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Full Length Research Paper

A study of the development and utilization of major functional compounds from different cultivars of bamboo leaves

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A reversed phase high performance liquid chromatography (RP-HPLC) method was described for simultaneous determination of seven effective constituents (orientin, isoorientin, vitexin, isovitexin, chlorogenic acid, caffeic acid and ferulic acid) in Bamboo leaf from nine cultivars of *Phyllostachys pubescens.* The seven compounds were simultaneously determined by HPLC with Sunfire C_{18} ODS column (4.6 × 250 mm, 5 µm) by gradient elution using acetonitrile (A) and 0.8% acetic acid (B) as the mobile phase. The flow rate was 1.0 mL·min^{-1}; the detection wavelength was 335 nm with column temperature at 25°C. Finally, these effective components were separated clearly and respectively in 28 min, the linear ranges of orientin, isoorientin, vitexin, isovitexin,chlorogenic acid, caffeic acid and ferulic acid were 0.093 to 0.623 µg (r = 0.9994), 0.058 to 0.385 µg (r = 0.9994), 0.045 to 0.301 µg (r = 0.9987), 0.041 to 0.272 µg (r = 0.9992), 0.059 to 0.396 µg (r = 0.9997), 0.046 to 0.312 µg (r = 0.9992), 0.103 to 0.684 µg (r = 0.9983), respectively; The average recoveries (n = 3) were 98.75, 98.34, 98.32, 99.70, 98.61, 98.20 and 99.31%, respectively. The validated method is simple, accurate and can be widely applied to quantification of the four flavonoids and three phenolic acid compounds in 9 different cultivars of *P. pubescens.*

Key words: Reversed phase high performance liquid chromatography (RP-HPLC), orientin, isoorientin, vitexin, isovitexin, chlorogenic acid, caffeic acid, ferulic acid.

INTRODUCTION

Phyllostachys pubescens Mazelex H.de Lehaie, taking up about 70% of bamboo in China, is one of the most important resource of economic bamboo species with its fast growth rate, high yield, wide use and broad planting area (Zhang and He, 2006).

Bamboo leaves contain comparatively high volume of flavonoids, such as orientin, isoorientin, vitexin and isovitexin, as well as phenolic acids, such as chlorogenic acid, caffeic acid and ferulic acid. These components have generated particular interest with regard to human health effects including antioxidant, anti-aging, antitumor, antiviral and antibacterial activities, and protection of cardiovascular diseases, cancer prevention, etc. (Zhang et al., 2004; Lu et al., 2005; Zhang et al., 2002; Tang et al., 2007). Thus, they can be used as natural antioxidant, antiseptics, food additive and so on. *P. pubescens* Mazelex H.de Lehaie produced in Zhejiang Province is a famous economic bamboo. It is documented in Chinese National Medicine Assembly as a main source of *Phyllostachys* bambusoideae. A large amount of *P. pubescens* in Zhejiang of China, one of the famous and main origins, are used clinically and cultivated to many

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Table 1. Composition of mobile phase with gradient elution program.

Time/min	A% (Acetonitrile)	B% (Acetic acid/water)
0-2	10-10	90-90
2-6	10-14	90-86
6-16	14-17	86-83
16-23	17-19	83-81
23-28	19-23	81-77

countries and regions.

After retrieving the literature published in recent twenty years, it is very difficult to identify the cultivars for *P. pubescens* based on their appearances, because the appearances of *P. pubescens* from the 15 cultivars are very similar (Guo et al., 2009). However, fewer researches focused on the simultaneous determination of these flavonoids and phenolic acids from bamboo using high performance liquid chromatography (HPLC) method. The aim of this study was to establish a method for determination of these seven effective compounds from nine different cultivars of *P. pubescens,* which set up a quality evaluation system of different cultivars of bamboo; therefore, this paper provides evidence for major functional compounds from different cultivars of bamboo leaves and lays a foundation for its further development and utilization.

MATERIALS AND METHODS

Reagents, standards and plant materials

Orientin, isoorientin, vitexin, isovitexin, chlorogenic acid, caffeic acid and ferulic acid standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The HPLC grade methanol and acetonitrile were purchased from Tedia (Tedia Co., OH, USA), while the HPLC grade acetic acid was 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).

Nine samples from different cultivars of *P. pubescens* Mazelex bamboo (*Phyllostachys heterocycla* cv. *Pubescens, Phyllostachys heterocycla* cv. *Gracilis, Phyllostachys heterocycla* cv. *Tao Kiang, Phyllostachys heterocycla* cv. *Heterocycla, Phyllostachys heterocycla* cv. *Luteosulcata, Phyllostachys heterocycla* cv. *Viridisulcata, Phyllostachys heterocycla* cv. *Obliquinoda*, *Phyllostachys heterocycla* cv. *Pachyloen and Phyllostachys heterocycla cv. Tubaeformis*) were gathered in the bamboos expo garden of Anji in Zhejiang Province, China. Those samples were identified by Xin-chun Lin Professor from Zhejiang Agricultural and Forestry University. The collected samples were dried for 2 h at 55°C and then ground into powder by 40 mesh sieves (Li et al., 2008).

Preparation of standard solution

The reference standards mixed solution containing orientin, isoorientin, vitexin, isovitexin, chlorogenic acid, caffeic acid and ferulic acid standards were diluted in methanol to 0.0311, 0.0192, 0.0151, 0.0136, 0.0198, 0.0156 and 0.0342 mg·mL^{-1}, respectively. 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 sample solution

The nine pulverized samples of *P. pubescens* from different cultivars in Zhejiang Province were accurately weighed (approximately 1.0 g), soaked 2 h in 10 mL of 70% methanol, and ultrasonic-extracted (KQ2200DE ultrasonic cleaning instrument (Kunshan Shumei Ultrasonic Instrument Co., Kunshan, China) with 30 ml of 70% methanol for two times (with a 40 min interval). The extracts of leaves were concentrated into dryness by the rotary evaporator, and then dissolved them to volumetric flasks of 25 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.

Instrumentation and HPLC chromatographic conditions

The HPLC system consisted of a Waters 2695 to 2996 system (Waters Corp., MA, USA), equipped with a binary solvent manager, an auto-sampler and a Waters 2996 diode array detector (DAD), was used for liquid chromatographic analysis. The column used was Sunfire C_{18} ODS (250 × 4.6 mm, 5 um, Waters Corp., MA, USA). The mobile phase consisted of (A) acetonitrile and (B) acetic acid/water (0.8:100, v/v). The gradient elution is optimized as Table 1. The flow rate was 1.0 mL/min, and the column temperature was 25°C. The injection volume was 10 µL. The detection wavelength was 335 nm.

RESULTS AND DISCUSSION

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 result indicated that the ultrasonic extracted for 1 h chosen as extracting method. And then the pulverized samples were ultrasonic extracted for 1 h with 100, 70 and 50% (v/v) of ethanol and methanol, respectively. Comparing with the rest solvents and methods, more peaks were obtained in the extracts that ultrasonic extracted for 1 h in 70% methanol. According to the principle that more chemical composition should be retained to evaluate traditional

Table 2. Calibration curves of eight effective compounds given as regression equation, correlation coefficient and linear response range.

Compound	Regression equation	Correlation coefficient (r)	Llinear response range (μg)
Orientin	$Y = 2.72 \times 10^{6}$; $X - 4.75 \times 10^{4}$	0.9998	0.093 - 0.623
Isoorientin	$Y = 1.92 \times 10^{6}$; $X - 3.08 \times 10^{4}$	0.9999	0.058 - 0.385
Vitexin	$Y = 2.01 \times 10^{6}$; $X - 1.28 \times 10^{4}$	0.9987	0.045 - 0.301
Isovitexin	$Y = 3.22 \times 10^{6}$; $X - 2.69 \times 10^{4}$	0.9992	0.041 - 0.272
Chlorogenic acid	$Y = 1.69 \times 10^{6}$; $X - 2.40 \times 10^{4}$	0.9999	0.059 - 0.396
Caffeic acid	$Y = 6.02 \times 10^{6}$; $X - 7.32 \times 10^{4}$	0.9993	0.046 - 0.312
Ferulic acid	$Y = 2.38 \times 10^{6}$; $X - 5.93 \times 10^{4}$	0.9998	0.103 - 0.684

Chinese medicine, 70% methanol was finally chosen as the extracting solvent and ultrasonic extracting for 1 h was as the extracting method.

Optimization of chromatographic conditions

Chromatograph column, column temperature, monitoring wave length and mobile phase were selected that provided the best results in chromatographic finger-printing analysis (Yuan et al., 2008; Li et al., 2004; Zhang et al., 2008). High performance liquid chromatography with ODS column is 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. Therefore, different types of column were tested. The property of separation of the Sunfire C_{18} ODS column for flavanones of bamboo is best than that of XBridge C_{18} ODS column. Comparing the chromatograms at three different temperatures 25, 30 and 40°C, after overall consideration of the analysis time and separating effect, the column temperature was set at 25°C.

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 added a certain percentage of acid into the mobile phase to improve the resolutions of chromatographic peaks. Different mobile phase 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) and acetonitrile-phosphoric acid/water (0.2:100, v/v), etc., (Lan et al., 2005; Wang et al., 2007; Zhou et al., 2008; Quercia et al., 1978). Finally, acetonitrile-acetic acid/ water (0.8:100, v/v) were selected as an appropriate mobile phase with gradient elution, which gave good resolution and shortest analysis time.

Selection of detection wavelength

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 simultaneous determination of seven effective con-stituents. Chromatogram at 335 nm showed the most abundant components information and the steadiest baseline than at other wavelength. Finally, we chose 335 nm as the monitoring wavelength.

Methodology validation

The assay linearity was determined by analysis of six different concentrations of the standard solutions. The standard curves were obtained by plotting peak area (y) vs. nominal concentration [x (μg/mL)] of each compound and were fitted to linear regression y = ax+b. Concentration of these marker sunstances in samples were calculated from this regression analysis. The regression equation, correlation coefficient, linear response range of orientin, isoorientin, vitexin, isovitexin, chlorogenic acid, caffeic acid and ferulic acid are showed in Table 2.

All tests were carried out on the sample extract solutions prepared as described in section preparation of standard solution and sample solution. The injection precision was determined by replicating HPLC injections of the same sample solution six times in a day and the peak area of each characteristic peak were calculated for the estimation of precision of sample. The results of the relative standard deviation (RSD) were 1.159, 0.365, 0.408, 0.457, 0.505, 0.740 and 0.730%, respectively. The sample stability test precision was determined with measurements from a single sample solution stored at room temperature for 0, 4, 8, 12, 18, and 24 h. And the results of RSD were 0.964, 0.198, 0.429, 0.518, 0.851, 0.213 and 0.871%.

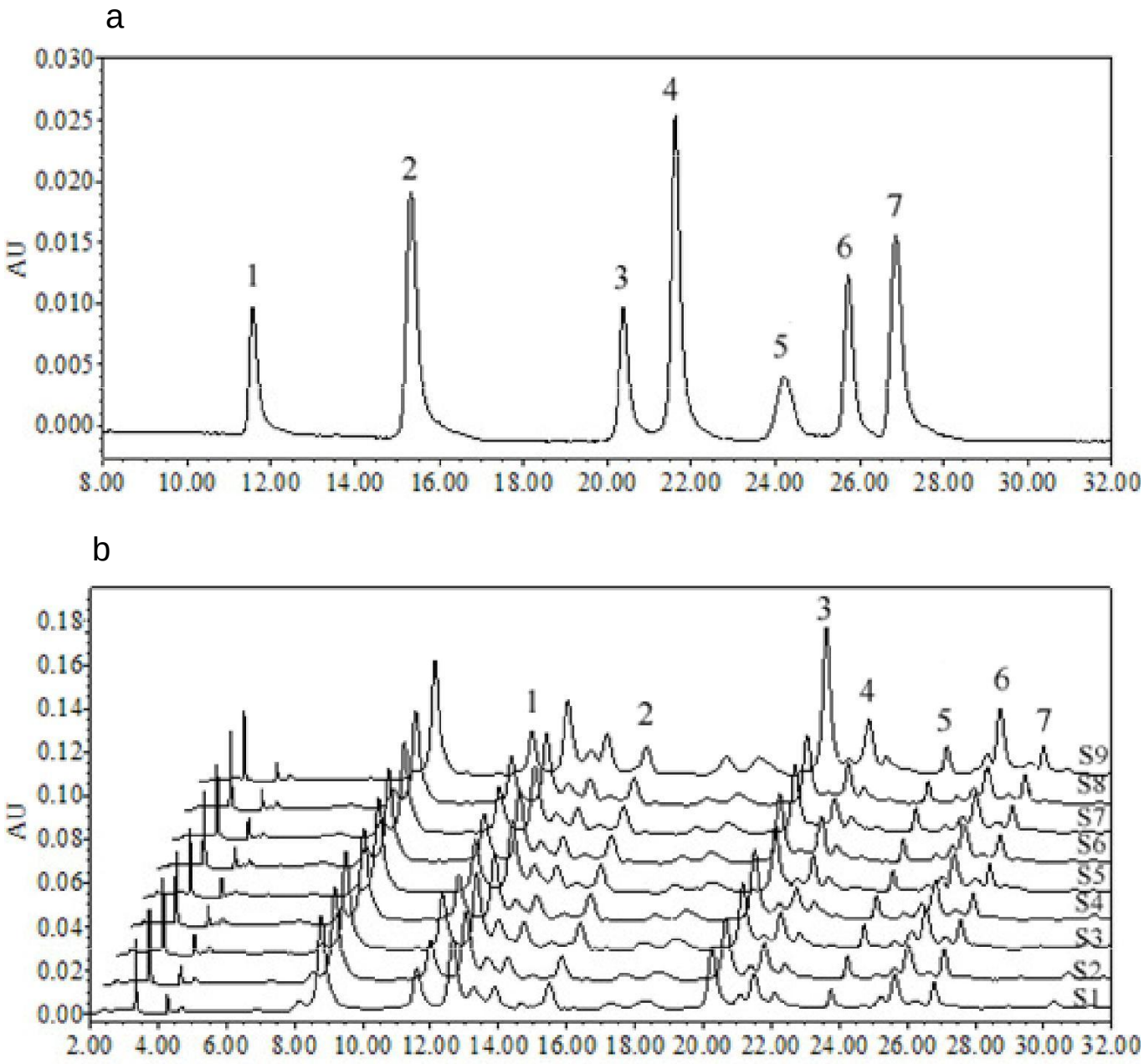

Figure 1. HPLC chromatograms of standard solution (a) and nine samples (b) of seven effective compounds. . 1. Chlorogenic acid; 2. Caffeic acid; 3. Isoorientin; 4. Orientin; 5. Vitexin; 6. Isovitexin;7. Ferulic acid. S1: *Phyllostachys heterocycla* cv. *Pubescens.* S2: *Phyllostachys heterocycla* cv. *Gracilis.* S3: *Phyllostachys heterocycla* cv. *Tao Kiang* S4: *Phyllostachys heterocycla* cv.*Heterocycla.* S5: *Phyllostachys heterocycla* cv.*Luteosulcata*. S6: *Phyllostachys heterocycla* cv. *Viridisulcata.* S7: *Phyllostachys heterocycla* cv. *Obliquinoda.* S8: *Phyllostachys heterocycla* cv.*Pachyloen.* S9: *Phyllostachys heterocycla* cv. *Tubaeformis.*

The repeatability was assessed by analyzing six independently prepared samples of bamboo-leaf samples. And results of RSD of repeatability were 0.947, 0.965, 1.004, 0.416, 1.201, 1.458 and 0.553%, respectively. The recoveries of the seven compounds and the RSDs were 98.75, 0.851, 98.34, 1.092, 98.32, 1.142, 99.70, 0.232, 98.61, 1.315, 98.20, 1.813, 99.31 and 0.545%. The peak area of each characteristic peak were calculated for the estimation of precision, stability, repeatability and recoveries and all the results of the relative standard deviation (RSD) were no more than 3%. Thus, all results indicated that the quality of the studied samples and the HPLC-DAD measurements were stable and under control.

Table 3. Quantitative values of four flavonoids and three phenol acids in leaf of nine samples (n = 3).

Cultivars	Chlorogenic acid	Caffeic acid	Isoorientin	Orientin	Vitexin	Isovitexin	Ferulic acid
P. pubescens	0.192	0.081	0.433	0.155	0.137	0.175	0.096
P. gracilis	0.315	0.086	0.469	0.185	0.147	0.188	0.116
P. Tao Kiang	0.283	0.093	0.439	0.166	0.148	0.186	0.099
P. heterocycla	0.304	0.090	0.450	0.172	0.145	0.185	0.109
P. luteosulcata	0.225	0.091	0.505	0.186	0.126	0.183	0.102
P. viridisulcata	0.248	0.087	0.510	0.199	0.145	0.188	0.098
P. obliquinoda	0.275	0.089	0.548	0.139	0.139	0.185	0.099
P. pachyloen	0.194	0.085	0.436	0.151	0.136	0.177	0.092
P. tubaeformis	0.295	0.099	0.684	0.203	0.151	0.197	0.098

Determination of the seven effective compounds in different cultivars of *Phyllostachys pubescens* samples

The nine sample solutions of *P. pubescens* were prepared and analyzed, and chromatograms of the samples were recorded at 335 nm, which is shown in Figure 1. The RT (retention time) of chlorogenic acid, caffeic acid, isoorientin, orientin, vitexin, isovitexin, and ferulic acid were 11.531, 15.401, 20.415, 21.509, 24.276, 25.788 and 26.943 min, respectively. Adequate separation was achieved and the quantitative values of seven effective compounds can be referred in Table 3. The nine cultivars of bamboo used in this experiment are all varieties of *P. pubescens.* It is hard to discriminate the differences between these varieties merely from their appearances. However, the test results indicate that, in the same period and place, the types of flavonoids and phenolic acids are similar in different varieties of bamboo leaves while chlorogenic acid, orientin and isoorientin differ. Compared the seven effective constituents, all leaves contain more chlorogenic acid and isoorientin but less ferulic acid and caffeic acid. The experiment also proves that the total contents of those effective components vary little in different varieties, ranking as: *P. tubaeformis >P. viridisulcata >P. obliquinoda >P. luteosulcata >P. gracilis >P. heterocycla >P. Tao Kiang >P. pubescens >P. pachyloen.*

Conclusion

The HPLC method established in this paper measures simultaneously the content of seven constituents, namely chlorogenic acid, caffeic acid, isoorientin, orientin, vitexin, isovitexin, ferulic acid, in leaves of nine different cultivars of *P. pubescens*, finding that all the seven components exist in the nine samples. Among the three kinds of phenolic acids and four flavonoids, chlorogenic acid and isoorientin are contained more while ferulic acid and caffeic acid are less. Nevertheless, the overall volumes of three phenolic acid compounds and four flavonoids in the nine leaves are similar. Hence, the paper aims to set a liable foundation for the further development and application of the chemistry components in *P. pubescens*. The method used in the experiment is simple, speedy, accurate with high repetitiveness and liability. The result can be commonly used in quality control and appraisal of bamboo leaves and its preparations. It offers a reference to further understand the distributional pattern of phenolic acids and flavonoids in bamboo leaves and to its future development and utilization.

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