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Optimization of Ultrasonic-Assisted Enzymatic Extraction of Freeze-Dried Sea Buckthorn (*Hippophae rhamnoides* L.) Berry Oil Using Response Surface Methodology

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Abstract: The ultrasound-assisted extraction (UAE) of oil has received immense importance nowadays because of the enormous benefits the process offers. However, the literature evaluating this process is scarce for sea buckthorn oil. Furthermore, to date, to the best of our knowledge, a study evaluating the combined use of enzymes and UAE for this oil is lacking. In this study, oil from freeze-dried sea buckthorn berries was extracted using ultrasound-assisted enzymatic extraction (UAEE) and the effect of variables (time, enzyme concentration, and solvent to sample ratio) was evaluated on oil yield and its physiochemical properties (acid value, peroxide value, iodine value, density, and color). The optimum conditions were determined using the response surface methodology. The optimum conditions established were 5.08 mL/g, 14.65 min, and 3.13 U/g for a solvent to sample ratio, ultra-sonication time, and enzyme units, respectively. The oil yield was 18.32%. Physicochemical parameters were found better in UAEE oil than in the Soxhlet-extracted oil. Gas chromatography detected relatively higher levels of fatty acids, including palmitic, palmitoleic, and oleic acids in UAEE oil. The optimum conditions were also verified for adequacy by validation and results were matched with predicted values with 0.8 to 1.5 error %, which states that the model can be utilized to predict oil yield percentage.

Keywords: fatty acid composition; FAME; physicochemical characteristics; predictive model; Soxhlet extraction; ultrasonication

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1. Introduction

Sea buckthorn (family *Elaeagnaceae*) is a hardy bush possessing smooth black or brown bark and yellow or orange to red berries [1]. It is naturally distributed in Asia and Europe, with more than 200 genotypes of plants found in the Laddakh region of India [2]. These berries remain throughout winter on the plant and bear smooth, brown and shiny seeds [1,3]. The high organic acid content provides these berries with a sour taste [3], while lipophilic and hydrophilic components make the fruit highly nutritious. Sea buckthorn

berries are used in various food preparations, such as jams, juices, jellies, oil, and other dietary supplements [4]. The oil obtained from ripe berries often offer advantages of high nutritional components, such as vitamins, fatty acids, trace elements, and other bioactive compounds [5]. These phytochemicals provide several health benefits, such as antimicrobial, antioxidant, anticarcinogenic, and anti-inflammatory effects [6]. Additionally, it has the potential to reduce diabetes, skin, and cardiovascular diseases [7]. Normally, the sea buckthorn oil is extracted from the pulp and seed through several methods. With respect to quality and composition, seed oil and pulp oil differ in fatty acid composition, and appearance as well as in bioactive compounds [8]. Pulp oil is dark orange, rich in palmitoleic acid, and possesses a characteristic smell and sour taste compared to sea buckthorn seed oil [9,10].

Despite these advantages, the sea buckthorn berries are very difficult to harvest due to the plants' thorn arrangement. The commonly identified way to obtain berries is to remove the entire branch; hence, it is harvested only once in a year or two [11], which limits the scope of any wastage or loss during processing. Furthermore, this highlights that the oil extraction must be performed to achieve the maximum possible yield. Mechanical cold pressing is a commonly employed method for oil extraction from sea buckthorn, which delivers a yield of 12.5 g/100 g seeds [12]. The oil derived from pulp, on the other hand, requires an additional step of filtration [13]. The other methods involved are solvent extraction, screw pressing, and aqueous extraction methods; however, these methods are linked with several disadvantages, such as time consumption, less oil yield, and labor intensiveness [14,15].

However, very few studies have been reported on the extraction of sea buckthorn oil through the UAE method. Sanwal et al. [16] evaluated different parameters (such as temperature, time, solvent–sample ratio and extraction efficiency) of the UAE method for the extraction of sea buckthorn oil from seeds. Isopenco et al. [17] compared the UAE and the process through response surface methodology (RSM). No study has been published on the combined use of enzymes and UAE for oil extraction from sea buckthorn, i.e., ultrasound-assisted enzymatic extraction (UAEE) [18]. The literature review highlighted that few studies made a comparison between the most commonly used extraction procedure, i.e., Soxhlet, and UAE for sea buckthorn oil extraction. The application of an effective optimization tool, such as RSM, not only saves time, but also reduces the cost of the experiment by decreasing the number of trials while establishing a mathematical model to identify the interaction between different variables [6]. No study has yet been conducted on sea buckthorn oil extraction by the UAEE technique using RSM.

The present study aimed to fill the identified gaps stated above and in the published literature by comparing two different sea buckthorn oil extraction processes, i.e., UAEE and solvent extraction. Oil yield and physicochemical characteristics were determined. Considering that the UAEE method has not been explored to date, the effect of the different variables (time, enzyme units, and solvent to sample ratio) were evaluated, and optimization of the process conditions using RSM followed by validation was performed.

2. Materials and Methods

2.1. Materials

Freeze-dried sea buckthorn berries were procured from Adept Impex Private Limited, Agra, Uttar Pradesh, India and samples were prepared by cleaning whole berries by removing dirt and other extraneous matter. Berries were then dried at a temperature of 35 °C using a hot air dryer (Sisco Instruments, New Delhi, India) and dried samples were ground and sieved using 60 mesh size (pore size 0.3 mm) to obtain a uniform particle size.

2.2. Chemicals and Reagents

All chemicals used were of analytical grade. Petroleum ether, n-hexane (GC grade), pectinase, chloroform, phenolphthalein, potassium hydroxide pellets, potassium iodide, sodium thiosulphate, potassium dichromate, soluble starch, iodine crystals, boron trifluo-

ride in methanol (14%), sodium chloride, and sodium hydroxide pellets were purchased from SRL Pvt. Ltd. (Mumbai, India). Ethanol, bromine, methanol, and glacial acetic acid were purchased from Fisher Scientific (Waltham, MA, USA). The fatty acid methyl esters (FAME) mixture (C4–C24) was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.3. Oil Extraction

The extraction of oil from sea buckthorn was mainly facilitated by the non-polar solvent (n-Hexane) owing to the presence of non-polar compounds in sea buckthorn at high levels [19]. Moreover, studies have shown the presence of a high amount of pectin (up to 15%) in sea buckthorn [20]. Hence, in the present study, n-hexane and pectinase were mainly used for the extraction of oil from sea buckthorn.

2.3.1. Soxhlet Extraction

The Soxhlet extraction of oil from whole sea buckthorn berries was performed using the method of Zhang et al. [21] with slight modifications. Berry powder (5 g) was weighed in a thimble and placed in a Soxhlet apparatus. Then, 250 mL of n-hexane was poured into a pre-weighed flask and connected to the condenser. The extraction process was carried out for 8 h at 50 °C. A rotary vacuum evaporator (Buchi, R210, Switzerland) was used to concentrate the solution at 40 °C. The concentrated oil solution (%) was weighed using a standard formula.

$$\text{Oil content } (\%) = \frac{\text{Final weight of flask} - \text{Initial weight of flask}}{\text{Weight of sample}} \times 100$$

2.3.2. Ultrasonic-Assisted Enzymatic Extraction (UAEE)

An ultrasonic system (Sonica LCC Q500, Qsonica, Newton, CT, USA) with a probe-type tip (1 cm diameter) was used for the oil extraction from sea buckthorn seed powder. Variables used for the UAEE were: extraction time (θ): 4–20 min; solvent to sample ratio (R): 5:1 to 6:1 (mL:g); and enzyme concentration: 2–10 units (Table 1). Based on the previous reports and preliminary trials, the temperature (T): 50 °C; ultrasound power (P): 500 W; and frequency: 20 KHz, were fixed. The powder (15 g) was mixed with 15 mL of n-hexane, and sonication was performed considering the different experimental conditions defined, based on preliminary trials. The optimum conditions (pH 5, temperature 50 °C, and time 120 min) were identified for pectinase enzyme activity. A specific amount of pectinase enzyme units (in acetate buffer solution) was added to the ultra-sonicated solvent–sample mixture followed by incubation at 50 °C for 120 min in an incubator shaker (Innova 42, New Brunswick Scientific, Enfield, CT, USA). Samples were later centrifuged at 10,000× g for 15 min and the oil layer was carefully separated using a micropipette. Samples were prepared in triplicates and the oil yield percentage was determined using the following formula:

$$\%\ \text{Oil yield} = \frac{\text{Weight of oil extracted}}{\text{Weight of sample}} \times 100$$

Table 1. Experimental ranges and levels of three variables in a central composite design.

Code	Variable	Levels				
		$-\alpha$	−1	0	+1	$+\alpha$
X_1	Solvent/sample ratio (mL/g)	4	4.5	5	5.5	6
X_2	Time in (minute)	5	10	15	20	25
X_3	Enzyme (unit)	1	2	3	4	5

RSM Design for the Optimization of Oil Extraction

The effects of independent variables on process output and central composite design (CCD) was prepared in Design-Expert software (version 11, Stat-Ease, Minneapolis, MI, USA) after obtaining the results of a single factor experiment.

The second-degree polynomial surface model, derived from the design, consists of 1 intercept term, 3 square terms, 3 interaction terms, and 3 linear terms, as given in Equation (1).

$$Y = b0 + b1X_A + b2X_B + b3X_c + b11X_A^2 + b22X_B^2 + b33X_C^2 + b12X_AX_B + b13X_AX_c + b23X_BX_c \quad (1)$$

where, Y is the dependent variable (sea buckthorn oil yield); b0 is constant; b1, b2, and b3 are the linear coefficients; b11, b22, and b33 are the quadratic coefficients; b12, b13, and b23 are the interaction coefficients; and X_A, X_B, and X_C are the coded values of the independent variables.

As stated, the relative effect of the process variables (time, enzyme units, and solvent to sample ratio) on the responses were studied (Table 2) and the oil extraction process was optimized. Oil yield was the response studied.

Table 2. Central composite design model with response.

	Factor 1	Factor 2	Factor 3	Response
Run	**A: Solvent/Ratio (mL/g)**	**B: Time (Min)**	**C: Enzyme (Units/g)**	**Yield (%)**
1	4.5	10	4	17.73
2	5	15	1	17.40
3	4.5	10	2	17.46
4	5.5	10	2	17.63
5	5	15	3	18.34
6	5.5	10	4	17.90
7	5.5	20	2	17.65
8	4.5	20	2	17.65
9	5	15	3	18.39
10	5	15	3	18.28
11	5	5	3	16.95
12	5	15	3	18.36
13	5	15	5	17.60
14	5	25	3	16.83
15	4	15	3	17.73
16	6	15	3	17.90
17	5.5	20	4	17.58
18	5	15	3	18.22
19	4.5	20	4	17.59

2.4. Analysis of Physicochemical Properties

The physicochemical properties of sea buckthorn oil derived through the Soxhlet and UAEE methods were analyzed in terms of acid value, iodine value, peroxidase value, oil density, and tristimulus color values.

2.4.1. Density

Oil density was determined using the method described by Thimmaiah [22], with slight modifications. To determine the density, two mL of a specific gravity bottle was weighed and recorded (initial weight). A specific gravity bottle was filled with distilled water and the excess sample was removed by whipping with tissue paper. The weight of the sample was measured and recorded (final weight). The same procedure was repeated for the oil sample to determine the weight of the oil. The analysis was done in triplicate. The density of the sample was calculated and expressed as mass per unit volume of the sample by using the following equation:

$$\text{Density (g/cm}^3\text{)} = (\text{Mass of sample/Mass of distilled water}) \times \text{Density of water}$$

2.4.2. Acid Value

Acid number is defined as the mg KOH required to neutralize the free fatty acids present in one gram of the sample and determined by titrating the oil in an alcoholic medium against standard potassium hydroxide solution. The method given by Thimmaiah [22], with slight modification, was used to determine acid value. The oil sample (2.5 g) was weighed and transferred to a 250 mL conical flask. Then, 50 mL of freshly prepared neutral solvent was added to the oil sample and 1 mL of phenolphthalein indicator solution was added. The solution was titrated against 0.1 N KOH with vigorous shaking during titration until a pink color was obtained. A blank (without oil sample) was run against 0.1 N KOH and recorded for correction. Titrations were done in triplicate. The acid value was calculated according to the following equation:

$$\text{Acid value (mg KOH/g)} = \frac{56.1 \times \text{V} \times \text{N}}{\text{W}}$$

where,

V = Standard KOH volume used in mL
N = Normality of KOH solution
W = Weight of sample in grams

2.4.3. Peroxide Value

The peroxide values of oil samples were determined using the method described by FSSAI [23]. The peroxide value was determined by measuring peroxides present in the oil using titration against thiosulphate in the presence of potassium iodide. The oil sample (5 g) was weighed and transferred to a 500 mL conical flask. Acetic acid chloroform solvent mixture (30 mL) was added to the solution and swirled to dissolve the oil completely. Then, 0.5 mL of saturated KI solution was added and the solution was allowed to stand for 1 min with occasional shaking, and then 30 mL of distilled water was added. The solution was titrated against sodium thiosulphate solution until the yellow color was almost gone. A starch solution indicator (0.5 mL) was added and the titration was continued with vigorous shaking to liberate all of the iodine from the chloroform layer until a blue color disappeared. A blank was also conducted with the absence of oil, and titrations were done in triplicate. The peroxide value was calculated and expressed as milliequivalent of peroxide oxygen per kg sample (meq/kg) using the following equation:

$$\text{Peroxide value (meq/kg oil)} = \frac{\text{Titre} \times \text{N} \times 100}{\text{Weight of sample}}$$

where,

Titre = sodium thiosulphate used in mL (blank corrected), N = normality of sodium thiosulphate solution.

2.4.4. Iodine Value

The iodine values of the oil samples were determined using the method described by FSSAI [23]. For the iodine value, the degree of oil unsaturation was measured, which is defined as the number of grams of iodine absorbed by 100 g of oil. For this, 0.2 g of oil sample was weighed into a 500 mL stoppered conical flask. A total of 20 mL of chloroform was added to the sample and dissolved properly to completely mix the sample with the chloroform. Hanus iodine solution (25 mL) was added to the solution and mixed well and kept for 30 min in the dark. A KI solution of 20 mL was added after 30 min. The solution was titrated against 0.1 N sodium thiosulphate solution using starch as an indicator with vigorous shaking to liberate the iodine from the chloroform layer. A blank was also run

with the absence of oil, and titration was done in triplicate. The iodine value was calculated and expressed as grams of iodine per 100 g of oil using the following equation:

$$\text{Iodine value } (\text{g } I_2/100 \text{ g oil}) = \frac{\text{A } \times \text{ N } \times 0.1269 \times 100}{\text{Weight of sample}}$$

where,

A = mL of $Na_2S_2O_3$ used (blank conducted),
N = normality of $Na_2S_2O_3$ solution.

2.4.5. Color

The color of oil was determined using a chromameter (Konica Minolta CR-400, Tokyo, Japan) and was presented in the Munsell notation using the method of Sagar and Pareek [24]. L^* represents darkness or lightness, the value of a^* represents greenness (−) or redness (+), and the value of b^* represents yellowness (+) or blueness (−) with chroma and hue angle.

2.5. Estimation of Fatty Acids in the Oil

FA analysis was carried out in a gas chromatograph (Scion, 456-GC, Livingston, UK) equipped with a flame-ionization detector (FID) and fused silica capillary cis/trans column SP 2560 (100 m × 250 µm internal diameters × 0.20 µm film) (Supelco, Bellefonte, PA, USA). FAME was prepared by weighing extracted oil (350 mg) in a 50 mL conical flask with 7 mL of boron trifluoride. A 0.5 N methanolic sodium hydroxide solution (6 mL) was added to the flask. The flask was fitted to the condenser and boiled until the oil droplets disappeared. Then, 5 mL of heptane and boron trifluoride were mixed into the boiling solution. A total of 1 mL from the upper layer of the heptane solution was then transferred into a test tube with anhydrous sodium sulphate. The obtained solution was injected into the GC column. The fatty acid composition was determined by comparing their retention times with those of known standards (fatty acid mixture: C4–C24) and the concentration of individual fatty acids was determined using the percentage (%) of the total area under the peak.

2.6. Conditions of GC Instrumentation

The fatty acid methyl esters were run on gas chromatography (GC), which was equipped with a flame-ionization detector. Fused silica capillary cis/trans column SP 2560, 100 m × 250 µm internal diameters × 0.20 µm film was used (Supelco, Bellefonte, PA, USA). The following temperature program was used for the optimal separation of fatty acids including trans fatty acids: the initial oven temperature was 140 °C, the hold time was 5 min, the ramp was 1 °C/min; the final temperature was 250 °C, the hold time was 25 min, and the total run time was 120 min. The injector port was 225 °C, the detector port was 260 °C, and the gas rates used were 0.3 mL/min carrier gas (nitrogen), 15 mL/min make-up gas (nitrogen), and 35 and 350 mL/min flame gases hydrogen and air, respectively. A split ratio of 1:10 and an injection volume of 1 µL were used.

2.7. Statistical Analyses

All of the experiments were performed in triplicate and one-way analysis of variance (ANOVA) and Duncan's test (post hoc) were employed for determining the significance difference ($p \leq 0.05$) using IBM® SPSS statistics (version 20, IBM, Armonk, NY, USA). The data were expressed as mean ± standard deviation (S.D).

3. Results

3.1. Soxhlet Extraction and Physiochemical Properties of Oil

The yield of oil extracted from sea buckthorn berries was found to be 18.39%. The acid value, an indicator of oil purity, was found to be 9.2 ± 0.104 mg KOH/g. The iodine value, which represents the relative level of unsaturation in oil, was found to be 123.5 ± 2.291 g I_2/100 g. The peroxide value (related parameter of rancidity) was

6.4 ± 0.100 meq/kg, whereas, the density of the Soxhlet extract oil was found to be 0.82 ± 0.026 g/cm^3. The mean *L**, *a**, *b**, hue, and chroma of the oil extracted from the process were 29.53, 1.83, −2.06, 311.62, and 2.75. Furthermore, Soxhlet-extracted oil possessed 10.75 ± 0.108 mg/g linoleic and 1.46 ± 0.005 mg/g stearic acids.

Kaushal and Sharma [25] analyzed iodine concentration in the seed oil of *H. salicifolia* and *H. rhamnoides* extracted using a Soxhlet in petroleum ether and reported an iodine value in the range of 150–154 g I_2/100 g, which is higher than the values observed in the present study. Further, Kaushal and Sharma [25] found a higher peroxide value for *H. salicifolia* (18.30 ± 0.44 meq/kg) and *H. rhamnoides* (17.50 ± 0.17 meq/kg). Pavlović et al. [26] extracted sea buckthorn oil by employing a supercritical CO_2 extraction (the other most prevalent sea buckthorn oil extraction process) and obtained an 11.60% oil yield, which was 36.92% lower than the yield obtained in the present study. Furthermore, the fatty acids, such as palmitic, palmitoleic, and oleic acids formed 35, 20, and 32–35% in oil. Yang and Kallio [13] obtained an oil yield of 11.3% from seeds by juice extraction and freeze-drying process.

3.2. Ultrasound-Assisted Enzyme Extraction (UAEE)

3.2.1. Response Surface Optimization of Ultrasonic Extraction Condition

A total of 19 runs were conducted and the experimental order was randomly arranged. In all of the experimental runs, the maximum sea buckthorn oil yield recorded was 18.38% under experimental conditions; 15 min ultrasonic time, 5:1 mL/g solvent sample ratio, and three enzyme units per gram. The model adequacy was explained by analysis of variance and the data are presented in Table 3. The model fit summary is presented in Table 4. The F-value, which was 175.51, and the *p*-value, which was less than 0.0001, showed that the model is significant. The F-value and *p*-value (Table 3) and R^2 value (Table 5) mean the regression model is valid and the fitting degree is good.

Table 3. Analysis of variance for quadratic model.

Source	Sum of Squares	df	Mean	F-Value	*p*-Value
Model	3.44	9	0.3824	175.51	<0.0001 **
A-solvent/ratio	0.0269	1	0.0269	12.35	0.0066
B-Time	0.0148	1	0.0148	6.78	0.0285 *
C-Enzyme	0.0408	1	0.0408	18.73	0.0019 **
AB	0.0150	1	0.0150	6.87	0.0277 *
AC	0.0000	1	0.0000	0.0167	0.9000
BC	0.0555	1	0.0555	25.46	0.0007 **
A^2	0.3625	1	0.3625	166.39	<0.0001 **
B^2	2.98	1	2.98	1368.10	<0.0001 **
C^2	0.9660	1	0.9660	443.39	<0.0001 **
Residual	0.0196	9	0.0022		
Lack of Fit	0.0014	5	0.0003	0.0593	0.9958
Pure Error	0.0183	4	0.0046		
Corrected Total	3.46	18			

* significant ($p \leq 0.05$), ** more significant ($p \leq 0.01$).

Table 4. Model adequacy tested.

Source	Sum of Squares	df	Mean	F-Value	*p*-Value	
Mean vs. Total	5985.15	1	5985.15			
Linear vs. Mean	0.0825	3	0.0275	0.1221	0.9456	
2FI vs. Linear	0.0705	3	0.0235	0.0852	0.9668	
Quadratic vs. 2FI	3.29	3	1.10	503.14	<0.0001	Suggested
Cubic vs. Quadratic	0.0000	4	0.0000	0.0000	1.0000	Aliased
Residual	0.0196	5	0.0039			
Total	5988.61	19	315.19			

Table 5. Fit statistics summary.

Standard deviation	0.0467
Mean	17.75
Coefficient of variance %	0.2630
R^2	0.9943
Adjusted R^2	0.9887
Predicted R^2	0.9889
Adequate precision	43.7038

The model is significant when the R^2 value is greater than 70% [27]. Model terms with a *p*-value less than 0.05 showed a significant effect on oil terms. In this case, significant model terms are A, B, C, AB, BC, A^2, B^2, and C^2, and the model terms with $p > 0.05$ had no significant effect on oil yield by the following quadratic Equation (2):

$$Y = 18.32 - 0.0410\,X_A - 0.0304\,X_B + 0.0505\,X_C - 0.1237\,X_A^2 - 0.3548\,X_B^2 - 0.2020\,X_B^2 - 0.0433\,X_AX_B - 0.0021\,X_AX_C - 0.0833\,X_BX_C \quad (2)$$

where, X_A is solvent to sample ratio mL/g, X_B is ultrasonication time, and X_C is enzyme units/g. The orthogonal design contains an intercept, which is the average response of all runs. The variance inflation factor (VIF) of orthogonal variables will be one, and greater than one indicates multicollinearity; the higher the VIF the more severe the correlation of factors.

Isopenco et al. [17] utilized RSM to optimize the extraction efficiency of sea buckthorn oil using the UAE method and identified the second-order quadratic regression model as the best fit, having the highest F value of 36.51 and lowest *p*-value of about <0.0001. The results reported 50 °C, 13.77 W/cm^2, and 10 min as the optimum extraction condition. Similarly, Xu et al. [28] utilized RSM to derive whole berry sea buckthorn oil using the supercritical carbon dioxide extraction method. The linear effect of pressure on oil yield was observed at low-pressure values, while a negative linear effect was observed for temperature. On the other hand, the interactive effect of both pressure and temperature had a positive effect on oil yield. The extraction time and flow rate selected, based on optimization, were 82 min and 17.0 L/h, with optimum pressure of 27.6 MPa and 34.5 °C temperature; resulting in an oil yield of 208.0 g/kg.

3.2.2. Optimization of Sea Buckthorn Oil Yield Conditions

Design-Expert software version 11 was used to make 3D surface plots. These plots were drawn by varying two factors and keeping other variables constant. The results of sea buckthorn oil yield extraction, as affected by solvent sample ratio mL/g, ultra-sonication time, and enzyme units per gram, are given in a 3D surface plot.

The effect of the solvent sample ratio and time at a fixed enzyme unit of 3.314 on sea buckthorn percent oil yield is presented as a 3D surface plot (Figure 1a), which indicated that sea buckthorn oil yield percentage increased with an increase in the solvent ratio. Furthermore, percent oil yield is increased when time is increased from 10 to 14.6, after this range a slight decrease in oil yield was observed. This can be explained by the phenomenon that the cell wall of the seed is completely ruptured and the impurities (insoluble substances, suspended lipids, and cytosol) become suspended in the solvent, which results in lower permeability of the solvent [29,30] and also re-absorption of oil, due to an increase in surface area of the cell wall [30].

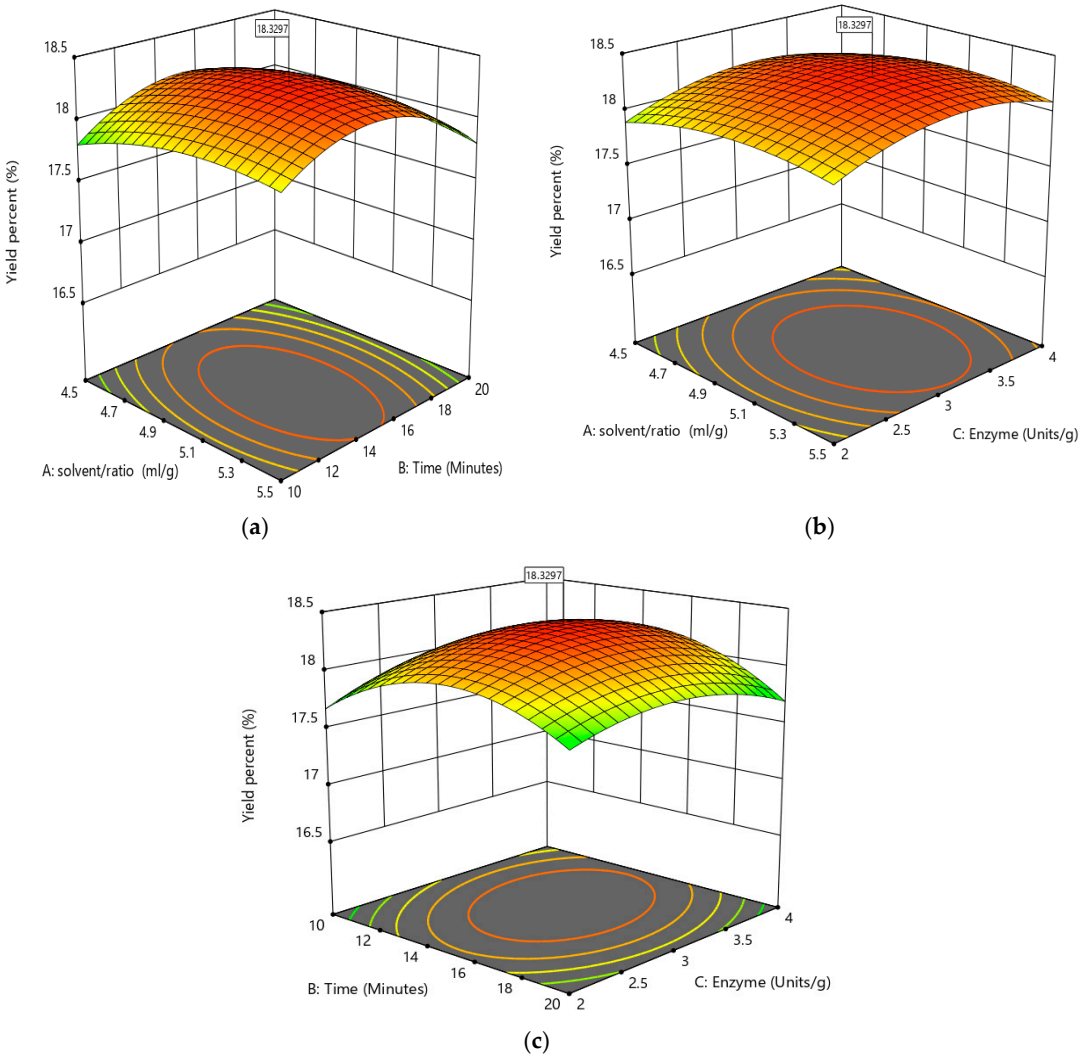


Figure 1. Three-dimension plots of response surface: (**a**) effect of solvent sample ratio and time; (**b**) effect of enzyme units and solvent sample ratio; (**c**) effect of time and enzyme units.

The effects of enzyme units and solvent to liquid ratio on sea buckthorn oil yield percentage at a constant time of 14.65 are presented in Figure 1b. It was observed that oil yield percentage increased with the increase in enzyme units and solvent/sample ratio. Figure 1c shows a 3D surface plot of percent oil yield as a function of time and enzyme units at a fixed solvent sample ratio of 5.08 mL/g. It can be observed that sea buckthorn oil yield percentage increased with the increase in enzyme units per gram and ultrasonic time. However, ultrasonic time, after reaching a point (14.65 min), decreases the oil yield percentage.

The optimum conditions given by the Design-Expert 11 software for UAEE were a solvent sample ratio of 5.08 mL/g, an ultra-sonication time of 14.65, and 3.13 U/g enzyme units. Under these parameters, the predicted sea buckthorn oil yield percentage was 18.329%. A validation of the predicted conditions was then done to check the reliability of the model. Therefore, the experiments were conducted in triplicate using the above conditions, and it was observed that experimental values were near to the predicted values and the error identified was due to the difference in uniformity of the sample. Error % was between 0.30 and 3.75%, which indicated that the predicted conditions are verified and can be used to obtain optimum responses [31].

3.2.3. Physicochemical Properties of UAEE Extracted Sea Buckthorn Oil

The UAEE method led to a yield of 26.13%, which was 1.42-fold higher than the yield from the Soxhlet method. A significant difference ($p \leq 0.05$) was observed between the values of UAEE- and Soxhlet-extracted oil. Sanwal et al. [16] achieved a yield of 6.87 g using UAE of seed oil from sea buckthorn. The optimum conditions reported in the study were 50 °C, 8.28 min, 552 W ultrasound power, and 10:1 (mL:g) solvent to sample ratio. In another study, an extraction efficiency of about 87.4% was obtained through UAE [17], keeping optimum conditions as 40 °C temperature, 13.77 W/cm^2 UI, and 10 min extraction time. As observed, the variability obtained in the yield is due to the diverse solvent ratio, temperature, cultivar type, and extraction conditions used in these studies [32].

The acid value of oil extracted by UAEE was found to be 1.35-fold lower than the Soxhlet-extracted oil (with a significant difference of $p \leq 0.05$), indicating more purity in the oil [33]. Furthermore, a peroxide value of about 4.1 meq/kg was obtained for UAEE-extracted oil in comparison to 6.4 meq/kg of Soxhlet-extracted oil. Munkhbayar et al. [33] reported a lower peroxide value (2 meq/kg) for pulp oil using enzymatic treatment, which means the oil was more susceptible to rancidity. Isopenco et al. [17] reported 1.2 and 0.9 meq active oxygen/kg peroxide value of UAE- and Soxhlet-extracted sea buckthorn oil, respectively. It can be concluded that the green or novel techniques have the potential to retain antioxidants during extraction, which are associated with a lower peroxide value [1].

Another physical parameter for sea buckthorn quality is the color of the oil because it affects the organoleptic attributes of the sample [34]. The oil color values were determined as L^*, a^*, b^*, hue, and chroma. UAEE oil showed a higher value of a^* (3.62 ± 0.02), hue angle (349.97 ± 0.26), and chroma (3.67 ± 0.02), while the Soxhlet-derived oil had the maximum level of L^* and b^* (Table 6). A significant difference ($p \leq 0.05$) can be seen between the values of samples which may be corresponded to the level of carotenoids in the extracts obtained from two different methods. Recently, Hussain et al. [6] also conducted an experiment on the UAE of dietary fiber from sea buckthorn pomace using the RSM experiment and reported relatively higher values, i.e., 54.71 ± 0.72, 52.35 ± 1.04, and 79.28 ± 0.62 for L^*, a^*, and b^*, respectively. Moreover, Aaby et al. [35] investigated the effect of temperature (80 °C, 10 min) on the color of sea buckthorn puree and reported a lower value for L^* (4.0) and chroma (0.2).

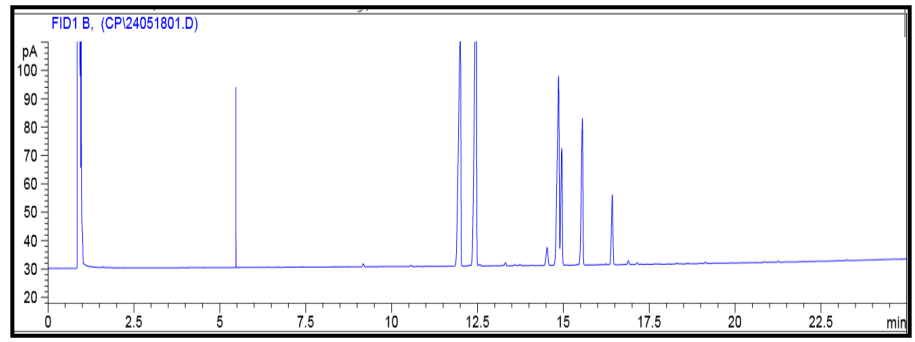
Table 6. Physicochemical characteristics, fatty acid composition, and tristimulous color values of sea buckthorn oil.

	Retention Time	
	Soxhlet Extraction	**UAEE**
Physicochemical characteristics		
Acid value (mg KOH/g)	9.2 ^{b} ± 0.104	6.77 ^{a} ± 0.305
Iodine value (g I_2/100 g)	123.5 ^{b} ± 2.291	138.67 ^{a} ± 3.512
Peroxide value (meq/kg)	6.4 ^{b} ± 0.100	4.33 ^{a} ± 0.252
Density (g/cm^3)	0.82 ^{b} ± 0.026	0.92 ^{a} ± 0.015
Fatty acid composition (mg/g)		
Palmitic acid	25.29 ^{b} ± 0.295	25.22 ^{a} ± 0.301
Stearic acid	1.46 ^{b} ± 0.005	1.54 ^{a} ± 0.008
Palmitoleic acid	32.36 ^{b} ± 0.510	31.76 ^{a} ± 0.503
Oleic acid	24.46 ^{b} ± 0.203	17.20 ^{a} ± 0.172
Linoleic acid	10.75 ^{b} ± 0.108	10.81 ^{a} ± 0.115
Linolenic acid	4.83 ^{b} ± 0.102	4.83 ^{a} ± 0.104
Color values		
L^*	29.53 ^{b} ± 0.0152	27.45 ^{a} ± 0.0360
a^*	1.83 ^{b} ± 0.02	3.62 ^{a} ± 0.02
b^*	−2.06 ^{b} ± 0.0351	−0.64 ^{a} ± 0.0208
Hue (h^0)	311.62 ^{b} ± 0.678	349.97 ^{a} ± 0.268
Chroma (C^*)	2.75 ^{b} ± 0.0238	3.67 ^{a} ± 0.0232

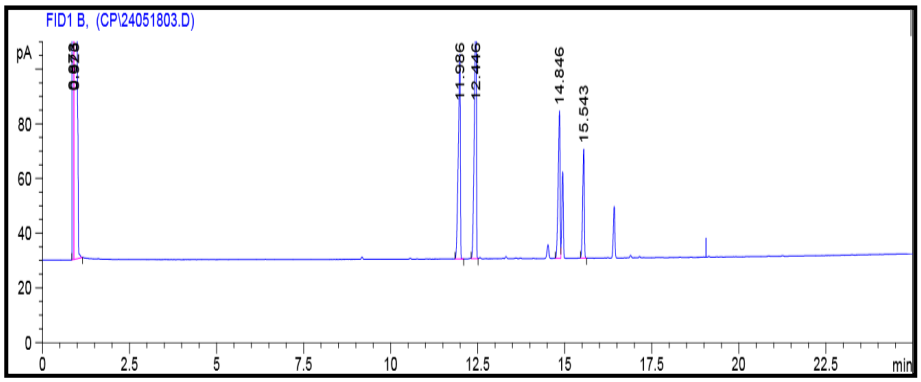
Values are expressed in Mean ± standard deviation. The values with ^{a} and ^{b} superscripts in a column indicate a significant difference ($p < 0.05$). n = 3.

3.2.4. Fatty Acid Composition of Extracted Oil

The fatty acid composition of sea buckthorn oil is given in Table 6 and the chromatograph is presented in Figure 2. Palmitoleic, palmitic, and oleic acids were the predominant fatty acids in UAEE oil, whereas Soxhlet-extracted oil had a higher level of linoleic (10.81 mg/g) and stearic acids (1.54 mg/g), which agrees with Dulf et al. [8]. Similarly, Isopencu et al. [17] applied RSM to optimize the UAE conditions for oil extraction from the seeds of sea buckthorn and compared its fatty acid analysis composition with Soxhlet-extracted oil. It was reported that the palmitic, palmitoleic, and oleic acids were in a higher range in UAE oil than the Soxhlet-extracted sample. This is consistent with the results of the present study. Usually, sea buckthorn oil contains a range of saturated and unsaturated fatty acids that depends upon the source materials, i.e., seed, fruit, and pomace [36].



(**a**)



(**b**)

Figure 2. Chromatogram of sea buckthorn oil extracted by (**a**) the Soxhlet method and (**b**) ultrasonication-assisted enzyme extraction.

Dąbrowski et al. [37] extracted flesh oil from five Polish cultivars, namely 'Botaniczeskaja Ljubitelskaja', 'Golden Rain', 'Luczystaja', 'Maryna' and 'Prozracznaja', of sea buckthorn and estimated the fatty acids using gas chromatography. They identified a total of seven fatty acids, in which, palmitoleic (29.0–32.8 g/100 g) and palmitic (37.7–41.3 g/100 g) were the dominant in all of the cultivars, which supports the present study. In a recent study, Patra et al. [36] extracted oil using a microwave-assisted extraction method and reported that the palmitic was the prime fatty acid in the pomace (44.1%) and seed (49.1%) of sea buckthorn, followed by oleic acid, i.e., 35.5% and 29.6%, respectively. During a comparison of UAEE with supercritical CO_2 extraction (SCCE) of sea buckthorn berry oil [26] and Soxhlet extraction, the results reported that the palmitic acid content was more in the Soxhlet extraction than in SCCE. Palmitoleic acid and linoleic acid in UAEE and Soxhlet extraction oil were found to be similar, but higher than the reported value of SCCE. Geographical region, growing conditions, and the experimental set were the crucial factors for controlling the fatty acid content in sea buckthorn. Oleic acid in UAEE was less when compared to SCCE, but more when compared with Soxhlet extraction. Stearic acid and

linolenic acid were found to be almost similar in all three methods. Munkhbayar et al. [33] have reported the presence of similar fatty acids (the ones obtained in the present study) in sea buckthorn oil extracted through enzymatic analysis and Soxhlet analysis. However, the levels of the fatty acids varied with the type of extraction method used, i.e., palmitoleic acid was prominent in enzymatic treatment, while palmitic acid was higher in the traditional Soxhlet method.

4. Conclusions

Sea buckthorn is a very important fruit with a lot of applications in pharmaceutical and nutraceutical areas and can be grown in adverse conditions. It was found that this model was adequate to predict the optimum oil yields from dried sea buckthorn berries. Color coordinates L^* were lower than the oil extracted from the Soxhlet extraction method, but a^*, b^*, hue, and chroma values were higher in the oil extracted by the UAEE method. Palmitic, palmitoleic, and oleic acids were present in higher concentrations, and stearic and linoleic acids were present in lower concentrations in oil extracted by the UAEE method compared to the Soxhlet extraction method.

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