A novel high-performance liquid chromatography fingerprint approach to discriminate *Phyllostachys pubescens* from China

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ABSTRACT

Background: A high-performance liquid chromatography fingerprint of different variants of *Phyllostachys pubescens* was developed for the source discrimination and quality control of *Phyllostachys pubescens* medicines produced in Zhejiang Province, China. **Materials and Methods:** Samples of *Phyllostachys pubescens* collected from nine cultivars in Zhejiang Province were used to establish the fingerprint. Taking the chromatographic peak (isoorientin) as the reference peak, the chromatographic column was Sunfire C_{18} ODS (250 mm × 4.6 mm, 5 μm) at a column temperature of 25°C. The mobile phase was acetonitrile 0.8% acetic acid water solution (in a gradient elution mode) at a flow rate of 1 ml/min. The detection wavelength was 330 nm. **Results:** 20 common peaks were established in the chromatographic fingerprints of *Phyllostachys pubescens*. And then by comparing the UV and standard substance with those of the authentic standards and literature, eight main peaks in the fingerprints were identified. Data of nine cultivars were evaluated statistically using similarity analysis, hierarchical cluster analysis (HCA) in order to establish a similarity standard of fingerprint for quality control of *Phyllostachys pubescens*, then to classify the samples. The similarity indexes were all above 0.95 between reference chromatogram and each sample's chromatogram. **Conclusion:** in this study, the methods established are reliable, and could be used to evaluate the quality and to identify the species of *Phyllostachys pubescens* in the future.

Key words: Fingerprint, high-performance liquid chromatography, *Phyllostachys pubescens*

INTRODUCTION

Phyllostachys pubescens Mazelex H.de Lehaie is an evergreen plant belonging to the genus *Phyllostachys* of *Bambusoideae* in *Gramineae*. It is one of the most important resources of economic bamboo species, because of its resource advantage, such as rapid growth speed, high yield, extensive use, short crucial period for forest formation, and strong regeneration capacity.[1]

The leaf of bamboo, which contains flavonoids, phenolic acids, and coumarin lactone, is widely used as traditional Chinese medicinal material. In modern researches, the components of *Phyllostachys pubescens* have showed many activities, such as antibacteriostasis, antioxidant activity, resisting oxidation and aging, scavenging of free radical activity, enhancing the activity of SOD and GSH-PX.[2]

Phyllostachys pubescens Mazelex H.de Lehaie produced in Zhejiang Province is a famous economic bamboo, and it is documented in Chinese National Medicine Assembly as a main source of *Phyllostachys bambusoideae*. A large amount of *Phyllostachys pubescens* in Zhejiang, one of the famous and main origins, are used clinically and cultivated to many countries and regions. Therefore, it is necessary to develop a good method for the quality control of *Phyllostachys pubescens*.

Of the literature published in the recent 20 years, to our knowledge, no literature has been documented where an high-performance liquid chromatography (HPLC) fingerprint was used to evaluate the quality of *Phyllostachys pubescens*. In addition, we found that in the previous

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investigation it is very difficult to identify the cultivars for *Phyllostachys pubescens* based on their appearances, because the appearances of *Phyllostachys pubescens* from the 12 cultivars are very similar[3] On the other hand, as shown in the literature,[4-7] statistical analysis based on the data of samples, such as similarity analysis, hierarchical cluster analysis, can provide reliable methods to evaluate and classify the samples. In order to obtain a holistic quality evaluation and an effective species discrimination method of different cultivars *Phyllostachys pubescens*, an HPLC fingerprinting method will be established in this work.

MATERIALS AND METHODS

Instrumentation
A Waters 2695-2996 system (Waters Corp., MA, USA), equipped with a binary solvent manager, an auto-sampler and a photodiode array detector (PDA), was used for liquid chromatographic analysis. A KQ2200DE ultrasonic cleaning instrument (Kunshan Shumei Ultrasonic Instrument Co., Kunshan, China) was used for extraction.

Reagents and materials
Orientin, isoorientin, vitexin, isovitexin and *p*-coumaric acid, chlorogenic acid, caffeic acid and caffeic acid, ferulic acid standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The HPLC grade methanol was purchased from Tedia (Tedia Co., OH, USA), while the HPLC grade formic acid were from Merck (Merck Co., Darmstadt, Germany). All the other chemicals and solvents used in sample preparation were of analytical grade. Deionized water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

The *Phyllostachys pubescens* samples, from nine cultivars, were collected respectively from Linan and Anji countries in Zhejiang Province, and all identified as the dried leaf of *Phyllostachys pubescens* by Professor Xin-chun Lin in Zhejiang Agricultural and Forestry University [Table 1]. Then the

samples were processed, pulverized and dried, and then sifted through the 40-mesh sieve for later use.[8]

High-performance liquid chromatography chromatographic conditions
The separation was performed on a Sunfire C18 ODS (250 mm×4.6 mm, 5 μm) column. The mobile phase consisted of (A) acetonitrile and (B) acetic acid/water (0.8:100, v/v). The gradient elution was optimized as shown in Table 2. The flow rate was 1.0 ml/min, and the column was maintained at 25°C. The monitoring wave length was set at 330 nm.

Preparation of the standard solution
A standard mixture containing orientin, isoorientin, vitexin, isovitexin, *p*-coumaric acid, chlorogenic acid, caffeic acid and caffeic acid, ferulic acid standards was prepared in methanol. The solutions were filtered through a 0.22 μm syringe filter, and an aliquot (10 μl) of each filtrate was subjected to HPLC analysis.

Preparation of the sample solution
The eight pulverized samples of *Phyllostachys pubescens* from different cultivars in Zhejiang Province were accurately weighed (approximately 1.0 g), soaked for 2 hours in 10 ml of 70% ethanol, and ultrasonic-extracted with 30 ml of 70% ethanol for two times (after a 40-minute interval). The extracts of leaves were concentrated into dryness by the rotary evaporator, and then dissolved to volumetric flasks of 50 ml methanol. The extracted solutions were filtered through a 0.22 μm syringe filter, and an aliquot (10 μl) of each filtrate was subjected to HPLC analysis.

Data analysis
Similarity analysis was performed by the professional software Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A), which was recommended by SFDA of China. The software was used to employ the correlative coefficient in evaluating the similarities of different chromatogram. The

Table 1: Representative samples of *Phyllostachys pubescens* Mazelex H.de Lehaie investigated in this study

Sample No.	Herbal species	Collection site	Collection time
S1	*Phyllostachys heterocycla* cv. *Pubescens*	Lin An Country, Zhejiang Province	2010-04-22
S2	*Phyllostachys heterocycla* cv. *Obliquinoda*	An Ji Country, Zhejiang Province	2010-05-10
S3	*Phyllostachys heterocycla* cv. *Heterocycla*	An Ji Country, Zhejiang Province	2010-05-10
S4	*Phyllostachys heterocycla* cv. *Tao Kiang*	Lin An Country, Zhejiang Province	2010-04-22
S5	*Phyllostachys heterocycla* cv. *Pachyloen*	An Ji Country, Zhejiang Province	2010-04-28
S6	*Phyllostachys heterocycla* cv. *Viridisulcata*	An Ji Country, Zhejiang Province	2010-05-10
S7	*Phyllostachys heterocycla* cv. *Gracilis*	Lin An Country, Zhejiang Province	2010-04-22
S8	*Phyllostachys heterocycla* cv. *Tubaeformis*	An Ji Country, Zhejiang Province	2010-04-28
S9	*Phyllostachys heterocycla* cv. *Luteosulcata*	An Ji Country, Zhejiang Province	2010-05-10

Table 2: Composition of mobile phase with gradient elution program

T/min	A (%)	B (%)
0-2	10-10	90-90
2-6	10-14	90-86
6-16	14-17	86-83
16-23	17-19	83-81
23-30	19-23	81-77
30-42	23-31	77-69
42-50	31-90	69-10

hierarchical cluster analysis of samples were performed using SPSS software (SPSS for Windows 17.0, SPSS Inc., USA)

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Chromatograph column, column temperature, monitoring wave length, and mobile phase were selected that provided the best results in chromatographic fingerprinting analysis.[9-11] Two high-performance liquid chromatography with ODS column are recommend for separation of flavonoids and phenol acids. Because of the similar interaction with the column which results from their similar chemical structures, it is challenging to develop a best chromatograph and separate condition. Different types of columns were tested. The property of separation of the Sunfire C_{18} ODS column for flavanones of bamboo is better than that of XBridge C_{18} ODS column. Comparing the chromatograms at three different temperatures 25°C, 30°C, and 40°C, we found no obvious separation difference, but after overall consideration of the analysis time and separating effect, the column temperature was set at 25°C.

Selection of an appropriate detection wavelength was of great importance to ensure precise detection of some essential constituents and to achieve more peaks. Waters 2996 Photo-Diode Array detector (PDA) was used in the analysis, and full scan runs were made initially to select the optimum wavelength that provided the best results in chromatographic fingerprinting analysis. Chromatogram at 330 nm showed the most abundant components information and the steadiest baseline comparing with the other wavelengths. Finally we chose 330 nm as the monitoring wavelength.

The effect of the composition of mobile phase on the chromatographic separation of the samples was investigated in this study. With the consideration of the fact shown in the literature, there are many kinds of flavonoids and phenolic acids in bamboo leaf, while some of them are isomers, so we tried to add a certain percentage of acid into the mobile phase to improve the resolutions of chromatographic peaks.[12] Different mobile phases were tried, such as methanol-water, acetonitrile-water, formic acid/methanol (0.1:100, v/v)-formic acid/water (0.1:100,v/v), acetonitrile-acetic acid/water (0.8:100,v/v), acetonitrile-phosphoric acid/water (0.2:100,v/v), etc. Finally, acetonitrile-acetic acid/water (0.8:100, v/v) was selected as an appropriate mobile phase with gradient elution, which gave good resolution and shortest analysis time.

Optimization of extracting conditions

With methanol as the extracting solvent, the pulverized samples were reflux extracted, soxhlet extracted, and ultrasonic extracted, respectively. According to the withdrawal rates of flavonoids, the ultrasonic extracted for 1 hour was chosen as extracting method. And then the pulverized samples were ultrasonic extracted for 1 hour with 100%, 70%, 50% (v/v) of ethanol and methanol, respectively. Comparing with the other solvents and other methods, more peaks were obtained in the extracts that ultrasonic extracted for 1 hour in 70% ethanol. According to the principle that more chemical composition should be retained to evaluate traditional Chinese medicine, 70% ethanol was finally chosen as the extracting solvent and ultrasonic extracting for 1 hour was as the extracting method.

Methodology validation

All tests below were carried out on the sample extract solutions prepared as described in the sections "Preparation of the standard solution" and "Preparation of the sample solution." The injection precision was determined by replicating HPLC injections of the same sample solution six times per day. Precision of sample stability was determined with measurements from a single sample solution stored at room temperature for 0, 4, 8, 12, 18, and 24 hours. The repeatability 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 and all the results of the relative standard deviation (RSD) of RRT and RPA were found not to exceed 3.0%. Thus, all results indicated that the quality of the studied samples and the UPLC-DAD measurements were stable and under control.

High-performance liquid chromatography fingerprint analysis

The nine sample solutions of *Phyllostachys pubescens* and standard substances were prepared and analyzed, and chromatograms of the samples were recorded in 50

minutes, which shown in Figure 1. According to the results of HPLC fingerprint, we found 20 common peaks. Among the characteristic peaks, peak 10 at retention time of 19.06 minutes, which was identified as isoorientin contrasting with the isoorientin standard, was designated as the reference peak. Because it appears with a stable time and a large area in the chromatogram, the RSD value is below 3.0%. Also its comparative standing time of common peaks approximately fits the requirements of "Technological Requirements of Fingerprint Chromatography Research of Traditional Chinese Medicinal Injection."

The literature shows that isoorientin is a kind of flavone rich in *Phyllostachys pubescens*. Isoorientin is one of the main active components in medicinal herbs, featuring with broad pharmacological activity, antioxidant, liver protective activity.[13] It can also improve the blood circulation and ease cardiovascular and cerebrovascular diseases. So as an object of reference, isoorientin can be used in the identification and quality appraisal of *Phyllostachys pubescens*.

Relative peak area and similarity of the sample
The nine sample solutions of *Phyllostachys pubescens* were prepared and analyzed, and chromatograms of the samples were recorded at 330 nm. The chromatographic peaks in different samples with the same relative retention time were defined as the characteristic peaks. Twenty peaks were identified as the characteristic peaks. Among the characteristic peaks, peak 10 at retention time of 19.07 minute was designated as the reference peak for the calculation of RPA. The relative retention time (RRT) and relative retention area (RPA) of these 20 peaks in

nine samples were shown in Table 3. The data of the chromatographic fingerprints were imported into the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004 A) software. In this study, a similarity analysis based on matching against the median of the fusion vectors of all the samples was performed. The reference chromatogram was generated by the median method. The similarity indexes of the nine samples are listed in Table 4.

Identification of chromatographic peaks
Standard substances coupled with LC-PDA had been widely used for qualitative or quantitative analysis of components in analytes separated by LC. In this study, standard substances coupled were employed to analyze the components in *Phyllostachys pubescens* separated by UV spectrum. Eight components were unambiguously identified in the samples of *Phyllostachys pubescens*. They were orientin (peak11), isoorientin (peak10), vitexin (peak12), isovitexin (peak15), *p*-coumaric acid (peak14), chlorogenic acid (peak3), caffeic acid (peak7), and ferulic acid (peak17). The other compounds of common peaks were needed to be identified in a further study. The HPLC chromatogram of sample No. 5 acquired at 330 nm is shown in Figure 2. The result indicated that all of the different cultivars of *Phyllostachys pubescens* possess high flavonoids and phenolics contents and strong bioactivities.

Similarity analysis
Compared with the reference chromatogram generated by the median method, the similarity indexes of the nine samples collected from different cultivars were all
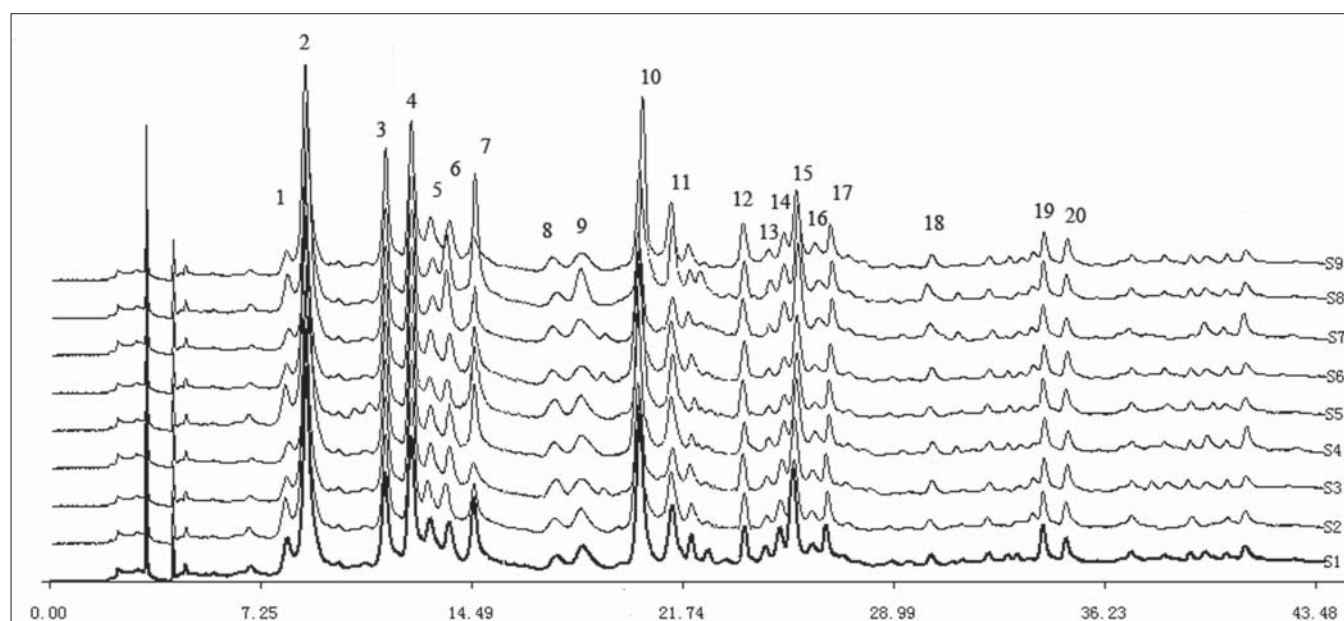

**Figure 1:** Chromatogram of the fingerprints of the nine samples

Table 3: The calculation result of the relative peak area of the communion peak

RPT	RSD%	RPA									RSD%
		S1	S2	S3	S4	S5	S6	S7	S8	S9	
0.049	2.45	0.100	0.173	0.142	0.149	0.090	0.142	0.148	0.049	0.169	27.542
0.099	1.15	0.996	0.926	0.996	0.949	0.888	1.078	1.019	0.921	1.115	10.902
0.148	0.66	0.523	0.514	0.498	0.466	0.498	0.506	0.509	0.418	0.519	7.125
0.197	1.07	0.824	0.723	0.718	0.703	0.811	0.753	0.771	0.576	0.799	6.015
0.247	1.2	0.409	0.379	0.364	0.376	0.382	0.366	0.361	0.260	0.397	10.252
0.296	0.55	0.338	0.372	0.363	0.356	0.389	0.391	0.263	0.309	0.377	5.793
0.345	0.38	0.410	0.364	0.329	0.358	0.419	0.414	0.373	0.419	0.422	18.386
0.395	0.63	0.184	0.198	0.182	0.174	0.171	0.207	0.178	0.212	0.191	19.820
0.444	0.7	0.211	0.190	0.199	0.197	0.186	0.204	0.208	0.244	0.233	21.946
0.493	0.49	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	13.783
0.543	0.39	0.463	0.468	0.431	0.456	0.437	0.491	0.453	0.353	0.493	4.015
0.592	0.36	0.381	0.380	0.409	0.374	0.365	0.461	0.384	0.292	0.444	5.245
0.641	0.35	0.066	0.041	0.040	0.038	0.042	0.071	0.039	0.025	0.055	24.834
0.691	0.32	0.132	0.118	0.131	0.114	0.115	0.150	0.128	0.108	0.140	8.853
0.740	0.35	0.472	0.497	0.471	0.492	0.460	0.548	0.479	0.368	0.539	3.906
0.789	0.19	0.089	0.073	0.072	0.078	0.076	0.110	0.084	0.045	0.090	13.503
0.839	0.5	0.262	0.266	0.282	0.268	0.241	0.306	0.264	0.225	0.293	7.735
0.888	0.34	0.046	0.039	0.046	0.037	0.043	0.046	0.048	0.050	0.040	22.256
0.937	0.18	0.204	0.192	0.192	0.192	0.183	0.235	0.200	0.129	0.227	5.971
0.987	0.27	0.105	0.105	0.100	0.101	0.095	0.113	0.102	0.120	0.107	20.226

RSD: Relative standard deviation, RPA: Relative peak area

Table 4: The calculation result of similarity of communion peak

	S1	S2	S3	S4	S5	S6	S7	S8	S9	Reference
S1	1.000	0.973	0.921	0.949	0.985	0.94	0.972	0.921	0.923	0.988
S2	0.973	1.000	0.891	0.981	0.966	0.904	0.953	0.972	0.965	0.983
S3	0.921	0.891	1.000	0.977	0.926	0.99	0.915	0.944	0.961	0.987
S4	0.949	0.981	0.977	1.000	0.949	0.994	0.992	0.969	0.983	0.954
S5	0.985	0.966	0.926	0.949	1.000	0.943	0.965	0.923	0.965	0.971
S6	0.94	0.904	0.99	0.994	0.943	1.000	0.903	0.942	0.934	0.986
S7	0.972	0.953	0.915	0.932	0.965	0.903	1.000	0.973	0.923	0.997
S8	0.921	0.972	0.944	0.969	0.923	0.942	0.973	1.000	0.928	0.953
S9	0.923	0.965	0.961	0.983	0.965	0.996	0.923	0.928	1.000	0.964
Reference	0.986	0.988	0.937	0.954	0.971	0.951	0.971	0.953	0.964	1.000
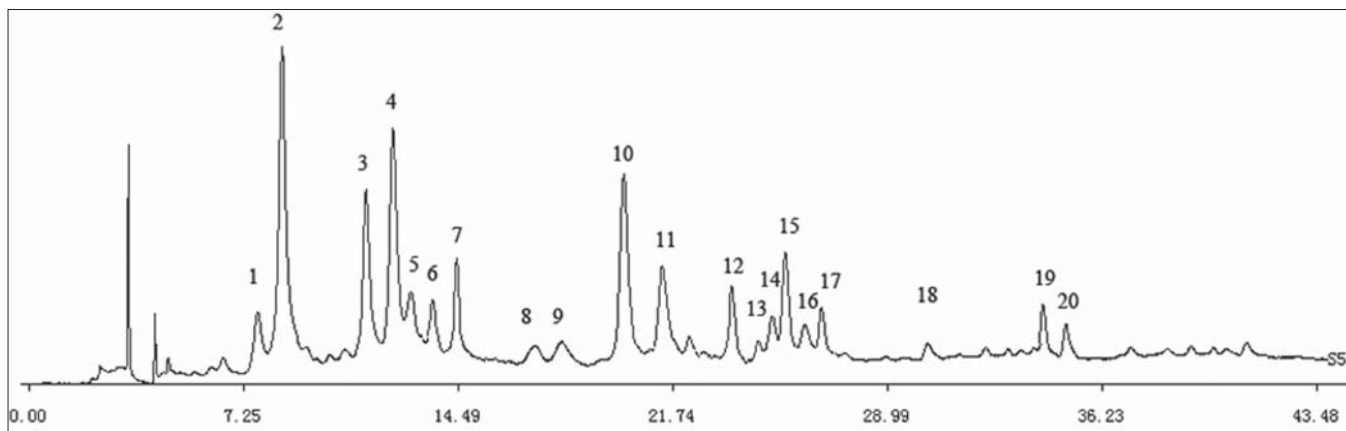
Figure 2: The HPLC chromatogram of sample No. 5 acquired at 330 nm (3. chlorogenic acid; 7. caffeic acid; 10. isoorientin; 11. orientin; 12. vitexin; 14. p-coumaric acid; 15. isovitexin; 17. ferulic acid)

above 0.95. It showed that each of the nine *Phyllostachys pubescens* samples had a high similarity matching against the reference chromatogram. That means that the qualities of nine *Phyllostachys pubescens* samples collected from different cultivars in Zhejiang Province were similar. The result was consistent with the study on traditional taxonomy of bamboo resources. Doctors indiscriminately treated *Phyllostachys pubescens* from *different cultivars* as *Phyllostachys pubescens* in clinical use in a long time. But through careful observations, there were some unobvious differences between the samples from different cultivars that the similarity from *Phyllostachys heterocycla* cv. *Pubescens* and *Phyllostachys heterocycla* cv. *Pachyloen* was above 0.98, while the sample similarity from *Phyllostachys heterocycla* cv. *Pubescens* and *Phyllostachys heterocycla cv. Tubaeformis* was 0.92. On the contrary, the similarities of samples from different cultivars have no clear regularity. The twenty characteristic peaks in chromatograms were the main components of *Phyllostachys pubescens*. In practice of quality control, the herbs which have the smallest similarity indexes, or are below a certain value, can be regarded as not qualified. Therefore, for quality control of *Phyllostachys pubescens*, 0.95 was set as an appropriate value, and it is easy to find that the samples of *Phyllostachys pubescens* with similarity indexes above 0.95 have reliable qualities.

Hierarchical cluster analysis

As shown by the information listed in the section "Reagents and materials," the nine *Phyllostachys pubescens* samples studied here were collected from different cultivars. It would therefore be of interest to see if the sample set can be further divided into subgroups based on hierarchical cluster analysis (HCA). The RPA of 20 characteristic peaks of nine samples were input the SPSS software and formed a 9×20 matrix. Distances among the nine samples were calculated using the SPSS software, with between-groups linkage as the cluster method, squared Euclidean distance as the measure interval. It was clear that the nine samples could be divided into three clusters at a rescaled distance of 5: sample S8 could be formed as cluster 1; samples S1-S5 could be grouped into cluster 2; the others could be grouped in to cluster 3. The dendrogram constructed according to hierarchical cluster analysis, based on the squared Euclidean distance, shows the relationships among the *Phyllostachys pubescens* samples collected from nine cultivars [Figure 3].

From the features of appearance, the main difference between *Phyllostachys heterocycla* cv. *Luteosulcata* and *Phyllostachys heterocycla* cv. *Viridisulcata* is the color of the stalk. Similar to the appearance analysis, the hierarchical cluster analysis in this paper also shows the high similarity between these two cultivars. Likewise, the main external difference between *Phyllostachys heterocycla* cv. *Pubescens* and
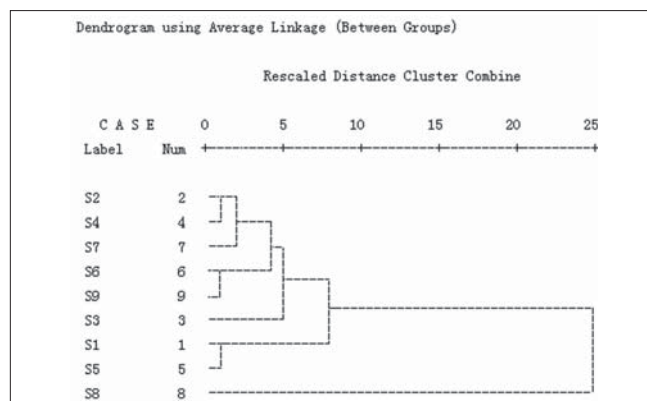

Figure 3: Results of hierarchical cluster analysis of nine samples

Phyllostachys heterocycla cv. *Tubaeformis* is the thickness of the stalk wall while other characteristics are almost the same, which was also testified in the hierarchical cluster analysis in this paper while the relationship of *Phyllostachys heterocycla* cv. *Tubaeformis* was far with the other cultivars of *Phyllostachys Pubescens*. Therefore, the researching results of hierarchical cluster analysis and forestry taxology of bamboo resources are consistent. Hierarchical cluster analysis thus showed that the nine samples could be separated into three distinct groups based on their compositional fingerprints.

CONCLUSION

An HPLC method was developed for the fingerprint of *Phyllostachys pubescens* collected from nine cultivars in Zhejiang Province. Nine samples were analyzed by this method, and the chromatographic data were processed by similarity analysis and hierarchical cluster analysis. Components in 8 of 20 characteristic peaks were identified, which were orientin, isoorientin, vitexin, isovitexin, *p*-coumaric acid, chlorogenic acid, caffeic acid, and ferulic acid. The result of the similarity analysis proves that the *Phyllostachys pubescens* samples have relatively constant quality, and this HPLC fingerprinting method could be used in the quality control as a powerful tool. The result of hierarchical cluster analysis indicates that the components in *Phyllostachys pubescens* from the nine cultivars have very similar varieties and contents.

The HPLC fingerprinting method and the species discriminating method of *Phyllostachys pubescens* established in this study are precise and efficient, and hence could be used in the quality control and species identification of *Phyllostachys pubescens*.

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