Research Article

Extraction of *Nitraria tangutorum* seed lipid using different extraction methods and analysis of its fatty acids by HPLC fluorescence detection and on-line MS identification

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The seed lipid of *Nitraria tangutorum* was extracted by supercritical carbon dioxide extraction, microwave-assisted reflux extraction, ultrasound-assisted extraction, or solvent reflux extraction. The experimental parameters of supercritical carbon dioxide extraction including pressure, temperature, particle size, and extraction time were investigated. A facile and sensitive method for the simultaneous determination of 30 saturated and 9 unsaturated fatty acids by HPLC with fluorescence detection after pre-column derivatization was developed. Fatty acid derivatives were separated on a reversed-phase Eclipse XDB-C_8 column in conjunction with gradient elution. Identification of fatty acid derivatives was carried out by on-line APCI/MS in positive-ion mode. Excellent quantitative linear responses of the 39 fatty acids were observed in the range of 0.014 to 14 μmol/L with correlation coefficients higher than 0.9992. Limits of detection were in the range of 0.32–3.7 nmol/L (S/N = 3). The fatty acids in *Nitraria tangutorum* seed lipid with or without saponification extracted by the four different methods were determined and compared. The results indicated that the mass percentage of unsaturated fatty acids (mainly oleic acid, linoleic acid and linolenic acid) in *Nitraria tangutorum* seed lipid was up to 79%, and the best method was supercritical carbon dioxide extraction.

Keywords: Extraction / Fatty acids / Fluorescence detection / HPLC / *Nitraria tangutorum* Bobr.

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1 Introduction

The *Nitraria* genus, which belongs to the Zygophyllaceae family, is a kind of shrub that produces esculent berries and is widely distributed in the Middle East, Central Asia and the northwest region of China. This kind of shrub is well adapted to grow under bad living conditions such as arid and saline-alkali soils. Due to its natural capacity to withstand wind and sand, it is often used for sand and soil conservation [1]. *Nitraria* plants are the main dominant species in desert areas on the Qinghai-Tibetan Plateau. Both *Nitraria tangutorum* Bobr. and *Nitraria sibirica* Pall. are the naturally distributed species in this area, and the former is a special species of China [2]. It was reported that the fruits and seeds of *Nitraria tangutorum* were used among village folks to cure the weaknesses of spleen and stomach, indigestion, neurasthenia and cold [3], and its leaves were used to treat convulsion, neuralgia, arrhythmia, *etc.* [4]. In addition, its fruits were effective in decreasing the blood lipid levels and had anti-oxidative effects [5]. Although flavonoids and alkaloids in *Nitraria tangutorum* were studied and reported [3, 4], there have been almost no other reports about their chemical composition.

Fatty acids, especially unsaturated fatty acids, play an important role in the regulation of a variety of physiological and biological functions in living organisms [6]. For *Nitraria tangutorum*, fatty acids mainly exist in the lipid of seeds. Tra-

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ditional extraction methods for seed lipid are ultrasound-assisted extraction (USE) [7] and solvent reflux extraction (SRE) [8], but these methods need organic solvents and a high temperature which possibly results in oxidation of the long-chain unsaturated fatty acids. Microwave-assisted reflux extraction (MWRE) [9] is rapid and effective; however, it needs polar solvents and especially organic solvents, which is disadvantageous for the the production of foods, drugs and health products. Supercritical carbon dioxide extraction (SCCE) [10, 11] is one of the most popular methods for lipid extraction, mainly because of its low temperature needs, high efficiency, and anaerobic conditions. Therefore, in this paper, we studied the experimental parameters of SCCE and simultaneously evaluated MWRE, USE and SRE for the purpose of comparison.

Also, the analysis of fatty acids in the seed lipid is equally important for the study and exploitation of *Nitraria tangutorum*. Therefore, a sensitive method that can simultaneously determine saturated and unsaturated fatty acids will be indispensable for the analysis and control of raw materials and products from *Nitraria tangutorum*. Most fatty acids show neither UV or visible absorption nor fluorescence; thus their detection measuring absorbance is fairly difficult [12]. However, easily detectable fatty acid derivatives by methyl or ethyl esterification using GC or GC/MS have been broadly reported [13, 14]. In contrast to GC, use of HPLC allows fatty acids to be converted into a large number of different derivatives [15]. Derivatization can overcome some problems such as tailing peaks and low detector sensitivity by the formation of less polar derivatives, which can be more easily and sensitively analyzed by HPLC with UV or fluorescence detection. Therefore, derivatization of these analytes with labeling reagents has been widely adopted. Up to now, a great many derivatization reagents have been developed; however, it has been reported that many of these reagents have limitations in their applications, such as low detection sensitivity, short detection wavelengths, poor stability, tedious analytical procedures, and serious interferences in biological sample analysis [16].

Recently, we synthesized acridone-9-ethyl-*p*-toluenesulfonate (AETS) [6, 17], 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) [18, 19], 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP) [20, 21] and 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-d]imidazol-1-yl) ethyl-*p*-toluenesulfonate (ANITS) [22] for the determination of saturated fatty acids or bile acids. The aims of the present work were to (1) develop an SCCE technique for extracting lipid from *Nitraria tangutorum* seed, and (2) to develop a sensitive method for the simultaneous determination of 30 saturated and 9 unsaturated fatty acids. The fluorescence detection sensitivity for fatty acids was compared with those when using AETS [6, 17], BDETS [18, 19] and 2-(2-naphthoxy)-ethyl-2-(piperidino)-ethanesulfonate (NOEPES) [23] as labeling reagents.

2 Materials and methods

2.1 HPLC-APCI/MS instrumentation

The HPLC system was an Agilent HP 1100 series and consisted of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a fluorescence detector (FLD) (model G1321A). The HPLC system was controlled by HP Chemstation software. The mass spectrometer, a 1100 Series LC/MSD Trap-SL (ion trap) from Bruker Daltonics (Bremen, Germany) was equipped with an atmospheric pressure chemical ionization (APCI) source. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. The ion source conditions were: APCI in positive-ion mode; nebulizer pressure 413.69 MPa; dry gas temperature 350 °C; dry gas flow rate 5.0 L/min; Vap temperature 450 °C; corona current (nA) 4000 (pos); capillary voltage 3500 V. Derivatives were separated on an Eclipse XDB-C_8 column (150 × 4.6 mm, 5 μm; Agilent) by gradient elution. A Paratherm U2 electronic water bath (Hitachi, Tokyo, Japan) was used to control the temperature. The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL, USA).

2.2 Chemicals

Standards of 30 saturated fatty acids (C_1–C_{30}) were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Nine unsaturated fatty acid standards including 12-octadecenoic acid (18:1), 9,12-octadecadienoic acid (18:2), 8,11,14-octadecatrienoic acid (18:3), 11-eicosenoic acid (20:1), 6,9,12,15-arachidonic acid (20:4), 5,8,11,14,17-eicosapentaenoic acid (20:5), 12-docosenoic acid (22:1), 2,5,8,11,14,17-docosahexenoic acid (22:6), and 20-tetracosenoic acid (24:1) were purchased from Sigma (St. Louis, MO, USA). Spectroscopically pure acetonitrile was purchased from Merck (Germany). *N*,*N*-Dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Jining Chemical Reagent Co. (Shandong, Jining, China) and treated with 5-Å molecular sieves, and then redistilled prior to use. Benzene, toluene, tetrahydrofuran, potassium carbonate, pyridine and chloroform were of analytical grade and obtained from Shanghai Chemical Reagent Co. Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were of analytical grade unless otherwise stated.

2.3 Materials of *Nitraria tangutorum*

The plant materials were collected from Dulan county, Qinghai province, in September 2004. *Nitraria tangutorum* seeds were dried under a stream of nitrogen and ground to pow-

dered samples. In all cases, dried and powdered *Nitraria tangutorum* material was used.

2.4 Extraction of *Nitraria tangutorum* seed lipid

The seed lipid of *Nitraria tangutorum* was extracted by SCCE, MWRE, USE or SRE. Chloroform was chosen as solvent for MWRE, USE and SRE according to our research experience [20, 21]. Their extraction procedures are as follows:

(1) Extraction procedures of SCCE were conducted in a flow circulatory extraction apparatus. In every experiment, 300-g samples of powdered seeds were used. The extraction capacity was 1000 mL and the flow rate of carbon dioxide was 40 kg/h. Carbon dioxide was pumped into the extractor from a 6-MPa pressurized bottle. Pressure was maintained constant at 6.7 and 6.5 MPa in separator I and II, respectively. The extractor and separators were jacketed to maintain constant temperatures at 40 and 30 °C, respectively. The lipid was collected every 15 min from the two separators and the carbon dioxide was cooled and recycled into the system. Successively collected lipids were weighed and analyzed.

(2) To a 100-mL round-bottom flask, 0.5 g pulverized *Nitraria tangutorum* seed and 25 mL chloroform were added and the sample was extracted for 20 min at 65 °C under reflux with the microwave power at 500 W. After the contents were filtered, the chloroform was evaporated to dryness in a rotary vacuum evaporator at 60 °C and then the seed lipid was gained.

(3) To a 50-mL round-bottom flask, 0.5 g pulverized *Nitraria tangutorum* seed and 25 mL chloroform were added and the sample was sonicated for 2 h at room temperature. After the contents were filtered, the chloroform was evaporated to dryness in a rotary vacuum evaporator at 60 °C and then the seed lipid was gained.

(4) To a 50-mL round-bottom flask, 0.5 g pulverized *Nitraria tangutorum* seed and 25 mL chloroform were added. The contents were rapidly heated to reflux for 2 h with vigorous stirring. After cooling, the contents were filtered. The chloroform was evaporated to dryness in a rotary vacuum evaporator at 60 °C and then the seed lipid was gained.

2.5 Saponification of seed lipid

To a 10-mL test tube, 0.1 g seed lipid and 2.0 mL potassium hydroxide/methanol solution (2 mol/L) was added. After sealing, the test tube was immersed in a water bath at 90 °C for 2 h. After cooling, the contents were transferred to a centrifugal test tube, added with 2 mL water, and adjusted to pH 7.0 with 6 mol/L 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-μm nylon membrane filter, and stored at –10 °C until HPLC analysis.

2.6 Preparation of standard solutions

The labeling reagent solution (5.0×10^{-2} mol/L) was prepared by dissolving 246 mg TSPP in 10 mL of DMF. The corresponding derivatization reagent solution of low concentration (0.01 mol/L) was obtained by diluting the stock solution with DMF. Individual standards of fatty acids were prepared by dissolving the fatty acid in HPLC-grade acetonitrile to a concentration of 1.0×10^{-2} mol/L. For long-chain fatty acids (*i.e.* $> C_{15}$), the individual stock solution was prepared by dissolving the fatty acid in DMF and diluting with the mixed solvent of acetonitrile/DMF (1 : 1 vol/vol), owing to their poor solubility. Standards of 39 mixed fatty acids (1.0×10^{-4} mol/L) were prepared by diluting the corresponding individual stock solutions with acetonitrile. When not in use, all reagent solutions were stored at 4 °C in a refrigerator until HPLC analysis.

2.7 Derivatization of fatty acids

To a solution consisting of 50 μL of standard fatty acid mixture in a 2-mL vial, 100 μL derivatization reagent solution, 10 mg anhydrous K_2CO_3 and 200 μL DMF were added. The vial was sealed and allowed to react in a water bath at 90 °C for 30 min with shaking in 5-min intervals. After the reaction was completed, the mixture was taken to cool at room temperature. A 750-μL mixed solution of acetonitrile and DMF (CH_3CN/DMF, 1 : 1 vol/vol) was added to dilute the derivatization solution. The diluted solution (10 μL, 36 pmol) was injected directly as a representative chromatographic analysis. Actually, each injected amount from 0.14 to 143 pmol (injected volume 10 μL) could be obtained by appropriate dilution. The derivatization scheme of fatty acids with TSPP is shown in Fig. 1.

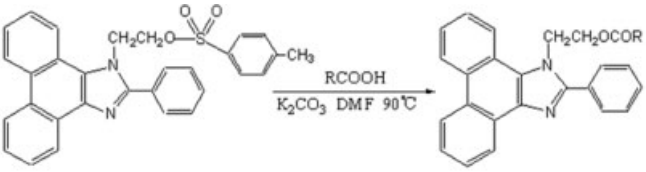

Figure 1. Derivatization scheme of fatty acids with TSPP.

2.8 HPLC separation conditions

HPLC separation of 39 fatty acid derivatives was carried out on a reversed-phase Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm; Agilent) by gradient elution. Eluent A was 50% acetonitrile; eluent B was 50% acetonitrile containing 20 mmol/L ammonium formate buffer (pH 3.7); eluent C was a mixed solution of acetonitrile and DMF (ACN/DMF 100 : 2 vol/vol); eluent D was a mixed solution of acetonitrile and DMF (ACN/DMF 100 : 30 vol/vol). Before injection of the next sample, the column was equilibrated with mobile phase A

for 10 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} 260 nm and λ_{em} 380 nm, respectively. The gradient elution program was as follows: 0–4 min = 95% A + 5% C, 4.2–8 min = 95% B + 5% C, 8.5 min = 75% B + 25% C, 15 min = 50% B + 50% C, 50 min = 100% C, 60–75 min = 100% D.

2.9 Quantitative analysis

Quantitative conversion of fatty acids from the extracts of *Nitraria tangutorum* seed to their TSPP derivatives was ensured by using an excess of TSPP. All fatty acids were quantified using the external standard method with detection at 380 nm. The calibration curves for each fatty acid derivative were obtained by linear regression, plotting peak area *versus* concentration.

3 Results and discussion

3.1 Optimization of SCCE parameters and comparison with MWRE, USE and SRE

For SCCE, the experimental parameters such as pressure, temperature, time, and particle size were evaluated. When the seed lipid was extracted from seed powders at 10, 15, 20, 25 and 30 MPa, at a temperature of 45 °C and a carbon dioxide flow rate of 40 kg/h, the results indicated that the extraction yield (calculated as [lipid weight (g)/pulverized seed weight (g)] × 100%) increased with increasing pressure from 10 to 20 MPa; however, the extraction yield was invariable after 20 MPa. For practical applications, 20 MPa should be employed. The effects of extraction temperature on the lipid yield were investigated at 30, 35, 40, 45 and 50 °C (the pressure was maintained at 20 MPa, and the CO_2 flow rate at 40 kg/h). The seed lipid yield increased slowly with increasing temperature, ultimately reaching a maximum at 40 °C. Additionally, the particle size of the pulverized seed sample had a critical impact on the extraction efficiency. We investigated it at a given pressure (20 MPa), temperature (40 °C) and extraction time (120 min). Particle sizes of 0.5, 0.9 and 2.0 mm in diameter brought extraction yields of 26, 9.7 and 5.4%, respectively. Obviously, the smallest particle size gave the highest lipid yield. However, we could not use smaller samples than 0.5 mm, because the smaller particles can form a bedsheet and increase the resistance of the mass transfer. Therefore, a suitable particle size of 0.5 mm in diameter was employed. In addition, for extraction time, the extraction process was usually composed of three stages. The first stage was the rapid extraction of the free solute. At this stage, the extraction yield increased rapidly with time, corresponding to 0–30 min in our experiment. The second stage represented the transition of surface to internal diffusion and the extraction yield increased slowly, corresponding to 30–75 min. In the third stage the internal diffusion and the extraction yield tended to be saturated [24, 25], corresponding to 75–120 min. Because the density of supercritical carbon dioxide is close to a liquid, and its diffusion constant and viscosity are close to a gas, it not only has good extraction efficiency similar to organic solvents but also has a better mass transfer ability than organic solvents. In our experiment, the entire extraction process was almost completed within 90 min. Under the above-mentioned optimal conditions, the extraction yield of SCCE could reach 26%.

For the other three extraction methods (MWRE, USE and SRE), the routine conditions were used as described in the experimental section, and the extraction yields for them were as follows: MWRE 9.8%, USE 6.5%, and SRE 11%. From the extraction yields, we can see that SCCE had the highest extraction yield. Moreover, SCCE did not use organic solvents and performed under relatively low extraction temperatures. All these characteristics of SCCE make it very popular in the food, medicine, and health product fields.

3.2 Derivatization conditions

The optimization process of TSPP derivatization with 26 fatty acids (C_1–C_{26}) has been reported by our laboratory [21]. After our careful study, we found that the derivatization conditions of TSPP with 39 saturated and unsaturated fatty acids were similar to that. Therefore, we do not give unnecessary details in this paper. The optimal derivatization conditions were as follows: TSPP reacted with fatty acids in DMF at 90 °C for 30 min in the presence of 10 mg potassium carbonate with the addition of a fivefold molar reagent excess to total molar fatty acids.

3.3 HPLC separation and MS identification

On a reversed-phase Eclipse XDB-C_8 column, several gradient elution programs were investigated to ensure satisfactory HPLC separation. Ammonium formate buffer was used to control the pH during HPLC separation, resulting in a faster separation and a lower and more stable chromatogram baseline. To achieve optimal separation, the pH value of mobile phase B was tested. Separation of the derivatized long- and short-chain fatty acids can be accomplished at acidic conditions with pH 3.7. With pH <3.5, most of the fatty acids were resolved, except for C_1 and compound B; B and C_2 partially co-eluted. In comparison with pH 3.7, the eluent at pH >4.0 resulted in an obvious increase in retention time for most of the fatty acid derivatives; at the same time, compounds C and C_4 co-eluted. After further experiments, it was found that if the pH value of mobile phase B was adjusted to 3.7, a satisfactory resolution for the 39 fatty acid derivatives was obtained within 75 min with the shortest retention time values and the sharpest peaks. In fact, the addition of DMF to mobile phases C and D could raise the solubility of the fatty acid derivatives to obtain fast separation with sharp peaks. To

achieve optimal separation, the added amount of DMF in mobile phases C and D was confirmed, with optimization as described above. With the optimal chromatographic conditions as described above, the chromatogram of the 39 standard fatty acid derivatives as shown in Fig. 2 was obtained.

The ionization and fragmentation of the isolated TSPP-fatty acid derivatives was studied by on-line MS with the APCI source in the positive-ion mode. As expected, the TSPP-fatty acid derivative produced an intense molecular ion peak at *m/z* $[M+H]^+$. With MS/MS analysis, the collision-induced dissociation spectra of the molecular ion at *m/z* $[M+H]^+$ produced specific fragment ions at *m/z* $[M'+CH_2CH_2]^+$ and *m/z* 295.0 (M' was the molecular mass of the corresponding fatty acid). The specific fragment ion *m/z* 295.0 was the corresponding protonated TSPP core structure moiety and *m/z* $[M'+CH_2CH_2]^+$ was the corresponding protonated fatty acid moiety. Selected reaction monitoring, based on the *m/z* $[M+H]^+ \rightarrow$ *m/z* $[M'+CH_2CH_2]^+$ and *m/z* 295.0 transition, was specific for TSPP-fatty acid derivatives. There was no detectable signal from the blank water sample using this transition. Although other endogenous acidic compounds present in the sample were presumably co-extracted and derivatized by the TSPP reagent, no disturbance was observed due to the highly specific parent mass-to-charge ratio (*m/z* $[M+H]^+$) and the characteristic product ions *m/z* $[M'+CH_2CH_2]^+$ and *m/z* 295.0. To reduce the disturbance from other unknown components present in the sample to a minimum, gradient elution with HPLC for the separation and determination of derivatized fatty acids was an efficient method. The characteristic fragment ions of *m/z* 295.0 (molecular core structure) and *m/z* $[M'+CH_2CH_2]^+$ came from the cleavage of the N-CH_2CH_2OCO bond. With the APCI source in positive-ion mode, the intense ion current signals should be attributed to the introduction of two weak basic nitrogen atoms in the TSPP molecular core structure, resulting in high ionizing efficiency. The MS, MS/MS analysis and the cleavage mode for the TSPP-18:1 derivative are shown in Fig. 3A–C. All molecular ions $[M+H]^+$ for the 39 fatty acid derivatives are shown in Table 1.

3.4 Comparison of the fluorescence sensitivity with AETS and BDETS

As observed, the molecular structure of TSPP played the same role in the esterification with fatty acids as do those of AETS [6, 17] and BDETS [18, 19]. The relative detector responses of TSPP, AETS and BDETS for the individual derivatized fatty acids were investigated. As expected, the fluorescence responses for representative C_{10}–C_{20} fatty acid derivatives using AETS and BDETS as labeling reagent were at least 2–4-fold and 3–8-fold, respectively, lower than that obtained by TSPP. This was probably due to the fact that TSPP has a larger molar absorbance coefficient (ε) that made it more sensitive for the detection of derivatized fatty acids [AETS: $\varepsilon = 5.72 \times 10^4$ L mol^{-1} cm^{-1} (255 nm); BDETS: $\varepsilon = 2.54 \times 10^4$ L mol^{-1} cm^{-1} (249 nm); TSPP: $\varepsilon = 6.0 \times 10^4$ L mol^{-1} cm^{-1} (259 nm)]. The difference in the molar absorbance coefficient may be attributed to the TSPP molecular structure, in which the n-π conjugation system is dramatically augmented due to the introduction of a phenylimidazole-[4,5-f]-9,10-phenanthrene function group into the labeling reagent molecule, which makes it more sensitive for the fluorescence detection of fatty acid derivatives.

3.5 Reproducibility, accuracy, linearity and detection limits

A standard solution consisting of 39 fatty acids (1×10^{-4} mol/L) was prepared, and the method reproducibility was examined by injecting quantitative fatty acid derivatives for six

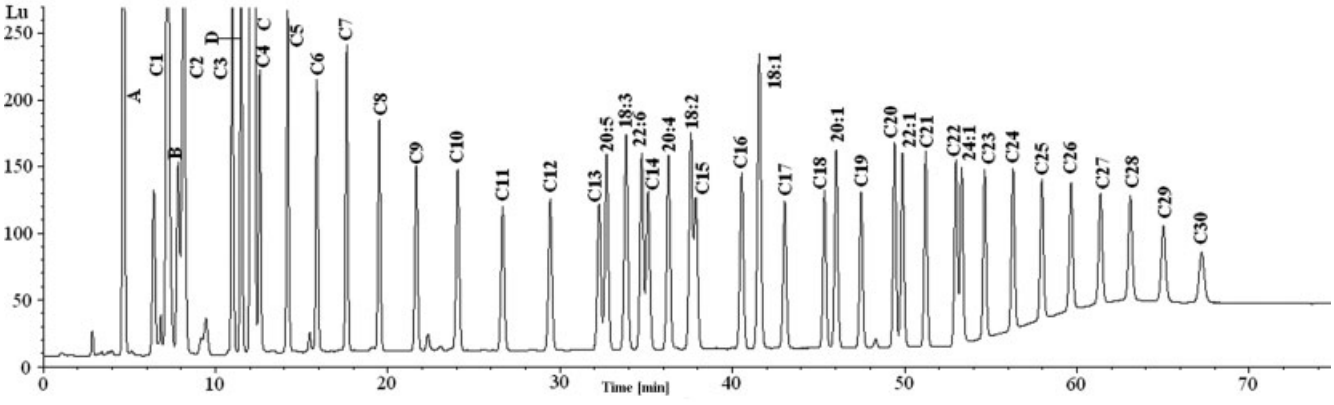

Figure 2. Chromatogram of standard fatty acid derivatives (36 pmol). Chromatographic conditions as described in the experimental section. Peaks: C_1–C_{30} are 30 kinds of saturated fatty acids containing 1–30 carbons, respectively. The other nine unsaturated fatty acids are listed in Section 2.2; (A) 1-ethanol-2-phenylimidazole[4,5-f]9,10-phenanthrene); (B) 2-phenylimidazole-[4,5-f]9,10-phenanthrene; (C) TSPP; (D) impurity.

Table 1. Linear regression equations, correlation coefficients, detection limits, MS data of fatty acid derivatives, and repeatability for peak area and retention time (n = 6).

Fatty acids	$Y = AX + B$, Y: peak area; X: injected amounts (pmol)	Correlation coefficients	MS $[M+H]^+$	Detection limits [fmol]	Retention time RSD [%]	Peak area RSD [%]
C_1	$Y = 170.26X + 109.23$	0.9999	367.1	3.2	0.37	0.19
C_2	$Y = 125.46X + 213.41$	0.9995	381.2	8.3	0.41	0.65
C_3	$Y = 72.09X + 73.26$	0.9998	395.1	10	0.35	0.46
C_4	$Y = 51.78X + 28.69$	0.9998	409.2	14	0.32	0.83
C_5	$Y = 62.53X + 27.39$	0.9997	423.2	15	0.19	0.46
C_6	$Y = 50.51X + 23.36$	0.9998	437.2	12	0.18	0.69
C_7	$Y = 58.58X + 25.90$	0.9998	451.2	18	0.12	0.57
C_8	$Y = 49.22X + 16.41$	0.9999	465.2	17	0.099	0.56
C_9	$Y = 44.58X + 11.85$	0.9999	479.2	27	0.089	0.44
C_{10}	$Y = 48.63X + 17.56$	0.9999	493.3	24	0.073	0.31
C_{11}	$Y = 41.23X + 10.71$	0.9998	507.3	31	0.081	0.44
C_{12}	$Y = 44.97X + 9.61$	0.9999	521.3	29	0.063	0.38
C_{13}	$Y = 44.29X + 7.63$	0.9999	535.4	29	0.056	0.24
20:5	$Y = 56.58X + 18.23$	0.9999	623.2	18	0.053	0.22
18:3	$Y = 64.21X + 13.37$	0.9997	599.2	17	0.059	0.25
22:6	$Y = 56.11X + 20.44$	0.9999	649.2	18	0.058	0.29
C_{14}	$Y = 47.61X + 16.48$	0.9999	549.4	26	0.044	0.25
20:4	$Y = 56.78X + 16.61$	0.9999	625.2	17	0.073	0.31
18:2	$Y = 65.41X + 12.76$	0.9998	601.2	16	0.035	0.22
C_{15}	$Y = 41.69X + 30.75$	0.9994	563.4	27	0.043	0.14
C_{16}	$Y = 52.07X + 11.55$	0.9999	577.4	22	0.031	0.095
18:1	$Y = 85.58X + 25.01$	0.9998	603.2	14	0.029	0.12
C_{17}	$Y = 42.61X + 3.47$	0.9999	591.4	25	0.026	0.17
C_{18}	$Y = 42.31X + 12.84$	0.9997	605.4	23	0.019	0.16
20:1	$Y = 52.32X + 20.59$	0.9998	631.2	16	0.027	0.17
C_{19}	$Y = 40.55X + 11.83$	0.9999	619.5	24	0.020	0.16
C_{20}	$Y = 51.23X + 16.39$	0.9999	633.5	22	0.043	0.36
22:1	$Y = 48.77X + 17.98$	0.9996	659.3	22	0.084	0.52
C_{21}	$Y = 49.36X + 15.25$	0.9992	647.6	23	0.072	0.88
C_{22}	$Y = 49.01X + 15.60$	0.9999	661.6	25	0.095	1.3
24:1	$Y = 45.28X + 26.56$	0.9999	687.3	25	0.11	1.1
C_{23}	$Y = 44.21X + 26.47$	0.9999	675.6	24	0.12	1.4
C_{24}	$Y = 44.71X + 26.16$	0.9998	689.6	20	0.19	1.9
C_{25}	$Y = 41.19X + 29.44$	0.9998	703.6	21	0.23	2.1
C_{26}	$Y = 39.03X + 30.49$	0.9997	717.6	23	0.27	2.3
C_{27}	$Y = 35.87X + 22.73$	0.9998	731.5	24	0.24	2.2
C_{28}	$Y = 36.89X + 25.48$	0.9999	745.6	30	0.25	2.6
C_{29}	$Y = 31.59X + 26.27$	0.9998	759.5	32	0.27	3.0
C_{30}	$Y = 24.82X + 41.08$	0.9993	773.6	37	0.24	2.7

times (injected amount 50 pmol, 10 μL). The RSD of the peak areas and retention times were from 0.095 to 3.0% and from 0.019 to 0.41% (Table 1), respectively. Precision and accuracy: Six replicates (n = 6) at 0.1, 1.0 and 5.0 μmol/L of the 39 fatty acids were used to make low- to high-range concentrations. The mean accuracy ranged from 91 to 105%, with the largest mean RSD <6.8%.

Based on the optimum derivatization conditions, the linearity of the 39 fatty acids was evaluated in the range of 0.014–14 μmol/L (injection volume 10 μL, injected amount from 143 to 0.14 pmol with a 1021-fold concentration range). The calibration graph was established with the peak area (Y) *versus* the fatty acid injection amount (X, pmol). The linear regression equations are shown in Table 1. All of the fatty acids were found to give excellent linear responses with correlation coefficients in the range of 0.9992–0.9999. By injecting 0.14 pmol fatty acid derivatives (10 μL, 14 nmol/L), the detection limits were calculated as 0.14/(peak height/3 × noise) at a signal-to-noise ratio of 3, which was from 3.2 to 37 fmol with an average of 21 fmol (Table 1). When using AETS, BDETS and

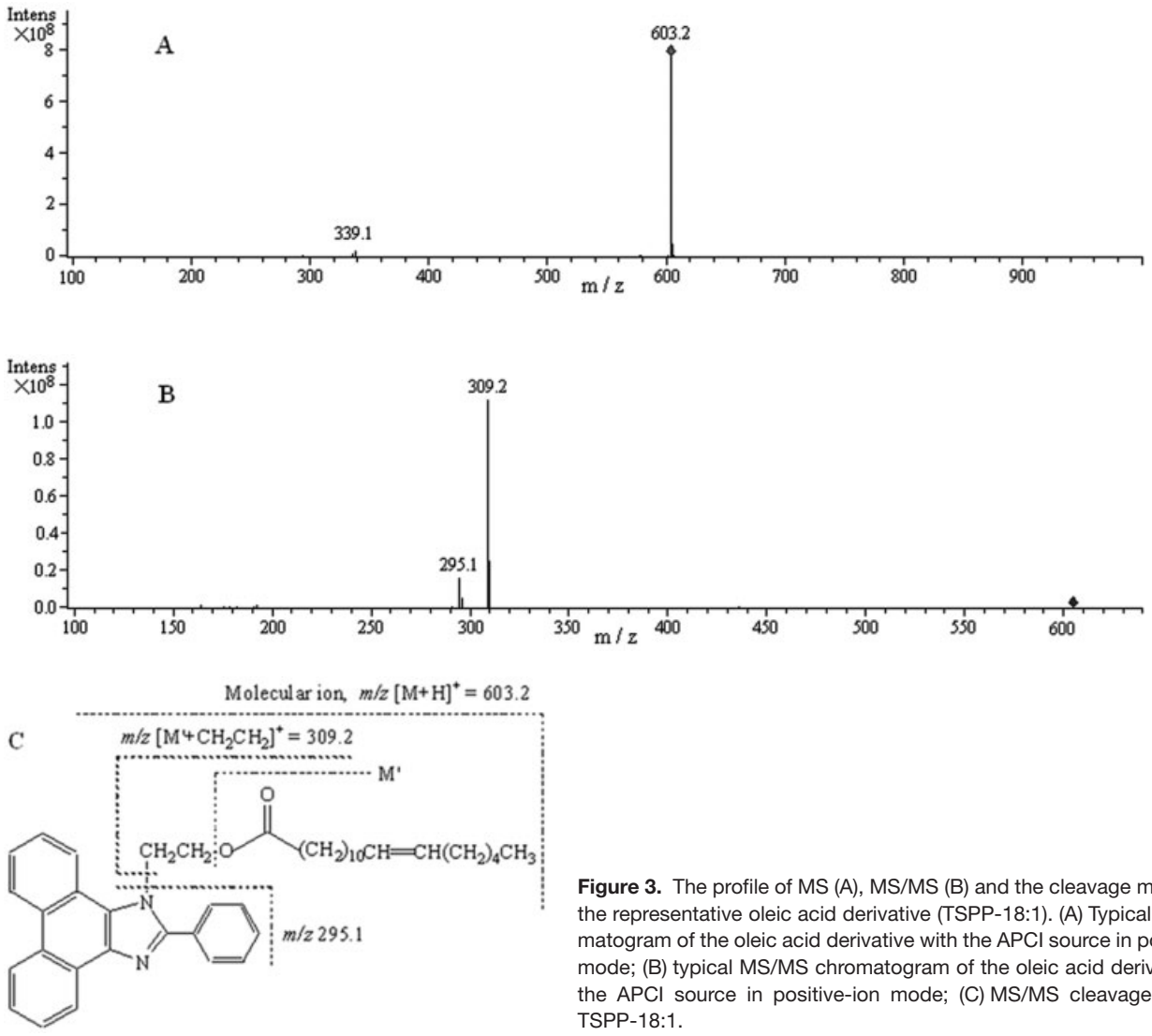

Figure 3. The profile of MS (A), MS/MS (B) and the cleavage mode (C) of the representative oleic acid derivative (TSPP-18:1). (A) Typical MS chromatogram of the oleic acid derivative with the APCI source in positive-ion mode; (B) typical MS/MS chromatogram of the oleic acid derivative with the APCI source in positive-ion mode; (C) MS/MS cleavage mode of TSPP-18:1.

NOEPES as labeling reagents, the detection limits (injected volume 10 μL) were 12–44 fmol for AETS with an average of 25 fmol [6], 25–80 fmol for BDETS with an average of 47 fmol [18], and 56 fmol for NOEPES [23]. The lowest detection limits and the average detection limits of TSPP decreased by 4–8-fold and 1–3-fold, respectively, in comparison with those of AETS, BDETS and NOEPES.

3.6 Analysis of seed lipid of *Nitraria tangutorum* and recovery

A representative chromatogram of fatty acids in the seed lipid of *Nitraria tangutorum* by SCCE with fluorescence detection is shown in Fig. 4. Chromatographic peaks were identified by contrasting their retention times with those of a standard chromatogram, and simultaneously confirmed by on-line APCI/MS identification. All fatty acids were quantified by linear regression equations. The fatty acid compositions with saponification from the seed lipid of *Nitraria tangutorum* extracted by SCCE, MWRE, USE and SRE, and the free fatty acid compositions from the seed lipid extracted by SCCE, are shown in Table 2. The results indicate that (1) unsaturated fatty acids (mainly 18:3, 18:2 and 18:1) are the main fatty acids in *Nitraria tangutorum* seed lipid, which possess a high mass percentage of 79% compared to total fatty acids. (2) The contents of fatty acids after saponification are about 60–80 times higher than those of free fatty acids in the extracts of SCCE. This may be due to the fact that most fatty acids in *Nitraria tangutorum* seed exist as esters such as acylglycerols, methyl esters or ethyl esters. (3) The contents of unsaturated fatty acids in the seed lipid extracted by MWRE, USE and SRE are obviously lower than those extracted by SCCE. This

Table 2. Contents of fatty acids in the seed lipid of *Nitraria tangutorum* by four extraction methods (n = 3).

Fatty acids	SCCE with saponification [mg/g]	MWRE with saponification [mg/g]	USE with saponification [mg/g]	SRE with saponification [mg/g]	SCCE without saponification [mg/g]
C_1	3.3	3.1	3.0	3.0	0.056
C_2	3.3	3.1	3.0	3.0	0.055
C_3	0.87	0.82	0.79	0.77	0.015
C_4	1.3	1.3	1.2	1.2	0.022
C_5	1.5	1.4	1.3	1.3	0.024
C_6	2.5	2.4	2.2	2.2	0.042
C_7	1.7	1.6	1.5	1.5	0.026
C_8	2.1	2.0	1.9	1.8	0.032
C_9	1.3	1.2	1.2	1.1	0.020
C_{10}	0.43	0.40	0.38	0.38	0.007
C_{11}	0	0	0	0	0
C_{12}	0.83	0.78	0.75	0.73	0.013
C_{13}	0	0	0	0	0
20:5	0	0	0	0	0
18:3	27	26	27	26	0.33
22:6	0	0	0	0	0
C_{14}	4.6	4.3	4.1	4.0	0.059
20:4	6.5	6.1	5.9	5.8	0.084
18:2	562	548	548	548	6.8
C_{15}	0	0	0	0	0
C_{16}	94	95	94	96	1.5
18:1	170	166	169	166	2.1
C_{17}	4.8	4.4	4.3	4.2	0.070
C_{18}	47	50	48	49	0.70
20:1	5.5	5.1	4.9	4.8	0.081
C_{19}	0	0	0	0	0
C_{20}	9.3	8.6	8.4	8.2	0.14
22:1	0	0	0	0	0
C_{21}	2.7	2.5	2.4	2.4	0.40
C_{22}	12	11	10.4	10	0.17
24:1	0	0	0	0	0
C_{23}	1.9	1.8	1.7	1.7	0.028
C_{24}	11	10	9.8	9.5	0.16
C_{25}	0	0	0	0	0
C_{26}	0	0	0	0	0
C_{27}	0	0	0	0	0
C_{28}	0	0	0	0	0
C_{29}	0	0	0	0	0
C_{30}	0	0	0	0	0
U/T	79%	78%	78%	78%	74%
U/S	3.8	3.6	3.7	3.6	2.9

U/T, Unsaturated/total fatty acids (%); U/S, unsaturated/saturated fatty acids.

might be due to the different extraction mechanisms of these four methods: MWRE and USE proceed with the assistance of microwave and ultrasound, and MWRE and SRE proceed under solvent reflux at 60 ° or higher temperature; SCCE proceeds with supercritical carbon dioxide fluids at 40 °C, so this experimental condition can avoid the interaction of atmospheric oxygen with the unsaturated fatty acids of the seed lipid.

The analytical recoveries of the 39 fatty acids were investigated by the addition of known amounts of standard solution (10 μL, 1.0×10^{-4} mol/L) to the pulverized *Nitraria tangutorum* seed, whose contents of fatty acids were known by calculation from linear regression equations. MWRE and derivatization was done under the same optimal conditions as mentioned above, and the analyses were carried out in three

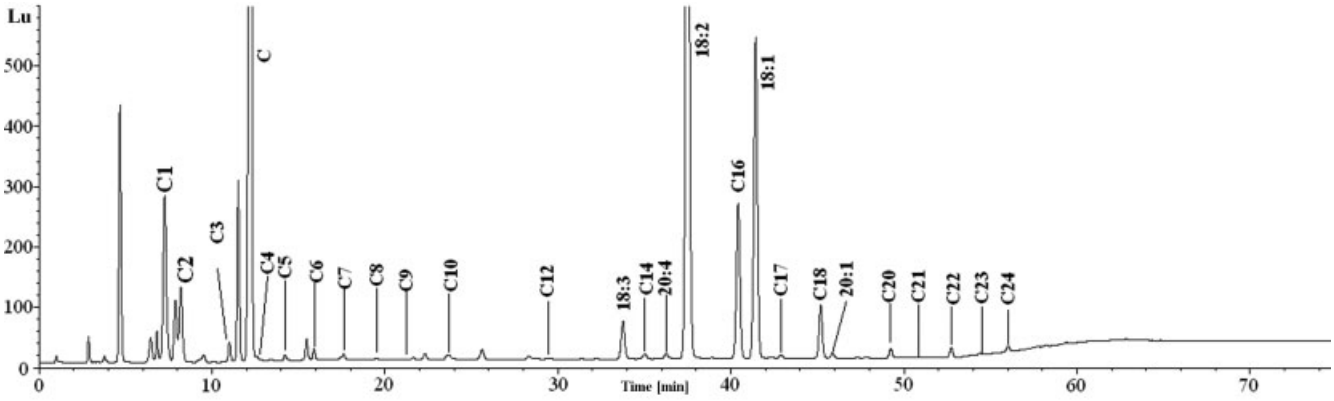

Figure 4. Representative chromatogram of fatty acids in the seed lipid of *Nitraria tangutorum* seeds by SCCE.

duplicates. The experimental recoveries were in the range of 89–105%.

4 Conclusions

In this study, simultaneous determination of 39 fatty acids extracted from pulverized *Nitraria tangutorum* seed using TSPP as derivatization reagent, with HPLC fluorescence detection and post-column MS identification, could be successfully achieved. The SCCE is of higher efficiency and has better characteristics for application in industrial products regarding chemical compositions and extraction for natural medicines or foods than MWRE, USE and SRE. The established method could be hopefully applied to the extraction and determination of fatty acids from various drugs, plants and biochemistry samples.

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Conflict of interest statement

The authors have declared no conflict of interest.

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