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Preparative Separation and Identification of the Flavonoid Phlorhizin from the Crude Extract of *Lithocarpus Polystachyus* Rehd

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Abstract: The flavonoid phlorhizin is abundant in the leaves of Sweet Tea (ST, *Lithocarpus polystachyus* Rehd). Phlorhizin was preparatively separated and purified from a crude ST extract containing 40 % total flavonoids by static adsorption and dynamic desorption on ADS-7 macroporous resin and neutral alumina column chromatography. Only water and ethanol were used as solvents and eluants throughout the whole separation and purification process. Using a phlorhizin standard as the reference compound, the target compound separated from the crude ST extracts was analyzed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (EIS-MS) and identified as 99.87% pure (by HPLC-UV) phlorhizin. The results showed that 10 g of the target compound could be obtained from 40 g of the crude extracts in a single operation, indicating a 40% recovery. Therefore, this represents an efficient and environmentally-friendly technology for separating and purifying phlorhizin from ST leaves.

Keywords: Phlorhizin; separation; *Lithocarpus polystachyus*; macroporous resin

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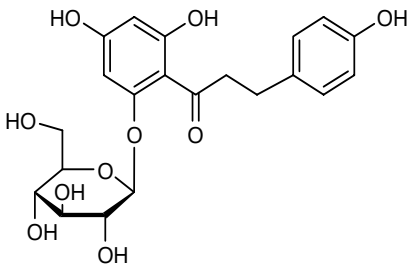
Introduction

1-[2-(β-D-Glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone (phlorhizin, Figure 1), a valuable polyphenolic compound, belongs to the dihydrochalcones, a class of flavonoids. It is generally known that phlorhizin is the principal phenolic glucoside in apple trees [1-3]. Since it was isolated from the bark of the apple tree in 1835 [4], phlorhizin has been used as a pharmaceutical and tool for physiology research for more than 150 years. Its principal pharmacological action is to produce renal glycosuria and block glucose transportation by inhibition of the sodium-linked glucose transporters (SGLTs) [5-9]. In recent years, beside hypoglycemia and hypolipemia [10-12], a series of new bioactive functions of phlorhizin have been found, such as inhibition of lipid peroxidation [13], prevention of bone loss [14], enhancement of memory [15, 16], inhibition of growth of human colon cancer cells, [17, 18]. As quoted by Ehrenkranz: "The use of phlorhizin may provide the molecular basis for the clinical observation that 'an apple a day keeps the doctor away'…" [4], and phlorhizin is attracting the attention of more researchers than ever before.

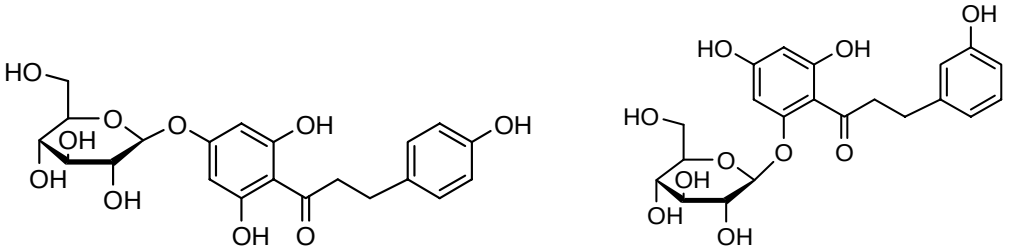
Figure 1. Chemical structure of phlorhizin.



However, because commercial phlorhizin is currently produced from the root bark of apple trees, which can only be harvested once in the plant's life cycle, its production cost is very high and thus its uses are seriously limited. This high production cost hinders the discovery of further and wider applications of phlorhizin. *Lithocarpus polystachyus* Rehd is a shrub distributed widely throughout the mountainous regions in southern China. Its tender leaves, called Sweet Tea (ST) in southern China, can be harvested two or three times a year and have been commonly used as a sweet tonic beverage and traditional herb [19], taken for hundreds of years without evidence of adverse effects or toxicity. During research seeking sweet compounds in the tender leaves, abundant phlorhizin was found, at amounts of up to around 7 % [20-23], and thus ST was found to be a potentially new substitute for the root bark of apple trees as a rich and cheaper source for phlorhizin production. Nevertheless, there are numerous difficulties for the separation of high purity phlorhizin from ST, because phlorhizin exists in ST together with many other polyphenolic compounds with similar properties, such as anthocyanin [24], and phloretin-4-, and 3-hydroxyphlorhizin D-glucoside [22, 25] (Figure 2), so aside from some historical research reporting some analytical scale extractions and separations of phlorhizin from ST [20-23, 25, 26], no information on preparative separations of this compound from ST is available. Furthermore, in the reported methods of phlorhizin extraction and separation, large volumes of hazardous organic solvents, such as diethyl ether, ethyl acetate, methanol, acetone and chloroform were used, thus these methods would be considered environmentally unfriendly. Therefore, finding an efficient and simple technology to separate phlorhizin from ST leaves is a key step for the efficient

utilization of ST as a new source of phlorhizin, and an environmentally-friendly production way is much more important nowadays.

Figure 2. Chemical structures of phloretin-4-D-glucosid (**A**) and 3-hydroxy phlorhizin glucoside (**B**).



In our previous experiments a crude extract was obtained by a microwave-assisted extraction method, in which only water was used as solvent. In the present study, a preparative separation of phlorhizin from the crude extract of ST was performed. A simple technology for efficient separation of phlorhizin in which only water and ethanol were used as solvents and eluants was optimized through a great deal of systematic experimentation. The identity of the target compound was confirmed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (EIS-MS) methods.

Results and Discussion

Separation of the crude extract with ADS-7 resin: effect of adsorption time

When flavonoids are adsorbed by a resin, the amount of free flavonoid in the solution is reduced and the concentration will obviously decline. Figure 3 shows the influence of the adsorption time on the resin adsorption of flavonoids. As the adsorption time increased from 0 to 2 hr, the free flavonoid concentration in the solution was sharply reduced, showing that the amount of flavonoid adsorbed on the resin increased rapidly. Then, between 2-3 hr, the adsorption apparently slowed down, and after 3 hr no further decline in the free flavonoid concentration was detected, which indicated that the adsorption equilibrium was reached. As a result, we selected 2 hr as the optimum adsorption time.

Effects of elution speed and volume of desorption solution (Bed Volume, BV) on desorption ratio (DR)

As shown in Figure 4, the maximum DRs were not affected significantly by different elution rates. However, to reach the same maximum DR a larger volume of desorption solution was needed at faster elution rates than at slower ones. In another words, it took less time to reach the same DR at a faster elution speed than at a slower one.

Balancing these three factors: high DR, low volume of desorption solution and short elution time, we defined 1.0 BV/h and 3 BV as the optimum conditions. Under these optimum conditions, the resin separated product (RSP) was obtained, and its total flavonoid content reached 88 %.

Figure 3. Influence of time on the resin adsorption of flavonoids.

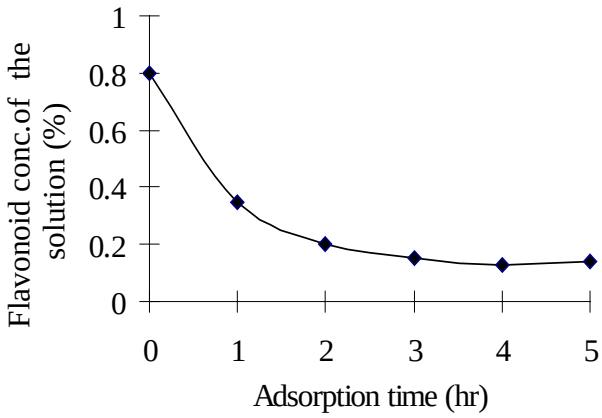
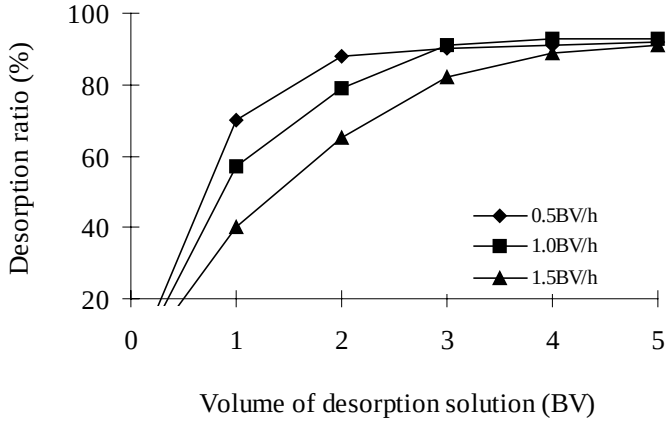


Figure 4. Effect of elution speed and volume of desorption solution on the resin column desorption.

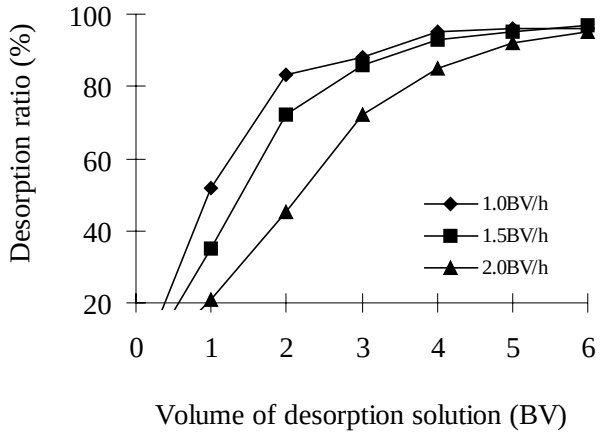


Purification of RSP by neutral alumina column

Figure 5 presents the effects of elution rate and volume of desorption solution on purification of the target compound from RSP by a neutral alumina column. The alumina column was packed with RSP as described in the Experimental section, and was washed with 65 % aqueous ethanol solution at rates of 1.0, 1.5 and 2.0 BV/h, respectively. When the maximum desorption ratio was reached, about 5 BV of desorption solution was used at 2.0 BV/h, and about 4 BV of desorption solution was used at elution rates of both 1.0 and 1.5 BV/h. However, it took 2.5 h at 2.0 BV/h, 2.7 h at 1.5 BV/h, and about 4 h at1.0 BV/h, respectively, to reach the maximum desorption ratio. Therefore, we defined an elution rate of 1.5 BV/h and about 2.7 BV of 65 % aqueous ethanol solution as the optimum elution conditions.

Using the optimized methodology described above and under the conditions described in the Experimental section, 10 g of light yellow target compound were obtained from 40 g of the deep brown crude extracts in a single operation (the recovery thus reached 25 %). Since the 40 g of crude extract in turn came from 300 g of dried ST leaves, the calculated recovery of the target compound from ST leaves was 3.3 %.

Figure 5. Effect of elution speed and volume of desorption solution on the neutral alumina column desorption.



Purity and molecular identification of the target compound

TLC

The target compound was compared with a reference sample of phlorhizin on a polyamide thin layer chromatography plate, as described in the Experimental. Three plates containing both the target compound and the phlorhizin standard on the same starting line were eluted with three kinds of eluants. The TLC results showed only one spot on each of these eluted plates, and the shape, color and R_f of the target compound spot were the same as that of the phloridzin standard on each of the plates, which indicated that the target compound was of very high purity and very likely identical to the phloridzin standard.

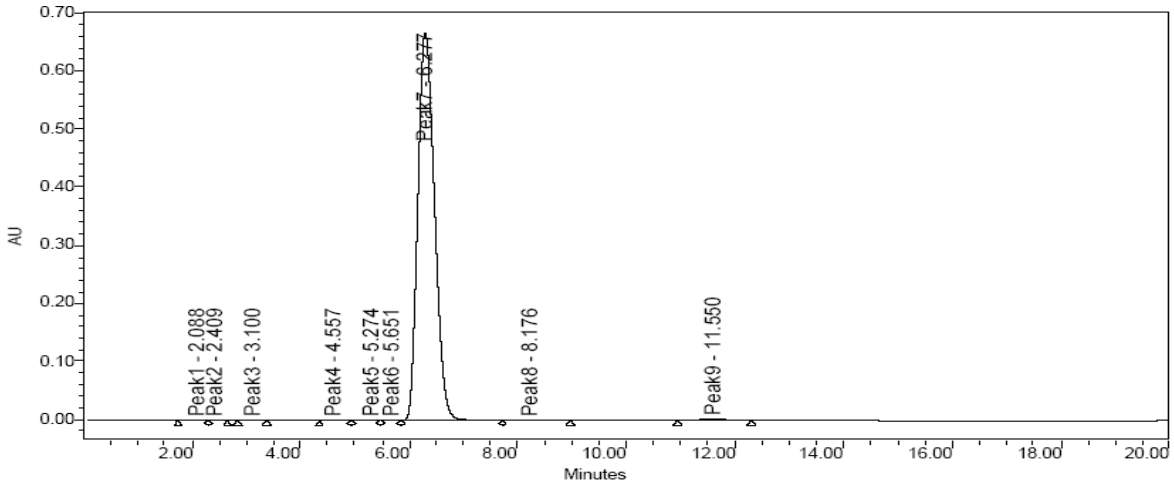
HPLC

The HPLC data of the target compound and the phlorhizin standard are listed in Table 1. As shown, the HPLC-UV purity of the target compound was 99.87 %, although it was noted that the retention time of the target compound was slightly different from that of a phloridzin standard, a fact that might be due to slight differences in the operating conditions. Figure 6 shows the HPLC profile of a mixture of equal amounts of the phlorhizin standard and the target compound. Only one main peak with a retention time of 6.277 min and 98.74 area % was observed, indicating that the target compound was identical to the phloridzin standard by HPLC.

Table 1. HPLC data of the target compound and a phlorhizin standard.

Peak	RT (min)	% Area	% Height
Target compound	6.6	99.87	99.89
Phlorhizin standard	6.3	96.66	97.32

Figure 6. HPLC chromatogram of a mixture of equal amounts of a phlorhizin standard and the target compound.



ESI-MS

The ESI-MS spectra of the target compound (A) and the phlorhizin standard (B) are shown in Figure 7. The molecular mass of the phlorhizin dihydrate standard is 472 Da, and the expected $[M-H]^-$ negative ion was observed in the spectra at m/z 471. Loss of two H_2O molecules (36 Da) from this ion gave a $[M-2H_2O-H]^-$ ion at m/z 435. Cleavage of a glucose fragment without a OH substituent (163 Da) from the pseudomolecular ion at 435 m/z and protonation resulted in an ion with m/z 273. These characteristic ions can be observed in both Figures 6A and 6B, as the data of the $[M-H]^-$ ions of both the two compounds displayed the same features, which confirms that the target compound and the phlorhizin standard are the same compound.

Conclusions

Sweet Tea (*Lithocarpus polystachyus* Rehd) provides a very rich source of the flavonoid phlorhizin, a very valuable bioactive compound and therefore development of an efficient and simple extraction and separation technique for this compound is essential to exploit this rich potential resource. In this paper, phlorhizin was efficiently separated and purified from the crude ST extracts by a relatively simple method, and at the same time, no any organic solvent except for ethanol was used in the whole process, thus the described approach is also environmentally friendly.

Experimental

General

Standards of the compounds phlorhizin and phloretin were purchased from Sigma. Rutin was purchased from Shanghai Chemical Co. (Shanghai, P.R. China); all other chemicals were of analytical grade. All solutions were filtered through 0.45 μm membranes (Fisher Scientific) before HPLC analysis. Macroporous ADS-7 resin was supplied by Nankai-Hecheng Science & Technology Limited Company (Tianjin, China) and its physical properties are listed in Table 2.

Table 2. Physical characteristics of macroporous ADS-7 resin *

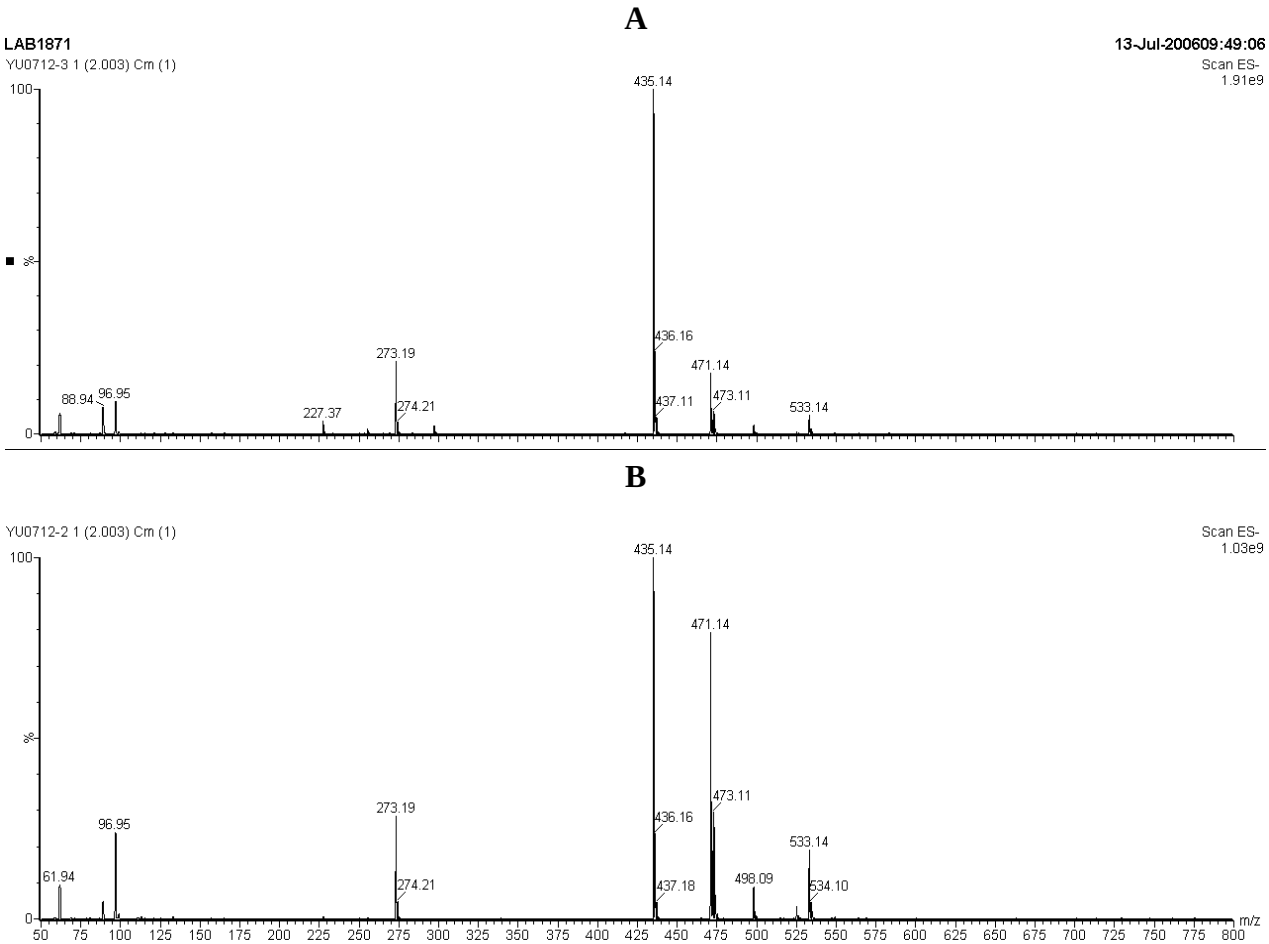
Ave. pore diam.(nm)	Ave. bead diam.(mm)	Surface area (m^2/g)	Water content (%)	Polarity
25-30	0.3-1.0	100-150	60-70	Polar

* The resin can be used directly without pre-treatment.

Crude ST extracts

Crude ST extracts were obtained as described in our previous work (in press) by a microwave-assisted extraction (MAE) without use of an organic solvent. The crude extracts were of a dark brown color, and the total flavonoid content reached 40 %.

Figure 7. ESI-MS spectra of the $[M-H]^-$ ion of (A) the target compound and (B) the phlorhizin standard.



Static adsorption and dynamic desorption by the resin: static adsorption

A pre-weighed amount of resin was placed in an Erlenmeyer flask. Then, three times the amount of an aqueous solution of ST extract was added into the flask. The flask was then shaken at room temperature (25-30 °C) on a rotator operated at 120 rpm.

Dynamic desorption

Dynamic desorption experiments were carried out in a 5 cm x 60 cm glass column packed with the extract adsorbed resin. The bed volume (BV) of the resin was 200 mL. The column was washed first with deionized water till the eluent was colorless, and then desorbed with aqueous ethanol solution (80:20, v/v). The eluents were concentrated and dried under vacuum to give the resin separated product (RSP).

Dynamic elution on an alumina column

A glass column (5 cm x 60 cm) was wet-packed with neutral alumina, which required about 200 mL BV. Then RSP (about 20 g) was dissolved in a small amount of 65 % aqueous ethanol solution and placed on the top of the neutral alumina column. The loaded column was eluted with 65 % aqueous ethanol solution, the eluent was collected, concentrated and crystallized from 40 % aqueous ethanol solution at 4 °C. The crystals were dried and weighed before further analyses. The desorption ratio is given by the following expression: $D = C_d V_d / C_R W_R \times 100$, where D is the desorption ratio (%); C_d is the concentration of flavonoid in the desorption solution (mg/mL); Vd is the volume of the desorption solution; C_R is the flavonoid content (mg/g) of RSP loaded on the resin column, and W_R is the weight (g) of the RSP.

Analytical methods: Determination of total flavonoid concentrations

These measurements were conducted as described by Liu, *et al.* [27]. An aliquot of flavonoid solution was added to a 25 mL flask containing 5 % $NaNO_2$ (w/w, 0.75 mL) and allowed to react for 6 min, then 10 % $Al(NO_3)_3$ (w/w, 0.75 mL) was added, and 6 minutes later, 4 % NaOH (w/w, 4 mL) was added. After mixing, an aqueous solution of 30 % ethanol was added to the flask, which was made up to the mark. The solution was allowed to stand for 10 min at room temperature, and the absorbance at 500 nm was determined on a BECKMAN 7400 spectrophotometer. The total flavonoid concentration was calculated using rutin as the calibration standard. A good linear relationship was obtained over the range of 0.0016 – 0.040 mg/mL, and the regression equation was: $y = 0.0059x + 0.0176$ ($R^2 = 0.9948$, n=5), where y is the concentration of total flavonoids (mg/mL) and x is the absorbance at 500 nm.

TLC analysis

The sample and standard were co-spotted at the base of polyamide thin layer chromatography plates (10.0 cm x 20 cm, produced by Sijia Biochemistry Plastic Company, Taizhou, Zhejiang, P.R. China.) that were then developed with three different eluants: (1) 100:2 95 % aqueous ethanol/acetic acid; (2) 6:2:2:1 water/1-butanol/acetone/acetic acid, and (3) 10:8:1 acetone/water/acetic acid. The eluted plates were dried, stained with 1 % aluminium chloride in ethanol, and observed under 280 nm and 360 nm UV light.

HPLC analysis

All chromatographic analyses were conducted on a Summit HPLC system (Dionex, Germany), equipped with a C-18 reversed-phase column (Diamond, 150 mm x 4.6 mm i.d., 5 μm particle size). The mobile phase consisted of a 60:40 (v/v) mixture of methanol/water at a flow rate 1.5 mL/min and the column effluent was monitored at 283 nm with a UV diode-array detector. The column was maintained at ambient temperature (25-30 °C).

EIS-MS analysis

Mass spectrometry experiments were performed on a ZQ2000 mass spectrometer (Waters, USA), equipped with an electrospray source. MS parameters were as follows: negative ionization mode, capillary, 2.89 kV; source temperature, 100°C; desolvation temperature, 250 °C; mass range, 50 to 800 m/z.

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Sample Availability: Contact the authors.