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Optimization of Extraction Parameters and Effect of Different Solvent Systems on The Omega-3 Fatty Acids Content of Algal Oil *(Nannochloropsis sp.)*

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Abstract

Ultrasonically-assisted algal oil (*Nannocholoropsis sp*.) extraction (UAE) was optimized using Response Surface Methodology (RSM) and hexane. Extraction variables were determined as extraction time, temperature, and solvent:biomass ratio. Optimization was made by aiming both maximum oil yield and omega-3 fatty acid (ω-3 FA) content at the same time. The optimum conditions were determined to be 44.30 ℃, 62.46 min, 19.9:1 g/ml. The extraction time and temperature significantly affected the yield and ω-3 FA content, whereas solvent: biomass ratio did not affect the range of values tested for each of the variables ($p<0.05$). Then, under these optimum conditions, UAE was applied using selected solvents of different polarities (hexane, chloroform, methanol, ethanol and 2-propanol). The effects of different solvents on the oil yield, ω-3 FA content, oxidation, and nutritional properties of algal oil were investigated. Methanol was found to be more efficient than other solvents considering oil yield and ω-3 FA, especially eicosapentaenoic acid (EPA) content (14.46%). Although methanol and chloroform are widely used in extraction, their toxicity limits their use in the food industry. Considering the oil yield and ω-3 FA content of non-toxic solvents, it was determined that 2-propanol was more preferable due to its high ω-3 FA content.

Keywords: Edible algal oil, Ultrasonic assisted extraction, RSM optimization, Solvent polarity, Omega-3 fatty acid

Research article
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INTRODUCTION

Microalgae are called single-celled, photosynthetic, and aquatic organisms ranging in size from 1 to 100 μm (Saber et al., 2016). Although it varies according to the species, microalgae are good sources of proteins, lipids, carbohydrates and vitamins. Owing to these properties, they are widely used in cosmetics, pharmaceutical, animal feed, biofuel, and food industries (Gong et al., 2011; Rebolloso-Fuentes et al., 2001; Spolaore et al., 2006). *Nannochloropsis sp*., a type of microalgae, is used in the production of algal oil as an alternative to fish oil due to its abundant content of polyunsaturated fatty acids (PUFA), especially omega-3 (ω-3) fatty acids (FA) (Ryckebosch et al., 2014b).

Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), which are among the ω-3 FA, are important fatty acids with many health-beneficial properties. These essential fatty acids, which the body cannot produce and must be taken by foods, are important in the regulation of physiological functions, including blood pressure, brain and eye development, and in the prevention of some diseases such as cardiovascular diseases, hypertension, and diabetes (Fedorova-Dahms et al., 2011; Joumard-Cubizolles et al., 2017; Kaushik et al., 2015).

Fish oils are the primary sources of ω-3 FA. However, negative factors such as unwanted fishy odor, risk of heavy metals, and fat-soluble contaminants limit the consumption of fish oil (Khozin-Goldberg et al., 2011; Li et al., 2019; Oterhals et al., 2010). Due to the popularity of diets belonging to different diet groups such as vegan or vegetarian, there have been studies in recent years on the use of microalgae as an alternative ω-3 source to fish oil (Katiyar and Arora, 2020; Ryan and Symington, 2015). In addition, since microalgae are the primary producers of ω-3 in the marine environment, they can be considered the key to a sustainable PUFA source (Ryckebosch et al., 2014).

Some studies have already been performed on the lipid extraction from microalgae, although most of them focused on biodiesel production (Archer et al., 2019; Li et al., 2019; Wang et al., 2016). Traditionally, the extraction of various bioactive compounds from natural products has been carried out many industries using different methods and solvents. In the lab-scale, conventional extraction methods such as maceration and soxhlet extraction have many disadvantages like a large amount of solvent utilization, long extraction time, and lower extraction yield (Bermúdez Menéndez et al., 2014).

The ultrasound-assisted extraction (UAE) method is an inexpensive and simple substitute for traditional extraction techniques (Dey and Rathod, 2013). UAE uses acoustic cavitation for producing cavitation bubbles that implode resulting in high shear forces. This helps in disrupting the cell wall allowing the solvent to penetrate the material and increases the contact surface area between the solvent and compound of interest, resulting in increased mass transfer along with good mixing (Vinatoru et al., 1997). Extraction temperature, time, along with other factors such as solvent concentrations, pH, solid-liquid ratio, particle size which affect the extraction of bioactive compounds (Ma et al., 2008, Maran et al., 2017). Response surface methodology (RSM) can be employed to optimize extraction conditions for algal oil where extraction temperature, time, and solvent:biomass ratio serve as independent variables that influence responses in a given set of experiments (Myers and Montgomery, 2002; Belwal et al., 2016).

The aim of this study was to determine the optimum extraction conditions for independent oil extraction variables (extraction temperature, time, and solvent:biomass ratio) and validate the optimized conditions based on the combination of algal oil yield and its ω-3 FA content. Furthermore, polar and non-polar solvents including hexane, methanol, ethanol, chloroform, and 2-propanol were tested to increase further extraction yield and oil quality under optimum extraction condition from *Nannochloropsis sp.*

MATERIALS and METHODS

Materials

Freeze-dried *Nannochloropsis sp.* powder were obtained from BluebioTech Int. (Kaltenkirchen, Germany). All chemicals were purchased from Merck (Merck Chemicals Co. (Darmstadt, Germany) and Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Chemical properties of solvents (hexane, methanol, ethanol, chloroform, and 2-propanol) were given in Table 1.

Table 1. Chemical properties of solvents used in extraction

Solvent	Polarity Index	Dipole Moment	Dielectric constant	Boiling point	Viscosity (40°C)
Hexane	0.1	0	1.9	68.9	0.26
Chloroform	4.1	1.1	4.8	61.7	0.47
Methanol	5.1	1.70	32.6	65	1.70
Ethanol	4.6	1.70	24.3	78.5	0.83
2-propanol	3.9	1.66	18.3	82.4	1.35

Methods

Chemical composition

The total protein content of *Nannochloropsis sp.* was determined according to the method of A.O.A.C. (AOAC, 1984); moisture and ash contents were determined according to the methods of Helrich (Helrich, 1990). Total lipid content was analyzed using the method of Bligh and Dyer (AOAC, 1984). The oil yield (% of the dry algae biomass) was quantified gravimetrically.

Experimental design

The test ranges of extraction temperature, time, and solvent: biomass ratios on the algal oil yield and ω-3 FA content were chosen based on our preliminary work. They were 25-65 °C for extraction temperature, 30-90 min for extraction time, and 10:1-30:1 (ml:g) for solvent (hexane): biomass ratio. Optimization was made by targeting both maximum oil yield and maximum omega-3 content at the same time. These three variables at three levels were applied to Box-Behnken Design (Box et al., 1960) to generate a second-order polynomial Equation 1.

$$Y = \beta 0 + \sum \beta i\, Xi + \beta ii\, X2i + \sum \beta ij\, Xi\, Xj, \quad (1)$$

In the equation *Y* stands for the experimental response; β0, βi, βii, and βij are constants and regression coefficients of the model; and *Xi* and *Xj* are uncoded values of independent variables. The experimental design and responses (oil yield and its ω-3 FA content) are given in Table 2.

The responses obtained from the experimental design were subjected to multiple nonlinear regressions using the software Design-Expert 9.0. (Topuz et al., 2016). Optimum conditions were determined by the computer program by entering the maximum and minimum points of the variables.

Table 2. Box–Behnken experimental design and responses

	Experimental design						Responses	
Test	Max-min. points			X_1 (g: mL)	X_2 (°C)	X_3 (min.)	Oil yield (%)	Omega-3 fatty acid* content (%)
A_1	-1	-1	0	1:10	25	60	18.26±1.13	33.04±0.43
A_2	0	-1	-1	1:20	25	30	18.67±0.86	33.40±0.65
A_3	+1	-1	0	1:30	25	60	18.81±0.78	32.59±0.56
A_4	0	-1	+1	1:20	25	90	18.89±0.82	32.06±0.74
A_5	+1	0	+1	1:30	45	90	20.44±1.05	31.04±0.60
A_6	+1	0	-1	1:30	45	30	19.61±0.63	31.76±0.43
A_7	0	0	0	1:20	45	60	19.88±0.67	31.75±0.84
A_8	0	0	0	1:20	45	60	19.88±0.93	31.60±0.66
A_9	-1	0	-1	1:10	45	30	18.98±0.79	31.78±0.72
A_{10}	0	0	0	1:20	45	60	20.33±1.03	31.45±0.53
A_{11}	-1	0	+1	1:10	45	90	20.41±0.45	31.40±0.44
A_{12}	0	+1	-1	1:20	65	30	20.90±0.56	30.92±0.75
A_{13}	0	+1	+1	1:20	65	90	21.60±0.71	30.30±0.56
A_{14}	+1	+1	0	1:30	65	60	21.19±0.84	30.61±0.34
A_{15}	-1	+1	0	1:10	65	60	21.05±0.58	30.81±0.52

X_1: biomass:solvent (hexane) ratio; X_2: Extraction temperature; X_3: Extraction time
*Omega-3 fatty acids: C18:3 (alpha-linolenic acid, ALA), C20:3, C20:5 (Eicosapentaenoic acid, EPA), C22:6 (Docosahexaenoic acid, DHA).

Ultrasound-assisted extraction

UAE of algal oil was performed using an ultrasonic homogenizer (Sonopuls HD 4200, 200W, Bandelin GmbH & Co, Berlin, Germany) equipped with a 13 mm diameter tip at a frequency of 20 kHz. Hexane was used as a solvent in the optimization step of the study. And ethanol, methanol, chloroform, and 2-propanol were selected as additional extracting solvents to be evaluated. The extraction was carried out at the required temperature and time according to the experimental design. After the extraction, the mixture was centrifuged and filtered through Whatman No. 1. The solvent was evaporated with a rotary evaporator (Heidolph, HeiVAP Advantage, Schwabach, Germany) at 50°C. Then, nitrogen was injected into the flask and oils were stored at amber-colored flasks at -45 °C.

Fatty acid composition

The fatty acid methyl esterification procedure was carried out according to Ozogul (Ozogul and Ozogul, 2007). The algal oil (10 mg) dissolved in 2-mL n-heptane was mixed with 4-mL 2-M methanolic KOH and centrifuged at 4,000 rpm for 10 min. The upper layer was injected into a gas chromatograph. The fatty acid composition analyses were performed in duplicate and the results were given in chromatography area % as mean values.

Gas Chromatography Conditions: Gas chromatography (Perkin Elmer, Clarus 500, Waltham, MA, USA) equipped with a BPX70 silica column (50 m x 0.22 mm, film thickness 0.25 µm; SGE Inc., Victoria, Australia) and a flame ionization detector was used. The oven temperature was started at 140 ℃ for 5 min, raised to 200 ℃ at a rate of 4 ℃/min and ended at 220 ℃ with an increase of 1℃/min. The injection temperature was 220℃ and the carrier gas, Helium flow rate, was 1.0 ml/min. The detector temperature was set at 280 ℃ with a split ratio of 1:50.

Oil oxidation analysis

Peroxide value

Peroxide analysis was performed using the method described by A.O.A.C (A.O.A.C, 1990). The oil was dissolved by adding chloroform: acetic acid (1:1.5) solution and 1 ml of potassium iodide on the sample of algae (1 g). After standing for 5 minutes in the dark, some water, and 1% starch solution were added as an indicator. The mixture was titrated with 0.01 N sodium thiosulfate conjugate. The obtained results are calculated according to the formula given below.

$$PV\ (meq/kg) = (V\text{-}B \times Nf / W) \times 1000 \quad (2)$$

V: volume of sodium thiosulfate;
B:used sodium thiosulfate,
W: sample weight;
Nf: the normality of sodium thiosulfate.

Para-anisidine value (p-Av)

The value of *p*-Av, the indicator of secondary oxidation product, was determined according to the methods of Frankel (1984). 0.5 g algal oil was dissolved in 25 ml n-hexane (A1). 5 ml of the solution was taken and 1 ml *p*-Av standard (Merck 800458, Germany) was added and kept at room temperature for 10 minutes in the dark (A2). The *p*-Av value of the samples was determined by the following formula with the aid of the absorbance values obtained by spectrophotometric measurements at 350 nm. Due to the dark color of the algal oil, 1/10 dilution was applied in the *p*-Av analysis based on the colorimetric measurement.

$$p\text{-}Av = 25\ (1.2 \times (A2 - A1)) / \text{sample weight} \quad (3)$$

Nutritional properties

The effect of algae oil extracted with solvents selected from *Nannocholoropsis sp*. on nutritional quality and chronic heart health was evaluated by two separate indexes given below. These indexes were calculated using the equations described by Ulbricht (1991).

$$\text{Atherogenicity index (AI)} = \frac{[(C12{:}0 + (4 \times C14{:}0) + C16{:}0)]}{(\Sigma\text{MUFA} + \Sigma\omega\text{-}6 + \Sigma\omega\text{-}3)} \quad (4)$$

$$\text{Thrombogenicity index (TI)} = \frac{(C14{:}0 + C16{:}0 + C18{:}0)}{[(0.5 \times \Sigma\text{MUFA}) + (0.5 \times \Sigma\omega\text{-}6) + (3 \times \Sigma\omega\text{-}3) + (\Sigma\omega\text{-}3 / \Sigma\omega\text{-}6)]} \quad (5)$$

C12:0 (Dodecanoic acid); C14:0 (Tetradecanoic acid); C16:0 (Hexadecanoic acid); C18:0 (Octadecanoic acid); C18:1ω9 (9-octadecanoic acid); C18:2ω6 (9,12-octadecadienoic acid); C18:3ω3 (9,12,15-octadecatrienoic acid); C20:4ω6 (5,8,11,14-eicosatetraenoic acid); C20:5ω3 (5,8,11,14,17-eicosapentaenoic acid); ω-3 (Omega-3); ω-6 (Omega-6).

STATISTICAL ANALYSIS

The findings were subjected to analysis of variance using the Design Expert Statistical Program (Stat-Ease Inc. Minneapolis, USA) and SAS 9.0 (Statistical Analysis System, Cary, NC, USA) packet programs and significant differences were determined by the Duncan Multiple Comparison Test. All analyses were performed in duplicate.

RESULTS and DISCUSSION

The composition of dried *Nannochloropsis sp.* algae biomass was found to contain 35.7% carbohydrate, 31.3 % protein, 23.1% oil, 9.9% ash, and 5.7% moisture. In a similar study, Babuskin et al. (2014) was found the oil and protein contents of *Nannochloropsis oculata* as 22.1 and 36.0, respectively. Total oil and protein contents of *Nannocholoropsis sp.* in the study are compatible with the literature.

Optimization and verification of algal oil extraction conditions

Dried *Nannochloropsis sp.* algae were extracted using hexane under the conditions in Table 2. determined by the Design Expert program, and oil yield and ω-3 FA contents were examined. The optimum extraction conditions were determined as 44.30 °C extraction temperature, 62.46 min extraction time and 1:19.9 g/ml biomass:solvent ratio. Under these conditions, the computer program calculated both the oil yield and ω-3 FA content to be highest as 19.79% and 32.03%, respectively. To verify the predicted result with the practical value, extractions were performed using the obtained optimal conditions.

The experimental results had no significant difference according to the predicted results ($p<0.05$). The mean values of oil yield (19.96%) and ω-3 FA content (32.21%) obtained from real extractions demonstrated the validity of the RSM model for maximal oil yield and ω-3 FA content (Table 3).

Table 3. Predicted and experimental optimum conditions for maximum yield and omega-3 fatty acid content

	Optimum conditions			Responses	
	X_1 (g/ml)	X_2 (°C)	X_3 (min)	Oil yield (%)	Omega-3 fatty acids* content (%)
Predicted**	1/19.9	44.30	62.46	19.79[a]	32.03[a]
Experimental***	1/19.9	44.30	62.46	19.96±0.71[a]	32.21±0.44[a]

X_1: Biomass: solvent (hexane) ratio; X_2: Extraction temperature; X_3: Extraction time,
* Omega-3 fatty acids: C18:3 (ALA), C20:3, C20:5 (EPA), C22:6 (DHA),
**Predicted using ridge analysis of response surface quadratic model,
*** The values represent means ± standard deviation, *n*:3. The different letters in the same column show the values were significantly different according to Duncan's multiple range test ($p<0.05$).

The results of the ANOVA test according to the quadratic equations of Design Expert for the extraction oil yield and ω-3 FA are given in Table 4. According to the Analysis of variance (ANOVA) probability values in the model, it was found that while extraction temperature and time had a significant effect on oil yield and ω-3 FA, solvent:biomass ratio was ineffective in the range of values tested for each of the variables ($p<0.05$).

Table 4. Analysis of variance (ANOVA) probability of oil yield and ω-3 FA.

Source	**Sum of squares**		**Df**		**Mean square**		**F-value**		**p-value**	
	Oil yield	ω-3 FA	Oil yield	ω-3 FA	Oil yield	ω-3 FA	Oil yield	ω-3 FA	Oil yield	ω-3 FA
Model	14.46	10.46	6	6	2.41	1.73	39.62	46.44	<0.0001	<0.0001
X_1 (g/ml)	0.2278	0.1326	1	1	0.2278	0.1326	3.75	3.55	0.089	0.0962
X_2 (°C)	12.78	8.93	1	1	12.78	8.93	210.08	239.08	<0.0001	<0.0001
X_3 (min)	1.26	1.17	1	1	1.26	1.17	20.78	31.35	0.0019	0.0005
$X_1 X_2$	0.0420	0.0156	1	1	0.042	0.0156	0.6910	0.4185	0.4299	0.5358
$X_1 X_3$	0.0900	0.0289	1	1	0.0900	0.0289	1.48	0.7741	0.2585	0.4046
$X_2 X_3$	0.0576	0.1296	1	1	0.0576	0.1296	0.9471	3.47	0.3590	0.0994
Residual	0.4865	0.2987	8	8	0.0608	0.0373	-	-	-	-
Pure error	0.1350	0.0450	2	2	0.0675	0.0225	-	-	-	-
R^2	0.976	0.9721	6	6						
Adjusted R^2	0.943	0.9512	1	1						
Predicted R^2	0.873	0.8703	1	1						
Adeq precision	19.72	21.80	1	1						

X_1: Solvent: biomass ratio (g/ml), X_2: Temperature (°C), X_3: Time (min.).
$p<0.05$ indicate model terms are significant.
Df: Degree of freedom.

The Predicted R^2 of oil yield (0.8733) is in reasonable agreement with the Adjusted R^2 (0.9430). The Predicted R^2 of ω-3 FA (0.8703) is in reasonable agreement with the Adjusted R^2 (0.9512), too; i.e. the difference is less than 0.2 (Design-Expert 8.7.1).

Adeq Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The calculated ratio for oil yield (19.72) and ω-3 FA (21.80) indicates an adequate signal. This model can be used to navigate the design space (Design-Expert 8.7.1).

Effect of extraction variables on yield and ω-3 FA content of algal oil

The effect of solvent:biomass ratio, extraction temperature, and extraction time on oil extraction yield and ω-3 FA content *Nannocholoropsis sp.* are shown in Figure 1. The extracted total lipid yield of biomass varied between 18.26 and 21.60%. The total ω-3 FA content ranged between 30.30 and 33.40%.

As can be seen in Figure 1, increasing the temperature by fixing the extraction time at 60 min resulted in a linear increase in oil yield (Fig. 1a) and a decrease in ω-3 FA (Fig. 1d). The increase in the solvent: biomass ratio slightly increased the oil yield and the ω-3 FA content much in the range of values tested for each of the variables. Increasing both variables together increased the oil yield up to 21.6.

At the fixed temperature (45 °C), increasing extraction time increased the oil yield (Fig. 1b). the increasing rate of The solvent:biomass, slightly increased the oil yield and ω-3 FA (Fig.1b/1e). At the fixed solvent:biomass ratio (1:20), the increase in extraction temperature increased the oil yield (Fig. 1c) in contrast to the ω-3 FA content (Fig.1f). When both variables increased together, a positive acceleration was observed in the oil yield increase.

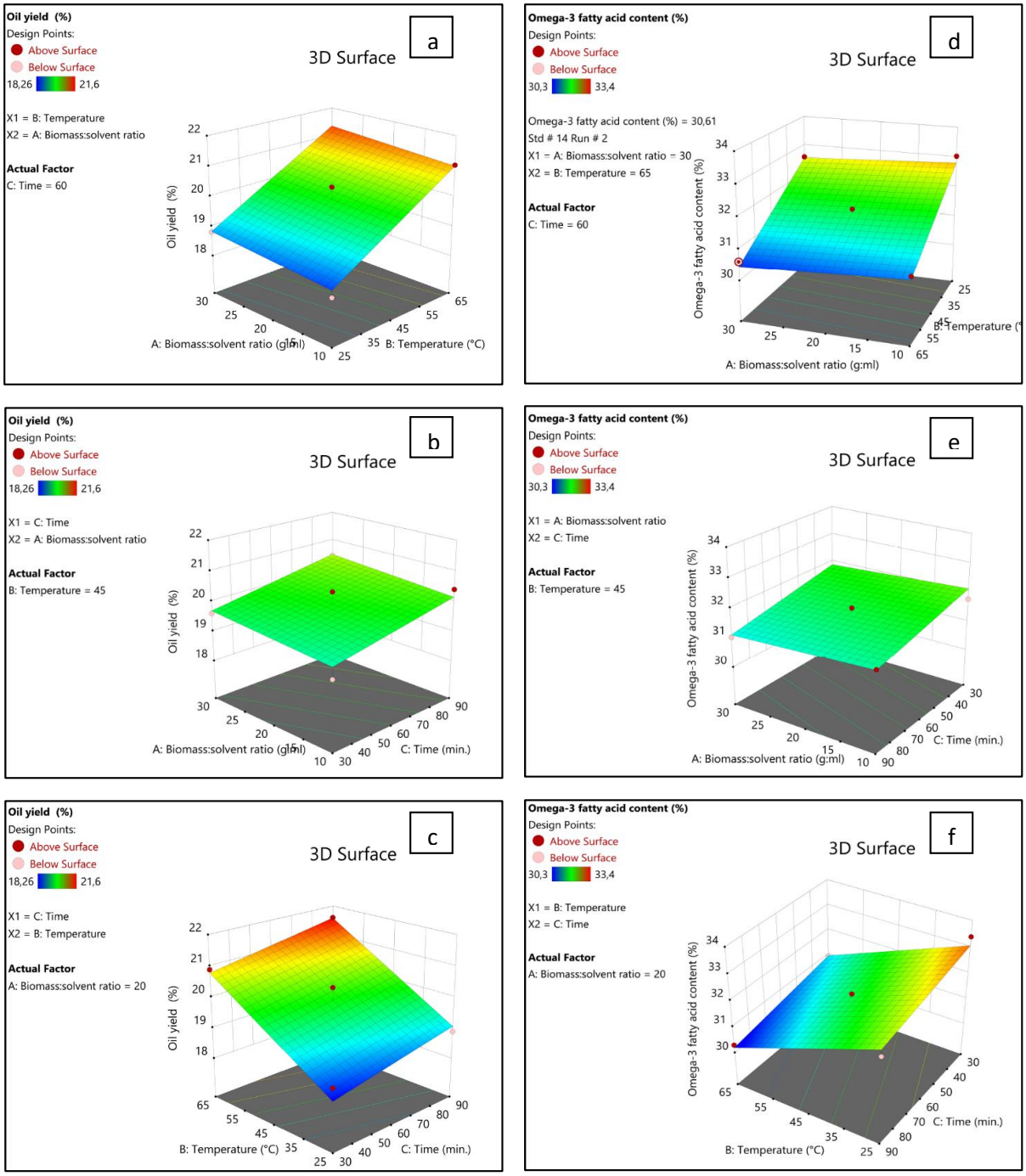

Figure 1. Response surface plots for maximum oil yield (a, b, c) and ω-3 FA content (d, e, f) of algal oil

Effect of solvent type on the yield of algal oil

The oil extraction yields of *Nannocholoropsis sp.* at optimum conditions are shown in Table 5. The solvent type significantly ($P<0.05$) affected the yield of algal oil. Oil yields of extractions made using the solvents having different polarity, varied between 19.40 and 27.61%. Methanol had the significantly highest oil yield while, ethanol had the lowest.

Also, there was no significant difference between the oil yield of hexane and ethanol. In addition, chloroform had a significantly higher oil yield than 2-propanol. Although methanol and chloroform were effective solvents, especially the toxicity of chloroform limiting its use in the food industry. However, these solvents are used in pharmacology and cosmetic technology.

Table 5. Effect of solvent polarity on the oxidative stability, yield and ω-3 FA of algal oil

Solvents	Oil yield (g/100g)	ω-3 FA (g/100g)	PV (meq O_2/kg)	p-Av
Hexane	20.12±0.35^{d}	30.75±0.49^{c}	2.12±0.20^{c,b}	1.40±0.05^{a}
Chloroform	25.62±0.26^{b}	34.93±0.25^{a}	1.45±0.03^{c}	1.61±0.04^{a}
Methanol	27.61±0.12^{a}	30.45±0.39^{c}	2.11±0.00^{c,b}	1.15±0.68^{a}
Ethanol	19.4±0.41^{d}	34.23±0.21^{b}	2.32±0.05^{b}	1.29±0.11^{a}
2-propanol	23.73±0.21^{c}	34.81±0.23^{a}	3.97±0.43^{a}	1.56±0.53^{a}

The values represent means ± standard deviation. Means with different letters (a, b, c) in the same columns are significantly different ($p<0.05$).

There are different opinions in the literature about the use of solvents for oil extraction one by one or as a mixture. In a study conducted by Balasubramanian, it was stated that the use of polar and apolar solvent mixtures provides higher oil yield than using only polar or only apolar solvents (Balasubramanian et al., 2013).

However, in another study, it was reported that a mixture of hexane and ethanol (1: 1, v / v) provided less lipid yield than extraction using only hexane as solvent (Shen et al., 2009). Mixed solvents were not used in this study, but it was found that oil yield and ω-3 FA content did not show a linear increase with the polarity index.

Effect of solvents polarity on the oxidation of algal oil

The effects of selected solvents on the peroxide (PV) and para anisidine (*p*-Av) values of algal oil extracted at optimum conditions are shown in Table 5. Oxidative stability can be assessed by analyzing primary and secondary oxidation products (measured by PV and anisidine *p*-Av, respectively) (Balboa et al., 2014). The solvent type significantly ($p<0.05$) affected PV values of algal oil whereas, had not affected the *p*-Av values The highest PV was obtained with 2-propanol, while the lowest PV was obtained with chloroform. PV values of hexane, methanol and ethanol, there was no significant difference ($p<0.05$). Both PV (1.45-3.97 meq/kg) and *p*-Av (1.15-1.61) oxidation values were lower than reported by Liu et al. (2018) (7.23-9.06 meq/kg and 1.9-7.9, respectively). It can be said that methanol, chloroform and hexane relatively retards oil oxidation compared to the other solvents.

Effect of solvent polarity on the fatty acid composition of algal oil

The FA profile of algal oils extracted with the solvents selected under optimum conditions is shown in Table 6. As it was shown in Table 6, there was no significant difference between both of the SFA and MUFA contents of hexane and chloroform ($p<0.05$). It was also found that hexane and chloroform had significantly higher SFA and MUFA content than other solvents. In this study, similar to Schambach et al. (2020), it was obtained that a high amount of palmitic (16: 0) and palmitoleic acid (16: 1) as well as minor amounts of myristic acid (14:0). The Oleic acid (18:1) content was relatively higher in this study.

Table 6. Effect of solvent polarity on the fatty acid profile of algal oil

Fatty acids (%)	**Solvents**				
	Hexane	**Chloroform**	**Methanol**	**Ethanol**	**2-propanol**
C14:0	3.28±0.25	4.44±0.03	5.09±0.04	4.47±0.04	3.76±0.05
C15:0	0.34±0.07	0.45±0.01	0.44±0.01	0.46±0.00	0.43±0.01
C16:0	37.89±0.52	37.93±0.12	33.49±0.15	34.57±0.25	33.85±0.19
C17:0	0.41±0.08	0.42±0.03	0.30±0.05	0.45±0.00	0.32±0.00
C18:0	1.41±0.08	1.25±0.12	0.35±0.05	1.26±0.01	1.11±0.02
ΣSFA	***44.42±1.17^{a}***	***45.31±0.00^{a}***	***40.04±0.28^{b}***	***42.27±0.28^{b}***	***41.03±0.28^{b}***
C16:1	29.36±1.02	30.48±0.12	29.25±0.26	28.72±0.24	27.79±0.24
C17:1	0.54±0.06	nd	nd	nd	nd
C18:1 n-9	11.78±1.57	12.35±0.13	9.64±0.36	10.11±0.04	11.01±0.04
ΣMUFA	***41.67±0.85^{a}***	***42.83±0.01^{a}***	***38.89±0.14^{b}***	***38.83±0.39^{b}***	***38.80±0.19^{b}***
C18:2 n-6	0.94±0.11	1.08±0.07	1.36±0.01	0.82±0.01	1.05±0.07
C18:3 n-6	0.45±0.11	0.67±0.00	0.69±0.06	0.48±0.01	0.73±0.06
C18:3n-3	1.21±0.06	0.78±0.01	0.51±0.06	2.52±0.01	2.72±0.03
C20:3 n-3	2.50±0.02	1.32±0.49	2.81±0,02	2.54±0.01	3.93±0.06
C20:4n-6	2.08±0.16	nd	nd	nd	nd
C20:5 n-3(EPA)	5.83±0.07	7.19±0.08	14.46±0.23	10.41±0.31	9.09±0.19
C22:6 n-3(*DHA*)	0.21±0.01	0.27±0.02	0.76±0.03	0.43±0.02	0.32±0.02
ΣPUFA	***13.23±0.33^{c}***	***11.30±0.22^{d}***	***20.57±0.08^{a}***	***17.19±0.19^{b}***	***17.83±0.01^{b}***
Σn-6	3.47	1.75	2.05	1.3	1.78
Σn-3	9.75	9.56	18.54	15.9	16.06
n-6/n-3	0.35	0.18	0.11	0.08	0.10
AI	0,92	1,03	0,91	0,94	0,86
TI	0,78	0,77	0,45	0,5	0,5
Undefined	0.68	0.56	0.50	1.71	2.34

The values represent ± standard errors, *n*:3 per experimental replicate; Means within the same row (a, b, c, d) with different letters are different ($p<0.05$).

SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, AI: Atherogenicity index; TI: Thrombogenicity index.

PUFA contents of the samples varied between 11.30 and 20.57. While there was no significant difference between ethanol and 2-propanol, a significant difference was observed between other solvents ($p<0.05$). *Nannochloropsis sp*. is one of the potential genera of microalgae having higher EPA (C20:5), biomass, and lipid productivities (Chua et al., 2020). EPA, which is one of the valuable ω-3 FA found in algal oil, was the major PUFA in all oil samples in the study. The EPA content varied between 5.83% and 14.46%. Figueiredo, in his study investigating the effect of different extraction methods on the ω-3 FA content of *Nannochloropsis oceanica*, obtained the highest EPA content (18.4%) from the dichloromethane/methanol mixture (Figueiredo et al., 2019).

In this study, methanol was found to be the most effective solvent for the extraction of EPA and ω-3 FA (14.46 ±0.23 EPA/g dry biomass). In addition, it was determined that the algae oil extracted with methanol (20.57%) had the highest PUFA content, while the oil extracted with chloroform (11.30%) had the lowest PUFA content. However, the use of methanol in the food industry is not safe. Therefore, it is considered more appropriate to use ethanol and 2-propanol, which do not differ statistically, in the production of ω-3 FA.

Nutritional properties of algal oil

AI and TI values provide information about the nutritional quality of FA and the effect of chronic heart health. Algal oil, AI and TI values and PUFA/SFA and n6/n3 ratios are given in Figure 2. It is reported that long-chain PUFA, especially EPA and DHA, have protective effects against heart diseases. In a clinical study of over a thousand patients, it was reported that supplying patients with 1g/day of ω-3 FA resulted in a large reduction in death, cardiovascular disease, and heart failure by 20, 30, and 45%, respectively (Punia et al., 2019). World health organization and food and agriculture organization experts stated daily intake of EPA+DHA as at least 250 mg, while the American Heart Association reported 500 mg/day for adults (Martins et al., 2013). It is recommended to occur. N6/n3 ratio should be less than 4, n3/n6 should be greater than 6, and PUFA / SFA should be greater than 0.4 (Topuz et al., 2017).

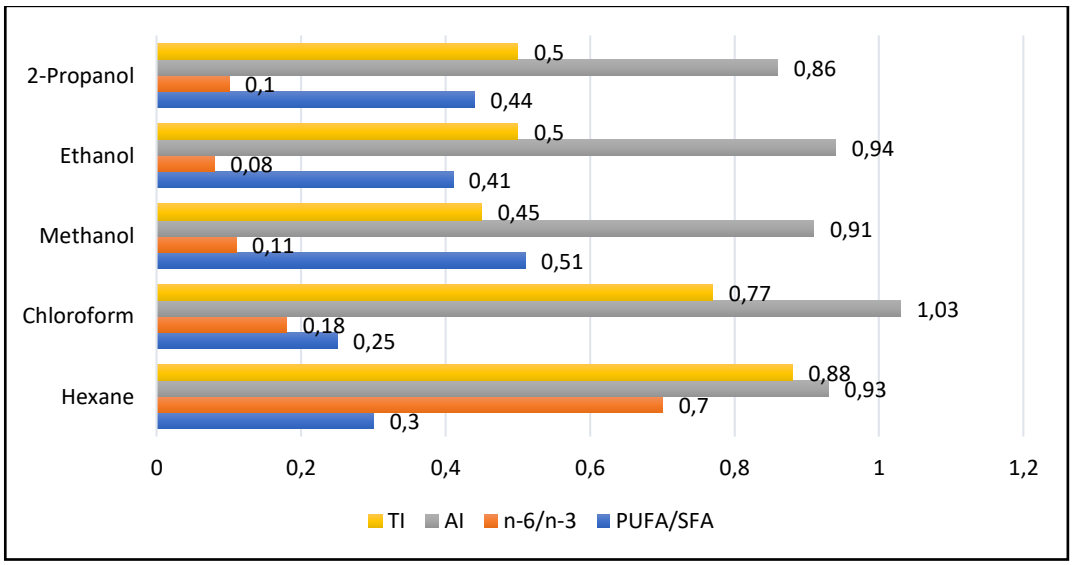

Figure 2. Nutritional properties of algal oil extracted using different polarity solvents
TI: Thrombogenicity index, AI: Atherogenicity index,

n-6: ω-6 FA, n-3: ω -3 FA, PUFA: Polyunsaturated fatty acid, SFA: Saturated fatty acid

The PUFA/SFA ratio as a measure of the propensity of diet to influence the incidence of coronary heart disease should be replaced by the atherogenic index (AI) and thrombogenic index (TI) (Ulbricht, 1991). The algal oils extracted with methanol and hexane, which had relatively high amounts of EPA, led to the desired AI and TI values of 1.43 and 0.31; followed by 1.40 and 0.29, respectively. These findings in accordance with the results of the previous study performed by Mitra (Mitra and Mishra, 2019).

It is seen that the oil extracted with methanol has the highest PUFA content (20.50%), and the lowest (11.30%) of the oil extracted with chloroform. When Figure 3 is examined, it is seen that the solvents in accordance with the specified reference values are methanol, ethanol and 2-propanol. When algal oil and fish oil were compared in terms of essential fatty acid, it was observed that there was no significant difference, and the EPA content was higher in *Nannocholoropsis sp.* than in fish oil.

Currently, there are many studies on ω-3 FA from fish (especially EPA and DHA). However, there are relatively few studies on the beneficial effects of algal oil on metabolism, and the use of algal oil as part of the modern diet is gaining acceptance. As supported in the literature, algal oil has a high EPA content and is an alternative source of ω-3 FA for daily consumption in terms of nutritional quality (Chen et al., 2007; Kumari et al., 2013; Mitra et al., 2015).

CONCLUSION

The optimization of ultrasound/hexane-assisted extraction conditions of edible algal oil from *Nannocholoropsis sp.* was performed employing RSM and using hexane. Extraction temperature (44.30℃) and time (62.46 min) affected the oil yield and its ω-3 FA content, whereas the solvent:biomass ratio (19.9:1 g/ml) did not affect the range of values tested for each of the variables. The oil yield and ω-3 FA content were highly dependent on the solvent, used in oil extraction. Methanol had the highest oil yield , whereas the highest ω-3 FA content were obtained in oil samples extracted with 2-propanol and chloroform. Although chloroform/methanol is the most frequently lipid solvent mixture used due to its fast and quantitative extraction, its biggest disadvantage is the high toxicity of chloroform. When the oil yield and ω-3 FA content of non-toxic solvents (hexane, ethanol and 2-propanol) were compared, it was found that 2-propanol was more suitable for use in the food industry owing to its higher ω-3 FA content. The results from this study may help extract ω-3 FA (especially EPA)-rich edible oil from microalgae adequately and efficiently.

REFERENCES

AOAC. 1984. Official Methods of Analysis (14th edn.), *J. Assoc.Off. Ana. Chem*., Washington DC, p. 1018.

AOAC. 1990. Official Methods of Analysis, *J. Assoc.Off. Ana. Chem*, Arlington, VA: Author.

Archer L., Mc Gee D., Paskuliakova A., McCoy G. R., Smyth T., Gillespie E. & Touzet N. 2019. Fatty acid profiling of new Irish microalgal isolates producing the high-value metabolites EPA and DHA, *Algal Research*, 44, 101671.

Babuskin S., Krishnan K. R., Saravana Babu P. A., Sivarajan M. & Sukumar M. 2014. Functional foods enriched with marine microalga Nannochloropsis oculata as a source of ω-3 fatty acids, *Food Technology and Biotechnology*, 52(3), 292-299.

Balasubramanian R. K., Doan T. T. Y. & Obbard J. P. 2013. Factors affecting cellular lipid extraction from marine microalgae, *Chemical Engineering Journal*, 215, 929-936.

Balboa E. M., Soto M. L., Nogueira D. R., González-López N., Conde E., Moure A. ... & Domínguez H. 2014. Potential of antioxidant extracts produced by aqueous processing of renewable resources for the formulation of cosmetics, *Industrial Crops and Products*, 58, 104-110.

Belwal T., Dhyani P., Bhatt I. D., Rawal R. S. & Pande V. 2016. Optimization extraction conditions for improving phenolic content and antioxidant activity in Berberis asiatica fruits using response surface methodology (RSM), *Food Chemistry*, 207, 115-124.

Bermúdez Menéndez J. M., Arenillas A., Menéndez Díaz J. Á., Boffa L., Mantegna S., Binello A. & Cravotto G. 2014. Optimization of microalgae oil extraction under ultrasound and microwave irradiation, *Journal of Chemical Technology & Biotechnology*, 89(11), 1779-1784.

Bligh E. G. & Dyer W. J. 1959. A rapid method of total lipid extraction and purification, *Canadian journal of biochemistry and physiology*, 37(8), 911-917.

Box G. E. & Behnken D. W. 1960. Some new three level designs for the study of quantitative variables, *Technometrics*, 2(4), 455-475.

Chen G. Q., Jiang Y. & Chen F. 2007. Fatty acid and lipid class composition of the eicosapentaenoic acid-producing microalga, Nitzschia laevis, *Food chemistry*, 104(4), 1580-1585

Chua E. T., Dal'Molin C., Thomas-Hall S., Netzel M. E., Netzel G. & Schenk P. M. 2020. Cold and dark treatments induce omega-3 fatty acid and carotenoid production in Nannochloropsis oceanica, *Algal Research*, 51, 102059.

Dey S., & Rathod V. K. 2013. Ultrasound assisted extraction of β-carotene from Spirulina platensis, *Ultrasonics Sonochemistry*, 20(1), 271-276.

Fedorova-Dahms I., Marone P. A., Bauter M. & Ryan A. S. 2011. Safety evaluation of DHA-rich Algal Oil from Schizochytrium sp, *Food and chemical toxicology*, 49(12), 3310-3318.

Figueiredo A. R., da Costa E., Silva J., Domingues M. R. & Domingues P. 2019. The effects of different extraction methods of lipids from Nannochloropsis oceanica on the contents of omega-3 fatty acids, *Algal Research*, 41, 101556.

Frankel E. N. 1984. Lipid oxidation: mechanisms, products and biological significance, *Journal of the American Oil Chemists' Society*, 61(12), 1908-1917.

Gong Y., Hu H., Gao Y., Xu X. & Gao H. 2011. Microalgae as platforms for production of recombinant proteins and valuable compounds: progress and prospects, *Journal of industrial microbiology & biotechnology*, 38(12), 1879-1890.

Guillard R. R. 1975. Culture of phytoplankton for feeding marine invertebrates. In Culture of marine invertebrate animals (pp. 29-60), *Springer*, Boston, MA.

Helrich K. 1990. Official methods of Analysis, *J. Assoc.Off. Ana. Chem*, Volume 2.

Joumard-Cubizolles L., Lee J. C. Y., Vigor C., Leung H. H., Bertrand-Michel J., Galano J. M. ... & Gladine C. 2017. Insight into the contribution of isoprostanoids to the health effects of omega 3 PUFAs, *Prostaglandins & Other Lipid Mediators*, 133, 111-122.

Katiyar R. & Arora A. 2020. Health promoting functional lipids from microalgae pool: A review, *Algal Research*, 46, 101800.

Kaushik P., Dowling K., Barrow C. J. & Adhikari B. 2015. Microencapsulation of omega-3 fatty acids: A review of microencapsulation and characterization methods, *Journal of functional foods*, 19, 868-881.

Khozin-Goldberg I., Iskandarov U. & Cohen Z. 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology, *Applied microbiology and biotechnology*, 91(4), 905.

Kumari P., Bijo A. J., Mantri V. A., Reddy C. R. K. & Jha B. 2013. Fatty acid profiling of tropical marine macroalgae: an analysis from chemotaxonomic and nutritional perspectives, *Phytochemistry*, 86, 44-56.

Li X., Liu J., Chen G., Zhang J., Wang C. & Liu B. 2019. Extraction and purification of eicosapentaenoic acid and docosahexaenoic acid from microalgae: A critical review, *Algal Research*, 43, 101619.

Liu L., Qu X., Li X., Bora A. F. M., Chen P., Wang H. & Wang C. 2018. Effect of exopolysaccharides-producing strain on oxidation stability of DHA micro algae oil microcapsules, *Food bioscience*, 23, 60-66.

Ma Y., Ye X., Hao Y., Xu G., Xu G. & Liu D. 2008. Ultrasound-assisted extraction of hesperidin from Penggan (Citrus reticulata) peel, *Ultrasonics Sonochemistry*, 15(3), 227-232.

Maran J. P., Manikandan S., Nivetha C. V. & Dinesh R. 2017. Ultrasound assisted extraction of bioactive compounds from Nephelium lappaceum L. fruit peel using central composite face centered response surface design, *Arabian Journal of Chemistry*, 10, S1145-S1157.

Martins D. A., Custódio L., Barreira L., Pereira H., Ben-Hamadou R., Varela J. & Abu-Salah K. M. 2013. Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae, *Marine drugs*, 11(7), 2259-2281.

Mitra M. & Mishra S. 2019 A comparative analysis of different extraction solvent systems on the extractability of eicosapentaenoic acid from the marine eustigmatophyte *Nannochloropsis oceanica*, *Algal Research*, 38, 101387.

Mitra M., Patidar S. K. & Mishra S. 2015. Integrated process of two stage cultivation of Nannochloropsis sp. for nutraceutically valuable eicosapentaenoic acid along with biodiesel, *Bioresource Technology*, 193, 363-369.

Myers R. H., Montgomery D. C. & Anderson-Cook C. M. 2002. Process and product optimization using designed experiments, *Response surface methodology*, 2, 328-335.

Oterhals Å., Kvamme B. & Berntssen M. H. 2010. Modeling of a short-path distillation process to remove persistent organic pollutants in fish oil based on process parameters and quantitative structure properties relationships, *Chemosphere*, 80(2), 83-92.

Özogul Y. & Özogul F. 2007. Fatty acid profiles of commercially important fish species from the Mediterranean, Aegean and Black Seas, *Food Chemistry*, 100(4), 1634-1638.

Punia S., Sandhu K. S., Siroha A. K. & Dhull S. B. 2019. Omega 3-metabolism, absorption, bioavailability and health benefits–A review, *PharmaNutrition*, 10, 100162.

Rebolloso-Fuentes M. M., Navarro-Pérez A., García-Camacho F., Ramos-Miras,J. J. & Guil-Guerrero, J. L. 2001. Biomass nutrient profiles of the microalga Nannochloropsis, *Journal of Agricultural and Food Chemistry*, 49(6), 2966-2972.

Ryan L. & Symington,A. M. 2015. Algal-oil supplements are a viable alternative to fish-oil supplements in terms of docosahexaenoic acid (22: 6n-3; DHA), *Journal of functional foods*, 19, 852-858.

Ryckebosch E., Bermúdez S. P. C., Termote-Verhalle R., Bruneel C., Muylaert K., Parra-Saldivar R. & Foubert I. 2014. Influence of extraction solvent system on the extractability of lipid components from the biomass of Nannochloropsis gaditana, *Journal of Applied Phycology*, 26(3), 1501-1510.

Ryckebosch E., Bruneel C., Termote-Verhalle R., Muylaert K. & Foubert I. 2014b. Influence of extraction solvent system on extractability of lipid components from different microalgae species, *Algal Research*, 3, 36-43.

Saber M., Nakhshiniev B. & Yoshikawa K. 2016. A review of production and upgrading of algal bio-oil, *Renewable and Sustainable Energy Reviews*, 58, 918-930.

Schambach J. Y., Finck A. M., Kitin P., Hunt C. G., Hanschen E. R., Vogler B. ... & Barry A. N. 2020. Growth, total lipid, and omega-3 fatty acid production by Nannochloropsis spp. cultivated with raw plant substrate, *Algal Research*, 51, 102041.

Shen Y., Pei Z., Yuan W. & Mao E. 2009. Effect of nitrogen and extraction method on algae lipid yield, *International Journal of Agricultural and Biological Engineering*, 2(1), 51-57.

Spolaore P., Joannis-Cassan C., Duran E. & Isambert A. 2006. Commercial applications of microalgae, *Journal of bioscience and bioengineering*, 101(2), 87-96.

Topuz O. K., Gokoglu N., Yerlikaya P., Ucak I. & Gumus B. 2016. Optimization of antioxidant activity and phenolic compound extraction conditions from red seaweed (Laurencia obtuse), *Journal of Aquatic Food Product Technology*, 25(3), 414-422.

Topuz O. K., Yerlikaya P., Yatmaz H. A., Kaya A. & Alp A. C. 2017. Polyunsaturated fatty acid (PUFA) contents of meat and egg of rainbow Trout fish (Oncorhynchus mykiss), *Scientific Papers*. Series D. Animal Science, 312-315.

Ulbricht T. L. V. & Southgate D. A. T. 1991. Coronary heart disease: seven dietary factors, *The lancet*, 338(8773), 985-992.

Vinatoru M., Toma M., Radu O., Filip P. I., Lazurca D. & Mason T. J. 1997. The use of ultrasound for the extraction of bioactive principles from plant materials, *Ultrasonics sonochemistry*, 4(2), 135-139.

Wang S., Zhu J., Dai L., Zhao X., Liu D. & Du W. 2016. A novel process on lipid extraction from microalgae for biodiesel production, *Energy*, 115, 963-968.