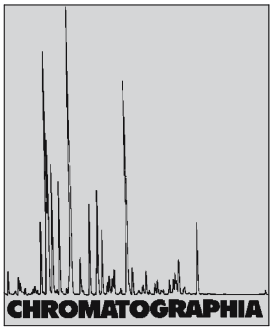
Determination of 30 Free Fatty Acids in Two Famous Tibetan Medicines by HPLC with Fluorescence Detection and Mass Spectrometric Identification



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Jing Sun[1,2], Guichen Chen[1,✉], Xianen Zhao[1,2], Wenhua Xu[1,2], Guoying Zhou[1,2], Youji Han[1,2], Jinmao You[3]

[1] Research Center of National Tibetan Medicine, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, 59 Xiguan Street, Xining 810008, People's Republic of China; E-Mail: gcchen@nwipb.ac.cn
[2] Graduate School of the Chinese Academy of Sciences, Beijing 100049, People's Republic of China
[3] College of Chemistry Science, Qufu Normal University, Qufu 273165, People's Republic of China

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Abstract

A simple and sensitive high-performance liquid chromatographic (HPLC) method with fluorescence detection and mass spectrometric identification has been developed for analysis of 30 long-chain and short-chain free fatty acids (FFAs). The fatty acids were derivatized to their esters with 1-[2-(*p*-toluenesulfonate)ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP) in *N*,*N*-dimethylformamide (DMF) at 90 °C with anhydrous K_2CO_3 as catalyst. A mixture of C_1–C_{30} fatty acids was completely separated within 60 min by gradient elution on a reversed-phase C_8 column. Qualitative identification of the acids was performed by atmospheric-pressure chemical ionization mass spectrometry (APCI–MS) in positive-ion mode. The fluorescence excitation and emission wavelengths were 260 and 380 nm, respectively. Quantitative determination of the 30 acids in two Tibetan medicines *Gentiana straminea* and *G. dahurica* was performed. The results indicated that the medicines contained many FFAs. Linear correlation coefficients for the FFA derivatives were >0.9991. Relative standard deviations (RSDs, $n = 6$) for the fatty acid derivatives were <3%. Detection limits (at a signal-to-noise ratio of 3:1) were 3.1–38 fmol. When the fatty acid derivatives were determined in the two real samples results were satisfactory and the sensitivity and reproducibility of the method were good.

Keywords

Column liquid chromatography–mass spectrometry
Fluorescence detection
Free fatty acids
Gentiana straminea
G. dahurica

Introduction

Free fatty acids (FFAs) occur widely in living organisms and are important in the regulation of a variety of physiological and biological functions [1]. Although most fatty acids neither fluoresce nor absorb light in the ultraviolet or visible regions of the spectrum, they are readily derivatized and analysis of the methyl esters, for example, has been performed by gas chromatography (GC) [2] and supercritical-fluid chromatography [3]. Compared with GC, high-performance liquid chromatography (HPLC) of FFAs can be performed with many more derivatives [4–9]. Derivatization can be used to overcome problems such as peak-tailing by formation of less polar compounds which can be analyzed easily by HPLC [10]. Low detection sensitivity can be overcome by derivatization of the FFAs with a fluorescence-labeling reagent then quantitative analysis by HPLC with fluorescence detection. This technique, which is more sensitive and selective than GC, has been widely adopted and used for quantitative determination of FFAs with high sensitivity in a variety of biological samples. Precolumn derivatization is currently a popular method. A variety of fluorescent

reagents, including 3-bromoacetyl-6,7-methylenedioxycoumarin [11], have been used for labeling of FFAs; FFAs have also been derivatized with diazomethane to furnish the methyl esters [12]. Although these compounds react with carboxyl groups under mild conditions at room temperature, without the need for catalysts, shortcomings of the reagents have been reported, for example the need to use short detection wavelengths, poor stability of the reagents, and serious interferences in analysis of real samples. Lu et al. [13] used NOE-PES sulfonic acid to determine FFAs; the derivatization procedure is, however, performed in benzene or methylbenzene, in the presence of KOH, with a crown ether as catalyst, and the derivatization solution must be pretreated before HPLC separation.

In this study a new fluorescence reagent, 1-[2-(*p*-toluenesulfonate)ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP), in the presence of K_2CO_3 as catalyst, at 90 °C in *N*,*N*-dimethylformamide (DMF) as solvent has been used for pre-column derivatization. The solution after derivatization can be injected directly. The main merits of the method are the simple derivatization procedure and that it is unnecessary to remove excess reagent or solvents after derivatization. Complete and reproducible separation of a multi-component mixture (C_1–C_{30}) of fatty acid derivatives has been achieved by use of gradient elution.

Gentiana straminea Maxim. and *G. dahurica* Fischer. (Gentianaceae), two famous traditional Tibetan medicines, are perennial herbs distributed in the high mountains and alpine environment of the Qinghai–Tibet Plateau at altitudes from 2,000 to 5,000 m [14]. They have traditionally been used as medicinal plants to treat cuts, bacteriosis, neurasthenia, dephlogistication, and rheumatism, and to relieve wounds and sores [15, 16]. The dried root is the part most commonly used in medicine; the bioactive constituents of the root are mainly iridoid glycosides which have the properties of cleansing the blood and kidneys and relieving indigestion, and can be used to treat jaundice and rheumatism [17, 18]. In recent years studies of these two species, especially the root extracts, have focused on determination of their active constituents and assessment of their pharmacological effectiveness [16, 19–22]. Although studies have recently been conducted on the association of physiology and reproductive biology with trace levels of minerals in *G. straminea* and *G. dahurica* [23–28], there have been very few reports of studies of other nutritious substances, for example FFAs, in the plants. Investigation of the composition and concentration of FFAs in *G. straminea* and *G. dahurica* is therefore important. The FFA content of the plants could be used to characterize and evaluate the quality and effectiveness of a medicine, and quantitative determination of FFAs may reveal other beneficial aspects of herbal medicines, and enable these to be exploited [2]. The objective of this work was to use the new fluorescence reagent TSPP to label 30 long-chain and short-chain fatty acids to obtain more basic quantitative and qualitative information about *G. straminea* and *G. dahurica*.

Experimental

Chemicals and Materials

G. straminea and *G. dahurica* were collected in September 2004 at five different locations at an altitude of 2,300–4,500 m. At each sampling site the two plants were harvested from different positions more than 1 m apart, and at least 20 individual plants were collected randomly to ensure representative sampling. Botany department voucher specimens were authenticated by Professor Guichen Chen and deposited in Northwest Institute of Plateau Biology, Chinese Academy of Sciences (Xining, China).

HPLC-grade acetonitrile (spectroscopically pure) was purchased from Merck (Germany). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Chloroform, DMF, and $HCOONH_4$, were analytical grade.

Standards of the 30 FFAs were purchased from Shanghai Chemical Reagent (China). Individual stock solutions of the FFAs (5.0×10^{-3} mol L^{-1}) were prepared in 1:1 (*v*/*v*) acetonitrile–DMF. Mixed 1.0×10^{-4} mol L^{-1} standard solutions of the fatty acids for HPLC analysis were prepared from the stock solutions by dilution with the same solvent mixture. All solutions were stored at 4 °C under refrigeration.

TSPP was synthesized in our laboratory as described below.

Synthesis of the Labeling Reagent TSPP

TSPP was synthesized, as described below, by use of a literature method [29].

Synthesis of phenylimidazole-[4,5-*f*]-9,10-phenanthrene: phenylimidazole-[4,5-*f*]-9,10-phenanthrene was synthesized by a modification of a method described elsewhere [30]. 9,10-Phenanthraquinone (16 g), benzaldehyde (10 mL), and ammonium acetate (120 g) were well mixed in a 500-mL round-bottomed flask. Glacial acetic acid (300 mL) was added and the contents of the flask were then rapidly heated to 80–90 °C, with stirring, and maintained at this temperature for 3 h. After cooling, the pH of the solution was adjusted to 7–8 by addition of aqueous ammonia. 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 5:1 (*v*/*v*) acetonitrile–DMF to afford slightly yellow crystals, yield 92%.

Synthesis of 1-(2-hydroxyethyl)-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (EPP): EPP was conveniently synthesized by a modification of a method described elsewhere [31]. Phenylimidazole-[4,5-*f*]-9,10-phenanthrene (12 g), ethylene carbonate (4.5 g), a trace amount of KOH (0.2 g), and DMF (120 mL) were mixed in a 500-mL round-bottomed flask and rapidly heated to reflux for 6 h with vigorous stirring. After cooling, the contents were poured into 300 mL water. The precipitated solid was recovered by filtration and washed successively with water and 3:2 (*v*/*v*) ethanol–water. The crude product was dried at room temperature for 48 h and recrystallized twice from 5:1 (*v*/*v*) acetonitrile–DMF to afford a white acicular crystals, 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), 3,193.13 (–OH); 1,603.5 (Ph–C = N–); 1,559.6, 1,525.5, 1,496.8 (Ph); 1,448.7; 1,397.6, 1,362.8 (C–H); 1,063.3 (C–O), 1,031.1, 770.9, 749.3, 722.4, 731.1. *m*/*z* $[M + H]^+$, 339.0.

Synthesis of 1-[2-(p-toluenesulfonate) ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP): a mixture of 1-(2-hydroxyethyl)-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (1.5 g) and pyridine (10 mL) was added dropwise within 30 min, with vigorous stirring, at 0 °C, to a solution of *p*-toluenesulfonyl chloride (2.53 g) in pyridine (40 mL) in a 100-mL

round-bottomed flask. 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 then poured into 100 mL ice water and stirred vigorously for 0.5 h. The precipitated solid was recovered by filtration, washed with water, and dried at ambient temperature for 48 h. The crude product was recrystallized twice from acetonitrile to give white 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), 3,114.7 (Ph–N–CH_2–); 1,625.3, 1,609.4 (Ph–N=N–), 1,545.3, 1,509.6 (Ph), 1,444.9, 1,399.2, 1,378.7 (C–H), 1,355.7 (–C–SO_2–); 1,190.2, 1,176.9 (Ph–S–), 1,094.4, 1,014.2, 908.8, 781.3, 754.3, 705.2. m/z $[M + H]^+$, 493.0.

TSPP solution (1.0×10^{-3} mol L^{-1}) was prepared by dissolving 4.92 mg TSPP in 10 mL anhydrous acetonitrile prepared by distilling HPLC-grade acetonitrile from P_2O_5.

Sampling and Extraction of FFAs

After transport to the laboratory the roots were cut from the plants, mixed, rinsed gently, to protect the fragile surface, with tap water then deionized water, and dried at 50 °C until constant weight. All samples were then ground in a stainless-steel mill. Dried and pulverized *G. straminea* and *G. dahurica* plant root was then extracted with chloroform by use of two different techniques.

Ultrasound-Assisted Extraction

A sample (0.2 g) of the pulverized plant material and chloroform (5.0 mL) were placed in a 10-mL round-bottomed flask. The flask was immersed in an ultrasonic water-bath (Kunshan Instrumental, Kunshan, Zhejiang Province, China) and sonicated for 20 min. Two samples were each extracted three times and the extracts were combined.

Extraction by Shaking

Plant samples (0.2 g) were extracted three times, each time for 20 min, with chloroform (5.0 mL), by use of a mechanical shaker, and the extracts were combined.

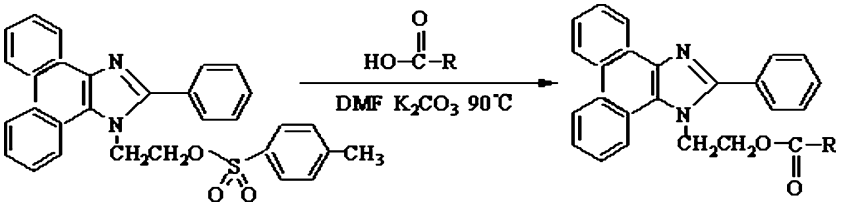

Fig. 1. Schematic diagram of the derivatization of FFAs with TSPP

Derivatization of Standards and Samples

Standards

DMF (200 µL), 50 µL of the mixed solution of the free fatty acids (1.0×10^{-4} mol L^{-1}), 150 µL derivatization reagent (TSPP) solution, and 10 mg anhydrous K_2CO_3 catalyst were placed in a vial. The vial was sealed and placed in a water-bath at 90 °C for 30 min with shaking every 5 min. When reaction was complete the mixture was left to cool to room temperature. Aqueous acetonitrile solution [1:1 (v/v), 100 µL] was added to dilute the derivatization solution and the diluted solution (10 µL) was injected directly into the chromatograph. The derivatization procedure is shown schematically in Fig. 1.

Samples

Pyridine (1.5 mL) was added to the combined extracts and the mixture was ultrasonicated for 20 s to transform FFAs into their organic salts. The solvent was then evaporated under a stream of nitrogen gas. The residue was dissolved in 500 µL HPLC-grade DMF and derivatization of the extract solution was performed as described above.

Chromatography and Mass Spectrometry

Chromatography was performed with an Agilent 1100 series chromatograph equipped with a quaternary pump (model G1311A), a vacuum degasser (model G1322A), a fluorescence detector (FLD model G1321A), an autosampler (model G1329A), a thermostatically controlled column compartment (model G1316A), and a diode-array detector (DAD model G1315A). The injection volume was 10 µL. Reversed-phase chromatography

Table 1. Program used for gradient elution from the Eclipse XDB-C_8 column

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

Mobile phase components: A, 1:1 (v/v) CH_3CN–water; B, 1:1 (v/v) CH_3CN–water containing 0.2 mol L^{-1} $HCOONH_4$ buffer; C, 100:2 (v/v) CH_3CN–DMF, D, 100:30 (v/v) CH_3CN–DMF

of the 30 FFA derivatives was performed at 30 °C on a 4.6 mm × 150 mm, 5 µm particle, Eclipse XDB-C_8 column (Agilent) with a quaternary gradient prepared from 1:1 (v/v) CH_3CN–water (component A), 1:1 (v/v) CH_3CN–water containing 0.2 mol L^{-1} $HCOONH_4$ buffer (component B), 100:2 (v/v) CH_3CN–DMF (component C), and 100:30 (v/v) CH_3CN–DMF (component D). The gradient elution program is presented in Table 1; the flow-rate was 1.0 mL min^{-1}. The fluorescence excitation (λ_{ex}) and emission wavelengths (λ_{em}) were 260 and 380 nm, respectively. The HPLC system was controlled by use of HP Chemstation software.

Mass spectrometry was performed with an 1100 series LC–MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) equipped with atmospheric-pressure chemical ionization (APCI). The APCI source was operated with a nebulizer pressure of 60 psi, a dry-gas temperature of 350 °C, and a dry gas flow of 5.0 L min^{-1}. The APCI Vap temperature was 450 °C, the corona current 4,000 nA (positive), and the capillary potential 3,500 V. The mass spectrometer was controlled by use of Esquire-LC NT software, version 4.1.

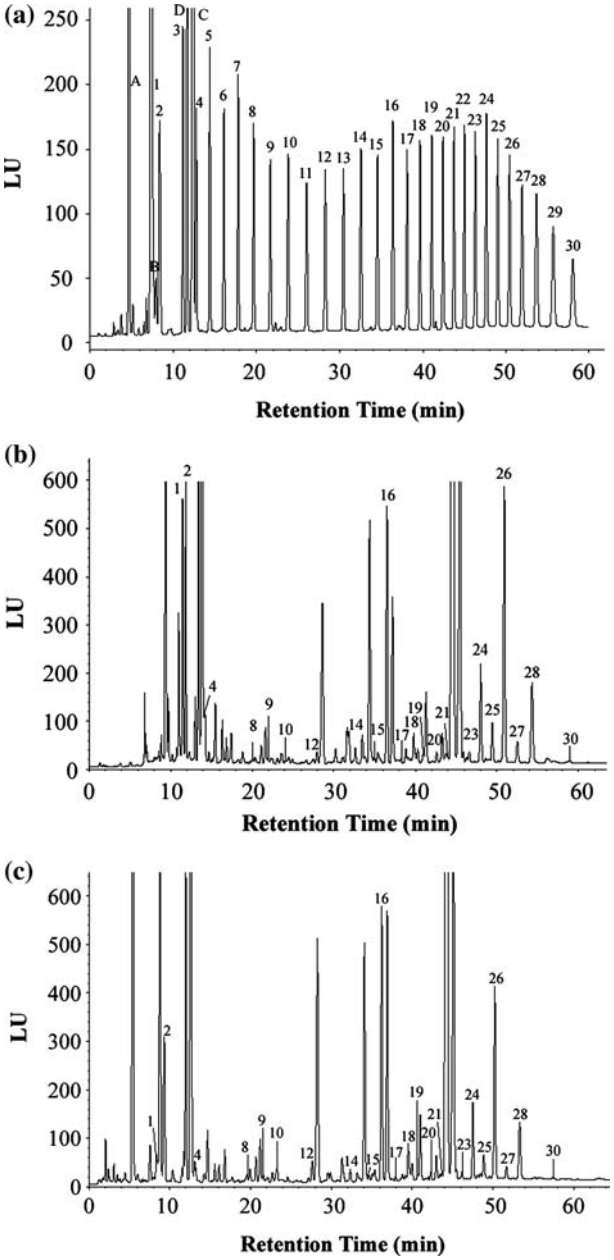

Fig. 2. **a** Chromatogram obtained from long-chain and short-chain fatty acid derivatives (amount injected 50 pmol). **b** Chromatogram obtained from FFAs in *Gentiana straminea* root. **c** Chromatogram obtained from FFAs in *G. dahurica* root. Eclipse XDB-C_8 column (4.6 mm × 150 mm, 5 µm particles); flow rate 1.0 mL min^{-1}; column temperature 30 °C; λ_{ex} 260 nm, λ_{em} 380 nm. Peaks: **1** = formic acid; **2** = acetic acid; **3** = propionic acid; **4** = butyric acid; **5** = valeric acid; **6** = hexanoic acid; **7** = heptanoic acid; **8** = octanoic acid; **9** = pelargonic acid; **10** = decanoic 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** = eicosanoic acid; **21** = heneicosanoic acid; **22** = docosanoic acid; **23** = tricosanoic acid; **24** = tetracosanoic acid; **25** = pentacosanoic acid; **26** = hexacosanoic acid; **27** = heptacosanoic acid; **28** = octacosanoic acid; **29** = nonacosanoic acid; **30** = dotriacontanoic acid; **A** = 1-(2-hydroxyethyl)-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (*EPP*); **B** = 2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene; **C** = reagent peak; **D** = impurity peak

Quantitative Analysis

Quantitative conversion of the FFAs in extracts of *G. straminea* and *G. dahurica* to their TSPP derivatives was guaranteed by use of excess TSPP. All FFAs in *G. straminea* and *G. dahurica* plant roots were quantified by use of the external standard method with fluorescence detection as described above. Calibration plots for each TSPP FFA derivative were obtained by linear regression, by plotting peak area against concentration.

Results and Discussion

Stability and Spectral Characteristics of TSPP

TSPP was synthesized by condensation of 1-(2-hydroxyethyl)-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene and *p*-toluenesulfonyl chloride in pyridine and the product was obtained by washing with water and recrystallization from acetonitrile. The wavelength of maximum UV absorption (λ_{max}) of TSPP in acetonitrile was 259 nm and the molar absorbance, e, was 6.0×10^4 L mol^{-1} cm^{-1}. The fluorescence excitation (λ_{ex}) and emission (λ_{em}) wavelengths (in acetonitrile) were 260 and 380 nm, respectively.

Optimization of the Extraction

Two methods for extraction of FFAs from *G. straminea* and *G. dahurica* were evaluated by comparison of the detector responses obtained from analysis of the derivatized FFAs. The results indicated the fatty acids were extracted most efficiently from *G. straminea* and *G. dahurica* samples by use of ultrasonication. Lower efficiency was usually observed if extraction was performed by shaking. In tests with different solvents the efficiency of extraction was highest when chloroform was used, possibly because the FFAs are more soluble in chloroform than in methanol or acetonitrile. In general, the solubility of the FFAs in the solvents tested seemed to decrease in the order chloroform > methanol > acetonitrile. All subsequent experiments in this study were therefore performed using ultrasonic extraction with chloroform as solvent.

LC Separation and Mass Spectrometric (MS) Identification

An Eclipse XDB-C_8 column was used with gradient elution. Several gradient programs were investigated to ensure satisfactory HPLC separation in the shortest time. Gradient elution was per-

formed as indicated in Table 1. Complete baseline resolution of the 30 fatty acid derivatives was achieved in 60 min with short retention times and sharp peaks. DMF was used in mobile phase components C and D to promote elution during HPLC separation, because DMF increases the solubility of fatty acid derivatives, enabling rapid separation with sharp peaks; use of DMF also resulted in a low, very stable baseline. To achieve the optimum separation the amount of CH_3CN added to mobile phase component B was tested and use of 50% (*v*/*v*) CH_3CN was found to enable separation of the derivatized long-chain fatty acids. A chromatogram showing complete baseline resolution of all the fatty acid derivatives is given in Fig. 2a.

Ionization and identification of the isolated TSPP derivatives of the fatty acids by mass spectrometry with APCI detection in positive-ion detection mode were studied. As expected, the TSPP–fatty acid derivatives produced an intense molecular-ion peak at *m*/*z* $[M + H]^+$. In tandem mass spectrometry (MS–MS) analysis of the fatty acid derivatives, collision-induced dissociation spectra of *m*/*z* $[M + H]^+$ produced specific fragment ions at *m*/*z* $[M' + CH_2CH_2]^+$ and *m*/*z* 295.1 (where M′ in the characteristic fragment *m*/*z* $[M' + CH_2CH_2]^+$ is the molecular mass of the fatty acid). The specific fragment ion *m*/*z* 295.1 was from the molecular core of the TSPP, and arose from cleavage of the N–CH_2CH_2OCO bond. Selected reaction monitoring based on *m*/*z* $[M + H]^+ \rightarrow$ *m*/*z* $[M' + CH_2CH_2]^+$ and the *m*/*z* 295.1 transition was specific for fatty acid derivatives. No detectable signal for this transition was obtained from a blank water sample. Although other endogenous acidic compounds present in natural environmental samples were presumably co-extracted and derivatized by the TSPP, no interference was observed, because of 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 transitions. HPLC with gradient elution enabled efficient separation and analysis of the derivatized fatty acids and reduced disturbance from other, unknown, components of the sample to a minimum. With APCI in positive-ion mode the intense ion-current signals for the fatty acid derivatives could be attributed to introduction of the two weakly basic nitrogen atoms in the corresponding TSPP molecular core structure, resulting in highly efficient production of the *m*/*z* 295.1 ion. The mode of cleavage and results from MS–MS analysis for a representative derivative, that of the C_{24} acid, are shown in Fig. 3a–c. All molecular ions $[M + H]^+$ and the corresponding specific fragment ions for the 30 fatty acid derivatives are shown in Table 2. Although MS is not the most sensitive detector for HPLC, it can provide suf-

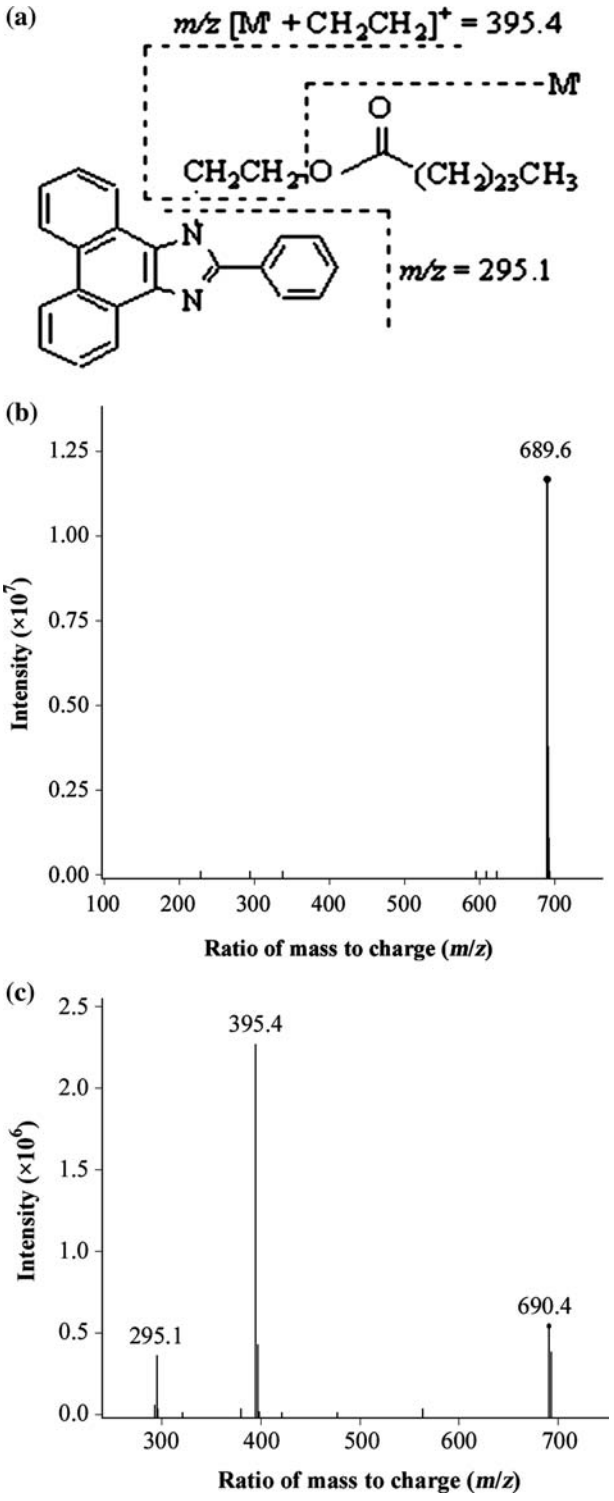

Fig. 3. **a** The mode of cleavage. **b** Typical full-scan mass spectrum of the TSPP derivative of tetracosanoic acid in the range 100–1,000 amu with APCI in positive-ion mode. **c** Typical MS–MS spectrum of the TSPP derivative of tetracosanoic acid in the range 100–850 amu with APCI in positive-ion mode; the fragment ions were *m*/*z* 395.4 and *m*/*z* 295.1

Table 2. MS data for the TSPP derivatives of the fatty acids

FFA	Molecular ion $[M + H]^+$	Characteristic fragment ion, m/z $[M' + CH_2CH_2]^+$	Specific fragment ion, molecular core structure
C_1	367.1	–	295.1
C_2	381.2	–	295.1
C_3	395.1	–	295.1
C_4	409.2	–	295.1
C_5	423.2	–	295.1
C_6	437.2	–	295.1
C_7	451.2	–	295.1
C_8	465.2	–	295.1
C_9	479.2	–	295.1
C_{10}	493.3	–	295.1
C_{11}	507.3	–	295.1
C_{12}	521.3	227.1	295.1
C_{13}	535.4	241.2	295.1
C_{14}	549.4	255.2	295.1
C_{15}	563.4	269.2	295.1
C_{16}	577.4	283.3	295.1
C_{17}	591.4	297.2	295.1
C_{18}	605.4	311.2	295.1
C_{19}	619.5	325.3	295.1
C_{20}	633.5	339.3	295.1
C_{21}	647.6	353.3	295.1
C_{22}	661.6	367.4	295.1
C_{23}	675.6	381.3	295.1
C_{24}	689.6	395.4	295.1
C_{25}	703.6	409.4	295.1
C_{26}	717.6	423.4	295.1
C_{27}	731.5	437.5	295.1
C_{28}	745.6	451.4	295.1
C_{29}	759.5	465.4	295.1
C_{30}	773.6	480.0	295.1

Table 3. Linear regression equations, correlation coefficients, detection limits, and repeatability of peak areas and retention times ($n = 6$)

FFA	$Y = AX + B$	Correlation coefficient	Detection limit (fmol)	Retention time RSD (%)	Peak area RSD (%)
C_1	$Y = 743.5X + 211.6$	0.9993	3.1	0.33	0.20
C_2	$Y = 173.4X + 49.36$	0.9996	23	0.39	0.70
C_3	$Y = 150.9X + 40.65$	0.9995	11	0.43	0.63
C_4	$Y = 93.2X + 30.25$	0.9995	15	0.19	0.39
C_5	$Y = 139.3X + 33.25$	0.9991	14	0.17	0.46
C_6	$Y = 114.3X + 30.55$	0.9998	13	0.18	0.78
C_7	$Y = 133.3X + 31.22$	0.9998	16	0.30	0.61
C_8	$Y = 119.7X + 35.36$	0.9997	18	0.046	0.92
C_9	$Y = 101.3X + 26.12$	0.9999	28	0.088	0.22
C_{10}	$Y = 113.5X + 31.56$	0.9996	23	0.067	0.43
C_{11}	$Y = 97.5X + 24.52$	0.9997	31	0.081	0.37
C_{12}	$Y = 105.3X + 28.25$	0.9996	28	0.063	0.38
C_{13}	$Y = 106.2X + 27.34$	0.9997	29	0.045	0.21
C_{14}	$Y = 113.6X + 30.64$	0.9995	27	0.063	0.29
C_{15}	$Y = 108.1X + 31.01$	0.9995	26	0.055	0.47
C_{16}	$Y = 128.1X + 35.35$	0.9992	23	0.047	0.35
C_{17}	$Y = 103.7X + 30.11$	0.9995	24	0.031	0.23
C_{18}	$Y = 106.5X + 30.59$	0.9995	22	0.024	0.22
C_{19}	$Y = 101.6X + 32.23$	0.9992	25	0.028	0.18
C_{20}	$Y = 102.2X + 33.71$	0.9993	22	0.051	0.53
C_{21}	$Y = 105.3X + 34.56$	0.9992	26	0.083	1.03
C_{22}	$Y = 109.7X + 39.57$	0.9993	23	0.11	1.11
C_{23}	$Y = 114.3X + 31.75$	0.9998	27	0.23	1.62
C_{24}	$Y = 131.7X + 32.29$	0.9995	22	0.22	1.43
C_{25}	$Y = 121.7X + 28.22$	0.9997	20	0.30	1.53
C_{26}	$Y = 118.4X + 22.12$	0.9999	24	0.26	2.40
C_{27}	$Y = 121.4X - 11.34$	0.9993	25	0.20	1.37
C_{28}	$Y = 119.3X + 35.78$	0.9995	38	0.33	2.40
C_{29}	$Y = 102.8X + 33.76$	0.9997	33	0.28	2.38
C_{30}	$Y = 82.1X + 31.52$	0.9996	34	0.21	2.76

X is the amount injected (pmol) and Y is the peak area

ficient data to enable structure determination and quantification of the amounts of the fatty acids in biological samples. Use of MS coupled with liquid chromatography has been widely used for analysis of complex mixtures of compounds commonly found in biological matrices. The MS data discussed above ensured correct identification of the analytes both in standards and samples.

Comparison of TSPP and 9-(2-Hydroxyethyl) carbazole

Experiments have been performed in our laboratory to compare the sensitivity of detection after use of TSPP and 9-(2-hydroxyethyl)carbazole (HEC) for derivatization [4]. C_{20}–C_{30} long-chain fatty acids were derivatized under the respective optimum conditions and the same volume of the derivatization solution was injected, using the respective detection wavelength. It has been documented that use of TSPP results in greater sensitivity than use of HEC; TSPP can therefore be used as an effective fluorescence reagent for derivatization of FFAs [32].

Reproducibility, Precision, Calibration, and Detection Limits

A standard solution containing the C_1–C_{30} FFAs at 3.0×10^{-6} mol L^{-1} was used to examine the repeatability of the method (the amount of each fatty acid injected was 50 pmol). RSDs of peak areas and retention times were less than 0.43 and 2.76%, respectively. Six replicate injections ($n = 6$) of 0.1, 1.0, and 5.0 μmol L^{-1} of the C_1–C_{30} fatty acids were performed to determine the precision and accuracy of the method for low, medium, and high concentrations. The mean inter-day accuracy ranged from 88.6 to 106.8% and the largest mean CV was $< 7.2\%$. The mean inter-day precision for all the standards was $< 6.2\%$ of the expected concentration. Recoveries were determined from values obtained after analysis of *G. straminea* and *G. dahurica* plants; amounts were calculated by use of calibration plots constructed after derivatization of fatty acid standards. Known amounts of the 30

Table 4. Amounts (μg g^{-1}) of the FFAs in the roots of *Gentiana straminea* and *G. dahurica* (mean ± SEM, $n = 3$)

FFA	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean
Gentiana straminea roots						
C_1	0.587 ± 0.033	1.080 ± 0.073	0.602 ± 0.048	0.348 ± 0.056	0.222 ± 0.043	0.568
C_2	11.280 ± 0.042	5.471 ± 0.314	5.956 ± 0.612	4.614 ± 0.677	2.913 ± 0.521	6.047
C_3	0	0	0	0	0	0
C_4	0.200 ± 0.011	0.683 ± 0.071	0.082 ± 0.011	0.112 ± 0.013	0.175 ± 0.023	0.250
C_5	0	0	0	0	0	0
C_6	0	0	0	0	0	0
C_7	0	0	0	0	0	0
C_8	0.088 ± 0.009	0.019 ± 0.007	0.014 ± 0.006	0.006 ± 0.001	0.175 ± 0.017	0.060
C_9	0.102 ± 0.012	0.189 ± 0.036	0.705 ± 0.107	0.663 ± 0.099	0.281 ± 0.046	0.388
C_{10}	0.005 ± 0.001	0.018 ± 0.006	1.415 ± 0.528	3.548 ± 0.876	0.596 ± 0.095	1.116
C_{11}	0	0	0	0	0	0
C_{12}	0.077 ± 0.009	0.509 ± 0.079	0.692 ± 0.130	0.648 ± 0.143	1.188 ± 0.565	0.623
C_{13}	0	0	0	0	0	0
C_{14}	0.740 ± 0.004	1.454 ± 0.063	2.661 ± 0.519	2.621 ± 0.643	1.970 ± 0.622	1.889
C_{15}	0.007 ± 0.003	0.053 ± 0.011	0.389 ± 0.093	1.287 ± 0.272	0.154 ± 0.038	0.378
C_{16}	13.883 ± 0.133	22.748 ± 0.837	53.011 ± 6.771	70.605 ± 4.337	40.476 ± 6.334	40.145
C_{17}	0.052 ± 0.008	0.014 ± 0.004	1.643 ± 0.698	0.897 ± 0.114	0.471 ± 0.081	0.615
C_{18}	1.774 ± 0.043	3.896 ± 0.089	5.064 ± 1.306	4.932 ± 0.712	9.264 ± 0.992	4.986
C_{19}	0.030 ± 0.007	0.629 ± 0.103	0.363 ± 0.111	0.009 ± 0.001	1.646 ± 0.553	0.535
C_{20}	0.478 ± 0.047	3.253 ± 0.318	2.190 ± 0.736	2.574 ± 0.374	5.423 ± 0.986	2.784
C_{21}	0.010 ± 0.002	0.197 ± 0.039	0.353 ± 0.088	0.184 ± 0.076	1.015 ± 0.331	0.352
C_{22}	0	0	0	0	0	0
C_{23}	0.159 ± 0.029	2.611 ± 0.082	2.776 ± 0.911	2.295 ± 0.412	4.648 ± 0.876	2.498
C_{24}	4.672 ± 0.118	30.692 ± 2.319	49.331 ± 2.374	43.090 ± 1.331	35.727 ± 2.839	32.702
C_{25}	1.582 ± 0.085	12.851 ± 1.107	21.034 ± 2.556	13.883 ± 3.111	13.368 ± 2.476	12.544
C_{26}	16.753 ± 0.055	98.752 ± 3.496	149.933 ± 2.476	110.201 ± 2.438	88.520 ± 3.337	92.832
C_{27}	1.551 ± 0.032	8.106 ± 1.039	9.504 ± 2.334	6.178 ± 1.998	6.195 ± 0.728	6.307
C_{28}	6.974 ± 0.111	33.960 ± 2.314	33.656 ± 3.876	21.618 ± 2.316	27.598 ± 1.106	24.761
C_{29}	0	0	0	0	0	0
C_{30}	0.087 ± 0.019	0	1.042 ± 0.088	0.984 ± 0.114	1.405 ± 0.371	0.704
Gentiana dahurica roots						
C_1	0.260 ± 0.032	0.373 ± 0.045	0.108 ± 0.031	0.033 ± 0.012	0.157 ± 0.043	0.186
C_2	4.539 ± 0.128	5.190 ± 0.230	4.294 ±0.537	4.442 ± 0.356	3.528 ± 0.312	4.399
C_3	0	0	0	0	0	0
C_4	0.249 ± 0.043	0.354 ± 0.068	0.131 ± 0.037	0.125 ± 0.079	0.043 ± 0.011	0.180
C_5	0	0	0	0	0	0
C_6	0	0	0	0	0	0
C_7	0	0	0	0	0	0
C_8	0.435 ± 0.086	1.029 ± 0.018	0.678 ± 0.017	0.287 ± 0.059	1.470 ± 0.228	0.780
C_9	0.420 ± 0.079	0.764 ± 0.092	1.162 ± 0.234	0.371 ± 0.072	0.549 ± 0.076	0.653
C_{10}	0.614 ± 0.089	1.034 ± 0.068	0.776 ± 0.081	2.136 ± 0.151	0.701 ± 0.092	1.052
C_{11}	0	0	0	0	0	0
C_{12}	1.542 ± 0.231	3.305 ± 0.030	2.898 ± 0.402	2.717 ± 0.442	2.311 ± 0.201	2.555
C_{13}	0	0	0	0	0	0
C_{14}	1.401 ± 0.331	1.237 ± 0.219	1.377 ± 0.246	1.825 ± 0.556	1.408 ± 0.512	1.450
C_{15}	0.434 ± 0.111	0.213 ± 0.038	0.748 ± 0.157	0.788 ± 0.201	0.836 ± 0.269	0.604
C_{16}	29.017 ± 1.221	35.744 ± 0.062	56.626 ± 0.337	45.073 ± 3.728	48.772 ± 1.619	43.046
C_{17}	0.201 ± 0.029	0.356 ± 0.050	0.478 ± 0.078	0.575 ± 0.069	0.449 ± 0.058	0.412
C_{18}	5.165 ± 0.032	8.730 ± 0.114	3.505 ± 0.045	6.266 ± 0.039	14.852 ± 0.369	7.704
C_{19}	0.127 ± 0.023	0.182 ± 0.037	1.179 ± 0.061	0.033 ± 0.010	1.957 ± 0.079	0.696
C_{20}	2.551 ± 0.146	2.727 ± 0.311	4.245 ± 0.851	1.381 ± 0.066	8.143 ± 0.037	3.809
C_{21}	0.323 ± 0.077	0.192 ± 0.043	1.396 ± 0.028	0.170 ± 0.036	2.025 ± 0.110	0.821
C_{22}	0	0	0	0	0	0
C_{23}	2.969 ± 0.078	3.242 ± 0.765	0.006 ± 0.001	1.959 ± 0.411	4.242 ± 0.879	2.483
C_{24}	11.745 ± 2.374	16.041 ± 0.204	28.894 ± 0.034	18.019 ± 0.032	18.818 ± 0.011	18.703
C_{25}	4.182 ± 0.984	7.256 ± 0.037	13.484 ± 0.220	6.027 ± 0.018	7.137 ± 0.042	7.617
C_{26}	32.767 ± 0.059	56.649 ± 0.028	119.992 ± 0.234	59.378 ± 0.076	47.955 ± 0.040	63.348
C_{27}	2.570 ± 0.073	4.556 ± 0.123	10.625 ± 0.059	4.276 ± 0.013	2.733 ± 0.010	4.952
C_{28}	16.790 ± 0.033	24.747 ± 0.078	72.994 ± 0.032	23.821 ± 0.033	11.729 ± 0.062	30.016
C_{29}	0	0	0	0	0	0
C_{30}	2.048 ± 0.063	2.384 ± 0.061	7.575 ± 0.048	1.444 ± 0.028	0.848 ± 0.055	2.860

fatty acids were added to two identical *G. straminea* and *G. dahurica* plant samples. The samples were extracted in accordance with the method described in the experimental section, the FFAs were derivatized with TSPP, and chromatographic analysis was performed in duplicate. Recoveries were in the range 90.3–103.5% ($n = 3$). The linearity of the procedures was evaluated in the range 8.0×10^{-3} to 20 μmol L^{-1} after use of the optimum derivatization conditions; the injection volume was 10 μL and the amount injected was from 200.0 pmol to 24.4 fmol, i.e. a 8,197-fold range of concentrations. Calibration plots of peak area (y) against fatty acid concentration (x, pmol, amount injected) were established. The linear regression equations obtained are listed in Table 3. Excellent linear responses were obtained for all the

fatty acids in this range of concentrations; correlation coefficients were >0.9991. At higher concentrations nonlinearity was observed. Detection limits (signal-to-noise ratio 3:1) calculated after injection of 1.0 pmol of each derivatized fatty acid were from 3.1 to 38 fmol.

Sample Analysis

Typical chromatograms obtained from analysis, with fluorescence detection, of FFAs extracted from *G. straminea* and *G. dahurica* are given in Fig. 2b and c, respectively. Simultaneous determination of 30 FFAs in these two plant medicines was easily achieved by using TSPP as the derivatization reagent. The FFAs were identified by use of on-line post-column mass spectrometric analysis. The method was suitable for determination of these components in the roots of two medicinal plants and results were satisfactory. The amounts of the 30 FFAs in these two plants are listed in Table 4. Amounts of long-chain FFAs with an even number of carbon atoms are clearly much higher than amounts of the adjacent FAAs with odd numbers of carbon atoms; amounts of C_{16}, C_{24}, C_{26}, and C_{28} are particularly high. Amounts of FFAs in *G. straminea* roots were much higher than in *G. dahurica* roots, and amounts of the fatty acids in plants of the same species from the five different sampling locations were significantly different. The geographical region in which plants are collected has been found to be an important factor in determining the amounts of chemical compounds in herbal medicines [33, 34]. It is probable that environmental conditions, for example soil type and meteorological factors, affect the amounts of the fatty acids.

Conclusions

Simultaneous quantification by fluorescence detection and MS identification of 30 FFAs in two medicinal plants, *G. straminea* and *G. dahurica*, has been accomplished successfully. Derivatization of FFAs with TSPP is simple and inexpensive and results in sensitive detection. Under the proposed conditions complete derivatization of long chain ($>C_{20}$) fatty acids can be achieved, and under the HPLC conditions used the derivatives of the C_1–C_{30} FFAs were separated in 60 min by use of gradient elution. This method can be used for analysis of carboxylic acids in medicinal plants. Further studies on simultaneous identification and quantification of unsaturated fatty acids by use of this method are currently in progress in our laboratory.

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