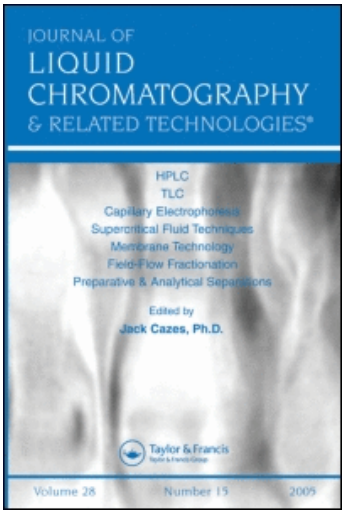

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Isolation of Two Sucrose Esters from *Polygala tenuifolia* by High Speed Countercurrent Chromatography

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Abstract: Two sucrose esters, 3,6′-disinapoyl sucrose and tenuifoliside A were isolated from the *n*-butanol extract of the cortexes of *Polygala tenuifolia* Willd. by high speed countercurrent chromatography (HSCCC) in one or two steps. The two-phase solvent systems used were composed of chloroform–methanol–water (3:3.5:2 v/v) and ethyl acetate-*n*-butanol-ethanol-water (4:0.6:0.6:5). From 300 mg crude fraction, 3,6′-disinapoyl sucrose (40.1 mg) and tenuifoliside A (8.1 mg) were obtained at above 93% purity by HPLC analyses, and their structures were identified by IR, ^{1}H NMR, ^{13}C NMR, and MS spectra.

Keywords: High speed countercurrent chromatography, *Polygala tenuiflolia*, 3,6′-Disinapoyl sucrose, Tenuifoliside A

INTRODUCTION

The roots of *Polygala tenuifolia* Willd., "yuanzhi", is a well-known traditional Chinese medicine used as an expectorant, tonic, sedative, and for preventing dementia.[1] Xanthones, saponins, and sucrose esters had been isolated from this plant.[2–5] As one of the effective components of *Polygala tenuifolia*, sucrose esters had the activating action on cholineacetyltransferase, which

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could be a cerebral function improver, improving different cerebral function disorders such as cerebral infarction or dementia.[6,7]

The separation and purification of sucrose ester from *Polygala tenuiflolia* using the conventional methods is tedious, and which always includes multiple purification methods, such as silica gel column chromatography, preparative TLC, and HPLC purification.[4,8] Moreover, in the previous separation process, these sucrose ester compounds with a phenolic group and cinnamoyl double bond in the structures were found to be unstable, and their structures were easily changed through long time silica gel column chromatography.

High speed countercurrent chromatography (HSCCC), being a liquid–liquid partition chromatographic technique without solid stationary phase, eliminates irreversible adsorption of the sample onto the solid support and avoids the adsorption losses and the formation of artifacts.[9] Therefore, the method has been widely used for the preparative separation of phenolic compounds, such as flavonoids, hydroxyanthraquinones, and lignans.[10–12] Compared with the traditional solid–liquid column chromatography, it yields a higher recovery and efficiency.

The present paper describes the successful preparative separation and purification of two sucrose esters from the partially purified *n*-butanol extract of *Polygala tenuiflolia* by high speed countercurrent chromatography, which provides a simple and rapid separation process of those variable compounds.

EXPERIMENTAL

Apparatus

The analytical HSCCC instrument employed in the present study is a Model GS 20 analytical high speed countercurrent chromatograph produced by Beijing Institute of New Technology Application (Beijing, China). The apparatus holds a pair of column holders symmetrically on the rotary frame, at a distance of 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 50 m long, 0.85 mm i.d. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 40 mL. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal, and the revolution speed of the apparatus was performed at 1600 rpm in the present study.

Preparative HSCCC was performed using a Model GS10A multilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a PTFE multilayer coil of 110 m × 1.6 mm i.d. with a total capacity of 230 mL. The β value of the preparative column ranged from 0.5 to 0.8, and the revolution speed was used at 800 rpm in this experiment.

The pump we used is a Model NS-1007 constant-flow pump, and the detector is a Model 8823A-UV detector, both manufactured by Beijing Institute of New Technology Application (Beijing, China). A manual sample injection valve was used with a 2.0 mL loop for the analytical HSCCC and a 20 mL loop for the preparative HSCCC (Tianjin High-New Science & Technology Co., Tianjing, China). The chromatograms were recorded in a Model 3057 recorder (Sichuan Instrument Factory, Chongqing, China). The high performance liquid chromatography (HPLC) equipment used was an Agilent 1100 series system, which consisted of a G 1322A degasser, a G1311A quat pump, a G1311A Als, a G1316A column, and a G1315B DAD detector (Waldbronn, Germany). All NMR data were recorded on a Bruker AMX 300 (Karlsruhe, Germany).

Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Acetonitrile used for HPLC analysis was of Burdick & Jackson brand high purity solvent, purchased from Beijing Honghao Weiye Science and Technology Develop Co. (Beijing, China). D101 resin was purchased from Tianjin Chemical Co. (Tianjin, China). The cortexes of *P. tenuifolia* were collected from Taiyuan herbal drug company (Taiyuan, China) in Oct, 2000.

Extraction of Crude Samples

The air-dried cortexes of *P. tenuifolia* (11 kg) were ground and refluxed with 95% EtOH (77 L) three times each for 3 h. The solution was combined and evaporated *in vacuo* to yield 4.9 kg of residue, a portion (363 g) of which was suspended in water (6 L) and extracted successively with petroleum (12 L), $CHCl_3$ (17 L), and *n*-butanol (12 L). The *n*-butanol extract (110 g) was subjected to a macroporous resin D101 column (8.5 × 36 cm). The adsorbed material was eluted with H_2O (5.0 L), 40% (4.8 L), and 70% EtOH (3.1 L), successively, which yielded 27.8 g of 40% eluate and 18.8 g of 70% eluate. Portions of the 40% eluate were subjected to HSCCC.

Preparation of Two-Phase Solvent System and Sample Solution

In the present study, we selected a two-phase solvent system composed of chloroform-methanol-water (3:3.5:2, v/v) and ethyl acetate-*n*-butanol-ethanol-water (4:0.6:0.6:5, v/v). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude sample in the mobile phase solvent system used for separation.

HSCCC Separation Procedure

Analytical HSCCC was used for selecting a suitable solvent system for the separation of the sample. In each analytical separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 1600 rpm, while the lower phase (mobile phase) was pumped into the column at a flow-rate of 1.0 mL.min^{-1}. After the mobile phase emerged and hydrodynamic equilibrium was established in the column, about 1 mL of the sample solution containing 10 mg of the sample was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile.

The preparative separation was similarly carried out in the preparative HSCCC, using a 20 mL loop (300 mg crude in 20 mL mobile phase), at a flow rate of 2.0 mL.min^{-1} and 800 rpm revolution speed.

HPLC Analyses and Identification of HSCCC Fractions

The crude 40% eluate of *Polygala tenuifolia* and each HSCCC peak fraction were analyzed by HPLC. The analyses were performed with a Waters symmetry ODS C_{18} column (250 × 4.6 mm I.D.) at column temperature of 30°C. The mobile phase composed of acetonitrile–water (23:77, v/v) was eluted at a flow rate of 1.0 mL/min, and the effluent monitored at 311 nm by a DAD detector.

Identification of the target compounds, tenuifoliside A and 3,6′-disinapoyl sucrose (Fig. 1) were based on the spectra analyses of MS, IR, ^{1}H NMR, and ^{13}C NMR.

RESULTS AND DISCUSSION

The 40% ethanol eluate of the *n*-butanol extract of *Polygala tenuifolia* was analyzed by HPLC, and the result indicated that it contained several compounds, among which tenuifoliside A represented 3.8% and 3,6′-disinapoyl sucrose represented 16.0% of the total (Fig. 2A).

In order to achieve an efficient resolution of the target compounds, different solvent systems were examined at different volume ratios, such as ethyl acetate-*n*-butanol-water (10:1:8), ethyl acetate-ethanol-water (5:1:5), ethyl acetate-*n*-butanol-ethanol-water (4:0.6:0.6:5, 10:0.6:0.6:10, 2.5:0.6:0.6:5) and *n*-hexane-ethyl acetate-*n*-butanol-water (5:5:1:8), but the resolution of the

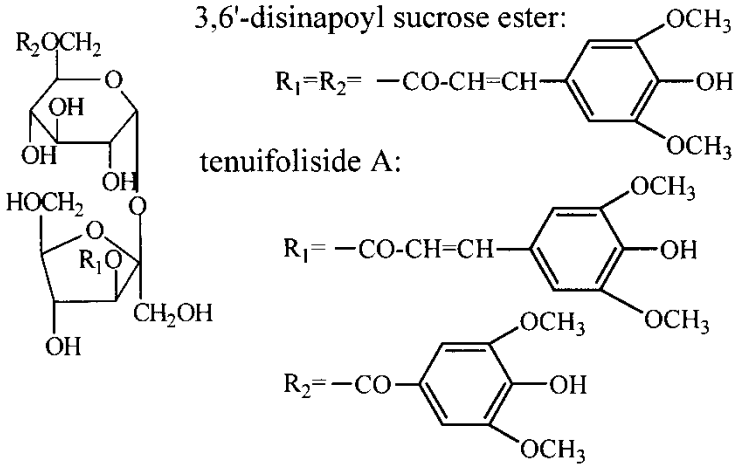

Figure 1. Structures of 3,6′-disinapoyl sucrose and tenuifoliside A.

target compounds was poor, which often mixed with other compounds. So, another solvent system of chloroform-methanol-water was tested and the result indicated that chloroform-methanol-water at volume ratio of 3:3.5:2 could separate the sucrose esters efficiently (Fig. 3). But when detecting the purity of tenuifoliside A (peak 1 of the first HSCCC chromatogram), we found there was a small peak ahead of the tenuifoliside A in the HPLC analysis result (Fig. 2B). So, the second HSCCC separation was performed on this fraction, and this time ethyl acetate-*n*-butanol-ethanol-water (4:0.6:0.6:5) was chosen as the solvent system (Fig. 4). After this separation, the purity of tenuifolisde A was increased to a higher content, but the small peak still existed in the front of tenuifoliside A. When the ^{1}H NMR spectrum of tenuifoliside A was carried out, there were some very weak signals that *Z*-cinnamoyl existed. It was reported that *E*- and *Z*-cinnamoyl readily interchanged in daylight,[13] so we deduced that the small peak which was difficult to separate was, perhaps, the isomer of tenuifoliside A, that is 3′-*Z*-3,4,5-trimethoxycinnamoyl-6-p-hydroxybenzoyl sucrose, which perhaps transformed from tenuifoliside A during the course of the separation. In order to prove this deduction, the solution of tenuifoliside A was placed under the UV light for 1.5 h, and then detected in the same HPLC condition. The results showed that the peak area of tenuifoliside A was decreased, while the area of the peak before A was increased (Fig. 5), which was similar to the transformation of *E*- and *Z*-saponins with the similar cinnamoyl substituents isolated from *Polygala senega*.[14] This transformation often occurs in solution, so it is necessary to perform the isolation as soon as possible, and evaporate the solution to dryness immediately after the separation.

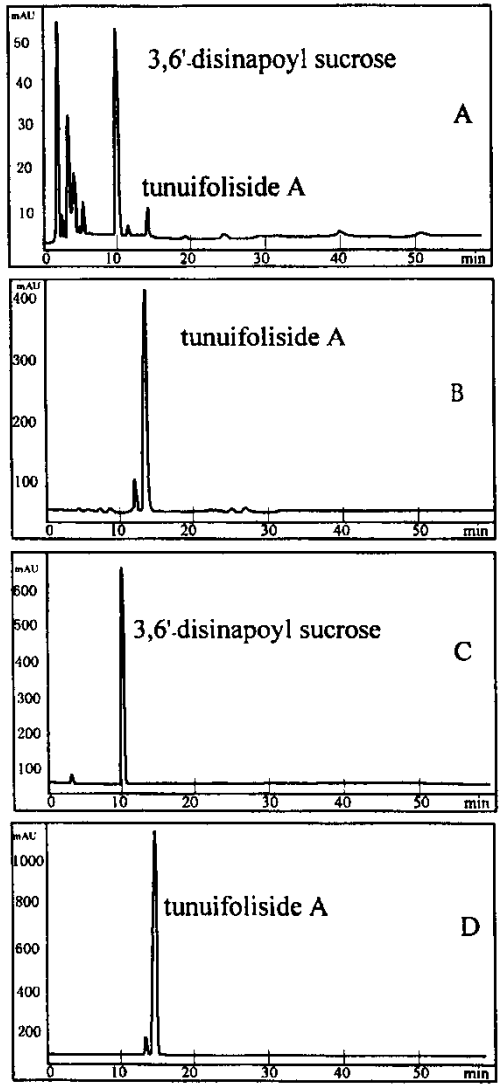

Figure 2. The results of HPLC analysis of the crude sample and purified HSCCC peaks. (A) Crude sample; (B) peak 1 in Fig. 3; (C) peak 2 in Fig. 3; (D) peak 2 in Fig. 4. Column: Waters Symmetry Rp-18 (5μm, 250 mm × 4.6 mm I.D); column temperature: 30°C; mobile phase: acetonitrile–water (23:77, v/v); flow-rate: 1.0 mL.min^{-1}.

Moreover, in the course of the separation, we found that a chloroform-methanol-water system was very suitable for the separation of the sucrose esters, because some other compounds with more polarity had been retained in the stationary phase and could be directly removed from the column by the pressurized air. This suggested that the process of de-salts, sugars, and

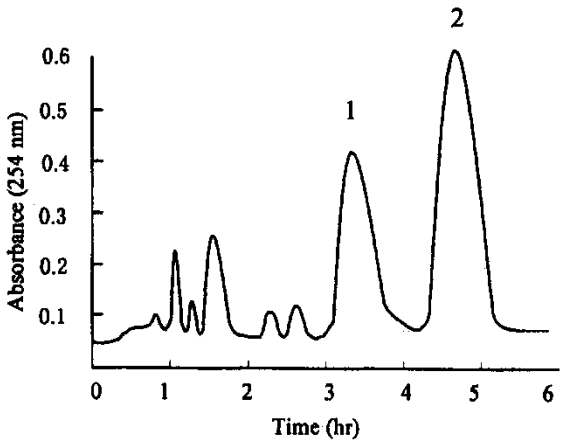

Figure 3. Chromatogram of the 40% ethanol eluate of the *n*-butanol extract of *Polygala tenuifolia* by preparative HSCCC. Peak 1: tenuifiliside A; peak 2: 3,6′-disinapoyl sucrose. solvent system: chloroform-methanol-water (3:3.5:2, v/v); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 2.0 mL.min^{-1}; revolution speed: 800 rpm; sample: 300 mg dissolved in 20 mL lower phase; retention of the stationary phase: 60.8%.

some other polar compounds by macroporous resin column chromatography could be omitted, which could further simplify the purification process of sucrose esters.

A total amount of 40.1 mg of 3,6′-disinapoyl sucrose was obtained from 300 mg of the crude sample, and its purity was increased from 16.0% to 95.3% after only a one-step separation; at the same time, a 8.1 mg of tenuifoliside A was also obtained from the same sample, and its purity was increased from 3.8% to 93.6% by a two step HSCCC (Fig. 2C, 2D). Since the existence of the aforementioned conformational transformation, the purities of sucrose esters with single conformers which we have isolated are not very high.

The structural identification of 3,6′-disinapoyl sucrose and tenuifoliside A were carried out by MS, IR, ^{1}H NMR, and ^{13}C NMR spectra as follows: 3,6′-Disinapoyl sucrose: FAB-MS m/z: 753 $[M-H]^{-}$. IR ν_{max}^{KBr}cm^{-1}: 3405 (OH), 1699 (C═O), 1631 (C═C), 1601, 1515, 1460 (aromatic ring). ^{1}H-NMR (300 MHz, acetone-d_6) δ: 7.70 (1H, d, J = 15.9 Hz, H-γ of sinapoyl), 6.54 (1H, d, J = 15.9 Hz, H-β of sinapoyl), 7.05 (2H, s, H-2, 6 of sinapoyl), 3.89 (6H, s, OMe-sinapoyl); 7.63 (1H, d, J = 15.9 Hz, H-γ of sinapoyl), 6.45 (1H, d, J = 15.9 Hz, H-β of sinapoyl), 7.03 (2H, s, H-2, 6 of sinapoyl), 5.46(1H, d, J = 7.5 Hz, H-3 of fru), 5.47(1H, d, J = 3.3 Hz, H-1 of α-glc), 3.87 (6H, s, OMe-sinapoyl), 3.85 (6H, s, OMe-sinapoyl'). ^{13}C-NMR (75 MHz, acetone-d_6) δ: 92.5(C-1 of Glc), 73.9(C-2 of Glc), 74.8(C-3 of Glc), 71.3(C-4 of Glc), 72.1(C-5 of Glc), 64.5(C-6 of Glc), 65.8(C-1 of fru), 104.8(c-2 of fru), 79.8(C-3 of fru), 73.0(C-4 of fru), 84.3(C-5 of fru),

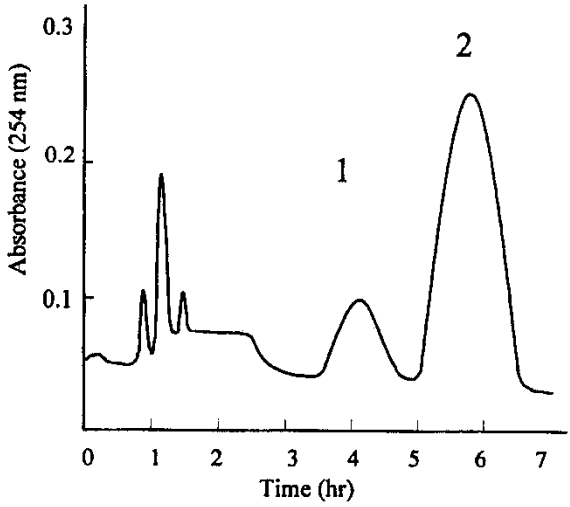

Figure 4. Chromatogram of second separation of tenuifoliside A by preparative HSCCC. Peak 1: unknown peak; peak 2: tenuifoliside A. solvent system: ethyl acetate–n-butanol-ethanol–water (4:0.6:0.6:5, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 mL.min^{-1}; revolution speed: 800 rpm; sample: 18.4 mg dissolved in 2 mL lower phase; retention of the stationary phase: 46.1%.

62.7(C-6 of fru), 166.8(C-α of sinapoyl), 115.7(C-β of sinapoyl), 146.2(C-γ of sinapoyl), 126.0(C-1 of sinapoyl), 106.8(C-2, 6 of sinapoyl), 148.9 (C-3, 5 of sinapoyl), 139.4(C-4 of sinapoyl), 56.7(OMe of sinapoyl), 167.7(C-α of sinapoyl'), 116.0(C-β of sinapoyl'), 147.0(C-γ of sinapoyl'), 126.2(C-1 of sinapoyl'), 107.0(C-2, 6 of sinapoyl'), 148.9 (C-3, 5 of sinapoyl'), 139.4(C-4 of sinapoyl'), 56.7(OMe of sinapoyl'). Compared with the literature data, the ^{1}H NMR and ^{13}C NMR data are in agreement with those of 3,6′-disinapoyl sucrose.[4]

Tenuifolisde A: FAB-MS m/z: 683 $[M + H]^+$. IR $\boldsymbol{\nu}_{max}^{KBr}$ cm^{-1}: 3406 (OH), 1703 (C═O), 1595, 1509, 1459 (aromatic ring). ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.71 (1H, d, J = 15.9 Hz, H-γ of sinapoyl), 6.54 (1H, d, J = 15.9 Hz, H-β of sinapoyl), 6.95 (2H, s, H-2, 6 of 3,4,5-trimethoxycinnamate), 3.86 (6H, s, OMe × 2), 3.78 (3H, s, OMe); 6.81 (1H, d, J = 8.4 Hz, H-2, 6 of p-hydroxybenzoyl), 7.89 (1H, d, J = 8.4 Hz, H-3, 5 of p-hydroxybenzoyl); 5.48(1H, d, J = 7.8 Hz, H-3 of fru), 5.49 (1H, d, J = 3.6 Hz, H-1 of α-glc). ^{13}C-NMR (75 MHz, CD_3OD) δ: 93.1(C-1 of Glc), 73.1(C-2 of Glc), 74.9(C-3 of Glc Glc-3), 71.6(C-4 of Glc), 72.5(C-5 of Glc), 65.1(C-6 of Glc), 65.6(C-1 of fru), 104.8(c-2 of fru), 79.7(C-3 of fru), 74.0(C-4 of fru), 84.1(C-5 of fru), 63.4(C-6 of fru), 167.7(C-α of 3,4,5-trimethoxycinnamoyl), 117.8(C-β of 3,4,5-trimethoxycinnamoyl), 147.2(C-γ of 3,4,5-trimethoxycinnamoyl),

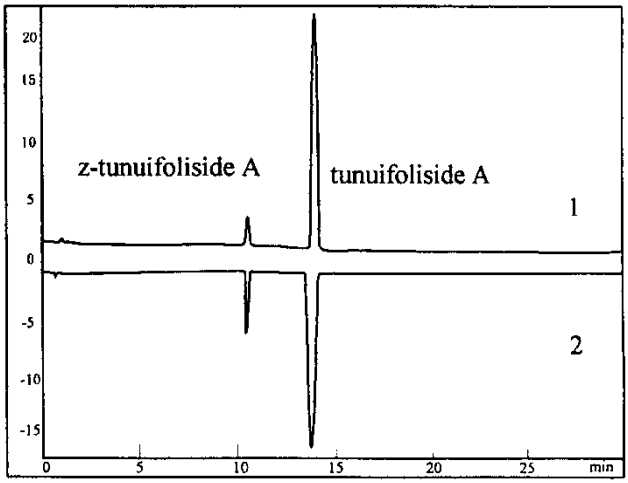

Figure 5. The results of HPLC analysis of tenuifoliside A at diffirent times. (1) chromatogram of fresh aqueous solution of tenuifoliside A; (2) chromatogram of fresh aqueous solution of tenuifoliside A irradiated under UV for 1.5 h. HPLC condition was same as those in Fig. 2.

131.5(C-1 of 3,4,5-trimethoxycinnamoyl), 106.9(C-2, 6 of 3,4,5-trimethoxycinnamoyl), 154.8(C-3, 5 of 3,4,5-trimethoxycinnamoyl), 141.3(C-4 of 3,4,5-trimethoxycinnamoyl), 56.8, 61.1(OMe of 3,4,5- trimethoxycinnamoyl), 168.1(C-α of p-hydroxybenzoyl), 122.0(C-1 of p-hydroxybenzoyl), 133.0(C-2, 6 of p-hydroxybenyl), 116.2 (C-3, 5 of p-hydroxybenzoyl), 163.6(C-4 of p-hydroxybenzoyl). Compared with the literature data, the ^{1}H NMR and ^{13}C NMR data are in agreement with those of 3′-*E*-3,4,5-trimethoxycinnamoyl-6-p-hydroxybenzoyl sucrose, namely tenuifoliside A.[4]

The present study demonstrates that HSCCC is a fast and effective methodology, very suitable for a highly selective preparation of variable sucrose esters from the crude fraction of the cortexes of *Polygala tenuifolia.*

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