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Extraction of *Nitraria tangutorum* seed oil by supercritical carbon dioxide and determination of free fatty acids by HPLC/APCI/MS with fluorescence detection

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Abstract

The seed oil from *Nitraria tangutorum* samples was obtained by supercritical carbon dioxide extraction methods. The extraction parameters for this methodology, including pressure, temperature, particle size and extraction time, were optimized. The free fatty acids in the seed oil were separated with a pre-column derivation method and 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) as a labeling regent, followed by high-performance liquid chromatography (HPLC) with fluorescence detection. The target compounds were identified by mass spectrometry with atmospheric pressure chemical ionization (APCI in positive-ion mode). HPLC analysis shows that the main compositions of the seed oil samples were free fatty acids (FFAs) in high to low concentrations as follows: linoleic acid, oleic acid, hexadecanoic acid and octadecanoic acid. The assay detection limits (at signal-to-noise of 3:1) were 3.378–6.572 nmol/L. Excellent linear responses were observed, with correlation coefficients greater than 0.999. The facile BDETS derivatization coupled with mass spectrometry detection allowed the development of a highly sensitive method for analyzing free fatty acids in seed oil by supercritical CO_2 extraction. The established method is highly efficient for seed oil extraction and extremely sensitive for fatty acid profile determination.

Keywords: Supercritical carbon dioxide extraction; *Nitraria tangutorum* Bobr.; Seed oil; Free fatty acids; HPLC/APCI/MS

1. Introduction

The genus *Nitraria* (Zygophyllaceae) is a shrub that bears edible berries and is widely distributed in the Middle East, central Asia, and the northwest region of China. Among the *Nitraria* (*N.*) species, only *Nitraria tangutorum* Bobr. grows in China, especially in the desert areas of Qinghai-Tibetan Plateau. A main goal of a *N. tangutorum* Bobr. forest is to conserve the soil and water from the wind-blown sand [1,2]. In addition, its leaves, fruits and seeds are often used in folklore medicines such as antispasmodic, antineuropathic, and anti-arrhythmicagent [3,4] to cure weaknesses in the spleen and stomach, including indigestion, neurasthenia and cold [5,6]. And the fruits have the effect of anti-oxidation and decreasing blood lipid level [7]. However, the effective components of *N. tangutorum* are not clear.

Processes for extracting oils from tissues using supercritical carbon dioxide ($SCCO_2$) are potential alternatives to conventional expeller-pressing and organic solvent extraction methods using *n*-hexane, ethanol or other organic solvents. Compared to conventional solvent extraction techniques, supercritical CO_2 extraction leaves non-toxic residues. Currently, supercritical extraction of solid matters has rarely been applied continuously to large-scale plant processing because of the numerous difficulties associated with the uninterrupted transport of solid material through the high-pressure extractor. Indeed, only a semi-batch mode extractor with the supercritical fluid flowing through a fixed bed of material is used industrially for decaffeinating tea and coffee. Extracting oil from seeds with CO_2 is gaining popularity due to the requirement of a lower number of authorized solvents in the food industry [8].

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Seed oil from *N. tangutorum* contains abundant fatty acid stores that play important roles at trace levels. Because seed oil fatty acids regulate a variety of physiological and biological functions, investigating their composition is of vital industrial importance. Most fatty acids do not show natural absorption or fluorescence in the vis/UV spectra. Thus, detectable fatty acid derivatives, such as methyl esters, obtained with gas chromatography (GC) or GC/mass spectrometry (MS) have been reported [9]. In contrast with GC, use of high-performance liquid chromatography (HPLC) allows the fatty acids to be converted to a large number of different derivatives [10]. If one uses the less polar labeling reagent derivatives, some problems such as tailing peaks and low detector sensitivity can be overcome by HPLC analysis. And with the advent of soft ionization techniques, HPLC–MS has become a powerful analytical tool in phytochemistry due to its sensitivity, rapidity, specificity, and low levels of sample consumption compared with conventional HPLC.

The aims of the present work were to: (1) develop a fast, simple and high efficiency supercritical technique for extracting seed oil from *N. tangutorum* and (2) establish a HPLC–MS analysis procedure for free fatty acids using 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) as a labeling regent.

2. Experimental procedures

2.1. HPLC–APCI–MS instrumentation

HPLC–MS analysis was performed using a LC/MSD-Trap-SL liquid chromatography/mass spectrometer (1100 Series LC/MSD Trap, a complete LC/MS/MS). The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an APCI source. The mass spectrometer system was controlled by Esquire-LC NT software (Version 4.1). All HPLC system devices were from the HP1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), a fluorescence detector (FLD; model G1321A) and a diode array detector (DAD; model G1315A). Ion source type, APCI (in positive-ion detection mode); nebulizer pressure, 60 psi; dry gas temperature, 350 °C; dry gas flow, 5.0 L/min. APCI vap temperature 350 °C; corona current (nA), 4000 (pos); capillary voltage, 3500 V. Derivatives were separated on a reversed-phase Eclipse XDB-C8 column (150 mm × 4.6 mm, 5 μm, Agilent). The HPLC system was controlled by HP Chemstation software. A 30-min gradient elution (A: 50% acetonitrile containing 30 mmol/L ammonium formate, pH 3.7; B: 100% acetonitrile) was selected for separating the free fatty acid (FFA) derivatives. The fluorescence spectra were obtained on a 650-10 S fluorescence spectrophotometer (Hitachi, Seisakusho, Tokyo, Japan). A Paratherm U_2 electronic water bath (Hitachi, Tokyo, Japan) was used to control temperature. Excitation and emission bandpass were set at 15 nm. Maximum fluorescence responses of derivatives were achieved at the excitation wavelength of 260 nm and emission wavelength of 380 nm (no correction). The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL).

2.2. Regents

Ethanol was purchased from Jining Chemical Reagent Co. (Shandong, China). HPLC grade acetonitrile was obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All fatty acids were purchased from Sigma Reagent Co. (USA). All other reagents were of analytical grade unless otherwise stated. CO_2 (99.99% purity), contained in a cylinder with an eductor tube, was obtained from Fushan Co. (Yantai, China). BDETS was synthesized in our laboratory, as described previously [11].

2.3. Plant materials

The plant materials were collected from Dulan county, Qinghai province in September 2004. *N. tangutorum* seeds were dried under a stream of nitrogen and broken into powdered samples. The oil content was 15.84%, as determined by hydrodistillation. In all cases, dried and powdered *N. tangutorum* material was used.

2.4. Supercritical CO_2 extraction

Extraction measurements were conducted in a semi-batch flow extraction apparatus as shown in Fig. 1. The extraction capacity was 1000 mL and a maximum flow rate of CO_2 was 50 kg/h. CO_2 was pumped into the extractor (E) from a 6 MPa-pressurized bottle (Q). The extractor pressure was regulated by valve 2. The separators S_1 and S_2 were regulated with valves 3 and 4, respectively. Pressure was maintained constantly at 6.7 and 6.5 MPa in separators S_1 and S_2, respectively. The extractor and separators were jacketed to maintain a constant temperature. The temperature in S_1 and S_2 was maintained at 30 °C.

In all experiments, 300 g samples of powdered seeds were placed in the extractor cylinder, and filter mesh screens were placed at both ends of the cylinder to prevent any carry over of particles. The cylinder was placed into the temperature-

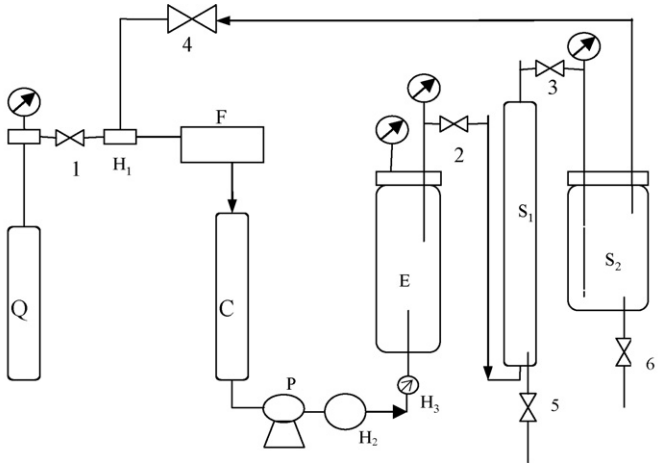

Fig. 1. Schematic diagram of the experimental apparatus. Q: CO_2 bottle; H_1: filter; F: refrigerator tank; C: fluid CO_2 cylinder; P: pump; H_2: heater; H_3: mass flowmeter; E: extractor; S_1, S_2: separators; 1–6: valves.

controlled chamber, and great care was taken to ensure the air was purged through the filter before the extraction began. The $SCCO_2$ was pumped at a constant flow rate and directed to the bottom of the extractor for up-flow configuration. The supercritical phase from the extractor was passed through the valves, into which the pressure was throttled gradually and serially via two separators. The oil was collected every 15 min from the two separators and the CO_2 was cooled and recycled into the system. Successive collected samples were weighed and analyzed.

2.5. Preparation of standard solutions

The derivatization reagent solution 1.0×10^{-3} mol/L was prepared by dissolving 3.26 mg of BDETS in 10 mL of anhydrous acetonitrile prepared by distilling the HPLC grade acetonitrile dried with P_2O_5. Individual stock solutions of the fatty acids were prepared by dissolving each fatty acid in DMF and diluting with acetonitrile to a concentration of 1.0×10^{-3} mol/L. The low fatty acid concentrations (individual concentrations of 5.0×10^{-5} mol/L) were prepared for HPLC analysis by diluting the corresponding stock solutions (1.0×10^{-3} mol/L) with HPLC-grade acetonitrile. All reagent solutions were stored at 4 °C.

2.6. Derivatization of fatty acids

A 10 mg K_2CO_3 and 200 μL DMF was added to 100 μL of a standard fatty acid mixture in a 1 mL vial. The vial was sealed and allowed to react in a water bath at 90 °C with shaking in 5 min intervals for 30 min. After the reaction was completed, the mixture was cooled at room temperature. A 200-μL volume of the acetonitrile solution (CH_3CN/H_2O 1:1, v/v) was added to dilute the derivatization solution. The diluted derivatized solution (10 μL) was injected directly into the chromatograph. The derivatization procedure is shown in Fig. 2.

2.7. Separation of free fatty acid derivatives with HPLC

HPLC separation of BDETS derivatives was conducted on an Eclipse XDB-C8 column with a gradient elution. Eluent A was 50% (v/v) of acetonitrile containing 30 mmol/L ammonium formate (pH 3.7); B was 100% (v/v) of acetonitrile. The percentage of the mobile phase was changed after injection as follows: 0–40% (B) from 0 to 12 min; 40–80% (B) from 12 to 15 min; 80–100% (B) and from 15 to 25 min; 100% (B) maintained for 5 min. Before injecting each subsequent sample, the column was equilibrated with the mobile phase A for 10 min. The flow rate was maintained at 1.0 mL/min and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at $\lambda_{ex} = 333$ nm and $\lambda_{em} = 390$ nm, respectively [11]. A complete baseline resolution for all fatty acid derivatives under these conditions is shown in Fig. 3.

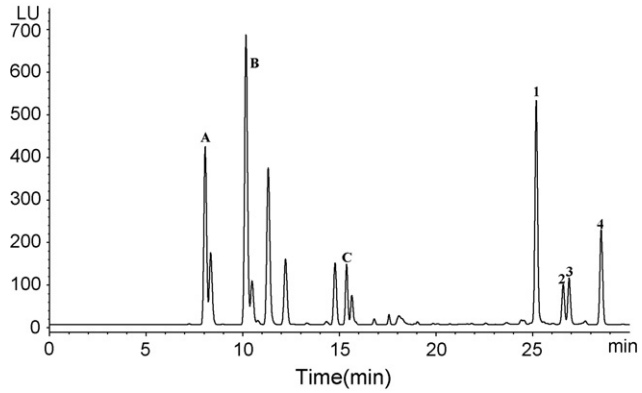

Fig. 3. Chromatogram of standard free fatty acid derivatives (100 pmol). Chromatographic conditions: column temperature was maintained at 30 °C; excitation wavelength $\lambda_{ex} = 333$ nm; emission wavelength $\lambda_{em} = 390$ nm. Eclipse XDB-C_8 column (4.6 mm × 150 mm, 5 μm); flow rate: 1.0 mL/min; Peaks: 1–4 denoted the peak of linoleic acid, hexadecanoic acid, oleic acid and octadecanoic acid, respectively. A, B and C represent the peak of 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS), unidentified and reagent, respectively.

2.8. 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 diagnosis 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.

As expected, the BDETS-fatty acid derivative produced an intense molecular ion peak at m/z $[MH]^+$. With MS/MS analysis of the fatty acid derivatives, the collision-induced dissociation

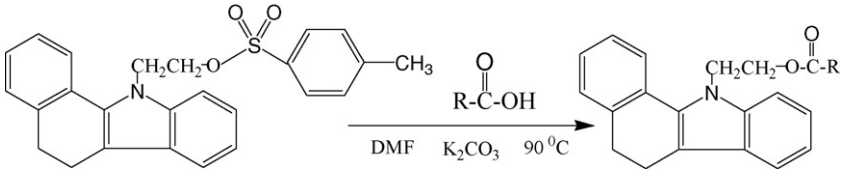

Fig. 2. Derivatization scheme of fatty acids with 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluene-sulfonate (BDETS).

spectra of m/z $(MH)^+$ produced the specific fragment ions at m/z $[M' + CH_2CH_2]^+$ and m/z 264.1 and m/z 246.0. The M′ in characteristic fragment m/z $[M' + CH_2CH_2]^+$ corresponded to the molecular mass of the fatty acids; the specific fragment ion m/z 264.1 was from the molecular core structure. The selected reaction monitoring, based on the m/z $[MH]^+ \rightarrow m/z$ $[M' + CH_2CH_2]^+$ and m/z 264.1 transition, was specific for fatty acid derivatives. There was no detectable signal from the blank water sample using this transition. Although other endogenous acidic compounds present in natural environmental samples were presumably co-extracted and derivatized by BDETS, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ions in the m/z $[M' + CH_2CH_2]^+$, m/z 264.1 and m/z 246.0 transition. The characteristic fragment ion of m/z 264.1 came from the cleavage of NCH_2CH_2O-CO, and m/z 246.0 was ascribed to the loss of H_2O molecules from the fragment ion of m/z 264.1 (RCH_2CH_2OH, here R is a molecular core structure) that created a more steady structure $RCH{=}CH_2$. With APCI in positive-ion detection mode, intense ion current signals for derivatized fatty acid derivatives should be attributed to the introduction of one weak basic nitrogen in the corresponding BDETS molecular core structure, resulting in high ionization efficiency. The cleavage mode and MS/MS analysis for the fatty acid derivatives is shown in Figs. 4 and 5, respectively. All molecular ions $[MH]^+$ and corresponding specific fragment ions for fatty acid derivatives are shown in Table 1.

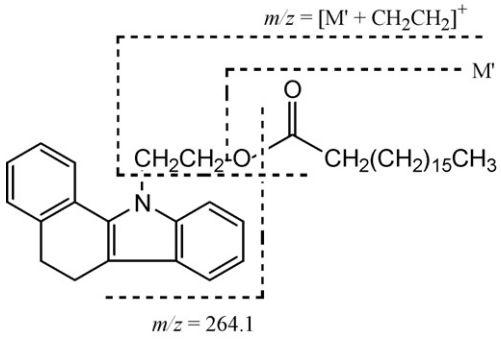

Fig. 4. The MS/MS cleavage mode of BDETS-fatty acid derivative.

2.9. Quantitative analysis

Quantitative conversion of FFAs in seed oil to their BDETS derivatives was guaranteed by using an excess of BDETS. All fatty acids were quantified in seed oil using the external standard method with detection fluorescence spectra at 390 nm. The calibration curves for each BDETS fatty acid derivative were obtained by linear regression plotting peak area versus concentration.

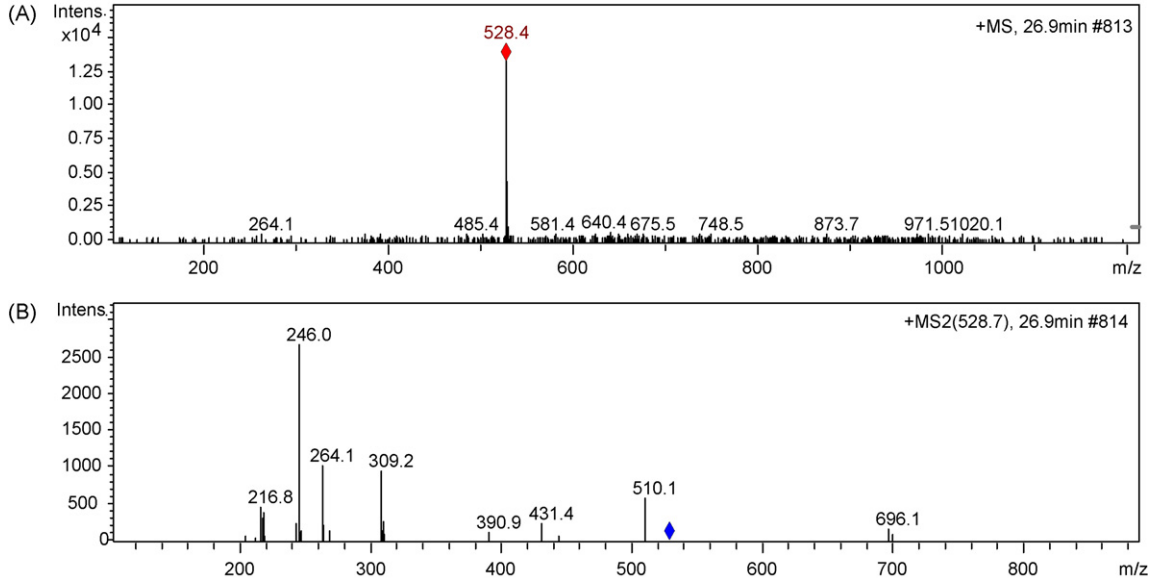

Fig. 5. The profile of the molecular ion chromatogram and scanning of the isolated representative derivative of $C_{18:1}$ fatty acid with BDETS as labeling agent. Typical MS chromatogram of BDETS-$C_{18:1}$ ester from full scanning range from 200 to 1000 amu under APCI in positive-ion detection mode (A: MS; B: MS/MS).

Table 1
MS data of BDETS fatty acid derivatives

Fatty acid	Molecular ion $[MH]^+$	Characteristic fragment ions m/z $[M' + CH_2CH_2]^+$	Specific fragment ions, molecular core structure
$C_{18:2}$	526.4	508.3 ($[MH]^+$-H_2O); 307.2	264.1, 246.0
$C_{16:0}$	502.4	283.2	264.1, 246.0
$C_{18:1}$	528.4	510.1($[MH]^+$-H_2O); 309.2	264.1, 246.0
$C_{18:0}$	530.5	311.3	264.1, 246.0

Table 2
Linear regression and detection limits

FFA	Linear regression	Regression coefficients	Detection limits (nmol/L)
Linoleic acid	$Y = 35.71X - 12.07$	0.9994	3.38
Hexadecanoic acid	$Y = 18.22X - 41.25$	0.9991	4.73
Oleic acid	$Y = 18.23X - 56.71$	0.9995	5.65
Octadecanoic acid	$Y = 23.63X - 13.64$	0.9990	6.59

2.10. Reproducibility, calibration and detection limits

We first prepared a standard solution of fatty acids (3.0×10^{-6} mol/L) and examined the reproducibility of the method. We then injected 50 pmol of each fatty acid into the HPCL scanner and recorded the resulting peaks and retention times of the relative standard deviations (RSDs). These were found to range from 1.45 to 2.01% and 0.024 to 0.074%, respectively. The linearities of the procedures were found to range from 9.766×10^{-3} to 20 μmol/L (the injection volume was 10 μL and the injected amount ranged from 400 pmol to 75 fmol with a 5300-fold concentration range). The calibration graph was established with the peak area (*y*-axis) versus the fatty acid concentration (*x*-axis: pmol, injected amount), and the linear regression equations were obtained as shown in Table 2. All of the fatty acids provided excellent linear responses, with correlation coefficients greater than 0.9990. The linear relationships at the higher concentrations were not tested. For the 1.0 pmol injections, the calculated detection limits (at signal-to-noise of 3:1) of all of the derivatized fatty acids ranged from 3.38 to 6.59 nmol/L.

3. Results and discussion

3.1. Supercritical CO_2 extraction

3.1.1. Effect of pressure

Pressure effects on the oil yields have been studied. We extracted the seed oil from unsieved seed powders at 10, 15, 20, 25 and 30 MPa at a temperature 45 °C, and CO_2 flow rate of 40 kg/h. It was found that the extraction yield increased with increasing pressure, from 10 to 20 MPa (see Fig. 6). However, the extraction yield saturated after the pressure reached 20 MPa. This may be explained by the solubility of the oil components in CO_2. For practical applications, 20 MPa should be employed.

3.1.2. Effect of temperature

The effects of extraction temperature on the oil yield were investigated at 30, 35, 40, 45 and 50 °C (in all cases, the pressure was maintained at 20 MPa, and the CO_2 flow rate at 40 kg/h). As shown in Fig. 7, the seed oil yield increased slowly with increasing temperatures, ultimately reaching a maximum at 40 °C. Such a weak dependence of oil yield on temperature is due to the retrograde solubility. On one hand, the density of the supercritical CO_2 decreased with increasing temperatures, and served to decrease the solubility of seed oil. On the other hand, the pressure of the solute in supercritical CO_2 mixtures increased concomitantly with an increase in temperature, thus improving solubility [12].

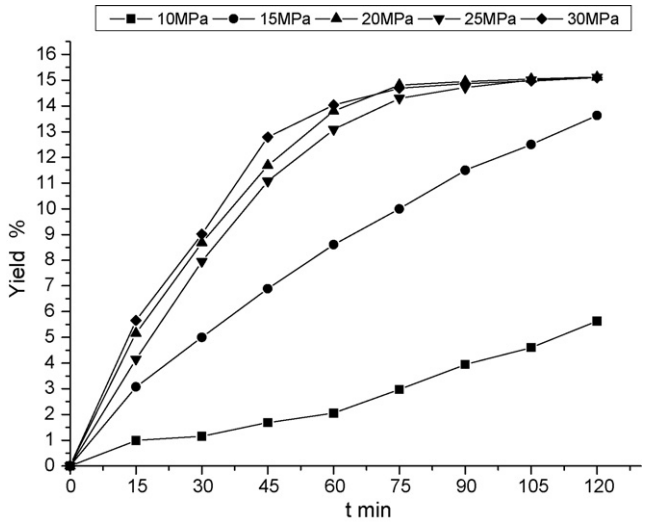

Fig. 6. Extraction rate at different pressures. The temperature was set to 40 °C, and the flow rate was 40 kg/h; particle size: unsieved.

3.1.3. Effect of particle size

Apart from pressure and temperature, sample particle size has a critical impact on the extraction efficiency. In the present work, we investigated the effect of particle size on the extraction efficiency at a given pressure (20 MPa) and temperature (40 °C). The particle sizes of 0.5, 0.9 and 2.0 mm diameters yielded, respectively, 25.9, 9.7 and 5.36% of the samples at a dynamic extraction time of 120 min. The smaller the particle size, the higher the oil yield. However, we could not use the very small samples, because these can form a bedsheet and increase resistance of the mass transfer. Therefore, a suitable particle size should be employed.

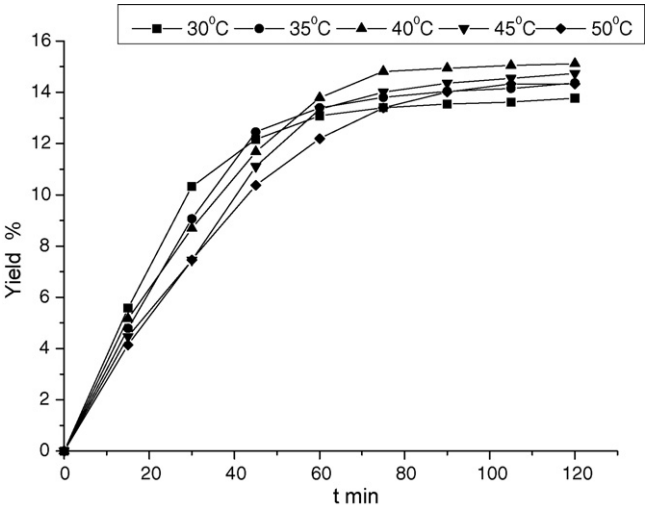

Fig. 7. Extraction rate at different temperatures. The pressure was set to 20 MPa and the flow rate was 40 kg/h; particle size: unsieved.

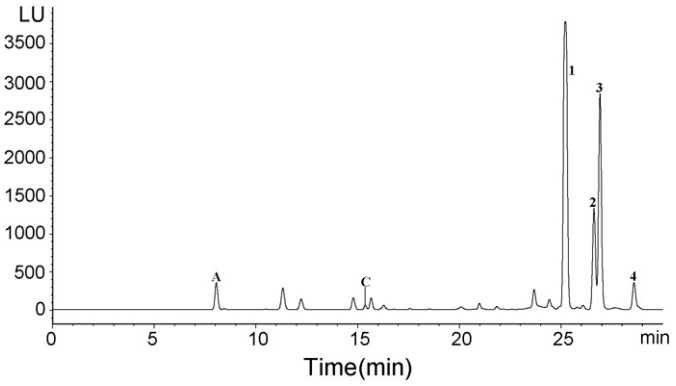

Fig. 8. Chromatogram of FFA derivative in extracted oil from *Nitraria tangutorum* seeds. Column temperature: 30 °C; excitation wavelength λ_{ex} = 333 nm; emission wavelength λ_{em} = 390 nm; Eclipse XDB-C_8 column: 4.6 mm × 150 mm, 5 μm; flow rate: 1.0 mL/min; Peaks as described in Fig. 5.

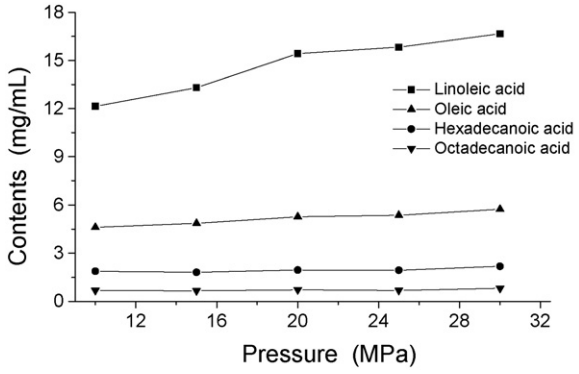

Fig. 9. Effect of extraction pressure on the free fatty acids in the seed oil. The data are averaged values of three independent samples, and the relative standard deviations are in the range of 0.04–0.11. Extraction conditions: the temperature was set to 40 °C, and the flow rate was 40 kg/h; particle size: unsieved.

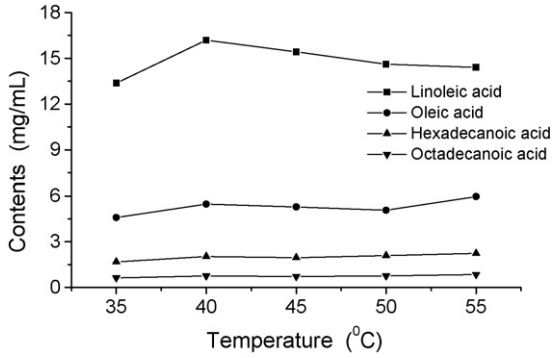

Fig. 10. Effect of extraction temperature on the free fatty acids in the seed oil. The data are average values of three independent samples, and the relative standard deviations are in the range of 0.05–0.13. Extraction conditions: the pressure was set to 20 MPa, and the flow rate was 40 kg/h; particle size: unsieved.

3.1.4. Effect of extraction time and the quality of oil

Usually, the extraction process is composed of three stages, as shown in Fig. 7. The first stage is the rapid extraction of the free solute. At this stage, the extraction yield increases rapidly with time, corresponding to the initial of the yield–time curve from 0 to 30 min. The second stage represents the transitional of surface and internal diffusions and the extraction yield increases slowly. This stage corresponds to the yield–time curve from 30 to 75 min. The third stage is when the internal diffusion and the extraction yield tend to saturated, as shown in Fig. 7 (75–120 min) [13–19]. The time consumed in the first extraction stage, however, depends on the solute solubility in supercritical CO_2, as well as the initial particle size. In this experiment (Fig. 7), the entire extraction process was almost completed within 90 min. The color of the extracted oil samples changed with extraction time from a whitish-clear to a yellow product. In the initial stages, most volatile components of oil are extracted. Later, the mixtures of oil and colorful pigments of the seed covers were extracted.

3.2. Analysis of the seed oil samples using HPLC–APCI–MS

The FFAs in the extracted oil samples were determined by HPLC. A representative FFAs chromatogram is shown in Fig. 8. The FFA components at the various extraction pressures are shown in Fig. 9. The amount of linoleic acid ($C_{18:2}$) in the extracted seed oil increased with increasing extraction pressures, while the other three fatty acids displayed only a weak pressure dependency. Similarly, the FFA components at different extraction temperatures are shown in Fig. 10. The linoleic acid ($C_{18:2}$) was found to be temperature-sensitive and corresponded to a optimal temperature of 40 °C. However, this was not the case for the other three fatty acids, the yields for which were not temperature-dependent. We refer this to the different solubility of linoleic acid at supercritical CO_2.

Additionally, we observed that the sample sizes affected the extraction efficiency. The 0.9 mm diameter size samples yielded optimal linoleic acid and oleic acid concentrations, whereas smaller particle sizes yielded the highest hexadecanoic acid returns. Optimal yields of octadecanoic acid, however, relied upon the largest starting samples. These results are shown in Table 3.

Table 3
The FFA contents in the oil extracted from different particle size samples

Diameter (mm)	Contents (mg/mL)			
	Linoleic acid	Oleic acid	Hexadecanoic acid	Octadecanoic acid
0.5	17.120	5.987	2.112	0.797
0.9	17.875	6.236	2.068	0.788
2.0	16.953	5.949	1.811	0.833

4. Conclusion

In this present work, the seed oil of *N. tangutorum* was extracted using supercritical CO_2. The experimental results show that the process parameters effect obviously on the extraction efficiency. The optimal extraction pressure and temperature was determined to be 20 MPa and 40 °C, respectively. When the rate of fluid CO_2 flow is 40 kg/h, the extraction time is 90–120 min. In addition, the FFAs concentrations in the

extracted seed oil were determined by pre-column derivatization (using BDETS as the labeling reagent) using HPLC with florescence detection, and identified by MS/APCI. We found that the main compositions of the seed oil samples extracted under the optimal conditions were FFAs in high to low concentrations as follows: linoleic acid (16.20 mg/mL), oleic acid (5.47 mg/mL), hexadecanoic acid (2.03 mg/mL) and octadecanoic acid (0.76 mg/mL). These developed methods provide a template in analyzing seed oil.

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