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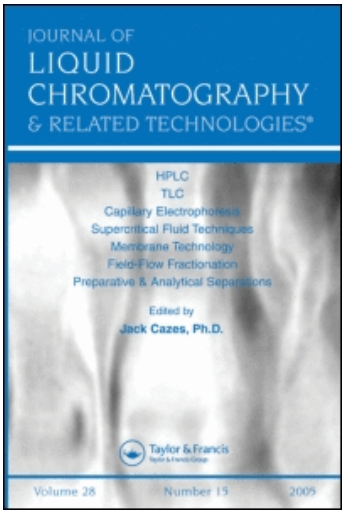

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Preparative Isolation and Purification of Amygdalin from *Prunus armeniaca* L. with High Recovery by High-Speed Countercurrent Chromatography

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Abstract: The bioactive component, amygdalin, was successfully separated from the crude extract of *Prunus armeniaca* L. using high-speed countercurrent chromatography (HSCCC) with high recovery. A polar two-phase solvent system, containing *n*-butanol-ethyl acetate-water (4 : 1 : 6, v/v/v) was run on a preparative scale. The lower phase was used as the mobile phase in the head to tail elution mode. A 208 mg quantity of the crude extract, containing 36.1% amygdalin, was loaded onto a 270 mL HSCCC column, which yielded 69 mg amygdalin of 98.7% purity with 90.7% recovery. The chemical structure of amygdalin was determined by electrospray ionization MS-MS and ^{1}H NMR.

Keywords: Countercurrent chromatography, Preparative chromatography, Plant materials, Pharmaceutical analysis, *Prunus armeniaca* L., Amygdalin

INTRODUCTION

Amygdalin, also called vitamin B17, has the following empirical formula: $C_{20}H_{27}NO_{11}$ (Fig. 1). It is the main bioactive component in the traditional

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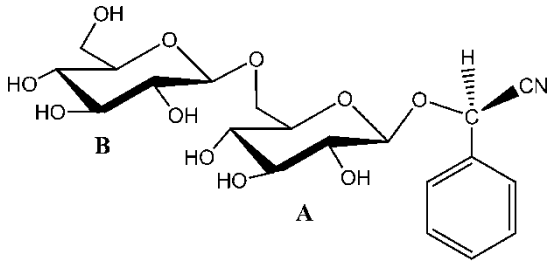

Figure 1. Chemical structure of amygdalin.

Chinese medicinal herb *Prunus armeniaca* L.[1] Amygdalin can be used in medicine for preventing and treating migraine, hypertension, chronic inflammation, and other reaction source diseases. Most important of all, amygdalin is used as a medicament for the treatment of cancer.[2–4] There have been many research reports concerning so-called anti-tumor effects of loquat seed and its principle composition for anti-cancer activity is generally considered to be amygdalin.[5] In addition, amygdalin can be used as a cerebral function improver that is effective as a therapeutic agent for cerebrovascular lesions such as psychogenic symptoms, nerve symptoms, subjective symptoms, and daily life activity disorder.[6] In view of these pharmacological effects, an efficient method for the preparative separation and purification of amygdalin from natural sources is warranted. The conventional methods, such as column chromatography and crystallization are tedious and often require several steps. High-speed countercurrent chromatography (HSCCC) uses no solid support, so the adsorbing effects on stationary phase material and artifact formation can be eliminated. This technique has the maximum capacity with an excellent sample recovery, and it can be employed for preparative-scale separation in a completely straightforward manner. Furthermore, it permits introduction of crude samples directly into the hollow column.[7] Apricot kernels and bitter almond kernels (*Prunus armeniaca* L.) have a content of amygdalin of about 3–4% by weight.[2] The present paper reports the successful preparative separation and purification of amygdalin from kernels of the traditional Chinese medicinal herb *Prunus armeniaca* L. by HSCCC.

EXPERIMENTAL

Reagents and Materials

All organic solvents used for HSCCC were of analytical grade and were purchased from Hangzhou HuiPu Chemical Factory (Hangzhou, China). Acetonitrile used for HPLC analysis was of chromatographic grade. *Prunus armeniaca* L. was purchased from Wulin Drugstore (Hangzhou, China).

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Apparatus

A Model TBE-300A high-speed countercurrent chromatograph (Shanghai Tauto Biotechnique, Shanghai, China), equipped with three preparative multilayer coils (270 mL, wound with 1.6 mm I.D. PTFE tubing) was used. The β values of this column range from 0.46 to 0.73 ($\beta = r/R$, $R = 6.5$ cm, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder shaft and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 rpm and 1,000 rpm. The columns of the HSCCC were installed in a vessel that was retained at 35°C by a Model HX-1050 constant-temperature controller (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Shengyitong Technique Co. Ltd., Beijing, China). Continuous monitoring of the effluent was achieved with a Model UV-II detector monitor (Shanghai Institute of Biochemistry of the Academy of Science, Shanghai, China) at 254 nm. A manual sample injection valve with a 20 mL loop (Shanghai Tauto Biotechnique, Shanghai, China) was used to introduce the sample into the column. A Sepu3000 workstation (Hangzhou PuHui Technology, Hangzhou, China) was employed to record the chromatogram. Eluate was collected with a Model BSZ-100 fraction collector (Shanghai Huxi Tech, Shanghai, China), 6 mL for each fraction.

The high-performance liquid chromatograph (HPLC) used was a CLASS-VP Ver. 6.1 system (Shimadzu, Japan) comprised of a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver. 6.1 workstation.

Extraction of Crude Sample

Seeds, 100 g, of *Prunus armeniaca* L. were extracted three times with 75% aqueous ethanol under refluxing (1 h, 1 h, 1 h). The ethanol extracts were combined and concentrated to dryness under reduced pressure, yielding 6.98 g of ethanol extract. Then, it was redissolved in water and extracted with ether and *n*-butanol, consecutively. The *n*-butanol extract was concentrated to dryness under reduced pressure, yielding 1.25 g dried powder that was directly subjected to HSCCC.

Selection of Two-Phase Solvent System

A number of two-phase solvent systems were tested, by changing the volume ratio of the solvent, to obtain the optimum composition that gave suitable

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Table 1. The *K* (partition coefficient) values of amygdalin in different solvent systems

Solvent system	*K*
n-Butanol-water (1 : 1)	0.26
n-Butanol-0.3 mol/l ammonium acetate (1 : 1)	0.23
n-Butanol-0.3 mol/l sodium chloride (1 : 1)	0.14
n-Butanol-0.3 mol/l sodium dihydrogen phosphate (1 : 1)	0.20
n-Butanol-0.2% aqueous trifluoroacetic acid (1 : 1)	0.28
n-Butanol-acetic acid-water (4 : 1 : 5)	0.32
n-Butanol-ethyl acetate-water (3 : 2 : 5)	0.51
n-Butanol-ethyl acetate-water (3 : 3 : 5)	0.62
n-Butanol-ethyl acetate-water (4 : 1 : 5)	0.41
n-Butanol-ethyl acetate-water (4 : 1 : 6)	0.46

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partition coefficient (*K*) values. The measurement of the *K* values was performed as follows:[8] About 1 mg of *n*-butanol extract was added to the two mutually equilibrated solvent phases (2 mL each) in a stoppered test tube (10 mm × 120 mm). It was then thoroughly mixed with a vortex to equilibrate the contents. After settling, the upper and lower phases were analyzed by HPLC to obtain the partition coefficient of the target compound. *K* was expressed as the peak area of the target compound in the upper phase divided by that in the lower phase (see Table 1).

Preparation of Two-Phase Solvent System and Sample Solution

The selected solvent system was thoroughly equilibrated in a separation funnel by repeatedly vigorously shaking at the same temperature as in the HSCCC column. The two phases were separated just prior to use. The upper phase was used as the stationary phase, while the lower phase was used as the mobile phase. The sample solution was prepared by dissolving the crude sample in the mixture of the lower phase and the upper phase (1 : 1, v/v) of the solvent system used for HSCCC separation.

Separation Procedure

HSCCC was performed as follows. The multilayer-coiled column was first entirely filled with the upper phase as a stationary phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow-rate of 1.50 mL/min, while the apparatus was run at a revolution speed of 850 rpm. After hydrodynamic equilibrium was reached, as

indicated by a clear mobile phase eluting at the tail outlet, the sample solution (208 mg dissolved in 8 mL of a mixed solution of lower phase and upper phase (1 : 1, v/v) of the solvent system) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was manually collected according to the elution profile and analyzed by HPLC. After the separation was completed, retention of the stationary phase was measured by collecting the column contents, by forcing them out of the column with pressurized air.

HPLC Analysis and Identification of HSCCC Peak Fractions

The *n*-butanol extract from *Prunus armeniaca* L. and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a Shim-Pack CLC-ODS C_{18} column (250 mm × 6 mm I.D.). The mobile phase, composed of acetonitrile–water (17 : 83, v/v), was eluted at a flow-rate of 0.6 mL/min, and the effluent was monitored with a Shimadzu SPD10Avp UV detector at 254 nm. Routine sample calculations were made by comparison of the peak area with that of the standard.

Identification of HSCCC peak fractions was carried out by electrospray ionization (ESI) MS-MS and ^{1}H NMR. ESI-MS-MS was performed with an API-2000 mass spectrometer (Applied Biosystems, America). ^{1}H NMR spectra were recorded with a Bruker Avance 400 MHz spectrometer (Bruker Corporation, America) with TMS (tetramethylsilane) as the internal standard.

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RESULTS AND DISCUSSION

Selection of Suitable Two-Phase Solvent System for HSCCC

Successful separation of the target compound using HSCCC depends on the selection of suitable solvent systems, which should be focused on the following points: (1) no decomposition or denaturation of the sample; (2) sufficient sample solubility; (3) suitable partition coefficient (K) values of the target compound (i.e., usually between 0.2 and 5); (4) satisfactory retention of the stationary phase; and (5) the settling time of the solvent system should be short (i.e., $\leq$30 s).[9] A series of experiments was performed to determine the optimal two-phase solvent system; their K values were measured and are summarized in Table 1. Amygdalin is a highly polar compound and it is freely soluble in water, even in 0.3 mol/L aqueous ammonium acetate, 0.3 mol/L aqueous sodium chloride, and 0.3 mol/L sodium dihydrogen phosphate. As shown in Table 1, the K values of amygdalin in the solvent systems including *n*-butanol-water (1 : 1), *n*-butanol-0.3 mol/L ammonium acetate (1 : 1), *n*-butanol-0.3 mol/L sodium chloride (1 : 1), and *n*-butanol-0.3 mol/L

sodium dihydrogen phosphate (1:1) were similarly small, which led to amygdalin and other compounds being eluted together, close to the solvent front, and resulted in poor resolution. When acetic acid or trifluoroacetic acid was added to the solvent system consisting of *n*-butanol-water (1 : 1), the *K* value of amygdalin slightly improved, but it still could not separate the target compound well. As shown in the Table 1, the solvent system *n*-butanol-ethyl acetate-water (3 : 3 : 5, v/v/v) gives a suitable *K* value of 0.62, as does the solvent system *n*-butanol-ethyl acetate-water (3 : 2 : 5, v/v/v). However, the settling time for these solvent systems is over 3 min, which would directly lead to poor retention of stationary phase. Finally, the solvent system *n*-butanol-ethyl acetate-water (4 : 1 : 6) would satisfy the above points and our experiment clearly demonstrated that amygdalin could be well separated from the crude extract by HSCCC. Though the *K* value of amygdalin in the system of *n*-butanol-ethyl acetate-water (4 : 1 : 6) was smaller than that of the above two systems, the settling time decreased.

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Separation of Amygdalin with HSCCC

The crude sample was dissolved in 8 mL of a mixture of both lower phase and upper phase (1 : 1, v/v) of the solvent system used for the HSCCC separation. A 208 mg quantity of *n*-butanol extract was separated by HSCCC, which yielded 69 mg of amygdalin with 98.7% purity and 90.7% recovery. The retention of the stationary phase was 26.9%, and the separation time was 180 min for a complete separation run. Figure 2 shows the result obtained from 208 mg of the *n*-butanol extract of *Prunus armeniaca* L. by preparative HSCCC.

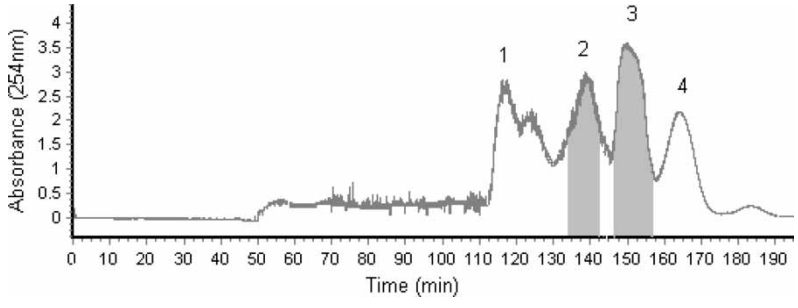

Figure 2. Chromatogram of the crude sample from *Prunus armeniaca* L. by HSCCC. Peak 1: unknown compounds; Peak 2: unknown compounds; Peak 3: amygdalin; Peak 4: unknown compounds. Solvent system: *n*-butanol–ethyl acetate-water (4 : 1 : 6, v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 1.5 ml/min; revolution speed: 850 rpm; sample: 208 mg dissolved in 8 ml mixture solution of lower phase and upper phase (1 : 1, v/v) of the solvent system; retention of the stationary phase: 26.9%.

HPLC Analysis of the Crude Sample and HSCCC Peak Fractions

The *n*-butanol extract of *Prunus armeniaca* L. was analysed by HPLC. The result indicated that, under UV 254 nm monitoring, the crude contained several compounds, among which amygdalin represented 36.1% of the total. After HSCCC separation, the fractions containing amygdalin were collected. The analysis indicated that the Peak 3 fraction contained amygdalin, which weighed 69 mg, and was over 98.7% pure, and the Peak 2 fraction contained an unknown compound which weighed 9.5 mg, with over 92.2% purity, as determined by HPLC (Fig. 3).

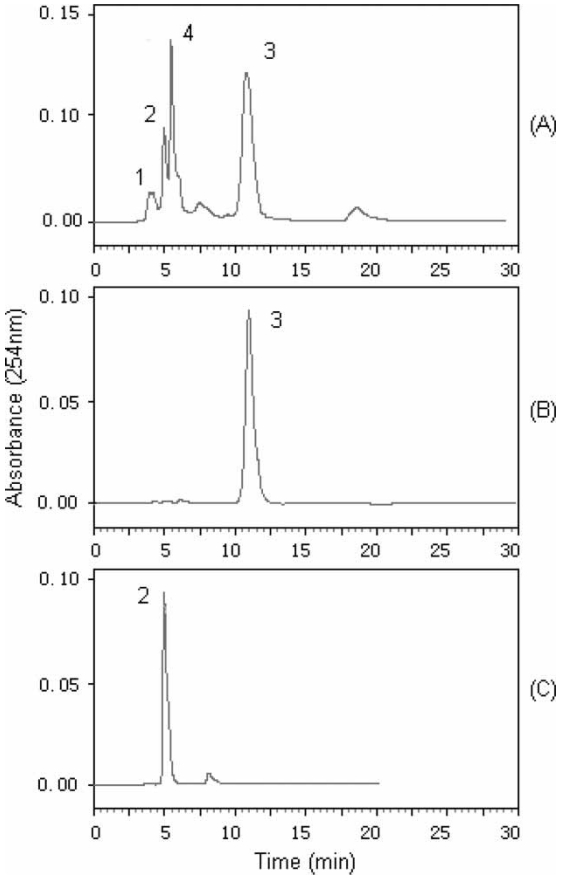

Figure 3. Results of HPLC analyses of the crude sample of *Prunus armeniaca* L. and its HSCCC fraction. Column: Shim-Pack CLC-ODS C_{18} column (250 mm × 6 mm I.D.); mobile phase: acetonitrile–water (17 : 83); flow-rate: 0.6 ml/min; UV detector: 254 nm. (A) The crude sample; (B) HSCCC fraction from Peak 3; (C) HSCCC fraction from Peak 2 (Fig. 2).

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Structural Identification

The structural identification of amygdalin was carried out by ESI-MS-MS and ^{1}H NMR spectra. Peak 3: white needle crystals, positive ESI-MS m/z: 479.9 $(M + Na)^+$, 457.9, MS-MS fragmentation of m/z 457.9: 458.2 $(M + H)^+$, 296.2 (M-glc + H)$^+$, 163.0, 144.9. ^{1}H NMR (DMSO-d_6) δ ppm: 7.58 (2H, m, H-ortho of phenyl), 7.48 (2H, m, H-meta of phenyl), 7.47 (1H, m, H-para of phenyl), 6.01 (1H, s, methine), 4.42 (1H, d, $J = 7.78$, H-A_1), 4.24 (1H, d, $J = 5.27$, H-B_1), 3.00 $\sim$ 4.02 (12H, m, H-A_{2-6} and -B_{2-6}), 4.52 (1H, s, OH-B_6), 4.95 $\sim$ 5.35 (6H, m, OH-A_{2-4} and -B_{2-4}). After comparing the data with spectral information from literature,[10–12] Peak 3 was confirmed as amygdalin.

The result of our studies described above clearly demonstrated that HSCCC is successful in the preparative separation of amygdalin from the crude extract of *Prunus armeniaca* L., with high recovery.

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