International Journal of Nutrition Sciences

Journal Home Page: ijns.sums.ac.ir

ORIGINAL ARTICLE

One-Step Preparative Separation of Flavone and Isoflavone Glycosides from *Sophora Japonica* Fruit by High-Speed Counter-Current Chromatography Based on COSMO-RS Model

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ARTICLE INFO

Keywords:
Flavonoids
Isoflavones
Sophora japonica
Counter-current chromatography
Fruit

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Received: April 2, 2021
Revised: August 5, 2021
Accepted: August 17, 2021

ABSTRACT

Background: *Sophora japonica* L. (*Leguminosae*), a well-known traditional medicine in Asia, is officially listed in the Chinese and European Pharmacopoeia. Its buds and fruits have been used as medicinal agents with significant biological activity. Pharmacologic studies and clinical practice have demonstrated that they have beneficial effects in the treatment of many diseases. Herein, we developed a simpler and effective separation method of flavones and isoflavones from *S. japonica* fruit by one-step HSCCC based on the COSMO-RS model.

Methods: HSCCC was applied for the preparative separation and purification of five active compounds from *S. japonica* L. fruit (three flavone glycosides and two isoflavone glycosides). Under the assistance of a COSMO-RS, one-step separation with the two-elution solvent system that was designed.

Results: The first elution was conducted with the lower phase of ethyl acetate-*n*-butanol-water (5:1:5, v/v), and the second elution was performed with the same one containing 4.0 mmol L^{-1} of $NH_3 \cdot H_2O$. From 200 mg of crude extract, 11.2 mg of kaempferol-3-O-sophoroside (I), 3.2 mg of rutin (II), 3.4 mg of kaempferol-3-O-α-L-ramnopyranosyl-(1–6)-β-D-glucopyranoside (III), 11.5 mg of sophorabioside (IV), and 10.1 mg of sophoricoside (V) were obtained. Their purity values were 98.1%, 97.6%, 99.5%, 99.2%, and 98.3%, respectively.

Conclusion: The five targets of I-V can be used as reference substances for chromatographic purposes as well as for the further physiological studies. The developed method is instructive for the separation of other flavonoids too.

Please cite this article as: Wang Z, Liu Y, Fan C, Cao X. One-Step Preparative Separation of Flavone and Isoflavone Glycosides from *Sophora Japonica* Fruit by High-Speed Counter-Current Chromatography Based on COSMO-RS Model. Int J Nutr Sci. 2021;6(3):148-154. doi: 10.30476/IJNS.2021.92070.1146.

Introduction

Sophora japonica L. (*Leguminosae*), a well-known traditional medicine in Asia, is officially listed in the Chinese and European Pharmacopoeia (1). Its buds and fruits have been used as medicinal agents with significant biological activity. Pharmacologic

studies and clinical practice have demonstrated that they have beneficial effects in the treatment of bleeding hemorrhoids, hematemesis, hematuria, hemorrhinia, uterine or intestinal hemorrhage, leukorrhea, metrorrhagia, pyoderma, hypertension, conjunctivitis, arteriosclerosis, and dizziness (1-3). Besides, it can prevent weight gain in high-fat diet-induced obese mice (4). The main components of *S. japonica* L. are flavones and isoflavones or their glycosides, such as rutin, sophorabioside, and sophoricoside (5-7). Sophoricoside is an isoflavone glycoside present in *S. japonica*. It has been known for its anti-inflammatory, anti-oxidant, anti-diabetic, estrogenic activity, and immunomodulatory activity (8). Sophoricoside also has a hepatic protective effect in high fructose (HF) diet-fed mice, which can be a novel natural flavonoid for alleviating HF-induced liver injury (9). These bioactive substances can interact with other compounds, such as protein, carbohydrate, acid, and so on. Component interaction is closely related to the change of characteristics in flavonoids. *In vivo*, it may change a variety of flavonoid's physiological activities (10).

There are two methods for the isolation and purification of flavonoid compounds from *S. japonica* L. including conventional column chromatography and high-speed counter-current chromatography (HSCCC); while the former one requires tedious procedures resulting in low recoveries of the products. HSCCC, a support free liquid-liquid partition chromatographic technique eliminates the irreversible adsorption of the compounds to solid supports and has an excellent sample recovery when compared to some conventional methods (11). It is used more and more frequently in separation of various natural and synthetic products (12-14).

Herein, a separation method of five flavonoid compounds from *S. japonica* by HSCCC was proposed (15). However, it contained several steps including macroporous resin column separation and two-step HSCCC separation methods using different solvent systems. The conductor-like screening model for real solvents (COSMO-RS) is a kind of molecular modeling tool combining quantum chemical calculation with statistical thermodynamics (16). It can predict partition coefficients and efficiently selects solvents without experimental data (17). Herein, we developed a simpler and effective separation method of flavones and isoflavones from *S. japonica* fruit by one-step HSCCC based on the COSMO-RS model.

Materials and Methods

Ethanol, ethyl acetate, *n*-butanol, aqueous ammonia ($NH_3 \cdot H_2O$, 25%, w/w) were analytical grade and purchased from Beijing Chemical Works (Beijing, China). Methanol applied for high performance liquid chromatography (HPLC) analysis was HPLC grade (Fisher, America). Ultrapure water obtained by Milli-Q water purification system (18.2 Ω m^{-1}, Millipore, USA) was applied for all solutions and dilutions. The dried fruits of *S. japonica* L. (herbarium number: 890180-2) were purchased from a drug store (Beijing Tong Ren Tang Chinese Medicine Co., Ltd).

HSCCC instrument named TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation columns connected in series (I.D. of the tubing=1.6 mm, total volume=260 mL) and a 20 mL sample loop was employed. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal (β=r/R, where r is the distance from the coil to the holder shaft). The revolution speed of the apparatus could be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulating instrument (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the experimental temperature. The HSCCC system was equipped with a constant-flow pump, an 8823B-UV Monitor at 254 nm, and a N2000 workstation (Zhejiang University, Hangzhou, China).

The high performance liquid chromatography (HPLC) equipment (Agilent 1100 HPLC system) was consisted of quaternary Pump (G1311A), DAD detector (G1315B), injection valve (Rheodyne 7725i) with a 20 μL loop, degasser (G1332), and HPLC workstation (Agilent). The nuclear magnetic resonance (NMR) spectrometer was utilized in the study (Bruker AV 600 NMR system, Bruker Inc., Germany). Plant disintegrator (HY-04B Beijing Huanyatiayuan mechanical technology Co., LTD, Beijing, China) was applied for the sample disintegration.

Sample preparation was done according to the previous published work (15). The dried fruit of *S. japonica* L. was ground to about 40 mesh by the plant disintegrator (HY-04B), The powder (20 g) was extracted twice (40 min each time, 40°C) with 100 mL of 70% ethanol by ultrasonic cleaning instrument sonication (KQ-400DB, 40 KHZ, 400W, KunShan Ultrasonic Instruments Co., Ltd., Kunshan, China). The extracts were combined and evaporated to dryness by rotary evaporation at 45°C under reduced pressure. About crude sample (10 g)

was obtained and stored in a refrigerator (4°C) for further separation.

The selection of a two-phase solvent system was entirely conducted with the BIOVIA software (COSMOtherm 2020, version 20.0). The geometry optimization and screening charge density of all molecular structures were carried out using BIOVIA (Tmolex 2021, version 21.0) with basis set (BP86/TZVP). The equilibrium compositions of each phase in solvent systems were obtained by the liquid extraction module. Partition coefficient (K) and resolution (α) values were predicted based on the reported work (18, 19). Dissociation constants (p*K*a or p*K*b) values of modifier were considered during the calculations (acetic acid: 4.79; ammonia: 4.74).

The two-phase solvent system in the present study was ethyl acetate-*n*-butanol-water (5:1:5, v/v). The lower phase was used as the mobile phase a, and the upper phase was used as the stationary phase. The mobile phase b was prepared by mixing the mobile phase a and $NH_3 \cdot H_2O$. The final concentration of ammonia was 4.0 mmol L^{-1}. First, the coiled column was entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 850 rpm, while the lower phase was pumped into the column at a flow rate of 2.0 mL min^{-1}. After reaching the hydrodynamic equilibrium, the sample solution was injected through the injection valve. The sample solution was prepared by dissolving the crude extract (200 mg) in a solution composed of the upper and lower phases (8 mL, 1:1, v/v). The effluent from the outlet of the column was continuously monitored at 254 nm with a UV detector after sample loading. Mobile phase a was changed to mobile phase b and the flow rate was increased to 3.0 mL min^{-1} at 290 min for shortening the separation time. Peak fractions were collected according to the elution profile.

The crude sample and each peak fraction obtained by HSCCC were analyzed by HPLC. HPLC analysis was performed (Ultimate XB-C18, 250 mm × 4.6 mm i.d. 5 μm, Welch Materials, Inc.) at 25°C.

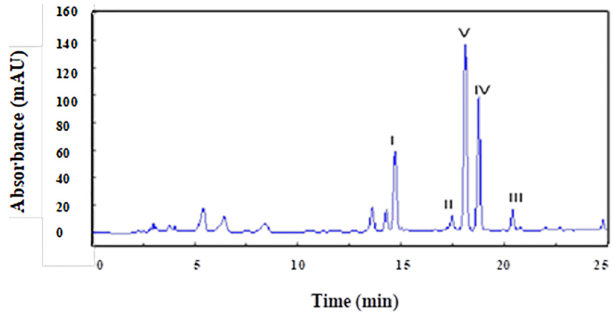

Figure 1: Chemical structures and sigma surfaces of target compounds.

Methanol (A) and water (B) were utilized as the mobile phase in the gradient-elution mode as 0-5 min, 30% A; 5-15 min, 30-50% A; and 15-30 min, 50-100% A. The flow rate of the mobile phase was 1.0 mL min^{-1}. The effluents were monitored at 280 nm. The identification of HSCCC peak fractions was carried out by electrospray mass spectrometry (ESI-MS) and NMR.

Results

As illustrated in Figure 1 for selection of two-phase solvent systems by COSMO-RS model, the crude extract contained several compounds, which were almost consistent with the reported work (20). Then these compounds were identified by ESI-MS. The preliminary structures could be obtained as I, $C_{27}H_{30}O_{16}$, 609 (M-H); II, $C_{27}H_{30}O_{16}$, 609 (M-H); III, $C_{27}H_{30}O_{15}$, 593 (M-H); IV, $C_{27}H_{30}O_{14}$, 577 (M-H); V, $C_{21}H_{20}O_{10}$, 431 (M-H). After quantum chemistry calculation, their sigma surfaces were shown in Figure 2. A series of solvent systems were chosen as candidates according to the published literature (15, 20). Table 1 shows the predicted K values in different solvent systems. K values of five target compounds were presented. The *n*-heptane-ethyl acetate-methanol-water (1:5:0.5:6, v/v) quaternary solvent system presented low retention capacity for four target compounds, and this would result in rapid elution and poor resolution. The K value

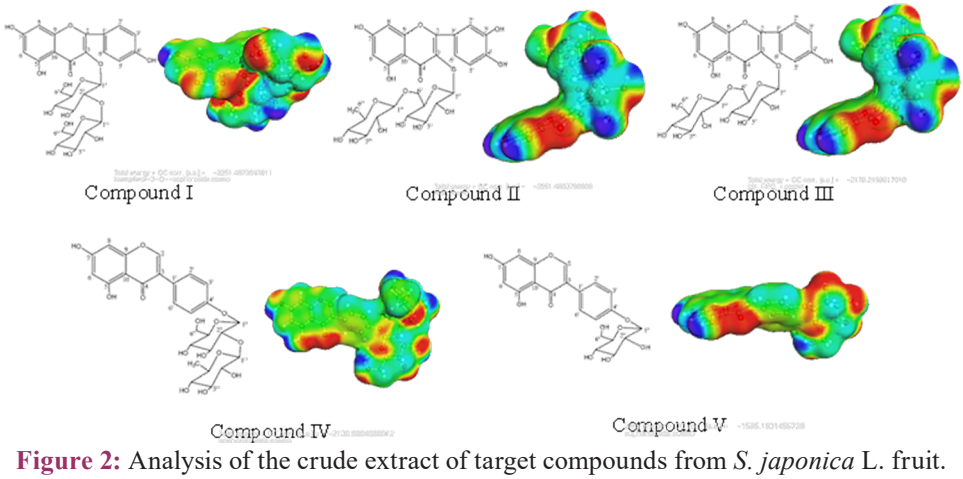

Figure 2: Analysis of the crude extract of target compounds from *S. japonica* L. fruit.

Table 1: Predicted partition coefficient (K) values of targets by different solvent systems.

Solvent systems	Volume ratio (v/v)	K_I	K_{II}	K_{III}	K_{IV}	K_V
n-heptane-ethyl acetate-methanol-water	1:5:0.5:6	0.31	0.17	0.35	0.20	42.98
Ethyl acetate-water	5:5	3.57	2.96	3.63	5.53	6.08
Ethyl acetate-methanol-water	5:1:5	2.12	2.06	2.11	2.21	2.26
Ethyl acetate-*n*-butanol-water	5:1:5	4.04	3.52	3.98	4.31	4.64
Ethyl acetate-*n*-butanol-water (2 mmol L^{-1} acetic acid)	5:1:5	4.09	3.78	4.04	4.2	4.54
Ethyl acetate-*n*-butanol-water (2 mmol L^{-1} $NH_3 \cdot H_2O$)	5:1:5	4.08	3.68	4.04	4.13	4.46
Ethyl acetate-*n*-butanol-water (4 mmol L^{-1} $NH_3 \cdot H_2O$)	5:1:5	3.97	3.63	3.92	4.11	4.45

I: Kaempferol-3-O-sophoroside; II: Rutin; III: Kaempferol-3-O-α-L-ramnopyranosyl-(1–6)-β-D-glucopyranoside; IV: Sophorabioside; V: Sophoricoside.

Table 2: Predicted resolution (α) values of targets by different solvent systems.

Solvent systems	Volume ratio (v/v)	$\alpha_{II/I}$	$\alpha_{III/II}$	$\alpha_{IV/III}$	$\alpha_{V/IV}$
Ethyl acetate-methanol-water	5:1:5	1.03	1.03	1.05	1.02
Ethyl acetate-*n*-butanol-water	5:1:5	1.15	1.13	1.08	1.08
Ethyl acetate-*n*-butanol-water (2 mmol L^{-1} $NH_3 \cdot H_2O$)	5:1:5	1.11	1.10	1.02	1.08
Ethyl acetate-*n*-butanol-water (4 mmol L^{-1} $NH_3 \cdot H_2O$)	5:1:5	1.09	1.08	1.05	1.08

I: Kaempferol-3-O-sophoroside; II: Rutin; III: Kaempferol-3-O-α-L-ramnopyranosyl-(1–6)-β-D-glucopyranoside; IV: Sophorabioside; V: Sophoricoside.

of the compound V was large in the quaternary solvent system, and the binary one (ethyl acetate-water) shared the same problem.

This indicated the strong retention capacity in the stationary phase and long-time elution during the separation. The predicted α values in Table 2 showed that ethyl acetate-*n*-butanol-water was better than ethyl acetate-methanol-water. Furthermore, the addition of modifiers in ethyl acetate-*n*-butanol-water solvent system reduced the K values (especially for the compound V). Additionally, we could also find that the α values of the compound I, II, III, IV decreased with an increase in the concentration of ammonia and that of the compound V remained unchanged. The $NH_3 \cdot H_2O$ with lower K values toward targets was finally selected as a modifier in the solvent system. Regarding separation by high-speed counter-current chromatography, when *n*-hexane-ethyl acetate-methanol-water (1:5:0.5:6, v/v) was used as the two-phase system, the entire elution process took about 2 hours. However, the chromatogram (Figure 3) shows merely two main peaks. Moreover, as can be seen from Figure 4 (A), ethyl acetate-*n*-butanol-water (5:1:5, v/v) ternary solvent system is suitable for the separation of the compound I, II, III, IV.

However, there was still an obvious impurity in the front of the compound V. Meanwhile, it took almost 10 hours to complete the whole elution of five targets. The five target compounds were successfully separated, and the elution time was almost 8 hours with a flow rate of 2.0 mL min^{-1}. Different flow rates (2.0 and 3.0 mL min^{-1}) were evaluated. However, it was found that the compounds I, II, III, and IV

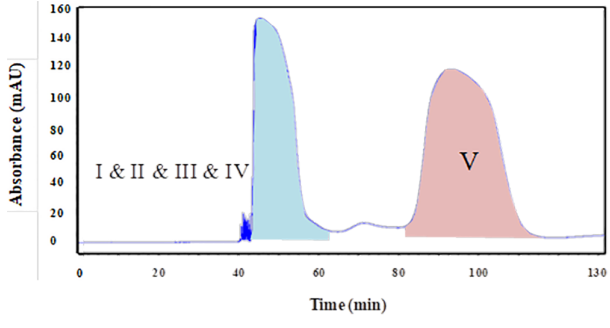

Figure 3: The chromatogram of separating target compounds by counter-current chromatography with solvent system *n*-hexane-ethyl acetate-methanol-water (1:5:0.5:6, v/v). Conditions: column volume: 260 mL; flow rate: 2 mL min^{-1}; rotation speed: 850 rpm; sample loading amount: 100 mg; detection: 254 nm; retention of stationary phase: 77.8%.

cannot get complete separation when the two elution processes all used 3.0 mL min^{-1}. It took less than 7 hours for the whole HSCCC separation by stepwise increasing the flow rate to 3.0 mL min^{-1}, and the mobile phase was changed at the same time (Figure 4 (B)). Five fractions (I: 11.2 mg, II: 3.2 mg, III: 3.4 mg, IV: 11.5 mg, and V: 10.1 mg) were obtained from 200 mg crude extract in almost 400 minutes. Their purity values were 98.1%, 97.6%, 99.5%, 99.2%, and 98.3%, respectively (Figure 4 (C)). The chemical structures of the five targeted compounds isolated by HSCCC were identified by ^{1}H-NMR and ^{13}C-NMR (Supplementary Materials).

Discussion

Partition coefficient (K) is the most important factor in the counter-current chromatography (CCC)

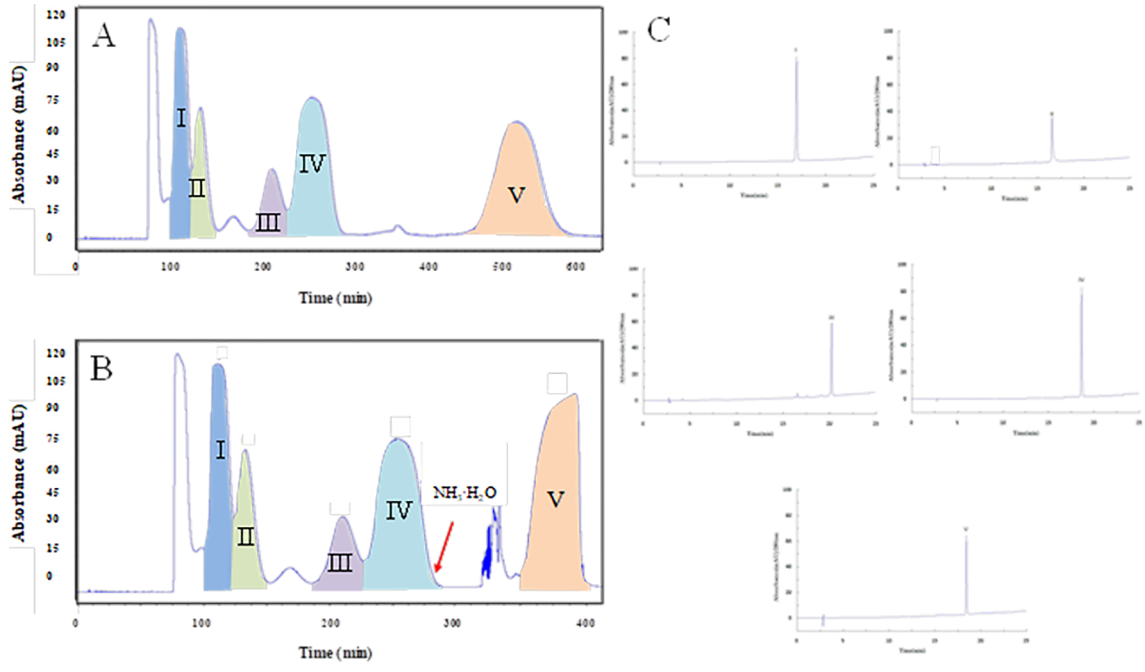

Figure 4: The chromatogram of separating target compounds by counter-current chromatography with mobile phase **a** (A), mobile phase **a** and **b** (B) and high performance liquid chromatography (HPLC) analysis of each fractions (C). Conditions: solvent system: mobile phase **a** (ethyl acetate-*n*-butanol-water, 5:1:5, v/v) and mobile phase **b** (ethyl acetate-*n*-butanol-4 mmol L^{-1} $NH_3 \cdot H_2O$, 5:1:5, v/v); column volume: 260 mL; flow rate: 2 mL min^{-1} for mobile phase **a** and 3 mL min^{-1} for mobile phase **b**; rotation speed: 850 rpm; sample loading amount: 200 mg; detection: 254 nm; retention of stationary phase: 52.0% for mobile phase **a** and 53.7% for mobile phase **b**.

separation experiment, since the retention of different targets mainly depends on K. Targets with smaller K values that are quickly eluted with the selected mobile phase, while those with larger K values will have longer retention time (21). The ammonium hydroxide was as an eluter in the mobile phase. Increasing the concentration of eluter will result in a higher concentration in eluted fraction and shorter retention time of the analyte (22). Therefore, a one-step separation procedure with the two elution solvent systems can be designed based on the prediction results of COSMO-RS. The first elution can be conducted with the lower phase of ethyl acetate-*n*-butanol-water (5:1:5, v/v); and the second elution can be performed with the same lower phase containing 4.0 mmol L^{-1} of $NH_3 \cdot H_2O$.

In the CCC separation experiment, the HPLC analysis demonstrated that the compound I, II, III, and IV gathered in the front peak, and the compound V was in the second peak without satisfied purity. This was consistent with the predicted results obtained by COSMO-RS. In order to obtain the compound V with high purity, the mobile phase was changed to the modified one (acetic acid, 2 mmol L^{-1}) after the elution of the compound IV. But it cannot get satisfactory separation effects. On the contrary, when aqueous ammonia (2 mmol L^{-1}) was employed in the selected system, an obvious separation trend between the compound V and the main impurity was observed. To shorten the separation time and get rid of the interference, the amount of $NH_3 \cdot H_2O$ was increased to 4.0 mmol L^{-1}.

Conclusion

In this study, three flavonoid glycosides (kaempferol-3-O-sophoroside, rutin, and kaempferol-3-O-α-L-ramnopyranosyl-(1–6)-β-D-glucopyranoside) and two isoflavonoid glycosides (sophorabioside, and sophoricoside) of *S. japonica* L. fruit were successfully isolated and separated by HSCCC in one single run. The separation procedure with two elution solvent systems was designed with the assistance of COSMO-RS. The first elution was conducted with the lower phase of ethyl acetate-*n*-butanol-water (5:1:5, v/v), and the second elution was performed with the same lower phase containing 4.0 mmol L^{-1} of $NH_3 \cdot H_2O$. With increasing the flow rate from 2.0 to 3.0 mL min^{-1} after peak IV, the entire separation process took less than 7 hours. The purity values of obtained targets were over 97%, which can be used as reference substances for chromatographic purposes as well as for the further physiological studies. It seems that the developed method is instructive for the separation of other flavonoids too.

Acknowledgement

The authors acknowledge financial support of this work by National Natural Science Foundation of China (32102069) and Beijing Natural Science

Foundation and Beijing Municipal Education Committee (KZ202010011017).

Conflict of Interest

None declared.

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