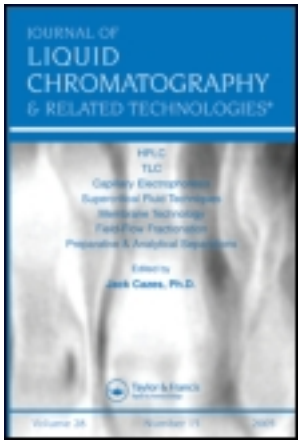

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ljlc20

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Accepted author version posted online: 23 Apr 2012. Version of record first published: 13 Aug 2012

To cite this article: Cuihua Song, Caiqing Zhang, Zhiwei Sun, Jinmao You & Yourui Suo (2012): DETERMINATION AND IDENTIFICATION OF FATTY ACIDS IN MICROULA SIKKIMENSIS SEED OIL USING 1,2-BENZOCARBAZOLE-9-ETHYL-P-TOLUENESULFONATE AS A NOVEL LABELING REAGENT BY HPLC WITH FLUORESCENCE DETECTION AND APCI-MS, Journal of Liquid Chromatography & Related Technologies, 35:14, 2066-2080

To link to this article: http://dx.doi.org/10.1080/10826076.2011.627621

Journal of Liquid Chromatography & Related Technologies, 35:2066–2080, 2012

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826076.2011.627621

DETERMINATION AND IDENTIFICATION OF FATTY ACIDS IN *MICROULA SIKKIMENSIS* SEED OIL USING 1,2-BENZOCARBAZOLE-9-ETHYL-P-TOLUENESULFONATE AS A NOVEL LABELING REAGENT BY HPLC WITH FLUORESCENCE DETECTION AND APCI-MS

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□ *Using 1,2-benzocarbazole-9-ethyl-p-toluenesulfonate (BCETS) as the labeling reagent, a sensitive method for the determination of fatty acids by high performance liquid chromatography (HPLC) with fluorescence detection has been developed. The BCETS could easily and quickly label fatty acids in the presence of the K_2CO_3 catalyst at 90° C for 30 min in N,N-dimethylformamide solvent. All the tested fatty acid derivatives were separated on a reversed-phase Eclipse XDB-C_8 column by HPLC in conjunction with gradient elution. The identification was carried out by post-column atmospheric pressure chemical ionization mass spectrometry (APCI/MS) in positive ion detection mode. The fluorescence excitation and emission wavelengths of the derivatives were at λ_{ex} 279 nm and λ_{em} 380 nm, respectively. Linear correlation coefficients for all fatty acid derivatives are more than 0.9994. Detection limits, at a signal-to-noise ratio of 3:1, were 0.21–0.99 μg/L for the labeled fatty acids. In this study, fatty acids from the extracted* Microula sikkimensis *Hemsl (*M. sikkimensis *H.) seed were sensitively determined. It was found that the seed oil mainly contained Oleic acid, Linoleic acid, α-Linolenic acid, Hexadecanoic acid, Stearidonic acid, γ-Linolenic acid, and Eicosenoic acid.* M. sikkimensis *H. seed oil was rich in unsaturated fatty acids and may be used as high-quality edible oil for its health benefits.*

Keywords derivatization, fatty acids, fluorescence detection, high performance liquid chromatography, mass spectrometry, *Microula sikkimensis* Hemsl seed oil

C. Zhang and C. Song contributed equally to this work.

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INTRODUCTION

Microula sikkimensis genus is a member of the *Microula* Benth. family. It is a rare wild plant found around the Qing-Tibet Plateau and is widely distributed in Bhutan, Sikkim, Nepal, and the northwest region of China. There are twenty-nine species known worldwide, of which twenty-five are found in China.[1] *Microula sikkimensis* Hemsl (*M. sikkimensis* H.) is the natural distributing species in Qinghai Province of China on Qing-Tibetan Plateau. This plant is an excellent and edible oil plant with health benefits. The lipids it contains are important in medicinal treatments for cardiovascular and hepatic diseases.[2] It has been reported that the seed oil of *M. sikkimensis* H. is effective in preventing and treating experimental hyperlipemia in rats.[3] Although some chemical constituents in the seed were isolated and reported,[4] literature reports on fatty acid composition of *M. sikkimensis* H. seed oil are relatively poor.[5,6]

Most methods used for the determination of fatty acids are based on liquid-liquid extraction using methyl tert-butyl ether, derivatization to the respective methyl-, trimethylsilyl-esters, and analysis using gas chromatography (GC) analysis.[7,8] Although GC methods accompanied by derivatization are extensively used for carboxylic acid analyses, there are some disadvantages, particularly with respect to heat-labile polyenic fatty acid methyl esters. On the other hand, high performance liquid chromatography (HPLC) becomes an important means for the separation of multi-components in complex matrices. However, many of biologically important carboxylic acids do not exhibit efficient detection properties, such as absorption in ultraviolet-visible regions and luminescence. Furthermore, absorbance of underivatized carboxylic acids near 205 nm cannot be recommended, because the solvents added to the mobile phase absorb at the detection wavelength. An increasingly popular way to enhance the sensitivity and selectivity of detection for carboxylic acids is chemical derivatization of the carboxyl moiety with a suitable chromophore, fluorophore, and electrophore. Therefore, derivatization of these analytes with labeling reagents, especially for sensitive fluorescent detection, has been widely adopted in the past three decades.[9–15] However, it has been reported that these reagents have limitations in their applications such as low detection sensitivity, short detection wavelengths, poor stability, tediously analytical procedure, and serious interferences in the biological sample analyses.[16] More recently, we have synthesized acridone-9-ethyl-p-toluenesulfonate,[17,18], 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-d]imidazol-1-yl) ethyl-p-toluenesulfonate,[19] 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-*f*]-9,10–phenanthrene,[20] and 1,2-benzo-3, 4-dihydrocarbazole-9-ethyl-p-toluenesulfonate[21] for the sensitive determination of saturated fatty acids. In this work, A novel reagent 1,2-benzo-9-ethyl-*p*-toluenesulfonate (BCETS) for labeling fatty acids was synthesized and evaluated. The introduction of the 1,2-benzocarbazole

functional group into the labeling reagent molecule dramatically augmented the *n*-p conjugation and was favorable for the sensitive determination of fatty acids with fluorescence detection. Also, it was the first time that the fatty acids from M. sikkimensis H seed oil were determined by HPLC with fluorescence detection. The results indicated that *M.* sikkimensis *H.* seed oil was rich in unsaturated fatty acids and may be used as high-quality edible oil for its health benefits.

MATERIALS AND METHODS

Instruments

The HPLC system was Agilent 1100 series (Hewlett-Packard, CA, USA) and consisted of a 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 mass spectrometer, an 1100 Series LC/MSD Trap-SL (Bruker Daltonik, Bremen, Germany), was equipped with an atmospheric pressure chemical ionization (APCI) source (model G1947A). The HPLC system was controlled by HP Chemstation software. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. Derivatives were separated on a reversed phase Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent) by a gradient elution. The mobile phase was filtered through a 0.2 mm nylon membrane filter (Alltech, Deerfield, IL). A HA120-50-2 supercritical fluid extraction instrument (Huaan scientific research devices Co. Ltd., Nantong, Jiangsu, China) was employed. The extraction capacity was 1000 mL and a maximum flow rate of CO_2 was 50 kg/h.

Chemicals and Materials

Standards of saturated fatty acids (C5-C24) were purchased from Shanghai Chemical Reagent Co (Shanghai, China). Nine unsaturated fatty acid standards including Oleic acid (C18:1 ω9), Linoleic acid(C18:2 ω6), α-Linolenic acid(C18:3 ω3), Eicosenoic acid (C20:1 ω9), Arachidonic acid (20:4 ω6), Eicosapentanoic acid(C20:5 ω3), Docosenoic acid(C22:1 ω9), Docosahexanoic acid(C22:6 ω3;), and Tetracosenoic acid(C24:1 ω9) were purchased from Sigma Co (St. Louis, MO, USA). Spectroscopically pure acetonitrile was purchased from Germany (Merck, KGAa, Germany). *N,N*-dimethylformamide (DMF), potassium carbonate (K_2CO_3) and chloroform were of analytical grade obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were also of analytical grade

unless otherwise stated. The plant materials were collected from Qinghai province and were identified by Professor Changfan Zhou in Northwest Plateau Institute of Biology, Chinese Academy of Sciences.

The labeling reagent 1,2-benzocarbazole-9-ethyl-*p*-toluenesulfonate (BCETS) was synthesized and purified in the authors' laboratory. To a solution of 3.7 g *p*-toluenesulfonyl chloride in 30 mL pyridine (0°C) in a 100-mL of round-bottomed flask, a mixture of 1,2-benzo-carbazole-9-ethanol[22] (5 g) in 50 mL of pyridine was added dropwise within 30 min under vigorous stirring, After stirring at 0°C for 4 hr, the contents were left to stand at ambient temperature for another 4 h with vigorous stirring. The mixture was then transferred into a 100 mL of ice water with vigorous stirring for 30 min; the precipitated solid was recovered by filtration, washed with the distilled water, and dried at ambient temperature for 48 hr. And then, the precipitated solid were recrystallized twice with methanol to give the white crystals 6.4 g, yield (71.7%), m.p.155.8–156.5°C, Found (%): C 72.32, H 4.98, N 3.35, O 11.56, S 7.79; calculated (%): C 71.94, H 5.52, N 3.36, O 11.51, S 7.67. IR (KBr): 3053.20(Ph-N-CH_2-); 2998.66, 2953.25, 2937.57, 2898.95, 2836.57(Ph); 1594.93(Ph); 1468.75, 1368.70(C-H), 1360.36(-C-SO_2-); 1175.50, 1095.42(Ph-S-), 974.15, 745.16, 769.8; MS: m/z $[M+H]^+$: 416.

Supercritical CO_2 Extraction

Supercritical CO_2 extraction offers numerous potential advantages over conventional extraction processes, including non-toxic, non-explosive, environmental friendly, cost-effective, lower consumption of organic solvent, time-saving, and high selectivity. In this work, extraction was conducted in a semi-batch flow extraction apparatus which had been described in previous literature.[21] In this work, the supercritical CO_2 extraction conditions for *M. sikkimensis H* seed oil was set according to the literature with proper modification.[21] In practical extraction, the powdered *M. sikkimensis H* seeds (300 g) with particle size at 0.5–0.9 mm diameter were used. The flow rate of CO_2 was 40 kg/h; the extraction pressure was set at 25 Mpa; the extractor was maintained at 40°C; and the extraction time was 90 min. Under this conditions, the extraction yield attained 35.0% (calculated as: [oil weight (g)/pulverized seed weight (g)] × 100%).

Treatment of the Oil Sample

For the determination of free fatty acid in the oil samples, the diluted oil with DMF was directly used for derivatization procedure. To determine the total fatty acids in the seed oil, a saponification procedure was carried out as described as follows. 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 hr. After cooling to ambient temperature, 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 derivatization and HPLC analysis.

Preparation of Standard Solutions

The labeling reagent solution (5.0×10^{-2} mol/L) was prepared by dissolving 246 mg of BCETS 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., >C15), 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 29 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 solutions were stored at 4°C in a refrigerator until HPLC analysis.

Pre-column Derivatization of Fatty Acids

The derivatization was preceded according to the similar method we described previously.[19] To a 2-mL vial, 50 µl BCETS, 10 mg K_2CO_3, 100 µL of the saponified oil sample (or the diluted oil with DMF) and 200 µL DMF was successively added. 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 to room temperature. A 200 µL volume of the aqueous acetonitrile solution (1:1, *v*/*v*) was added to dilute the derivatization solution. The diluted solution (10 µL) was injected directly into the chromatographic system. The derivatization procedure is shown in Figure 1.

Separation and Identification of Fatty Acid Derivatives with HPLC-APCI-MS

The HPLC separation of BCETS derivatives was performed on an Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 µm, Agilent) with a gradient elution. Eluent A was 50% aqueous acetonitrile containing 30 mM

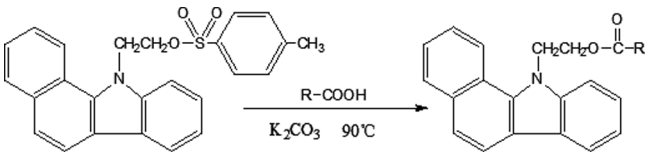

FIGURE 1 Derivatization scheme of fatty acids with BCETS.

ammonium formate (pH 3.7); B was acetonitrile. Gradient: 0–30 min, 55%–85% B; 30–50 min, 85%–100% B; and 100% B were maintained for 10 min. The flow rate was kept at 1.0 mL/min and the column temperature was set at 30°C. Maximum fluorescence responses of derivatives were achieved at the excitation wavelength of 279 nm and emission wavelength of 380 nm. Chromatographic peaks were identified by spiked the working standard with each individual fatty acid in turn, and simultaneously confirmed by mass spectrometry. Ion source conditions: 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 4000 nA (pos); capillary voltage 3500 V.

Quantitative Analysis

Quantitative conversion of fatty acids from the extracts of *M. sikkimensis* H. seed to their BCETS derivatives was ensured by using an excess of BCETS. Fatty acids were quantified using the external standard method, and the calibration curves for each fatty acid derivative were obtained by linear regression plotting peak area versus concentration. For the rough quantification of unexpected fatty acid components, the peak area normalization was carried out in chromatographic window of 20–60 min.

RESULTS AND DISCUSSION

Chromatographic Separation and MS/MS Identification

The retention time of fatty acids on reversed phase column may be predicted by semi-empirical means. Since the interaction of the hydrocarbon chain with the alkylsilica stationary phase arises primarily from dispersion (induced dipole) forces, the logarithm of the capacity factor of saturated fatty acids is linearly related to the solute carbon number. Based on the linear plots constructed from the saturated fatty acids eluted, the equivalent chain length values of unsaturated fatty acids can be calculated.[23–26] Generally, each double bond produces a reduction in retention time

approximating that of two fewer methylene groups; this, therefore, results in close elution of fatty acids such as the C16:0 and C18:1. Such difficult-to-separate combinations have been referred to as "critical pairs,"[26] and also include C20:0 and C22:1, C22:0, and C24:1, and C14:0 and C20:4. On the other hand, although reversed-phase selectivity stems from the structural differences in the carbon backbone of the analyte fatty acids, chromophoric derivatization could change the polarity of the analyte as a whole and, thus, change the chromatographic resolution.[27] In this work, an Eclipse XDB-C_8 column (150 × 4.6 mm i.d, 5 μm, Agilent) was used in conjunction with a gradient elution as described in Experimental section, and all fatty acid derivatives gave a satisfactory resolution (see Figure 2). The results indicated that the BCETS derivatization was helpful for improving the resolution of critical pairs in HPLC analysis of fatty acids.

At the same time, the BCETS-fatty acid derivatives were identified by on-line post-column APCI-MS in positive-ion detection mode. The BCETS-fatty acid derivative produced an intense molecular ion peak at m/z $[M+H]^+$. MS and MS/MS data for all the fatty acid derivatives are shown in Table 1. These intense ion current signals for fatty acid derivatives should be attributed to the introduction of the weakly basic nitrogen atoms in the corresponding BCETS molecular core structure, resulting in high ionization efficiency. The characteristic fragment ion of m/z 217 (benzocarbozole

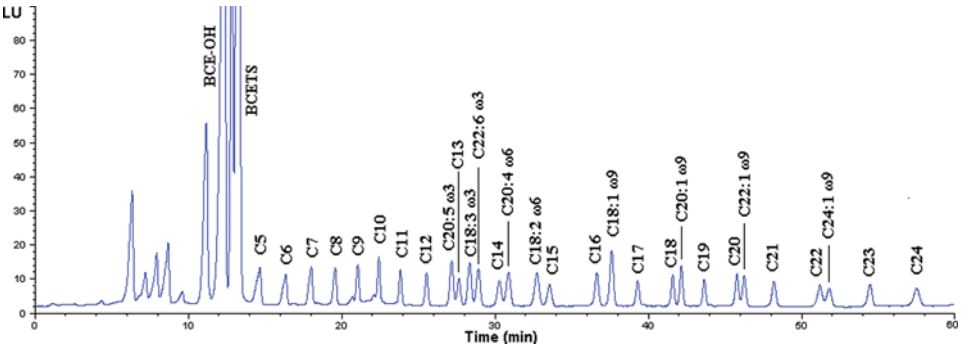

FIGURE 2 Chromatogram of a mixture of BCETS derivatized fatty acid standards. Chromatographic conditions: Column temperature at 30°C; excitation wavelength λex 279 nm, emission wavelength λem 380 nm; Eclipse XDB-C_8 column (4.6 × 150 mm, 5 μm); flow rate = 1.0 mL/min. Peak labels: Valeric acid (C5); Hexanoic acid (C6); Heptoic acid (C7); Octoic acid (C8); Pelargoic acid (C9); Decoic acid (C10); Undecanoic acid (C11); Dodecanoic acid (C12); Tridecanoic acid (C13); Eicosapentanoic acid (C20:5 ω3); α-Linolenic acid (C18:3 ω3); Docosahexanoic acid (C22:6ω3); Tetradecanoic acid (C14); Arachidonic acid (C20:4ω6); Linoleic acid (C18:2ω6); Pentadecanoic acid (C15); Hexadecanoic acid (C16); Oleic acid (C18:1 ω9); Heptadecanoic acid (C17); Octadecanoic acid (C18); Eicosenoic acid (C20:1 ω9); Nonadecanoic acid (C19); Eicosoic acid (C20); Docosenoic acid (C22:1 ω9); Heneicosoic acid (C21); Docosanoic acid (C22); Tetracosenoic acid (C24:1 ω9); Tricosanoic acid (C23) ; Tetracosanoic acid; (C24). 1,2-benzocarbazole-9-ethyl-*p*-toluenesulfonate (BCETS); 1,2-benzo-carbazole-9-ethanol(BCE-OH). (Color figure available online.)

TABLE 1 MS and MS/MS Data for BCETS Derivatized Fatty Acids

Fatty acids	Retention time (min)	$[M+H]^+$	MS/MS Data and Corresponding Cleavage Mode				
			C-N bond	C-O bond	O-CO bond	$[MH-H_2O]^+$	Charge-Remote fragmentation
C5	14.642	346.2	216.6, 129.2	243.8	261.4		
C6	16.335	360.2	216.6, 133.2	243.8	261.4		
C7	17.992	374.1	216.8, 157.6	243.5	261.3		
C8	19.559	388.3	216.6, 171.3	243.8	261.2		
C9	21.037	402.3	216.6, 185.4	243.7	261.3		
C10	22.399	416.3	216.8, 199.5	243.4	261.4		
C11	23.812	430.1	216.7, 213.4	243.6	261.4		
C12	25.515	444.3	216.8, 227.3	243.7	261.3		
C13	27.620	458.3	216.6, 231.2	243.7	261.5		
C14	30.263	472.3	216.4, 255.3	243.7	261.4		
C15	33.534	486.3	216.8, 269.6	243.5	261.4		
C16	36.613	500.2	216.7, 283.5	243.6	261.4		
C17	39.275	514.4	216.7, 297.1	243.8	261.5		
C18	41.565	528.3	216.5, 311.3	243.7	261.5		
C19	43.615	542.4	216.6, 325.1	243.5	261.4		
C20	45.748	556.3	216.5, 339.4	243.8	261.3		
C21	48.169	570.3	216.8, 353.2	243.6	261.1		
C22	51.165	584.1	216.6, 367.5	243.6	261.3		
C23	54.418	598.4	216.7, 371.2	243.8	261.3		
C24	57.477	612.3	216.6, 395.4	243.7	261.4		
C20:5 ω3	27.148	546.1	216.6, 328.6	243.8	261.9	528.5	355.9, 381.1, 396.0, 410.7, 421.8, 437.1, 449.1, 461.7, 475.9, 490.6
C22:6 ω3	28.890	571.9	216.6, 354.9	243.7	261.8	554.0	381.9, 407.8, 425.6, 449.2, 461.9, 476.0, 487.5, 501.5, 516.1, 527.6
C20:4 ω6	30.854	547.7	216.7, 330.9	243.7	261.6	530.3	356.8, 370.3, 382.9, 395.6, 426.5, 435.8, 450.2, 463.7, 478.9, 491.0
C18:3 ω3	28.342	521.9	215.7, 304.9	243.8	261.7	504.0	369.5, 383.8, 397.8, 411.9, 427.4, 437.8, 452.0, 466.1, 480.9, 494.4
C18:2 ω6	32.703	523.9	216.6, 306.7	243.7	261.9	505.8	384.0, 397.7, 411.5, 425.7, 439.7
C18:1 ω9	37.589	525.8	216.5, 309.0	243.6	261.3	507.7	389.6, 427.6, 441.1, 463.7
C20:1 ω9	42.129	553.9	216.9, 336.9	243.8	261.5	435.8	389.3, 455.5
C22:1 ω9	46.222	581.7	216.8, 365.6	243.4	261.2	547.2	389.4, 455.8, 483.4
C24:1 ω9	51.755	609.8	216.6, 393.3	243.6	261.4	591.8	389.4, 526.1, 583.1

molecular core structure) came from the cleavage of the N-CH_2 bond, and the specific fragment ions at m/z $[MH-217]^+$ was the corresponding fatty acid moiety released from quasi-molecular ion by losing the benzocarbozole core structure. The specific fragment ion at m/z 244 and m/z 262 aroused from the cleavage of CH_2-O bond and O-CO bond, respectively. The selected reaction monitoring, based on the m/z $[M+H]^+ \rightarrow m/z$ 217, m/z 244, and m/z 262 transition, was specific for fatty acid derivatives. For the MS/MS of

unsaturated fatty acids, the collision-induced dissociation produced the fragment ions $[MH\text{-}H_2O]^+$ by losing H_2O molecules, which was a specific fragment ion for the identification of unsaturated fatty acid derivatives. Also, the charge-remote fragmentations were observed in the MS/MS of unsaturated fatty acid derivatives, which mainly aroused from α-cleavage with the rearrangement of α-hydrogen, and *β*-cleavage with the rearrangement of *γ*-hydrogen.[28] The MS, MS/MS, and cleavage mode for representative BCETS-C18:3 *ω*3 derivative are shown in Figure 3.

Method Validation

The calibration curves for each BCETS-fatty acid derivative were obtained by linear regression plotting peak area versus concentration. The calibration graph was established with the peak area (y axis) versus the fatty acid concentration (x axis: pmol, injected amount), and all of the fatty acids provided excellent linear responses, with correlation coefficients >0.9991. The limit of detection (LOD) was established based on a signal-to-noise ratio of 3, and the LODs of 29 analytes ranged from 0.21–0.99 μg/L. The limit of quantification (LOQ) was established based on a signal-to-noise ratio of 10, and the LOQs ranged from 0.7 to 3.3 μg/L. The details of LODs and LOQs are shown in Table 2. These results indicated that the proposed method was

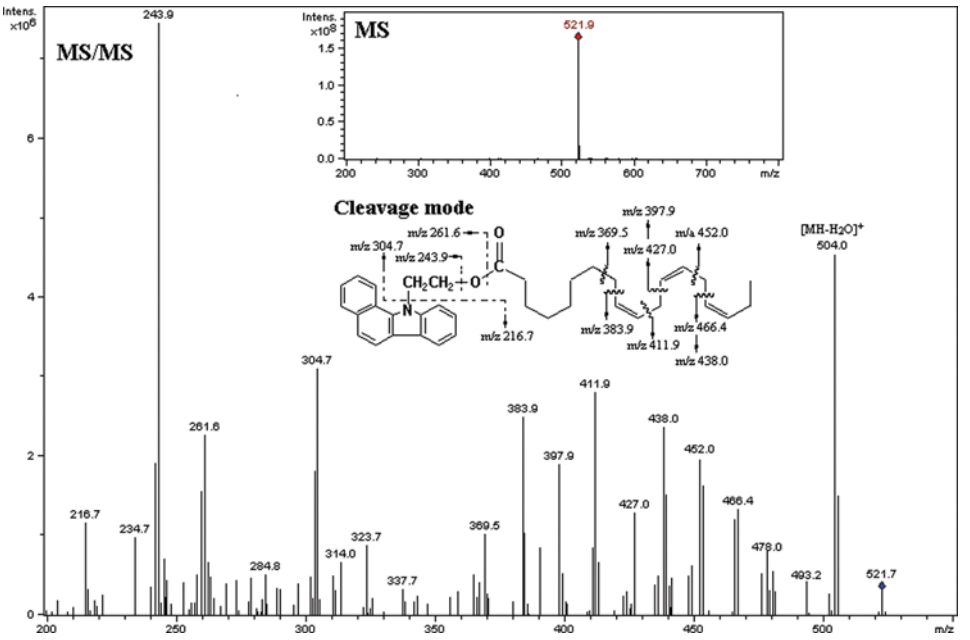

FIGURE 3 The representative MS, MS/MS and cleavage mode for BCETS labeled C18:3 *ω*3. (Color figure available online.)

sensitive enough to detect the fatty acid at the concentration of μg/L level. The attractive sensitivities were mainly contributed to the introduction of 1,2-benzocarbazole functional group into the labeling reagent molecule, which dramatically augmented the n-π conjugation, and thus improved the fluorescent properties of labeling reagent.

The repeatability of the peak current was estimated by making repetitive injections of a standard acid derivatives mixture solution (50 pmol for each analyte) under the selected optimum conditions ($n = 6$). The relative standard deviations (RSDs) of the peak areas and retention time for 29 acid derivatives were 0.47–1.92% and 0.02–0.19%, respectively (the details

TABLE 2 Linear Regression Equations, Correlation Coefficients, Detection Limits, Repeatability and Recoveries

					Precision ($n = 6$) RSD (%)		Recovery ($n = 3$) (%)	
Fatty acids	Regression equation[a]	Correlation coefficient	LOD[b] (μg/L)	LOQ[c] (μg/L)	Retention time	Peak area	Mean	RSD
C5	$y = 1.50\,x + 2.10$	0.9998	0.21	0.70	0.083	0.47	92.3	1.9
C6	$y = 1.18\,x - 1.07$	0.9997	0.27	0.90	0.075	0.70	93.4	2.1
C7	$y = 1.21\,x + 5.32$	0.9994	0.22	0.73	0.110	0.59	96.2	1.8
C8	$y = 1.05\,x + 7.02$	0.9999	0.46	1.53	0.097	0.58	101.5	2.6
C9	$y = 0.84\,x + 5.16$	0.9997	0.31	1.03	0.092	0.49	102.7	2.3
C10	$y = 0.90\,x + 4.23$	0.9995	0.34	1.13	0.073	0.60	104.1	2.4
C11	$y = 0.89\,x - 1.93$	0.9994	0.38	1.26	0.084	0.50	97.6	1.9
C12	$y = 0.93\,x + 0.38$	0.9998	0.38	1.27	0.062	0.47	98.7	1.7
C20:5 ω3	$y = 1.19\,x + 2.90$	0.9996	0.49	1.63	0.061	0.68	97.3	2.8
C13	$y = 0.60\,x + 3.47$	0.9994	0.73	2.43	0.056	0.72	98.4	2.9
C18:3 ω3	$y = 1.23\,x + 3.60$	0.9996	0.44	1.46	0.059	0.65	102.3	2.7
C22:6 ω3	$y = 1.09\,x + 4.20$	0.9998	0.53	1.76	0.059	0.60	101.4	3.1
C14	$y = 0.74\,x + 4.49$	0.9994	0.47	1.56	0.044	0.67	99.8	1.9
C20:4 ω6	$y = 1.08\,x + 2.65$	0.9991	0.46	1.53	0.072	0.73	102.9	2.1
C18:2 ω6	$y = 1.42\,x - 5.38$	0.9996	0.40	1.33	0.037	0.67	103.6	2.6
C15	$y = 0.87\,x - 1.81$	0.9994	0.29	0.97	0.041	0.79	98.4	2.0
C16	$y = 1.26\,x - 3.36$	0.9994	0.28	0.93	0.035	0.81	99.0	2.4
C18:1 ω9	$y = 1.84\,x - 3.47$	0.9994	0.35	1.17	0.029	0.74	100.7	2.7
C17	$y = 0.86\,x - 3.63$	0.9998	0.33	1.10	0.027	0.69	100.2	2.4
C18	$y = 0.92\,x - 2.24$	0.9999	0.34	1.13	0.029	0.78	101.4	2.2
C20:1ω9	$y = 1.14\,x - 2.76$	0.9995	0.48	1.60	0.037	0.20	104.3	2.1
C19	$y = 0.82\,x - 4.19$	0.9996	0.32	1.07	0.021	0.79	101.2	2.6
C20	$y = 0.74\,x + 0.01$	0.9997	0.46	1.53	0.045	0.80	102.8	2.9
C22:1ω9	$y = 0.76\,x - 1.56$	0.9998	0.63	2.10	0.088	0.62	101.5	3.1
C21	$y = 0.97\,x - 2.09$	0.9999	0.51	1.70	0.072	0.88	104.1	2.3
C22	$y = 0.67\,x - 2.45$	0.9997	0.56	1.87	0.095	1.25	103.2	2.4
C24:1ω9	$y = 0.54\,x + 0.87$	0.9996	0.75	2.50	0.180	1.14	100.2	3.2
C23	$y = 1.03\,x - 5.89$	0.9994	0.99	3.30	0.120	1.38	102.1	3.1
C24	$y = 1.01\,x - 2.48$	0.9999	0.91	3.03	0.190	1.92	103.4	2.9

[a]X: injection amount (pmol); Y: peak area.
[b]At a signal-to-noise ratio of 3.
[c]At a signal-to-noise ratio of 10.

are presented in Table 2). Precision of the method was evaluated by six replicates ($n = 6$) determination of known concentrations of 29 fatty acids in triplicate over 3 consecutive days (0.1, 1.0, and 5.0 mmol/L of fatty acids were used to make the low to high range concentrations). The mean inter-day precision for all standards was $\leq$2.8%, and the mean inter-day precision was $\leq$3.5%. The precision data exhibited in the present study showed that it was feasible to determine the aforementioned analytes by the developed HPLC-FLD method.

Accuracy was evaluated with the recoveries from identical *M. sikkimensis* H. seed oil samples. To evaluate the accuracy of the method, the recovery experiments were conducted under the conditions described in the Materials and Methods section ($n = 3$). Recovery was determined by a standard addition method (all 29 acids were added at the level of 100 mmol/L), and the mean recoveries ranged from 92.3 to 104.3% (see Table 2). The results indicated that the method was sufficiently accurate for the simultaneous determination of the aforementioned acids.

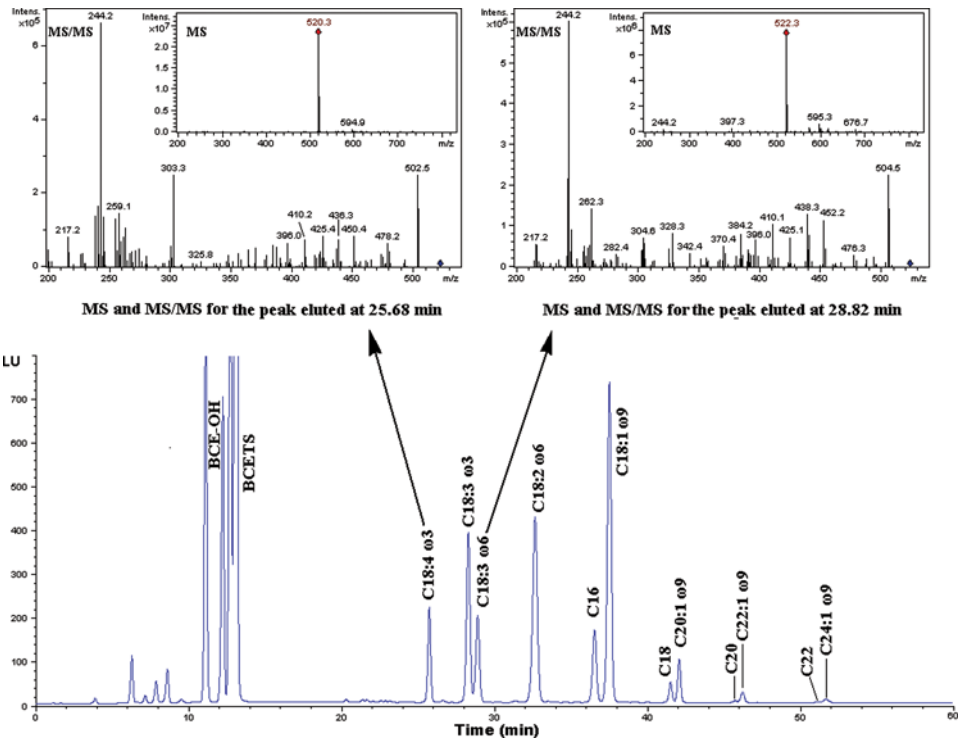

FIGURE 4 Chromatogram for fatty acids from *M. sikkimensis* H. seed oil The chromatographic conditions and peak labels are the same as Figure 2; in addition, Stearidonic acid (C18:4 ω3) and γ-Linolenic acid (C18:3 ω6) were identified with APCI-MS, and the corresponding MS and MS/MS profiles are also given. (Color figure available online.)

Fatty Acids Composition of *M. sikkimensis* H. Seed Oil

The chromatogram for the analysis of free fatty acids from *M. sikkimensis* H. seed oil is shown in Figure 4. As expected, the fatty acids are simultaneously separated with a satisfactory resolution. These chromatographic peaks were identified by retention time, as well as post-column APCI-MS. It was found that the main fatty acids in *M. sikkimensis* H. seed oil were C18:3 ω3, C18:2 ω6, C18:1ω9, C16, C18, and C20:1ω9. The contents of these fatty acids were quantified using the external standard method, and the detailed compositional data are shown in Table 3. In addition, two unexpected fatty acids components were observed, and the corresponding MS and MS/MS are also shown in Figure 4. According to these mass spectral data, the peaks eluted at 25.68 min and 28.82 min were identified as stearidonic acid (C18:4 ω3; $[M+H]^+$ m/z 520.3) and γ-linolenic acid(C18:3 ω6; $[M+H]^+$ m/z 522.3), respectively. Stearidonic acid (C18:4 ω3) is a polyunsaturated fatty acid that constitutes the first metabolite of α-linolenic acid (C18:3 ω3) in the metabolic pathway leading to eicosapentaenoic acid (C20:5 ω3) and docosahexaenoic acid (C22:6 ω3).[29] And, γ-linolenic acid (C18:3 ω6) is unique among the omega-6 polyunsaturated fatty acids in its potential to suppress tumor growth and metastasis.[30] These two fatty acids recently have received increased attention because of their various

TABLE 3 Content of Free and Total Fatty Acids from *M. sikkimensis* H. Seed Oil ($n=3$)

	Free fatty acids		Total fatty acids	
Components	Absolute content (mg/g)	Relative content[a] (%)	Absolute content (mg/g)	Relative content[a] (%)
C18:4 ω3	[b]	3.80	[b]	7.12
C18:3 ω3	6.08	17.66	168.40	15.34
C18:3 ω6	[b]	4.64	[b]	8.21
C18:2 ω6	6.67	22.15	210.80	22.16
C16	2.91	9.40	70.9	7.23
C18:1 ω9	7.68	32.78	235.23	31.63
C18	1.02	2.14	24.38	1.61
C20:1 ω9	1.25	2.97	47.23	3.41
C20	0.34	0.52	4.26	0.20
C22:1 ω9	0.61	0.92	22.92	1.01
C22	0.14	0.16	3.27	0.10
C24:1 ω9	0.57	0.57	15.14	0.45
saturated fatty acids	4.41	12.22	103.26	9.14
unsaturated fatty acids	20.97[c]	85.49	699.72[c]	89.43

[a]Peak area percentage (calculated by the peak area normalization in chromatographic window of 20–60 min).

[b]C18:4 ω3 and C18:3 ω6 were only quantified by peak area normalization, and the absolute contents were not obtained.

[c]C18:4 ω3 and C18:3 ω6 are not included.

physiological functions in the human body. For the rough quantification of these two fatty acid components, the area normalization method was carried out for the peaks eluted in the chromatographic window of 20–60 min, and the results are listed in Table 3. The quantitative results indicated *M. sikkimensis* H.seed oil is rich in unsaturated fatty acids. The mass percentage of C18:1ω9, C18:2 ω6, C18:3 ω3, C20:1ω9, C22:1ω9, and C24:1ω9 were 23.5%, 21.0%, 16.8%, 4.7%, 2.3%, and 1.5%, respectively. And, the stearidonic acid and γ-linolenic acid accounted for 7.12% and 8.21%, respectively (peak area percentage).

CONCLUSIONS

In this work, a simple method for the determination of fatty acids using BCETS as labeling reagent by HPLC with fluorescence detection had been developed. It has been demonstrated that the BCETS derivatization procedure exhibited three advantages in analyzing fatty acids by HPLC: (1) the BCETS derivatization could change the polarity of the analyte as a whole and thus, the chromatographic resolution for critical pairs was improved; (2) the BCETS derivatization provided fatty acids with the fluorescence group of benzocarbozole, which made it possible as the fatty acids can be sensitively detected with fluorescence detection at fmol levels; and (3) the fatty acids can also be sensitively identified by on-line APCI-MS, which profit from the introduction of a weakly basic nitrogen atom by a BCETS derivatization procedure. Furthermore, the components of *M. sikkimensis* H. seed oil was identified and determined with the proposed method. The results indicated that the seed oil mainly contained C18:1 ω3, C18:2 ω6, C18:3 ω3, C16, C18:4 ω3, C18:3 ω6, and C20:1ω9. The contents of these unsaturated fatty acids were over 85%. *M. sikkimensis* H. seed oil was rich in unsaturated fatty acids and may be used as a high-quality edible oil for its health benefits.

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