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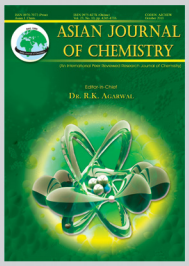

Separation of Syringin and Flavonoids from *Saussurea involucrata* by Macroporous Resin

L.Y. YAO[1,2], L.W. WANG[1], B. ZHAO[1,*], X.F. YUAN[1], X.D. WANG[1], Y.C. WANG[1], H.M. SU[3] and S.M. NIU[4]

[1]National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, 100190 Beijing, P.R. China
[2]Graduate University of Chinese Academy of Sciences, 100049 Beijing, P.R. China
[3]Xinjiang Fukang Forest Farm, 831500 Fukang, P.R. China
[4]Administration of Xinjiang Tianchi Lake National Natrue Reserve Areas, 831500 Fukang, P.R. China

*Corresponding author: Fax: +86 10 62574372; Tel: +86 10 82627059; E-mail: bzhao@home.ipe.ac.cn

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The performance and separation characteristics of nine macroporous resins for the enrichment and purification of flavonoids and syringin from planted *Saussurea involucrata* extracts were evaluated. The experimental results showed that a resin labeled X-5 offered higher adsorption and desorption capacities and higher adsorption speed for syringin and flavonoids than other resins and its adsorption data fit to the Freundlich isotherm. The dynamic adsorption and desorption experiments were carried out in the column packed with X-5 resin to optimize the separation process. An enriched flavonoids extract without syringin and an enriched syringin extract with 80.3 % purity could be separated from crude *Saussurea involucrata* extracts in one run. The results showed that X-5 resin had a good ability to separate syringin and flavonoids from planted *Saussurea involucrata*. This method would be very useful and has important significance for the protection of wild *Saussurea involucrata*.

Key Words: Adsorption, Flavonoids, Macroporous resin, *Saussurea involucrata,* Syringing.

INTRODUCTION

Saussurea involucrata is a well-known Chinese traditional medicine for the treatment of inflammation, arthritis, menstrual problems and regulating menstrual cycles[1]. Modern pharmacological studies have demonstrated that it possesses some activities such as free radical scavenging, antifatigue, antiinflammation, anticancer and immunomodulation[2-5]. The extracts of *S. involucrata* are mainly composed of flavonoids, which are the active components, including apigenin, kaempferide, acacetin, luteolin, quercetin, apigenin-5,6-dimethoxy-flavone, apigenin-6-methoxy-flavone, rutin, *etc*.[6-8]. Syringin, one of the phenolic glucosides isolated from *S. involucrata*, has various medicinal applications. Recent studies have shown that syringin and its derived sinapyl alcohol had tumor necrosis factor α-secretion inhibiting effect, an anti-hypersensitivity effect as well as antiinflammatory effects on autoimmune diseases[9,10].

Currently, there are a few reports on separation methods available for the enrichment and purification of flavonoids in *S. involucrata*[6-8,11]. The separation and purification are directly related to the product quality and clinic curative effect. Though the conventional methods for separating flavonoids from the extracts are normally carried out by means of solid-liquid extraction or liquid-liquid extraction, followed by a column chromatography, these separation processes were not particularly effective in the decrease of energy consumption and labor intensiveness. Alternatively, the adsorption-desorption process on macroporous resins is one of the more efficient methods with a moderate purification effect and can be used for the recovery and concentration of plant secondary metabolites. Macroporous resins are durable, polar, non-polar or slightly hydrophilic polymers having high adsorption capacity with possible recovery of the adsorbed molecules, relative low cost and easy regeneration[12]. In recent years, macroporous resin adsorption technology is gaining popularity in pharmaceutical applications and has also been used for the purification of flavonoids[13-15].

Saussurea involucrata grows on high mountains and in severely cold areas. The inhabitation is thus very rigorous. Driven by commercial incentives, the wild population of this plant has been threatened with depletion in recent years due to excessive harvesting. It has been listed as a protected plant by Chinese government. In previous study, we established a method for rapid and efficient multiplication of the *Saussurea involucrata* plants and DNA polymorphisms and genetic

variations were investigated using randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers to control the genetic quality of this traditional Chinese medicinal plant[16]. The HPLC fingerprints of *Saussurea involucrata* for the quick quality control was also established, 13 characteristic peaks were indicated on HPLC fingerprints among 10 batches of *S. involucrata* samples including planted and wild from different areas in Xinjiang[17]. The present study has been focused on separation of flavonoids and syringin produced in planted *S. involucrata* as to substitute use of wild plants. There is no report on separation for syringin and flavonoids in the *S. involucrata* crude extract so far. In the present study, experiments were carried out to investigate the adsorption and desorption properties of nine different macroporous resins and develop an efficient method for the preparative separation of syringin and flavonoids with the optimal resin. The information in this study is significant in the selection of adsorption resins for enrichment and purification of flavonoids extracts from *S. involucrata* or other herbal materials in general.

EXPERIMENTAL

Ethanol, phosphoric acid, sodium nitrite, aluminum nitrate and sodium hydroxide (all AC grade) were purchased from the local market and used without further purification. Methanol and acetonitrile were HPLC grade (Fisher, USA). Standard samples of hispidulin, rutin, quercitrin and syringin were purchased from the National Institute of the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China.

S. involucrata was collected from Tianchi snow lotus plant base in Tianshan region (Xinjiang, China), dried to constant weight and pulverized and then stored in an airtight bag before the experiments.

Adsorbents: Macroporous resins including ADS-8, ADS-17, AB-8, NKA, NKAII and X-5 were supplied by Nankai Hecheng S & T (Tianjin, China), HPD100, HPD400 and HPD600 were obtained from Cangzhou Bon Chemical Co. Ltd., China.

Adsorption ratio, adsorption capacity and desorption ratio of resins were determined in this study and polarity, surface area and pore-size were provided by the manufacturers (Table-1). 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, then, dried at 60 ºC under vacuum. Prior to use in the adsorption experiments, preweighed amounts of adsorbents were wetted with ethanol and subsequently the ethanol was thoroughly replaced with deionized water.

Preparation of crude *Saussurea involucrata* extracts: *Saussurea involucrata* was dried at room temperature, powdered by a disintegrator and then sieved (40-80 mesh). *S. involucrata* powder (10 g) was extracted by 300 mL of ethanol-water (50:50, v/v) solution in an ultrasonic bath for 0.5 h, repeated two times. The extraction solution was centrifuged at 5000 rpm for 10 min. The supernatant was concentrated to 1/10th of the original volume by removing the ethanol in a rotary evaporator at 65 ºC.

TABLE-1
PHYSICAL PROPERTIES OF THE MACROPOROUS RESINS

Resin	Polarity	Surface area (m^2/g)	Pore radius (Å)	Granularity (mm)
ADS-8	Non-polar	450-500	120-160	0.3-1.2
ADS-17	Moderate polar	90-120	250-300	0.3-1.2
AB-8	Moderate polar	480-520	130-140	0.3-1.25
NKA	Non-polar	570-590	200-220	0.3-1.25
NKAII	Polar	160-200	145-155	0.3-1.25
X-5	Non-polar	500-600	290-300	0.3-1.25
HPD100	Non-polar	650-700	90-100	0.3-1.25
HPD400	Moderate polar	500-550	75-80	0.3-1.25
HPD600	Polar	500-550	100-120	0.3-1.25

Analytical methods

Determination of concentrations of total flavonoids: UV-VIS analysis was applied to determine the content of total flavonoids[15]. The level of total flavonoids was analyzed by the chemical colourimetric technique utilizing reaction of $AlCl_3$ with phenolic hydroxyl group in basic solution. Maximum absorbance was at 495 nm, thereby being detectable by an UV-VIS spectrophotometer. A good linear relationship was obtained over the range of 0.005-0.5 mg/mL and the regression equation was $y = 0.0064x$ ($R^2 = 0.9992$, $n = 6$), where y is the absorbance at 495 nm, x is the concentration of flavonoids (mg/mL) and R is the regression coefficient.

HPLC analysis: Quantification of the syringin concentration was carried out by HPLC. The HPLC system (Model LC-20AT, Shimadzu, Japan) used for the determination was consisted of a system controller and SPD-M20A diode array detector. Sample analysis was performed on a Diamonsil TM Diamond C_{18} column (5 µm, 250 mm × 4.6 mm) with a gradient elution. The gradients were formed by varying the proportion of water (A) and acetonitrile (B). The elution procedure was: 0-25 min, 10-30 % of B; 25-30 min, 30 % of B; 30-60 min, 30-40 % of B. The flow rate was 1.0 mL/min. The retention time of syringin was 15.28 min. The column temperature was maintained at 30 ºC.

Static adsorption and desorption tests: The static adsorption tests of *S. involucrata* extracts were performed as follows: 1 g hydrated resin and 25 mL aqueous solution of crude extracts were added into a 100 mL Erlenmeyer flask. The flasks were then shaken (120 rpm) at 25 ºC for 12 h. Total flavonids and syringin in the solution after adsorption were analyzed by UV-VIS and HPLC method respectively. After the adsorption reached equilibrium, the appropriate volume of 90 % ethanol was added to the separated adsorbents, shaking at 25 ºC for 24 h, the desorption solution was also analyzed. The preliminary selection of these resins was evaluated by the adsorption capacities and the ratios of adsorption and desorption.

The selectivity and adsorption capacity of different resins for syringin and flavonoids were evaluated by their adsorption isotherms, fitness to Freundlich equation and desorption properties. The adsorption and desorption properties under different conditions including solution concentration and ethanol-water ratios used for desorption were also compared.

Dynamic adsorption and desorption tests: Dynamic adsorption experiments were carried out in a glass column (12 mm × 500 mm) wet-packed with the selected resin. The

bed volume (BV) of the resin was 20 mL and the packed length of resin bed was 18 cm (equal to 10 g of dry resin). The feed rate was 1.0 mL/min. The syringin and total flavonoids contents in the effluent liquid were analyzed by spectrophotometer and HPLC, the eluted aliquots collected at 5 mL intervals by a BSZ-100 auto-fractional collector (Shanghai, China). When adsorption equilibrium was reached, the adsorbate laden column was washed first with deionized water (3 BV), then eluted by ethanol-water (30:70, 2 BV; 80:20, 3 BV, v/v) solution, the flow rate of elution solvent was 1 mL/min. All the dynamic experiments were performed at room temperature. The concentrations of syringin and flavonoids in each part of the collected desorption solution were determined. The eluate was concentrated and dried under vacuum before further analysis.

Adsorption capacity, ratios of adsorption and desorption: The following equations were used to quantify the capacity of adsorption as well as the ratios of adsorption and desorption.

$$q_e = \frac{(c_o - c_e)v_i}{w} \quad (1)$$

$$E = \frac{c_o - c_e}{c_o} \times 100\% \quad (2)$$

where, q_e is the adsorption capacity at adsorption equilibrium (mg/g resin); E is the adsorption ratio (%); c_o and c_e are the initial and equilibrium concentrations of solute in the solution, respectively (g/mL); v_i is the volume of the initial sample solution (mL) and W is the weight of dry resin (g).

$$D = \frac{c_d - v_d}{(c_o - c_e)v_i} \times 100\% \quad (3)$$

where, D is the desorption ratio (%); c_d is the concentration of solute in the desorption solution (g/mL); v_d is the volume of the desorption solution (mL); c_o, c_e and v_i are the same as above.

RESULTS AND DISCUSSION

Selection of the resins: Resins with non-polarity or weak-polarity revealed stronger adsorption of non-polar substances. Besides, the dimensional structure of resin is another important factor affecting adsorption performance[18]. As shown in Table-2, the adsorption and desorption ratios of X-5 and NKA resins are considerably higher than those of other resins. This correlates with the polarity of the resins and the chemical properties of the adsorbed substance. The high adsorption capacities of HPD100, HPD400 and HPD600 may because of their big surface area and small pore radius, yet their desorption ratios were lower. Hence, X-5 and NKA resins were selected to conduct a further investigation in their adsorption and desorption behaviours towards syringin and flavonoids.

Adsorption kinetics on X-5 and NKA resins: Generally, the resins with different physical and chemical properties show different dynamics of adsorption. So it is necessary to investigate the factors involved in dynamics of adsorption. The kinetics curves of flavonoids and syringin adsorption on X-5 and NKA resins were obtained. As can be seen in Figs.1 and 2, for the two resins, the adsorption capacities increased with the extension of adsorption time, reached equilibrium in about 4 h. In the first 2 h the adsorption capacities increased rapidly and then increased slowly and reached equilibrium at 4 h. At any time the adsorption capacity of syringin on X-5 was higher than that of NKA. In the comprehensive consideration of the adsorption capacity and desorption ratio, X-5 resin was selected as the most suitable resin for the separation of flavonoids and syringin and used in the following tests.

TABLE-2
RESULTS OF ADSORPTION CAPACITIES, ADSORPTION AND DESORPTION RATIOS OF DIFFERENT RESINS

Resin	Adsorption capacity (mg/g resin)		Adsorption ratio (%)		Desorption ratio (%)	
	(1)	(2)	(1)	(2)	(1)	(2)
ADS-8	11.20	2.52	54.6	84.2	59.2	83.5
ADS-17	14.63	8.27	24.6	83.5	33.8	78.1
AB-8	12.37	7.00	51.5	76.5	63.1	79.8
NKA	16.25	8.25	85.2	86.1	86.4	90.5
NKAII	11.65	9.75	47.7	75.5	46.1	56.3
X-5	16.40	9.26	80.5	88.3	91.2	94.4
HPD100	16.00	7.10	68.4	80.0	76.1	80.4
HPD400	14.50	9.33	69.2	85.2	74.6	79.8
HPD600	13.25	8.54	66.0	84.7	73.7	81.3

(1) Total flavonoids; (2) Syringin

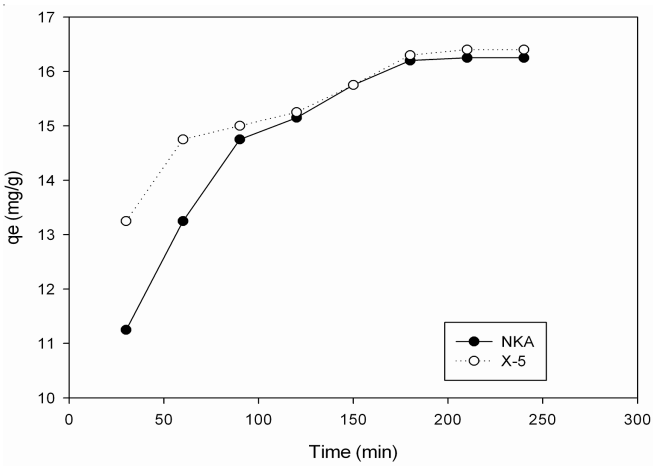


Fig.1. Adsorption kinetics of flavonoids

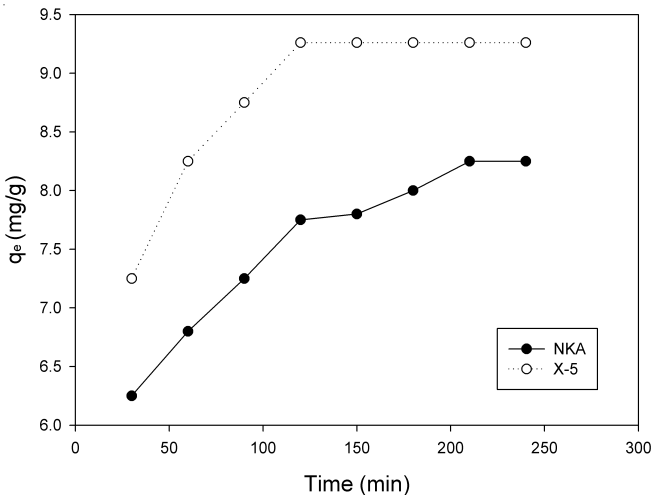


Fig. 2. Adsorption kinetics of syringin

Adsorption isotherms: Equilibrium adsorption isotherms were studied at 25 ºC (Figs. 3 and 4). The initial concentration of syringin in corresponding solution was 0.11, 0.16, 0.24,

TABLE-3
PHYSICAL PROPERTIES OF THE MACROPOROUS RESINS (25 ºC)

Constituents	Langmuir	R^2	Freundlich	R^2
Flavonoids	$q_e/c_e = 0.0576\ c_e + 0.000541$	0.9726	$q_e = 24.0\ c_e^{0.1725}$	0.9504
Syringin	$q_e/c_e = 0.1069\ c_e + 0.00012$	0.9499	$q_e = 12.08\ c_e^{0.0833}$	0.9870

0.36 and 0.54 mg/mL, respectively. The initial concentration of total flavonoids in the corresponding solution was 0.44, 0.66, 1.00, 1.49 and 2.24 mg/mL, respectively.

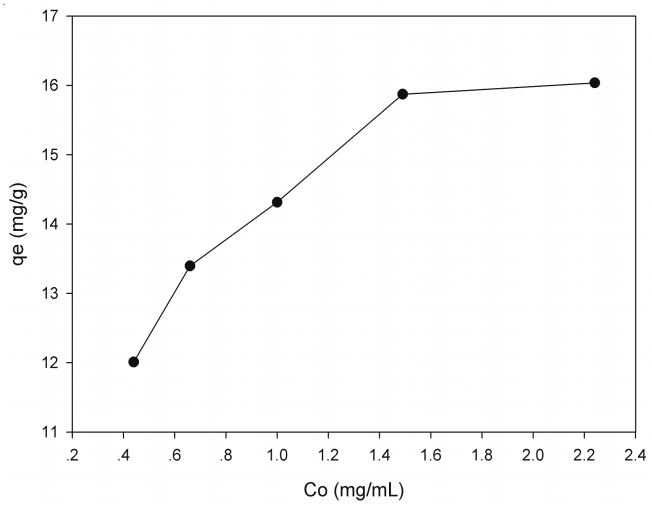


Fig. 3. Adsorption isotherms of flavonoids on X-5

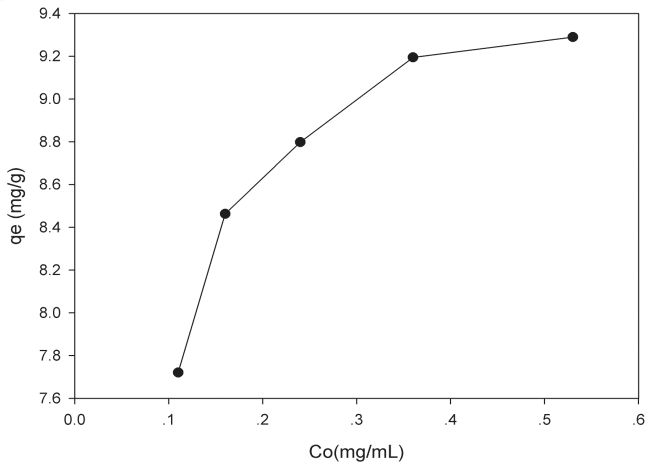


Fig. 4. Adsorption isotherms of syringin on X-5

As shown in Fig. 3 and 4, for both flavonoids and syringin, the adsorption equilibrium reaches the saturation plateau when the initial concentration of flavonoids is 1.49 mg/mL and the corresponding initial concentration of syringin is 0.36 mg/mL. Thus, the concentrations of flavonoids and syringin in the feed solution were selected at 1.49 and 0.36 mg/mL, respectively.

The equilibrium experimental data were fitted to the Langmuir (4) and Freundlich (5) equations to describe the interaction of solutes with the resin:

$$\frac{c_e}{q_e} = \frac{c_e}{q_o} + \frac{1}{Kq_o} \quad (4)$$

where, K is adsorption equilibrium constant, q_o is theoretically calculated maximum adsoption capacity (mg/g resin).

$$q_e = Kc_e^n \quad (5)$$

where, K is Freundlich constant *i.e.*, an adsorption capacity and n is an empirical constant related to the magnitude of the adsorption driving force[14].

Langmuir isotherm model assumes monomolecular layer adsorption with a homogeneous distribution of adsorption energies and without mutual interaction between adsorbed molecules. Langmuir eqn. (4) was converted to the linearized form with c_e and c_e/q_e as independent variable, the experimental data were statistically analyzed and R^2 was obtained.

The Freundlich model is used extensively in the physical adsorption and chemical adsorption and can be used to describe the adsorption behaviour of monomolecular layer as well as that of the multi-molecular layer. It assumes a heterogeneous distribution among the adsorption sites at different energies. It is a two-parameter model widely employed for many different adsorbate/adsorbent systems for liquid and gas phase adsorption[19].

A linearized form of Freundlich eqn. (5) can be written as:

$$\ln q_e = \ln K + n \ln c_e \quad (6)$$

the K and n can be obtained from the intercept and slope, respectively and the linear regression from a plot of $\ln q_e$ *versus* $\ln c_e$. The Langmuir and Freundlich parameters were summarized in Table-3.

In general, in Freundlich eqn., $q_e = Kc_e^n$, the adsorption can take place easily when n value is between 0.1 and 0.5, but not easy to happen if n is between 0.5 and 1. However, it is very difficult to occur[20] if n value exceeds 1. In Table-3, n values are all between 0.1 and 0.5 and indicate that the adsorptions of flavonoids and syringin on X-5 resin can take place easily and the resin is appropriate for the separations of both flavonoids and syringin.

Static desorption on X-5 resin: The proper desorption solution was chosen according to the polarity of resins and the solubility in the desorption solution. Flavonoids and syringin dissolved easily in methanol, ethanol, acetone and other organic solvents. Considering the production cost and safety, ethanol-water solution was chosen as desorption solution. The adsorption process was conducted by the procedure described in section 2.5. After adsorption equilibrium was reached, the adsorbates had been desorbed for 6 h in shakers at 25 ºC using 20 mL of 20, 30, 40, 60, 70 and 80 % aqueous ethanol solutions, respectively. With increasing ethanol concentrations, the desorption ratio of both flavonoids and syringin increased accordingly. The maximum desorption ratio of flavonoids and syringin was 91.2 and 94.4 % respectively, when using ethanol at a concentration of 80 %. (Fig. 5).

Flavonoids is low polar, resulting in its highly adsorptive interactions with X-5 resin. It could not easily be eluted even by 30 % ethanol solution. In contrast, the desorption ratio of syringin on X-5 by 30 % ethanol was 92 %. As a result,

flavonoids and syringin were well separated on the resin. Therefore, ethanol-water (30:70, 80:20, v/v) solution was selected as the appropriate desorption solution in the following tests.

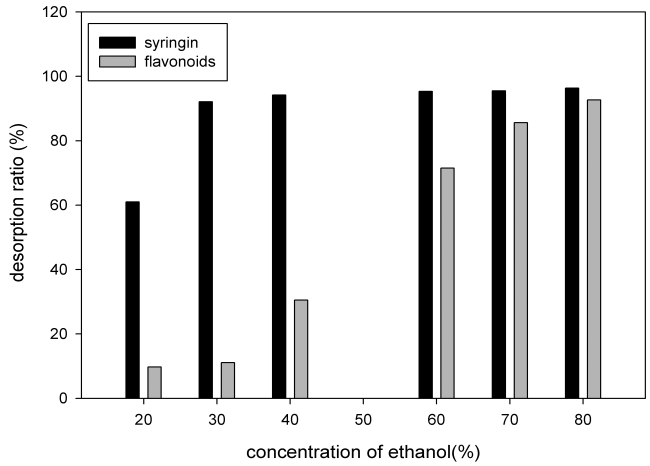


Fig. 5. Effects of ethanol concentration on the desorption ratios of flavonoids and syringin on X-5 resin

Dynamic adsorption on X-5: The dynamic adsorption results on X-5 are summarized in Table-4. As shown in Table- 4, the highest adsorption capacity is observed when the initial concentration of flavonoids and syringin are 1.51 and 0.78 mg/mL, respectively. The processing volume of sample solution on X-5 resin was determined as 100 mL and initial concentration of flavonoids was 1.51 mg/mL. When the feed concentration was low, the amount of the adsorbate relative to active sites was low and adsorption increased proportionally with increasing concentration of the flavonoids and syringin. However, with further increase of feed concentration, the amount of impurities in the crude extract also increased and the active site to adsorbate ratio reduced. These impurities would compete for active sites on the resins with flavonoids and syringin, each in their own concentration-dependent rate, resulting in the observed lower adsorption of the target solute[14].

TABLE-4
BREAKTHROUGH VOLUME AND QUANTITIES OF SYRINGIN AND FLAVONOIDS ON X-5 RESIN AT DIFFERENT FEED CONCENTRATIONS UNDER DYNAMIC ADSORPTION CONDITIONS

Constituents	C_o (mg/mL)	Breakthrough volume (mL)	Adsorbed quantity (mg)
Flavonoids	2.26	65	146.0
	1.51	105	158.5
	1.00	155	154.5
	0.67	200	132.0
Syringin	0.78	65	50.5
	0.52	105	54.4
	0.34	155	52.1
	0.23	200	46.0

Dynamic desorption on X-5: The dynamic desorption curves on X-5 resin were obtained based on the volume of desorption solution and the concentration of the solute in eluent under 30 % ethanol-water solution (2 BV) for syringin and 80 % ethanol solution (3 BV) for flavonoids. Gradient elution can make the desorption more efficient, as can be seen in Fig. 6, syringin and flavonoids could completely be desorbed from X-5 resin with approximately 100 mL (5 BV) of desorption solution. After elution, most syringin adsorbed on X-5 resin was eluted by 30 % ethanol-water solution (2 BV) and most flavonoids was eluted by 80 % ethanol-water solution (3 BV).

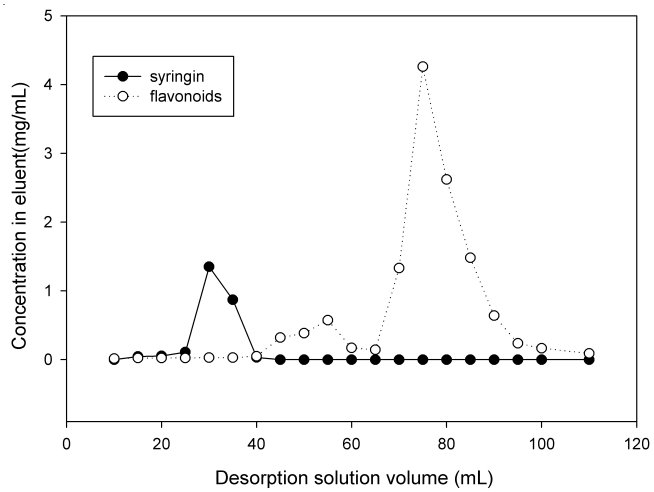
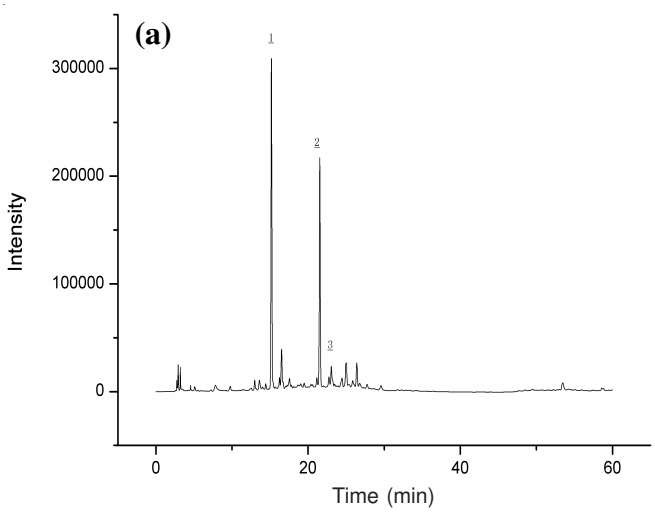


Fig. 6. Dynamic desorption curves of flavonoids and syringin on column packed with X-5 resin (gradient conditions: 30 % ethanol-water solution, 2 BV; 80 % ethanol solution, 3 BV)

The chromatograms of the tested samples before and after treatment with X-5 resin are shown in Fig. 7. When 30 % ethanol is used, it can be seen that syringin is completely separated from the *S. involucrata* crude extract while the major flavonoids remained on the X-5 resin. The desorbed solution was freeze-dried after removing ethanol. By comparison, it can be seen that some impurities in the crude extracts were removed and the relative peak area of rutin increased significantly after the separation on X-5 resin.

After dynamic separation on X-5 column chromatography, chlorogenic acid and some guaianolides compounds were removed. The recovery of syringin (purity > 80.3 %, HPLC) after elution by the eluent of 30 % ethanol was 78.36 %. Most of the flavonoids were eluted by the eluent of 80 % ethanol. The content of total flavonoids reached 90.5 % in the product



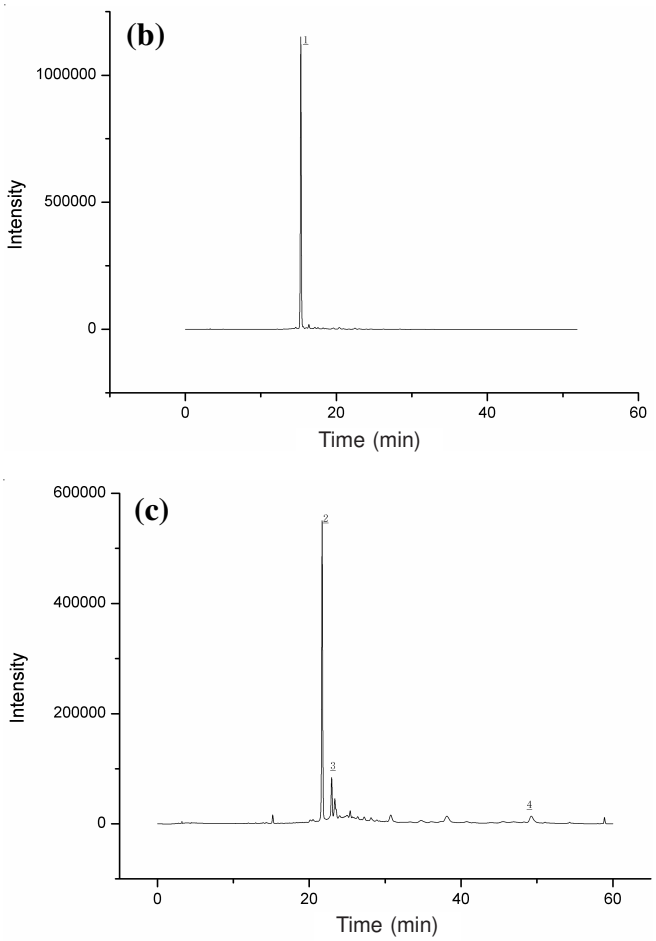


Fig. 7. Chromatograms of samples before treatment (a) and samples eluted by 30 % ethanol (b), by 80 % ethanol (c): 1. syringin, 15.28 min; 2. rutin, 22.4 min; 3. quercitin, 23.4 min; 4. hispidulin, 49.2 min

and the recovery of flavonoids was 83.6 %. X-5 resin was regenerated by flowing 60 mL NaOH solution (0.5 M, 3 BV) through the glass column at a flow rate of 2 mL/min and subsequently washed thoroughly by pure water before its reuse. The resin exhibited excellent reusability and no remarkable change was observed on the separation performance during 10 successive separation cycles.

Conclusion

The preparative separation process of syringin and flavonoids from planted *S. involucrata* crude extract with macroporous resin was successfully developed. Among the nine macroporous resins tested, X-5 gave the best separation efficiency because of its high surface area, optimum average pore diameter and appropriate surface functional residues. The adsorption rate curves indicated that the adsorption on X-5 was fitted to Langmuir and Freundlich equation. Column packed with X-5 resin was used to enrich and purify syringin and flavonoids through gradient elution. Syringin was obtained in high recovery (78.36 %) and high purity (80.3 %) by X-5 resin adsorption followed by desorption with 30 % ethanol. The contents of total flavonoids reached 90.5 % in the 80 % ethanol aqueous elute. In conclusion, this adsorption-desorption method was useful in the enrichment and purification of flavonoids from planted *S. involucrata*. This adsorption method is superior because of its procedural simplicity, lower cost, high efficiency and it may provide the references for the large-scale production. According to this approach, planted *S. involucrata* has the potential to replace the wild *S. involucrata* in medical use, further studies on biological activities of flavonoids from the planted *S. involucrata* need to be studied for promoting development and utilization of the resource.

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