Simultaneous Determination of Flavonoids from Bamboo Leaf Extracts Using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

An analytical method for the simultaneous determination of ten major functional flavonoids (isoorientin, orientin, vitexin, isovitexin, apigenin, luteolin, tricetin, quercetin, rutin, and kaempferol) in different bamboo species was developed by liquid chromatography-tandem mass spectrometry. Chromatographic separation was carried out on a reversed-phase C-18 column with acetonitrile and water as the mobile phases. Detection was performed in negative ion electrospray ionization mode using multiple reaction monitoring mode. The correlation coefficients for the calibration curves ranged from 0.9955 to 0.9997. The limit of detection ranged from 1 to 45 ng/ml. The applicability of this analytical approach was confirmed by the successful analysis of real leaf samples of four bamboo species, family Poaceae: *Pleioblastus amarus* (Keng) Keng f., *Phyllostachys glauca* McClure, *Phyllostachys edullis* (Carrière) J.Houz, and *Indocalamus latifolius* (Keng) McClure. The total flavonoid contents were 3321.09, 3095.96, 4037.33, and 2808.42 mg/kg for *P. amarus*, *P. glauca*, *P. edullis*, and *I. latifolius*, respectively.

Keywords Bamboo leaf flavonoids · LC–MS–MS · Multiple reaction monitoring · Different ethanol extracts

Introduction

Bamboo, an important building material and a potential source of bioactive substances, is widely distributed in the tropics and subtropics (Lu et al. 2005). Bamboo leaves have been used as food and folk medicine for more than 1000 years in China (Lu et al. 2005; Gong et al. 2015). Numerous studies have shown that the major bioactive components of bamboo leaf extract are flavonoids including kaempferol (1), rutin (2), orientin (4), isoorientin (8), vitexin (9) and isovitexin (10), and quercetin (5) (Zhang et al. 2005; Lee et al. 2010; Wang et al. 2010; 2012; Wu et al. 2012; Ma

et al. 2012; Mao et al. 2013). Previous studies have reported that flavonoid-rich bamboo leaf extracts have a variety of biological effects including antioxidant, anticancer, antibacterial, antiviral, anti-inflammatory, and antimutagenic effects (Lu et al. 2005; An et al. 2012; Van Hoyweghen et al. 2012). These extracts can also be used as ingredients in dietary supplements and food additives (Lu et al. 2005; Zhang et al. 2007; Koide et al. 2011; Gong et al. 2015), in part, due to the antioxidant activity of bamboo leaves. Antioxidants from bamboo leaves can be added to the puffed grain food (corn, rice, wheat, and amaranth, *inter alia*, for snacks and food product) meat products and oils, as authorized by the Ministry of Health, PR China (Lu et al. 2005). Bamboo leaf flavonoids are also important in fat resistance and effective in the treatment of cancer and aging (Hu et al. 2000).

Concerning the extensive utilization of bamboo leaf flavonoids in the functional food industry and commercial products, it is necessary to develop a method to quantitatively determine active flavonoids and ensure quality control of these extracts. Some analytical methods have been reported for the simultaneous quantitation of two or four flavonoid constituents, including 8, 10, 4, and 9 in bamboo leaf extracts. Analytical methods include thin-layer chromatography (TLC) (Wang et al. 2012), high-performance liquid chromatography HPLC (Zhang et al.

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2005), high-performance capillary electrophoresis (HPCE) (Lu et al. 2005), high-performance thin-layer chromatography (HPTLC) (Sun et al. 2010; Jian et al. 2011), and high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Pereira et al. 2010). However, to date, liquid chromatography-tandem mass spectrometry (LC-MS-MS) methods have not been developed for the simultaneous analysis of the ten major flavonoids in bamboo leaves. LC-MS-MS has been recognized as a powerful analytical tool for the fast measurement of various analytes, such as polar, nonvolatile, high molecular mass compounds in natural product research compared to reversed-phase standalone HPLC coupled with diode array detection (Zu et al. 2006). In this study, a simple, sensitive, and throughout LC-MS-MS method was first developed and validated for the simultaneous determination of ten flavonoids: kaempferol (1), rutin (2), tricetin (3), orientin (4), quercetin (5), luteolin (6), apigenin (7), isoorientin (8), vitexin (9), and isovitexin (10), which are generally considered to be the major constituent flavonoids of bamboo leaves (Yang et al. 2017), in a single run and to demonstrate the applicability of the method for the analysis of leaf samples from four species of bamboo.

- 1 $R_1=R_2=R_3=R_4=H$; $R_5=OH$
- 2 $R_2=R_3=R_4=H$; $R_1=OH$; $R_5=OGlu_6^{-1}Rha$
- 3 $R_1=R_2=OCH_3$; $R_3=R_4=R_5=H$
- 4 $R_2=OH$; $R_1=R_3=R_5=H$; $R_4=Glu$
- 5 $R_2=R_5=OH$; $R_1=R_3=R_4=H$
- 6 $R_1=OH$; $R_2=R_3=R_4=R_5=H$
- 7 $R_1=R_2=R_3=R_4=R_5=H$
- 8 $R_2=OH$; $R_1=R_4=R_5=H$; $R_3=Glu$
- 9 $R_1=R_2=R_3=R_5=H$; $R_4=Glu$
- 10 $R_1=R_2=R_4=R_5=H$; $R_3=Glu$

Materials and Methods

The ten flavonoid compounds were purchased from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China) with a purity above 98%. Leaves of four bamboo species, *Pleioblastus amarus* (Keng) Keng f., *Phyllostachys glauca* McClure, *Phyllostachys edulis* (Carrière)

J. Houz, and *Indocalamus latifolius* (Keng) McClure, were collected from Changning County, Sichuan Province, China in November, 2016 and identified by Guanghui Lai, Guangde Forestry and Grassland Bureau (Anhui, China). Vouchers registered as CDBI0152311 and CDBI0152265 for *P. amarus* and *I. latifolius* were deposited in the herbarium of Chengdu Institute of Biology, Chinese Academy of Sciences. Vouchers registered as SM720601535 and LBG00120870 for *P. heterocycle* and *P. glauca* were deposited in the Chongqing Academy of Chinese Materia Medica herbarium and the Lushan Botanical Garden, Chinese Academy of Sciences herbarium, respectively. Dried bamboo leaf powder (10.0 g) was macerated in a mixture (100 ml) of ethanol (EtOH)/H₂O (3:2) and treated with a sonicator (250 W, KQ-500, Kunshan Ultrasonic Instrument Co., Ltd.) for 30 min at 50 °C. After repeated extraction ($n=3$), the extracted solution obtained by filtration was concentrated under reduced pressure and then diluted with water to 50 ml. The extract, after extraction with petroleum ether (3 × 150 ml), was evaporated to dryness with a rotary evaporator and subjected to a macroporous resin column (Diaion HP-20, Mitsubishi Chemical Corp., Tokyo, Japan) at a flow rate of 10 ml/min using H₂O/EtOH (100:0, 85:15, 70:30, 50:50, 30:70, 5:95 v/v) as the solvent to produce six extracts. The solvent was removed under reduced pressure. The residue was reconstituted in 65% EtOH/H₂O (10 ml), filtered through a 0.22-μm cellulose membrane, transferred into glass vials, and stored at −20 °C until analysis.

LC-MS-MS analysis was conducted by using a Bruker Advance UPLC system coupled with a Bruker EVOQ Elite triple quadrupole mass spectrometer (Bruker, Fremont, CA), which was connected to an electrospray ionization (ESI) source. The samples were injected onto an Agilent Eclipse XDB C-18 (4.6 × 150 mm, 5 μm) column. The flow rate was 0.8 ml/min with a split ratio of 1:1, and the column temperature was set at 40 °C. Each sample was analyzed at least three times with an injection volume of 2 μl. The ESI source was operated in negative ion mode, and full scan mass spectral data were acquired over a range from mass-to-charge (m/z) 50 to 800. Negative ESI mode was selected as the ionization mode for further optimization experiments. Multiple reaction monitoring (MRM) mode using the m/z transitions of the precursor and product ions was applied to enhance sensitivity. The optimized mass spectrometric parameters were as follows: nebulizer gas flow, 60 ml/min; probe gas flow, 50 ml/min; cone temperature, 300 °C; cone gas flow, 20 ml/min; and probe temperature, 300 °C.

To estimate the analytical parameters of the methodology for bamboo samples, intra-day precision, limits of detection and quantification, matrix effects, and recovery were evaluated. Five consecutive injections of each bamboo leaf

extracts were performed on the same day to evaluate the method's precision. The results are expressed as relative standard deviations (RSDs). Calibration curves were established by injecting pooled standard solutions prepared from the standard mixtures. Calibration curves were constructed by plotting the concentrations of each target analyte versus the target analyte peak area using a linear regression analysis. A six-point calibration curve in the range of 0.1–50 µg/ml was generated with correlation coefficients (R^2) between 0.9955 and 0.9997. The detection limits (LODs) and quantification limits (LOQs) were estimated at signal-to-noise (S/N) ratios of 3 and 10, respectively. Recovery tests were carried out (five replicates) by spiking the compounds at three levels in the range of the calibration curves (1, 10, and 50 µg/ml, final concentration added) in the bamboo leaf samples before extraction, and a procedural blank was also carried out. Five replications of 10.0 g well-homogenized bamboo leaf composites were extracted and cleaned

up with the procedures described above. The matrix effect was evaluated by comparing the slopes of the calibration curves obtained from the spiked bamboo leaf samples with the slopes of the calibration curves obtained from the standards. The signal of the analyte was considered enhanced if the quotient of the spiked sample curve slope and the standard curve slope was higher than 1, whereas the signal of the analyte was considered suppressed if the value was lower than 1.

Results and Discussion

Individual target compound solutions were directly injected into the ESI–MS–MS system to identify the precursor and product ions under negative ionization modes. After choosing the precursor ions and product ions, the parameters for collision energy (CE) were

Fig. 1 The extracted ion chromatograms of ten flavonoids. **a** Reference standards and **b** a mixture for one of the bamboo leaf extracts (*Pleioblastus amarus*). Kaempferol (**1**, $R_t=21.18$ min), rutin (**2**, $R_t=12.06$ min) 0.85 µg/ml; triclin (**3**, $R_t=20.7$ min) 0.0635 µg/ml; orientin (**4**, $R_t=7.25$ min) 5.25 µg/ml; quercetin (**5**, $R_t=19.07$ min)

0.35 µg/ml; luteolin (**6**, $R_t=18.53$ min) 0.13 µg/ml; 0.0635 µg/ml; apigenin (**7**, $R_t=20.97$ min), 0.275 µg/ml; isoorientin (**8**, $R_t=6.19$ min) 1.5 µg/ml; vitexin (**9**, $R_t=10.98$ min) 2.5 µg/ml; isovitexin (**10**, $R_t=11.71$ min) 6.5 µg/ml

Table 1 Intra-day precision, linearity, limits of detection (LOD), and limits of quantification (LOQ) of the analytes with LC–MS/MS

Compound	Linearity		LOQ (ng/ml)	LOD (ng/ml)	Intra-day precision (RSD %)
	Range (µg/ml)	R^2			
Apigenin	0.1–50	0.9993	3	1	1.12
Quercetin	0.1–50	0.9991	20	5	2.21
Rutin	0.1–50	0.9985	100	30	1.38
Luteolin	0.1–50	0.9971	5	2	3.12
Kaempferol	0.1–50	0.9987	5	2	2.34
Vitexin	0.1–50	0.9997	100	35	2.18
Isovitexin	0.1–50	0.9993	150	45	1.36
Orientin	0.1–50	0.9991	55	15	3.26
Isoorientin	0.1–50	0.9978	90	25	5.33
Tricin	0.1–50	0.9955	10	3	3.45

further optimized to obtain the maximum response of the precursor and product ions (Table S1). In this study, different mobile phases, such as methanol (MeOH), acetonitrile (MeCN), and a mixture of MeOH and MeCN (50:50, v/v) with different compositions (ammonium acetate, formic acid at various concentrations), were tested. As expected, the addition of ammonium acetate into the mobile phases resulted in good peak shapes and enhanced the detection sensitivity for the majority of the target compounds. Finally, gradients consisting of 0.05% ammonium acetate in acetonitrile (mobile phase A) and 0.05% ammonium acetate in water (mobile phase B) were chosen as the appropriate mobile phases for routine LC–MS–MS analyses of the target compounds. The optimized gradient elution for LC separation was as follows: 0 ~ 15.00 min, 15% A; 15.01 ~ 25.00 min, 35% A; 25.00 ~ 27.00 min, 35% A ~ 100% A; 27.00 ~ 28.00 min, 100% A ~ 15% A. The equilibration time before the next run was 6 min. Under the described chromatographic conditions, all the compounds were eluted in a total run time of 25 min. Figure 1 shows a chromatogram for all

standard mixture after being optimized. Flavonoids were eluted in the order of isoorientin (**8**, R_t = 6.19 min), orientin (**4**, R_t = 7.25 min), vitexin (**9**, R_t = 10.98 min), isovitexin (**10**, R_t = 11.71 min), rutin (**2**, R_t = 12.06 min), luteolin (**6**, R_t = 18.53 min), quercetin (**5**, R_t = 19.07 min), tricetin (**3**, R_t = 20.7 min), apigenin (**7**, R_t = 20.97 min), and kaempferol (**1**, R_t = 21.18 min).

Analytical parameters, such as the linearity, precision of the method, and LOD, were calculated for the developed LC–ESI–MS–MS method. The intra-day RSD values ranged from 1.12 to 5.33% and were always < 10%, indicating good intra-assay variation (Table 1). For the linearity study, calibration curves were determined for all the individual analytes in the six calibration standards (0.1–50 µg/ml) with correlation coefficients ranging between 0.9955 and 0.9997 (Table 1). The LOD and LOQ values for the target compounds ranged from 1 to 45 ng/ml and from 3 to 150 ng/ml, respectively (Table 1). The recoveries of the bamboo leaf flavonoids ranged from 68.45 to 112.31%, with standard deviations ranging from 2.3 to 9.1% for the 0.1, 0.2, and 0.4 µg/ml spiking tests (n = 5; Table S2). The results of the

Fig. 2 Total flavonoids in different ethanol fractions of four bamboo leaf crude extracts

method validation revealed good sensitivity, precision, and accuracy for the simultaneous determination of all of the target compounds by the developed method.

The developed LC–MS–MS method was finally applied to the determination of ten major flavonoids from four bamboo leaf extracts (*P. amarus*, *I. latifolius*, *P. heterocycle*, *P. glauca*) (Table S3) and the corresponding desorption solutions (EtOH concentration: 0, 15, 30, 50, 70, and 95%, v/v) of the four bamboo leaf extracts (Table S4, 5, 6, 7). As summarized in Table S3, all four bamboo leaf extracts contained compounds 1–4, and the concentrations of these ten flavonoids were similar among the four species. Compounds 3, 4, and 9 were identified as major components in all the bamboo leaf extracts (Fig. S1), which is in agreement with previous studies (Jiao et al. 2007; Van Hoyweghen et al. 2012). The total concentrations of the flavonoids were 3321.09, 3095.96, 4037.33, and 2808.42 mg/kg in *P. amarus*, *I. latifolius*, *P. heterocycle*, *P. glauca* respectively (Table S3). The most abundant flavonoids that were determined in the four bamboo leaf extracts were 3, 2, and 4. It has been reported that these compounds are used as markers for the determination of bamboo leaf flavonoids in commercial products (Jiao et al. 2007; Zhang et al. 2005). Fig. S1 shows the compositions and contents of the ten flavonoids in different ethanol extracts of leaves from four species of bamboo. All the target analytes were detected in only the 30% and 50% EtOH extracts, and 2 and 4 were the dominant compounds in all of the examined samples. The highest content of total flavonoids was detected in the 50% EtOH extract (Fig. 2) in all of the examined samples. This finding can also provide information for the further purification of flavonoid-rich extracts. The compositions and distributions of the detected flavonoids were similar in the leaf extracts from the four species of bamboo (Fig. 2). The information obtained in this study provides further support for the development of bamboo leaf extract as food supplements.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s43450-021-00158-1>.

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Data Availability Some or all data, models, or code that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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