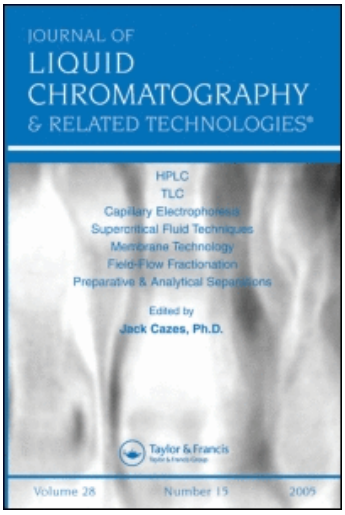

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HPLC-FLUORIMETRIC METHOD FOR ANALYSIS OF FREE FATTY ACIDS IN *STELLERA CHAMAEJASMA L*

Cuihua Song,[1] Zhiwei Sun,[2,3] Lian Xia,[1,2,3] Yourui Suo,[2] and Jinmao You[1,2]

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□ *A sensitive HPLC method for the determination of fatty acids using 2-(2-(pyren-1-yl)-1H-benzo[d]imidazol-1-yl) ethyl-p-toluenesulfonate (PBIOTs) as a novel labeling reagent with fluorescence detection has been developed. PBIOTs 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. Fatty acids derivatives were separated on a reversed phase Eclipse XDB-C_8 column by HPLC in conjunction with gradient elution. The corresponding derivatives were identified by post-column APCI-MS in positive ion detection mode. PBIOTs-fatty acid derivatives gave an intense molecular ion peak at m/z $[M+H]^+$; with MS/MS analysis, the collision induced dissociation spectra of m/z $[M+H]^+$ produced the specific fragment ions at m/z $[M+H–319]^+$ and m/z 319.0 (here, m/z 319 is the core structural moiety of the PBIOTs molecule). The fluorescence excitation and emission wavelengths of the derivatives were $\lambda_{ex}=350$ nm and $\lambda_{em}=402$ nm, respectively. Linear correlation coefficients for all fatty acid derivatives were ≥ 0.9985. Detection limits for the labeled fatty acids, at a signal-to-noise ratio of 3:1, were in the range of 10.32– 44.28 fmol. From the extracts of* Stellera chamaejasma L*, free fatty acids were sensitively determined.*

Keywords 2-(2-(pyren-1-yl)-1*H*-benzo[*d*]imidazol-1-yl) ethyl-p-toluenesulfonate (PBIOTs), atmospheric pressure chemical ionization mass spectrometer (APCI-MS), fatty acids (FAs), fluorescence detection (FLD), high performance liquid chromatography (HPLC), *Stellera chamaejasma L*

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INTRODUCTION

Stellera chamejasme L plants, which are widespread in the east of Tibet in China and belongs to the family of thymelaeaceae, is a well recognized traditional Chinese herbal medicine.[1] It has long been used for the treatment of hydrothorax, ascites, carbuncles, cough, scrofula, bacteriosis, pulmonary tuberculosis, pneumonia, and neurasthenia, etc.[2,3] Recently, it has been found to posses obvious antitumor and antiviral, especially anti-HIV, activities.[4,5] It has been reported that *Stellera chamejasme L.* plants may be useful in the treatment of a malignant disease.[6] Although many chemical components have been elucidated in the root of *Stellera chamejasme L* plants, such as lipophilic terpenoids, flavonoids, phenolic compounds, and steroids,[7–10] the literatures on the multiple components of fatty acids (FAs), especially very long chain fatty acids (VLCFAs $>C_{16}$) in *Stellera chamejasme L* plants have not been elucidated in detail. VLCFAs play physiologically important roles at trace levels in the regulation of a variety of physiological and biological functions. The investigation of the composition of FAs in *Stellera chamaejasme L* plants is of equal importance. Most FAs show neither natural absorption in the UV regions nor fluorescence naturally; however, easily detectable fatty acid derivatives by methyl esterification with GC or GC/MS have been reported.[11,12] In contrast with GC, use of HPLC allows the fatty acids to be converted to a large number of different derivatives.[13] Derivatization can overcome some problems such as tailing peaks, and low detector sensitivity by the formation of less polar compounds. Therefore, derivatization of these analytes with labeling reagents, especially for the fluorescence detection, has been widely adopted, such as 4-bromomethyl-7-methoxycoumarin (BrMMC),[14] 9-anthryldiazomethane (ADAM),[15] 4-(1-methylphenanthro [9,10-*d*]imidazole-2-yl)benzohydrazide (MPIB-Hz),[16] and so on. Derivatization with these reagents produces derivatives with high molar absorption coefficients in the ultraviolet, as well as high response factors in fluorescence. However, it has been reported that these reagents have limitations in their applications, such as short detection wavelengths, poor stability, tediously analytical procedure, and serious interferences in the biological sample analyses.[13]

In our previous studies, we had described the synthesis of a number of fluorescence labeling reagents and their applications for the determination of saturated FAs, such as acridone-9-ethyl-p-toluene sulfonate (AETS),[17] 2-(2-(anthracen-10-yl)-1*H*- naphtho[2,3-*d*]imidazol-1-yl)ethyl-*p*-toluenesulfonate (ANITS),[18] and 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP).[19] The aims of the present works are to develop a new labeling reagent 2-(2-(pyren-1-yl)-1*H*-benzo[*d*]imidazol-1-yl) ethyl-*p*-toluenesulfonate (PBIOTs), and simultaneously analyze the

saturated and unsaturated FAs extracted from *Stellera chamaejasme L.* HPLC separation coupling with APCI-MS identification for PBIOTs-FA derivatives is accomplished on a reversed phase Eclipse XDB-C_8 column. Linearity, detection limits, and precision of the procedure are also determined.

EXPERIMENTAL

Chemicals

Saturated fatty acids (C_5-C_{30}) used as standards were of chromatographic grade and purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Unsaturated fatty acids standards including 12-octadecenoic acid ($C_{18:1}$), 9,12-octadecadienoic acid ($C_{18:2}$), 9,12,15-octadecatrienoic acid ($C_{18:3}$), 6,9,12,15-arachidonic acid ($C_{20:4}$), 5,8,11,14,17-eicosapentaenoic acid ($C_{20:5}$), 2,5,8,11,14,17-docosahexenoic acid ($C_{22:6}$) were purchased from Sigma Co. (St. Louis, MO, USA). Spectroscopically pure acetonitrile was purchased from Germany (Merck, KGAa). N,N-dimethylformamide (DMF) was purchased from Jining Chemical Reagent Co. (Jining, Shandong, China) and treated with 5 Å molecular sieve, and then redistilled prior to use. HPLC grade acetonitrile (CH_3CN) was purchased from Yucheng Chemical Reagent Co. (Shandong Province, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were of analytical grade unless otherwise stated. *Stellera chamaejasme L* plant samples were obtained from Yushu County in August 2008 (Qinghai Province, China), and was identified by professor Changfan Zhou (Northwest Plateau Institute of Biology, Chinese Academy of Sciences).

Instruments and Conditions

Experiments were performed using Agilent 1100 Series LC/MSD Trap SL (Hewlett-Packard, CA, USA). All the HPLC system devices were 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). The mass spectrometer (model G2445D) 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. PBIOTs-FAs derivatives were separated on a reversed phase Eclipse XDB-C_8 column (150 × 4.6 mm, 5 μm, Agilent) by a gradient elution, and the eluents components and the gradient program was shown in Table 1. The flow rate was constant at 1.0 mL/min and the column temperature

TABLE 1 Gradient Elution Program

Time (min)	A* (%)	B* (%)	C* (%)
0	50	50	0
12	15	85	0
18	10	90	0
23	0	75	25
30	0	68	32
35	0	55	45
40	0	55	45

*Eluent A was water; eluent B was acetonitrile; eluent C was the mixed solvent of acetonitrile and DMF(1:1, *v*/*v*).

was set at 30°C. The maximum excitation and emission wavelengths were set at λ_{ex} 350 nm and λ_{em} 402 nm. The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL). 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 (nA) 4000 (pos); capillary voltage 3500 V.

Semi-preparative HPLC separation was used to obtain the single PBIOTs-C_{16} derivative, which was used to test the fluorescence properties. The semi-preparative HPLC system was Waters Delta 600 (Waters, Japan) and consisted of an online degasser, a Waters 600 controller with Waters 2489 UV/visible detector, and an auto fraction collector. Reverse phase semi-preparative HPLC separation was performed on a SunFire™ Prep-C_{18} column (10 × 150 mm, 10 μm, Made in Ireland) with Zorbax PrepHT guard cartridge columns. The derivatized PBIOTs-C_{16} solution (1000 μL, 1.0×10^{-3} M) was injected into the semi-preparative HPLC system. An isocratic elution with acetonitrile at 2 mL/min was carried out, and the PBIOTs-C_{16} derivative fraction was eluted within the chromatographic window of 9–12 min. The collected PBIOTs-C_{16} fraction was made up to total volume of 25 mL with acetonitrile. The corresponding PBIOTs-C_{16} concentration was 4.0×10^{-5} M. This solution was used to evaluate the fluorescence properties of the representative fatty acid derivatives. The fluorescence excitation and emission were recorded on F-7000 fluorescence spectrophotometer (Hitachi, Seisakusho, Tokyo, Japan) and the slits were both set at 5 nm.

Synthesis of the Labeling Reagent PBIOTs

PBIOTs was synthesized according to the routes in Fig. 1, and the detailed procedures were as follow:

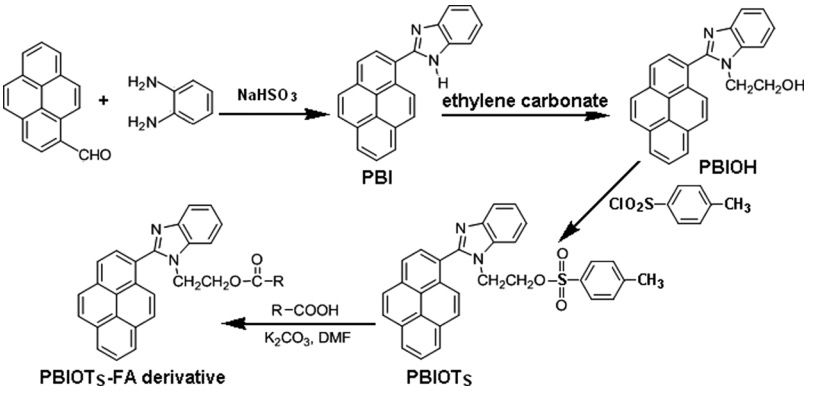

FIGURE 1 The synthesis route of PBIOTs and the derivatization scheme of PBIOTs with fatty acids.

(1) Synthesis of 2-(Pyren-1-yl)-1H-benzo[d]imidazole (PBI)

2-(pyren-1-yl)-1H-benzo[d]imidazole was synthesized as follows: 1, 2-Diaminobenzene (4.5 g) and ethanol (200 mL) were fully mixed in a 500 mL round bottom flask. A mixture of pyrene-1-carbaldehyde (5.0 g) and $NaHSO_3$ (5.2 g) in 80 mL ethanol was added dropwise with vigorous stirring. The contents were heated to reflux for 4 h with vigorous stirring. After cooling, the solution was concentrated using a rotary evaporator. The residue was poured into 500 mL water with vigorous stirring. The precipitated solid was recovered by filtration, washed with water, and dried at room temperature for 48 h. The crude product was recrystallized twice from a mixed solvent of methanol and *N,N′*-dimethylformamide (DMF) (5:1, *v*/*v*) to afford yellow crystals (6.5 g); yield 94.0%.

(2) Synthesis of 2-(2-(Pyren-1-yl)-1H-benzo[d]imidazol-1-yl) ethanol (PBIOH)

2-(pyren-1-yl)-1H-benzo[d]imidazole (6.2 g), ethylene carbonate (2.9 g), and KOH (0.2 g) were dissolved together in 80 mL DMF in a 250 mL round bottom flask and rapidly heated to reflux for 2.5 h with vigorous stirring. After cooling, the contents were poured into 300 mL water with vigorous stirring. The precipitated solid was recovered by filtration, washed successively with water. The crude product was dried at room temperature and recrystallized twice from acetonitrile/DMF mixed solvent (5:1, *v*/*v*) to afford yellow crystals (7.7 g); yield 82.8%.

(3) Preparation of 2-(2-(Pyren-1-yl)-1H-benzo[d]imidazol-1-yl) ethyl-p-toluenesulfonate (PBIOTs)

To a solution of 2-(2-(pyren-1-yl)-1*H*-benzo[*d*]imidazol-1-yl) ethanol (2.6 g) in 50 mL pyridine (0°C) in a 100 mL round bottom flask, p-toluenesulfonyl chloride (5.0 g) was added in batches with vigorous

stirring. After stirring at 0°C for 6 h, the contents were kept at ambient temperature for another 4 h with stirring. The contents were transferred into ice water with vigorous stirring for 0.5 h; the precipitated solid was filtrated, washed with water, and dried at ambient temperature for 48 h. The crude product was recrystallized twice from acetonitrile to give slightly yellow crystals (3.4 g); yield 90.7%. m.p. > 210°C. Found, C 74.42, H 4.69, N 5.41, S 6.20, O 9.30; calculated, C 74.40, H 4.68, N 5.42, S 6.21, O 9.29; IR (KBr): 3043.30 ($\nu_{\text{Ar-H}}$), 2984.30 ($\nu_{\text{R-H}}$), 1598.97, 1527.05, 1482.94, 1455.23 (ν_{Ph}), 1358.28 ($\nu_{\text{-C-SO2-}}$), 1189.75, 1176.12($\nu_{\text{Ph-S-}}$); APCI-MS (positive mode), m/z: 516.5; MS/MS: m/z: 318.6.

Preparation of Standard Solutions

The standard FAs for HPLC analysis at individual concentration of 1.0×10^{-4} mol/L were prepared by dilution of the corresponding stock solution (1.0×10^{-2} mol/L) with the mixed solvent of acetonitrile/DMF (9:1, v/v). PBIOTs solution (5.0×10^{-2} mol/L) was prepared by dissolving 258.1 mg PBIOTs in 10 mL DMF. The corresponding low concentration of derivatization reagent solution (5.0×10^{-3} mol/L) was obtained by diluting the stock solution with DMF. When not in use, all solutions were stored at 4°C in a refrigerator until HPLC analysis.

Extraction of Fatty Acids from *Stellera chamaejasme L* Roots

After transport to the laboratory, the roots of *Stellera chamaejasme L* were cut from the plants, washed with tap water then deionized water, and dried at 50°C until constant weight. All samples were then ground in a stainless steel mill. Dried and pulverized *Stellera chamaejasme L* was then extracted with chloroform by use of two different techniques. (1) Ultrasound Assisted Extraction: A sample (0.2 g) of the pulverized plant material and chloroform (5.0 mL) were placed in a 10 mL round bottomed flask. The flask was immersed in an ultrasonic water bath and sonicated for 20 min. The samples were each extracted three times and the extracts were combined. (2) Extraction by Shaking: Plant: samples (0.2 g) were extracted three times, each time for 20 min, with chloroform (5.0 mL), by use of a mechanical shaker, and the extracts were combined. The final solution was evaporated to dryness by a stream of nitrogen gas at ambient temperature. The residue was redissolved in 1.0 mL DMF and stored at 4°C until HPLC analysis.

Derivatization Procedure

To a solution containing 50 μL of a standard FAs mixture in a 2 mL vial, 140 μL derivatization reagent solution, 10 mg K_2CO_3 and 210 μL DMF were

added. The vial was sealed and allowed to react in a water bath at 90°C for 40 min with shaking in 5 min. intervals. The derivatization procedure is shown in Fig. 1. After the reaction was completed, the mixture was taken to cool at room temperature. A 600 μL volume of acetonitrile solution (CH_3CN/DMF, 1:1, *v*/*v*) was added to dilute the derivatization solution. The diluted solution was injected directly into the chromatograph (10 μL).

RESULTS AND DISCUSSION

Ultraviolet and Fluorescence for PBIOTs Derivatives

For the determination of maximum absorption wavelength, and molar absorption coefficients (ε) of PBIOTs-FA derivatives, the representative PBIOTs-C_{16} solution at concentration of 1.5×10^{-5} mol/L was used to test UV properties (PBIOTs-C_{16} was obtained by a preparation scale derivatization and purified by a semi-preparative HPLC system, as above described in Experimental section). The ultraviolet absorption of PBIOTs-C_{16} derivatives was evaluated in acetonitrile solvent systems. The absorption was obtained with the scanning range in 200 to 400 nm. PBIOTs-C_{16} derivatives exhibited two main absorption bands (at 276 nm and 342 nm), and the maximum molar absorption coefficient (ε) in acetonitrile mediums were $3.37 \times 10^4\,L\,mol^{-1}\,cm^{-1}$ (342 nm). No obvious blue or red shifts were observed in aqueous acetonitrile solvents (0–100%). The excitation and emission spectra of the representative PBIOTs-C_{16} derivative were recorded by a fluorescence spectrophotometer (the PBIOTs-C_{16} solution used for the fluorescent test were at concentration of $1.5 \times 10^{-7}\,mol\,L^{-1}$ in acetonitrile). The fluorescence spectra of PBIOTs-C_{16} exhibited the maximum excitation λ_{ex} and the maximum emission λ_{em} at 352 nm and 403 nm, respectively. The excitation and emission wavelengths in aqueous acetonitrile solution (50–100%, *v*/*v*) exhibited no obvious blue or red shift. An increase in the acetonitrile concentration from 50 to 100% resulted in about 7.8–12.4% increases in fluorescence intensities.

Optimal Derivatization

PBIOTs plays the same esterification with fatty acids as do ANITS, as they provide the same active functional group of p-toluenesulfonate. Therefore, the derivatization reaction of PBIOTs with FAs was optimized according to the similar procedures to those described for ANITS in our previous works.[18] The results indicated that DMF was the best cosolvent for the derivatization procedure, and about 10 mg of K_2CO_3 was the best basic catalyst. The peak heights for all derivatized FAs reached maximum and

constant at 90°C after heating for 30 min. The addition of 5- to 6-fold molar excess reagent to total molar FAs can guarantee maximum and constant response of FA derivatives, and further increasing the excess of reagent beyond this level had no significant improvement in peak responses. To an unknown concentration of FAs in real samples, complete derivatization was guaranteed by using an excess of PBIOTs until constant peak intensity for detector responses.

Optimization of the Extraction

Two methods for extraction of free FAs from *Stellera chamaejasma L* were evaluated by comparison of the detector responses obtained from analysis of the derivatized free FAs. The results indicated the fatty acids were extracted most efficiently from *Stellera chamaejasma L* samples by use of ultrasonication. Lower efficiency was usually observed if extraction was performed by shaking. All subsequent experiments in this study were therefore performed using ultrasonic extraction with chloroform.

Chromatographic Separation and APCI-MS Identification

The simultaneous separation of C_1-C_{30} saturated fatty acid derivatives in combination with gradient elution could be achieved using aqueous acetonitrile and a mixed solvent of acetonitrile/DMF (1:1, *v*/*v*) as eluents as was previously reported.[20] However, the retention of unsaturated was not studied. In this work, besides saturated fatty acids, six unsaturated fatty acids were also studied. The separation of fatty acid derivatives could be carried out by different columns such as BDS-C_{18}, ODS-C_{18}, XDB-C_8, and so on; however, the separation of the fatty acid derivatives on the Eclipse XDB-C_8 column gave the best results. Therefore, an Eclipse XDB-C_8 column (150 × 4.6 mm i.d, 5 mm; Agilent) was selected in conjunction with gradient elution. The gradient elution was carried out as described in the Experimental section, and the elution gave a good separation with the shortest retention values and the sharpest peaks in a single run. The chromatogram of all fatty acid derivatives is shown in Fig. 2. For the coeluted peaks, like C_{14} with $C_{20:4}$, satisfactory resolution could be achieved with a very slow gradient elution within 70 min, which would hardly be acceptable for routine analysis work. Also, APCI-MS identifications of FAs from real samples indicated that the $C_{20:4}$ were not the most important component in *Stellera chamaejasme L.* Therefore, the subsequent experiments were carried out with good separation under the proposed conditions in Experimental section.

The ionization and fragmentation of the PBIOTs-FA derivatives were studied by mass spectrometry under APCI in positive ion detection mode.

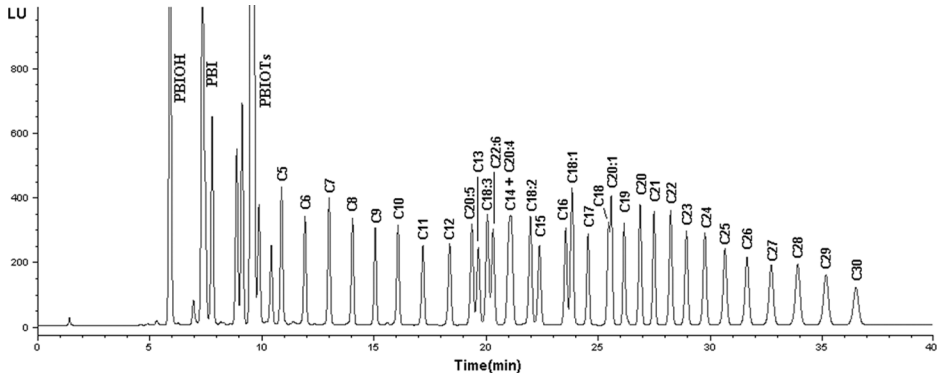

FIGURE 2 Chromatogram of fatty acid derivatives. (injection amount 50 pmol). Peaks as follows: C_5 (valeric acid); C_6 (hexanoic acid); C_7 (heptoic acid); C_8 (octoic acid); C_9 (pelargoic acid); C_{10} (decylic acid); C_{11} (undecanoic acid); C_{12} (dodecanoic acid); C_{13} (tridecanoic acid); C_{14} (tetradecanoic acid); C_{15} (pentadecanoic acid); C_{16} (hexadecanoic acid); C_{17} (heptadecanoic acid); C_{18} (octadecanoic acid); C_{19} (nonadecanoic acid); C_{20} (eicosoic acid); C_{21} (heneicosoic acid); C_{22} (docosanoic acid); C_{23} (tricosanoic acid0; C_{24} (tetracosanoic acid); C_{25} (pentacosanoic acid); C_{26} (hexacosanoic acid); C_{27} (heptacosanoic acid); C_{28} (octacosanoic acid); C_{29} (nonacosanoic acid); C_{30} (dotriacontanoic acid); $C_{18:1}$ (12-octadecenoic acid); $C_{18:2}$ (9,12-octadecadienoic acid); $C_{18:3}$ (9,12,15-octadecatrienoic acid); $C_{20:4}$ (6,9,12,15-arachidonic acid); $C_{20:5}$ (5,8,11,14,17-eicosapentaenoic acid); $C_{22:6}$ (2,5,8,11,14,17-docosahexenoic acid).

As expected, PBIOTs-FA derivatives produced an intense molecular ion peak at m/z $[M+H]^+$. With APCI in positive ion detection mode, intense ion current signals for FA derivatives should be attributed to the introduction of the weakly basic nitrogen atoms in the corresponding PBIOTs molecular core structure, resulting in high ionization efficiency. All molecular ions $[M+H]^+$ and corresponding specific fragment ions for the FA derivatives are shown in Table 2. With MS/MS analysis of the FA derivatives, the collision induced dissociation spectra of m/z $[M+H]^+$ produced the specific fragment ions at m/z 319 and m/z $[M+H–319]^+$. The specific fragment ion m/z 319 was the protonated molecular core structure moiety resulting from the cleavage of $N-CH_2CH_2$ bond, and the specific fragment ion at m/z $[M+H–319]^+$ was the corresponding protonated fatty acid moiety. For unsaturated fatty acids derivatives, the fragment ions resulting from cleavage of C–C bonds in the charge remote terminal can also be observed in their MS/MS, which did not occur in case of saturated fatty acid derivatives. The representative MS/MS profile and corresponding cleavage mode for PBIOTs-$C_{18:3}$ derivatives were shown in Fig. 3. The selected reaction monitoring, based on the m/z $[M+H]^+ \rightarrow$ m/z $[M+H–319]^+$ and m/z 319 transitions, was specific for the fatty acid derivatives. Although other endogenous acidic compounds present in natural environmental samples were presumably coextracted and derivatized by the PBIOTs reagent, no interference was observed due to the highly specific parent

TABLE 2 MS and MS/MS Data for Fatty Acid Derivatives

Fatty acids	Retention time (min)	Molecular ion $[M+H]^+$	MS/MS Data
Saturated fatty acids			
C_5	10.89	446.5	318.4, 344.8, 128.6
C_6	11.93	460.5	318.6, 344.7, 142.5
C_7	13.00	474.6	318.5, 344.5, 156.6
C_8	14.05	488.6	318.5, 344.6, 170.6
C_9	15.05	502.6	318.5, 344.7, 184.5
C_{10}	16.06	516.7	318.6, 345.2, 198.6
C_{11}	17.18	530.7	318.6, 344.4, 212.7
C_{12}	18.37	544.7	318.6, 344.4, 226.7
C_{13}	19.65	558.7	318.5, 344.5, 240.5
C_{14}	20.86	572.7	318.6, 344.9, 254.6
C_{15}	22.38	586.7	318.5, 344.7, 268.7
C_{16}	23.55	600.8	318.5, 344.7, 282.7
C_{17}	24.54	614.8	318.5, 345.5, 296.7
C_{18}	25.47	629.1	318.7, 345.0, 310.8
C_{19}	26.14	642.8	318.5, 344.5, 324.8
C_{20}	26.85	656.8	318.6, 344.4, 338.8
C_{21}	27.49	671.0	318.6, 344.4, 352.7
C_{22}	28.20	684.7	318.5, 344.7, 366.8
C_{23}	28.94	698.7	318.6, 344.8, 380.7
C_{24}	29.76	712.9	318.5, 345.0, 394.7
C_{25}	30.66	726.7	318.5, 345.1, 408.6
C_{26}	31.64	740.8	318.6, 344.9, 422.6
C_{27}	32.72	754.8	318.5, 344.7, 436.5
C_{28}	33.90	768.8	318.6, 344.9, 450.7
C_{29}	35.15	782.7	318.6, 344.8, 464.7
C_{30}	36.48	796.6	318.6, 344.9, 478.7
Unsaturated fatty acids			
$C_{18:1}$	23.82	626.7	318.7, 345.0, 308.8, 528.7, 514.6, 50.8, 608.7
$C_{18:2}$	21.97	624.7	318.7, 344.8, 306.7, 498.8, 512.9, 526.5, 540.6, 565.1, 607.0
$C_{18:3}$	20.05	623.0	318.8, 344.7, 304.7, 409.1, 424.9, 452.9, 473.0, 491.8, 511.8, 529.9, 562.7, 578.8, 603.7
$C_{20:4}$	21.11	648.8	318.6, 344.6, 330.8, 496.6, 483.9, 471.3, 506.0, 527.5, 536.8, 551.2, 564.7, 579.9, 592.0, 619.8, 631.3
$C_{20:5}$	19.36	646.7	318.7, 344.5, 328.8, 456.9, 482.1, 497.0, 511.7, 522.8, 538.1, 551.0, 562.7, 576.9, 591.6, 529.5
$C_{22:6}$	20.30	672.8	318.8, 344.7, 354.8, 482.9, 510.6, 523.0, 536.0, 544.8, 550.2, 562.7, 576.5, 590.0, 602.7, 616.0, 630.5, 655.7, 665.0

mass-to-charge ratio and the characteristic product ions in the m/z $[M+H–319]^+$ and m/z 319 transition.

Calibration, Detection Limits, Precision, and Accuracy

Based on the optimum derivatization conditions, the linearities of the procedures were evaluated in the range of 2×10^{-2} to 2×10^{-5} mmol/L

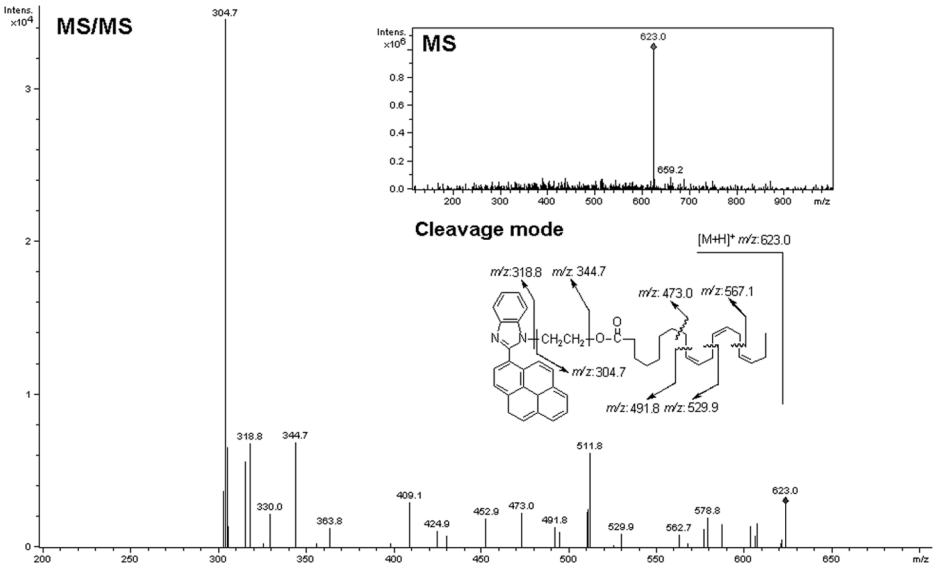

FIGURE 3 The MS profile and MS/MS cleavage mode of the representative 9,12,15-octadecatrienoic acid derivative (PBIOTs-$C_{18:3}$).

(injection volume 10 μL, corresponding injected amount from 200.0 pmol to 0.2 pmol). the peak area (Y) versus fatty acid concentration (X: pmol, injected amount) of each analyte were subjected to regression analysis to obtain the calibration equations and correlation coefficients, and the results of regression analysis on calibration curves are summarized in Table 3. The results showed that within the concentration range there was an excellent correlation between the peak current and the concentration of each analyte. The limit of detection (LOD) was established based on a signal-to-noise ratio of 3, and the LODs of 32 analytes ranged from 10.32 to 44.28 fmol. The limit of quantification (LOQ) was established based on a signal-to-noise ratio of 10, and the LOQs ranged from 34.4 to 147.6 fmol. The details of LODs and LOQs were shown in Table 3. 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 32 acid derivatives were 0.28–0.62% and 0.054–0.098%, respectively (the details are presented in Table 3). Precision of the method was evaluated by six replicates ($n = 6$) determination of known concentrations of 32 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 interday precision ranged from 2.0 to 2.8%, and the mean interday precision for all standards was from 2.7 to 3.5%. The precision data exhibited in the

TABLE 3 Linear Regression Equations, Correlation Coefficients, Detection Limits, and Repeatability for Peak Area and Retention Time

Fatty acids	Linear regression equation*	Correlation coefficient (R)	LOD** (fmol)	LOQ** (fmol)	RSD (%) ($n=6$)	
					Retention time	Peak area
C_5	$Y=66.09X+14.91$	0.9999	10.32	34.4	0.082	0.42
C_6	$Y=53.34X+11.09$	0.9999	11.29	37.63	0.098	0.45
C_7	$Y=64.13X+9.82$	0.9998	11.85	39.5	0.083	0.39
C_8	$Y=50.34X+18.01$	0.9997	15	50	0.072	0.47
C_9	$Y=45.78X+10.14$	0.9999	13.91	46.37	0.091	0.56
C_{10}	$Y=52.62X+10.82$	0.9999	12.63	42.1	0.089	0.44
C_{11}	$Y=44.47X+9.14$	0.9999	13.33	44.43	0.078	0.62
C_{12}	$Y=47.07X+18.51$	0.9998	12.15	40.5	0.081	0.59
$C_{20:5}$	$Y=50.36X+43.30$	0.9990	14.12	47.07	0.084	0.47
C_{13}	$Y=40.91X+28.73$	0.9995	14.33	47.77	0.072	0.54
$C_{18:3}$	$Y=51.59X+72.76$	0.9985	14.77	49.23	0.083	0.53
$C_{22:6}$	$Y=44.38X+43.84$	0.9995	16.84	56.13	0.089	0.56
C_{14}	$Y=54.90X+13.27$	0.9996	11.43	38.1	0.065	0.42
$C_{20:4}$	$Y=64.90X+17.24$	0.9999	12.36	41.66	0.078	0.45
$C_{18:2}$	$Y=70.35X+10.63$	0.9994	11.71	39.03	0.076	0.64
C_{15}	$Y=48.34X+13.34$	0.9999	16.57	55.0	0.079	0.46
C_{16}	$Y=56.62X+11.68$	0.9997	16.41	54.6	0.068	0.61
$C_{18:1}$	$Y=92.62X+35.72$	0.9985	11.3	37.67	0.073	0.62
C_{17}	$Y=47.19X+16.28$	0.9998	12.31	41.03	0.065	0.61
C_{18}	$Y=59.39X+18.83$	0.9996	12.15	40.5	0.059	0.64
C_{19}	$Y=51.09X+8.55$	0.9999	14.33	47.76	0.078	0.63
C_{20}	$Y=57.29X+20.83$	0.9999	13.71	45.7	0.073	0.67
C_{21}	$Y=56.84X+20.19$	0.9998	12.97	43.23	0.079	0.25
C_{22}	$Y=55.79X+18.63$	0.9997	12.8	42.67	0.072	0.34
C_{23}	$Y=54.52X+22.43$	0.9997	15.24	50.8	0.067	0.32
C_{24}	$Y=58.03X+18.70$	0.9997	14.12	47.06	0.076	0.29
C_{25}	$Y=48.16X+20.39$	0.9997	17.14	57.14	0.072	0.33
C_{26}	$Y=39.50X+16.73$	0.9997	21.33	71.1	0.075	0.39
C_{27}	$Y=30.74X+13.78$	0.9997	29.09	96.97	0.078	0.25
C_{28}	$Y=28.53X+13.33$	0.9997	35.56	118.5	0.072	0.28
C_{29}	$Y=22.10X+12.91$	0.9996	43.64	145.5	0.064	0.31
C_{30}	$Y=18.63X+14.07$	0.9999	44.28	147.6	0.054	0.37

*Y: peak area; X: injected amount (pmol).

**LODs and LOQs were established based on a signal-to-noise ratio of 3 and 10, respectively.

present study showed that it was feasible to determine the above analytes by the developed HPLC-FLD method. Accuracy was evaluated with the recoveries from identical samples. To evaluate the accuracy of the method, the recovery experiments under the optimum conditions were conducted with the extracts from root ($n=3$). Recovery was determined by a standard addition method (all 32 acids were added at the level of 100 μmol/L), and the mean recoveries were ranged from 91.7 to 99.2%. The results indicated that the method was sufficiently accurate for the simultaneous determination of the above acids.

Comparison of PBIOTs with the Previously Reported Labeling Reagents

Comparing the proposed method with those involving previous labeling reagents, such as BrMMC,[14] ADAM,[15] MPIB-Hz,[16] AETS,[17] ANITS,[18] and TSPP,[19] PBIOTs exhibits superior properties including convenient derivatization procedure and excellent fluorescent property. ADAM can react smoothly with carboxyl acids under mild conditions, but ADAM cannot be stored for long periods as a solution, or even as a solid. The reagent often requires purification just before use with a suitable method such as column chromatography. The derivatization with BrMMC is carried out at 60°C in acetone or toluene solvent in the presence of K_2CO_3 and phase transfer agent (e.g., 18- crown-6). Also, the derivatization solution must be pretreated in order to remove the toluene and the phase transfer agent. Derivatization of PBIOTs with fatty acids can be accomplished within 30–40 min at 90°C and gives almost the theoretic yields of derivatives in DMF in the presence of the K_2CO_3 catalyst. Also, the derivatization solution of PBIOTs can be directly injected without pretreatment operation prior to HPLC.

The new labeling reagent PBIOTs for FAs showed good similarity with AETS, ANITS, and TSPP, and the detection limits were also in femtomol level, which is equivalent with the previous ones. PBIOTs-FA derivatives exhibit another attractive advantage over the others with maximum $\lambda_{ex}/\lambda_{em}$ at 350/402 nm, especially for the cases that VLCFA were concerned. To compare PBIOTs with our previous reagents, we respectively employed PBIOTs, AETS, ANITS, and TSPP as labeling reagents to analyze the mentioned 32 FAs by HPLC with gradient elution and fluorescence detection. In order to elute VLCFA derivatives out of the reversed phase columns within the shortest analysis time, the appropriate amount of DMF (CH_3CN/DMF, 1:1, *v*/*v*) was added to the strong eluents. Detections were carried out at each maximum $\lambda_{ex}/\lambda_{em}$ (for PBIOTs-FA, 350/402 nm; for AETS-FA, 252/430 nm; for TSPP-FA, 260/380 nm; for ANITS-FA, 250/512 nm). In the cases of AETS, TSPP, and ANITS as labeling reagents, the chromatographic baseline drifted upwards with the increasing percentage of DMF in the mixed eluent during the gradient elutions; when DMF reaches about 15–20% in mixed eluent, it become so serious that the peaks of VLCFAs can hardly be exactly integrated with less than 1 pmol injection; Fortunately, when PBIOTs was used as labeling reagent, it gave a pretty chromatogram with a relatively plain baseline, which is significant to guarantee accurate quantification trace amounts of free VLCFA from real samples. This advantage of PBIOTs probably can be attributed to the fact that PBIOTs-FA derivatives have relatively long excitation wavelengths of 350 nm, which is about 100 nm longer than those of the other tested reagents.

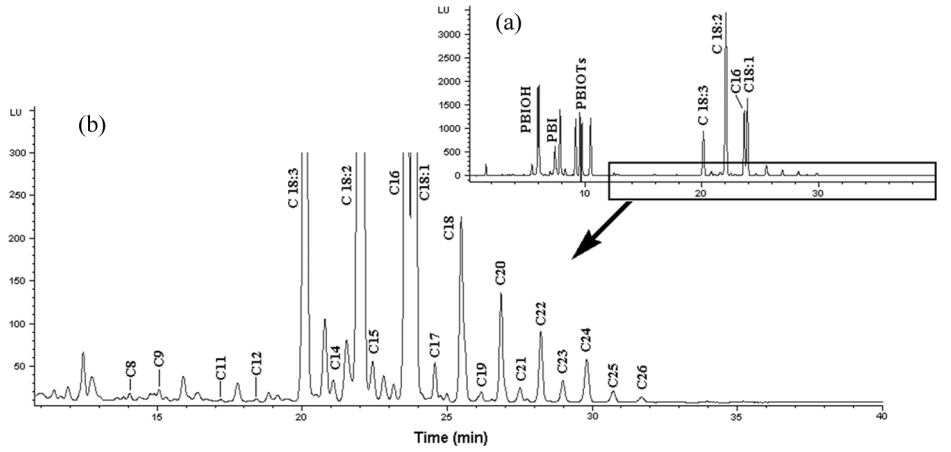

FIGURE 4 Chromatogram of FAs from extracts of the root of *Stellera chamaejasme L* plant (a: Full scale chromatogram; b: the enlarged chromatographic window ranging from 11 to 40 min; symbols are same as Fig. 2).

Analysis of Samples

The chromatograms for the analysis of free fatty acids extracted from the root of *Stellera chamaejasme L* plants were shown in Fig. 4. Free fatty acids compositional data from extracted root of the *Stellera chamaejasme L* plants

TABLE 4 The Contents of Fatty Acids Obtained From Extracts of the Root of *Stellera chamaejasme L* Plant

Fatty acids	Content (μg/g)	Recovery (%)	Fatty acids	Content (μg/g)	Recovery (%)
Saturated fatty acids					
C_5	*	96.6	C_{18}	58.86 ± 2.57	96.9
C_6	*	97.2	C_{19}	6.40 ± 0.36	95.7
C_7	*	97.5	C_{20}	57.97 ± 3.68	97.4
C_8	*	98.4	C_{21}	7.52 ± 0.36	96.5
C_9	*	99.2	C_{22}	43.13 ± 2.88	97.2
C_{10}	*	98.0	C_{23}	14.69 ± 0.63	96.8
C_{11}	*	96.3	C_{24}	33.43 ± 1.24	96.1
C_{12}	*	97.5	C_{25}	10.69 ± 0.48	95.8
C_{13}	*	97.4	C_{26}	5.96 ± 0.32	95.6
C_{14}	63.26 ± 3.47	96.9	C_{27}	*	94.5
C_{15}	17.74 ± 0.92	96.7	C_{28}	*	95.7
C_{16}	554.02 ± 27.3	92.8	C_{29}	*	95.4
C_{17}	16.42 ± 0.94	97.6	C_{30}	*	94.8
Unsaturated fatty acids					
$C_{18:3}$	475.96 ± 30.5	93.6	$C_{20:4}$	9.786 ± 0.51	98.7
$C_{18:2}$	1386.92 ± 46.2	91.7	$C_{20:5}$	*	99.2
$C_{18:1}$	410.14 ± 23.3	93.5	$C_{22:6}$	*	96.4

*Components were not determined or blow LOQ.

were shown in Table 4 (data were average values of five runs). The results indicate that the predominate contents of free FAs in the root of the *Stellera chamaejasme L* plant are $C_{18:1}$ (475.96 (g/g), $C_{18:2}$ (1386.92(g/g), $C_{18:3}$ (410.14 (g/g), and C_{16} (554.02 (g/g). In the next place, the saturated VLCFAs (C_{17}-C_{26}) can be observed. For the saturated VLCFAs, the content of VLCFAs with an even number carbon is obviously higher than those with an odd number carbon. This data would be of great importance for all around exploiter of the source of *Stellera chamaejasme L.*

CONCLUSION

The novel reagent PBIOTs for the labeling of the fatty acids was synthesized and evaluated. The introduction of the pyrenyl functional group into the labeling reagent molecule dramatically augments the conjugation system, which is favorable for the sensitive determination of free fatty acids with fluorescence detection. At the same time, the PBIOTs molecule contains a basic nitrogen atom in its PBI moiety. Therefore, PBIOTs-FA derivatives are ready to accept H^+ to form adducted ions $[M+H]^+$ in the APCI chamber, which is favorable for sensitive APCI-MS identification. Based on the derivatization with PBIOTs, a HPLC-FLD method for analyzing fatty acids was established and evaluated. The established method was sensitive, accurate, and reliable. It was also applied to analysis of free fatty acids from *Stellera chamaejasma L*, which gave satisfactory results. The established method can also be applied to the determination of various drugs and plants containing free fatty acids.

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REFERENCES

1. Feng, B.M.; Pei, Y.H.; Hua, H.M. A new biflavonoid from *Stellera chamaejasme L.* Chin. Chem. Lett. **2004**, *15*, 61–62.
2. Jiang, Z.-H.; Tanaka, T.; Sakamoto, T.; Kouno, I.; Duan, J.-A.; Zhou, R.-H. Biflvanones, diterpenes and coumarins from *Stellera chamaejasme.* Chem. Pharm. Bull. **2002**, *50*, 137–139.
3. Feng, W.; Yoshinori, F.; Masanori, O.; Tomoyuki, T.; Xu, J.; Mitsuzi, Y.; Tetsuro, I. Antitumor principles of *Stellera chamaejasme L.* Chin. J. Cancer Res. **1997**, *9*, 89–94.
4. Feng, W.J.; Ikekawa, T.; Yoshida, M. The antitumor activities of gnidimacrin isolated from *Stellera chamaejasme L.* Chin. J. Cancer Res. **1996**, *8*, 101–104.

5. Yoshida, M.; Feng, W.J.; Saijo, N.; Ikekawa, T. Antitumor activity of daphnane-type diterpene gnidimacrin isolated from *Stellera chamaejasme L.* Int. J. Cancer **1996**, *66*, 268–270.
6. Fan, J. General research situation of antitumor activity and mechanism of *Stellera chamaejasme L.* J. Clin. Res. **2007**, *24*, 2001–2003.
7. Jian, L.; Jia, H.; Pang, L.; Ding, L.; Zhang, Q. Ultrasound-assisted extraction of diterpenoids Jolkinolide B from Euphorbia fischeriana Steud. Fine Chemicals **2007**, *24*, 972–974.
8. Xu, S.; He, H.; Zeng, X.; Guo, S.; Liu, Y. Isolation and identification of triterpenoids and acids from Euphorbia fischeriana Steud. Nat. Prod. Res. Dev. **2003**, *15*, 396–397.
9. Zheng, B.; Hu, H. Changes of flavonoids content in *Stellera chamaejasme* in Songnen grassland of Heilongjiang Province. J. NE. Forest. Univ. **2006**, *34*, 59–60.
10. Feng, B.; Gong, X.; Shi, Li.; Jiang, G.; Pei, Y.; Wang, Y. Studies on phenolic compounds from *Stellera chamaejasme*. China J. of Chin. Mater. Med. **2008**, *33*, 403–405.
11. Gamazo-Vázquez, J.; García-Falcón, M.S.; Simal-Gándara, J. Control of contamination of olive oil by sunflower seed oil in bottling plants by GC-MS of fatty acid methyl esters. Food Contr. **2003**, *14*(7), 463–467.
12. Kulig, C.C.; Beresford, T.P.; Everson, G.T. Rapid, accurate, and sensitive fatty acid ethyl ester determination by gas chromatography-mass spectrometry. J. Lab. Clin. Med. **2006**, *147*(3), 133–138.
13. Toyo'oka, T. Fluorescent tagging of physiologically important carboxylic acids, including fatty acids, for their detection in liquid chromatography. Anal. Chim. Acta **2002**, *465*, 111–130.
14. Wolf, J.H.; Korf, J. 4-bromomethyl-7-methoxycoumarin and analogues as derivatization agents for high-performance liquid chromatography determinations: A review. J. Pharm. Biomed. Anal. **1992**, *10*, 99–107.
15. Yoshida, T.; Uetake, A.; Yamaguchi, H.; Nimura, N.; Kinoshita, T. New preparation method for 9-anthryldiazomethane (ADAM) as a fluorescent labeling reagent for fatty acids and derivatives. Anal. Biochem. **1988**, *173*, 70–74.
16. Iwata, T.; Hirose, T.; Nakamura, M.; Yamaguchi, M. 4-(1-Methylphenanthro[9,10-d]-imidazol-2-yl)-benzohydrazide as derivatization reagent for carboxylic acids in high-performance liquid chromatography with conventional and laser-induced fluorescence detection. Analyst **1994**, *119*, 1747–1751.
17. Li, Y.; Zhao, X.; Ding, C.; Wang, H.; Suo, Y.; Chen, G.; You, J. HPLC-APCI-MS determination of free fatty acids in Tibet folk medicine lomatogonium rotatum with fluorescence detection and mass spectrometric identification. J. Liq. Chromatogr. & Rel. Technol. **2006**, *29*, 2741–2751.
18. You, J.M.; Zhu, F.; Zhao, W.C.; Zhao, X.E.; Suo, Y.R.; Liu, S. Analysis of saturated free fatty acids from pollen by HPLC with fluorescence detection. J. Eur. J. Lipid Sci. **2007**, *109*, 225–236.
19. You, J.M.; Zhao, X.E.; Suo, Y.R.; Li, Y.L.; Wang, H.L.; Chen, G.C. Determination of long-chain fatty acids in bryophyte plants extracts by HPLC with fluorescence detection and identification with MS. J. Chromatogr. B **2007**, *848*, 283–291.
20. Sun, J.; Chen, G.; Zhao, X.; Xu, W.; Zhou, G.; Han, Y.; You, J. Determination of 30 free fatty acids in two famous Tibetan medicines with fluorescence detection and mass spectrometry identification. Chromatographia **2007**, *65*, 469–476.