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Optimization of supercritical carbon dioxide (CO_2) extraction of sardine (*Sardinella lemuru* Bleeker) oil using response surface methodology (RSM)

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SUMMARY: Oil was extracted from freeze-dried sardine (*Sardinella lemur*) fillets using supercritical carbon dioxide (SC-CO_2) and a few milliliters of ethanol were optimized with response surface methodology (RSM). The impact of extraction pressure (200–400 bars) and temperature (40–70 °C) were studied on the total extraction yields, ratios of Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA). The results were compared with those of Soxhlet and modified Kinsella methods (MKM). The oils obtained using the SC-CO_2 and MKM methods were significantly (P<0.05) higher in oil yield (8.04% and 6.83%), EPA (5.43% and 5.45%) and DHA (18.76% and 18.54%), respectively, compared to the Soxhlet yield (5.10%), EPA (2.17%) and DHA (06.46%). Of the two independent variables, pressure had a critical effect on yield while EPA and DHA ratios were notably influenced by temperature. The combined optimal values were pressure at 328 bars and temperature at 40 °C, with corresponding responses of 7.20%, 5.68% and 20.09% for yield, EPA and DHA, respectively. The experimental values in this study were reasonably comparable to their predicted counterparts.

KEYWORDS: *Docosahexaenoic acid; Eicosapentaenoic acid; Fatty acid composition; Fish oil; Response surface methodology; Supercritical carbon dioxide extraction*

RESUMEN: ***Optimización de la extracción mediante dióxido de carbono supercrítico (CO2) de aceites de sardinas (*Sardinella lemuru *Bleeker) usando la metodología de superficie de respuesta (RSM)***. El aceite se extrae de filetes de sardinas (*Sardinella lemur*) liofilizando, mediante dióxido de carbono supercrítico (SC-CO_2) y unos mililitros de etanol, optimizándose mediante la metodología de superficie de respuesta (RSM). Se ha estudiado la influencia de la presión de extracción (200–400 bars) y la temperatura (40–70 °C) sobre los rendimientos de extracción total, y sobre las relaciones de ácido eicosapentaenoico (EPA) y ácido docosahexaenoico (DHA). Los resultados se compararon con los obtenidos mediante extracción con Soxhlet y el método de Kinsella modificado (MKM). Los aceites obtenidos mediante SC-CO_2 y métodos MKM fueron significativamente (P<0.05) superiores en rendimientos de aceite (8,04% y 6,83%), EPA (5,43% y 5,45%) y DHA (18,76% y 18,54%), respectivamente, en comparación con rendimientos mediante Soxhlet (5,10%), EPA (2,17%) y DHA (06,46%). De las dos variables independientes, la presión tuvo un efecto crítico sobre el rendimiento, mientras que los porcentajes de EPA y DHA estuvieron notablemente influenciados por la temperatura. Los valores óptimos fueron para una presión de 328 bar y una temperatura de 40 °C, y sus correspondientes respuestas fueron 7,20%, 5,68% y 20,09% para el rendimiento, EPA y DHA, respectivamente. Los valores experimentales de este estudio fueron los previstos y son comparables razonablemente con sus homólogos.

PALABRAS CLAVE: *Aceite de pescado; Ácido docosahexaenoico; Ácido eicosapentaenoico; Composición de ácidos grasos; Extracción con dióxido de carbono supercrítico; Metodología de superficie de respuesta*

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1. INTRODUCTION

Fish and its products utilized for human consumption in different ways (fresh or frozen, whole or fish fillet) contribute to the nutrition and health of a considerable portion of the world's population to provide vital nourishment, especially proteins, fats, vitamins and minerals.

Fats from fatty fish species consist of an important dietary source of ω-3 long-chain polyunsaturated fatty acids (PUFAs) namely eicosapentaenoic acid (EPA, 20:5ω-3), and docosahexaenoic acid (DHA, 22:6ω-3), which have shown potential benefits on the adequate growth of children and prevention of cardiovascular diseases and cancer (Shahidi and Miraliakbari, 2004). Fish fat also contributes to energy supplies and assists in the proper absorption of fat soluble vitamins namely A, D, E, and K in humans (Banda-Nyirenda *et al*., 2009). Health organizations have established specific guidelines for the general population to increase the intake of ω-3 PUFAs; for example, WHO advises that the total ω-3 PUFA intake should cover 1–2% of human energy, and the American Heart Association and the Scientific Advisory Committee of Nutrition (U.K.) recommend eating fish, particularly fatty fish, at least two times a week (Pazos *et al*., 2008).

With growing public awareness of these clinical benefits of fish lipids mainly their contents of PUFAs, particularly EPA (C20:5 n-3) and DHA (C22:6 n-3, there is a drastic need to develop efficient extraction methods to obtain these important fatty acid components. The extraction and purification of the lipids by conventional methods, such as Soxhlet extraction, vacuum distillation, urea crystallization or conventional crystallization involve some problems due to the toxicity or flammability of the solvents. These methods may have adverse health effects. They may also cause the decomposition of the PUFAs as they are used with high-temperatures during processing (Létisse *et al*., 2006). These drawbacks can be avoided by using supercritical carbon dioxide (SC-CO_2) procedure, a more appropriate technique for the extraction and fractionation of edible oils containing thermo and light susceptible components like PUFAs. The extraction can be carried out at low temperatures away from light, besides this, carbon dioxide (with critical temperature, pressure and density of 31.18 °C, 72.0 bar 0.47 g·cm^{-3}, respectively) is safe (generally recognized as safe, GRAS from the US Food and Drug Administration, FDA), residue free, non-flammable, inexpensive and environmentally friendly (Pyo and Oo, 2007). SC-CO_2 extracted products are excellent in quality fresh-like products and are comparable to natural foods which are free of biological impurities, have a longer shelf life, and the ability to fractionate extracts in a single step besides a feasibility to extract various products by simply adjusting operating conditions (Martínez, 2008, Lang and Wai, 2001).

This study aimed to obtain oil extracts from freeze-dried sardine fillets using a supercritical fluid method with CO_2 as the extraction solvent and EtOH as the co-solvent for the recovery of these extracts. The impact of extraction pressure and temperature on total extraction yields and fatty acid profiles was studied with a focus on the ratios of EPA and DHA. The results were compared with those of Soxhlet and modified Kinsella extraction methods.

2. MATERIALS AND METHODS

2.1. Raw material

Whole fish samples (sardines) were purchased from Pasar Borong, a whole sale local market at Pugung, Selangor, Malaysia. Chemicals and solvents were either of analytical or HPLC grade were purchased from Fisher Scientific Chemical Co. (Loughborough, England) and Merck (Darmastadt, Germany).

2.2. Preparation of fish

The fresh sardine samples purchased from Pasar Borong were kept in plastic bags and transported in an insulated icebox to the laboratory. The samples were immediately de-headed, gutted, and washed with copious amounts of cool water, and the flesh and bones were then separated using a de-boner (model- FD 6, Safe World Food-Tech Pvt. Ltd., Klang, Selangor Darul Ehsan, Malaysia). The flesh was stored at −25 °C and then freeze dried (Model: LABCONCO, USA) at a drying temperature of −40 °C under a 0.133 bar vacuum. The moisture content of the freeze dried samples was determined (data not given) and kept in desiccators until use.

2.3. Proximate analysis of the fish

Before oil extraction, the fish was analyzed for its proximate composition such as moisture, lipid, ash and protein contents to indicate its initial nutritional qualities. Moisture, oil and ash contents were determined as described by AOAC (AOAC, 1990) with slight modifications for Soxhlet oil extraction. The oven-drying method (105 °C) was used for moisture content and furnace at 550 °C for ash content. Protein content was determined according to Pomeranz and Meloan (Pomeranz and Meloan, 2000).

2.4. Extraction of sardine oil by soxhlet

Soxhlet extractions were carried out (in triplicate) as described by AOAC, 1990 with minor modifications. Five grams of freeze dried fillet were extracted using 200 mL of petroleum ether for eight hours. The extracted lipids were evaporated under vacuum at 60 °C using a rotary evaporator (Rotavapor R-210, Büchi, Switzerland) and then were placed in an oven at 30 °C for 1 h before they were transferred into desiccators and reweighed. The extracted lipids were transferred into a brown bottle, flashed with nitrogen gas and stored at −25±1 °C.

2.5. Extraction of sardine oil by the modified Kinsella method (MKM)

Freeze dried sardine fillets were extracted using modified Kinsella (1977) method (MKM) by Kim *et al*. (1991). Initially, samples were homogenized for 2 min using a warring blender with chloroform and methanol in the proportion of 1 g tissue: 1 mL chloroform: 2 mL methanol. An additional equivalent amount of chloroform and de-ionized water (1 mL·g^{-1} tissue) was added and the mixture was homogenized for 30 sec. The mixture was filtered through a Whatman No.1 filter paper on a No.3 Buchner funnel with a slight suction. The mixture was transferred to a decanter flask and was left to stand for a few minutes to complete the separation and clarification. The lower clear phase (chloroform and lipid) was poured into a conical flask. The extract was then concentrated using a vacuum rotary evaporator (Rotavapor R-210, Büchi, Switzerland). The extracted lipid was transferred into a brown bottle, flashed with nitrogen gas and stored at −25±1 °C until further analysis. The total lipid content was calculated gravimetrically as below:

$$\text{Total lipid content (\%)} = W_1/W_S*100$$

Where: W_1 = weight of dried lipid (g) and W_S = the weight of sample (g).

2.6. Supercritical carbon dioxide (SC-CO_2) extraction of sardine oil

The supercritical fluid extractor (SFE) used was ABRP200, Pittsburgh, PA, USA with a 500 mL extractor vessel attached (Figure 1). Parameters were selected from a software (ICE) program, which permitted to set and control the extraction status. The liquid CO_2 was pressurized to the desired pressure and heated to the targeted temperature with a pressure pump (P-50, Pittsburg, PA, USA) to reach the supercritical state prior to passing it into the extraction vessel. During this step, the pump head temperature was decreased to 4 °C. Then, the system was equilibrated until pressure, CO_2 rate and temperature became constant to begin the extraction. Absolute ethanol (EtOH) was used as the co-solvent and was fixed at a flow rate of 3 mL·min^{-1}.

The duration of the static and dynamic extraction times was fixed at 30 and 80 min, respectively. The thimble containing the sample (40 g) was placed in the extraction chamber equipped with a heating jacket. The components were then extracted by the pre-heated supercritical CO_2 and entered the trap through a nozzle where CO_2 was depressurized. The extraction was performed under various experimental conditions as generated by the RSM design. EtOH was removed from the extracts by vacuum evaporation using a rotary evaporator. The extracts were then placed in the oven at 30 °C for 30 min before being transferred into desiccators for final constant weight. The extracts were transferred into brown glass bottles, flashed with nitrogen and stored in a freezer of −25±1 °C until further analysis.

2.7. Identification of fatty acid (FA) profile by gas chromatography (GC)

The fatty acid (FA) composition of the lipid extracts was analyzed based on the Christie (1993) method using Agilent gas chromatography (G1530N, USA). The column used was BPX-70 (60 m×0.32 mm i.d., 0.25 μm film thickness) with the phase composition 90% biscyanopropyl; 10% cyanopropyl phenyl polysiloxane from SGE, Melbourne, Australia. A100 μL aliquot of the test sample was thoroughly mixed by dissolving 50 μL of sample into 950 μl of n-hexane, and 50 μL of sodium methoxide was added to prepare the FA methyl esters (FAMEs). The mixture was then shaken vigorously using an autovortexer (Stuart, UK) for 30 s and stored for 5 min so that it formed two layers. The clear upper layer containing the FAMEs (1 μL) was pipetted off and injected into the gas chromatograph. The oven temperature was set at 115 °C, held for 2 min, raised to 180 °C at a rate of 8 °C·min^{-1} and held for 10 min to be finally raised to 240 °C at a rate of 8 °C·min^{-1} and held for 10 min and the carrier gas (helium) at a rate of 1.6 mL·min^{-1} was flashed through. The FA

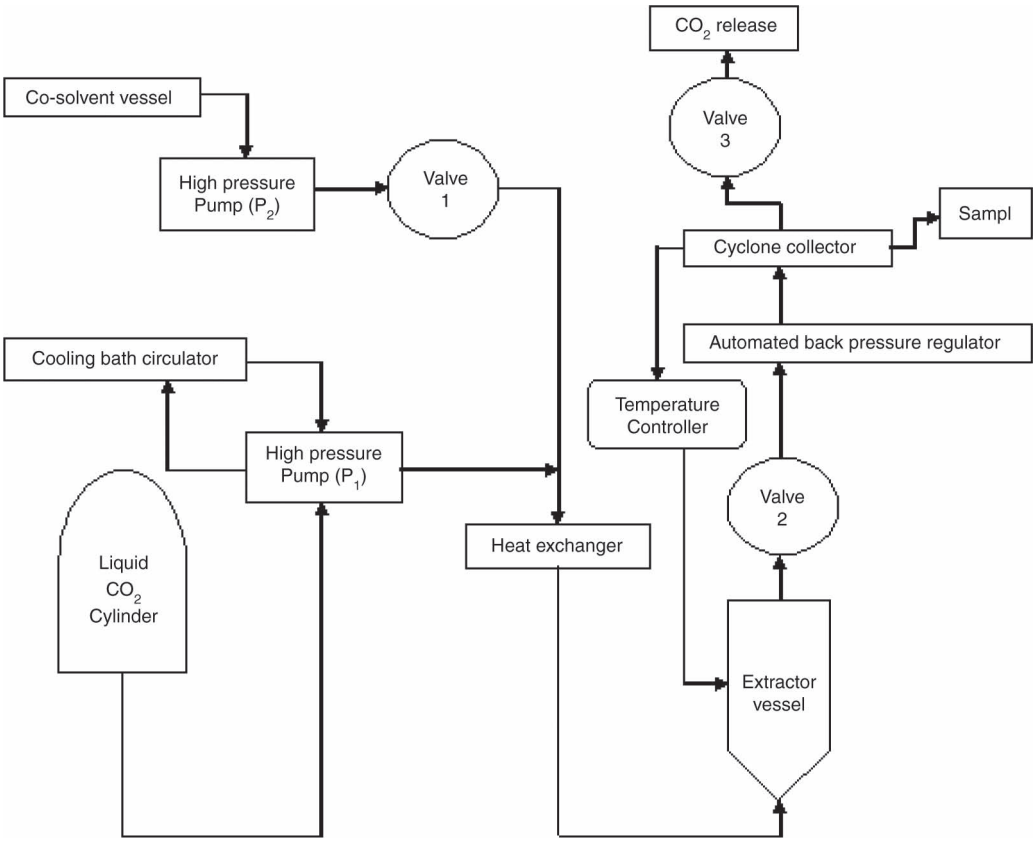

FIGURE 1. Schematic Diagram of Supercritical Fluid Extractor.

components were identified based on the standard mixture of FAMEs containing 37 mixed fatty acids.

2.8. Experimental design and statistical analysis

Response surface methodology (RSM) was used to determine the optimum conditions for yield EPA and DHA of sardine oil extracted using SFE. The experimental design and statistical analysis were carried out using the Minitab V. 14 statistical package (Minitab Inc., PA,USA). Central composite design (CCD) with cube points was selected to evaluate the effects of two independent variables (extraction temperature and pressure), coded as X_1 and X_2, respectively, on the yield EPA and DHA of the SC-CO_2 extracted sardine oil. The minimum and maximum values for the extraction temperature were set at 40 to 70 °C whereas pressure was in the range of 200 to 400 bars. It should be noted that using CCD with cube, out range values called "star points" could be found to predict the optimum point in case it lies out of the selected range. Optimization of the two independent variables was achieved by maximizing the three dependent variables i.e. yield, EPA and DHA to achieve highest values using a MINITAB numerical response optimizer. The whole design consisted of 13 combinations including five replicates of the center point as in Table 1, (Myers, 2002). The ANOVA tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all the terms in the polynomial were analyzed statistically by computing the F-value at a probability (p) of 0.001, 0.01 or 0.05 of the SC-CO_2 extracted sardine oil. The statistically found non-significant (p>0.05) terms were removed from the initial models and only significant (p<0.05) factors were involved in the final reduced model. Experimental data were fitted to the following second order polynomial model and regression coefficients were obtained. The generalized second-order polynomial model proposed for the response surface analysis was given as follows

$$y = \beta_0 + \sum_{i=1}^{k} \beta ixi + \sum_{i=1}^{k} \beta iixi^2 X_i^2 + \sum_{i}^{K-1} \sum_{j}^{k} \beta ijxixj \quad \text{Eq. (1)}$$

Where β_0, βi, βii, βij were regression coefficients for intercept, linear, quadratic and interaction terms,

Table 1. Comparison of yield and n-3 PUFAs (EPA and DHA) obtained via SC-CO_2 with those of Soxhlet and MKM

				Responses								
		Parameters		Yield (%)			EPA (%)			DHA (%)		
Techniques	Run	X_1	X_2	y_0	y_1	y_0–y_1	y_0	y_1	y_0–y_1	y_0	y_1	y_0–y_1
SC-CO_2 Extraction	1	400	40	6.04	6.64	−0.06	5.45	5.51	−0.06	20.23	20.69	0.05
	2	300	76	5.32	6.09	−0.13	4.98	5.11	−0.13	15.70	15.69	0.01
	3c	300	55	8.16	7.95	−0.09	5.39	5.48	−0.09	18.38	18.82	−0.44
	4	441	55	5.76	5.42	0.02	5.54	5.51	0.02	19.12	18.86	0.25
	5c	300	55	8.07	7.95	0.00	5.48	5.48	0.00	19.01	18.82	0.18
	6c	300	55	8.14	7.95	0.09	5.57	5.48	0.09	18.89	18.82	0.06
	7	400	70	5.43	5.18	0.04	5.61	5.56	0.04	16.50	16.94	−0.44
	8c	300	55	7.62	7.95	0.01	5.49	5.48	0.01	19.06	18.82	0.23
	9	300	33	6.15	5.73	0.01	5.86	5.84	0.01	20.35	20.25	0.09
	10	158	55	2.39	3.08	−0.02	5.06	5.08	−0.02	17.38	17.53	−0.15
	11c	300	55	7.78	7.95	0.10	5.58	5.48	0.10	18.79	18.82	−0.03
	12	200	70	6.21	5.24	0.07	4.78	4.70	0.07	16.40	15.99	0.40
	13	200	40	3.39	3.27	−0.03	5.74	5.77	−0.03	19.01	19.22	−0.21
Soxhlet extraction[1]				5.10±0.10			2.17±0.55			6.46±2.36		
MKM extraction[1]				6.83±0.15			5.43±0.05			18.54±1.68		

[1]Each value is the mean ±S.D; n=3; c: center point; SC-CO_2: supercritical carbon dioxide; PUFAs: poly unsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; MKM: modified Kinsella method "otherwise called Blight and Dyer (1959)"; y_0: experimental value; y_1: predicted value; y_0–y_1: residue.

respectively. X_I and Xj were coded values of the independent variables, while k was the number of the tested factors (k=2).

For the söxhlet and modified Kinsella methods, triplicate extractions of each were considered and their means were compared with the SC-CO_2 optimized sardine oil.

3. RESULTS AND DISCUSSION

3.1. Moisture content, protein, fat and ash of sardine fillets

Moisture protein, ash and lipid contents are generally used as indicators of nutritional values of fish (Stansby, 1962). A high content of water in fish was correlated with low protein and lipid contents (Dempson *et al*., 2004). The moisture, protein, crude lipid and ash contents of the Malaysian sardine fillets were 77.8±0.2, 15.4±0.51, 5.1±0.10 and 1.4±0.17, respectively. These values were within the normal ranges for sardine which were reported by Payne *et al*. (1999), taking into account that lipid levels as well as other energy parameters fluctuate seasonally (Payne *et al*., 1999). Despite differences in region, which may lead to a variation in parameters (Çelik *et al*., 2005), our findings were in a close agreement with those reported by Serdaro Lu and Feleko Lu (2005), Payne *et al*. (1999), and Fernandes *et al*. (2014).

3.2. Response surface methodology (RSM) model fitness

The ranges of each independent variable (pressure and temperature) which affected extraction efficiency of EPA and DHA ratios were chosen. In this study, the upper and lower values for the parameters were set at +alpha (+α=1.414) and –alpha (−α=1.414), hence, all the factor levels were selected within the limits that were practical with SC-CO_2 (above critical temperature of 31 °C and critical pressure of 72 bars) and desirable. In RSM, natural variables are transformed into coded variables that have been defined as dimensionless with mean zero and same standard deviation (Liyana-Pathirana and Shahidi, 2005).

The linear, quadratic and interaction effects of supercritical pressure and temperature on sardine oil extraction efficiency, and the ratio of EPA and DHA are shown in Table 1. The results suggest that the models fitted for response variables were empirically adequate due to their high coefficient of determination (R^2>0.92), which means that more than 92% of the response variation could be explained as a function of the two SC-CO_2 parameters (Pressure and temperature). The highly adjusted R^2 (>0.89) as well as insignificance of any lack of fit in the data also indicated its reliability.

Using multiple regression analysis, the relationship between the tested parameters and the

responses were explained in equations (Eq. 2, 3, and 4 for yield, EPA and DHA, respectively).

$$Yield = -34.8 + 0.151\ X_1 + 0.678\ X_2 - 0.000185\ X_1^2 - 0.00453\ X_2^2 - 0.000572\ X_1\ X_2 \quad \text{Eq. (2)}$$

$$EPA = 8.25 - 0.00346\ X_1 - 0.0730\ X_2 - 0.000009\ X_1^2 + 0.000187\ X_1\ X_2 \quad \text{Eq. (3)}$$

$$DHA = 14.8 + 0.0235\ X_1 + 0.101\ X_2 - 0.000031\ X_1^2 - 0.00189\ X_2^2 \quad \text{Eq. (4)}$$

3.3. Optimization procedures

Multiple response optimizations were used to assess the optimum levels of the parameters which could achieve the desirable response areas (Mirhosseini *et al.*, 2008). Besides the numerical optimization (Figure 2), the 3D plots (Figure 3) which were advocated for the graphical interpretation of the interaction effect of independent variables on the dependent variables (Montgomery and Wiley, 2001) were also considered using the Minitab software to locate the exact optimum point of independent variables and to obtain overall joint optimized values using the Minitab program response optimizer. The overall optimal values for maximum SC-CO_2 extraction efficiency and n-3PUFAs ratios (EPA and DHA) of pressure and temperature were anticipated to be at pressure

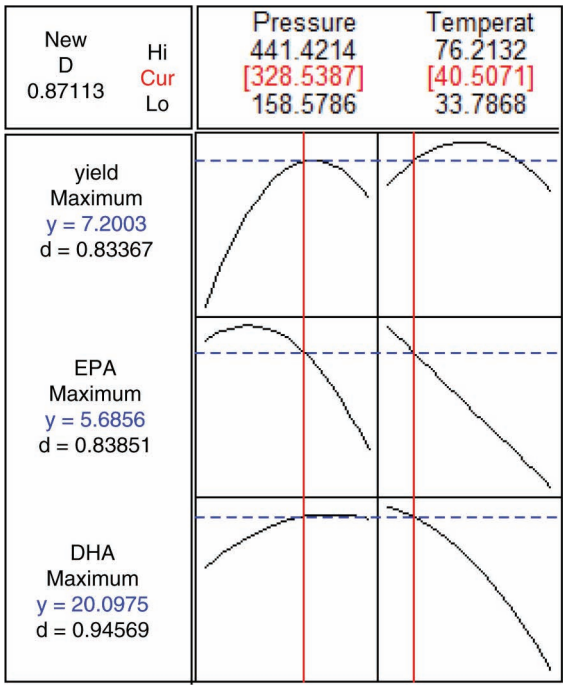

FIGURE 2. Combined optimum conditions of yield (%), EPA (%) and DHA (%) ratios of SC-CO_2 extracted sardine oil.

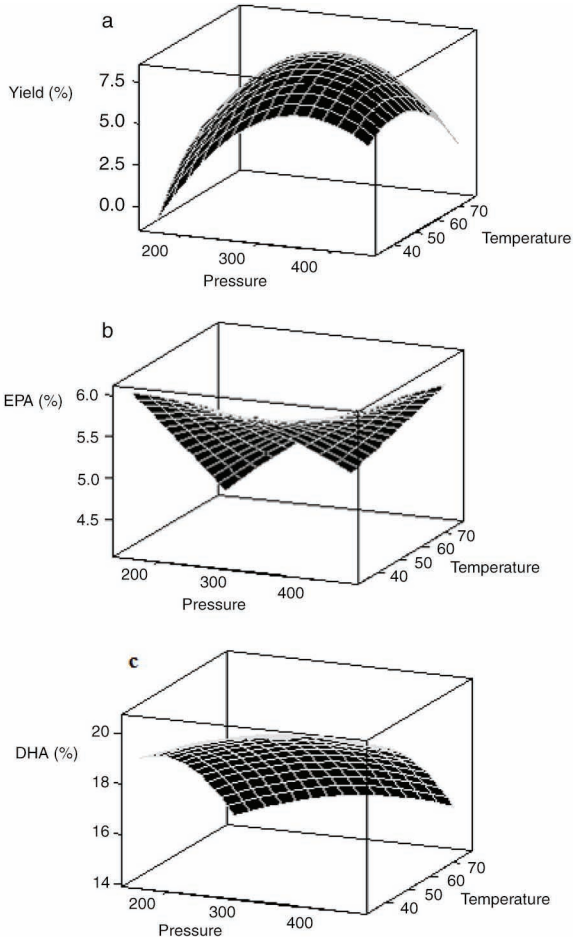

FIGURE 3. Surface plot of pressure and temperature influence on sardine fillet oil (a) extraction yield, (b) EPA ratio and (c) DHA ratio

of 328 bars and temperature of 40 °C as shown in Figure 2. Under the optimized conditions, the corresponding predicted dependent variables for yield, EPA and DHA were 7.20, 5.68 and 20.09%, respectively.

3.4. Verification of the final reduced models

The fitness of the response surface equation was checked from the error rate between experimental and predicted values of the reduced response regression models (Tables 2 and 3). The experimental and predicted values of yield, EPA and DHA obtained from equations 2, 3 and 4 are presented in Table 1. For each of the experimental values, Y_0 was compared with the predicted values, Y_1 calculated from the equation. A close agreement between the experimental and predicted values was noted and no significant ($p>0.05$) difference was found between those values, thus suggesting the adequate fitness of the response equations.

TABLE 2. Regression coefficients and analysis of variance of the reduced regression models for total yields

	Regression coefficient (β)		
Term	**yield**	**EPA**	**DHA**
cons	−34.7800	8.247	14.77
X_1	0.1507	−0.003	0.023
X_2	0.6783	−0.073	0.100
X_1^2	−0.0002	−8.804	−3.136
X_2^2	0.0045	*	−0.001
X_1X_2	−0.0006	>0.001	*
Regression model (R^2)	0.9250	0.947	0.968
Regression	0.0001	>0.001	>0.001
lack-of-fit	0.1100	0.343	0.306
Adj R-sq	0.8710	0.92	0.952

Cons: constant; X_1: pressure (bars); X_2: temperature (°C); *: its values were not significant and thus reduced from the model; EPA: eicosapentaenoic acid and DHA: docosahexaenoic acid.

3.5. Extraction of the oil

The sardine oils obtained from the various SC-CO_2 (run orders 1–13) and solvent (Soxhlet or MKM) extracts are given in Table (1). Although some SC-CO_2 runs (center points) gave higher extraction efficiency than MKM, overall results of both were statistically similar (p>0.05) and the two techniques gave a higher yield (>6%) than that of the söxhlet extraction (5.1%). SC-CO_2 extraction results were in agreement with those reported by Létisse *et al*. (2006). The MKM method initially established by Bligh and Dyer (1959) could be extracted with all lipids, including polar lipids, phospholipids and possibly lipids bound with other components from cellular membranes and with high yield. Yet application of this method for food supplementation has raised questions on its safety owing to toxicity of the solvents used (Létisse *et al*., 2006). Hence, the SC-CO_2 method using the environmentally-friendly and non-toxic CO_2 with small milliliters of non-hazardous solvents like ethanol or even nil could be a more attractive technique, provided that the conditions whereby maximum yield of lipid and/or fatty acids can be achieved, are optimized.

3.6. Impact of supercritical pressure and temperature on the dependent variables

The individual influence and efficient interaction between pressure and temperature were investigated. The impact of pressure and temperature on the SC-CO_2 sardine oil extraction was determined at pressures of 200 and 400 bars, temperatures of 40 and 70 °C respectively, and at a constant CO_2 flow rate (18 g·min^{-1}). Similar trends of pressure and temperature influence on the extracts with SC-CO_2 were observed in previous studies (Mariod *et al*., 2010, Jamilah *et al*., 2011). The high extraction yield (>8%) was obtained at 300 bars and 55 °C followed by 200 bars and 70 °C, while the lower yield was obtained at 158 bars and 55 °C. Based on these results a significant (p<0.05) effect of pressure and temperature on the sardine extraction efficiency was obvious. At low and extremely high pressures (200>p>350 bars), the oil extraction efficiency was decreased. The consequence of the extraction pressure and temperature on the yield of sardine oil at a steady CO_2 flow rate (18 g·min^{-1}) is demonstrated in Figure 3a. In view of the above results, it is clear that there was a significant joint (p<0.05) effect between the two parameters; i.e., when low pressure (200 bars) but high temperature (70 °C) and vice versa (400 bars and 40 °C) was applied, a good yield of 6.21% and 6.04, respectively (Table 1) was achieved. A reciprocal impact of SFE pressure-temperature inter-relationship, similar to the present findings, was reported by Wie *et al*. (2009). By increasing the temperature or pressure of the solvent, the rate of extraction with SC-CO_2 can be

TABLE 3. Significant probability (p-values and t-ratio) of the independent variable effects in the final reduced models of sardine oil from SFE

		Main effects		Quadratic effects		Interaction effects
Variable		X_1	X_2	X_1^2	X_2^2	X_1X_2
yield	p-value	>0.001[a]	0.002[b]	>0.001[a]	0.004[a]	0.034[c]
	t-ratio	−6.335	7.854	4.909	−7.481	−2.631
EPA	P-value	0.001[b]	>0.001[a]	0.028[c]	*	>0.001[a]
	t-ratio	4.940	−8.300	−2.680	*	6.43
DHA	p-value	0.003[b]	>0.001[a]	0.031[c]	0.007[b]	*
	t-ratio	4.235	−14.466	−2.620	0.119	*

EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; X_1 and X_2: the main effects; X_1^2 X_2^2: the quadratic effect and x_1x_2: the interaction effect of pressure (bars) and temperature (°C), respectively; *: its values were not significant and thus reduced from the model. Values with small superscript letters are statistically significant at [a]p<0.001, [b]p<0.01, [c]p<0.05.

improved (Zaidul *et al*., 2007). The center points (300 bars and 55 °C), however, showed the highest yields of sardine oil extraction with SC-CO_2. This was in line with what Pan *et al*. (2012) reported.

Despite the fact that the comparison of fatty acid composition in fish oils is quite difficult due to several probable affecting factors like season (Celik, 2008, Rasoarahona *et al*., 2005, Shirai *et al*., 2002) the EPA and DHA overall values extracted with MKM or SC-CO_2 in this study were in good agreement with those of South east Asian waters sardines (Chaijan *et al*., 2006). A significant (p<0.05) effect of pressure and temperature, with the latter being more influential on EPA and DHA, was noted. There was an inverse relationship of temperature increment with EPA (Figure 3b) and DHA (Figure 3c). For instance, when an extraction temperature as low as 33 °C with an intermediate pressure of 300 bars (Table 1) was used, the highest values of EPA (5.84) and DHA (20.35) were achieved. In contrast, the lowest portions were reached with high temperatures of up to 70 °C and a low pressure at 200 bars (Table 1). The severe susceptibility of poly unsaturated fatty acids to higher temperatures or light is a well addressed concern.

3.7. Fatty acid profile

Lipids from freeze-dried sardine extracted through various extraction methods (Soxhlet, MKM and SC-CO_2) were analyzed in order to determine the relationship between changes in the lipid fatty acid (FA) profile and method of extraction. The extraction efficiency (%) of the sardine oil obtained from SC-CO_2 was varied based on the extraction conditions (Table 1), therefore, triplicate analyses of SC-CO_2 optimum extraction efficiency (pressure: 321 bars and temperature 54 °C with a corresponding yield of 8.04%) were compared with the conventional extraction methods. The percentage values of methyl ester FA analyses in the söxhlet, MKM and SC-CO_2 extracted oils are presented in Table (4), while their typical chromatographic peaks are shown in Figure 4. No significant difference (p>0.05) in FA composition between MKM and SC-CO_2 was apparent. However, both formers were significantly

TABLE 4: Fatty acid profile of sardine (*Sardinella lemuru*) fillet lipids as affected by different extraction methods

Fatty acids	Average RT	Extracted by Soxhlet (%)	Extracted by MKM (%)	Extracted by ^{B}SC-CO_2 (%)
C14:0	4.97±0.01	6.79±1.56^{a}	5.24±0.36^{a}	5.37±0.35^{a}
C15:0	6.35±0.01	1.77±0.28^{a}	1.38±0.08^{a}	1.33±0.07^{a}
C16:0	8.04±0.04	30.93±3.46^{a}	29.89±1.17^{a}	29.57±1.02^{a}
C16:1n-7	8.41±0.03	6.12±0.64^{a}	5.17±0.31^{a}	5.12±0.18^{a}
C17:0	9.47±0.01	3.01±0.02^{a}	2.39±0.15^{a}	2.30±0.05^{a}
C17:1	9.87±0.01	0.68±0.20^{a}	0.55±0.05^{a}	0.63±0.02^{a}
C18:0	11.18±0.05	15.14±1.33^{a}	11.19±0.35^{a}	10.97±0.02^{a}
C18:1 n-9c	11.53±0.03	14.37±0.86^{a}	12.05±0.40^{a}	12.46±0.03^{a}
C18:2 n- 6c	11.63±0.03	2.89±0.18^{a}	2.48±0.09^{a}	2.34±0.07^{a}
C20:4 n- 6	16.48±0.01	0.94±0.20^{a}	2.40±0.11^{b}	2.19±0.03^{b}
C20:5 n- 3 (EPA)	**18.01±0.03**	2.17±0.55^{a}	5.43±0.05^{b}	5.45±0.14^{b}
C22:5-n-6	21.72±0.01	0.68±0.24^{a}	1.63±0.14^{a}	1.65±0.09^{a}
C22:5 n- 3	22.70±0.01	0.57±0.24^{a}	1.57±0.14^{a}	1.81±0.30^{a}
C22:6 n- 3 (DHA)	**23.04±0.05**	6.46±2.36^{a}	18.54±1.68^{b}	18.76±1.28^{b}
Others		7.46	0.09	0.05
∑ SFA		65.07	50.09	49.54
∑ MUFA		21.17	17.77	18.21
∑ PUFA		13.71	32.05	32.20
∑ n-6 FA		4.51	6.51	6.18
∑ n-3 FA		9.20	25.54	26.02
∑ n-3/n-6		2.03	3.92	4.21
∑ DHA/EPA		2.98	3.41	3.44

Values are means + S.D; n=3; means within each row with different lower case superscripts are significantly (p<0.05) different; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: poly unsaturated fatty acids; n-6 FA: omega-6 fatty acids; n-3FA: omega-3 fatty acids; ∑: Total; MKM: modified Kinsella method; RT: retention time; SC-CO_2: supercritical carbon dioxide; ^{B}SC-CO_2 parameters: pressure=321 bars; temperature=54 °C; time=80 min; CO_2 flow rate=18 g/min.

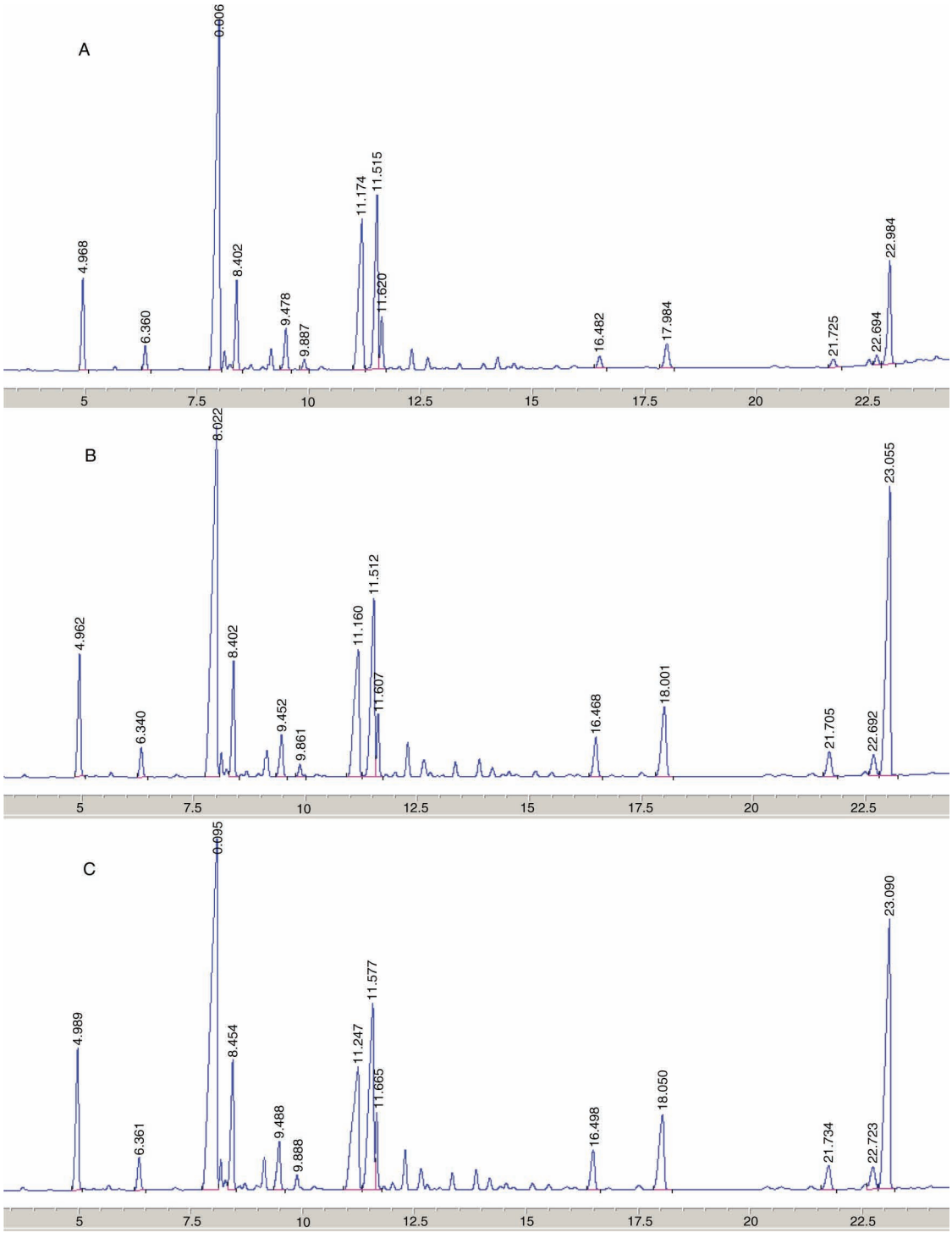

FIGURE 4. Chromatograph peaks of the fatty acid composition of *S. lemuru* oil obtained via (A) SOXHLET, (B) MKM and (C) SC-CO_2 [pressure =321 bars; temperature =54 °C; time =80 min; CO_2 flow rate =18 g·min^{-1}; modifier (EtOH) =3 mL/min]. For identification refer to the retention time in Table (4)

(p<0.05) different from the soxhlet extracted oil FA composition. The prolonged extraction time (8 h) at a relatively high temperature (60 °C) with Soxhlet had perhaps decreased the heat sensitive unsaturated fatty acids, especially the more susceptible PUFAs such as, EPA and DHA in the oil. From the analysis, the main components of sardine oil are shown in Table 4.

4. CONCLUSIONS

Among the three extraction methods (Soxhlet, MKM and SC-CO_2) compared for their extraction efficiency and their recovery of PUFAs particularly EPA and DHA, MKM and SFE (32.05 and 32.2%, respectively) exhibited similar results in this regard, nevertheless, certain points in SC-CO_2 showed higher but not significant yield and/or PUFAs than MKM, depending on pressure and/or temperature. However, a slightly lower yield and remarkably decreased ratios of PUFAs (yield=5.1±0.1% and PUFAs=13.7%) compared to those of MKM and SC-CO_2 (yield=6.83±0.15 and 6.46±2.36%, respectively) were found with Soxhlet extraction. Although good yields could be found using MKM, criticism about safety aspects due to the harmful extraction solvents has been raised, thus turning to SC-CO_2 which is safe, residue free, non-flammable and uses inexpensive CO_2 seems a more attractive choice recently for the extraction of lipids, nutraceuticals and bioactive compounds from diverse sources.

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