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Original Article

Extraction of *Microula sikkimensis* seed oil and simultaneous analysis of saturated and unsaturated fatty acids by fluorescence detection with reversed-phase HPLC

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ABSTRACT

The seed oil of *Microula sikkimensis* Hemsl. was extracted by supercritical carbon dioxide extraction, microwave-assisted reflux extraction, ultrasound-assisted extraction and solvent reflux extraction. The experimental parameters of supercritical carbon dioxide extraction which is classic in these extraction techniques including pressure, temperature, particle size and extraction time were optimized. A simple and sensitive method for the simultaneous determination of 39 kinds of saturated and unsaturated fatty acids in seed oil using high performance liquid chromatography (HPLC) with fluorescence detection following pre-column derivatization with 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP) has been developed. Fatty acid derivatives were separated on a reversed-phase Eclipse XDB-C_8 column in conjunction with a gradient elution. Quantitative linear range of 39 fatty acids was 0.014–14.29 μmol/L, and excellent linear responses were observed with correlation coefficients more than 0.9992. Detection limits were in the range of 3.24–36.97 fmol (10 μL, S/N 3:1). Stability of derivatives, method repeatability and recovery were evaluated and the results were excellent for efficient HPLC analysis. Fatty acids in *M. sikkimensis* Hemsl. seed oil with or without saponification by different extraction methods were analyzed and compared. The facile TSPP derivatization coupled with HPLC fluorescence detection allowed for the quantitation of short- and long-chain fatty acids from biological and natural environmental samples.

1. Introduction

Microula sikkimensis genus is a member of the *Microula* Benth. family. It is a kind of rare wild oil plant around Qing-Tibet Plateau and is widely distributed in Bhutan, Sikkim, Nepal and the northwest region of China in places such as Qinghai and Tibet. There are 29 species known worldwide, of which 25 are found especially in China (Editorial Committee of Chinese Plant, 1990; Wang et al., 2003). *M. sikkimensis* Hemsl. (*M. sikkimensis* H.) is the natural distributing species in Qinghai Province of China on Qing-Tibetan Plateau. The seeds of *M. sikkimensis* H. contain abundant oil, more than 80% of which are unsaturated fatty acids such as linolenic acid and linolic acid. This plant is an excellent and edible oil plant with health benefits. Furthermore, the lipids it contains are important in medicinal treatments for cardiovascular and hepatic diseases (Liu et al., 2006). It has been reported that the seed oil of *M. sikkimensis* H. was effective in preventing and treating experimental hyperlipemia in rats (Li et al., 1999). Although eight chemical constituents (β-sitosterol, lupeol, betulinol, stigmasta-4-ene-3-one, stigmasta-4-ene-3,6-dione, 6β-hydroxylstigmasta-4-ene-3-one, normilin, rutaevin) in the seed of *M. sikkimensis* H. were isolated and reported (Zheng et al., 2003), there have been almost no reports about the fatty acid composition in this plant.

Fatty acids, especially unsaturated fatty acids, are important as nutritional substances and metabolites in living organisms. Many kinds of fatty acids play an important role in the regulation of a variety of physiological and biological functions (Zhao et al., 2006). For *M. sikkimensis* H., fatty acids exist mainly in the lipids of the seed oil. The traditional extraction methods for the seed oil are ultrasound-assisted extraction (USE) (Jerkovic et al., 2007) and solvent reflux extraction (SRE) (Custódio et al., 2007), but these methods require organic solvents and high temperatures, which can possibly cause oxidation of the long-chain unsaturated fatty acids. Microwave-assisted reflux extraction (MWRE) (Chen et al.,

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2008) is rapid and effective; however, it requires polar solvent – especially organic solvent – which is disadvantageous for the development of foods, drugs and health products. Recently, supercritical carbon dioxide extraction (SCCE) (Cao et al., 2007; Landberg et al., 2007) has become one of the most popular methods for lipid extraction because of its low temperature and high efficiency. Therefore, in this paper, we carefully studied the extraction parameters of SCCE for the extraction of seed oil of *M. sikkimensis* H., and simultaneously evaluated the MWRE, USE and SRE methods for the purpose of comparison.

Moreover, an analysis of the fatty acids in *M. sikkimensis* H. seed oil is equally important. Therefore, it is necessary to develop a sensitive method for the simultaneous determination of both saturated and unsaturated fatty acids. Most fatty acids show neither natural absorption in visible or UV regions nor fluorescence, thus their detection of trace levels using absorptiometry is fairly difficult (Ingalls et al., 1984). However, easily detectable fatty acid derivatives by methyl or ethyl esterification with GC or GC–MS have been reported (Gamazo-Vázquez et al., 2003; Kulig Clark et al., 2006). In contrast with GC, the use of HPLC allows the fatty acids to be converted to a large number of different derivatives (Christie, 1987). Derivatization can overcome some problems such as tailing peaks and low detector sensitivity by the formation of less polar compounds, which can be more easily analyzed by LC. Therefore, derivatization of these analytes with labelling reagents has been widely adopted because HPLC with UV, especially fluorescence detection, has higher sensitivity. However, Toyo'oka (2002) have reported that many of these reagents have limitations in their applications such as low detection sensitivity, short detection wavelengths, poor stability, tedious analytical procedure and serious interferences in the biological sample analyses.

More recently, we synthesized acridone-9-ethyl-*p*-toluenesulfonate (AETS) (Zhao et al., 2006; Li et al., 2006) and 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) (Shi et al., 2005; You et al., 2004) for the sensitive determination of 19 free fatty acids and 10 bile acids. The aims of the present work are: (1) to develop a sensitive method using 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP) (You et al., 2007; Zhao et al., 2007) as labelling reagent for the simultaneous determination of saturated and unsaturated fatty acids and (2) to develop an efficient extraction technique for the lipids (seed oil) in *M. sikkimensis* H. The fluorescence detection sensitivity of TSPP for fatty acids was compared with that of AETS (Zhao et al., 2006; Li et al., 2006), BDETS (Shi et al., 2005; You et al., 2004) and NOEPES (Lu et al., 2000).

2. Materials and methods

2.1. Instrumentation

The HPLC system was an Agilent HP 1100 series and consisted of an on-line 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, an 1100 Series LC/MSD Trap-SL (ion trap) 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. Ion source conditions were: APCI in positive-ion mode; nebulizer pressure 413.69 MPa; dry gas temperature 350 °C dry gas flow 5.0 L min^{-1}; vap temperature 450 °C; corona current (nA) 4000 (pos); capillary voltage 3500 V. Derivatives were separated on an Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent Co.) by a gradient elution. A Paratherm U2 electronic water bath (Hitachi, Tokyo, Japan) was used to control temperature. The mobile phase was filtered through a 0.2-mm nylon membrane filter (Alltech, Deerfield, IL).

2.2. Chemicals

Standards of 30 saturated fatty acids (C1–C30) were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Nine unsaturated fatty acid standards including 12-octadecenoic acid (C18:1), 9,12-octadecadienoic acid (C18:2), 8,11,14-octadecatrienoic acid (C18:3), 11-eicosenoic acid (C20:1), 6,9,12,15-arachidonic acid (C20:4), 5,8,11,14,17-eicosapentaenoic acid (C20:5), 12-docosenoic acid (C22:1), 2,5,8,11,14,17-docosahexenoic acid (C22:6), and 20-tetracosenoic acid (C24:1) were purchased from Sigma Co. (St. Louis, MO, USA). Spectroscopically pure acetonitrile was purchased from Merck Co. (Germany). N,N-dimethylformamide (DMF) and dimethyl-sulfoxide (DMSO) were purchased from Jining Chemical Reagent Co. (Shandong, Jining, China) and treated with 5 Å molecular sieve, and then redistilled prior to use. Benzene, toluene, tetrahydrofuran, potassium carbonate, pyridine and chloroform were of analytical grade obtained from Shanghai Chemical Reagent Co. (Shanghai, China). AETS, BDETS and TSPP were synthesized in our laboratory. TSPP has good stability as solid or in anhydrous acetonitrile or methanol solution. The fluorescence excitation and emission wavelengths of TSPP were at λ_{ex} = 260 and λ_{em} = 380 nm, and exhibited no obvious blue- or red-shift in different concentrations of acetonitrile or methanol (0–100%). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were all of analytical grade unless otherwise stated.

2.3. Plant materials of M. sikkimensis H.

The plant materials were collected from Qinghai province in October 2006. *M. sikkimensis* H. seeds were dried under a stream of nitrogen and ground into powdered samples with 0.5 mm, 0.9 mm and 2.0 mm diameters for SCCE, and 0.5 mm diameter for MWRE, USE and SRE. In all cases, dried and powdered *M. sikkimensis* H. material was used.

2.4. Extraction of seed oil from M. sikkimensis H. samples

The seed oil of *M. sikkimensis* H. was extracted by supercritical carbon dioxide extraction (SCCE), microwave-assisted reflux extraction (MWRE), ultrasound-assisted extraction (USE) and solvent reflux extraction (SRE). Chloroform was chosen as the extraction solvent for MWRE, USE and SRE because it could give satisfactory extraction yields for short- and long-chain fatty acids (Zhao et al., 2007). The extraction procedures of the four methods were as follows:

(1) Extraction procedures of SCCE were conducted in a flow extraction apparatus. In every experiment 300 g samples of powdered seeds were used. 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 from a 6 MPa-pressurized bottle. 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. 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) To a 100-mL-round-bottom flask, 0.5 g pulverized *M. sikkimensis* H. seed and 25 mL chloroform were added, and the sample was extracted for 20 min at 65 °C under reflux with the

microwave power set to 500 W. After the contents were filtered, the chloroform was evaporated to dryness in a rotary vacuum evaporator at 60 °C, leaving the extracted seed oil.

(3) To a 50 mL of round-bottom flask, 0.5 g pulverized *M. sikkimensis* H. 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, leaving the extracted seed oil.

(4) To a 50-mL-round-bottomed flask, 0.5 g pulverized *M. sikkimensis* H. 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, leaving the extracted seed oil.

2.5. Saponification of seed oil

To a 10-mL test tube, 0.1 g seed oil and 2.0 mL potassium hydroxide/methanol solution (2 mol/L) 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 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-mm nylon membrane filter, and stored at −10 °C until HPLC analysis.

2.6. Preparation of standard solutions

The labelling reagent (TSPP) solution (5.0×10^{-2} mol/L) was prepared by dissolving 246 mg of 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP) in 10 mL of DMF. Corresponding derivatization reagent solution of low concentration (0.01 mol/L) was obtained by diluting the stock solution with DMF. Individual standard of fatty acids was 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, v/v) owing to their poor solubility. Standards of 39 mixed fatty acids (1.0×10^{-4} mol/L) were prepared by diluting corresponding individual stock solution with acetonitrile to a concentration of 1.0×10^{-4} mol/L. When not in use, all reagent solutions were stored at 4 °C in a refrigerator until HPLC analysis.

Table 1
Gradient elution program.

Time (min)	A (%)	B (%)	C (%)	D (%)
0	95	0	5	0
4	95	0	5	0
4.2	0	95	5	0
8	0	95	5	0
8.5	0	75	25	0
15	0	50	50	0
50	0	0	100	0
60	0	0	0	100
75	0	0	0	100

2.7. Derivatization

To a solution consisting of 50 μL of standard fatty acids 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 at 5-min intervals. After the reaction was completed, the mixture was taken to cool at room temperature and diluted appropriately for HPLC analysis. The derivatization procedure is shown in Fig. 1.

2.8. Chromatographic 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 mm, Agilent Co.) by a gradient elution. Eluent A was 50% of acetonitrile; B was 50% acetonitrile containing 20 mmol/L ammonium formate buffer (pH 3.7); C was a mixture solution of acetonitrile and DMF (ACN/DMF, 100:2, v/v); D was a mixed solution of acetonitrile and DMF (ACN/DMF 100:30, v/v). 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 and λ_{em} 380 nm, respectively. Gradient elution program is presented in Table 1.

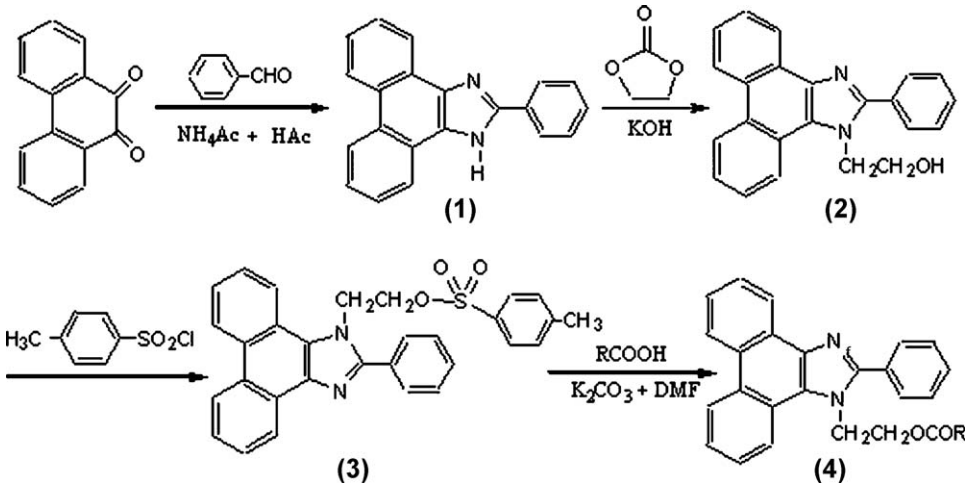

Fig. 1. The synthesis of TSPP and the derivatization procedure of TSPP with fatty acids. (1) Phenylimidazole-[4,5-f]-9,10-phenanthrene; (2) 1-(ethanol)-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (EPP); (3) 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP); (4) corresponding derivative.

2.9. Quantitative analysis

Quantitative conversion of fatty acids from the extracts of *M. sikkimensis* H. 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. Extraction of seed oil

For SCCE, the experimental parameters such as pressure, temperature, time, and particle size were evaluated. When the seed oil was extracted from seed powders at 10, 15, 20, 25 and 30 MPa at 45 °C with CO_2 flow rate of 40 kg/h, the extraction yield (calculated as: [oil weight (g)/pulverized seed weight (g)] × 100%) increased from 10 to 20 MPa; above 20 MPa, the extraction yield was invariable. Therefore, 20 MPa should be employed. The effects of temperature were investigated at 30, 35, 40, 45 and 50 °C with the pressure maintained at 20 MPa and the CO_2 flow rate at 40 kg/h. The seed oil yield increased slowly with increasing temperatures, and reached a maximum at 40 °C. Besides pressure and temperature, particle size of pulverized seed sample had a critical impact on the extraction efficiency. We investigated it at a given pressure (20 MPa) and temperature (40 °C). The particle sizes of 0.5 mm, 0.9 mm and 2.0 mm diameters yielded 33.9, 17.7 and 13.36% of extracted oil at an extraction time of 120 min, respectively. Obviously, the smallest particle size gave the highest oil yield. However, we could not use smaller samples because these could form a layer and increase the resistance of the mass transfer. Therefore, a suitable particle size at 0.5–0.9 mm diameter was employed. In addition, for extraction time, the entire extraction process was almost completed within 90 min. Under the above-mentioned optimal conditions, the extraction yield of SCCE may attain 34.0%.

For the three other extraction methods (MWRE, USE and SRE), the routine conditions were used as described above in Section 2, and the extraction yields for them were as follows: MWRE, 13.12%; USE, 7.35%; and SRE, 12.16%.

From the extraction yields of four methods, we can see that SCCE has the highest extraction yield. Moreover, it uses no organic solvents and works under relatively lower extraction temperature. All these characteristics of SCCE make it increasingly popular for the analysis of food, medicine, health products, etc.

3.2. Derivatization

The optimization process of derivatization of TSPP with 26 fatty acids (C1–C26) has been reported by our laboratory (Zhao et al., 2007). After our careful study, we found that the derivatization conditions of TSPP with 39 kinds of saturated and unsaturated fatty acids were similar to our earlier study. Therefore, we do not give unnecessary details in the present 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 fivefold molar reagent excess to total molar fatty acids.

3.3. HPLC separation and MS identification

An Eclipse XDB-C_8 column was selected in conjunction with a gradient elution, and several programs were investigated to ensure satisfactory HPLC separation. The optimal gradient elution was carried out as described in Table 1. To achieve optimal separation, the choice of pH value of mobile phase B was tested. The results indicated that separation of the derivatized long- and short-chain fatty acids can be accomplished at acidic condition with pH 3.7. In fact, the addition of DMF in mobile phase C and D could raise solubility of fatty acid derivatives to obtain fast separation with sharp peaks. With the optimal chromatographic conditions described above, a satisfactory resolution for 39 fatty acid derivatives was obtained within 75 min with the shortest retention-time values and the sharpest peaks.

The ionization and fragmentation of the isolated TSPP-fatty acid derivatives were studied by using mass spectrometry, with atmospheric pressure chemical ionization (APCI) source in 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 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 295.0 (M′ was molecular mass of fatty acid). The specific fragment ion m/z 295.0 was the protonated TSPP core structure moiety, and the ion m/z $[M'+CH_2CH_2]^+$ was the protonated fatty acid moiety. With APCI in positive-ion mode, intense ion current signals should be attributed to the introduction of two weak basic nitrogen atom in TSPP molecular core structure which results in highly ionizable efficiency. The MS and MS/MS analysis and cleavage mode for a representative TSPP-C20 derivative is shown in Fig. 2(A–C).

3.4. Comparison of the fluorescence sensitivity with AETS and BDETS

Under the optimal experimental conditions, fluorescence responses for representative C10–C20 fatty acid derivatives using AETS and BDETS as labelling reagent were, respectively, at least two- to fourfold and three- to eightfold lower than those 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 molar absorbance coefficient may be attributed to the introduction of a phenylimidazole-[4,5-f]-9,10-phenanthrene function group into TSPP molecular structure, by which the n–π conjugation system is dramatically augmented.

3.5. Reproducibility, accuracy, linearity and detection limits

A standard solution consisting of 39 fatty acids was prepared, and the method reproducibility was examined by injecting quantitative fatty acid derivatives 6 times (injected amount 50 pmol, 10 μL). The RSDs of the peak areas and retention times were 0.095–2.96 and 0.019–0.41%, respectively. Precision and accuracy: 6 replicates ($n = 6$) at 0.1, 1.0 and 5.0 μmol/L of 39 fatty acids were used to make the low- to high-range concentrations. The mean accuracy ranged from 91.2 to 104.5% with the largest mean RSD < 7.5%.

Based on the optimum derivatization conditions, the linearities of 39 fatty acids were evaluated in the range of 0.014–14.29 μmol/L. The calibration graph was established with the peak area (y) versus fatty acid injection amount (x: pmol). All the fatty acids were found to give excellent linear responses over this range with correlation coefficients of 0.9992–0.9999. With 1.0 pmol injection for each derivatized fatty acid, the calculated detection limits (S/N = 3:1) were from 3.24 to 36.97 fmol with an average of 21.33 fmol. With AETS, BDETS and NOEPES as labelling reagents, the detection limits were, 12.3–43.7 fmol for AETS (Zhao et al., 2006) with an average of 24.6 fmol, 24.8–80.37 fmol for BDETS (Shi et al., 2005) with an average of 46.5 fmol, and 56 fmol for NOEPES (Lu et al., 2000). The lowest detection limits and the average

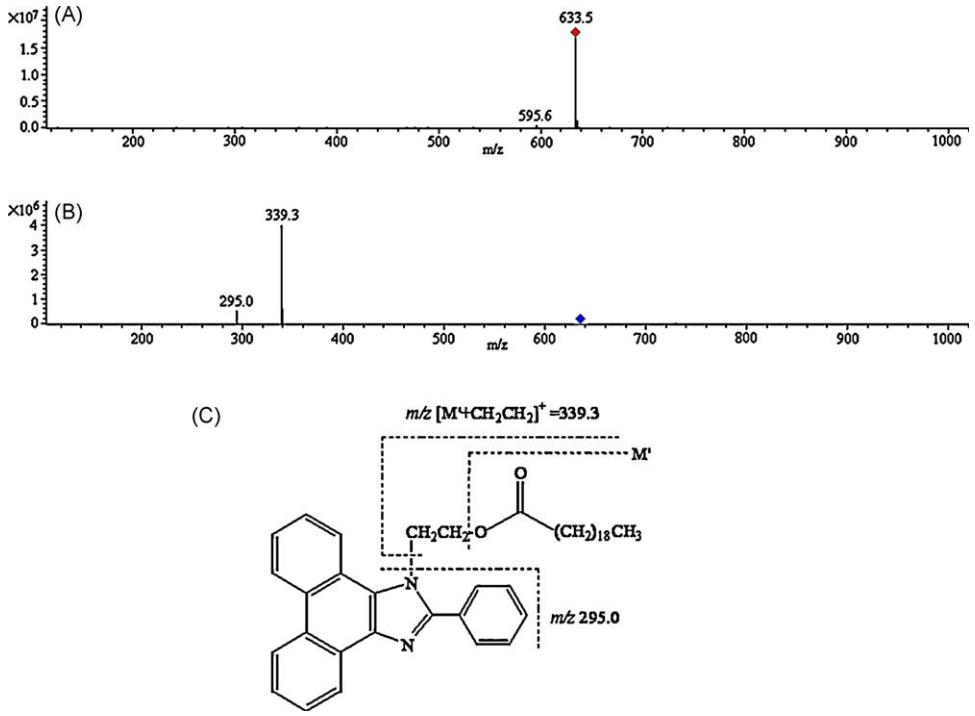

Fig. 2. The profile of MS and MS/MS of the representative n-eicosoic acid derivative (TSPP-C20). (A) Typical MS chromatogram of n-eicosoic acid derivative from full scanning range from 100 to 1000 amu with APCI source in positive-ion mode; (B) typical MS/MS chromatogram of n-eicosoic acid derivative from full scanning range from 100 to 1000 amu with APCI in positive-ion mode; fragment ions, *m*/*z* 339.3 and *m*/*z* 295.0; (C) the MS/MS cleavage mode of TSPP-C20 derivative.

detection limits of TSPP, respectively, decreased by four- to eightfold and one- to threefold in comparison with those of AETS, BDETS and NOEPES per 10 μL injection.

3.6. Analysis of *M. sikkimensis* H. samples and recovery

The representative chromatogram of fatty acids in the seed oil of *M. sikkimensis* H. seeds by SCCE with fluorescence detection is shown in Fig. 3. Chromatographic peaks were identified by contrasting their retention times with those of standard peaks and simultaneously confirming them through on-line MS identification. Fatty acid compositions after saponification from the seed oil of *M. sikkimensis* H. seed by SCCE, MWRE, USE and SRE, and the free fatty acid compositions without saponification from the seed oil of SCCE, are shown in Table 2. The results indicate that (1) the main fatty acids in *M. sikkimensis* H. are, C18:1 257.15 mg/g (28.57%, mass percent); C18:2 210.80 mg/g (23.43%); C18:3 179.04 mg/g (19.89%); C16 113.16 mg/g (12.57%); C18 34.48 mg/g (3.83%); (2) the contents (mg/g) of fatty acids after saponification are about 50–65 times higher than those of free fatty acids. This may be due to the fact that most fatty acids in *M. sikkimensis* H. seed exist in the formation of esters such as glyceride, methyl ester or ethyl ester; and (3) the mass percents (%, ratio of the mass of a fatty acid with that of all fatty acids) of unsaturated fatty acids (C18:3, C18:2, C18:1) in the seed oil by MWRE, USE and SRE, are obviously lower than those that result from SCCE, but this finding is the opposite for C16 and C18. This may be due to the different extraction mechanism of these four methods: MWRE and USE proceed under the assistance of microwave and ultrasound, and the MWRE and SRE proceed with the solvent reflux at 60 °C or higher temperature; SCCE proceeds with supercritical carbon dioxide fluids as extracting solvent under 40 °C, and this experimental condition avoids the interface of atmospheric oxygen with the unsaturated fatty acids in the seed oil.

The recoveries of 39 fatty acids were investigated by the addition of known amounts of standard solution (10 μL, 1.0×10^{-4} mol/L) to pulverized samples of *M. sikkimensis* H. seed. The MWRE and derivatization were the same as optimal conditions above, and the analyses were carried out in three duplicates. The experimental recoveries were in the range of 89.12–105.7%.

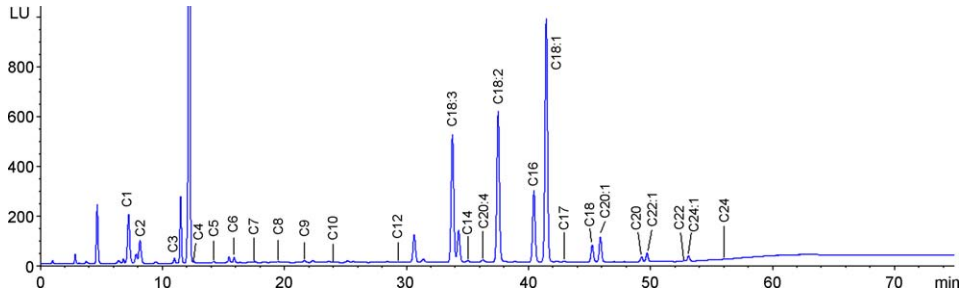

Fig. 3. The representative chromatogram of fatty acids in the seed oil of *M. sikkimensis* H. seeds by supercritical carbon dioxide extraction. Peaks: C1–C30 are 30 kinds of saturated fatty acids containing corresponding carbon atom from 1 to 30, 9 kinds of unsaturated fatty acids are listed in experimental; (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 2
Contents of fatty acids in *M. sikkimensis* H. seed oil by four extraction methods ($n = 3$).

Fatty acids	SCCE with saponification, % (mg/g)[a]	MWRE with saponification, % (mg/g)[a]	USE with saponification, % (mg/g)[a]	SRE with saponification, % (mg/g)[a]	SCCE without saponification, % (mg/g)[a]
C1	0.31 (2.80)	0.31 (2.63)	0.32 (2.44)	0.32 (2.57)	0.40 (0.056)
C2	0.24 (2.16)	0.24 (2.03)	0.24 (1.88)	0.24 (1.98)	0.31 (0.043)
C3	0.084 (0.75)	0.085 (0.71)	0.087 (0.65)	0.087 (0.69)	0.11 (0.015)
C4	0.063 (0.57)	0.064 (0.54)	0.066 (0.49)	0.065 (0.52)	0.082 (0.011)
C5	0.049 (0.44)	0.049 (0.42)	0.051 (0.38)	0.051 (0.41)	0.064 (0.0088)
C6	0.27 (2.47)	0.27 (2.33)	0.28 (2.15)	0.28 (2.28)	0.36 (0.049)
C7	0.043 (0.39)	0.043 (0.36)	0.045 (0.33)	0.044 (0.35)	0.052 (0.0072)
C8	0.15 (1.35)	0.15 (1.27)	0.15 (1.17)	0.15 (1.23)	0.18 (0.025)
C9	0.23 (2.03)	0.23 (1.89)	0.24 (1.77)	0.23 (1.85)	0.27 (0.037)
C10	0.028 (0.25)	0.028 (0.24)	0.029 (0.22)	0.029 (0.23)	0.034 (0.0047)
C11	0	0	0	0	0
C12	0.14 (1.26)	0.14 (1.17)	0.14 (1.08)	0.14 (1.14)	0.16 (0.023)
C13	0	0	0	0	0
C20:5	0	0	0	0	0
C18:3	19.89 (179.04)	19.77 (164.71)	19.76 (148.60)	19.74 (157.55)	19.81 (2.75)
C22:6	0	0	0	0	0
C14	0.27 (2.39)	0.27 (2.25)	0.27 (2.06)	0.27 (2.20)	0.26 (0.036)
C20:4	0.55 (4.91)	0.55 (4.62)	0.56 (4.22)	0.56 (4.52)	0.54 (0.075)
C18:2	23.43 (210.80)	23.28 (193.94)	23.26 (174.96)	23.24 (185.50)	23.32 (3.24)
C15	0	0	0	0	0
C16	12.57 (113.16)	12.77 (106.37)	12.94 (97.32)	13.04 (104.11)	12.52 (1.74)
C18:1	28.57 (257.15)	28.40 (236.57)	28.04 (210.86)	28.02 (223.72)	28.45 (3.95)
C17	0.21 (1.86)	0.21 (1.75)	0.21 (1.60)	0.21 (1.69)	0.21 (0.028)
C18	3.83 (34.48)	3.89 (32.41)	3.94 (29.65)	3.93 (31.37)	3.82 (0.53)
C20:1	4.81 (43.25)	4.88 (40.65)	4.95 (37.19)	4.93 (39.35)	4.78 (0.66)
C19	0	0	0	0	0
C20	0.88 (7.92)	0.89 (7.44)	0.91 (6.81)	0.90 (7.21)	0.87 (0.12)
C22:1	1.83 (16.48)	1.86 (15.49)	1.88 (14.17)	1.88 (14.99)	1.82 (0.25)
C21	0	0	0	0	0
C22	0.15 (1.32)	0.15 (1.24)	0.15 (1.14)	0.15 (1.20)	0.15 (0.020)
C24:1	1.24 (11.17)	1.26 (10.49)	1.27 (9.61)	1.27 (10.16)	1.24 (0.17)
C23	0	0	0	0	0
C24	0.16 (1.42)	0.16 (1.34)	0.16 (1.22)	0.16 (1.29)	0.16 (0.022)
C25	0	0	0	0	0
C26	0	0	0	0	0
C27	0	0	0	0	0
C28	0	0	0	0	0
C29	0	0	0	0	0
C30	0	0	0	0	0

[a] Mass percent (%, ratio of the mass of a fatty acid with that of all fatty acids), absolute content (mg (fatty acid)/g (oil)).

4. Conclusion

In this study, simultaneous determination of 39 fatty acids extracted from pulverized *M. sikkimensis* H. seed using TSPP as derivatization reagent with HPLC fluorescence detection and on-line MS identification has been successfully achieved. The SCCE method is more efficient and more compatible for chemical compositions extraction in natural medicines or foods than MWRE, USE and SRE. It is the authors' hope that the established method can be applied to the extraction and determination of fatty acids from various drugs, plants and biochemistry samples.

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