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Abstract

Silver ion chromatography, utilizing columns packed with silver ions bonded to silica gel, has proved to be an invaluable technique for the analysis of some positional isomers. In this work, silver ion chromatography by combination with online heart-cutting LC-LC technique for the preparative separation of two sesquiterpenes positional isomers from a natural product was investigated. On the basis of the evaluation that silver ion content impacts on the separation, the laboratory-made silver ion columns, utilizing silica gel impregnated with 15% silver nitrate as column packing materials, were used for peak resolution improvement of these two isomers and the preparative separation of them in heart-cutting LC-LC. The relationship among the maximal sample load, flow rate and peak resolution in the silver ion column were optimized, and the performance of the silver ion column was compared with conventional C_{18} column and silica gel column. Based on the developed chromatographic conditions, online heart-cutting LC-LC chromatographic separation system in combination with a silica gel column and a silver ion column that was applied to preparative separation of these two isomers from a traditional Chinese medicine, *Inula racemosa* Hook.f., was established. The results showed that the online heart-cutting LC-LC technique by combination of a silica gel column and a silver ion column for the preparative separation of these two positional isomers from this natural plant was superior to the preparative separation performed on a single-column system with C_{18} column or silica gel column.

Keywords: Positional isomers; Argentation; Peak resolution enhancement; Heart-cutting LC-LC; Preparative separation; Natural product

1. Introduction

Silver ion chromatography, utilizing columns packed with silver ions bonded to a silica gel or similar substrate, has proved to be an invaluable technique for the analysis of complex triacylglycerol mixtures, fatty acids, lipids and some positional isomers [1-4]. This application based on the mechanism that the different number, or/and geometrical configuration, or/and position of unsaturated C-C bonds provide the different amount of π donors. Since silver ions act as π acceptors while unsaturated C-C bonds act as π donors, causing molecules that contain fewer π donors or do not contain π donors to be eluted firstly on silver ion chromatography [5].

Although silver ion chromatography by a single-column system is inexpensive and easy to operate for the trace separation and determination of active ingredients, or preparative separation compounds from simple sample on small-scale [6], it still restricts to the large-scale preparative separation of compounds from complex natural products. Due to adsorption of excessive impurities, the action of silver ions becomes worse, causing the decrease of column separation efficiency, even resulting in bleeding of silver ions and affecting the UV detector. In addition, a single-column system sometimes has limited sample capacity and poor relative peak separation [7]. Notably, heart-cutting two-dimensional liquid chromatography (LC-LC) offers the probability to improve these drawbacks based on a tandem combination of two independent liquid phase separation systems [8-11].

Inula racemosa Hook.f., a medicinal plant, is widely distributed in Europe, Asia and Africa [12]. Its root as a traditional medicine has been most frequently applied for issues related to peptic disorders, phlegm, detumescence, inflammatory and vermifuge [13]. Alantolactone (AL) and isoalantolactone (IS) (Figure.1) are two sesquiterpenes positional isomers and have abundant content in this medicinal plant. Previous studies suggested that they show many biological activities [14-25], especially in killing of cancer cells. In addition, researchers also have examined the relationship between their structural modifications and biological activities [26-28]. These studies suggested that AL and IS may be good potential lead compounds for future anticancer agent development or act as chemical templates for the design, synthesis, and semisynthesis of new substances. Nevertheless, further studies are required in preclinical and clinical applications and to explain their potential role. For these studies, it certainly will need a large number of materials. In the current ways, acquisition of AL and IS from natural plants is a short-cut. Therefore, a robust method for the preparative separation of AL and IS from this medicinal plant may be necessary. Unfortunately, the structural similarity of AL and IS increases the preparative separation difficulty of them from this natural plant. Although the traditional separation methods used in the trace separation and determination of these two compounds have been performed with capillary electrophoresis (CE) [29], gas chromatography (GC) [30] and RP-HPLC [31, 32], when these methods are used to isolate AL and IS from *Inula racemosa* Hook.f. on a larger scale, it becomes difficult due to the limitation of the resolution between their peaks. Hence, the

establishment of an effective method for increase of the peak resolution and its application to their preparative separation from this plant becomes a key problem. Since AL and IS constitute a pair of positional isomers related to C=C bond, silver ion chromatography provides the probability to improve the separation of them (Figure.1). Therefore, the aim of this study was to improve the separation of these two isomers by silver ion chromatography in preparative scale and obtain them from a crude extract of *Inula racemosa* Hook.f. by preparative separation with silver ion chromatography technique in combination with online heart-cutting LC-LC technique.

2. Experimental

2.1. Materials and reagents

The dried roots of *Inula racemosa* Hook.f. were purchased from Bai Ding (Tibet, China). Alantolactone and isoalantolactone were purchased from National Institute of Food and Drug Control for the quantification and qualitative study (Beijing, China). Silver nitrate was of chemical grade (Zhengzhou Kaidi Chemical Products Co., Ltd, Henan, China). Silica gel (5 ~ 10 μm particles and 10 ~ 40 μm particles) was purchased from Qingdao Yida Silica Reagent Factory (Fujian, China). Ethyl acetate, n-hexane, 95% ethanol and acetonitrile reagents were of analytical grade and purchased from Xi'an Chemical Reagent Factory (Shaanxi, China).

2.2. Sample preparation

The dried roots of *Inula racemosa* Hook.f. were pulverized and sieved through a screen (100 ~ 200 mesh). 100 g of this powder was extracted with 1000 ml of 95% ethanol solution by ultrasonic treatment for 30 min at 45 ℃ and 100 kHz/450 W (Bandelin Sonorex, Germany) [33] and repeated three times. The extracts were concentrated on a rotavapor at 45 °C (REC32E, Shanghai Yarong Instrument, China). The residues were dissolved in n-hexane/ethyl acetate (100 mL: 70/30, v/v) solution to get sample solutions for the preparative separation, and the contents of AL and IS in this sample solution were 25.62 mg/mL and 31.32 mg/mL, respectively.

The stock solution for the analysis of AL and IS was prepared by dissolving AL and IS into n-hexane/ethyl acetate (70/30, v/v) and mixing at concentration containing AL of 25.86 mg/mL and IS of 30.26 mg/mL.

2.3. Column packing

The laboratory-made silver ion column, utilizing silica gel impregnated with silver nitrate as

column packing materials, was prepared for the preparative separation in heart-cutting LC-LC. The silica gel impregnated with silver nitrate was prepared by the method reported previously with slightly modification [34] (Figure.2). To avoid waste and the risk of blindness, the silver ion content in silica gel impregnated with silver nitrate for the impact on the separation of these two isomers was first observed on silver ion TLC plate [34]. Then the sliver ion columns (Stainless steel tube, Jiangsu Hanbang Co. Ltd., China) were slurry packed with ethyl acetate using a CST chromatographic column packing machine (KeSheng Experimental Equipment Co., Ltd., Suzhou, China) according to the operating instructions, and the performance of the prepared silver ion column for the preparative separation of these two isomers was investigated and compared with C_{18} column on preparative HPLC instrument (Two Alltech-627 high-pressure pumps equipped with a Model-500 UV-VIS detector, Alltech, USA).

2.4. Connections of the heart-cutting LC-LC system

The connections of the heart-cutting LC-LC system are illustrated in Figure.3. The column temperatures were controlled at 30 ℃ using an independent QT-330 chromatographic column thermostat (Henan Jiuhegongchuang co., Ltd., China). Two NP7000-C high-pressure pumps equipped with a NU3010-C UV-VIS detector (Jiangsu Hanbang Technology Co., Ltd., Jiangsu, China) were involved in the first dimension, and the UV detector was set at 260 nm. The system control and data collection were carried out using an EasyChrom-1000 chromatographic workstation. A silica gel column (22 mm i.d. × 250 mm, 5 μm, Grace, USA) as a preparative column was used for the first-dimension separation, while a 22 mm i.d. × 20 mm silica gel column as pre-column was equipped between the sample loop and the preparative column. In addition, two Alltech-627 high-pressure pumps equipped with a Model-500 UV-VIS detector (Alltech, USA) were used in the second dimension, and the UV detector was set at 260 nm. The system control and data acquisition were performed by using an AllChrom Plus server workstation. The second-dimension separation was performed on a laboratory-made sliver ion column (22 mm i.d. × 250 mm).

2.5. Procedure for preparative separation

First-dimension unit separation– N-hexane (A)/ethyl acetate (B) (v/v) were employed as the mobile phase. 10 mL of the sample solution was injected into a 20 mL sample loop while valve A

was set at position B. Then the elution program was carried out by the EasyChrom-1000 chromatographic workstation: 0–15 min (Valve A at position A; Flow rate, 12 mL/min; 85% A/15% B; When the peak of the fraction containing AL and IS started to emerge, valve B and valve A were all switched to position B: the fraction containing AL and IS was cut and transferred onto the silver ion column via the automatic FC-AS-312 component collector), and 15.1–25 min (Valve A at position B; flow rate, 15 mL/min; 0% A/100% B) and 25.1–30 min (Valve A at position B; flow rate, 15 mL/min; 85% A/15% B).

Second-dimension unit separation– When all of the fraction containing AL and IS from the first-dimension column had been completely transferred onto the second-dimension column, the following elution program was run with a mobile phase composition of (A) n-hexane/(B) ethyl acetate: 0–22 min (Valve B at position A; Flow rate, 10 mL/min; 75% A/25% B; AL and IS were automatically collected by the component collector on the second-dimension unit), 22.1–25 min (Valve B at position B; Flow rate, 15 mL/min; 0% A/100% B) and 27.1–32 min (Valve B at position B; Flow rate, 15 mL min^{-1}; 75% A/25% B).

2.6. Quantitative HPLC analysis

The crude *Inula racemosa* Hook.f. extract and the prepared compounds were analyzed by a commercially available sliver ion column (4.6 mm i.d. × 250 mm, ChromSpher Lipids CP28313, Varian, USA) in the analytical HPLC instrument (Waters 2695 HPLC system equipped with a Waters 2487 UV-VIS detector controlled through an Empower chromatographic workstation, Waters, USA). The concentration range of calibration solutions obtained by diluting the stock solutions at the range of 5172.0-323.25 μg/mL for AL and 6052.0-378.25 μg/mL for IS. Chromatographic separation was performed using an isocratic elution at a flow rate of 1.0 mL/min with mobile phase 75/25 (n-hexane/ethyl acetate). The column temperature was controlled at 30 °C, and the effluents were monitored at 260 nm by a UV detector.

3. Results and discussion

3.1. Silver ion content in the improvement of separation

For the preparative separation of AL and IS, the goal in the present study was to obtain them with high efficiency. Since low sample load reduces the efficiency in preparative separation of AL and IS, we were concerned about the increase of the maximal sample load. It is well known that

the resolution has great influence on the maximal sample load, as well as run times, equilibration times and solvent consumption. With increase of sample load, the resolution decreases [35]. The decreased resolution limits the increase of the maximal sample load and throughput per unit time. Therefore, the enhancement of the resolution between AL and IS may improve the maximal sample load.

The amount of silver ion in the silica gel impregnated with silver nitrate is vital for the improvement of the resolution. Therefore, the investigation on the content of silver nitrate may be necessary. TLC technique in the evaluation of the effect of silver ion content is easy and convenient in this study. In the TLC separation, the developing solvent was composed of n-hexane/ethyl acetate (80/20). The TLC plates were sprayed with an ethanol solution containing 10% sulfuric acid and then heated in an IR drier until obvious colour appeared. As shown in Fig.4, AL and IS could not be isolated completely on the TLC plates when no silver nitrate added into silica gel (Figure.4-1 and Figure.4-3), whereas these two isomers on TLC plates by using silica gel (10 ~ 40 μm particles) impregnated with silver nitrate showed a good separation (Figure.4-2 and Figure.4-4 - Figure.4-9). Since the level of silver nitrate impregnation recommended has varied from 0.5-40% [36] and the case where we have found 10% of impregnation is more effective for separations of isomers in column separation [37], 6%, 10% and 15% silver nitrate to improve the separation were investigated in the present study. From the obtained thin-layer chromatograms (Figure.4), with the increase of the silver nitrate content, the amount of sample load increased with complete separation. Since 15% silver nitrate facilitated the isolation more effectively than other ratios with the increase of sample load (Figure.4-3 - Figure.4-9), silica gel (5 ~ 10 μm particles) impregnated with 15% silver nitrate as column packing materials was prepared in the following study. Whether more than 15% silver nitrate is more effective in preparative separation of these two isomers needs to be explored.

3.2. Sliver ion column for the separation of two sesquiterpenes positional isomers

Even though these two positional isomers achieved good separation on silver ion TLC plates with 15% of impregnation, whether the preparative separation in HPLC, utilizing silver ion column packed with this impregnation (15% silver nitrate), could acquire improvement was unknown. Based on the TLC analysis, the investigation was first performed on a single

laboratory-made 4.6 mm i.d. × 250 mm sliver ion column with mobile phase 80/20 (n-hexane/ethyl acetate, v/v) at the flow rate of 1 mL/min, where it obtained a good peak resolution, and a satisfactory peak shape and separation times within 20 μL of the stock solution (Figure.5a), whereas peak resolution decreased when the sample load increased to 80 μL of the stock solution (Figure.5b). Since the mobile phase with n-hexane/ethyl acetate (80/20, v/v) shortened their separation times and reduction of ethyl acetate concentration (Such as reduction of 5% ethyl acetate) in mobile phase has a great influence on the retention times, the optimization of the mobile phase composition for the increase of sample load was not carried out. Furthermore, considering that reduction of the flow rate may improve the peak resolution and then increase the sample load [38], the mobile phase 80/20 (n-hexane/ethyl acetate, v/v) at the flow rate of 0.8 mL/min and 0.6 mL/min were evaluated in the following experiment, respectively. When the flow rate decreased to 0.8 mL/min, a complete resolution could be obtained within 180 μL of the stock solution (Figure.6), while the retention times of these two isomers slightly delayed but there was no obvious changes in the peak shape. When the sample load continued increasing, bad resolution appeared (Figure.6). In a view of this, the flow rate reduced to 0.6 mL/min, in which the maximal sample load increased to 480 μL of the stock solution with a complete resolution (Figure.5c and Figure.6). However, the peaks seem to be flat and trailing, and the retention times delayed markedly (Figure.5c). The reason may be the decrease in the elution strength of solvent at the low flow rate. Therefore, the mobile phase was optimized for n-hexane/ethyl acetate (75/25), which reduced peaks trailing and retention times (Figure.5d) and given a complete resolution.

Fig.6 shows the relationship among the resolution, sample load and flow rate. With the increase of sample load, the peak resolution decreased. The increased flow rate also reduced the peak resolution and leaded to the decrease of the peak height. Although the decrease of the flow rate made peak width increase slightly, it obtains a good resolution (≥ 1.5) with the increase of sample load. When the sample volume increased to 480 μL of the stock solution, the resolution between the AL and IS nearly equals to 1.5 at the flow rate of 0.6 mL/min, so the sample load didn't increase any more.

In order to validate the recovery and repeatability under the developed conditions using the prepared silver column, 480 μL of the stock solution was separated on the 4.6 mm i.d. × 250 mm

column with n-hexane/ethyl acetate (75/25) at the flow rate of 0.6 mL/min. By six replicate experiments, the recovery of AL was 95.3% with RSD of 1.3% and that of IS was 95.1% with RSD of 1.6%, while the repeatability was determined with the values of RSD of 1.09% and 1.23%. The results showed a satisfactory recovery and repeatability.

3.3. Conventional separation methods

C_{18} and silica gel packing materials are commonly used for preparative separation. Evidently, compared with silica gel, silica gel impregnated with silver nitrate achieved good separation with the increase of sample load (Figure.4). To further evaluate the performance of the laboratory-made silver ion column (4.6 mm i.d. × 250 mm), a same size C_{18} column (4.6 mm i.d. × 250 mm) for preparative separation of AL and IS was compared with the silver ion column (Chromatographic conditions were presented in supplementary material). Although a satisfactory separation achieved on C_{18} column using a higher concentration of water in the mobile phase, it sacrificed a lot of time and still had limited sample treatment capacity because of the limitation of the peak resolution. However, the silver ion column reduced the retention times and increased the maximal sample load with good resolution, compared to C_{18} column (Table. 1 and Figure.7).

3.4. Application of heart-cutting LC-LC system in the preparative separation

The online heart-cutting LC-LC technique was applied to the preparative separation of these two positional isomers from this natural plant. Although AL and IS could not be separated completely with silica gel materials, the fraction of AL and IS achieved good separation (Figure.4-1 and Figure.8a). Therefore, a silica gel columns was equipped on the first-dimension separation to isolate the fraction of AL and IS from the crude extract, while a laboratory-made 22 mm i.d. × 250 mm sliver ion column was selected for the second-dimension separation to further separate AL and IS. The elution conditions both in the first dimension and the second dimension were developed based on the above analysis. The maximal sample load and the flow rate for the selected silver ion column in LC-LC separation were estimated by applying the linear amplification analysis (About 10.9 mL of the stock solution and flow rate of 14 ml/min) [39], and then the diameter of the silica gel column used in first-dimension separation was chosen based on the requirement for making full use of the selected silver ion column in the advantage of the high sample load. For this reason, if 11 mL of the crude extract solution can be treated by silica gel

column equipped on the first dimension, the sample load amount loaded on the selected silver ion column transferred from the first dimension may be close to its maximal sample load in LC-LC preparative separation. Therefore, two silica gel columns, with different diameters (10 mm i.d. × 250 mm, 22 mm i.d. × 250 mm), were tested respectively. The results showed that the 22 mm i.d. × 250 mm column was superior to another one, in which it could treat 10 mL of the crude extract solution with n-hexane/ethyl acetate (85/15, v/v) at the flow of 12 mL/ min (Figure.8b-d). Hence, a 22 mm i.d. × 250 mm silica gel column with n-hexane/ethyl acetate (85/15, v/v) as eluent at the flow of 12 mL/ min was used for the first-dimension separation.

Although 10 mL of the crude extract solution could be treated in the first-dimension separation (Figure.9a), a bad separation appeared in the second-dimension separation with n-hexane/ethyl acetate (75/25, v/v) at the flow of 14 mL/ min. However, when the flow rate in the second-dimension separation decreased to 10 mL/min, the peaks of these two isomers obtained a baseline separation (Figure.9b). In addition, to maintain the column separation performance per unit time, the columns restored by elution using 100% ethyl acetate at the end of separation each time. Using multicycle and consecutive separation, 2.3 g of AL with a purity of 97.8% and 2.8 g of IS with a purity of 98.8% were obtained from of the crude extract within 8.5 hours. In an LC-LC separation model, the preparative separation achieved high-efficiency compared with conventional methods.

Moreover, the crystals obtained from 90% ethanol were analysed by X-ray diffraction. Full crystallographic details for the compounds reported in this paper were deposited in the Cambridge Crystallographic Data Centre as Supplementary Publication No. CCDC 1438651 for AL (1) [40] and No. CCDC 1438652 for IS (2) [41]. Copies of these data can be obtained upon application to the CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [*http://www.ccdc.cam.ac.uk*].

4. Conclusion

Silver ion chromatography was demonstrated to be an effective technique for improvement of the separation of AL and IS in preparative scale. Online heart-cutting LC-LC technique by combination of the silica gel column and silver ion column was successfully applied to preparative separation of AL and IS from a crude extract of *Inula racemosa* Hook.f. This study offers a rapid and effective approach for the preparative separation of these two positional isomers from natural

product.

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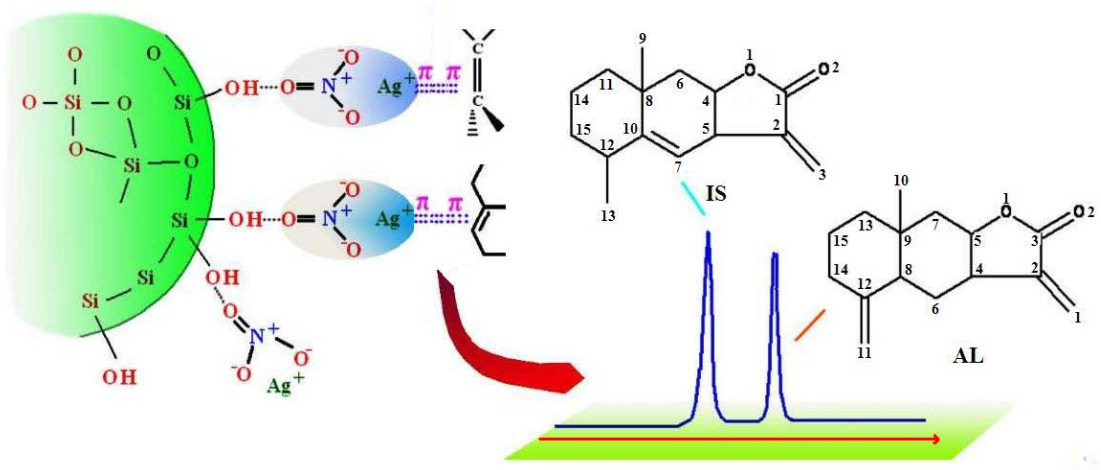

Figure.1 Schematic illustration of sliver ion chromatography for the separation of alantolactone (AL) and isoalantolactone (IS), and their chemical structures.

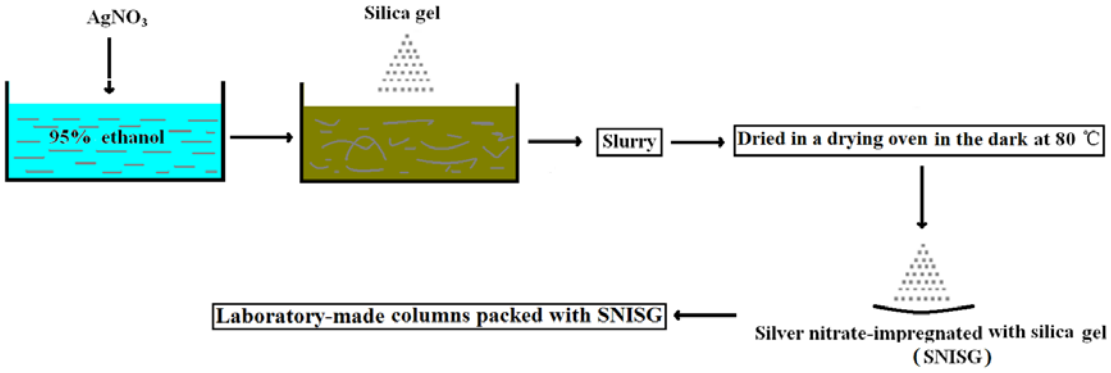

Figure.2. Schematic illustration of the preparation of silver ion columns packed with silica gel impregnated with silver nitrate (SNISG).

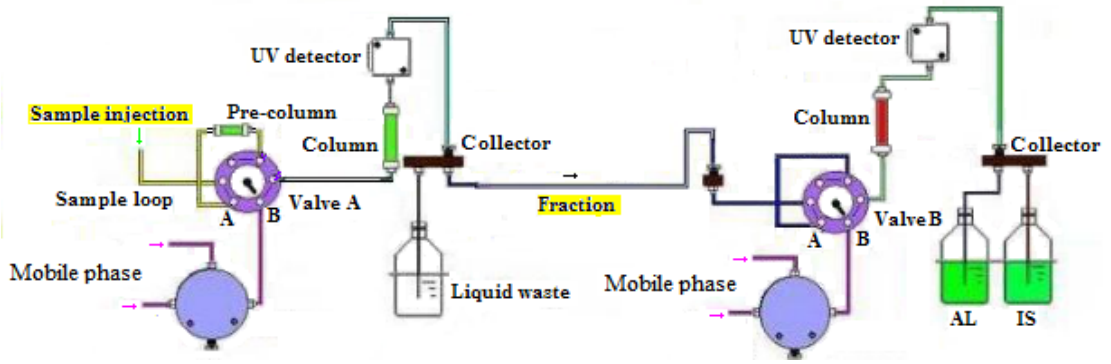

Figure.3. Schematic illustration of the instrumental configurations used for online heart-cutting LC-LC preparative separation.

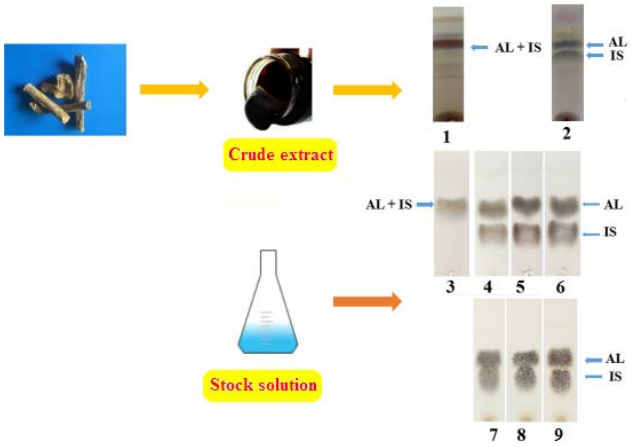

Figure.4. 0% (1, 3), 6% (4, 7), 10% (5, 8) and 15% (6, 9) silver nitrate as additives: (3-6), 10 μL of the stock solution was separated on silver ion TLC plates; (7-9), 20 μL of the stock solution was separated on silver ion TLC plates; (1), the crude extract was separated on silica gel TLC plate; (2), the crude extract was separated on silver ion TLC plate (Impregnation with 6% silver nitrate).

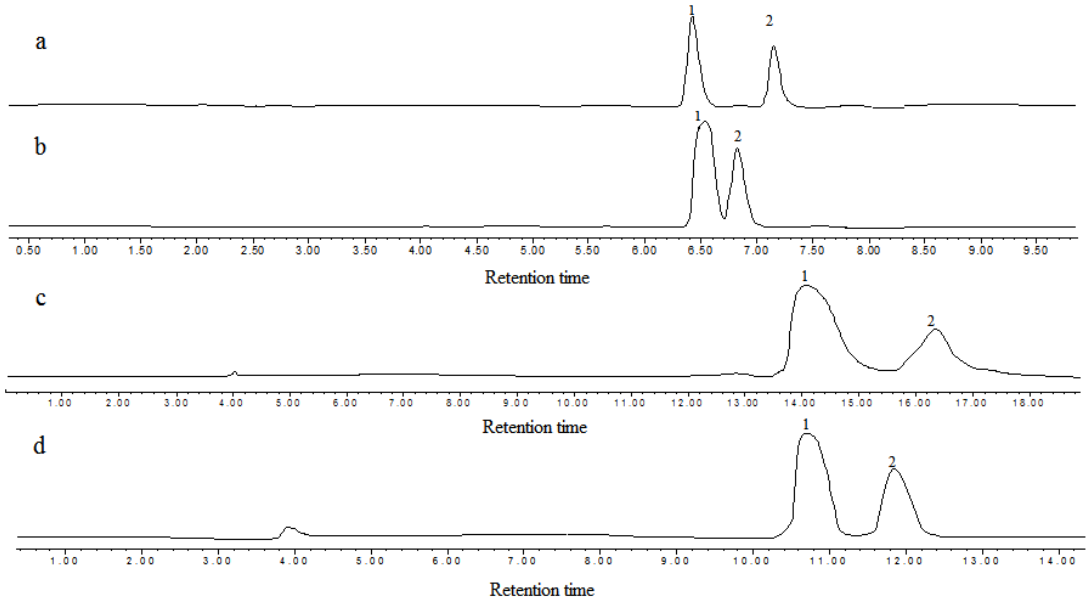

Figure.5. (1) AL and (2) IS. (a) 20 μL of the stock solution was separated with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 1 mL/min; (b) 80 μL of the stock solution was separated with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 1 mL/min; (c) 480 μL of the stock solution was separated with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 0.6 mL/min; (d) 480 μL of the stock solution was separated with mobile phase 75/25 (v/v) for n-hexane/ethyl acetate at the flow of 0.6 mL/min. Detection: UV 260 nm. Column: Laboratory-made 4.6 mm i.d. × 250 mm sliver ion column.

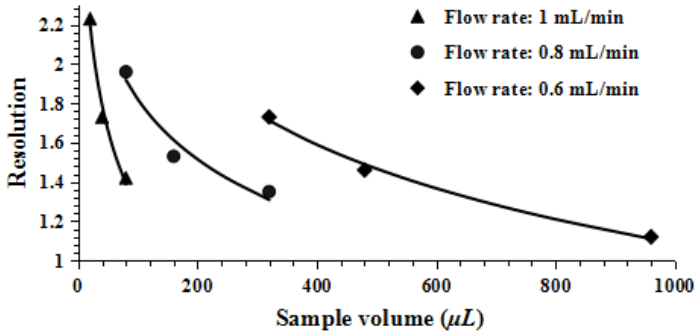

Figure. 6. The relationship among the resolution, sample load and flow rate. Column: Laboratory-made 4.6 mm i.d. × 250 mm sliver ion column. Mobile phase: n-hexane/ethyl acetate. Detection: UV 260 nm. Sample: the stock solution.

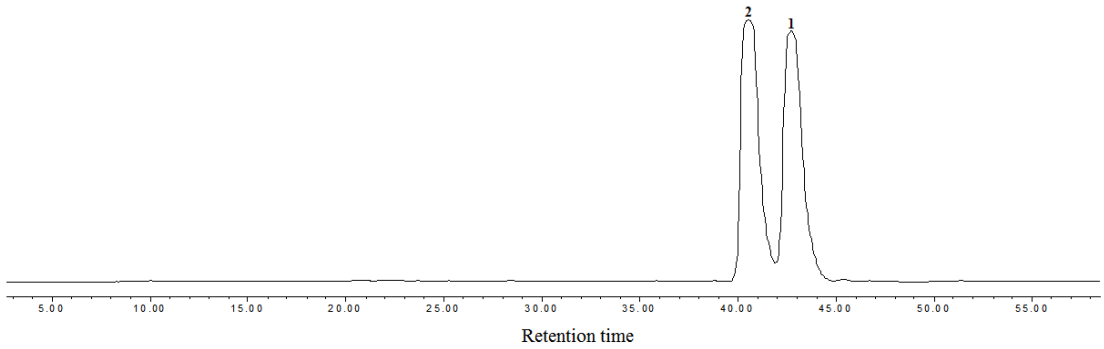

Figure.7. (1) AL and (2) IS. Chromatogram: Separation of AL and IS with mobile phase 65:35 (v/v) for acetonitrile:water. Flow rate: 0.6 mL/min. Detection: UV 220 nm. Column: Commercially available C18 column. Injection volume: 80 μL of the stock solution.

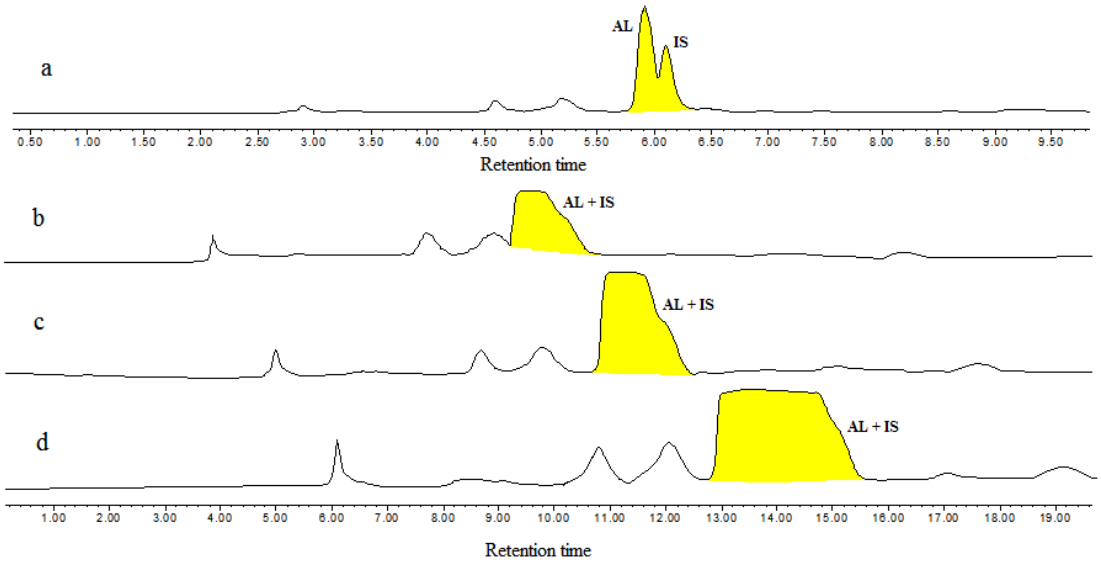

Figure.8. (a) 10 μL of the crude extract solution was separated on a 4.6 mm i.d. × 250 mm column with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 1 mL/min. (b) 6 mL of the crude extract solution was separated on a 10 mm i.d. × 250 mm column with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 4 mL/min; (c) 6 mL of the crude extract solution was separated on a 22 mm i.d. × 250 mm column with mobile phase 80/20 (v/v) for n-hexane/ethyl acetate at the flow of 15 mL/min; (d) 10 mL of the crude extract solution was separated on a 22 mm i.d. × 250 mm column with mobile phase 85/15 (v/v) for n-hexane/ethyl acetate at the flow of 12 mL/ min. Detection: UV 260 nm. Column: Commercially available silica gel column.

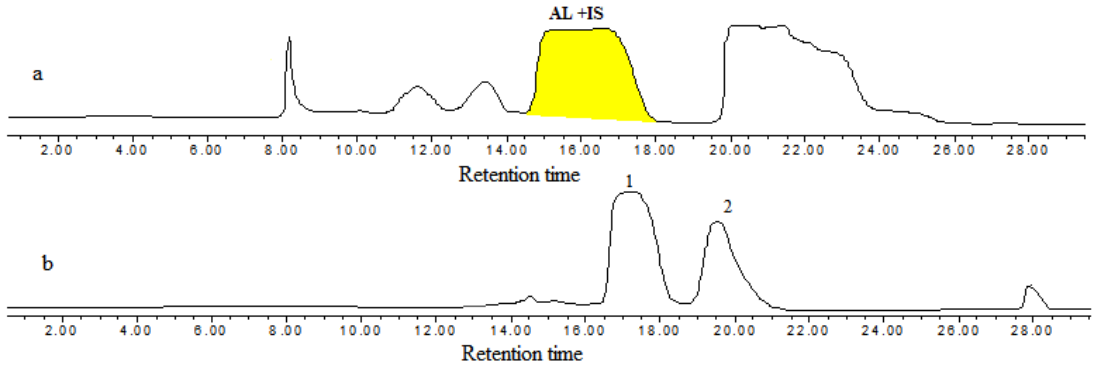

Figure.9. (1) AL and (2) IS. Chromatogram of online heart-cutting LC-LC separation: (a) the first-dimension separation was carried out on a silica gel column (22 mm i.d. × 250 mm) with mobile phase 85/15 (v/v) for n-hexane/ethyl acetate at the flow of 12 mL/min, and (b) the second-dimension separation was performed on a laboratory-made sliver ion column (22 mm i.d. × 250 mm) with mobile phase 75/25 (v/v) for n-hexane/ethyl acetate at the flow of 10 mL/min. Detection: UV 260 nm..