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Application of preparative high-speed counter-current chromatography/preparative high-performance liquid chromatography mode in rapid separation of saponins

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Abstract

Combined with preparative high-performance liquid chromatography, high-speed counter-current chromatography was employed for isolation and purification of saponins from *Gypsophila paniculata* L. *n*-Hexane–*n*-butanol–methanol–0.02% TFA (1:9:1:9, v/v) was employed as solvent system and 210 nm was chosen as the wavelength of ultraviolet detection for the first time. The research tried to compare HSCCC with prep-HPLC, and further integrated their advantages to improve separation efficiency. Five known triterpene saponins were identified by ^{13}C NMR and ESI-MS and their purities were all above 96%. The results demonstrated that adopted method was a feasible, economical and efficient technique for rapid preparative isolation of saponins.

Keywords: High-speed counter-current chromatography; Preparative high-performance liquid chromatography; *Gypsophila paniculata* L.; Saponins

1. Introduction

Saponins are an important group of natural products consisting of a triterpenoid or steroid aglycone attached with one or more sugar chains, they distribute in nature widely and show diverse bioactivities [1–3]. Separation and purification of saponins have been concerned by related researchers for a lot of years. Because of their relative higher polarity, traditional solid/liquid chromatography (SLC) could not always obtain satisfactory resolution. Moreover, irreversible adsorption and artifact formation are nearly fatal to separation of saponins. Therefore more efficient and safety methods need to be explored further. Preparative high-performance liquid chromatography (prep-HPLC) is becoming a powerful tool gradually in virtue of its superexcellent efficiency and higher recovery. But it is more expensive and is also complex for operators. Plentiful raw samples would make columns polluted and overloaded. Meanwhile separation outcome depends on columns severely.

With the development of separation technique, high-speed counter-current chromatography (HSCCC) went upon the stage as a new kind of support-free all-liquid partition chromatography. It could bring on higher sample recovery than SLC, and receive larger amount of sample injection than HPLC; multiform relative pure substances can be obtained at one time in large amount. Furthermore its separation efficiency exceeds droplet counter-current chromatography (DCCC) and centrifugal partition chromatography (CPC), which are based on same partition mechanism and were ever applied in separation of saponins. Basing on above advantages, HSCCC has been successfully used for separation of bioactive saponins in recent years [4–17]. Because saponins always have few chromophores in their structures and show very weak UV absorption, evaporative light scattering detection (ELSD) and thin layer chromatography (TLC) were ever employed to reveal separation results in the previous HSCCC study (see Fig. 1). TLC is cheaper and easier to operate, but it cannot give researchers timely and visualized chromatogram. ELSD is an universal and non-specific detector, however it would consume some sample. Moreover split valve could result in instability of flow rate and optimal split ratio is needed to explore. UV detector, as the most popular detector in HSCCC, was employed to guide separation of saponins

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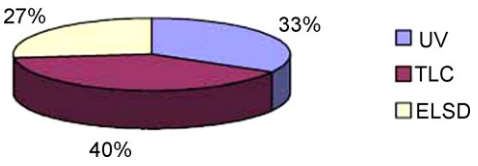


Fig. 1. Three detection methods for separation of saponins in HSCCC and their applied percentage.

sometimes. But the selected detection wavelengths were not in the region of greatest absorption of saponins, which meant the sensitivity might be lower. It was reported that the UV detection in the range from 200 to 210 nm is usually used in HPLC analysis for most saponins and the sensitivity was satisfactory [18], but it has not ever been employed in HSCCC. So UV detection at 210 nm was applied in the present paper to monitor separation of saponins on HSCCC for the first time. Considering complementary action between HSCCC and prep-HPLC studied in the previous work [19–21], the method of preparative HSCCC guided by UV detection at end absorption and combined with prep-HPLC was established for rapid separation and purification of saponins from the ethanol extract of *Gypsophila paniculata* L.. As a result, five triterpene saponins were obtained eventually and their chemical structures were shown in Fig. 2.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study was TBE-300 high-speed counter-current chromatography (Tauto Biotechnique, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.5 mm, total volume = 300 ml) and a 20 ml sample loop. The revolution radius was 5 cm, and the values β ($\beta = r/R$, 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) of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. The system was also equipped with one S-1007 constant flow pump (Shenyitong Tech & Exploitation, Beijing, China), a C-635 UV photometer (Buchi, Switzerland). The data were collected with the model N2000 chromatography workstation (Zhejiang University, Hangzhou, China). The analytical and preparative HPLC equipment were Agilent 1100 series system and Agilent HPLC workstation (Agilent, USA). Nuclear magnetic resonance (NMR) spectrometer was Bruker AM-500 MHz (Bruker, Switzerland). ESI-MS was Agilent 1100 Series LC-MS Trap SL (Agilent, USA).

2.2. Reagents

All solvents used for HSCCC were of analytical grade (Hanbon Sci & Tech, Jiangsu, China). Methanol for HPLC was of chromatographic grade (Hanbon Sci & Tech), MCI gel CHP-20P (Mitsubishi chemical, Japan) was used for enrichment of saponins and water was redistilled water.

The dried roots of *G. paniculata* L. were collected from Yunnan province and identified by professor Mian Zhang, Department of Pharmacognosy, China Pharmaceutical University.

2.3. Preparation of crude extract

200 g dried roots of *G. paniculata* L. were powdered and extracted by 75% ethanol (2 l) and the extract was concentrated to form a syrup. The syrup was dissolved in 1 l water by sonication and partitioned with ethyl acetate and *n*-butanol of equal volume three times successively. Then *n*-butanol solution was concentrated and about 20 g residue was obtained ultimately. In order to enrich the target components and remove impurities, the residue of *n*-butanol was loaded on MCI gel CHP-20P column (200 g, 75–150 μm) and eluted with distilled water, 30% and 75% methanol successively. The 75% methanol effluent was collected and evaporated at 65 °C under reduced pressure and about 680 mg residue was obtained. All the residues were stored in a refrigerator (5 °C) for further use.

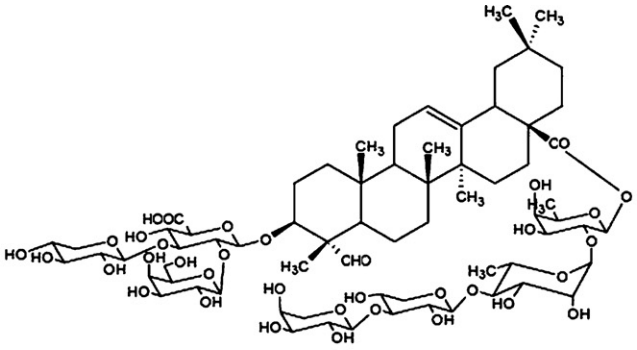
2.4. Preparation of two-phase solvent system and sample solution

In the present study, the two-phase solvent system composed of *n*-hexane–*n*-butanol–methanol–0.02% TFA (1:9:1:9, v/v) was used for HSCCC separation. Each component of solvent system was added to a separatory funnel and thoroughly equilibrated at room temperature for 12 h. The upper phase and lower phase were separated and degassed by sonication for 30 min shortly before use.

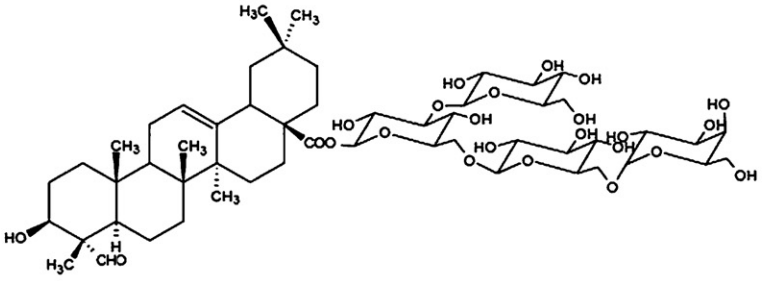
For HSCCC separation of refined *n*-butanol fraction, the sample solution was prepared by dissolving 220 mg of enriched saponins in 5 ml of the lower phase.

2.5. Preliminary fractionation by HSCCC

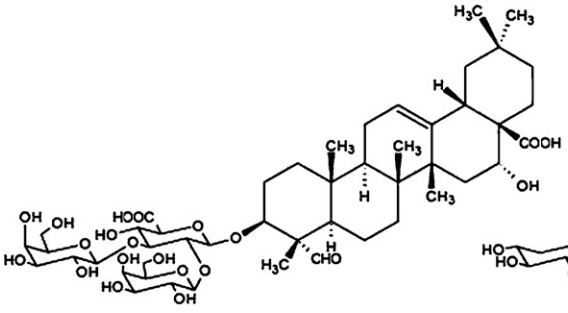
In the separation process, the coiled column was first entirely filled with the upper organic stationary. Then the apparatus was rotated at 850 rpm, while the lower aqueous mobile phase was pumped into the column at flow rate of 2.0 ml/min. After the mobile phase front emerged and the system established a steady state hydrodynamic equilibrium, the sample solution was injected into the separation column through the injection valve. The effluent from the outlet of the column was monitored with UV detector at 210 nm. The HSCCC chromatogram was shown in Fig. 3. Peak fractions I–IV were manually collected according to the chromatogram. When general separation was finished, the residual solution in the column was blown out by nitrogen gas and collected too. Above five fractions were concentrated under reduced pressure and their residuals were dissolved in methanol for subsequent HPLC analysis.



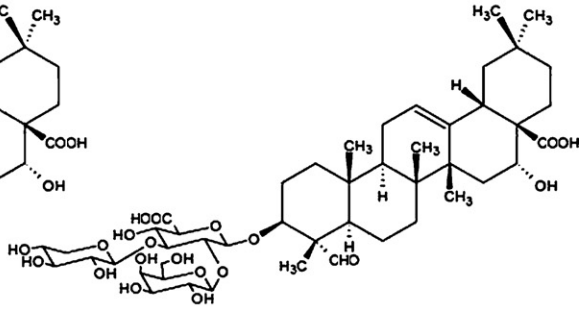
Compound 1



Compound 2



Compound 3



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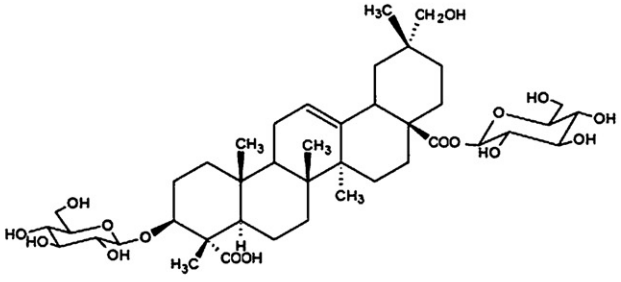

Compound 5

Fig. 2. Chemical structures of compounds **1**–**5**.

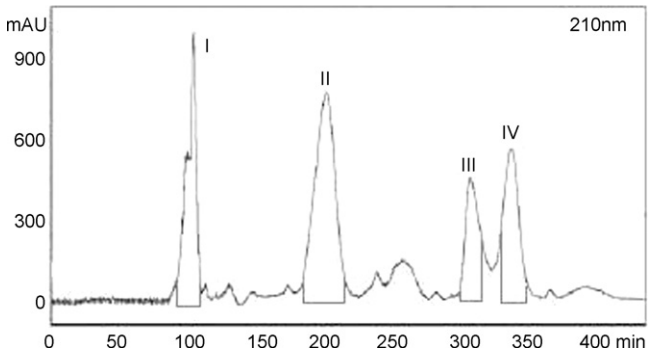

Fig. 3. HSCCC chromatogram of refined *n*-butanol fraction. Conditions: two-phase solvent system, *n*-hexane–*n*-butanol–methanol–water (1:9:1:9, v/v); stationary phase, upper organic phase; mobile phase, lower phase; flow rate: 2 ml/min; revolution speed, 850 rpm; sample size, 220 mg of ether extracts dissolved in 5 ml of the lower phase; detection wavelength, 210 nm; separation temperature was under room temperature (20–25 °C); retention of the stationary phase, 43%.

2.6. HPLC analysis and identification of HSCCC peak fractions

The HPLC analysis of refined *n*-butanol fraction and HSCCC peak fractions were performed with a Shim-Pack CLC-ODS (150 mm × 4.6 mm i.d., 5 μm) column at room temperature. Mobile phase was methanol–0.02% TFA in gradient mode as follows: 5:95–100:0 in 50 min. Injection was made through 100 μl loop. The effluent was monitored at 210 nm and the flow rate was kept at 1.0 ml/min constantly.

2.7. Subsequent purification by prep-HPLC

Quantities of 20–30 mg of the fraction I in Fig. 2 were further purified by prep-HPLC on a Shim-Pack CLC-ODS (250 mm × 20 mm i.d., 5 μm) column at room temperature. Mobile phase was methanol–0.02% TFA (30:70, v/v) in isocratic mode and injection was made through 900 μl loop. The effluent was monitored at 210 nm and the flow rate was kept at 10 ml/min constantly. At last compound **1** (16.3 min) was obtained from this fraction as shown in Fig. 4.

3. Results and discussion

3.1. Selection of fore treatment process

The roots of *G. paniculata* L. possess rich sources of triterpene saponins, and these saponins can be perfectly extracted by 50–75% ethanol in previous study. Finally 75% ethanol was selected for less water-soluble impurity in extracts. In previous study [4–17], acetone precipitation, D101 resin, ODS, even silica gel column chromatography were used to remove the fat soluble components and acquire total saponins of certain purity after crude extract was obtained. Compared with those methods, MCI gel CHP-20P showed its unique advantages and was ever successfully used in enrichment of saponins [22]. So the 75% ethanol extract was partitioned with ethyl acetate and *n*-butanol firstly, and then the *n*-butanol extract was further subjected to MCI gel CHP-20P column and eluted by methanol–water with increasing strength. As a result, five major saponins, with a considerable amount, were enriched from the total ethanol extract. Basing on the HPLC conditions in Section 2.6 and external standard method, the purities of five compounds before and after enrichment by MCI column were 1.2, 1.9, 0.8, 1.3, 0.9% and 10.1, 18.8, 9.7, 11.3, 10.5% respectively.

3.2. HPLC analysis and identification of HSCCC peak fractions

The refined *n*-butanol fraction and each peak fraction of HSCCC were analyzed by HPLC. Because there was greater difference in the polarity of major constituents, gradient elution was employed to obtain the optimum resolution and shorten analysis time. 210 nm was still chosen for the wavelength of UV detection. Since acetonitrile gives lower absorption at lower wavelength than methanol, the gradient mode of acetonitrile–water is supposed to overcome the severe drift of baseline. But poor resolution was obtained when acetonitrile was employed in the experiment, so methanol had to be chosen finally. Trace of TFA was also used to inhibit the tailing of peaks. According to the adopted conditions and area normalization method the purities of the five compounds were all above 96% and their HPLC chromatograms and corresponding UV absorption were shown in Fig. 5. Identification of the HSCCC

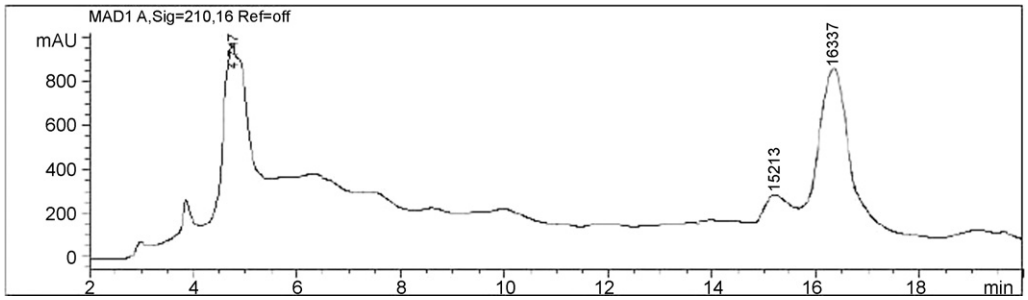

Fig. 4. Preparative HPLC chromatogram of purification of compound **1**. Column: Shimadzu VP-ODS (250 mm × 20 mm i.d., 5 μm); mobile phase: methanol–0.02% TFA (30:70); flow rate: 10 ml/min; detection wavelength: 210 nm.

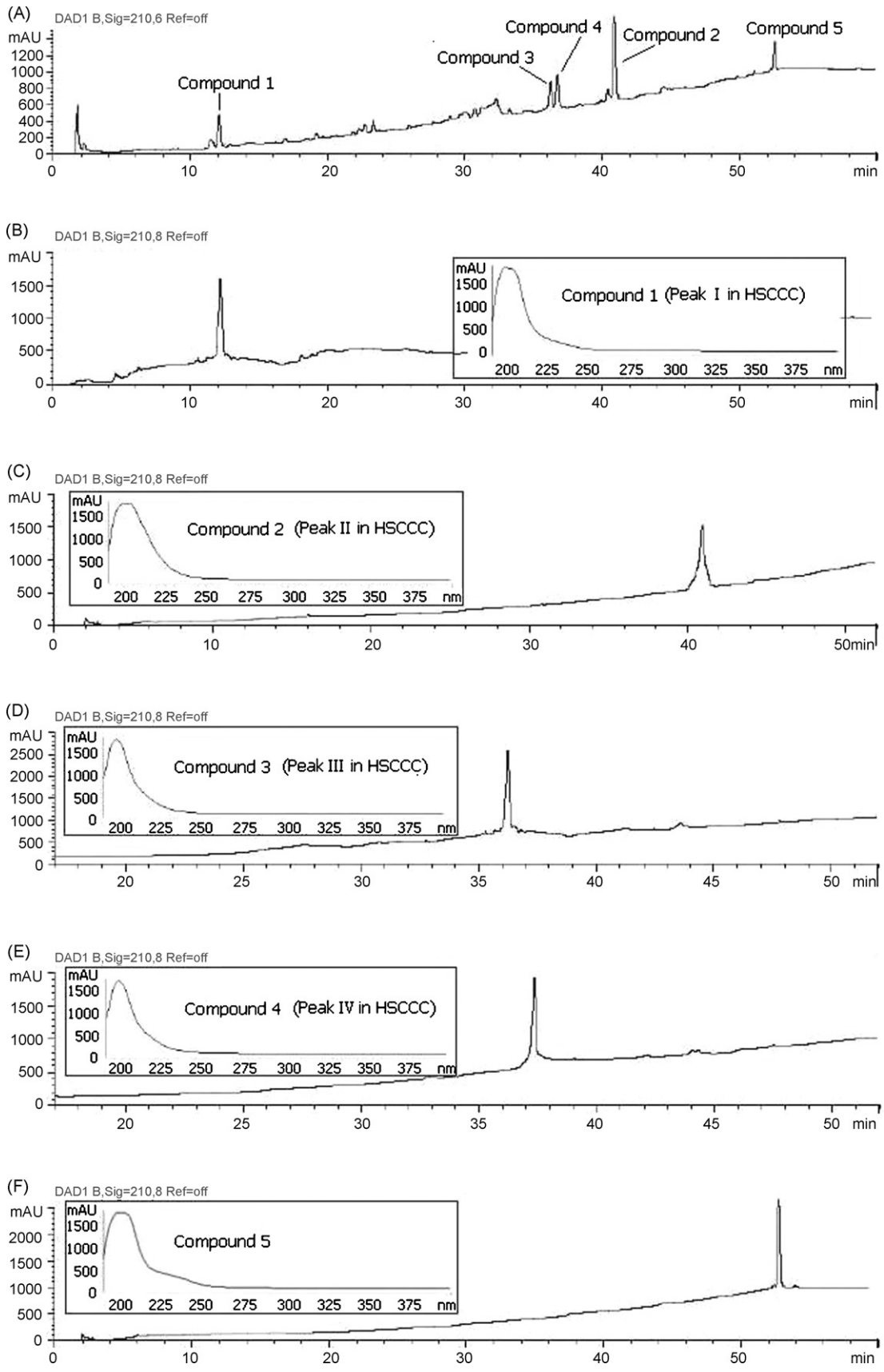

Fig. 5. Analytical HPLC chromatograms of *n*-butanol fraction (A) and HSCCC peak fractions together with their UV absorbance as follows: (B), peak I (compound **1**); (C), peak II (compound **2**); (D), peak III (compound **3**); (E), peak IV (compound **4**); (F), compound **5**. Column, Shimadzu VP-ODS (150 mm × 4.6 mm i.d., 5 μm); mobile phase, methanol–0.02% TFA (methanol: 5–100% in 50 min); flow rate, 1.0 ml/min; detection wavelength, 210 nm.

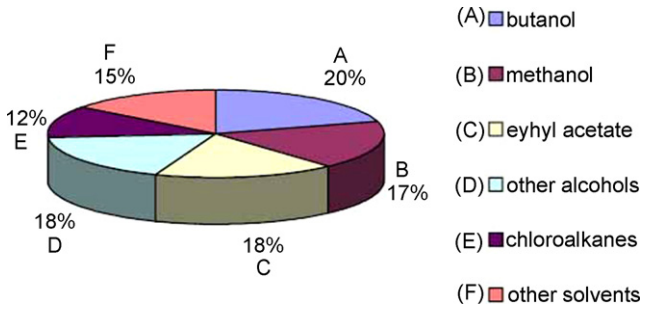

Fig. 6. Various organic solvents used in separation of saponins on HSCCC and their applied percentage.

peak fractions were based on retention time together with data of ^{13}C NMR and ESI-MS.

3.3. Selection of two-phase solvent system

Successful separation by HSCCC largely depends upon the selection of suitable two-phase solvent systems. In previous research of saponins separation [4–17], many different organic solvents were ever selected as components of solvent systems, among them *n*-butanol, methanol, ethyl acetate and chloroform were used frequently (see Fig. 6). However, some common solvents with longer cut-off wavelength, just like ethyl acetate (254 nm), chloroform (245 nm) and acetone (330 nm), could not be chosen as components of solvent system when eluents were detected in the end absorption range (200–220 nm). This would not result in great impediment, because there were many other suitable solvents waiting for selection. In order to eliminate disturbance as much as possible and obtain relative sensitive detection, solvents with shorter cut-off wavelength should be adopted. Water, acetonitrile, saturated hydrocarbons, alcohols, etc., were all perfect transparent solvents in short wavelength range. As most saponins had certain partition coefficient between water and *n*-butanol, the two solvents were suggested to play the role of principal components and a small quantity of saturated hydrocarbons, alcohols or acetonitrile were added to adjust surface tension and dielectric constant.

On the basis of above principles, several kinds of solvent systems were tested in this experiment, such as *n*-hexane–*n*-butanol–methanol–0.02% TFA (0:9:1:9, 1:9:1:9, 2:9:1:9, 1:9:2:9, v/v), *n*-hexane–*n*-butanol–ethanol–0.02% TFA (1:9:1:9, v/v) and *n*-hexane–*n*-butanol–acetonitrile–0.02% TFA (1:9:1:9, v/v). *K*-values of target compounds were measured as important index to predict the performance of tested systems and they were shown in Table 1. Ethanol system was abandoned firstly because severe emulsification occurred and the volume of upper phase was smaller, which might result in poor retention of the stationary phase. As for acetonitrile system, its separation performance was similar to methanol system, but acetonitrile was not preferred for its much more expense and higher boiling point than methanol. In the system of *n*-hexane–*n*-butanol–methanol–0.02% TFA (0:9:1:9), the great mass of saponins distributed in upper stationary phase.

Table 1
The *K*-values of target components measured in different solvent systems

Solvent system		*K*-value			
		System A	System B	System C	System D
Refined *n*-butanol fraction	I	0.68	0.31	0.24	<0.2
	II	1.95	1.64	1.61	1.12
	III	3.06	2.21	2.12	1.85
	IV	3.31	2.46	2.30	1.92
	V	>5	>5	>5	>5

System A, *n*-hexane–*n*-butanol–methanol–0.02% TFA (0:9:1:9, v/v); system B, *n*-hexane–*n*-butanol–methanol–0.02% TFA (1:9:1:9, v/v); system C, *n*-hexane–*n*-butanol–methanol–0.02% TFA (2:9:1:9, v/v); system D, *n*-hexane–*n*-butanol–methanol–0.02% TFA (1:9:2:9, v/v). Experimental protocol: 4 ml of each phase of the equilibrated two-phase solvent system was added to approximately 8 mg of crude sample placed in a 10 ml test tube. The test tube was caped and shaken vigorously for 2 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficient (*K*). The partition coefficient (*K*) value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

Meanwhile when the ratio of methanol was increased, the color of upper layer solution would deepen, which indicated more impurity and pigment existed in the solution. So *n*-hexane–*n*-butanol–methanol–0.02% TFA (1:9:1:9, v/v) was used as the two-phase solvent system ultimately. Four peaks marked as peak I, II, III and IV (in Fig. 3) were collected, among them peaks II–IV were pure comparatively. Because the separation mechanism between HSCCC and HPLC is completely different, the order of these four peaks in Fig. 3 is not completely in accordance with that in Fig. 5A. Finally the residual solution in the separation column was blown out and another compound was obtained. The sample of peak I was subjected to prep-HPLC to be purified further.

3.4. Comparison and combination of HSCCC and HPLC

Definitely, HSCCC is not the right tool for analytical purposes. However, as summarized in Table 2, it enables dirty material to be handled directly. Compared with HSCCC, prep-

Table 2
Comparison with HSCCC and prep-HPLC

Items for comparison	HSCCC	Prep-HPLC
Separation theory	Liquid–liquid partition	Liquid–solid partition
Solvent system	More	Less
Sample quantity	Larger	Smaller
Sample complexity	Higher	Lower
Each separation time	Longer	Shorter
Recoveries of the targets	Higher	Lower
Pre-processing time	Shorter	Longer
Elution mode	More	Less
Reproducibility among columns	Better	Worse
Cost	Lower	Higher

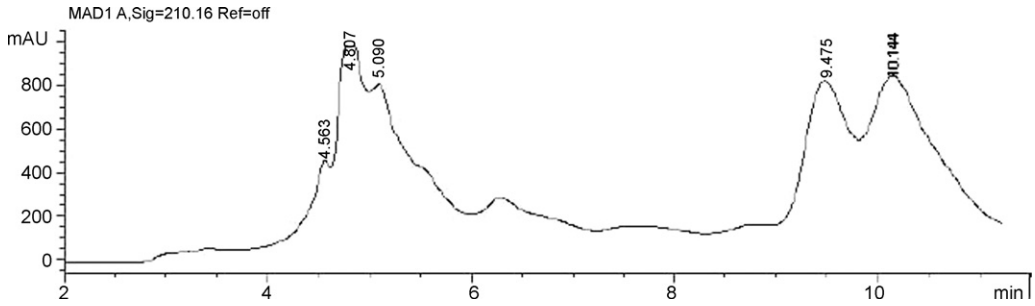

Fig. 7. Preparative HPLC chromatogram of separation of compounds **2** and **3**. Column, Shimadzu VP-ODS (250 mm × 20 mm i.d., 5 μm); mobile phase, methanol–0.02% TFA (75:25); flow rate, 10 ml/min; detection wavelength, 210 nm.

HPLC requires a sample treatment before separation, which enables very efficient purification of high value materials in one step. Taking into account the time required for the purification steps before prep-HPLC, the productivity of HSCCC is higher than that of HPLC. Furthermore, the separation mechanism of HSCCC is completely different from HPLC, it can give another prospective way when satisfied separation cannot be realized by methanol, acetonitrile or other solvents on C_{18} or C_8 columns. In the study, the same quantity of mixture of compounds **2** and **3**, whose retention time were very similar on HPLC, was isolated by HSCCC and prep-HPLC respectively in order to compare their separation performance preliminarily. As shown in Fig. 7, the retention time of two compounds were 9.4 and 10.1 min, respectively basing on optimized HPLC conditions. It was obvious that the resolution was better in HSCCC ($Rs > 1$) than prep-HPLC ($Rs < 1$).

Certainly application of prep-HPLC had its optimal time in the combinatorial method too. Because stepwise and gradient elution cannot be adopted on HSCCC with detection in short wavelength range because they will result in baseline shift. For this reason sometimes it cannot achieve complete separation of mixture in one step and in short time on HSCCC. Then the rapidity superiority of prep-HPLC could be utilized after the steps of preliminary fractionation are finished and the multicomponent sample is simplified. Basing on the combination of their advantages, compound **1** was obtained through initial enrichment of HSCCC and further rapid purification of prep-HPLC.

Through the described study about HSCCC and HPLC, the inner relation of their mobile phase was also revealed initially. It was found that, for the diverse polarity of target saponins, the isolation abilities of the solvent systems composed by various proportion of *n*-hexane, *n*-butanol, methanol and water in HSCCC might be related with elution strength of methanol–water with different concentration on reversed phase column in HPLC. If the saponins had suitable *K*-values (0.2–5) in solvent system of *n*-hexane–*n*-butanol–methanol–0.02% TFA/water (1:9:1:9), which might have 3–6 sugars and make up of the main body of total saponins, they could be separated efficiently by 60–80% methanol on prep-HPLC by the test. Through this potential bridge between HSCCC and prep-HPLC, it is rather convenient to establish the isolation conditions for most saponins.

3.5. The structural identification

The structural identification of peak fractions was performed with ESI-MS and ^{13}C NMR. Data of each compound were given as follows:

Data of compound **1** (7.9 mg): ESI-MS (*m/z*): 1525 ($[M–H]^-$); ^{13}C NMR (125 MHz, pyridine-d_5): 38.1 (C-1), 25.2 (C-2), 84.5 (C-3), 55.1 (C-4), 48.8 (C-5), 20.6 (C-6), 32.7 (C-7), 40.3 (C-8), 47.9 (C-9), 36.2 (C-10), 23.7 (C-11), 122.5 (C-12), 144.0 (C-13), 42.2 (C-14), 28.1 (C-15), 23.7 (C-16), 46.9 (C-17), 41.8 (C-18), 46.4 (C-19), 30.8 (C-20), 33.9 (C-21), 32.5 (C-22), 210.3 (C-23), 11.3 (C-24), 15.7 (C-25), 17.3 (C-26), 26.1 (C-27), 176.5 (C-28), 33.3 (C-29), 23.8 (C-30), 104.2 (C-1 of GlcA), 78.7 (C-2 of GlcA), 86.1 (C-3 of GlcA), 70.9 (C-4 of GlcA), 77.6 (C-5 of GlcA), 171.4 (C-6 of GlcA), 105.4 (C-1 of Glc), 75.9 (C-2 of Glc), 78.4 (C-3 of Glc), 72.5 (C-4 of Glc), 78.6 (C-5 of Glc), 63.1 (C-6 of Glc),104.5 (C-1 of Gal), 73.7 (C-2 of Gal), 75.8 (C-3 of Gal), 70.2 (C-4 of Gal), 76.6 (C-5 of Gal), 61.9 (C-6 of Gal), 105.1 (C-1 of Xyl), 75.5 (C-2 of Xyl), 78.6 (C-3 of Xyl), 71.4 (C-4 of Xyl), 67.5 (C-5 of Xyl), 105.2 (C-1 of Xyl'), 75.5 (C-2 of Xyl'), 79.6 (C-3 of Xyl'), 70.8 (C-4 of Xyl'), 67.3 (C-5 of Xyl'), 95.1 (C-1 of Fuc), 75.3 (C-2 of Fuc), 75.6 (C-3 of Fuc), 72.8 (C-4 of Fuc), 72.2 (C-5 of Fuc), 17.3(C-6 of Fuc), 102.1 (C-1 of Xyl), 71.3 (C-2 of Xyl), 82.3 (C-3 of Xyl), 78.8 (C-4 of Xyl), 69.2 (C-4 of Xyl), 19.0 (C-5 of Xyl). Comparing the above data with Ref. [23], compound **1** was identified as 3-*O*-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl gypsogenin 28-*O*-β-D-glucopyranosyl-(1 → 3)-[β-D-xylopyranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside.

Data of compound **2** (15.2 mg): ESI-MS (*m/z*): 1117 ($[M–H]^-$); ^{13}C NMR (125 MHz, pyridine-d_5): 38.7 (C-1), 27.2 (C-2), 71.3 (C-3), 56.1 (C-4), 47.6 (C-5), 21.4 (C-6), 32.7 (C-7), 40.1 (C-8), 47.9 (C-9), 36.2 (C-10), 23.8 (C-11), 122.5 (C-12), 144.5 (C-13), 42.2 (C-14), 28.4 (C-15), 23.2 (C-16), 47.1 (C-17), 41.8 (C-18), 46.3 (C-19), 30.8 (C-20), 34.1 (C-21), 32.5 (C-22), 207.6 (C-23), 9.7 (C-24), 15.7 (C-25), 176.3 (C-28), 33.3 (C-29), 23.7 (C-30), 95.2 (C-1 of Glc), 72.7 (C-2 of Glc), 88.7 (C-3 of Glc), 69.3 (C-4 of Glc), 77.6 (C-5 of Glc), 69.4 (C-6 of Glc), 105.8 (C-1 of Glc'), 75.4 (C-2 of Glc'), 78.7 (C-3 of Glc'), 71.5 (C-4 of Glc'), 78.6 (C-5 of Glc'), 62.8 (C-6 of Glc'), 105.7 (C-1 of Glc"), 75.0 (C-2 of Glc"), 78.3 (C-3 of Glc"), 71.7 (C-4 of Glc"), 76.6 (C-5 of Glc"),

68.5 (C-6 of Glc"), 100.5 (C-1 of Gal), 70.7 (C-2 of Gal), 71.8 (C-3 of Gal), 71.2 (C-4 of Gal), 72.6 (C-5 of Gal), 62.9 (C-6 of Gal). The results were very similar to those in ref. [24], compound **2** corresponded to gypsogenin α-D-galactopyranosyl-(1 → 6)-[β-D-glucopyranosyl-(1 → 3)]-β-D-glucopyranosyl ester.

Data of compound **3** (8.3 mg): ESI-MS (*m/z*): 985 ($[M-H]^-$); ^{13}C NMR (125 MHz, pyridine-d_5): 38.1 (C-1), 23.7 (C-2), 84.3 (C-3), 55.1 (C-4), 48.6 (C-5), 20.4 (C-6), 32.7 (C-7), 40.1 (C-8), 48.9 (C-9), 36.2 (C-10), 25.5 (C-11), 122.5 (C-12), 145.5 (C-13), 42.1 (C-14), 36.4 (C-15), 74.7 (C-16), 47.2 (C-17), 41.3 (C-18), 47.3 (C-19), 31.4 (C-20), 36.1 (C-21), 32.8 (C-22), 209.6 (C-23), 11.2 (C-24), 15.7 (C-25), 179.9 (C-28), 33.3 (C-29), 24.7 (C-30), 103.8 (C-1 of GlcA), 78.4 (C-2 of GlcA), 87.7 (C-3 of GlcA), 71.5 (C-4 of GlcA), 77.6 (C-5 of GlcA), 171.8 (C-6 of GlcA), 104.5 (C-1 of Gal), 73.7 (C-2 of Gal), 75.8 (C-3 of Gal), 70.2 (C-4 of Gal), 76.6 (C-5 of Gal), 61.9 (C-6 of Gal), 105.1 (C-1 of Gal'), 73.1 (C-2 of Gal'), 77.5 (C-3 of Gal'), 70.2 (C-4 of Gal'), 75.5 (C-5 of Gal'), 62.1 (C-6 of Gal'). Compared with the data given in Ref. [24], compound **3** corresponded to 3-*O*-β-D-galactopyranosyl-(1 → 2)-[β-Dgalactopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl quillaic acid.

Data of compound **4** (8.7 mg): ESI-MS (*m/z*): 953 ($[M+Cl]^-$); ^{13}C NMR (125 MHz, pyridine-d_5): 38.0 (C-1), 23.7 (C-2), 84.3 (C-3), 55.0 (C-4), 48.6 (C-5), 20.6 (C-6), 32.6 (C-7), 40.4 (C-8), 48.9 (C-9), 36.2 (C-10), 25.6 (C-11), 122.5 (C-12), 145.5 (C-13), 41.5 (C-14), 36.4 (C-15), 74.8 (C-16), 47.5 (C-17), 42.3 (C-18), 47.3 (C-19), 31.4 (C-20), 36.1 (C-21), 32.8 (C-22), 209.6 (C-23), 11.2 (C-24), 15.8 (C-25), 180.0 (C-28), 33.3 (C-29), 24.7 (C-30), 104.1 (C-1 of Glc A), 78.8 (C-2 of Glc A), 86.1 (C-3 of Glc A), 71.5 (C-4 of Glc A), 77.6 (C-5 of Glc A), 171.8 (C-6 of Glc A), 104.5 (C-1 of Gal), 73.7 (C-2 of Gal), 76.8 (C-3 of Gal), 70.2 (C-4 of Gal), 75.6 (C-5 of Gal), 62.0 (C-6 of Gal), 105.1 (C-1 of Xyl), 75.5 (C-2 of Xyl), 78.6 (C-3 of Xyl), 70.8 (C-4 of Xyl), 67.5 (C-5 of Xyl). The data were very similar to those in Ref. [25] and it corresponded to gypsogenin 3-*O*-β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucuronopyranoside.

Data of compound **5** (8.8 mg): ESI-MS (*m/z*): 825 ($[M-H]^+$); ^{13}C NMR (125 MHz, pyridine-d_5): 39.3 (C-1), 26.5 (C-2), 86.4 (C-3), 54.0 (C-4), 52.1 (C-5), 21.6 (C-6), 33.1 (C-7), 40.4 (C-8), 48.2 (C-9), 36.9 (C-10), 23.8 (C-11), 144.5 (C-13), 42.5 (C-14), 28.5 (C-15), 23.8 (C-16), 47.5 (C-17), 41.3 (C-18), 41.3 (C-19), 36.4 (C-20), 29.1 (C-21), 32.3 (C-22), 186.6 (C-23), 13.2 (C-24), 16.3 (C-25), 17.4 (C-26), 26.5 (C-27), 176.8 (C-28), 74.0 (C-29), 19.5 (C-30), 104.8 (C-1 of Glc), 75.8 (C-2 of Glc1), 77.5 (C-3 of Glc), 71.8 (C-4 of Glc), 77.6 (C-5 of Glc), 63.0 (C-6 of Glc), 95.8 (C-1 of Glc'), 74.1 (C-2 of Glc'), 78.8 (C-3 of Glc'), 71.5 (C-4 of Glc'), 79.0 (C-5 of Glc'), 62.6 (C-6 of Glc'), which matched with the reported data given in Ref. [26], compound **5** corresponded to dianoside C.

4. Conclusions

In order to make the separation of saponins by HSCCC more convenient, new detection condition and assorted solvent system were developed. Using of solvent systems composed of solvents with shorter cut-off wavelength make detection in end absorption range feasible for the first time in the study, and it is very meaningful to those laboratories without ELSD. On the other hand, HSCCC + prep-HPLC has become a new and superior separation mode for saponins over other chromatographic methods for complementary action between the two methods. Initial preparation preformed by HSCCC enriched certain polarity bands containing the chosen targets, followed with further purification by high resolution prep-HPLC procedures with 5 μm ODS column, the high purity of saponins could be obtained from plants. Its most significant advantage was that almost quantitative mass balance of components and better resolution were achieved simultaneously in short time. The relationship between elution solvents of HSCCC and prep-HPLC was revealed, that was if the saponins had suitable *K*-values (0.2–5) in solvent system of *n*-hexane–*n*-butanol–methanol–0.02% TFA (1:9:1:9), they could be separated efficiently by 60–80% methanol on HPLC. It would fasten the establishment of separation conditions for most saponins on HSCCC or prep-HPLC.

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