African Journal of Biotechnology Vol. 7 (13), pp. 2147-2155, 4 July, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Isolation of flavonoids from mulberry (*Morus alba* L.) leaves with macroporous resins

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Accepted 20 May, 2008

Flavonoids are major active constituents in mulberry (*Morus alba* L.) leaves and possess various pharmacological activities. In the present study, the extraction technology and preparative separation of total flavonoids (TFs) in mulberry leaves extracts by macroporous resins were studied systematically, and the column packed with selected resin was used to perform dynamic adsorption and desorption tests to optimize the separation process. The research results indicated that the maximal yield of TFs was achieved when mulberry leaves were extracted under the optimized conditions of a pH of 8, a solid-liquid ratio of 1:40, a temperature of 90°C and a time of 4 h. H103 resin is the most appropriate for the separation of TFs from other components in mulberry leaves extracts, which adsorption behavior can be described with Langmuir and Freundlich isotherms and two-step adsorption kinetics model. The optimum parameters for TFs separation by H103 were achieved, after one run treatment. The recovery and purity of TFs in the final product were 90.57 and 76.33%, respectively. In conclusion, the preparative separation of TFs in mulberry leaves can be easily and effectively done by H103 resin.

Key words: Optimization, isolation, flavonoids, *Morus alba* (L.), mulberry leaves, macroporous resin.

INTRODUCTION

Mulberry, *Morus alba* L., as a non-toxic natural therapeutic agent, belongs to the family of Moraceae, and has been cultivated in many Asian countries such as China, India, Korea, Japan and Thailand where the leaves were used as food for silkworms (Nuengchamnong et al., 2007). There is an increasing interest on mulberry leaves because the leaves were found to have hypoglycemic, hypotensive, diuretic, bacteriostatic and antivirotic properties and they have been applied widely in clinic, which has important values to gerontal diseases and delayed consenescence (Chu et al., 2006). The mulberry leaves are rich in flavonoids, alkaloids and polysaccharides components which are known as the most potently major active compounds by chemical constituent investigations. Among those, the TFs in mulberry leaves were contained rutin, quercetin, isoquercitrin and quercetin 3-(6-malonylglucoside) (Lee et al., 2007). Flavonoids have found applications in food and pharmaceutical industries for their valuable properties, and adsorbent resins have been utilized to separate and concentrate these products from the natral matrixes (Fu et al., 2005; Qi et al., 2007).In particular, the medical applications of total flavoniods of mulberry leaves have provoked much interest, and its leaves have been brought to market as a product of traditional Chinese medicine (TCM) that possesses notable biological activities.

Nowadays, the traditional separation methods about the flavonoids of mulberry leaves used so far were limited to simple techniques such as extraction and precipitation leading to low recoveries. Hence a more efficient and simple purification method for TFs in mulberry leaves is required. The purification method of macroporous resin is a new promising technology in bioengineering, and has successfully applied in the preparative separation of TFs in natural products (Aehle et al., 2004; Fu et al., 2005; Zhang and Li, 2006; Qi et al., 2007).Similarly, such a method is extremely useful for the extraction and separation of the TFs from mulberry leaves. Therefore, the objectives of the present study were to investigate the adsorption and desorption properties of TFs from mulberry leaves on different macroporous resins, and

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Table 1. Extraction of TFs from mulberry leaves by L_9 (3^4).

Level	Factors				Yield of TFs (%)
	Temperature (℃)	Solid-liqid ratio	Extraction time (h)	pH	
1	70	1:20	2	7	2.78
2	70	1:30	4	8	3.77
3	70	1:40	6	9	3.35
4	80	1:20	4	9	3.47
5	80	1:30	6	7	3.22
6	80	1:40	2	8	4.02
7	90	1:20	6	8	3.58
8	90	1:30	2	9	3.32
9	90	1:40	4	7	4.10
T_1	9.90	9.83	10.13	10.11	
T_2	10.71	10.32	11.34	11.38	
T_3	11.01	11.47	10.15	10.13	
X_1	3.30	3.28	3.38	3.37	
X_2	3.57	3.44	3.78	3.79	
X_3	3.67	3.82	3.38	3.38	
R	0.37	0.55	0.41	0.42	

develop an efficient method for the preparative separation of TFs from mulberry leaves with the resin. The results in this study are significant for the preparative separation of TFs from other plants in general.

MATERIALS AND METHODS

Chemicals and reagents

Rutin standard was brought from National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). All other reagents were of analytical grade and deionized water was purified by a Milli-Q water-purification system from Millipore (Bedford, MA, USA). Appropriate amount of standard was dissolved in ethanol to yield the stock solutions at concentration of 0.5 mg/ml for rutin.

Adsorbents

Macroporous resins including AB-8, X-5, S-8, H103, NKA, NKA-9, NKA-II, D101, D4020 and D3520 were purchased from Nankai Hecheng Science and Technology (Tianjin, China).

The adsorbent beads were pre-treated to remove the monomers and porogenic agents trapped inside the pores during the synthesis process. All resins were soaked in 95% ethanol, shaken for 24 h and washed by deionized water thoroughly (Juang and Shiau, 1999).

The moisture contents of the test resins were determined by drying the beds at 60℃ to constant weight in dry oven.

Optimization of extraction technology of TFs from mulberry leaves

Mulberry leaves were collected during the summer season in West campus, Jiangsu University of Science and Technology, Jiangsu province, China. The material was dried at 60℃, powered by a herb disintegrator (Qinzhou Sanyang Package Equipment Co., Ltd) and then sieved (60 mesh).

As shown in Table 1, the extraction technology of TFs from mulberry leaves was optimized by orthogonal experiments L_9 (3^4). Mulberry leaves powder (500 g) was extracted under optimum process conditions. The extracts were purified by membrane filtration and then transferred to a rotary evaporator device (R200, Büchi, Switzerland) and concentrated under vacuum to the contents of TFs 6.05 mg/ml (pH 6), stored at 4℃.

Static adsorption and desorption tests

The static adsorption tests of mulberry leaves extracts on macroporous resins were performed as follows: 1 g sample of hydrated test resins were placed in flasks with a lid; then 50 ml of sample solutions of mulberry leaves extracts (TFs concentration 6.05 mg/ml) were added. The flasks were then shaken (100 rpm) for 24 h at 25℃. The solutions after adsorption were analyzed by chromatometry.

The desorption processes were performed as follows: after reaching adsorption equilibrium, the resins were first washed by deionized water and then desorbed with 30 ml ethanol water (30:70, 60:40, 100:0) solutions, respectively. The flasks were then shaken (100 rpm) for 24 h at 25℃. The desorption solutions were analyzed by chromatometry.

The selectivity of resins was based on the capacities of adsorption and desorption, and radio of desorption. The adsorption kinetic curves of TFs on the preliminarily selected H103 resin was studied according to the method described above; the respective concentrations in the sample solutions were monitored at certain time intervals till equilibrium.

The test for equilibrium adsorption isotherms on H103 and AB-8 resins were conducted by mixing 50 ml sample solutions of mulberry leaves extracts at different concentrations with 0.5 g (dry weight) resins, and then shaking for 6 h at a temperature of 25, 30 and 35℃. The initial and equilibrium concentrations at different temperatures were determined by chromatometry.

The test for adsorption kinetics on H103 resin were conducted by mixing 75 ml sample solutions of mulberry leaves extracts (the concentration of TFs 6.05 mg/ml) with 1 g (dry weight) resin, and then shaking (100 rpm) for 10 h at a temperature of 25℃. The remaining concentrations at different times were determined by chromatometry.

The adsorption and desorption properties of the selected H103 resin under different conditions including initial concentration of sample solution, temperature, and concentration of ethanol for desorption were also evaluated.

Dynamic adsorption and desorption tests

Dynamic adsorption and desorption experiments were carried out on glass columns (25 × 400 mm) wet-packed with 16 g (dry weight) of selected H103 resin, and the packed length of resin was 80 mm. Sample solution flowed through the glass column at the flow rate of 1 ml/min and the concentration of TFs in the effluent liquid were monitored at certain time intervals by chromatometry. For adsorptive equilibrium, the adsorbated column was washed first by deionized water, and then eluted by ethanol water (60:40) solution. The flow rate was 1 ml/min, and the concentration of TFs in desorption solution was determined by chromatometry.

Determination of TFs

The TFs content was determined by the aluminum trichloride method of Wang et al. (2008).

Adsorption capacities, adsorption radio, desorption radio and recovery

The following equations were used to quantify the capacities of adsorption and desorption as well as the ratio of desorption.
Adsorption evaluation:

$$q_e = \frac{(C_0 - C_e)V_0}{m} \quad (1)$$

$$E(\%) = \frac{C_0 - C_e}{C_0} \times 100\% \quad (2)$$

where q_e is the adsorption capacity at adsorption equilibrium (mg/g resin); E the adsorption ratio (%); C_0 and C_e the initial and equilibrium concentrations of solute in the solution, respectively (mg/ml); V_0 the volume of the initial sample solution (ml) and m is the weight of dry resin (g).
Desorption evaluation:

$$R = \frac{C_d V_d}{C_0 V_0} \times 100\% \quad (3)$$

$$D = \frac{C_d V_d}{(C_0 - C_e)V_0} \times 100\% \quad (4)$$

where R is the recovery (%); C_d the concentration of the solute in the desorption solution (mg/ml); V_d the volume of the desorption solution (ml); D the desorption ratio (%); C_0 ,C_e and V_0 are the same as described above.

Adsorption equations

The equilibrium experimental data were fitted to the Henry, Langmuir and Freundlich equations to describe the interaction of solutes with the resin (Jung et al., 2001).

Adsorption kinetics models

The experimental data of adsorption rate constant were fitted to the two-step adsorption kinetics model to describe the adsorption kinetic property of solutes with the resin:

$$q_t = q_e - A_1 e^{-k_1 t} - A_2 e^{-k_2 t} \quad (5)$$

$$dq_t / dt = k_i (q_e - q_t) + c_i \quad i = 1, 2 \quad (6)$$

where A_1 and A_2 are frequency factors; k_1 and k_2 are adsorption rate constants; q_t is the adsorption quantity (mg/g) (Nagaoka and Imae, 2003).

RESULTS AND DISCUSSION

Optimum process conditions for extracting TFs from mulberry leaves

As can be seen from Table 1, the results showed the influence order of different factors on the yield of TFs from mulberry leaves: solid-liqid ratio > pH > extraction time > extraction temperature. The optimum technological conditions for extracting TFs from mulberry leaves were: pH 8; solid-liquid ratio, 1:40; temperature, 90°C; and time, 4 h. the maximal yield of TFs was 4.77% when mulberry leaves were extracted under the optimized conditions.

Adsorption capacity, adsorption ratio, desorption radio and recovery

The adsorption capacities of S-8, H103 and AB-8 resins toward TFs of mulberry leaves were considerably higher than those of other resins (Figure 1). However, the recovery and desorption ratio of S-8 resin was the lowest in all resins (Figure 2). On the contrary, although the ad-sorption capacities of H103 and AB-8 resins were not the highest, the recovery and desorption ratio were higher, which correlates with the capacities of the resins and the chemical features of the adsorbed substance. Therefore, H103 and AB-8 resins were selected to further investigate their adsorption behavior toward TFs of mulberry leaves.
Different concentrations of ethanol solutions were used to perform desorption tests in order to choose proper desorption solution. With the increasing of the ethanol concentration, the desorption ratios on all resins increased accordingly and reached their peak value at the concentration of 60%, then decreased with the concentration of ethanol (Figure 2). Therefore, ethanol water (40:60) solution was selected as the appropriate desorption solution and was used in the subsequent assays.

Adsorption isotherms on AB-8 and H103 resin

Equilibrium adsorption isotherms were conducted at tem-

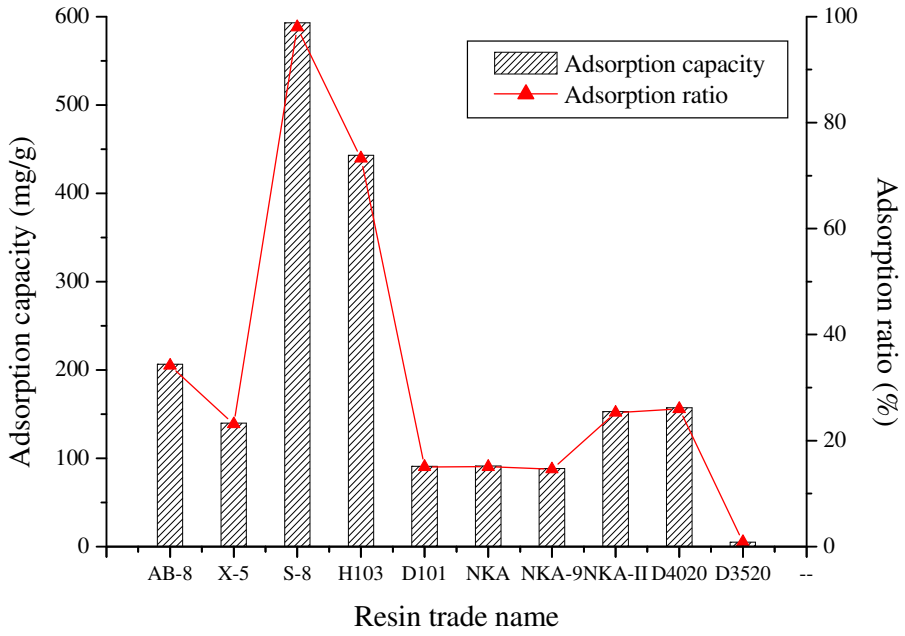


Figure 1. Adsorption capacity and adsorption ratio of TFs in mulberry leaves on different resins.

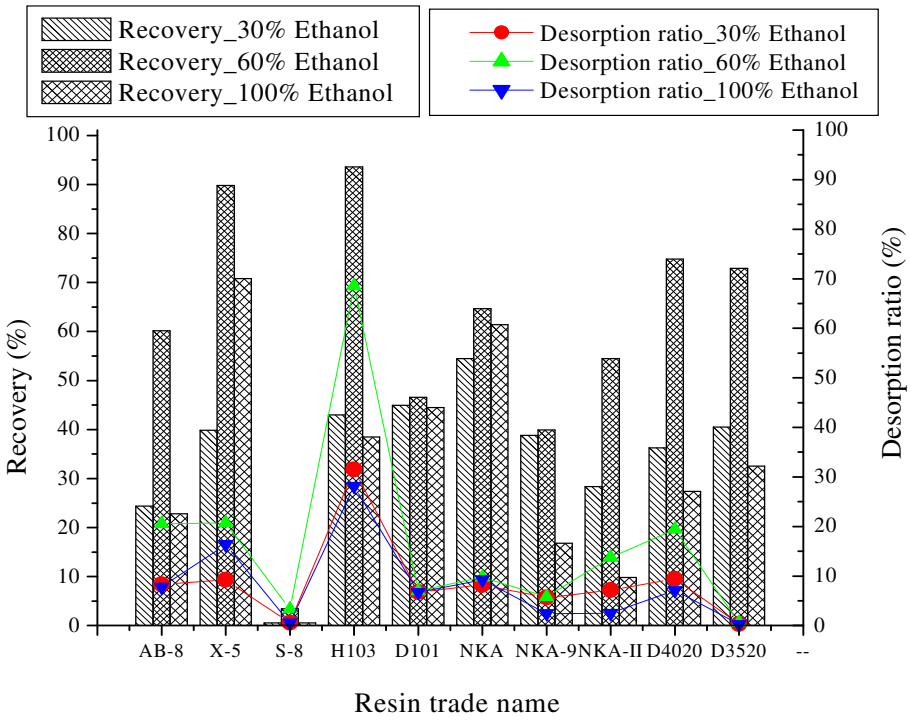
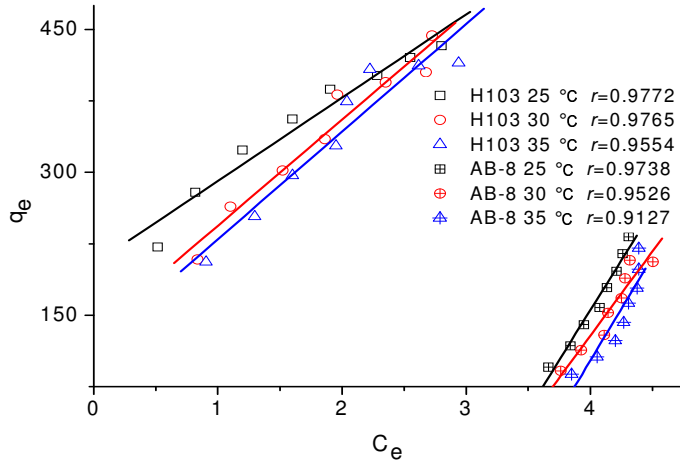
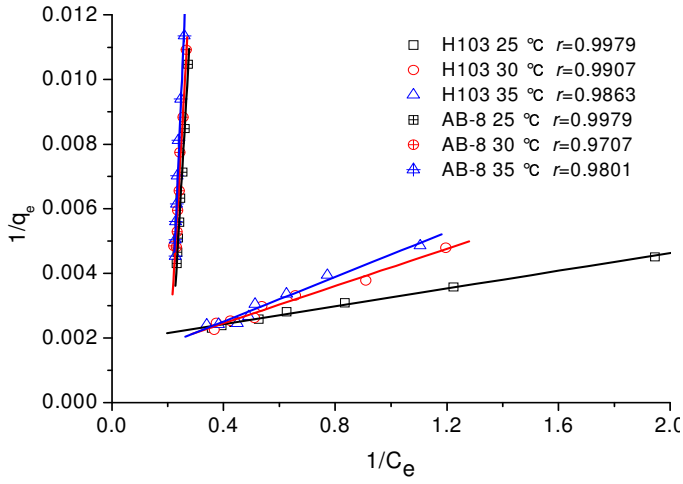


Figure 2. Recovery and desorption ratio of TFs in mulberry leaves on different resins.

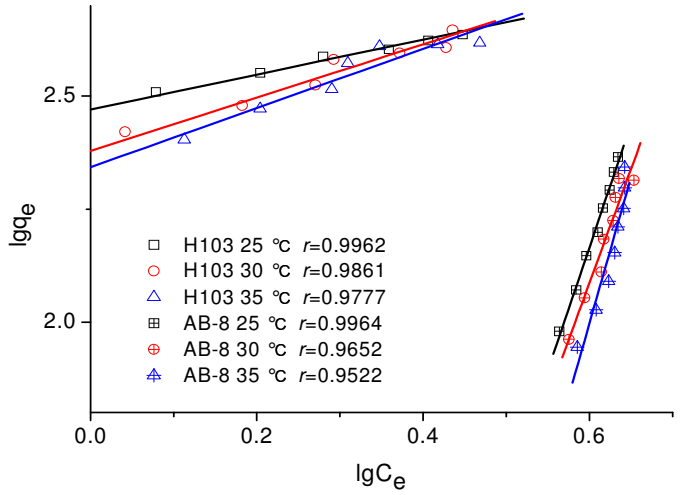
peratures of 25, 30 and 35℃. For the two resins, the adsorption capacity increased with the initial concentration of TFs in mulberry leaves, and reached the saturation plateau when the initial concentrations of TFs in mulberry leaves were 3.0 and 4.4 mg/ml, respectively (Figure 3). It can also be seen from Figure 3, within the temperatures investigated, at the same initial concentration, the adsorption capacities decreased with the increasing temperature; the adsorptive speed was slower than desorptive speed for TFs in mulberry leaves, which



(a) Henry isotherm



(b) Langmuir isotherm



(c) Freundlich isotherm

Figure 3. Adsorption isotherms for TFs in mulberry in mulberry leaves on H103 and AB-8 resins.

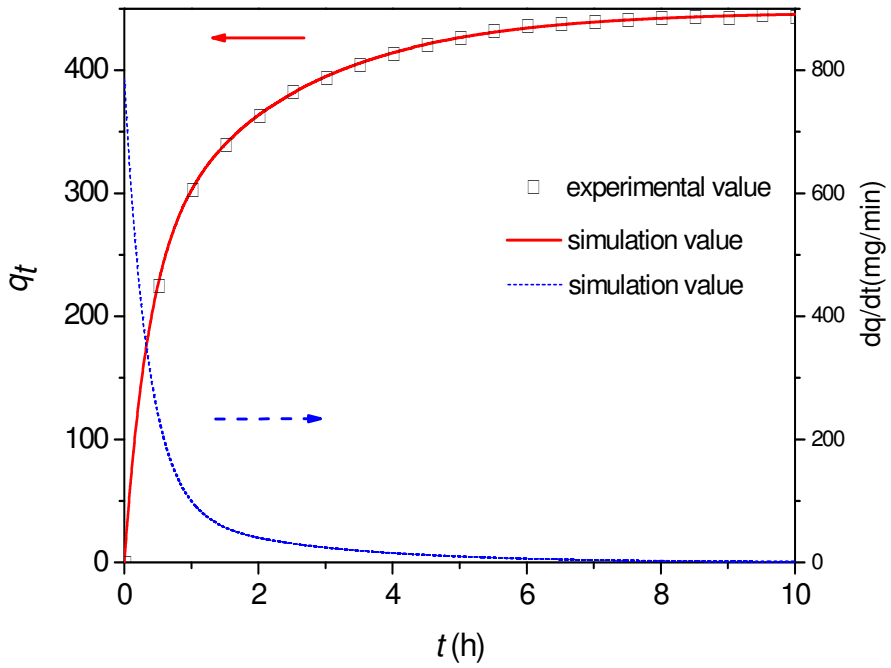


Figure 4. Adsorption kinetics and adsorption rate curves of TFs from mulberry leaves.

indicated that the adsorption was a thermopositive process. Therefore, 25℃ was used in the subsequent assays.

The Henry, Langmuir and Freundlich isotherms are the best known and the most often used isotherms for the adsorption of solutes from a solution in the bio-separation process. The Henry, Langmuir and Freundlich parameters were obtained from the Henry, Langmuir and Freundlich equations, and summarized in Figure 3. The correlation coefficients of the Henry, Langmuir and Freundlich equations on H103 resin were slightly higher than AB-8 resin, especially the equilibrium adsorption isotherm of H103 that highly fit to the Langmuir and Freundlich models. The Langmuir model assumes monomolecular layer adsorption with a homogeneous distribution of adsorption energies and without mutual interaction between adsorbed molecules, while the Freundlich model can be used to describe the adsorption behavior of monomolecular layer as well as that of the muli-molecular layer (Fu et al., 2005).The equilibrium adsorption behavior of H103 fit both models. In the comprehensive consideration of the adsorption capacity and desorption ratio, H103 resin was selected as the most suitable resin for the separation of TFs in mulberry leaves and was used in the subsequent assays.

Adsorption kinetics on H103 resin

The adsorption capacity of H103 resin increased with adsorption time, reaching equilibrium at about 5 h (Figure 4). In first 2 h, the adsorption capacity increased rapidly, after 2 h the slopes, at different times which indicate the adsorption ratio, varied little.

Experimental and theoretical studies of adsorption kinetics in various solute-solution systems have been the subject of so many papers; one of the most famous is probably the pioneering paper published by Lagergren in 1898 (Lagergren et al., 1898).Since that time, a variety of theoretical expressions have been proposed to describe the observed adsorption kinetics. Some of them were purely empirical and some were based on certain physical models of adsorption kinetics. Although one may face a variety of physical situations, such a two-step kinetic mechanism should be very typical of many adsorption systems, because the kinetics of molecule sorption from aqueous solutions by solids can be considered as a two-step process (Rudzinski and Plazinski, 2008).

The adsorption process is the distribution of the solute molecules between the adsorbents and the liquid phase (Scordino et al., 2003). In the current paper, we apply two-step adsorption kinetics to the analysis of the above kinetics data. The analytical curves of the kinetics were calculated, and computer simulations were carried out to interpret the experimental data of TFs adsorption on H103. The adsorption rate constants were obtained from two-step adsorption kinetics model; the kinetics parameters were described as Table 2. As can be seen from Figure 5, for the two steps, the adsorption rate constants $k_1 > k_2$. The results demonstrated a similar two-step adsorption kinetics model as "fast followed by slow" in the adsorption process of TFs on H103 resin.

Table 2. Adsorption Kinetics parameters in the two-step adsorption kinetics model.

Parameters	Value	Std Error	t-value	95% CLs		P>|t|
q_e	447.63	0.67	669.53	446.21	449.04	0.00
A_1	207.89	4.48	46.39	198.39	217.39	0.00
k_1	0.46	0.01	39.33	0.43	0.48	0.00
A_2	239.77	4.98	48.15	229.21	250.32	0.00
K_2	2.88	0.09	32.08	2.69	3.07	0.00

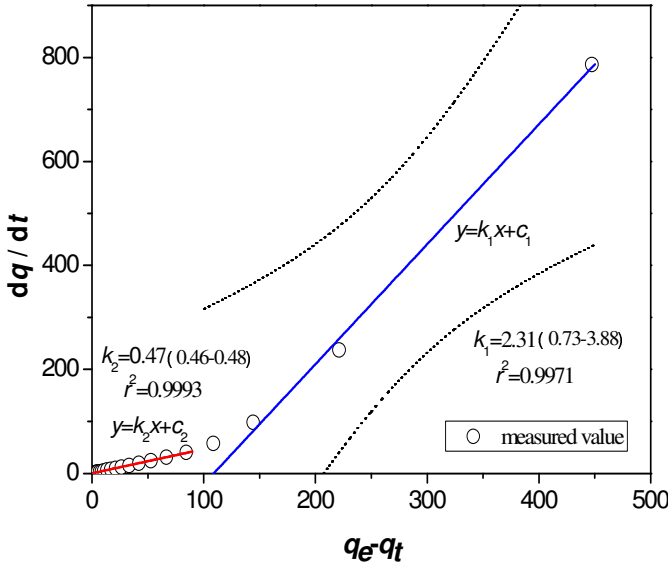


Figure 5. Adsorption rate constants in two-step adsorption kinetics model on H103 resin.

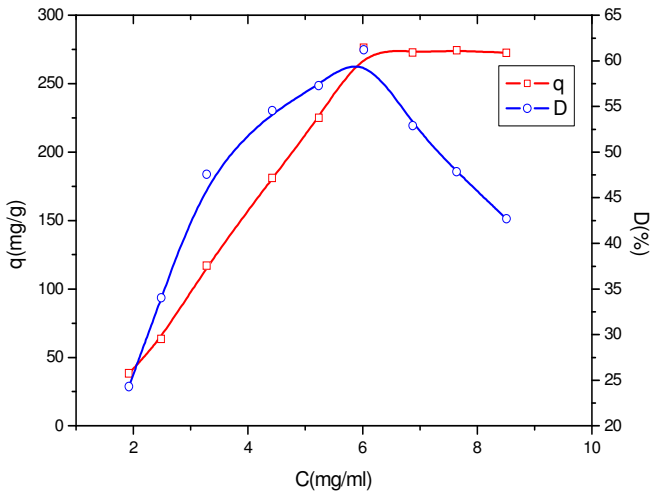


Figure 6. Adsorption capacity and desorption ratio of H103 resin under different TFs concentration.

There are two adsorption mechanisms that should be considered in the adsorption kinetics of TFs on H103 resin: In the first mechanism, the surface coverage by the adsorbed TFs molecules increases relatively rapid at early stage due to the formation of first adlayer and then gradually at longer time scale by the formation of the second adlayer. The total coverage depends on the degree of the contribution of two adsorption processes. On the other hand, in the second mechanism, the maximum coverage happens by the excess adsorption at the early stage. The excess TFs molecules are desorbed at the long time scale, accompanying with the rearrangement to the monolayer (Nagaoka and Imae, 2003). Therefore, adsorption kinetics process for TFs in mulberry leaves on H103 resin fitted to two-step adsorption kinetics model.

Effect of concentration of sample solution on H103 resin

The concentration of sample solution influences the adsorption capacity and adsorption ratio on H103 resin (Figure 6). For TFs in mulberry leaves, the highest adsorption capacity appeared at the concentration of sample solution of 6.02 mg/ml, and then decreased with the increase of the concentration of sample solution. Hence, the concentration of sample solution was adjusted to about 6.02 mg/ml for the subsequent assays.

Dynamic leakage curve on H103 resin

The dynamic leakage curves on H103 resin were obtained for TFs in mulberry leaves based on the volume of effluent liquid and the concentration of solute therein. The flow rate of sample solution is one of the important factors influencing the dynamic leakage curves; there was a linear relationship between $\ln[C/(C_0-C)]$ and t (Wang et al., 2008), as shown in Figure 7.

As the flow rate of sample solution increased, the dynamic leakage time decreased (Figure 7); on the contrary, the adsorption capacity increased. In the comprehensive consideration of the dynamic leakage time, adsorption capacity and regression coefficient of linear relationship between $\ln[C/(C_0-C)]$ and t, 9.0 ml/min was selected the most suitable flow rate of sample solution for the dynamic adsorption of TFs in mulberry leaves and was used in the subsequent assays.

Table 3. Dynamic desorption process parameters in the Pearson IV equation.

Flow rate of desorption solution (ml/min	r^2	DF Adj r^2	Fit Std Err	F-value
4.5	0.9957	0.9943	21.33	923.21
7.5	0.9826	0.9746	48.29	158.34

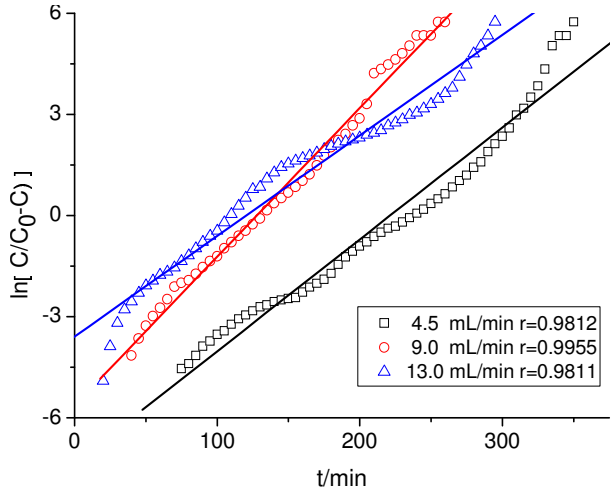


Figure 7. Linear relationship between ln[C/(C_0-C)] and t in dynamic leakage process of TFs in mulberry leaves on column packed with H103 resin under different flow rate of 6.05 mg/ml TFs solution

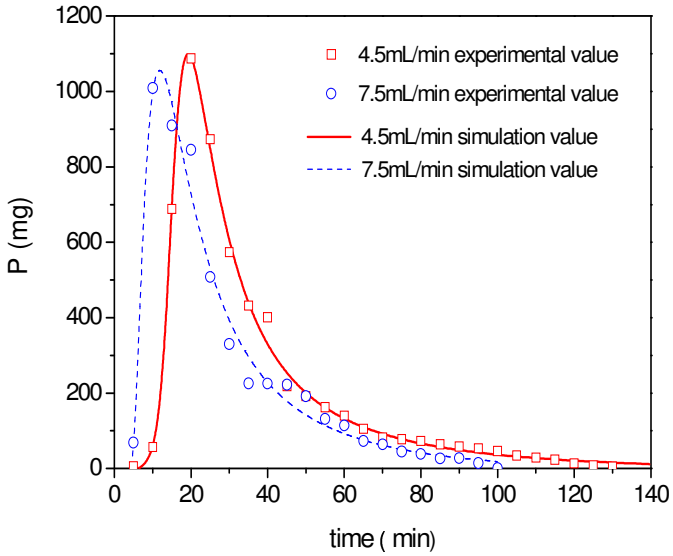


Figure 8. Dynamic desorption curves of TFs in mulberry leaves on column packed with H103 resin under different flow rate of 60% ethanol.

Dynamic desorption curve on H103 resin

The dynamic desorption curves on H103 resin were obtained based on desorption time and quality of solute therein. The flow rate of desorption solution is one of the important factors influencing the dynamic desorption curves, and the recovery and purity of TFs in mulberry leaves decreased with the flow rate of desorption solution (Figure 8). From the figure, it is shown that the data of dynamic desorption curves fitted to Pearson IV equation which parameters were described in Table 3. The experiment results shown that when the flow rate of desorption solution was 4.5 ml/min, the recovery and purity of TFs in the final product were 90.57 and 76.33%, respectively. The dynamic desorption curves equation was: $y = a + b\,(1 + n^2)^{-e} \exp(-f\,(\text{atan}(n) + \text{atan}(f / (2e)))) / (1 + f^2 / (4e^2))^{-e}$, where n = (x-df /(2e) - c) /d, a = -14.53, b = 1113.87, c = 19.28, d = 5.14, e = 0.96, f = -2.35, r^2 = 0.9957. In the comprehensive consideration of the dynamic desorption time, the stimulated results by Pearson IV equation, recovery and purity of TFs in mulberry leaves, 4.5 ml/min was selected the most suitable flow rate of desorption solution for the dynamic desorption of TFs in mulberry leaves.

Conclusions

The preconcentration and separation method of TFs from mulberry leaves with macroporous resins was successfully achieved in this study. The maximal yield of TFs was obtained when mulberry leaves were extracted under the optimized conditions of pH of 8, a solid-liquid ratio of 1:40, a temperature of 90℃ and a time of 4 h. Among the ten resins investigated, H103 resin offers the best separation capacity for TFs from other components in mulberry leaves extracts. Process parameters including concentration and flow rate of sample solution, concentration and flow rate of desorption solution, temperature, etc., were optimized for the most effective separation of TFs in mulberry leaves on H103 resin. The equilibrium experimental data of adsorption of TFs in mulberry leaves on H103 resin at different temperatures fitted to Langmuir and Freundlich isotherms. The adsorption kinetics process for TFs in mulberry leaves on H103 resin fitted to two-step adsorption kinetics model. Through one run treatment on the column packed with H103 resin under optimal conditions, the recovery and purity of TFs in the final product were 90.57 and 76.33%, respectively. The adsorption method is more advanced than the traditional methods in the separation of TFs in mulberry leaves extracts, because of its lower cost and high efficiency. The techniques in this study can be applied for the separation of other flavonoids in other natural products.

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