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Short communication

Isolation and purification of iridoid glycosides from *Gardenia jasminoides* Ellis by isocratic reversed-phase two-dimensional preparative high-performance liquid chromatography with column switch technology

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Abstract

A two-dimensional column-switching system without sample loop trapping, where two columns were switched directly via a six-port two-position switching valve, was successfully applied for the first time to the isolation and purification of six iridoid glycosides including geniposide, gardenoside, shanzhiside, scandoside methyl ester, deacetyl-asperulosidic acid methyl ester and genipin-1-β-D-gentiobioside from *Gardenia jasminoides* Ellis, a plant used in the traditional Chinese medicine. The introduction of the six-port switching valve instead of sample loop assured 100% recovery from the first dimension to the second, and the injection volumes of the second dimension could reach 20 ml. In this mode of operation, the sample size of the two-dimensional approach was more than 1.3 times that of conventional gradient methods with even less solvent consumption. And the simultaneous operations of the two dimensions allowed the cycle time to be less than 19 min, compared with that (90 min) in the gradient elution single-dimension mode of operation. All of the six isolated iridoid glycosides were isolated at high purities of over 99% with approximately 96% recoveries.
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Keywords: Preparative chromatography; Column switch; *Gardenia jasminoides* Ellis; Iridoid glycoside; Two-dimensional chromatography

1. Introduction

Gardenia is a popular ornamental shrub found worldwide. The fruits of *Gardenia jasminoides* Ellis (Rubiaceae) (Chinese herbal name is Zhi Zi) has been used for the remedy for hepatic pain due to cirrhosis, abdominal pain due to dysentery, anti-phlogistics, diuretic, laxative, choleretic, and homeostatic purposes in the treatment of trauma by external application [1]. The major effective constituents of Gardenia fruits are iridoid glycosides such as geniposide, gardenoside, shanzhiside, scandoside methyl ester, deacetyl-asperulosidic acid methyl ester and genipin-1-β-D-gentiobioside [2–8]. These compounds may be responsible for the biological activities of this drug [9–14] and their rapid and accurate purification is of great significance in the quality control of this natural drug and its formulations. The chemical structures of iridoid glycosides were shown in Fig. 1.

At present, iridoid glycosides are commercially purified from *G. jasminoides* Ellis by several steps such as crystallization and chromatography [3–8]. All these conventional methods are tedious, time consuming, thus, are not suitable for large-scale isolation.

Using a two-dimensional approach in which the objective of the first dimension is to allow only the components of interest to be transported to the second column, could increase the peak capacity of the separation system and in the meantime allows isocratic conditions to be employed [15–17]. However, the sample pre-concentration or sample accumulation procedure using an enrichment column may lead to a consumption of cycle time. In a previous study, the development of a parallel two-dimensional system was discussed [18], however, the injection volumes were somehow limited due to the limitations in injection system that restricted continuous operation at higher injection loading. Moreover, the recoveries in the first dimension were limited to around 75% due to the requirements to overfill the injection loop. Such an approach was not considered robust and productive enough

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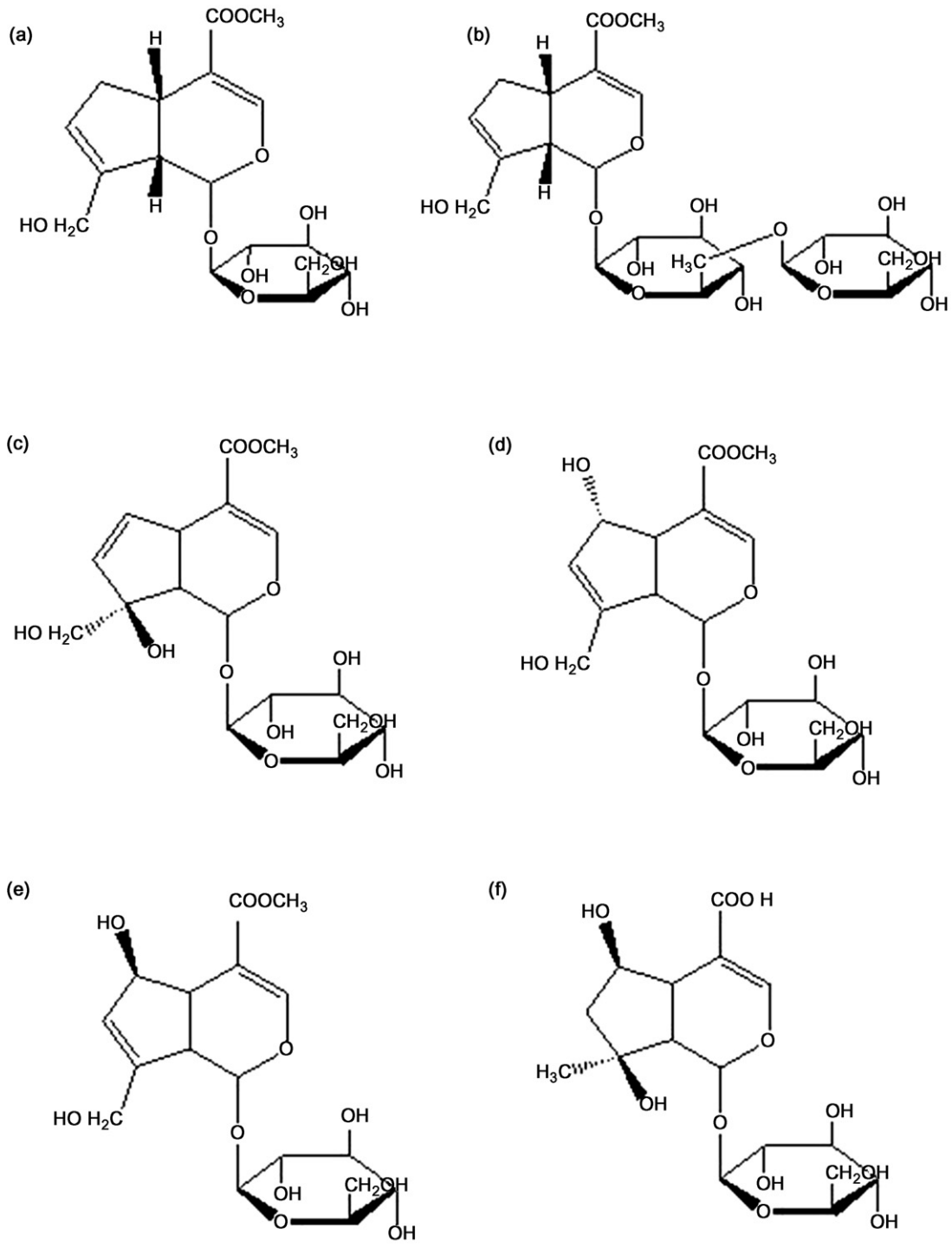

Fig. 1. Chemical structures of the iridoid glycosides identified from *Gardenia jasminoides* Ellis. (a) Geniposide; (b) genipin-1-β-D-gentiobioside; (c) gardenoside; (d) deacetyl-asperulosidic acid methyl ester; (e) scandoside methyl ester; (f) shanzhiside.

to employ routinely for processing large quantities of pure compounds.

In this paper, a two-dimensional reversed-phase HPLC separation system, where two columns were operated independently via two six-port two-position switching valves, was successfully applied for the first time to the isolation and purification of six iridoid glycosides from *G. jasminoides* Ellis.

2. Experimental

2.1. Materials and reagents

Ethanol used for extraction was of analytical grade and purchased from China Medicine (Group) Shanghai Chemical Reagent Corporation (PR China). Acetonitrile used for HPLC was of chromatographic grade (Merck, Germany). Reverse

osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions.

The *G. jasminoides* Eillis was purchased from a local drug store and identified by Dr. Luping Qin (Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, China).

2.2. Apparatus

Gradient one-dimensional chromatographic experiments were performed on a Dionex HPLC system (Dionex Corporation, USA) including P680 pump, ASI-100 automated sample injector, thermostatted column compartment and PDA-100 photodiode array detector. Chromeleon software (Version 6.50) was used for evaluation and quantification.

Isocratic two-dimensional chromatographic experiments were performed on a Shimadzu HPLC system incorporating two Waters 515 pumps (Waters, USA), two SPD-10A UV-vis detectors (Shimadzu, Japan), two CTO-10AS column ovens (Shimadzu, Japan), two six-port, two-position valve including one switch valve and one injection valve with a 5000 μl sample loop (C2-2006, Unimicro Technologies, China), and the model N2000 workstation (Zhejiang University, China). Valve switching was controlled via the onboard Millennium^{32} software.

2.3. Sample preparation

About 10 g of dried fruit of *G. jasminoides* Eillis was chopped and extracted three times by reflux with 100 ml volume of 50% ethanol in a haven for 2 h. After filtration, the extract was combined and evaporated to dryness by rotary evaporation at 60 °C under reduced pressure. Regardless of whether gradient one-dimensional or isocratic two-dimensional methods were employed the sample solution was prepared by dissolving the extract in 100 ml of HPLC mobile phase.

2.4. Gradient elution one-dimensional separations procedure

Optimized mobile phase conditions were scouted at an analytical scale and fine-tuned in the semi-prep and overload modes of separation reported in this communication using a pair of columns equipped with the same stationary phase.

An YMC ODS-C_{18} column (250 mm × 4.6 mm i.d.; 5 μm, YMC, Japan) was used for analytical scale separation with a 50 μl injection volume and an YMC ODS-C_{18} column (250 mm × 10 mm i.d.; 5 μm, YMC, Japan) was used for semi-preparative scale separation with a 1.5 ml injection volume. Both of the analytical and the semi-preparative HPLC separation are performed under the same conditions except for the different flow-rates, which was 1.0 ml/min for the former and 4.7 ml/min for the latter. Column temperature was set at 25 °C. A linear gradient elution of A (CH_3COOH:H_2O = 0.1:100) and B (CH_3COOH:CH_3CN = 0.1:100) was used as follows: an isocratic elution with 7% B in the first 15 min, and a linear gradient from 7 to 20% B from 15 to 60 min. A photodiode array detector was used for detection in order to gain a greater understanding of the sample complexity. The DAD recorded UV spectra in the range from 190 to 350 nm, and the HPLC chromatogram was monitored at 238 nm.

2.5. Isocratic two-dimensional separations procedure

Isocratic two-dimensional HPLC was performed with a reversed-phase Lichrospher C_{18} (100 mm × 10.0 mm i.d.; 5 μm) (Hanbang Science, China) using 7% acetonitrile (including 0.1% acetic acid) as the first dimension mobile-phase at a flow-rate of 9.8 ml/min, and an YMC ODS-C_{18} column (250 mm × 10 mm i.d.; 5 μm, YMC, Japan) using the same mobile-phase in the second dimension at a flow-rate of 4.0 ml/min.

Fig. 2 shows the schematic diagram of the hyphenated two-dimensional HPLC system. The heart of the system was the

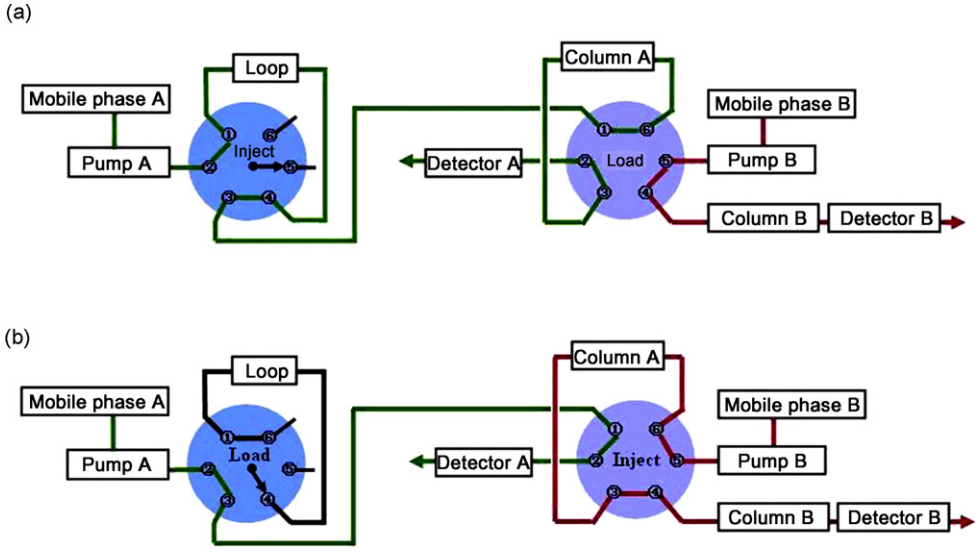

Fig. 2. Schematic diagram of the two-dimensional HPLC system. Column A: column in the first separation dimension; Column B: column in the second dimension. (a) System configuration for elution on column A. (b) System configuration for elution on column A and column B.

switch valve instead of sample loop. The six-port, two-position valve was used for the column-switch between the two dimensions. When triggered, the mobile phase B eluted through not only the second column but also the first column. That is to say, the fraction was directly switched into the second dimension from the first dimension, thus was not restricted by volume. After a period of time, the valve was triggered back and the two columns went back to work independently.

2.6. HPLC–DAD purity analysis and identification of preparative HPLC peak fractions

Each peak fraction obtained was analyzed on a Dionex HPLC system, and all of the chromatography conditions were the same with those in the gradient elution one-dimensional separation procedure.

Identification of the reparative HPLC pure fraction was carried out by UV (Cary-50, Varian), IR (Hitachi 275-50), MS (Finnigan MAT 711), ^{1}H NMR and ^{13}C NMR (Varian Unity Inova–500).

3. Results and discussion

3.1. Scale-up studies of gradient elution one-dimensional separations in preparative-scale

This paper presents a scale-up of HPLC method development procedure using a pair of columns equipped with the same stationary phase where only analytical scale data are used to predict optimal preparative operation conditions for the separation of six iridoid glycosides. To scale up to the preparative separation, 30 times of the injection volume and 4.7 times of the flow rate were performed with other HPLC conditions remain constant.

The wide range in retention of the many different constituents is emphasized by the long gradient running time (nearly 60 min). The long cycle time (the whole cycle time was approximately 90 min) also included re-equilibration using six column volumes of the initial mobile phase during which the chromatographic system underwent the injection process. A substantial reduction in the peak capacity and a relatively poor resolution of the first four components were observed for a further sample loading.

3.2. Isocratic two-dimensional separations with column switch technology

To reduce the cycle time, isocratic elution mode is favored to the gradient one. Fig. 3a illustrates the one-dimensional isocratic chromatographic separation of the crude extract of *G. jasminoides* Ellis following a 2.0 ml injection of above-mentioned sample solution. The total run-time was approximately 22 min.

Due to the wide variety of polarities in the sample, resolutions of components with similar relative migration rates would be poor in the mode of isocratic single-dimensional separations to minimize the flushing time that the system would require to remove strongly retained species (the resolution of shanzhiside, deacetyl-asperulosidic acid methyl ester, gardenoside and scandosidemethyl ester would be poor). Using a two-dimensional approach, however, allows isocratic conditions to be employed. Four of the target components that could not be resolved in the first dimension were switched onto the second dimension. As was shown in Fig. 3b, the same mobile-phase in isocratic single-dimensional separations was used to elute the extract on column A and the flow-rate was increased to 9.8 ml/min, which allows crude separation. During the period from 1.5 to 6.5 min, the sample region containing the above four components (shanzhiside, deacetyl-asperulosidic acid methyl ester, gardenoside and scandosidemethyl ester) was switched onto the second column B through a six-port two-position valve (Fig. 3c).

To minimize the cycle time, the void time in both of the two dimensions and the time required for the injection process were taken into consideration. Therefore, the cycle time was 19 min, even though the first-dimensional separation required 22 min to be completed and the second dimension almost 25 min. The injection of a second sample onto the first column could be performed before all sample components were eluted from the first column.

Traditionally, the valve used to heat-cut between the two columns was an injection valve, and the sample fraction containing the component of interest in the first dimension was heart-cut to a sample loop prior to loading onto the second dimension [18–20]. In such systems, only the fraction remained in the sample loop can be flushed into next dimension. Obviously, this would ultimately limit its potential. In our paper, all of the sample fractions containing the components of interest in the first dimension were directly switched onto the second dimension without sample loop. The valve used between the two columns is a six-port switching valve instead of injection valve ensured 100% recovery from the first dimension to the second. The four iridoid glycosides in the first dimension of this separation eluted in the region between approximately 1.5 and 6.5 min, thus, the switch volumes of the second dimension could reach 20 ml.

3.3. Significant advantages of the column switch mode over trapping loop in separation efficiency

In references mentioned above [18,21,22], due to the problem of band broadening in the first dimension, the isocratic elution mode and the limitation of heart-cut volume would also lead to a poor resolution of other compounds in the first dimension as has been mentioned above.

However, in our paper, it was the non-restriction of switch volume that ensured nice resolution of some target components in the first dimension. Excellent separation efficiency (short elution time) was also obtained in spite of the consideration of the resolutions in the first dimension.

From 10 g crude materials of *G. jasminoides* Ellis, six iridoid glycosides (280 mg geniposide, 41.9 mg gardenoside, 32.6 mg shanzhiside, 31.6 mg scandoside methyl ester, 43.6 mg deacetyl-asperulosidic acid methyl ester and 65.6 mg genipin-1-β-D-gentiobioside) were obtained in a single run. HPLC analysis of each peak fractions of this preparative HPLC system revealed that the purities of all the six iridoid glycosides were over 99%.

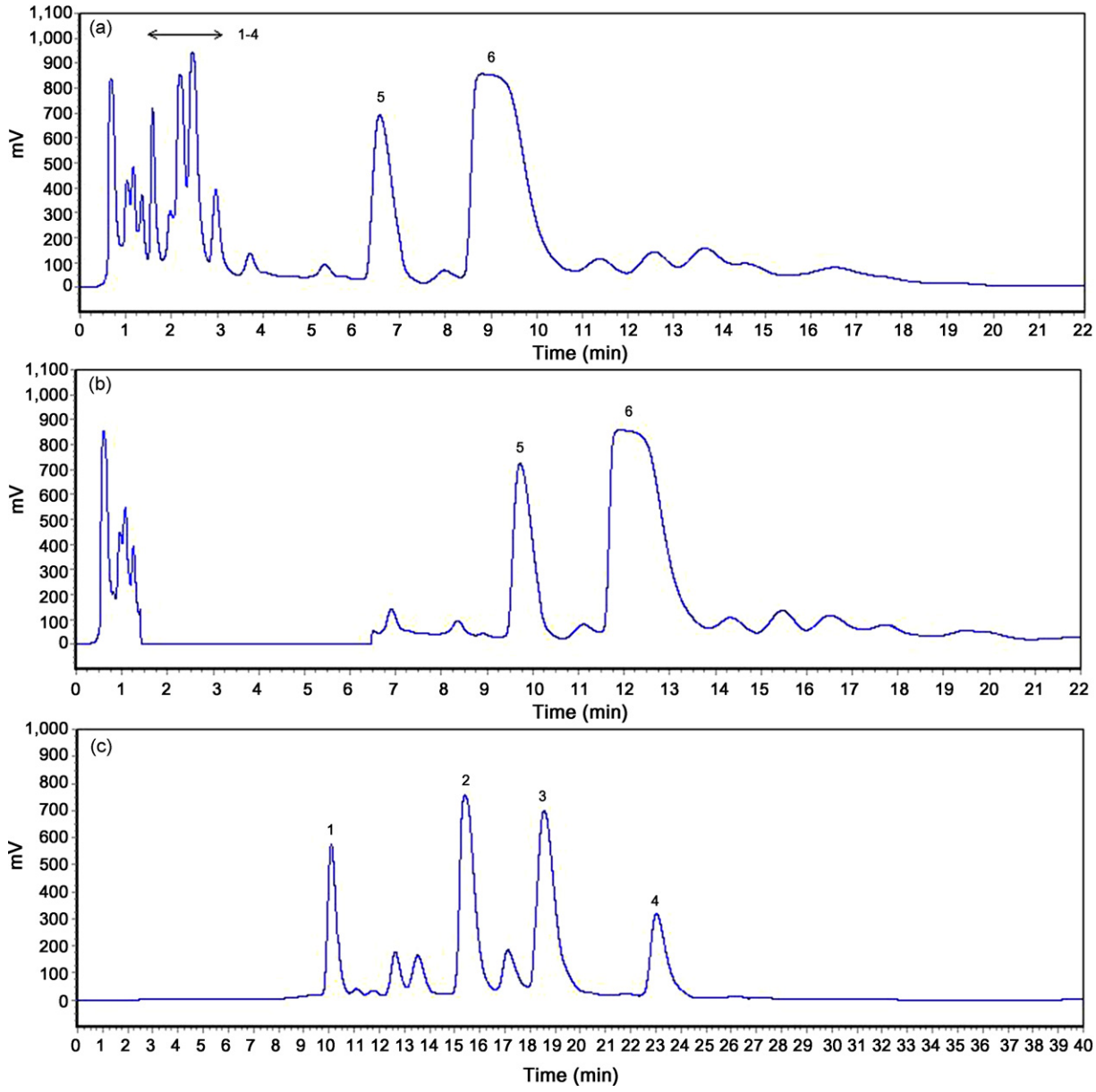

Fig. 3. Chromatograms illustrating the separation of the components in the extract from *Gardenia jasminoides* Ellis. The injection volumes were 2.0 ml, detection at 238 nm. (a) Chromatogram illustrating the separation of the entire sample components on a Lichrospher C_{18} (100 mm × 10.0 mm i.d.; 5 μm). Mobile phase: 7% acetonitrile (including 0.1% acetic acid); flow-rate: 9.8 ml min^{-1}. (b) chromatogram illustrating the separation of partial sample components on a Lichrospher C_{18} (100 mm × 10.0 mm i.d.; 5 μm) in the first dimension. Mobile phase: 7% acetonitrile (including 0.1% acetic acid); flow-rate: 9.8 ml min^{-1} (c) chromatogram illustrating the separation of target four components on an YMC ODS column (250 mm × 10 mm, 5 μm) in the second dimension. Mobile phase: 7% acetonitrile (including 0.1% acetic acid); flow-rate: 4.0 ml min^{-1}; switch volumes: 20 ml. 1 = shanzhiside; 2 = deacetyl-asperulosidic acid methyl ester; 3 = gardenoside; 4 = scandosidemethyl ester; 5 = genipin-1-β-D-gentiobioside; 6 = geniposide.

Table 1
Characteristics of two-dimensional and gradient elution separations of six iridoid glycosides from *Gardenia jasminoides* Ellis

System	Purity (%)	V_i (ml)	Δt_c (min)	t (h)	V_s (ml)	Y_i (%)
Two-dimensional	>99	2.0	19	16	918	>96
One-dimensional gradient	>95	1.5	90	100	2890	>88

V_i, injection volume (dissolved in HPLC mobile phase); Δt_c, cycle time; t, total time (10 g crude herb); V_s, total solvent consumption (10 g crude herb); Y_i, recovery yield.

3.4. Comparison between the isocratic two-dimensional and the gradient elution one-dimensional separation modes

Apart from the much higher production rate of the two-dimensional method, the solvent consumption of it (918 ml) was also only 31% of that (2890 ml) in the gradient one-dimensional mode. Details of each process are presented in Table 1. Quite clearly the two-dimensional system out performed those in a single dimension.

3.5. The structural identification

Identification of the pure products was performed by MS, ^{1}H NMR and ^{13}C NMR analysis. Comparing with the reported data, the MS, ^{1}H NMR and ^{13}C NMR data agree with those of geniposide, gardenoside, scandoside methyl ester, deacetyl-asperulosidic acid methyl ester, shanzhiside and genipin-1-β-D-gentiobioside [3,4,6].

4. Conclusion

A two-dimensional reversed-phase HPLC separation system, where two columns were operated independently via two six-port two-position switching valves, was successfully applied for the first time to the isolation and purification of six iridoid glycosides from *G. jasminoides* Ellis.

It is envisioned that this approach could be adopted as a new technical platform for a wide range of different preparative HPLC rapid and automated purification system to isolate chemical constituents from TCM in general.

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