

Determination of Ruscogenin in *Ophiopogonis Radix* by High-performance Liquid Chromatography-evaporative Light Scattering Detector Coupled with Hierarchical Clustering Analysis

Chun-Hua Liu¹, Ming Li¹, Ya-Qian Feng¹, Yuan-Jia Hu², Bo-Yang Yu^{1,3}, Jin Qi¹

¹Department of Complex Prescription of TCM, Jiangsu Key Laboratory of TCM Evaluation and Translational Research, China Pharmaceutical University, Nanjing 211198, ²State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao 999078, ³State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 211198, PR China

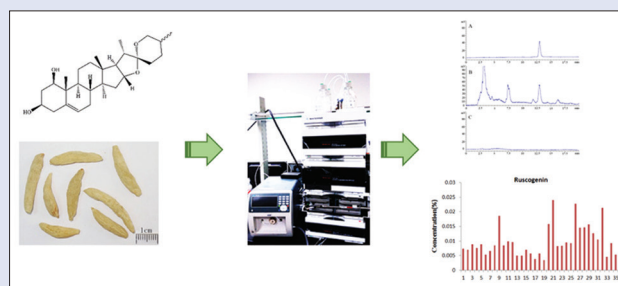
ABSTRACT

Background: *Ophiopogonis Radix* is a famous traditional Chinese medicine. It is necessary to establish a suitable quality control methods of *Ophiopogonis Radix*. **Objective:** To investigate the quality control methods of *Ophiopogonis Radix* by high-performance liquid chromatography (HPLC) coupled with evaporative light scattering detector (ELSD). **Materials and Methods:** A rapid and simple method, HPLC coupled with ELSD, was applied to determine ruscogenin in 35 batches of *Ophiopogonis Radix* samples. Orthogonal tests and single factor explorations were used to optimize the extraction condition of ruscogenin. The content of ruscogenin in different origin was further analyzed by hierarchical clustering analysis (HCA). **Results:** The ruscogenin was successfully determined by HPLC-ELSD with a two-phase solvent system composed of methanol-water (88:12) at a flow rate 1.0 ml/min, column temperature maintained at 25°C, detector draft tube temperature at 42.2°C, nebulizer gas flow rate at 1.4 L/min, and the gain at 8. The result showed the good linearity of ruscogenin in the range of 40.20–804.00 µg/ml ($R^2 = 0.9996$). Average of recovery was 101.3% (relative standard deviation = 1.59%). A significant difference of ruscogenin content was shown among 35 batches of *Ophiopogonis Radix* from different origin, varied from 0.0035% to 0.0240%. HCA based on the content of ruscogenin indicated that *Ophiopogonis Radix* in different origin was mainly divided into two clusters. **Conclusion:** This simple, rapid, low-cost, and reliable HPLC-ELSD method could be suitable for measurement of ruscogenin content ratios and quality control of *Ophiopogonis Radix*. **Key words:** Hierarchical clustering analysis, high-performance liquid chromatography-evaporative light scattering detector, *Ophiopogonis Radix*, ruscogenin

SUMMARY

• *Ophiopogonis Radix* is an important Traditional Chinese Medicine (TCM) to treat and prevent cardiovascular diseases and acute or chronic inflammation for thousands of years. Steroidal saponins were known as the dominant active components for their significant cardiovascular activity, and the most steroid saponin of them is ruscogenin. Therefore, ruscogenin was chosen as the marker component for evaluating the quality of *Ophiopogonis Radix*.

This study focused on establishing a stable, low-cost, simple and practical method of HPLC-ELSD to determine the ruscogenin content, and 35 batches of samples of *Ophiopogonis Radix* were determined. Meanwhile, these results were analyzed by hierarchical clustering analysis and the methodology validation was based on USP34-NF-29 <1225>. Results showed that this analysis method was simple and stable, which would provide an important reference to establish the quality control methodology for other herb preparations and formulas containing *Ophiopogonis Radix*.



Correspondence:

Prof. Bo-Yang Yu,
Department of Complex Prescription of TCM, Jiangsu Key Laboratory of TCM Evaluation and Translational Research and State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 211198, PR China.
E-mail: boyangyu59@163.com.
Dr. Jin Qi,
Department of Complex Prescription of TCM, Jiangsu Key Laboratory of TCM Evaluation and Translational Research, China Pharmaceutical University, Nanjing 211198, PR China.
E-mail: yaoyuelingxing@163.com
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INTRODUCTION

Ophiopogonis Radix, a popular traditional Chinese medicine (TCM), was used to treat and prevent cardiovascular diseases and acute or chronic inflammation for thousands of years,^[1,2] consisting of the dried tuberous root of *Ophiopogon japonicus* (L.f.) Ker-Gawl (known as Maidong).^[3] *O. japonicus* is an evergreen perennial widely distributed in South China, especially in Zhejiang Province (Zhe Maidong in China) and Sichuan Province (Chuan Maidong in China).^[2,4]

On the basis of previous phytochemical studies, steroidal saponins, homoisoflavonoids, and saccharides have been reported as the major components in *O. japonicas*.^[5-9] In these components, steroidal saponins were known as the dominant active components for their significant cardiovascular activity,^[1] and the most steroid saponin of them is ruscogenin [Figure 1].^[10] Ruscogenin was used to treat acute and chronic

inflammatory diseases^[11,12] and cardiovascular diseases such as arrhythmia, angina, and thrombosis.^[13-15] To ensure their clinical efficacy, quality control of *O. japonicus* is of great importance. Given this, ruscogenin was chosen as the marker component for evaluating the quality of *Ophiopogonis Radix*.

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Ruscogenin along with its glycoside saponins are difficult to be detected by UV detector due to the lack of UV chromophore. ELSD is a universal and nonspecific mass detector based on the detection of solute molecules by light scattering after nebulization and evaporation of the mobile phase.^[16,17] Therefore, high-performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD), a practical and low-cost method, serves as a complementary tool for the detection of steroid saponin.

In the past years, it is a challenge for the quality control of *Ophiopogonis Radix* because of the lower content of individual steroidal saponin and homoisflavonoid. Although they may be detected by mass spectrometry (MS),^[6] there are not enough equipments in every laboratory because of immense expense. ELSD could overcome this problem. Several studies have been conducted on the determination of *Ophiopogonin D* or *Ophiopogonin D'* by ELSD,^[18,19] total saponins by UV-VIS spectrophotometer,^[8,20,21] homoisflavonoids by HPLC,^[22] diosgenin, and ruscogenin by nonaqueous capillary electrophoresis,^[23] and fingerprint analysis have been used in some investigations.^[17,24] However, until now few studies^[25] focus on determination of ruscogenin in *Ophiopogonis Radix* by HPLC-ELSD.

In the present study, the total ruscogenin after hydrolysis was determined by HPLC-ELSD, suitable extraction conditions of ruscogenin were systematically optimized, the content in different origin was analyzed and the quality of *Ophiopogonis Radix* was evaluated combining hierarchical clustering analysis (HCA).

MATERIALS AND METHODS

Plant materials

Thirty-five batches of *Ophiopogonis Radix* were collected from different provinces in China, including Jiangsu, Anhui, Guizhou, Yunnan, Zhejiang, Guangxi, Sichuan, Chongqing, Jiangxi, and Hunan. All samples were identified by Prof. Bo-Yang Yu, the voucher specimen (Number: 20111008) was deposited in the Herbarium of Department of Traditional Chinese Medicine of China Pharmaceutical University. Standard of ruscogenin was prepared in our laboratory and identified with purity no <98%.

Chemicals

HPLC-grade methanol was purchased from Yuwang Group (Shandong, China); ultrapure water was prepared by Milli-Qsystem (Milford, MA, USA); other chemicals and solvents were analytical grade. Sulfuric acid was bought from Nanjing Chemical Reagent Co., Ltd. (Jiangsu, China); sodium hydroxide was bought from Xilong Reagent Co., Ltd. (Guangdong, China); diethyl ether was bought from Nanjing Chemical Reagent Co., Ltd. (Jiangsu, China).

Instruments

Agilent 1260 HPLC System (Agilent Technologies, Palo Alto, CA, USA) comprises a quaternary solvent delivery system, an on-line degasser,

an auto-sampler, a column temperature controller, and an analytical workstation (Agilent LC B.04.03 ChemStation) coupled with detector Alltech 3300 ELSD (Alltech, USA).

Chromatographic conditions

The analytical column was a Merck RP-C₁₈ (4.6 mm × 250 mm, 5 μm) with C₁₈ guard column. The mobile system consisted of methanol-water (88:12) at a flow rate of 1.0 ml/min. Column temperature was maintained at 25°C. The draft tube temperature was at 42.2°C, the nebulizer gas flow rate was at 1.4 L/min, the gain was at 8. The solvent was filtered through a 0.22 μm filter and degassed. The sample injection volume was 10 μl. The total acquisition time was 25 min.

Reference standard solution preparation

Standard stock solutions with known concentrations were obtained by dissolving accurately weighted ruscogenin in methanol.

Optimization of extraction condition

As, the extraction of ruscogenin was affected by many factors, so orthogonal designs were taken to optimize the extraction condition, including optimization of acid hydrolysis conditions and extraction of ruscogenin.

Optimization of acid hydrolysis conditions

According to related literatures and previous preliminary experiments,^[25] orthogonal design 1 was applied to select the acid hydrolysis conditions of ruscogenin in *Ophiopogonis Radix*. 2.0 g *Ophiopogonis Radix* was transferred to a suitable flask, and 25 ml sulfuric acid with different concentration was respectively added to hydrolysis for different time in water bath with different temperature according to factors [Table 1]. Then the extracting solution was adjusted to neutral with 16% NaOH and filtered. The residue was collected and dried at 60°C and then Soxhlet extracted with 70 ml petroleum ether for 6 h. Petroleum ether layer was collected and evaporated to dryness. Then the residue was transferred to a 2 ml volumetric flask and diluted to 2 ml with methanol.

Table 1: Orthogonal factors and levels (acid hydrolysis)

Levels	Factors		
	Sulfuric acid concentration (%) (A)	Hydrolysis temperature (°C) (B)	Hydrolysis time (h) (C)
1	2	80	4
2	3	90	6
3	4	100	8

Table 2: L₉ (3⁴) results of orthogonal design 1

Number	Sulfuric acid concentration (A)	Hydrolysis temperature (B)	Hydrolysis time (C)	Error (D)	Ruscogenin (%)
1	1	1	1	1	0.00076
2	1	2	2	2	0.0019
3	1	3	3	3	0.0021
4	2	1	2	3	0.0017
5	2	2	3	1	0.0021
6	2	3	1	2	0.0059
7	3	1	3	2	0.0022
8	3	2	1	3	0.0026
9	3	3	2	1	0.0026
Average 1	0.002	0.002	0.003	0.002	
Average 2	0.003	0.002	0.002	0.003	
Average 3	0.002	0.004	0.002	0.002	
Range	0.001	0.002	0.001	0.001	

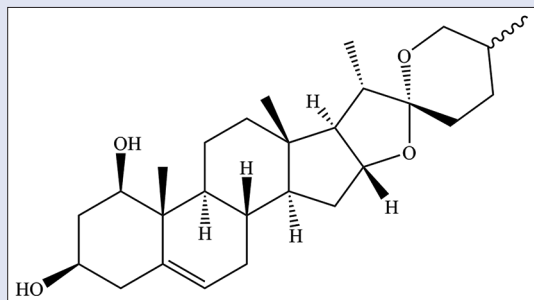


Figure 1: The structure of ruscogenin

The obtained solution was passed through a membrane filter (0.45 μm), and the first part of the filtrate was discarded, then the successive filtrate was collected as the sample solution. Ten microliters sample solution and standard solution were injected into the chromatography, in which the chromatograms were recorded, and the content of ruscogenin was calculated in *Ophiopogonis Radix* coupled with visual analysis and variance analysis. The results of the orthogonal test and extreme difference analysis were presented in Table 2. All the analysis of variance were performed by the statistical software SPSS version 19.0 (SPSS Inc., Chicago, Illinois, USA) and the result was listed in Table 3.

Optimization of extraction of ruscogenin

Orthogonal design 2 was applied to select the best extraction condition of ruscogenin. During this procedure, the steps before being extracted with petroleum ether were similar to "Optimization of acid hydrolysis conditions," and just the acid hydrolysis was performed using certain factors with 25 ml 3% H_2SO_4 and being refluxed in boiling water bath for 6 h. After being adjusted to neutral with 16% NaOH and filtered, residue was collected and dried at 60°C and then extracted with 70 ml different solvents, different methods, and different time as factors [Table 4]. The rest steps were same as the corresponding process of "Optimization of acid hydrolysis conditions." The results of orthogonal test 2 and extreme difference analysis were presented in Table 5. The analysis of variance [Table 6] was performed by statistical software SPSS version 19.0 (SPSS Inc.).

Table 3: Analysis of variance

Source	Type III sum of squares	df	Mean square	F	Significant
Corrected model	1.236E-5 ^a	6	2.060E-6	1.079	0.554
Intercept	5.299E-5	1	5.299E-5	27.768	0.034
Sulfuric	3.961E-6	2	1.981E-6	1.038	0.491
Temperature	6.288E-6	2	3.144E-6	1.648	0.378
Time	2.108E-6	2	1.054E-6	0.552	0.644
Error	3.816E-6	2	1.908E-6		
Total	6.916E-5	9			
Corrected total	1.617E-5	8			

^a $R^2=0.764$ (adjusted $R^2=0.056$)

Table 4: Factors and levels

Levels	Factors		
	Extraction solvents (A)	Extraction methods (B)	Time (h) (C)
1	Petroleum ether	Soxhlet extraction	2
2	Cyclohexane	Reflux	4
3	Ether	Sonication	6

Table 5: L_9 (3^4) results of orthogonal design 2

Number	Solvents (A)	Extraction methods (B)	Extraction time (C)	Error (D)	Ruscogenin (%)
1	1	1	1	1	0.0029
2	1	2	2	2	0.0032
3	1	3	3	3	0.0032
4	2	1	2	3	0.0055
5	2	2	3	1	0.0056
6	2	3	1	2	0.00054
7	3	1	3	2	0.0059
8	3	2	1	3	0.0036
9	3	3	2	1	0.0038
Average 1	0.003	0.005	0.002	0.004	
Average 2	0.004	0.004	0.004	0.003	
Average 3	0.004	0.003	0.005	0.004	
Range	0.001	0.002	0.003	0.001	

Single factor experiment

To select better extraction method, the different hydrolysis time was investigated based on orthogonal design 1, compared in these experiments (4 h, 6 h) as shown in Table 7, and the different extraction solvents (cyclohexane, ether) were analyzed on the basis of orthogonal experiment 2 [Table 8].

Sample solutions preparation

Sample solutions preparation were based on above optimized conditions as following: About 2.0 g *Ophiopogonis Radix*, powdered to pass through a 24 mesh sieve and accurately weighed, was transferred to a 100 ml glass-stoppered conical flask. Twenty-five milliliters 3% H_2SO_4 was accurately added to reflux in boiling water bath for 6 h. Extracting solution was adjusted to neutral with 16% NaOH and then filtered. The collected residue, dried at 60°C, was soxhlet extracted with 70 ml ether for 6 h. Ether layer was collected, and evaporated to dryness and the residual were transferred to a 2 ml volumetric flask and diluted to 2 ml with methanol, which passed through a membrane filter (0.45 μm porosity), discarding the first part of the filtrate and collecting the successive filtrate as the sample solution. Ten microliters sample solution and standard solution were injected into the chromatography as "Chromatographic conditions," in which the chromatograms were recorded, and the content of ruscogenin of *Ophiopogonis Radix* was calculated. Thirty-five batches of *Ophiopogonis Radix* samples were extracted with this same method.

Statistical analysis

Statistics was analyzed with SPSS version 19.0 statistical software (SPSS, USA).

RESULTS

Optimization of extraction condition

The results [Table 2] showed that hydrolysis temperature has a notable influence on the acid hydrolysis of saponins from *O. japonicus*. Besides, visual analysis [Table 2] stated that the influence to the mean extraction yields of the ruscogenin decreases in the order: B > A \geq C according

Table 6: Analysis of variance

Source	Type III sum of squares	df	Mean square	F	Significant
Corrected model	1.843E-5 ^a	4	4.607E-6	4.755	0.080
Intercept	0.000	1	0.000	134.450	0.000
Solvents	0.000	0			
Methods	7.985E-6	2	3.992E-6	4.121	0.107
Time	1.044E-5	2	5.221E-6	5.389	0.073
Error	3.875E-6	4	9.689E-7		
Total	0.000	9			
Corrected total	2.230E-5	8			

^a $R^2=0.826$ (adjusted $R^2=0.652$)

Table 7: Results of different hydrolysis time ($n=3$)

Hydrolysis time (h)	Ruscogenin (%)	RSD (%)
4	0.0068	10.27
6	0.0070	1.35

RSD: Relative standard deviation

Table 8: Results of different extraction solvents ($n=3$)

Solvents	Ruscogenin (%)	RSD (%)
Cyclohexane	0.00658	3.53
Ether	0.00702	1.35

RSD: Relative standard deviation

to the *R* values, and the best combination of test factors was A2B3C1. However, based on analysis of variance [Table 3], A, B, and C have no significant difference. Therefore, to ensure the repeatability and stability of the method, further experiments as "Investigation of hydrolysis time" were applied to optimize the hydrolysis time.

Optimization of extraction of ruscogenin

Extraction time have a notable influence on the extraction of ruscogenin [Table 5]. Besides, visual analysis [Table 5] stated that the influence to the mean extraction yields of the ruscogenin decreases in the order: C > B > A according to the *R* values, and the best combination of test factors was A2B1C3 or A3B1C3. However, based on analysis of variance [Table 6], A, B and C have no significant difference. Therefore, to select the best extraction method and ensure the stability, further experiments were performed to compare A2B1C3 with A3B1C3 as "Investigation of extraction solvents."

Single factor experiment

Investigation of hydrolysis time

The results [Table 7] showed that 6 h was appropriate for hydrolysis because its relative standard deviation (RSD) was not more than 2.0%. So, the optimal conditions were determined: 25 ml, 3% sulfuric acid concentration, refluxed in the boiling water bath for 6 h.

Investigation of extraction solvents

Solution ether was appropriate [Table 8] because of its RSD < 2.0% as well as relatively high content of ruscogenin, so ether was chosen for further study.

Determination of ruscogenin in Ophiopogonis Radix

Method validation of ruscogenin: Precision, linearity, accuracy, specificity, stability, detection limit, quantitation limit, and ruggedness.

The method was validated through investigation on the precision, linearity, accuracy, specificity, stability, and ruggedness.

Precision (repeatability and intermediate precision)

Repeatability was assessed with 9 sample solutions of low, medium, and high levels (1.0, 2.0 and 3.0 g respectively), each with three triplicates. The content of ruscogenin was calculated with the external reference method [Table 9]. The RSDs of inner-day variation of three levels were in the range from 0.84% to 4.40% for the content of ruscogenin, and average RSD was 3.53%.

Intermediate precision was performed through investigation of 3 different days, 3 different analysts and 3 different columns, each with 3 triplicates [Tables 10-12]. These results showed good precision.

Linearity and range

Good linearity was shown with correlation 0.9996 [Table 13].

Accuracy

Accuracy was calculated as the percentage of recovery by the assay of the known added amount of ruscogenin in the sample.

Three different concentrations (low, medium, and high) of reference standard were spiked to 2.0 g of Ophiopogonis Radix, all in triplicates. Average recovery was 101.32% [Table 14].

Specificity

The integration peak in the chromatogram of the sample solution was corresponding in time to the peak in the chromatogram of

Table 9: Repeatability

	Weight of drug (mg)	Peak area	Content (%)	Average (%)	RSD (%)	Average (%)	RSD (%)
Low	1.0000	381.40	0.009132	0.008740	4.40	0.008996	3.53
	0.9999	356.40	0.008727				
	1.0003	334.62	0.008362				
Medium	2.0033	1067.48	0.009089	0.009005	2.70		
	2.0022	1085.14	0.009195				
	2.0046	1006.36	0.008731				
High	3.0009	1976.34	0.009170	0.009243	0.84		
	3.0010	2026.32	0.009325				
	3.0017	1997.82	0.009234				

RSD: Relative standard deviation

Table 10: Intermediate precision-different days

	Weight of drug (mg)	Peak area	Content (%)	Average (%)	RSD (%)	Average (%)	RSD (%)
Day 1	2.0058	1031.89	0.008874	0.008685	1.90	0.008419	3.71
	2.0028	984.252	0.008610				
	2.0057	979.721	0.008571				
Day 2	2.0060	880.935	0.007980	0.008309	3.81		
	2.0030	984.718	0.008612				
	2.0046	939.189	0.008336				
Day 3	2.0037	883.394	0.008044	0.008263	3.89		
	2.0034	986.822	0.008622				
	2.0031	909.143	0.008162				

RSD: Relative standard deviation

Table 11: Intermediate precision-different analysts

	Weight of drug (mg)	Peak area	Content (%)	Average (%)	RSD (%)	Average (%)	RSD (%)
Analyst 1	2.0037	904.516	0.008132	0.008328	2.92	0.008252	3.75
	2.0034	924.282	0.008252				
	2.0031	982.708	0.008599				
Analyst 2	2.0058	820.044	0.007607	0.008062	4.92		
	2.0028	922.048	0.008241				
	2.0057	940.296	0.008338				
Analyst 3	2.0039	993.775	0.008661	0.008365	3.46		
	2.0036	941.168	0.008352				
	2.0033	896.116	0.008083				

RSD: Relative standard deviation

Table 12: Intermediate precision-different column

	Weight of drug (mg)	Peak area	Content (%)	Average (%)	RSD (%)	Average (%)	RSD (%)
Column 1	2.0030	916.300	0.008206	0.008345	1.44	0.008284	1.29
	2.0034	951.900	0.008416				
	2.0031	951.000	0.008412				
Column 2	2.0028	901.500	0.008117	0.008193	0.93		
	2.0028	913.800	0.008819				
	2.0027	926.900	0.008270				
Column 3	2.0029	949.100	0.008402	0.008313	0.96		
	2.0036	923.597	0.008247				
	2.0033	930.500	0.008289				

RSD: Relative standard deviation

Table 13: Linearity and range

Analyte	Regression equation	<i>R</i> ²	Linearity range (µg/ml)	Residual sum of squares
Ruscogenin	y=0.6705x-4.7693	0.9996	40.20-804.00	3.692

standard solution. No such peak of that retention time appeared in the chromatogram of blank solvent [Figure 2].

Stability

The stability of sample solution was investigated by comparing the peak areas and retention time of ruscogenin in the chromatograph of the same sample solution, after stored at room temperature for different time (0, 2, 4, 6, 8, 12, 24 h). Stability was evaluated by calculating the RSD of peak area and retention time. The RSD were 0.61% and 0.46%, respectively [Table 15]. The analyses were found to be stable within 24 h in the sample solution.

Detection limit

According to USP34 NF 29 chapter <1225>, detection limit was 0.0804 µg/ml and when the concentration was 0.0804 µg/ml, signal to noise ratios were about 3:1.

Quantitation limit

According to USP34 NF 29 chapter <1225>, when the concentration was 0.1608 µg/ml, signal to noise ratios were about 10:1. Quantitation limit was 0.1608 µg/ml.

Ruggedness

The ruggedness of the established method was evaluated by examining its stability with small variations of procedural parameters, including pH of mobile phase, ratio of components in mobile phase, ELSD detector, different columns, flow rate, injection volume, and column temperature. Results of ruggedness indicated that the drift tube temperature of ELSD should be not lower than 42.2°C; the pH of mobile phase, ratio of components in mobile phase, flow rate, injection volume, and column temperature can be slightly adjusted for system suitability.

Content of ruscogenin in 35 batches of *Ophiopogonis Radix*

Sample solutions of 35 batches of *Ophiopogonis Radix* were determined

using the validated chromatographic method for ruscogenin. Its content varied from 0.0035% to 0.0240% [Table 16, Figures 3 and 4].

Hierarchical clustering analysis

In order to assess the variation of *Ophiopogonis Radix* in different origin, HCA was performed based on the content of ruscogenin from HPLC-ELSD profiles. Between groups linkage method was performed, and Squared Euclidean distance was selected as a measurement. In HCA dendrogram, the shorter distance between two samples indicated their higher similarity and the samples clustered into the same group were the most similar ones.^[26] The results [Figure 5] demonstrated that the 35 batches of samples were obviously divided into two main clusters according to their contents. Sample 1–19 were belonging to cluster-I, which were mainly commercial *Ophiopogonis Radix* (Chuan Maidong) although collected from different province, whereas sample 20–35 were belonging to cluster-II, which were mainly collected from Zhejiang Province and partly from cultivation base in Sichuan Province.

Table 14: Recovery test

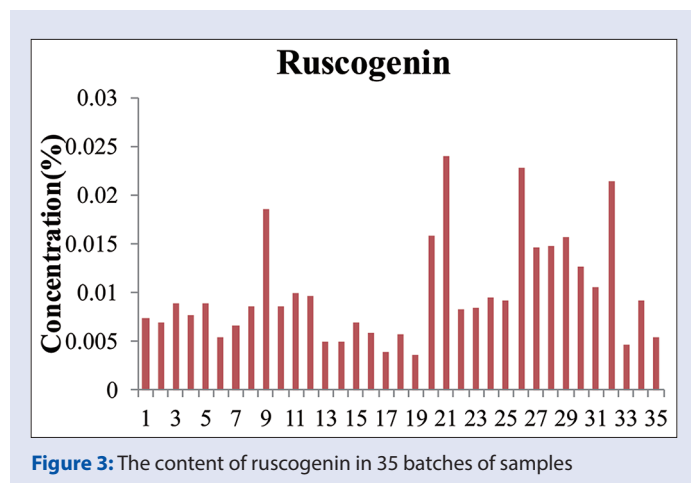
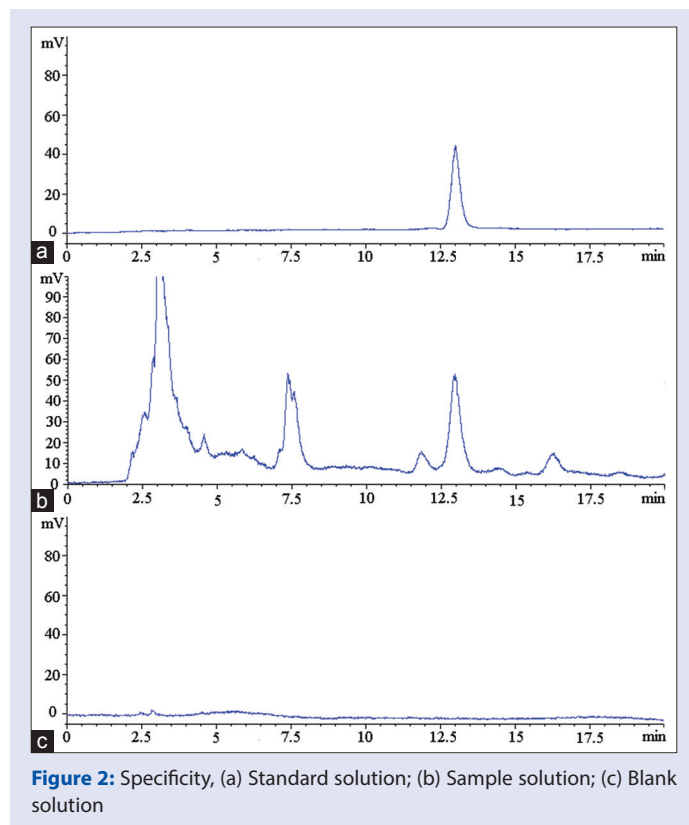
	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)	Average (%)	RSD (%)
Low	0.180	0.150	0.333	102.10	100.47	1.49
	0.180	0.150	0.328	99.16		
	0.180	0.150	0.330	100.13		
Medium	0.180	0.180	0.366	103.11	102.31	0.97
	0.180	0.180	0.366	102.62		
	0.180	0.180	0.362	101.19		
High	0.180	0.220	0.408	103.45	101.14	2.10
	0.180	0.220	0.402	100.70		
	0.180	0.220	0.399	99.27		
Average (%)	101.32					
RSD (%)	1.59					

RSD: Relative standard deviation

Table 15: Stability

Time (h)	Peak area	Retention time (min)
0	596.500	13.000
2	597.852	12.957
4	596.984	12.926
6	598.635	12.925
8	606.973	12.857
12	599.604	12.876
24	601.886	12.905
Average	599.711	12.926
RSD (%)	0.61	0.46

RSD: Relative standard deviation



DISCUSSION

Optimization of high-performance liquid chromatography-evaporative light scattering detector condition

HPLC, a simple, stable, and durable analysis techniques, is widely applied to assess the types and content of chemical compositions

Table 16: Content of 35 batches of opiopogons radix (n=2)

Origin	Ruscogenin (%)	RSD (%)
Huayuanzhen	0.007350	0.08
Anhuiyishengtang	0.006965	0.12
Hunan zhenxing	0.008926	0.16
Guangmingxiang	0.007620	4.20
Guangxi yulin I	0.008873	1.36
Xuzhoupenzu	0.005413	0.55
Yunnan baiyao	0.006599	0.05
Bozhouruicao	0.008504	0.15
Wuxi	0.010854	1.85
Guizhou	0.008485	0.24
Giangxiyulin II	0.009861	0.01
Yishengtang	0.009618	2.44
Chongqing I	0.004986	2.64
Jiangxi II	0.005001	1.03
Huayuanzhen	0.006938	0.38
Laomaxiang	0.005786	1.10
Bozhouguoyuan	0.003801	3.70
Hunan II	0.005707	0.91
Chongqing II	0.003509	2.53
Huayuanzhen	0.015788	0.54
Anxianhuagaizhen	0.023970	0.19
Sichuan	0.008284	0.69
Huayuanzhen	0.008394	2.82
Huayuanzhen	0.009503	1.23
Huayuanzhen	0.009225	0.90
Cixifuhai	0.022728	1.83
Cixidonghai	0.014551	0.47
Cixidonghai	0.014712	0.72
Songjiacun II	0.015698	2.03
Cixifuhaihuangtang	0.012621	1.61
Songjiacun I	0.010583	1.25
Hangzhou 12081	0.021383	0.78
Zhejiang 11003	0.004586	1.62
Huayuanzhen	0.009225	0.90
Shengshan	0.005315	2.64
Average	0.009973	
80% average	0.007978	

RSD: Relative standard deviation

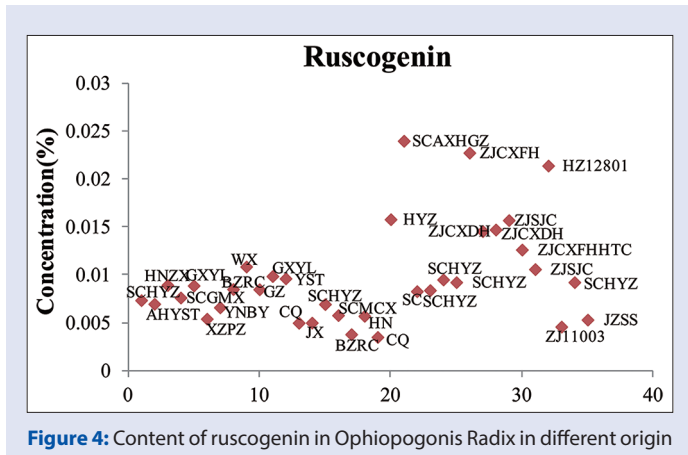


Figure 4: Content of ruscogenin in Ophiopogon Radix in different origin

in TCMs. HPLC can be equipped with multiple detectors, such as a UV detector, ELSD, fluorescence detector and MS, of which the UV detector is most frequently used.^[27] Given that ruscogenin lack of UV chromophore, HPLC coupled with ELSD was applied to our research. In order to achieve rapid and stable analysis of ruscogenin in Ophiopogon Radix, ELSD different nebulizer-gas flow rate (1.3, 1.4, 1.5, 1.6 L/min), and drift tube temperature (39.2°C, 40.6°C, 42.2°C, and 45°C) were compared according to the value of signal-to-noise ratio. These results indicated that the value of signal-to-noise ratio was better when the nebulizer-gas flow rate was at 1.4 L/min and drift tube temperature was at 42.2°C. Therefore, they were chosen for the analysis.

To obtain the chromatograms with the good separation, different chromatographic condition, including mobile phase system (methanol- and acetonitrile-water), pH of mobile phase (water, 0.02% aqueous solution of formic acid, 0.10% aqueous solution of formic acid, and 0.3% aqueous solution of triethylamine), methanol ratio (85%, 88%, and 90%), flow rate (1.0, 1.1, and 1.2 ml/min), column temperature (15°C, 25°C, and 30°C), and injection volume (5, 10, 15 and 20 µl) were compared and analyzed. As a result, based on system suitability parameters (tailing factor, resolution, and number of theoretical plates), methanol-water (88-12), flow rate 1.0 ml/min, column temperature 25°C, and injection volume 10 µl were finally chosen.

Optimization of sample preparation

In order to obtain better extraction condition, variables involved in the procedure such as hydrolysis and extraction method (extraction firstly, then hydrolysis) and different acid (hydrochloric acid, sulfuric acid)

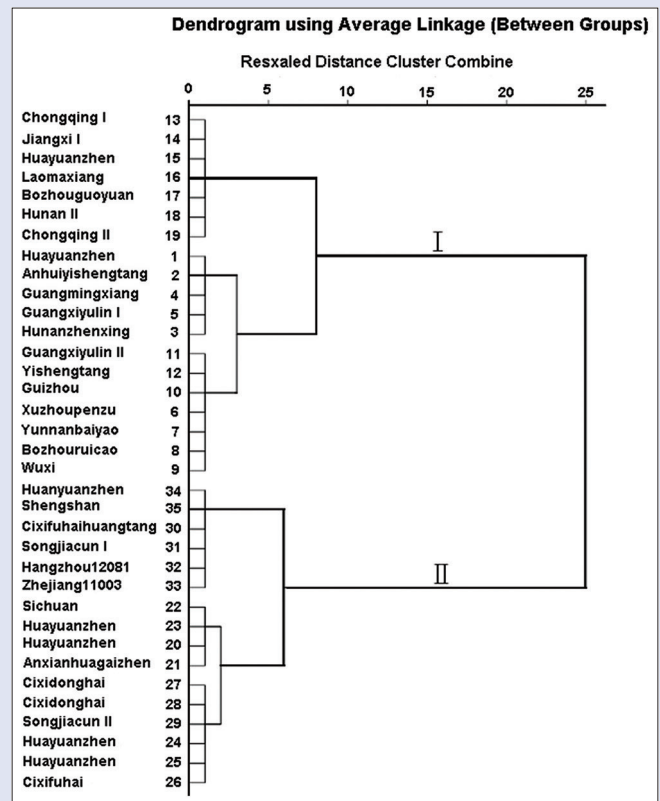


Figure 5: Dendrograms of hierarchical cluster analysis for the 35 tested samples of Ophiopogon Radix

were optimized in preliminary experiment. About 2.0 g *Ophiopogonis Radix*, powdered to pass through a 24 mesh sieve, accurately weighed, was Soxhlet extracted with 70 ml methanol for 4 h once, then solution was collected, evaporated to dryness, and residue was hydrolyzed with 25 ml 3% H₂SO₄ in water bath for 4 h, and 16% NaOH was added to adjust neutral. Then the hydrolysate was extracted with 50 ml petroleum ether for 3 times, and petroleum ether layer was collected and evaporated to dryness. The rest steps were similar with corresponding programs in "Sample solutions preparation." This result suggested that more impurity was included in a sample solution with this method, compared with the procedure of hydrolysis firstly, then extraction as "Optimization of extraction condition" of the methods section. Moreover, the preliminary experiment also investigated the hydrolysis of different acid (2.22% hydrochloric acid, 3% sulfuric acid), the other steps were same as corresponding "Optimization of extraction condition" of methods section. The result indicated that content of ruscogenin with 3% sulfuric acid was more stable (the RSD was <2%, while more than 10% with hydrochloric acid, *n* = 3). Thus, sulfuric acid was chosen for further experiments. Based on these preliminary experiments, orthogonal tests, and single factor explorations were designed in a present experiment to select the best extraction condition.

Validation of methodology

According to USP34-NF-29 chapter <1225>, precision (repeatability and intermediate precision), linearity, accuracy, specificity, stability, detection limit, quantitation limit, and ruggedness were investigated. Average RSD of repeatability was 3.53%; average RSD of intermediate precision-different days, different analysts and different column were 3.71%, 3.75%, and 1.29%, respectively. The calibration curves exhibited good linearity ($R^2 = 0.9996$) in the range of 40.20–804.00 g/ml. Average of recovery was 101.3% (RSD = 1.59%, *n* = 9). Stability RSD of the area and retention time was 0.61% and 0.46%. The detection limit was 0.0804 µg/ml and quantitation limit was 0.1608 µg/ml. Results of ruggedness indicated that the drift tube temperature of ELSD detector (Alltech ELSD 3300) should be not lower than 42.2°C. The pH of mobile phase, the ratio of components in the mobile phase, flow rate, injection volume, and column temperature can be slightly adjusted for system suitability. These indicated that the analysis method was simple, feasible, and stable.

Hierarchical clustering analysis

Based on the content of ruscogenin in *Ophiopogonis Radix*, HCA was performed to analyze results of 35 batches of samples (*Ophiopogonis Radix*). These results illustrated that samples could be appropriately divided into two main clusters related to their contents and the difference of content in samples was significant in a different origin. It is well known that types and quantities of chemical components in medicinal plants are probably considerably affected on collection at different times and from different localities.^[26,28] This reason is related to different environments, soil texture condition, growing time, harvesting time, and cultivated techniques. Therefore, in order to control the quality of *Ophiopogonis Radix*, it is necessary to establish a stable and simple analysis method. In the present study, a practical and the low-cost technique could evaluate the quality through systematically optimization of extraction condition, chromatographic condition, and method validation.

CONCLUSION

In the present work, an impersonal, valid, low-cost and the stable analysis method was developed and applied to the quality control of the *Ophiopogonis Radix* through systematically optimization of extraction condition, chromatographic condition and method validation. Sample solutions of 35 batches of *Ophiopogonis Radix* were determined using the validated chromatographic method for ruscogenin. The content of

ruscogenin varied from 0.0035% to 0.0240%. Our results also showed that HPLC-ELSD enjoyed the practical advantages of simple analysis procedure and reduced quantity of cost consumption for the quality control of *Ophiopogonis Radix*. Therefore, the method developed in this study would provide an important reference to establish the quality control method for other herb preparations and formulas containing *Ophiopogonis Radix*.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Bo-Yang Yu



Jin Qi

ABOUT AUTHORS

Bo-yang Yu, Jiangsu Key Laboratory of TCM Evaluation and Translational Research, Department of Complex Prescription of TCM, China Pharmaceutical University, Nanjing 211198; State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 211198, PR China.

Jin Qi, Jiangsu Key Laboratory of TCM Evaluation and Translational Research, Department of Complex Prescription of TCM, China Pharmaceutical University, Nanjing 211198, PR China.