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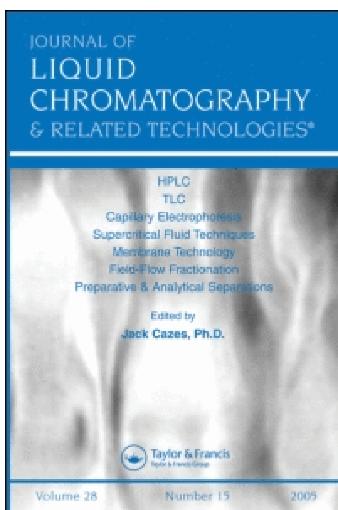

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Determination of Free Fatty Acids in Soil and Bryophyte Plants by Precolumn Derivatization via HPLC with Fluorescence Detection and Tandem Mass Spectrometry (HPLC-MS/MS)

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Determination of Free Fatty Acids in Soil and Bryophyte Plants by Precolumn Derivatization via HPLC with Fluorescence Detection and Tandem Mass Spectrometry (HPLC-MS/MS)

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Abstract: A sensitive method for the determination of 30 kinds of free fatty acids (FFAs, C_1-C_{30}) with 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenan- threne (TSPP) as labeling reagent and using high performance liquid chromatography with fluorescence detection and identification by online postcolumn mass spectrometry with atmospheric pressure chemical ionization (APCI) source in positive-ion mode (HPLC/MS/APCI) has been developed. TSPP could easily and quickly label FFAs in the presence of K_2CO_3 catalyst at 90°C for 30 min in N,N-dimethylformamide (DMF) solvent, and maximal labeling yields close to 100% were observed with a 5-fold excess of molar reagent. Derivatives were stable enough to be efficiently analyzed by high performance liquid chromatography. TSPP was introduced into fatty acid molecules and effectively augmented MS ionization of fatty acid derivatives and led to regular MS and MS/MS information.

The collision induced cleavage of protonated molecular ions formed specific fragment ions at m/z $[MH]^+$(molecular ion), m/z $[M' + CH_2CH_2]^+$(M′ was molecular mass of the corresponding FFA) and m/z 295.0 (the mass of protonated molecular core structure of TSPP). Fatty acid derivatives were separated on a reversed-phase Eclipse XDB-C_8 column (4.6 × 150 mm, 5 μm, Agilent) with a good baseline resolution in combination with a gradient elution. Linear ranges of 30 FFAs are 2.441×10^{-3} to 20 μmol/L, detection limits are 3.24 ~ 36.97 fmol (injection volume 10 μL, at a signal-to-noise ratio of 3, S/N 3:1). The mean interday precision

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ranged from 93.4 to 106.2% with the largest mean coefficients of variation (R.S.D.) <7.5%. The mean intraday precision for all standards was <6.4% of the expected concentration. Excellent linear responses were observed with correlation coefficients of >0.9991. Good compositional data could be obtained from the analysis of extracted fatty acids from as little as 200 mg of bryophyte plant samples.

Therefore, the facile TSPP derivatization coupled with HPLC/MS/APCI analysis allowed the development of a highly sensitive method for the quantitation of trace levels of short and long chain fatty acids from biological and natural environmental samples.

Keywords: 1-[2-(*p*-Toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP), Derivatization, High performance liquid chromatography, Fluorescence detection, Fatty acids, Mass spectrometry

INTRODUCTION

Fatty acids are widely distributed in nature and important as nutritional substances and metabolites in living organisms. Many kinds of fatty acids are presented in biological specimens and they play physiologically 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. But, in fact, 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 show sharp, acrid, and unpleasant tastes 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 bisbibenzyl 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 using absorptiometry is fairly difficult.[6] However, easily detectable fatty acid derivatives by methyl or ethyl esterification with GC or GC/MS have been reported.[7,8] In contrast with GC, use of HPLC allows the fatty acids to be converted to a large number of different derivatives.[9] 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 has been widely adopted since HPLC with UV, especially fluorescence detection, has higher sensitivity. These reagents commonly used include coumarin type derivatives,[10]–[16] diazomethane-type reagents such as 9-anthryldiazomethane (ADAM)[17]–[18] and 1-pyrenyldiazomethane (PDAM),[19] quinoxalinone derivatives.[20]–[22] benzofurazan-type reagents,[23]–[25] sulfonate ester reagents 2-(2-naphthoxy)-ethyl-2- (piperidino)-ethanesulfonate

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(NOEPES),[26] 2-(2,3-naphthalimido)- ethyl trifluoromethane- sulfonate (NE-OTF),[27] and 2-(2,3-anthracene -dicarboximido) ethyl trifluoromethane-sulfonate (AE-OTF),[28] benzohydrazide-type reagents such as 4-(1-methylphenanthro [9,10-d]imidazole-z2-yl)benzohydrazide (MPIB-hydrazide),[29] and 4-(5,6-Dimethoxy-2-benzimidazoyl)- benzohydrazide) (DMBI-hydrazide),[30] and so on. However, it has been reported that these reagents have limitations in their applications such as low detection sensitivity, short detection wavelengths, poor stability, tediously analytical procedure, and serious interferences in the biological sample analyses.[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 19 fatty acids or bile acids. In this study, the aims of the present work are to develop a new labeling reagent 1-[2-(*p*-toluenesulfonate)- ethyl]-2-phenylimidazole- [4,5-f]-9,10-phenanthrene (TSPP) and establish a simple and sensitive method for the determination of 30 FFAs extracted from bryophyte plants and soil. The optimal derivatization conditions such as catalyst, reaction time, and solvent system were investigated. The fluorescence detection sensitivity for FFAs was compared with AETS,[32,33] BDETS,[34] and NOEPES[26] as labeling reagents. Linearity, reproducibility, detection limits, and precision of the procedure were also investigated. HPLC separation with tandem mass spectrometry identification for 30 FFA derivatives was accomplished in combination with a gradient elution on a reversed-phase Eclipse XDB-C_8 column. The determination of FFAs in bryophyte plants and soil using the proposed method was satisfactory.

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EXPERIMENTAL

Instrumentation

All the HPLC system devices were from the 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 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: APCI in positive ion mode; nebulizer pressure 413.69 MPa; dry gas temperature, 350°C; dry gas flow, 5.0 L/min. APCI Vap temperature 450°C; corona current (nA) 4000 (pos); capillary voltage 3500 V. Derivatives were separated on a reversed-phase Eclipse XDB-C_8 column (150 × 4.6 mm, 5 μm, Agilent Co.) by 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 mm nylon membrane filter (Alltech, Deerfiled, IL).

Prior to its use, the instrument was checked to meet the sensitivity defined by the manufacturer. The FL was calibrated and tested using the FL diagnosis procedure of the ChemStation software for HP1100 system. The HP1100 LC/MSD Trap SL was calibrated with APCI tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated, so that mass accuracy, specification, and sensitivity were achieved over the entire mass range. APCI source and instrument parameters were optimized by infusing the TSPP derivatives that were isolated from an HPLC column using fluorometric detection into the online post-column mass spectrometry.

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Chemicals

Standards of 30 FFAs (C_1-C_{30}) were purchased from Shanghai Chemical Reagent Co (Shanghai, China). Spectroscopically pure acetonitrile was purchased from Merck Co. (Germany). N,N-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were purchased from Jining Chemical Reagent Co (Shandong, Jining, China) and treated with 5Å 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). 1,2-Benzo-3,4- dihydrocarbazole-9-ethyl-*p*- toluenesulfonate (BDETS)[35] synthesized in our laboratory was used to compare the fluorescence sensitivity with TSPP. Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were of analytical grade unless otherwise stated. Bryophyte plant samples (**A**: *Entodon macropodus* (Changbai Mountain, Jilin, China); **B**: *Dicranum aesium* (Huanglong, Sichuan, China) were friendly obtained from College of Life Science, Qufu Normal University (Shandong, China). Soil was collected from Qufu Normal University (Shandong, China).

Preparation of Standard and Sample Solutions

The labeling reagent (TSPP) solution (5.0×10^{-2} mol/L) was prepared by dissolving 246 mg of 1-[2-(p-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10- phenanthrene (TSPP) in 10 mL of DMF. Corresponding derivatization reagent solution of low concentration (5.0×10^{-3} mol/L) was obtained by diluting the stock solution with DMF. The individual standard of fatty acids was prepared by dissolving the fatty acid in HPLC grade acetonitrile to a concentration of 1.0×10^{-2} mol/L. For long chain fatty acids (i.e., $>C_{15}$), the individual stock solution was prepared by dissolving the fatty acid in DMF and diluting with the mixed solvent of acetonitrile/DMF (1:1, *v/v*)

owing to their poor solubility. Standards of mixed 30 FFAs were prepared by diluting the corresponding individual stock solution with acetonitrile to a concentration of 1.0×10^{-4} mol/L. When not in use, all reagent solutions were stored at 4°C in a refrigerator until HPLC analysis.

The Bryophyte plant was washed using deionized water and then dried under a stream of nitrogen gas. To a 50 mL round bottom flask, 200 mg pulverized bryophyte and 10 mL chloroform were added. After the contents of the flask were vortexed for 0.5 min, the flask was immersed in a sonicator water bath and the sample was sonicated in 30 s intervals for 2 min. The sample was allowed to incubate at room temperature for 24 h. The contents were filtered, and the residues were extracted with another 10 mL of chloroform according to the procedure as described above, the combined chloroform was added to 1.0 mL of pyridine and sonicated for 30 s. The chloroform was then evaporated to dryness with a stream of nitrogen gas. The residue was redissolved in 500 μL DMF, filtered through a 0.2 mm nylon membrane filter, and stored at −10°C until HPLC analysis.

Soil (20 g) and 40 mL chloroform were added into a 250 mL round bottom flask. The contents of the flask were vortexed for 0.5 min, and then sonicated for 30 min. The contents were filtered, and the residues were extracted with another 40 mL of chloroform according to the procedure as described above, the combined chloroform was added to 1.0 mL of pyridine and sonicated for 30 s. The chloroform was then evaporated to dryness with a stream of nitrogen gas. The residue was redissolved in 600 μL of DMF, filtered through a 0.2 μm nylon membrane filter, and stored at −10°C until HPLC analysis.

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Derivatization of Standard and Sample

To a solution consisting of 50 μL of standard fatty acids mixture in a 2 mL vial, 150 μL derivatization reagent solution, 10 mg anhydrous K_2CO_3, and 200 μL DMF were added, respectively. The vial was sealed and allowed to react in a water bath at 90°C for 30 min with shaking at 5 min intervals. After the reaction was completed, the mixture was cooled at room temperature. A 600 μL mixture solution of acetonitrile and DMF (CH_3CN/DMF, 1:1, *v*/*v*) was added to dilute the derivatization solution. The diluted solution (10 μL, 50 pmol) was injected directly for chromatographic analysis. The derivatization procedure is shown in Figure 1. The derivatization of the extracted sample solutions was the same as above.

Chromatographic Conditions

HPLC separation of 30 FFAs derivatives was carried out on a reversed-phase Eclipse XDB-C_8 column (150 × 4.6 mm, 5 mm, Agilent Co.) by a gradient

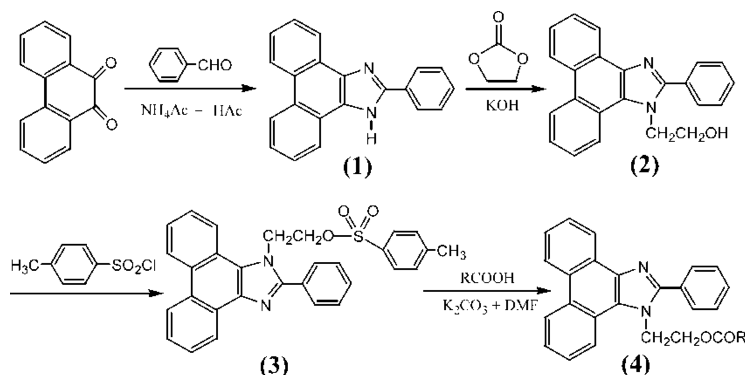

Figure 1. The scheme of derivatization procedure for the labeling of FFAs using TSPP. (**1**): Phenylimidazole-[4,5-f]-9,10- phenanthrene; (**2**): 1-(Ethanol)-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (EPP); (**3**): 1-[2- (p-Toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP);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

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elution. Eluent A was 50% of acetonitrile; B was 50% acetonitrile containing 0.2 mol/L ammonium formate buffer (pH 3.7); C was a mixture solution of acetonitrile and DMF (ACN/DMF, 100:2, *v*/*v*); D was a mixed solution of acetonitrile and DMF (ACN/DMF 100:30, *v*/*v*). Before injection of the next sample, the column was equilibrated with mobile phase A for 10 min. The flow rate was constant at 1.0 mL/min and the column temperature was set at 30°C. The fluorescence excitation and emission wavelengths were set at λ_{ex} 260 and λ_{em} 380 nm, respectively. The gradient elution program is presented in Table 1.

Table 1. Gradient elution program

Time (min)	A (%)	B (%)	C (%)	D (%)
0	95	0	5	0
4	95	0	5	0
4.2	0	95	5	0
8	0	95	5	0
8.5	0	75	25	0
15	0	50	50	0
40	0	0	100	0
48	0	0	0	100
65	0	0	0	100

Eluent A, B, C, D as chromatographic conditions part.

Synthesis of Labeling Reagent (TSPP)

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

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 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, the pH of the solution was adjusted to 7~8 with ammonia water. 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 (acetonitrile/DMF 5:1, *v/v*) to afford a slight yellow crystal, yield 92%.

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 conveniently synthesized by a modified method.[35] Phenylimidazole- [4,5-f]-9,10-phenanthrene (12 g), ethylene carbonate (4.5 g), trace amount of KOH (0.2 g), and DMF (120 mL) were mixed in a 500 mL round bottom 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% 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 (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

To a solution of 40 mL pyridine (0°C) consisted of 2.53 g toluenesulfonyl chloride in a 100 mL round bottom 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, within 30 min with 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 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 ambient temperature for 48 h. The crude products were recrystallized twice from acetonitrile to give the white

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crystals 1.76 g, 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.

RESULTS AND DISCUSSION

Stability and Spectral Properties of TSPP

When an anhydrous solution of TSPP in acetonitrile or methanol was stored under refrigeration for two 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 to 400 nm. Maximum ultraviolet absorption responses were observed at the wavelengths of 259 nm and 320 nm, respectively. The molar absorption coefficients (ε) of TSPP reagent in acetonitrile solution (acetonitrile/water, 2:1, v/v) are $\varepsilon = 6.0 \times 10^4$ L mol^{-1} cm^{-1} (259 nm), 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 $\lambda ex = 260$ and $\lambda em = 380$ nm, respectively, and exhibited no obvious blue- or red-shift with different concentrations of acetonitrile or methanol (0–100%).

Optimal Extraction

In order to achieve the highest extraction yields, two extraction methods for FFAs in bryophyte plant samples were evaluated by comparing the fluorescence detector responses obtained by the analysis of the derivatized fatty acids from the extracted samples. The results indicated that the highest extraction efficiency of FFAs in bryophyte plant samples was achieved by ultrasonication extraction. In most cases, lower extraction efficiency was observed for the shaken extraction. With various extraction solvents, the highest extraction efficiency for FFAs was observed using the chloroform as an extraction solvent because the solubility of FFAs in chloroform was higher than that in single methanol or acetonitrile. Subsequently, all experiments in this study were performed by the ultrasonication extraction using chloroform as the extraction solvent.

Optimal Derivatization

The effect of the reaction temperature on the derivatization yields was evaluated from 30°C to 95°C. As observed, fluorescence responses reached

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the 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 dimethylsulfoxide (DMSO). DMF resulted in the most intense fluorescence response in the chromatogram. In addition, DMF was used as the derivatization cosolvent in preference to other solvents, as it easily 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 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 = or >10 mg, and with a further excess of K_2CO_3, the detector responses did not significantly increase. Although NOEPES, NE-OTF, AE-OTF have been developed as sulfonate ester reagents for the determination of fatty acids, crown ether and potassium carbonate were usually used in the derivatization procedure with toxic toluene as solvents, 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 remarkably different in the presence or absence of 18-crown-6. A constant fluorescence intensity was achieved with the addition of 5-fold molar reagent excess to total molar fatty acids, 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 for detector responses.

HPLC Separation and MS/MS Identification

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 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 more rapid separation and a lower and more stable chromatogram 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 can be accomplished at acidic condition with pH 3.7. With pH <3.5, most of the fatty acids were resolved with an exception being C_1 and compound B; B and C_2, respectively, partially coeluted. In comparison with pH 3.7, eluent at pH >4.0 resulted in an obvious increase in retention time value for most of the fatty acid

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derivatives, at the same time, compound **C** and C_4 was coeluted. After further experiments, it was found that if the pH value of mobile phase **B** was adjusted to 3.7, a complete baseline resolution for 30 FFA standard derivatives was obtained within 65 min, with the shortest retention time values and the sharpest peaks. In fact, the addition of DMF in mobile phase C and D could raise the solubility of fatty acid derivatives to obtain fast separation with sharp peaks. To achieve optimal separation, the added amount of DMF in mobile phase C and D was confirmed with optimization as described above. With the optimal chromatographic conditions described above, the chromatogram of a complete baseline resolution for all FFA derivatives is shown in Figure 2. Under all these conditions, all 30 fatty acids from real samples were separated with a good baseline resolution.

The ionization and fragmentation of the isolated TSPP-FFA derivatives was studied by mass spectrometry with the atmospheric pressure chemical ionization (APCI) source in positive-ion mode. As expected, the TSPP-fatty acid derivative produced an intense molecular ion peak at *m/z* $[MH]^+$. 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*

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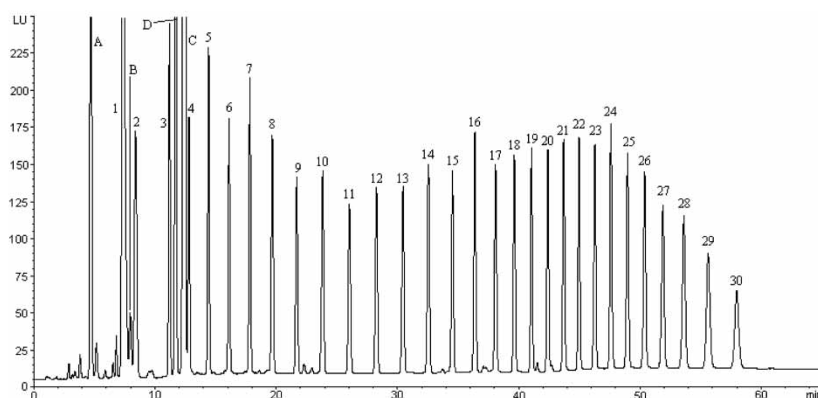

Figure 2. Chromatogram of 30 standard fatty acid derivatives (50 pmol). Chromatographic conditions: Column temperature at 30°C; excitation wavelength λ_{ex} 260 nm, emission wavelength λ_{em} 380 nm; Eclipse XDB-C_8 column (4.6 × 150 mm, 5 μm); flow rate = 1.0 mL min^{-1}; Peaks: 1, formic acid; 2, acetic 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; 27, heptacosanoicacid; 28, octacosanoic acid; 29, nonacosanoic acid; 30, dotriacontanoic 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, not identification).

$[M' + CH_2CH_2]^+$ and m/z 295.0. The M′ was molecular mass of the corresponding FFA; the specific fragment ion m/z 295.0 was the corresponding protonated molecular core structure moiety. The specific fragment ions at m/z $[M' + CH_2CH_2]^+$ was the corresponding protonated fatty acid moiety. The selected reaction monitoring, based on the m/z $[MH]^+ \rightarrow m/z$ $[M' + CH_2CH_2]^+$ and m/z 295.0 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 the sample were presumably coextracted and derivatized by the TSPP reagent, no disturbance was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ion in the m/z $[MH]^+$®m/z $[M' + CH_2CH_2]^+$ and m/z 295.0 transition. To reduce the disturbance to minimum from other unknown components presented in the sample, the gradient elution with HPLC for the separation and determination of derivatized fatty acids was an efficient method. The characteristic fragment ion of m/z 295.0 (molecular core structure) and m/z $[M' + CH_2CH_2]^+$ came from the cleavage of the N-CH_2CH_2OCO bond. With APCI in positive ion mode, intense ion current signals should be attributed to the introduction of two weak basic nitrogen atoms in the TSPP molecular core structure, resulting in highly ionizable efficiency. The MS/MS analysis and cleavage mode for a representative TSPP-C_{20} derivative is shown in Figure 3 (A, B, C). All molecular ions $[MH]^+$ for 30 fatty acid derivatives are shown in Table 2.

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Comparison of the Fluorescence Sensitivity of TSPP, AETS, and BDETS

As observed, the molecular structure of TSPP underwent the same esterification with fatty acids as do 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 ($\lambda_{ex}/_{em}$: 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 reagents were, respectively, at least $2 \sim 4$-fold and $3 \sim 8$-fold lower than that of those obtained by TSPP. This was probably due to the fact that TSPP has a larger molar absorbance coefficient (ε() that made it more sensitive for detection of derivatized fatty acids (AETS: $\varepsilon = 5.72 \times 10^4$ L mol^{-1} cm^{-1} (255 nm); BDETS: $\varepsilon = 2.54 \times 10^4$ L mol^{-1} cm^{-1} (249 nm); TSPP: $\varepsilon = 6.0 \times 10^4$ L mol^{-1} cm^{-1} (259 nm)). The difference in molar absorbance coefficient may be attributed to the TSPP molecular structure, in which n-π conjugation system is dramatically augmented due to introducing a phenylimidazole-[4,5-f]-9,10- phenanthrene function group into the labeling reagent that makes it more sensitive for the fluorescence detection of FFA derivatives.

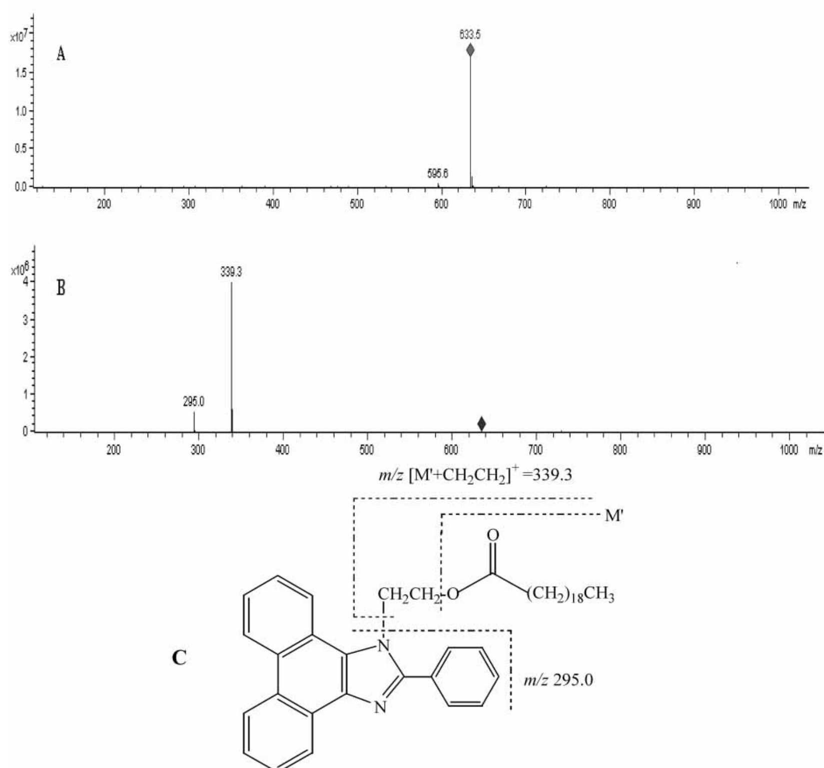

Figure 3. The profile of molecular ion chromatogram and MS/MS scanning of the isolated representative *n*-eicosoic acid derivatives (TSPP-C_{20}). (**A**) Typical HPLC-MS chromatogram of fatty acid derivatives from full scanning range from 100 to 1000 amu with APCI in positive-ion mode. (**B**) Typical MS/MS chromatogram of eicosoic acid derivative from full scanning range from 100 to 1000 amu with APCI in positive-ion mode; Fragment ions, m/z 339.3 and m/z 295.0. (**C**) The MS/MS cleavage mode of TSPP-C_{20} derivative.

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Reproducibility, Precision, Linearity, and Detection Limits

A standard solution consisting of C_1–C_{30} fatty acids (5×10^{-5}mol/L) 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 (R.S.D.s) of the peak areas and retention times are from 0.15 to 2.56% and from 0.019 to 0.41% (shown in Table 2), respectively. Precision and accuracy: Six replicates (n = 6) at 0.1, 1.0, and 5.0 μmol/L of 30 fatty acids were used to make the low to high range concentrations. The mean interday accuracy ranged from 93.4 to 106.2% with the largest mean coefficients of variation (R.S.D.) $<$7.5%. The

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Table 2. Linear regression equations, correlation coefficients, detection limits, MS dada of 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	Correlation coefficients	Detection limits (fmol)	MS $[M+H]^+$	Retention time R.S.D. (%)	Peak area R.S.D. (%)
C_1	$Y = 736.5X + 208.6$	0.9995	3.24	367.1	0.37	0.19
C_2	$Y = 169.1X + 54.55$	0.9991	24.28	381.2	0.41	0.65
C_3	$Y = 147.9X + 43.74$	0.9995	10.14	395.1	0.35	0.46
C_4	$Y = 94.80X + 31.45$	0.9994	14.44	409.2	0.32	0.83
C_5	$Y = 141.3X + 35.15$	0.9997	14.99	423.2	0.19	0.46
C_6	$Y = 113.2X + 32.55$	0.9997	12.23	437.2	0.18	0.69
C_7	$Y = 132.2X + 31.11$	0.9998	18.39	451.2	0.12	0.57
C_8	$Y = 120.2X + 36.46$	0.9997	16.66	465.2	0.099	0.56
C_9	$Y = 102.6X + 28.21$	0.9996	27.22	479.2	0.089	0.44
C_{10}	$Y = 112.3X + 30.46$	0.9996	24.71	493.3	0.073	0.31
C_{11}	$Y = 95.73X + 25.42$	0.9996	31.17	507.3	0.081	0.44
C_{12}	$Y = 105.3X + 28.25$	0.9996	28.77	521.3	0.063	0.38
C_{13}	$Y = 105.2X + 26.44$	0.9997	28.86	535.4	0.056	0.24
C_{14}	$Y = 114.9X + 31.94$	0.9995	26.31	549.4	0.044	0.25

(*continued*)

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Table 2. Continued

FFA	Y = A*X+B X: Injected amount (pmol); Y: Peak area	Correlation coefficients	Detection limits (fmol)	MS $[M+H]^+$	Retention time R.S.D. (%)	Peak area R.S.D. (%)
C_{15}	$Y = 109.7X + 30.01$	0.9995	26.28	563.4	0.043	0.17
C_{16}	$Y = 128.5X + 36.37$	0.9995	21.71	577.4	0.031	0.15
C_{17}	$Y = 104.6X + 30.99$	0.9995	25.08	591.4	0.026	0.17
C_{18}	$Y = 107.2X + 29.59$	0.9995	23.34	605.4	0.019	0.16
C_{19}	$Y = 102.5X + 31.35$	0.9992	24.44	619.5	0.020	0.16
C_{20}	$Y = 102.8X + 32.74$	0.9993	22.17	633.5	0.043	0.36
C_{21}	$Y = 106.7X + 34.49$	0.9992	23.46	647.6	0.072	0.88
C_{22}	$Y = 110.6X + 38.75$	0.9993	24.72	661.6	0.095	1.25
C_{23}	$Y = 113.8X + 30.77$	0.9993	24.36	675.6	0.12	1.36
C_{24}	$Y = 131.4X + 31.19$	0.9995	19.97	689.6	0.20	1.56
C_{25}	$Y = 122.8X + 27.39$	0.9997	20.63	703.6	0.23	2.04
C_{26}	$Y = 119.34X + 23.39$	0.9999	22.61	717.6	0.27	2.24
C_{27}	$Y = 122.3X - 10.56$	0.9993	23.96	731.5	0.24	2.13
C_{28}	$Y = 118.6X + 35.99$	0.9995	29.39	745.6	0.25	2.40
C_{29}	$Y = 103.1X + 34.97$	0.9994	32.21	759.5	0.27	2.56
C_{30}	$Y = 82.02X + 31.47$	0.9994	36.97	773.6	0.24	2.35

mean intraday precision for all standards was <6.4% of the expected concentration.

Based on the optimum derivatization conditions, the linearities of 30 FFAs ($C_1 \sim C_{30}$) were evaluated in the range of 2.441×10^{-3} to 20 μmol/L (injection volume 10 μL, injected amount from 200.0 pmol to 24.41 fmol with a 8193-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 0.9991 ~ 0.9999. The linear relationships for higher concentrations were not tested over the linearity range. With a 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 3.24 to 36.97 fmol with an average of 18.8 fmol (injection volume 10 μL, shown in Table 2). The limits of quantitation (LOQ) were also tested as the lowest concentration in the mixture of the derivatized fatty acid standards. The concentration ranges are from 2.441×10^{-3} for C_5 to 2.652×10^{-3} μmol/L for C_{30} (injection volume 10 μL). The detection limits using TSPP as derivatization reagent were, respectively, compared with AETS, BDETS, and NOEPES. With AETS, BDETS, and NOEPES as labeling reagents, the detection limits (injected volume 10 μL) were, respectively, 12.3 ~ 43.7 fmol for AETS[32] with an average of 24.6 fmol, 24.8 ~ 80.37 fmol for BDETS[34] with an average of 46.5 fmol, and 56 fmol for NOEPES.[26] The lowest detection limits and the average detection limits, respectively, decreased by 4–8-fold and 1-3-fold in comparison with those obtained using AETS, BDETS, and NOEPES as labeling reagents per 10 μL injection.

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Analysis of Samples and Recovery

The chromatogram for the analysis of FFAs extracted from bryophyte plants (**A**: *Entodon macropodus* (Changbai Mountain, Jilin, China); **B**: *Dicranum aesium* (Huanglong, Sichuan, China)) and soil (**C**) with fluorescence detection, are shown in Figure 4 (**A**, **B**, **C**), respectively. Chromatographic peaks were identified by contrasted chromatograms of real samples with that of fatty acid standards by retention time, and, simultaneously, confirmed by mass spectrometry 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 reagents. All fatty acids were quantified by linear regression equations. FFAs compositional data from extracted bryophyte plants and soil are shown in Table 3. The results indicated that the main FFAs with higher contents in A and B bryophyte plants were, respectively, C_{14} 0.1670, 10.56 μg/g; C_{16} 103.7, 74.78 μg/g; C_{18} 13.43, 21.43 μg/g; C_{20} 3.104, 10.05 μg/g; C_{22} 10.67, 11.72 μg/g; C_{24} 30.48,

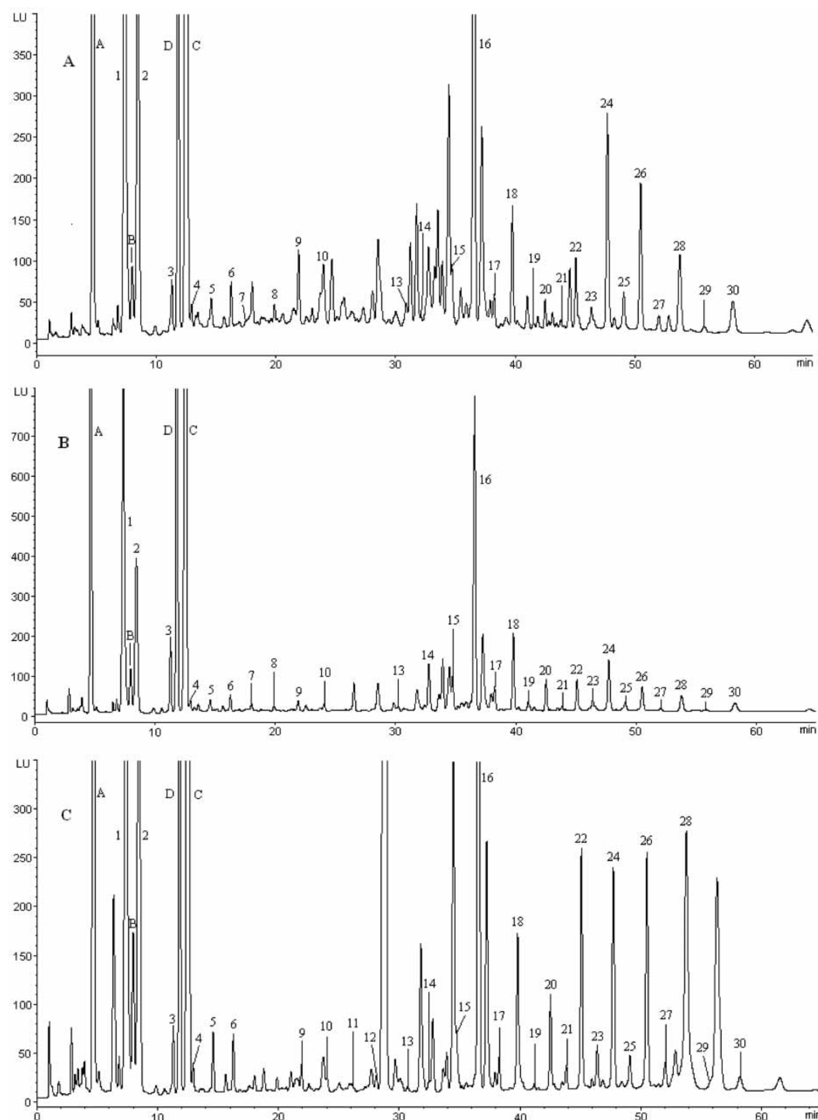

Figure 4. Chromatogram of derivatived FFAs from extracts of two bryophyte plants and soil (**A**) *Entodon macropodus*; (**B**) *Dicranum aesium*; (**C**) Soil. Chromatographic conditions and peaks as Figure 2.

18.42 μg/g; C_{26} 27.22, 11.91 μg/g; C_{28} 19.87, 9.944 μg/g; C_{30} 16.85, 10.92 μg/g. The contents of FFAs were about 100 times higher in two bryophyte plants than in soil. It was obvious that the contents of FFAs with even numbers of carbon in a series (C_{14}~C_{30} fatty acids) were higher than

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Table 3. Contents of free fatty acids from two bryophyte plants and soil (data are average values of three runs)

FFA	Soil (μg/g)	*Entodon macropodus* (μg/g)	*Dicranum aesium* (μg/g)
C_1	0.01154	0.9451	0.9861
C_2	0.01256	0.7059	0.8934
C_3	0.009206	0.6521	0.5611
C_4	0.001782	0.2108	0.5973
C_5	0.002193	0.9075	0.7949
C_6	0.002255	1.177	1.541
C_7	0	0.1312	0.4529
C_8	0	0.5852	0.5341
C_9	0.005589	4.662	1.535
C_{10}	0.005644	2.746	0.8689
C_{11}	0.005923	0	0
C_{12}	0.004103	0	0
C_{13}	0.001011	0.9623	0.6508
C_{14}	0.007815	0.1670	10.56
C_{15}	0.008857	1.998	4.593
C_{16}	0.1526	103.7	74.78
C_{17}	0.003449	2.617	4.391
C_{18}	0.1054	13.43	21.43
C_{19}	0.001864	0.6929	0.5624
C_{20}	0.009172	3.104	10.05
C_{21}	0.003726	0.4835	0.6018
C_{22}	0.01366	10.67	11.72
C_{23}	0.005104	2.361	1.415
C_{24}	0.01302	30.48	18.42
C_{25}	0.004964	6.793	3.937
C_{26}	0.01319	27.22	11.91
C_{27}	0.003864	3.275	1.806
C_{28}	0.01413	19.87	9.944
C_{29}	0.002923	1.588	0.7891
C_{30}	0.01008	16.85	10.92

that of those with odd numbers of carbon in their neighbor. That might be related to their physiological function.

The recoveries of 30 FFAs were investigated by the addition of known amounts of 30 FFAs standard solutions (10 μL, 1.0×10^{-4} mol/L) into the bryophyte plants whose contents of 30 FFAs were known by calculating from linear regression equations. The extraction and derivatization were the same as the optimal conditions above, and the analyses were carried out in 3 duplicates. The experimental recoveries were in the range of 89.62∼105.3%.

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CONCLUSION

In this study, simultaneous determination of 30 FFAs extracted from two bryophyte plants and soil using TSPP as precolumn derivatization with HPLC fluorescence detection and tandem mass spectrometry identification could be successfully achieved. The derivatization and HPLC separation conditions of the reagent TSPP for the labeling 30 FFAs, and MS/MS identification of FFA derivatives by molecular ion and its fragment ions, were evaluated. The introduction of the phenylimidazole-[4,5-f]-9,10-phenanthrene functional group into the TSPP 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 FFAs with fluorescence detection. At the same time, the TSPP molecule contained two weak basic nitrogen atoms in its molecular core structure so that the TSPP reagent and its FFA derivatives exhibited high mass spectrometric ion current signals, which could be hopefully applied to identification of trace amounts of fatty acids by APCI in positive ion mode. The new labeling reagent TSPP for FFAs showed good similarity with AETS, BDETS, and NOEPES, but had higher sensitivity, and the detection limits were in femtomol level. The HPLC separation for FFA derivatives showed good repeatability. The TSPP reagent and its hydrolysis products did not interfere with the separation of 30 FFA derivatives by optimal gradient elution. The established method could be hopefully applied to the determination of FFAs from various drugs, plants, and biochemistry samples.

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