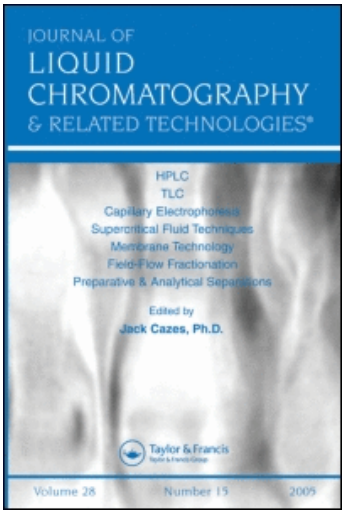

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Preparative Isolation and Purification of Four Compounds from Chinese Medicinal Herb *Gentiana Scabra* Bunge by High-Speed Countercurrent Chromatography

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Abstract: High-speed countercurrent chromatography (HSCCC) was applied to the separation and purification of 8-hydroxy-10-hydrosweroside, swertiamarin, and trifloroside from the Chinese medicinal herb *Gentiana scabra* Bunge. Fifty milligrams of crude extracts were separated using n-hexane-1-butanol-methanol-0.4% acetic acid in water (1.4:8:3:15.5, v/v) as the two-phase solvent system yielding 8 mg of 8-hydroxy-10-hydrosweroside, 18 mg of swertiamarin, 11 mg of trifloroside, and 4 mg of an unknown compound with the purity of 96.7, 98.4, 97.1, and 96.2%, respectively, in a one step separation. The chemical structures of these components were identified by ESI-MS.

Keywords: *Gentiana scabra* Bunge, High speed countercurrent chromatography (HSCCC), 8-Hydroxy-10-hydrosweroside, Swertiamarin, Trifloroside

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INTRODUCTION

Gentiana scabra Bunge, a well known traditional Chinese drug with a long history of use for the treatment of pneumonia, bronchitis, tuberculosis, and inflammation of the gallbladder and liver.[1] Many species of *Gentiana* contain a variety of compounds such as iridoid and secoiridoid components, xanthones, flavonoids, triterpenoids, and alkaloids.[2–7] The major components of *Gentiana*, including gentiopicrin, swertiamarin, and sweroside, each have their own specific pharmaceutical activities.

Although, the preparative separation and purification of the components from *Gentiana* by conventional methods such as column chromatography have been reported, the methods are tedious and require multiple chromatography steps, and, in addition, the adsorptive effects onto the stationary support matrix and artifact formation are serious.[8] High-speed countercurrent chromatography (HSCCC) is a kind of liquid–liquid partition chromatography method that uses no solid support matrix.[9] It eliminates irreversible adsorptive loss of samples onto the solid support matrix used in conventional chromatography. Hence, the technique allows complete recovery of the sample, and is suitable for preparative separations of gram quantity of samples.[10]

In this paper, the two-phase solvent system composed of n-hexane-1-butanol-methanol-0.4% acetic acid in water at a volume ratio of 1.4:8:3:15.5, was used for preparative separation and purification of 8-hydroxy-10-hydrosweroside, swertiamarin, trifloroside, and an unknown compound from *Gentiana scabra* Bunge by HSCCC. The purities of these compounds were determined by HPLC, and their structures (Figure 1) were identified by ESI-MS.

EXPERIMENTAL

Apparatus

The HSCCC instrument employed in the present study is a GS10A-2 high speed countercurrent chromatograph (Beijing Institute of New Technology Application, Beijing, China). The apparatus holds a multilayer coil separation column prepared from 110 m long, 1.6 mm ID PTFE (polytetrafluoroethylene) with a total capacity of 230 mL. The β value of this preparative column varied from 0.5 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The rotation speed of the apparatus can be regulated from 0 to 1000 rpm. The system was also equipped with one NS-1007 constant flow pump, a Model 8823A-UV monitor operating at 254 nm, and a Yakogawa 3057 recorder.

The HPLC system used in this study consists of a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SPD-M10Avp UV detector, an

injection valve (Model 7726) with a 20 mL loop, a system controller (SCL-10AVP), and a Shimadzu LC Solution Workstation (Shimadzu, Japan). The column applied in this work is a Diamonsil C18 column (250 mm × 4.6 mm ID, 5 μm, Dikma Technologies china).

Reagents

All organic solvents used for HSCCC were of analytical grade (Tianjing Damao Reagent Factory, Tiangjing, China). Methanol used for HPLC analysis was of chromatographic grade (DIKMA Filiale in Guangzhou, China). Raw plant material of *Gentiana scabra* Bunge was purchased from Guangzhou Traditional Chinese Medicine Company, Guangzhou, China.

Extraction of Crude Samples

Gentiana scabra Bunge was dried at a constant temperature of 60°C for 2 hours, then ground to powder (about 30 mesh) with a disintegrator. The powder (200 g) was extracted with 1000 mL of 95% ethanol at 78°C three times. The extraction duration was 5 h, 6 h, and 6 h, respectively. The filtrate was pooled and condensed off the solvent by evaporating under a reduced pressure, yielding 135 g of a crude extract. The extract was dissolved in water (200 mL) and then extracted, first by petroleum ether (b.p. 60–90°C) and then, by 1-butanol three times (200 mL, 150 mL, 150 mL, respectively). The butanol extract was evaporated to dryness yielding 50 g of a crude sample. It was stored in a refrigerator at 4°C for further purification by HSCCC.

Selection of the Two-Phase Solvent System

The composition of the two-phase solvent system was selected according to the partition coefficient (K) of the target compounds in the crude sample. The partition coefficients were determined by HPLC as follows: a suitable amount of the crude sample was dissolved in 2 mL of aqueous phase of the pre-equilibrated two-phase solvent system. The solution was determined by HPLC at 254 nm and the peak area was recorded as A_1. Then, an equal volume of the organic phase was added to the solution and mixed thoroughly. After settling, the aqueous phase was similarly analyzed by HPLC and the peak area was recorded as A_2. The partition coefficient ($K_{upper/lower}$) was obtained by the following equation:

$$K = (A_1 - A_2)/A_2 \quad (1)$$

HSCCC Separation

In this work, n-hexane-1-butanol-methanol-0.4% acetic acid in water, at a volume ratio of 1.4:8:3:15.5 was selected as a two-phase solvent system. The solvent system was prepared by delivering all the solvents to a separation funnel according to the volume ratios and thoroughly equilibrating by repetitive vigorous shaking. After being thoroughly equilibrated, the upper and lower phases were separated and degassed by sonication for 30 min prior to use.

The sample solution was prepared by dissolving the crude extract in an equal volume of each phase of the solvent system used for HSCCC separation.

In the separation process, the multilayer coiled column was first entirely filled with the upper phase as a stationary phase. Then the apparatus was rotated at 750 rpm. The lower phase was then pumped into the head end of the column inlet at a flow-rate of 1.5 mL/min. After hydrodynamic equilibrium was reached, the sample solution (50 mg dissolved in 2 mL of a 1:1 mixture of each phase of the solvent system) was injected through the sample port. The effluent from the tail end of the column was continuously

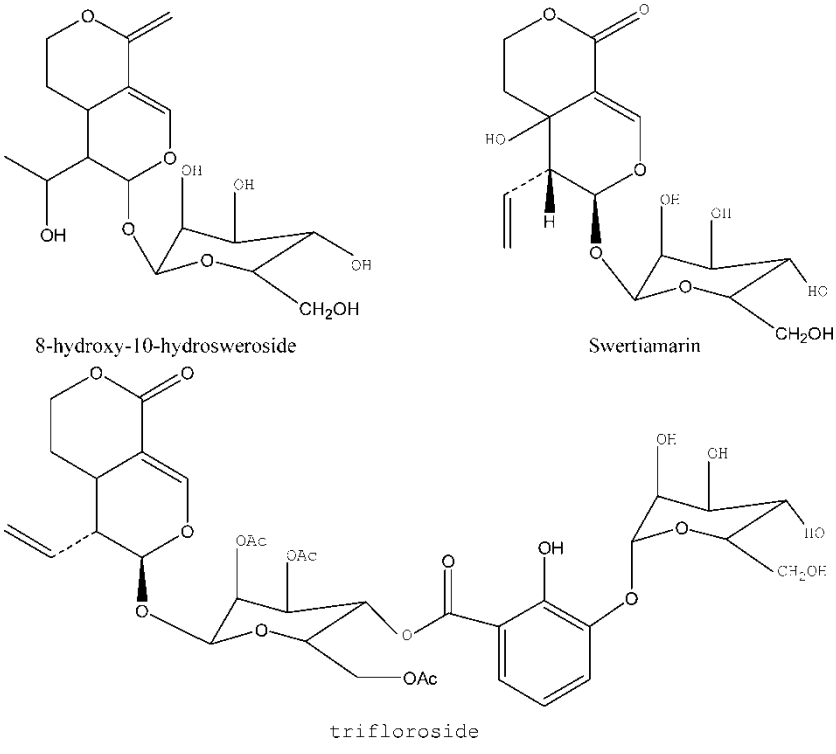


Figure 1. Chemical structures of target compounds from *Gentiana scabra* Bunge.

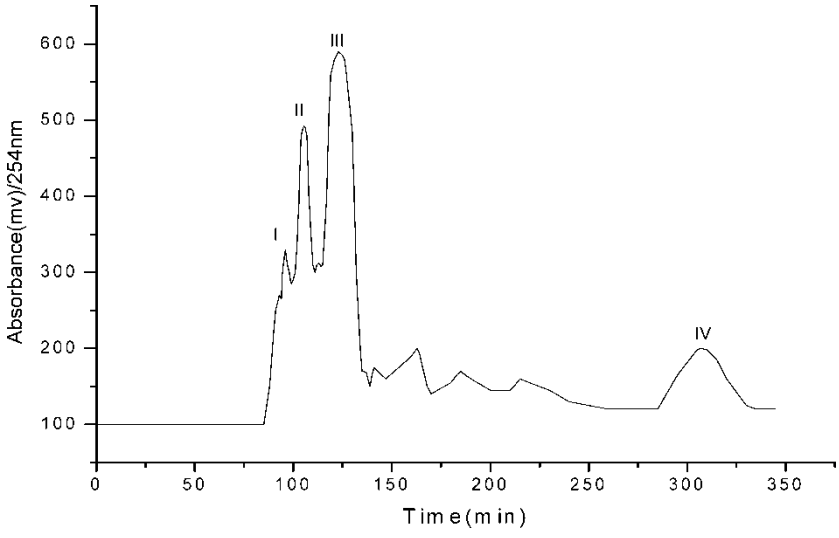


Figure 2. HSCCC chromatograms of crude extracts of *Gentiana scabra* Bunge. Peak I: unknown compound, Peak II: 8-hydroxy-10-hydrosweroside, Peak III: swertiamarin, Peak IV: trifloroside. HSCCC condition: solvent system: n-hexane-n-butanol-methanol-0.4% acetic acid water (1.4:8:3:15.5, v/v); mobile phase: the lower phase; flow rate: starting at 1.5 mL/min (3 h), then 2.5 mL/min; revolution speed first 750 rpm, then 800 rpm; detection wavelength: 254 nm; sample size: 50 mg of crude sample dissolved in 2 mL of the lower phase.

monitored with a UV detector at a wavelength of 254 nm. The flow rate of the lower mobile phase was maintained at 2.5 mL/min until the three major fractions were eluted (about 3 hours). Each peak fraction was manually collected according to the elution profile and finally determined by HPLC.

HPLC Analysis and Identification of HSCCC Peak Fractions

The partially purified extract of *Gentiana scabra* Bunge and each peak fraction obtained from HSCCC were analyzed by HPLC. The analyses were performed with a Diamonsil C18 column. Methanol–0.4% acetic acid in water was used as the mobile phase in a gradient mode (methanol: starting at 10%; 20 min, 25%; 30 min, 55%; 45 min, 55%; 60 min, 80%; 61 min, 100%; 80 min, 100%; 81 min, stop). The flow rate of the mobile phase was 0.8 mL/min, while the effluent was monitored by a dual wavelength absorbance detector at 254 nm.

Identification of HSCCC peak fractions was carried out by ESI-MS, a Finnigan LCQ Deca ion trap mass XP MAX spectrometer equipped with an electrospray ionization source (Thermo Finnigan, San Jose, CA, USA).

RESULTS AND DISCUSSION

Optimization of HPLC Condition

Several elution systems were tested for HPLC separation of the crude sample using methanol–water, methanol–acetic acid, gradient elution of methanol–acetic acid, etc. When methanol–0.4% acetic acid in water was used as the mobile phase in a gradient mode (starting with methanol at 10%; 20 min, 25%; 30 min, 55%; 45 min, 55%; 60 min, 80%; 61 min, 100%; 80 min, 100%; 81 min, stop), good results were obtained. The crude extract and peak fractions obtained by HSCCC were analyzed by HPLC under this optimum analytical condition.

Selection of Two-Phase Solvent System and Other Conditions of HSCCC

Successful separation by HSCCC depends upon the selection of a suitable two- phase solvent system, which provides an ideal range of the partition coefficient (K) for the targeted compounds. The ideal K value of the compound separated by HSCCC is between 0.5 and 1.0. If the $K_{stationary/mobile} \gg 1$, the separation time will be too long and HSCCC peaks will be broadened.

In this study, a series of two-phase solvent systems was tested to optimize K values for three target compounds by the HPLC: they include n-hexane-ethyl acetate-methanol-water, ethyl acetate-1-butanol-methanol-water, ethyl acetate-methanol-water, 1-butanol-methanol-0.4% acetic acid in water, and n-hexane-1-butanol-methanol-0.4% acetic acid in water. The $K_{upper/lower}$ values obtained from these solvent systems are summarized in Table 1. The K values of the target compounds in the n-hexane-ethyl acetate-methanol-water, ethyl acetate-1-butanol-methanol-water, and ethyl acetate-methanol-water solvent systems were very small, indicating that the target compounds mainly partitioned in the aqueous phase. Next, the solvent systems, which have a slightly higher polarity were selected. When 1-butanol-methanol-water (5:1:5 or 5:1.5:5, v/v) was used as the two-phase solvent system, the phase separation time was too long. Since a low concentration of acetic acid could improve the phase separation and the retention of the stationary phase,[11] 0.4% acetic acid was added to the solvent system in a subsequent study. Although, the 1-butanol–methanol–0.4% acetic acid (4:1:5, v/v) gave suitable K values, when applied to the HSCCC separation it resulted in low retention of the stationary phase ($<10\%$). The addition of a small amount of *n*-hexane to 1-butanol–methanol–0.4% acetic acid, however, improved the retention of the stationary phase. As shown in Table 1, n-hexane-1-butanol-

Table 1. *K* values of three compounds in various two-phase solvent systems

Solvent system (v/v)	*K* value (upper/lower)		
	II	III	IV
H–EA–M–W (5:5:5:5)	∼0[a]	∼0	0.71
H–EA–M–W (4:5:4:5)	∼0	∼0	0.84
EA–M–W* (10:1:3:10)	∼0	∼0	1.4
B–M–W (4:1:5)	0.51	0.78	1.2
H-B–M–W* (1:4:2:5)	0.15	0.35	0.48
H-B–M–W* (1:4:2.5:6)	0.32	0.42	0.66
H-B–M–W* (0.5:3:2:6)	0.33	0.41	1.02
H-B–M–W* (2.5:6:4:10)	0.59	0.39	0.96
H-B–M–W* (1.5:6:3.5:10)	0.19	0.37	1.79
H-B–M–W* (1.5:6:4:11)	0.21	0.41	2.57
H-B–M–W* (1.4:8:3:15.5)	0.29	0.54	3.46

H: *n*-Hexane, EA: Ethyl acetate, M: Methanol, B: 1-butanol, W*: 0.4% acetic acid in water, W: Water, II: 8-hydroxy-10-hydrosweroside, III: swertiamarin, IV: trifloroside. [a]∼0 indicates that the partition coefficient is too small to be measured.

methanol-0.4% acetic acid in water (1.4:8:3:15.5, v/v) shows a suitable range of K values for three target compounds, and it was successfully used in the HSCCC separation.

Besides K values, other conditions such as the revolution speed of the separation column and flow rate of the mobile phase were also investigated. The results indicated that reducing the flow rate improved the retention of the stationary phase while it broadened the peaks; and when the flow rate was increased, the loss of stationary phase became significant. The rotary speed of the separation coil also improves the retention of the stationary phase. Considering the above aspects, the present separation was started at a flow rate of 1.5 mL/min and at revolution speed of 750 rpm, and after the three major fractions were eluted in about 3 hours, the flow rate of the mobile phase was changed to 2.5 mL/min and revolution speed to 800 rpm to save the HSCCC separation time. The retention percentage of the stationary phase was estimated to be 54% when the initial hydrodynamic equilibrium was reached. The typical HSCCC chromatogram was shown in Figure 2. From 50 mg of the crude sample, four target compounds were purified in a one step separation, yielding 4 mg of an unknown compound (Peak I), 8 mg of 8-hydroxy-10-hydrosweroside (Peak II), 18 mg of swertiamarin (Peak III), and 11 mg of trifloroside (Peak IV). The purities of these compounds were 96.7, 98.4, 97.1, and 96.2%, respectively, as determined by HPLC (Figure 3).

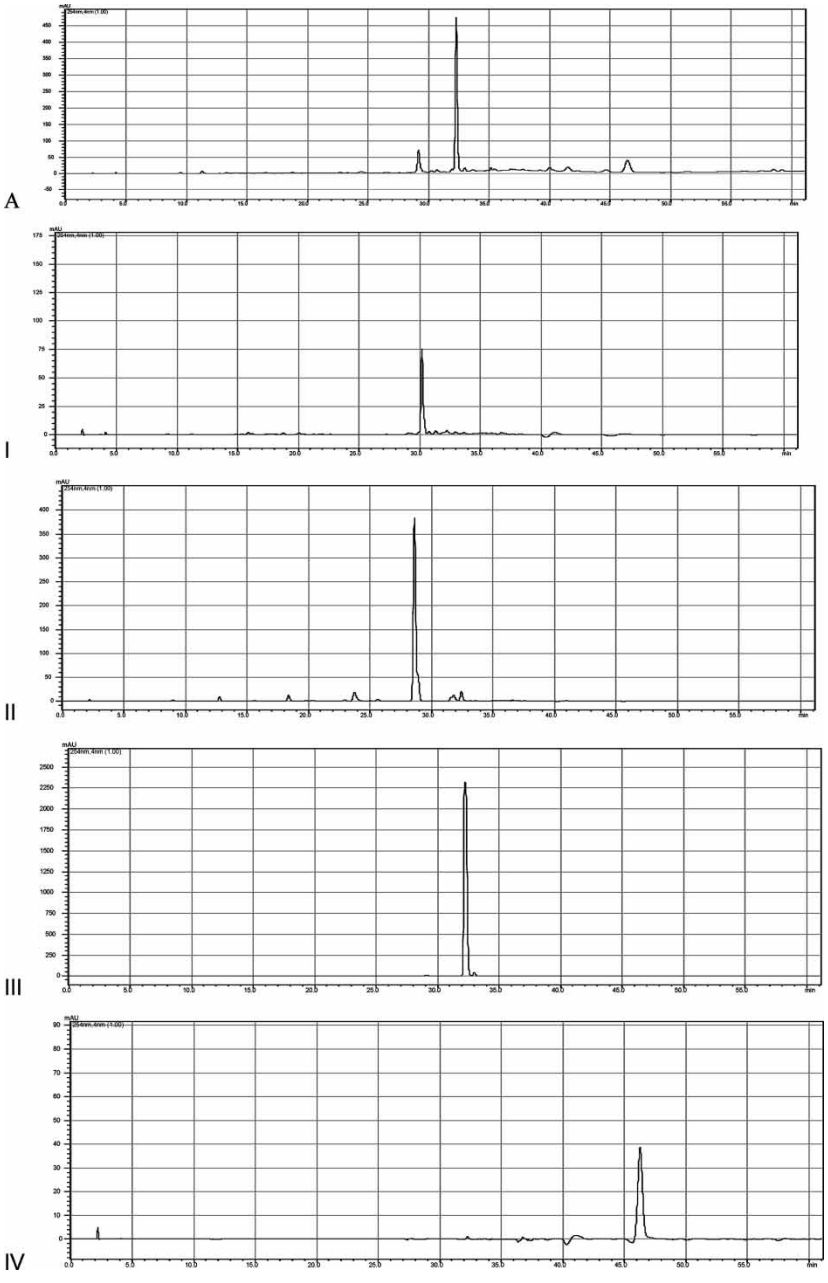
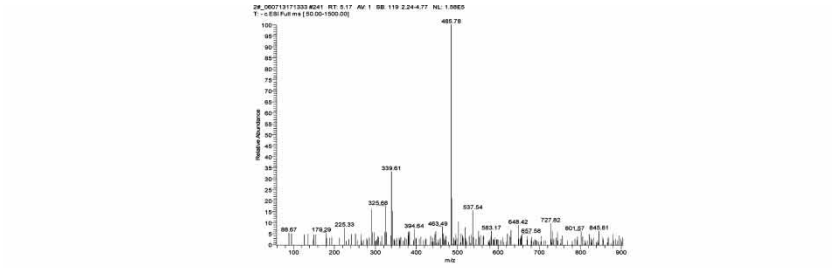
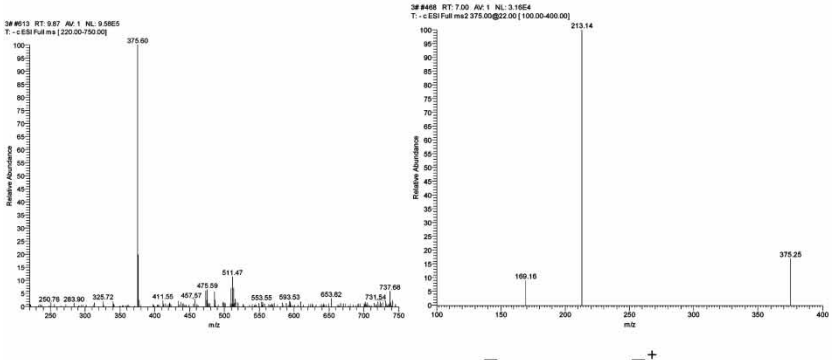
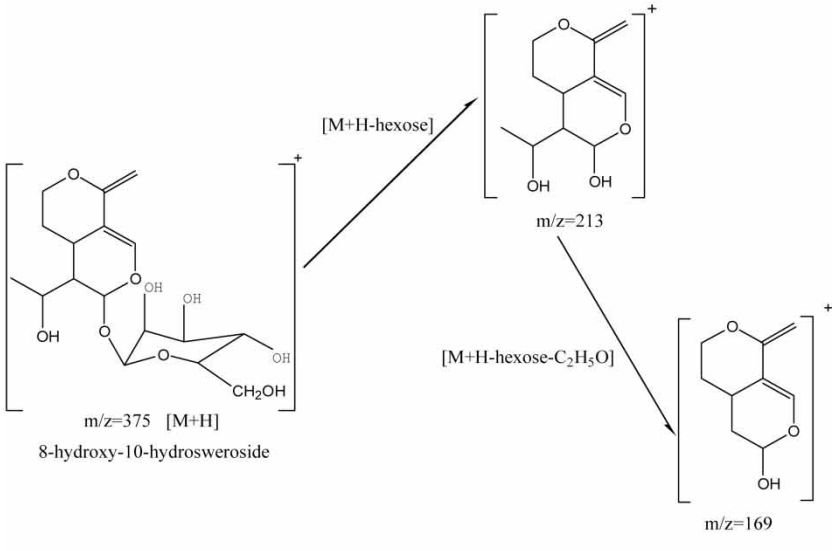


Figure 3. HPLC chromatograms of crude extracts from *Gentiana scabra* Bunge (A) and HSCCC peak fractions (I–IV). Column: Diamonsil C18 column (250 × 4.6 mm ID, 5 μm); mobile phase: methanol-0.4% acetic acid water (methanol: starting at 10%; 20 min, 25%; 30 min, 55%; 45 min, 55%; 60 min, 80%; 61 min, 100%; 80 min, 100%; 81 min, stop); flow rate: 0.8 mL min^{-1}; detection wavelength: 254 nm.



Peak I





Peak II

Figure 4. The mass spectra and the structure analysis of the compound separated from *Gentiana scabra* Bunge by HSCCC.

(*continued*)

Structural Identification

The chemical structure of each peak fraction obtained by HSCCC was identified according to its EIS-MS data: Peak I in Figure 3: ESI-MS, m/z 485 $[M-H]^-$. Peak II in Figure 3: negative ESI-MS, m/z 375 $[M+H]^+$, EIS-MS^2: m/z213, 169. Peak III in Figure 3: negative ESI-MS, m/z 415 $[M]^+$, EIS-MS^2: m/z 355, 179. Peak IV in Figure 3: negative ESI-MS, m/z 781$[M-H]^-$, EIS-MS^2: m/z 739,619, 315.

The mass spectrogram of Peak II shows that it was 8-hydroxy-10-hydrosweroside with m/z values of 375 $[M+H]^+$, EIS-MS^2: m/z 213 $[M+H$-hexose$]^+$, 169 $[M+H$-hexose-$C_2H_5O]^+$. The mass spectrogram of Peak II shows that it was swertiamarin with m/z values of 415 $[M+Na+H_2O]^-$, EIS-MS^2: m/z 355 $[M-OH-H]^-$, 179 [hexose]$^-$, while the mass spectrogram of peak IV is almost identical to the literature[4] values of trifloroside with its m/z of 781$[M-H]^-$, EIS-MS^2: m/z 619 $[M-H-$hexose$]^-$.

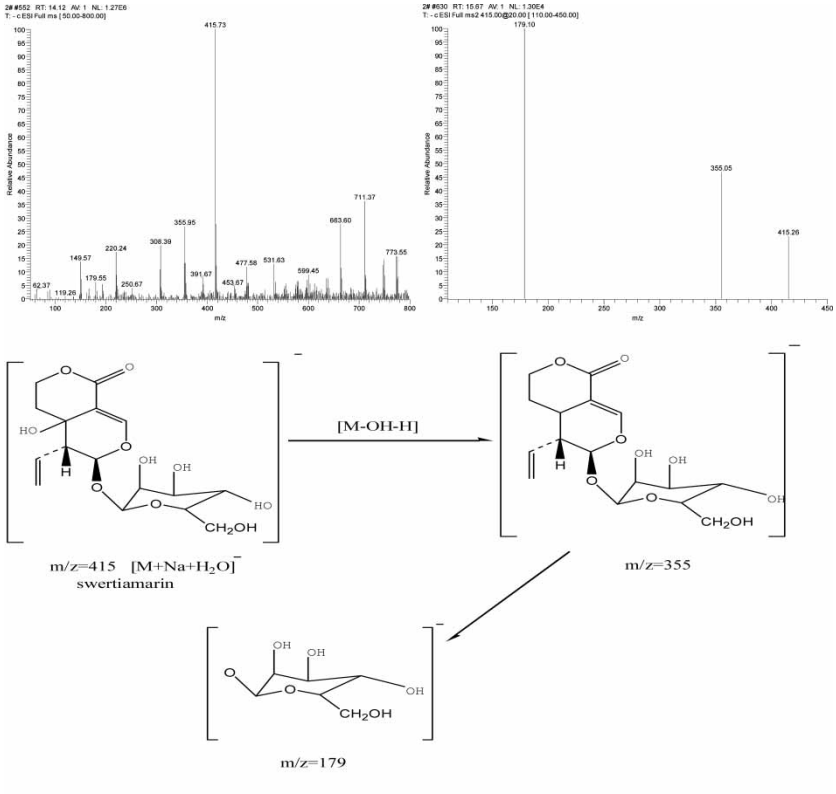


Figure 4. Continued.

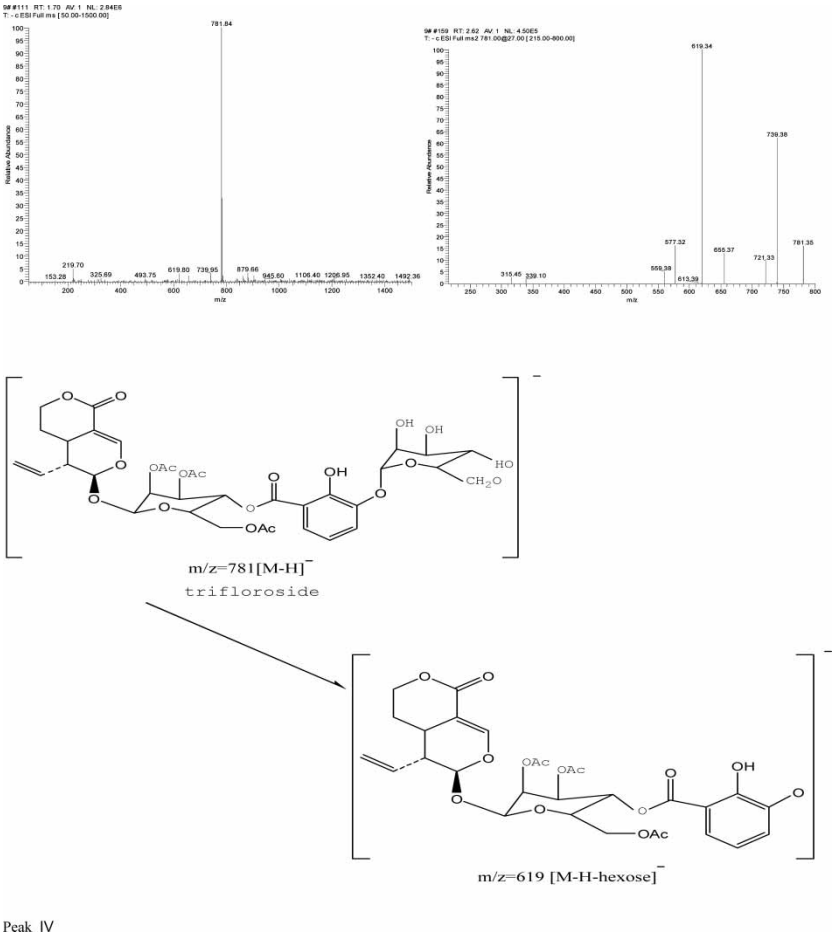


Figure 4. Continued.

Peak I was an unknown compound. The mass spectrogram and the structure analysis are shown in Figure 4.

CONCLUSION

We have successfully purified, for the first time, 3 target compounds from *Gentiana scabra* Bunge using HSCCC. The two-phase solvent system was selected according to the partition coefficients of the target compounds by HPLC. Under the optimized solvent system composed of n-hexane-1-butanol-methanol-0.4% acetic acid in water (1.4:8:3:15.5, v/v), 50 mg of the crude sample was separated, yielding 8 mg of 8-hydroxy-10-hydrosweroside,

18 mg of swertiamarin, 11 mg of trifloroside, and 4 mg of an unknown compound with the purity of 96.7, 98.4, 97.1, and 96.2%, respectively, in a one step separation. The method will be useful for separation of various other compounds from the crude extracts of natural products.

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