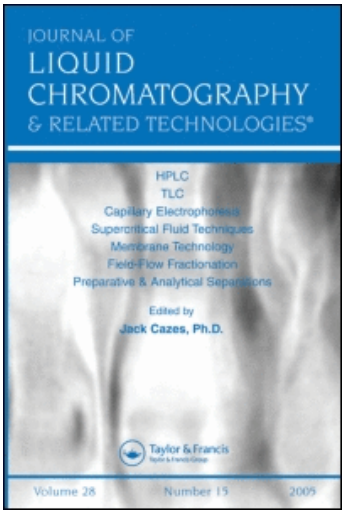

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Quality Evaluation of *Rhodiola crenulata*: Quantitative and Qualitative Analysis of Ten Main Components by HPLC

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Abstract: A high performance liquid chromatographic method has been performed for the simultaneous identification and quantification of ten bioactive components in three botanical materials of *Rhodiola* genus (*R. crenulata*, *R. sachliensis* and *R. dumulos*). Eight samples of *Rhodiola* genus prepared from three different botanical materials were investigated by the proposed method, and the variance was revealed by the calculated results of these contents and similarity values for ten main flavonoids. The proposed method is simple, effective, and suitable for the evaluation of this traditional Chinese medicine.

Keywords: *Rhodiola rosea* L, *Rhodiola crenulata*, Flavonoid, HPLC, Qualitative evaluation, Simultaneous analysis

INTRODUCTION

The plants of *Rhodiola* genus (Crassulaceae), are perennial herbaceous plants and mainly distributed in southwest China, including Yunnan province, Sichuan province, Tibetan Autonomous region, et al.[1,2] It was reported that

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they had similar effects as *Panax ginseng* and *Acanthopanax senticosus*.[3] *Rhodiola crenulata* (Hook. F. et Thoms.) H. Ohba, as a traditional Chinese medicine (TCM) named *Radix et Rhizoma Rhodiolae crenulatae*[4] had the properties of benefiting vital energy and promotion of blood circulation. The rhizome of Rhodiola rosea L. has been used as a tonic and antiaging agent since ancient times.[5] Recently it has been reported that *Rhodiola* genus showed the effect of anti-fatigue, anti-anoxia, and improving memory ability.[3] And, they were also used to increase physical endurance and longevity, and treat depression, anemia, and nervous system disorders.[5] As a traditional regional herbal remedy, it has also been used by Tibetans in many characteristic ways, such as clearing heat in the lungs, eliminating toxins from the body, treating various epidemic diseases, edema of limbs, traumatic injuries, and burns.[6]

As recorded in *China Pharmacopoeia*,[4] only salidroside was determined to control the quality of this medicinal material. So far, it is well known that multiple constituents are responsible for the therapeutic effects of TCM,[7] thus it seems necessary to determine bioactive components as much as possible to ensure the quality of *Rhodiola crenulata*. In recent years, methods for multi-component analysis have increasingly been founded as a credible solution for the analysis of a complex system in TCM. Multi-component analysis of bioactive compounds in TCM has been reported with high resolution and wide application.[8,9] The method to elucidate and control the multiple constituents of this drug is urgently required, so as to ensure efficacy, safety, and batch to batch uniformity.

In this paper, we developed a simple and accurate HPLC method to simultaneously determine the ten main active compounds, including Rhodioloside (HJT1), Kaempferol-3 -sophoroside (HJT2), Kaempferol-3-O-α-rhamnopyranosyl $(1 \rightarrow 4)$-β-D-glucopyranoside (HJT3), Kaempferol-3-rungioside (HJT4), Kaempferol-3-O-L-rhamnopyranoside (HJT5), Rhodiosin (HJT6), Rhodionin (HJT7), Kaempferol-4′-O-α-L-rhamnopyranoside (HJT8), Kaempferol (HJT9), and Rhodiolin (HJT10), and elucidated the variance in three botanical materials of *Rhodiola* genus, including *R. crenulata, R. sachliensis*, and *R. dumulosa*.

EXPERIMENTAL

Apparatus, Reagents, and Materials

An LC2010A*HT* HPLC system coupled with a DAD detector was used (Shimadzu Corporation, Kyoto, Japan). HPLC grade acetonitrile, methanol, triethylamine, and acetic acid were purchased from Merck Company (Merck, Darmstadt, Germany). Ultrapure water was prepared by a Milli-Q50 SP Reagent Water System (Millipore Corporation, MA, USA) for the preparation of samples and buffer solutions. Other reagents were of analytical grade.

Eight batches of samples were purchased from different habitats of China and belong to three different crude materials of *Rhodiola* genus, including *R. crenulata* (lot No. 061006, 061215), *R. sachliensis* (lot No. 061103, 061104, and 061219), *R. dumulosa* (lot No. 061113, 061126, and 061221). The voucher specimen of these collections has been identified and deposited at Herbarium of School of Pharmacy, Second Military Medical University, Shanghai, P. R. China.

Extraction and Isolation Procedures

The medicinal material (10 kg, Lot No. 061006) was powdered and extracted with 95% ethanol ultrasonically for 3 h (50 L × 3). The supernatants were filtered and evaporated to dryness (3654 g) under reduced pressure. The extract (3600 g) was dissolved in 6 L of deionized water and fractionated three times by petroleum ether (5 L × 3), ethyl acetate (5 L × 3), and n-butanol (5 L× 3), then, the solutions were evaporated to obtain the fractions weighted 31 g, 1450 g, 1840 g, respectively. There were bioactive components for increased physical endurance and longevity in ethyl acetate and n-butanol investigated by pharmacological tests,[10–12] so we carried on a systematical separation to elucidate the constituents in these two fractions. The ethyl acetate part was admixed with equal amounts of silica gel particles, eluted by chloroform-methanol with the gradient elution of 10:1 → 5:1 → 4 1 → 2:1 → 1:1, and further separations were performed on reverse phase preparative HPLC by gradient elution of methanol-water. We obtained HJT5, HJT6, HJT7, HJT8 in elution fraction of 5:1, obtained HJT1, HJT5, HJT7 in elution fraction of 4:1, HJT7, HJT9, and HT10 in elution fraction of 2:1. The n-butanol part was isolated and eluted on HP-20 macroporous resin by different proportions of ethanol-water. We obtained HJT3 in the 40% ethanol solvent and HJT4 in the 50% ethanol solvent by polyamide column separation, and obtained HJT2 in the 60% ethanol part by reverse phase preparative HPLC purified repeatedly. The chemical structures (shown in Figure 1) of the ten above mentioned components were identified by spectral analysis and the previous reports,[13,14] and the purities exceeded 99%.

Preparation of Standard Solutions

Each compound (including HJT1, HJT2, HJT3, HJT4, HJT5, HJT6, HJT7, HJT8, HJT9, and HJT10) was accurately weighted, then dissolved in methanol and diluted to appropriate concentration, respectively. A mixed standard solution, containing 0.312 mg/mL of HJT1, 0.200 mg/mL of HJT2, 0.202 mg/mL of HJT3, 0.202 mg/mL of HJT4, 0.202 mg/mL ofHJT5, 0.400 mg/mL of HJT6, 0.504 mg/mL of HJT7, 0.201 mg/mL of HJT8, 0.301 mg/mL of HJT9, 0.207 mg/mL of HJT10, was prepared in

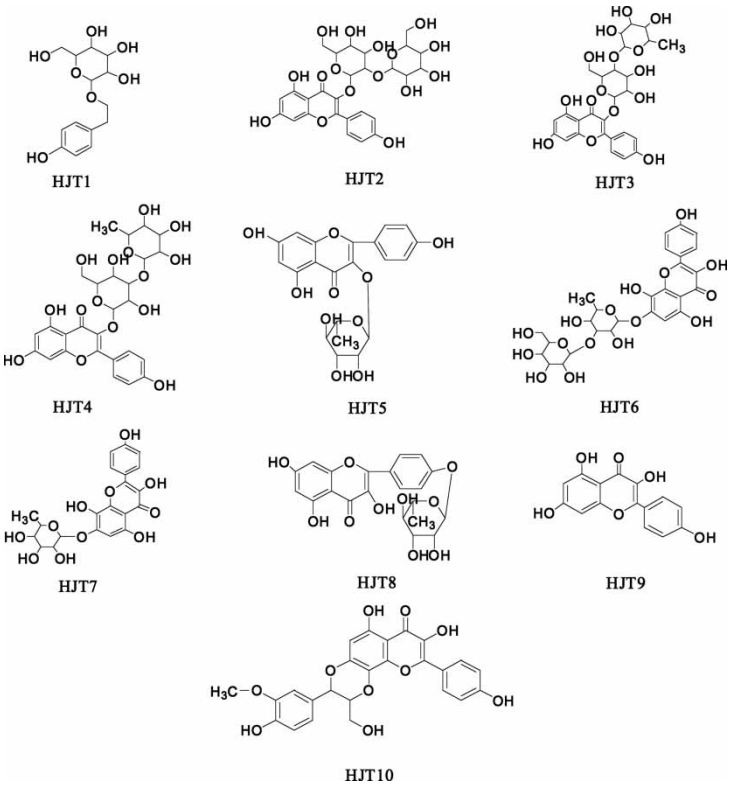

Figure 1. Chemical structures of the ten investigated compounds from *Rhodiola crenulata*. HJT1: Rhodioloside; HJT2: Kaempferol-3–Sophoroside; HJT3: Kaempferol-3-O-α-rhamnopyranosyl $(1 \rightarrow 4)$-β-D–glucopyranoside; HJT4: Kaempferol-3-rungioside; HJT5: Kaempferol-3-O- L-rhamnopyranoside; HJT6: Rhodiosin; HJT7: Rhodionin; HJT8: Kaempferol-4′-O- α-L-rhamnopyranoside; HJT9: Kaempferol; HJT10: Rhodiolin.

methanol. A set of standard solutions were also prepared by the appropriate dilution of the stock solution with methanol, containing 31.2–624 μg/mL of HJT1, 20.0–400 μg/mL of HJT2, 20.2–404 μg/mL of HJT3, 20.2–404 μg/mL of HJT4, 20.2–404 μg/mL of HJT5, 40.0–800 μg/mL of HJT6, 50.4–1008 μg/mL of HJT7, 20.1–402 μg/mL of HJT8, 30.1–602 μg/mL of HJT9, 20.7–414 μg/mL of HJT10. All the solutions were stored in the refrigerator at 4°C before analysis.

Preparation of Samples

All medical materials were collected at October 2006, and dried at 60°C until constant weight. Each dried material was pulverized to 100 mesh. Approximately

1.0 g pulverized powder was accurately weighted and then extracted with 70% ethanol (50 mL) ultrasonically 2 times for 30 min. The supernatant solution was filtrated through a 0.45 μm syringe filter (Type Millex-HA, Millipore, USA) and the aliquots (20 μL) were subjected to HPLC analysis.

HPLC-DAD Analysis

A LC2010A*HT* HPLC system coupled with a DAD detector (Shimadzu Corporation, Kyoto, Japan) was used for quantitative determination of the ten compounds. The separation was performed on an Inertsil C_{18} column (5 μm, 4.6 × 250 mm, GL Science Inc, Japan) with the column temperature set at 25°C. The mobile phase consisted of water containing (A) 0.3% (v/v) acetic acid (adjusted to pH 5.0 by triethylamine) and (B) acetonitrile. A gradient program was used as follows (v/v): 0–60 min, B 10%–33%; 60–70 min, B 33%–70%; 70–75 min, B 70%–100%. The flow rate was 0.8 mL/min, and the injection volume was 10 μL. The investigated compounds were determined at the wavelength of 265 nm. The chromatograms were acquired with an LCsolution workstation (Shimadzu Corporation, Kyoto, Japan).

RESULTS AND DISCUSSION

Optimization of Sample Preparation and Chromatographic Separation Conditions

Extraction method, extraction solvent, and extraction time were investigated so as to obtain the best extraction efficiency. The results suggested that ultrasonic extraction was more effective with little interference. Therefore, the ultrasonic extraction was chosen as the optimization method. Methanol, 35%, 70% methanol, and 95% methanol were selected as extraction solvents to optimize the efficiency of the extraction. The results showed that 70% methanol was most suitable for extraction of the investigated compounds.

Approximately 1.0 g samples were accurately weighted and extracted with 50 mL methanol for 30, 60, 90, and 120 min, respectively, to determine optimal extraction time. It was shown that the maker compounds were almost completely extracted within 60 min. Hence, 60 min was chosen as optimal extraction time.

In order to identify the target compounds and get high resolution, three mobile phases (phosphonic acid-triethylamine buffer-acetonitrile, 0.1% formic acid-acetonitrile, and 0.3% acetic acid-acetonitrile, v/v) were investigated. The HPLC-UV chromatogram acquired showed baseline separation of

the compounds by gradient elution of 0.3% acetic acid aqueous solution-acetonitrile on an octadecylsilyl column.

DAD detection was employed at the wavelength range of 190–400 nm and the UV spectra of 70% ethanol extract from *Rhodiola crenulata* were investigated. It was found that the chromatogram at 265 nm could properly represent the profile of the constituents (shown in Figure 2). Compared with the UV spectra and reported data (λ max) of principal rhodioloside in *Rhodiola rosea*,[15–17] the detection wavelength was set at 278 nm as one of the maximum absorption wavelengths for rhodioloside. However, as shown in Figure 2, the global chromatogram of the 70% methanol extract of *Rhodiola crenulata* showed good separation and high sensitivity at the wavelength of 265 nm. Therefore, the wavelength of 265 nm was selected, not only for rhodioloside but also for other flavonoids. The developed method provided significantly high resolution and sensitivity in most contents of *Rhodiola crenulata.*

Method Validation

Linearity

The stock solutions containing ten main components were prepared and diluted to appropriate concentration for the construction of calibration curves. The linearity calibration curves were constructed by at least six assays of each reference compound. The regression equation was calculated in the form of $y = ax + b$, where y and x were the values of peak area and concentration of each reference compound, respectively. Results of the regression analyses and the correlation coefficients (r^2) were listed in Table 1. The high correlation coefficient values ($r^2 > 0.9990$) indicated good linearity between their peak areas (y) and investigated compound concentrations (x, μg/mL) in relatively wide concentration ranges. The limits of detection (LOD) were also determined with a signal-to-noise ratio of 3 and ranged from 2.5 ng to 24 ng at the wavelength of 265 nm, which showed a high sensitivity at these chromatographic conditions.

Precision and Accuracy

The reproducibility (relative standard deviation, R.S.D.) of the proposed method in terms of the contents of five replicate injections was detected in intra-day and inter-day ($n = 5$) for ten reference compounds, respectively. As listed in Table 2, both intra-day and inter-day reproducibility (R.S.D.) of the ten investigated compounds were less than 1.62%.

Recovery

The recoveries of the ten active components were determined by the method of standards addition. Suitable amounts (about 50% of the content) of the ten

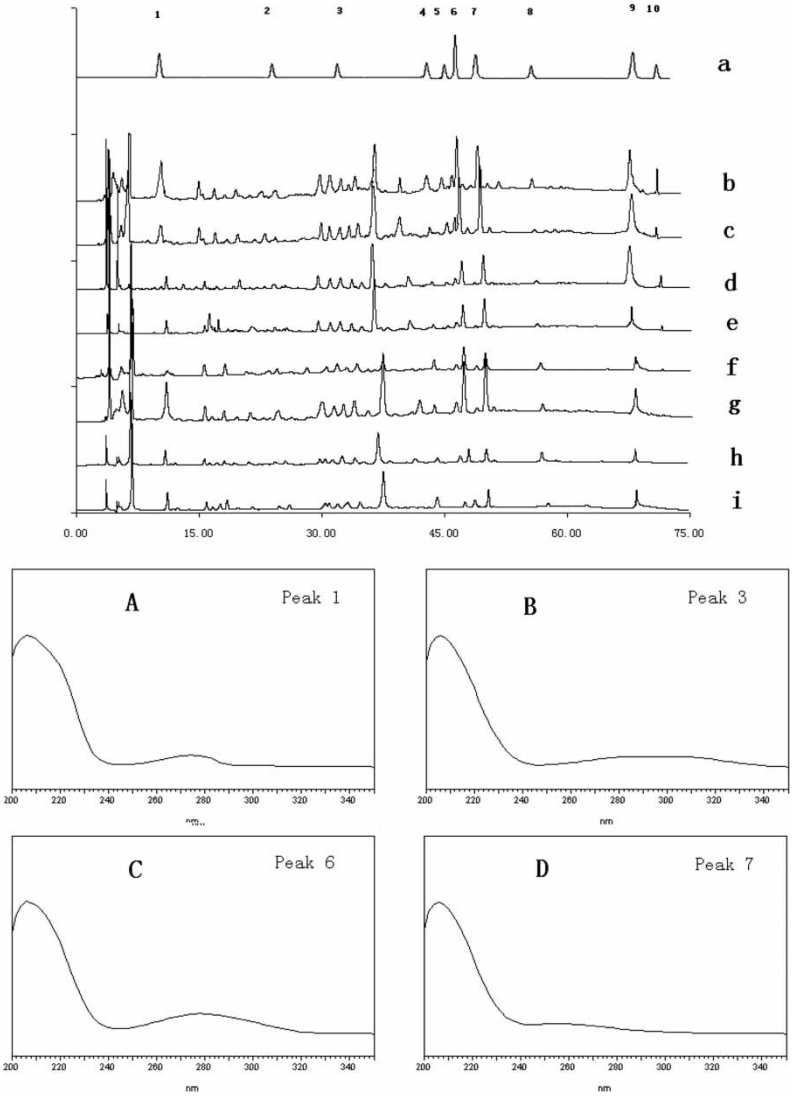

Figure 2. Chromatograms of (a) the mixture of ten standard compounds, (b) the ten investigated compounds in 3 different botanic materials of *Rhodiola rosea* L. (precedence ordering as Table 5 listed), and UV spectra of main compounds, Rhodioloside (A), Kaempferol-3-O-α-rhamnopyranosyl $(1 \rightarrow 4)$-β-D–glucopyranoside (B), Rhodiosin (C), Rhodionin (D) by DAD detector.

compounds were spiked into a sample of *Rhodiola crenulata*, which were determined previously. The mixture was extracted and analyzed by using the proposed sample preparation procedure. For comparison, an unspiked sample was concurrently prepared and analyzed simultaneously. As shown

Table 1. Statistical results of linear regression equation analysis in the determination of the ten flavonoid glycosides

		Regression equation				
Investigated compound	Retention time (min)	Linear range (μg/mL)	Slope (a)	Intercept (b)	r^2 (n = 6)	LOD (ng)
HJT1	10.97	31.2–624	7.85×10^6	5778.6	0.9990	4.0
HJT2	24.08	20.0–400	4.19×10^7	−105440	0.9996	18
HJT3	32.03	20.2–404	2.99×10^6	−14891	0.9994	6.4
HJT4	43.43	20.2–404	1.83×10^6	4965.8	0.9994	16
HJT5	46.24	20.2–404	2.75×10^6	3592.2	0.9998	9.0
HJT6	47.10	40.0–800	7.92×10^5	2287.7	1.0000	3.1
HJT7	49.72	50.4–1008	2.96×10^5	20326	0.9996	2.5
HJT8	56.20	20.1–402	2.66×10^6	−102.99	0.9998	8.6
HJT9	67.93	30.1–602	2.66×10^5	−21327	0.9996	3.9
HJT10	71.03	20.1–401	2.99×10^6	1506.3	0.9990	24

In the regression equation $y = ax + b$, y refers to the peak area (A), x concentration of the reference flavonoids (μg/ml), r^2 the correlation coefficient of the equation and LOD is the limit of detection (S/N = 3).

in Table 3, the mean recoveries of the compounds were 98.9–102.4%, with R.S.D. values ranged from 0.9% to 2.6% ($n = 5$).

Determination of Ten Main Bioactive Components in Eight Samples

The ten predominant bioactive components in *Rhodiola crenulata* were simultaneously determined by the proposed HPLC-UV method at the conditions described above in each sample. The quantitative analyses were performed by

Table 2. Statistical results of precision of the ten flavonoid glycosides ($n = 5$)

	Intra-day precision		Inter-day precision	
Compound	Content (mg/g)	R.S.D. (%)	Content (mg/g)	R.S.D. (%)
HJT1	10.1	0.73	10.0	1.56
HJT2	5.67	0.78	5.63	1.33
HJT3	0.31	0.33	0.30	1.30
HJT4	0.15	0.43	0.14	1.26
HJT5	0.22	0.71	0.20	0.95
HJT6	2.31	0.48	2.34	1.46
HJT7	4.77	0.39	4.75	1.29
HJT8	0.08	0.29	0.07	0.97
HJT9	0.73	0.78	0.72	1.37
HJT10	0.11	0.46	0.11	1.62

Table 3. Statistical results of recovery of the ten flavonoid glycosides ($n = 5$)

Compound	Added amount (mg)	Recorded amount (mg)	Calculated recovery (%)	Mean recovery (%)	R.S.D. (%)
HJT1	1.318	1.316 ± 0.024	99.2, 100.9, 101.7, 98.5, 99.2	99.9	1.3
HJT2	0.983	0.981 ± 0.016	99.6, 100.9, 100.7, 98.2, 99.4	99.8	1.1
HJT3	1.090	1.102 ± 0.037	103.0, 99.3, 104.1, 101.5, 97.7	101.7	2.6
HJT4	0.775	0.772 ± 0.013	99.5, 101.1, 99.2, 97.9, 100.6	99.7	1.3
HJT5	1.130	1.158 ± 0.039	101.8, 99.0, 103.3, 104.3, 103.8	102.4	2.1
HJT6	1.200	1.209 ± 0.033	98.0, 98.2, 103.8, 101.9, 102.0	100.8	2.5
HJT7	1.538	1.527 ± 0.035	98.2, 103.0, 97.3, 98.0, 100.0	99.3	2.3
HJT8	0.621	0.614 ± 0.015	101.9, 99.4, 96.1, 97.1, 100.2	98.9	2.4
HJT9	1.111	1.104 ± 0.026	96.8, 97.2, 99.9, 101.3, 101.9	99.4	2.3
HJT10	0.321	0.324 ± 0.003	100.3, 101.6, 99.7, 101.9, 100.6	100.8	0.9

means of the external standard methods, and the calculated results were listed in Table 4 and Table 5. Among the ten components, the contents of HJT1, HJT6, and HJT7 were higher than the other seven flavonoids, with average values of 10.1 mg/g, 2.32 mg/g, and 4.77 mg/g, respectively. The calculated total content of these ten components in the samples from Yunnan province was higher than that in any other samples, which is the main habitat as reported. For the three species of botanic materials *Rhodiola rosea* L., the total contents of flavonoids in *Rhodiola crenulata* was higher than those in the other two genus, especially, HJT2 in *Rhodiola crenulata* is very high, while in others very low or nearly undetected, and the content of rhodioloside was significantly varied from habitat to habitat. Further researches should be carried on to reveal the relationship between the amount of flavonoids and pharmacological effects in *Rhodiola rosea* L.

Similarity Analysis and Quality Evaluation of *Rhodiola rosea* L

The eight samples were examined under the proposed method, and the results matched the criterion stipulated in *Chinese Pharmacopoeia*l. The acquired

Table 4. Contents (mg/g) of the ten flavonoid glycosides in the eight samples (mean ± deviation, $n = 3$)

Sample no.	Content of each flavonoid glycosides in eight samples (mg/g)									
	HJT1	HJT2	HJT3	HJT4	HJT5	HJT6	HJT7	HJT8	HJT9	HJT10
1	10.1 ± 0.32	2.46 ± 0.16	0.28 ± 0.07	0.16 ± 0.05	0.27 ± 0.02	3.32 ± 0.21	4.77 ± 0.29	0.06 ± 0.01	0.72 ± 0.04	0.09 ± 0.01
2	4.07 ± 0.19	1.27 ± 0.13	0.27 ± 0.02	0.13 ± 0.02	0.36 ± 0.06	4.02 ± 0.26	4.00 ± 0.31	0.02 ± 0.00	0.50 ± 0.09	0.04 ± 0.01
3	1.38 ± 0.04	0.91 ± 0.03	0.17 ± 0.01	0.10 ± 0.00	0.21 ± 0.05	2.51 ± 0.17	3.01 ± 0.25	0.02 ± 0.01	0.52 ± 0.06	0.03 ± 0.00
4	1.44 ± 0.13	0.24 ± 0.03	0.17 ± 0.04	0.09 ± 0.01	0.17 ± 0.03	2.44 ± 0.22	2.64 ± 0.20	0.01 ± 0.00	0.12 ± 0.01	0.01 ± 0.00
5	0.76 ± 0.02	1.58 ± 0.13	0.11 ± 0.01	0.15 ± 0.03	0.21 ± 0.03	2.69 ± 0.17	2.39 ± 0.13	0.03 ± 0.01	0.08 ± 0.02	0.01 ± 0.00
6	10.4 ± 0.35	2.93 ± 0.13	0.25 ± 0.04	0.14 ± 0.01	0.23 ± 0.03	3.06 ± 0.26	3.58 ± 0.27	0.03 ± 0.00	0.14 ± 0.02	—
7	1.44 ± 0.11	0.09 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.29 ± 0.02	1.69 ± 0.15	1.71 ± 0.21	0.04 ± 0.01	0.09 ± 0.02	—
8	2.90 ± 0.04	0.34 ± 0.03	0.06 ± 0.01	0.14 ± 0.01	0.18 ± 0.02	1.13 ± 0.18	1.37 ± 0.11	0.01 ± 0.00	0.10 ± 0.01	—

Table 5. Origin of samples of *Rhodiola rosea* L. obtained from different locations in China

Sample no.	Place of purchase	Species
1	Kunming City, Yunnan Province	*Rhodiola crenulata*
2	Guangzhou City, Guangdong Province	*Rhodiola crenulata*
3	Luoyang City, Henan Province	*Rhodiola sachliensis* A. Bor.
4	Yong'antang drug store, Beijing	*Rhodiola sachliensis* A. Bor.
5	Hefei City, Anhui Province	*Rhodiola sachliensis* A. Bor.
6	Nanchang City, Jiangxi Province	*Rhodiola dumulosa(Franch) Fu*
7	Qingdao City, Shandong Province	*Rhodiola dumulosa(Franch) Fu*
8	Weihai City, Shandong Province	*Rhodiola dumulosa(Franch) Fu*

chromatograms strictly revealed the differences of compound contents among batches, the qualification of the ten marker compounds might confirm the differences, but it was not enough for a complex prescription of TCM. Similarity measurements of the HPLC-UV chromatograms between a test sample and a reference sample is often employed to quantitatively conduct quality evaluation. Similarity value is most commonly calculated by the congruence coefficient, as expressed by the following formula:[18]

$$r = \sum_{i=1}^{n} x_i x_i^0 / \sqrt{\sum_{i=1}^{n} x_i^2 \sum_{i=1}^{n} x_i^{0^2}}$$

where r is the similarity value between the chromatograms of sample x and that of reference sample x^0, and x_i, x_i^0 denote the ith peak areas of these two chromatograms, respectively.

In this study, the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A, Chinese Pharmacopoeia Committee, Beijing, China) was used to achieve the similarity values as listed in Table 6, during which the mean data of the eight chromatograms was used as a reference. The similarity values varied in the range of 0.541–0.939, and it was concluded that the differences between batches were acquired. The botanic materials, *R. dumulosa* and *R. sachliensis*, have relatively high similarity values, which matches the determination results described above. From the similarity evaluation results, we can easily evaluate the quality of each sample. The botanic material with low similarity value and low flavonoid content are considered to have comparatively poor bioactivity, and is not suitable to be used in a clinic. We think that the variation of *Rhodiola* genus might be caused by the difference of botanic material, the habitat, harvest time, and the storage process.

Table 6. Similarity values of difference batches of *Rhodiola* genus

Lot no.	061006	061215	061103	061104	061219	061113	061126	061221	Reference
061006	1								
061215	0.806	1							
061103	0.722	0.796	1						
061104	0.696	0.750	0.888	1					
061219	0.687	0.876	0.836	0.809	1				
061113	0.650	0.711	0.785	0.765	0.851	1			
061126	0.472	0.753	0.583	0.522	0.753	0.541	1		
061221	0.567	0.842	0.713	0.643	0.841	0.620	0.939	1	
Reference	0.886	0.948	0.892	0.860	0.928	0.857	0.743	0.832	1

Reference, reference chromatogram defined by the mean data of the eight samples.

CONCLUSION

This is the first report on validation of an analytical method for qualification and quantification of flavonoids in *Rhodiola* genus. The proposed method makes it possible to simultaneously determine different structural multi-components in one run with acceptable levels of linearity, precision, repeatability, and accuracy. The method has been applied successfully to simultaneously quantify ten bioactive components in *Rhodiola* genus. The results demonstrate that the proposed method could be readily utilized as a quality control method for *Rhodiola* genus.

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