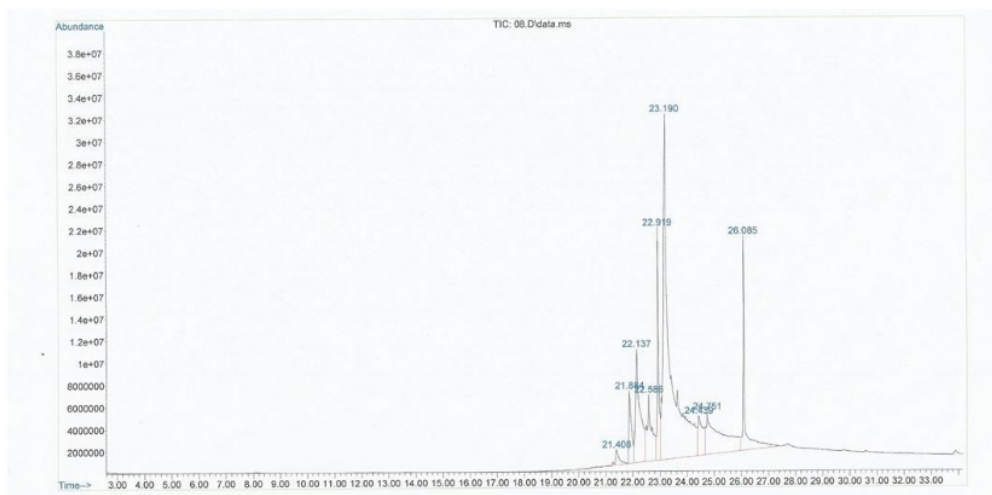


Figure 5. Chromatogram of an extract (research path 2 at 5,000 rpm and 30 minutes) at retention time 26.085 min indicating the existence of squalene

The three different pre-treatment scenarios performed previously were observed further to study their effects on the squalene content in the

extract through GC-MS analysis and the results of these analyses are depicted in Figure 6.

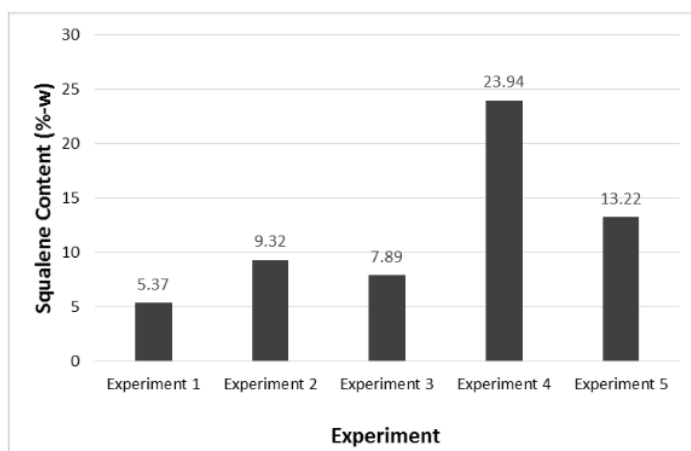


Figure 6. Squalene content in extracts resulting from 5 experiments based on GC-MS Result. Experiment 1: saponification process with 50 %-w/v KOH continued by LLE process (representing the research path 1). Experiment 2 and 3: saponification process with 50 %-w/v KOH, continued with centrifugation at 5,000 rpm for 30 minutes, and LLE (representing research path 2 in two replications). Experiment 4 and 5: saponification process with 50 %-w/v KOH, followed by centrifugation at 5,000 rpm for 30 minutes, second step of saponification with 50 %-w/v KOH, and LLE Process (representing research path 3 in two replications).

Figure 6 shows that the squalene content in the extract resulting from single stage extraction was increased from 5.370 % (research path 1) to 9.320 % (research path 2). However, the highest squalene content was found in experiment 4 (research path 3), where up to 23.940 % squalene content was obtained. Even though the replication of GC-MS analysis of the same research path (experiment 5) did not deliver as high squalene content as in experiment 4, it still showed an increase in squalene content compared to other experiments.

There are several reasons why research path 3 delivered highest squalene content. This research path combined a centrifugation and double saponification as pre-treatment of PFAD, and was proven to be able to remove a large portion of FFA in it. After the implementation of this combination of pre-treatment, the FFA content was reduced down to 0.3 ± 0.06 %. During extraction, squalene was expected to be more soluble in DCM, while soap phase will be distributed more in water phase. The extraction solvent DCM was chosen, because it had been proven by Yusuf (2015) to be the best solvent in extracting squalene from PFAD. However, FFA is also found to be highly soluble in non-polar organic solvent (Astuti, *et. al.*, 2010), so that it might also be found in DCM phase during extraction. Hence, reducing the amount of FFA as much as possible prior to the extraction will favor the selectivity of squalene.

Moreover, the centrifugation was also capable in removing a large portion of glycerol and soaps, which are not desired to enter the LLE process. Removing these impurities has been proven to have significant effect on the squalene content in the final extract. Hence, based on this result, an optimum pre-treatment process for squalene extraction from PFAD is decided to follow the research path 3, which combines a centrifugation and double saponification process.

Multiple Stage Extraction

Multiple stage process (Figure 2) was applied in this study in order to determine how far the squalene content can be increased by applying the previously chosen pre-treatment method. The first three stages were the preliminary stages, and for the analysis, stage 4 until 6 were observed. Each circle of the process represents a single stage extraction conducted in a separator funnel, where layers of extract and raffinate would be formed. The extract resulted from stage 4 to 6 were then analyzed using GC-MS to determine their squalene content. Additionally, extract coming from the first step was also analyzed in order to make a before-and-after comparison of the squalene content. The GC-MS results of these extracts are summarized in the Figure 7 below.

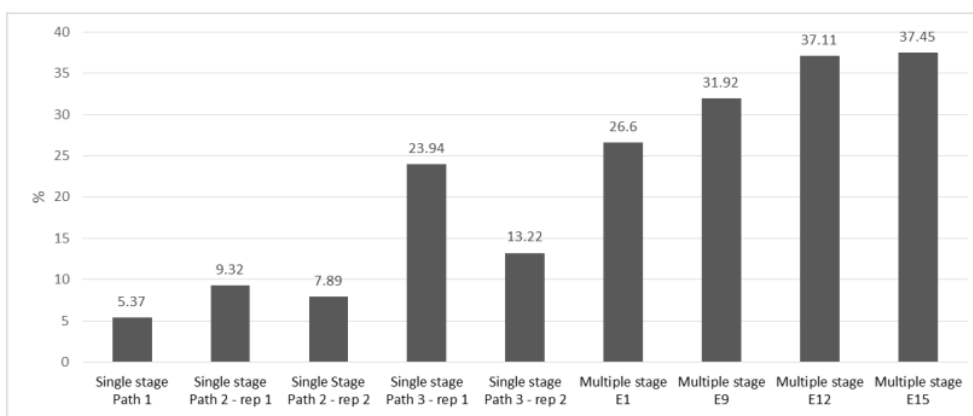


Figure 7. Comparison of squalene content in single and multiple stage extraction based on GC-MS result

As mentioned in previous section of this paper, extract E1 was analyzed to represent the single LLE using research path 3 as pre-treatment, whereas extracts E9, E12 and E15 were analyzed to represent the results of multiple stage LLE. The extract E15 is considered as the resulting final extract approximating real condition in a continuous multiple stage LLE process. Even though the experiment labelled with Single Stage Path 3 in Figure 7 was conducted with the exactly same procedure with E1, the GC-MS result showed slightly different value in squalene content. This could be caused since there was possibly a slight difference in the PFAD sample, as the waste specification taken from the palm oil industry might differ from day to day, depending on many factors during the production process.

The final squalene content resulting from the multiple stage process (extract E15) was 37.450 %, which was significantly higher than the squalene content in the extract obtained from single-stage extraction, which was 26.600 %. It has been shown, that the application of pre-treatment processes combined with multiple stage extraction was proven to be able to significantly increase the squalene content in the extract. However, the removal of FFA prior to the LLE process did not necessarily reduce its content in the extract to the lowest possible amount. A relatively large amount of FFA was still detected in the GC-MS analysis results²³ which were mostly the oleic and palmitic acid. In order to reduce the FFA content in the extract even further, hence, increasing the squalene content, the application of other separation techniques need to be explored. An extract purification must also be taken into consideration, in order to remove impurities from the squalene extract.

CONCLUSION

This research has proven that by applying centrifugation process in a combination with double saponification, the free fatty acid (FFA) content in PFAD can be reduced down to 0.3 ± 0.06 %-w. Further combination between this pre-treatment and a multiple stage extraction process was found to be able to increase the squalene content in the extract up to 37.450 %-w. Compared

to a single stage extraction with saponification alone as pre-treatment, this number shows an increase of squalene content of around six folds. It was also concluded that despite this positive result, there exists a big room for improvement to be explored in future works. The impurities were still found as majority in the extract, and mostly was identified as free fatty acids. This indicates that the removal of FFA in the pre-treatment must be supported also with extract purification at the end.

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