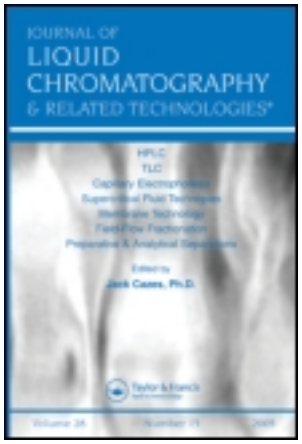

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PREPARATIVE ISOLATION AND PURIFICATION OF LUTONARIN AND SAPONARIN FROM BARLEY SEEDLINGS BY HSCCC

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□ *In order to isolate large amounts of pure lutonarin and saponarin, a high performance method for isolation and purification of these two flavonoids from barley (*Hordeum vulgare*) seedlings was successfully established by utilizing high-speed counter-current chromatography (HSCCC). Separation was performed with a two-phase solvent system: ethyl acetate/n-butanol/water (3:2:5, v/v/v). A total of 24 mg lutonarin and 14 mg saponarin was obtained in one-step separation from 100 mg sample. High performance liquid chromatography (HPLC) analysis showed that the purity of each of the two compounds was over 98%. Their chemical structures were confirmed by UV, ^{1}H NMR, and ^{13}C NMR data.*

Keywords barley seedlings, *Hordeum vulgare*, HPLC, HSCCC, lutonarin, saponarin

INTRODUCTION

Barley (*Hordeum vulgare*), a well-known local crop of Qinghai-Tibet Plateau and officially listed in the Flora Republicae Popularis Sinicae,[1] is traditionally used as a staple in South West China provinces such as Xizang, Sichuan, Qinghai, and Yunnan and has positive effects in the treatment of diabetes, hypertension, tumors, and liver disorders. Previous studies have focused primarily on nutritional and feed value.[2–6] With the increased public interest in health-promoting compounds, the bioactive compounds of barley have captured people's attention. Among barley's compounds, flavonoids have been identified as related to antioxidant activity.[7–10] Recent research on the flavonoids in barley seedlings showed that they were suitable for health care products due to their positive effects on antihypoxia and antifatigue. Both of these conditions are of great

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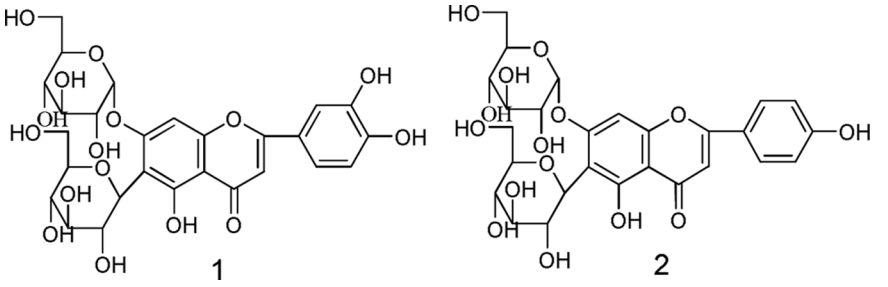

FIGURE 1 Chemical structures of lutonarin (1) and saponarin (2).

significance for people living in the plateau. Lutonarin and saponarin are the major flavonoids found in barley seedlings.[11] With the increase in research applications of lutonarin and saponarin, the demands for highly pure amounts of these compounds are rapidly increasing. However, until recently, only limited scientific information was available on the isolation and purification of these compounds.

HSCCC is a support-free, liquid–liquid, partition chromatographic technology.[12] It eliminates the irreversible adsorption of a sample onto a solid support and has an excellent sample recovery. It is gaining increasing interest and is used more and more frequently in the isolation of bioactive components from crude materials.

Lutonarin and saponarin (structures shown in Figure 1) are structurally similar flavonoids. Although numerous studies have reported the separation of flavonoids by HSCCC,[13–17] to our knowledge, no paper had reported on the use of HSCCC for the isolation and purification of mixtures of lutonarin and saponarin. The purpose of this study was to develop a high-performance method using HSCCC for isolating these individual compounds.

EXPERIMENTAL

Apparatus

The preparative HSCCC instrument employed in the study was a TBE-300A high-speed countercurrent chromatography (Tauto Biotech Co., Shanghai, China). The apparatus was equipped with a polytetra fluoroethylene, three preparative coils (i.d. of the tubing = 1.6 mm, total volume = 280 mL), and a 20 mL sample loop. The revolution radius was 5 cm. The β values of the preparative column ranged from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The revolution

speed of the apparatus can be regulated with a speed controller in the range between 0 and 1,000 rpm. The HSCCC system was equipped with a model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China), a model 8823B UV detector (Beijing Huanyu Science Company, Beijing, China) operating at 280 nm, and a model N2000 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by the LX-300 constant temperature circulating implement (Beijing Changliu Instruments Co. Ltd., Beijing, China).

The high-performance liquid chromatography (HPLC) analysis was performed on an Agilent 1200 system (Agilent Technologies Co. Ltd., USA) equipped with G1354A solvent delivery unit, G1315B UV-vis photodiode array detector, G1316A column thermostat, G1313A autosampler, Phenomenex luna C_{18} (4.6 × 150 mm, 5 μm) analytical column, and an Agilent HPLC workstation.

The nuclear magnetic resonance (NMR) spectrometer was a Mercury-400BB NMR spectrometer (Varian Co. Ltd., USA).

Reagents and Materials

All organic solvents used for the preparation of the crude extract and HSCCC separation were of analytical grade and purchased from Tianjin Fuyu Chemical Co. (Tianjin, China). Methanol used for HPLC was of chromatographic grade purchased from Yuwang Chemical Ltd. (Shandong, China). Dimethyl sulfoxide (DMSO-d_6) was used as the solvent for NMR determination. Barley seedlings were collected from Qinghai province in June 2010 and were dried in the shade at room temperature.

Preparation of Crude Sample

Dried barley seedlings (200 g) were ground into a powder and extracted three times with 12 L of 30% ethanol at 90°C. The solid-liquid ratio was 1:20 for three extractions. The extraction time for the three extractions was 2 hr, 2 hr, and 1 hr, respectively. All the filtrates were combined and concentrated by rotary evaporation at 60°C under reduced pressure. The resulting 5 L of residue was cooled for 24 hr at 4°C and precipitation was filtered and dried, and then 1.5 g of brown powder sample was obtained for subsequent isolation and purification.

Selection of Two-Phase Solvent System

The two-phase solvent system was selected according to the partition coefficient (K) of the target components of the sample. The K values were

determined by HPLC analysis in the following manner. A suitable amount of sample was added into a series of solvent systems, followed by shaking the solution fully. Then, equal volumes of the upper and lower phases were separately evaporated to dryness. The residues were diluted into 2 mL of methanol and analyzed by HPLC. The *K* value was defined as the peak area of the compound in the upper phase divided by the peak area in the lower phase.

Preparation of the Two Phase Solvent System and Sample Solution

The two-phase solvent system used was composed of ethyl acetate/*n*-butanol/water (3:2:5, v/v/v). Each solvent was added to a separatory funnel and thoroughly equilibrated at room temperature. The upper phase and the lower phase were separated and degassed by ultrasonic bath for 30 min shortly before use. The upper phase was used as the stationary phase and the lower phase was used as the mobile phase. The sample solution was prepared by dissolving 100 mg of brown powder sample in 20 mL of the lower phase.

HSCCC Separation Procedure

HSCCC separation was performed as follows: the multiple-coiled column was first entirely filled with the upper phase. Then, the lower phase was pumped into the head end of the inlet column at a flow-rate of 1.6 mL/min, while the coils were rotated at 900 rpm. After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet, 20 mL of sample solution was injected using an injection valve. The effluent from the tail end of the column was continuously monitored with UV detection at 280 nm. After the sample injection, the data were collected immediately. The fractions were collected manually according to the chromatogram and then evaporated under reduced pressure. The residues were dissolved in 30% ethanol for subsequent purity analysis by HPLC.

HPLC Analysis

The partially purified sample and each peak fraction obtained by HSCCC were all analyzed on an Eclipse XDB C_{18} column (250 mm × 4.6 mm I.D., 5 μm). A methanol-water system was used as the mobile phase in the gradient elution mode (methanol concentration from 38% to 62% in 14 minutes). The column temperature was set at 25°C. The flow rate of the mobile phase was 1.0 mL/min, and 10 μL aliquots were injected into the column. The effluents were monitored at 270 nm by a UV detector.[18]

Identification of HSCCC Peak Fractions

Identification of HSCCC peak fractions was carried out by ^{1}H NMR, ^{13}C NMR. The NMR spectra were obtained on a Mercury-400BB NMR spectrometer with tetramethylsilane (TMS) as an internal standard.

RESULTS AND DISCUSSION

A successful separation of target compounds using HSCCC requires a careful search for a suitable two-phase solvent system to provide an ideal range of partition coefficients for the applied material. A *K* within the range of 0.5–2.0 is appropriate for HSCCC separation. A smaller *K* value elutes the solute closer to the solvent front with lower resolution, while a larger *K* value tends to give better resolution but broader, more dilute peaks due to a longer elution time.[19] Moreover, the target compounds must be stable and soluble in such a solvent system, and the solvent system must separate clearly and quickly into two phases. Based on the aforementioned principles, three solvent systems composed of ethyl acetate/methanol/water, ethyl acetate/*n*-butanol/methanol/water, and ethyl acetate/*n*-butanol/water were selected according to the polarity of the target compounds.[20] Their *K* values were measured and are summarized in Table 1. When ethyl acetate/methanol/water solvent systems with different volume ratios, ranging from 5:1:5, 5:1.5:5 and 5:2:5, were tried, the *K* values were suitable, but the retention of the stationary phase was below 30%. With the addition of *n*-butanol to ethyl acetate/methanol/water, the system improved the retention of the stationary phase, but was not suitable for the *K* values of the target compounds. When methanol was eliminated and an ethyl acetate/n-butanol/water system was used, the *K* values were not only suitable, but also the retention of the stationary phase was greatly improved and the target compounds could be well-separated. Furthermore, the target compounds were freely soluble in these solvent systems. Of the

TABLE 1 The *K* Values of the Target Components in Several Solvent Systems

Solvent System	K_1	K_2
ethyl acetate/methanol/water (5:1:5, *v*/*v*/*v*/*v*)	0.98	1.02
ethyl acetate/methanol/water (5:1.5:5, *v*/*v*/*v*/*v*)	1.23	0.99
ethyl acetate/methanol/water (5:2:5, *v*/*v*/*v*/*v*)	1.65	1.31
ethyl acetate/*n*-butanol/methanol/water (5:0.5:2:5, *v*/*v*/*v*/*v*)	2.52	1.80
ethyl acetate/*n*-butanol/methanol/water (5:1:1.5:5, *v*/*v*/*v*/*v*)	2.43	1.74
ethyl acetate/*n*-butanol/methanol/water (5:2:1:5, *v*/*v*/*v*/*v*)	2.37	1.68
ethyl acetate/*n*-butanol/water (4:1:5, *v*/*v*/*v*/*v*)	2.28	1.52
ethyl acetate/*n*-butanol/water (3:2:5, *v*/*v*/*v*/*v*)	2.09	1.21
ethyl acetate/*n*-butanol/water (2:3:5, *v*/*v*/*v*/*v*)	1.76	0.99

three different volume ratios used, 4:1:5, 3:2:5, and 2:3:5, the first one required the smallest preparative time but resulted in the worst separation, while the third provided the best separation but the required the longest preparative time. In addition, a high ratio of *n*-butanol increased the difficulty in the 2:3:5 ratio sample recovery. Taking all these observations into account, the two-phase solvent system composed of ethyl acetate-n-butanol-water at a ratio of 3:2:5 was employed.

In addition to the *K* values of solvent system, other important parameters, including the flow rate of the mobile phase and the revolution speed of the apparatus, were studied. A preliminary test was conducted at two flow rates, 1.6 and 2.0 mL/min, and it was determined that the former rate gave a higher retention of the stationary phase and better sample separation. Two different revolution speeds, 800 and 900 rpm, were also used to separate samples at each of the two flow rates. Higher retention of the stationary phase and better sample separation were obtained at 900 rpm. A flow rate of 1.6 mL/min and a revolution speed of 900 rpm were therefore chosen for the present study, and the retention of the stationary phase was 42% (Figure 2). A low flow rate and a high revolution speed helped to increase the retention volume of the stationary phase and resulted in improved peak resolution.[19,21] The peak fractions separated by HSCCC was analyzed by HPLC and the chromatogram is shown in Figure 3. Utilizing the aforementioned procedures, 24 mg of lutonarin and 14 mg saponarin, with the purity up to 98%, were separated and purified by HSCCC

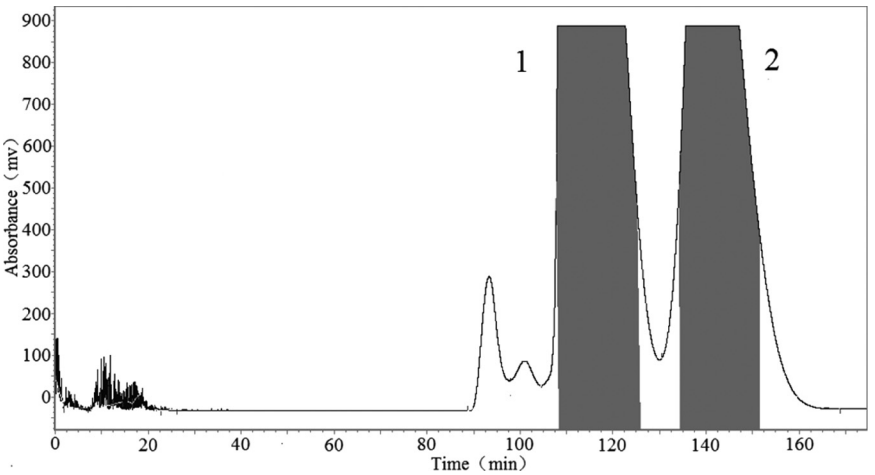

FIGURE 2 HSCCC chromatogram of crude extract from Barley seedlings. Two phase solvent system: ethyl acetate/*n*-butanol/water 3:2:5, v/v/v); mobile phase, the lower phase; flow rate 1.6 mL min^{-1}; revolution speed, 900 rpm; detection wavelength, 280 nm; separation temperature, 25°C; sample size, 100 mg sample dissolved in 20 mL of the lower phase. Retention of the stationary phase: 42%.

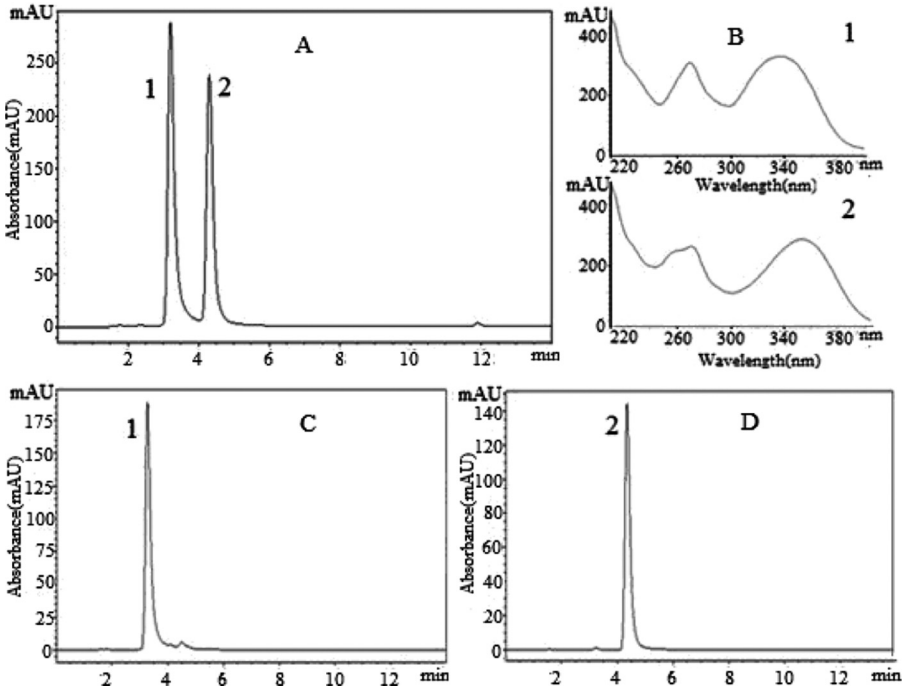

FIGURE 3 HPLC chromatogram of crude sample (A), lutonarin (C), and saponarin (D) and UV spectrum of lutonarin and saponarin (B); lutonarin (1) and saponarin (2); Column: Eclipse XDB C_{18} column (250 mm × 4.6 mm I.D., 5 μm); mobile phase: methanol and water in gradient mode (methanol: water (v/v) from 38: 62 to 62: 38, in 13 min); flow rate: 1.0 mL min^{-1}; detection wavelength: 270 nm; column temperature: 30°C.

from 100 mg brown powder sample. These compounds can be used as reference sample to control the quality of barley products.

The chemical structures of peak fractions separated by HSCCC were identified according to their UV, ^{1}H NMR, ^{13}C NMR. Peak 1 and 2 were assigned to lutonarin and saponarin. The data of each peak fraction were given as follows:

Peak 1 in Figure 2: $C_{27}H_{30}O_{15}$, $UV_{\lambda max}$: 270, 350 nm. APCIMS (positive ion mode) m/z: 610.7$[M+H]^{+}$, 449.0$[M+H-162]^{+}$; APCIMS (negative ion mode) m/z: 609.0$[M-H]^{-}$, 447.2$[M-H-162]^{-}$. ^{1}HNMR(DMSO-d_6, 400 MHz)δ: 7.42(1H,dd,J = 8.0,2.0 Hz,H-6′), 7.40(1H,d,J = 2.0 Hz,H-2′), 6.94(1H,d,J = 8.0 Hz,H-5′), 6.70(1H, s,H-3), 6.60(1H,s,H-8), 4.98(1H,d,J = 7.0 Hz,G1-1), 4.70(1H,d,J = 6.8 Hz, G2-1); ^{13}CNMR(DMSO-d_6, 100 MHz)δ: 182.8(C-4), 164.8(C-7), 162.4(C-2), 160.4(C-9), 156.5(C-5), 150.2(C-4′), 146.7(C-3′), 121.0(C-6′), 119.5(C-1′), 116.1(C-5′), 112.7(C-2′), 110.5(C-6), 102.4(C-10), 101.2(G1-1), 100.8(C-3), 93.6(C-8), 81.0(G2-5), 79.0(G2-3), 77.2(G1-5), 76.0(G1-3), 73.9(G1-2), 72.7(G2-1), 70.9(G2-2), 70.3(G1-4), 69.6(G2-4), 60.7(G2-6), 60.3(G1-6).

Peak 2 in Figure 2: $C_{27}H_{30}O_{15}$. $UV_{\lambda max}$: 270, 340 nm. APCIMS (positive ion mode) m/z: 594.9$[M+H]^+$, 433.2$[M+H\text{-}162]^+$; APCIMS (negative ion mode) m/z: 593.0$[M\text{-}H]^-$, 431.2$[M\text{-}H\text{-}162]^-$. ^{1}HNMR(DMSO-$d_6$, 400 MHz)$\delta$: 7.98(2H,d,J = 8.8 Hz,H-2′,6’), 6.95(2H,d,J = 8.8 Hz,H-3′,5’), 6.93(1H,s,H-3), 6.88(1H,s,H-8), 4.98(1H,d,J = 7.0 Hz,G1-1), 4.72(1H,d,J = 6.8 Hz,G2-1); ^{13}CNMR(DMSO-d_6, 100 MHz)δ: 182.1(C-4), 164.2(C-2), 162.5(C-7), 161.3(C-4′), 159.3(C-9), 156.4(C-5), 128.6(C-2′,6’), 120.9(C-1′), 116.0(C-3′,5’), 108.9(C-6), 104.2(C-10),103.2(C-3), 101.2(G1-1), 93.7(C-8), 80.9(G2-5), 78.9(G2-3), 77.2(G1-5), 75.7(G1-3), 73.8(G1-2), 72.9(G2-1), 70.8(G2-2),70.6(G1-4),69.6(G2-4), 60.6(G2-6), 60.3(G1-6).

CONCLUSION

This study demonstrates that HSCCC is a simple, fast, and efficient method for preparative isolation of lutonarin and saponarin with high purities in one-step separation from the extracts of barley seedlings. These compounds with high purities may be used as reference substance for further chemical research and pharmacological studies. As the lack of standard compounds became the main limitation on the research and development of natural plant,[22] this method has good potential on the preparation of standards from nature products, especially on the quality control of barley products.

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