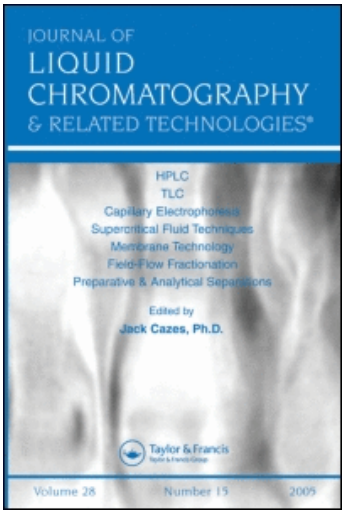

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 10 December 2004

To cite this Article Du, Qizhen , Jerz, Gerold and Winterhalter, Peter(2005) 'Preparation of Three Flavonoids from the Bark of *Salix alba* by High-Speed Countercurrent Chromatographic Separation', Journal of Liquid Chromatography & Related Technologies, 27: 20, 3257 — 3264
To link to this Article: DOI: 10.1081/JLC-200034917
URL: http://dx.doi.org/10.1081/JLC-200034917

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 27, No. 20, pp. 3257–3264, 2004

Preparation of Three Flavonoids from the Bark of *Salix alba* by High-Speed Countercurrent Chromatographic Separation

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ABSTRACT

The main flavonoids from the bark extract of *Salix alba* (Salicaceae) were separated on preparative scale using high-speed countercurrent chromatography (HSCCC). In each separation, 1.0 g crude extract was applied to yield pure eriodictyol (120 mg), 5,7-dihydroxychromen-4-one (29.5 mg), and naringenin (50 mg), respectively, while water–methanol–ethyl acetate–*n*-hexane (3 : 2 : 2 : 2, v/v) was used for a two-phase solvent system. The chemical structures of three flavonoids were elucidated by

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DOI: 10.1081/JLC-200034917 1082-6076 (Print); 1520-572X (Online)

means of electrospray ionization ion trap multiple mass spectrometry (ESI-MS–MS), as well as ^{1}H-, ^{13}C-, and DEPT-NMR spectroscopy.

Key Words: *Salix alba*; Salicaceae; Flavonoids; Chromenone; High-speed countercurrent chromatography; Preparative isolation; ESI-MS-MS; NMR.

INTRODUCTION

Bioactive constituents of the bark of *Salix alba* (white willow bark), such as salicin, salicylic alcohol, and acylated salicin derivates are well known for treatment of rheumatic fever and subacute bacterial endocarditis. In addition, there is a high content of flavonoids in the bark of all *Salix* species. Flavonoids present in *S. caprea* wood were reported to have antifungal and antimicrobial properties.[1,2] From the view point of chemotaxonomy, white willow bark seems to possess a similar flavonoid spectrum known as *S. caprea* wood.[3] This paper describes the preparation of three flavonoids (Fig. 1) from white willow bark by separation with high-speed countercurrent chromatography (HSCCC).[4] HSCCC is an all-liquid chromatographic system based on fast partitioning of the analytes between two immiscible liquid solvent phases. This eliminates irreversible adsorbing, and artifact formation, which often occurs during the separation of polyphenols. The chemical structures of two flavanonols and one chromenone component were elucidated by means of electrospray ionization mass spectroscopy (ESI-MS), and nuclear magnetic resonance (NMR) spectroscopy.

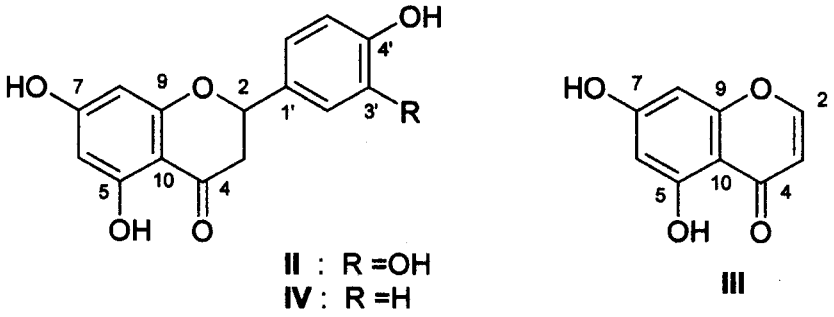

Figure 1. Phenolic constituents separated by HSCCC from the ethyl acetate partition of *S. alba* bark extract. Compounds **II**, eriodictyol; **III**, 5,7-dihydroxy-chromen-4-one; **IV**, naringenin.

EXPERIMENTAL

Extraction of Flavonoids

The material of investigation was an industrially prepared crude ethanolic extract of *S. alba* (Kinglong Biological Products Co. Ltd, Changsha, China), which was prepared by extraction of *S. alba* bark with 80% ethanol at 60°C, evaporated at 50°C under reduced pressure, and the concentrated solution was spray-dried at 60°C. The powderous extract (50 g) was dissolved in 1000 mL of water, and extracted two times with an isovolumetric amount of ethyl acetate. The organic phases were combined, and evaporated under reduced pressure at 40°C to give a slurry. Freeze-drying yielded 11.6 g of a flavonoid mixture, which was directly submitted to the HSCCC-separation.

HSCCC

The HSCCC instrument was a multilayer coil countercurrent chromatograph, manufactured by P.C. Inc. (Potomac, MD), equipped with a 385-mL coil column made of a polytetrafluoroethylene tubing (2.6 mm, I.D.). The mobile phase was delivered by a Biotronik HPLC pump BT 3020 (Jasco, Gross-Umstadt, Germany). The HSCCC experiment was performed with a two-phase solvent system composed of water–methanol–ethyl acetate–*n*-hexane (3 : 2 : 2 : 2, v/v). After thorough equilibration of the four solvents in a separatory funnel, the two resulting phases were separated shortly before use. The multilayer coil column was entirely filled with the upper organic phase as the stationary phase. Then the apparatus was rotated at 650 rpm for equilibration of the system. For a single run, 1.0 g of the dried flavonoid mixture was dissolved in 40 mL of mobile phase. Injection of the sample to the HSCCC system was done by a teflon sample loop, followed by immediate pumping of mobile phase at a flow rate of 3.0 mL/min. The effluent stream was continuously monitored with a Knauer UV-VIS variable wavelength detector (Berlin, Germany), and 10 mL fractions were collected with a Superfrac fraction collector (Pharmacia, Uppsala, Sweden).

HPLC Analysis

The HPLC system was composed of a Knauer HPLC pump 64, a manual injector, an ODS column (Ultrasphere, 5 μm, 250 × 4.6 mm^2, Beckman, Germany), a Knauer variable wavelength monitor, and a data processing system. The applied gradient was composed of methanol and water, with

initially 30% methanol for 10 min, increasing to 50% methanol in 10 min, then up to 70% methanol in 10 min. The flow rate was 1 mL/min and the detecting wavelength was 254 nm.

Electrospray Ionization-MS-MS (Syringe Pump)

All electrospray ionization (ESI-MS-MS) experiments were performed on a Bruker Esquire LC-MS ion trap multiple mass spectrometer (Bremen, Germany) in positive and negative ionization mode analyzing ions up to m/z 2200. During ESI-MS, and MS-MS fragmentation studies, the purified samples were introduced via a syringe pump at a flow rate of 240 μL/min. Drying gas was nitrogen (flow rate 7.0 L/min, 330°C), and nebulizer pressure was set to 5 psi. ESI-MS parameters (negative mode): capillary +4500 V, end plate +4000 V, cap exit −90 V, cap exit offset −60 V, skim 1 −30 V, skim 2 −10 V; ESI-MS parameters (positive mode): capillary −4500 V, end plate −4000 V, cap exit +90 V, cap exit offset +60 V, skim 1 +30 V, skim 2 +10 V. MS–MS experiments afforded fragmentation amplitude values between 0.8 and 1.2.

NMR-Analysis

^{1}H-, ^{13}C- and DEPT 90/135-NMR spectra were recorded in MeOH-d_4, and DMSO-d_6, on a Bruker AMX 300 (Karlsruhe, Germany) with 300 MHz for ^{1}H-, and 75.5 MHz for ^{13}C-measurements, respectively.

RESULTS AND DISCUSSION

Ethyl acetate was applied for liquid–liquid partitioning of 50 g of an ethanolic *S. alba* extract to yield 23.2% crude flavonoid material (11.6 g). Analytical HPLC analysis at $\lambda = 254$ nm confirmed three main components in the chromatogram, which were the target substances for the HSCCC separation.

The HSCCC chromatogram presented in Fig. 2 shows the separation of 1 g of *S. alba* flavonoid extract, achieved with the two-phase solvent system composed of water–methanol–ethyl acetate–*n*-hexane (3 : 2 : 2 : 2, v/v). The UV-trace at λ 254 nm detected four very abundant peaks I, II, III, and IV, with complete baseline separation between peaks III and IV. A selective fractionation was necessary, especially for the less resolved peaks I and II, as graphically shown in Fig. 2. Evaporation of the organic solvents occurred under reduced pressure, and the following freeze-drying yielded 613, 120,

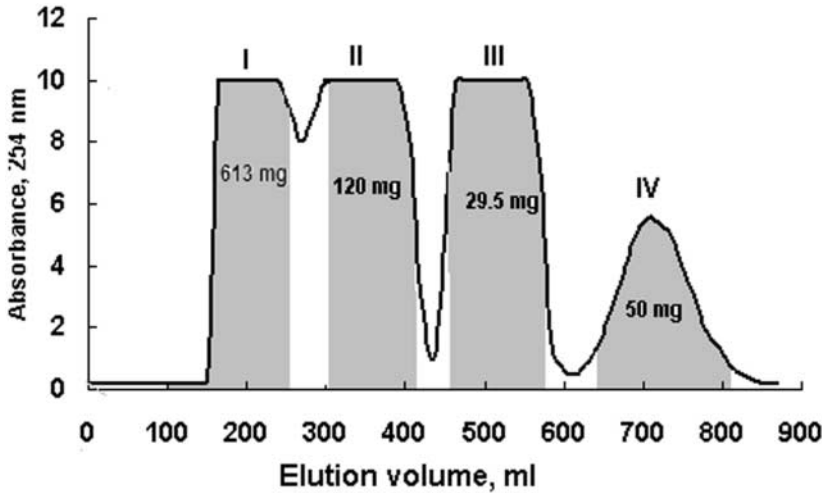

Figure 2. High-speed countercurrent chromatogram (UV-trace at λ 254 nm) of the ethyl acetate partition of *S. alba* bark extract. Solvent system: water–methanol–ethyl acetate–*n*-hexane (3 : 2 : 2 : 2, v/v); mobile phase: aqueous lower phase; stationary phase: upper organic phase; flow rate: 3 mL/min; retention of stationary phase after separation: 58%.

29.5, and 50 mg for the fractions I, II, III, and IV, respectively. HPLC analysis of the four components indicated that peak I consisted of a highly complex mixture, while components II, III, and IV, corresponding to peaks 13, 12, and 14, respectively, gave single peaks at the given analytical conditions (Fig. 3). Obviously, the elution order of peaks in HSCCC based on partition is different from HPLC based on adsorption, which depends on molecular structure and polarity. The preparative HSCCC resulted in a good separation of the most abundant components in the ethyl acetate partition of *S. alba*; high sample loading capacity, minimum of sample clean-up, no irreversible adsorption effects of analytes to solid phase column material, and complete sample recovery.[5]

Structure confirmation of the pure compounds **II**, **III**, and **IV** was done by ^{1}H-, ^{13}C-, DEPT-NMR, and ESI-MS-MS. Spectroscopical data of eriodictyol (**II**), 5,7-dihydroxy-chromen-4-one (**III**), and naringenin (**IV**) are given below, and were in excellent accordance with published reference data.[6–11]

Eriodictyol (**II**): white powder, ESI-MS (pos.) m/z: 289 $[M+H]^+$, 311 $[M+Na]^+$, MS^2 fragmentation of m/z 289: 179, 163, 153; ESI-MS (neg.) m/z: 575 $[2M-H]^-$, 287 $[M-H]^-$, MS^2 fragmentation of m/z 575: 287; MS^3 fragmentation of m/z 287: 151; MS^3 fragmentation of m/z 151: 106. ^{1}H-NMR (MeOH-d_4): δ 2.70 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 3.0$ Hz,

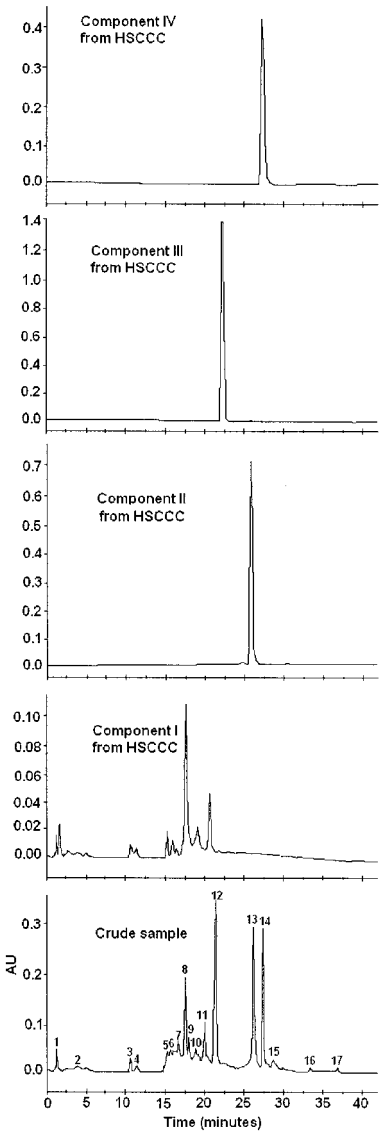

Figure 3. HPLC analysis of the crude sample and the fractions (I–IV) from the HSCCC separation. Column: ODS column (Ultrasphere, 5 μm, 250 × 4.6 mm^2, Beckman, Germany); gradient elution: 0–10 min, 30% methanol–70% water; 10–20 min, 50% methanol–50% water; 20–30 min, 70% methanol–30% water; detecting wavelength: λ 254 nm; peak 12: 5,7-dihydroxy-chromen-4-one (**III**); peak 13: eriodictyol (**II**); peak 14: naringenin (**IV**).

H-3_{eq}), 3.05 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 3.0$ Hz, H-3_{ax}), 5.27 (1H, dd, $J_1 = 13.0$ Hz, $J_2 = 3.0$ Hz, H-2), 5.88 (1H, d, $J = 2.0$ Hz, H-6), 5.90 (1H, d, $J = 2.0$ Hz, H-8), 6.78–6.79 (2H, H-5′,H-6′), 6.91 (1H, s_{br}, H-2′). ^{13}C-NMR (DMSO-d_6): δ 42.2 (C-3), 78.3 (C-2), 94.8 (C-8), 95.7 (C-6), 101.7 (C-10), 114.2 (C-2′), 115.3 (C-5′), 117.8 (C-6′), 129.4 (C-1′), 145.1 (C-3′), 145.6 (C-4′), 162.8 (C-9), 163.4 (C-5), 166.6 (C-7), 196.2 (C-4).

5,7-Dihydroxy-chromen-4-one (**III**): white powder, ESI-MS (pos.) m/z: 179 $[M + H]^+$; ESI-MS (neg.) m/z: 177 $[M - H]^-$, MS^2 fragmentation of m/z 177: 133 $[M\text{-}44]^-$. ^{1}H-NMR (MeOH-d_4): δ 6.18 (1H, d, $J = 6.0$ Hz, H-3), 6.21 (1H, d, $J = 2.0$ Hz, H-6), 6.33 (1H, d, $J = 2.0$ Hz, H-8), 7.95 (1H, d, $J = 6.0$ Hz, H-2). ^{13}C-NMR (MeOH-d_4): δ 95.1 (C-8), 100.3 (C-6), 106.8 (C-10), 111.7 (C-3), 158.0 (C-2), 159.9 (C-9), 163.5 (C-5), 166.2 (C-7), 183.6 (C-4).

Naringenin (**IV**): white powder, ESI-MS (pos.) m/z: 273 $[M + H]^+$, MS^2 fragmentation of m/z 273: 153. ESI-MS (neg.) m/z: 271 $[M - H]^-$, MS^2 fragmentation of m/z 271: 151; MS^3 fragmentation of m/z 151: 106, MS^4 fragmentation of m/z 106: 63. ^{1}H-NMR (MeOH-d_4): δ 2.70 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 3.0$ Hz, H-3_{eq}), 3.10 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 13.0$ Hz, H-3_{ax}), 5.34 (1H, dd, $J_1 = 13.0$ Hz, $J_2 = 3.0$ Hz, H-2), 5.88 (1H, d, $J = 2.0$ Hz, H-6), 5.90 (1H, d, $J = 2.0$ Hz, H-8), 6.82 (2H, AA′–BB′, H-3′, H-5′), 7.31 (2H, AA′–BB′, H-2′, H-6′). ^{13}C-NMR (MeOH-d_4): δ 44.0 (C-3), 80.5 (C-2), 96.2 (C-8), 97.1 (C-6), 103.4 (C-10), 116.4 (C-3′, C-5′), 129.0 (C-2′, C-6′), 131.1 (C-1′), 159.0 (C-4′), 164.9 (C-9), 165.5 (C-5), 168.4 (C-7), 197.8 (C-4).

In summary, this study presents an efficient method of preparing eriodictyol (**II**), 5,7-dihydroxychromen-4-one (**III**), and naringenin (**IV**) from the bark of *S. alba*, which is now available for evaluation of pharmacological effects of white willow bark. This single-step HSCCC separation of an enriched flavonoid crude extract of the bark of *S. alba* bark, clearly demonstrates the high capabilities of this technique to isolate bioactive components in larger amounts.

ACKNOWLEDGMENTS

Qizhen Du is grateful for financial support by the "Alexander-von-Humboldt Foundation."

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Received June 20, 2004
Accepted July 28, 2004
Manuscript 6427