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Determination of long-chain fatty acids in bryophyte plants extracts by HPLC with fluorescence detection and identification with MS

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Abstract

A sensitive method for the determination of long-chain fatty acids (LCFAs) (>C20) using 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP) as tagging reagent with fluorescence detection and identification with post-column APCI/MS has been developed. The LCFAs in bryophyte plant samples were obtained based on distillation extraction with 1:1 (v/v) chloroform/methanol as extracting solvent. TSPP could easily and quickly label LCFAs at 90 °C in the presence of K_2CO_3 catalyst in DMF. Eleven free LCFAs from the extracts of bryophyte plants were sensitively determined. Maximal labeling yields close to 100% were observed with a five-fold excess of molar reagent. Separation of the derivatized fatty acids exhibited a good baseline resolution in combination with a gradient elution on a reversed-phase Eclipse XDB-C_8 column. Calculated detection limits from 1.0 pmol injection, at a signal-to-noise ratio of 3, were 26.19–76.67 fmol. Excellent linear responses were observed with coefficients of >0.9996. Good compositional data were obtained from the analysis of the extracted LCFAs containing as little as 0.2 g of bryophyte plant samples. Therefore, the facile TSPP derivatization coupled with HPLC/APCI/MS analysis allowed the development of a highly sensitive method for the quantitation of trace levels of LCFAs from biological and natural environmental samples.
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Keywords: 1-[2-(*p*-Toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP); Derivatization; HPLC; Fatty acids; Mass spectrometry

1. Introduction

The phytochemistry of bryophytes has been neglected for a long time because they are very small and difficult to collect in large amounts as pure samples. However, the bryophytes have been used as medicinal plants to cure cuts, external wounds, bacteriosis, pulmonary tuberculosis, neurasthenia, pneumonia, neurasthenia, etc. It is estimated that 80% of the bryophytes show sharp, acrid, and unpleasant taste that are not damaged by insect, bacteria, snails and mammals [1]. Although many chemical components have been elucidated in the bryophyte plants such as lipophilic terpenoids [2,3], acetogenins [4], and bis-bibenzyl compounds [5], literature reports on LCFAs (>C20) in bryophyte plants are relatively poor. LCFAs play physiologically important roles at trace levels in the regulation of a variety of physiological and biological functions. The investigation of the composition of LCFAs in bryophyte plants is of equal importance. Most fatty acids show neither natural absorption in the visible or UV regions nor do they fluorescence naturally; however, easily detectable fatty acid derivatives by methyl esterification with GC or GC/MS have been reported [6,7]. In contrast with GC, use of HPLC allows the fatty acids to be converted to a large number of different derivatives [8]. Derivatization can overcome some problems such as tailing peaks, and low detector sensitivity by the formation of less polar compounds, which can be more easily analyzed by LC. Therefore, derivatization of these analytes with labeling reagents, especially for the fluorescence detection, has been widely adopted. These reagents commonly used include coumarin-type derivatives [9–16]; diazomethane-type reagents such as 9-anthryldiazomethane (ADAM) [17,18] and 1-pyrenyldiazomethane (PDAM) [19]; quinoxalinone derivatives [20–23]; benzofurazan-type reagents

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[24–26]; sulfonate ester reagents such as 2-(2-naphthoxy)-ethyl-2-(piperidino)-ethanesulfonate (NOEPES) [27], 2-(2,3-naphthalimino)-ethyl trifluoromethane-sulfonate (NE-OTF) [28] and 2-(2,3-anthracene -dicarboximido) ethyl trifluoromethanesulfonate (AE-OTF) [29]; benzohydrazide-type reagents such as 4-(1-methylphenanthro [9,10-d]imidazole-2-yl)benzohydrazide (MPIB-hydrazide) [30] and 4-(5,6-Dimethoxy-2-benzimidazoyl)-benzohydrazide) (DMBI-hydrazide) [31] and so on. However, it has been reported that these reagents have limitations in their applications, such as short detection wavelengths, poor stability, low detection sensitivity, tediously analytical procedure, and serious interferences in the biological sample analyses [32].

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), which used DMF replacing crown ether and benzene or toluene as solvents to label LCFAs; (2) To develop a fast and simple technique for high extraction efficiency of LCFAs in bryophyte plants; different extraction methods such as ultrasonic-assisted solvent extraction, distillation extraction and shaking extraction are tested. The optimal derivatization and extraction conditions are evaluated. HPLC separation coupling with APCI/MS identification for long-chain fatty acid derivatives is accomplished in combination with a gradient elution on a reversed-phase Eclipse XDB-C_8 column. The fluorescence detection responses are compared to those obtained with NE-OTF [28] and 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) [33] as labeling reagent previously reported. Linearity, detection limits and precision of the procedure are also determined. The proposed method used TSPP as labeling reagent for the determination of LCFAs from the bryophyte plants extracts is satisfactory.

2. Experimental

2.1. Chemicals

Saturated fatty acids (C_{20}–C_{30}) used as standards were of chromatographic grade and purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Spectroscopically pure acetonitrile was purchased from Germany (Merck, KGAa). *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 (K_2CO_3), pyridine, 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. Bryophyte plant samples (A) *Homomallium connexum* (*Card.*) Broth (Zoucheng, Shandong, China); (B) *Actinothuidium hookeri* (Jiuzhaigou, Sichuan, China); (C) *Neckera pennata* (Jiuhaigou, Sichun, China) were obtained from College of Life Science, Qufu Normal University (Shandong, China).

2.2. HPLC instrumentation and conditions

The HPLC system was Agilent HP 1100 series (Waldbronn, Germany) 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 1100 Series LC/MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with an atmospheric pressure chemical ionization (APCI) source. The HPLC system was controlled by HP Chemstation software. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. 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. Derivatives were separated on a reversed-phase Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent) by a gradient elution. Eluent A was pure acetonitrile; B was a mixed solvent of acetonitrile and DMF (acetonitrile/DMF, 1:1, v/v). Before injection of the next sample, the column was equilibrated with Eluent A. Gradient conditions: initial = 100% A; 15 min = 100% B (kept for 10 min). The flow rate was constant at 1.0 mL/min and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} 260 and λ_{em} 380 nm for the 650-10 S fluorescence spectrophotometer (Hitachi, Seisakusho, Tokyo, Japan) and the bandpass were both set at 15 nm. A Paratherm U_2 electronic water-bath (Hitachi, Tokyo, Japan) was used to control temperature for fluorescence scanning. 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.

2.3. Synthesis of labeling reagent 1-[2-(p-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP)

2.3.1. Synthesis of phenylimidazole-[4,5-f]-9,10-phenanthrene

Phenylimidazole-[4,5-f]-9,10-phenanthrene (1) was synthesized by a modified method previously described as follows [34]: 9,10-Phenanthraquinone (16 g), benzaldehyde (10 mL) and ammonium acetate (120 g) were fully mixed in a 500-mL of round-bottom flask. After glacial acetic acid (300 mL) was added, the contents of the flask were rapidly heated to 80–90 °C with stirring for 3 h. After cooling, pH of solution 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 mixed solvent (5:1, v/v) to afford a slight yellow crystal, yield 92%, m.p. 262 °C. Found, C 84.61, H 4.71, N 9.05; Calculated, C 85.69, H 4.79, N 9.52; IR (KBr): 3146.52 (N–H), 1536.87 (Ph), 1457.98, 1347.37 (C–H), 772.96, 750.61, 718.99, 692.17. APCI/MS: *m*/*z*: 295.1 $[M + H]^+$.

2.3.2. Synthesis of 1-[ethanol]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (EPP)

1-[Ethanol]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (EPP) (2) was conveniently synthesized by a modified method [33]. Phenylimidazole-[4,5-f]-9,10-phenanthrene (12 g), KOH (0.2 g) and DMF (120 mL) were mixed in a 500-mL of round-bottom flask and rapidly heated to reflux for 6 h with vigorous stirring. After cooling, the contents were transferred into a 300 mL of water. The precipitated solid was recovered by filtration, washed successively with water, 60% ethanol solution (ethanol/water 3:2, v/v). The crude product was dried at room temperature for 48 h and recrystallized twice from acetonitrile/DMF mixed solvent (acetonitrile/DMF, 5:1, v/v) to afford a white acicular type crystal, yield 90%, 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 (phenyl–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*: 339.0 $(M+H)^+$.

2.3.3. Preparation of TSPP (3)

To a solution of 3.82 g toluenesulfonyl chloride in 20 mL pyridine (0 °C) in a 100-mL of round-bottom flask, a mixture of 1-[ethanol]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (6.00 g) in 10 mL of pyridine was added dropwise within 30 min under vigorous stirring. After stirring at 0 °C for 4 h, the contents were kept at ambient temperature for another 4 h with vigorous stirring. The contents was transferred into a 100 mL of ice water with vigorous stirring for 0.5 h, the precipitated solid was recovered by filtration, washed with the distilled water and dried at ambient temperature for 48 h. The crude products were recrystallized twice from acetonitrile to give the white crystals 7.8 g, yield 90%, 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 (phenyl–N–CH_2–); 1625.3, 1609.4 (phenyl–N=N–), 1545.3, 1509.6 (phenyl), 1444.9, 1399.2, 1378.7(C–H), 1355.7 (–C–SO_2–); 1190.2, 1176.9 (phenyl–S–), 1094.4, 1014.2, 908.8, 781.3, 754.3, 705.2. *m/z* $(M+H)^+$, 493.0.

2.4. Preparation of standard solutions

TSPP solution (1.0×10^{-3} mol/L) was prepared by dissolving 4.92 mg TSPP in 10 mL anhydrous acetonitrile prepared by distilling HPLC grade acetonitrile dried with P_2O_5. Individual stock solutions of the LCFAs (1.0×10^{-3} mol/L) were prepared in acetonitrile/DMF (1:1, v/v) and diluted to 5.0×10^{-5} mol/L concentration for HPLC analysis with the same solvent composition. When not in use, all reagent solutions were stored at 4 °C in a refrigerator.

2.5. Extraction of LCFAs from bryophyte plant samples

Bryophyte plants (10 g) were, respectively, washed with 40 mL each of 0.1 M hydrochloric acid, 0.1 M sodium hydroxide, and de-ionized water. The washed bryophyte plant was dried under reduced pressure. In all cases, as a source dried and pulverized bryophyte plant material (0.2 g) and as an extraction solvent chloroform or chloroform/methanol (1:1, v/v) were used.

1. Ultrasound-assisted extraction: To a 25 mL of round-bottom flask, 0.2 g pulverized bryophyte and 5.0 mL chloroform was added and the sample was sonicated for 20 min. The bryophyte plant sample was extracted three times. The contents were combined.
2. Shaking extraction: The bryophyte plant sample (0.2 g) was extracted three times (20 min for each extraction) with 5.0 mL of chloroform in a mechanical shaker and the extracts were combined.
3. Distillation extraction: To a 25 mL of round-bottom flask, 0.2 g pulverized bryophyte and 5.0 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 25 mL of round-bottom flask. The distillation extraction was repeated three times with 5.0 mL of chloroform and the extracts were combined.
4. Distillation extraction with mixed solvent: To a 25 mL of round-bottom flask, 0.2 g pulverized bryophyte and 5.0 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 25-mL of round-bottom flask. The extraction was repeated three times with 5.0 mL of methanol/chloroform (1:1, v/v) and the extracts were combined.

Each extracts above mentioned was filtered and evaporated to dryness by a rotary vacuum evaporator at 60 °C. The residue was re-dissolved in 2.0 mL methanol, and filtered through a 0.2-μm nylon membrane filter. A 1.0-mL of this methanol solution was applied to a C_{18} SPE column (500 mg of bed) that was previously conditioned with 10 mL methanol followed by 20 mL water. The column was washed with 20 mL water followed by 10 mL of 50% aqueous methanol in order to remove excessive interferences; the LCFAs were eluted with 10 mL mixed solvent of methanol and chloroform (methanol/chloroform, 5:1, v/v). The final solution was evaporated to dryness by a rotary vacuum evaporator at 60 °C. The residue was re-dissolved in 1.0 mL DMF and stored at 4 °C until HPLC analysis.

2.6. Derivatization

To a solution containing 20 μL of a standard fatty acids mixture in 1-mL vial, 100 μL reagent solution, 10 mg K_2CO_3 and 200 μL DMF was added, respectively. The tube was sealed and allowed to place in water bather 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 acetonitrile solution (CH_3CN/H_2O 1:1, v/v) was added to dilute the derivatization solution. The diluted solution (10 μL) was injected directly to the HPLC system. The derivatization procedure is shown in Fig. 1.

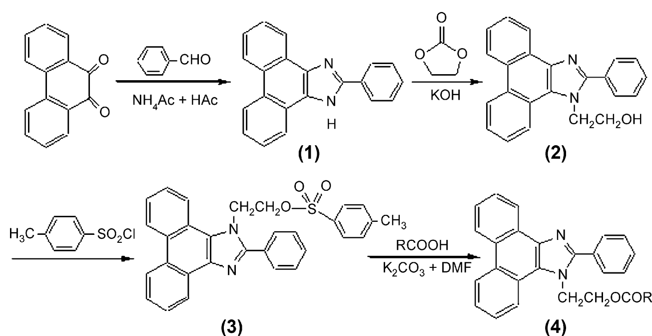

Fig. 1. The scheme of derivatization procedure for the labeling of long-chain fatty acids. (1): Phenylimidazole-[4,5-f]-9,10-phenanthrene; (2): 1-(Ethanol)-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (EPP); (3): 1-[2-(*p*-Toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP); (4): Corresponding derivative.

2.7. Quantitative analysis

Quantitative conversion of LCFAs from the bryophyte plant extracts to their TSPP derivatives was guaranteed by using an excess of TSPP. All fatty acids were quantified in bryophyte plants using the external standard method with detection at 390 nm. The calibration curves for each TSPP long-chain fatty acid derivative were obtained by linear regression plotting peak area versus concentration.

3. Results and discussion

3.1. Ultraviolet absorption of the 1-[2-(p-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP)

The ultraviolet absorption of 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP) was investigated in acetonitrile. The absorption wavelength of TSPP was obtained with the scanning range 200–400 nm. Maximum ultraviolet absorption responses were observed at 259 and 320 nm, respectively. The molar absorption coefficients (ε) of TSPP reagent in acetonitrile are $\varepsilon = 6.0 \times 10^4\ \mathrm{L\,mol^{-1}\,cm^{-1}}$ (259 nm), $1.8 \times 10^4\ \mathrm{L\,mol^{-1}\,cm^{-1}}$ (320 nm), respectively.

3.2. Fluorescence TSPP derivatives

The excitation and emission spectra of the representative TSPP derivatives (C_{20}–C_{24} fatty acids) were collected using the scanning mode of the fluorescence detector (single derivative was obtained according to the derivatization method and purified by a C_{18} SPE column as previously described in experimental section). Maximum fluorescence responses of TSPP derivatives were achieved at the excitation wavelength of 260 nm and emission wavelength of 380 nm (no correction). The excitation and emission wavelengths in acetonitrile or methanol solution (0–100%) exhibited no obvious blue- or red-shift. Fluorescence intensities of derivatives is minimally quenched by inorganic anions such as sulfate, nitrate, and phosphate, organic anions such as bicarbonate and citrate, and monovalent and divalent cations that are abundant in biological fluids.

Fluorescence intensity of reagent TSPP itself showed a dramatically quenching relative to that of its derivatives. This is probably due to the fact that the presence of sulfur atoms in TSPP molecular core structure results in a significant quenching in emission intensity. The ratio $I_{em}/I'_{em} = 4.3 : 1$ was observed (here, I_{em} and I'_{em} were, respectively, relative fluorescence intensity of derivative and TSPP itself). The steady-state fluorescence intensity of representative TSPP-ester (C_{24}-ester) was investigated at different temperatures in acetonitrile. The temperature was tested in 10 °C increments from 35 to 75 °C. Emission spectra were recorded using the maximum excitation wavelength (260 nm) (the profile no shown). The results indicated that the emission intensities decreased with increasing temperature, and the relative fluorescence intensity changed from 184 to 146 with the temperature increasing from 30 to 70 °C. Probably due to the loss of excited-state energy through hydrogen bonding or due to the protonation in strong hydrogen-bonding solvents led to a corresponding decrease in emission intensity. A kinetic analysis of fluorescence intensity of representative TSPP-ester (C_{24} fatty acids) at the different temperatures (30–70 °C in 10 °C increments) led to a linear correlation between ln I_{em} and $1/T$. (I_{em}: Relative fluorescence intensity; T: Thermodynamic temperature). The slopes of the working curves showed the emission stabilization energies were 2.146 Kcal/mol ($R^2 = 0.9958$, in acetonitrile).

3.3. Optimal derivatization

Time course studies on the derivatization reaction of fatty acids with TSPP were investigated 90 °C. As observed, the fluorescence responses reached the maximum at 30 min, indicating that the reagent rapidly reacted with fatty acids under these mild conditions to afford fluorescent derivatives. Dioxane, benzene, toluene, acetonitrile, tetrahydrofuran (THF), *N,N′*-dimethylformamide (DMF), dichloromethane, ethyl acetate, chloroform, dimethyl-sulfoxide (DMSO) and acetone were investigated as reaction co-solvents for derivatization. DMF resulted in the most intense fluorescence responses in chromatogram. In addition, DMF was used as the derivatization co-solvent in preference to other solvents as it easily avoided the problem of precipitation of hydrophobic long-chain fatty acid derivatives. Several basic 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 for the derivatization. The results indicated that K_2CO_3 was the best basic catalyst and gave the highest detection responses. 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 $K_2CO_3 > 10$ mg, and an excess 20 mg of K_2CO_3 did not significantly increase the detector responses. The peak heights for all derivatized fatty acids were constant at 90 °C after heating for 30 min. Although NE-OTF, 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 toxic toluene as solvents, which the derivatization solution is usually required to treat prior to chromatographic analysis. With DMF solvent in our experiments, the detection responses were no remarkable difference in the presence or absence of 18-crown-6. A constant fluorescence intensity was achieved with the addition of 5–6-fold molar reagent excess to total molar fatty acids, and further increasing the excess of reagent beyond this level had no significant effect on yields. To an unknown concentration of sample, complete derivatization was guaranteed by using excess of TSPP until constant peak intensity for detector responses.

3.4. Optimal extraction

Four methods for the extraction of LCFAs in bryophyte plant samples were evaluated by the comparison the fluorescence responses obtained by the analysis of the derivatized LCFAs from the extracted samples. The results indicated that the highest extraction efficiency of LCFAs in bryophyte plant samples was achieved by distillation extraction. This was probably due to the fact that solubility of LCFAs ($>C_{20}$) in hot organic solvents was higher than that in normal temperature. The analysis of the results obtained for ultrasonication extraction shows that the extraction efficiency of LCFAs was between distillation extraction and shaking extraction. In most cases, a lower efficiency of extraction was observed for shaking extraction. With various solvents as extraction solution, the highest efficiency of extraction was observed using the mixed methanol and chloroform (methanol/chloroform, 1:1, v/v). This was not surprising, since the solubility of LCFAs in methanol/chloroform (1:1, v/v) was higher than that in single methanol or chloroform. In general, in the used solvents, solubility of each tested LCFAs (C_{20}–C_{30}) in solvents appeared to follow the pattern methanol/chloroform (1:1, v/v) > chloroform > methanol > acetonitrile. The total extracted amount of LCFAs using methanol/chloroform as extraction solvent in combination with distillation method was 1.3-fold higher than that of obtained by ultrasonication. Subsequently all experiments, in this study, were performed by the distillation extraction with methanol/chloroform (1:1, v/v) as extraction solvent.

3.5. Chromatographic separation and MS/MS analysis

An Eclipse XDB-C8 column was selected in conjunction with a gradient elution, several programs were investigated to ensure satisfactory HPLC separation within the shortest time. The gradient elution from A to B within 15 min was found to give the best separation with the shortest retention values and the sharpest peaks. Under these conditions, all C_{20}–C_{30} fatty acid derivatives from real sample were separated with a good baseline resolution. DMF was used in mobile phase B to increase elution during HPLC separation, resulting in a lower and more stable chromatogram baseline for the separation of long-chain fatty acid derivatives. In fact, the addition of DMF in mobile phase B can raise solubility of fatty acid derivatives to obtain fast separation with sharp peaks. To achieve optimal separation, the added amount of DMF in mobile phase B was tested. With gradient elution program as described in experimental section, separation of the derivatized LCFAs can be accomplished using mobile phase B containing 50% (v/v) of DMF. Chromatogram of a complete baseline resolution for all fatty acid derivatives (C_{20}–C_{30}) is shown in Fig. 2A.

The ionization and fragmentation of the isolated TSPP-fatty acid derivatives were studied by mass spectrometry with atmospheric pressure chemical ionization detection in positive-ion detection mode. As expected, the TSPP-fatty acid derivative produced an intense molecular ion peak at m/z $[MH]^+$. With MS/MS analysis of fatty acid derivatives, the collision-induced dissociation spectra of m/z $[MH]^+$ produced the specific fragment ions at m/z $[M' + CH_2CH_2]^+$ and m/z 295.1. The M′ was corresponding the molecular mass of LCFAs; the specific fragment ion m/z 295.1 was corresponding the protonated molecular core structure moiety. The specific fragment ions at m/z $[M' + CH_2CH_2]^+$ was corresponding the protonated fatty acid moiety. The selected reaction monitoring, based on the m/z $[MH]^+ \rightarrow m/z$ $[M' + CH_2CH_2]^+$ and m/z 295.1 transition, was specific for fatty acid derivatives. There was no detectable signal from the blank water sample using this transition. Although other endogenous acidic compounds present in natural environmental samples were presumably coextracted and derivatized by TSPP reagent, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ions in the m/z $[M' + CH_2CH_2]^+$ and m/z 295.1 transition. To reduce the disturbance to minimum from other unknown components presented in sample, the gradient elution with HPLC for the separation and determination of derivatized LCFAs was an efficient method. The characteristic fragment ion of m/z 295.1 (molecular core structure) came from the cleavage of $N-CH_2COO$ bond. With APCI in positive-ion detection mode, intense ion current signals for long-chain fatty acid derivatives should be attributed to the introduction of 2 weak basic nitrogen in corresponding TSPP molecular core structure resulting in high ionization efficiency. The cleavage mode and MS/MS analysis for a representative TSPP-C_{24} derivative is shown in Fig. 3(A–C). All molecular ions $[MH]^+$ and corresponding specific fragment ions for C_{20}–C_{30} fatty acid derivatives are shown in Table 1.

3.6. Comparison of the responses between TSPP and BDETS for fluorescence

As observed, the molecular structure of TSPP that it plays the same esterification with fatty acids as do of NE-OTF [27] and BDETS [32]. Relative responses of TSPP ($\lambda_{ex}/\lambda_{em}$: 260/380 nm) and BDETS ($\lambda_{ex}/\lambda_{em}$: 333/390 nm) for the individual derivatized fatty acids (C_{20}–C_{30}) are investigated. As expected, fluorescence responses for C_{20}–C_{30} fatty acid derivatives using BDETS as labeling reagent are, at least, 8–10-fold lower than that of those obtained by TSPP. The fluorescence responses for NE-OTF fatty acid derivatives are 1.6–2.4-fold lower relative to that of TSPP derivatives. This is probably due to the fact that TSPP has the large molar absorbance that make it more sensitive for the detection of derivatized fatty acids (BDETS: $\varepsilon = 2.54 \times 10^4$ L mol^{-1} cm^{-1} (249 nm); TSPP: $\varepsilon = 6.0 \times 10^4$ L mol^{-1} cm^{-1} (259 nm)). The difference in molar

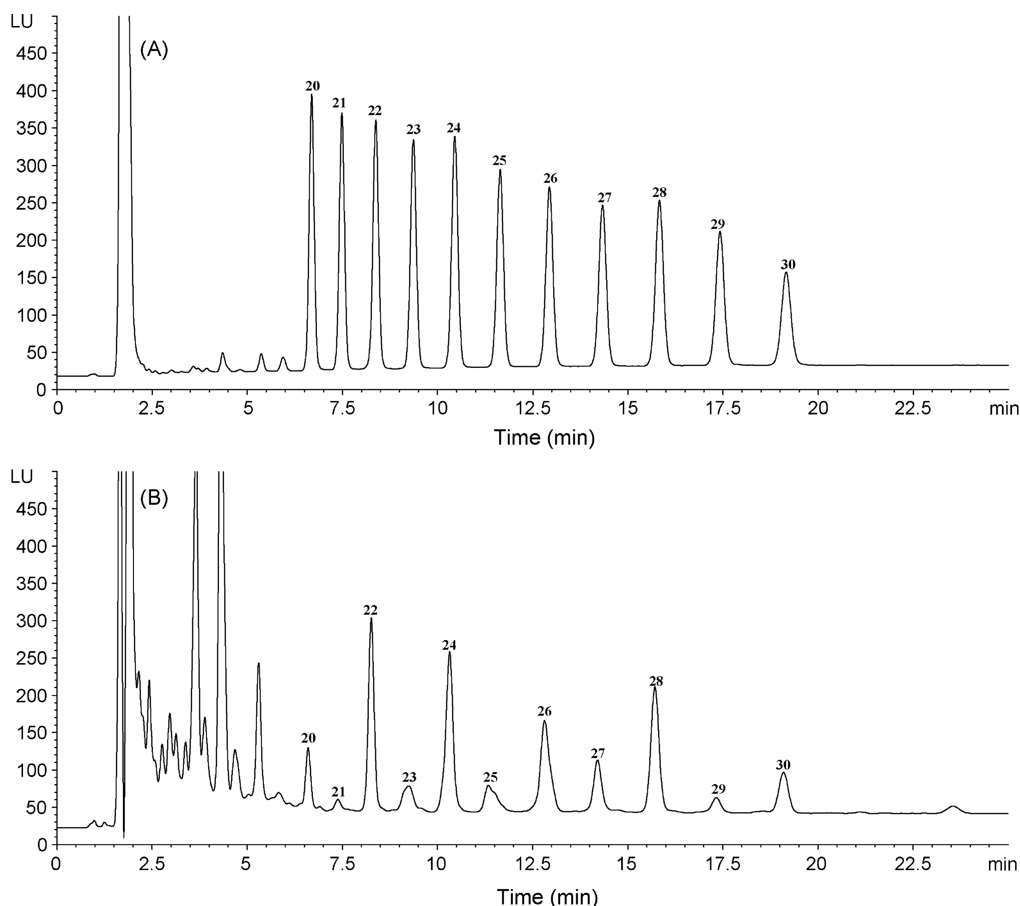

Fig. 2. (A) Chromatogram of a mixture of long-chain fatty acid standards; (B) chromatogram of free long-chain fatty acids from Neckera pennata extracts. Chromatographic conditions: Column temperature at 30 °C; excitation wavelength λ_{ex} 260 nm, emission wavelength λ_{em} 380 nm; Eclipse XDB-C_8 column (4.6 × 150 mm, 5 μm); flow rate = 1.0 mL min^{-1}; Peaks: 1-C_{20} (eicosoic acid); 2-C_{21} (heneicosoic acid); 3-C_{22} (docosanoic acid); 4-C_{23} (tricosanoic acid); 5-C_{24} (tetracosanoic acid); 6-C_{25} (pentacosanoic acid); 7-C_{26} (hexacosanoic acid); 8-C_{27} (heptacosanoicacid); 9-C_{28} (octacosanoic acid); 10-C_{29} (nonacosanoic acid); 11-C_{30} (dotriacontanoic acid).

absorbance may be attributed to the TSPP molecular structure, in which its n–π conjugation system is dramatically augmented due to introduction of a phenylimidazole-[4,5-f]-9,10-phenanthrene function group into the labeling reagent molecule that makes it more sensitive for the fluorescence detection of fatty acid derivatives.

3.7. Reproducibility, precision, calibration and detection limits

A standard solution containing C_{20}–C_{30} fatty acids (3.0×10^{-6} mol/L) was prepared and the method repeatability was examined by injecting each fatty acid (correspond-

Table 1
MS data of TSPP fatty acid derivatives

Fatty acid	Free fatty acid molecular weight	Molecular ions of derivatives $[MH]^+$	MS/MS spectra of TSPP derivatives[a]	
			$[M' + CH_2CH_2]^+$	Molecular core
C_{20}	312	633.6	339.4	295.2
C_{21}	326	647.6	353.4	295.2
C_{22}	340	661.6	367.4	295.2
C_{23}	354	675.6	381.4	295.1
C_{24}	368	689.6	395.4	295.1
C_{25}	382	703.6	409.5	295.2
C_{26}	396	717.6	423.5	295.1
C_{27}	410	731.5	437.5	295.2
C_{28}	424	745.6	451.5	295.1
C_{29}	438	759.5	465.5	295.2
C_{30}	452	773.6	479.5	295.2

[a] M′: corresponding molecular weight of free fatty acids.

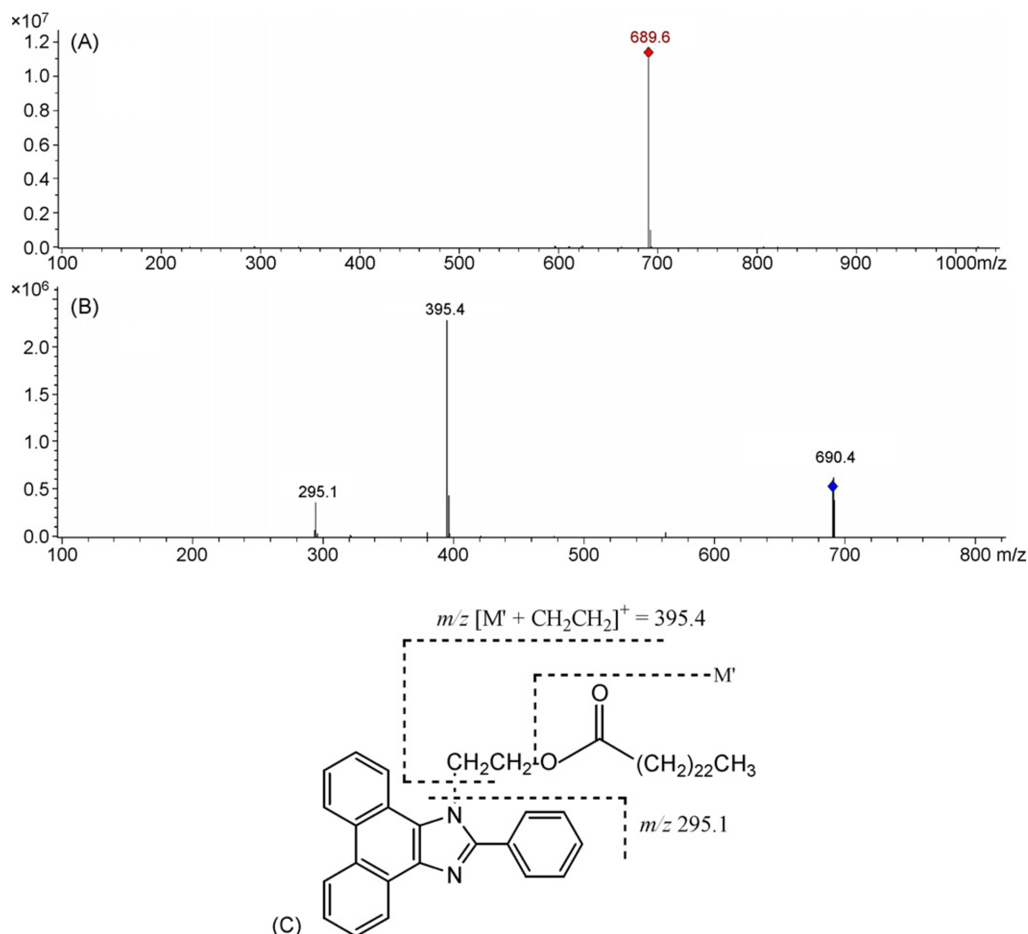

Fig. 3. The profile of molecular ion chromatogram and scanning of the isolated representative n-C_{24} acid derivative (TSPP-C_{24}). (A) Typical LC/MS profile of n-tetracosanic acid derivative (TSPP-C_{24}) from full scanning range from 100 to 1000 amu with APCI in positive-ion detection mode. (B) Typical APCI-MS/MS profile of n-tetracosanic acid derivative (TSPP-C_{24}) from full scanning range from 100 to 850 amu with APCI in positive-ion detection mode; Fragment ions, m/z 395.4 and m/z 295.1. (C) The MS/MS cleavage mode of TSPP-C_{24} derivative.

ing injected amount 50 pmol). The relative standard deviations (R.S.D.s) of the peak areas and retention times varied from 0.10 to 2.44% and from 0.019 to 0.40%, respectively. The reproducibility of the method was evaluated by the determination of known concentrations of 11 fatty acids (concentrations are 50 and 100 ng mL^{-1}, respectively) in quintuplicate over 2 days. The standard deviations (S.D.) of actual determined values were between 0.4–1.8. The corresponding coefficients of variation (R.S.D.) were between 0.8 and 2.4%. Precision and accuracy: Six replicates ($n = 6$) at 0.1, 1.0, and 5.0 μmol/L of C_{20}–C_{30} fatty acids were used to make the low to high-range concentrations. The mean interday accuracy ranged from 92.6 to 106.8% with

Table 2
Linear regression equations, correlation coefficients, detection limits, and repeatability of peak area and retention time ($n = 6$)

FFA	$Y = AX + B$, X: injected amount (pmol), Y: peak area	Correlation coefficients	Detection limits (fmol)	Retention time R.S.D. (%)	Peak area R.S.D. (%)
C_{20}	$Y = 70.93X + 25.35$	0.9996	26.19	0.074	0.31
C_{21}	$Y = 68.53X + 21.53$	0.9997	27.56	0.082	0.44
C_{22}	$Y = 71.73X + 21.67$	0.9997	28.76	0.12	0.37
C_{23}	$Y = 68.76X + 20.66$	0.9997	29.58	0.16	0.24
C_{24}	$Y = 74.11X + 21.52$	0.9997	32.32	0.14	0.55
C_{25}	$Y = 66.88X + 20.95$	0.9997	37.22	0.19	0.64
C_{26}	$Y = 64.77X + 19.68$	0.9997	41.83	0.23	0.85
C_{27}	$Y = 61.33X + 17.15$	0.9998	55.99	0.23	1.75
C_{28}	$Y = 65.50X + 17.99$	0.9999	50.89	0.28	2.25
C_{29}	$Y = 55.36X + 15.02$	0.9998	63.09	0.32	2.56
C_{30}	$Y = 42.26X + 10.14$	0.9998	76.67	0.34	3.55

Table 3
Contents of free fatty acids from three real bryophyte plant samples (data are average values of three runs)

FFA	*Homomallium connexum* (*Card.*) Broth. (μg/g)	(±) Error	*Actinothuidium hookeri* (μg/g)	(±) Error	*Neckera pennata* (μg/g)	(±) Error
C_{20}	4.34	0.32	28.85	1.88	10.46	1.04
C_{21}	0.19	0.06	3.53	0.16	2.39	0.21
C_{22}	7.84	0.34	23.05	0.68	42.66	1.26
C_{23}	1.24	0.04	6.42	0.32	9.04	1.00
C_{24}	9.39	0.39	25.19	1.28	47.35	1.24
C_{25}	1.68	0.05	5.23	0.66	14.09	1.48
C_{26}	8.35	0.22	11.63	1.02	42.89	1.64
C_{27}	0.95	0.08	1.31	0.07	22.31	1.28
C_{28}	13.18	0.16	4.63	0.16	54.85	1.86
C_{29}	1.02	0.06	0	0	9.24	1.02
C_{30}	8.22	1.02	3.55	0.42	33.10	1.40

the largest mean %CV < 6.8. The mean interday precision for all standards was <6.2% of the expected concentration. The recoveries were determined from values obtained following actual analysis of the bryophyte plants as calculated from the calibration graph constructed by using the performed fatty acid derivatives. In two identical bryophyte plant samples, known amount of 11 above-mentioned fatty acids were added. The samples were treated according to the method as described in text and derivatized with TSPP, and the analyses were carried out in duplicate. The experimental recoveries are in the range of 88–102%. Based on the optimum derivatization conditions, the linearities of the procedures were evaluated in the range of 9.766×10^{-3} to 20 μmol/L (injection volume 10 μl, corresponding injected amount from 200.0 pmol to 97.66 fmol with a 2048-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 >0.9996. The linear relationships for further higher concentrations were not tested due to over linearity range. With 1.0 pmol injection for each derivatized fatty acid, the calculated detection limits (at signal-to-noise of 3:1) were from 26.19 to 76.67 fmol. The limits of quantitation (LOQ) are also tested as the lowest concentration in the mixture of the derivatized fatty acid standards. The concentration ranges are from 9.766×10^{-3} for C_{10} to 9.846×10^{-3} μmol/L for C_{20} (injection volume 10 μL). The detection limits using TSPP as derivatization reagent are, respectively, compared with BDETS and NE-OTF. With BDETS and NE-OTF as labeling reagents, the detection limits were, respectively, from 124.8 to 180.37 fmol for BDETS and from 167.8 to 244.6 fmol for NE-OTF. The lowest detection limits decreased by 4–6-fold in comparison with those obtained using BDETS and NE-OTF as labeling reagents per 10 μL injection.

3.8. Analysis of samples

Bryophyte plants are one of the most important sources of bioactive components. Study on components of bryophyte plants has important significance. The chromatogram for the analysis of free LCFAs extracted from bryophyte plants *Neckera pennata* is shown in Fig. 2B (here, chromatograms for *H. connexum* (*Card.*) Broth, and *A. hookeri* are not shown). Free LCFAs compositional data from extracted bryophyte plants are shown in Table 3 (data are average values of three runs). The results indicate that the main contents of free LCFAs in bryophyte plants are C_{20} (4.34–28.85 μg/g,), C_{22} (7.84–42.66 μg/g), C_{24} (9.39–47.35 μg/g), C_{26} (8.35–42.89 μg/g), C_{28} (4.63–54.85 μg/g) and C_{30} (3.55–33.10 μg/g). It is demonstrated that the content of LCFAs with an even number carbon in a series (C_{20}–C_{30} fatty acids) is higher than that of those with odd number carbon. These data are of important value in order to obtain plentiful free LCFAs in bryophyte plants.

4. Conclusions

The method described in this paper produces a separation of the LCFAs obtained by distillation extraction with chloroform/methanol (1:1, v/v) as extraction solvent. The improved performance for the complete extraction of LCFAs in bryophyte plant samples has been demonstrated in detail. The reagent TSPP for the labeling of LCFAs obtained from bryophyte plant samples were evaluated. The introduction of the phenylimidazole-[4,5-f]-9,10-phenanthren functional group into labeling reagent molecule made the molecular n–π conjugation system to be augmented dramatically and was favorable for the sensitive determination of trace levels of long-chain fatty acid with fluorescence detection. At the same time, TSPP molecule contains two weak basic nitrogen atoms in its molecular core structure that make it can easily form more stable molecular ions and produces a favorable result for the sensitive identification of fatty acid derivatives under MS/APCI in positive-ion detection mode. One of the most attractive features of this established extraction method exhibits its simpleness and high extraction efficiency in order to obtain LCFAs in bryophyte plant samples. The new labeling reagent for long-chain fatty acid derivatization showed good correlation in comparison with BDETS and NE-OTF. Detection limits are in the femtomol range. The LC separation for the long-chain fatty acid derivatives shows good repeatability. Reagent and its hydrolysis products do not inter-

fere with the separation by a gradient elution. The established method can also be applied to the determination of various drugs and plants containing LCFAs.

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