



Analysis of Free Fatty Acid in *Rheum tanguticum* by HPLC-FLD with Pre-Column Derivatization

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Rheum tanguticum Maxim. ex Balf. is a famous traditional Chinese medicinal herb used in conditions including constipation and inflammation. Chemical compounds in *Rh. tanguticum* have been studied widely, but to date the free fatty acids have not been analyzed. In the present study, free fatty acids present in *Rh. tanguticum* were detected using a high-performance liquid chromatography fluorescence detection (HPLC-FLD) method coupled with pre-column derivatization. The labeling reagent 2-(5-benzocridine)ethyl-*p*-toluenesulfonate (BAETS) was used to derivatize with free fatty acids and the derivatives were separated on HPLC with gradient elution. This method showed high sensitivity, good stability and repeatability and was applied to the quantitative analysis of free fatty acids in *Rh. tanguticum* from different regions of Qinghai province. Fifteen saturated fatty acids and four unsaturated fatty acids were detected in these samples. The total content of free fatty acids ranged from 721.08 to 1105.14 $\mu\text{g g}^{-1}$. Hexadecanoic acid had the highest concentration (average 266.33 $\mu\text{g g}^{-1}$) and decoic acid the lowest (average 4.59 $\mu\text{g g}^{-1}$). Four unsaturated fatty acids, including oleic acid, linoleic acid, linolenic acid and hexadecenoic acid were found in *Rh. tanguticum*, with the ratios unsaturated fatty acids: total fatty acids of 31.47 to 47.25 %. The composition of free fatty acids *Rh. tanguticum* in are presented in this paper and these results may be useful for the consideration of the nutritional and medical potential of this species.

Keywords: *Rh. tanguticum*, Free fatty acids, HPLC fluorescence detection, Pre-column derivatization.

INTRODUCTION

Rhubarb is a frequently used traditional Chinese herbal medicine belonging to the family Polygonaceae. The roots and rhizomes of Rhubarb have been used in China for over 2000 years for its multiple medical properties, including purgative, antiinflammatory and hemostatic. Moreover, it is also an important component in many traditional prescriptions and Chinese patent medicines. *Rheum tanguticum* Maxim. ex Balf., a species endemic to the Tibetan Plateau, is one of the three genuine Rhubarbs officially listed in the Chinese Pharmacopoeia¹ and is traditionally considered better than the other two, *Rh. officinale* Baill. and *Rh. palmatum* Linn.² Due to its medical importance, the chemical compounds of *Rh. tanguticum* have been studied extensively, mainly anthraquinones, anthrones, tannins and stilbenes^{3,4}. However, the free fatty acids (FFA) of this species have not yet been reported.

Fatty acids are essential components to living organisms existing in nature, both as nutritional substances and as parts of other compounds like lipids. Researchers have found that fatty acids, especially unsaturated fatty acids (UFA), may have

bioactivities including hypolipidemic, anti-inflammatory and antiarrhythmic⁵⁻⁷. Considering the importance of fatty acids, we aimed to investigate the composition of free fatty acids in *Rh. tanguticum* in this study.

The most common methods to detect fatty acids are gas chromatography (GC) and gas chromatography-mass (GC-MS). Gas chromatography methods have been widely used in the determination of fatty acids in different research areas, but there are some limitations such as low sensitivity, or thermal instability from high temperature⁸. Compared with gas chromatography methods, high-performance liquid chromatography fluorescence detection (HPLC-FLD) coupled with pre-column derivatization can overcome these problems⁹. In this study, a HPLC-FLD method with the labeling reagent 2-(5-benzocridine)ethyl-*p*-toluenesulfonate (BAETS)¹⁰ was used to detect free fatty acids in *Rh. tanguticum*.

EXPERIMENTAL

All fatty acid standards and HPLC-grade acetonitrile were purchased from Sigma (USA). Water was purified by a Milli-Q system (USA). N,N-Dimethylformamide (DMF), potassium

carbonate, chloroform and other reagents were of analytical grade. The labeling reagent 2-(5-benzoacridine)ethyl-*p*-toluenesulfonate (BAETS) was synthesized in the authors' laboratory¹⁰. An Agilent 1100 series HPLC system was applied to the free fatty acids analysis, equipped with an online vacuum degasser, a quaternary pump, an auto sampler, a thermostated column compartment and a fluorescence detector (FLD). A reversed-phase Hypersil BDS C8 column (200 × 4.6 mm, 5 μm i.d., Dalian Elite, China) was used for the separation.

Preparation of solutions: Standard stock solution of fatty acid (1×10^{-2} mol L⁻¹) was dissolved with each standard in corresponding volume of acetonitrile. A mixed standard solution containing 34 fatty acids (1×10^{-4} mol L⁻¹) was prepared by dilution of each stock solution with acetonitrile. Derivatization reagent solution (3×10^{-5} mol L⁻¹) was obtained by dissolving 12.8 mg BAETS in 10 mL acetonitrile. When not in use, all solutions were stored at 4 °C in a refrigerator.

Preparation of samples: Samples of *Rh. tanguticum* were collected from Qinghai province during the harvesting time in 2011, including wild samples (DR1, DR2) from Dari, Guoluo region; wild (QL1) and cultivated samples (QL2) from Qilian, Haibei region. After collection, the samples were washed with pure water, dried and powdered to 0.2 mm. The prepared sample powder (50 mg) was put in a centrifuge tube, mixed with 5 mL chloroform. The mixture was extracted under the ultrasonic bath for 2 h and soaked for 12 h. It was centrifuged and 1 mL of the supernatant was collected and dried with nitrogen. The prepared sample was redissolved in acetonitrile for the derivatization procedure.

Derivatization procedure: To a vial containing standard fatty acid mixture or sample, 20 mg K₂CO₃, 200 μL DMF, 200 μL BAETS solution were added. The vial was sealed and the reaction was carried out in a water bath at 90 °C for 0.5 h. After cooling to room temperature, the solution was diluted with acetonitrile and filtered through a 0.2-μm membrane filter for HPLC analysis. The derivatization procedure is shown in Fig. 1.

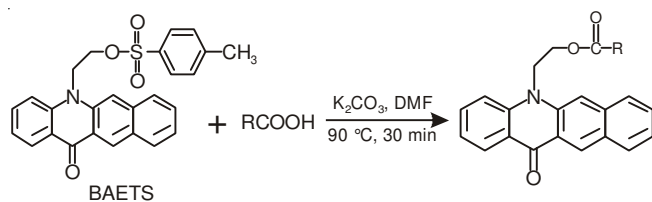


Fig. 1. Derivatization scheme of BAETS with fatty acid

HPLC conditions: HPLC separation was performed with a gradient elution. Eluent A was 5 % acetonitrile in water and eluent B was acetonitrile. The gradient condition was as follows: 45-83 % acetonitrile from 0 to 20 min and kept 10 min; 83-92 % acetonitrile from 30 to 50 min; 92-100 % acetonitrile from 50 to 55 min and kept 15 min. There was a 5 min equilibration with the initial mobile phase condition before the next injection. The injection volume was 10 μL. The flow rate was constant at 1 mL min⁻¹ and the column temperature at 30 °C. The fluorescence excitation and emission wavelengths were set at 272 nm and 505 nm, respectively.

RESULTS AND DISCUSSION

Optimization of solvents and HPLC conditions: Several solvents were tested to get the most satisfactory result. Compared with previous studies, buffer (e.g. ammonium formate, formic acid) and DMF were commonly used to improve separation between two peaks¹¹⁻¹³. However, to the best of our knowledge, both buffer and DMF are harmful to the column, or erode the HPLC system. To avoid these materials, a method of fatty acid analysis and protection of the equipment was achieved in the present study. Two solutions (ethanol and chloroform) were tested for extract efficiency. The results showed that although ethanol is environmentally friendly and does less harm to the human body, it achieved a low extraction rate. Consequently, chloroform was chosen to perform this experiment.

Method validation: The method was validated for linearity, precision, limits of detection (LOD) and recovery. Linearity was established with the regression of the peak area versus concentration of each fatty acid standard in the range of 0.125-20 μmol L⁻¹. The derivatives were found to give linear responses with correlation coefficients of > 0.998 3. The LOD at a signal to noise ratio (S: N) of 3:1 were in the range of 1.70-12.05 pmol mL⁻¹. The inter-day (n = 3) and intra-day (n = 3) precision for BAETS derivatives by the relative standard deviations (RSD) of the peak areas were less than 6.18 and 7.37 %, respectively. The experimental recoveries were in the range of 85.28-105.27 %. The method validation parameters are shown in Table-1 and the chromatogram of 34 standard fatty acid derivatives is shown in Fig. 2A.

Analysis of free fatty acids in *Rh. tanguticum*: Free fatty acids of *Rh. tanguticum* have been found in its volatile oil¹⁴. Eight free fatty acids were detected with GC-MS, including dodecanoic acid (C12), myristic acid (C14), octadecadienoic acid (C18:2), hexadecenoic acid (C16:1), pentadecanoic acid (C15), hexadecanoic acid (C16), heptadecanoic acid (C17) and octadecanoic acid (C18)¹⁴.

In the present study, 19 free fatty acids including 15 saturated fatty acids and 4 unsaturated fatty acids were determined in *Rh. tanguticum* using the described HPLC-FLD method, containing all free fatty acids detected with GC-MS except heptadecanoic acid. Saturated fatty acids ranged from C5 to C26, mainly with an even number of carbons, which was consistent with findings for other medical herbs^{9,11,12}. Unsaturated fatty acids contained octadecatrienoic acid (C18:3), hexadecenoic acid (C16:1), octadecadienoic acid (C18:2) and octadecenoic acid (C18:1). The analysis data are shown in Table-2 and representative chromatogram is in Fig. 2 B.

The total content of free fatty acids ranged from 721.08 to 1105.14 μg g⁻¹ and the ratios of unsaturated fatty acids: total fatty acids were between 31.47 and 47.25 %. The highest concentration of free fatty acids is hexadecanoic acid (C16), which was consistent with the previous study¹⁴ and the lowest concentration was decoic acid (C10), with an average content of 266.33 μg g⁻¹ and 4.59 μg g⁻¹, respectively. The order of average concentration of unsaturated fatty acids from high to low is C18:2, C18:1, C18:3 and C16:1.

The wild sample (QL1) collected in Qilian was found to have the highest concentration of total fatty acids, as well as

TABLE-1
LINEARITY, CORRELATION COEFFICIENT, PRECISION, LOD, AND RECOVERY FOR THE METHOD

Fatty acid	Linearity ^a	Correlation coefficient	Precision RSD (%)		LOD (pmol mL ⁻¹)	Recovery (%)
			Intra-day	Inter-day		
C5	y = 23.02x + 8.97	0.999 9	1.05	6.96	2.48	90.25
C6	y = 22.50x + 6.25	0.999 9	4.18	3.62	2.52	95.65
C7	y = 22.68x + 3.43	0.999 9	3.89	5.04	2.59	97.31
C8	y = 19.59x + 3.63	0.999 9	2.54	2.94	3.06	98.22
C9	y = 18.42x + 3.82	0.999 9	1.68	2.48	3.22	92.11
C10	y = 20.11x + 18.41	0.999 9	3.43	6.25	2.67	91.10
C11	y = 17.63x + 17.62	0.999 5	0.99	2.12	3.16	90.33
C12	y = 17.63x + 17.62	0.999 5	1.78	3.71	3.16	98.25
C13	y = 20.19x + 6.22	0.999 9	0.33	4.90	2.88	100.01
C18:3	y = 24.07x + 0.32	0.999 9	0.32	4.28	2.60	93.58
C22:6	y = 17.90x-5.64	0.999 8	0.43	4.21	3.42	100.28
C14	y = 22.05x-6.40	0.999 9	0.58	7.09	2.93	96.28
C20:4	y = 24.48x-6.46	0.999 9	0.60	7.21	2.55	92.84
C16:1	y = 27.52x + 3.43	0.999 9	0.66	6.71	2.29	102.56
C18:2	y = 24.97x-4.26	0.999 9	0.30	5.86	2.61	97.34
C15	y = 20.19x-11.90	0.999 9	4.09	7.22	3.15	90.92
C16	y = 23.54x-5.86	0.999 9	0.73	6.20	2.80	91.34
C18:1	y = 37.08x-4.12	0.999 9	1.96	5.82	1.70	91.61
C17	y = 18.95x-1.13	0.999 9	2.65	7.37	3.08	101.98
C18	y = 20.29x-5.20	0.999 9	0.46	4.96	3.48	88.65
C20:1	y = 21.47x-0.63	0.999 9	0.62	6.82	2.77	87.92
C19	y = 19.60x-18.05	0.999 9	0.68	7.11	3.69	105.27
C20	y = 18.94x + 5.66	0.999 9	0.60	5.50	2.75	85.28
C22:1	y = 19.97x-8.93	0.999 8	2.79	4.53	2.65	103.10
C21	y = 17.45x-13.12	0.999 9	1.45	4.33	3.96	99.20
C22	y = 16.64x-3.65	0.999 9	1.38	3.94	3.70	100.52
C23	y = 15.38x-9.97	0.999 7	1.34	3.11	3.68	96.71
C24	y = 16.43x-24.27	0.999 7	3.22	1.50	4.64	101.21
C25	y = 14.24x-15.28	0.999 9	1.57	1.61	4.93	99.20
C26	y = 12.71x-13.70	0.999 9	5.83	2.24	5.54	95.45
C27	y = 11.25x-18.51	0.999 6	1.12	2.31	6.57	97.81
C28	y = 11.35x-24.51	0.999 7	3.14	2.25	8.09	96.82
C29	y = 9.01x-22.74	0.998 3	2.11	1.18	8.53	97.41
C30	y = 5.84x-1.15	0.999 5	6.18	4.03	12.05	91.21

^aLinearity Y means peak area; X means injected amount, pmol 10 μ L

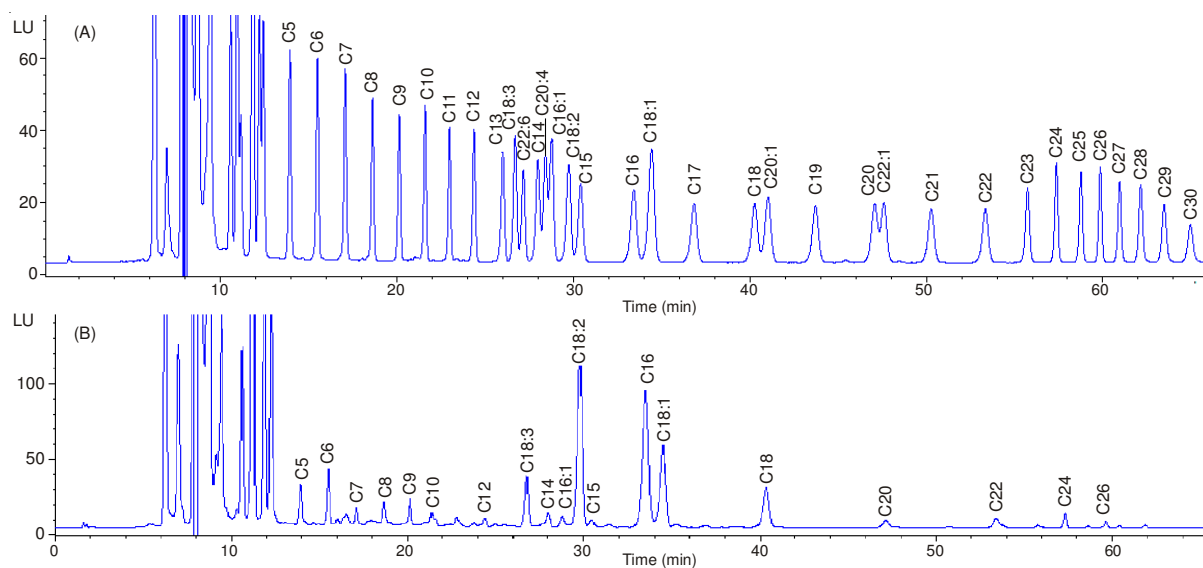


Fig. 2. Chromatograms for representative fatty acid derivatives of standards (A) and *Rh. tanguticum* (B); Abbreviations: C5 (pentanoic acid); C6 (hexanoic acid); C7 (heptanoic acid); C8 (caprylic acid); C9 (pelargonic acid); C10 (decanoic acid); C11 (undecanoic acid); C12 (dodecanoic acid); C13 (tridecanoic acid); C18:3 (8,11,14-octadecatrienoic acid); C22:6 (2,5,8,11,14,17-docosahexaenoic acid); C14 (myristic acid); C20:4 (6,9,12,15-arachidonic acid); C16:1 (2-hexadecenoic acid); C18:2 (9,12-octadecadienoic acid); C15 (pentadecanoic acid); C16 (hexadecanoic acid); C18:1 (12-octadecenoic acid); C17 (heptadecanoic acid); C18 (octadecanoic acid); C20:1 (11-eicosenoic acid); C19 (nonadecanoic acid); C20 (arachidic acid); C22:1 (12-docosenoic acid); C21 (heneicosoic acid); C22 (docosanoic acid); C23 (tricosanoic acid); C24 (tetracosanoic acid); C25 (pentacosanoic acid); C26 (hexacosanoic acid); C27 (carboeric acid); C28 (octocosanoic acid); C29 (motanic acid); C30 (myricyl acid)

TABLE-2
FREE FATTY ACID DETECTED IN *Rh. tanguticum*

Fatty acid	Content ($\mu\text{g g}^{-1}$)			
	DR1	DR2	QL1	QL2
C5	14.55	8.49	6.72	14.40
C6	12.71	11.36	4.41	15.10
C7	8.63	5.11	3.79	4.85
C8	11.39	5.51	5.59	10.16
C9	14.86	19.20	12.03	13.37
C10	4.77	4.41	4.49	4.69
C12	8.28	6.66	8.60	6.93
C18:3	34.50	35.55	95.79	21.89
C14	13.08	11.41	15.23	11.88
C16:1	11.41	7.54	8.71	14.51
C18:2	137.18	115.53	265.95	72.20
C15	13.21	9.67	9.55	9.41
C16	326.55	235.66	291.69	211.42
C18:1	191.16	93.34	151.78	118.33
C18	139.67	122.74	138.42	122.85
C20	15.46	12.92	14.34	12.54
C22	30.97	32.12	33.79	26.49
C24	17.36	20.82	15.45	12.92
C26	16.25	5.08	18.80	17.16
TFA ^a	1021.98	763.13	1105.14	721.08
UFA ^b	374.25	251.96	522.23	226.93
UFA/TFA ^c (%)	36.62	33.02	47.25	31.47

^aTFA: total free fatty acids, ^bUFA: unsaturated fatty acids, ^cUFA/TFA: ratio between unsaturated fatty acids and total fatty acids

unsaturated fatty acids, with a content of 1105.14 and 522.23 $\mu\text{g g}^{-1}$, respectively. In contrast, the cultivated sample (QL2) in Qilian had the lowest concentration of both total fatty acids and unsaturated fatty acids.

Conclusion

A sensitive and selective HPLC-FLD method coupled with fluorogenic derivatization for simultaneous determination of saturated and unsaturated fatty acids was applied to the determination of free fatty acids in *Rh. tanguticum*. Nineteen free fatty acids were detected, including 15 saturated fatty acids

and 4 unsaturated fatty acids and hexadecanoic acid was found to have the highest concentration. The method showed high sensitivity, allowed to react in a gentle condition and is practicable in laboratories where HPLC-FLD equipment is available. Moreover, it can be provided as a useful method for free fatty acids detection in plant materials.

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