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Analytical Methods

Isolation and purification of four flavone *C*-glycosides from antioxidant of bamboo leaves by macroporous resin column chromatography and preparative high-performance liquid chromatography

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Abstract

The present study investigated a robust method for the preparation of four flavone *C*-glycosides, i.e. orientin, homoorientin, vitexin and isovitexin, which were prepared from an ethanol aqueous extract, i.e. antioxidant of bamboo leaves (AOB), by AB-8 resin-based column chromatography and preparative high-performance liquid chromatography (HPLC) using a mobile phase consisting of 10% and 15% (v/v) of acetonitrile and 1% acetic acid. These flavone *C*-glycosides were further purified by the drowning-out crystallization method using methanol and water as drowning-out anti-solvents and salting-out agents, respectively. The purity was assessed by analytical HPLC and the confirmation of chemical structures was performed by IR, MS, NMR and UV spectroscopy. Orientin (49 mg), homoorientin (142 mg), vitexin (15 mg) and isovitexin (62 mg) were prepared from 6.5 g of crude column chromatography fraction obtained from 5 L of AOB concentrated solution. The present method is robust and suitable for preparing available quantities of pure flavone *C*-glycosides and the quantification of orientin, homoorientin, vitexin and isovitexin in bamboo leaves.
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Keywords: Flavone *C*-glycosides; Macroporous resin; Preparative HPLC; Drowning-out crystallization; Antioxidant of bamboo leaves

1. Introduction

Secondary plant metabolites are biologically active non-nutrients that have been associated with the protection of human health (Wiseman, 1999). Flavonoids, a large category of plant polyphenol secondary metabolites, are widely distributed in medicinal herbs, fruits, teas, etc. (Havsteen, 1983). Flavonoids, which are present in human diets, have generated particular interest with regard to human health effects including antioxidant activities (Bahorun, Luximon-Ramma, Crozier, & Aruoma, 2004; Burns et al., 2000), protection of cardiovascular diseases (Engler & Engler, 2006; Sesso, Gaziano, Liu, & Buring, 2003; Yochum, Kushi, Meyer, & Folsom, 1999), cancer prevention (Kosmider & Osiecka, 2004), etc. Flavone *C*-glycosides, an important subclass of the flavonoid family, were found in some plants, such as the tree *Pterocarpus marsupium* (Yadav & Singh, 1998) and the fruits of *Cucurbitaceae* (Abou-Zaid, Lombardo, Kite, Grayer, & Veitch, 2001). Various biological activities of flavone *C*-glycosides have been reported, including the antimicrobial activity of homoorientin (luteolin-6-glucoside) together with vitexin (apigenin-8-glucoside) against *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Afifi, Shervington, & Darwish, 1997) and the protective effects of luteolin-7-glucoside against liver injury caused by carbon tetrachloride in rats (Zheng, Sun, Xubo Li, Song, & Wang, 2004).

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Antioxidant of bamboo leaves (AOB), a pale brown powder extracted from bamboo leaves, was capable of blocking chain reactions of lipid autooxidation, chelating metal ions of transient state, scavenging nitrite compounds and blocking the synthetic reaction of nitrosamine (Lou, Zhang, Wu, Qi, & Zhuo, 2004). Moreover, AOB was shown to have strong antioxidant activity and inhibitory efficacy on transition metal ions and free radicals inducing deterioration of macromolecules *in vitro* (Hu, Zhang, & Kitts, 2000). In China, AOB has already been approved as a novel food antioxidant, which can be added into the puffed foods, aquatic products, meat products, edible oils, cereals, bakery foods, fruit and vegetable juices, tea beverage and fried foods authorized by Ministry of Health, China (Ministry of Health, 2003). The main functional components of AOB include flavonoids, lactones and phenolic acids while flavone *C*-glycosides are a group of representative flavonoids in AOB reported by Zhang et al. (2005). The chemical structures of four flavone *C*-glycosides found in AOB, i.e. orientin, homoorientin, vitexin and isovitexin, are shown in Fig. 1.

A metabolic study performed previously by our research group reported these four flavone *C*-glycosides were poorly absorbed in gastrointestinal tract (Zhang, Tie, Bao, Wu, & Zhang, 2007). Such poor absorption characteristic of flavone *C*-glycosides *in vivo* indicated that they may have potentials on pharmacological activities. Although various pharmacological effects of flavonoids have been reported in many peer-reviewed journals, few studies focused on the functional properties of flavone *C*-glycosides in animals or humans. Such research status may be partly ascribed to the source of flavone *C*-glycoside monomers. To cope with this problem, researchers found and optimized preparative technologies of flavonoids from various herbs. High-speed counter-current chromatography (HSCCC) (Du, Chen, Jerz, & Winterhalter, 2004; Sun, Sun, & Liu, 2007) and preparative column chromatography (Akiyama, Matsuoka, & Hayashi, 2002; Maciejewicz, 2001) are widely used. Preparative high-performance liquid chromatography (HPLC) and related technology is an effective method for the isolation and purification of active components from plant herbs. However, fewer researches optimized the isolation and purification method of flavone *C*-glycosides from plants using the preparative HPLC method compared to HSCCC and column chromatography (Miyake, Yamamoto, Morimitsu, & Osawa, 1997; Kumarasamy et al., 2004). The aim of this study was to establish a new method for the isolation and purification of four flavone *C*-glycosides (orientin, homoorientin, vitexin and isovitexin) from AOB using resin-based column chromatography and preparative HPLC technology.

2. Experimental

2.1. Chemicals

Orientin (5,7,3′,4′-tetrahydroxyflavone-8-glucoside), homoorientin (5,7,3′,4′-tetrahydroxyflavone-6-glucoside), vitexin (5,7,4′-trihydroxyflavone-8-glucoside) and isovitexin (5,7,4′-trihydroxyflavone-6-glucoside) (HPLC-grade) were purchased from Extrasynthese (Lyon, France). Acetonitrile and ethyl acetate (HPLC-grade both) were obtained from Tedia (Fairfield, OH, USA). Methanol (HPLC-grade) was purchased from Merck (Whitehouse Station, NJ, USA). All of other solvents and chemicals used in this study were of analytical grade. Redistilled water was used in the resin-based column chromatography while ultrapure water purified via the Milli-Q system (Millipore, Bedford, USA) was used during both preparative and analytical HPLC analysis. Stock and working standards of four flavone *C*-glycosides were prepared by dissolving these analytes in 50% (v/v) methanol aqueous solution. The standard solutions stored at 4 °C were stable for at least 3 months.

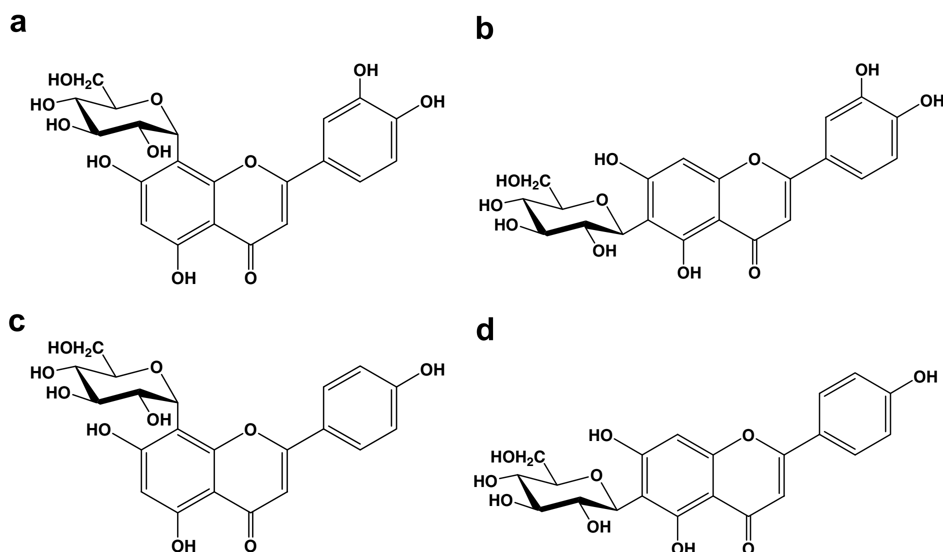

Fig. 1. Chemical structures of flavone *C*-glycosides in antioxidant of bamboo leaves (AOB). (a) Orientin, (b) homoorientin, (c) vitexin and (d) isovitexin.

2.2. Samples

The concentrated solution of antioxidant of bamboo leaves (AOB) was provided by Zhejiang University Innoessen Co. Ltd. (Hangzhou, Zhejiang, China). AOB was prepared from the bamboo leaves of *Phyllostachys nigra* var. *henonis* identified by Research Institute of Subtropical Forestry of the Chinese Academy of Forestry (Hangzhou, Zhejiang, China). Briefly, fresh bamboo leaves were collected during the autumn season in Anji district (Zhejiang, China) and then air dried. The coarse powder of bamboo leaves was obtained after comminution and filtration (20–40 mesh) and 10 g of powder was extracted for 1 h by 100 mL of 30% (v/v) ethanol aqueous solution using the hot reflux method. The filtrate was then isolated by membrane filtration to remove macro- and micro-molecular components such as polysaccharides and minerals and AOB concentrated solution was finally obtained after concentrating in vacuum, and submitted to the preparative resin-based column chromatography.

2.3. AB-8 resin-based column chromatography

The resin-based column chromatography was performed on a low-pressure glass chromatographic column (150 cm × 100 mm ID, Huamei Experiment Instrument Plant, Shanghai, China) filled with AB-8 macroporous adsorption resin (polystyrene resin, 0.3–1.25 mm particle size, Nankai University Chemical Plant, Tianjin, China). Such resin-based column needed to be pretreated prior to use. In detail, the chromatographic column was first activated with 24 L of 5% (v/v) hydrochloric acid and

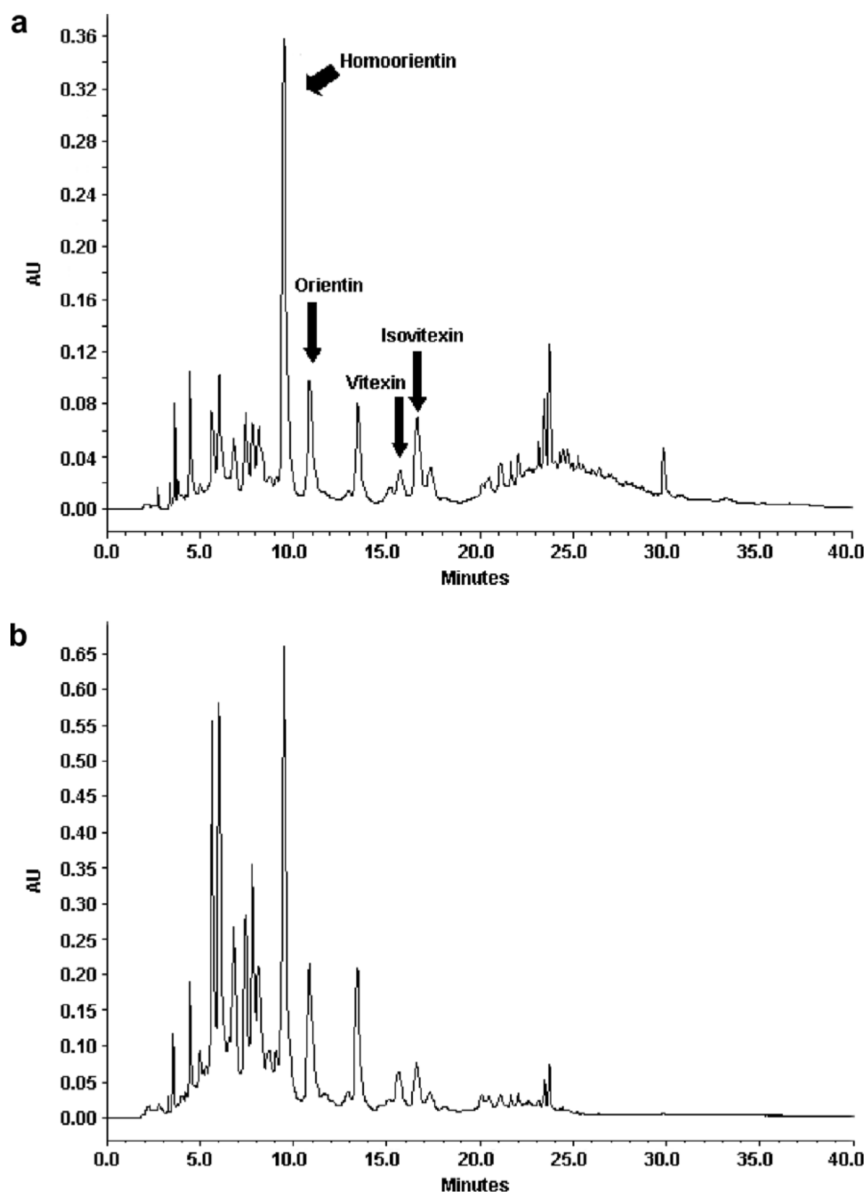

Fig. 2. The analytical HPLC chromatograms of different fractions from AOB by AB-8 resin-based column chromatography. (a) Original AOB dilute solution; (b) water fraction of AOB; and (c) 50% (v/v) ethanol fraction of AOB.

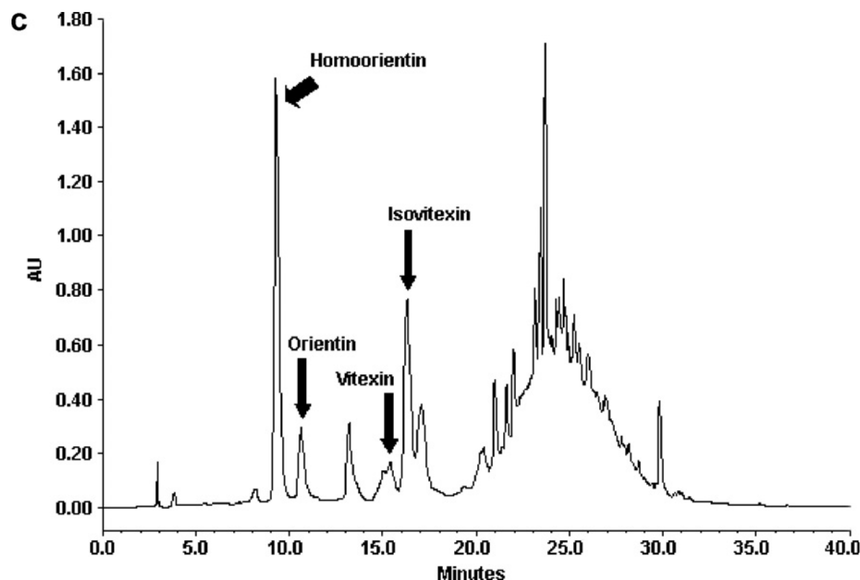

Fig. 2 (*continued*)

equilibrated with redistilled water to a neutral pH at a flow rate of 60 mL/min. Similarly, the column was then activated with 24 L of 2% (v/v) sodium hydroxide and equilibrated with redistilled water to a neutral pH at the same flow rate. Subsequently, 5 L of AOB concentrated solution was loaded onto the pretreated column and adsorbed statically overnight. The column was eluted with 25 L of redistilled water. Then, the flavone *C*-glycoside rich extract was eluted with 25 L of 50% (v/v) ethanol solution at a flow rate of 60 mL/min and this fraction was carefully collected. All portions of the above extract were then concentrated and dried by rotatory evaporator, and redissolved with 600 mL of 50% (v/v) methanol solution. Finally, this concentrated extract was submitted to the preparative HPLC analysis.

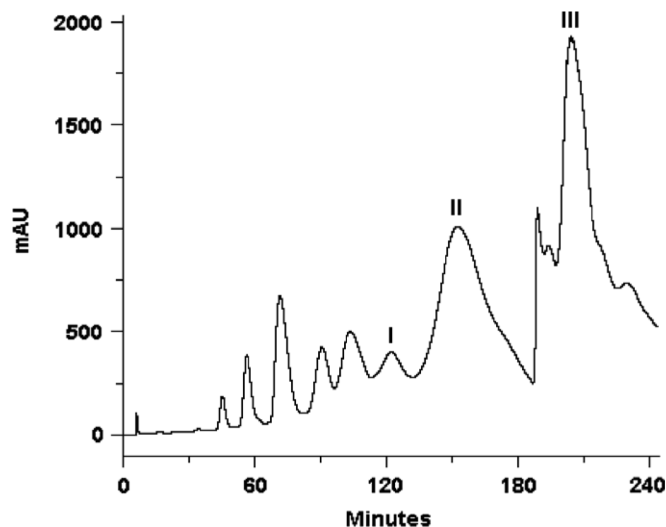

Fig. 3. The preparative HPLC chromatogram of the flavone *C*-glycoside rich fraction (50% ethanol fraction via resin-based column chromatography) from AOB. Fractions I–III were collected during preparative HPLC analysis manually. Fraction I, orientin; fraction II, homoorientin, orientin, vitexin; and fraction III, isovitexin.

2.4. Preparative HPLC separation

The chromatographic separation was performed on a Waters DeltaPrep 400 Preparative Chromatography system equipped with Waters Prep LC Controller and Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA). The preparative HPLC was performed on a Bondapak® C_{18} preparative column (300 mm × 7.8 mm I.D., 10 μm; Waters, Milford, MA, USA). The mobile phases included acetonitrile (solvent A) and 1% (v/v) acetic acid in water (solvent B). A gradient elution programme was used for present preparative separation as follows: 10% A (180 min), 10–15% A (0.1 min), 15% A (60 min). The flow rate was 25 mL/min while the detected wavelength was 330 nm. The injection volume was 10 mL. The preparative HPLC equipment was controlled by Waters Empower 2 Chromatography Data Software (Waters, Milford, MA, USA). The flavone *C*-glycoside peaks were collected manually.

2.5. Analytical HPLC detection

The analytical method for the determination of flavone *C*-glycosides in column chromatographic fractions, preparative HPLC fractions and further purification samples was performed according to our previous study, which optimized a robust HPLC method for the chromatographic separation of these four flavone *C*-glycosides with a good resolution ($R > 1.6$) (Zhang et al., 2005). Briefly, analytical HPLC was performed on a Waters 2695 HPLC chromatograph (Waters, Milford, MA, USA) with a Luna C_{18} column (5 μm, 250 mm × 4.6 mm ID) protected by a RP_{18} guard column (5 μm, 4.0 mm × 3.0 mm ID), both purchased from Phenomenex (Torrance, CA, USA). A gradi-

ent programme was used with the mobile phase, combining solvent A (acetonitrile) and solvent B (1% (v/v) acetic acid adjusted to pH 3.0 with sodium hydroxide) as follows: 15% A (15 min), 15–40% A (10 min), 40% A (9 min), 40–15% A (6 min). The flow rate was 1.0 mL/min. The injection volume was 30 μL while the column temperature was maintained at 40 °C. Signal was monitored at 330 nm with the diode array detector (DAD).

2.6. Further purification of flavone C-glycosides

The fractions, which represented four flavone *C*-glycosides collected from the eluent of preparative HPLC, were concentrated and dried by rotatory evaporator, respectively. Each residue was redissolved with 10 mL of methanol and transferred into a centrifuge tube. An equal volume of ultrapure water (10 mL) was added into the centrifuge tube, which was then placed overnight at 4 °C. The analytes were largely precipitated via the drowning-out crystallization and then centrifuged (10,000 r/min, 20 min) at 4 °C with a Microfuge 18 Beckman Coulter centrifuge (Beckman Coulter Inc., Fullerton, CA, USA). The supernatant was carefully removed and the final product was obtained after washing and drying.

2.7. Identification of flavone C-glycosides

The preparative monomers of flavone *C*-glycosides were identified by IR, UV, MS, ^{1}H NMR and ^{13}C NMR spectrometry. IR analysis was performed on a Nexus 670

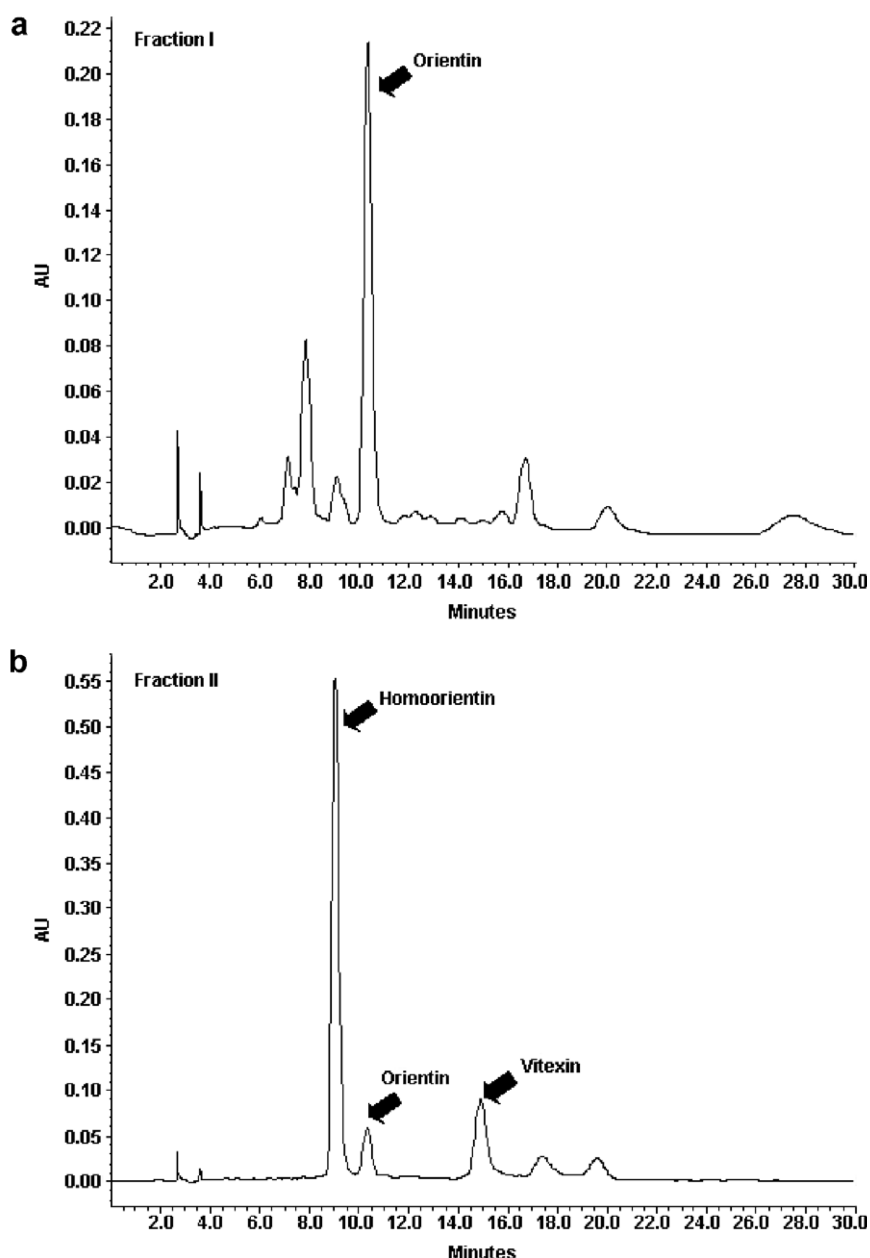

Fig. 4. The analytical HPLC chromatograms of (a) fraction I, (b) fraction II and (c) fraction III collected during preparative HPLC analysis.

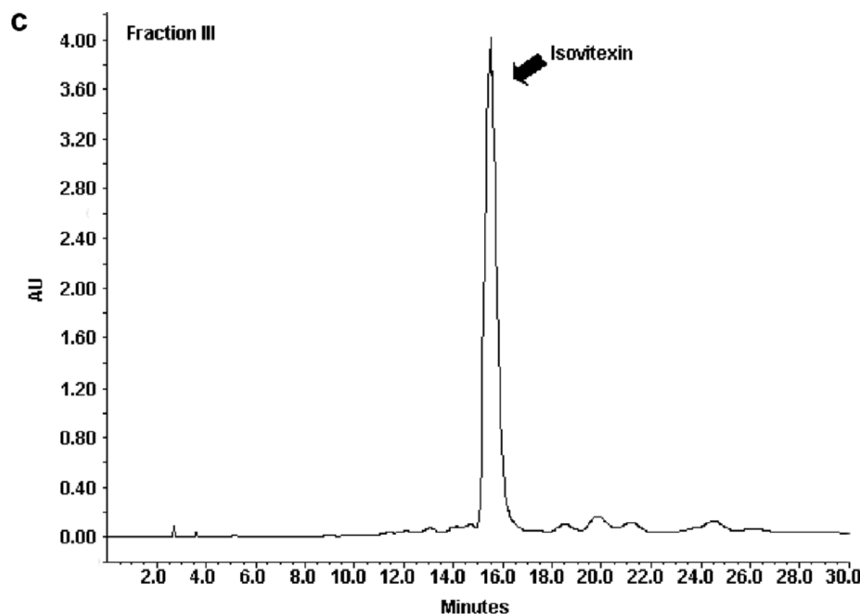

Fig. 4 (*continued*)

Flourier Transform Infrared Spectrometer (Thermo Nicolet, San Jose, CA, USA). UV data were obtained from HPLC-DAD analysis (Waters, Milford, MA, USA). Mass spectral data of preparative flavone *C*-glycosides were obtained with an APEX III Fourier-transform ion cyclotron resonance mass spectrometry (Bruker Daltonics Inc., Billerica, MA, USA). ^{1}H NMR and ^{13}C NMR spectrometry was recorded on an Avance DMX-500 (500 MHz) NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany).

3. Results and discussion

3.1. Resin-based column chromatography

Previous studies discussed the preparative isolation of flavone *C*-glycosides from plant herbs using the HSCCC or silica gel column chromatography method. Zhou, Peng, Fan, and Wu (2005) performed the isolation and purification of orientin and vitexin simultaneously using HSCCC technology. Afifi, Khalil, and Abdalla (1999) separated homoorientin from *Arum palaestinum* by silica gel column chromatography and further purified this compound by recrystallization. In the present work, AB-8 resin-based column chromatography was mainly used to isolate the total flavone *C*-glycosides fraction from AOB other than each flavone *C*-glycoside monomer. Polystyrene (AB-8) resin, which is a cross-link polymer with the attachment of abundant hydrophilic groups in its skeleton structure (Tang, Zhou, & Duan, 2001), has large capacities and high recovery yield for preparative separation of many biological products such as phenols and glycoside compounds due to its huge specific surface area and appropriate micropore volumes (Wang et al., 2005; Wu, Zhong, & Xi, 2004). Considering the main functional components in AOB including flavonoids, lactones and phenolic acids, the removal of impurity and extraction of flavone *C*-glycoside are required during the column chromatography. The HPLC separation of AOB dilute solution performed according to Section 2.5 is shown in Fig. 2a. Results indicated from the UV absorbance of DAD spectrum in each peak showed that most of phenolic acids were eluted before 9 min. Compared to phenolic acids, the polarity of four flavone *C*-glycoside subjects was smaller so that these compounds were eluted with a longer retention time (approximately range: 9–17 min). Under such conditions, AOB adsorbed in AB-8 resin was initially eluted with redistilled water to remove phenolic acids, minerals and sugars, and subsequently eluted with 50% (v/v) ethanol solution to obtain the flavone *C*-glycoside rich fraction. The HPLC chromatograms of the above-mentioned water and 50% (v/v) ethanol fraction from AOB were shown in Fig. 2b and c, respectively. Results of HPLC analysis indicated that most of phenolic acids were desorbed from AB-8 resin via water elution while trace levels of them were found in the 50% (v/v) ethanol fraction. Nevertheless, some other components, which were identified as coumaric compounds and weak polar flavonoids primarily elucidated by the UV absorbance of DAD spectrum in each peak, were also found in the 50% (v/v) ethanol fraction (range of retention time: 20–40 min). These impurities could then be further separated from the flavone *C*-glycoside rich fraction and removed by preparative HPLC. After concentrating and drying by rotatory evaporator, 6.5 g of total flavone *C*-glycoside rich fraction was obtained by AB-8 resin-based column chromatography.

3.2. Preparative HPLC separation

For the application of preparative HPLC technology, Kumarasamy et al. (2004) successfully optimized a preparative procedure regarding the isolation of four flavone *C*-glycosides including homoorientin, swertiajaponin, swertisin and isoscopatin-2″-β-D-glucopyranoside. Although previous studies reported the chromatographic methods regarding the separation of flavone *C*-glycosides including HSCCC and preparative HPLC (Miyake et al., 1997; Sun et al., 2007), few studies focused on the chromatographic methods regarding the isolation of multiple isomer pairs of flavone *C*-glycosides simultaneously. Furthermore, study on HPLC-MS using collision induced dissociation (CID) showed slight differentiation of the isomer pairs, i.e. homoorientin/orientin and isovitexin/vitexin, via comparing the CID-MS/MS spectra of particular fragments from the *C*-glycoside units (Pereira, Yariwake, & McCullagh, 2005). Therefore, it is a great challenge to isolate these two flavone *C*-glycoside isomer pairs simulta-

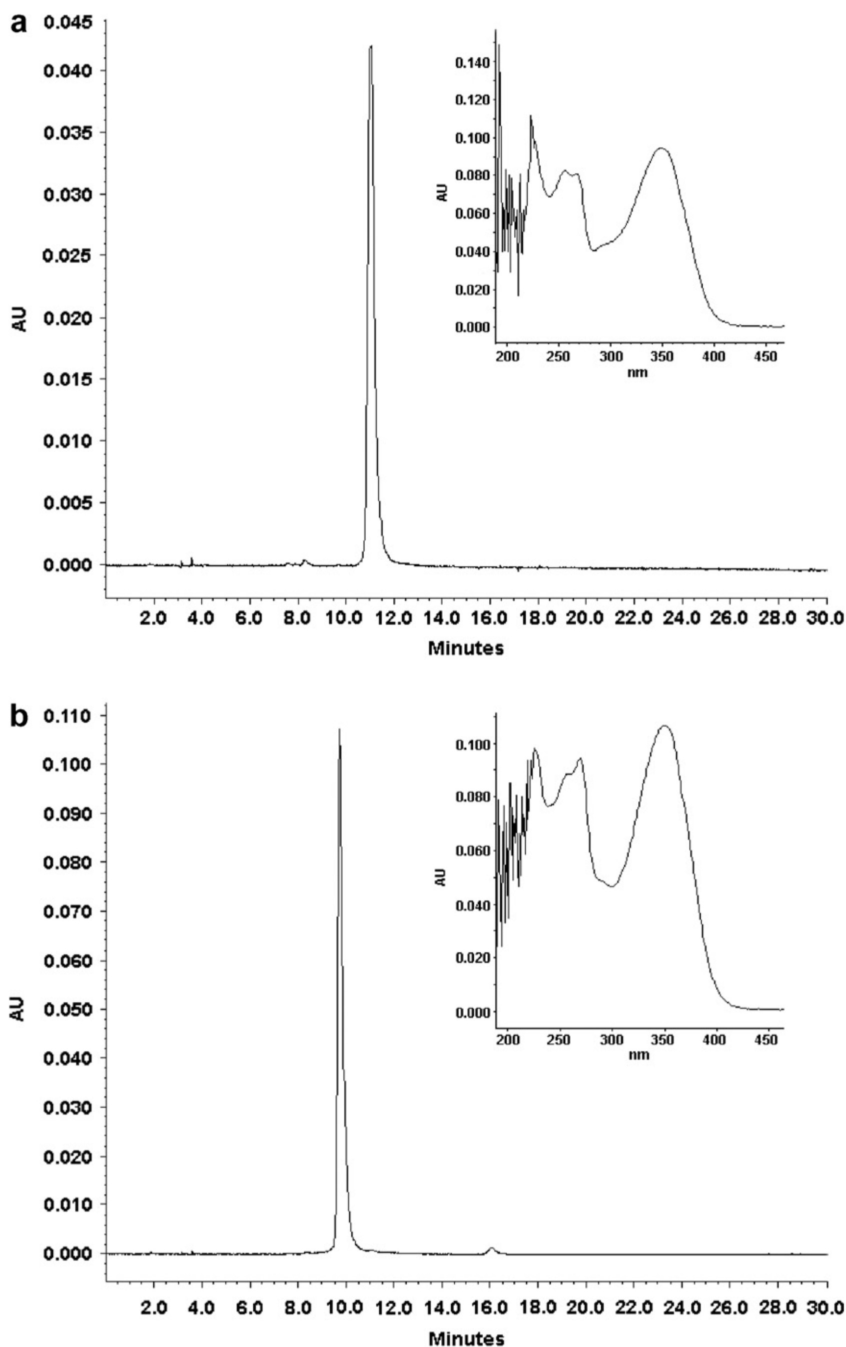

Fig. 5. The analytical HPLC chromatograms of (a) orientin, (b) homoorientin, (c) vitexin and (d) isovitexin purified by the drowning-out crystallization method.

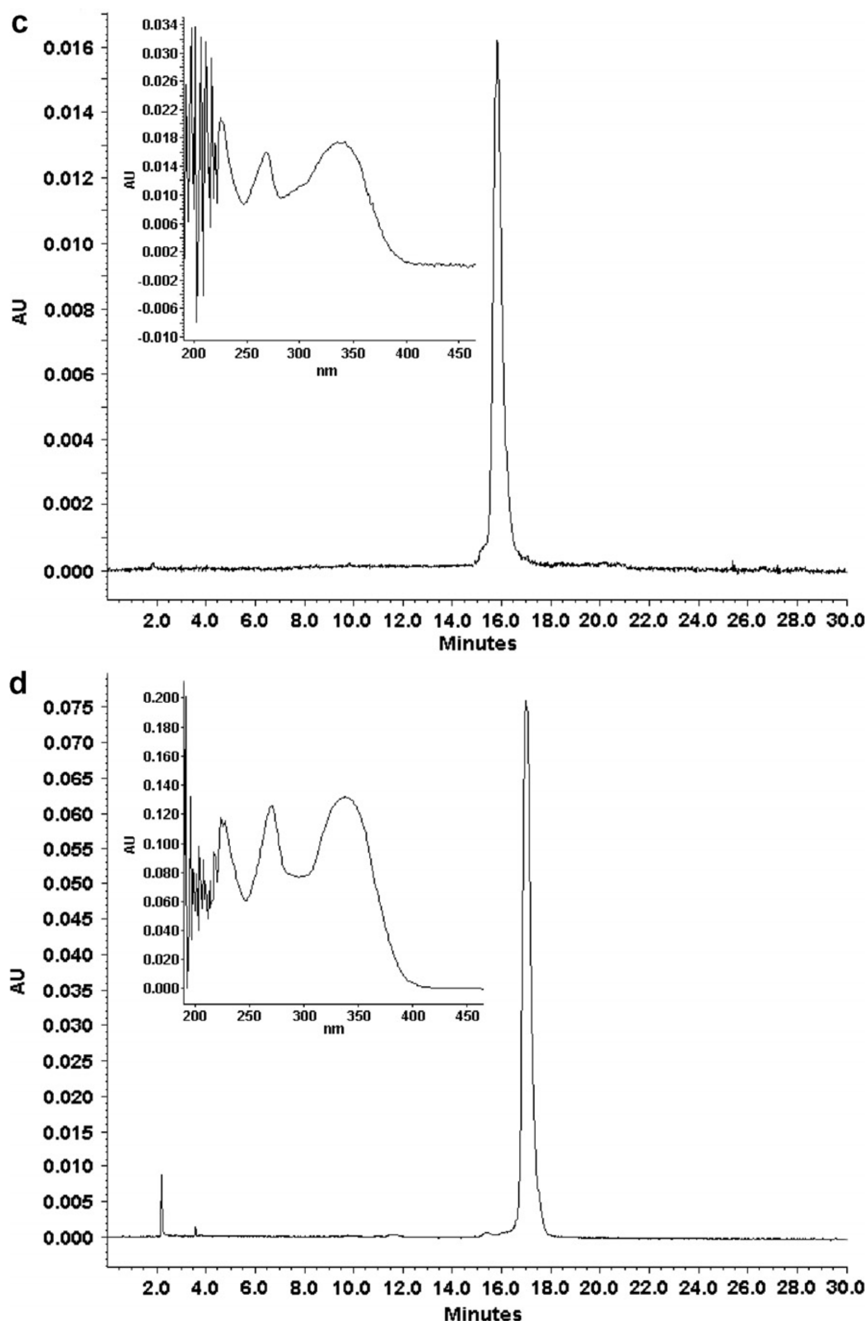

Fig. 5 (*continued*)

neously by preparative HPLC in the present work. Fig. 3 showed the preparative HPLC chromatogram of the flavone *C*-glycoside rich fraction (fractions I–III) from AOB using the gradient elution programme as described in Section 2.4. The purity of each collected flavone *C*-glycoside was determined by analytical HPLC as described in Section 2.5 (Fig. 4). Initially, all of peaks presenting in the preparative HPLC chromatogram were collected and identified according to their retention times and UV spectral data from the analytical HPLC system. Compared to the DAD spectra of four flavone *C*-glycoside standards, three peaks (fractions I–III) were identified as flavone *C*-glycosides in the present work (Figs. 3 and 4). In detail, the analytical HPLC chromatograms of these fractions showed that the main components of fractions I and III were identified as orientin and isovitexin, respectively. Nevertheless, some minor impurities were also found during the elution of these two fractions and should be removed via further purification treatments (Fig. 4a and c). For the components of fraction II, three flavone *C*-glycosides including homoorientin, orientin and vitexin were observed and homoorientin was recognized as the predominant compound

(Fig. 4b). Multiple components detected in this fraction may be ascribed to (i) an overlapped elution area between the fraction I (identified as orientin) and II, and (ii) a great peak width. Previous study demonstrated that the content of vtexin is much lower than homoorientin in AOB (Zhang et al., 2005). The elution of homoorientin covered the response of vitexin as a whole during preparative HPLC analysis. Therefore, a further purification procedure should be performed in order to separate the predominant homoorientin from other two flavone *C*-glycosides in the fraction II. Meanwhile, the isolation between orientin and vitexin in fraction II may be operated by a secondary preparative HPLC analysis.

3.3. Further purification and secondary preparative HPLC analysis

When fractions are collected from the preparative HPLC, it is then necessary to evaporate the mobile phase and consequently purify the chemicals studied in this work. To obtain flavone *C*-glycoside monomers with a high purity and remove minor impurities co-eluted from the preparative HPLC, all of the three peak fractions collected and concentrated previously were further purified by the drowning-out crystallization method as described in Section 2.6. Drowning-out is widely used as an alternative to crystallization processes for the isolation and separation of organic fine chemical processes such as pharmaceuticals due to its low cost, high energy efficiency and good sensitivity to operational conditions. It is considered as one of the most important separation techniques, especially when separation of solutes from multi-component solutions is required (Borissova, Dashova, Lai, & Roberts, 2004). Drowning-out is a reactive crystallization technique based upon three stages, i.e. dissolution in drowning-out anti-solvents, addition of salting-out agents and precipitation (Pina, Fernández-Dıaz, Prieto, & Veintemillas-Verdaguer, 2001). In the present work, the drowning-out method was used to further purify flavone *C*-glycosides while methanol and water were selected as the drowning-out anti-solvent and salting-out agent, respectively. Fig. 5a, b and d showed the analytical HPLC chromatograms of fractions I–III, which were concentrated and purified by the drowning-out crystallization method. Fractions I–III were identified as orientin, homoorientin and isovitexin. For the fractions I and III (Fig. 5a and d), purification processed by the drowning-out crystallization treatment improved the purity of two flavone *C*-glycosides compared to the HPLC chromatograms of fractions obtained from preparative HPLC (Fig. 4a and c). For the fraction II (Fig. 5b), homoorientin was precipitated by the drowning-out treatment due to its predominant content in this fraction. Subsequently, this suspension was carefully transferred into the tube and then the supernatant was separated via centrifugation. Meanwhile, the precipitation (homoorientin) was submitted to the analytical HPLC detection. Chromatographic results showed that the purity of homoorientin was greatly improved while other two flavone *C*-glycosides, i.e. orientin and vitexin, found in the fraction II originally were not detected (Fig. 5b), which indicated that these two flavonoids were dissolved in the supernatant of fraction II. A secondary preparative HPLC chromatogram of the concentrated solution of this supernatant was shown in Fig. 6. The fractions IV and V were identified as orientin and vitexin according to their retention time and UV spectral information from the DAD of analytical HPLC system, respectively. These two fractions were submitted to further purification via the drowning-out crystallization method as usual and their purities were also largely improved (Fig. 5a and c).

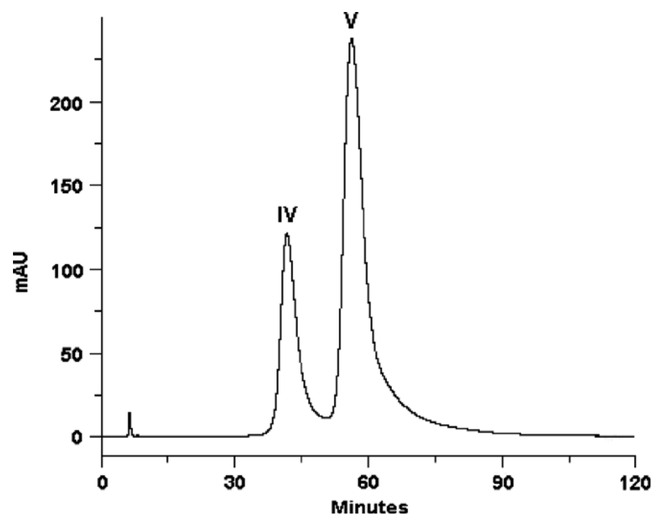

Fig. 6. The secondary preparative HPLC chromatogram of fraction II. Fraction IV, orientin; fraction V, vitexin.

3.4. Confirmation of flavone C-glycosides

To further confirm the chemical structures of preparative flavone *C*-glycosides, five fractions (I′–V′) after the drowning-out crystallization treatment were submitted to the IR, MS and NMR analysis. Compared to the IR, MS, ^{1}H NMR and ^{13}C NMR spectral data from previous publications (Kumarasamy et al., 2004; March et al., 2006; Peng, Fan, Hong, Chai, & Wu, 2005; Zou, Yang, Dong, Zhou, & Lin, 2005) and flavone *C*-glycoside standards in the present study, the fraction I′, II′, III′, IV′ and V′ were identified as orientin, homoorientin, isovitexin, orientin and vitexin, respectively. Furthermore, the fractions I′ and IV′ were merged together because these two fractions were both confirmed as orientin.

4. Conclusion

Four flavone *C*-glycosides, i.e. orientin, homoorientin, vitexin and isovitexin, were obtained from an ethanol aqueous extraction of bamboo leaves by AB-8 resin-based column chromatography and preparative HPLC using a mobile phase consisting of acetonitrile and 1% acetic acid and a gradient programme. These flavone *C*-glycosides

were further purified by the drowning-out crystallization method using methanol and water as drowning-out anti-solvents and salting-out agents, respectively. Orientin (49 mg), homoorientin (142 mg), vitexin (15 mg) and isovitexin (62 mg) were prepared from 6.5 g of crude column chromatography fraction obtained from 5 L of AOB concentrated solution. The purity was assessed by analytical HPLC and the confirmation of chemical structures was performed by IR, MS, NMR and UV spectroscopy. The present method is suitable for preparing available quantities of pure flavone *C*-glycosides and the quantification of orientin, homoorientin, vitexin and isovitexin in bamboo leaves.

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