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Effects of process parameters on EPA and DHA concentrate production from Atlantic salmon by-product oil: Optimization and characterization

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Abstract−Supercritical carbon dioxide (SC-CO_2) extracted Atlantic salmon frame bone oil (SFBO) was used for Eicosapentaenoic acid and Docosahexaenoic acid (EPA-DHA) concentrate production by urea complexation. Urea/fatty acids (2.5 to 4.0 w/w), crystallization temperature (−24 to −8 °C) and crystallization time (8 to 24 h) were studied by Box-Behnken Design (BBD) to maximize EPA-DHA content. Highest EPA-DHA content was 60.63% at urea/fatty acids 4.0 w/w, crystallization temperature −15.67 °C and crystallization time 8 h. EPA-DHA concentrate showed improvement of EPA-DHA from 6.39% in SFBO to 62.34%, increase of astaxanthin content from 21.33 μg/g in SFBO to 44.69 μg/g in EPA-DHA concentrate, no residual urea and reduction of many off-flavor compounds. The EPA-DHA yield showed an inverse relation with the urea/fatty acids, whereas its concentration increased proportionally with urea/fatty acids. Therefore, EPA-DHA concentrate produced from SFBO by urea complexation may be an efficient technique to provide *ω*-3 polyunsaturated fatty acids to the consumers.

Keywords: Salmon Frame Bone Oil, Eicosapentaenoic Acid, Docosahexaenoic Acid, Optimization, Volatile Organic Compounds

INTRODUCTION

Atlantic salmon (*Salmo salar*) is a popular fish that is considered to be healthy due to high protein, polyunsaturated fatty acids, special carotenoid pigment (astaxanthin) and vitamin D content. Salmon frame bone is a by-product of salmon fillet producing industry containing the vertebral column and attached muscle. The automated salmon filleting industry produces approximately 59-63% fillet, 9-15% frame bone, 10-12% head and 1-2% trimmings of total wet weight of fish [1]. The frame bone of salmon is reported to contain 22.65% lipid [2]. The salmon frame bone contains high amount of omega-3 polyunsaturated fatty acids (*ω*-3 PUFAs), mostly EPA and DHA, which are beneficial for cardiovascular, hyperglyceridemia and inflammatory problems. Many researchers have studied the bioactivity of *ω*-3 PUFAs such as antihypertensive, antioxidant, antiarthritis, anticancer, antidepression and antiaging effects [3]. Dietary guidelines of UK for cardiovascular disease suggested to intake long chain *ω*-3 PUFAs and encouraged to increase average *ω*-3 PUFAs from 0.1 to 0.2 g day^{-1} [4]. So, the demand for marine PUFAs is increasing day by day as the people are getting cautious about their health. The intaking PUFAs concentrate is suggested by the researchers as it is devoid of saturated fatty acids and allowing daily intake of total lipid minimum [5]. For the production of *ω*-3 PUFAs concentrate industrially, fish oil is preferably used as a raw material. At present, fish w-3 PUFA concentrates that contribute to the nutritional requirements are extracted from liver or muscle, rendering high cost for the consumers. Salmon fillet producing industries discard a high quantity of frame bone every year which can be utilized as a raw material of edible oil and *ω*-3 PUFAs production. Production of *ω*-3 PUFAs from salmon frame bone oil using a suitable method has potential for providing nutritional requirements, specially EPA-DHA of the growing population and valorization of salmon waste.

The available methods to produce EPA-DHA concentrates from marine oils include urea complexation, low-temperature winterization, enzymatic reaction, supercritical fluid extraction, molecular distillation etc. Among the above-mentioned techniques, urea complexation is convenient, cheap, efficient and simple for *ω*-3 PUFAs production [6]. To separate polyunsaturated fatty acids from saturated and monounsaturated fatty acids, urea complexation is a highly efficient method [7]. In urea crystallization procedure, saturated and monounsaturated fatty acids form complex with urea at cold condition and the polyunsaturated fatty acids dissolved in alcoholic solution are separated easily by filtration [8]. A very stable complex is formed at low temperature in urea crystallization at which filtration at very low temperature is not required [9]. To optimize the responsible variables, response surface methodology (RSM) is an effective method which determines the relationship between the independent variables and response of desired parameters [10]. For developing the optimization of the process parameters conveniently to produce EPA-DHA concentrated oil, Box-Behnken Design (BBD) of RSM was selected.

This research work was focused on the optimization of influencing process parameters such as urea/fatty acids (w/w), crystalli-

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zation temperature (°C) and crystallization time (h) to produce high EPA-DHA containing PUFAs concentrate by response surface methodology. Moreover, characterizations of the produced EPA-DHA concentrate were carried out on the basis of fatty acid composition, astaxanthin content, detecting residual urea and volatile organic compounds (VOCs) in produced EPA-DHA concentrate.

MATERIALS AND METHODS

1. Materials and Reagents

Carbon dioxide (CO_2) gas purity 99.99% was supplied by Korea Specialty Electronic Materials Co., Ltd. (KOSEM), Yangsan, Korea. Fatty acid methyl esters (FAME) and astaxanthin standard were purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA. Paradimethylaminobenzaldehyde (DMAB) was purchased from Fishers Scientific, New Jersey, USA. Urea purity >99.00% was purchased from Junsei Chemical Co., Ltd., Tokyo, Japan. Analytical or HPLC graded chemicals and solvents only were used in this study.

2. Collection of Sample

Atlantic salmon frame bone was collected from Seawell Co., Ltd. (fish imported from Norway), Haeundae-gu, Busan, Republic of Korea in January, 2016. The blood, slime and other dirt materials of sample were cleaned by washing with cold water (4 °C), drained and freeze dried (Scanvac, Cool safe 110-4, LaboGene, Denmark). The freeze-dried sample was ground by using an electric blender (Hanil, HMF-3260S, 2,000 mL, Seoul, Republic of Korea) and kept at −60 °C until oil extraction by SC-CO_2.

3. SC-CO_2 Extraction of SFBO

An SC-CO_2 system with extractor size 200 mL was loaded with 100 g of freeze-dried crushed sample. At the bottom and top of the extraction vessel, thin layers of cotton were placed to avoid entrance of sample through the gas line. The cap of the extraction vessel was plugged carefully to ensure no gas leakage. Liquid CO_2 was pumped by a high pressure pump (Milroyal, Milton Roy, USA) into the extraction vessel up to the desired pressure. The pressure of CO_2 was controlled using a back pressure regulator (BPR) (Tescom 54-2000 Series, Elk River, Minnesota, USA). The extraction vessel and separator were connected with two different water baths for maintaining temperature (MAT'L: SUS 304, Ilshin Autoclave Co. Ltd., Daejeon, Korea). Flow rate of CO_2 was controlled by a needle valve (HOKE 1300 series, CIRCOR Instrumentation, Spartanburg, South Carolina, USA) and was measured by a gas flow meter (Shinagawa, Tokyo, Japan). The temperature and pressure applied for SC-CO_2 extraction were selected from the previous research work [11] with some modifications, and the duration of oil extraction was fixed by repeated trials of finishing extraction. The temperature and pressure were maintained at 45 °C and 25 MPa, respectively, with constant 27 g/min flow rate of CO_2 for 2.5 h.

4. Preparation of Free Fatty Acids from SFBO

Preparation of free fatty acids for urea complexation was according to the method described by Wanasundara and Shahidi [12] with slight modifications. SFBO 50 g was measured, taken in a 500 mL size conical flask and added with 10 mg of butylated hydroxyanisole (BHA). Then 11.50 g potassium hydroxide (KOH), 22 mL distilled water and 132 mL aqueous ethanol (94% v/v) were added and mixed with the oil. A layer of nitrogen gas was made in the space of the conical flask and a water bath was used for heating the reactants at 62 °C for 1 h. After completing saponification, 100 mL of distilled water was added to the saponified mixture and 200 mL of hexane was mixed and discarded using a separatory funnel to remove unsaponifiable matter; this process was repeated two times. The aqueous fraction containing saponified materials was taken in a beaker and kept stirred at 250 rpm. Then, 3 N HCl was added gently to acidify until the pH reached to 1. Then 100 mL hexane was poured to the mixture, stirred for 5 mins to mix properly and was taken in a separator. The fatty acids aggregated in hexane were separated and moisture was removed over Na_2SO_4 (anhydrous). The fatty acids were recovered by evaporating hexane at 42 °C under vacuum system. To protect from oxidation or other biochemical changes, recovered free fatty acids were preserved at −60 °C.

5. Urea Complexation for EPA-DHA Concentrate Production

Urea complexation was applied for separating ω-3 PUFAs, specially EPA-DHA from other fatty acids following the method of Wanasundara and Shahidi [12] with some modifications. Exactly, 5 g of free fatty acids was taken in a 250 mL beaker. 20% (w/w) urea solution in 95% ethanol (v/v) was added maintaining different urea/fatty acids ratio (2.5 to 4.0 w/w) and heated at 65 °C under stirring by an electro-magnetic stirrer to make the whole mixture a transparent uniform solution. First, the urea-fatty acid mixture solution was kept at rest for 30 min at room temperature for crystal formation, then was placed inside the refrigerator at different temperatures (−24 °C to −8 °C) for different times (8 h to 24 h) to complete crystal formation. The urea-fatty acid crystal was brought out from the refrigerator and filtered immediately under suction using a Buchner funnel and filter paper. Distilled water of equal volume was added to the filtrate and 6 N HCl was added slowly to lower pH around 4.5. An equal volume of hexane was added immediately and the stirring was continued for 1 h covering the head opening of the beaker by aluminum foil paper to reduce solvent evaporation. The fatty acids were dissolved in the upper hexanic fraction, which was clearly separable from hydroethanolic residual urea containing bottom layer. The hexanic fraction was isolated using a separatory funnel, repeatedly washed with distilled water to remove residual urea and dried over Na_2SO_4 (anhydrous). A rotary vacuum evaporator was used to remove the solvent at 42 °C. A gas chromatographic (GC) analysis was done for the determination of fatty acids composition of produced EPA-DHA concentrate.

6. Optimization Procedures for Production of EPA-DHA Concentrate

6-1. Experimental Design and Statistical Analysis

The effects of process parameters and conditions to produce EPA-DHA concentrate from SFBO by urea complexation were evaluated by response surface methodology. The present experimental design was created with 17 design points, three numeric factors on three levels on the basis of Box-Behnken Design (BBD). Urea/fatty acids ratio (2.5 to 4.0 w/w), crystallization temperature (−24 to −8 °C) and crystallization time (8 to 24 h) were the three independent variables for this study. The parameters were normalized by forcing each of the coded variables from-1 to 1, as they can influence the response more similarly, and so the unit of the parameters was irrelevant [13].

Table 1. BBD with variable levels and coded values for maximizing EPA and DHA content by urea complexation

Run no.	Variable levels with coded values			(EPA+DHA)[d]	
	Ratio[a]	Temperature[b]	Time[c]	Actual value	Predicted value
1	2.5 (−1)	−24 (−1)	16 (0)	32.62	33.63
2	4 (+1)	−24 (−1)	16 (0)	55.34	54.76
3	2.5 (−1)	−8 (+1)	16 (0)	30.79	31.36
4	4 (+1)	−8 (+1)	16 (0)	57.2	56.18
5	2.5 (−1)	−16 (0)	8 (−1)	39.17	38.18
6	4 (+1)	−16 (0)	8 (−1)	60.02	60.62
7	2.5 (−1)	−16 (0)	24 (+1)	40.42	39.82
8	4 (+1)	−16 (0)	24 (+1)	62.34	63.33
9	3.25 (0)	−24 (−1)	8 (−1)	43.93	43.91
10	3.25 (0)	−8 (+1)	8 (−1)	42.64	43.05
11	3.25 (0)	−24 (−1)	24 (+1)	46.06	45.65
12	3.25 (0)	−8 (+1)	24 (+1)	45.65	45.67
13	3.25 (0)	−16 (0)	16 (0)	49.54	50.62
14	3.25 (0)	−16 (0)	16 (0)	50.59	50.62
15	3.25 (0)	−16 (0)	16 (0)	51.14	50.62
16	3.25 (0)	−16 (0)	16 (0)	50.78	50.62
17	3.25 (0)	−16 (0)	16 (0)	51.05	50.62

Note: Run no. 13-17 are the replications of central levels
[a]Urea:fatty acid (w/w)
[b]Crystallization temperature (°C)
[c]Crystallization time (h)
[d]Eicosapentaenoic acid and docosahexaenoic acid (%)

The variables were coded following the equation below [14]:

$$X_i = \frac{(x_i - x_o)}{\Delta x} \quad (1)$$

where, X_i=coded value, x_i=corresponding actual value, x_o=actual value in the center of the domain, and Δx=increment of x_i corresponding to a variation of 1 unit of X. Independent variables of the natural and coded values used in BBD are given in Table 1. The second-order polynomial model (Eq. (2)) fitted the response variables that usually narrate the relationship between the response and independent variables [15].

$$Y = \beta_0 + \Sigma_{i=1}^{3}\beta_i X_i + \Sigma_{i=1}^{3}\beta_{ii} X_i^2 + \Sigma_{i=1}^{3}\Sigma_{j=1}^{3}\beta_{ij} X_i X_j \quad (2)$$

where, Y=variables of response, X_i and X_j=independent variables affecting the response, β_o, β_i, β_{ii}, and β_{ij}=the intercept, linear, quadratic and interaction regression terms, respectively [16]. Optimum urea complexation conditions were determined considering the EPA-DHA concentration (%) as a response. Desirability functions were considered for multiple response treatments and optimal condition selection [17]. Design-Expert v. 7 Trail (Stat-Ease, Minneapolis, USA) was used for the analysis of experimental design and multiple linear regressions.

7. Analysis of Fatty Acid Composition

A gas chromatograph (6890 Agilent Technologies, Wilmington, USA), with a fused silica capillary column (100 m length×0.25 mm internal diameter, 0.2 μm of film, Supelco™, Bellefonte, USA) was used for the analysis of the fatty acids composition of normal SFBO and EPA-DHA concentrate. Fatty acid methyl esters of EPA-DHA concentrate and SFBO were prepared following the methods of the American Oil Chemists' Society (AOCS), Ce 2-66 (2) and (3), respectively [18]. The oven temperature was programmed to start at a constant temperature of 130 °C for 3 min, then increased to 240 °C at a rate of 4 °C/min. Then the temperature was kept constant at 240 °C for 10 min, the final temperature of both the injector and detector was maintained at 250 °C. The retention time of fatty acid methyl esters standard (Supelco™, Bellefonte, PA, USA) was compared to identify the fatty acid composition of the oil samples and quantitative results were presented as peak area (%).

8. Yield of EPA-DHA at Different Urea to Free Fatty Acid Ratio

EPA-DHA concentrate was produced at different urea/fatty acids ratio (2 to 5 w/w) at optimized crystallization temperature (−15.67 °C) and crystallization time (8 h). A relationship between the EPA-DHA yield (%) and EPA-DHA concentration (%) at different urea to free fatty acid ratio was determined.

9. Astaxanthin Content Determination by HPLC

Astaxanthin content of SFBO and EPA-DHA concentrate produced from urea complexation was determined according the method used by Ali-Nehari [19]. A Waters model 600E system controller (Milford, USA) high-performance liquid chromatograph (HPLC) equipped with a model 484 UV/VIS detector, and Eclipse Plus C18 column (5 μm, 4.6×250 mm, Agilent, USA) was used for astaxanthin content determination. Mobile isocratic phase consisted of acetonitrile, dichloromethane and ethanol at volume ratio of 5 : 10 : 85 and was eluted at 1 mL/min. Astaxanthin was detected at the wavelength of 470 nm and quantification was done based on the peak area comparing with standard astaxanthin. Acetonitrile was used to dissolve standard astaxanthin at different concentration (1-40 μg/mL) for preparing a standard curve (Fig. 1).

10. Determination of Urea Content in EPA-DHA Concentrate

Qualitative and quantitative estimation of urea in produced EPA-DHA concentrate was done according to the method described by Rames [20] with some modifications. For the estimation of urea in EPA-DHA concentrate, a test based on the use of p-dimethylaminobenzaldehyde (DMAB) was performed. The principle of this method is that at room temperature, a yellow complex is formed by urea in the presence of DMAB reagent in a low acidic solution. DMAB reagent is prepared in ethyl alcohol (1.6%, m/v) containing 10% (v/v) of concentrated hydrochloric acid (HCl). 3 g of EPA-

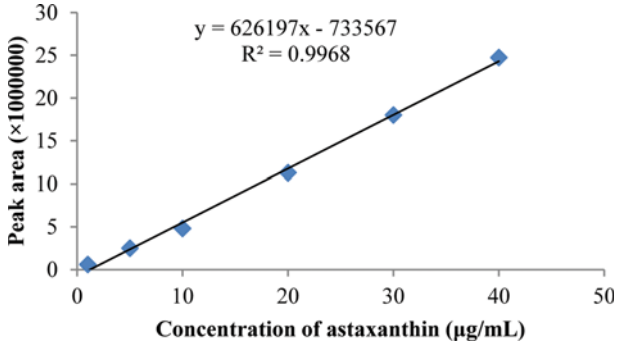

Fig. 1. Standard curve of astaxanthin.

DHA concentrate was added to 3 mL of distilled water and vortexed for 5 mins. The reagent with sample mixture was then centrifuged at 15,000 rpm for 10 min. 2 mL of clear aqueous part was collected by using a syringe and was added with 2 mL of DMAB solution and vortexed for 30 s. The mixture was kept at room temperature for 5 mins and the OD value was taken at 420 nm.

11. Volatile Organic Compound Analysis

Volatile organic compounds (VOCs) of normal SFBO and produced EPA-DHA concentrate were analyzed using GCMS (GCMS-QP2010, Shimadzu Co., Kyoto, Japan). 3 g of samples were taken into 250 mL capacity dark amber vials with high volume of head space (Supelco™ Inc., Bellefonte, PA, USA). Then, the vials were carefully capped with PTEE septum (QMX Laboratories Ltd., ESSX, UK) and kept inside a hot air oven at 40 °C for 30 min release volatile organic compounds. After heating, the sample vials were kept at room temperature for 20 min, and then the released volatile compounds were collected by using a vacuum pump and mass flow controller (AALBORG Instrument and Controls Inc., Orangeburg, USA) in a triple bed absorption tube (Tenax-TA, Supelco™ Inc., USA). An automatic thermal desorber (ATD; ATD-400, Perkin Elmer, UK) was used for collecting the VOCs from the tube which was connected with GCMS connected with an AT-1 column (60 m×0.32 mm×1.0 μm). As a carrier gas, helium was used at a flow rate 0.62 mL/min and inlet pressure was maintained at 15.7 psi. The thermal and other conditions maintained in this study were similar to Lee [21]. The quantification of VOCs was determined as the area % of the peaks.

RESULTS AND DISCUSSION

1. Yield of Oil

35.15% of the oil was obtained from the freeze-dried, crushed

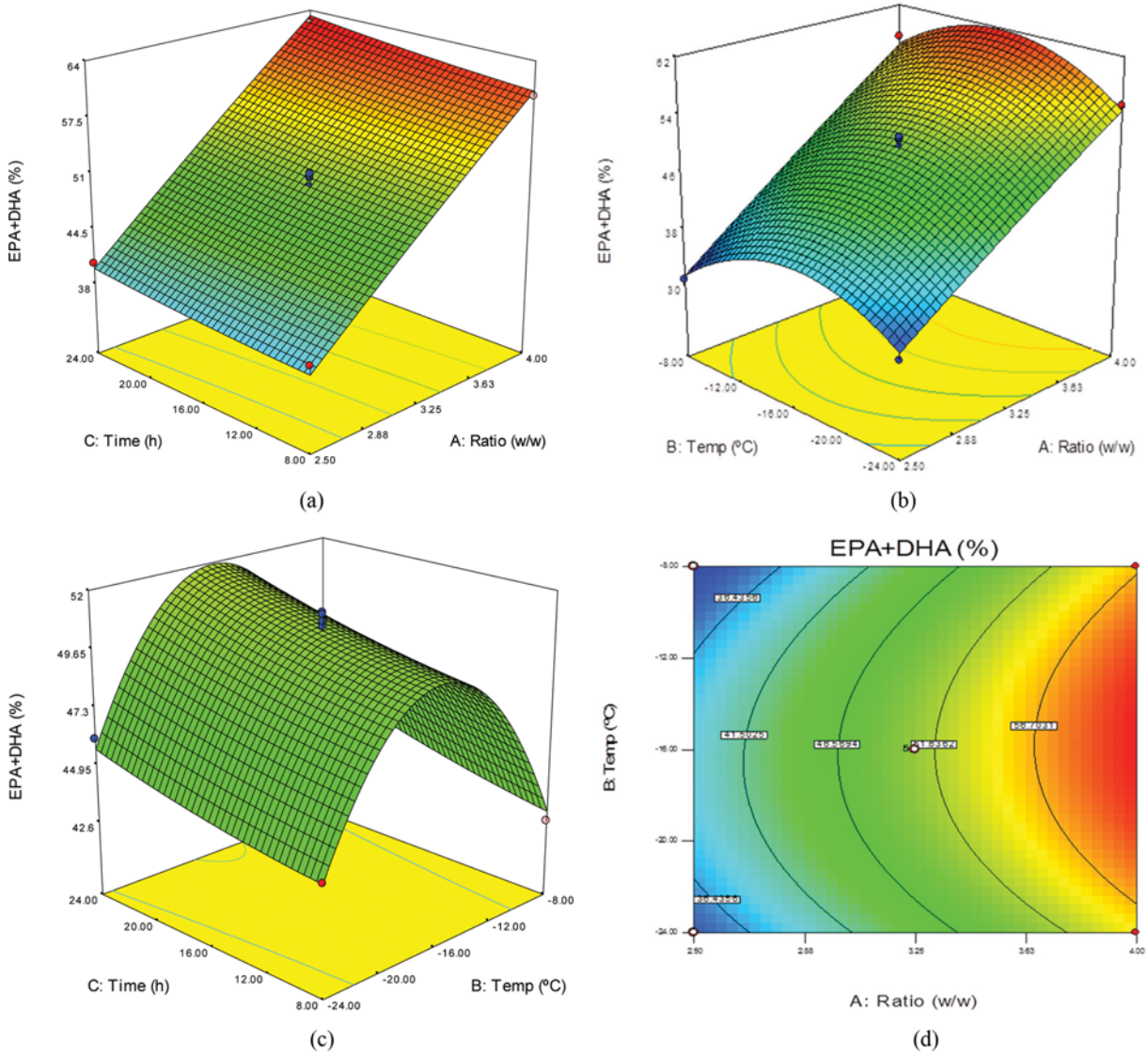

Fig. 2. Response surface plots representing interaction effects among the parameters: (a) Urea/fatty acids and crystallization time (b) urea/fatty acids and crystallization temperature (c) crystallization time and crystallization temperature (d) contour plots for the effects of urea/fatty acids and crystallization temperature.

salmon frame bone by the SC-CO_2 extraction process. The SC-CO_2 extraction method was applied for extraction SFBO in this experiment as a green technology for obtaining solvent contamination-free good quality oil. SC-CO_2 is a green extraction technique that is advantageous over conventional extraction techniques as no solvent residues are contained in the extracted product [22].

2. Optimization of the Parameters for Maximizing EPA-DHA Concentration

The values obtained from different urea complexation conditions in order to optimize process conditions by BBD of response surface methodology are given in Table 1. Response surface plots of studied influencing process parameters urea/fatty acids, crystallization time and crystallization temperature are plotted in Fig. 2. The values of EPA-DHA concentration obtained from the present experiment were found to vary from 30.79 to 62.34% (Table 1). The efficiency of increasing EPA-DHA concentration depends on the complex formation between saturated/mono or di-unsaturated fatty acids and urea. The higher EPA and DHA content of the non-urea complex fraction mostly depends on urea/fatty acids ratio and crystallization temperature in urea complexation [23]. Statistical analysis showed that urea to fatty acid ratio influenced significantly the EPA-DHA content and yield, which is why it is considered as the most crucial factor in urea complexation process. Secondly, crystallization temperature also played a role to increase the total EPA-DHA concentration, whereas crystallization time did not affect significantly. For increasing PUFAs by urea complexation, the important factors are to be considered as urea ratio to fatty acids and crystallization temperature as reported by Chin [24].

2-1. Fitting the Model for Analyzing Response Surface

Experimental data obtained in the present study were well-matched to a second-order polynomial equation (Eq. (3)). Statistical analysis was done for the production of multiple regression coefficient for regression tools to make the least residual possible [15]. The outcomes of the ANOVA (analysis of variance) are given in Table 2. The highest multiple determination (R^2) value for EPA-DHA concentrate was 0.998 (Table 3), indicating a well-fitted relation between the model equation and experimental values. The model regression was significant ($p<0.05$), which suggested the statistical acceptance of the arithmetical model for the response (Table 2). The lack of fit (F-value) for all the responses was not significant ($p>0.05$) (Table 2), confirmed by the F-value test. Considering all above conditions of ANOVA, Eq. (3) would be able to illustrate

Table 2. Analysis of variance (ANOVA) for response surface quadratic model for EPA-DHA concentrates

Source	Sum of squares	df	Mean square	F value	P-value (Prob>F)	Remark
Model	1237.29	9	137.48	130.28	<0.0001	Significant
A-Ratio (w/w)	1055.70	1	1055.70	1000.44	<0.0001	
B-Temp (°C)	0.35	1	0.35	0.33	0.5834	
C-Time (h)	9.48	1	9.48	8.99	0.0200	
AB	3.40	1	3.40	3.23	0.1155	
AC	0.29	1	0.29	0.27	0.6186	
BC	0.19	1	0.19	0.18	0.6813	
A^2	0.54	1	0.54	0.51	0.4983	
B^2	165.79	1	165.79	157.11	<0.0001	
C^2	0.21	1	0.21	0.20	0.6667	
Residual	7.39	7	1.06			
Lack of fit	5.74	3	1.91	4.64	0.0861	Not significant
Pure error	1.65	4	0.41			
Cor total	1244.68	16				

A, B, C, representing experimental variables

A=ratio of urea to fatty acids, B=crystallization temperature, and C=crystallization time

df=degree of freedom

Table 3. Fitting of the sequential model for EPA-DHA concentration

Source	Sum of squares	df	Mean square	F value	P-value Prob>F	R^2	Adjusted R^2	Predicted R^2	Press	Remark
Mean vs Total	38525.54	1	38525.54			0.856	0.822	0.735	329.57	
Linear vs Mean	1065.53	3	355.178	25.77	<0.0001	0.859	0.774	0.428	711.61	
2FI vs Linear	3.88	3	1.29	0.07	0.9727	0.994	0.986	0.924	94.39	
Quadratic vs 2FI	167.88	3	55.96	53.03	<0.0001	0.998	0.994		+	Suggested Aliased
Cubic vs Quadratic	5.73	3	1.91	4.64	0.0861					
Residual	1.65	4	0.41							
Total	39770.22	17	2339.42							

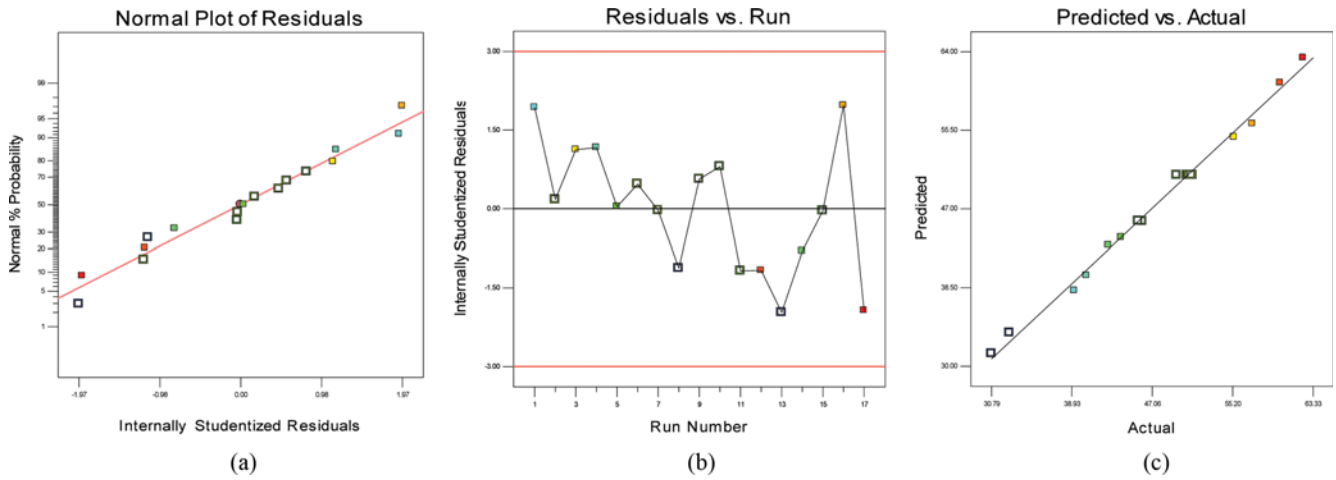

Fig. 3. Diagnostic plots for the adequacy of proposed model (a) plot of residuals for response (b) Internally studentized residuals plot and (c) The predicted values vs. the experimental values.

the performance of EPA-DHA concentrate production [25].

$$\text{EPA-DHA concentration (\%)} = -39.223 + 21.194R - 3.718Te - 0.066Ti + 0.154R*Te + 0.045R*Ti + 0.003Te*Ti - 0.636R^2 - 0.098Te^2 + 0.004Ti^2 \quad (3)$$

where, R=urea/fatty acids (w/w), Te=crystallization temperature (°C), and Ti=crystallization time (h).

2-2. Diagnostic of the Model Adequacy

The model adequacy for the present experiment was tested by preparing various linear diagnostic plots presented in Fig. 3. The normal percentage plot of residuals of response was laid around the horizontal line and showed no significant difference of the variation (Fig. 3(a)). The fitness of the constructed model was proved by the internally studentized residuals plot (Fig. 3(b)), where all the values took place inside the range (±3). Experimental and predicted data from the prepared model were fairly close and stayed a nearly straight line (Fig. 3(c)), which indicated the adequacy of the present model.

2-3. Optimization and Verification of the Model

In the current model, the predicted combination of the parameters was urea/fatty acids ratio 4 : 1, crystallization temperature −15.67 °C and crystallization time 8 h for obtaining maximum EPA-DHA concentration. At these conditions, the predicted value of EPA-DHA concentration was 60.63% (at desirability of 0.972). Desirability ramp was used for validating the optimum conditions of the model. At the optimized conditions, triplicate experiments were done for EPA-DHA concentrate production which contained EPA-DHA content 62.34%. In the desirability functions, the obtained results closely matched the predicted values at the optimized conditions with desirability functions, proving BBD could be used effectively as the plot of residuals was normally distributed. The internally studentized residuals remained adjacent to the horizontal line, indicating no digression of variance and satisfactory of the model fitting (Fig. 3(a)). All the data plots of the internally studentized residuals stayed within the range of ±3 (Fig. 3(b)), which also proved the verification of the proposed model. The experimentally obtained values and the model predicted values were quite closer to a straight line (Fig. 3(c)), again proving the verification of the model for optimizing the process parameters that affect EPA-DHA concentrate production.

3. Fatty Acid Composition of SFBO and EPA-DHA Concentrate

Fatty acid analysis of SFBO and EPA-DHA concentrate are presented in Table 4 and Supplementary Fig. 1. The table showed us that, the EPA and DHA content of SFBO was 6.39% which was increased to 62.34% in the EPA-DHA concentrate. SFBO contained 28.45% of saturated fatty acids and 11.08% of ω-3 polyunsaturated fatty acids, whereas the EPA-DHA concentrate produced by urea complexation procedure decreased the saturated fatty acids content to 5.74% and increased ω-3 polyunsaturated fatty acids to 66.52%. Urea complexation procedure was highly efficient to reduce the saturated fatty acids and increase polyunsaturated fatty acids. The saturated and monounsaturated fatty acids were easily complexed with urea and crystallized out on cooling and could subsequently be removed by filtration. In hot (62-65 °C) ethanolic solution of fatty acids, urea remains straight-chain organic molecular form which is transformed to hexagonal crystal form enclosing the fatty acid molecules during crystallization. Van der Waals forces, induced electrostatic attractions and London dispersion forces take part forming the complex [26], which depends upon size, shape and geometry of fatty acids. During crystallization from mixed fatty acid solution, the long and straight chain saturated and mono/di-unsaturated fatty acids are abducted with urea while the PUFAs remain in the alcoholic solution. When lower ratio of urea/fatty acids is used, the fatty acids compete among themselves for complexing with urea depending on the degree of unsaturation. Therefore, with decreasing the extent of unsaturation and increasing chain length, the tendency of fatty acids for urea complexation is increased. Thus, straight chain fatty acids are separated from the cyclic or branched ones [27]. Urea complexation is an exothermal process; therefore, the stability of the complexed compounds depends on the extent of heat formation. Fatty acid chain length increases the molar heat formation and vice versa. The tendency for urea complex formation is increased with decreasing the temperature; the optimum tempera-

Table 4. Fatty acid composition of SFBO and EPA-DHA concentrated oil

Sl. no.	Fatty acids	Retention time	Normal SFBO	EPA-DHA concentrated oil
			Area%	Area%
1.	Caprylicacid (C8:0)	12.393	10.15	nd
2.	Capricacid (C10:0)	14.110	2.03	nd
3.	Mystricacid (C14:0)	20.039	1.96	0.57
4.	Palmitic acid (C16:0)	23.409	10.88	0.88
5.	Palmitoleicacid (C16:1)	24.612	2.64	0.79
6.	Stearic acid (C18:0)	26.608	3.43	0.65
7.	Oleic acid (C18:1n9C)	27.718	32.39	5.17
8.	Elaidicacid (C18:1n9t)	27.801	3.21	0.44
9.	Linoleic acid (C18:2n6c)	29.170	17.90	6.46
10.	Arachidicacid (C20:0)	29.556	nd	1.94
11.	r-Linoleic acid (C18:3n6)	30.202	nd	1.31
12.	cis-11-Eicosenoic acid (C20:1)	30.514	1.72	1.3
13.	Linolenic acid (C18:3n3)	30.816	4.69	4.18
14.	Cis-11,14 Eicosadienoicacid (C20:2)	31.977	1.81	7.07
15.	Behenicacid(C22:0)	32.427	nd	0.42
16.	Cis-8,11,14-Eicosatrienoic acid (C20:3n6)	33.105	nd	0.91
17.	Tricosanoicacid (C23:0)	34.023	nd	1.28
18.	cis-13,16-Docosadienoic acid (C22:2)	34.965	0.78	3.43
19.	5,8,11,14,17-Eicosapentanoic acid (C20:5n3)	36.024	2.87	13.92
20.	Nervonicacid (C24:1)	36.488	nd	0.86
21.	4,7,10,13,16,19-Docosahexanoic acid (C22:6n3)	40.961	3.52	48.42
	Total		100.00	100.00
	ΣEPA+DHA		6.39	62.34
	ΣSFA		28.45	5.74
	ΣUFA		71.55	94.26
	Σω-3 PUFA		11.08	66.52

Note: EPA=eicosapentanoic acid, DHA=docosahexanoic acid, SFA=saturated fatty acids, UFA=unsaturated fatty acids, PUFA=poly unsaturated fatty acids, nd=not detected

ture depends on the particular PUFAs [28]. Thus, the role of temperature is important for crystallization of saturated long chain fatty acids properly avoiding PUFAs. The initial EPA-DHA content of oil is important for determining the EPA-DHA content of final product and yield. This study was based on the valorization of lower EPA-DHA containing salmon frame bone oil rendering lower yield of the final product (Fig. 4). Some researchers investigated the production of EPA-DHA rich 2-monoacylglycerol (2-MAG) by alcoholysis reaction of fish oil using *sn*-1,3 specific lipases. Marine lipids are characterized containing a major part of long chain EPA and DHA in the 2-position of the glycerol backbone. By enzymatic process, the fatty acids in 1,3-position are removed and then 2-MAG is obtained in hydro-ethanolic fraction, whereas other parts including fatty acids are discarded in the hexanic fraction. This method is suitable for obtaining a reasonable content of EPA-DHA concentrated oil when the initial content is high. Alcoholysis reaction of cod liver oil by Novozymes 435 enriched EPA and DHA content from 16.1% to 26.40% when ethanol (96% v/v) was used as reaction media [29]. EPA and DHA content was obtained as 40.80 and 44.90% from initial oil content 20.60% and 29.60% of cod liver oil and tuna oil, respectively by alcoholysis reaction catalyzed by Novozymes 435 [30]. Urea crystallization technique increased EPA-DHA concentration from 25.54% to 69.55% in oil of tuna fish (*Thunnus* sp.) canning by-product oil [31]. The total DHA and EPA increased to 85.02% from tuna oil obtained at a urea/fatty acids ratio of 15 (mole/mole), a crystallization temperature of −5 °C, and a crystallization time of 20 h [23].

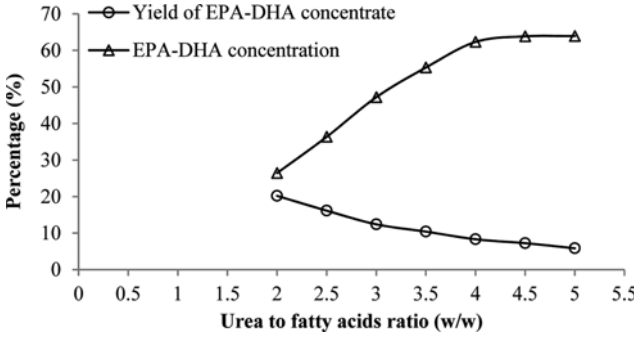

Fig. 4. Changes in the EPA-DHA yield and concentration at different urea/fatty acids ratio.

Table 5. Changes in volatile organic compounds of normal SFBO and EPA-DHA concentrate

Sl. no.	Compounds (area %)	RT	Normal SFBO	EPA-DHA concentrate
1.	Methanol	3.501	0.39	nd
2.	Ethyl alcohol	3.977	22.88	26.36
3.	*n-Pentane	4.514	0.44	nd
4.	*2-Pentene, (Z)-	4.764	0.06	nd
5.	*Acetic acid, methyl ester	5.007	0.12	nd
6.	Butanal	6.321	0.52	0.85
7.	*2-Butanone	6.546	1.46	nd
8.	n-Hexane	6.978	14.03	15.49
9.	2-Butanol	7.136	0.43	nd
10.	Trichloromethane	7.414	3.01	0.54
11.	*Butanal, 3-methyl-	9.027	1.34	0.43
12.	*Butanal, 2-methyl-	9.660	0.38	0.09
13.	1-Penten-3-one	11.197	0.09	nd
14.	*Disulfide, dimethyl	14.115	1.12	nd
15.	2-Methyl-3-vinyl-oxirane	14.124	nd	0.05
16.	*4-Pentenal	14.468	0.12	nd
17.	2-Pentenal, (E)-	14.500	0.00	0.11
18.	n-Hexanal	16.284	2.23	3.04
19.	*3-Pentanol	16.457	0.16	nd
20.	*n-Octane	16.822	0.17	0.16
21.	3-Octyne	17.253	0.10	nd
22.	*2-Hexenal	18.134	0.16	0.08
23.	n-Heptanal	19.664	0.18	0.15
24.	Alpha-phellandrene	20.917	0.14	nd
25.	1,3,6-Octatriene, 3,7-dimethyl-, (E)-	21.185	0.48	nd
26.	1-Heptanol	21.858	7.21	4.63
27.	Octanal	22.546	nd	0.18
28.	2-Bromooctane	24.362	0.05	nd
29.	Nonanal	26.215	0.15	0.12
30.	Butane,1,2,4-trichloro-heptafluoro-	45.407	nd	0.13
31.	Benzene, (1-butylnonyl)-	47.919	0.04	nd
32.	1-Ethyl-2-methyl-3-phenylindane	48.267	0.05	nd
33.	Benzylamine	48.472	0.22	nd
34.	2,4-Diphenyl-4-methyl-2(E)-pentene	48.553	0.21	nd
35.	2-Bromo dodecane	48.811	0.05	nd
36.	2,4-Diphenyl-4-methyl-2(E)-pentene	48.976	0.06	nd
37.	Benzophenone, 5-isopropyl-2-methyl-	48.976	0.06	nd
38.	*Benzene, (1-methylnonyl)-	49.349	0.04	nd
39.	1,6,6-Triphenyl-2-piperidinone	49.349	0.04	nd
40.	Tricyclo hexadeca-3, hexaen-2-one	49.590	0.08	nd
41.	5,6,11,12-Tetrahydrodibenzazocine	49.800	0.04	nd
42.	Butane, -tetrachloro-hexafluoro-	49.951	0.07	nd
43.	*Benzene,1,2-dimethoxy-4-propenyl)-	49.800	0.04	nd
44.	*Butane, trichloroheptafluoro-	50.607	0.57	nd
45.	*Butane,-tetrachloro-hexafluoro-	50.827	0.21	nd
46.	2-Methoxy-1-phenyl-1-propene	51.092	0.01	nd
47.	*Butane,1,2,4-trichloro-heptafluoro-	51.305	0.04	nd
48.	Dodecanoic acid, isooctyl ester	55.227	0.41	0.34

Note: nd=not detected, RT=retention time

*=Compounds claimed for the undesirable odor of fish oil [19,37]

4. Relation of Urea/Fatty Acids Ratio with EPA-DHA Yield and Concentration

The relationship between EPA-DHA yield (%) and EPA-DHA concentration (%) of SFBO at optimized crystallization temperature (−15.67 °C) and crystallization time (8 h) at different urea/fatty acids ratio is presented in Fig. 4. The figure shows that the EPA-DHA yield had an inverse relation with the urea/fatty acids, whereas the EPA-DHA concentration increased proportionally with urea/fatty acids. The highest EPA-DHA yield, 20.20%, was found at urea/fatty acids ratio 2 : 1, whereas the highest EPA-DHA concentration, 63.94%, was found at urea/fatty acids ratio 5 : 1. The highest yield of EPA-DHA concentrates was 52.80% at lowest urea/fatty acids ratio 1 : 1, and highest EPA-DHA concentration 61.60% at highest urea/fatty acids ratio 5 : 1 [12]. The content of DHA and EPA was inversely related to the liquid recovery yield [23]. This relation graph can be used for the production of EPA-DHA concentrate containing the desired concentration and yield of EPA-DHA by manipulating urea/fatty acids ratio at optimized crystallization temperature and crystallization time.

5. Astaxanthin Content in EPA-DHA Concentrate

Astaxanthin content in EPA-DHA concentrate was 44.69 μg/ g, whereas in SFBO contained 21.33 μg/g. The aggregation of astaxanthin in the EPA-DHA concentrate is due to the processing activity of SFBO during urea complexation. In fish oil, astaxanthin remains as an astaxanthin ester of fatty acids [32]. At the time of hydrolysis of SFBO for preparation of fatty acids, the astaxanthin esters were also hydrolyzed and liberated from the fatty acid parts. Saponification procedure was done for obtaining free astaxanthin [33]. In alcoholic solution, urea formed complex with saturated and mono/di-unsaturated fatty acids while the total astaxanthin was solubilized in solvent. Astaxanthin is highly soluble in organic solvents like acetone, ethanol, hexane and acetic acid but not in water [32]. After filtration of urea-saturated fatty acids complex, the PUFAs are collected through hexanic fraction from hydro-ethanolic fraction. At the time, astaxanthin was solubilized with hexane. Astaxanthin is a high potential bioactive compound and researchers recommended for taking 2-4 mg astaxanthin for an adult person/day [34].

6. Urea Content in EPA-DHA Concentrates

Qualitative and quantitative tests of urea showed no residue of urea in produced EPA-DHA concentrate. Actually, the urea formed a complex with saturated and mono/di-unsaturated fatty acids which was filtered by using filter paper. Urea is highly soluble in polar solvent, including water and insoluble in hexane. During purification procedure, the EPA-DHA was fractionated with hexane, which caused removal of residual urea with hydro-ethanolic part. Moreover, hexane fraction containing EPA-DHA concentrate was washed with distilled water repeatedly, which removed urea residues completely. There was no previous result available regarding availability of residual urea in EPA-DHA concentrate produced by urea complexation.

7. Changes in Volatile Organic Compounds (VOCs) in EPA-DHA Concentrate

The results of the VOCs of SFBO and producing EPA-DHA concentrate are presented in Table 5. The odor of fish oil produced by various volatile organic compounds is the major factor limiting its application in the food industry. Most of these volatile components were derived from oxidative degradation of PUFA, which are abundant in fish oil [35]. Major volatile compounds in fish oil are comprised of a number of alkenals and alkadienals. Oxidation of linolenic acids and PUFAs is supposed to form unsaturated aldehydes, such as (Z)-pent-2 enal and E-hex-2-enal. Dienals are highly oxidative and form another oxidative undesired compound after their generation. Decadienals once formed, degrade by cleavaging the bond between C4 and C5 to form hexanal, 2-butenal, hexane and but-2-en-1,4-dial [36]. In oxidized fish oil, (E,Z,Z)-deca-2,4,7-trienal and (E,E,Z)-deca-2,4,7-trienal are two compounds claimed to be responsible for the fishy flavor due to oxidation of linoleic acid. Decatrienal and tridecatetraenal are claimed to be the products responsible for autoxidation of methyl docosa-hexaenoate in oxidation of marine oils.

Many of the VOCs responsible for the undesirable fishy odor of SFBO decreased or disappeared in the EPA-DHA concentrate produced by urea complexation (Table 5). This might be caused either during the procedure of hydrolysis of the SFBO for fatty acids formation or washing with hydro-ethanolic solvents in the separation of EPA-DHA concentrate from urea or undesired oil residues. No previous reports were available regarding changes in volatile organic compounds (VOCs) in EPA-DHA concentrate.

CONCLUSION

Salmon frame bone is a rich source of marine lipid feasible to use for ω-3 polyunsaturated fatty acids (PUFAs) containing EPA-DHA concentrate production for human consumption. Box-Behnken Design of RSM was successfully applied for optimizing influential process parameters in urea complexation to produce EPA-DHA concentrated oil. Urea/fatty acids and crystallization time had a significant effect on EPA-DHA concentrate production. Produced EPA-DHA concentrate exhibited 62.34% of EPA and DHA at urea/fatty acids 4 : 1, crystallization temperature −15.67 °C and crystallization time 8 h. The oil quality regarding ω-3 fatty acids, VOCs and bioactive carotenoid pigment astaxanthin content was improved due to urea complexation. The absence of residual urea in the EPA-DHA concentrate rendering safety for consumption. EPA-DHA concentrate using salmon frame bone oil by urea complexation may play an important role providing food supplement as well as financial benefits in food and pharmaceutical industries.

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SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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Supporting Information

Effects of process parameters on EPA and DHA concentrate production from Atlantic salmon by-product oil: Optimization and characterization

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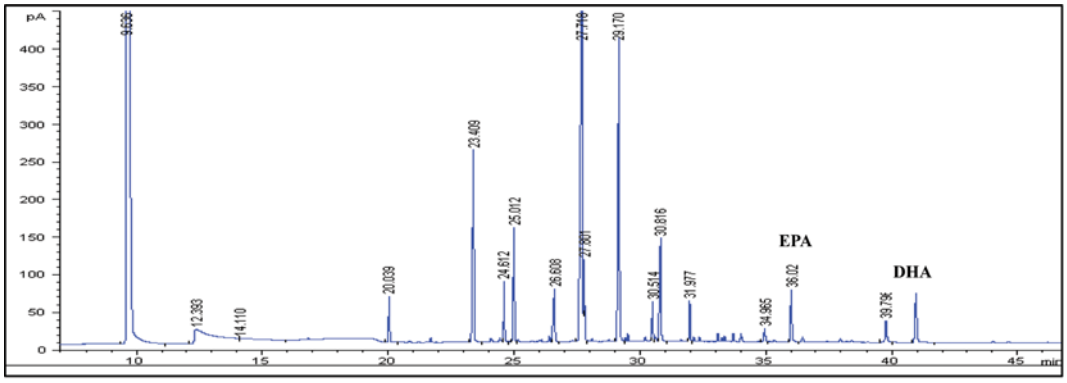
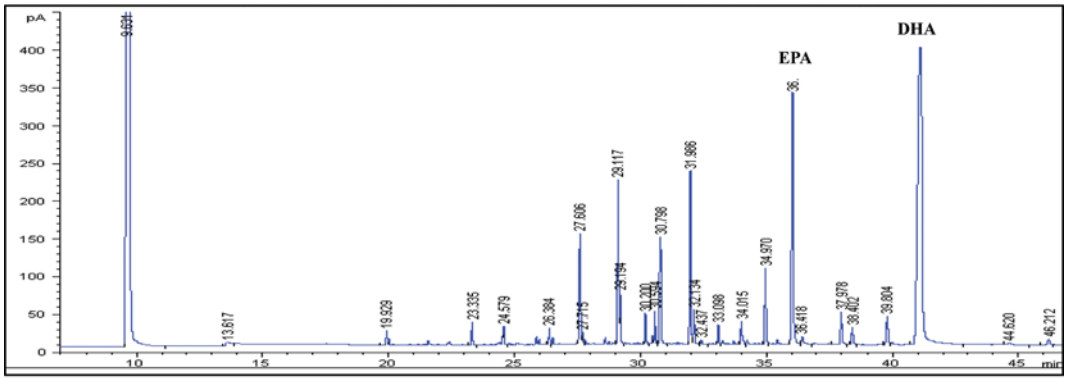


(a)



(b)

Fig. S1. Gas chromatogram (a) normal SFBO and (b) EPA-DHA concentrate.