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Research Article

Efficient purification of high-purity compounds from the stem of *Lonicera japonica* Thunb using two-dimensional preparative chromatography

Purification of high-purity compounds from traditional Chinese medicines (TCMs) plays an important role in investigating their bioactivity. Nevertheless, it is often quite difficult to isolate compounds with high purity because of the complexity of TCMs in chemical composition. In this work, a two-dimensional preparation method was successfully developed for the preparation of high-purity compounds from the stem of *Lonicera japonica* Thunb, based on two novel polar copolymerized RP stationary phases, XAqua C3 and XAqua C18. An XAqua C3 prep column was used to separate the sample in the first-dimensional preparation, and 14 g of sample was fractionated into eight fractions with a recovery of 82%. An XAqua C18 prep column was selected to prepare high-purity compounds in the second-dimensional preparation for its good orthogonality with the XAqua C3 stationary phase. As a result, major compounds in the sample were isolated with more than 99% purity. This method is a potent method to realize the efficient purification of compounds with high purity from the stem of *L. japonica* Thunb and it shows great potential in the separation of high-purity compounds from complex samples.

Keywords: High-purity compound / *Lonicera japonica* Thunb / Two-dimensional preparation / Traditional Chinese medicine
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1 Introduction

Lonicera japonica Thunb. (Caprifoliaceae), also known as Ren Dong, Japanese honeysuckle and Jin Yin Hua, is one of the most widely used traditional Chinese medicines (TCMs). Since 1995, *L. japonica* has been listed in the Pharmacopoeia of the People's Republic of China and more than 500 prescriptions containing *L. japonica* are used to treat various diseases in China. Modern pharmacological studies have proved that *L. japonica* possesses many biological functions, including cytoprotective [1], hepatoprotective [2], anti-inflammatory [3], antiviral [4], antibacterial [5], and antioxidant activity [6]. However, the extract of *L. japonica* is so complex that the chemical compositions responsible for the therapeutic effects are unclear yet. It is desirable to obtain compounds in *L. japonica* with enough purity and quantity for pharmacological activity research.

To date, isolating compounds from *L. japonica* still relies on traditional separation techniques [7–11]. These techniques are time consuming and laborious owing to successive extractions with solvents and consecutive column isolations. In recent years, many modern separation techniques have been utilized to prepare compounds from *L. japonica*, like high-speed counter-current chromatography [12, 13]. Although it gives high-separation efficiency, it performs separations over a period of hours, rather than tens of minutes typical for HPLC. Currently, preparative HPLC (prep-HPLC), as one of the most efficient techniques for the preparation of compounds in complex samples, provides a lot of advantages over other chromatographic techniques, such as high efficiency, high resolution, and good repeatability. It takes advantages of high-performance separation, online detection, and automatic control to the realize efficient preparation of target compounds [14]. Accordingly, prep-HPLC has received increasing attention from many phytochemists and pharmaceutical companies.

RPLC is a dominant separation mode and by estimation about 80% of all HPLC separations are presently performed by this technique, thanks to its high capacity, universality and

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Abbreviation: TCM, traditional Chinese medicine

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reproducibility [15]. It has been wildly applied in the separation of compounds from TCMs. However, a TCM sample may comprise hundreds and thousands of chemical constituents. It is tough to obtain high-purity compounds employing only 1D preparation due to limited chromatographic resolution and peak capacity.

Nowadays, two-dimensional HPLC (2D HPLC) has been perceived as a powerful tool to separate compounds from complex samples, since it made great improvement in separation selectivity and peak capacity [16, 17]. It has been proved that 2D-RP/RPLC is a practical separation method for the isolation of compounds from complex mixtures owing to its high-separation efficiency and broadest application [18, 19]. Nevertheless, 2D-RP/RPLC presents quite a challenge in terms of finding column combinations that are sufficiently orthogonal, which is a significant obstacle to the wider application of 2D-RP/RPLC [20]. Our previous work demonstrated that developing novel RP columns might be helpful to solve that problem [21, 22]. Besides, it is tough to separate polar compounds using 2D-RP/RPLC, due to poor retention of polar compounds on the RP columns [23]. In this study, based on two novel polar copolymerized reversed-phase stationary phases that largely improve the retention and selectivity for polar compounds [24], we have developed a preparative 2D-RP/RPLC method to realize high-performance separation of high-purity compounds from the target constituents in *L. japonica* Thunb, which will facilitate the study of its pharmacological activities.

2 Materials and methods

2.1 Apparatus and reagents

An industrial level prep-HPLC system was assembled in our laboratory. The system consists of two prep-HPLC pumps (DEAIC P280), a UV detector, a sample injector (Rheodyne 7725i) and a HPLC workstation (DEAIC EC2000).

Purification Factory is a preparative LC system, which consists of two 2525 binary gradient module (Waters, Milford, MA, USA), autosampler (Leap Technologies, Carrboro, NC, USA), a 2498 UV detector (Waters), and MassLynx software (Waters, V. 4.1).

Chromatographic analysis and separation were performed on an Alliance HPLC system consisting of a Waters 2695 HPLC pump and a Waters 2996 photodiode array detector. Data acquisition and processing were conducted by Waters Empower software.

Identification of pure compounds was carried out using MS and NMR spectroscopy. Mass spectrometry was performed on a Q-TOF Premier^{TM} instrument (Waters MS Technologies, Manchester, UK). ^{13}C-NMR spectra were measured on a Bruker DRX-400 spectrometer (^{13}C-NMR at 100 MHz), with CH_3OD as solvent.

All the columns used in this work were purchased from Acchrom (Beijing, China) and listed as follows: XUnion C18 (4.6 × 250 mm, 10 μm), XAqua C3 (4.6 × 250 mm, 10 μm), XAqua C18 (4.6 × 250 mm, 10 μm), XAqua C18 (4.6 × 150 mm, 5 μm), XAqua C3 (50 × 500 mm, 40 μm), XAqua C3 (100 × 260 mm, 15 μm), and XAqua C18 (20 × 250 mm, 10 μm).

Ethanol (95%) and methanol were of industrial grade. Methanol used for analytical HPLC was of chromatographic grade (Yuwang, China). Formic acid for prep-HPLC and analytical HPLC was of analytical grade (Supervision of Tianjin Kermel Chemical Reagents Development Centre) and HPLC grade (ACROS Organics, USA), respectively. Water for analysis and preparation was reverse osmosis Milli-Q Water (18.2 MΩ).

2.2 Sample preparation

The stem of *L. japonica* Thunb was collected from ShanDong province and authenticated by Mr. Xiaoping Yang, Xiyuan Hospital of China Academy of Traditional Chinese Medicine. One kilogram of the herb was decocted in 10 L of water for 60 min three times. After filtration, the three decoctions were combined and concentrated by rotary evaporation at 60°C in vacuum. The extract was dissolved in water/ethanol 30:70 v/v and kept for 24 h at 4°C. Then, the supernatant was rotary evaporated to the concentration at about 200 mg/mL and filtered through 0.45 μm membranes. The 500 mL of aqueous solution obtained from the extraction described above was prepared with five injections on an XAqua C3 prep column (50 × 500 mm, 40 μm), preconditioned successively with ethanol and distilled water (0.2% formic acid). The high-polarity constituents were removed by washing with distilled water (0.2% formic acid). Then the target constituents were eluted with 40% ethanol. The weak-polarity constituents were removed by elution with 90% ethanol. The target constituents were concentrated by rotary evaporation at 60°C in vacuum for the subsequent preparation.

2.3 Chromatographic conditions

The first-dimensional preparation was performed on an XAqua C3 prep column (100 × 260 mm, 15 μm). The mobile phase A1 was 0.2% v/v formic acid in water, and B1 was methanol. Gradient elution steps were as follows: 0–15 min, 10% B1; 15–37 min, 15% B1; 37–65 min, 20% B1; 65–75 min, 90% B1. The time of equilibrium took 15 min. The flow-rate was 330 mL/min. The Chromatogram was recorded at 255 nm.

The second-dimensional preparation was performed on an XAqua C18 prep column (20 × 250 mm, 10 μm). The mobile phases A2 and B2 were the same as A1 and B1, respectively. Different isocratic elution conditions were adopted to separate fractions collected from the first-dimensional preparation. Isocratic elution for fraction 1 was 30 min, 18% B2, for fraction 2 was 30 min, 23% B2, for fraction 3 was 30 min, 25% B2, for fraction 7 was 30 min, 28% B2 and for fraction 13 was 30 min, 30% B2. The flow-rate was 20 mL/min. The chromatogram was recorded at 255 nm.

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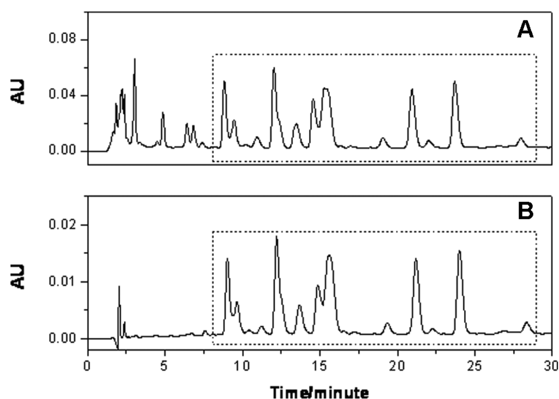

Figure 1. HPLC chromatograms for SPE fractions of *L. japonica* on XAqua C18 column (4.6 × 150 mm, 5 μm). (A) Crude sample; (B) 40% ethanol elution.

HPLC analysis of each fraction and the pure compounds was performed on an XAqua C18 column (4.6 × 150 mm, 5 μm). The flow-rate was 1 mL/min. The column temperature was kept at 30°C and the effluent was recorded at 255 nm. Mobile phase A was 0.2% v/v formic acid in water, and B was methanol. Gradient elution steps were as follows: 0–30 min, 15–30% B; 30–40 min, 90% B.

3 Results and discussion

3.1 Column selection

The main components of sample are kinds of relatively polar and medium polarity components in the extract. Prior to the 2D preparation, it is necessary to remove the other parts from the *L. japonica* extract. SPE would be an efficient method to solve this problem. As can be seen in Fig. 1, the dashed part was determined as main constituents of sample, which were totally eluted by 40% ethanol. The complexity of crude sample was reduced by means of SPE.

For the sake of constructing an orthogonal 2D-RP/RPLC to realize efficient preparation of high-purity compounds, several RP columns were tested to separate the compounds in the sample (Fig. 2). As can be seen in Fig. 2A, the target constituents were poorly retained on the XUnion C18 column. Contrastively, satisfactory separation qualities were obtained on the XAqua C3 and XAqua C18 columns (Fig. 2B and C), and distinct chromatographic patterns were observed on these two columns, indicating that they might exhibit different selectivity for the separation of compounds in the sample. According to the manufacturer, XAqua C3 and XAqua C18 are both polar copolymerized RP columns, but contain different contents of polar groups and types of alkyl chains. Hence, we speculated that the RP/RPLC based on these two columns could perform different selectivity in the sample separation. The XAqua C3 column was utilized in first-dimensional preparation to separate the sample. Subsequently, the XAqua C18 column was used in the second-dimensional preparation to prepare compounds with high purity for its high-separation efficiency and different selectivity.

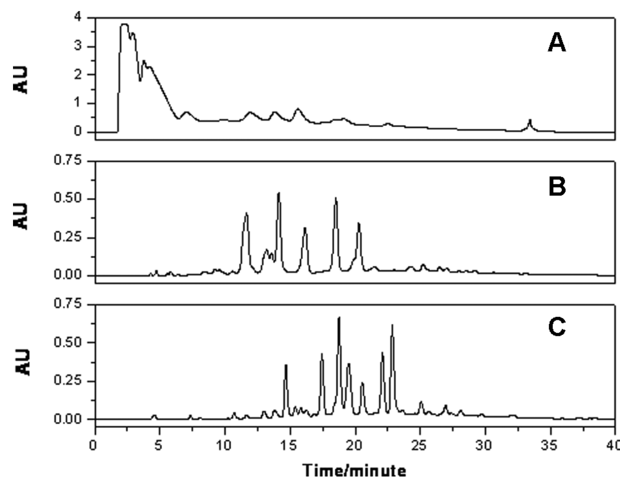

Figure 2. Unidimensional chromatograms of sample on (A) XUnion C18 column (4.6 × 250 mm, 10 μm); (B) XAqua C3 column (4.6 × 250 mm, 10 μm); (C) XAqua C18 column (4.6 × 250 mm, 10 μm). Conditions: mobile phase A: 0.2% formic acid aqueous solution, and B: MeOH; gradient: 0–40 min, 5%→70% B. Other conditions: the monitoring wavelength: 255 nm; flow rate: 1 mL/min and injection volume is 10 μL; column temperature: 30°C.

3.2 First-dimensional preparation

The chromatographic conditions of the first-dimensional preparation, including sampling amount, solvent flow rate, and gradient condition, were optimized in this work. Under the optimized conditions, the first-dimensional preparation was performed on an XAqua C3 prep column (Fig. 3). The sample loading was about 7 g. The fractions were collected according to their UV absorption intensity to improve the purity of compounds as much as possible. The 14 g sample was fractionated into eight fractions (fractions 1–8) with a recovery of 82%. Preparative columns with large id offered adequate sample loading, which was helpful to improve preparation efficiency. All fractions were dried by lyophilization. The weight of fractions 1, 2, 3, 7, and 8 was at the range from 0.4 to 1.5 g, and those would be separated in subsequent preparations.

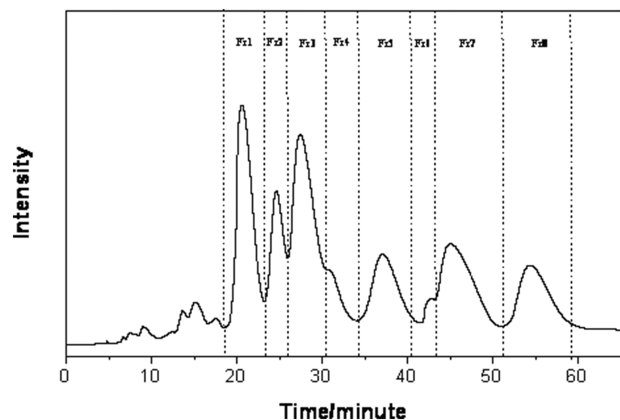

Figure 3. First-dimensional preparation of sample on XAqua C3 prep column.

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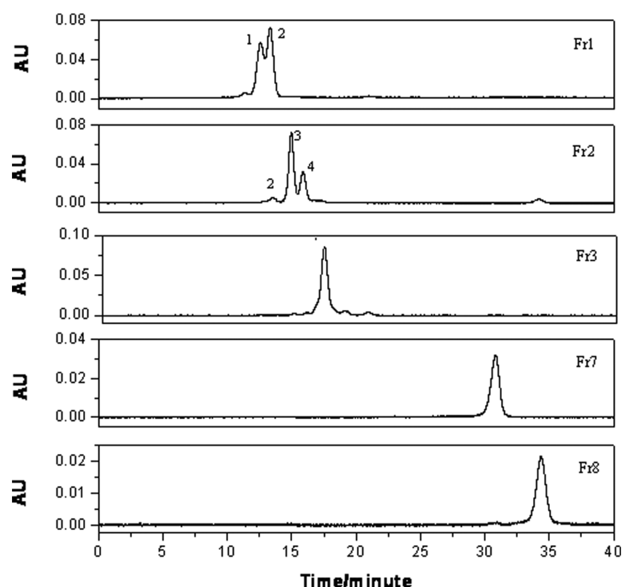

Figure 4. HPLC analysis of fractions 1, 2, 3, 7, and 8 on XAqua C3 column (4.6 × 250 mm, 10 μm). Conditions: mobile phase A: 0.2% formic acid aqueous solution, and B: MeOH; gradient: 0–10 min, 10% B; 10–25 min, 15% B; 25–40 min, 20% B. Other conditions are the same as those in Fig. 2.

3.3 Analysis of collected fractions on XAqua C3 column and XAqua C18 column

Fractions 1, 2, 3, 7, and 8 were analyzed on the XAqua C3 column and XAqua C18 column, respectively (Figs. 4 and 5). The comparison result between Figs. 4 and 5 showed that the elution order in these chromatograms was different from each other and much more peaks were observed on the XAqua C18 column compared to the XAqua C3 column, indicating that the 2D-RP/RPLC based on these two columns was reasonably orthogonal as expected [25]. It was noticed that good orthogonality was significant to achieve efficient preparation of high-purity compounds from the sample. For instance, peaks 1 and 2 in fraction 1 had a close retention time on the XAqua C3 column, but obtained satisfactory resolution on the Xaqua C18 column. These two compounds could be easily prepared with high purity in the second-dimensional preparation. If solely separated on the XAqua C3 column, these two peaks were tough to separate and collecte with favorable purity. Similar phenomena were observed with fractions 2 and 3. The coeluted compounds on the XAqua C3 column could be separated on the XAqua C18 column with satisfactory resolution benefiting from the different separation selectivity of the two columns. For instance, fraction 8 was a single peak on the XAqua C3 column. It might be regarded as high-purity compound in traditional separation techniques, where unidimensional HPLC preparation was commonly used as the final step to yield pure compounds. However, when fraction 8 was analyzed on the XAqua C18 column, about six peaks coeluted on the XAqua C3 column scattered in the chromatogram. Peaks 12 and 14 in fraction 8 could be directly prepared by optimizing chromatographic conditions. And some low-abundance components were able to be obtained by increasing sample amount. Thus, the XAqua C18 column would be utilized in the second-dimensional preparation for its different separation selectivity, which not only improved the preparation efficiency, but also provided a remarkable contribution to the purification of high-purity compounds from the complex sample. Besides, it was found that the purity of fraction 7 was more than 99% after the first-dimensional preparation as can be seen in Figs. 4 and 5.

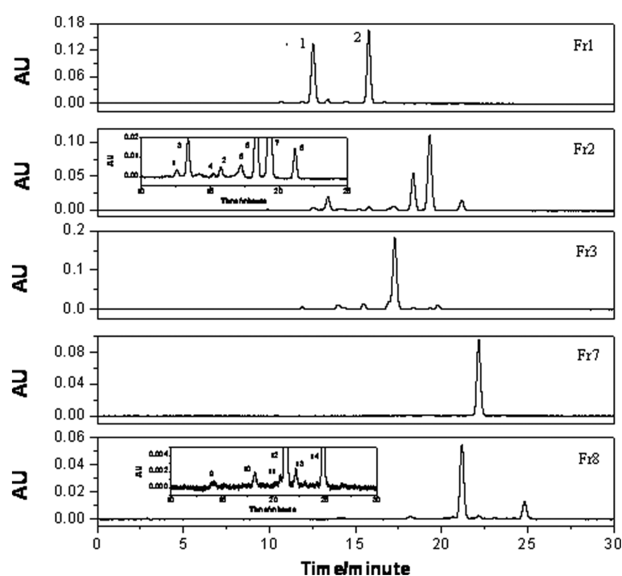

Figure 5. HPLC analysis of fractions 1, 2, 3, 7, and 8 on XAqua C18 column (4.6 × 250 mm, 10 μm). Conditions: mobile phase A: 0.2% formic acid aqueous solution, and B: MeOH; gradient: 0–30 min, 15%→50% B. Other conditions are the same as those in Fig. 2.

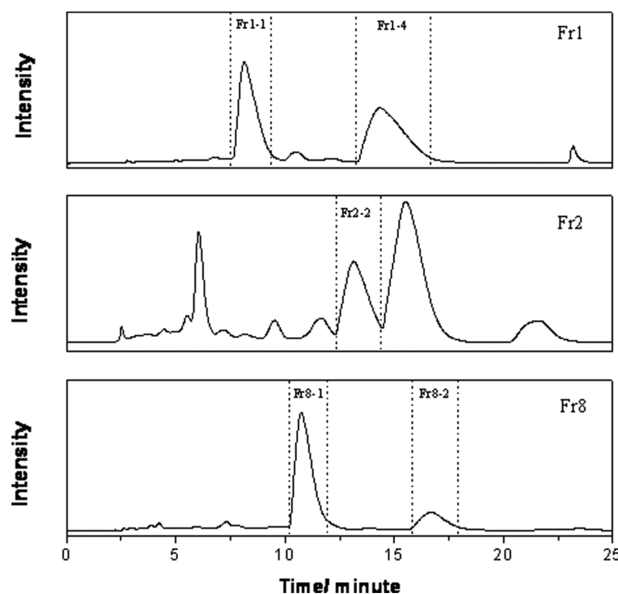

Figure 6. Second-dimensional preparation of fractions 1, 2, and 8 on XAqua C18 prep column.

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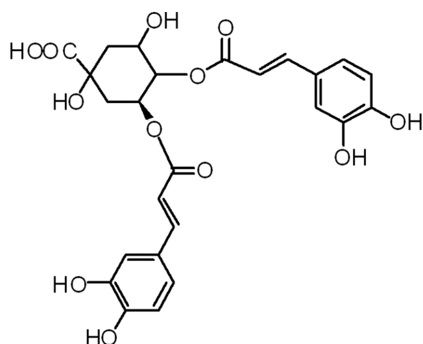
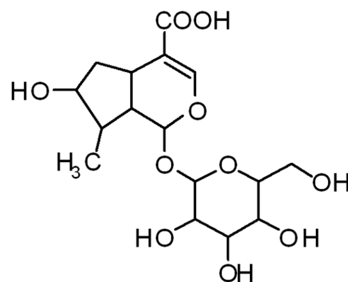
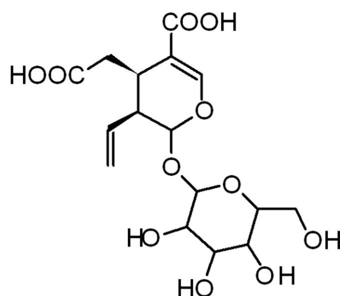


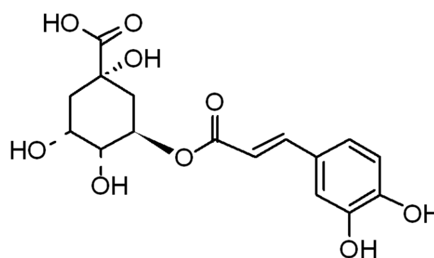
3,4-*O*-dicaffeoyl quinic acid
(Fr1-1)



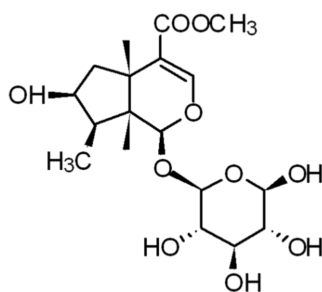
Loganic acid
(Fr1-4)



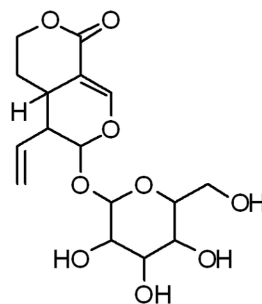
Secologanoside
(Fr2-2)



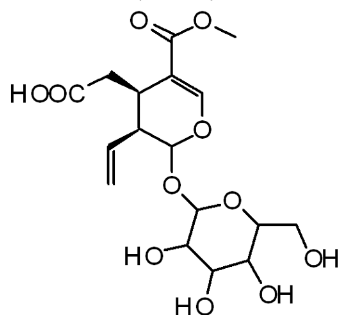
Chlorogenic acid
(Fr3-2)



Loganin
(Fr7-2)



Sweroside
(Fr8-1)



Secoxyloganin
(Fr8-2)

Figure 7. Structures of the separated compounds.

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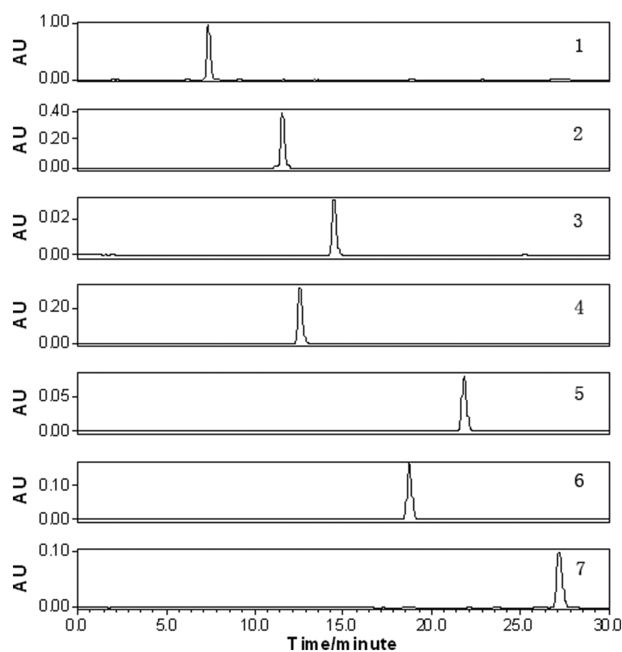

Figure 8. Purity evaluation of prepared compounds on XAqua C18 column (4.6 × 150 mm, 5 μm). Compounds 1 and 2 were isolated from the fraction 1; compound 3 was isolated from fraction 2; compound 4 was isolated from fraction 3; compound 5 was isolated from fraction 7; compounds 6 and 7 were isolated from fraction 8.

3.4 Second-dimensional preparation for compounds isolation

The second-dimensional preparation was performed on purification factory with an XAqua C18 prep column. It is necessary to optimize the chromatographic conditions to obtain satisfactory resolution. An approach used to transfer the operation conditions from analytical scale to preparative scale was developed in our previous work [26]. Based on the above method, the chromatographic conditions for each fraction were optimized on the XAqua C18 column, and then transferred to preparative scale with a minor revision. It was found that isocratic conditions were enough to provide satisfactory resolution for each fraction and were adopted in this work regarding eluent savings and avoiding time required for reconditioning in gradient elution mode. In addition, it is noteworthy that "heart-cutting" was used as the repeated separation strategy to insure the purity of compounds. The preparation chromatograms of fractions 1, 2, and 8 are shown in Fig. 6.

After isolating fractions 1, 2, and 8, five pure compounds were obtained with recoveries greater than 80% and dried by rotary evaporation at 60°C in vacuum. Although the purity of the fraction 3 was more than 80% after the first-dimensional preparation, it was purified in the second-dimensional preparation to improve the purity. In all, compounds **1** (Fr1-1, 154.6 mg), **2** (Fr1-4, 156.4 mg), **3** (Fr2-2, 44.9 mg), **4** (Fr3-1, 118.7 mg), **5** (Fr7, 440.9 mg), **6** (Fr8-1, 85.6 mg), and **7** (Fr8-2, 70.9 mg) had enough amount to be characterized by NMR spectroscopy. They were identified as 3,4-*O*-dicaffeoyl quinic acid, loganic acid, secologanoside, chlorogenic acid, loganin, sweroside, and secoxyloganin, respectively. The chemical structures of the prepared compounds are shown in Fig. 7 and details are listed in the Supporting Information. The purity of these compounds was checked by HPLC, shown in Fig. 8 and all compounds are of more than 99% purity.

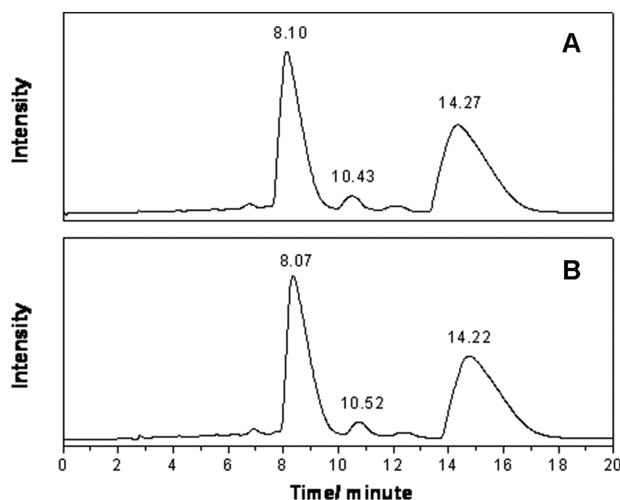

Figure 9. Second-dimensional preparation of fraction 1 on XAqua prep C18 column in different injections. (A) The 2nd injection and (B) the 12th injection.

Additionally, the repeatability of the system is another influential factor to prepare high-purity compounds, as good repeatability is able to minimize the cross of fractions as far as possible. It was investigated in this study. Taking fraction 2 as an example (Fig. 9), the values illustrated that the repeatability of the system was excellent. Minor compounds could be enriched by repeat collection as well.

4 Concluding remarks

A practical 2D-preparation method was developed for the purification of high-purity compounds from the target constituents in *L. japonica* Thunb. An XAqua C3 prep column was used to simplify the sample in the first-dimensional preparation. Then an XAqua C18 prep column was adopted to prepare compounds with high purity in the second-dimensional preparation. Five fractions collected from the first-dimensional preparation were analyzed on the XAqua C3 column and XAqua C18 column, showed good orthogonality of 2D preparation. And even coeluted compounds on the XAqua C3 column could be separated on the XAqua C18 column, which was attributed to the different separation selectivity of these two columns in terms of the sample separation. Benefiting from the good orthogonality and optimized collection mode, seven compounds with more than 99% purity were yielded in the second-dimensional preparation. And more pure compounds were able to be prepared and identified through increasing the sample amount. The overall results demonstrated that this method was quite

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useful for the preparation of high-purity compounds from polar and medium polarity constituents in *L. japonica*, but it might be relatively inefficient to separate other parts in the extract. We will develop appropriate methods to cope with them in our further study. More compounds with high purity could be isolated from TCMs using the established method, which would be helpful to explain pharmacological effects of TCMs to some extent.

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The authors have declared no conflict of interest.

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