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Quantitative and chemical profiles analysis of the root of *Morinda officinalis* based on reversed-phase high performance liquid chromatography combined with chemometrics methods

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A reversed-phase high performance liquid chromatography (HPLC) method was developed to evaluate the quality of *Morinda officinalis* through establishing chromatographic fingerprint and simultaneous determination of four anthraquinone compounds, namely 2-hydroxy-3-hydroxymethyl- anthraquinone, 2-hydroxy-1-methoxy-anthraquinone, rubiadin-1-methyl ether and rubiadin. The chromatographic separation was performed on C_{18} column with a gradient elution program using a mixture of acetonitrile and 0.2% aqueous phosphoric acid (v/v) as mobile phase. In fingerprint analysis, 16 peaks were selected as the characteristic peaks to evaluate the similarities of samples collected from different origins in China according to the State Food and Drug Administration (SFDA) requirements. Furthermore, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were also applied to evaluate the variation of chemical components among different sources of *M. officinalis* in China. This study indicated that the developed HPLC method, combined with chemometrics analysis, could be readily utilized to control the quality of *M. officinalis* and its related traditional Chinese medicinal (TCM) preparations.

Key words: *Morinda officinalis*, fingerprint analysis, anthraquinone compounds, chemometric analysis, quality control.

INTRODUCTION

Morinda officinalis How (Rubiaceae) is a small vine that grows widely in tropical and subtropical regions. In China, this plant is widely distributed and cultured in Guangdong, Fujian, Guangxi and Hainan Provinces, and has been recorded in pharmacopeia of the People's Republic of China since 1963 as a tonic to treat rheumatoid arthritis and impotence (China Pharmacopoeia Committee, 2010). In South China, Hong Kong and Macao, this plant has been developed into various health foods, such as "Ba-ji-tian wine", "Ba-ji-zi-bu Gao". In previous studies, it has been reported that the root of *M. officinalis* contains anthraquinone, iridoids, oligosaccharides and polysaccharides (Wu et al., 2009; Choi et al., 2005; Zhu et al., 2009) and have a variety of pharmacological activities including antiosteoporotic, antinociceptive, anti-inflammatory, antidepressant and enhancing immune

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system function (Bao et al., 2011; Choi et al., 2005; Li et al., 2003). Although, the application of the root of *M. officinalis* is growing steadily, there was no suitable method to effectively control the quality of the root of *M. officinalis*. In addition, most of the medicinal materials of the root of *M. officinalis* come from cultured plant, in which there are six kinds of farm races with different germplasm resources, leading to variations in the chemical composition and the amounts of major bioactive constituents.

In order to control the quality and clarify the differentiation of chemical constituents in *M. officinalis*, a high performance liquid chromatography-diode-array detection (HPLC-DAD) method was built for establishment HPLC profile of chemical constituents and simultaneous determination of four anthraquinone compounds (2-hydroxy-3-hydroxymethyl-anthraquinone, 2-hydroxy-1- methoxy-anthraquinone, rubiadin-1-methyl ether and rubiadin) in the root of *M. officinalis* from various sources. In our previous study, these four anthraquinone compounds were also isolated, and their potent antiosteoporotic activity was identified (Wu et al., 2009). In consideration of complexity of herb medicine, the HPLC chromatograms are complex multivariate data sets, so minor differences between similar chromatograms might be missed, the chemical pattern recognition methods, such as similarity analysis (SA), hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used to reasonably definite the class of the herbal medicine and to efficiently evaluate the differentiation of the root of *M. officinalis* samples. We expected that this HPLC method would be helpful for the quality control of *M. officinalis* in the future.

MATERIALS AND METHODS

Plant materials and reagents

Twenty-five (25) *M. officinalis* populations were collected from different regions of China, and all voucher specimens were taxonomically identified based on morphological characteristics by Professor Q. Y. Zhang and deposited at Herbarium of Department of Pharmacognosy, Second Military Medical University in Shanghai, China.

HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (USA). HPLC grade water was prepared using a deionized water treatment system (Millipore, Bedford, MA, USA). Analytical grade methanol, ethanol and phosphoric acid were purchased from Sinopharm Chemical Reagent Co. Ltd, Shanghai, China. All the solutions were filtered through 0.45 µm membranes (Schleicher and Schuell, Dassel, Germany) and degassed by ultrasonic bath before use.

Instrument and chromatographic conditions

The chromatographic separation was performed on an Angilent 1200 HPLC system (USA), equipped with a quaternary pump, an autosampler, a degasser, an automatic thermostatic column compartment, a DAD detector and a computer with a Chemstation software program for analysis of the HPLC data. Agilent Extend-C_{18} reversed-phase column (250 × 4.6 mm, 5 µm) together with a Phenomenex C_{18} guard column (10 × 4.6 mm, 5 µm) was used with column temperature set at 30°C. HPLC-DAD was used for purity assay of reference compounds.

The mobile phase consisted of acetonitrile (A) and 0.2% aqueous phosphoric acid (v/v, B) using a gradient program of 5 to 30% A in 0 to 15 min, 30 to 40% A in 15 to 35 min and 40 to 80% A in 35 to 80 min. This was followed by a 10 min equilibration period to the injection of each sample. The flow rate was 1 ml/min, and detection wavelength was set at 277 nm, an aliquot of 20 µl solution was injected for acquiring chromatograms.

Preparation of standard solutions

2-hydroxy-3-hydroxymethyl-anthraquinone, 2-hydroxy-1-methoxy-anthraquinone, rubiadin-1-methyl ether and rubiadin were isolated from *M. officinalis*. Their structures and the chromatogram of mixture standard compounds are shown in Figure 1. The purity of each compound was determined to be more than 98% by normalization of the peak area by HPLC. The reference compounds were accurately weighed and dissolved in methanol, diluted to appropriate concentration ranges for the establishment of calibration curves. All stock and working standard solutions were stored at 4°C till it used for analysis.

Preparation of sample solutions

The medicinal materials were milled into powder and dried to constant weight. 2.0 g fine powder was accurately weighed, and extracted twice with 100 ml of 95% ethanol by reflux for 2 h. The extracts were filtered and evaporated under vacuum; the residues were dissolved with 5 ml methanol solution and sonicated for 10 min. The sample solution was filtered through a 0.45 µm membrane filter prior to HPLC analysis and the injection volume was 20 µl.

Data analysis

The chromatographic profiles of all extracts were performed by professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A), which was recommended by the State Food and Drug Administration (SFDA) of China for evaluating similarities of chromatographic profiles of traditional Chinese medicinal (TCM) (Liang et al., 2004). In this study, HCA and PCA were used to assess HPLC fingerprints of all samples. The relative retention time (RRT) and relative peak areas (RPA) of each characteristic peak to reference peak were also calculated in the chromatograms.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

In order to achieve better chromatographic separation, various linear gradients of acetonitrile-water were investigated at a flow-rate of 1 ml/min. The following mobile phase systems were tried: (1) 5 to 100% methanol and 95 to 0% water in 80 min; (2) 10 to 55% methanol and 90 to 45% water in 80 min; (3) 5 to 80% methanol and 95 to 20% phosphoric acid (0.2%) in 80 min; (4) 10 to 75% methanol and 90 to 25% phosphoric acid (0.2%) in 75 min; (5) 5 to 80% acetonitrile and 95 to 20% in

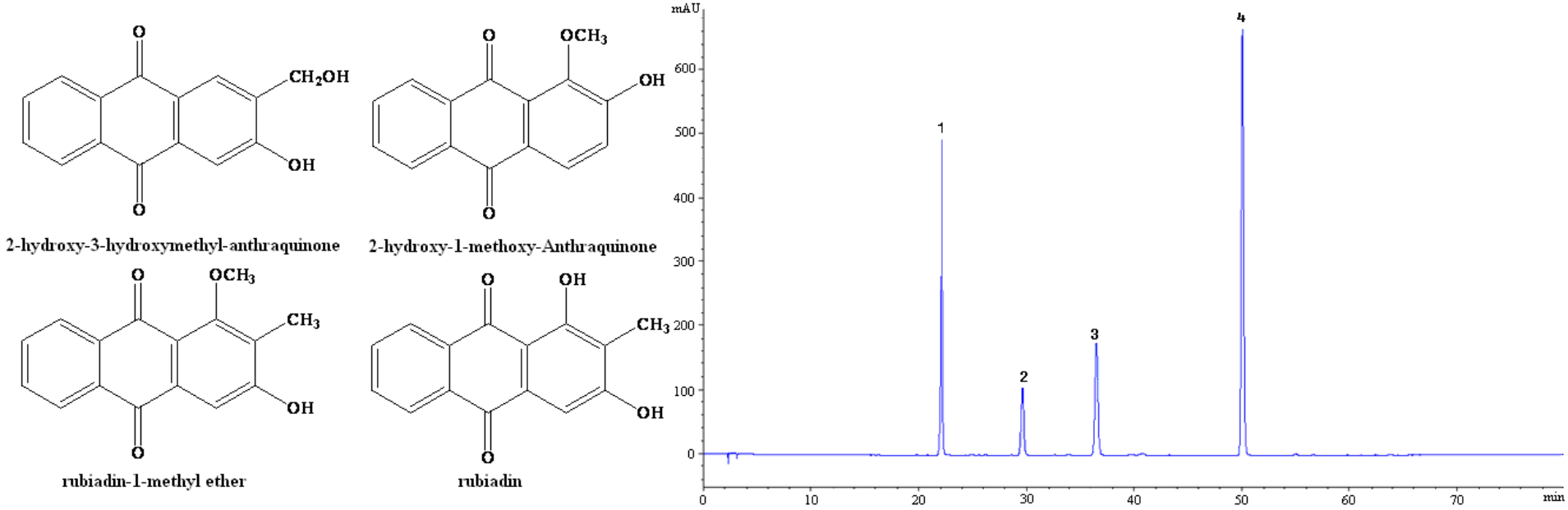

Figure 1. Chemical structures and HPLC chromatogram of mixture standard of 4 anthraquinones: (1), 2-hydroxy-3-hydroxymethethyl-anthraquinone; (2), 2-hydroxy-1-methoxy-anthraquinone; (3), rubiadin-1-methyl ether; (4), rubiadin.

water in 80 min; (6) 10 to 70% acetonitrile and 90 to 30% water in 75 min; (7) 5 to 80% acetonitrile and 95 to 20% phosphoric acid (0.2%) in 80 min; (8) 10 to 75% acetonitrile and 90 to 25% phosphoric acid (0.2%) in 70 min. The acetonitrile (A)-0.2% aqueous phosphoric acid (v/v, B) system was the ultimate choice, which produced a better resolution and stable baseline on the HPLC chromatographic profiles. In the process of gradient optimization, gradient time and initial composition of the mobile phase were taken into consideration. Finally, the gradient program was decided as 5 to 30% A in 0 to 15 min, 30 to 40% A in 15 to 35 min and 40 to 80% A in 35 to 80 min.

Due to a full-scan experiment of the four active components from 200 to 400 nm, 277 nm was selected as detection wavelength so that more characteristic peaks could be obtained, and the baseline was well improved on the chromatographic profiles.

Method validation of quantitative analysis

The sample for method validation was collected from Guangdong province, China, and identified as M. officinalis. The calibration curve was generated to confirm the linear relationship between the peak area and the concentrations of each reference compound in the test samples. The four standards of 2-hydroxy-3-hydroxymethyl-anthraquinone, 2-hydroxy-1-methoxy-anthraquinone, rubiadin-1-methyl ether and rubiadin were accurately weighed, dissolved, and diluted with methanol in a volumetric flask to obtain standard solutions for the calibration curves. A mixed solution containing 4 analytes at various concentrations were injected in triplicate. Calibration curves were peak area versus concentration for each analyte. The linear regression equations, correlation coefficients and ranges of calibration curves for the listed anthraquinone derivatives are shown in Table 1. The calibration curves showed good linear regression with correlation coefficient over 0.9998 within test ranges.

Intra- and inter-day variations were utilized to determine the precision. A sample of the medicinal

Table 1. Regression equation and correlation coefficient of calibration curves for four anthraquinones.

Compound	Regression equation	R	Linearity range (μg/ml)
2-Hydroxy-3-hydroxymethyl-anthraquinone	Y=21512 X + 23.704	0.9999	0.2-250
2-Hydroxy-1-methoxy-anthraquinone	Y=37260 X + 5.7713	0.9998	0.2-250
Rubiadin-1-methyl ether	Y=70335 X + 13.412	0.9999	0.2-250
Rubiadin	Y=71208 X + 3.7282	0.9998	0.2-250

Y, Peak area of anthraquinone derivatives; X, concentration of anthraquinone derivatives; R, correlation coefficient. Acquisition wavelength, 277nm.

Table 2. Intra- and inter–day precision and repeatability for the four anthraquinones in *M. officinalis* (n=5).

Compound	Intra-day (n = 5)		Inter-day (n = 5)		Repeatability (n = 5)	
	mg/g	RSD%	mg/g	RSD%	mg/g	RSD%
2-Hydroxy-3-hydroxymethyl-anthraquinone	0.0363	0.6	0.0351	1.6	0.0342	2.0)
2-Hydroxy-1-methoxy-anthraquinone	0.0300	0.6	0.0312	1.2	0.0321	1.6
Rubiadin-1-methyl ether	0.0884	0.7	0.0861	2.0	0.0853	2.3
Rubiadin	0.0205	0.6	0.0230	0.9	0.0205	1.8

Table 3. Recovery experiment of analytical method for four anthraquinones(n=6).

Compound	Oringinal (μg)	Spiked (μg)	Found (μg)	Recovery (%)	RSD (%)
2-Hydroxy-3-hydroxymethyl-anthraquinone	187.9	200.0	356.0	98.69	0.90
2-Hydroxy-1-methoxy- anthraquinone	321.4	400.0	718.4	99.67	1.97
Rubiadin-1-methyl ether	160.2	200.0	356.0	97.91	1.14
Rubiadin	42.5	40.0	44.1	99.74	0.41

The data was present as average of six determinations. Recovery (%) = [(found amount - original amount)/spiked amount] ×100. RSD (%) = (SD/mean) × 100.

material was prepared as previously described, and was subjected to HPLC analysis five times in the same day to evaluate intra-daily variation, and once each day for five consecutive days to assess inter-daily variation. In order to test the repeatability, five different working solutions from the same samples were analyzed. Variations were expressed as relative standard deviations (RSD). Table 2 showed the results of the tests of precision and repeatability, indicating that the RSD values of the overall intra- and inter-day variations were less than 2.0% and those of the repeatability were less than 2.3% for the analytes. The recovery test was determined by adding standard solution to the samples from the same batch of medicinal material. The mixtures were later processed according to the sample preparation procedure. The experiments were repeated five times. The recovery data are shown in Table 3. The average recoveries of the four anthraquinone compounds were 97.91 to 99.74% and their RSD values were less than 1.97%. Therefore, the HPLC-DAD method was precise, accurate and sensitive enough for simultaneously quantitative evaluation of four anthraquinone compounds in *M. officinalis*.

HPLC profile and quantitative analysis

To standardize the HPLC profile, 25 samples of *M. officinalis* were analyzed. Peaks that existed in all chromatograms of samples with reasonable heights and good resolutions were assigned as “characteristic peaks” for the root of *M. officinalis*. As shown in Figure 2, there were 16 distinct characteristic peaks (from peak 1 to 16) in the HPLC fingerprint common patterns from the 25 samples of *M. officinalis* and the overlapped chromatogram of the investigated samples is shown in Figure 3. Peaks purity was identified by comparison of retention time and DAD spectra. A peak 6 was assigned as the reference peak, which had the highest peak area, and a moderate retention time in the simulative mean chromatograms of *M. officinalis* from different sources. RRT and RPA of the characteristic peaks are shown in Table 4. The similarities of chromatograms of 25 samples comparing with the reference fingerprint, which was developed with the median of all chromatograms, are shown in Table 5. From Tables 4 and 5, a visible variation was observed among the samples. The area of

Table 4. RRT and RPA of characteristic peaks in 25 samples of *M. officinalis*.

Peak	RRT		RPA	
	Means	RSD (%)	Means	RSD (%)
1	0.61	0.11	0.165	99.6
2	0.77	0.15	0.115	92.6
3	0.81	0.23	0.691	101.1
4	0.86	1.10	0.320	89.1
5	0.93	0.22	0.167	146.1
6	1	0.16	1	35.8
7	1.05	0.19	0.109	189.2
8	1.12	0.16	0.390	77.6
9	1.37	0.22	0.210	28.2
10	1.51	0.18	0.043	63.7
11	1.57	0.13	0.043	57.7
12	1.73	0.11	0.033	42.0
13	1.75	0.13	0.032	37.9
14	1.77	0.13	0.045	106.1
15	1.81	0.13	0.027	37.1
16	1.87	0.11	0.029	152.8

Table 5. The similarities of chromatograms of 25 samples.

Collected location	Similarities
Gaoyao, Guangdong	0.914
Zhaoqing, Guangdong	0.647
Wuhua, Guangdong	0.936
Deqing, Guangdong	0.793
Qionghai, Hainan	0.941
Wanning, Hunan	0.904
Zijin, Guangdong	0.936
Nanjin, Fujian	0.588
Meizhou, Guangdong	0.952
Heping, Guangdong	0.929
Kunming, Yunnan#	0.904
Yangjiang, Guangdong	0.983
Qingyuan, Guangdong	0.807
Ganzhou, Jiangxi#	0.941
Pinghe, Fujiang	0.975
Yunfu, Shandong#	0.854
Wuping, Fujian	0.950
Jiujiang, Jiangxi#	0.337
Fengkai,Shandong#	0.966
Baise, Guangxi	0.958
Yongding, Fujian	0.886
Shanghang, Fujian	0.968
Jieyang, Guangdong	0.858
Jingxi, Guangxi	0.957
Yunan, Shandong#	0.936

peaks 1, 2, 3, 5, 7, 14 and 16 varied remarkably, and the RSD of RPA were 99.6, 92.6, 101.1, 146.1, 189.2, 106.1 and 152.8%. Similarity of different samples comparison with the standard fingerprints ranged from 0.337 to 0.983, and the similarity of sample 2, 4, 8, 13 and 18 were less than 0.85, indicating that the chemical composition and content in the *M. officinalis* varied significantly.

According to the contents and pharmacological properties of major constituents in *M. officinalis*, the peaks of 2-hydroxy-3-hydroxymethyl-anthraquinone, 2-hydroxy- 1-methoxy-anthraquinone, rubiadin-1-methyl ether and rubiadin were chosen as reference peak. The contents of four anthraquinone derivatives of 25 samples of *M. offcinalis* from different sources in China were determined using the establishing HPLC method. The results are shown in Table 6. The quantitative analysis results showed that the samples from different sources generally contained the four anthraquinone derivatives. The content ranges of four anthraquinone compounds in the collected samples of *M. officinalis* were 2.02 to 111.15 µg/g (2-hydroxy-3-hydroxymethyl-anthraquinone), 3.67 to 211.57 µg/g (2-hydroxy-1-methoxy-anthraquinone), 1.94 to 98.56 µg/g (rubiadin-1-methyl ether) and 0.78 to21.53 µg/g (rubiadin), respectively. Among the four compounds, the contents of 2-hydroxy-1-methoxy-anthraquinone and rubiadin-1-methyl ether in all samples were generally more than other anthraquinones. The results in Table 6 showed that the contents of each anthraquinone indifferent samples were significantly different, which were consistent with that of HPLC fingerprint analysis.

Hierarchical cluster analysis (HCA)

In order to assess the resemblance and differences of these samples, a hierarchical agglomerative clustering

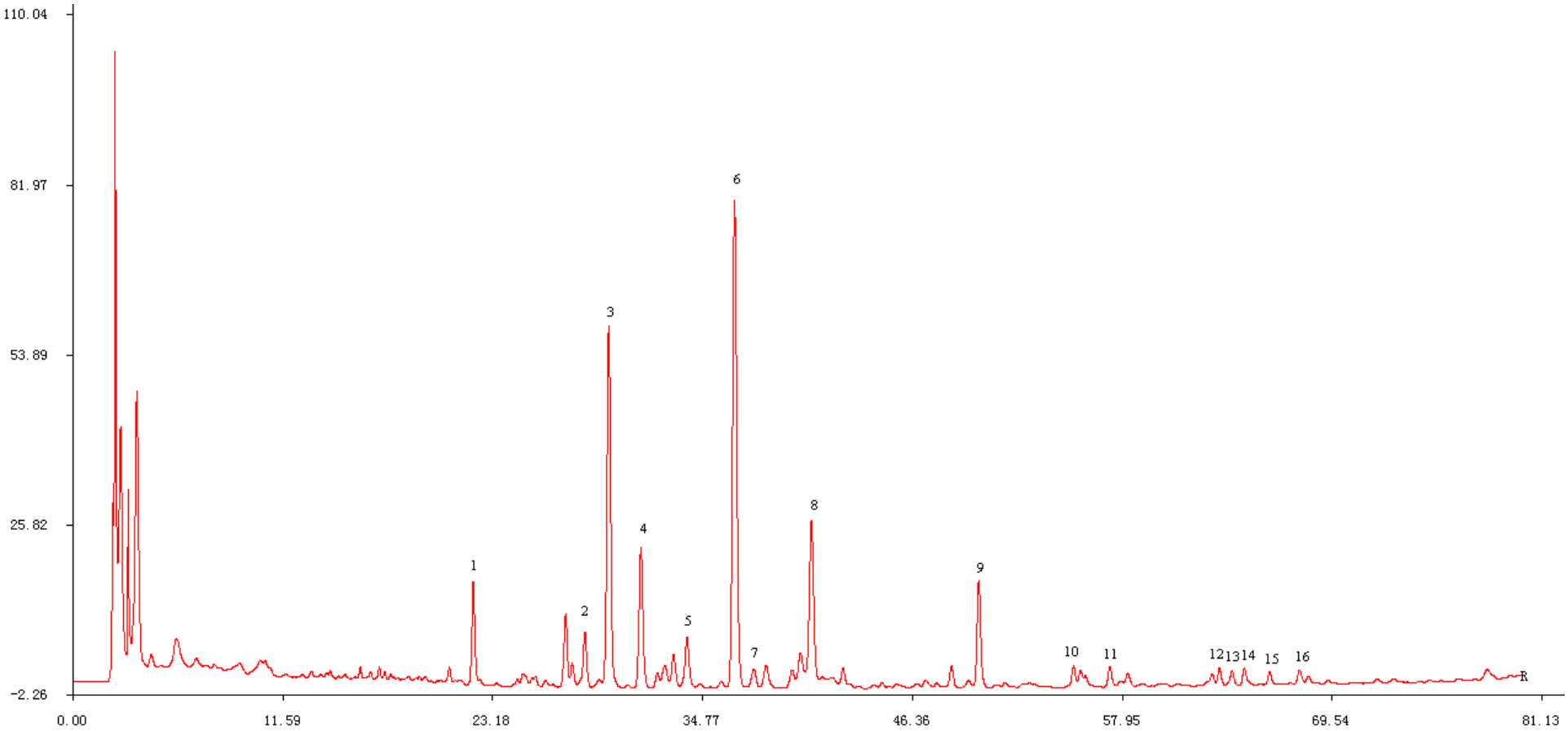

Figure 2. Average artificial HPLC fingerprint common pattern of *M. officinalis* based on 25 samples.

analysis of root of *M. officinalis* samples was performed based on the RPA of all the 16 characteristics chromatographic peaks. The RPA of characteristics constituents in 25 samples of root of *M. officinalis* from various sources formed a matrix of 16x25. The results of HCA are shown in Figure 4, from which the quality characteristics were revealed more clearly. The results of the HCA showed that the samples from different sources could be divided into four quality clusters. Cluster I was distinguished as rubiadin-1-methyl ether-rich chemotype, which contained more rubiadin-1-methyl ether than other anthraquinones. Cluster II was distinguished as 2-hydroxy-1-methoxy-anthraquinone – rich chemotype, which contained more 2-hydroxy-1-methoxy-anthraquinone than other anthraquinones. The cluster III only include sample 18, with higher amount of total anthraquinones and some compounds that peaks 1, 2, 3, 4, 8 and 10 stand for. The cluster IV only include sample 8, in which the content of anthraquinones was least, and only reached to 9.52 μg/g. These results indicated that HCA could be used to distinguish the root of *M.*

Figure 3. Overlapped HPLC chromatogram for fingerprint analysis of *M. officinalis* from different origins.

officinalis with different quality.

Principal component analysis (PCA)

In order to evaluate the discrimination ability of these characteristic components, the PCA was performed on the RPA of 16 characteristics chromatographic peaks in the HPLC profiles using software of SPSS 16.0 (SPSS for Windows 16.0, SPSS Corporation, USA). In the scores plot, samples were mapped in the space spanned by the first two principal components PC1 versus PC2, they could explain over 67% of the variability. The score plot of the first two principal components (Figure 5) showed the clear differentiation of the samples as grouped by PCA. The scatter points showed that the samples could

Table 6. Contents (μg/g) of four anthraquinones in different samples of *M. officinalis* (n = 3).

Collected location	Collection time	1	2	3	4	Total
Gaoyao, Guangdong	August, 2008	18.19	15.18	44.23	10.28	87.88
Zhaoqing, Guangdong	August, 2008	94.21	163.38	80.45	21.53	359.57
Wuhua, Guangdong	August, 2008	9.07	10.26	38.83	6.95	65.11
Deqing, Guangdong	August, 2008	111.15	211.57	79.99	15.34	418.05
Qionghai, Hainan	September, 2008	11.34	21.76	23.24	5.19	61.53
Wanning, Hunan	September, 2008	5.61	21.63	16.57	5.46	49.27
Zijin, Guangdong	August, 2008	36.88	71.25	41.15	10.06	159.34
Nanjin, Fujian	October, 2008	3.13	3.67	1.94	0.78	9.52
Meizhou, Guangdong	August, 2008	9.65	17.76	41.23	10.99	79.63
Heping, Guangdong	August, 2008	9.93	58.71	37.99	7.11	113.74
Kunming, Yunnan#	September, 2008	37.92	38.40	98.56	13.98	188.86
Yangjiang, Guangdong	August, 2008	18.04	24.57	26.83	5.76	75.2
Qingyuan,Guangdong	August, 2008	72.15	175.79	68.32	16.28	332.54
Ganzhou, Jiangxi#	September, 2008	4.99	37.32	48.46	6.62	97.39
Pinghe, Fujiang	October, 2008	0.84	14.07	26.07	4.19	45.17
Yunfu, Shandong#	September, 2008	17.04	98.55	52.00	11.48	179.07
Wuping, Fujian	October, 2008	2.91	35.47	18.92	4.10	61.4
Jiujiang, Jiangxi#	September, 2008	24.42	192.55	28.87	7.71	253.55
Fengkai, Shandong#	September, 2008	5.69	32.52	43.18	7.12	88.51
Baise, Guangxi	September, 2008	4.65	14.02	38.01	5.14	61.82
Yongding, Fujian	October, 2008	2.02	4.51	22.17	3.76	32.46
Shanghang, Fujian	October, 2008	3.28	14.22	28.81	4.73	51.04
Jieyang, Guangdong	August, 2008	11.02	56.71	41.30	8.22	117.25
Jingxi, Guangxi	August, 2008	9.06	39.44	84.31	12.68	145.49
Yunan, Shandong#	October, 2008	10.14	58.79	82.25	14.49	165.67

[a]The data was present at average of duplicates.1, 2-hydroxy-3-hydroxymethyl anthraquinone; 2, 2-hydroxy-1-methoxy-anthraquinone; 3, Rubiadin-1-methyl ether; 4, Rubiadin; #, indicate that the medicinal material were bought from labled location.

be classified into two groups, which were marked as group I to II according to different chemical composition and content, respectively. Group I consisted of samples containing more rubiadin-1-methyl ether, and group II consisted of the samples containing more 2-hydroxy-1-methoxy-anthraquinone. In addition, samples2, 4 and 13 were not included in the group I nor in group II; these samples had a very high content of anthraquinones, which were correspondence with the SA and same to HCA. The results of HCA and PCA could be validated with each other and provided more references for the quality evaluation of root of *M. officinalis*.

Summary of results analysis

The method presented here was used for the first time to determine the four major constituents in the roots of *M. officinalis* from different sources. Several conclusions can be drawn from our results: Chemometrics analysis indicated that the quality of medicinal materials of *M. officinalis* had no significant relativity with geographic location. HCA and PCA could distinguish these samples as different chemical-types but not different geographic population. *M. officinalis* grows in Fujian, Guangdong, Guangxi and Hainan province in China, and these areas have closely geographic distance, and are similar in climates. Therefore, the qualities of *M. officinalis* in these areas have no significant difference. However, *M. officinalis* for medicinal purpose mainly come from cultured plant, and have various farm races commonly existing in growing base, so the quality difference were related with their different germplasm sources and genetic diversity.

SA showed that samples 2, 4, 8, 13 and 18 had little similarity with its similarity, respectively as 0.647, 0.793, 0.588, 0.807, and 0.337. In view of results of content analysis, the samples 2, 4, 13 and 18 had a higher content of anthraquinones with their total content, respectively as 359.57, 418.05, 332.57 and 253.55 μg/g; sample 8 had lowest content of anthraquinones with its total content only as 9.52 μg/g. These results also explained why samples 8 and 18, respectively were grouped as different type in HCA analysis, and samples

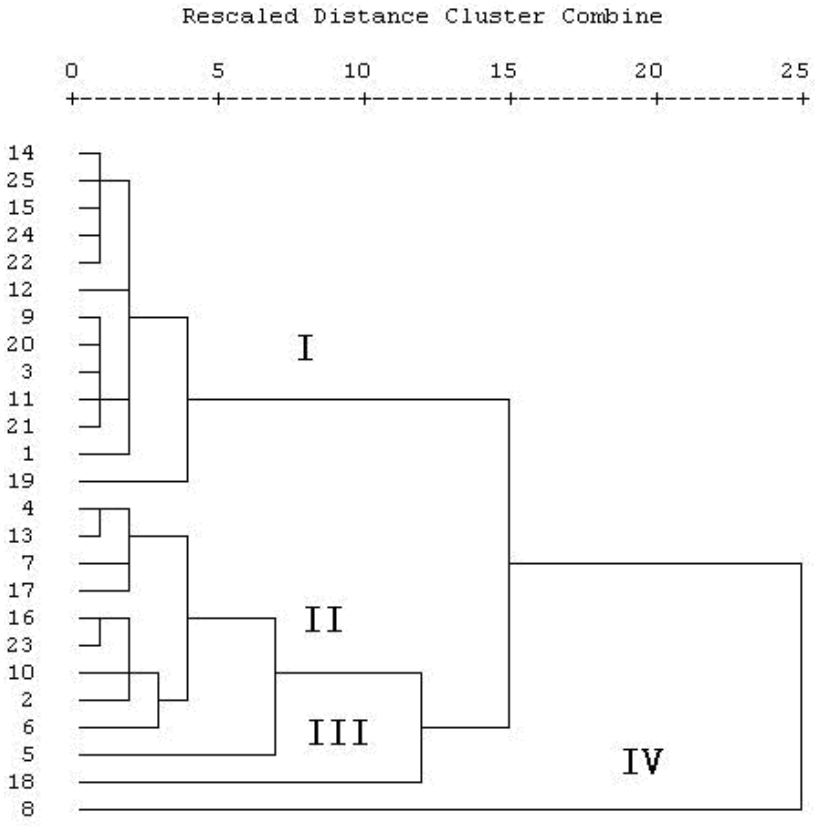

Figure 4. Results of HCA of 25 *M. officinalis* samples (dendrogram using average linkage between groups).

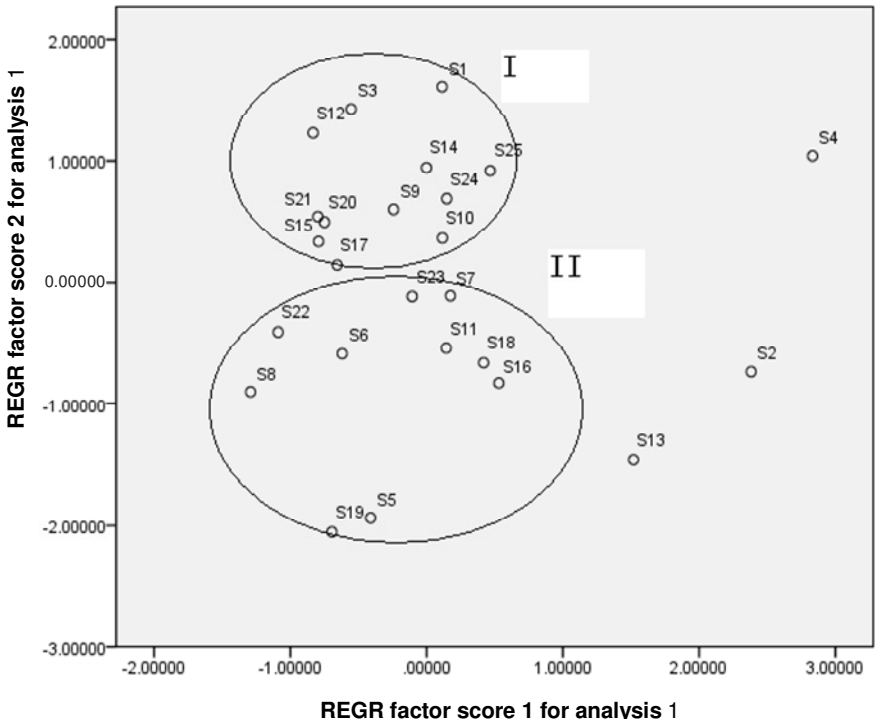

Figure 5. PCA projection of the 25 samples, PC1 and PC2 are the first two principal components.

2, 4 and 13 were not included in the groups I and II in PCA analysis.

Conclusions

A HPLC-DAD chromatographic method was established for the quality control of *M. officinalis*. The method was well validated by systematically comparing chromatograms of all samples from different sources, and certified helpfully to improve the quality control. HPLC fingerprint profiles of the root of *M. officinalis* samples were similar by visual inspection and there were many variations in the chemical contents of four anthraquinones and morphology due to differences of the original plant. Chemometrics methods should be applied with the HPLC fingerprint techniques for analysis of chemical variation of *M. officinalis* samples. In this work, it has been showed that chemometrics techniques such as SA, HCA and PCA were able to classify samples objectively and successfully in accordance with their chemical constituents and content. Furthermore, the method developed in this study will provide an important reference to establish the quality control method for other related TCM preparations.

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