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Full Length Research Paper

Preparative enrichment and separation of isoliquiritigenin from licorice extracts with macroporous resins

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In the present study, a green and efficient method for the enrichment and separation of isoliquiritigenin (ISL) from licorice, a well known medicinal and food herb, was critically investigated. Firstly, the performances of adsorption properties for eight ADS macroporous resins were carried out, in order to choose the best resin. And ADS-F8 resin offered the best separation power for ISL, its adsorption equilibrium data fitted well to the Langmuir and Freundlich isotherms. Dynamic adsorption and desorption tests were carried out on columns packed with ADS-F8 resin to obtain optimal parameters for separating ISL. The results demonstrated that under optimum parameters for adsorption on ADS-F8 resin were sample solution ISL concentration 0.25 mg/ml, feed volume 2 BV, flow rate 1.5 ml/min, temperature 20°C; while for desorption were elution solution aqueous-ethanol (20:80, v/v) 2 BV, flow rate 1.5 ml/min. The purity of ISL after being treated was increased 7.82-fold from 3.55 to 27.77% with the recovery yield of 80.1%. This separation method is easy and effective, and will provide a potential approach for the large-scale ISL production.

Key words: Preparative separation, isoliquiritigenin; licorice, ADS-F8 resin, adsorption, desorption.

INTRODUCTION

Licorice, a plant of ancient origin, is widely used as dietary supplement in two primary forms: roots (rhizomes) and extracts (Al-Bachir et al., 2004). Both products have been approved to use in foods by most national and supranational regulatory agencies. Licorice derivatives have been given generally recognized as safe (GRAS) status in the USA in 1985 and were largely used as flavouring and sweetening agents in confectionery and other food products, such as beverages and chewing gum (Gabriele et al., 2001; Ariño et al., 2007). Besides being a popular food additive, licorice is also one of the most widely used traditional Chinese medicines (TCMs). It exerts antitussive, expectorant and antipyretic actions and can be used to treat diseases such as cough, pharyngitis, bronchitis, ague, phthisis, gastric ulcer, etc. (Ma et al., 2005; Cao et al., 2004). Among the active components in licorice and its extracts, isoliquiritigenin (ISL, Figure 1) is the remarkable one and has been reported to possess strong biological activity (Kim et al., 2010; Liu et al., 2008).

ISL was reported to possess antioxidative and superoxide scavenging activities (Haraguchi et al., 1998), antiplatelet aggregation effect (Tawata et al., 1992), estrogenic property (Tamir et al., 2001), and inhibitory

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Nomenclature: ***A,*** a parameter which relates to the adsorption energy; C_0, the initial concentrations of solute in the solution (mg/ml); ***Ce,*** equilibrium concentrations of solute in the solution (mg/ml); C_X, the concentration of the solute in the desorption solution (mg/ml); ***D,*** the desorption ratio (%); ***E,*** the adsorption ratio (%); ***K,*** the Freundlich constant; ***m,*** the dry weight of the corresponding resin (g); ***Q,*** the maximum adsorptive capacity; ***Qe,*** the adsorption capacity at adsorption equilibrium (mg/g dry weight of resin) ***Vi,*** the volume of the initial feed solution (ml); V_d, the volume of the desorption solution
1/*n,* an empirical constant.

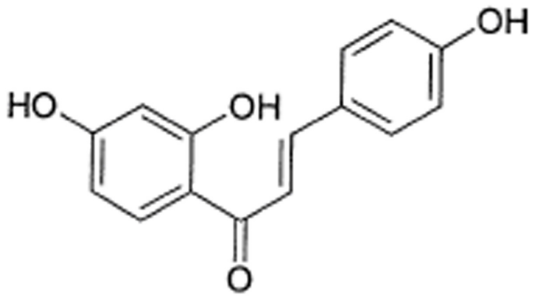

Figure 1. Chemical structure of isoliquiritigenin.

effect on xanthine oxidase activity *in vitro* (Kong et al., 2000). Recently, it was found that ISL had good effects on inhibiting proliferation, inducing apoptosis and locking cell cycle progression in the G1 phase against the human non-small cell lung cancer A549 cell line (Hsu et al., 2004). It induced cell cycle arrest and p21 expression in human lung cancer cells (Ii et al., 2004), apoptosis and p53-expression in Hep G2 cells (Hsu et al., 2005), and also induces apoptosis by depolarizing mitochondrial membranes in prostate cancer cells (Jung et al., 2006).

Due to the fact that natural ISL was low content in licorice and the isolation was difficult, therefore, the development of an efficient separation method turns to be of extreme importance. Macroporous resins are durable polar, non-polar or slightly hydrophilic polymers having high adsorption capacity with possible recovery of the adsorbed molecules. Purification through adsorption is based on differences in molecular weight, polarity, or shape of different molecules from the solution, which leads to differences in affinity for the adsorbent (Liu et al., 2004). Compared to the normal chromatography separation with silica gel, alumina, polyamide and liquid-liquid partition methods, macroporous resin is more efficient presenting a moderate purification effect, relatively fast, low cost and results in higher recovery of the products. Macroporous resins have been successfully applied for recovery of anthocyanins from grape pomace extracts (Kammerer et al., 2005), and separation of madecassoside and asiaticoside from *Centella asiatica* extracts (Jia et al., 2008). Fu et al. (2005) have studied the application of macroporous resins in the separation of licorice flavonoids and glycyrrhizic acid. Using XDA-1 resin, glycyrrhizic acid was well separated from the total flavonoids, however, no single flavonoid with higher content was obtained. Moreover, the parameters for dynamic adsorption and desorption tests were not optimized. In our previous study (Fu et al., 2006), some commonly used resins were tested for the separation of ISL, these resins were broad-spectrum and the adsorption capacities were not satisfactory.

ADS resins are a series of macroporous adsorptive resins developed by Nankai Hecheng Ltd. (Tianjin, China) with specificity to flavonoids, they can selectively adsorb flavonoids from aqueous solutions as well as non-aqueous systems. In the present study, some ADS resins were investigated for their adsorption and desorption properties of ISL. According to our results, an efficient method for the preparative enrichment and separation of ISL from licorice extracts with the optimal ADS-F8 resin was developed. Higher ISL content in the product was obtained with good recovery after being treated with ADS-F8 resin. The developed method can be referenced for separation of ISL in large scale or in others raw material in general.

MATERIALS AND METHODS

Reagents and adsorbents

Isoliquiritigenin (4,2',4'-trihydroxychalcone) was purchased from Sigma (Spain). Cellulase (>1000 u/mg) was obtained from Shanghai WeeBeyond Scientific & Trade Co., Ltd. (Shanghai, China) and dissolved in water to yield a solution at the concentration of 0.4 mg/ml. Methanol of HPLC grade was purchased from J & K Chemical Ltd. (China). All other reagents were analytical grade and the deionized water was purified by a Milli-Q Water Purification system (Millipore, MA, USA). All solutions were filtered through 0.45 µm membranes (Fisher Scientific, USA) before HPLC analyses.

Eight macroporous resins including ADS-8, ADS-17, ADS-21, ADS-F8, ADS-7, ADS-5, ADS-31 and ADS-13 were supplied by Nankai Hecheng Ltd. (Tianjin, China). Table 1 summarized the data offered by the manufacturers on the physical properties of the resins. The resins were pre-treated by 1 mol/l HCl and NaOH solutions successively to remove the monomers and porogenic agents trapped inside the pores during the synthesis process, and then dried at 60°C under vacuum (Grzegorczyk and Carta, 1996). Prior to use in the adsorption experiments, pre-weighed amounts of resins were wet with ethanol, and then by deionized water (Juang and Shiau, 1999).

HPLC analysis of ISL

Jasco HPLC system (Model 7800, Japan) equipped with thermostatic controlled column chamber, injection valve with a 10 µl sample loop was used to perform the analysis of ISL. All the modules were controlled by HPLC System Manager Window-based software. Chromatographic separation of ISL was carried out on HiQ Sil C18V reversed-phase column (250 × 4.6 mm i.d., Kya Tech, Japan) packed with 5 µm diameter particles, fitted with suitable guard column. The mobile phase was methanol-water-phosphoric acid (50:49.8:0.2, v/v/v), the flow rate was 1 ml/min, the column temperature was maintained at 30°C, the detection wavelength was 360 nm, and the retention time was 36.75 min. The working calibration curve based on ISL standard solutions showed good linearity over the range of 0.035-0.60 mg/ml. The regression line for ISL was $Y = 53831491.39X - 74737.83$, ($R^2$ =0.9991, n = 8), where Y was the peak area of ISL and X was the concentration of ISL (mg/ml).

Preparation of crude licorice extracts

The licorices (*Glycyrrhiza uralensis* Fisch.) were purchased from medicinal materials market of Harbin, China, and authenticated by Prof. Shao-Quan Nie from the Key Laboratory of Forest Plant

Table 1. Physical properties of the tested macroporous resins.

Trade name	Surface area (m^2/g)	Average pore diameter (nm)	Particle diameter (mm)	Polarity
ADS-5	520 - 600	25.0 - 30.0	0.3 - 1.25	Non-polar
ADS-8	450 - 500	12.0 - 16.0	0.3 - 1.25	Non-polar
ADS-17	90 - 120	25.0 - 30.0	0.3 - 1.25	Moderate-polar
ADS-11	190 - 220	25.0 - 30.0	0.3 - 1.25	Polar
ADS-21	80 - 100	15.0 - 20.0	0.3 - 1.25	Polar
ADS-31	60.9	12.6	0.3 - 1.25	Polar
ADS-F8	100 - 120	15.0 - 20.0	0.3 - 1.25	Polar
ADS-7	100 - 120	25.0 - 30.0	0.3 - 1.25	Strong-polar

Ecology, Ministry of Education, Northeast Forestry University, China. Voucher specimens were deposited in the herbarium of this Key Laboratory. The dried licorices were powdered and sieved through a 20-60 mesh metal sieve to achieve a standard size of particles. Enzyme assisted extraction of ISL was conducted by adding 500 ml aqueous solution of cellulase (0.4 mg/ml) to 100 g licorice sample. The mixture was kept in an Erlenmeyer flask and allowed to react for 24 h at 35°C with 100 rpm (round per minute) agitation. 2000 ml ethanol was added, followed by ultrasonic extraction at room temperature for 10 min, the extraction procedure was repeated for three times. The extracted solutions were centrifuged at 4000 rpm for 10 min at room temperature using a centrifuge (22R, Heraeus Sepatech, Germany). The supernatants were concentrated to dryness under vacuum in a rotary evaporator (RE-52AA, Shanghai Huxi Instrument Co., China) and then dissolved in aqueous-ethanol (85:15, v/v) solution to get a sample solution of licorice extracts at the concentration of 0.3 mg/ml for ISL.

Static adsorption and desorption experiments

Pre-weighed amounts of hydrated resins (equal to 3.0 g dry resin) were put into 150 ml in Erlenmeyer flasks with 0.3 mg/ml ISL sample solutions in static adsorption-desorption experiments. The flasks were then shaken (100 rpm) for 12 h at 20°C. After adsorption equilibrium was reached, the resins were desorbed for 6 h in shakers at 20°C using 100 ml aqueous-ethanol solutions at different concentrations, respectively. The adsorption capacities of different resins towards ISL were evaluated and the adsorption equilibrium isotherms were conducted by static adsorption equilibration. Equilibrium experiments for each adsorbent-ISL system were carried out at 20, 30 and 45°C by shaking the flasks at 100 rpm for 6 h. The solution was separated from the resin and the equilibrium concentration of ISL in solution was determined by HPLC.

Dynamic adsorption and desorption experiments

Dynamic adsorption-desorption experiments were carried out on glass columns (2 × 30 cm) wet-packed with ADS-F8 resin. The ISL concentration in sample solution was 0.25 mg/ml. The bed volume (BV) of resin was about 27 ml and the packed length of resin bed was 8.6 cm. The adsorption performance at two different feed flow rate, 1.5 ml/min and 3.0 ml/min, were compared. The effluents were collected at 10 min intervals and the concentration of ISL was monitored by HPLC. The loading of the sample solution was stopped when the concentration of ISL in effluent reached the initial concentration. The adsorbate-laden column was washed first with deionized water, and then desorbed with aqueous-ethanol (20:80, v/v) solution at the flow rate of 1.5 and 3.0 ml/min. The eluents were collected at 5 ml intervals and the concentration of ISL was monitored by HPLC.

Capacity of adsorption, ratios of adsorption and desorption

The following equations are used to quantify the adsorption capacity as well as the adsorption and desorption ratio:

Adsorption evaluation

$$Qe = [(C_0 - Ce) \times Vi] / m \quad (1)$$

$$E\,(\%) = [(C_0 - Ce) / C_0] \times 100 \quad (2)$$

Desorption evaluation

$$D\,(\%) = C_X V_d / [(C_0 - Ce) \times V_i] \times 100 \quad (3)$$

Langmuir and Freundlich equations

Langmuir equation:

$$1/Qe = 1/(Q\,aCe) + 1/Q \quad (4)$$

Freundlich equation:

$$\ln Qe = 1/n \ln Ce + \ln K \quad (5)$$

Where K is the Freundlich constant that is an indicator of adsorption capacity, and $1/n$ is an empirical constant related to the magnitude of the adsorption driving force (Jung et al., 2001).

RESULTS AND DISCUSSION

Adsorption kinetics

Adsorption kinetics curves were obtained for ISL on the eight macroporous resins. The adsorption process comprises three steps when the distribution of the solute molecules between the adsorbent and the liquid phase: mass transfer of solute molecules from the solution to the adsorbent particle surface, and then diffusion within internal structure to the adsorption site, finally rapid uptake (Scordino et al., 2003). As can be seen from Figure 2, for all the eight resins, the adsorption capacity increased with adsorption time. At the first 3 h, the slope

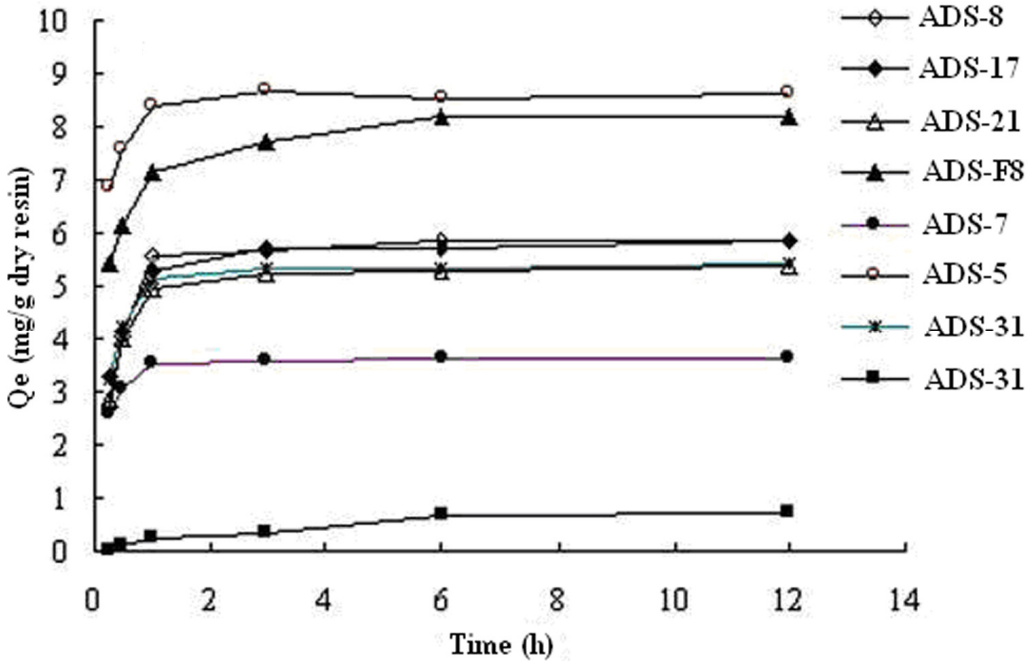

Figure 2. Static adsorption kinetics curves of ISL on eight different resins.

of the tangent on the kinetics curves which indicates the adsorption rate decreased rapidly, and then leveled off after 3 h, and reached equilibrium at about 6 h. The fast adsorption process in the first 3 h was due to high diffusivity of ISL into micropores of the resins in the bulk solution, and the slow adsorption process after 3 h was due to the high intraparticle mass transfer resistance within the macroporous resins. Hence, the static adsorption time for ISL can be selected as 6 h.

Selection of optimal resin

The adsorption and desorption processes were conducted by the procedure described in static adsorption and desorption experiments. Table 2 shows the adsorption and desorption ratio of ISL. ISL is a moderate-polar compound because of the presence of three hydroxyl groups, resulting in its highly adsorptive interaction with the polar ADS-F8 resin. Simultaneously, the desorption ratio of ISL on ADS-F8 resin by aqueous-ethanol (20:80, v/v) solution was 83.15% and the content of ISL was 24.1%, which were much higher than those of other resins, indicating that ISL was well separated on ADS-F8 resin. Considering this, ADS-F8 resin was selected as the most suitable resin and used in the following tests.

Adsorption isotherms on ADS-F8 resin

Equilibrium adsorption isotherms were obtained by contacting 150 ml sample solutions at different concentrations with ADS-F8 resins in a shaker with the temperature controlled at 20, 30 and 45°C. The initial concentration of ISL in the sample solutions were 0.035, 0.067, 0.121, 0.182, 0.243, and 0.300 mg/ml, respectively.

As can be seen from Figure 3, the adsorption reached the saturation plateau when the initial concentration of ISL was 0.243 mg/ml. Thus, the concentration of ISL in the feed solution was selected as 0.25 mg/ml.

Table 3 showed the parameters of Langmuir and Freundlich equations for ISL on ADS-F8 resin at different temperatures. The Langmuir equation is based on a theoretical model, which assumes that adsorption occurs onto specific sites of a uniform energetic surface, without interactions between adsorbed molecules, and adsorption confined to monolayer layers. On the other hand, the Freundlich equation is used extensively in the physical adsorption and chemical adsorption and can be applied to describe the adsorption behaviour of monomolecular layer as well as that of the multi-molecular ones.

As shown in Table 3, within the temperature investigated, the adsorption equilibrium data at 20°C correlated better with the Langmuir and Freundlich equations, the correlation coefficients were 0.9990 and 0.9838, respectively. These can describe the better adsorption behaviour of ISL on ADS-F8 resin. It could be observed that at the same initial concentration, the adsorption capacity of ADS-F8 resin decreased with the increasing of temperature (Figure 3). Therefore, 20°C was selected as the proper temperature for adsorption of ISL on

Table 2. Adsorption-desorption data of ISL on eight macroporous resins (n=3).

Trade name	Adsorption capacity (mg/g)	Adsorption ratio (%)	Desorption capacity (mg/g)	Desorption ratio (%)	Content (%)
ADS-8	5.85	46.73	4.41	75.45	8.35
ADS-17	5.71	45.62	4.30	75.22	9.52
ADS-21	5.31	42.42	2.53	47.57	9.95
ADS-F8	8.17	65.27	7.12	83.15	24.10
ADS-7	3.60	28.76	2.30	63.62	9.91
ADS-5	8.54	68.23	6.56	76.85	4.40
ADS-31	5.36	42.82	1.13	21.07	4.24
ADS-13	0.65	5.19	0.52	80.14	3.70

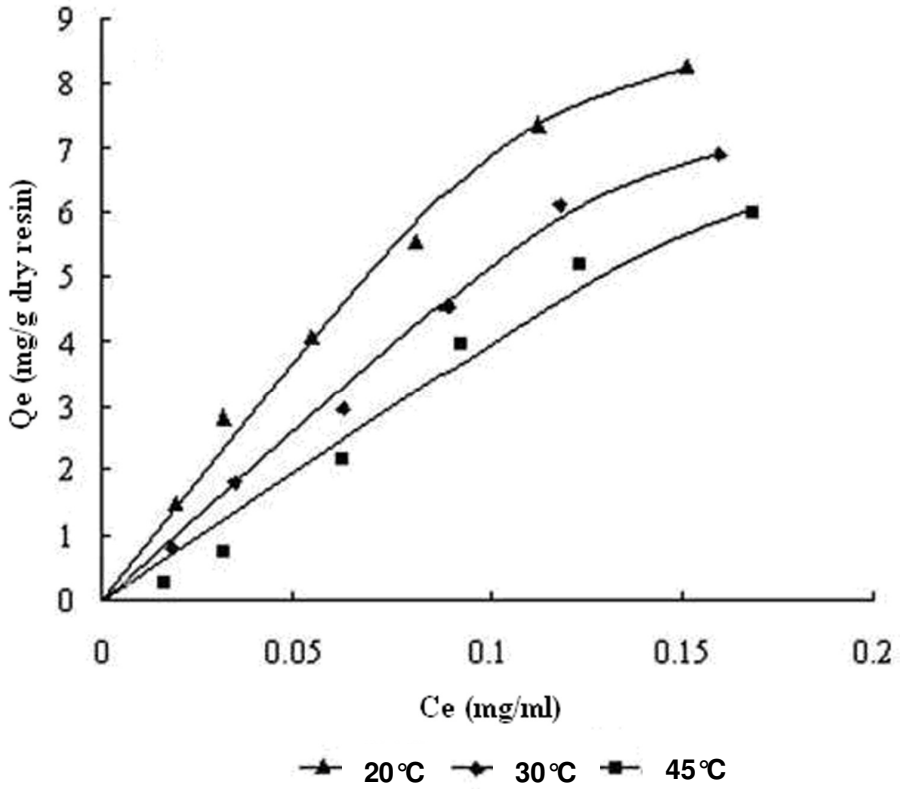

Figure 3. Adsorption isotherms of ISL on ADS-F8 resin at different temperatures.

on ADS-F8 resin and was used in the following tests.

Static desorption on ADS-F8 resin

It is known that resins have greater adsorption capacity and the adsorbates are much easier to be eluted from resins than other adsorbents such as alumina, silica gel, etc. Considering the objective of our study was to obtain an enriched preparation of ISL for medicine industry, aqueous ethanol solutions were used instead of methanol.

It was found in our study, with the increase of the ethanol concentration, the desorption ratio of ISL increased accordingly. ISL could not be detected in the eluent from ADS-F8 resin when the ethanol concentration was lower than 20%. When the concentration was increased up to 80%, the desorption ratio of ISL on ADS-F8 was 83.15%, and the ISL content in product was 24.1%, which were higher than those obtained by other ethanol concen-trations (data not shown). Therefore, aqueous-ethanol (20:80, v/v) solution was selected as the

Table 3. Langmuir and Freundlich adsorption isotherm parameters for ISL on ADS-F8 resin.

Temperature (℃)	Langmuir equation	Correlation coefficient R^2	Freundlich equation	Correlation coefficient R^2
20	1/Qe = 0.0098/Ce + 0.0573	0.9990	ln Qe = 0.7682 ln Ce + 3.6232	0.9838
30	1/Qe = 0.0232/Ce - 0.0363	0.9899	ln Qe = 1.0147 ln Ce + 3.9080	0.9883
45	1/Qe = 0.0681/Ce - 0.4632	0.9765	ln Qe = 1.4075 ln Ce + 4.5428	0.9832

Ce, equilibrium concentrations of solute in the solution (mg/ml)
Qe, the adsorption capacity at adsorption equilibrium (mg/g dry weight of resin)

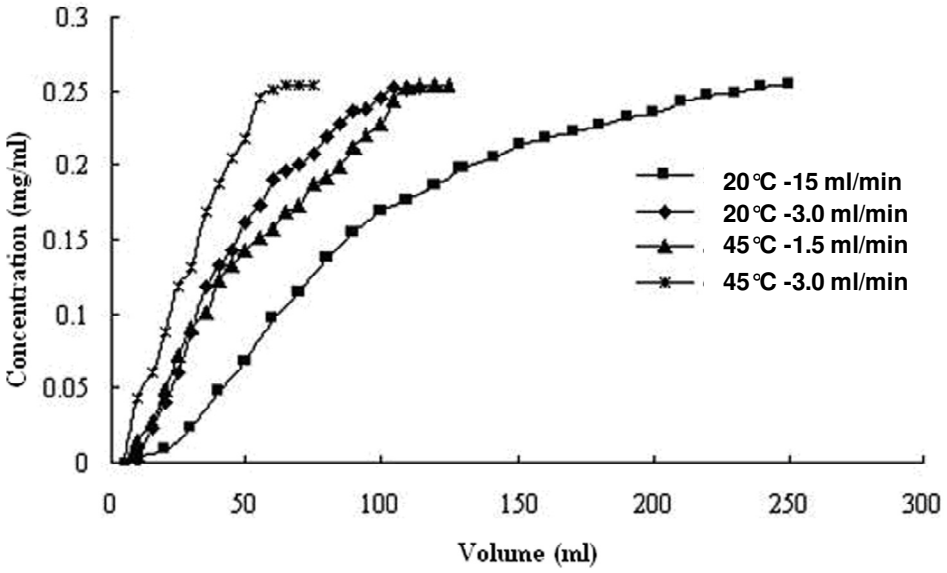

Figure 4. Dynamic leakage curves of ISL on ADS-F8 resin.

appropriate desorption solution.

Dynamic leakage curves on ADS-F8 resin

The experimental leakage curves for ISL on ADS-F8 resin were constructed and shown in Figure 4. At the same flow rate, when the operation temperature was low, the interactions between ISL molecules and the active sites of resin were more sufficient and the adsorption capacity was higher. It can be also observed that lowering the feeding flow rate postponed the reaching to the saturation plateau. However, within the investigated temperature, for both the flow rate, the resin bed reached the saturation plateau at the same concentration. Therefore, for the more processing volume of sample solution and more sufficient adsorption, 1.5 ml/min was selected as the best sample flow rate, and the operation temperature was maintained at 20℃ for further experiments.

Under this condition, when the ISL concentration in the effluent reaches 20% of its initial concentration, it can be considered the adsorption of ISL on ADS-F8 resin reached equilibrium, the feed volume of sample solution was 50 ml, about 2 BV.

Dynamic desorption curves on ADS-F8 resin

As can be seen from Figure 5, 1.5 ml/min was appropriate for desorption of ISL from ADS-F8 resin in this study, since at this flow rate 50 ml (2 BV) of desorption solution could elute ISL thoroughly, whereas it needed 65 ml at 3.0 ml/min.

The desorption solution were collected, analyzed by HPLC and dried under vacuum. The ISL content, defined as the wt% of ISL in the product, was calculated. The content of ISL increased 7.82-fold from 3.55 to 27.77%, and the recovery yield of ISL was 80.1%. Figure 6 illustrated the HPLC chromatograms of the test samples of licorice extracts before and after treatment with ADS-F8 resin. As was shown, majority of impurities were removed from the extracts while the peak of ISL was heightened in the product and its relative area increased

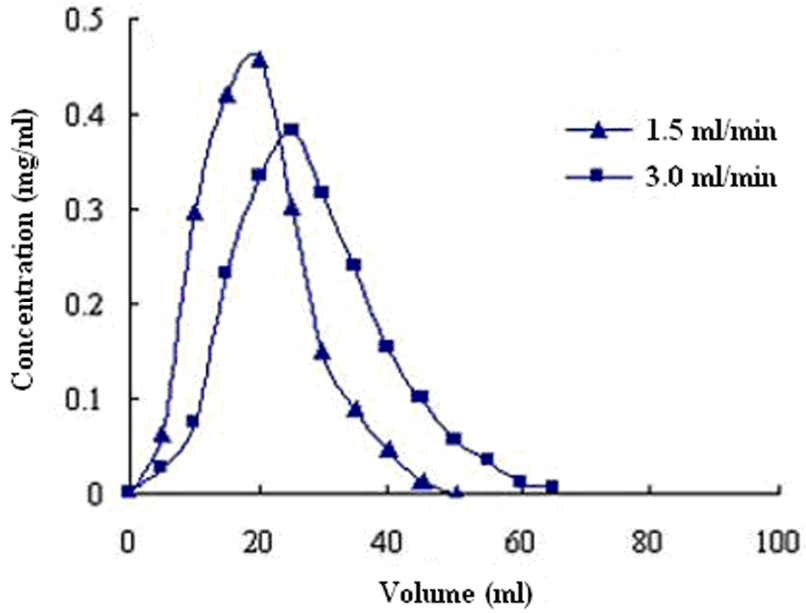

Figure 5. Dynamic desorption curves of ISL on ADS-F8 resin.

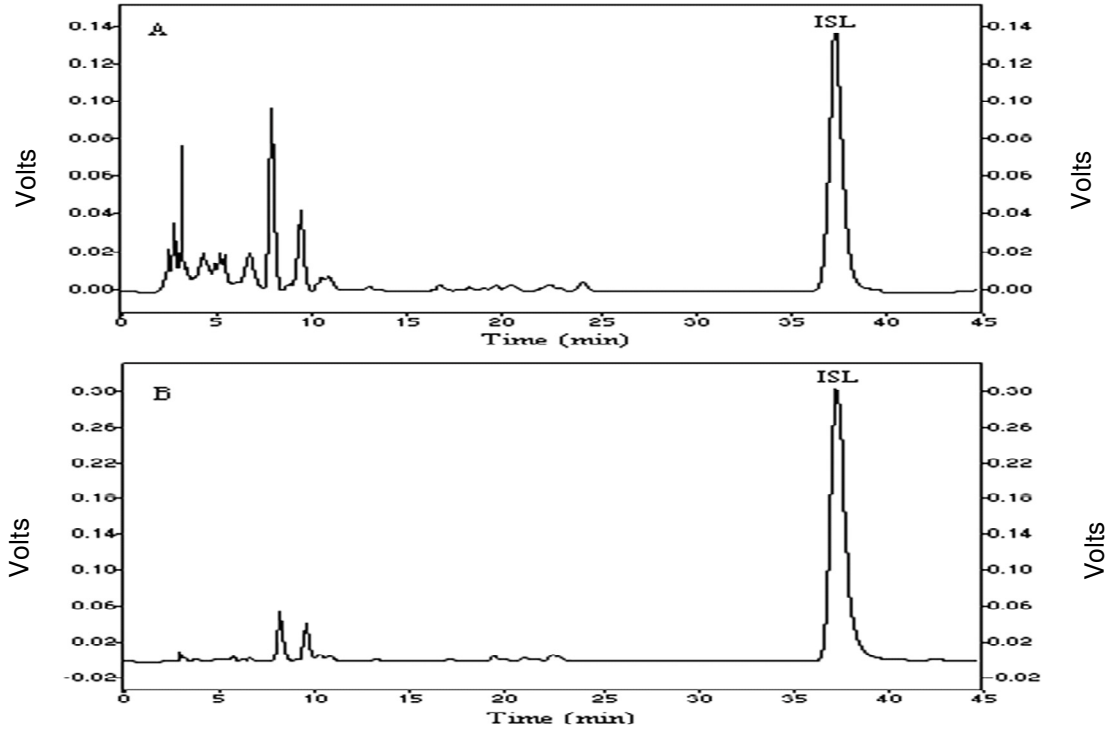

Figure 6. HPLC chromatograms of sample solution of licorice extracts (A) and desorption solution (B) from ADS-F8

markedly.

Our results revealed that the optimum parameters for the preparative separation of ISL with ADS-F8 macroporous resin as follows:

Adsorption: sample solution ISL concentration 0.25 mg/ml, feed volume 2 BV, flow rate 1.5 ml/min, temperature 20℃; Desorption: elution solvent aqueous-ethanol (20:80, v/v), volume 2 BV, flow rate 1.5 ml/ min.

Conclusions

In the present study, the preparative enrichment and separation of ISL was successfully achieved from licorice extracts on ADS macroporous resins. According to the research results, ADS-F8 resin offered the best separation power for ISL among the eight resins investigated. Within the temperature studied (20 to 45°C), lower temperature exhibited a better separation power for ISL on ADS-F8 resin. Under optimal conditions, the content of ISL in the product was increased 7.82-fold, from 3.55 to 27.77%, and the recovery yield was 80.1%. The application of macroporous resins as separation of ISL for crude extracts should have some advantages, such as its simple operation, low cost, high efficiency, and it may provide scientific references for the large scale ISL production.

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