Squalene recovery from palm fatty acid distillate using supercritical fluid extraction

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Abstract

The objective of this research is recovery of squalene from palm fatty acid distillate (PFAD) using supercritical fluid extraction (SFE). The extraction process was performed by using the SFE and recovered squalene was analyzed from total lipid extracts using HPLC. A central composite design was used to study the effect of SFE variables namely pressure (X_1) from 200 to 400 bar and temperature (X_2) from 40 to 60^{0}C on the total lipid extracts (Y_1) and squalene concentration (Y_2) and optimize the SFE conditions. The nonlinear regression equations were significantly ($p < 0.05$) fitted for both responses with high R_2 (> 0.965), which had no indication of lack of fit. The results indicated that a combined pressure (200 bar) and temperature (50^{0}C) was predicted to provide the optimum region in terms of total lipid extracts (13.84 ± 0.52%) and squalene concentration (418.31 ± 18.40 ppm) studied.

Introduction

Squalene is a natural lipid belonging to the triterpene hydrocarbon group (2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexane) with a symmetrical 30-carbon polyprenyl compound and containing six isoprene units (Bhattacharjee and Shingal, 2003). The chemical structure of squalene is given in Figure 1. It is a colourless oil that extracts in semi-solid phase with faint odour and taste (Bhattacharjee and Shingal, 2003). Apart from that, it is low density and lighter than water with a specific gravity of 0.9 (Channon, 1926).

Squalene is known as a biochemical intermediate synthesis of cholesterol and other steroids (Redyy and Couvreur, 2009). Sixty percent of dietary squalene is absorbed and distributed in human tissue (Vazquez *et al*., 2007). In human, squalene is synthesized in the liver and the skin, and then transported in the blood (Reddy and Couvreur, 2009). Squalene is used in clinical and daily usage such as detoxification factor, skin and eye antioxidant, provide cells with oxygen, bactericidal and fungicidal agent, antistatic and emollient in pharmaceutical and cosmetics, fine chemical, magnetic tape and also as low temperature lubricants (Bahttacahrjee and Shingal 2003).

The name squalene was given because of its occurrence in shark liver oil (*Squalus* spp.), which is the richest and primary source of squalene (Vazquez *et al*., 2007). However, the limitation of squalene usage is because of the uncertainty of its availability as a result of international concern to protect marine animal (Bondioli *et al*., 1993). Squalene can be found as a minor constituent in plant oils such as olive oil, palm oil, amaranth oil, wheat germ oil and also in vegetable oil (Bhattacharjee and Shingal, 2003). However, squalene content in olive, wheat germ and rice bran oils are in the 0.1 – 0.7% ranges which are not high enough to be considered as a viable source (Schnetzler and Breene, 1994).

It was reported palm fatty acid distillate (PFAD) contains high amount of squalene up to 1.03% w/w more than other common vegetable oil (Posada *et al*., 2007). PFAD is a by-product of physical refining of palm oil and it could become one of the most valuable sources of squalene. Approximately 3.66 tonnes PFAD are produced for every 100 tonnes of crude palm oil (Chu *et al*., 2004). In 2002, Malaysia produced 500 metric tons of PFAD from crude palm oil (Sundram *et al*., 2002).

SFE can be defined as a process of separating multi components mixture into two different types which are component volatiles and specific interactions between the mixture component and the supercritical fluid solvent (McHugh, 1990). SFE operating conditions are chosen in order to achieve

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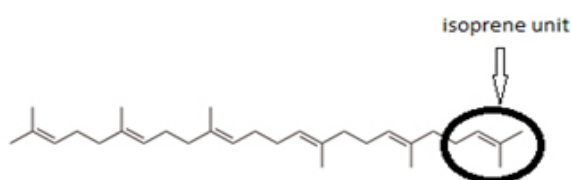Email: badli@putra.upm.edu.my*

Figure 1. Chemical structure of squalene

Table 1. Levels of independent variables established according to the CCD

Independent variable	Coded Factor	Level		
		Low (-1)	Med (0)	High (1)
Pressure (bar)	X_1	200	300	400
Temperature (°C)	X_2	40	50	60

the selective extraction of interested compounds and minimizing the simultaneous extraction of undesirable residue (Reverchon and De Marco, 2006). The solubility of the sample also plays an important role in affecting the SFE operation as well as pressure and temperature. SFE can be applied to food and pharmaceutical applications involving processing and handling of complex, thermolabile bioactive components (Mishra *et al*., 1993). The mystical properties of supercritical fluids can be described as offering the promise of quick extraction, improved separations, lower operating costs, and broad industrial applications to name just a few. It also offers rapidity, flexibility, inexpensive and more environmentally friendly than organic solvent and ability to allow the analysis of substances which cannot be analysed by gas chromatography (GC) (Smith, 1988; Güçlü-Üstündağ and Temelli, 2004). The main objective of this study is to investigate the effect of SC-CO$_2$ variables namely, pressure and temperature on the total lipid extracts and squalene concentration from PFAD and to optimize processing procedure to extract squalene from PFAD using SFE.

Materials and Method

Materials and reagents

Palm fatty acid distillate (PFAD) (iodine value, IV, 64.3 g I$_2$/100 g oil; slip melting point, SMP, 47.5°C) in semi-solid phase was obtained from Golden Jomalina Food Industries Sdn. Bhd., Teluk Panglima Garang, Selangor, Malaysia. Commercial immobilized, *Candida antartica* lipase (Novozyme 435®) was obtained from Novo Nordisk, Bagsvaerd, Denmark. The n-hexane of analytical grade and 100% acetonitrile of HPLC grade were obtained from MERCK, (Frankfurter, Darmstadt, Germany). Squalene of 98-100% purity was purchased from

Sigma Chemical Co., (St. Louis, MO, USA). Commercial grade liquefied carbon dioxide (purity, 99.9%), supplied in cylinder with dip tube, was purchased from MOX-LINDE Gases Sdn. Bhd., (Petaling Jaya, Selangor, Malaysia). Nucleosil C18 column (particle size 5μm, 125 x 4.0 mm i.d) was purchased from Jones Chromatography, (Reading, Berkshire, England).

Sample preparation

Enzymatic hydrolysis (Chu *et al*., 2003) is one of the processes used to concentrate squalene from PFAD in the presence of a catalyst to yield free acids. It was conducted under the following conditions: a mixture of 50 g melted PFAD, 50 ml distilled water and 1.0% w/w Novozyme 435 lipase was stirred magnetically. Stirring took place in a stoppered, 250 ml conical flask under nitrogen, which was placed in a 65±1°C water bath in the dark. Reaction was stopped after 6 hours by filtering out the immobilised lipase using a two layer cheese cloth. A sample was used for squalene determination.

Experimental design using response surface methodology (RSM)

The central composite design (CCD) was applied in the experimental design chosen to study the responses, namely total lipids extract, Y$_1$ and squalene concentration, Y$_2$. The independent variables were X$_1$ and X$_2$ representing pressure and temperature, respectively. The settings for the independent variables were as follows (low/high values): pressure (200-400 bar) and temperature (40-60°C). Each variable was coded at three levels: -1, 0, and +1, giving them a range of 200 – 400 bar and 40 – 60°C, respectively, (Table 1). The extraction time applied for this design was 90 min. In order to allow the estimation of the pure error, six replicate runs at the centre point (0, 0) of the design were considered. The experimental design was developed to (i) find a relationship between each response and two independent variables and (ii) to determine the optimum level of the independent variables which will result in the desired goals.

Statistical analysis

MINITAB release 14 was performed using statistical analysis software to calculate the optimization point. A quadratic polynomial regression model was assumed for predicting both Y responses, namely Y$_1$ and Y$_2$. Equation 1 has been proposed for each responses of Y.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum\sum \beta_{ij} X_i X_j \qquad (1)$$
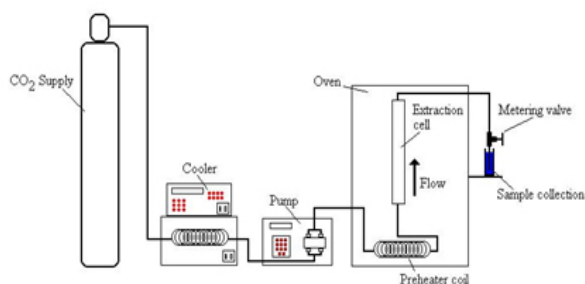
Figure 2. Schematic diagrams of supercritical carbon dioxide extraction (Zaidul *et al*., 2007)

Where β_0 is an intercept; β_i, first-order model coefficient, β_{ii}, quadratic coefficient for the ith variable, β_{ij}, interaction coefficient for the interaction of variables i and j; Xi and Xj are independent variables (Choon-Hui *et al*., 2009). The model was evaluated using the coefficient of determination (R^2) and the analysis of variance (ANOVA). Contour plots were developed using the fitted full quadratic polynomial equations obtained by holding one of the independent variables at a constant value and changing the level of the other variables (Chu *et al*., 2003).

Verification of model

Experiments for optimum point were performed in triplicate and each set of yields were averaged. The result were then test using two sample t-test at the least significant difference at the 95 % confident level was calculated for each yield.

Supercritical fluid extraction
The experimental setup for the supercritical fluid extraction

(SFE) process is shown in Figure 2. It is consisted of a 50 ml volume extractor vessel (model EV-3, JASCO Corporation, Tokyo) with 13 mm diameter and 320 mm height, high-pressure pump (model PU-1580, JASCO Corporation, Tokyo), automated back pressure regulator (model BP-1580-81, JASCO Corporation, Tokyo) and a chiller (Yih Der, B/L-730).

The liquid CO_2 was compressed to the desired pressure and then continuously pumped through the extractor at 3 ml/min for 90 min extraction time. The extraction occurred during the continuous extraction until the desired time was completed. The use of rigid materials such as glass beads with the sample allowed a proper flow rate of CO_2 and it also can maintain the desired permissibility of the particle (Wang and Weller, 2006). Meanwhile glass wool was used at the both inlet and outlet of the vessel to prevent any possible carryover of solid material. In order to maintain the desired condition of the extraction, the extraction vessel was equipped with a temperature controller and pressure valves at both inlet and outlet. The extraction product was only collected at the end of the experiment via a valve which was located at the bottom of the separator and the total amount of solute collected was weighed using a balance (Mettler Toledo, model AG 204) with an accuracy of ± 0.0001g.

Result and Discussion

Model fitting

The matrix of optimisation is shown in Table 2. All experiments were conducted in random order. Parameter ranges were made based on the preliminary study of the extraction process using SC-CO_2. The response surface analysis allowed the development of the polynomial regression relationship where each response variable (Y_i) was assessed as a function of pressure (X_1) and temperature (X_2). Table 3 presents the estimated regression coefficients of the response models, along with the corresponding R^2 values and lack of fit tests.

Table 3 shows the significant ($p < 0.05$) regression relationship between the independent variables (pressure and temperature) and the responses (total lipid extracts and squalene concentration). The response surface analysis offered high R^2 values ranging from 0.965 to 0.991 (Table 3). It indicates that at least 95% of the variation of the response variables could be accurately explained by the reliable regression models relating the independent variables (pressure and temperature) and responses (total lipid extracts and squalene concentration). Therefore, this finding exhibits a satisfactory fitness of the response surface models employed for describing the response variations as a function of two independent variables (pressure and temperature) (Table 3). These indicate that the generated models sufficiently explained the data variation and significantly represent the actual relationships between the reaction parameters.

According to the Table 4, the main effect of pressure and temperature had significant ($p < 0.05$) effects on the total lipid extracts and squalene concentration. It shows the highest R^2 and p values for lack of fit test and lowest p value for regression for full quadratic regression model compared to other regression model. It should be noted that the polynomial regression equation is significant ($p < 0.05$) only for a selected range (Hamed *et al*., 2007). It does not exist in the factor ranges and cannot be extrapolated beyond these ranges (Montgomery, 2001). As shown in Table 4, both main effects of pressure and temperature had the most significant

Table 2. Matrix of the CCD

Run	Pressure (bar)	Temperature (°C)	Total lipid extract (%)	Squalene (ppm)
1	**230**	**57**	**19.6967**	**436.08**
2	370	57	70.0478	340.25
3	300	60	47.2167	375.00
4	200	50	13.3189	431.25
5*	300	50	32.6874	368.42
6	370	43	47.0458	379.83
7	230	43	16.8030	426.42
8	300	40	20.2570	405.58
9	400	50	70.3584	336.83
10*	300	50	31.9724	381.92
11*	300	50	31.0923	384.75
12*	300	50	30.1136	397.25
13*	300	50	30.1268	398.58
14*	300	50	32.5229	389.58

* central point for centre composite design (CCD)

Table 3. Regression coefficients, R^2, adjusted R^2, probability values, and lack of fit for two dependent variables[a]

Regression coefficients	Total lipid extracts (Y_1)	Squalene concentration (Y_2)
b_0	32.226	389.41
b_1	20.158	-34.494
b_2	8.003	-9.146
b_1^2	5.262	
b_{12}	5.027	-12.310
R^2	**0.991**	**0.965**
R^2 (adj)	0.985	0.950
regression (P value)	**0.000[b]**	**0.000[b]**
lack of fit (P value)	**0.048[c]**	**0.593[c]**

[a]Key: bi the estimated regression coefficient for the main effects; b_{ii} the estimated regression coefficient for the quadratic effects; b_{ij} the estimated regression coefficient for the interaction effects; 1, pressure and 2, temperature. [b]Significant ($p < 0.05$). [c]No significant ($p > 0.05$)

Table 4. F ratio and p value for each independent variable effects in the polynomial response surface models[a]

Variables		Main effects		Quadratic effects		Interaction effect
		X_1	X_2	X_1^2	X_2^2	X_1X_2
Total lipid extracts (Y_1)	P value	0.000[b]	0.000[b]	0.000[b]	0.107	0.001[b]
	F ratio	873.853	137.734	57.078	3.415	27.175
Squalene concentration (Y_2)	P value	0.000[b]	0.005[b]	0.756	0.145	0.007[b]
	F ratio	229.341	16.12	0.104	2.692	14.600

[a]Key: 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 $p < 0.05$

($p < 0.05$) effect on total lipid extracts (Y_1) and squalene concentration (Y_2).Therefore, pressure and temperature should be considered as critical parameter in order to extract total lipid and squalene. The interaction effect of pressure and temperature also significantly ($p < 0.05$) affected both response variables (Table 4).

Response analysis
Effect of temperature on total lipid extracts and squalene concentration

Figures 3, show the effects of pressure and temperature on total lipids extracts and squalene concentration, respectively. It shows that total lipid
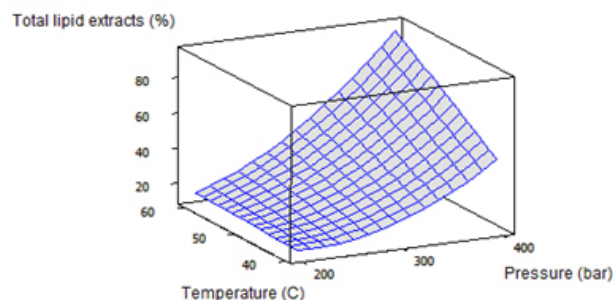

Figure 3(a). Response surface plot for the effect of pressure and temperature on total lipid extract (%)
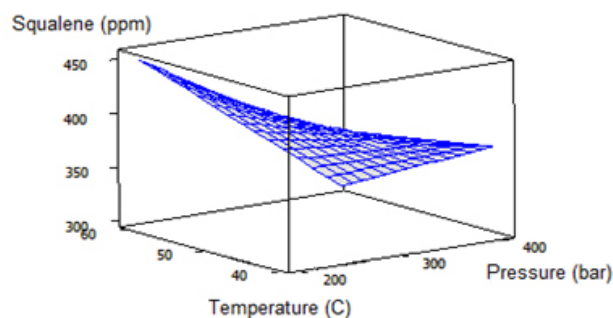

Figure 3(b). Response surface plot for the effect of pressure and temperature on squalene concentration (ppm)

extracts and squalene concentration are strongly nonlinear in the studied independent variable ranges. Figure 3 (a) presents the effect of temperature on total lipid extracts at 40, 50 and 60 °C and constant pressure of 200, 300 and 400 bar. Reverchon and De Marco (2006) reported, the tendency of compounds to be extracted passing through the supercritical fluid will increase with the increasing temperature. This is because the increase in temperature can increase the vapour pressure of oil since more molecules have enough energy to escape from the oil at high temperature.

According to the Figure 3 (a), the total lipid extracts had increased with combined effects of pressure and temperature. The result shows that the extraction yield increased as temperature increased from 40 to 60°C which could indicate that temperature enhances desorption of the oil from the PFAD into the CO_2. This can be explained in a way that increasing temperature increases the vapour pressure of the oil. Thus enhancing the contact between the oil and SC-CO_2 (Reverchon and De Marco, 2006).

Figure 3 (b) presents the effect of temperature on squalene concentration. Density of CO_2 at constant pressure decreases with increasing temperature and hence reduces the solvent power of SC-CO_2. Roop *et al.* (1989) reported a moderate increase in temperature can lead to a large decrease in SC-CO2 density, with a consequent reduction in oil solubility. The result shows squalene concentration decreased as the temperature was increased from 40 to 60°C

Table 5. Experimental and predicted values for the response variables[a]

Run	Total Lipid Extracts[b] (w%)			Squalene Concentration[b] (ppm)		
	Y_0	Y_i	$Y_0 - Y_i$	Y_0	Y_i	$Y_0 - Y_i$
1	32.523	32.187	0.336	389.580	395.427	-5.847
2	30.127	32.187	-2.060	398.580	395.427	3.153
3	47.046	44.576	2.469	379.830	382.389	-2.559
4	16.803	14.315	2.488	426.420	426.757	-0.337
5	30.114	32.187	-2.074	397.250	395.427	1.823
6	19.697	20.267	-0.570	436.080	433.085	2.995
7	70.048	70.636	-0.588	340.250	339.478	0.772
8	31.092	32.265	-1.173	384.750	383.393	1.357
9	13.319	14.281	-0.962	431.250	432.174	-0.924
10	47.217	43.583	3.634	375.000	370.459	4.541
11	20.257	20.948	-0.691	405.580	396.327	9.253
12	31.972	32.265	-0.293	381.920	383.393	-1.473
13	70.358	71.295	-0.937	336.830	334.611	2.219
14	32.687	32.265	0.422	368.420	383.393	-14.973

[a]Y_0, experimental value; Y_i, predicted value; and $Y_0 - Y_i$, residue. [b]No significant ($p>0.05$) difference between experimental (Y_0) and predicted (Y_i)
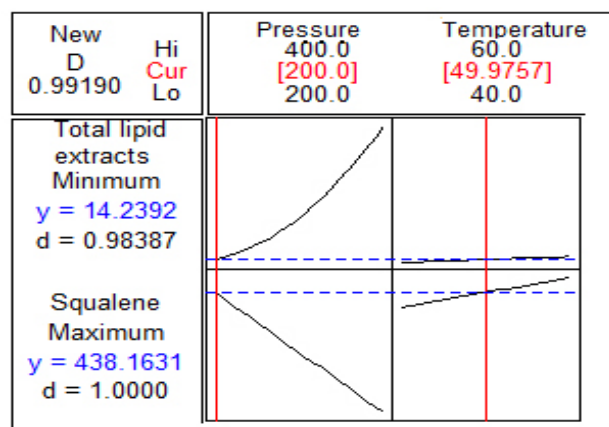

Figure 4. Response optimization of the effects of 200 bar and 50ºC on total lipid extract (%) and squalene concentration (ppm)

at constant pressure. Squalene concentration had decreased gradually with increasing temperature. It proves that the fluid solvating power decreased with the increase in temperature.

Effect of pressure on total lipid extracts and squalene concentration

Figure 3 (a) presents the effect of pressure on total lipid extracts. The result shows the total lipid extracts increases with increasing pressure from 200 to 400 bar and with increasing temperature at constant pressure. It proves that, at a constant temperature, an increase in the pressure will increase the density of the SC-CO_2, hence increasing the solvent strength of SC-CO_2. Luque De Castro *et al*. (1994) reported that, the distance between the molecules decreased as the density increased. Thus, the interaction between the oil and CO_2 increased, leading to greater solubility of the analytes in CO_2. An increase in pressure will also accelerate mass transfer oil into the solvent in the supercritical extractor vessel system and improve the extraction yield.

In contrast, the increase in pressure as well

as the temperature increase lead to a decrease in squalene concentration (Figure 3 (b)). Therefore, it is concluded that the squalene concentration is negatively influenced by the pressure and temperature. The positive effect of pressure could be due to the selectivity of SC-CO_2 for squalene is decreasing, as there is an increased possibility of co-extraction of other hydrocarbons at high pressure. This might be because increasing pressure will cause the increase in fluid density, which alters solubility of solute. As an example, Gomes *et al*. (2007) found that the oil solubility will improve when the CO_2 density increased with the rise in pressure. On comparison, squalene extracts from yeast using SC-CO_2 at 150 and 250 bar were colourless to pale yellow and less viscous and at 350-355 bar, the extract was distinctly brownish and more viscous (Bhattacharjee and Shingal, 2003). The results also show the colour and viscosity of total extracts obtained at 200 and 300 bars was pale yellow and less viscous. In contrast, at pressure 300 to 400 bars, the colour was strong yellow to brownish and more viscous compared to the total extracts less than 300 bar. This suggests the presence of other compounds in the total extracts when the extract pressure was increased up to 300 bar.

Optimization condition

Graphical and numerical optimization were carried out to determine an optimum set level of pressure and temperature resulting in the desirable total lipid extracts and squalene concentration. There are a number of combinations of pressure and temperature that could give minimum total lipid extracts with the highest concentration of squalene. A numerical optimization was also carried out to determine the exact value of multiple response optimization resulting in the desirable goals. The multiple response optimization shows that the overall optimum region was achieved at pressure of 200 bar and temperature 50ºC (Figure 4). Under this optimum condition, the corresponding predicted response value for total lipid extracts is 14.24% and squalene concentration is 438.16 ppm (Figure 5).

Verification of the Model

Table 5 shows the experimental and predicted values. The experimental response values were found to be in agreement with the predicted values. There are no significant ($p > 0.05$) difference between the experimental and predicted values. The results indicated that the corresponding experimental value for total lipid extracts and squalene concentration were 13.84 ± 0.52% and 418.31 ± 18.40 ppm,

respectively. From the optimum region, the closeness between the experimental and the predicted values from the model verified the accuracy of the response surface models fitted to the experimental data.

Conclusion

As a novel extraction method, SFE of total lipid extracts and squalene concentration was optimized by central composite design. The lowest total lipid extracts (13.84 %) with a highest squalene concentration (418.31 ppm) were obtained at 200 bar and 50 ^{0}C for a 90 min extraction time. Based on the analysis of variance (ANOVA), pressure and temperature had a significant effect on the total lipid extracts and squalene concentration. Results of this study can be used as a guide for a pilot scale production of squalene from PFAD using SFE for commercialization purpose. Pressure swing technique which is pressurization-depressurization steps can be applied in SFE process. This will be able to reduce the amount of CO_2 used and increase the efficiency of the extraction process.

Acknowledgements

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