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Preparative separation of lithospermic acid B from *Salvia miltiorrhiza* by polyamide resin and preparative high-performance liquid chromatography

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ABSTRACT

Adsorption on polyamide resin was investigated as a means of separating lithospermic acid B (LAB) from a crude extract of the roots of the traditional Chinese medicine *Salvia miltiorrhiza* Bunge ("Danshen"). Variables affecting adsorption capacity (solution pH, contact time on resin, initial LAB concentration) were studied. Adsorption was strongly dependent upon the initial concentration of LAB and pH. In all conditions, the polyamide resin gave optimal adsorption of LAB at an initial concentration of 2.66 mg/mL and pH <3.0. The adsorption isotherm correlated well with the Langmuir-type adsorption isotherm. Maximal adsorption capacity was calculated to be 380 mg/g at pH 2.0 and 25 °C. LAB purity of 85.30% could be obtained by polyamide resin adsorption followed by elution with 70% ethanol solution, and the recovery was 87.1%. After preparative HPLC, the maximum HPLC purity obtained was 99.28% with a recovery of 75.2%. This method provides an efficient and low-cost method for LAB purification for industrial applications.

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1. Introduction

The traditional Chinese medicine (TCM) Danshen is also known as Radix (dried root) *Salvia miltiorrhiza* Bunge. It has been extensively used for the treatment and prevention of disorders of the cardiovascular system [1]. The active constituents of Danshen are divided into two groups: phenolic acids (which are water-soluble) and tanshinones (which are lipophilic) [2]. Most research has focused on the aqueous fractions of Danshen because water decoction is the commonly used method for the preparation of TCMs for human consumption [3]. The putative active components of its aqueous extract are danshensu (3,4-dihydroxyphenyl lactic acid), protocatechuic aldehyde, lithospermic acid, salvianolic acid A and lithospermic acid B (LAB; also termed salvianolic acid B) [4–6]. LAB (Fig. 1) is the most abundant and bioactive compound among water-soluble phenolic acids [7], and has strong antioxidant and free-radical scavenging activity [8,9]. LAB has been selected as the reference standard component for the quality control of Danshen end-products in the 2010 edition of the Chinese Pharmacopoeia.

Preparation of pure LAB is difficult because it is readily degraded, particularly at high temperature and in aqueous solutions of high pH [10]. High-speed counter-current chromatography has recently been used to yield pure LAB [11–13], but this method is unsatisfactory because of high cost and low output, and is unsuitable for large-scale preparation of LAB. Liquid adsorption chromatography on oligo-β-cyclodextrin-substituted agarose gel media has been another approach for LAB purification [14]: the recovery was 73.6% with a purity of 98.0%. Kan et al. [15] used microsphere resin chromatography combined with microbial biotransformation for LAB purification from crude extract, and achieved a yield of 75.0% and purity of 99.0%. Lee et al. [16] recently achieved a multi-step procedure for large-scale purification of LAB from Danshen using solvent fractionation and Sephadex LH-20 column chromatography followed by preparative high-performance liquid chromatography (HPLC): the recovery was 75.0% with purity of 99.2%. The methods mentioned above can provide high-purity LAB, but access to expensive media, the time-consuming procedure, and consumption of organic solvents has limited these approaches. It is necessary to develop an environmentally friendly and low-cost method to purify LAB in the food and pharmaceutical industries.

In this report, polyamide resin (Nylon-6) as a potential chromatographic medium and ethanol as the elution solvent were assessed for the separation of LAB from the crude extract of Danshen. The sorption conditions of LAB were studied and optimized by static and dynamic methods. The kinetics of the adsorption process and adsorption isotherms were exploited to improve this process. Based on this knowledge, a new adsorption chromatographic method was achieved for the isolation and purification of LAB from Danshen using polyamide resin and preparative HPLC.

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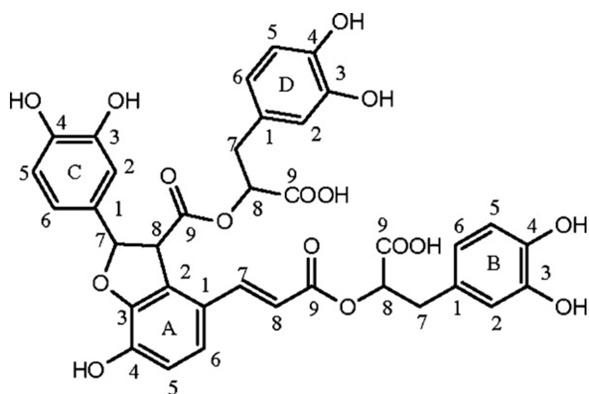

Fig. 1. Structure of LAB.

2. Experimental

2.1. Materials and reagents

Danshen was purchased from the Danshen Cultivation Base of Anguo (Hebei Province, PR China). Polyamide resin (Nylon-6; particle size, 75–150 μm; surface area, 5–10 m^2/g) was purchased from the New Pharmaceutical Company (Wuhan, China). The resin was not treated before use in adsorption and purification experiments. LAB with a purity of 98.5% was obtained from the National Institute for the Control of Biological and Pharmaceutical Drugs (Beijing, China). HPLC-grade acetonitrile and methanol were purchased from Tedia Company Incorporated (Fairfield, OH, USA). All other chemicals were of analytical grade. Deionized water was prepared using a Millipore water purification system (Millipore Company, Billerica, MA, USA).

2.2. HPLC analyses and validation

The analytical method for the determination of LAB was undertaken according to a previously published method [17] with some modification. Briefly, analytical HPLC was carried out on Agilent 1100 Series with a G1314A UV–Vis detector using an Agilent Zorbax Eclipse XDB-C8 column (150 mm × 4.6 mm; 5 μm). The HPLC system was controlled by a personal computer employing HP Chemstation software. The flow rate was 1.0 mL/min. Solvent A (1%, v/v aqueous acetic acid) and solvent B (acetonitrile:methanol = 3:2, v/v) were used as the mobile phase. The gradient was as follows: 0–10 min, linear change from 25% B to 35% B; 10–12 min, linear change from 35% B to 25% B; then the system was maintained at 25% B for 5 min. The temperature was maintained at 30 °C. The detection wavelength was 288 nm, and the injection volumes were 10 μL.

The working calibration curve based on LAB standard solutions showed good linearity over the range 0.05–8.00 mg/mL. The regression line for LAB was $y = 12178.2x - 1229.58$ ($r = 0.9991$, $n = 9$), where y is the peak area (mAU) of LAB and x is the concentration of LAB (mg/mL). The limit of detection (LOD) and limit of quantification (LOQ) were 0.75 μg/mL and 1.87 μg/mL determined at signal-to-noise ratios (S/N) of 3 and 10, respectively.

The intra-day precision of the method was determined using multiple analyses ($n = 6$) of the standard solution at three concentrations (low, medium, high) within 1 day. As shown in Table 1, the relative standard deviation (RSD) was <3.0% and the accuracies were in range 98.00–100.87%.

To determine the repeatability of the analysis method, six samples from the same crude extract (10.0 mg contained ∼4 mg LAB) were dissolved to 5.0 mL and determined using the developed method. The repeatability (RSD, $n = 6$) was 3.36%.

Table 1
Intra-day precision of the developed method ($n = 6$).

Concentration (mg/mL)	Detected (mg/mL)	Accuracy (%)	RSD (%)
0.05	0.049 ± 0.00123	98.00	2.51
4.00	3.96 ± 0.00628	99.00	0.16
8.00	8.07 ± 0.00804	100.88	0.09

A known amount of the mixed standard (1.25 mg) was added to a certain amount of the crude extract (5.0 mg) quantified previously, and then mixture was dissolved to 5.0 mL and determined using the developed method. The experiment recovery was 101.44% with a RSD value of 2.68%.

The stability of LAB was investigated at different pH values within 24 h. The results showed that the sample solution would be stable within 24 h with a RSD < 1.17% at pH 0.43–4.72. At pH 6.88, LAB is stable only within 9 h with a RSD of 0.93%. Data acquisition was analyzed using origin V 7.0 software (OriginLab, Northampton, MA, USA).

2.3. Adsorption experiments

Sorption equilibrium experiments were conducted in a closed 250 mL Erlenmeyer flask. This was achieved by shaking 0.200 g of polyamide resin in 100 mL aqueous solutions containing LAB at 150 rpm for a predetermined time. Sorption experiments were carried out in an incubator at controlled temperature. The initial pH of the solution was adjusted to the desired pH by adding hydrochloric acid or sodium hydroxide solution to the medium. pH measurements were done using a pH meter (Sartorius, Goettingen, Germany). Initial and equilibrium LAB concentrations in aqueous solutions were determined by HPLC. The adsorption capacity [18] of LAB was calculated using the following Eq. (1):

$$q_t = \frac{(c_0 - c_t)v_0}{m_s} \quad (1)$$

where q_t is the adsorption capacity of LAB onto polyamide resin (mg/g), c_0 and c_t are the LAB concentration in solution (mg/mL) initially and at any time (t), respectively, v_0 is the volume of the solution (mL) and m_s is the dry mass of the polyamide resin (g).

2.4. Desorption experiments

Desorption studies were done in a closed 250 mL Erlenmeyer flask by shaking 1.00 g of polyamide resin in 100 mL LAB solution at an initial concentration of 3.91 mg/mL at 150 rpm for 12 h. The polyamide resin was then separated by centrifugation for 3 min at 5000 rpm. After separation of the polyamide resin, it was agitated with 100 mL of different concentrations of ethanol solution for 4 h. The desorption ratio was evaluated using the following Eq. (2):

$$\text{DR}(\%) = \frac{c_d v_d}{(c_0 - c_e)v_i} \times 100\% \quad (2)$$

where DR (%) is the desorption ratio, c_d is the concentration of LAB in the desorption solution (mg/mL), v_d is the volume of desorption solution, c_0 is the initial concentration of LAB, c_e is equilibrium LAB concentration, and v_i is the volume of the initial solution.

2.5. Preparation of crude aqueous extract of Danshen

The dried roots of Danshen were ground into ∼0.42 mm powder (30.0 g). They were extracted twice with 300 mL of deionized water at pH 2.0 by microwave-assisted extraction using a domestic microwave oven (Wanjiale, Zhejiang, China) for 3 min under an output power of 650 W at <60 °C. The extraction solution was centrifuged for 3 min at 5000 rpm and the supernatant separated using

a polyamide resin column. As a control sample, 20 mL supernatant was freeze-dried. The purity of LAB was 6.20%.

2.6. Elution profile of LAB on polyamide resin

To study the elution profile of LAB in the chromatographic separation, adsorption of LAB from the supernatant was carried out on a glass column (600 mm × 25 mm, ID) packed with 20.0 g polyamide resin; the volume of the stationary phase was ~100 mL. The column was preconditioned for chromatography by pre-equilibrating in 500 mL deionized water. Next, 100 mL of 2.66 mg/mL LAB solution was loaded onto the column at a constant flow rate of 1.0 mL/min. First, 500 mL of deionized water (pH 2.0) were used to remove polysaccharides and proteins, and then the column was washed by sequentially passing 500 mL aqueous ethanol of 20 (pH 2.0), 50 (pH 2.0), 70 (pH 5.0) and 90% (pH 5.0, v/v). The effluents from the column were recovered using a collector in 100 mL aliquots. The LAB concentrations in each fraction were determined by HPLC. The percent recovery of LAB was calculated using the following Eq. (3):

$$\text{Recovery}(\%) = \frac{\text{total LAB eluted}}{\text{total LAB loaded}} \times 100\% \quad (3)$$

2.7. Purification by preparative HPLC and identification of the pure compound

Semi-preparative HPLC was developed on a Shim-pack PREP-ODS column (20 mm × 250 mm, 15 μm) using a LC-6AD system (Shimadzu, Japan). The flow rate was 8.0 mL/min. Solvent A (1%, v/v aqueous acetic acid) and solvent B (acetonitrile:methanol = 3:2, v/v) were used as the mobile phase. The processing conditions for the linear gradient elution of LAB were: 0–25 min, linear change from 55% B to 70% B; room temperature; the detection wavelength was 288 nm; and the injection volumes were 1.0 mL.

Identification of the pure LAB was carried out on a triple-quadrupole mass spectrometer (Finnigan MAT TSQ; Finnigan MAT, San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) interface. Auxiliary gas and sheath gas were at 20 and 40 arbitrary units, respectively. The mass spectrometer was programmed to perform full scans between 120 *m/z* and 1200 *m/z* in negative ion mode. The spray voltage was 4.5 kV, and the capillary temperature was 325 °C.

3. Results and discussion

3.1. Effect of initial concentration on adsorption

The initial study was done to determine the adsorption kinetics of LAB, as well as to choose the contact time to be used in the next steps of the optimization. Adsorption kinetics were much faster for a concentration of 2.66 mg/mL than for lower concentrations, an asymptotic curve was reached within 240 min of contact time, and the adsorption equilibrium was established (Fig. 2). A contact time of 240 min was therefore considered to be a reasonable contact time in this study. Adsorption capacity was strongly dependent upon the initial concentration of LAB. Adsorption capacity was 69, 196, and 376 mg/g at an initial LAB concentration of 0.16, 0.43, and 2.66 mg/mL, respectively, at 25 °C. This effect may be because, when LAB concentration increases, the adsorption may be from monolayer coverage to multilayer coverage due to strong interactions between hydrogen bonds among free-state LAB at low pH.

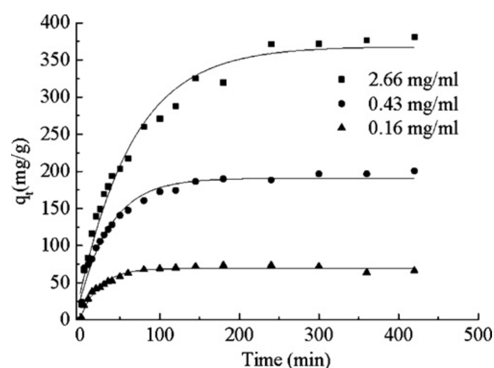

Fig. 2. Adsorption kinetics of LAB at different concentrations.

3.2. Adsorption kinetics

To clarify the kinetic characteristics of the adsorption, two well-known kinetic models (pseudo first-order kinetic model and pseudo second-order kinetic model) were applied to evaluate experimental data. The linear form of these two models is given as (4) and (5):

$$\log(q_e - q_t) = \log q_e - \left(\frac{k_1}{2.303}\right) t \quad (4)$$

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left(\frac{1}{q_e}\right) t \quad (5)$$

where q_e and q_t are the adsorption capacity of the LAB adsorbed (mg/g) at equilibrium and certain time t (min), respectively. k_1 is the adsorption rate constant (min^{-1}) of the pseudo first-order sorption reaction, and k_2 (g/mg min) is the rate constant of the pseudo second-order sorption reaction. According to the plots of the two linear models, the rate constants and theoretical equilibrium sorption capacities, q_e (theoretical), can be calculated from the slopes and intercepts. Constants related to these models are given in Table 2.

After comparison of the results with the correlation coefficients (Table 2), the correlation coefficients for the second-order kinetic model obtained at higher concentrations were higher than those for the first-order model. As can be seen from Table 2, the values of q_e (theoretical) and q_e (experimental) of the first-order mode (except for the concentration of 0.16 mg/mL) are not in good agreement with each other. This suggests that the adsorption of LAB onto polyamide resin does not fit to the pseudo first-order kinetic model. For the pseudo second-order kinetic model, theoretical and experimental q_e values are in a good accordance with each other.

Table 2
First-order and second-order rate constants.

Sample		Pseudo first-order			Pseudo second-order		
Initial concentration (mg/mL)	q_e (experimental)[a] (mg/g)	k_1 (min^{-1})	q_e (theoretical)[b] (mg/g)	R^2	k_2 ($\times 10^5$) (g/mg min)	q_e (theoretical)[b] (mg/g)	R^2
2.66	375.55	0.012	322.84	0.988	5.26	421.94	0.996
0.43	195.5	0.018	152.05	0.989	2.19	209.64	0.991
0.16	68.75	0.037	62.95	0.991	1.03	71.22	0.995

[a] q_e (experimental) is the experimental equilibrium adsorption capacity of LAB.
[b] q_e (theoretical) is the theoretical equilibrium adsorption capacity of LAB.

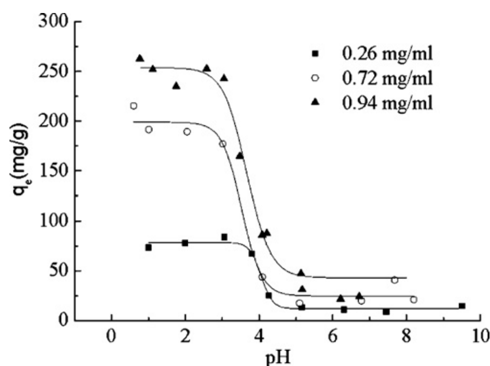

Fig. 3. Effect of pH on adsorption of LAB at different concentrations.

This suggests that the adsorption of LAB onto the polyamide followed pseudo second-order type kinetics. It is known that pseudo second-order kinetics provide the best fits to the experimental data for sorption systems in which chemisorption is significant in the rate-controlling step.

3.3. Effect of pH on adsorption

The optimal pH range for the higher adsorption capacity of LAB was pH < 3.0 (Fig. 3). A lower adsorption capacity of LAB was observed at pH >∼5.0. LAB is a dicarboxylic acid (pK_{a1} and pK_{a2} were 3.14 and 4.52, respectively) [17]. After adjustment of the solution to pH < 3.1, the carboxyl groups would be freed to readily form hydrogen bonds with polyamide resin. Solubility and adsorption are inversely related; at pH > 4.5, the carboxyl group of LAB would be present as carboxylate and would show high water solubility, resulting in a low tendency to hydrogen-bond. Hence, adsorption capacity can be controlled by solution pH in further chromatographic processes.

3.4. Adsorption isotherm

A sorption isotherm was constructed (Fig. 4). The trend of the curve of an adsorption isotherm provides interesting qualitative information regarding the nature of solute–area interactions [19]. The most popular classification of solute adsorption isotherms in aqueous solutions was established by Giles et al. [20] and is based on the configuration of the initial part of the curve. Adsorption isotherms obtained in this work were of type L2 (i.e., Langmuir class). They had a linear initial part showing high diffusion of LAB into the polyamide resin, and tending to an asymptotic region. This suggests that there was no strong competition between solvent and solute for occupation of adsorption sites [21] at low pH. The experimental points were fitted using the following formula (6):

Table 3
Langmuir constants for the adsorption of LAB on polyamide at various temperatures.

Temperature (°C)	Concentration (mg/mL)	b[a]	q_m[b] (mg/g)	R^2
25	0.118–1.574	12.05	490.26	0.999
35	0.240–2.61	6.12	483.07	0.995
45	0.127–1.84	3.38	474.71	0.987

[a] b are constants.
[b] q_m is the maximum adsorption capacity of LAB.

$$\frac{c_e}{q_e} = \frac{1}{q_m b} + \frac{1}{q_m} c_e \quad (6)$$

where q_e is the equilibrium adsorption capacity of LAB onto polyamide resin (mg/g), c_e is the equilibrium LAB concentration in the solution (mg/mL), q_m is the maximum adsorption capacity of LAB on polyamide resin, and b are constants.

The best fit was obtained using the Langmuir model ($R^2 > 0.996$). This suggested that saturation of adsorption sites by LAB in a monolayer could occur at a low concentration of LAB. The maximum adsorption capacity of LAB was calculated to be 380.2 mg/g. All these isotherms were fitted using the Langmuir equation to the experimental data. The correlation coefficient, b, and q_m calculated for these isotherms using a linear regression procedure for LAB at different temperatures are listed in Table 3.

As evident from the R^2 values in Table 3, the Langmuir isotherm yielded best fits to the experimental data. The values of the isotherm parameter b showed a decrease with an increase in temperature, which accounts for the exothermic nature of the ongoing process. Similarly, the values of q_m decreased with increases in temperature, indicating that adsorption is favored at lower temperatures.

3.5. Desorption studies

The results of desorption are shown in Table 4. The desorption ratio of LAB increased with increasing ethanol concentration. A desorption ratio >95% was obtained by >60% of aqueous ethanol solution, indicating that LAB was well separated on the polyamide resin.

3.6. Elution chromatogram of LAB

The elution profile of LAB from polyamide resin is shown in Fig. 5. Most of the LAB was released within fractions 13–21. The 70% aqueous fraction was collected and combined after HPLC analyses. The LAB fraction was evaporated under reduced pressure at 60–70 °C, and the residues freeze-dried. The target compound was

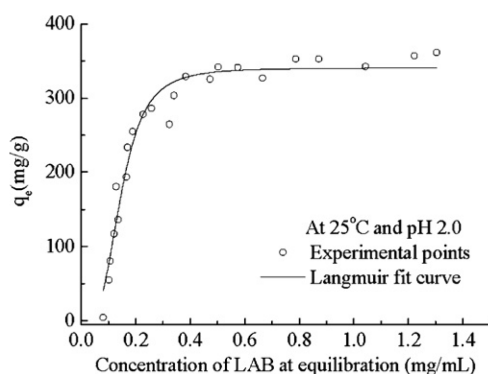

Fig. 4. Non-linear regression of experimental data obtained for the adsorption isotherms of LAB using polyamide resins according to the Langmuir model.

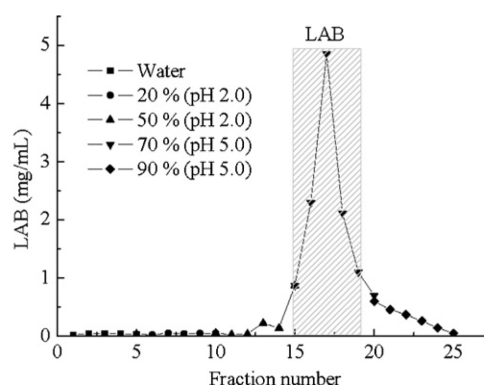

Fig. 5. Elution chromatogram of LAB.

Table 4
Effect of ethanol concentration on the desorption ratio.

Ethanol (v/v, %, pH 5.07)	20	40	60	80	95
DR (%)[a]	50.46 ± 3.16	71.25 ± 1.87	95.43 ± 2.45	95.30 ± 2.32	98.7 ± 2.23

Results are means ± SD ($n = 3$).
[a] *DR* (%) is the desorption ratio.

obtained with 85.3% purity and 87.1% recovery. Compared with the 6.2% purity in the crude extract, the fold purity was increased 13.75-fold in the elution process. With LAB, there are other phenolic acids present in the aliquots collected after column chromatography, which decreases the overall percent HPLC purity of LAB. To further purify LAB, preparative HPLC was carried out.

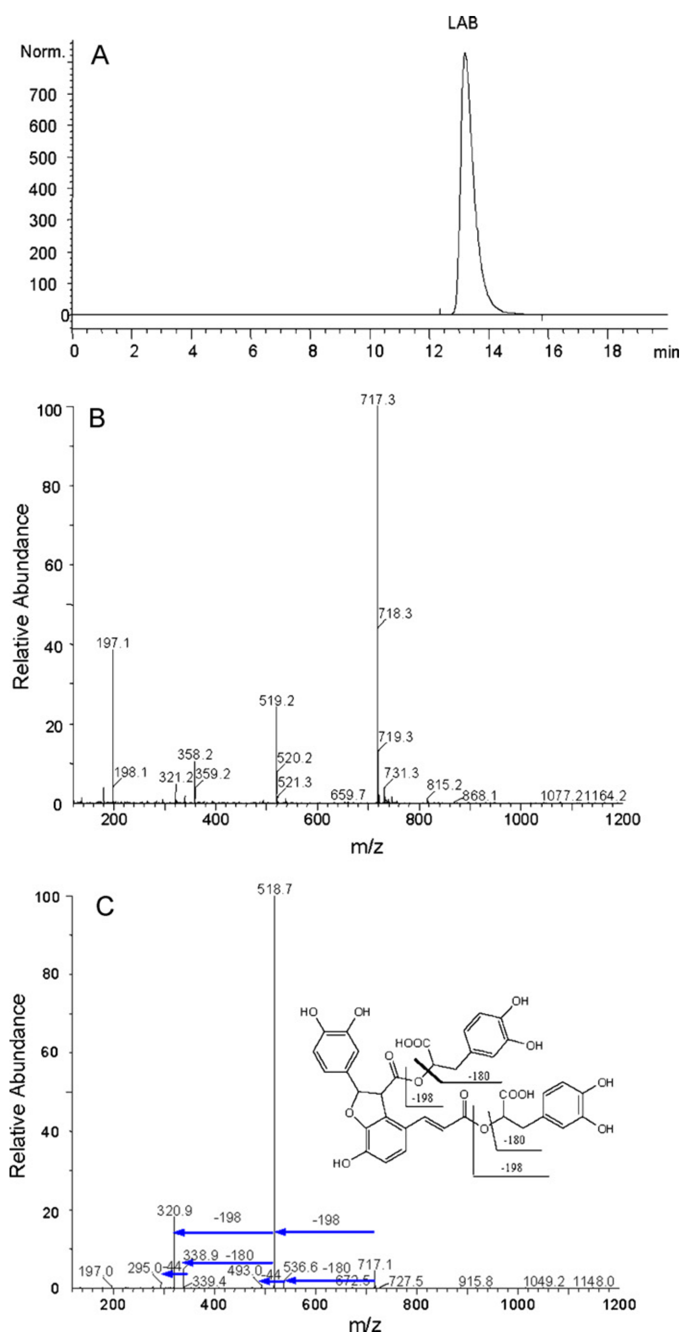

Fig. 6. HPLC chromatogram (A), mass spectrum of the isolated compound (B), and mass spectrum of the $[M-H]^-$ ion (*m/z* 717) of isolated compound (C).

3.7. Purification by preparative HPLC and identification

After purification with preparative HPLC, the purity of the LAB increased up to 99.28%, and the fold-purity increased up to 16.01, but the recovery decreased to 75.2%. The HPLC chromatogram and negative ion mass spectra of the isolated compound are presented in Fig. 6. The big ion $[M-H]^-$ at *m/z* 717 with a molecular mass of 718 was observed in its mass spectrum (Fig. 6B). According to Zeng et al. [22], characterization of the MS/MS spectrum of LAB should include the neutral loss of Danshensu (DSS, 198u), caffeic acid (CA, 180u) and CO_2 (44u). Those characterizations were demonstrated in the MS/MS spectrum of the isolated compound (Fig. 6C). For example, the spectrum of the $[M-H]^-$ ion at *m/z* 717 exhibited ions at *m/z* 519 and 537, corresponding to losses of one DSS (198u) and CA (180u), respectively. The characteristic fragment at *m/z* 321 resulted from the successive loss of two DSS (198u); the fragment at *m/z* 493 resulted from losses of CA (180u) and CO_2 (44u); and the fragment at *m/z* 295 resulted from losses of DSS (198u), CA (180u) and CO_2 (44u). These data corresponded with the values obtained by Zeng and Cui et al. [22,23]. Consequently, the isolated compound was unambiguously identified as LA B.

4. Conclusion

Summarizing data on the separation of LAB from the crude extract, we concluded that the adsorption process correlated well with the Langmuir-type adsorption isotherm. The maximal capacity of adsorption was calculated to be 280.2 mg/g. The optimal pH was <3.0 because the carboxyl group of LAB would be present in a free state; this decreases solubility and increases the chances of forming hydrogen bonds with the polyamide resin. Higher capacity was obtained with a higher initial concentration of LAB. LAB with a purity of 85.3% and recovery of 87.1% can be obtained by polyamide resin as a chromatographic medium under optimal adsorption and elution conditions. Further purification using preparative HPLC yielded LAB with a purity of 99.28% and total recovery of 75.2%. Compared with conventional preparation methods, the method described herein is suitable for large-scale preparation of LAB.

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