JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Separation and Purification of Tricin from an Antioxidant Product Derived from Bamboo Leaves

JINGJING JIAO,† YU ZHANG,† CHENGMEI LIU,‡ JIE'ER LIU,† XIAOQIN WU,† AND YING ZHANG*,†

Department of Food Science and Nutrition, School of Biosystems Engineering and Food Science, Zhejiang University, 268 Kaixuan Road, Hangzhou 310029, Zhejiang, China and Sino-German Food Engineering Center, Nanchang University, 235 East Nanjing Road, Nanchang 330047, Jiangxi, China

Tricin (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone) occurs in its glycosidic form in rice bran and other grass species such as wheat, barley, and maize. Tricin is considered sufficiently safe for clinical development as a cancer chemopreventive agent, therefore it can be used for cancer prevention. This study established a new method for the preparation of tricin from bamboo leaves as an alternative to traditional methods such as chemical synthesis via the Baker−Venkata−Raman reaction between acetylsyringic acid and phloroacetophenone. Tricin was prepared from an antioxidant product that was derived from bamboo leaves (AOB) by extraction with aqueous ethanol. A concentrated solution of this product was obtained and then processed by polystyrene (AB-8) resin column chromatography and preparative high-performance liquid chromatography (HPLC) with 30% (v/v) acetonitrile in 1% (v/v) acetic acid as the mobile phase. The collected tricin-rich fraction was further sequentially purified by dialysis membrane separation and drowning-out crystallization methods. The purity was assessed by analytical HPLC with 25% (v/v) acetonitrile in 1% (v/v) acetic acid as the mobile phase, and the chemical confirmation was evaluated by infrared, mass, nuclear magnetic resonance, and ultraviolet spectroscopies. Tricin (3.09 g) was prepared from 174 g of a crude column chromatography fraction obtained from 5 L of AOB concentrated solution. The present method is appropriate for preparing quantities of pure tricin, which can be used for the quantification of tricin in various plant herbs and further for pharmaceutical/biomedical applications and evaluation.

KEYWORDS: Tricin; resin column chromatography; preparative HPLC; dialysis membrane separation; drowning-out crystallization; antioxidant product derived from bamboo leaves

INTRODUCTION

Flavonoids, a large category of plant polyphenol secondary metabolites and biologically active non-nutrients, are widely distributed in medicinal herbs, fruits, teas, etc (*1*). The health effects of flavonoids in human diet have attracted wide attention during these years. Many studies attempted to characterize potential cancer chemopreventive properties of various extract ingredients from plants and reported positive effects of flavonoids on cancer prevention (*2*, *3*). Among these pharmacologically identified active agents, there is an important flavone aglycone, that is, tricin (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone), the chemical structure of which is shown in **Figure 1**.

Tricin occurs in its glycosidic form in rice bran and other grass species such as wheat, barley, and maize (*4*). A previous safety evaluation study tentatively demonstrated that tricin can be considered as a cancer chemopreventive agent for the clinical development due to its validated safety (*5*). Many original contributions reported the great potential of tricin on cancer prevention. Hudson et al. (*6*) demonstrated that tricin interfered potently with the growth of human-derived malignant MDA-MB-468 breast cancer cells, but was much less growth-inhibitory in HBL-100 cells. Cai et al. (*7*) further reported great growth-inhibitory and cell cycle-arresting properties of tricin in human MDA-MB-468 breast cancer cells in vitro. However, the potent breast tumor cell growth-inhibitory activity of tricin in vitro does not directly translate into activity in the nude mouse bearing the MDA-MB-468 tumor. Nevertheless, the high levels of tricin found in the gastrointestinal tract after dietary intake render exploration of its ability to prevent colorectal carcinogenesis. Mechanistic studies found that the ability of tricin to inhibit cyclooxygenase enzymes and attenuation modulation of cyclooxygenase-mediated prostaglandin production may contribute to its chemopreventive efficacy on intestinal carcinogenesis and prostatic cancer respectively (*8*, *9*).

Although the cancer chemopreventive property of tricin was investigated in animals or human cancer cell lines, very little is known about the pharmacology of tricin other than that it

* To whom correspondence should be addressed. Phone: +86 571 8697 1388, Fax: +86 571 8604 9803, E-mail: y_zhang@zju.edu.cn.
† Zhejiang University.
‡ Nanchang University.

10.1021/jf0716533 CCC: $37.00
Published on Web 11/15/2007

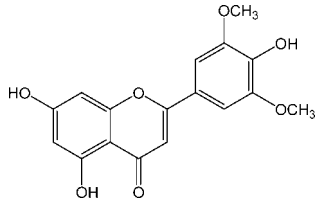

Figure 1. Chemical structure of tricin (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone).

possesses anticlonogenic activity in human-derived colon and breast cancer cells in vitro (*6*) and antineoplastic properties in mice with the P_{388} leukemia (*10*). Meanwhile, few studies focused on other pharmacological activities of tricin. Such research status may partly be ascribed to the source of tricin, which is mainly derived from chemical synthesis, more so than from natural herbs (*11, 12*). Actually, tricin is commonly distributed in the plant kingdom. However, few studies reported the separation and purification methods of tricin from various plants. Published studies used the combined technology of silica gel column chromatography and thin-layer chromatography (TLC) to separate tricin from aerial parts of alfalfa (*Medicago sativa* L.) and endophyte-infected blue grass (*Poa ampla*) on a small scale (*13, 14*). Our previous study found tricin in bamboo leaves (*Phyllostachys nigra* var. *henonis*) (*15*). The antioxidant product derived from bamboo leaves (AOB), a pale brown powder extracted from bamboo leaves, was capable of blocking chain reactions of lipid autoxidation, chelating metal ions of transient state, scavenging nitrite compounds, and blocking the synthetic reaction of nitrosamine, as testified by our previous study (*16*). Moreover, AOB was shown to have strong antioxidant activity and inhibitory efficacy on transition metal ion and free-radical induced deterioration of macromolecules in vitro (*17*). AOB has been listed in the national standards (i.e. GB2760) as a kind of food antioxidant in China. Additionally, it is allowed to be added into puffed foods, aquatic products, meat products, edible oils, cereals, bakery foods, fruit and vegetable juices, tea beverages, and fried foods by the Ministry of Health, China (*18*). The main functional components are flavonoids, lactones, and phenolic acids, whereas tricin is one of the representative flavones in AOB. Preparative high-performance liquid chromatography (HPLC) is an effective method for the isolation and purification of active components from plant herbs. Previous studies validated an analytical HPLC method for the determination of tricin in various samples (*4, 19, 20*), which supplied some recommendations for the chromatographic separation of tricin. However, few studies reported the isolation and purification of tricin from plants using the preparative HPLC method. The aim of this study was to optimize a new method for the isolation of tricin from AOB using the resin column chromatography and preparative HPLC technologies and the purification of tricin using the dialysis membrane separation and drowning-out crystallization methods.

MATERIALS AND METHODS

Chemicals. An authentic sample of tricin (approximately 2.5 mg) was prepared by TLC from the bamboo leaves of *Phyllostachys nigra* var. *henonis* and identified by infrared (IR), Ultraviolet (UV), mass (MS), ^{1}H nuclear magnetic resonance (NMR), and ^{13}C NMR spectrometries in our previous study (*15*). Acetonitrile (HPLC-grade) was purchased from Tedia (Fairfield, OH, USA), and methanol (HPLC-grade) was obtained from Merck (Whitehouse Station, NJ, USA). All other solvents and chemicals used in the present work were of analytical grade. Redistilled water was used in the resin column chromatography, whereas ultrapure water purified via the Milli-Q system (Millipore, Bedford, USA) was used in both preparative and analytical HPLC. Stock and working standards of tricin were prepared by dissolving the authentic sample of tricin in acetonitrile. The standard solutions were stable for at least 3 months when stored at 4 °C.

Samples. The concentrated AOB solution was provided by Zhejiang University Innoessen Co., Ltd. (Hangzhou, China). AOB was prepared from the bamboo leaves of *Phyllostachys nigra* var. *henonis*, identified by the Research Institute of Subtropical Forestry of the Chinese Academy of Forestry (Hangzhou, China). Briefly, fresh bamboo leaves were collected during the autumn season in the Anji district (Zhejiang Province, China) and air-dried. The coarse powder of bamboo leaves was obtained after comminution and filtration (20–40 mesh), and this powder was extracted over 1 h by a 30% (v/v) ethanol aqueous solution using the hot reflux method with a solid-to-liquid ratio of 1:10 (m/v). The filtrate was then isolated by membrane filtration to remove macro- and micromolecular components such as polysaccharides and minerals. Finally, 5 L of AOB concentrated solution (specific density: 1.06 g/mL) was obtained after concentrating in vacuum, and the resin was submitted to resin-based column chromatography.

AB-8 Resin Column Chromatography. The resin column chromatography was performed on a low-pressure glass chromatographic column (150 cm × 100 mm I.D., Hua Mei 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 a resin column should be preactivated before use. In detail, the chromatographic column was first activated with 24 L of 5% (v/v) hydrochloric acid and was equilibrated with redistilled water to a neutral pH at a flow rate of 60 mL/min. Similarly, the column was subsequently activated with 24 L of 2% (v/v) sodium hydroxide and then equilibrated with redistilled water to a nearly neutral pH at the same flow rate. Then, 5 L of AOB concentrated solution was loaded onto the pretreated column and was statically adsorbed overnight. The column was initially eluted with 25 L of 10% (v/v) ethanol solution to remove some impurities in AOB, such as phenolic acids, minerals, and sugars. The column was then eluted with 25 L of 50% (v/v) ethanol solution to remove some minor flavone aglycone or glycoside constituents, which were not investigated in this study. Then, the tricin-rich extract was eluted with 25 L of 70% (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, dried by rotatory evaporator, and redissolved with HPLC-grade acetontrile. Finally, this concentrated extract was submitted to the preparative HPLC analysis.

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 × 7.8 mm I.D., 10 μm; Waters, Milford, MA, USA). The mobile phases were acetonitrile and 1% (v/v) acetic acid in water using an isocratic elution program (30/70). The flow rate was 25 mL/min, and 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 tricin peak was manually collected.

Analytical HPLC Detection. The analytical method for the determination of tricin in AOB, column chromatographic fractions, preparative HPLC fractions, and further purification samples was performed according to previous publications (*4, 20*) with some modifications. Briefly, analytical HPLC was performed on a Waters 2695 HPLC chromatograph (Waters, Milford, MA, USA) with a Luna C_{18} column (5 μm, 250 × 4.6 mm I.D.) protected by a RP_{18} guard column (5 μm, 4.0 × 3.0 mm I.D.), both purchased from Phenomenex (Torrance, CA, USA). Solvent A (acetonitrile) and solvent B (1% (v/v) acetic acid adjusted to pH 3.0 with sodium hydroxide) were selected as the mobile phases. For the analysis of column chromatographic fractions, a gradient elution program was used, i.e. 15% A (15 min), 15–40% A (10 min), 40% A (9 min), 40–15% A (6 min). For the analysis of preparative HPLC fractions and further purification of samples, an isocratic elution method (A/B = 25/75) was used. The flow rate was 1.0 mL/min. The

injection volume was 30 μL, and the column temperature was maintained at 40 °C. The signal was monitored at 330 nm using the diode array detector (DAD).

Further Purification of Tricin. The dialysis membrane separation and drowning-out crystallization methods were continuously used for the further purification of tricin in this study. The fraction, which represented tricin collected from the eluent of preparative HPLC, was concentrated and dried by a rotatory evaporator. Then, the residue was redissolved with methanol, and the solution was transferred into a dialysis bag (1 m × 34 mm, MW3500). The dialysis bag was placed overnight in a beaker filled with HPLC-grade methanol (200 mL) to let tricin molecules pass through the dialysis bag. The permeation solution was then concentrated to approximately 10 mL by the rotatory evaporator and was transferred into a centrifuge tube. An equal volume of ultrapure water (10 mL) was added into the centrifuge tube, which was then placed at 4 °C overnight. The analytes were largely precipitated via crystallization, and the mixture was then centrifuged (10000 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.

Identification of Tricin. The preparative tricin was identified by IR, UV, MS, ^{1}H NMR, and ^{13}C NMR spectrometries. IR analysis was performed on a Nexus 670 Flourier-transform IR spectrometer (Thermo Nicolet, San Jose, CA, USA). UV data were obtained from HPLC with DAD analysis (Waters, Milford, MA, USA). Mass spectral data of the preparative tricin and the authentic sample of tricin 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).

RESULTS AND DISCUSSION

Polystyrene Resin Column Chromatography. In the present work, the polystyrene resin column chromatography method was mainly used to isolate a crude tricin-rich fraction from AOB. A previous study preliminarily investigated the preparative isolation of flavonoids by polystyrene resin column chromatography (*21*). Also, applications of some other types of column chromatography for the preparative study have been reported. For instance, Bouaziz et al. (*22*) reported an extraction method for the separation of flavonoids from olives by Sephadex column chromatography. Maciejewicz (*23*) used the silica gel column chromatography to isolate two flavonoid aglycones, pilloin and pinostrobin chalcone, from propolis. Polystyrene (AB-8) resin, which is a cross-link polymer with the attachment of hydrophilic-rich groups in its skeleton structure (*24*), has large capacities and high recovery yield for many biological products such as phenols and glycoside compounds because of its huge specific surface area and appropriate micropore volumes (*25*). However, such excellent characteristics of AB-8 resin lead to a comprehensive adsorption of most of the components in AOB. Therefore, an optimized choice of desorption solvent and its safety should both be taken into consideration. Because the main functional components in AOB includes flavonoids, lactones, and phenolic acids, the separation among these components is required during the column chromatography. The analytical HPLC chromatogram of AOB dilute solution is shown in **Figure 2a**. Results indicated from the UV absorbance of DAD scanned spectrum in each peak showed that most small molecular phenols were eluted before 9 min, whereas lactones and flavonoids with a strong or moderate polarity were subsequently eluted within the retention time range of 9−17 min. For the desorption process, AOB adsorbed in AB-8 resin was first eluted with 10% (v/v) ethanol solution to remove small molecular phenols, minerals, and sugars, and subsequently eluted with 50% (v/v) ethanol solution to remove most of lactones and some other flavonoid glycosides. Finally, the tricin-rich fraction was desorbed with 70% (v/v) ethanol solution and was collected for further isolation. The HPLC chromatograms of 10, 50, and 70% (v/v) ethanol fractions from AOB by resin column chromatography are shown in **Figure 2b−d**, respectively. Results of analytical HPLC demonstrated that most phenols were desorbed from AB-8 polystyrene resin via 10% ethanol elution and some other components, which were identified as coumaric compounds and other flavonoid glycosides elucidated by the UV absorbance of the DAD spectrum in each identified peak, were found in the 50% ethanol fraction. A predominant content of tricin was detected in the 70% ethanol fraction (**Figure 2d**). However, some minor impurities remaining in the resin were also coeluted in this fraction. These impurities could then be further separated from the tricin-rich fraction and removed by preparative HPLC. After concentrating and drying by the rotatory evaporator, 174 g of the crude tricin-rich fraction was obtained by AB-8 resin column chromatography. Unfortunately, some dark colloidal polymers simultaneously that coeluted in the 70% ethanol fraction were mixed with the tricin-rich fraction. These polymers could not be detected by HPLC because they were not absorbed by the UV spectrum, and should be removed during subsequent purification processes.

Separation of Tricin by Preparative HPLC. Figure 3 shows the preparative HPLC chromatogram of the crude tricin-rich fraction extracted from AOB with the mobile phase of acetonitrile/1% (v/v) acetic acid in water (30:70). In the present study, the crude tricin-rich fraction obtained from the concentrated resin column chromatographic products was dissolved in acetonitrile prior to the injection of preparative HPLC. The elution of the crude tricin-rich fraction was greatly affected in the initial stage of preparative HPLC because of a large injection volume (10 mL), which induced a temporary increase of acetonitrile in the mobile phase. Such temporary change of the mobile phase inevitably led to a very short retention of tricin accompanying with coelutions, which would reduce the preparative purity of tricin. To cope with this problem, we initially used water as the mobile phase (holding 5 min) at the beginning of preparative process to dilute the concentration of acetontrile from the injected sample solvent, and then we adjusted the mobile phase to a required acetonitrile level. Using such a mobile phase recipe, the preparative HPLC chromatogram of the crude tricin-rich fraction is presented in **Figure 3a**. The retention time of tricin was approximately 45 min. Although a reasonable retention of tricin during the preparative process was achieved, the peak shape of tricin seems unacceptable. Most parts of the tricin-rich fraction were covered by the strong background interference. It was difficult to collect the tricin-rich fraction and avoid adulteration with impurities. Such results may be ascribed to the change of mobile phase, which led to a wide-range elevation of the HPLC baseline. Alternatively, we diluted the injected sample with water to adjust the concentration of acetonitrile in the sample solvent to a requested level (30%), the same as the percentage in the mobile phase. Using this recipe, the preparative HPLC chromatogram of the crude tricin-rich fraction is presented in **Figure 3b**. The retention time of tricin was also close to 45 min. Compared to the previous recipe, although the injected concentration of crude tricin-rich fraction was reduced using this dilution method, the tricin peak in the preparative HPLC chromatogram was much more obvious, and the background interference was also greatly alleviated. These two significant improvements were convenient for the collection of the subject product. Moreover, the acetonitrile concentration

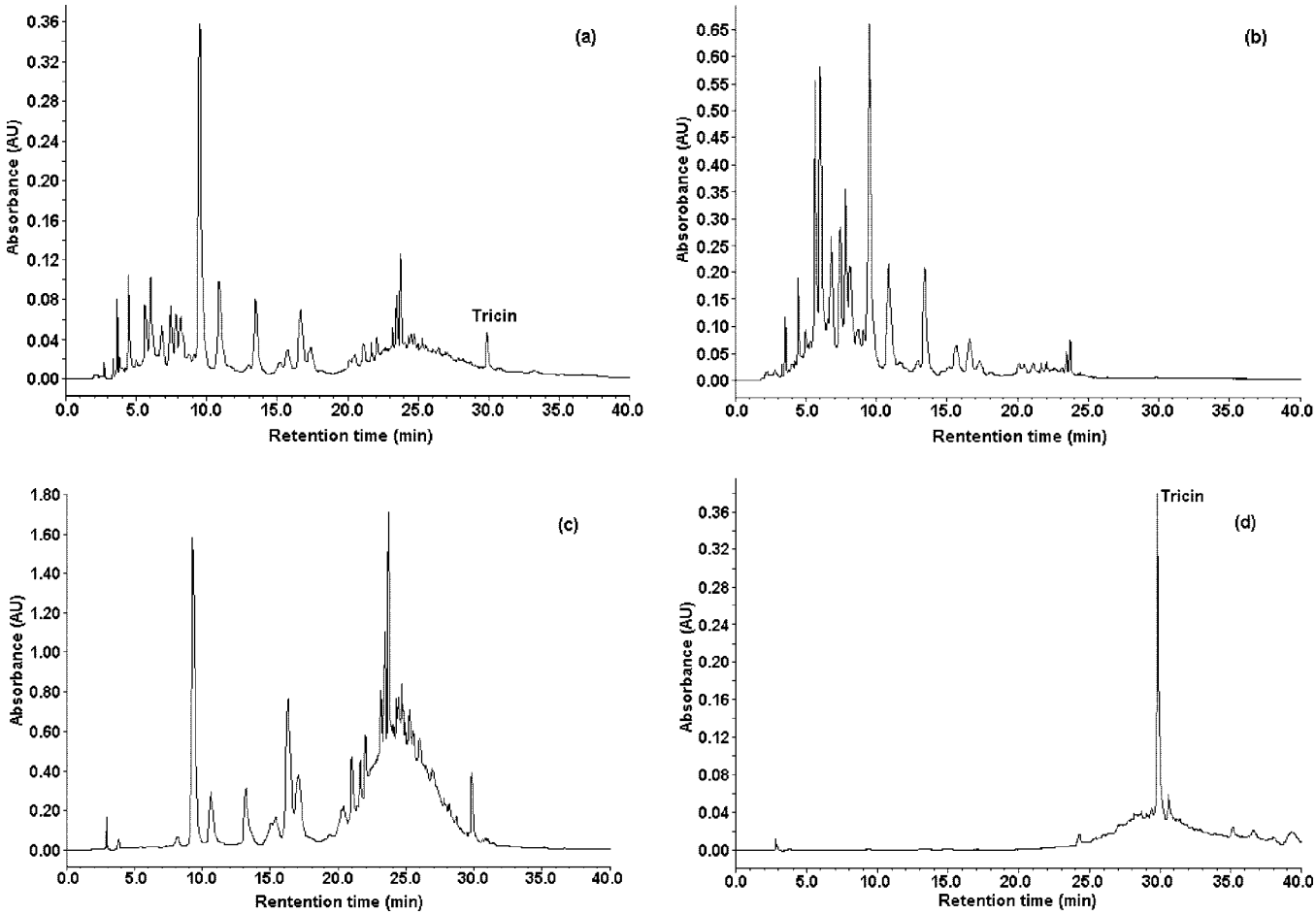

Figure 2. The analytical HPLC chromatograms of different fractions from AOB by AB-8 resin column chromatography. (**a**) original AOB dilute solution; (**b**) 10% (v/v) ethanol fraction; (**c**) 50% (v/v) ethanol fraction; (**d**) 70% (v/v) ethanol fraction. HPLC column: Luna C_{18} column (5 μm, 250 $\times$ 4.6 mm I.D.). Mobile phase and gradient programme: A (acetonitrile) and B (1% (v/v) acetic acid); 15% A (15 min), 15–40% A (10 min), 40% A (9 min), 40–15% A (6 min). Flow rate: 1.0 mL/min. Injection volume: 30 μL. Column temperature: 40 °C. Wavelength monitoring: 330 nm.

in the mobile phase was optimized in this study. Initially, 10, 20, 30, 40, and 50% (v/v) were chosen as the candidate levels. Results indicated that a more reasonable retention and a better separation (with capacity factor $k' > 1.6$) were simultaneously achieved using the 30% acetonitrile level as the mobile phase as compared to the other candidate levels.

A reverse-phase HPLC technique was used to identify the preparative tricin-rich fraction. The identification was based on comparisons of the chromatographic retention time and UV absorbance spectra of the preparative tricin with that of the authentic sample. The actual content of tricin in AOB was quantified as 1.1% (m/m) by the analytical HPLC method using the TLC prepared tricin as the standard. A representative analytical HPLC chromatogram of tricin collected by preparative HPLC is shown in **Figure 4a**. Initially, all peaks, which were presented in the preparative HPLC chromatogram, were collected and identified by their retention time and UV spectral data from the DAD of the analytical HPLC system. Compared to the UV absorbance spectra of the authentic sample, tricin was identified among the present peaks in the preparative HPLC chromatogram (**Figures 3** and **4**). Although few impurities were detected by analytical HPLC, some dark colloidal polymers that simultaneously coeluted during the collection process were inevitably mixed with the tricin-rich fraction as described above. Therefore, a further purification procedure should be performed to separate the predominant tricin from these dark polymers.

Further Preparative Purification of Tricin. When the tricin-rich fraction was collected from the preparative HPLC, it was then necessary to remove the mobile phase and to consequently purify the chemical. Initially, we tried to use the drowning-out crystallization method directly after preparative HPLC to further purify the tricin-rich fraction, which was collected and concentrated in advance. Drowning-out is extensively used as an alternative during crystallization processes for the isolation and separation of organic fine chemical processes such as pharmaceuticals because of its low cost, high energy efficiency, and good sensitivity to operational conditions. It is considered as one of the most important separation techniques (*26*). Drowning-out is a reactive crystallization technique based upon three stages, that is, dissolution in drowning-out antisolvents, addition of salting-out agents, and precipitation (*27*). Tricin is poorly soluble in water, so it can be precipitated when using water as the salting-out agent. Unfortunately, dark colloidal polymers were simultaneously precipitated. Such phenomenon was ascribed to the similar solubility between tricin and polymers and demonstrated that the drowning-out method was unable to be used for the purification of tricin before the polymers were removed. Because a great physical discrepancy was presented between these two compounds, the dialysis membrane separation method was then considered before a drowning-out procedure was performed. Using a dialysis bag (1 m $\times$ 34 mm, MW3500), it was obviously found that the dark colloidal polymer molecular was unable to pass through the dialysis membrane. The permeation solution was then concentrated. After the removal of the dark polymers, the drowning-out crystallization method could then be used for the precipitation of tricin using methanol

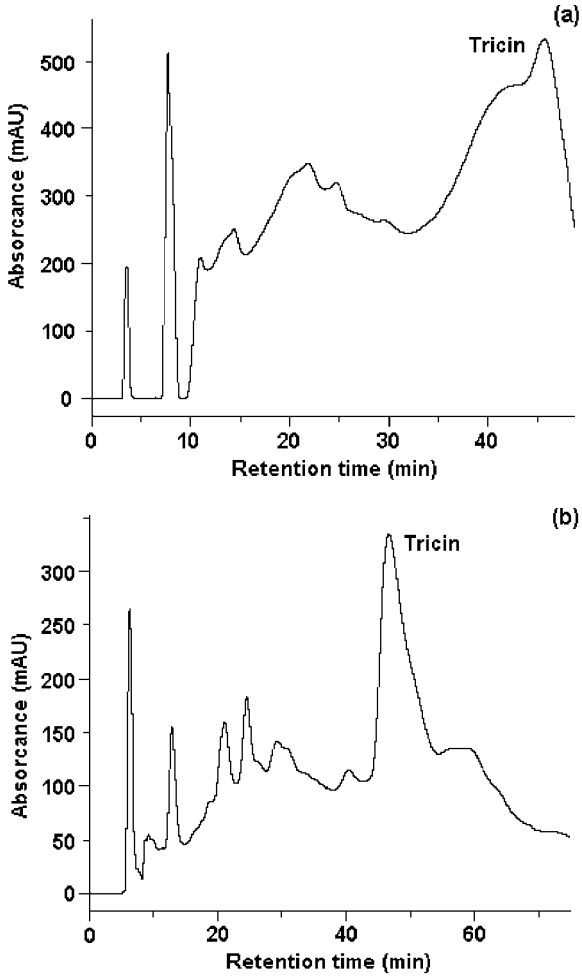

Figure 3. The preparative HPLC chromatograms of the tricin-rich fraction (70% ethanol fraction via AB-8 resin column chromatography) from AOB. (**a**) Recipe 1: using water as the mobile phase (holding 5 min) at the beginning of preparative process to dilute the concentration of acetontrile from the injected sample solvent, and then adjusting the mobile phase to a required acetonitrile level. (**b**) Recipe 2: diluting the injected sample with water to adjust the concentration of acetonitrile in the sample solvent to a requested level (30%), the same as the percentage in the mobile phase. The tricin-rich fraction was collected during preparative HPLC analysis manually. Preparative HPLC column: Bondapak C_{18} column (10 μm, 300 $\times$ 7.8 mm I.D.). Mobile phase: A (acetonitrile)/B (1% (v/v) acetic acid) = 30/70. Flow rate: 25 mL/min. Injection volume: 10 mL. Wavelength monitoring: 330 nm.

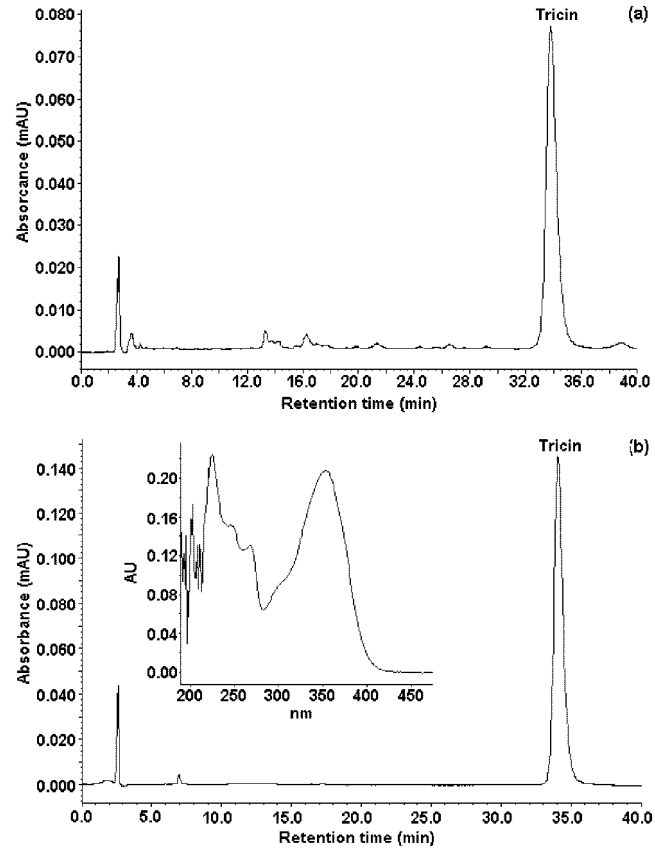

Figure 4. The analytical HPLC chromatograms of tricin collected during preparative HPLC analysis (**a**) before purification and (**b**) after purification. HPLC column: Luna C_{18} column (5 μm, 250 $\times$ 4.6 mm I.D.). Mobile phase: solvent A (acetonitrile)/solvent B (1% (v/v) acetic acid) = 25/75. Flow rate: 1.0 mL/min. Injection volume: 30 μL. Column temperature: 40 °C. Wavelength monitoring: 330 nm.

and water as the drowning-out antisolvent and salting-out agent, respectively. **Figure 4b** presents the analytical HPLC chromatogram of tricin after purification. Additionally, results demonstrated that the impurities that coeluted with tricin during the preparative HPLC were greatly reduced. The purity of final preparative tricin was calculated as approximately 96% using the authentic sample of tricin as the standard.

Chemical Confirmation of Tricin. To further confirm the chemical structure of preparative tricin, the final product after the dialysis membrane separation and drowning-out crystallization treatments was submitted to IR, MS, and NMR analysis. The confirmed information is as follows: Tricin, pale yellow crystal; mp. 287–289 °C; UV λ_{max} (MeOH) 225, 269, 352 nm; IR (KBr) υ_{max} 3410, 2940, 2840, 1665, 1610 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.33 (2H, s, H-2′, H-6′), 6.98 (1H, s, H-3), 6.56 (1H, d, J = 2.0 Hz, H-8), 6.21 (1H, d, J = 2.0 Hz, H-6), 3.90 (6H, s, $-OCH_3 \times 2$); ^{13}C NMR (DMSO-d_6, 500 MHz) δ 182.3 (C, C-4), 164.6 (C, C-2), 164.2 (CH, C-7), 161.9 (CH, C-9), 157.8 (C, C-5), 148.7 (C $\times$ 2, C-3′, C-5′), 140.3 (C, C-4′), 120.9 (C, C-1′), 104.8 (CH $\times$ 2, C-2′, C-6′), 104.2 (C, C-10), 104.1 (CH, C-3), 99.3 (CH, C-6), 94.7 (CH, C-8), 56.8 ($-OCH_3 \times 2$); Hrms (negative ion mode) m/z 329.0650 $[M - H]^-$ (calcd for $C_{17}H_{13}O_7$, 329.0661). Compared to the IR, MS, ^{1}H NMR, and ^{13}C NMR spectral data from previous publications (*12, 28–30*) and the authentic sample of tricin in the present study, the final product was identified as 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (tricin).

Comparison between the Preparative HPLC Method and Synthetic Approach. The present study described a preparative HPLC method to separate and purify tricin from AOB. Compared to the normal synthetic approach, the yield of the preparative method (0.73%) is higher. The yield of the synthetic approach via the Baker−Venkata−Raman reaction between acetylsyringic acid and phloroacetophenone can be calculated as 0.55% if following the synthesis steps according to Kuwabara et al. (2003) (*12*). In view of the number of steps, there are four steps in the preparative method (resin column chromatography, preparative HPLC, dialysis membrane separation, and drowning-out crystallization) and more than five steps in the synthesis method. In view of cost, the solvents used in the preparative method include ethanol, NaOH, HCl, acetic acid, and acetontrile, and most of them are cheap. However, the amount used is large. The solvents used in the synthesis method include oxalyl chloride, acetylsyringic acid, and trihydroxyacetophenone, and most of them are expensive. However, the amount used is not very large. Moreover, the source of materials

in the preparative method is potentially sustainable and cheap because bamboo leaves are a byproduct or even a waste product of bamboo usage. Overall, we believe both methods are appropriate for the preparation of tricin. Using the preparative method, we can obtain the requested material under a relatively safe condition. Using the synthesis method, we can prepare the requested material with a very high purity. However, the solvents of synthetic approach cost a lot, and the method is not very environmentally friendly nor safe. Furthermore, tricin with a 96% purity, as obtained by the preparative HPLC method, is enough for pharmaceutical/biomedical applications and evaluation.

In conclusion, this study established a new method for the preparation of tricin from bamboo leaves other than traditional methods such as the chemical synthesis via the Baker−Venkata−Raman reaction between acetylsyringic acid and phloroacetophenone (*12*). Previous publications reported that a large amount of tricin or its derivatives is present in the bamboo shoots sheath (*31*) and in bamboo grasses (*32*). At present, tricin was found in bamboo leaves and was obtained from AOB by AB-8 resin column chromatography to aquire a tricin-rich fraction; preparative HPLC was performed for the removal of impurities absorbed under ultraviolet detection with 30% (v/v) acetonitrile in 1% acetic acid as the mobile phase. The tricin-rich fraction was then sequentially purified by the dialysis membrane separation method, for the removal of dark colloidal polymers that are not absorbed under ultraviolet detection, and the drowning-out crystallization method, for the acquisition of crystal monomer. Pure tricin was obtained in a 0.73% (7.3 mg/g) overall yield from an AOB concentrated solution. Finally, tricin (3.09 g) was prepared from 174 g of a crude column chromatography fraction obtained from 5 L of AOB concentrated solution. The confirmation of purity and chemical structure was evaluated by analytical HPLC and IR, MS, NMR and UV spectroscopies, respectively. The present method is appropriate for preparing quantities of pure tricin, which can be used for the quantification of tricin in various plant herbs and further for pharmaceutical/biomedical applications and evaluation.

LITERATURE CITED

(1) Peterson, J.; Dwyer, J. Flavonoids: Dietary occurrence and biochemical activity. *Nutr. Res. (N.Y.)* **1998**, *18*, 1995–2018.

(2) Ross, J. A.; Kasum, C. M. Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Ann. Rev. Nutr.* **2002**, *22*, 19–34.

(3) Galati, G.; O'Brien, P. J. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. *Free Radic. Biol. Med.* **2004**, *37*, 287–303.

(4) Cai, H.; Steward, W. P.; Gescher, A. J. Determination of the putative cancer chemopreventive flavone tricin in plasma and tissues of mice by HPLC with UV-visible detection. *Biomed. Chromatogr.* **2005**, *19*, 518–522.

(5) Verschoyle, R. D.; Greaves, P.; Cai, H.; Borkhardt, A.; Broggini, M.; D'Incalci, M.; Riccio, E.; Doppalapudi, R.; Kapetanovic, I. M.; Steward, W. P.; Gescher, A. J. Preliminary safety evaluation of the putative cancer chemopreventive agent tricin, a naturally occurring flavone. *Cancer Chemother. Pharmacol.* **2006**, *57*, 1–6.

(6) Hudson, E. A.; Dinh, P. A.; Kokubun, T.; Simmonds, M. S. J.; Gescher, A. Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiol. Biomarkers Prev.* **2000**, *9*, 1163–1170.

(7) Cai, H.; Hudson, E. A.; Mann, P.; Verschoyle, R. D.; Greaves, P.; Manson, M. M.; Steward, W. P.; Gescher, A. J. Growth-inhibitory and cell cycle-arresting properties of the rice bran constituent tricin in human-derived breast cancer cells in vitro and in nude mice in vivo. *Br. J. Cancer* **2004**, *91*, 1364–1371.

(8) Cai, H.; Al-Fayez, M.; Tunstall, R. G.; Platton, S.; Greaves, P.; Steward, W. P.; Gescher, A. J. The rice bran constituent tricin potently inhibits cyclooxygenase enzymes and interferes with intestinal carcinogenesis in Apc^{Min} mice. *Mol. Cancer Ther.* **2005**, *4*, 1287–1292.

(9) Al-Fayez, M.; Cai, H.; Tunstall, R.; Steward, W. P.; Gescher, A. J. Differential modulation of cyclooxygenase-mediated prostaglandin production by the putative cancer chemopreventive flavonoids tricin, apigenin and quercetin. *Cancer Chemother. Pharmacol.* **2006**, *58*, 816–825.

(10) Lee, K.-H.; Tagahara, K.; Suzuki, H.; Wu, R.-Y.; Haruna, M.; Hall, I. H.; Huang, H.-C.; Ito, K.; Iida, T.; Lai, J.-S. Antitumor agents. 49. Tricin, kaempferol-3-O-β-D-glucopyranoside and (+)-nortrachelogenin, antileukemic principles from Wikstroemia indica. *J. Nat. Prod.* **1981**, *44*, 530–535.

(11) Nagarathnam, D.; Cushman, M. A short and facile synthetic route to hydroxylated flavones. New syntheses of apigenin, tricin, and luteolin. *J. Org. Chem.* **1991**, *56*, 4884–4887.

(12) Kuwabara, H.; Mouri, K.; Otsuka, H.; Kasai, R.; Yamasaki, K. Tricin from a *Malagasy Connaraceous* plant with potent antihistaminic activity. *J. Nat. Prod.* **2003**, *66*, 1273–1275.

(13) Ju, Y.; Sacalis, J. N.; Still, C. C. Bioactive flavonoids from endophyte-infected blue grass (*Poa ampla*). *J. Agric. Food Chem.* **1998**, *46*, 3785–3788.

(14) Stochmal, A.; Simonet, A. M.; Macias, F. A.; Oleszek, W. Alfalfa (*Medicago sativa* L.) flavonoids. 2. Tricin and chrysoeriol glycosides from aerial parts. *J. Agric. Food Chem.* **2001**, *49*, 5310–5314.

(15) Zhang, Y. Studies on health functional factors of bamboo leaves. PhD dissertation, Department of Food Science and Engineering, Wuxi University of Light Industry, 1995.

(16) Lou, D. D.; Zhang, Y.; Wu, X. Q.; Qi, J. J.; Zhuo, Y. X. Application of antioxidant of bamboo leaves (AOB) in Weixin western sausages. *Chin. Food Fermentat. Ind.* **2004**, *30*, 13–17.

(17) Hu, C.; Zhang, Y.; Kitts, D. D. Evaluation of antioxidant and prooxidant activities of bamboo *Phyllostachys nigra* var. Henonis leaf extract in vitro. *J. Agric. Food Chem.* **2000**, *48*, 3170–3176.

(18) Standardization Administration of the People's Republic of China (SAPRC). The hygienic standards for the use of food additives (GB2760). 2004–2006.

(19) Estiarte, M.; Peñuelas, J.; Cañigueral, S.; Casals, I. A reverse-phase HPLC method for tricin separation from wheat leaves. *Cereal Chem.* **1997**, *74*, 495–496.

(20) Cai, H.; Verschoyle, R. D.; Steward, W. P.; Gescher, A. J. Determination of the flavone tricin in human plasma by high-performance liquid chromatography. *Biomed. Chromatogr.* **2003**, *17*, 435–439.

(21) Tomás-Barberán, F. A.; Blázquez, M. A.; Garcia-Viguera, C.; Ferreres, F.; Tomás-Lorente, F. A comparative study of different Amberlite XAD resins in flavonoid analysis. *Phytochem. Anal.* **1992**, *3*, 178–181.

(22) Bouaziz, M.; Grayer, R. J.; Simmonds, M. S. J.; Damak, M.; Sayadi, S. Identification and antioxidant potential of flavonoids and low molecular weight phenols in olive cultivar Chemlali growing in Tunisia. *J. Agric. Food Chem.* **2005**, *53*, 236–241.

(23) Maciejewicz, W. Isolation of flavonoid aglycones from propolis by a column chromatography method and their identification by GC-MS and TLC methods. *J. Liq. Chromatogr. Relat. Technol.* **2001**, *24*, 1171–1179.

(24) Tang, Z. G.; Zhou, R. Q.; Duan, Z. T. Adsorption and desorption behavior of taurine on macroporous adsorption resins. *J. Chem. Technol. Biotechnol.* **2001**, *76*, 752–756.

(25) Wang, X.; Li, F. W.; Sun, Q. L.; Yuan, J. P.; Jiang, T.; Zheng, C. C. Application of preparative high-speed counter-current chromatography for separation and purification of arctiin from *Fructus Arctii*. *J. Chromatogr. A* **2005**, *1063*, 247–251.

(26) Borissova, A.; Dashova, Z.; Lai, X.; Roberts, K. J. Examination of the semi-batch crystallization of benzophenone from saturated methanol solution via aqueous antisolvent drowning-out as monitored in-process using ATR FTIR spectroscopy. *Cryst. Growth Des.* **2004**, *4*, 1053–1060.

(27) Pina, C. M.; Fernández-Díaz, L.; Prieto, M.; Veintemillas-Verdaguer, S. Metastability in drowning-out crystallization: precipitation of highly soluble sulphates. *J. Cryst. Growth* **2001**, *222*, 317–327.

(28) Chung, I. M.; Hahn, S. J.; Ahmad, A. Confirmation of potential herbicidal agents in hulls of rice. *Oryza sativa. J. Chem. Ecol.* **2005**, *31*, 1339–1352.

(29) Nakajima, Y.; Yun, Y. S.; Kunugi, A. Six new flavonolignans from *Sasa veitchii* (Carr.) Rehder. *Tetrahedron* **2003**, *59*, 8011–8015.

(30) Xu, D. H.; Li, B. J.; Liu, Y.; Huang, Z. S.; Gu, L. Q. Identification of rice (*Oryza sativa* L.) signal factors capable of inducing Agrobacterium vir gene expression. *Sci. China Ser. C-Life Sci.* **1996**, *39*, 8–16.

(31) Katsuzaki, H.; Sakai, K.; Achiwa, Y.; Imai, K.; Komiya, T. Isolation of antioxidative compounds from bamboo shoots sheath. *J. Jpn. Soc. Food Sci. Technol.* **1999**, *46*, 491–493.

(32) Tanaka, N.; Wada, H.; Fuchino, H.; Inoue, T.; Yasuda, D.; Kanda, S.; Kiyokawa, C.; Ashida, N.; Suzuki, T.; Yamazaki, K.; Hakamatsuka, T. Constituents of bamboos and bamboo grasses. *J. Pharm. Soc. Jpn.* **1998**, *118*, 332–337.

Received for review June 6, 2007. Revised manuscript received October 8, 2007. Accepted October 10, 2007.

JF0716533