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ORIGINAL PAPER

Compositional and Antioxidant Activity Analysis of *Zanthoxylum bungeanum* Seed Oil Obtained by Supercritical CO_2 Fluid Extraction

Lian Xia · Jinmao You · Guoliang Li · Zhiwei Sun · Yourui Suo

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Abstract Supercritical CO_2 fluid extraction (SFE-CO_2) of *Zanthoxylum bungeanum* (*Z. bungeanum*) seed oil was investigated. To optimize the SFE process, three-level Box-Behnken factorial design and response surface methodology (RSM) were applied to optimize the extraction conditions, including pressure, temperature and amount of modifier. The optimum conditions were as follows: extraction pressure, 29.28 MPa; extraction temperature, 41.19 °C; and the added amount of modifier, 10.94%. The experimental results showed that the maximum extraction yield was $21.85 \pm 0.23\%$ ($n = 3$) under the proposed conditions. The compositional analysis of *Z. bungeanum* seed oil was performed by HPLC-FLD-MS using a new labeling reagent of 2-(11*H*-benzo[a]carbazol-11-yl)-ethyl-4-methyl benzenesulfonate (BCETS). The results indicated that the *Z. bungeanum* seed oil contained mainly unsaturated fatty acids, including C18:3, C22:6, C20:4, C18:2, C18:1 and C20:1, which accounted for 84.0% (mass percentage) of the total amount. The antioxidant activity of seed oil obtained by Box-Behnken design concerning the DPPH radical was investigated, and this indicated that the pressure and the amount of added modifier had positive effects on the antioxidant activity, but the effect of the temperature elevation is complicated, depending on the nature of the extracted contents.

Keywords Supercritical CO_2 fluid extraction · *Zanthoxylum bungeanum* seed oil · Fatty acids composition · HPLC-FLD-MS · Antioxidant activity

L. Xia · J. You · G. Li · Z. Sun · Y. Suo
Northwest Plateau Institute of Biology,
The Chinese Academy of Sciences, Xining 810001, China

L. Xia · J. You (✉)
Key Laboratory of Life-Organic Analysis of Shan Dong Province, College of Chemistry Science,
Qufu Normal University, Qufu 273165, China
e-mail: Jmyou6304@163.com

L. Xia · G. Li · Z. Sun
Graduate University of Chinese Academy of Sciences,
Beijing 100049, People's Republic of China

Introduction

The plants of *Zanthoxylum* (Rutaceae) are aromatic trees and shrubs, native to warm temperate and subtropical areas worldwide. In Asia, this genus is often found in the Himalayan region and additionally in Central, South, Southeast and East Asia [1]. The fruits of *Zanthoxylum*, called "Huajiao" in Chinese, and their pericarps are especially used as a peppery spice in indigenous kitchens in China and as a folk medicine for the treatment of stomachache, toothache, abdominal pain, ascariosis, diarrhea and dysentery [2]. It can also be used to promote digestion and as a topical antibacterial agent for treatment of infected wounds [3]. Chang et al. [4] reported that the chloroform-soluble part of *Zanthoxylum* showed significant activity of anti-HBV DNA replication. The essential oils have shown anthelmintic effects [5]. Unfortunately, the seeds of *Zanthoxylum* as a byproduct are often used as fertilizer or fuel, and have an estimated annual production potential of 1 million metric tons in China. The fruits of *Zanthoxylum bungeanum* (*Z. bungeanum*) Maxim are the most popular huajiao commercial product, called "da hong pao" (big red robe). This species is native to southwestern China in the provinces Sichuan, Yunnan, Guizhou, Tibet, Guanxi and Guandong. The Hanyuan area in the province of Sichuan is well-known for producing the best quality da hong pao.

Yamazaki et al. [6] reported that methanol extracts of *Z. piperitum DC* (Japanese pepper) fruit possessed notable antioxidant activity. Their results also demonstrated that the higher amounts of polyphenols and flavonoids in the seeds of *Z. piperitum DC* may contribute to the stronger antioxidant activity of the fruits. However, most parts of *Zanthoxylum* fruits are seeds that are rich in oil, and the large amount of unsaturated fatty acids may also possess potentially notable antioxidant activity. To the best of our knowledge, studies on the chemical composition of seed oil from the Chinese pepper of *Z. bungeanum* and its antioxidant activity have not been reported yet.

Supercritical CO_2 fluid extraction (SFE-CO_2), offering numerous potential advantages over conventional extraction processes, has been widely used for seed oil extractions [7]. For possible industrial applications, the optimization and assessment of the extraction process with mathematical modeling seem to be essential. In classical methods, process parameters are optimized by conducting experiments concentrating on one factor at a time, which is troublesome and time-consuming, and also ignores the interaction effect of parameters. Compared to the classical methods, response surface methodology (RSM) is more efficient, requires fewer data and provides interaction effects on the response. It has been extensively applied to optimize processing parameters in the production of food, drugs, etc. [8, 9].

Quantitative determination of the fatty acid composition is of vital industrial importance and is helpful to control the quality of seed oil. In most cases, LC with UV-vis detection is the prevailing technique. However, fatty acid molecules show neither natural UV absorption nor fluorescence; therefore, derivatization of these analytes with labeling reagents, especially for the sensitive fluorescent detection, has been widely adopted.

The aims of this work are: (1) to optimize the SFE conditions, including extraction pressure, extraction temperature, the added amount of modifier and the interaction effect of the parameters using RSM; (2) to determine the fatty acid composition of *Z. bungeanum* seed oil by HPLC-FLD-MS using 2-(11*H*-benzo[a]carbazol-11-yl) ethyl 4-methyl benzenesulfonate (BCETS) as a new labeling regent; (3) to investigate the effect of the extraction condition on the antioxidant activity of the oils obtained by Box-Behnken design.

Materials and Chemicals

Plant Material

Seeds of *Z. bungeanum* were collected from Hanyuan county, Sichuan province, China, in September 2009 and authenticated by Prof. Chang-Fan Zhou at the Northwest Plateau Institute of Biology, Chinese Academy of Sciences. The seeds were ground in a rotary mill and then sieved (20–30 mesh).

Chemicals

Chromatograph-grade acetonitrile and ethanol were from Jining Chemical Reagent Co. (Shandong, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and all fatty acid standards were purchased from Sigma Reagent Co. (St. Louis, MO). CO_2 (99.99% purity), contained in a cylinder with an eductor tube, was obtained from Jinan Gas Co. (Shandong, China). 2-(11*H*-Benzo[a]carbazol-11-yl) ethyl-4-methylbenzenesulfonate (BCETS) was synthesized in our laboratory. The synthesis of BCETS is reported elsewhere. All other reagents were of analytical grade unless otherwise stated.

Experimental Procedures

Supercritical CO_2 Fluid Extraction

The extraction measurements were performed on an SFE121-50-1 device (Hua'an Supercritical Fluid Extraction Corp., Nantong, China). The schematic flow diagram was described in detail in a previous study [10].

A series of trials were carried out with varying conditions of pressure, temperature and added amounts of modifier. Table 1 shows the specified extraction conditions. Each extraction run lasted for 90 min; after that, the oil was recovered in the first separator (set at 60 °C and 7 MPa) and weighed to obtain the yield, while modifier and volatile compounds were collected in the second separator (set at 20 °C and 4–6 MPa).

Experimental Design and Statistical Analysis

The single-factor experimental designs (extracting pressure, extracting temperature, extracting time and the added amount of modifier) were carried out before RSM experiments (data not shown). The extraction was then carried out at a constant flow rate of 25 l/h. Based on single-factor designs, a three-variable, three-level Box-Behnken design was applied to optimize the extraction conditions in order to obtain high oil recovery of *Z. bungeanum*. The three independent variables set were extraction pressure (MPa, X_1), extraction temperature (°C, X_2) and amount of modifier (%, X_3). Each variable was set at the three levels, and a total of 17 experiments were designed (Table 1). Each experiment was performed in triplicate, and the average oil

Table 1 Experimental matrix and values of observed responses (including extraction yield and antioxidant activity)

Run	Pressure (MPa, X_1)	Temperature (°C, X_2)	Modifier (%, X_3)	Extraction yield (%)		Inhibition percentage (%)
				Experimental	Predict	
1	22.5 (0)	30 (−1)	0 (−1)	11.66	11.54	81.5
2	30 (+1)	50 (+1)	7.5 (0)	16.83	17.61	83.2
3	22.5 (0)	30 (−1)	15 (+1)	11.61	12.40	83.7
4	30 (+1)	40 (0)	0 (−1)	18.45	18.46	83.3
5	22.5 (0)	40 (0)	7.5 (0)	19.72	19.07	82.1
6	22.5 (0)	50 (+1)	0 (−1)	10.59	9.80	81.6
7	15 (−1)	40 (0)	0 (−1)	4.91	5.80	78.1
8	22.5 (0)	40 (0)	7.5 (0)	18.82	19.07	82.6
9	22.5 (0)	40 (0)	7.5 (0)	17.98	19.07	82.1
10	15 (−1)	30 (−1)	7.5 (0)	5.28	4.50	81.6
11	15 (−1)	40 (0)	15 (+1)	9.91	9.90	81.9
12	30 (+1)	30 (−1)	7.5 (0)	16.49	16.59	84.4
13	15 (−1)	50 (+1)	7.5 (0)	5.53	5.42	82.3
14	30 (+1)	40 (0)	15 (+1)	22.39	21.51	85.3
15	22.5 (0)	40 (0)	7.5 (0)	19.15	19.07	82.9
16	22.5 (0)	50 (+1)	15 (+1)	15.97	16.08	84.2
17	22.5 (0)	40 (0)	7.5 (0)	19.66	19.07	82.2

(−1) Low level; *(0)* medium level; *(+1)* high level

recovery (%) was taken as the response, Y. Regression analysis was performed for the experiment data and was fitted into the empirical second-order polynomial model, as shown in the following equation:

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

where β_0, β_i, β_{ii} and β_{ij} were regression coefficients in the intercept, linear, quadratic and interaction terms, respectively; X_i and X_j were the independent variables. A software Design-Expert 7.1.3 Trial (State-Ease, Inc., Minneapolis, MN) was used to obtain the coefficients of the quadratic polynomial model. The quality of the fitted model was expressed by the coefficient of determination R^2, and its statistical significance was checked by an F test.

Fatty Acid Composition Analysis

Preparation of Standard Solutions

BCETS solution (2.0×10^{-3} mol/l) was prepared by dissolving 8.32 mg BCETS in 10 ml anhydrous acetonitrile prepared by distilling HPLC grade acetonitrile dried with P_2O_5. Individual stock solutions of the fatty acids (1.0×10^{-3} mol/l) were prepared in ACN/DMF (1:1, v/v) and diluted to a concentration of 5.0×10^{-5} mol/l with the same solvent composition. When not in use, all reagent solutions were stored at 4 °C in a refrigerator.

Saponification of Seed Oil

To a 10-ml test tube, 0.1 g seed oil and 2.0 ml potassium hydroxide/methanol solution (2 M) were added. After being sealed, the test tube was immersed in a water bath at 90 °C for 2 h. After cooling, the contents were transferred into a centrifugal test tube, to which 2 ml water was added, and pH was adjusted to 7.0 with 2 M hydrochloric acid solution. This solution was extracted with chloroform three times (3 ml × 3). The combined chloroform was filtered and evaporated under a stream of nitrogen. The residue was re-dissolved in 50 ml DMF, filtered through a 0.2-mm nylon membrane filter and stored at −10 °C until derivatization.

Derivatization of Fatty Acids

To a solution containing 20 µl of a standard fatty acid mixture (or hydrolysis solution of the above) in a vial, 130 µl reagent solution, 10 mg K_2CO_3 and 200 µl DMF were added, respectively. The vial was sealed and placed in a water bath at 80 °C, and shaken at 5-min intervals for 30 min. After the reaction was completed, the mixture was cooled to room temperature. Then, 200 µl DMF was added to dilute the derivatization solution. The diluted solution (10 µl) was injected directly into the HPLC system. The derivatization procedure is shown in Fig. 1.

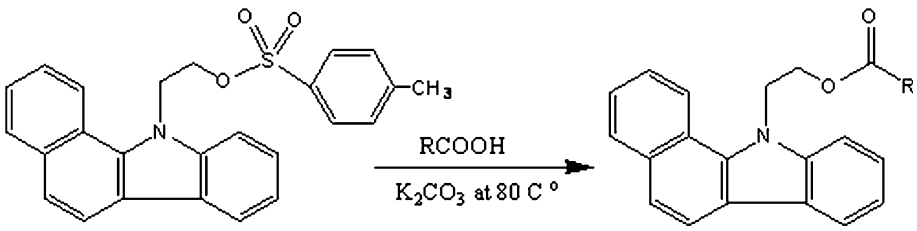

Fig. 1 Derivatization scheme of BCETS with fatty acids

Separation of Fatty Acid Derivatives with HPLC

HPLC separation of fatty acid derivatives was carried out on a reversed-phase Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent) with a gradient elution. Eluent A was water, B was a mixed solvent of ACN and DMF (1:1, v/v), and C was acetonitrile (100%). The flow rate was constant at 1.0 ml/min, and the column temperature was set at 30 °C. The injection volume was 10 μl. The fluorescence excitation and emission wavelengths were set at λ_{ex} 279 nm and λ_{em} 380 nm, respectively. The gradient elution programs were as follows: 45–10% A, 50–80% B, 5–10% C from 0 to 30 min; 10–3% A, 80–87% B and C remained at a constant flow of 10% from 30 to 40 min; 3–2% A, 87–88% B, and C also remained at a constant flow of 10% from 40 to 50 min; 2–0% A, 88–85% B, 10–15% C from 50 to 70 min.

Derivative Identification with MS/APCI

Prior to its use, the HP1100 LC/MSD-SL was checked to ensure that it met the sensitivity requirements defined by the manufacturer. The DAD and FLD were calibrated and tested using the appropriate diagnostic procedure of the ChemStation software for the HP1100 system. The HP1100 LC/MSD-SL was calibrated with an APCI tuning solution (Agilent Technology, Palo Alto, CA). The mass spectrometer was calibrated to ensure that the mass accuracy specifications and sensitivity were achieved over the entire mass range. The APCI source and instrument parameters were optimized by infusing the derivatives isolated from an HPLC column with FLD detection, and into the post-column, on-line mass spectrometer. The ionization and fragmentation of the isolated derivative were studied by mass spectrometry with APCI in positive-ion detection mode.

Quantitative Analysis

Quantitative conversion of fatty acids in seed oil to their BCETS derivatives was guaranteed by using an excess of BCETS. All fatty acids in seed oil were quantified using the external standard method with detection fluorescence spectra at 380 nm. The calibration curves for each BCETS fatty acid derivative were obtained by linear regression plotting peak area versus concentration.

Tocopherols, Saponification Value and Unsaponifiable Matter Analysis

The determination was completed according to the GB/T 12388-1990 "Method for determination of retinol and tocopherol in food," GB/T 5534-1995 "Animal and vegetable fats and oils–determination of saponification value" and GB/T 5535.2-1998 "Animal and vegetable fats and oils–Determination of unsaponifiable matter—Part 2: method using hexane extraction."

Analysis of Antioxidant Activity

The antioxidant activity was assessed by using DPPH radical-scavenging assay. The scavenging activity of seed oil towards the DPPH radical was measured by the method of Amarowicz et al. [11] with slight modification. All tests were carried out in triplicate.

In brief, 0.1 ml of seed oil was added to 2.4 ml of 0.0004% DPPH in ethanol. The mixture was then shaken vigorously and left in darkness for 60 min. Finally, the absorbance of the mixture was measured at 515 nm. Ascorbic acid (4 mg/ml) was used for reference. Antioxidant activity of the sample was calculated by:

$$\text{Inhibition percentage } (\%) = 100\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}$$

where A_{control} and A_{sample} are the absorbance values of the blank and tested samples.

Results and Discussion

Response Surface Analysis and Optimization of SFE Conditions

The SFE-CO_2 conditions for *Z. bungeanum* seed oil were optimized using different parameters by the combinations of the Box-Behnken design (3^3factorial). Table 1 presents the experiment design and corresponding response data for the oil extraction yield. The independent and dependent variables were analyzed to obtain a regression equation that could predict the response within the given range. The predicted second-order polynomial model was:

$$Y = 19.07 + 6.07X_1 + 0.48X_2 + 1.79X_3 + 0.023X_1X_2 - 0.26X_1X_3 + 1.36X_2X_3 - 3.29X_1^2 - 4.75X_2^2 - 1.86X_3^2$$

The regression coefficients of the intercept, linear, quadratic and interaction terms of the model were calculated using the least square technique and are presented in Table 2. Obviously, two linear parameters (pressure and the added amount of modifier), one interaction parameter (the interaction of temperature and the added amount of modifier) and all quadratic parameters were found to be significant at the level of $p < 0.05$ or $p < 0.01$, whereas the linear parameter of temperature and the other two interaction parameters (the interaction of pressure and temperature and the interaction of pressure and the added amount of modifier) were insignificant ($p > 0.1$). The results indicated that the pressure and the added amount of modifier were the major contributing factors to the oil extraction yield.

The analysis of variance (ANOVA) for the experimental results of the Box-Behnken design is shown in Table 2. The value of R^2 (0.9879) revealed that the experimental data were in good agreement with the predicted values of the yield of seed oil. The value of adj-R^2 (0.9724) suggested that the total variation of 97.24% for the yield of seed oil was attributed to the independent variables, and only about 2.76% of the total variation could not be explained by the model.

Lack of fit was an indication of the failure of a model that represented the experimental data at the points that were not included in the regression or variations in the models that cannot be accounted for by random error. If there was a significant lack of fit that could be indicated by a low probability value, the response predictor was discarded [12]. The F value for the lack of fit was insignificant ($p > 0.05$), meaning that this model was sufficiently accurate for predicting the relevant responses. The coefficient of variation (CV) of <6.48% indicated that the model was reproducible [13].

To further validate optimal values, the first partial derivative of the regression equation was taken and made to be zero. Calculating the equation gave the following results: $X_1 = 0.905$, $X_2 = 0.119$ and $X_3 = 0.459$. The optimal values of the variables given by the software were as follows: extraction pressure, 29.28 MPa; extraction temperature, 41.19 °C; and the added amount of modifier, 10.94%. Under these proposed conditions, the model gave the predicted value of Y (the extraction yield) as 22.25%.

To compare the predicted result with the practical value, experimental validation was performed using the optimized conditions, and the mean oil yield was 21.85 ± 0.23% ($n = 3$). The good correlation between these results confirmed that the response model was adequate to reflect the expected optimization.

Fatty Acid Composition Analysis

The fatty acid composition of the seed oil obtained under optimum SFE-CO_2 conditions was determined and quantified by HPLC-FLD-MS with pre-column derivatization.

Table 2 Estimated regression coefficients for the quadratic polynomial model and ANOVA for the experimental results

Parameter	Estimated coefficients	Standard error	DF*	Sum of squares	F value	Prob > F
β_0	19.07	0.42	1	499.88	63.68	<0.0001*
Linear parameters						
β_1	6.07	0.33	1	294.54	337.69	<0.0001*
β_2	0.48	0.33	1	1.87	2.15	0.1862
β_3	1.79	0.33	1	25.49	29.23	<0.0010*
Interaction parameters						
β_{12}	0.023	0.47	1	2.16E-003	2.48E-003	0.9617
β_{13}	−0.26	0.47	1	0.28	0.32	0.5885
β_{23}	1.36	0.47	1	7.35	8.43	0.0229
Quadratic parameters						
β_{11}	−3.29	0.46	1	45.51	52.18	0.0002*
β_{22}	−4.75	0.46	1	94.94	108.85	<0.0001*
β_{33}	−1.86	0.46	1	14.59	16.73	0.0046*
Residual			7	6.11		
Lack of fit			3	4.08	2.68	0.1824
Pure error			4	2.03		
R^2	0.9879		Adjusted R^2	0.9724		
CV (%)	6.48					

* Significance with $p < 0.01$

The BCETS-fatty acid derivatives were studied by mass spectrometry (MS) with the APCI ion source in positive ion detection mode. As expected, the BCETS-fatty acid derivative produced an intense molecular ion peak at *m/z* $[M + H]^+$. With MS-MS analysis of fatty acid derivatives, the collision-induced dissociation spectra of *m/z* $[M + H]^+$ produced the specific fragment ions at *m/z* $[M' + CH_2CH_2]^+$ and *m/z* 216.6 (M′: corresponding molecular mass of the fatty acids). The specific fragment ion at *m/z* 216.6 was from the protonated molecular core structure moiety. The specific fragment ions at *m/z* $[M' + CH_2CH_2]^+$ corresponded to the protonated fatty acid moiety. The selected reaction monitoring, based on the *m/z* $[M + H]^+ \rightarrow m/z$ $[M' + CH_2CH_2]^+$ and *m/z* 216.6 transition, was specific for fatty acid derivatives. In most cases, the collision-induced dissociation spectra of *m/z* $[M + H]^+$ for the unsaturated fatty acid derivatives produced specific fragment ions by losing H_2O molecules, giving the ion at *m/z* $[MH–H_2O]^+$, which was a specific fragment ion for the identification of unsaturated fatty acid derivatives. The MS-MS analysis and corresponding cleavage mode for a representative BCETS-$C_{18:1}$ derivative are shown in Fig. 2a–c. MS and MS-MS data for all the fatty acid derivatives are shown in Table 3.

The chromatogram of fatty acid standards is shown in Fig. 3a, and the chromatogram for the analysis of fatty acids of the seed oil samples under optimal SFE conditions is shown in Fig. 3b. The calibration graph was established with the peak area (*y* axis) versus the fatty acid concentration (*x* axis: pmol, injected amount) (see Table 3). The fatty acid contents of the seed oil are shown in Table 4.

Moreover, the validation of the method was investigated. The relative standard deviations (RSDs) of peak areas and retention times were 0.04–0.51 to 0.15–2.68%,

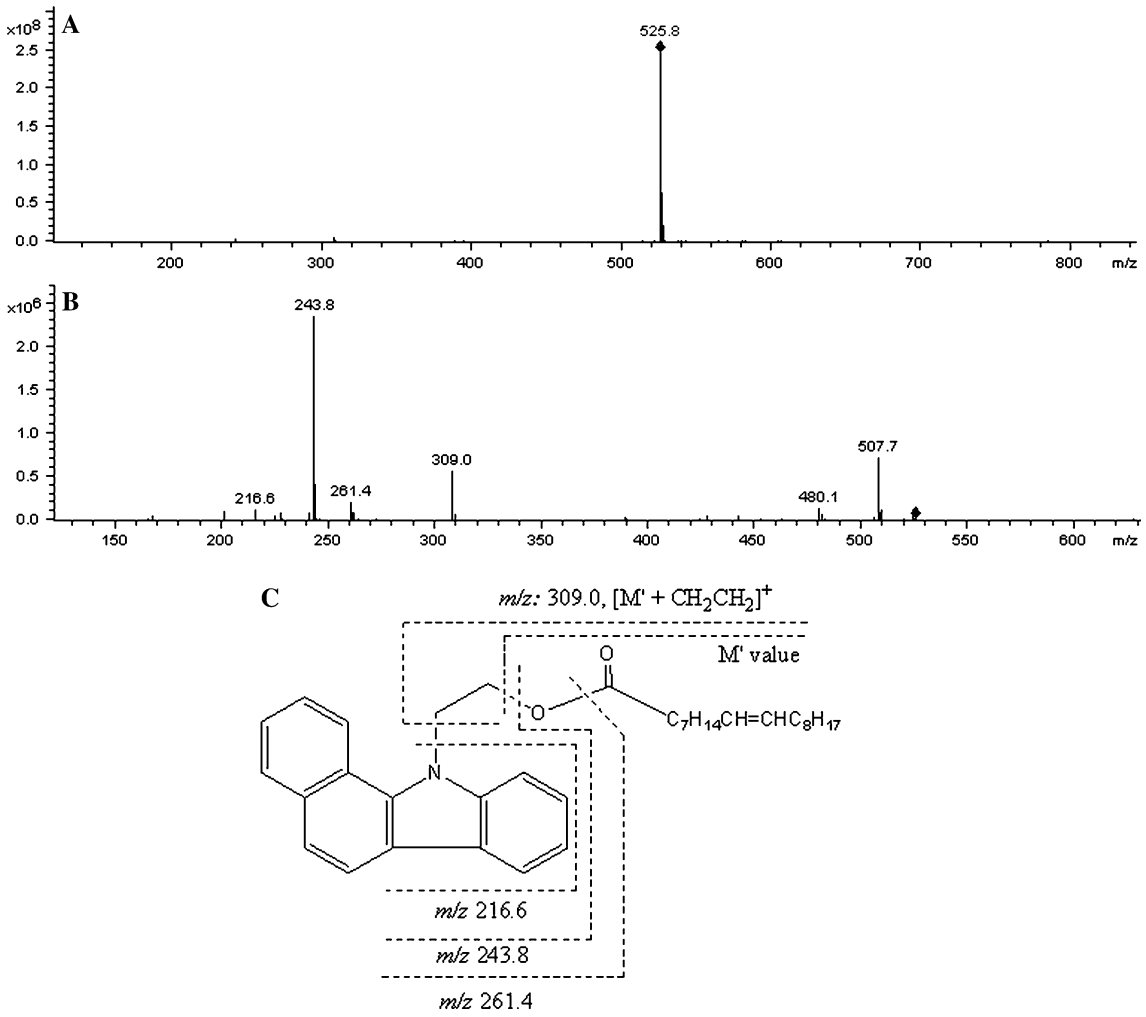

Fig. 2 The profile of the molecular ion chromatogram and scanning of the isolated representative n-C18:1 acid derivative (BCETS-C18:1). **a** Typical LC-MS profile of n-C18:1 acid derivative (BCETS-C18:1) from the full scanning range from 100 to 800 amu with APCI in positive-ion detection mode. **b** Typical APCI-MS-MS profile of n-C18:1 acid derivative (BCETS-C18:1) from full scanning range from 100 to 600 amu with APCI in positive-ion detection mode. **c** The MS-MS cleavage mode of BCETS-C18:1 derivative

Table 3 Linear regression equations, correlation coefficients, detection limits, repeatability and MS data of BCETS fatty acid derivatives

Fatty acids	Regression equation	Detection limit/fmol	Correlation coefficients	RSD% ($n = 6$)		Molecular ion $[MH]^+$	Characteristic fragment ions m/z $[M' + CH_2CH_2]^+$
				Retention time	Peak area		
C5	$Y = 1.50X + 2.10$	20.79	0.9998	0.21	0.47	346.2	216.6, 129.2
C6	$Y = 1.18X - 1.07$	23.67	0.9997	0.18	0.70	360.2	216.6, 133.2
C7	$Y = 1.21X + 5.32$	17.07	0.9994	0.13	0.59	374.1	216.8, 157.6
C8	$Y = 1.05X + 7.02$	32.06	0.9999	0.10	0.58	388.3	216.6, 171.3
C9	$Y = 0.84X + 5.16$	20.25	0.9997	0.09	0.49	402.3	216.6, 185.4
C10	$Y = 0.90X + 4.23$	19.76	0.9995	0.073	0.40	416.3	216.8, 199.5
C11	$Y = 0.89X - 1.93$	20.25	0.9994	0.084	0.50	430.1	216.7, 213.4
C12	$Y = 0.93X + 0.38$	18.92	0.9998	0.062	0.47	444.3	216.8, 227.3
C20:5	$Y = 1.19X + 2.90$	16.32	0.9996	0.061	0.38	546.1	216.6, 328.6, 528.2
C13	$Y = 0.60X + 3.47$	34.19	0.9994	0.056	0.22	458.3	216.6, 231.2
C18:3	$Y = 1.23X + 3.60$	15.65	0.9996	0.059	0.25	521.9	215.6, 304.9, 503.9
C22:6	$Y = 1.09X + 4.20$	16.19	0.9998	0.059	0.30	572.1	215.4, 354.9, 553.8
C14	$Y = 0.74X + 4.49$	20.79	0.9994	0.044	0.27	472.3	216.4, 255.6
C20:4	$Y = 1.08X + 2.65$	15.18	0.9999	0.072	0.33	547.9	217.0, 330.9, 529.9
C18:2	$Y = 1.42X - 5.38$	14.26	0.9996	0.037	0.27	523.9	216.6, 306.7, 516.0
C15	$Y = 0.87X - 1.81$	12.06	0.9994	0.041	0.19	486.3	216.8, 269.6
C16	$Y = 1.26X - 3.36$	10.79	0.9994	0.035	0.11	500.2	216.7, 283.5
C18:1	$Y = 1.94X - 3.47$	12.32	0.9994	0.029	0.14	525.8	216.7, 309.0, 507.7
C17	$Y = 0.86X - 3.63$	12.06	0.9998	0.027	0.19	514.4	216.7, 297.1
C18	$Y = 0.92X - 2.24$	12.06	0.9999	0.019	0.18	528.3	216.5, 311.3
C20:1	$Y = 1.14X - 2.76$	15.34	0.9995	0.037	0.20	553.9	216.5, 337.4, 536.0
C19	$Y = 0.82X - 4.19$	10.79	0.9996	0.021	0.19	542.4	216.6, 325.1
C20	$Y = 0.74X + 0.01$	14.65	0.9997	0.045	0.40	556.3	216.5, 339.4
C22:1	$Y = 0.76X - 1.56$	18.74	0.9998	0.088	0.52	582.6	216.8, 365.6, 564.3
C21	$Y = 0.97X - 2.09$	15.78	0.9999	0.072	0.88	570.3	216.8, 353.2
C22	$Y = 0.67X - 2.45$	16.34	0.9997	0.095	1.25	584.1	216.6, 367.5
C24:1	$Y = 0.54X + 0.87$	20.48	0.9996	0.18	1.14	610.5	216.6, 393.3, 592.1
C23	$Y = 1.03X - 5.89$	28.05	0.9994	0.12	1.38	598.4	216.7, 371.2
C24	$Y = 1.01X - 2.48$	24.75	0.9999	0.19	1.92	612.3	216.6, 395.4
C25	$Y = 1.08X - 11.64$	25.62	0.9996	0.24	2.30	626.5	216.8, 409.1
C26	$Y = 0.91X - 4.33$	26.79	0.9997	0.23	2.96	640.3	216.6, 423.0
C27	$Y = 0.87X - 4.49$	27.13	0.9996	0.25	2.23	654.6	216.6, 437.2

respectively. In the calibration graph, all of the fatty acids provided excellent linear responses, with correlation coefficients of >0.9994 (see Table 3). The results indicated that this method was sensitive and rapid for analysis of fatty acids.

The experiment results showed the saponification value and unsaponifiable matter of the *Z. bungeanum* seed oil obtained by SFE-CO_2 were 193.4 mg/g and 0.58%, respectively, and the main fatty acids were C18:3, C22:6, C20:4, C18:2, C16:0, C18:1, C18:0 and C20:1. The corresponding mass percentages (%) were 27.01, 0.46, 5.54, 27.81, 12.37, 23.81, 1.81 and 1.19, respectively. Obviously, the *Z. bungeanum* seed oil was rich in unsaturated fatty acids, accounting for 84.0% (mass percentage) of the total amount, which was one of the main reasons for its strong antioxidant activity.

Effect of SFE-CO_2 Condition on the Antioxidant Activity of the Seed Oil

The antioxidant activity of the *Z. bungeanum* seed oil obtained by Box-Behnken design was measured with the scavenging activity towards the DPPH radical, which varied with the changes of extraction conditions. The inhibition percentage of the oil varyied from 78.1 to 85.3% under the different extraction conditions, and the results are

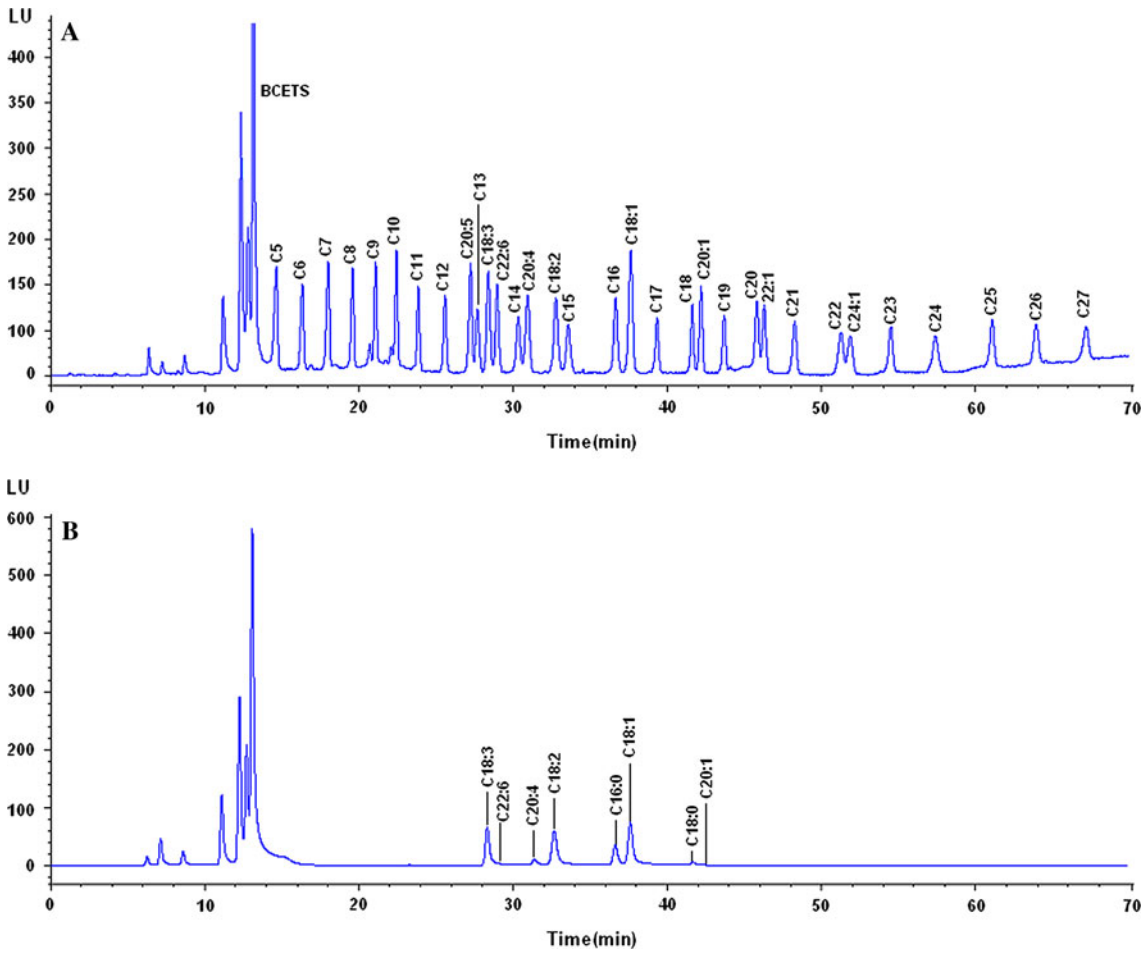

Fig. 3 **a** Chromatogram of a mixture of fatty acid standards; (**b**) chromatogram of fatty acids from *Z. bungeanum* seed oil. Chromatographic conditions: Column temperature at 30 °C; excitation wavelength λ_{ex} 279 nm, emission wavelength λ_{em} 380 nm; Eclipse XDB-C_8 column (4.6 × 150 mm, 5 μm); *flow rate* 1.0 ml min^{-1}; *peak labels*: *C5* (valeric acid); *C6* (hexanoic acid); *C7* (heptoic acid); *C8* (octoic acid); *C9* (pelargoic acid); *C10* (decoic acid); *C11* (undecanoic acid); *C12* (dodecanoic acid); *C13* (tridecanoic acid); *C20:5* (5,8,11,14,17-eicosapentaenoic acid); *C18* 3 (8,11,14-octadecatrienoic acid); *C22* 6 (2,5,8,11,14,17-docosahexenoic acid); *C14* (tetradecanoic acid); *C20* 4 (6,9,12,15-arachidonic acid); *C18* 2 (9,12-octadecadienoic acid); *C15* (pentadecanoic acid); *C16* (hexadecanoic acid); *C18* 1 (12-octadecenoic acid); *C17* (heptadecanoic acid); *C18* (octadecanoic acid); *C20* 1 (11-eicosenoic acid); *C19* (nonadecanoic acid); *C20* (eicosoic acid); *C22* 1 (12-docosenoic acid); *C21* (heneicosoic acid); *C22* (docosanoic acid); *C24* 1 (20-tetracosenoic acid); *C23* (tri-cosanoic acid); *C24* (tetracosanoic acid); *C25* (pentacosanoic acid); *C26* (hexacosanoic acid); *C27* (heptacosanoic acid)

presented in Table 1. The effect of SFE-CO_2 conditions on the antioxidant activity of the seed oil is shown in Fig. 4.

Figure 4a shows the effect of the extraction pressure and temperature on the antioxidant activity at a fixed modifier amount of 7.50%. With a definite extraction temperature, pressure had a positive linear effect on the antioxidant activity. The antioxidant activity increased significantly with increasing extraction pressure, most likely due to the increase of solvent density resulting in the improvement of the antioxidant component, such as tocopherols solubility, which was coextracted during the oil extraction. The tocopherol content data of the *Z. bungeanum* seed oil extracted by SFE are shown in Table 4. However, the extraction temperature showed different results compared to extraction pressure. Antioxidant activity decreased with increasing extraction temperature and reached a minimum value, followed by an enhancement with its further increase, probably due to a higher temperature resulting in lower extraction recovery of polyunsaturated fatty acids and their derivatives of amine because of a decrease in solubility [14]. On the other hand, higher temperature was beneficial to the extraction of terpenoids and volatile oils that have antioxidant activity owing to the increase in its volatility [15]. Hence, the effect of temperature elevation was difficult to predict because of its dependence on the nature of the sample.

With a fixed extraction temperature of 40 °C, the interaction impact on the antioxidant activity of the extraction pressure and the amount of modifier are shown in Fig. 4b. At a given amount of modifier of ethanol, the

Table 4 Fatty acid composition, tocopherol content, saponification value and unsaponifiable matter of *Z. bungeanum* seed oil extracted by SFE

Matters	Content	Relative content (%)
C18:3 Linolenic acid	14.64 mg/ml	27.78
C22:6 Docosahexenoic acid	0.30 mg/ml	0.57
C20:4 Arachidonic acid	3.11 mg/ml	5.89
C18:2 Linoleic acid	13.88 mg/ml	26.33
C16:0 Palmitic acid	6.94 mg/ml	13.18
C18:1 Oleic acid	12.04 mg/ml	22.86
C18:0 Stearic acid	1.44 mg/ml	2.73
C20:1 Eicosenoic acid	0.35 mg/ml	0.66
Total of all fatty acids	52.69 mg/ml	100
α-Tocopherols	27.3 mg/100 g	98.6
γ-Tocopherols	0.4 mg/100 g	1.4
Total of all tocopherols	27.7 mg/100 g	100
Saponification value	193.4 mg/g	
Unsaponifiable matter	0.58%	

antioxidant activity increased rapidly with increasing extraction pressure, and with a given extraction pressure, antioxidant activity increased with an increasing amount of modifier within the investigated range, probably because the modifier of ethanol could extract the antioxidant component of polyphenols and flavonoids well [16, 17].

With a fixed extraction pressure of 22.5 MPa, the interaction impact of the extraction temperature and amount of modifier on the antioxidant activity is shown in Fig. 4c. At a given amount of modifier, the antioxidant activity declined with increasing temperature and reached the lowest value, followed by an enhancement with its further increase. With a given temperature, the antioxidant activity of the oil was increased with an increasing amount of modifier.

Conclusions

In this study, the green SFE-CO_2 extraction process was used for the recovery of seed oil from *Z. bungeanum*, and the seed oil demonstrated marked antioxidant activity in the DPPH radical-scavenging assay. The effects of SFE-CO_2 conditions on the extraction yield and the antioxidant activity of the *Z. bungeanum* seed oil as well as its chemical composition were evaluated. Both the extraction yield and the antioxidant activity were strongly dependent on the pressure and the amount of modifier. The composition analysis indicated that the *Z. bungeanum* seed oil was rich in unsaturated fatty acids, the linolenic acid, linoleic acid and oleic acid accounted for 78.63% (mass percentage) of the total amount.

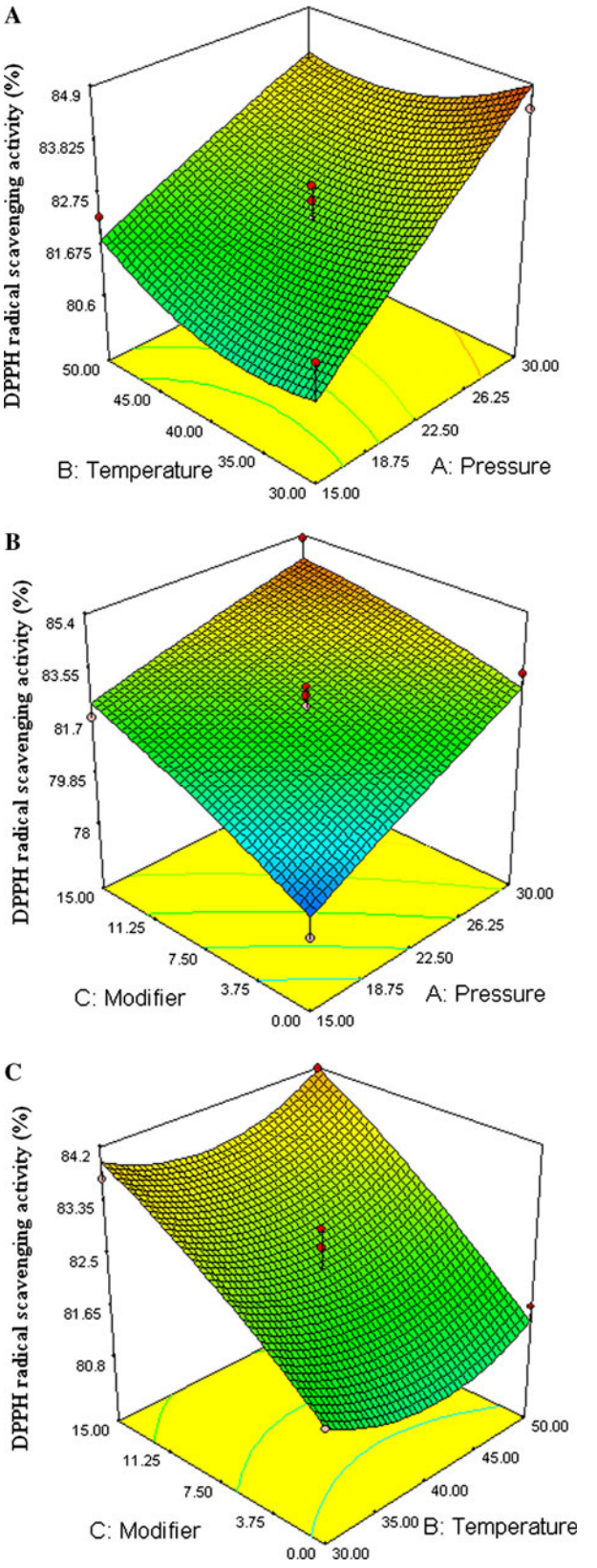

Fig. 4 Response surface plots of the oil antioxidant activity affected by extraction pressure, extraction temperature and the added amount of modifier

Z. bungeanum seed oil showed marked antioxidant activity, and the antioxidant activity was enhanced with increases of the extraction pressure and amount of modifier. However, the antioxidant activity of the seed oil decreased with increasing extraction temperature and reached a minimum value, followed by an enhancement with its further increase. Based on the results obtained, we can conclude that *Z. bungeanum* seed oil might play a potential role as a health-promoting agent with antioxidant activity in human diets, as well as provide valuable natural antioxidants for the pharmaceutical industry, which would contribute to the sustainable use of *Z. bungeanum* seed as an agricultural resource. The methods developed here provide a template for extracting and analyzing seed oil and making use of its antioxidant activity.

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