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HPLC-APCI-MS Determination of Free Fatty Acids in Tibet Folk Medicine *Lomatogonium rotatum* with Fluorescence Detection and Mass Spectrometric Identification

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Abstract: A simple and sensitive method for the determination of free fatty acids (FFAs) using acridone-9-ethyl-p-toluenesulfonate (AETS) as a fluorescence derivatization reagent by high performance liquid chromatography (HPLC) has been developed. Free fatty acid derivatives were separated on an Eclipse XDB-C_8 column with a good baseline resolution and detected with the fluorescence of which excitation and emission wavelengths of derivatives were set at $\lambda_{ex} = 404$ and $\lambda_{em} = 440$ nm, respectively. Identification of 19 fatty acid derivatives was carried out by online post-column mass spectrometry with an atmospheric pressure chemical ionization (APCI) source under positive-ion detection mode. Nineteen FFAs from the extract of *Lomatogonium rotatum* are sensitively determined. The results indicate that the plant *Lomatogonium rotatum* is enriched with an abundance of FFAs and FFAs of higher contents, which mainly focus on even carbon atoms, C_{14}, C_{16}, and C_{18}. The validation of the method including linearity, repeatability, and detection limits was examined. Most linear

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correlation coefficients for fatty acid derivatives are >0.9989, and detection limits (at signal-to-noise of 3:1) are 12.3–43.7 fmol. The relative standard deviations (RSDs) of the peak areas and retention times for 19 FFAs standards are <2.24% and 0.45%, respectively. The established method is rapid and reproducible for the separation determination of FFAs from the extract of *Lomatogonium rotatum* with satisfactory results.

Keywords: HPLC-APCI-MS, FFAs, AETS, Fluorescence detection, Derivatization, *Lomatogonium rotatum*

INTRODUCTION

Lomatogonium rotatum, belonging to the family of *Lomatogonium* in Gentianaceae, is a Tibetan herbal medicine growing on the Qinghai-Tibet Plateau. There are about 18 species of this genus recorded in the world and about 17 species are found in China. The aerial parts of *Lomatogonium rotatum* are used in Tibetan medicine to treat liver, gall bladder, and spleen diseases. The major and pharmaceutically active constituents in *Lomatogonium rotatum* include xanthones, flavonoids, and iridoids.[1–3] Pharmacological studies indicate that xanthones have various biological effects such as anti-inflammatory, anti-virus, hepatoprotective activity, and exciting the central nervous system.[4] In the last few years, routine analysis of this species had focused on the determination of these pharmaceutically active constituents.[5,6] However, there are few publications on some other nutritious substances in *Lomatogonium rotatum*, such as FFAs. Similarly, FFAs play physiologically important roles at trace levels in the regulation of a variety of physiological and biological functions.[7] The quantitative determination of FFAs may reveal and exploit other beneficial aspects of herbal medicines. Most fatty acids show neither natural absorption in the visible or UV regions, nor do they fluoresce naturally. However, easily detectable fatty acid derivatives as methyl esterification with gas chromatography (GC) have been reported,[8] even supercritical fluid chromatography (SFC).[9] In contrast with GC, use of HPLC allows the fatty acids to be converted to a large number of different derivatives.[10–13] 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 HPLC.[12] Recently, derivatization of FFAs with labeling reagents acridone-9-ethyl-p-toluenesulfonate (AETS) has been adopted to determinate FFAs from soil and bryophyte samples.[14]

The aim of the present work is to utilize the fluorescence derivatization reagent, AETS, to label long- and short-chain fatty acids from the extract of *Lomatogonium rotatum*, and to determine them quantitatively and qualitatively by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS).

EXPERIMENTAL

Instrumentation

Experiments were performed using the HP 1100 series equipped with a quaternary pump (model G1311A), a vacuum degasser (model G1322A), a fluorescence detector (FLD) (model G1321A), an autosampler (model G1329A), and a thermostated column compartment (model G1316A). 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 conditions: nebulizer pressure 0.42 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 an Eclipse XDB-C_8 column (4.6 mm × 150 mm, 5 μm, Agilent Co.). The HPLC system was controlled by HP Chemstation software. The ultrasonic cleaner was purchased from Kunshan Instrumental Co (Kunshan, Zhejiang Province, China).

Chemicals

Standards of 19 FFAS were purchased from Shanghai Chemical Reagent Co. HPLC grade acetonitrile (spectroscopically pure acetonitrile) was purchased from Merck Co (Germany). Water was purified on a Milli-Q system (Millipore, Bedford, MA). All other reagents were of analytical grade, including chloroform, DMF, pyridine. Fluorescence derivatization reagent acridone-9-ethyl-p-toluenesulfonate (AETS) was synthesized by Xian'en Zhao.[14]

Preparation of Standard and Sample Solutions

The standard fatty acids for HPLC analysis at individual concentrations of 1.0×10^{-4} mol/L were prepared by dilution of the corresponding stock solutions (1.0×10^{-2} mol/L) with the acetonitrile/DMF (1:1, *v*/*v*). The AETS solution (0.05 mol/L) was prepared by dissolving 0.1965 mg AETS in 10 mL DMF. Individual stock solutions of the fatty acids (1.0×10^{-4} mol/L) were prepared in acetonitrile/DMF (1:1, *v*/*v*). When not in use, all reagent solutions were stored at 4°C in a refrigerator until HPLC analysis.

The *Lomatogonium rotatum* plant sample was collected from Qinghai-Tibet Plateau in September, 2004. After transporting it to the laboratory, the sample was washed using deionized water and dried at 50°C until constant weight was obtained, then ground through a stainless steel mill for analysis.

The pulverized plant sample (0.18 g) and 5.0 mL chloroform was added to a 10 mL round bottom flask. The flask was immersed in a sonicator water bath and the sample was sonicated for 20 min. The extraction was repeated two times and the extracts were combined. To each of the combined contents, 1.5 mL pyridine was added, respectively. The mixture was then ultrasonicated for 20 seconds in order to transferr FFAs into their corresponding organic salts. Finally, the solvent was evaporated under a stream of nitrogen gas. The residue was redissolved in 500 μL DMF until HPLC analysis.

Derivatization of Standard and Sample

DMF (180 μL), 50 μL mixed fatty acids (1.0×10^{-4} mol/L), 120 μL devivatization reagent solution (5.0×10^{-3} mol/L), and 10 mg anhydrous K_2CO_3 catalyst were consecutively added into a vial. The vial was sealed and allowed to react in water bath at 85°C with shaking for 45 min. After the reaction was completed, the mixture was cooled at room temperature. A volume (1.05 mL) of the acetonitrile solution (CH_3CN/H_2O 1:1, v/v) was added to dilute the derivatization solution. The diluted solution (10 mL) was injected directly onto the chromatograph. 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 19 FFAs derivatives was carried out on a reversed phase Eclipse XDB-C_8 column kept at 30°C with a quaternary gradient elution. The mobile phases were (A) CH_3CN/H_2O 20:80 (V/V), (B) CH_3CN, and were pumped at 1.0 mL/min flow rate. The injection volume was 10 μL. The fluorescence excitation and emission wavelengths were set at λex 404 and λem 440 nm, respectively. The gradient elution program was presented in Table 1.

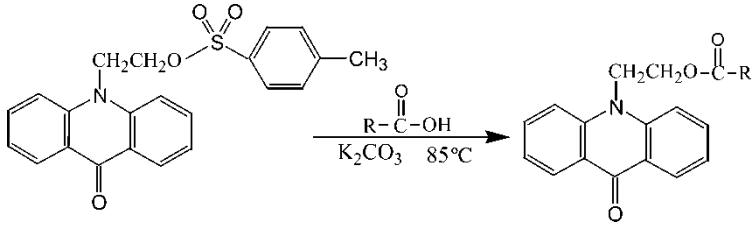

Figure 1. Derivatization scheme of acridone-9-ethyl-p-toluenesulfonate (AETS) with fat acids.

Table 1. Chromatographic gradient program eluted on Eclipse XDB-C_8 column

Time (min)	A (%)	B (%)
0	85	15
20	75	25
50	0	100
65	0	100

(A) CH_3CN/H_2O 20:80 (V/V); (B) CH_3CN.

RESULTS AND DISCUSSION

Optimal Extraction

Two methods for the extraction of FFAs in a *Lomatogonium rotatum* plant sample were evaluated by comparing the detector responses obtained by the analysis of the derivatized fatty acids. The results indicated that the highest extraction efficiency of FFAs in a *Lomatogonium rotatum* plant sample was achieved by ultrasonication extraction. In most cases, a 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.

LC Separation and MS Identification

An Eclipse XDB-C_8 column was selected in conjunction with gradient elution; several programs were investigated to ensure satisfactory HPLC separation within the shortest time. The gradient elution was carried out as described in Table 1. A complete baseline resolution for 19 FFAs derivatives was obtained within 55 min with the shortest retention values and the sharpest peaks. The chromatogram of a complete baseline resolution for 19 FFAs derivatives is shown in Figure 2.

The ionization and fragmentation of the isolated AETS fatty acid derivatives were studied by mass spectrometry with APCI detection in positive ion detection mode. As expected, the AETS 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 195.8. The M' in characteristic fragment m/z $[M' + CH_2CH_2]^+$ corresponded

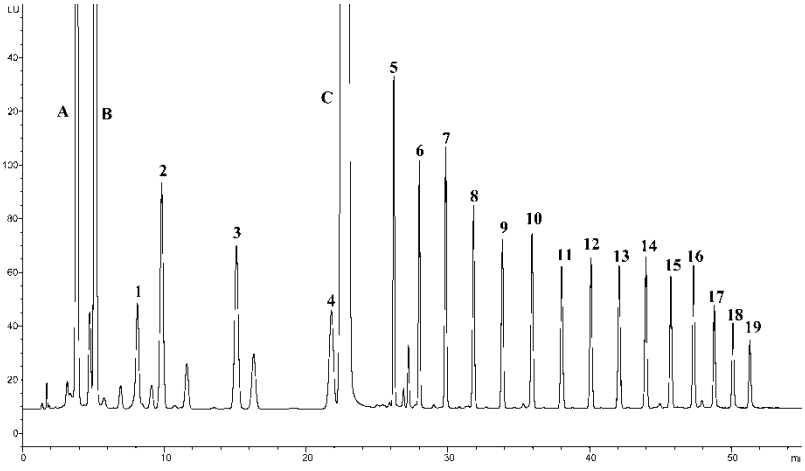

Figure 2. Chromatogram of standard fatty acid derivatives (corresponding injected amount 35.7 pmol). Chromatographic conditions: Column temperature at 30°C; excitation wavelength λ_{ex} 404 nm, emission wavelength λ_{em} 440 nm; Eclipse XDB-C_8 column (4.6 × 150 mm, 5 mm); flow rate = 1.0 mL min^{-1}·1, 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. A, acridone -9-ethanol; B, acridone; C, reagent peak.

to the molecular mass of fatty acids; the specific fragment ion m/z 195.8 was from the molecular core structure. The selected reaction monitoring, based on the m/z $[MH]^{+®}$ m/z $[M' + CH_2CH_2]^+$ and m/z 195.8 transition, was specific for fatty acid derivatives. Although other endogenous acidic compounds in plant samples were presumably co-extracted and derivatized by the AETS 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 195.8 transition. To minimize the disturbance from other unknown components presented in the sample, gradient elution with HPLC for the separation and determination of derivatized fatty acids was an efficient method. The characteristic fragment ion of m/z 195.8 (molecular core structure) came from the cleavage of the N-CH_2CH_2OCO bond. With APCI in positive ion detection mode, intense ion current signals for fatty acid derivatives should be attributed to the introduction of the weak basic nitrogen in corresponding AETS molecular core structure resulting in high ionization efficiency. The cleavage mode and MS/MS analysis for a representative C_9-derivative is shown in Figure 3. All molecular ions $[MH]^+$ of 19 fatty acid derivatives are shown in Table 2.

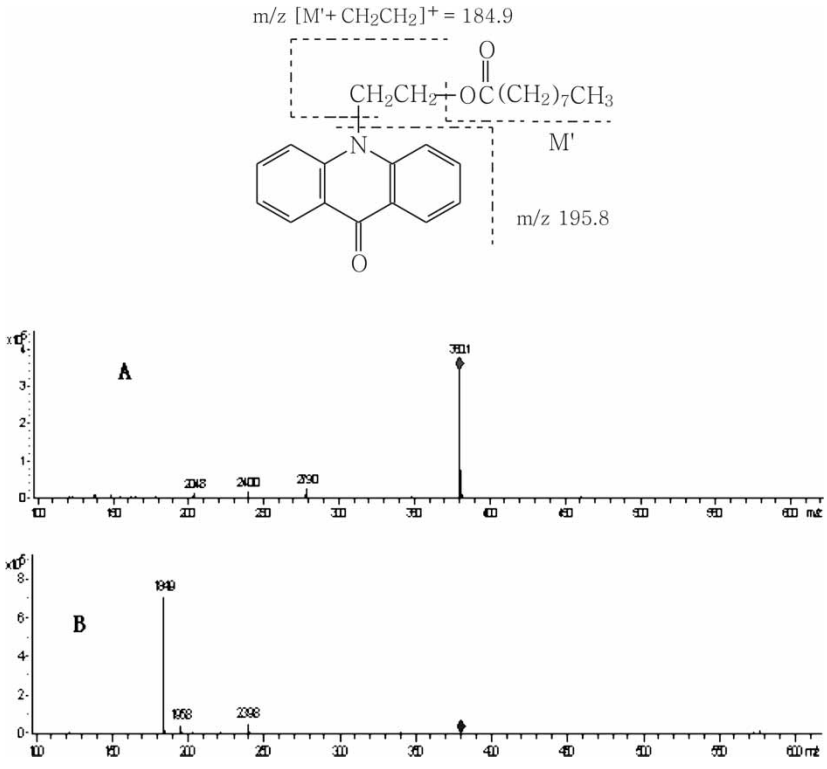

Figure 3. The profile of molecular ion chromatogram and scanning of the isolated representative n-pelargoic acid derivatives. (A) Typical MS chromatogram of n-pelargoic acid derivative from full scanning range from 100 to 600 amu with APCI at positive-ion detection mode. (B) Typical MS/MS chromatogram of n-pelargoic acid derivative from full scanning range from 100 to 600 amu with APCI in positive-ion detection mode; Fragment ions, m/z 184.9 and m/z 195.8.

Linearity, Reproducibility, and Detection Limits

Based on the optimum derivatization conditions, the linearities of the procedures were evaluated in the range of 200.0 pmol to 97.66 fmol (injection volume 10 μL). 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 FFAs were found to give excellent linear responses over this range, with correlation coefficients >0.9989. With 1.0 pmol injections for each derivatized fatty acid, the calculated detection limits (at signal-to-noise of 3:1) are from 12.3 to 43.7 fmol. Preparing a standard solution containing C_1-C_{19} FFAs (1.0×10^{-4} mol/L), the method repeatability was examined (corresponding injected amount 50 pmol for each fatty acid). The relative standard deviations (RSDs) of the

Table 2. Linear regression equations, correlation coefficients, detection limits, mass spectral data 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	Detection limits (fmol)	First step MS $(M+1)^+$	Retention time RSD (%)	Peak area RSD (%)
C_1	Y = 61.15X + 31.02	0.9997	20.64	268.0	0.3779	0.08915
C_2	Y = 37.49X + 18.32	0.9998	12.46	282.0	0.4028	0.6464
C_3	Y = 43.28X + 20.36	0.9998	15.15	296.0	0.4489	0.4593
C_4	Y = 29.24X + 12.92	0.9998	33.27	310.0	0.4162	0.8305
C_5	Y = 35.43X + 16.11	0.9998	17.47	324.1	0.1922	0.4619
C_6	Y = 27.94X + 13.56	0.9997	13.43	338.1	0.08422	0.6899
C_7	Y = 31.59X + 15.47	0.9998	12.28	352.1	0.04593	0.7719
C_8	Y = 25.95X + 13.06	0.9998	13.54	366.1	0.03029	0.6565
C_9	Y = 22.64X + 11.61	0.9997	14.65	380.1	0.02092	0.6351
C_{10}	Y = 24.34X + 12.73	0.9998	14.44	394.2	0.01256	0.7125
C_{11}	Y = 19.79X + 10.61	0.9998	15.30	408.2	0.007141	0.6369
C_{12}	Y = 20.57X + 10.97	0.9998	14.57	422.2	0.001318	0.5755
C_{13}	Y = 19.43X + 9.716	0.9998	14.54	436.2	0.006597	0.5355
C_{14}	Y = 19.51X + 9.148	0.9998	15.21	450.3	0.007375	0.5523
C_{15}	Y = 16.48X + 7.878	0.9997	18.49	464.3	0.009331	0.04369
C_{16}	Y = 15.44X + 8.873	0.9997	16.83	478.3	0.01069	0.7538
C_{17}	Y = 11.15X + 6.207	0.9997	24.67	492.3	0.01616	0.4746
C_{18}	Y = 8.287X + 5.368	0.9995	32.94	506.4	0.01949	1.336
C_{19}	Y = 5.533X + 3.628	0.9989	43.69	520.4	0.02018	2.236

peak areas and retention times are less than 2.236% and 0.4489%, respectively.

Determination of Sample

The chromatogram for the analysis of FFAs extracted from *Lomatogonium rotatum* with fluorescence detection was given in Figure 4. The simultaneous determination of 19 FFAs in this herbal medicine can be easily achieved using AETS as derivatization reagents. As can be seen in this study, the established method was suitable for the determination of these components from the extracted medicinal plant with satisfactory results. The content of 19 FFAs in this plant was also presented as shown in Table 3. Obviously, in this herbal medicine sample, the content of long chain fatty acids with even carbon atoms were much higher than those with each adjacent odd carbon atom, and relatively higher contents of fatty acids mainly focused on even carbon atoms, especially C_{14}, C_{16}, and C_{18}. In order to examine the reliability of the method, the recoveries of 19 FFAs standards were

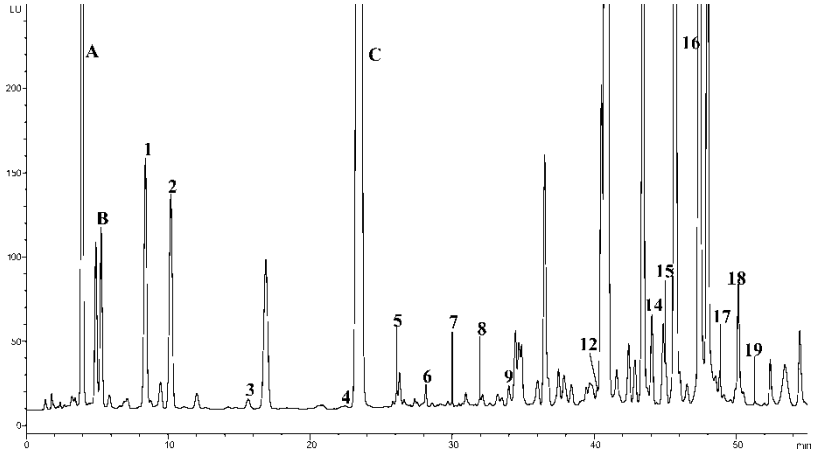

Figure 4. Chromatogram of free fatty acids in *Lomatogonium rotatum*. Chromatographic conditions and peaks as Figure 2.

investigated. The recovery was determined by the addition of known amounts of 19 FFAs standard solutions into the plant sample, and then extraction and derivatization at the same optimal conditions stated above. The recoveries of 19 FFAs were found to be in the range of 97.6–102.8%.

CONCLUSION

In this study, simultaneous determination of 19 FFAs in Tibetan folk medicine, *Lomatogonium rotatum*, can be successfully accomplished. The

Table 3. The content of free fatty acids in *Lomatogonium rotatum*

FFAs	Content (μg/g)	FFAs	Content (μg/g)
C_1	1.962	C_{11}	0
C_2	3.991	C_{12}	0.408
C_3	0.191	C_{13}	0
C_4	0.082	C_{14}	7.978
C_5	0.153	C_{15}	0.817
C_6	0.472	C_{16}	213.860
C_7	0.019	C_{17}	3.812
C_8	0.199	C_{18}	26.634
C_9	0.920	C_{19}	0.780
C_{10}	0		

results indicate that the *Lomatogonium rotatum* plant contain high amount of FFAs besides the active constituents of xanthones, iridoids, etc., as previously reported, and FFAs of higher contents mainly focus on that of even carbon atoms C_{14}, C_{16}, and C_{18}. This method is simple, sensitive, and suitable for the determination of FFAs in this herbal medicine sample.

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