Chemical Fingerprint and Quantitative Analysis for the Quality Evaluation of *Platycladi cacumen* by Ultra-performance Liquid Chromatography Coupled with Hierarchical Cluster Analysis

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Abstract

*Platycladi cacumen* (dried twigs and leaves of *Platycladus orientalis* (L.) Franco) is a frequently utilized Chinese medicinal herb. To evaluate the quality of the phytomedicine, an ultra-performance liquid chromatographic method with diode array detection was established for chemical fingerprinting and quantitative analysis. In this study, 27 batches of *P. cacumen* from different regions were collected for analysis. A chemical fingerprint with 20 common peaks was obtained using the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A). Among these 20 components, seven flavonoids (myricitrin, isoquercitrin, quercitrin, afzelin, cupressus flavone, amentoflavone and hinokiflavone) were identified and determined simultaneously. In the method validation, the seven analytes showed good regressions (R ≥ 0.9995) within linear ranges and good recoveries from 96.4% to 103.3%. Furthermore, with the contents of these seven flavonoids, hierarchical clustering analysis was applied to distinguish the 27 batches into five groups. The chemometric results showed that these groups were almost consistent with geographical positions and climatic conditions of the production regions. Integrating fingerprint analysis, simultaneous determination and hierarchical clustering analysis, the established method is rapid, sensitive, accurate and readily applicable, and also provides a significant foundation for quality control of *P. cacumen* efficiently.

Introduction

*Platycladus orientalis* (L.) Franco is a distinct genus of evergreen coniferous tree in the cypress family Cupressaceae, and is widely distributed in China. Besides serving as an evergreen tree species in the garden, it is also a source of Chinese Medicine. Its dry twig and leaf, *Platycladi cacumen* (PC, also called Cebaiye in China), are often collected from summer to autumn and dried in the shade. In China, PC has been used as a traditional Chinese medicine (TCM) for thousands of years. It was firstly recorded in “Shen Nong Ben Cao Jing”, and is now still listed in the *Chinese Pharmacopoeia* (1). It has been utilized to cool the blood to stanch bleeding, resolve phlegm, suppress cough, promote hair growth and blacken hairs (1). In recent years, PC has been found to have anti-inflammatory (2–4), anti-oxidant (5, 6), anticancer (7, 8), diuretic (9), hair growth promoting (10), anti-fibrotic (11) and anti-hyperuricemic activities (12), and also have some effect on the nervous system (13, 14).
Medicine (15) and other literatures (16, 17), it is recorded alone or in combination with other herbal medicines in the treatment of bacillary dysentery, chronic bronchitis, purpura, alopecia, seborrheic dermatitis, pulmonary tuberculosis, pertussis, duodenal ulcer and so on.

Responsible for the various bioactivities, flavonoids are considered as the material basis responsible for the efficacy of PC. There are a large amount of flavonoid glycosides, including myricitrin, isoquercitrin, quercitrin and afzelin. Besides these flavonoid glycosides, biflavonoids, at high contents, are another kind of flavonoids worthy of attention. Among these biflavonoids, cupressuflavone, amentoflavone and hinokiflavone are the representative ones. These seven flavonoids have been proved by lots of literatures to exert the anti-oxidant (18), hair growth promoting (19), neuroprotective (20, 21), sedative (22), anti-inflammatory (23), anti-tumor (24–27), anti-diabetics (29, 30), anti-viral (31) effects and so on.

Because of their various pharmacological activities and higher contents, these flavonoids in PC are considered as the bioactive components and key markers of quality evaluation. However, in Chinese Pharmacopoeia (1), only quercitrin is used as chemical marker for quality evaluation of PC. To our knowledge, the integral quality of a herbal medicine could not be reflected by a single component. So, it is unreasonable to evaluate the quality of PC with a single bioactive compound. Recently, there have been some reports on the quality analysis of PC by HPLC or CZE through determination of one or a few of these seven flavonoids (32–36). Compared with conventional HPLC, ultra-performance liquid chromatography (UPLC) has been more and more popularly applied in TCM studies because of its superior separation performance, faster analysis time and less mobile phase consumed. Despite these, there has been no research reported on the chemical fingerprint and quantitative analysis for the quality evaluation of PC using UPLC.

Meanwhile, as a kind of chemical component in TCM, flavonoids are the main secondary metabolites of many herbal medicines. Their contents are related to the environment of the planting area, such as temperature, humidity, altitude, soil and so on. In China, from west to east and from south to north, natural conditions differ so much in different provinces or areas that significant differences may be present in the contents of the same compounds in the same herbal medicines from different regions.

So, in this study, we aim to establish an UPLC-DAD method for fingerprint analysis and quantitative analysis of the seven flavonoids in PC as mentioned. The analysis method, coupled with hierarchical clustering analysis (HCA), is expected to be able to distinguish the differences among PC samples from different regions and to be useful for quality evaluation of PC in the future.

### Experimental

#### Plant materials

Twenty seven batches of PC were collected from different regions of China and prepared in the laboratory according to the processing method described in the Chinese Pharmacopoeia (2015 Edition). All

![Figure 1](image_url)
the voucher specimens were then authenticated by Professor Wu Qin-nan, the botanist of the College of Pharmacy at Nanjing University of Chinese Medicine (Nanjing, China) and deposited at the herbarium at the above location.

Chemicals and reagents

The standards of myricitrin (C_{21}H_{20}O_{12}, 93.8%), isoquercitrin (C_{21}H_{20}O_{12}, 92.9%), quercitrin (C_{21}H_{20}O_{11}, 92.7%) and amentoflavone (C_{22}H_{24}O_{10}, 90.2%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The other standards, afzelin (C_{21}H_{20}O_{10}), cupressusflavone (C_{20}H_{19}O_{10}) and hinokiflavone (C_{20}H_{19}O_{10}) were purchased from Nanjing Jin Yibai Biological Technology Co., Ltd. With UPLC, their purities were determined as 96.4%, 92.5% and 95.1%, respectively. The structures of these seven components are shown in Figure 1.

HPLC-grade methanol was obtained from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water was obtained with a Milli-Q system (Bedford, MA, USA). Formic acid of analytical grade was purchased from Anaqua Chemicals Supply Inc. Ltd (Houston, TX, USA). Other chemicals and solvents used in this study were of analytical grade.

Instrument and chromatographic conditions

All analysis were performed on a Shimadzu LC-20A series LC system (Kyoto, Japan), which included a quaternary solvent delivery system, an online degasser, a conditioned autosampler and a diode array detector. For chromatographic separation, a Waters BEH C_{18} column (100 × 2.1 mm, 1.7 µm) (Waters Corporation, Milford, MA, USA) was used and its temperature was set at 30°C. The mobile phase consisted of methanol (A) and 0.2% formic acid (v/v, B) with flow rate of 0.2 mL/min. The gradient program was: 0–2 min, 5–30% A; 2–10 min, 30–37% A; 10–15 min, 37–67% A; 15–20 min, 67–85% A; 20–22 min, 85%–95% A; 22–25 min, 95% A; 25–30 min, 95%–5% A. The injected volume was 2 µL and detection wavelength was 340 nm.

Preparation of standard solutions

Because of their characteristic solubilities, cupressusflavone, amentoflavone and hinokiflavone were accurately weighed and dissolved in dimethylsulfoxide before being diluted with methanol in volumetric flasks, while the other four flavonoid glycosides were dissolved directly in methanol, respectively. Then, for establishing the calibration curves, the seven stock standard solutions were mixed and diluted with methanol to prepare the mixed stock standard solution (myricitrin 230.73 µg/mL, isoquercitrin 21.09 µg/mL, quercitrin 439.86 µg/mL, afzelin 55.43 µg/mL, cupressusflavone 49.86 µg/mL, amentoflavone 74.87 µg/mL and hinokiflavone 102.23 µg/mL). Figure 2 shows the chromatogram of the standard solution of these seven analytes.

Preparation of sample solutions

Before preparation of sample solutions, the dried PC was ground to powder and passed through a 60-mesh sieve. An accurately weighed 1.0 g of the dried powder was introduced into a 100 mL stopper conical flask, mixed with 50 mL of ethanol–water (75:25) and weighed. The mixture was heated under reflux for 2 h. After cooling to room temperature, it was weighed accurately again. Loss of weight due to evaporation was replenished by adding an appropriate amount of ethanol–water (75:25). The extraction solution was mixed well and filtered. Two milliliter of the filtrate was dissolved with 10 mL of ethanol–water (75:25). The resulting solution was used as the sample solution. All the solutions were stored at 4°C and were filtered through 0.22 µm syringe filters before being injected into the UPLC. The injection volume was 2 µL.

Methods

Validation for fingerprint analysis

PC sample of Batch 5 was selected for the method validation. A same solution of this batch was analyzed with six consecutive injections for precision test. At different times (0, 2, 4, 8, 16, 24 and 48 h) after preparation, this same solution was also analyzed to test the stability. The repeatability was evaluated by analyzing six samples of the same batch.

Validation for quantitative analysis

Linearity, precision, repeatability, stability and recovery tests were carried out for the validation of the UPLC quantitative analysis method.

Calibration curves and linearity

The mixed stock standard solution was diluted with methanol to obtain five standard solutions with appropriate and proportional concentrations of each analyte for establishing the calibration curves. Each solution was analyzed for three times. The peak area (Y, arbitrary units) and concentration (X, µg/mL) were subjected to linear regression analysis.

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) for each flavonoid were separately determined at signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively.

Precision, repeatability and stability

A sample solution of Batch 5 was prepared as described above and was subjected to UPLC analysis six times to evaluate the intra-day precision. The inter-day precision was investigated by analyzing the same sample solution on three consecutive days. Six different sample solutions of the same batch were prepared with the same method to assess the repeatability. Stability was assessed by analyzing the same sample solution after storing at 0, 1, 2, 4, and 8 h. Relative standard deviation (RSD) was used as variation to evaluate the precision, repeatability and stability.

Recovery

In the recovery test, the standard addition method was used to evaluate the accuracy of the established method. The standard solutions of seven flavonoids were added to the samples of Batch 5 at the level equivalent to 100% of the known amount. The extraction and analysis were performed according to the proposed procedure. The recovery was evaluated by the following formula: [detected amount (µg) − original amount (µg)] × 100%/spiked amount (µg).

Data analysis

As recommended by Chinese Pharmacopoeia Commission, “Similarity Evaluation System for Chromatographic Fingerprint of
Traditional Chinese Medicine” (SES, Version 2004A), was employed as the software for fingerprint analysis. With SES, similarities of different chromatograms were calculated for the quality evaluations of 27 batches. Furthermore, a software package (SPSS 20.0) was used to perform HCA.

Results
Optimization of UPLC-DAD conditions
To obtain optimum chromatographic separation of peaks within a short time, the column, mobile phase and detection wavelength were investigated. Firstly, several different UPLC columns were tested to compare resolution. Baseline separation of the constituents was obtained on a Waters Acquity C18 column. Secondly, the mobile phase composition was optimized. Although methanol–water system is widely used as the mobile phase in chromatographic experiments (37, 38), the separation and the shapes of the characteristic peaks obtained were not satisfactory. According to the literatures (34–36) and our previous experience, addition of an acid to the mobile phase could be helpful in achieving better separation and peak shape for flavonoids. Thus, formic acid was added to the mobile phase. Thirdly, after a full-scan experiment of the seven flavonoids from 200 to 400 nm, 340 nm was chosen as the best detection wavelength, so that the characteristic peaks could be observed, and the baseline was stable in the chromatographic profiles.

Optimization of extraction conditions
To extract sufficiently all the analytes in PC, steam distillation extraction (SDE) and ultrasonic extraction (UE) were evaluated to compare their yields. The contents of the seven flavonoids obtained by SDE were higher than those by UE. Furthermore, extraction solvents (methanol–water, ethanol–water) with different ratios (100:0, 75:25, 50:50, 25:75), extraction number (1, 2) and extraction time (1, 2, 3 h) were also investigated. The results showed that there were higher extraction yield and lower toxicity by using ethanol–water (75:25, v/v) as extraction solvent. Therefore, SDE with ethanol–water (75:25, v/v) for 2 h once was employed as the optimum extraction condition.

Method validation for fingerprint analysis
In the stability and precision tests, the similarities of the different chromatograms were 1.000. In the reproducibility test, the chromatographic similarities of six samples from Batch 5 were 0.998, 0.998, 0.999, 1.000, 0.999 and 0.998. All the RSDs of relative peak areas and relative retention times of the characteristic peaks were <3% (S1, S2). Therefore, the analytical method used in this study is reproducible, and the samples are stable during the test period.

Method validation for quantitative analysis
For each analyte, the peak area (Y, arbitrary units) and concentration (X, μg/mL) were subjected to linear regression analysis. All standard compounds showed good linearity (R ≥ 0.9995). The regression equation, correlation coefficient (R), linear range, LOD and LOQ are listed in Table I.

In the precision, repeatability and stability experiments, each RSD value was less than 3.0% (Table II). In the recovery experiments, the average recoveries of the analytes were between 96.37% and 103.28% with RSD values less than 3.0% (Table III). All the results above indicated that the analytical method established was accurate, stable and reliable.

UPLC fingerprint analysis
After the chromatographic profiles of 27 batches of PC from different regions were investigated and compared, the UPLC reference fingerprint of PC was established (Figure 3) with Median method using SES software (Version 2004A). A total of 20 peaks were visible and assigned as the characteristic peaks in the analysis of the 27 batches. By comparing with the retention times and UV spectra of the standards, peaks 5, 6, 9, 10, 12, 13 and 15 were identified as myricitrin, Figure 2. The chromatogram of the mixed standard solution at 340 nm (Peak 5-myricitrin, Peak 6-isoquercitrin, Peak 9-quercitrin, Peak 10-afzelin, Peak 12-cupressuflavone, Peak 13-amentoflavone and Peak 15-hinokiflavone).
is the dendrogram of the 27 samples

The similarities between the reference fingerprint and chromatograms of the samples were calculated, which varied from 0.503 to 0.955 (Table IV). Each sample exhibited varying similarities with other samples. The results showed that the intrinsic qualities of these samples were different to some extent.

Quantitative analysis
After the seven bioactive flavonoids were identified in the fingerprint analysis, their contents in the 27 batches of PC from different regions were analyzed by the validated determination method (Table IV). This quantitative analysis was performed by the external standard method with calibration curves.

Hierarchical cluster analysis
As a multivariate analysis technique, HCA is often used to discriminate different samples. In this study, HCA was performed based on the contents of the seven main flavonoids. To minimize the effect caused by some notable differences in content among the seven compounds, standard normal variant transformation of these data was applied before HCA. During analysis, between-groups linkage was used as the cluster method and squared euclidean distance was set as the interval. Figure 4 is the dendrogram of the 27 samples after hierarchical cluster analysis. The dendrogram showed that all the samples would be classified into five groups. Among them, No. 4, 8, 9, 16, 22, 24 were in Cluster I, while No. 2, 3, 5, 6, 10, 11, 14, 15, 18, 23, 25, 27 in Cluster II and No. 1, 7, 12, 13, 19, 20, 21 in Cluster III. No. 17 and 26 belonged to Cluster IV and V, respectively.

Discussion
In this study, a novel UPLC-DAD method for fingerprint analysis and simultaneous determination of four flavonoid glycosides and three biflavonoids in PC has been established and validated, which is reported for the first time. Totally 27 batches of PC, collected from different regions, were successfully analyzed by the method coupled with hierarchical cluster analysis. In the fingerprint analysis, 20 characteristic peaks were found. As a result, a reference fingerprint profile was obtained and similarities of 27 samples ranged from 0.503 to 0.955.
In the fingerprint profile, seven flavonoids, including myricitrin, isoquercitrin, quercitrin, afzelin, cupressusflavone, amentoflavone and hinokiflavone, were identified and quantitatively analyzed in the samples. Among them, quercitrin was found with the highest content in each sample. So, it was proved rational that quercitrin was selected as chemical marker in the Chinese Pharmacopoeia (1). For the three biaflavonoids, content of either amentoflavone or hinokiflavone was found to be 10× higher than that of cupressusflavone.
All the contents of these seven flavonoids were more than 0.1 mg/g, and some even were nearly or above 1 mg/g. The results showed that it was reasonable to evaluate quality of PC with the four flavonoid glycosides and three biflavonoids using the UPLC method.

Based on the various contents of the seven flavonoids, all the 27 batches were clustered into five groups by HCA. The result, with some territoriality, indicated that the five categories were almost consistent with geographical positions and climatic conditions of the production regions. For example, Cluster I represented the region of South China along Wuyi Mountains, and Cluster II corresponded to most parts of North China and around Taihang Mountains, while Cluster III covered Northeast of China.

With this UPLC-DAD method, several advantages can be observed compared to traditional HPLC methods. Often in TCM analyses, lengthy chromatographic times and high mobile phase consumption were necessary to achieve the separation required. In this paper, the proposed UPLC-DAD method required shorter analysis time with less mobile phase consumption to achieve the needed separation and accuracy to provide both qualitative and quantitative analysis of TCM. Due to its reduced analysis time and solvents consumption, along with better separation efficiency, the UPLC method will be an effective alternative to previous HPLC methods in TCM researches.

### Supplementary Data

Supplementary data are available at *Journal of Chromatographic Science* online.

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### References


11. Wei, J.J.; Clinical study on treatment of 62 cases of Baixiefeng with oral medicine; *Journal of Anhui Traditional Chinese Medical University*, (2006); 14: 346–347.


15. Dong, H.M., Sheng, Z.H., Ge, E.N.; Determination of quercitrin from *Thuja orientalis* leaves by capillary-zone electrophoresis; *Chinese Journal of Experimental Traditional Medical Formulae*, (2010); 16: 73–75.


19. Dong, H.M., Sheng, Z.H., Ge, E.N.; Determination of quercitrin from *Thuja orientalis* leaves by capillary-zone electrophoresis; *Chinese Journal of Experimental Traditional Medical Formulae*, (2010); 16: 73–75.


22. Dong, H.M., Sheng, Z.H., Ge, E.N.; Determination of quercitrin from *Thuja orientalis* leaves by HPLC; *Journal of Shanghai College of Traditional Chinese Medicine*, (2010); 3: 85–86.


