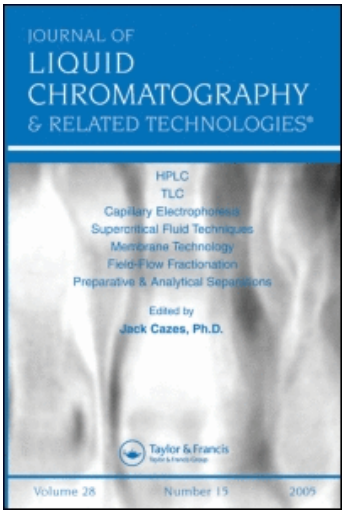

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Preparative Isolation and Purification of Two Closely Related Glycosidic Flavonoids from *Exocarpium Citri* Grandis by High-Speed Countercurrent Chromatography

Yong Liang[a]; Zhaofeng Huang[a]; Hongwei Chen[a]; Tianyou Zhang[b]; Yoichiro Ito[c]
[a] Institute of Analytical Chemistry, School of Chemistry and Environment, South China Normal University, Guangzhou, P. R. China [b] Beijing Institute of New Technology Application, Beijing, P. R. China [c] Center for Biochemistry and Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

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Preparative Isolation and Purification of Two Closely Related Glycosidic Flavonoids from *Exocarpium Citri* Grandis by High-Speed Countercurrent Chromatography

Yong Liang, Zhaofeng Huang, and Hongwei Chen
Institute of Analytical Chemistry, School of Chemistry and Environment, South China Normal University, Guangzhou, P. R. China

Tianyou Zhang
Beijing Institute of New Technology Application, Beijing, P. R. China

Yoichiro Ito
Center for Biochemistry and Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract: High-speed countercurrent chromatography (HSCCC) was successfully used for the isolation and purification of two closely related glycosidic flavonoids from *Exocarpium Citri* Grandis. n-Hexane-1-butanol–methanol–0.5% acetic acid (1:3:1:4, v/v) was used as the two-phase solvent system. From 50 mg of crude extracts of *Exocarpium citri* Grandis, 28.8 mg of naringin, 1.4 mg of rhoifolin, and 5.7 mg of an unknown compound were obtained with the purity of 97.1%, 95.5%, and 97.5%, respectively, in a single run. Two flavonoids fractions were characterized by ESI-MS confirming that the data was identical to the literature values.

Keywords: *Exocarpium citri* Grandis, Countercurrent chromatography, Naringin, Rhoifolin

Address correspondence to Yoichiro Ito, Center for Biochemistry and Biophysics, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 50, Room 3334, 50 South Drive MSC 8014, Bethesda, MD 20892-8014, USA. E-mail: itoy2@mail.nih.gov

INTRODUCTION

Exocarpium citri Grandis (*Juhong* in Chinese), dried ripe fruit peel of *Citrus reticulata* Blanco, is one of the most popular Chinese herbal drugs, officially listed in the Chinese Pharmacopoeia.[1] People usually use it to clear away anemofrigid cough, laryngeal itching cough with excessive sputum, dyspepsia and alcoholic diarrhea, chest tightness, and nausea. The active components of *Exocarpium citri* Grandis include naringin, rhoifolin, neohesperidin, and poncirin. Naringin is one of the main components of citrus flavonoids, which have been shown to possess biological activities such as anti-inflammatory properties, cholesterol lowering, and immune system modulation.[2] Recently, naringin was reported to display numerous biological effects such as antioxidant, hypocholesteremic, anti-atherogenic, and favoring drug absorption.[3] Rhoifolin also has a protective effect on the renal cellular membrane.[4] The chemical structures of naringin and rhoifolin are shown in Figure 1.

It is difficult to use conventional methods to separate and purify naringin and rhoifolin from *Exocarpium citri* Grandis, due to their close mutual similarity in chemical structure, acidity, and polarity. However, we have found that it is quite easy to purify each compound by high-speed countercurrent chromatography (HSCCC).

HSCCC[5] is a unique support free liquid–liquid partition chromatographic technique. Therefore, it eliminates the risk of irreversible adsorption of sample components on the solid support matrix used in the conventional chromatographic column. In some previous studies, this technique was applied successfully to the separation and purification of active components from traditional Chinese herbs and other natural produces, such as flavonoids,[6–8] alkaloids,[9–11] and coumarins.[12–14]

Electrospray ionization mass spectrometry (ESI-MS) is a soft ionization technique that can be used to produce ions even from thermally labile, nonvolatile, polar compounds. Since it can provide a direct means for measurement of molecular weight, it has been widely used for the analysis of natural products in either positive or negative ion mode.

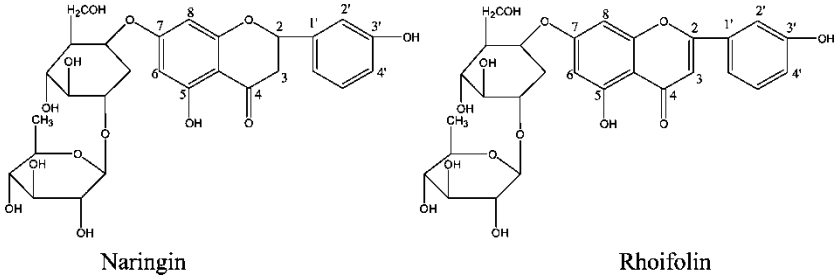

Figure 1. Chemical structures of naringin and rhoifolin.

In this work, we describe HSCCC separation of naringin, rhoifolin, and one unknown compound from 1-butanol crude extract of *Exocarpium citri* Grandis. High performance liquid chromatography (HPLC) was used for selecting the two-phase solvent system and determining the purity of compounds. Identification of HSCCC peak fractions was carried out by ESI-MS in negative mode analyses.

EXPERIMENTAL

Apparatus

The preparative HSCCC instrument employed in this study is a Model GS10A-2, with a multilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a polytetrafluoroethylene multilayer coil of 110 m × 1.6 mm, I.D. with a total capacity of 230 mL. The β value of the 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 is adjustable from 0 to 1000 rpm, and 850 rpm was used in this work. The system was also equipped with one NS-1007 constant flow pump, a Model 8823A-UV monitor operating at 254 nm, a Yakogawa 3057 recorder, and a manual injection valve with a 2 mL (for the analytical HSCCC) or 20 mL sample loop (for the preparative HSCCC).

The HPLC system was a Shimadzu LC-10AVP system consisting of two LC-10ATVP solvent delivery units, a UV–Vis photodiode array detector (SPD-M10AVP), an injection valve (Model 7726) with a 20 mL loop, a system controller (SCL-10AVP), a column oven (CTO-10ASVP), and a degasser (DGU12A). Evaluation and quantification were made by a Class-VP-LC workstation (Shimadzu, Kyoto, Japan). The column was a reversed phase ODS column (150 mm × 4.6 mm i.d., 5 μm, Shimadzu, Japan).

Reagents

All organic solvents used for HSCCC were of analytical grade. Acetonitrile used for HPLC analysis was of chromatographic grade and was purchased from DIKMA filiale in Guangzhou, China. *Exocarpium Citri* Grandis was purchased from Guangzhou Traditional Chinese Medicine Company, Guangzhou, China.

Extraction of Crude Samples

Exocarpium citri Grandis was dried at a constant temperature of 60°C, and then pulverized to about 30 mesh with a disintegrator. The powder (100 g)

was extracted with 500 mL of 80% ethanol under reflux three times. The extraction duration was 3, 2, and 1 h, respectively. The extract solution was then evaporated to remove ethanol under a reduced pressure. The aqueous solution was extracted with an equal volume of 1-butanol. Finally, the extracting solution was evaporated to dryness yielding about 9.12 g of flavonoid glycosides. It was stored in a refrigerator at 4°C for further purification by HSCCC.

Preparation of Two-Phase Solvent System and Sample Solutions

In this work, *n*-hexane-1-butanol–methanol–0.5% acetic acid (1:3:1:4, v/v) was selected as a two-phase solvent system. The solvent mixture was prepared by adding all the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated 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 crude extract sample solution (50 mg) was prepared by dissolving the crude extract in 2 mL of each phase, total volume of 4 mL, of the solvent system used for HSCCC separation.

Selection of Two-Phase Solvent System

n-Hexane-1-butanol–methanol–0.5% acetic acid was used as the two-phase solvent system for HSCCC. The composition of the two-phase solvent system was selected according to the partition coefficient (K) of the target compounds in the crude sample extracted from *Exocarpium citri* Grandis. The K values were determined by HPLC as follows: A small amount of crude sample was dissolved in 5 mL of aqueous phase of the pre-equilibrated two-phase solvent system. The solution was determined by HPLC 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 the equilibration was established, the solute concentration in the aqueous phase was determined by HPLC again and the peak area was recorded as A_2. The partition coefficient K (upper phase/lower phase) was obtained by the following equation: $K = (A_1 - A_2)/A_2$.

HSCCC Separation Procedure

HSCCC was performed as follows. The multilayer coiled column was first entirely filled with the upper phase as a stationary phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow rate of 1.5 mL/min, while the apparatus was run at a revolution

speed of 850 rpm. After the hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (50 mg dissolved in 4 mL of 1:1 volume mixture of each phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at the wavelength of 254 nm. Each peak fraction was manually collected according to the elution profile and later analyzed by HPLC.

HPLC Analysis and Identification of CCC Peak Fractions

The partially purified extract of *Exocarpium citri* Grandis and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a Vp-ODS column (150 mm × 4.6 mm i.d., 5 μm, Shimadzu, Japan). Acetonitrile–0.01% phosphoric acid was used as the mobile phase in gradient mode (acetonitrile: 0–5 min, 10%; 5–6 min, 21%; 6–25 min, 30%). The flow rate of the mobile phase was 0.8 mL/min., and the effluent was monitored by a dual wavelength absorbance detector.

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

RESULTS AND DISCUSSION

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

The selection of the two-phase solvent system, which provides suitable *K* values for the target compounds, is the most important step in performing HSCCC. In this work, a variety of solvent systems such as chloroform–methanol–water, n-hexane–ethyl acetate–methanol–water, ethyl acetate–1-butanol–water, 1-butanol–methanol–0.5% acetic acid, and n-hexane–1-butanol–methanol–0.5% acetic acid have been tested as the two-phase solvent system, the results of which are summarized in Table 1. According to the *K* values shown in Table 1, it can be seen that there was obvious disparity among the *K* values of target compounds in chloroform–methanol–water (4:3:2). In *n*-hexane–ethyl acetate–methanol–water (1:1:1:1), all *K* values became quite small. When ethyl acetate–1-butanol–water (3:2:5) was used, all *K* values became too high. As the relative volume of 1-butanol was reduced, these *K* values approached unity, while the *K* values of peaks I and II were too close to be resolved. It can be concluded that acetate–1-butanol–water is unsuitable for HSCCC separation of the compounds in *Exocarpium citri* Grandis.

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

Solvent system (v/v)	*K*-value		
	I	II	III
C–M–W (4:3:2)	6.37	17.65	0.75
H–EA–M–W (1:1:1:1)	0.015	0.019	0.066
EA–B–W (3:2:5)	7.76	5.17	10.25
EA–B–W (4:1:5)	3.89	2.85	6.82
EA–B–W (5:0.5:5)	1.13	1.10	1.98
EA–W (1:1)	0.24	0.24	1.85
B–M–0.5%HAC (5:1:5)	3.29	2.67	4.10
H–B–M–0.5%HAC (1:4:2:6)	1.28	0.87	2.01
H–B–M–0.5%HAC (1:2:1:3)	0.71	0.38	1.26
H–B–M–0.5%HAC (2: 5:2:7)	0.93	0.47	1.64
H–B–M–0.5%HAC (1:3:1:4)	1.28	0.77	2.08

n-Hexane: H, Chloroform: C, Ethyl acetate: EA, Methanol: M, 1-butanol: B, Acetic acid: HAC, Water: W, I: naringin, II: rhoifolin, III: unknown compound.

In 1-butanol–methanol–0.5% aqueous acetic acid (5:1:5), the target compounds still mainly partitioned in the upper phase. However, with an addition of *n*-hexane to the solvent system, both partition and phase separation were improved. When n-hexane–1-butanol–methanol–0.5% acetic acid (1:4:2:6) was used, the settling time of the two-phases became too long, resulting in serious loss of the stationary phase in HSCCC separation. When n-hexane–1-butanol–methanol–0.5% acetic acid (2:5:2:7) was used, the purity of naringin was unsatisfactory. Finally, n-hexane–1-butanol–methanol–0.5% acetic acid (1:3:1:4) gave a satisfactory separation of three compounds within an acceptable elution time.

The influences of revolution speed and flow rate of the mobile phase on HSCCC peak resolution were investigated as well. At a flow rate of 1.5 mL/min and a revolution speed of 850 rpm, the retention percentage of the stationary phase was about 54%, indicating that a good separation could be obtained. The crude samples from *Exocarpium citri* Grandis were separated and purified under this optimal HSCCC condition.

The HSCCC chromatogram of *Exocarpium citri* Grandis is shown in Figure 2A, where three components were obtained with the yields of 28.8 mg of naringin (Peak I, collected during 132–155 min), 1.4 mg of rhoifolin (Peak II, collected during190–215 min), and 5.7 mg of unknown compound (Peak III, collected during 415–475 min) from 150 mg of crude sample. Purities of these compounds were estimated to be 97.1%, 95.5% and 97.5%, respectively, by using the HPLC area normalization method (Figure 2B).

Structural Identification

Due to their acidic nature, flavonoids usually give higher ion abundances upon deprotonation in the negative ESI mode than via protonation in the positive ESI mode. Identification of HSCCC peak fractions was carried out by ESI-MS in negative mode analyses as follows:

HSCCC peak I in Fig. 3: negative ESI-MS, m/z 577 (M−H).
HSCCC peak II in Fig. 3: negative ESI-MS, m/z 579 (M−H).
HSCCC peak III in Fig.3: negative ESI-MS, m/z 653 (M−H).

The mass spectrum of Peak I shows that it is identical to the literature[15] values of rhoifolin [Fig. 4(a)] with m/z of 577 (M−H) and 269.4 ([M-Rham-Glc-H]$^-$), while the mass spectrogram of peak II is

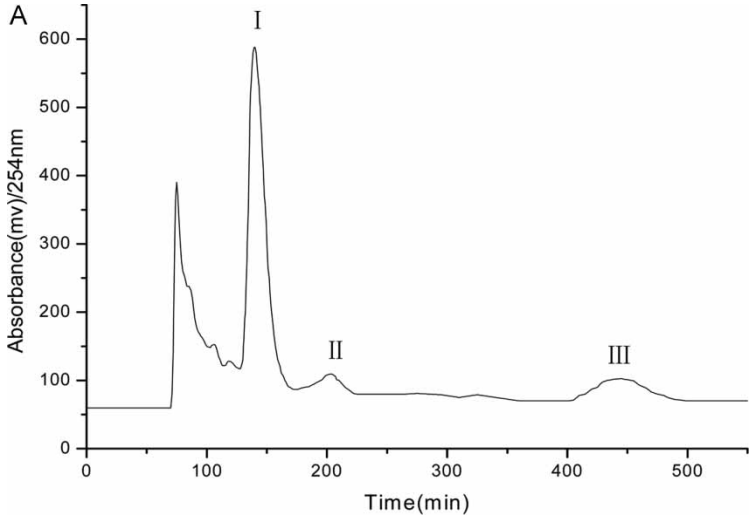

Figure 2. A. Chromatogram of the partially purified extract from *Exocarpium citri* Grandis by preparative HSCCC. Peak I: naringin; Peak II: rhoifolin; Peak III: unknown compound. Solvent system: *n*-hexane−1-butanol−methanol−0.5% acetic acid (1:3:1:4, v/v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 1.5 mL/min; revolution speed: 850 rpm; sample size: 50 mg; sample loop: 2 mL; retention of the stationary phase: 54%. B. HPLC chromatograms of crude extract from *Exocarpium citri* Grandis and HSCCC peak fractions. From top to bottom: Crude extract from *Exocarpium citri* Grandis, peaks I−III of HSCCC in A. HPLC conditions: Column: reversed-phase ODS column (150 mm × 4.6 mm i.d., 5 μm, Shimadzu, Japan); solvent system: acetonitrile−0.01% phosphoric acid eluted in a gradient mode (acetonitrile: 0−5 min, 10%; 5−6 min, 21%, 6−25 min, 30%). The flow rate of the mobile phase was 0.8 mL/min.

(*continued*)

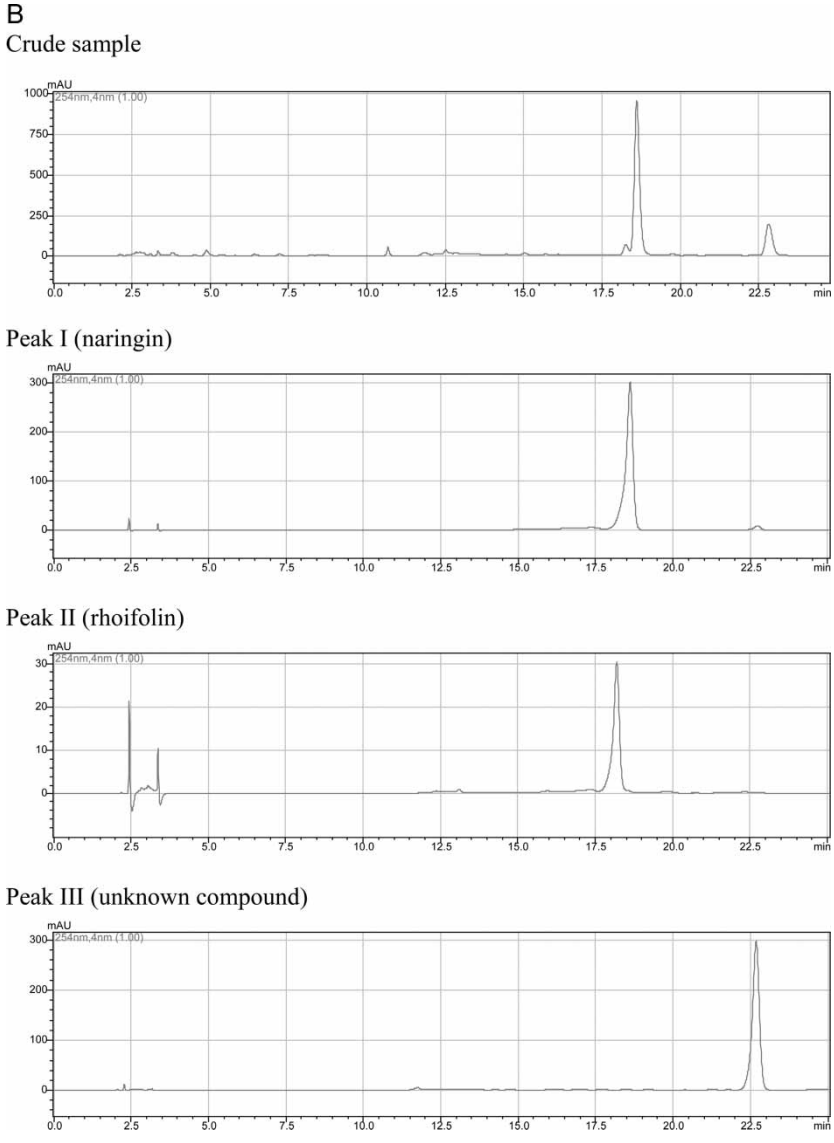

Figure 2. Continued.

almost identical to the literature[16] values of naringin [Fig. 4(b)] with its m/z values of 579 (M−H), 459.3(M−C_8H_8O), and 271.3 ([M-Rham-Glc-H]$^-$). Peak III is an unknown compound with molecular mass of 654.

A hypothesized fragmentation mechanism of $[M-H]^-$ ion of HSCCC rhoifolin and naringin was shown in Fig. 4. As we can see that they have the same pathway and produce the aglycone ion and the corresponding

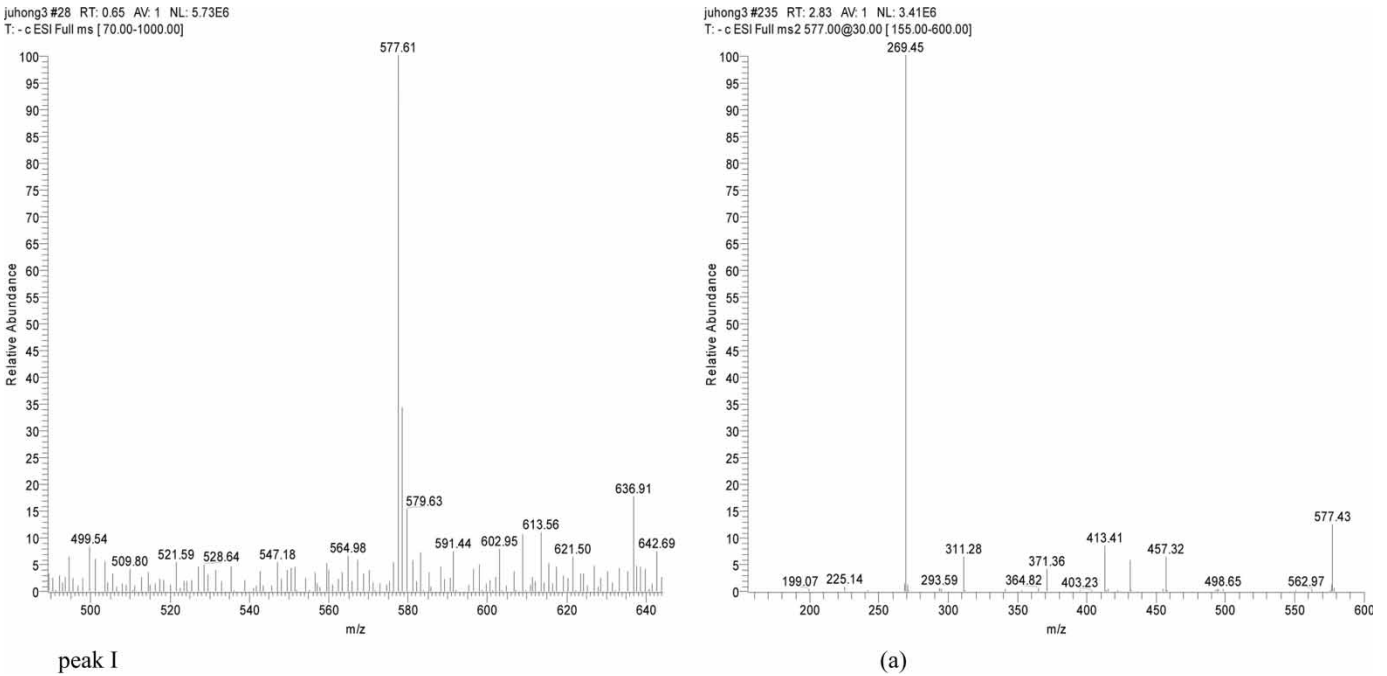

Figure 3a. Mass spectra of HSCCC peak I. (a) MS/MS spectrum of the $[M-H]^-$ ion of peak I (m/z 577).

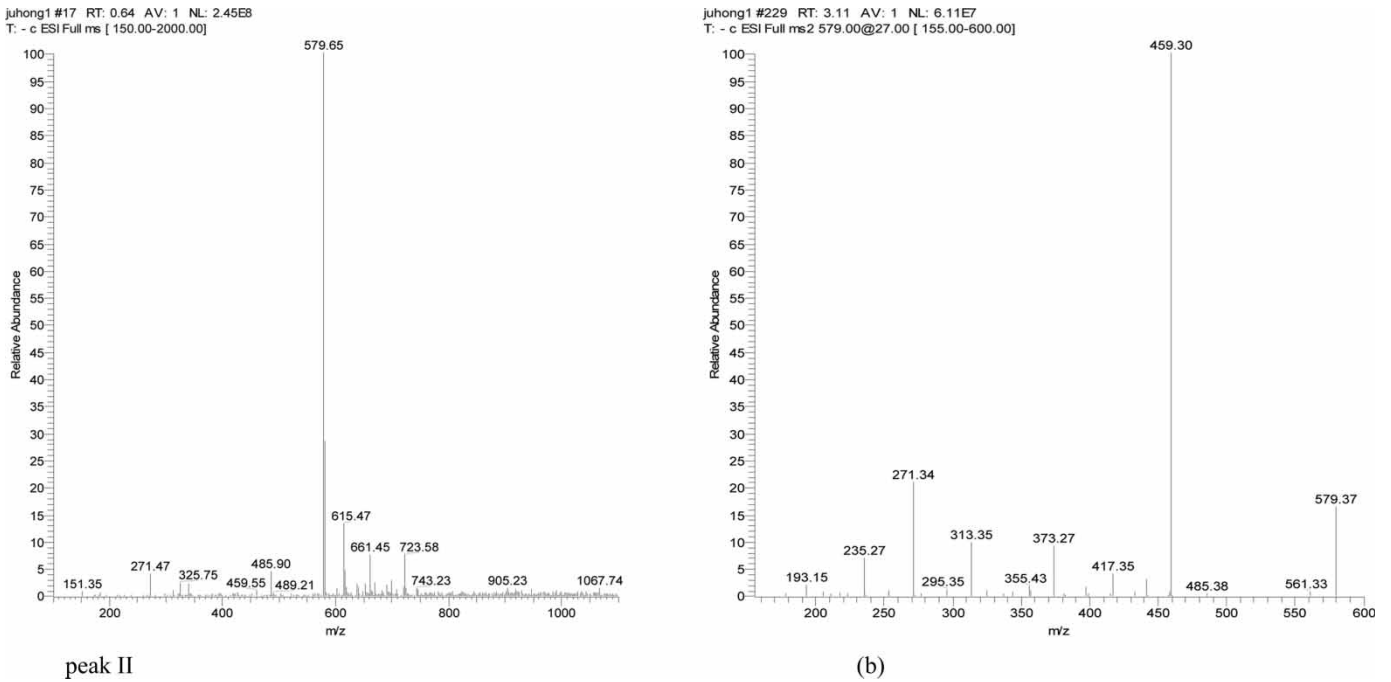

Figure 3b. Mass spectra of HSCCC peak II. (b) MS/MS spectrum of the $[M-H]^-$ ion of peak II (m/z 579).

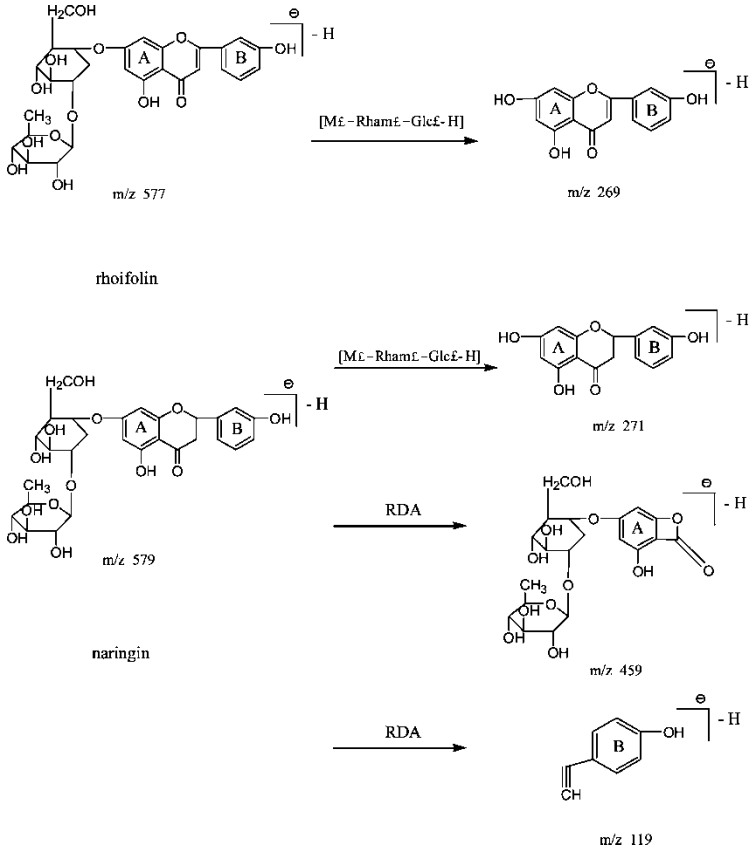

Figure 4. Proposed fragmentation mechanisms of $[M-H]^-$ ion of HSCCC rhoifolin and naringin. Glc: d-glucose, Rham: d-rhamnose.

flavonoid ion in the first process. But naringin also will process retro-Diels-Alder (RDA) cleavage and produces A^- and B^- ions by another pathway.

CONCLUSION

In this paper, two very closely related glycosidic flavonoids and one unknown compound were successfully separated at over 95% purity by HSCCC from a crude extract of *Exocarpium citri* Grandis using a two-phase solvent system composed of n-hexane-1-butanol–methanol–0.5% acetic acid (1:3:1:4, v/v). These compounds were characterized by ESI-MS and the data was identical to the literature values. The above results also indicate that HSCCC is an excellent technology to isolate closely related target components from a mixture of closely related compounds in a crude plant extract.

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