

Ultra-performance liquid chromatography fingerprinting for quality control of *Phragmitis rhizoma* (Lugen) produced in Baiyangdian

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ABSTRACT

Objective: To establish an ultra-performance liquid chromatography (UPLC) fingerprinting method for quality control of *Phragmitis rhizoma* from Baiyangdian. **Materials and Methods:** Ultrasonic extraction with 70% methanol was performed on 10 samples of *P. rhizoma* collected from 10 different villages in Baiyangdian. The sample solutions were analyzed by Waters UPLC equipped with the ACQUITY UPLC BEH C₁₈ column and photodiode array (PDA) detector, and gradient eluted with acetonitrile/water as the mobile phase. The flow rate was set to 0.1 mL/min; the column temperature was set to 25°C; and the detection wavelength was set to 285 nm. **Results:** The chromatograms of the 10 samples showed 27 common peaks, of which one was identified as the ferulic acid standard. The similarity indexes were all above 0.82. Hierarchical cluster analysis showed that the constituents and their quantities differed according to the diameter of the original plant, which is related to its age. **Conclusion:** The UPLC fingerprinting method had the advantages of being fast, accurate, and highly efficient; this indicated that it can be used for quality control of *P. rhizoma* produced in Baiyangdian. Also, the relation between the quality and diameter/age of the plant needs to be further investigated.

Key words: Baiyangdian, fingerprint, *Phragmitis rhizoma*, ultra-performance liquid chromatography

In recent years, traditional Chinese medicine has been gaining popularity and is widely used clinically because of its excellent qualities, such as low toxicity, less side-effects, good medical effects, and rare drug tolerance. It has long been proven that the major difference between traditional Chinese medicine and allopathic medicine is that the former consists of multiple components and has multiple targets in the body.

Traditionally, high-performance liquid chromatography (HPLC) fingerprinting is used for quality control of traditional Chinese medicines; however, this method cannot meet the requirements of high-throughput analysis and is greatly restricted, because its efficiency is low and the separating time is long, generally more than 1 h.^[1-3] Ultra-performance liquid chromatography (UPLC) can solve this

problem. Compared with the traditional HPLC method, it has the unparalleled advantages of ultra-high column efficiency, separation capability, and separation velocity. It has similar or better separating effects as compared to HPLC, and it can greatly reduce the operating time.^[4,5] Therefore, the UPLC fingerprinting technology has promising prospects and will certainly be used more and more widely as an ideal means of quality control in Chinese medicine.

Phragmitis rhizoma is the fresh or dried rhizome of *Phragmites communis* Trin.,^[6] which is commonly used in Chinese medicine. According to the records of the “Compendium of Materia Medica,” *P. rhizoma* is “sweet in taste and cold in nature, in lung and stomach channel,” which is described in theory of traditional Chinese medicine. It is used as an antiemetic, diuretic, and antipyretic. It is also used to treat polydipsia, cough, suppurative lung disease, and dysuria and other bladder abnormalities.

The Baiyangdian regions (Baoding, Hebei Province, China) are rich in *P. communis*, and have been bestowed the honorary title “land with the best reed in Hebei.” This province

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produces good quality *P. communis* in large quantities, and is an important source of *P. rhizoma* in the North of China.

After retrieving the literature published in the last 20 years, we found that no paper has reported the use of UPLC fingerprinting to evaluate the quality of *P. rhizoma* produced in Baiyangdian. This paper therefore, to our knowledge, is the first to present the method for obtaining UPLC fingerprints of *P. rhizoma* produced in Baiyangdian. We believe that this method will allow objective evaluation of the quality of *P. rhizoma* produced in Baiyangdian.

MATERIALS AND METHODS

Equipment

A Waters Acquity UPLC™ system (Waters Company, MA, USA), with a binary solvent manager, an automatic sample-feeding device, and a photodiode array detector (DAD) binary lineup examiner, was used for liquid chromatographic analysis. Shumei KQ2200DE ultrasonic cleaning instrument (Kunshan Shumei Ultrasonic Instrument Co., Kunshan, China) was used for extraction. The AUTO SCIENCE solvent filtration device, a drying oven (Senxin DGG-9240A), and Milli-Q super-purified water device from Millipore (Bedford, MA, USA) were the other instruments used.

Reagents and materials

Ferulic acid standard was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Peking, China). The batch number was 110773-200813, and the purity was more than 98%.

Ten samples were collected from ten villages around Baiyangdian, Hebei, China, and all were identified as the fresh rhizomes of *P. communis*. Then, the samples were dried, pulverized, and sieved (100 mesh sieve). All the voucher specimens were deposited in our laboratory. The 10 samples from different places are listed in Table 1.

HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany), and ultrapure water was made using the super water purification system. All the other reagents used were of analytical grade.

Ultra-performance liquid chromatography conditions

Sample solutions were analyzed using Waters Acquity UPLC with the BEH C₁₈ column (50 mm × 2.1 mm; 1.7 μm, Waters Company) and DAD detector. The mobile phase consisted of acetonitrile and water. The most suitable linear gradient elution program can be seen in Table 2. The flow rate used was 0.3 ml/min, and the temperatures of the chromatographic column and the automatic sample-feeding room were 30°C and 4°C, respectively. The DAD

Table 1: Representative samples of *Phragmites rhizoma* from Baiyangdian

Sample no.	Collection site	Diameter size*	Collection date
S1	Liutong	Small	05-16-2009
S2	Zhaobeikou	Big	05-16-2009
S3	Zhongqilizhuang	Small	05-18-2009
S4	Xidawu	Big	05-18-2009
S5	Dongdiantou	Big	05-18-2009
S6	Wangjiazhai	Big	05-19-2009
S7	Dizhuang	Big	05-27-2009
S8	Lianggou	Big	05-27-2009
S9	Liguangcuncun	Small	05-30-2009
S10	Shaozhuangzi	Big	06-03-2009

*"Diameter size" means the diameter of the stem of rhizome; "big" means that the diameter is bigger than 1 cm; "small" means that the diameter is smaller than 1 cm

Table 2: Linear gradient elution program for the mobile phase

Time (min)	(A) Water (%)	(B) Acetonitrile (%)
0	95	5
2	95	5
5	88	12
8	88	12
12	75	25
20	0	100

detector has a wavelength range of 190-400 nm; we used the wavelength 240 nm for our experiment.

Preparation of the standard and sample solutions

Ferulic acid standard was diluted with 50% methanol aqueous solution as the standard solution.

We weighed 0.5 g of the sample powder, extracted it with 15 ml methanol for 15 min by ultrasonic extraction, and then filtered it. The residue of the sample powder was extracted repeatedly with 15 ml methanol, and then filtered it. The twice filtrate were mixed together and then removed the solvent by rotary evaporation and then dissolved with 50% methanol aqueous solution into 5 ml. The solution was filtered through a 0.22-μm filtration membrane, and the filtrate obtained was used as the sample solution.

Assay method

The appropriate UPLC chromatographic conditions and run time were ascertained by trial and error. The blank sample solution (50% methanol/water) was analyzed under these conditions, and the baseline values were found to be steady. The sample solution (1 μl) was injected into the UPLC column for analysis. The chromatograms of the sample and standard solutions are shown in Figures 1 and 2, respectively.

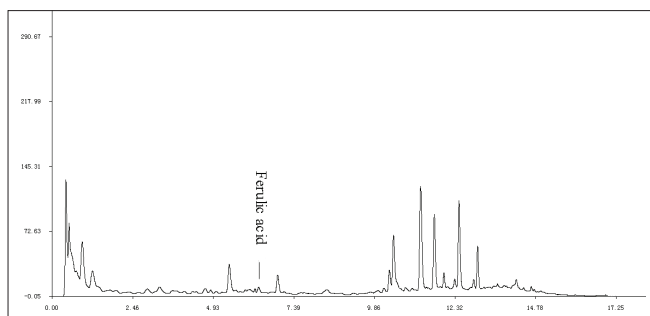


Figure 1: Ultra-performance liquid chromatography chromatogram of one sample. It showed most components information and the steadiest baseline than at other wavelength. The peak at retention time of 6.31 min was ferulic acid and designated as the reference peak for the calculation of relative peak area

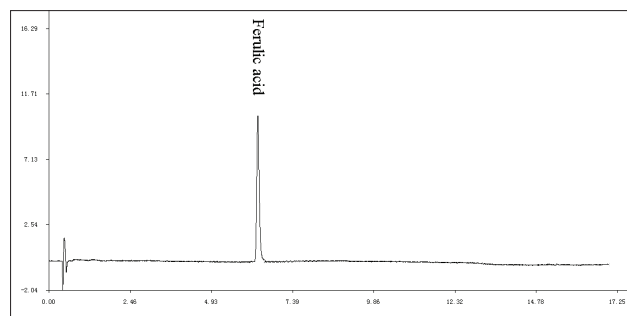


Figure 2: Ultra-performance liquid chromatography chromatogram of the ferulic acid standard. The peak at retention time of 6.31 min was ferulic acid and designated as the reference peak for the calculation of relative peak area

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Mobile phase

The effect of the composition of the mobile phase on the chromatographic separation of the samples was investigated. The compositions we investigated were as follows: Methanol–water, acetonitrile–water, formic acid/methanol (0.1:100, v/v)–formic acid/water (0.1:100, v/v). The results showed that the best resolution and shortest analysis time were achieved when the acetonitrile–water system was used.

Monitoring wave length

DAD was used in the analysis, and full-scan runs were performed initially to select the optimum wavelength that provided the best results. The results obtained at 285 nm showed the most accurate information about the components and had the steadiest baseline values. Therefore, this was selected as the monitoring wavelength.

Column temperature

We found no obvious separation difference between three temperatures 20°C, 25°C, and 30°C, but after considering the analysis time and separating effect, we set the column temperature to 25°C.

Optimization of extraction conditions

Extraction solvent

The pulverized samples were subject to ultrasonic extraction for 1 h with 100% ethanol, water, methanol, and *n*-butanol separately. The methanol extract showed a greater number of peaks. This reflected better retention of the chemical composition, which is important for the evaluation of traditional Chinese medicine. Methanol was, therefore, chosen as the extraction solvent.

Extraction method

With methanol as the extraction solvent, the pulverized samples were reflux extracted for 1 h, Soxhlet extracted for 1 h, ultrasonically extracted 1 time for 15 min, and ultrasonically extracted 2 times (15 min each time), respectively. Compared with the other methods, more peaks were obtained in lesser time with 2 times (15 min each time) of ultrasonic extraction. Therefore, this procedure was selected as the extraction method.

Validation of the methodology

All the tests below were carried out on the sample extract solutions prepared as described in Materials and Methods section. The injection precision was determined by taking the same sample solution and continually feed for 6 times. Sample stability was determined by taking the same sample solution and feeding at different time 0, 4, 8, 12, 18, 24 h. The repeatability of the experiment was assessed by analyzing six separate extract solutions of one sample.

The relative retention time (RRT) and relative peak area (RPA) of each characteristic peak were calculated for the estimation of precision, stability, and repeatability of the experimental steps. In terms of injection precision, the relative standard deviation (RSD) of RRT and RPA were found not to exceed 0.75% and 2.7%, respectively; in terms of sample stability, the RSD values were below 0.92% and 2.9%, respectively; and in terms of repeatability, the RSD values were less than 0.94% and 2.8%, respectively. Thus, all the results indicated that the quality of the studied samples and the UPLC-DAD measurements were stable and under control.

Calculation of the similarity indexes

The 10 sample solutions of *Phragmites rhizoma* were prepared and analyzed, and chromatograms of the samples were obtained at 285 nm [Figure 3]. The chromatographic peaks for different samples with the same RRT were defined as characteristic peaks. Twenty-seven peaks were identified as the characteristic peaks. Among the characteristic peaks, the peak at the retention time of 6.31 min, which was identified

as the ferulic acid standard, was designated as the reference peak for the calculation of RPA because it was an intense peak situated in the middle of the chromatogram. The data for the chromatographic fingerprints were imported into the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004 A) software (Chinese Pharmacopoeia Commission, Peking, China). These were then subject to a similarity analysis based on matching against the median of the fusion vectors of all the samples. A reference chromatogram was generated by the median method. The similarity indexes of the 10 samples are listed in Table 3.

Similarity analysis

Compared with the reference chromatogram, the similarity indexes of the 10 samples collected from the 10 villages around Baiyangdian were all above 0.820. The chromatograms for each of the 10 samples showed high similarity with the reference chromatogram. That is, the 10 samples had similar quality. However, careful observation showed that there were some unobvious differences between the samples with regard to diameter of the rhizome: The similarity indexes of samples with a “big” diameter were all above or equal to 0.898, while the indexes of samples with a “small” diameter were all less than 0.898 but above 0.820 [Figure 4]. This implies that there must be some differences in the types and quantities of chemical constituents between samples with a different diameter size. The quantities of the peaks and area of the peaks between 10 and 14 min in the 10 chromatograms were somewhat different.

Hierarchical cluster analysis

For the further analysis, the data for the 10 samples were processed by hierarchical cluster analysis. The RPA values of the 27 characteristic peaks of 10 samples were input into the SPSS software (SPSS for Windows 13.0, SPSS Inc., USA) to form a 10 × 27 matrix. Distance among the 10 samples was calculated using the SPSS software, with between-groups linkage as the cluster method, squared Euclidean distance as the measure interval, and range 0-1 as the standardized method. The results of the cluster

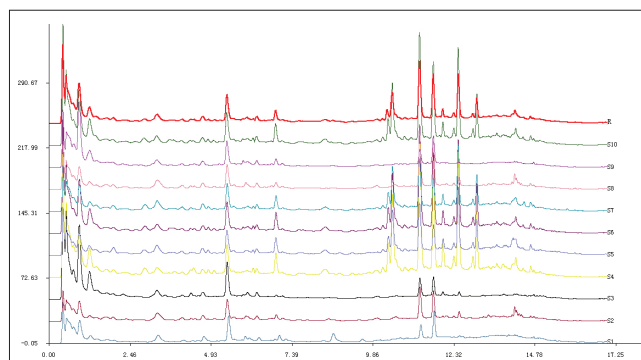


Figure 3: Chromatograms of the fingerprints of the 10 samples. The chromatograms of the 10 samples were obtained at 285 nm. Twenty-seven peaks were identified as the characteristic peaks

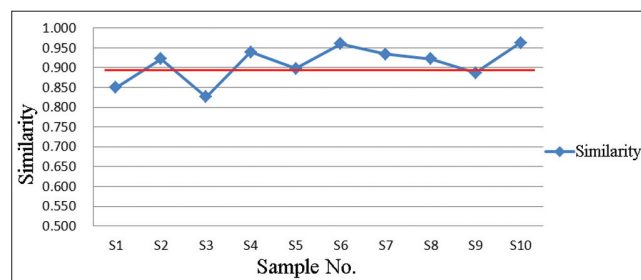


Figure 4: Line chart showing the results of the similarity analysis. The similarity indexes of the 10 samples were all above 0.820. The similarity indexes of samples with a “big” diameter were all above or equal to 0.898, while the indexes of samples with a “small” diameter were all less than 0.898 but above 0.820

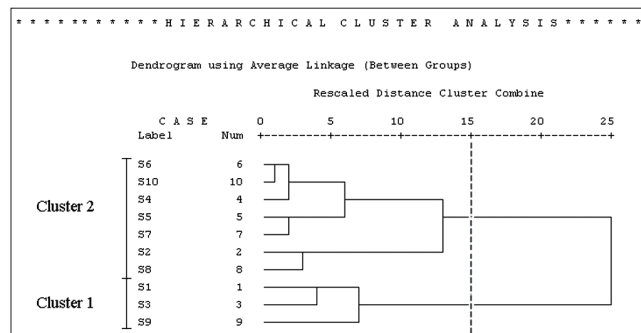


Figure 5: Results of the hierarchical cluster analysis The 10 samples could be divided into 2 clusters at a rescaled distance of 15: Cluster 1 – S1, S3, and S9; Cluster 2 – S2, S4, S5-S8, and S10

Table 3: Similarity indexes of the 10 samples of <i>Phragmitis rhizoma</i> from Baiyangdian											
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Reference
S1	1.000	0.889	0.924	0.726	0.678	0.750	0.690	0.834	0.896	0.767	0.850
S2	0.889	1.000	0.844	0.818	0.755	0.841	0.799	0.974	0.863	0.846	0.922
S3	0.924	0.844	1.000	0.699	0.656	0.725	0.650	0.780	0.902	0.753	0.826
S4	0.726	0.818	0.699	1.000	0.932	0.981	0.942	0.853	0.745	0.979	0.939
S5	0.678	0.755	0.656	0.932	1.000	0.923	0.933	0.777	0.699	0.922	0.898
S6	0.750	0.841	0.725	0.981	0.923	1.000	0.957	0.869	0.776	0.995	0.960
S7	0.690	0.799	0.650	0.942	0.933	0.957	1.000	0.822	0.714	0.946	0.934
S8	0.834	0.974	0.780	0.853	0.777	0.869	0.822	1.000	0.829	0.870	0.922
S9	0.896	0.863	0.902	0.745	0.699	0.776	0.714	0.829	1.000	0.793	0.887
S10	0.767	0.846	0.753	0.979	0.922	0.995	0.946	0.870	0.793	1.000	0.963
Reference	0.850	0.922	0.826	0.939	0.898	0.960	0.934	0.922	0.887	0.963	1.000

Table 4: Results of hierarchical cluster analysis

Sample no.	1:S1	2:S2	3:S3	4:S4	5:S5	6:S6	7:S7	8:S8	9:S9
Two clusters	1 ^a	2	1	2	2	2	2	2	1

^aThe same number indicates that the sample belongs to the same cluster

analysis are shown in Figure 5.

It was clear that the 10 samples could be divided into two clusters at a rescaled distance of 15: S1, S3, and S9 could be grouped as Cluster 1; S2, S4, S5-S8, and S10 could be grouped into Cluster 2 [Table 4]. This result was consistent with the groups that were formed when the samples were divided by their diameter. Thus, there is a correlation between the diameter of the samples and the types and quantities of chemical constituents they contain.

CONCLUSION

This experiment established the use of UPLC fingerprints for the evaluation of *Phragmites rhizoma* collected from 10 villages around Baiyangdian. Ten samples were analyzed by this method, and the chromatographic data were processed by similarity analysis and hierarchical cluster analysis. The results of the similarity analysis showed that the samples had relatively similar quality, and that this UPLC fingerprinting method could be used for quality control in Chinese medicine. Hierarchical cluster analysis suggested the presence of a correlation between the diameter size of samples and the types and quantities of chemical constituents they contained. *P. communis* is a perennial grass. It is therefore important to consider the growth characteristics of this species and identify the factors that affect the diameter of the stem of rhizome. While diameter is mainly related with the age of the plant, light, moisture, and soil also have some minor effects. This means that the chemical constituents are present in *Phragmites* rhizomes. This relationship between age and the quality of this herb needs to be studied in detail.

The UPLC fingerprinting technology established here has

the advantages of being fast, accurate, and highly efficient, and it can be used as an effective means of evaluating the quality of *Phragmites rhizoma* produced in Baiyangdian. Also, The UPLC fingerprinting technology can be used for evaluating herbs used in Chinese medicine.

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