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OPTIMISATION OF SQUALENE RECOVERY FROM PALM OIL BY-PRODUCT USING INTEGRATED SCCO$_2$-PRESSURE SWING

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ABSTRACT

Squalene is used as an ingredient in functional foods, pharmaceuticals, and cosmetics. Due to the limitation of squalene availability from predominant source which is shark liver oil and to sustain the environment by fully utilising the by-product of palm oil, an effort has been made to find a potential source of squalene as an alternative to shark liver oil. The motivation for this work is to optimise its extraction from an alternative source, a palm oil by-product known as palm fatty acid distillate (PFAD), using supercritical carbon dioxide (sc-CO$_2$) with the aid of a pressure swing technique. The measurements were performed using a sc-CO$_2$ extraction in a fixed bed at temperatures of 40°C, 50°C, and 60°C, pressures of 20, 30 and 40 MPa, and holding times of 20, 30 and 40 min; high performance liquid chromatography (HPLC) was used for squalene content analysis, optimised with a central composite design using research surface methodology (RSM). The second-order polynomial mathematical model adequately fitted the experimental results. The maximum squalene content from the model was predicted to be 356.24 ppm under the set of conditions with pressure of 23 MPa, temperature at 40°C, and 20 min holding time.

Keywords: squalene, palm fatty acid distillate, sc-CO$_2$, pressure swing technique.

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INTRODUCTION

The increasing number of uses of squalene in multiple global industries is alarming. Squalene is commonly associated with health benefits, and has pharmaceutical, cosmetic and food functional applications. For instance, it is used as an antioxidant in skin and eye creams, and as a bactericidal and fungicidal agent (Bhattacharjee *et al*., 2012). In addition, squalene is commonly used as a natural moisturiser or emollient agent in personal care products (Vázquez *et al*., 2007). As squalene is not very susceptible to peroxidation, it acts as a quencher of singlet oxygen in the skin (Ryan *et al*., 2007), and so is used, in dietary form, to protect human skin from lipid peroxidation caused by exposure to ultraviolet and other sources of oxidative damage.

However, the supply of squalene is limited. It is predominantly derived from animal products. Shark liver oil is the primary source of squalene, as it is approximately 80% squalene (Fornari *et al*., 2008). However, sharks are protected and this supply is not sustainable. Therefore, it is necessary to find alternative sources of squalene (Bhattacharjee *et al*., 2012). One such source is plant oils, for instance palm oil, wheat germ oil, rice bran oil, amaranth oil, and olive oil (Bhattacharjee *et al*., 2012; Bondioli *et al*., 1992; Fornari *et al*., 2008; Vázquez *et al*., 2007;

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Westerman *et al*., 2006). Squalene can be derived from palm fatty acid distillate (PFAD). PFAD is a low-value by-product obtained from the physical refining of palm oil during the fatty acid stripping and deodorisation stages (Al-Darmaki *et al*., 2012; Chin *et al*., 2012; Suleiman *et al*., 2012; Hosseini *et al*., 2015). In Malaysia, 649 459 t of PFAD were produced from the processing of 19.96 million tonnes of crude palm oil (CPO), accounting for 3.25% of the waste from the refining of a tonne of palm oil (MPOB, 2016). PFAD is commonly used as a feedstock for other industries, for example in the production of animal feeds and oleochemicals (Boonrod *et al*., 2017; Haslenda and Jamaludin, 2011). Even though, PFAD can be used in biorefinery, however, the main limitation is the transportation cost between plantation area and biorefinery (Koncsag *et al*., 2012). Squalene has been recovered from a by-product of the palm oil industry (Al-Darmaki *et al*., 2012; Gapor Md Top, 2010). Moreover, the use of an industrial by-product can increase profits and reduce the need for waste disposal (Riggi and Avola, 2008).

PFAD is 72.7%-92.6% free fatty acid (FFA), plus non-glycerides (1%-2.5%); the non-gylcerides are highly valuable phytochemicals, such as vitamin E (tocopherols) and squalene (Al-Darmaki *et al*., 2012; Gapor Md Top, 2010), which have wide applications in the food, cosmetics, and pharmaceuticals industries (Boonrod *et al*., 2017). The use of PFAD as an alternative source of such phytochemicals can lessen operation costs and sustain the market price of the end-products (Haslenda and Jamaludin, 2011).

The extraction and purification of squalene from PFAD using conventional methods like solvent extraction and steam distillation require many steps (Zaidul *et al*., 2007). Even though, the extraction of squalene using organic solvents might produce high extraction yield, however, this technique will give a severe impact towards environment (Corzzini *et al*., 2017). The use of toxic solvents such as hexane and chloroform in the conventional methods can be harmful to health and the environment (Mubarak *et al*., 2015). Furthermore, Ille (2000) argued that conventional methods tend to lose valuable volatile compounds during vacuum evaporation of the solvent, and the difficulty of removing the solvent from extracts may impact the yield and quality of the total production.

Extraction of valuable compounds using the conventional technology contributed to an intensifying problem of the environment. Limitations of using conventional extraction techniques can be overcome by using compressed gas as a solvent in the extraction process (Ille, 2000). Supercritical carbon dioxide (sc-CO_2) is an emerging tool for extraction due to its superior properties. Interestingly, for sc-CO_2 there is no distinction between the liquid and gas phases. The supercritical fluid has liquid-like density and gas-like viscosity (Brunner, 2005), and its fluid properties can be controlled simply by changing the temperature or pressure of the system without the need to cross any phase boundaries. Another important characteristic of the supercritical fluid is its dissolving power, which is dependent on its density, and this can be varied continuously by manipulating the temperature and pressure of the system in the vicinity of the critical point (Brunner, 2005). This in turn means that extraction times can be shorter and higher yields can be obtained, even for materials of high molecular weight, like waxes, paraffin, lipids, and resins (Azmir *et al*., 2013). A small isothermal rise in pressure near the critical point will significantly increase the fluid density (Brunner, 2005). However, this behaviour lessens further away from the critical point (Brunner, 2005).

Various researchers in the literature have studied the squalene recovery from olive oil deodoriser distillate (Paolo *et al*., 1993; Vázquez *et al*., 2007). Leng *et al*. (2008) studied the separation of squalene from PFAD using adsorption chromatography. The recovery of squalene from PFAD using sc-CO_2 was proposed in a previous paper (Suleiman *et al*., 2012). An extensive study on the supercritical fluid extraction and fractionation of squalene from PFAD using compresses carbon dioxide was done by Al-Darmaki *et al*. (2012). Herein, the focal aim is to investigate and optimise the recovery of squalene using sc-CO_2 integrated to a pressure swing technique in order to improve the extraction process and increase the yield (Zaidul *et al*., 2007). Pressure swing technique consists of pressurisation and depressurisation steps during the process of extraction using sc-CO_2. This technique initially has been proposed for the separation of cashew nut shell liquid from cashew nut shells (Smith Jr *et al*., 2003). The findings showed the yields had increased when applying pressure swing technique rather than constant pressure extraction and also reduced the consumption of CO_2 in the extraction process (Smith Jr *et al*., 2003). Zaidul *et al*. (2007) also investigated the use of pressure swing technique with sc-CO_2 in the separation of palm kernel oil from palm kernel and found out the yield was about double that obtained with continuous extraction. To our knowledge, there are no studies devoted on the squalene recovery from PFAD using sc-CO_2 with pressure swing technique.

MATERIALS AND METHODS

Materials and Analytical Methods

PFAD (iodine value, IV, 64.3 g I_2/100 g oil; slip melting point, SMP, 47.5°C) in semi-solid phase was supplied by Golden Jomalina Food Industries Sdn Bhd, Teluk Panglima Garang, Selangor, Malaysia.

Squalene with a purity of 98% was purchased from Sigma Aldrich. The *n*-hexane and 100% acetonitrile were purchased from Merck, Darmstadt, Germany. Commercial immobilised *Candida antartica* lipase (Novozyme 435) was purchased from Novo Nordisk, Bagsvaerd, Denmark. Carbon dioxide with a purity of 99.9% was purchased from Moxlinde Gases Sdn Bhd, Petaling Jaya, Selangor, Malaysia. All chemicals used were either of analytical or high performance liquid chromatography (HPLC) grades. Squalene content was measured by an HPLC analyser equipped with a UV detector (SPD-10AV VP UV Detector; Shimadzu) from Agilent Technologies. The analytical column was kept at 35°C and the UV-Vis detector was set at 208 nm. Identification was through spiking with a squalene standard solution and quantification was based on the use of a standard curve.

Experimental Set-up: Supercritical Fluid Extraction

A schematic diagram of the experimental set-up is presented in *Figure 1*. The maximum operating temperature and pressure were 100°C and 50 MPa, respectively. A high pressure pump (Jasco PU-1580 Intelligent HPLC Pump, Jasco Inc., Easton, USA) was fitted with a cooling jacket for the CO_2 supply. To cool the pump head, a low temperature bath circulator (Model 631D; Tech-Lab Manufacturing Sdn Bhd, Selangor, Malaysia) was used with a circulating mixture of (50%, v/v) ethylene glycol and deionised water. The volume of the extractor vessel (Model EV-3; Jasco Inc., Easton, USA) was 50 ml and the water bath was set to the desired temperature.

In this set-up, there were three independent variables: pressure (20, 30, 40 MPa), temperature (40°C, 50°C, 60°C), and holding time (20, 30, 40 min) for a 90 min dynamic extraction time. The liquid CO_2 was compressed to the desired pressure and continuously pumped through the extractor at 3 ml min^{-1}. The temperature was controlled using a water bath. *Figure 2* illustrates the experimental design. At a given pressure and temperature, the extractor containing PFAD was pressurised (step P1, *Figure 2*) with compressed CO_2 within 5 min, held for 20 min at the given conditions (step H1, *Figure 2*), and depressurised within 10 min (step D1, *Figure 2*). This pressure swing step was repeated three times before continuing with the continuous extraction for 60 min (step P4-C, *Figure 2*). Only the depressurisation steps and continuous extraction were taken into account for the extraction time, hence, the total extraction was 90 min. Note that the compressed CO_2 was not consumed during the holding steps (H_1, H_2, H_3). The yield was determined for each depressurisation step and at the end of the continuous extraction period.

Experimental Design

RSM was applied to optimise the process parameters for the sc-CO_2 extraction of squalene from PFAD with the aid of a pressure swing

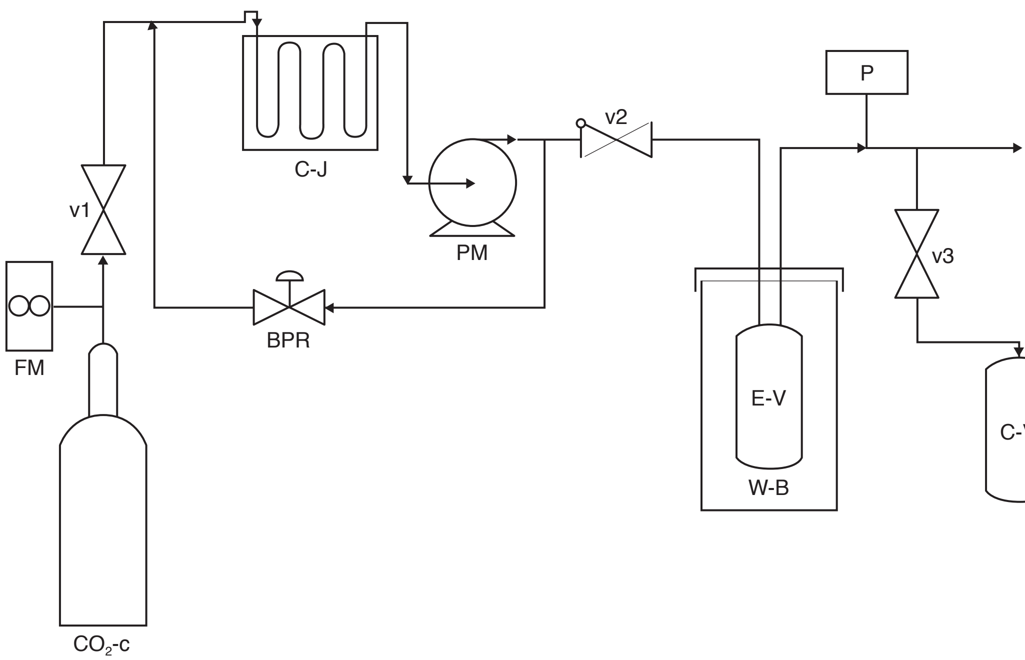

Figure 1. Supercritical carbon dioxide (sc-CO_2) controlled set-up with the aid of a pressure swing technique. The components are labelled as follows: CO_2-C (carbon dioxide cylinder); FM (flow meter); v1 and v3 (gate valve); v2 (check valve); C-J (cooling jacket); PM (pump); BPR (back pressure regulator); E-V (extractor vessel); W-B (water bath); P (pressure gauge); C-V (collector vessel).

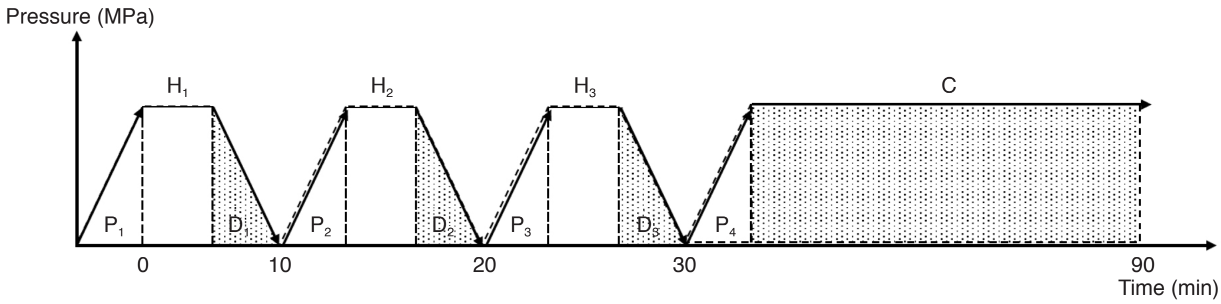

Figure 2. Experimental design for the extraction of squalene from palm fatty acid distillate (PFAD) using supercritical carbon dioxide (Sc-CO$_2$) with the aid of a pressure swing technique. The steps are labelled as follows: P_1, P_2, P_3, P_4: pressurisation steps of 5 min each; H_1, H_2, H_3: holding steps of 20 min; D_1, D_2, D_3: depressurisation steps of 10 min each (total extraction time is 30 min), which combined is the overall pressure swing step; C: continuous extraction for 60 min.

technique. The pressure (X_1), temperature (X_2), and holding time (X_3) were independent variables studied to optimise the squalene yield (Y). The independent variables were converted to range from -1 up to 1 for the appraisal of factors. *Table 1* lists the coded levels of the independent variables used in the RSM. The coded levels of the independent variables were chosen based on the preliminary experimental results. In order to minimise the effect of unexplained variability in the observed response due to extraneous factors, all experiments were performed in random order (Liu *et al*., 2009).

Herein, the experimental design was set up according to a central composite design (CCD). This design consists of three parts: (1) a full factorial or fractional factorial design; (2) an additional design, often a start design in which experimental points are at distance α from its centre; (3) a central point (Bezerra *et al*., 2008). CCD was chosen to ensure the availability of all information for analysis and a star design with three central points, as shown in *Table 2*. MINITAB release 14 was used for multiple regression analysis, analysis of variance (ANOVA), and to process the optimisation point. A quadratic polynomial regression model was used to predict response variables, *Y*, as a function of the parameters (independent variables) according to Equation (1):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum\sum \beta_{ij} X_i X_j \qquad (1)$$

where β_0 is an intercept, β_i, β_{ii}, and β_{ij} are first-order model, quadratic model, and interaction model coefficients, respectively. The X_i and X_j are the levels of the independent variables (Tan *et al*., 2009). The model was evaluated and the analysis included the overall model significance, associated probability, correlation coefficient, *R*, and determination coefficient, R^2. Response surface and contour plots were developed using the fully fitted quadratic polynomial equations obtained by holding one of the independent variables with the least effect on the response at a constant value and varying the levels of the other variables within the experimental range (Chu *et al*., 2004). All statistical tests were carried out with a confidence level of 95.0%.

RESULTS AND DISCUSSION

Fitting the Model

The squalene content obtained from all the experiments according to the research surface methodology (RSM) design is listed in *Table 2*. Experimental yields and squalene content were analysed to get a regression model. The regression model was used to estimate the predicted values, which were compared with the experimental values. In order to check its validity, *Table 3* presents the estimated coefficients of the quadratic regression model. A good model should have a significant effect ($p < 0.05$) in predicting the responses, while the lack-of-fit-test should not be significant ($p > 0.05$), and the coefficient of multiple determination ($R^2 = 0.982$) should be close to 1, to indicate the agreement between the predicted and experimental values.

The large value of R^2 (0.919) for squalene content in *Table 3* reveals the ability of the model to represent the experimental results. The ANOVA

TABLE 1. CODED LEVELS OF THE INDEPENDENT VARIABLES USED IN THE RESEARCH SURFACE METHODOLOGY (RSM) DESIGN

Coded-variables levels (Z_j)	Pressure (X_1, MPa)	Temperature (X_2, °C)	Holding time (X_3, min)
+1	40	60	40
0	30	50	30
-1	20	40	20

TABLE 2. EXPERIMENTAL SCHEME AND RESULTS OBTAINED FROM RESEARCH SURFACE METHODOLOGY (RSM) FOR THE YIELD AND SQUALENE CONTENT

No.	Pressure (Z_1)	Temperature (Z_2)	Holding time (Z_3)	Yield (%)	Squalene (ppm)
1	30	50	20	31.70	332.67
2	30	40	30	34.97	413.92
3	30	50	30	34.04	402.08
4	20	50	30	16.75	218.08
5	40	50	30	47.67	296.50
6	30	60	30	48.30	271.50
7	30	50	40	34.22	367.33
8	30	50	30	29.12	420.83
9	30	50	30	25.98	411.46
10	24	44	36	21.69	316.67
11	36	56	36	44.80	385.42
12	24	56	24	24.78	81.92
13	30	50	30	28.70	377.75
14	36	44	24	47.64	327.75
15	24	56	36	31.79	315.25
16	36	56	24	66.28	286.08
17	36	44	36	41.82	329.17
18	30	50	30	26.73	370.46
19	30	50	30	30.44	378.13
20	24	44	24	19.13	316.67

TABLE 3. REGRESSION COEFFICIENTS OF THE FITTED QUADRATIC EQUATION AND STANDARD ERRORS FOR THE YIELD AND SQUALENE CONTENT[a]

Regression coefficient	Yield	Squalene content
β_0	31.183	39.316
Linear		
β_1	11.523	31.948
β_2	4.435	-34.062
β_3	-1.021	29.302
Quadratic		
β_{11}	-	-53.876
β_{22}	4.671	-21.844
β_{33}	-	-19.110
Interaction		
β_{12}	-	31.344
β_{13}	-4.610	-
β_{23}	-	41.406
R^2	0.930	0.919
R^2 (adj)	0.889	0.829
Regression (P value)	0.000[b]	0.000[b]
Lack of fit (P value)	0.245[c]	0.090[c]

Note: [a]β_i - the regression coefficient for the main effects. β_{ii} - the estimated regression coefficient for the quadratic effects; β_{ij} - the estimated regression coefficient for the interaction effects; 1 - pressure, 2 - temperature and 3 - holding time. [b]Significant ($p < 0.05$). [c]Not significant ($p > 0.05$).

was also used to test for the effect of all parameters as linear, quadratic, or interaction coefficients on the response. As presented in *Table 4*, the linear term of temperature ($p < 0.004$) was found to be the most significant effect variable for the squalene content. This was followed by the linear terms of pressure ($p < 0.005$) and holding time ($p < 0.008$). The quadratic terms of pressure and temperature were also significant ($p < 0.001$ and $p < 0.031$, respectively). Finally, the interaction terms between temperature and holding time ($p < 0.005$) together with the interaction of pressure and temperature ($p < 0.020$) had a significant effect on the squalene content in PFAD.

Response Surface Analysis of the Model

The response surface of the model can be projected with three-dimensional plots by varying the two variables and keeping the other variables constant at the central point. *Figures 3* and *4* show the squalene content as a function of pressure, temperature, and holding time. Several experiments have been performed at different conditions in order to investigate the effect of parameters on the recovery of squalene from PFAD.

Figure 3 visualises the surface plot of the squalene content as a function of pressure and temperature at 30 min of fixed holding time. The influence of pressure had a negative quadratic effect on the squalene content. Results indicate that pressure has high impact on the squalene recovery. The squalene content increased with the rise of pressure from 20 to 30 MPa. This is most likely due to the increased CO_2 density as the pressure increased, which in turn increases the solubility. On the other hand, a further increase of pressure up to 40 MPa, resulting to a slightly decreased in the squalene content. This phenomenon can be related to the increased repulsive solute-solvent interactions in the highly compressed CO_2 (Gomes *et al*., 2007).

In the interaction between temperature and pressure, the squalene content rapidly decreases

TABLE 4. THE p-VALUE AND F RATIO FOR EACH INDEPENDENT VARIABLE EFFECT IN THE POLYNOMIAL RESPONSE SURFACE MODELS[a]

		Main effects			Quadratic effects			Interaction effects		
Variables		X_1	X_2	X_3	X_1^2	X_2^2	X_3^2	X_1X_2	X_1X_3	X_2X_3
Yield (Y_1)	P value	0.000[b]	0.003[b]	0.36	0.243	0.002[b]	0.165	0.603	0.009[b]	0.333
	F ratio	10.952	4.215	-0.97	1.261	4.605	1.527	0.541	-3.394	-1.031
Squalene content (Y_2)	P value	0.005[b]	0.004[b]	0.008[b]	0.000[b]	0.031[b]	0.052	0.020[b]	0.163	0.005[b]
	F ratio	3.825	-4.078	3.508	-6.419	-2.603	-2.277	2.907	-1.537	3.840

Note: [a]X_1 and X_2 the main effect of pressure and temperature, respectively. X_1^2 and X_2^2 the quadratic effect of pressure and temperature, respectively. X_1X_2 the interaction effect of pressure and temperature. [b]Significant at $p < 0.05$.

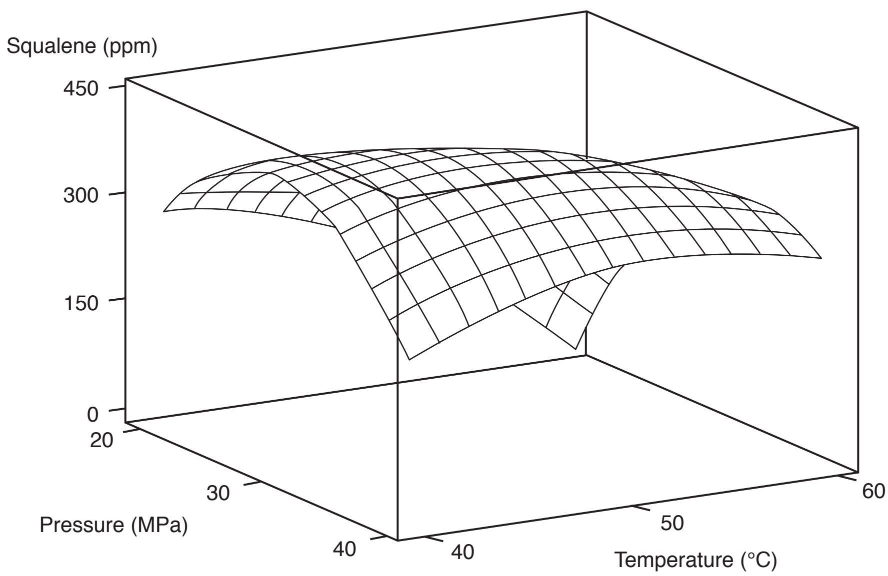

Figure 3. Response surface plot for the effect of pressure and temperature on the squalene content.

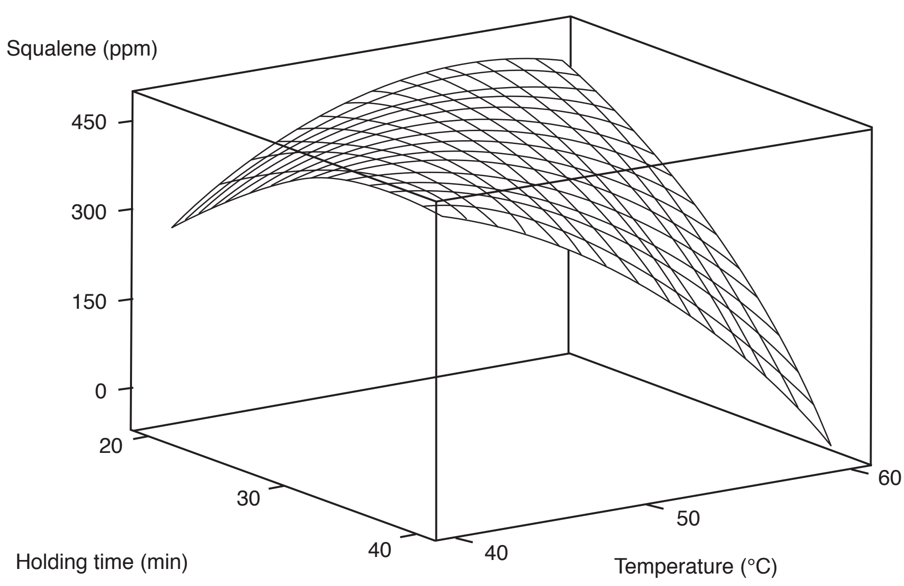

Figure 4. Response surface plot for the effect of holding time and temperature on the squalene content.

as the temperature increased at low pressures (*Figure 3*). The reduced density of CO_2 at higher temperatures might cause this trend to occur since increasing temperature leads to a decrease in the recovery of squalene. Herein, solvent selectivity plays an important role because it reduces the selectivity of sc-CO_2 towards squalene with increasing solubility (by increasing pressure and decreasing temperature) (Al-Darmaki *et al*., 2012). Nevertheless, as the pressure increases, particularly above 32 MPa, the reducing of squalene content becomes gradually with the increasing temperature. These results showed strong dependence of solubility on the pressure as compared to temperature. This is also reported by Hernández *et al*. (2010) working on the solubility of binary system squalene-CO_2.

To investigate the effect of temperature and holding time on the squalene content, pressure was kept at constant value of 30 MPa as shown in *Figure 4*. At a shorter holding time (20 min), the squalene content decreased as the temperature rose. In contrast, the squalene content increased as the temperature increased when the holding time was 40 min. In principle, the holding time in the pressure swing technique is based on the swelling phenomenon (Hernández *et al*., 2010; Patel *et al*., 2006; Smith *et al*., 1998; Subra *et al*., 1998). It has been reported that the sample volume expanded by up to 200% and 50% for *n*-decane and fish oil methyl esters, respectively, when applying this swing technique (Hernández *et al*., 2010; Smith *et al*., 1998). In addition, the effect of the swelling phenomenon on cashew nut shell was studied by Smith Jr *et al*. (2003) for each single depressurisation. The surface area of cashew nut shell increased the bulk CO_2 phase, and so improved the extraction yield (Smith Jr *et al*., 2003). Thus, the surface area available for sc-CO_2 to penetrate into PFAD increased during the holding time.

Optimisation was performed using a graphical optimisation method to predict the best extraction condition. It showed the overall optimum region was achieved at a pressure of 23 MPa and temperature of 40°C with 20 min holding time. Under this optimum set of conditions, the corresponding predicted response of yield and squalene content are 16.75% and 356.23 ppm, respectively. The predicted model was validated by experiments in triplicate at the optimal conditions. *Table 5* lists the verification results for the predicted model. The observed values are in a good agreement with the values predicted from the model.

TABLE 5. PREDICTED AND OBSERVED VALUES FOR THE SQUALENE EXTRACTED FROM PALM FATTY ACID DISTILLATE (PFAD) AT OPTIMUM CONDITIONS

Replication	Pressure (MPa)	Temperature (°C)	Holding time (min)	Yield (%)	Squalene content (ppm)
1	23	40	20	19.13	386.83
2	23	40	20	21.69	350.67
3	23	40	20	16.75	367.33

CONCLUSION

The second-order polynomial model adequately predicted the response variable of the squalene content for the integrated pressure swing and sc-CO_2 extraction from PFAD. The linear and quadratic of pressure, temperature and holding time had a significant effect on the squalene content. Furthermore, the interactions between pressure and temperature together with the interactions of temperature and holding time also significantly affected the squalene content. The predicted optimum extraction parameters using the graphical optimisation method were 23 MPa, 40°C, and 20 min holding time. Under such conditions, the yield and squalene content were 19.19±2.47% and 368.28±18.10 ppm, respectively. Even though, the yield extracts from this research is higher compared to the previous research (13.84%) using continuous sc-CO_2 extraction under optimum condition, the squalene content is slightly lower when applied pressure swing technique (Suleiman *et al.*, 2012). This might be due to the swelling phenomenon during the holding steps in pressure swing technique and further research on these findings is necessary. These findings also suggest that the recovery of squalene is potential for environmental and economical profitable waste management for an additional recovery of value compound from by-product.

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