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ORIGINAL PAPER

Trigonella foenum-graecum L. Seed Oil Obtained by Supercritical CO_2 Extraction

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Abstract Supercritical CO_2 extraction (SC-CO_2) of fenugreek (*Trigonella foenum-graecum* L.) seed oil and its chemical composition and antioxidant activity were investigated. A central composite design combined with response surface methodology was used to study extraction conditions including pressure, temperature, and time. The optimum extraction conditions were 28.5 MPa extraction pressure, 41 °C extraction temperature, and 118 min extraction time, where 3.78 % yield was predicted. Fenugreek seed oil extracted under optimum conditions by SC-CO_2 was mainly composed of 28.3 % C18:3, 33.45 % C18:2, 9.89 % C16, 8.1 % C18:1, 3.7 % C18, 0.71 % C20, and 0.61 % C22. The fenugreek oil was rich in unsaturated fatty acids (nearly 70 % of the total fatty acids), and polyunsaturated fatty acids accounted for 61.42 % (mass percentage) of the total amount. The 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity increased from 12.5 to 88.4 % when the concentration was increased from 1 to 12 mg/ml. The reducing power of the seed oil was concentration-dependent. The antioxidant activity of the supercritical fluid extraction extract was superior to those obtained by Soxhlet extraction.

Keywords Fenugreek (*Trigonella foenum-graecum* L.) · Seed oil · Supercritical carbon dioxide extraction · Response surface methodology · HPLC-FLD-MS · Antioxidant activity

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Introduction

Trigonella foenum-graecum L., commonly known as fenugreek, is an annual plant of the Trigonella Linn species in the Leguminosae family native to the area stretching from the eastern Mediterranean to central Asia and Africa, and much of this plant is cultivated in Pakistan, India, Canada, and China. The seeds are extensively used as a food spice in India, western Canada, Egypt, and China because of its strong flavor and aroma. In China, it is a medicinal and edible plant for making pancakes, and its seeds are included in the "Chinese Pharmacopoeia". Saponins [1], flavonoids [2, 3], galactomannans [4], trigonelline, and 4-hydroxyisoleucine [5] were isolated from fenugreek seeds in previous phytochemical research studies. The seeds are used in folk medicine because of their reported hypoglycemic [6], hypolipidemic, antidiabetic [7], liver-protective [8], renoprotective, anticancer [9], and antioxidant [10] effects.

Recent research has shown that fenugreek seed contains about 7 % lipids consisting mainly of unsaturated acids, namely linoleic, linolenic, and oleic acids [11, 12], and polyunsaturated fatty acids (PUFA) are present in significant amounts. Edible plants with high PUFA (particularly linoleic acid) content are recommended in the diets of people with high blood cholesterol [13]. Linoleic acid is a potential suppressor of tumor growth and metastasis, and

plays roles in preventing coronary heart diseases, hypertension, rheumatoid arthritis, ulcerative colitis [14], and other health issues. Therefore, the consumption of oils rich in polyunsaturated fatty acids is very important, because these oils can improve human nutrition by providing natural, plant-based sources of ω-3 fatty acids and antioxidants.

Similar to other seed oils, fenugreek seed oil is produced at laboratory scale by using organic solvent extraction. Organic solvent extractions, such as using petroleum ether extraction, are very time-consuming, leave behind toxic solvent residue, and cause degradation of unsaturated compounds because of heat [15]. Compared to conventional extraction methods, supercritical carbon dioxide extraction (SC-CO_2) possesses some excellent properties, including being non-toxic, non-flammable, cost-effective, and easily eliminated from extracts; it became an alternative method for extraction at the laboratory scale for extracting seed oils from natural plant sources including *Opuntia dillenii* Haw., *Xanthoceras sorbifolia* Bunge., *Cucurbita maxima*, and *Pistacia terebinthus* [16].

Quantitative determination of the fatty acid composition is very helpful in controling the quality of seed oil. In most cases, GC–MS is the prevailing technique but has some problems such as tailing peaks and low detector sensitivity in fatty acid analysis. Therefore, derivatization of these analytes with labeling reagents, especially for sensitive fluorescent detection, has been widely adopted in natural plants such as *Lomatogonium rotatum*, *Nitraria tangutorum*, and *Lycium barbarum.*

The objectives of the present study were to investigate SC-CO_2 oil extraction from fenugreek seed using pilot-scale extraction and to determine the effects of pressure, temperature, and time on oil yield. The quality of the SC-CO_2 extracted oil was also determined and compared to oil obtained by using Soxhlet extraction (SE). Furthermore, antioxidant activity of seed oil was determined by means of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay and reducing power assay.

Experimental Procedures

Materials

Seeds of *T. foenum-graecum* L. (fenugreek) were collected during the autumn of 2009 from the Datong area in Qinghai province in the northeastern part of the Tibetan Plateau, and dried at 60 °C for 24 h in a forced-air oven. The moisture and ash contents of the seeds were 7.68 and 3.05 %, respectively. The seeds were crushed, passed through a 40-mesh (0.63 mm) sieve, and stored at 4 °C before analysis. Fatty acid standards were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). DPPH was supplied by Sigma-Aldrich Co. (St. Louis, MO, USA), and spectroscopically pure acetonitrile was purchased from Merck (Merck KGaA, Darmstadt, Germany). 2-(5-Benzoacridine) ethyl-p-toluenesulfonate (BAETS) was synthesized following the method described by Li et al. [17]. All other chemicals and solvents were analytical grade and used without purification.

Supercritical Fluid Extraction with Carbon Dioxide

SC-CO_2 experiments were carried out following a previously described procedure [18]. The SC-CO_2 equipment (1-l capacity) used was model HA221-40-12 (Nantong Hua'an Co. Ltd, Jiangsu, China). Two-hundred grams of ground fenugreek seed was mixed with 5 mm of glass beads (10 g) in all experiment; then, they were placed in a stainless-steel extractor with filters at the top and bottom. After being cooled through a glycol chiller, liquefied CO_2 supplied from a gas cylinder was pumped into the extractor by using a high-pressure pump to the desired pressure. The SC-CO_2 flow was downward, and the flow rate was kept constant at 15 l/h. The pressure, temperature, and extraction time ranged between 20 and 30 MP, 35 and 50 °C, and 1 and 2 h, respectively. During extraction, the extraction pressure and CO_2 flow rate were controlled by adjusting the regulating valves, and the extraction temperature was regulated by using a temperature-controlled chamber wrapped outside of the extractor. Once the scheduled time was reached, the oil was collected from the first separator, while water and volatile components were recovered in the second one. The temperature and pressure of the first separator were 45 °C and 6 MPa, respectively, and the solvent-free oil was weighed to obtain yield. The oil was transferred to brown glass vials, flushed with nitrogen, sealed, and stored at −20 °C until the fatty acid composition and antioxidant activity were determined (about 25 days). All experiments were replicated three times. The yield of seed oil (%) was calculated using Eq. (1).

$$\text{yield of seed oil} = \frac{M_e}{M} \times 100\,\% \quad (1)$$

where M_e is the mass of extracted oil and M is mass of dried material.

Soxhlet Extraction

Soxhlet extraction was performed to compare extraction performance at laboratory scale with SC-CO_2. SE of fenugreek seed was conducted by using the method of Eller et al. [19] with some modifications. Fenugreek seeds (15 g) were extracted with 225 ml of petroleum ether (60–90 °C)

at 70 °C for 10 h by using a Soxhlet extractor. After extraction, the solvent was evaporated with a rotary evaporator at 40 °C. All experiments were replicated three times. The seed oil was transferred to brown glass vials, flushed with nitrogen, sealed, and stored at −20 °C until the fatty acid composition was determined and antioxidant activity was determined (about 25 days). The oil was weighed to calculate yield.

Experimental Design and Data Analysis

RSM combined with central composite design (CCD) was applied to optimize extraction pressure (X_1), temperature (X_2), and time (X_3) for SC-CO_2 of the fenugreek seed oil. In the CCD, 14 experiments and 6 replicates at the center were employed to fit the full quadratic equation model. The actual and coded levels of the independent variables used in the experimental design are shown in Table 1. The general equation and the coded variables equation follow reference [18] and are shown in Eq. (2):

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j}^{k} \beta_{ij} X_i X_j (k = 3) \quad (2)$$

where k is the numbers of variables; β_0 is the constant term; β_j, β_{jj}, and β_{ij} represent the coefficient of the first-order terms, quadratic terms, and interaction terms, respectively; and X_i and X_j are the independent coded variables.

The extraction pressure was varied from 20 to 30 MPa and temperature from 35 to 50 °C. All experiments were replicated three times, and the extraction yields are reported as mean values ± standard deviations. The response surface analysis procedure (Design-Expert 7.1.3 Trial, State-Ease, Inc., Minneapolis, MN, USA) was used for calculations and modeling of optimal conditions for SC-CO_2 extraction of fenugreek seed oil in the response surface regression procedure. Values of $P < 0.01$ were regarded as significant.

Table 1 Uncoded and coded levels of independent variables used in the RSM design

Coded-variables levels	Pressure (X_1, MPa)	Temperature (X_2, °C)	Time (X_3, min)
−1.682	16.6	30	39.5
−1	20	35	60
0	25	42.5	90
+1	30	50	120
+1.682	33.4	55	140.5
Δ_j	5	7.5	30

HPLC-FLD-MS Fatty Acid Composition Analysis

The HPLC system was an Agilent HP 1100 series (Agilent, Santa Clara, CA, USA) and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostat-controlled column compartment (model G1316A), and a fluorescence detector (FLD) (model G1321A). The HPLC system was controlled by HP Chemstation software. Fluorescence excitation and emission spectra were obtained with a F7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

The 18 standard fatty acids for HPLC analysis at individual concentration of 1.0×10^{-4} mol/l were prepared by diluting the corresponding stock solution (1.0×10^{-2} mol/l) with the mixed solvent of acetonitrile/DMF (9:11, v/v). 2-(5-Benzoacridine) ethyl-p-toluenesulfonate solution was prepared by dissolving 6 mg BAETS in 2 ml DMF. All solutions were stored at 4 °C in a refrigerator until used.

Ten-milligram seed oil samples were mixed with 0.5 ml of 2 M KOH methanol solution in a vial. The vial was sealed under a stream of nitrogen gas and sonicated for 2.0 min. Saponification was performed by heating at 90 °C for 2 h, then cooled to room temperature (20–25 °C). At the end of saponification, 0.5 ml of pure water was added. The pH of the saponified solution was adjusted to 7.0 with 6 M HCl. The mixture was added into 1 ml of trichloromethane and vortexed for 2.0 min, and then the lower organic phase was removed and placed into a 2-ml screw-capped amber vial; this process was repeated twice. The trichloromethane was removed and evaporated under a stream of nitrogen gas, and the fatty acids were redissolved with 2 ml of DMF and stored at 4 °C before HPLC derivatization.

DMF (150 μl), 20 μl of mixed free fatty acids (FFA) (1.0×10^{-4} mol/l), 130 μl of derivatization reagent solution, and 50 mg of an anhydrous K_2CO_3 catalyst were placed together in a vial. The vial was sealed and placed in a water bath at 90 °C with shaking in 5 min intervals for 40 min total duration. After the reaction was complete, the mixture was cooled to room temperature. The diluted solution (10 μl) was injected directly into the chromatograph. The derivatization procedure is shown in Fig. 1. Derivatization of the extracted sample solution was conducted as described above.

Derivatives were separated on a reversed phase Hypersil BDS-C8 column (200 mm × 4.6 mm, 5 μm; Dalian Elite Analytical Instruments Co., Ltd, Dalian, China) by a gradient elution. Eluent A was a mixed solvent of 30 % acetonitrile and ammonium formate (30 % acetonitrile/ammonium formate, 100:0.1, v/v); B was pure acetonitrile. The gradient conditions were as follows: initial = 52 % B; 35 min = 85 % B; 45 min = 100 % B (retained for

5 min). The flow rate was constant at 1.0 ml/min, and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} 272 and λ_{em} 505 nm for the F7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), and the bandpasses were both set at 15 nm. A Paratherm U2 electronic water bath (Hitachi, Tokyo, Japan) was used to control the temperature for fluorescence scanning. The mobile phase and sample were filtered through a 0.25-μm nylon membrane filter (Alltech, Deerfield, IL, USA).

FFA derivatives separated by HPLC were identified by online mass spectra equipped with an APCI source. The APCI probe was heated to 400 °C to ensure complete vaporization of the column effluent. Other mass spectra conditions were as follows: APCI source in positive-ion detection mode; 350 °C APCI Vap temperature; 413 kPa nebulizer pressure; 450 °C dry gas temperature; 5.0 l/min dry gas flow; 4,000 (pos) corona current (nA); 3,500 V capillary voltage.

The separation chromatogram of all standard fatty acid derivatives under these conditions is shown in Fig. 2, and the corresponding cleavage mode and MS/MS analysis for the representative BAETS-C20 derivative are shown in Fig. 1. MS data for all the fatty acid derivatives are shown in Table 2. Quantitative analysis of fatty acids in fenugreek seed oil to their BAETS derivatives was achieved by using

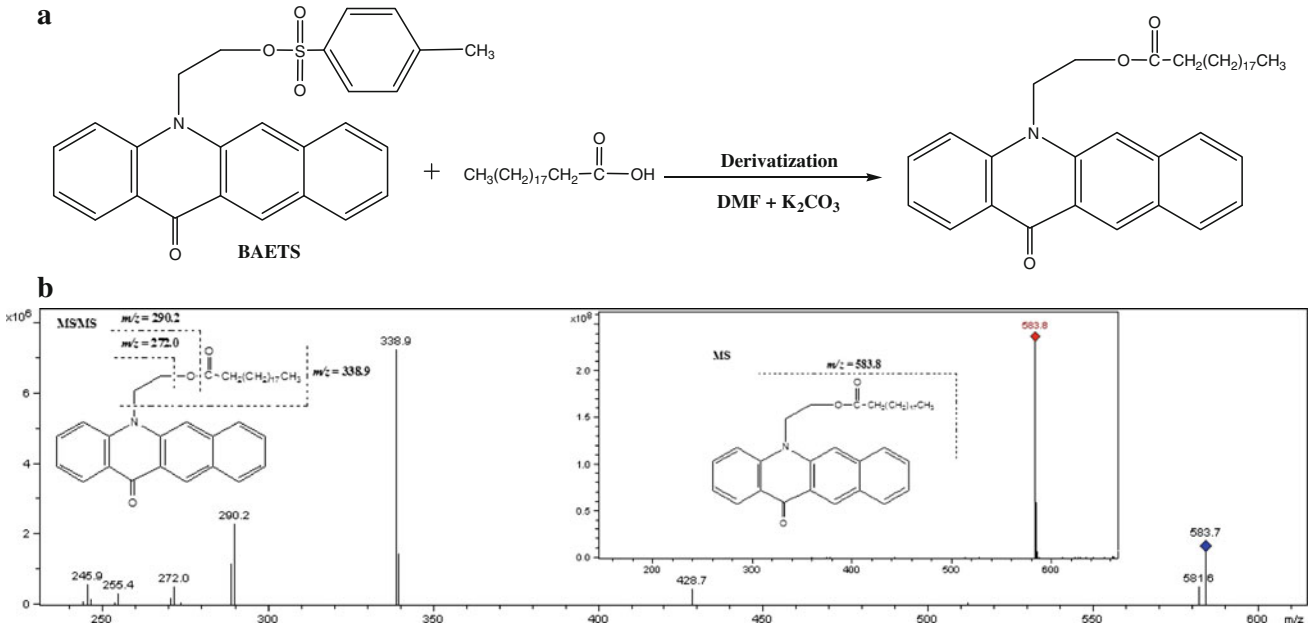

Fig. 1 Derivatization scheme of fatty acids with BAETS (**a**) and the cleavage mode of the typical BAETS-C20 fatty acid derivative (MS and MS/MS) (**b**)

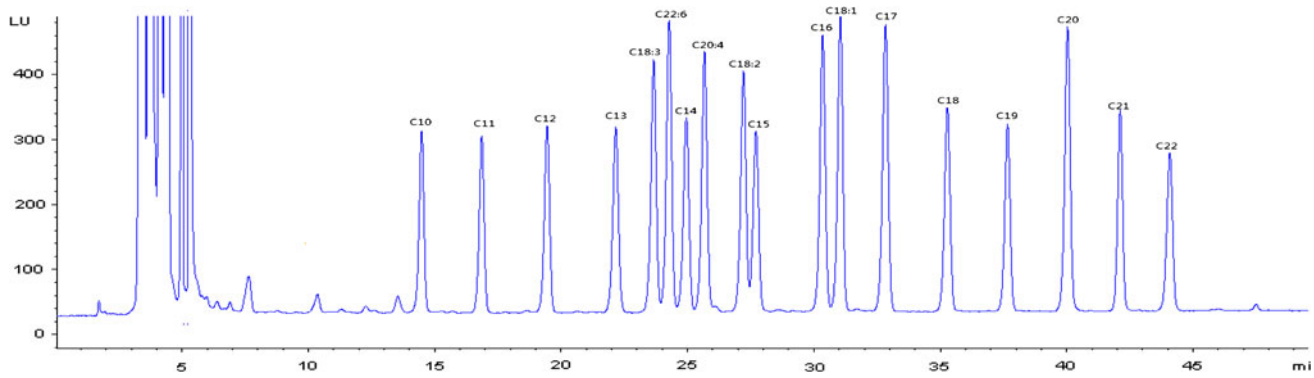

Fig. 2 Chromatogram of standard fatty acid derivatives C10 (decoic acid); C11 (undecanoic acid); C12 (dodecanoic acid); C13 (tridecanoic acid); C18:3 (linolenic acid); C22:6 (2,5,8,11,14,17-docosahexenoic acid); C14 (tetradecanoic acid); C20:4 (arachidonic acid); C18:2(linoleic acid); C15 (pentadecanoic acid); C16 (hexadecanoic acid); C18:1 (oleic acid); C17 (heptadecanoic acid); C18 (octadecanoic acid); C20:1 (11-eicosenoic acid); C19 (nonadecanoic acid); C20 (eicosoic acid); C21 (heneicosoic acid); C22 (docosanoic acid)

Table 2 Linear regression and detection limits of fatty acids

FFA	Molecular formula	FFA derivative $[M + H]^+$	Linear regression	Correlation coefficient (r)	Detection limits (fmol)
C10	$C_{10}H_{20}O_2$	443.8	$Y = 20.50X + 75.51$	0.9996	21.3
C11	$C_{11}H_{22}O_2$	458.0	$Y = 20.46X + 98.94$	0.9990	18.9
C12	$C_{12}H_{24}O_2$	471.8	$Y = 21.80X + 93.08$	0.9994	17.6
C13	$C_{13}H_{26}O_2$	486.1	$Y = 24.47X + 99.96$	0.9997	19.1
C18:3	$C_{18}H_{30}O_2$	552.3	$Y = 30.88X + 135.29$	0.9991	20.8
C22:6	$C_{22}H_{32}O_2$	600.3	$Y = 28.65X + 61.54$	0.9995	22.2
C14	$C_{14}H_{28}O_2$	500.1	$Y = 21.80X + 102.10$	0.9994	17.9
C20:4	$C_{20}H_{32}O_2$	576.3	$Y = 26.67X + 106.41$	0.9995	24.4
C18:2	$C_{18}H_{32}O_2$	554.3	$Y = 30.48X + 146.07$	0.9992	16.9
C15	$C_{15}H_{30}O_2$	514.2	$Y = 21.58X + 55.01$	0.9993	21.5
C16	$C_{16}H_{32}O_2$	528.5	$Y = 28.65X + 125.51$	0.9997	21.6
C18:1	$C_{18}H_{34}O_2$	555.6	$Y = 34.31X + 76.53$	0.9993	23.1
C17	$C_{17}H_{34}O_2$	542.1	$Y = 29.33X + 113.73$	0.9998	19.6
C18	$C_{18}H_{36}O_2$	556.4	$Y = 24.32X + 76.03$	0.9994	18.7
C19	$C_{19}H_{38}O_2$	570.0	$Y = 22.03X + 116.71$	0.9993	22.6
C20	$C_{20}H_{40}O_2$	583.8	$Y = 40.01X + 116.71$	0.9992	19.6
C21	$C_{21}H_{42}O_2$	598.0	$Y = 23.40X + 71.62$	0.9997	21.7
C22	$C_{22}H_{44}O_2$	613.5	$Y = 24.38X + 50.78$	0.9995	20.1

X injected amount (pmol); Y peak area

an excess of BAETS. All fatty acids were quantified in the seed oil by using the external standard method with fluorescence spectra at 505 nm. The calibration curve for each BAETS fatty acid derivative was obtained by using linear regression and plotting peak area versus concentration, as shown in Table 2.

Determination of Antioxidant Activity

The seed oils obtained by SE and SC-CO_2 with the optimal conditions were selected to determine antioxidant activity by using a DPPH radical-scavenging assay. All experiments were repeated three times, and the antioxidant activity was given as mean ± standard deviations. This method [20] was used with slight modifications in order to assess the DPPH free-radical scavenging capacity of fenugreek seed oils. The DPPH radical absorbs at 517 nm, and when reduced by an antioxidant, the absorbance value decreases. Briefly, 0.05 g of seed oil was mixed with 10 ml of ethanol/petroleum ether (1:1 v/v), then 1-ml aliquots at different concentrations (1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mg/ml) of the seed oil mixture were added to 5 ml of 0.025 % DPPH (Sigma-Aldrich, St. Louis, MO, USA) in methanol. The mixture was shaken vigorously and incubated in the dark for 30 min and then placed in an UV759 spectrophotometer (Shanghai Precision & Scientific Instrument Co., Ltd, Shanghai, China) to monitor absorbance at 517 nm. The test was replicated three times. The radical-scavenging activities of samples, expressed as percentage of DPPH radical scavenging effect, were calculated according to Eq. (3) [21].

$$\text{DPPH radical scavenging effect} = \left(1 - \frac{A_i}{A_j}\right) \times 100\,\% \tag{3}$$

where A_i is the absorbance at 517 nm of the control (DPPH solution without seed oil), and A_j is the absorbance of the test solution.

Reducing Power Assay

The reducing power assay and a UV–Visible spectrophotometer (UV759, Shanghai Precision & Scientific Instrument Co., Ltd, Shanghai, China) at 700 nm wavelength were used according to the method of Ahmadi et al. [22]. The SC-CO_2 extract obtained under optimum conditions and SE extract were dissolved in absolute alcohol to obtain sample solutions at different concentrations (1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mg/ml). Each sample solution (2.5 ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1 % potassium ferrocyanate [$K_3Fe(CN)_6$] solution. The reaction mixture was incubated in a water bath at 50 °C for 20 min, and 2.5 ml of 10 % trichloroacetic acid solution was added to the mixture to stop the reaction. The mixture was then centrifuged at 3,000 rpm for 10 min. The supernatant was gathered and mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % ferric chloride solution. The absorbance was measured at

700 nm. The increase of absorbance is synonymous of an increase in power reduction. The assays were carried out in triplicate, and the results are expressed as means ± standard deviations.

Results and Discussion

Yield of Seed Oil

Extraction temperature, pressure, and time were used as input process variables in SC-CO_2 extraction. The SC-CO_2 extraction yield data (wet basis) under different conditions are presented in Table 3. The fenugreek seed oil yield ranged from 2.70 to 3.67 %. The highest yield (3.67 % w/w) was obtained at 25 MPa, 42.5 °C, and 140 min. Soxhlet extraction has been traditionally used to determine total oil. The yield of oil from SE was 4.08 % (see Table 3) at 70 °C and 10 h. This indicated that SC-CO_2 extraction was about 80 % of that of SE.

Regression Modeling of SC-CO_2 Extraction

The oil yields obtained from all the CCD experiments are shown in Table 3. The second-order polynomial equation that fitted the coded variables followed previous studies [15]. The regression model for the relationship between oil yield (Y) and the coded values of independent variables of pressure (X_1), temperature (X_2), and time (X_3) and their interactions is shown in the following equation:

$$Y = -15.4680 + 0.6079X_1 + 0.4057X_2 + 0.0362X_3 - 3.8540 \times 10^{-3}X_1X_2 + 4.0791 \times 10^{-4}X_1X_3 + 2.8917 \times 10^{-4}X_2X_3 - 8.9636 \times 10^{-3}X_1^2 - 3.2060 \times 10^{-3}X_2^2 - 1.2917 \times 10^{-4}X_3^2$$

The regression coefficients for the second-order polynomial model is given in Table 4 and indicated that the quadratic parameters were significant ($P < 0.01$). The model predicting oil yield was adequate as indicated by error analysis that showed non-significant lack-of-fit ($P > 0.05$). The regression model for oil yield was highly significant ($P < 0.01$). The value of R^2 (0.9773) indicated that the experimental data for fenugreek seed oil yield were in good agreement with the predicted values. The F value for lack of fit was also insignificant ($P > 0.05$), meaning that this model was sufficiently accurate for predicting the responses. The coefficient of variation (CV) was 2.78 %, which indicated that the model had good precision and reliability.

Response Surface Analysis

Multiple regression coefficients obtained by employing a least squares technique to predict a second-order

Table 3 Experimental scheme and results obtained from RSM for the oil yield ($\bar{x} \pm s$, $n = 3$)

No.	Pressure (X_1, MPa)	Temperature (X_2, °C)	Time (X_3, min)	Oil yield (%)
1	1 (30)	1 (50)	1 (120)	3.36 ± 0.22
2	1	1	−1 (60)	2.74 ± 0.21
3	1	−1 (35)	1	3.75 ± 0.31
4	1	−1	−1	2.70 ± 0.10
5	−1 (20)	1	1	3.33 ± 0.22
6	−1	1	−1	2.78 ± 0.13
7	−1	−1	1	2.97 ± 0.21
8	−1	−1	−1	2.33 ± 0.12
9	1.682 (33.4)	0	0	3.22 ± 0.31
10	−1.682 (16.6)	0	0	2.56 ± 0.33
11	0 (25)	1.682 (55.1)	0	3.22 ± 0.34
12	0	−1.682 (29.9)	0	2.81 ± 0.20
13	0	0 (42.5)	1.682 (140.5)	3.67 ± 0.41
14	0	0	−1.682 (39.5)	2.73 ± 0.12
15	0	0	0 (90)	3.57 ± 0.30
16	0	0	0	3.45 ± 0.22
17	0	0	0	3.56 ± 0.31
18	0	0	0	3.49 ± 0.31
19	0	0	0	3.59 ± 0.13
20	0	0	0	3.46 ± 0.22
SE	0	70	600	4.08 ± 0.23

Table 4 Regression coefficients of predicated second-order polynomial model for the response variable

Source	Sum of squares	Degree of freedom	Mean squares	*F* value	*P* value
Model	9	3.3286	0.3698	47.7984	<0.0001
Lack of fit	0.0590	5	0.0120	3.23	0.1121
Pure error	0.0180	5	0.0036		
Total error	3.4100	19			
R^2	0.9773	Coefficient of variation (C.V, %)		2.78	

Table 5 ANOVA analysis of predicated second-order polynomial model for the response variable

Variables	Degree of freedom	Sum of Squares	Mean squares	*F* value	*P* value
X_1	1	0.3725	0.3725	48.1460	<0.0001
X_2	1	0.0943	0.0943	12.1873	0.0058
X_3	1	1.4509	1.4509	187.5153	<0.0001
X_1X_2	1	0.1663	0.1663	21.4952	0.0009
X_1X_3	1	0.0300	0.0300	3.8709	0.0775
X_2X_3	1	0.0339	0.0339	4.3767	0.0629

X_1 pressure; X_2 temperature; X_3 time

polynomial model for the oil yield and the ANOVA analysis of predicated second-order polynomial model are summarized in Table 5. Pressure and time had greater effects on oil yield than did temperature, and the interaction of pressure and temperature had the most significant effects compared to other combinations of extraction parameters. The linear, quadratic, and interaction effects of the independent variables were the primary factors affecting oil yield. Three-dimensional shaded surfaces of second-order polynomial models were used to predict the interactive effects of operational parameters for SC-CO_2 seed oil (Figs. 3–5).

Figure 3 is the three-dimensional response surface (a) and contour plots (b) showing the effects of pressure and temperature on the oil yield. Extraction pressure was one of the main parameters that influenced the composition of the extract. At a given temperature, oil yield increased with increasing pressure, especially at low pressure and temperature. Pressure had a positive linear effect on oil yield at low-pressure levels. This was likely due to improved oil solubility resulting from increased carbon dioxide density as pressure increased. If the temperature was greater than about 44 °C while the pressure was rising, seed oil yield increased much less. Once the pressure reached approximately 28 MPa, the oil yield decreased slightly. This was reflected in the plateau for oil yield at pressures exceeding 28 MPa or temperature exceeding 44 °C.

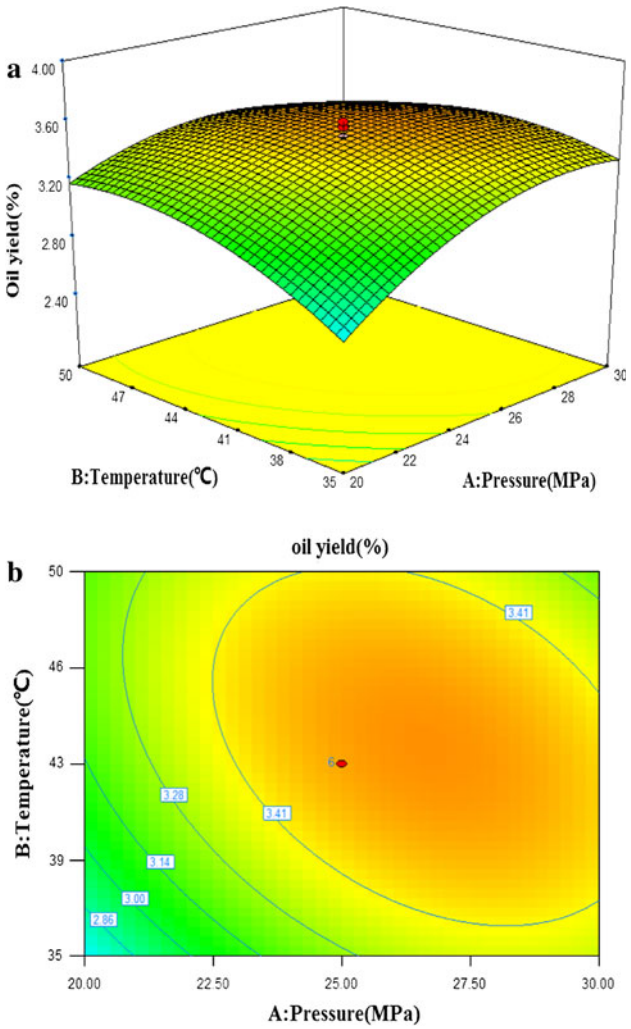

Fig. 3 Response surface (**a**) and contour plots (**b**) for the effect of temperature and pressure on the oil yield

Figure 4 is the three-dimensional response surface (a) and contour plots (b) showing the effects of pressure and extraction time on oil yield. Oil yield increased significantly with greater extraction times. From 60 to 120 min, seed oil yield increased substantially. At high pressure it increased less, and this may have been due to the fact that the seed oil was completely extracted. Although prolonging the extraction time increased oil yield, increasing time leads to higher costs and is not conducive to industrial production. Therefore, we must select a suitable time for SC-CO_2 extraction of fenugreek seed oil.

The effects of temperature on SC-CO_2 extraction were more difficult to determine than those of pressure because of two opposing effects on oil yield. First, the increase in temperature decreased the density of CO_2, leading to reduced solvent power. Second, the increase in temperature

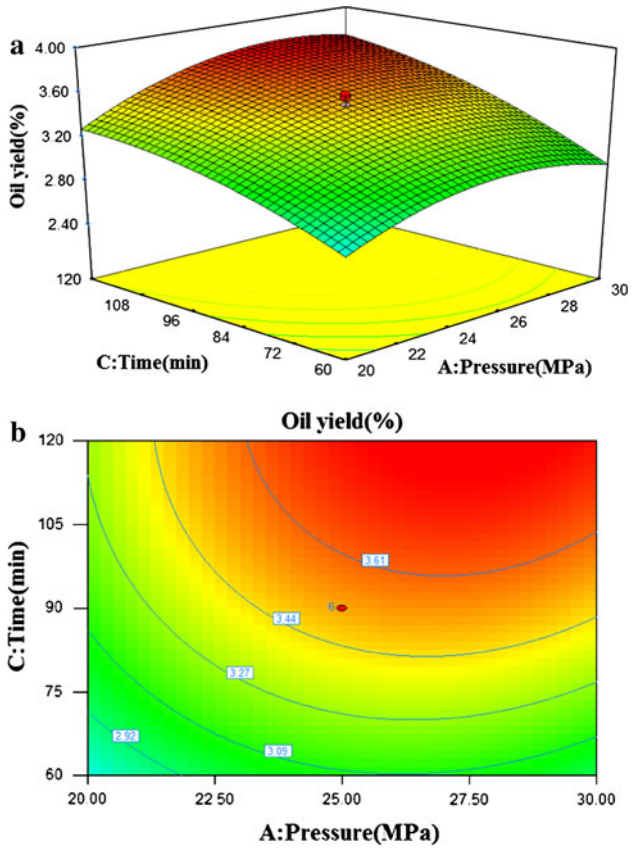

Fig. 4 Response surface (**a**) and contour plots (**b**) for the effect of pressure and extraction time on the oil yield

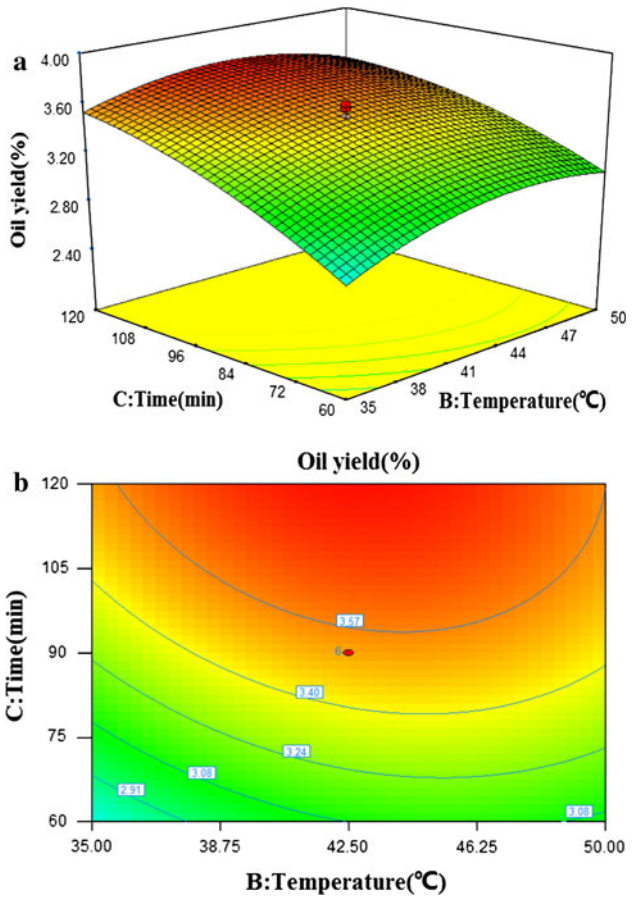

Fig. 5 Response surface (**a**) and contour plots (**b**) for the effect of temperature and extraction time on the oil yield

increased the vapor pressure of the solutes, thereby increasing oil solubility in SF-CO_2 [15]. Figure 5 shows the effects of temperature and extraction time on oil yield. Figure 5a shows that temperature was also a very important factor in extracting seed oil from fenugreek by SC-CO_2. Increasing oil yield with increasing temperature early in extraction was observed because the change of CO_2 density was less effective than that of solute vapor pressure. However, the trend reversed when temperature reached about 43.5 °C. Since the solubility of lipid largely depends on the balance between fluid density and solute vapor pressure, both must be controlled by fluid pressure and temperature.

Optimization and Verification of Optimized Models

The SC-CO_2 extraction conditions were considered optimum when oil yield reached the maximum value. Optimization was carried out by superimposing the contour plots for the oil yield (Y). The contour plot derived from the canonical analysis showed ellipsoidal contours at the maximum point. The center of these concentric circles was the optimum zone in which every point represented a combination of extraction parameters going maximum oil yield. According to the transforming equation of coded value, the optimum conditions were 28.4 MPa, 41.3 °C, and 118 min. Under these conditions, the maximum predicated value for oil yield was 3.78 %. In order to facilitate operation, process parameters were changed slightly, but oil yield was affected very little. Temperature of 41 °C, pressure of 28.5 MPa, and extraction time of 118 min are suitable considering the factors involved. The predicted values from the models were reasonably close to observed values.

Chemical Composition of Fenugreek Seed Oil

The linear regressions and detection limits of 18 fatty acids are shown in Table 2. The fenugreek seed oil obtained by SE and SC-CO_2 extraction gave similar HPLC chromatographic patterns. The fatty acid compositions of fenugreek seed oil extracted by SE and SC-CO_2 extraction are shown in Table 6.

The fatty acids from fenugreek seed oil extracted by SC-CO_2 and SE were mainly composed of C18:3, C18:2, C16, C18:1, C18, C20, and C22 (Fig. 6; Table 6). The lipids

Table 6 Fatty acids composition and content of fenugreek seed oil extracted by SFE and SE (mg/100 mg seed oil, $n = 3$)

Fatty acids	C18:3	C18:2	C16	C18:1	C18	C20	C22	UFA	PUFA	SFA
SFE[a]	28.31 ± 0.47	33.43 ± 0.79	9.89 ± 0.41	8.14 ± 0.58	3.68 ± 0.63	0.71 ± 0.15	0.61 ± 0.12	69.88 ± 1.84	61.74 ± 1.26	14.28 ± 1.31
SE[b]	9.91 ± 0.59	17.56 ± 0.65	6.40 ± 0.25	4.78 ± 0.17	2.22 ± 0.23	0.40 ± 0.11	0.41 ± 0.10	32.25 ± 1.41	27.47 ± 1.24	9.43 ± 0.69

UFA unsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *SFA* saturated fatty acids

[a] SFE supercritical fluid extraction (33.4 MPa, 42.5 °C, and 90 min)

[b] SE Soxhlet extraction (80 °C and 600 min)

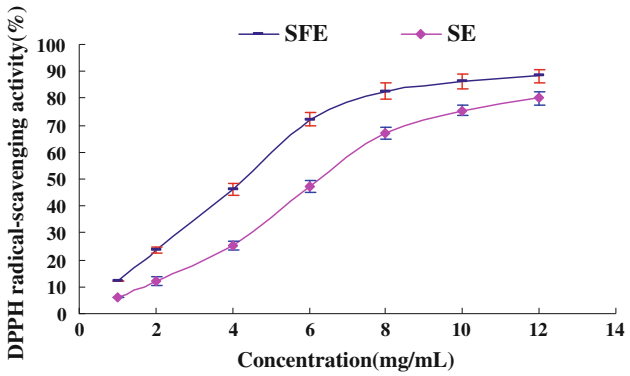

Fig. 6 Antioxidant activity of seed oil from fenugreek seed assessed by a DPPH radical-scavenging assay

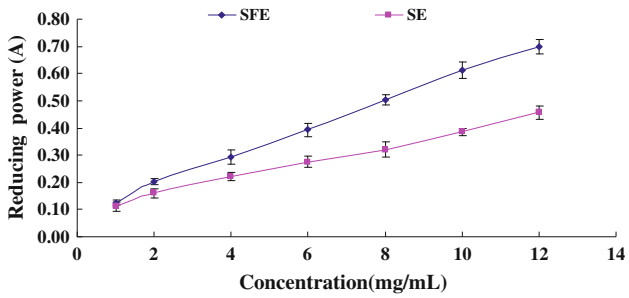

Fig. 7 Reducing power of various concentrations of SFE and SE extracts from fenugreek seed

contain abundant UFA in high to low concentrations as follows: linoleic acid (18:2), linolenic acid (18:3), and oleic acid (18:1). Fenugreek oil is rich in UFA (nearly 70 % of the total fatty acids), and the PUFAs constitute 61.7 % of the fatty acids. Furthermore, both UFA and PUFA contents were higher than by SE. The oil obtained by SC-CO_2 extraction was of better quality than that obtained by SE.

Antioxidant Activity

The DPPH radical-scavenging test is a commonly employed assay in antioxidant studies [23]. The antioxidant effects of plant extracts on DPPH radical scavenging may be due to their hydrogen-donating abilities, which reduce the stable violet DPPH radical to the yellow DPPH-H [21]. As shown in Fig. 6, the values for fenugreek seed oil ranged from 12.5 to 88.4 % when the concentrations varied from 1 to 12 mg/ml. Moreover, reduced extract concentration, reduced antioxidant activity, and the antioxidant activity of the extract were concentration-dependent.

The reducing power of various concentrations of the two fenugreek extracts is presented in Fig. 7. In this assay, the color of the test solution varies from yellow to different forms of green and blue, depending on the reducing power of each extract. Increasing absorbance at 700 nm indicates increased reducing ability. As shown in Fig. 7, the values for fenugreek seed oil ranged from 0.125 to 0.698 when the concentrations varied from 1 to 12 mg/ml. The reducing power of the SC-CO_2 and SE extracts increased with concentration. The SC-CO_2 extracts proved to be better sources of antioxidants than SE extract.

Conclusions

The best conditions for SC-CO_2 to extract fenugreek seed oil were 41 °C temperature, 28.5 MPa pressure, and 118 min extraction time, and the fenugreek seed oil contained approximately 60 % PUFA with abundant amounts of linoleic (18:2), linolenic (18:3), and oleic acids (18:1). The antioxidant activity of SC-CO_2 was concentration-dependent and higher than that of the SE extract. Therefore, SC-CO_2 represents a valuable alternative to the traditional extraction methods for the efficient extraction of fenugreek seed oil. The present investigation indicated that the oil extracts of fenugreek seed may have potential as food additives and health-promoting antioxidant agents in human diets.

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