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Separation and Purification of Ursolic Acid from *Cynomorium songaricum* Exacts with Macroporous Resins

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Abstract

Enrichment and purification of ursolic acid from *Cynomorium songaricum* exacts were studied using five macroporous resins. The static tests indicated that D101 resin was appropriate and its adsorption data were well fitted to the Langmuir and Freundlich isotherms. To optimize the separation process, dynamic adsorption and desorption tests were carried out. The optimal adsorption parameters were initial concentrations in sample solution of 3.8 mg/mL, pH of 6.0, sample loading amount of 5 BV, flow rate of 2 BV/h, and temperature of 25˚C. The optimal desorption parameters were 70% ethanol 5 BV, then 80% ethanol 10 BV, and flow rate of 2 BV/h. After one run treatment with AB-8 resin, the content of total flavonoids in the product increased from 9.83% to 67.8%, and the recovery yield was 84.02%. The results showed that AB-8 resin revealed a good ability to enrichment total flavonoids from *Cynomorium songaricum*, and the method can be referenced for the enrichment of total flavonoids from other materials.

Subject Areas

Biotechnology

Keywords

Ursolic Acid, Macroporous Resins, *Cynomorium songaricum* Rupr.

1. Introduction

Cynomorium songaricum Rupr., also known as "Suoyang" in China, has been widely used in traditional Chinese medicine, which is an obligate root parasitic plant. Its most common host is *Nitraria tangutorum* Bobr. [1], which plays an important ecological role as a windbreak and in sand fixation. It is mainly dis-

tributed in Northwestern China and Central Asia and is used as healthy foods and nutrients by local people [2]. Nowadays, the stems of *C. songaricum* are widely used as functional food ingredients or supplements. For example, the wine containing extract of *C. songaricum* as an ingredient is popular in China. Pharmacological research had demonstrated that anti-oxidation was one of the main activities of *C. songaricum* [3]. *C. songaricum* also has other activities, including anti-HIV [4], anti-aging [5], improving sexual function, and immunity [6].

Among the effective ingredients of *C. songaricum*, ursolic acid and polysaccharides are the most abundant effective components in *C. songaricum* [7]. In view of these well-known pharmacological properties, *C. songaricum* flavonoids have great potential to be used as a clinical therapeutic agent. Hence, more scientific researches are needed to confirm these bioactivities. Therefore, it is essential to apply low-cost and effective technology for the extraction and further purification of *C. songaricum* ursolic acid with good recovery and high content of flavonoids.

There are some methods for finding the concentration of active constituents from traditional Chinese herbs, such as solid-liquid extraction [8] [9] or solvent extraction [10] [11], column chromatography [12] [13] and the preparative high-speed counter-current chromatography [14] [15] [16]. However, these separation methods have limitations such as low extraction yield, the inclusion of many various steps, intense energy consumption, low efficiency and labor intensiveness. Comparatively, separation methods by macroporous resins are popular as a simple procedure, easy operation, low cost, high efficiency and easy generation [17] [18]. Therefore, there has been a growing interest in employing macroporous resins to separate bioactive components from crude extracts of herbal raw materials. For example, macroporous resins have been successfully used in the enrichment of polysaccharide from Astragalus extracts [19], pedunculoside and syringin from *Ilex rotunda* Thunb extracts [20], steviol glycosides from *Stevia rebaudiana* bertoni [21]. However, there is no report on using macroporous resins to enrich and separate ursolic acid from *C. songaricum* extracts so far.

In the present study, five resins with different polarities were used to investigate the adsorption and desorption properties of ursolic acid, and to develop a simple and efficient process for preliminary enrichment and separation of ursolic acid from *C. songaricum* extracts with the optimal resin. The parameters influencing the adsorption and desorption properties of ursolic acid were optimized and the experimental equilibrium data at different temperatures were fitted to Langmuir and Freundlich isotherms. The results from this study would be significant in order to develop a preparative method for the separation of ursolic acid from *C. songaricum* extracts.

2. Materials and Methods

2.1. Materials and Instruments

C. songaricum was obtained from microalgae engineering research center of

Gansu Province, China. The sample was dried at room temperature, powdered by an electric grinder and passed through a 40 mesh sieve. The rutin standard was purchased from the National Institute for the Control of Pharmaceutical Drugs in Beijing, China. All other chemicals used were of analytical-reagent grade.

Macroporous resins including NKA-9, NKA, D101, AB-8 and HPD-750 were purchased from the Chemical Plant of NanKai University (Tianjin, China). Their physical and chemical properties are listed in Table 1. They were pre-treated with HCl and NaOH solutions to remove the monomers and porogenicagents trapped inside the pores during the synthesis process. Prior to use, the resins were soaked in ethanol for 24 h, and subsequently was repeatedly washed with the ethanol until there was no residue after distillation, and finally washed with sufficient distilled water.

SP-721 spectrophotometer (Spectrum Instruments Corporation, Shanghai, China) was used for analysis of total flavonoids, RE-2000A rotary evaporator (Gongyi Jinghua Instrument Factory, Henan, China) was used for concentration of samples.

2.2. Preparation of Crude Ursolic Acid Extract

The dried powder was extracted twice with 1000 mL 100% ethanol at 65˚C for 3 h. The extract was centrifuged and the supernatant filtered through a 0.45um filter membrane, concentrated to remove the ethanol. The content of ursolic acid in extracts was 7.48%. The crude extract could be diluted by deionized water to obtain a different concentration used for the following experiments.

2.3. Determination of Ursolic Acid Content

The content of total flavonoids was determined by the colorimetric method with some modifications [22] [23]. The separations of ursolic acid were carried out using the ACE 5C18 250 × 4.6 mm (Advanced Chromatography Technologies, Aberdeen, Scotland) column. The mobile phase was composed of methanol and water (90/10, v/v). The flow rate was 0.6 mL/min and injection volume was 10 μL. Absorption was measured at 203 nm. Quantification was carried out by the external standard method and calibration curves were obtained.

2.4. Static Adsorption and Desorption Tests

2.4.1. Adsorption and Desorption Capacities, Desorption Ratio

The static adsorption and desorption tests were carried out in a water bath mode. 1 g resin was introduced into a 100 mL triangular flask. 30 mL of crude exact of ursolic acid (4 mg/mL) was added to each flask. The flasks were kept in the shaking water bath at 150 r/min and 25˚C until adsorption reaching equilibrium. After removed of the residual extract solutions, the adsorb ate-laden resins were washed with 15 mL deionized water for 3 times and desorbed with 50 mL 80% ethanol-water solution. During the desorption process, the flasks were shaken at 150 r/min, 25˚C for 12 h.

Table 1. Physical properties of the used macroporos resins.

Name	Particle diameter (mm)	Surface area (m^2/g)	Average pore diameter (nm)	Polarity
NKA-9	0.3 - 1.25	250 - 290	15.5 - 16.5	Polar
NKA	0.3 - 1.25	160 - 200	14.5 - 15.5	Polar
D101	0.3 - 1.25	≥400	9.0 - 11.0	Non-polar
AB-8	0.3 - 1.25	480 - 520	13.0 - 14.0	Weak-polar
HPD-750	0.3 - 1.25	650 - 700	8.5 - 9.0	Mild-polar

The selectivity of resins was based on the capacities of adsorption (Q_e), capacities of desorption (Q_d), and ratio of desorption (D), which were quantified according to the following equations:

$$Q_e = \left(C_0 - C\right)_e \times \frac{Vi}{W} \tag{1}$$

$$Q_d = \frac{C_d \times V_d}{W} \tag{2}$$

$$D = \frac{C_d \times V_d}{\left(C_0 - C_e\right)V_i} \times 100\% \tag{3}$$

where Q_e was the adsorption capacity at adsorption equilibrium(mg/g dry resin). Q_d was the desorption capacity after adsorption equilibrium (mg/g dry resin). C_0, C_e, and C_d were the initial, absorption equilibrium and desorption concentrations of analyte in the solutions, respectively (mg/mL). V_i and V_d were the volume of the initial sample and desorption solution (mL), respectively. W was the dry weight of resin (g), and D was the desorption ratio (%).

The impact of pH on the adsorption capacities of ursolic acid was carried out by mixing 1 g (dry weight) of hydrated selected resin with sample solutions (30 mL each) in the pH range of 4.0 - 8.0. The sample pH was adjusted to the desired value with hydrochloric acid or ammonia solution. Then, the flasks were shaken at 25˚C for 6 h.

2.4.2. Adsorption Kinetics

The adsorption kinetics curve of total flavonoids on the preliminarily selected resins was studied according to the aforementioned method. The concentrations of ursolic acid in liquid phase were monitored at equal time intervals till equilibration at 25˚C.

2.4.3. Adsorption Isotherms

In order to investigate the effect of initial concentration and temperature on the ursolic acid absorption, experiments of adsorption isotherm on selected resin were performed. Five aliquots of 30 mL sample solutions at different concentrations were contacted with pre-weighed amounts of hydrated resins (equal to 1 g dry resin) in a constant temperature shaker at 25˚C, 30˚C and 35˚C for 6 h.

The Langmuir and Freundlich models were used to evaluate the adsorption

behavior between adsorbate and adsorbent [24] [25] [26].

Langmuir isotherms:

$$Q_e = \frac{Q_m \times K_L \times C_e}{1 + K_L \times C_e} \tag{4}$$

Freundlich isotherms:

$$Q_e = K_F \times C_e^{1/n} \tag{5}$$

where Q_e and C_e were the same as those in Equation (1), K_L (mg/mL) and Q_m (mg/g resin) were the Langmuir constants. K_L was the dissociation constant of the adsorption interaction and Q_m was the maximum adsorption capacity. K_F (mg/mL) and n were the Freundlich constants.

2.4.4. Dynamic Adsorption and Desorption Tests

Dynamic adsorption and desorption experiments were carried out in a glass column (17 × 120 mm) wet-packed with the selected resin and the BV of the resin was 10 mL. The flow rate of sample solution was 2 BV/h through the glass column and the ursolic acid content in the effluent liquid was monitored by the colorimetric method till equilibrium adsorption. After adsorption equilibrium, the column was firstly washed with deionized water, and then eluted with different concentrations ethanol at the flow rate of 2.0 BV/h. Dynamic adsorption and desorption tests were repeated three times under optimal conditions, and the recovery of ursolic acid were calculated.

3. Result and Discussion

3.1. Adsorption and Desorption Capacities, Desorption Ratio

According to the "like dissolve like" rule, given ursolic acid contain non-polar phenyl group and polar multi-hydroxyl groups, either non-polar resins or polar resins were applicable to adsorption of ursolic acid. So, five macroporous resins ranging from non-polarity to polarity were employed for enrichment the ursolic acid.

As shown in Table 2, D101, AB-8 and HPD-750 exhibited adsorption capacities (97.2 mg/g resin, 90.6 mg/g resin and 80.26 mg/g resin, respectively) for ursolic acid. The result indicates that the three resins had good adsorption selectivity in contrast with other resins. However, for the desorption ratios of ursolic

Table 2. Adsorption capacities, adsorption and desorption ratios of total flavonoids on different macroporous resins.

Name	Q_e (mg/g resin)	Q_d (mg/g resin)	D (%)
NKA-9	45.02 ± 0.45	12.75 ± 0.24	28.32 ± 0.42
NKA	51.2 ± 0.35	17.22 ± 0.74	33.63 ± 0.17
D101	97.2 ± 0.47	85.23 ± 0.27	87.68 ± 0.14
AB-8	90.6 ± 0.19	76.10 ± 0.27	83.99 ± 0.27
HPD-750	80.26 ± 0.15	45.23 ± 0.22	56.31 ± 0.29

acid, they were 87.68% (D101), 83.99% (AB-8) and 56.31% (HPD-750), respectively, which illustrated that it was disadvantageous to the desorption of ursolic acid for D101 and AB-8.

Compared with other resins, D101 resin had a much higher adsorption capacity as well as the higher desorption ratio. Therefore, on full consideration of the adsorption/desorption properties and desorption ratio, D101 was selected as a suitable resin for separating ursolic acid in further study.

3.2. Effect of Sample Solution pH Value

The pH of the sample solution significantly affected the adsorption capacity of ursolic acid on D101 resin. As shown in **Figure 1(a)**, the adsorption capacity initially increased to reach its peak value at pH 6, and then decreased gradually. The result indicates that hydrogen bonding may play an important role in the adsorption process on D101 resin. At a higher pH value, the hydrogen bonding interactions were reduced, because the phenolic hydroxyl groups in ursolic acid dissociated to H^+ and their corresponding anions, thus resulting in the lower adsorption capacity. Hence, suitable pH value for the following tests was adjusted to 6.

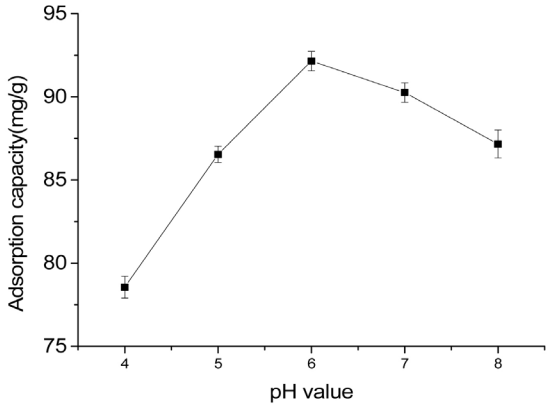
3.3. Adsorption Kinetics on D101 Resin

It is not enough for assessing the performance of a resin to use only static adsorption/desorption ratio tests. A suitable resin must also have high adsorption rates. The adsorption kinetics curves for the ursolic acid on D101 resin were obtained at 25˚C. As shown in **Figure 1(b)**, in the beginning, the adsorption capacity of D101 increased rapidly within 4 h, and then increased slowly, finally, reaching equilibrium at around 5 h. The fast initial step is likely due to the occurrence of adsorption in the easily accessible mesopores of the particles, proceeding with low mass transfer in the bulk solution. The later slower uptake, on the other hand, is indicative of processes with high intraparticle mass transfer resistance [27].

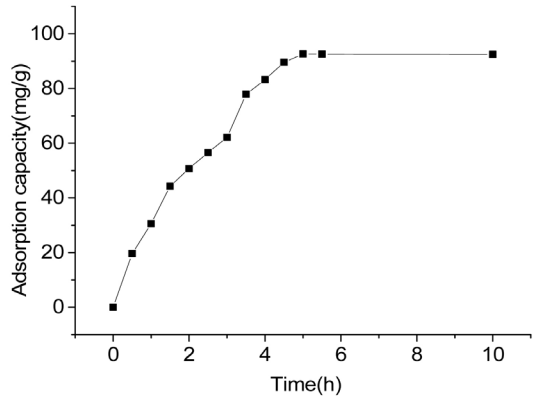
3.4. Adsorption Isotherms on D101 Resin

To further explore the adsorption properties of the ursolic acid on D101, equilibrium adsorption isotherms on D101 resin were investigated with various initial concentrations of ursolic acid at 25˚C, 30˚C and 35˚C. The initial concentrations of the ursolic acid in the solutions were 2.0, 2.7, 3.3, 3.8 and 4.0 mg/mL, respectively. As shown in **Figure 1(c)**, the adsorption capacity increased with the initial concentration and reached the saturation plateau when the initial concentration of the ursolic acid was 3.8 mg/mL. Thus 3.8 mg/mL ursolic acid was used as initial concentration of sample solution in the following tests.

In addition, for the interpretation of the equilibrated relationship between the solutes and the adsorbents, the Langmuir and Freundlich isotherms were used in this study. The Langmuir equation is the best known and the frequently used



(a)



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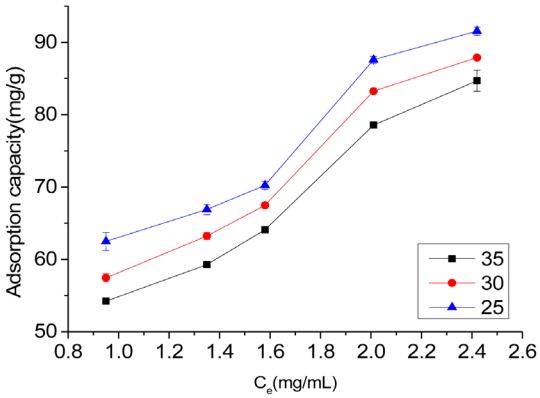

(c)

Figure 1. Effect of pH value on the adsorption capacity of ursolic acid on D101 resin (a); adsorption kinetics curve for theursolic acid on D101 resin at 25˚C (b); and adsorption isotherms for the ursolic acid on D101 resin at different temperatures (c) (25˚C, 30˚C and 35˚C).

equation to describe the adsorption behaviour of monomolecular layer [28], whereas the Freundlich equation is widely used to describe the adsorption behaviour of monomolecular layer as well as that of the multi-molecular layer [29]. **Table 3** listed the two isotherm equations at different temperatures and two important parameters: Q_m value (obtained from the Langmuir isotherm) and 1/n

Table 3. Langmuir and Freundlich parameters of ursolic acid on D101 resin at different temperature.

Temperature(˚C)	Langmuir equation	R^2	Freundlich equation	R^2
25	$C_e/Q_e = 0.0096\ C_e + 0.0084$	0.9962	$Q_e = 73.06\ C_e 0.3801$	0.9817
30	$C_e/Q_e = 0.0074\ C_e + 0.0079$	0.9934	$Q_e = 64.08\ C_e 0.4364$	0.9807
35	$C_e/Q_e = 0.0074\ C_e + 0.0088$	0.9923	$Q_e = 56.39\ C_e 0.4299$	0.9801

value (obtained from the Freundlich isotherm). The correlations (0.9923 - 0.9962) for ursolic acid on D101 indicated that the two models were suitable for describing the tested adsorption system in the concentration ranges studied.

Generally, in the Freundlich equation, the adsorption was easy to carry out when 1/n value was between 0.1 and 0.5, and it was difficult to take place if 1/n value was above 1 [30]. It can be seen from **Table 3**, the 1/n values were all between 0.3 and 0.5, indicated that the adsorption of the ursolic acid on D101 resin took place easily.

Within the ranges of temperatures investigated, the adsorption capacities decreased with the temperature increase (**Figure 1(c)**), which indicated the adsorption process was a thermopositive process. Similar results were obtained for the enrichment other compounds using macroporous resin [31]. Therefore, 25˚C was selected in the following experiments.

3.5. Dynamic Breakthrough Curve on D101 Resin

The dynamic leakage curve on D101 resin at the flow rate of 2 BV/h was obtained based on the volume of effluent liquid and the concentration of solute here in (**Figure 2**). In general, when the adsorption reaches the break point, the adsorption affinity decreases, even disappears, and the solutes leak from the resin. Hence, it is important to set up the leakage curve in order to calculate the quantity of resin, and the feed volume of sample solution. The adsorption capability is usually thought to reach the saturation when the concentration in effluent is 10% of the original concentration [32]. As shown in **Figure 2**, no obvious leakage of the ursolic acid in the effluent liquid was observed before 6 BV. Then, the concentration of the ursolic acid in the effluent liquid increased rapidly until it reached a steady plateau in 8 BV. The breakthrough point was captured when the feed volume of sample solution was approximate 5 BV (50 mL).

3.6. Dynamic Desorption Tests on D101 Resin

Dynamic desorption was performed with gradient eluent at the flow rate of 2 BV/h. Different elution solvents with the same volume of 5 BV were used to desorb the ursolic acid when the sample loading amount was 5 BV. At the 35% ethanol, the ursolic acid was hardly desorbed. When the ethanol concentration was over 35%, the desorption ability increased sharply and reached a peak value at 80% ethanol. Hence, a gradient elution procedure with 35% and 80% ethanol at a flow rate of 2 BV/h was applied for desorption of the ursolic acids. In

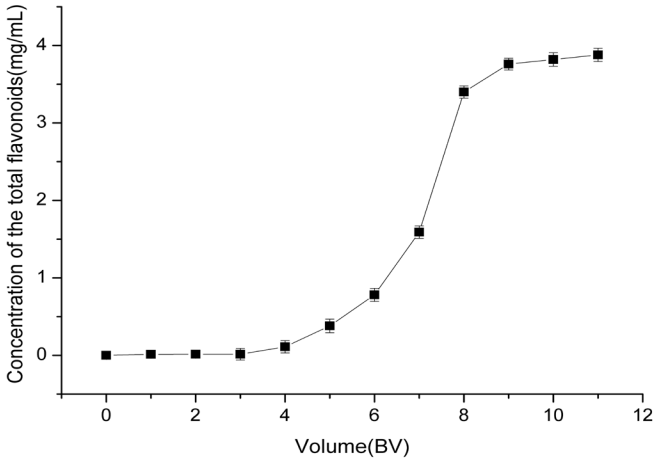

Figure 2. Dynamic breakthrough curves of ursolic acid on AB-8 resin.

Table 4. Results of gradient elution of the ursolic acids on column packed with D101 resin.

Concentration of ethanol (%)	Mass of dried residue (mg)	Mass of the ursolic acids (mg)	Content of the ursolic acids (%)	Recovery of the ursolic acids (%)
0	17.2 ± 0.25	0	0	
35	29.0 ± 0.31	0	0	
70	64.4 ± 0.19	28.6 ± 0.98	62.9 ± 0.91	
80	116.2 ± 0.35	95.3 ± 0.85	74.9 ± 1.51	
Collection of 60 - 70	180.6 ± 0.85	113.9 ± 0.75	63.06 ± 0.86	76.02 ± 1.22

addition, the elution volume of each ethanol concentration was modified under the guidance of absorbance.

The dried extract of *C. songaricum* (1.5 g) was dissolved in deionized water. The ursolic acids were enriched and purified from the sample solution with D101 resin. According to content analysis of ursolic acids, the elution scheme was modified as 5 BV deionized water, 5 BV 30% ethanol, 5 BV of 70% ethanol and 10 BV of 80% ethanol. As shown in **Table 4**, non-adsorption components were firstly washed out by 5 BV deionized water. Subsequently, some impurities were removed by elution of 5 BV 35% ethanol. The following 10 BV of 80% ethanol elution gave the product rich in the ursolic acids. After separation on D101 resin by the gradient elution, the content of the ursolic acids reached 63.06%, which was 10.1-fold to those in extract of C. *songaricum*, and the recovery yields were 76.02%.

4. Conclusions

The present study reported experimental data on the enrichment and separation of the ursolic acids from extracts of *C. songaricum* at various operating parameters, using five macroporous resins differing in chemical and physical properties. Among the resins used, the most effective resin (D101) was successfully applied

to obtain a product of the ursolic acids with higher content. The optimal enrichment and purification conditions for ursolic acid were confirmed as follows: for adsorption: concentration of ursolic acid in sample solution 3.8 mg/mL; pH 5; feed volume 5 BV; flow rate 2 BV/h; temperature 25˚C; for desorption: 70% ethanol 5 BV, then 80% ethanol 10 BV; flow rate 2 BV/h. The content of ursolic acids in the product was increased 10.1-fold from 7.84% to 63.06%, with a recovery yield of 76.02%.

In conclusion, this adsorption-desorption method using D101 resin is suitable for the separation of the ursolic acids from extracts of *C. songaricum* extract due to its simplicity of the method, high efficiency and likely ease in scaling-up.

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