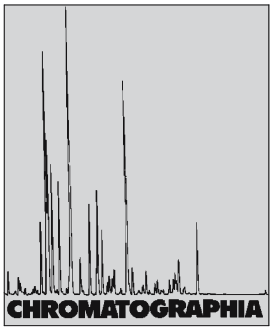
Determination of Free Fatty Acids in Bryophyte Plants and Soil by HPLC with Fluorescence Detection and Identification by Online MS



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Abstract

A method for the determination of long and short chain free fatty acids (FFAs), using 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP) as labeling reagent, has been developed. Identification of FFA derivatives was carried out by HPLC-MS with atmospheric pressure chemical ionization (APCI) in positive ion mode. Gradient elution on an Agilent Eclipse XDB-C_8 column gave good separation of the derivatives. Excellent linear responses were observed and good compositional data could be obtained from as little as 200 mg of bryophyte plants and soil samples. Facile TSPP derivatization coupled with HPLC-APCI-MS analysis allowed the development of a highly sensitive method for the quantitative analysis of trace level of FFAs from biological and natural environmental samples.

Keywords

Column liquid chromatography-mass spectrometry
Derivatization
Fatty acids
1-[2-(*p*-Toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP)
Bryophyte

Introduction

Fatty acids are widely distributed in nature and important as nutritional substances and metabolites in living organisms. Many kinds of fatty acids are present in biological specimens and they play important roles at trace levels in the regulation of a variety of physiological and biological functions. The phytochemistry of bryophytes has been neglected for a long time because they are small and difficult to collect in large amounts as pure samples. They have been used as medicinal plants to cure cuts, external wounds, bacteriosis, pulmonary tuberculosis, pneumonia, neurasthenia and so on. Moreover, it is estimated that 80% of the bryophytes have a sharp, acrid, and unpleasant taste and are not damaged by insects, bacteria, snails and mammals [1]. Although many chemical components have been elucidated in bryophyte plants such as lipophilic terpenoids [2, 3], acetogenins [4], and bisbiphenyl compounds [5], literature reports on FFAs in bryophyte plants are relatively poor. Most fatty acids show neither natural absorption in the visible or UV regions nor fluorescence, thus their detection of trace levels is fairly difficult [6]. However, easy detection of fatty acid derivatives by esterification followed by GC or GC-MS has been reported [7, 8]. In contrast with GC, HPLC allows the fatty acids to be converted to a large number of different derivatives [9] and derivatization with fluorescent labeling reagents has been widely adopted to give higher sensitivity [10–30]. However, it has been reported that many of these reagents have limitations in their applications such as low detection sensitivity, short detection wavelengths, poor stability, tedious analytical procedure and serious interferences in the biological sample analyses [31].

More recently, we synthesized acridone-9-ethyl-*p*-toluenesulfonate (AETS) [32, 33] and 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) [34, 35] for the sensitive determination of nineteen FFAs and ten bile acids. The aims of the present work are: (1) to develop a new labeling reagent 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP), using DMF replacing crown ether and benzene or toluene as solvents to label FFAs, and

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(2) to develop a fast and simple technique for high extraction efficiency of FFAs in bryophyte plants. Different extraction methods such as ultrasonic-assisted solvent extraction, distillation extraction and shaking extraction were tested to optimize derivatization and extraction conditions. HPLC separation coupled with on-line post-column APCI-MS identification was accomplished in combination with a gradient elution on an Eclipse XDB-C_8 column. The fluorescence detection sensitivity for FFAs was compared with AETS [32, 33], BDETS [34] and NOEPES [26] as labeling reagents. Linearity, detection limits and precision of the procedure were also determined.

Experimental

Chemicals

Saturated FFAs (C_1-C_{26}) used as standards were of chromatographic grade and purchased from Shanghai Chemical Reagent Co (Shanghai, China). Five unsaturated FFA standards including 9-hexadecenoic acid (C16:1), 12-octadecenoic acid (C18:1), 9,12-octadecadienoic acid (C18:2), 8,11,14-octadecatrienoic acid (C18:3), 6,9,12,15-arachidonic acid (C20:4) were purchased from Sigma Co (St. Louis, MO, USA) for the identification of peaks and the determination of recovery. Spectroscopically pure acetonitrile was purchased from Merck (Darmstadt, 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. 9-Fluorenemethanol (FM-OH) purchased from Sigma Co and 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS), synthesized in our laboratory [35], were used to compare the MS sensitivity with TSPP. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were of analytical grade unless otherwise stated. Bryophyte plant samples [(**a**) *Homomallium connexum* (Card.) Broth (Zoucheng, Shandong, China); (**b**) *Actinothuidium hookeri* (Jiuzhaigou, Sichuan, China); (**c**) *Neckera pennata* (Jiuzhaigou, Sichuan, China)] were kindly supplied by the College of Life Science, Qufu Normal University (Shandong, China). Soil samples were collected in the campus of Qufu Normal University.

Instrumentation and Conditions

The HPLC system was an Agilent HP 1100 series and consisted of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a fluorescence detector (FLD) (model G1321A). The mass spectrometer, a 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}. APCI vap temperature 450°C ; corona current (nA) 4000 (pos); capillary voltage 3,500 V. Derivatives were separated on an Eclipse XDB-C_8 column (150 × 4.6 mm, 5 µm, Agilent Technolgies, Waldbronn, Germany) by a gradient elution. The HPLC system was controlled by HP Chemstation software. A Paratherm U2 electronic water-bath (Hitachi, Tokyo, Japan) was used to control temperature. The mobile phase was filtered through a 0.2 µm nylon membrane filter (Alltech, Deerfiled, IL, USA).

Synthesis of Labeling Reagents

Synthesis of 2-Phenylimidazole-[4,5-f]-9,10-Phenanthrene

2-Phenylimidazole-[4,5-*f*]-9,10-phenanthrene was synthesized by a modified method according to the method as previously described [36] as follows: 9,10-phenanthraquinone (16 g), benzaldehyde (10 mL) and ammonium acetate (120 g) were fully mixed in a 500-mL of round-bottom flask. Glacial acetic acid (300 mL) was added and the contents of the flask were rapidly heated to 80∼90 °C with stirring for 3 h. After cooling, the pH was adjusted to 7∼8 with ammonium hydroxide. 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 acetonitrile/DMF (5:1, *v*/*v*) to give slightly yellow crystals in a 92% yield.

Synthesis of 1-[ethanol]-2-Phenylimidazole-[4,5-f]-9,10-Phenanthrene (EPP)

1-[ethanol]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (EPP) was synthesized by a modified method of that previously described [35]. 2-Phenylimidazole-[4,5-*f*]-9,10-phenanthrene (12 g), ethylene carbonate (4.5 g), 0.2 g of KOH and 120 mL DMF were mixed in a 500-mL of round-bottomed flask and rapidly heated to reflux for 6 h with vigorous stirring. After cooling, the contents were transferred into 300 mL of water. The precipitated solid was recovered by filtration, washed successively with water, 60% aqueous ethanol. The crude product was dried at room temperature for 48 h and recrystallized twice from acetonitrile/DMF (5:1, *v*/*v*) to afford white crystals in 90% yield, m.p. 274.5–275.8 °C. Found, C 81.60, H 5.34, N 8.30; Calculated, C 81.63, H 5.36, N 8.28; IR (KBr), 3193.13 (-OH); 1603.5 (ph-C = N-); 1559.6, 1525.5, 1496.8 (Ph); 1448.7; 1397.6, 1362.8 (C-H); 1063.3 (C-O), 1031.1, 770.9, 749.3, 722.4, 731.1. *m*/*z* $[M + H]^+$, 339.0.

Preparation of TSPP

A total of 2.53 g of toluenesulfonyl chloride were dissolved in 40 mL of pyridine in a 100-mL round-bottomed flask. A mixture of 1-[ethanol]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (1.5 g) and 10 mL of pyridine was added dropwise over a period of 30 min with vigorous stirring. After stirring at 0 °C for 4 h, the contents were kept at room temperature for another 4 h with vigorous stirring. The contents were transferred into 100 mL of ice water with vigorous stirring for 0.5 h; the precipitated solid was recovered by filtration, washed with water and dried at room temperature for 48 h. The crude products were recrystallized twice from acetonitrile to give 1.76 g of white crystals, yield 80.5 %, m.p. 173.7–174.6 °C. Found, C 73.13, H 4.90, N 5.71, S 6.52; Calculated, C 73.15, H 4.91, N 5.69, S 6.51. IR (KBr), 3114.7

(ph-N-CH2-); 1625.3, 1609.4 (ph-N = N-), 1545.3, 1509.6 (ph), 1444.9, 1399.2, 1378.7(C-H), 1355.7 (-C-SO2-); 1190.2, 1176.9 (ph-S-), 1094.4, 1014.2, 908.8, 781.3, 754.3, 705.2. m/z $[M+H]^+$, 493.0.

Preparation of Standard Solutions

The labeling stock solution (5.0×10^{-2} mol L^{-1}) 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. A lower concentration (5.0×10^{-3} mol L^{-1}) 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^{-1}. 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 acetonitrile/DMF (1:1, *v*/*v*) owing to their poor solubility. Standards of mixed 26 FFAs were prepared by diluting corresponding individual stock solutions with acetonitrile to a concentration of 1.0×10^{-4} mol L^{-1}. When not in use, all reagent solutions were stored at 4 °C in a refrigerator.

Extraction of FFAs from Bryophyte Plants and Soil Samples

Bryophyte plants (10 g) were washed with 40 mL each of 0.1 M hydrochloric acid, 0.1 M sodium hydroxide, and deionized water and then dried under a stream of nitrogen.

Ultrasound-Assisted Extraction

To a 50 mL of round-bottom flask, 200 mg pulverized bryophyte and 10 mL chloroform was added and the sample was sonicated for 20 min. The plant sample was extracted three times and the extracts combined.

Shaking Extraction

Two hundred milligram of the plant sample was extracted three times (20 min for each extraction) with 10 mL of chloroform in a mechanical shaker and the extracts were combined.

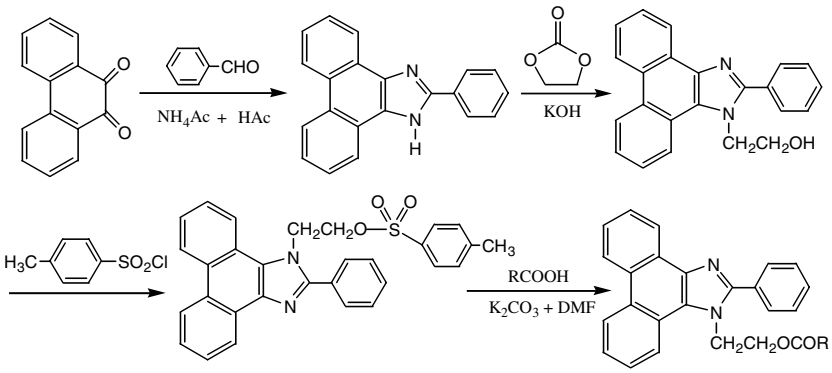

Fig. 1. Derivatization procedure using 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP) as labeling reagent

Distillation Extraction

To a 50 mL round-bottomed flask, 200 mg pulverized bryophyte and 10 mL chloroform were added. The contents were rapidly heated to reflux for 20 min with vigorous stirring. After cooling, the chloroform was transferred into a 100 mL round-bottomed flask. The distillation extraction was repeated three times with 10 mL of chloroform and the extracts were combined.

Distillation Extraction with Mixed Solvent

To a 50 mL round-bottomed flask, 200 mg pulverized bryophyte and 10 mL mixed solvent of methanol and chloroform (1:1, *v*/*v*) were added. The contents were rapidly heated to reflux for 20 min with vigorous stirring. After cooling, the solvent was transferred into a 100 mL round-bottomed flask. The extraction was repeated three times with 10 mL of methanol/chloroform and the extracts were combined.

Each extract obtained by the methods described above was filtered and evaporated to dryness in a rotary vacuum evaporator at 60 °C. The residue was redissolved in 1.0 mL DMF, filtered through a 0.2-μm nylon membrane filter, and stored at −10 °C until HPLC analysis. Soil samples were also extracted by the procedures described above.

Derivatization

To a solution consisted of 50 μL (1.0×10^{-4} mol L^{-1}) of a standard fatty acid mixture in a 2 mL vial, 130 μL (5.0×10^{-3} mol L^{-1}) TSPP solution, 10 mg K_2CO_3 and 170 μL DMF were added. The tube was sealed and allowed to react in a water bath at 90 °C with shaking for 30 min in 5 min intervals. After the reaction was completed, the mixture was cooled to room temperature. A 650-μL volume of acetonitrile/DMF (1:1, *v*/*v*) was added to dilute the derivatization solution. The diluted solution (10 μL, 50 pmol) was injected directly into the HPLC. The derivatization procedure is shown in Fig. 1. The derivatization of the extracted sample solutions was the same as for the standards.

Table 1. Gradient elution conditions for derivatized free fatty acids

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
40	0	0	100	0
45	0	0	0	100
60	0	0	0	100

Chromatographic Conditions

HPLC separation of 26 FFA derivatives was carried out on an Eclipse XDB-C_8 column (150 × 4.6 mm, 5 μm, Agilent) using gradient elution. Eluent A was 50% acetonitrile; B was 50% acetonitrile containing 0.2 mol L^{-1} ammonium formate buffer (pH 3.7); C was a mixture of acetonitrile and DMF (100:2, *v*/*v*); D was a mixture of acetonitrile and DMF (100:10, *v*/*v*). Before injection of the next sample, the column was equilibrated with mobile

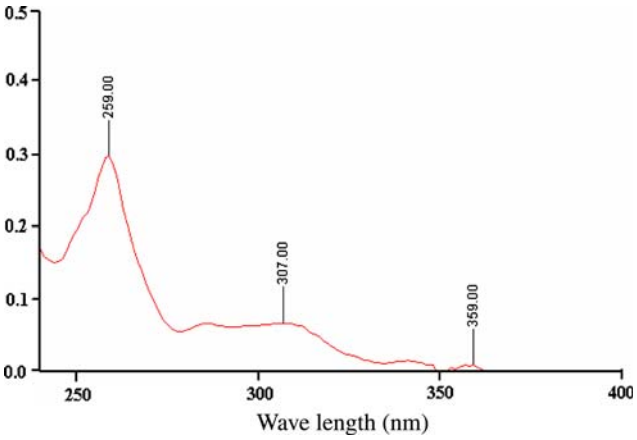

Fig. 2. The UVspectrum of TSPP in acetonitrile solution (ACN/H_2O = 2:1, *v*/*v*) TSPP concentration at 1.0×10^{-5} mol L^{-1}, measurement at room temperature; molar absorption coefficients: $\epsilon = 6.0 \times 10^4$ L mol^{-1} cm^{-1} (259 nm), $\epsilon = 1.8 \times 10^4$ L mol^{-1} cm^{-1} (307 nm)

phase A for 10 min. The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were λ_{ex} 260 and λ_{em}380 nm. The gradient elution program is presented in Table 1.

Quantitative Analysis

Quantitative conversion of FFAs from the bryophyte plants and soil extracts 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 FFA derivative were obtained by linear regression plotting peak area versus concentration.

Results and Discussion

Stability and Spectral Properties of TSPP

When an anhydrous solution of TSPP in acetonitrile or methanol was stored under refrigeration for 2 weeks, the derivatization yields for fatty acids were not obviously different. It was stable in water or common organic solvents. The ultraviolet absorption of TSPP was investigated in acetonitrile-water (2:1) solution with the scanning range in 200–400 nm. Maximum ultraviolet absorption responses were observed at the wavelengths of 259 and 320 nm, respectively (Fig. 2). The molar absorption coefficients (ϵ) of TSPP reagent in acetonitrile solution (acetonitrile/water, 2:1, *v*/*v*) are $\epsilon = 6.0 \times 10^4$ L mol^{-1} cm^{-1} (259 nm) and 1.8×10^4 L mol^{-1} cm^{-1} (307 nm), respectively. The fluorescence excitation and emission wavelengths of TSPP in acetonitrile or methanol solution were at λex = 260 and λem = 380 nm, respectively, and exhibited no obvious blue- or red-shift with different concentrations of acetonitrile or methanol (0–100%).

Optimization of Derivatization

The effect of the reaction temperature on the derivatization yields was evaluated from 30 ° to 95 °C. Fluorescence responses reached maximum at 90°C for 30 min indicating that TSPP reacts rapidly with FFAs under mild conditions to form fluorescence derivatives. Fluorescence responses were investigated with various derivatization solvent systems, such as dioxane, benzene, toluene, acetonitrile (ACN), tetrahydrofuran (THF), *N,N* dimethylformamide (DMF), dichloromethane, ethyl acetate, chloroform and dimethyl-sulfoxide (DMSO). DMF resulted in the most intense fluorescence responses. In addition, DMF was used as the derivatization co-solvent in preference to other solvents as it avoided the problem of precipitation of hydrophobic long-chain fatty acid derivatives. Several base catalysts including pyridine, 2-methylpyridine, triethylamine and 4-dimethylaminopyridine (DMAP), K_2CO_3, Na_2CO_3, $K_2C_2O_4$, $(CH_3)_4NCO_3$ and NaCN were evaluated as catalysts for the derivatization. The results indicated that K_2CO_3 was the best basic catalyst and gave the highest detector response. The effect of the added amount of K_2CO_3 on the derivatization was tested. Maximum and constant peak intensities could be attained with the added amounts of K_2CO_3 equal to or greater than 10 mg, and with a further excess of K_2CO_3, the detector responses did not significantly increase. Although NOE-PES, NE-OTF and AE-OTF have been developed as sulfonate ester reagents for the determination of fatty acids, crown ether and potassium carbonate are usually used in the derivatization procedure with toluene as solvent, in which the derivatization solution was usually required to be treated prior to chromatographic analysis. With DMF solvent in our experiments, the detection responses were not noticably different in the presence or absence of 18-crown-6. A constant fluorescence intensity was achieved with the addition of fivefold molar reagent excess and increasing the excess of reagent beyond this level had no significant effect on yields. To an unknown concentration of sample, such as the extracted bryophyte and soil samples, complete derivatization was guaranteed by using excess of TSPP until constant peak intensity was obtained.

Optimal Extraction

Four methods for the extraction of FFAs in bryophyte plants and soil samples were evaluated by comparing the fluorescence responses obtained by the analysis of the derivatized FFAs from the extracted samples. The results indicated that the highest extraction efficiency of FFAs in bryophyte plant samples was achieved by distillation extraction. This was probably due to the fact that solubility of FFAs in hot organic solvents was higher than that at room temperature. The analysis of the results obtained for ultrasonication extraction shows that the extraction efficiency of FFAs was between distillation extraction and shaking extraction. In most cases, a lower extraction efficiency was observed for shaking extraction. With various solvents as extraction solution, the highest efficiency of extraction was observed using methanol/chloroform (1:1, *v*/*v*). This was not surprising, since the solubility of FFAs especially of the long chain FFAs in methanol/chloroform was higher than that in a single solvent. The total extracted amounts of FFAs using methanol/chloroform as extraction

solvent in combination with distillation was 1.3 times greater than that of obtained by ultrasonication. All subsequent experiments in this study were performed by the distillation extraction with methanol/chloroform (1:1, *v*/*v*) as extraction solvent.

Chromatographic Separation and MS Analysis

An Eclipse XDB-C8 column was selected in conjunction with gradient elution. Several programs were investigated to ensure satisfactory separation in the shortest time. The optimal gradient elution was carried out as described in Table 1. Ammonium formate buffer was used instead of borate buffer to control pH during HPLC separation, resulting in a faster separation and a lower and more stable chromatographic baseline. To achieve optimal separation, the choice of pH value of mobile phase **B** was tested. Separation of the derivatized long and short chain fatty acids could be accomplished at pH 3.7. With pH less than 3.5, most of the fatty acids were resolved with the exception of C_1 and compound B; B and C_2, partially co-eluted. In comparison with pH 3.7, eluent B at pH > 4.0 resulted in an obvious increase in retention time for most of the fatty acid derivatives and compounds **C** and C_4 co-eluted. After further experiments, it was found that if the pH value of mobile phase **B** was adjusted to 3.7, a complete baseline resolution and sharp peaks for all 26 FFA derivatives were obtained within 60 min. The addition of DMF in mobile phases C and D raised the solubility of fatty acid derivatives to help attain fast separation and sharp peaks. With the optimal chromatographic conditions described above, the chromatogram of a complete baseline resolution for all FFA derivatives is shown in Fig. 3a. Under all these conditions, all 26 fatty acids from real samples were separated with a good baseline resolution.

The ionization and fragmentation of the TSPP-FFA derivatives were studied by mass spectrometry with an atmospheric pressure chemical ionization (APCI) source in positive-ion mode. The TSPP-FFA derivative produced an intense molecular ion peak at *m*/*z* $[MH]^+$. With MS-MS analysis of FFA derivatives, the collision-induced dissociation

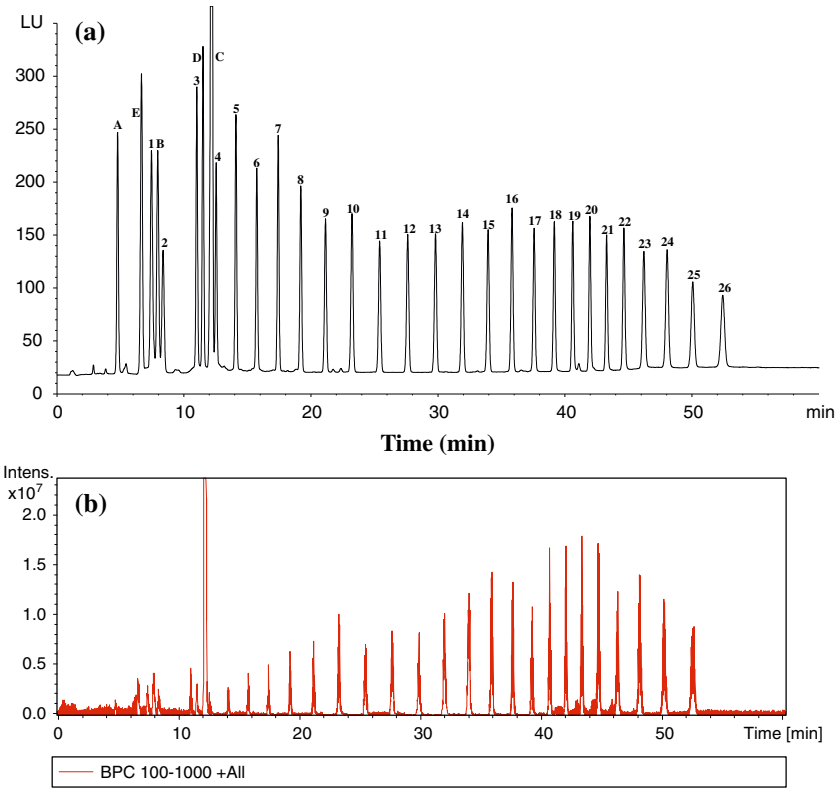

Fig. 3. Chromatogram (**a**) and MS total ion current spectrum (**b**) of standard free fatty acid derivatives. Chromatographic conditions: Column temperature at 30 °C; excitation wavelength λ_{ex} 260 nm, emission wavelength λ_{em} 380 nm; Eclipse XDB-C_8 column (150 × 4.6 mm, 5 µm, Agilent); flow rate 1.0 mL min^{-1}; Peaks: **1** = formic acid, **2** = acetic acid, **3** = propionic acid, **4** = butyric acid, **5** = valeric acid, **6** = hexanoic acid, **7** = heptoic acid, **8** = octoic acid, **9** = pelargoic acid, **10** = decoic acid, **11** = undecanoic acid, **12** = dodecanoic acid, **13** = tridecanoic acid, **14** = tetradecanoic acid, **15** = pentadecanoic acid, **16** = hexadecanoic acid, **17** = heptadecanoic acid, **18** = octadecanoic acid, **19** = nonadecanoic acid, **20** = eicosoic acid, **21** = heneicosoic acid, **22** = docosanoic acid, **23** = tricosanoic acid, **24** = tetracosanoic acid, **25** = pentacosanoic acid, **26** = hexacosanoic acid, **A** 1-ethanol-2-phenylimidazole[4,5-*f*]9,10- phenanthrene, **B** 2-phenylimidazole[4,5-*f*]9,10-phenanthrene; **C** TSPP, **D** and **E** impurity peaks

spectra of *m*/*z* $[MH]^+$ produced the specific fragment ions at *m*/*z* $[M'+CH_2CH_2]^+$ and *m*/*z* 295.0. M' is the molecular mass of the corresponding FFA; the fragment ion *m*/*z* 295.0 is the corresponding protonated molecular core structure moiety. The specific fragment ions at *m*/*z* $[M'+CH_2CH_2]^+$ are the corresponding protonated fatty acid moieties. The selected reaction monitoring, based on the *m*/*z* $[MH]^+ \rightarrow$ *m*/*z* $[M'+CH_2CH_2]^+$ and *m*/*z* 295.0 transition, is specific for fatty acid derivatives. The characteristic fragment ion of *m*/*z* 295.0 (molecular core structure) and *m*/*z* $[M'+CH_2CH_2]^+$ results from the cleavage of the N-CH_2CH_2OCO bond. With APCI in positive ion mode, intense MS total ion current signals were observed (Fig. 3b) and this should be attributed to the introduction of two weak basic nitrogen atom in the TSPP structure resulting in highly ionizable efficiency. There was no detectable signal from the blank water sample using this transition. Although other endogenous acidic compounds present in samples were presumably co-extracted and derivatized, no disturbance was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ion in the *m*/*z* $[MH]^+ \rightarrow$ *m*/*z* $[M'+CH_2CH_2]^+$ and *m*/*z* 295.0 transition. To reduce disturbances from other unknown compounds to a minimum, the gradient elution of the HPLC column was an efficient and helpful method. The MS and MS-MS analysis and the cleavage mode for a representative TSPP-C_{22} derivative are shown in Fig. 4a, b, c. All molecular ions $[MH]^+$ and the corresponding specific fragment ions for 26 fatty acid derivatives are shown in Table 2.

To enhance mass spectrometric sensitivity, we introduced a highly ionizable functional group 2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene which contains two weak basic nitrogen atoms into the

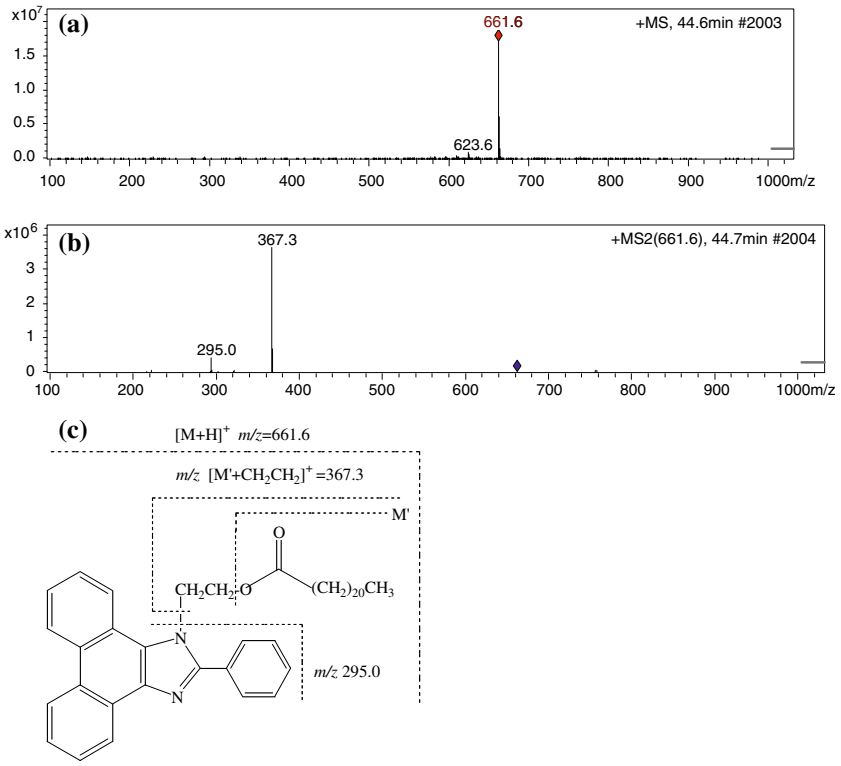

Fig. 4. The profile of molecular ion MS spectrum and MS-MS scanning of the isolated representative *n*- docosanoic acid derivatives (TSPP-C_{22}). **a** Typical HPLC-MS chromatogram of fatty acid derivatives over scanning range from 100 to 1,000 amu with APCI in positive-ion mode. **b** Typical MS-MS chromatogram of docosanoic acid derivative over scanning range from 100 to 1,000 amu with APCI in positive-ion mode; Fragment ions, *m/z* 367.3 and *m/z* 295.0. **c** The MS-MS cleavage mode of TSPP-C_{22} derivative

labeling reagent molecule. To prove its high ionization efficiency, the MS ion current intensities of 1-[ethanol]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (EPP-OH), 9-fluorenemethanol (FM-OH), 1,2-benzo-3,4-dihydrocarbazole-9-ethanol (BDC-OH) [35], 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) [35] and TSPP were compared by injecting the same amounts into the APCI source in positive ion mode under identical conditions. No molecular ion signal for FM-OH was observed, probably due to the fact that the FM-OH molecule does not contain an ionizable weak basic nitrogen atom. As observed in Fig. 5, $I_{EPP\text{-}OH}/I_{BDC\text{-}OH}$ = 12:1 ($I_{EPP\text{-}OH}$ and $I_{BDC\text{-}OH}$ are the MS ion current intensities with the same amount of sample), TSPP and BDETS also exhibited different ion current signals, I_{TSPP}/I_{BDETS} = 4:1(I_{TSPP} and I_{BDETS} are the ion current intensities with the same amount of sample). \the fact that TSPP and EPP-OH exhibit higher ion current signals is attributed to the introduction of two weak basic nitrogen atoms into the molecular core structure which were more easily protonated so as to result in high ionization efficiency.

Comparison of the Fluorescence Sensitivity of TSPP, AETS and BDETS

As observed, the molecular structure of TSPP esterified fatty acids as did AETS [32, 33], BDETS [34, 35] and NOEPES [26]. Relative detector responses of TSPP ($\lambda_{ex}/\lambda_{em}$: 260 / 380 nm), AETS ($\lambda_{ex}/\lambda_{em}$: 404 / 440 nm) and BDETS ($\lambda_{ex}/\lambda_{em}$: 333 / 390 nm) for the individual derivatized fatty acid were investigated. As expected, fluorescence responses for representative C_{10}-C_{20} fatty acid derivatives using AETS and BDETS as labeling reagent were at least 2∼4-fold and 3∼8-fold lower than that of those obtained with TSPP. This was probably due to the fact that TSPP has a larger molar absorbance coefficient (ϵ) [AETS: $\epsilon = 5.72 \times 10^4$ L mol^{-1} cm^{-1} (255 nm); BDETS: $\epsilon = 2.54 \times 10^4$ L mol^{-1} cm^{-1} (249 nm); TSPP: $\epsilon = 6.0 \times 10^4$ L mol^{-1} cm^{-1} (259 nm)]. The difference in molar absorbance coefficient may be attributed to the TSPP molecular structure, in which an n-π conjugation system is dramatically augmented due to the introduction of a 2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene functional group into the labeling reagent.

Reproducibility, Precision, Linearity and Detection Limits

A standard solution consisting C_1-C_{26} fatty acids (5×10^{-5} mol L^{-1}) was prepared, and the method reproducibility was examined by injecting quantitative fatty acid derivatives six times (corresponding injected amount 50 pmol, 10 µL). The relative standard deviations (RSDs) of the peak areas and retention times are from 0.10 to 2.44% and from 0.019 to 0.40%, respectively (Table 2). For precision and accuracy measurements, six replicates at 0.1, 1.0 and 5.0 µmol L^{-1} of the 26 FFAs were used to make the low to high range concentrations. The mean interday accuracy ranged from 94.6 to 103.8% with the largest mean coefficients of variation (R.S.D.) < 6.5%. The mean intraday precision for all standards was < 5.4% of the expected concentration.

Based on the optimum derivatization conditions, the linearities of the 26 FFAs (C_1∼C_{26}) were evaluated in the range of 4.883×10^{-3} to 20 µmol L^{-1} (injection volume 10 µL, injected amount from 200.0 pmol to 48.83 fmol over a 4096-fold concentration range). The calibration graph was established with the peak area (y) versus fatty acid concentration (x: pmol, injected amount). The linear regression equations are shown in Table 2. All of the fatty acids were found to give excellent linear responses over this range with correlation coefficients of 0.9996–0.9999. The linear relationships for higher concentrations were not tested. With 1.0 pmol injection for each derivatized fatty acid, the calculated detection limits (at signal-to-noise of 3:1, S/N = 3:1) are from 3.82 to 47.13 fmol with an average of 18.84 fmol (injection volume 10 µL Table 2). The detection limits using TSPP as derivatization reagent were compared with AETS, BDETS and NOEPES. With AETS, BDETS and NOEPES as labeling reagents, the detection limits (injected volume 10 µL) were 12.3∼43.7 fmol for

Table 2. Linear regression equations, correlation coefficients, detection limits, MS and MS/MS data for free fatty acid derivatives and repeatability for peak area and retention time ($n = 6$)

FFA	Y = A*X + B, X: Injected amount (pmol), Y: Peak area	Regression coefficient R	Detection limits (fmol)	Retention time (RSD%)	Peak area RSD(%)	Molecular ions MS	Specific fragment ions MS–MS
C_1	Y = 142.1X + 27.54	0.9998	3.82	0.36	0.19	367.1	295.0, Nd
C_2	Y = 106.90X-0.83	0.9999	22.75	0.40	0.65	381.2	295.0, Nd
C_3	Y = 123.60X + 20.06	0.9998	8.38	0.35	0.46	395.1	295.0, Nd
C_4	Y = 81.39X + 3.51	0.9998	19.45	0.32	0.83	409.2	295.0, 115.0
C_5	Y = 116.60X + 10.72	0.9997	11.37	0.19	0.46	423.2	295.0, 129.0
C_6	Y = 93.46X + 4.81	0.9997	22.67	0.18	0.68	437.2	295.0, 143.0
C_7	Y = 109.80X + 5.67	0.9998	15.96	0.14	0.57	451.3	295.0, 156.9
C_8	Y = 92.62X + 6.31	0.9999	19.94	0.10	0.55	465.2	295.0, 171.0
C_9	Y = 84.73X + 5.65	0.9997	19.65	0.092	0.44	479.3	295.0, 185.0
C_{10}	Y = 93.62X + 5.31	0.9998	21.04	0.073	0.31	493.3	295.0, 199.0
C_{11}	Y = 79.02X + 5.23	0.9998	31.27	0.081	0.43	507.3	295.0, 213.0
C_{12}	Y = 87.16X + 5.92	0.9998	21.23	0.063	0.37	521.3	294.9, 227.1
C_{13}	Y = 85.66X + 3.94	0.9998	27.59	0.056	0.24	535.4	295.1, 241.1
C_{14}	Y = 94.75X + 6.63	0.9998	21.53	0.044	0.25	549.4	294.9, 255.1
C_{15}	Y = 86.12X + 7.91	0.9997	26.67	0.043	0.14	563.4	295.0, 269.2
C_{16}	Y = 102.71X + 6.05	0.9997	20.41	0.031	0.10	577.4	295.0, 283.2
C_{17}	Y = 81.21X + 10.70	0.9996	23.77	0.026	0.17	591.5	295.0, 297.2
C_{18}	Y = 83.32X + 5.76	0.9998	26.11	0.019	0.16	605.5	295.1, 311.2
C_{19}	Y = 80.16X + 6.82	0.9998	28.20	0.020	0.16	619.5	295.0, 325.2
C_{20}	Y = 84.92X-0.17	0.9999	18.80	0.043	0.36	633.5	295.0, 339.3
C_{21}	Y = 75.85X + 2.67	0.9997	31.87	0.072	0.88	647.6	294.9, 353.3
C_{22}	Y = 89.81X + 4.14	0.9998	35.15	0.095	1.25	661.6	295.0, 367.3
C_{23}	Y = 84.73X + 5.28	0.9998	31.58	0.12	1.36	675.6	295.0, 381.3
C_{24}	Y = 104.30X + 10.37	0.9997	34.75	0.19	1.85	689.6	295.0, 395.3
C_{25}	Y = 83.66X + 5.23	0.9998	44.02	0.23	2.04	703.6	295.0, 409.4
C_{26}	Y = 79.29X + 7.23	0.9997	47.13	0.27	2.44	717.6	295.0, 423.4

Nd The specific fragment ions corresponding to the mass of the FFAs (C_1, C_2, C_3) were less than $m/z = 100$ which was the lowest mass limit of the MS detection, so they were not detected

AETS [32] with an average of 24.6 fmol, 24.8∼80.37 fmol for BDETS [34] with an average of 46.5, and 56 fmol for NOEPES [26]. The lowest detection limits and the average detection limits are 3–14-fold and 1–3-fold lower than those obtained using AETS, BDETS and NOEPES as labeling reagents per 10 μL injection.

Analysis of Samples and Recovery

The chromatogram for the analysis of FFAs extracted from bryophyte plants and soil samples [(**a**) *Homomallium connexum* (Card.) Broth; (**b**) *Actinothuidium hookeri*; (**c**) *Neckera pennata*; (**d**) Soil] with fluorescence detection are shown in Fig. 6a, b, c, d. Peaks were identified by comparison with retention times of reference materials and simultaneously confirmed by mass spectrometric identification. Quantitative derivatization of FFAs in the extracts of bryophyte plants and soil to their TSPP derivatives was guaranteed by using an excess of TSPP labeling reagent. All fatty acids were quantified by linear regression equations. FFAs compositional data

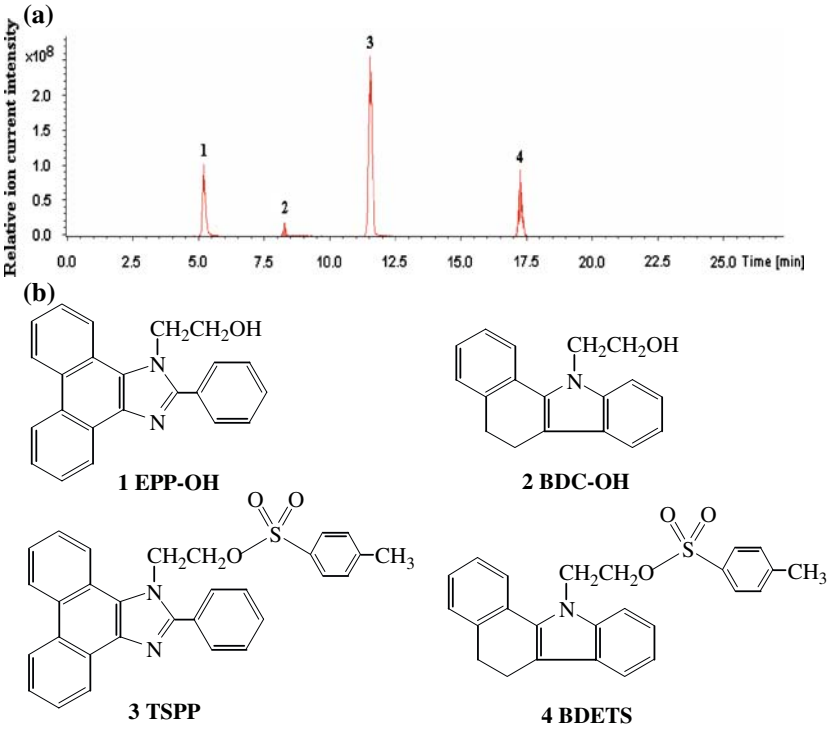

Fig. 5. The profile of MS total ion current spectrum (**a**) and the molecular structure (**b**) of the four isolated representative compounds. Scanning with APCI source in positive ion mode (concentration for each component was 5×10^{-5} mol L^{-1}, injection volume 10 μL, injected amount 500 pmol). Peaks: **1** = 1-[ethanol]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (EPP-OH), **2** = 1,2-benzo-3,4-Dihydrocarbazole-9-Ethanol (BDC-OH), **3** = 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP), **4** = 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS)

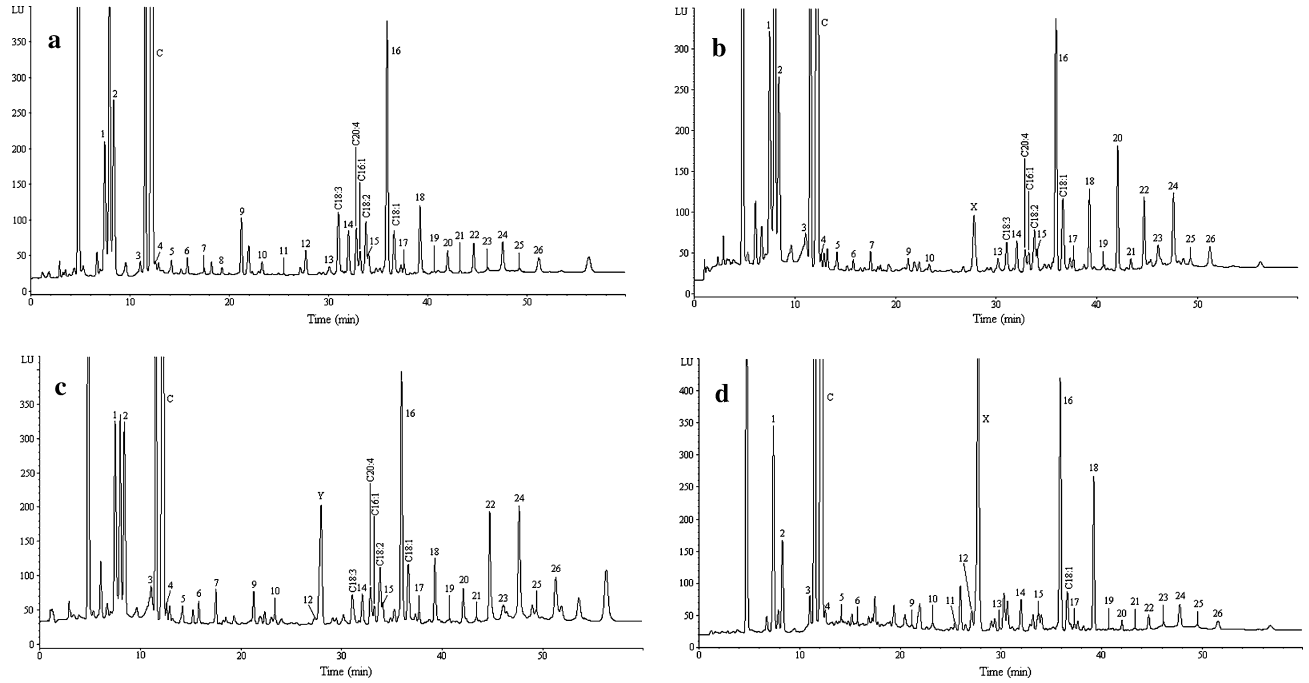

Fig. 6. Chromatogram of free fatty acids from three bryophyte plants and soil samples. **a** *Homomallium connexum* (Card.) Broth, **b** *Actinothuidium hookeri*, **c** *Neckera pennata*, **d** Soil. Chromatographic conditions: Column temperature at 30 °C; excitation wavelength λ_{ex} 260 nm, emission wavelength λ_{em} 380 nm; Eclipse XDB-C_8 column (4.6 × 150 mm, 5 μm); flow rate = 1.0 mL min^{-1}; Peaks: **C16:1** = 9-hexadecenoic acid, **C18:1** = 12-octadecenoic acid, **C18:2** = 9,12-octadecadienoic acid, **C18:3** = 8,11,14-octadecatrienoic acid, **C20:4** = 6,9,12,15-arachidonic acid, X and Y = unidentified, all the MS specific fragment ions were listed in Table 4, other peaks as Fig. 3

Table 3. Contents of free fatty acids from extracted three bryophyte plants and soil sample

FFA	*Homomallium connexum* (Card.) Broth (μg g^{-1})	*Actinothuidium hookeri* (μg g^{-1})	*Neckera pennata* (μg g^{-1})	Soil (μg g^{-1})
C_1	1.88	2.89	2.43	0.012
C_2	4.43	4.64	4.24	0.013
C_3	0.23	0.62	0.97	0.0032
C_4	0.16	0.32	0.57	0.0018
C_5	0.52	0.49	0.85	0.0012
C_6	0.75	0.50	1.17	0.0013
C_7	0.24	0.82	1.79	Nd
C_8	0.44	Nd	Nd	Nd
C_9	4.69	0.90	2.81	0.0016
C_{10}	1.02	0.51	0.81	0.0046
C_{11}	0.15	Nd	Nd	0.0036
C_{12}	2.79	Nd	0.49	0.0080
C_{13}	1.48	1.19	Nd	0.0010
C_{14}	5.22	3.46	3.90	0.018
C_{15}	0.91	1.11	0.87	0.0089
C_{16}	31.30	29.59	35.73	0.15
C_{17}	1.08	1.37	1.78	0.0034
C_{18}	11.27	12.24	11.65	0.12
C_{19}	0.074	1.06	0.55	0.0011
C_{20}	3.21	20.88	6.07	0.0072
C_{21}	0.52	1.75	0.914	0.0017
C_{22}	5.69	12.36	25.24	0.013
C_{23}	0.68	6.23	3.45	0.0041
C_{24}	6.04	13.68	27.62	0.023
C_{25}	0.89	1.61	1.54	0.0039
C_{26}	5.47	6.81	14.74	0.016

Nd Not detectable or with contents below the detection limits

from extracted bryophyte plants and soil are shown in Table 3. The results indicated that the main FFAs with higher contents in **a**, **b** and **c** bryophyte plants were, respectively, C_{14} (3.46–5.22 μg g^{-1}), C_{16} (29.56–35.73 μg g^{-1}), C_{18} (11.27–12.24 μg g^{-1}), C_{20} (3.21–20.88 μg g^{-1}), C_{22} (5.69–25.24 μg g^{-1}), C_{24} (6.04–27.62 μg g^{-1}) and C_{26} (5.47–14.74 μg g^{-1}). The contents of FFAs were about 100 times higher in three bryophyte plants than in soil. It was obvious that the contents of FFAs with an even number carbon in a series (C_{14}∼C_{26} FFAs) were higher than that of those with neighboring odd number carbon atoms. That might be related to their physiological function. In Fig. 6a, b, c, d, five unsaturated FFAs eluted after peaks 12, 14 and 16 were identified by online mass spectrometry and contrasting their retention time with unsaturated FFA standards (listed in Experimental part) which are marked in Fig. 6. The MS specific fragment ions of their TSPP derivatives in positive ion mode are listed in Table 4.

The recoveries of the FFAs were investigated by the addition of known amounts of standard solution (10 μL, 1.0×10^{-4} mol L^{-1}) into the bryophyte plant whose contents of FFAs were known by calculating from linear regression equations. The extraction and derivatization were the same as optimal conditions described above, and the analyses were carried out in three duplicates. The experimental recoveries were in the range of 89.62∼105.30%.

Table 4. The MS specific fragment ions of TSPP derivatives for some peaks in Fig. 6

FFA	MS	MS/MS
$C_{16:1}$	575.3	281.1, 295.1, 393.8, 448.2, 472.0, 557.5
$C_{18:1}$	603.4	295.0, 309.2, 321.2, 475.0
$C_{18:2}$	601.4	209.1, 289.3, 295.0, 296.0, 301.9, 307.2, 308.2, 461.5, 583.0
$C_{18:3}$	599.3	175.0, 184.8, 192.8, 248.9, 295.0, 296.0, 305.2, 306.0, 321.1, 449.4, 541.1, 555.5
$C_{20:4}$	625.4	185.0, 203.1, 226.9, 241.1, 270.1, 295.0, 320.9, 325.1, 331.2, 349.8, 366.2, 448.1, 554.1
X	621.3	181.0, 195.1, 210.1, 223.0, 265.0, 283.0, 327.2, 390.7
Y	777.6	226.0, 238.8, 267.2, 295.2, 308.2, 318.9, 320.2, 321.0, 339.0, 371.4, 403.1, 421.2, 465.1, 483.3, 759.5

Table 5. The recoveries of five unsaturated FFAs ($n = 3$)

Added amounts	C16:1 (%)	C18:1 (%)	C18:2 (%)	C18:3 (%)	C20:4 (%)
10 pmol	93.45	99.32	94.04	101.79	96.54
50 pmol	95.68	101.73	98.31	98.95	99.36
100 pmol	98.12	102.26	99.03	104.24	104.08

To prove whether the harsh conditions for derivatization would result in the destruction of all double bond systems in mono and polyunsaturated FFAs, the recoveries of five unsaturated FFAs (listed in Experimental part) were also investigated. The linear regression equations for them were first established with fatty acid concentration versus peak area using the optimum derivatization conditions, and a dried and pulverized bryophyte plant material (*Homomallium connexum* (Card.) Broth). After determination of the contents of five unsaturated FFAs in the bryophyte plant known amounts of FFA standards solution (containing 10, 50 and 100 pmol respectively) were added into 200 mg of plant material. The extraction and derivatization were the optimal conditions described above, and the analyses were carried out in three duplicates. The experimental recoveries were in the range of 93.45∼104.24% (Table 5). It is obvious that the double bond systems in unsaturated FFAs is not be destructed under the derivatization conditions used.

Conclusions

We have developed a new method for HPLC separation with fluorescence detection and online post-column mass spectrometry identification of 26 FFAs obtained by distillation extraction with chloroform/methanol (1:1, *v*/*v*) as extraction solvent. The improved performance for the complete extraction of FFAs in bryophyte plants and soil samples has been demonstrated. The derivatization and HPLC separation conditions of the reagent TSPP for the labeled FFAs, and MS-MS identification by molecular ions and their specific fragment ions, were evaluated. The new labeling reagent TSPP for FFAs show good similarity with AETS, BDETS and NOEPES but had a higher sensitivity and the detection limits in the femtomol level. TSPP and its hydrolysis products did not interfere with the separation of FFA derivatives by optimal gradient elution. The method could be applied to the determination of FFAs from various drugs, plants and biochemical samples.

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References

1. Asakawa Y (2001) Phytochemistry 56:297–312
2. Nagashima F, Tanaka H, Takaoka S (1997) Phytochemistry 45:353–363
3. Nagashima F, Tanaka H, Takaoka S (1997) Phytochemistry 45:555–558
4. Toyota M, Koyama H, Asakawa Y (1997) Phytochemistry 44:1261–1264
5. Hashimoto T, Irita H, Takaoka S, Tanaka M, Asakawa Y (2000) Tetrahedron 56:3153–3159
6. Ingalls ST, Minkler PE, Hoppel CL, Nordlander JE (1984) J Chromatogr A 299:365–376
7. Gamazo-Vázquez J, García-Falcón MS, Simal-Gándara J (2003) Food Control 14(7):463–467
8. Kulig Clark C, Beresford Thomas P, Everson Gregory T (2006) J Lab Clin Med 147(3):133–138
9. Christie WW (1987) HPLC and Lipids. 2nd edn. Pergamon Press, Oxford, UK, Chapter 7
10. Wolf JH, Korf J (1990) J Chromatogr A 502:423–430
11. Yoo JS, Mcguffin VL (1992) J Chromatogr A 627:87–96
12. Voelter W, Huber R, Zech K (1981) J Chromatogr A 217:491–507
13. Hayashi K, Kawase J, Yoshimura K, Ara K, Tsuji K (1984) Anal Biochem 136:314–320
14. Tsuchiya H, Hayashi T, Naruse H, Takagi N (1982) J Chromatogr A 234:121–130
15. Takadate A, Masuda T, Tajima C, Irikura M, Goya S (1992) Anal Sci 8(5):663–668
16. Takadate A, Masuda T, Murata C, Haratake C, Isobe A, Irikura M, Goya S (1992) Anal Sci 8(5):695–698
17. Yamaguchi Y, Tomita T, Senda M, Hirai A, Terano T, Tamura Y, Yoshida S (1986) J Chromatogr A 357:199–205
18. Yoshida T, Uetake A, Yamaguchi H, Nimura N, Kinoshita T (1988) Anal Biochem 173:70–74
19. Nimura N, Kinoshita T, Yoshida T, Uetake A, Nakai C (1988) Anal Chem 60(19):2067–2070
20. Yamaguchi M, Hara S, Matsunaga R, Nakamura M, Ohkura Y (1985) Anal Sci 1(3):295–296
21. Yamaguchi M, Hara S, Matsunaga R, Nakamura M, Ohkura Y (1985) J Chromatogr A 346:227–236
22. Iwata T, Inoue K, Nakamura M, Yamaguchi M (1992) Biomed Chromatogr 6(3):120–123
23. Toyo'oka T, Ishibashi M, Takeda Y, Imai K (1991) Analyst 116(6):609–614
24. Toyo'oka T, Ishibashi M, Takeda Y, Nakashima K, Akiyama S, Uzu S, Imai K (1991) J Chromatogr A 588:61–71
25. Toyo'oka T, Ishibashi M, Terao T, Imai K (1992) Biomed Chromatogr 6(3):143–148
26. Lu C, Wu H, Chen S, Kou H (2000) Chromatographia 51(5/6):315–321
27. Yasaka Y, Tanaka M, Shono T, Tetsumi T, Kawa J (1990) J Chromatogr A 508: 133–140
28. Akasaka K, Ohrui H, Meguro H (1993) Analyst 118:765–768
29. Iwata T, Hirose T, Nakamura M, Yamaguchi M (1994) Analyst 119(8):1747–1751
30. Iwata T, Nakamura M, Yamaguchi M (1992) Anal Sci 8(6):889–892

31. Toyo'oka T (2002) Anal Chim Acta 465:111–130
32. Zhao X, Wang H, Ding C, Suo Y, Sun J, Chen G, Sun X, You J (2006) Chinese J of Anal Chem 34(2):150–154
33. Li Y, Zhao X, Ding C, Wang H, Suo Y, Chen G., You J (2006) J Liq Chromatogr & Rel Technol 29:2741–2751
34. Shi Y, Ming Y, Zhao X, Cheng Y, Bai M, You J (2005) J Qufu Normal University 31(1):73–78
35. You J, Shi Y, Ming Y, Yu Z, Yi Y, Liu J (2004) Chromatographia 60:527–535
36. Kishikawa N, Wada M, Ohba Y, Nakashima K, Kuroda N (2004) J Chromatogr A 1057:83–88