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Semi-industrial isolation of salicin and amygdalin from plant extracts using slow rotary counter-current chromatography

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Abstract

Salicin in the bark extract of *Salix alba* and amygdalin in the fruit extract of *Semen armeniacae* were each separated by slow rotary counter-current chromatography (SRCCC). The apparatus was equipped with a 40-L column made of 17 mm i.d. convoluted Teflon tubing. A 500 g amount of crude extract containing salicin at 13.5% was separated yielding 63.5 g of salicin at 95.3% purity in 20 h using methyl *tert*-butyl ether–1-butanol (1:3) saturated by methanol–water (1:5) as a stationary phase and methanol–water (1:5) saturated by methyl *tert*-butyl ether–1-butanol (1:3) as a mobile phase. A 400 g amount of crude extract containing amygdalin at 55.3% was isolated to yield 221.2 g of amygdalin at 94.1% purity in 19 h using ethyl acetate–1-butanol (1:2) saturated by water as a stationary phase and water saturated by ethyl acetate–1-butanol (1:2) as a mobile phase. The flow rate of the mobile phase was 50 ml/min. The results show that industrial SRCCC separation of salicin and amygdalin is feasible using a larger column at a higher flow rate of the mobile phase.

Keywords: Salicin; *Salix alba*; Amygdalin; *Semen armeniacae*; Slow rotary counter-current chromatograph; Industrial isolation

1. Introduction

Salicin is well known for its effect on treating rheumatic fever and subacute bacterial endocarditis. Also salicin has been used as a traditional analgesic in Europe [1]. Salicin is a prodrug which is gradually hydrolyzed to aligenin by intestinal bacteria, and converted into salicylic acid after absorption. Thus it produces an antipyretic action without causing gastric injury [2]. Salicin is prepared from a decoction of the bark of *Salix alba* (white willow bark), by first precipitating the tannin by milk of lime, then evaporating the filtrate to a soft extract, and dissolving out the salicin by alcohol. This method causes lose of other bioactive compounds such as flavonoids [3].

Amygdalin is a commercially available natural cyanogenic glycoside which occurs in the seeds of Rosaceae, principally in the fruit of *Semen armeniacae* (bitter almonds) [4]. The isolation of this cyanogenic glycoside has been performed by column chromatography [5]. Counter-current chromatography (CCC) is an all-liquid chromatographic system based on fast partitioning effects of the analytes between two immiscible liquid phases, without adsorptive loss of the sample on the solid support used in the conventional liquid chromatography. Therefore, it is highly desirable to develop an industrial CCC for the separation of salicin and amygdalin.

The scaling-up of CCC for industrial use is very promising and challenging, though high-speed counter-current chromatography (HSCCC) is intensively used in preparative separation in laboratories. One way to scale up CCC is to utilize the slow rotary mode of coiled column, which was first described by Ito and Bhatnagar [6,7]. In this system, the best result was attained by rotating the coil slowly around

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its horizontal axis at a critical speed that yields high retention of the stationary phase. Recently an apparatus equipped with a 10-L capacity column made of 8.5 mm i.d. convoluted tubing was prepared in our laboratory. Using this apparatus, 150 g of crude tea extracts were successfully separated, yielding 40 g of biologically active epigallocatechin gallate (EGCG) in high purity at a recovery rate of 82.6% [8]. Then, we examined the chromatographic parameters using convoluted Teflon tubing with an average i.d. of 1.5 cm for developing a larger chromatographic column and found that it is feasible to scale up slow rotary counter-current chromatograph with larger i.d. tubing [9]. In the present study, an apparatus equipped with a 40-L capacity column is constructed and used for semi-industrial separation of salicin from the extract of white willow bark and amygdalin from the extract of bitter almond.

2. Experimental

2.1. Materials

Standard amygdalin were purchased from National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China). All solvents were of analytical grade, purchased from Hangzhou Chemicals Inc., China

The crude extract of salicin from the bark of *S. alba* was provided by Chashai Kinglong Bioproducts, Changshai, China. The extract contained salicin at 13.5%. The sample was directly used for SRCCC-separation.

The crude extract of amygdalin was prepared from dried fruits of *S. armeniacae*. A 1 Kg amount of the powdered fruits was extracted with 5 L of 90% ethanol at 85 °C for 30 min twice. The combined extract was evaporated into syrup. The syrup was defatted with ether, then dissolved into 1000 ml of ethanol at 80 °C. The ethanol solution was placed at −18 °C for crystallization, yielding 405 g of a crude extract containing amygdalin at 55.3%.

2.2. Slow rotary counter-current chromatograph

The slow rotary counter-current chromatograph used in the present study was constructed at the Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China. Figs. 1 and 2 show the sketch and photograph of the apparatus equipped with a 40-L column with three-layer coils made of 17 mm average i.d. convoluted Teflon tubing. The coil holder was 16 cm o.d. and 2.2 m long. A pair of rotary seals was used for input and output of mobile phase during the separation. The apparatus can be operated at a speed up to 150 rpm.

2.3. Preparation of solvent system and sample solution

The suitable solvent systems were selected by HSCCC separation of the sample on a 230 ml capacity column. For the

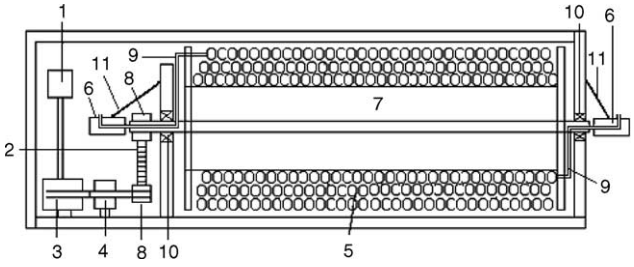

Fig. 1. Sketch of our slow rotary counter-current chromatograph equipped with a pair of rotary seals. 1, Speed controller; 2, transmission belt; 3, motor; 4, reducer; 5, coils; 6, rotary seal; 7, coil holder; 8, tooth gear; 9, connected tube; 10, bearing; 11, fixed tie of bearing.

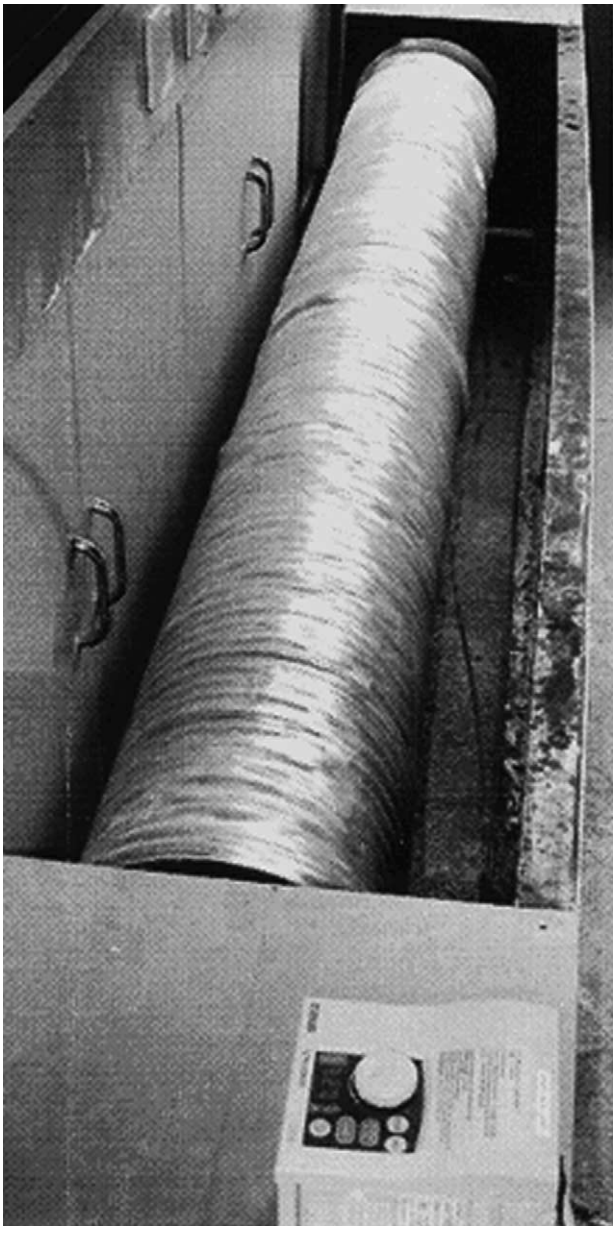

Fig. 2. Photograph of our slow rotary counter-current chromatograph equipped with a pair of rotary seals and a 40 L column.

separation of salicin the solvent system was methyl *tert*-butyl ether–1-butanol (1:3) saturated by methanol–water (1:5) used as the stationary phase and methanol–water (1:5) saturated by methyl *tert*-butyl ether–1-butanol (1:3) as the mobile phase. The sample solution was prepared by dissolving 500 g of the crude salicin extract in 5.0 L of mobile phase. For the separation of amygdalin, the solvent system was ethyl acetate–*n*-butanol (1:2) saturated by water used as the stationary phase and water saturated by ethyl acetate–1-butanol (1:2) as the mobile phase. The sample solution was prepared by dissolving 400 g of the crude amygdalin extract in 5.0 L of mobile phase.

2.4. System and procedure of semi-industrial separation

The semi-industrial separation system was composed of a K-1800 Knauer Wellchrom preparative HPLC pump (Germany), a 5-L sample loop made of 8 mm i.d. Teflon tubing, a B-684 Büchi collector (Switzerland) with 250 ml tube rack, an 8823 UV detector (China), and an LM17 recorder (China).

The separation experiment for each sample was conducted with the solvent system described above. The 40-L coil column was first entirely filled with stationary phase. Then the apparatus was rotated at a given rpm and the sample solution was injected into the CCC system through the Teflon sample loop with the mobile phase at a flow rate of 50.0 ml/min. Effluent was monitored with the UV detector at 254 nm and collected by the fraction collector into 250 ml fractions. The chromatograms were recorded with the recorder.

2.5. HPLC analysis

The HPLC system was composed of a Waters 510 pump, a manual injector, an ODS column (Ultrasphere, 5 μm, 250 mm × 4.6 mm, Beckman, Germany), a Waters 480 UV variable wavelength monitor and a data processing system. A gradient elution was performed for the separation of salicin with 5–50% methanol from 0 to 30 min. Detection wavelength was 254 nm. An isocratic elution of water–methanol–acetonitrile (70:25:5) was employed for the separation of amygdalin. Detection wavelength was 220 nm.

3. Resuslts and discussion

Fig. 3 shows the chromatogram of 500 g crude extract of salicin by SRCCC. HPLC analysis of the components revealed that component II was possibly salicin. Its purity was 87.6% determined by HPLC. The fractions were combined, decolorized with active carbon, and concentrated into syrup in vacuum at 50 °C. The syrup was freeze-dried to yield 64.3 g of light yellow powder containing salicin at 95.3% by HPLC (Fig. 4). A recrystallized product in acetone was used for confirmation of chemical structure. All the results from ESI-MS and ^{1}H, ^{13}C NMR showed the component was salicin: ESI-MS (pos. *m*/*z*): 309 $[M + Na]^+$, $[M]^+$ 286,

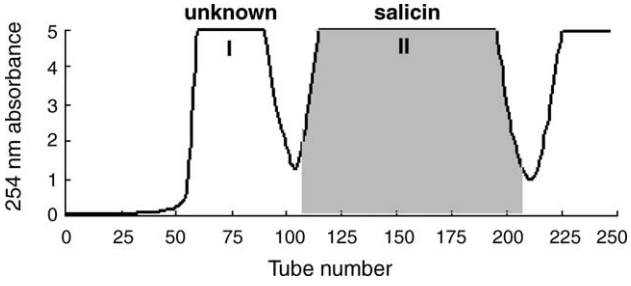

Fig. 3. SRCCC chromatogram of 500 g crude extract of salicin. Apparatus: slow rotary counter-current chromatograph with 40-L column; stationary phase: methyl *tert*-butyl ether–1-butanol (1:3) saturated with methanol–water (1:5); mobile phase: methanol–water (1:5) saturated by methyl *tert*-butyl ether–1-butanol (1:3); flow rate: 50 ml/min; retention of stationary phase: 63%; fraction volume: 250 ml; rotary speed: 60 rpm.

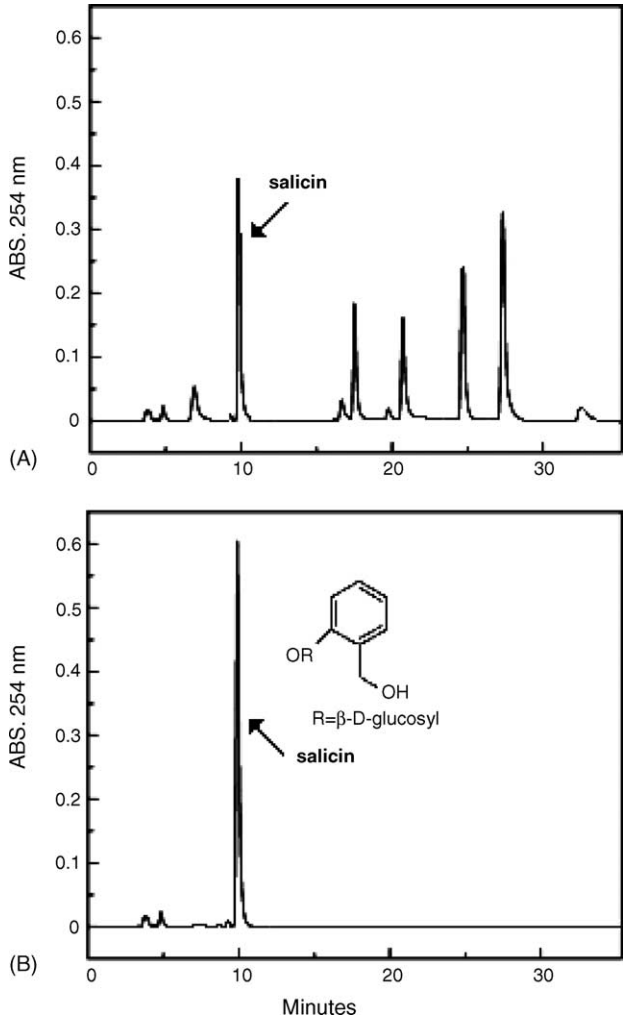

Fig. 4. HPLC analysis of the crude salicin extract and component II from SRCCC separation (Fig. 3). Column: ODS column (Ultrasphere, 5 μm, 250 mm × 4.6 mm); gradient elution: 5–50% methanol from 0 to 30 min; flow rate: 1 ml/min; detection wavelength: 254 nm. (A) Crude salicin extract; (B) component II from SRCCC separation.

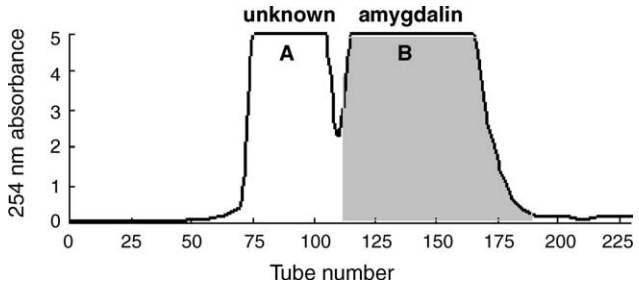

Fig. 5. SRCCC chromatogram of 400 g crude extract of amygdalin. Apparatus: slow rotary counter-current chromatograph with a 40-L column; stationary phase: ethyl acetate–1-butanol (1:2) saturated with water; mobile phase: water saturated with ethyl acetate–1-butanol (1:2); flow rate: 50 ml/min; retention of stationary phase: 55%; fraction volume: 250 ml; rotary speed: 50 rpm.

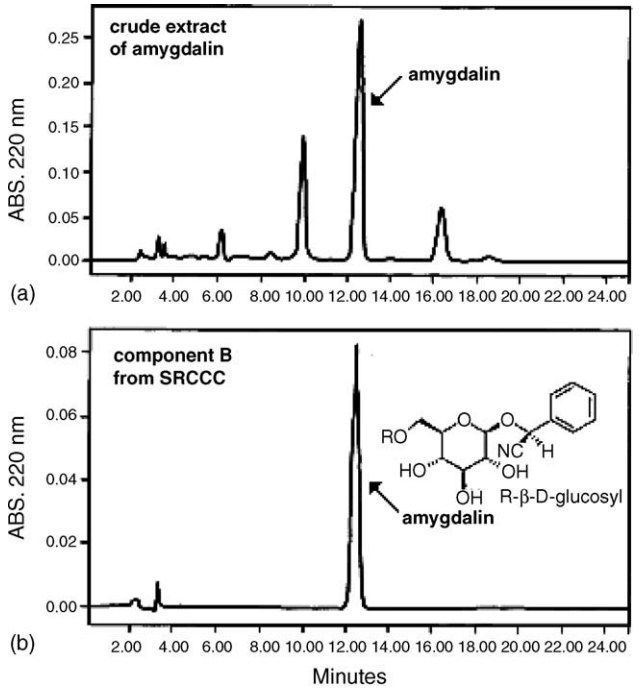

Fig. 6. HPLC analysis of the crude amygdalin extract and component B from SRCCC separation. Column: ODS column (Ultrasphere, 5 μm, 250 mm × 4.6 mm); elution: water–methanol–acetonitrile (70:25:5); flow rate: 1 ml/min; detection wavelength: 220 nm.

ESI-MS (neg.): 571.9 $[2M-H]^-$, 285 $[M-H]^-$. ^{1}H NMR (CD_3OD, 300 MHz): 3.28–3.50 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.64 (1H, dd, J_1 = 12.0; J_2 = 6.0 Hz, H-6′a), 3.83 (1H, dd, J_1 = 12.0; J_2 = 1.5 Hz, H-6′b), 4.51 (1H, d, J = 12.6 Hz; H-7b), 4.72 (1H, d, J = 12.6 Hz; H-7a), 4.81 (1H, d, J = 7.5 Hz; H-1′), 6.97 (1H, td, J_1 = 7.0; J_2 = 1.5 Hz, H-4), 7.14 (1H, dd, J_1 = 8.0; J_2 = 1.5 Hz, H-6), 7.19 (1H, td, J_1 = 8.0, J_2 = 1.5 Hz, H-5), 7.27 (1H, dd, J_1 = 7.0; J_2 = 1.5, H-3). ^{13}C NMR (CD_3OD, 75 MHz): 61.3 (C-7), 62.8 (C-6′), 71.6 (C-4′), 75.4 (C-2′), 78.3 (C-5′)*, 78.5 (C-3′)*, 103.7 (C-1′), 117.3 (C-6), 124.0 (C-4), 130.2 (C-3), 130.3 (C-5), 132.4 (C-2), 157.4 (C-1). (*^{13}C assignments may be interchanged.)

Fig. 5 shows the chromatogram of 400 g crude extract of amygdalin by our SRCCC system. HPLC analysis of the components revealed that component B was amygdalin. The fractions were combined and evaporated at 50 °C into syrup. The syrup was freeze-dried to yield 221.1 g of white powder which contained amygdalin at 94.1% by HPLC analysis (Fig. 6). A recrystallized product in ethanol was used for confirmation of chemical structure. The structure could be confirmed by following evidence. UV (λ^{nm}, MeOH): 252, 256, 268 and 269. ESI-MS (pos. *m*/*z*): 480 $[M+Na]^+$, 325 $[diglucoside]^+$. IRmax. (cm^{-1}): 3200–3600 (OH), 2962 (CH), 2231 (CN), 1645 (C=C) and 1095–1010 (C–O–C) is in accordance with the data of the standard amygdalin.

4. Conclusions

In the present studies 64.3 g of salicin and 221.1 g of amygdalin were purified in our SLCCC system each within 20 h. It may be possible to attain a larger yield using a crude sample prepurified by extraction. Moreover, a larger CCC column made of larger i.d. convoluted tubing will allow a larger sample load at a higher flow rate. Thus, industrial CCC separation of salicin and amygdalin is feasible with the present system.

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