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Separation and Purification of Flavonoid from Ginkgo Extract by Polyamide Resin

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An efficient separation process of flavonoid from Ginkgo extract was developed in this study. Polyamide resin offered the fine adsorption capacity, and its adsorption rate at 25°C fitted well to the Langmuir isotherm. Dynamic adsorption and desorption experiments were conducted to optimize the separation process of total flavonoids from the Ginkgo extract. After one run, the content of total flavonoids increased from 24.0% to 55.0%. The method will provide a potential approach for large-scale separation and purification of flavonoid for its wide pharmaceutical use.

Keywords flavonoid; Ginkgo extract; polyamide resin; separation

INTRODUCTION

Ginkgo biloba is considered the oldest surviving tree species, and the extract of the *Ginkgo biloba* leaves has been used for medicinal purpose in China for several thousands years (1). Ginkgo extract is available in either non-standardized form (2) or as a standardized extract containing 24% flavone glycosides and 6% terpene lactones (3,4). Ginkgo has gained broad acceptance among the general public, and broad distribution throughout the health food sector, drug stores, and supermarkets, due to the outstanding body of science supporting its use (5–9).

Separation methods based on synthetic adsorbents are gaining popularity in pharmaceutical applications and have also been used for polyphenols separation (5,6,10,11). Macroporous resin is one kind of adsorbent which is often used to separate flavonoids based on the polarity (12), sieve classification, hydrogen bonding interactions, and Van der waals forces (13). Polyamide resin is a polyamide powder with a large specific area and a considerable particle size processed from polyamide chip. It is extensively used for the separation of effective ingredients from natural plants (14,15). It has been used mostly based on the affinity of the hydrogen bonding interactions.

At present, liquid–liquid extraction and column chromatographic procedures are the main conventional protocols of extraction and separation techniques (16) and the characteristics of the adsorbent in column chromatographic procedures are very important for the separation effect. Flavonoid in the Ginkgo extract usually present as glycosides and their content normally reaches up to 30% (17) by the current conventional separation methods. The polyamide resin has specific selectivity for the phenolic acids, flavonoids, and quinines, and it could be an excellent adsorbent for the separation of functional compounds in the plant extract field. But our comprehensive literature review revealed that there was no report of detailed study on the kinetic characteristics and separation effect of the polyamide resin.

The objective of the current study was to assess the adsorption and desorption properties of Ginkgo flavonoid on polyamide resin. The physical models of the Langmuir equation and the Freundlich equation, HPLC method were used to analyze the kinetic properties and separation effect. The information is of significance for the preparative separation of Ginkgo extract or other herbal extract.

MATERIALS AND METHODS

Materials and Reagents

Ginkgo biloba extract (EGb) was provided by Yong Chun Tang Biological Science & Technology Co., Ltd. (Shandong, China); Polyamide resin (30–60 mesh) was acquired from Yantai Science & Biotechnology Co., Ltd. (Shandong, China).

All the other solvents/chemicals used were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Apparatus

R-201 rotary evaporator (Shanghai Shenshun Biotech Co., Ltd.); XT5502-R05C shaking water bath (Hangzhou Xutemp Temptech Co., Ltd., Hangzhou, China); Glass

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chromatographic columns (11 mm × 400 mm); BS2-100 fraction collector (Shanghai Qingpu Huxi Instruments Factory, China); UV-1600 spectrophotometer (Shanghai Mapada Instrument Co., Ltd., China); UV-detector (HD-3 UV-detector, Shanghai Huxi Analyses Instrument Company, China).

Experimental Procedures

The Pretreatment Method for the New Polyamide Resin

The new polyamide resin was first soaked in 90–95% ethanol solution with stirring constantly to drive the bubbles out. Then the resin was packed in the glass column and washed downwards with 90–95% ethanol solution until the eluent was clear and little residue was left after the eluent was dried. Then, the resin was washed with 2–2.5 bed volume of 5% sodium hydroxide, 1 bed volume of distilled water, 2–2.5 bed volume of 10% acetic acid solution in turn. At last it was washed with distilled water until the pH of the eluent was neutral. Then the resin subsequently was dried and kept for later use.

Static Adsorption and Desorption Tests on the Polyamide Resin

The static adsorption experiments were performed as follows: 1.0 g resin (dry weight basis) was introduced into a 100 mL conical flask. Then 30 mL of EGb solution (a portion of EGb was dissolved in 20% ethanol solution) with known flavonoid concentration was added to each flask. The flasks were kept in the shaking incubator set at 120 rpm, 25°C until adsorption equilibrium. The concentration of the flavonoid in the liquid phase was then analyzed by the colorimetric method.

The static desorption experiments were carried out as follows: the adsorbate-laden resins were first washed with deionized water and then desorbed in 30 mL of 30% ethanol solution. The flasks were shake incubated at 120 rpm, 25°C until desorption equilibrium. The concentration of the flavonoid in the liquid phase was analyzed by the colorimetric method.

The time courses of adsorption were evaluated using the Langmuir adsorption rate equation:

$$\ln\frac{q_e}{q_e - q_t} = kt + m \quad (1)$$

Where q_t and q_e were the adsorption quantity (mg/g) at time t (min) and at equilibrium, respectively; k was the equilibrium adsorption rate constant; and m was a constant.

The equilibrium adsorption isotherms on the resin were obtained based on the data of the initial and equilibrium concentration of flavonoid which were determined by the colorimetric method, and the degrees of fitness to the Langmuir equation and Freundlich equation were evaluated.

Dynamic Adsorption and Desorption Tests on the Polyamide Resin

Dynamic adsorption and desorption experiments were carried out in a glass column (11 mm × 400 mm) wet-packed with 5.0 g (dry weight basis) pretreated polyamide resin. The bed volume (BV) and length were 30 mL and 30 cm, respectively. The flow of samples in all cases was downward and it was monitored with a UV detector (HD-3 UV-detector, Shanghai Huxi Analyses Instrument Company, Shanghai, China). Sample solutions flowed through the glass column at 2 mL/min and the flavonoid concentration in the eluate was determined using the colorimetric method. After adsorption equilibrium, the adsorbate-laden column was washed successively by deionized water and then 30% ethanol solution, at a flow rate of 1.8 mL/min. The flavonoid concentration of the eluate was determined by the colorimetric method. The loading curve was obtained by the flavonoid concentration and the volume of the eluate. The eluate was concentrated by the rotary evaporator and dried in the vacuum oven. The residue was the purified product and weighed to determine the flavonoid content. Dynamic adsorption and desorption tests were repeated three times under optimal conditions.

Analysis of Flavonoid Concentration by Colorimetric Method

Flavonoid concentration was determined by using a colorimetric method described previously (17,18). Briefly, 0.25 mL of the extract or rutin standard solution was mixed with 1.25 mL of distilled water in a test tube, followed by addition of 75 μL of a 5% sodium nitrite solution. After 5 min, 75 μL of a 10% aluminum nitrate solution was added and the mixture was allowed to stand for a further 5 min before 0.5 mL of 1 M sodium hydroxide was added. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer (UV-1600, Shanghai Mapada Instrument Co. Ltd., China). Rutin was used as the standard for the calibration curve. Flavonoid concentration was calculated using the following linear equation based on the calibration curve:

$$A = 11.298C + 0.0071. \quad R^2 = 0.9998$$

Where A is the absorbance, C is the flavonoid concentration (mg/mL).

The colorimetric method was compared with the HPLC method, and the results showed that they had good linear relationship and the linear equation was $y = 1.0552x + 0.0172$ ($R^2 = 0.9987$). Where y was the HPLC value (mg/mL), x was the colorimetric value (mg/mL). The changes of the flavonoid concentration in the purification process

were determined by the colorimetric method due to its easiness and speediness.

Calculation of Adsorption Capacity, Adsorption, and Desorption Ratios

The capacity of adsorption, and the adsorption and desorption ratios were calculated as follows:

Adsorption evaluation:

$$q_e = (C_0 - C_e)\frac{V_i}{W} \tag{2}$$

$$E = \frac{(C_0 - C_e)}{C_0} \times 100\% \tag{3}$$

where q_e represented the adsorption quantity (mg/g) at equilibrium; E was the adsorption ratio (%), defined as the percent mass of total adsorbate adsorbed at equilibrium; C_0 and C_e were the initial and equilibrium concentrations of solutes in the solutions, respectively (mg/mL); V_i was the volume of the initial feed solution (mL) and W was the weight of the dry adsorbent (g).

Desorption evaluation:

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

Where D was the desorption ratio (%); C_d was the concentration of the solutes in the eluate (mg/mL); V_d was the volume of the eluate; C_0 and C_e were the initial and equilibrium concentrations of solutes in the solutions, respectively (mg/mL).

Langmuir Equation and Freundlich Equation

The equilibrium experimental data were fitted to the Langmuir (5) and Freundlich (6) isotherms to describe the interaction of solutes with the resin:

$$\frac{C_e}{q_e} = \frac{C_e}{q_{\max}} + \frac{1}{kq_{\max}} \tag{5}$$

$$q_e = aC_e^{1/n} \tag{6}$$

where q_e represented the adsorption quantity (mg/g resin) at equilibrium; C_e was the concentrations of solutes in the solutions at equilibrium; q_{max} was the theoretically calculated maximum adsorption capacity (mg/g resin); k was the adsorption equilibrium constant; a was the Freundlich constant, an indicator of adsorption capacity; and $1/n$ was an empirical constant related to the magnitude of the adsorption driving force.

Calculation of the Flavonoid Content of the Purified Product

The flavonoid content of the purified product using static and dynamic adsorption and desorption method was calculated using the following equation:

$$\text{Flavonoid content } \% = \frac{C_e V_d}{M} \times 100\% \tag{7}$$

Where C_e was the flavonoid concentration of static desorption solution or the flavonoid concentration of the total dynamic desorption eluate (mg/mL); V_d was the volume of the desorption solution (mL); M was the weight of the purified product (mg).

HPLC Analysis of Ginkgo Flavonoid

Samples for the flavonoid analysis were prepared using the method described by Hasler and Sticher (19) with some modifications. Samples (0.035 g) were transferred to a round bottom flask, then 20 mL of methanol and 5 mL of 25% HCl were added and the mixture was refluxed at 90°C for 30 min. The sample solutions were then suitably diluted with methanol and filtered through 0.45 μm membrane filters (Millipore, Nylon).

The analysis was performed by a high performance liquid chromatography system module 1100 (Agilent, Santa Clara, USA) equipped with photodiode array (PDA) detector. Diamonsil column (4.6 mm × 250 mm, DIKMA, Beijing, China) was used for separation and the column temperature was maintained at 30°C. The PDA detector was operated between 254 nm to 400 nm. Elution with solvent A (0.05% phosphoric acid) and solvent B (100% methanol) in a step gradient manner at a flow rate of 0.8 mL/min was carried out as follows: 0–13 min, 10%–80% B; 20–25 min, 80% B; 25–26 min, 80%–10% B. The sample injection volume was 10 μL. Peak identity was defined by the retention time observed for standard quercetin, kaempferol, and isorhamnetin. Flavonoids were quantified from peak area at 360 nm.

RESULTS AND DISCUSSION

Static Adsorption Test

The results of static adsorption and desorption tests showed (Table 1) that when the flavonoid concentration of the EGb solution varied from 0.35 mg/mL to 2.12 mg/mL, the adsorption capacity of the resin increased and the adsorption ratio decreased while the desorption ratio was slightly changed at about 35%, respectively. As the flavonoid concentration reached 1.41 mg/mL, the changes of the adsorption capacity and the adsorption ratio became slow. So, for static adsorption and desorption tests, the best concentration of the flavonoid was 1.41 mg/mL. The desorption solution (eluate) was concentrated and dried.

TABLE 1
Adsorption capacity, adsorption ratio, and desorption ratio of the polyamide resin in different concentration of flavonoid

Concentration (mg/mL)	0.35	0.88	1.41	1.77	2.12
Adsorption capacity (mg/g)	6.90	13.47	17.01	18.26	19.64
Adsorption ratio (%)	65.10	50.82	40.10	34.45	30.88
Desorption ratio (%)	37.13	36.85	36.21	35.84	35.50

The residue was the purified product using the static adsorption and desorption method.

Adsorption Isotherms

The equilibrium data can give information of the affinity between solutes and the adsorbent. The adsorption isotherm was used to describe the relationship between the concentration of the solute and liquid phase at equilibrium. The Langmuir isotherm and the Freundlich isotherm are the two best known and most often used isotherms for the adsorption of solutes from a solution. In general, the sorption isotherm of Langmuir assumes a monomolecular layer adsorption with a homogeneous distribution of adsorption energies and without mutual interaction between the adsorbed molecules. The sorption of Freundlich describes the equilibrium conditions of adsorption for a heterogeneous surface and does not imply the formation of a monolayer (5,12).

The equilibrium adsorption isotherm of Ginkgo flavonoid on the polyamide resin was constructed at 25°C, and the initial concentrations of the flavonoid were 0.35 mg/mL, 0.88 mg/mL, 1.41 mg/mL, 1.77 mg/mL, and 2.12 mg/mL, respectively. The adsorption capacity increased with the initial concentration, and reached the saturation plateau when the initial concentration of flavonoid reached 1.41 mg/mL. The curves generated from the data fittings to the Langmuir isotherm and Freundlich isotherm were shown in Fig. 1. Both model parameters and correlation factor (R^2) are listed in Table 2. Results showed that the correlation coefficients of both Langmuir (0.998) and Freundlich equations were rather high (0.980), but the Langmuir model described the adsorption behavior of the Ginkgo flavonoid on the polyamdie resin slightly better than the Freundlich equation. The theoretical maximum adsorption capacity q_{max} determined from the Langmuir equation was 23.53 mg/g resin, and the constant k, an indicator of the stability of the combination between the adsorbate and the adsorbent surface, was 3.17 mL/mg. In the Freundlich equation, the adsorption takes place easily when the $1/n$ value is between 0.1 and 0.5, and it is not easy to happen if $1/n$ value is above 1 (20). In Table 2, the $1/n$ value was 0.42, which indicated that polyamide resin was one kind of suitable adsorbent for the Ginkgo flavonoid.

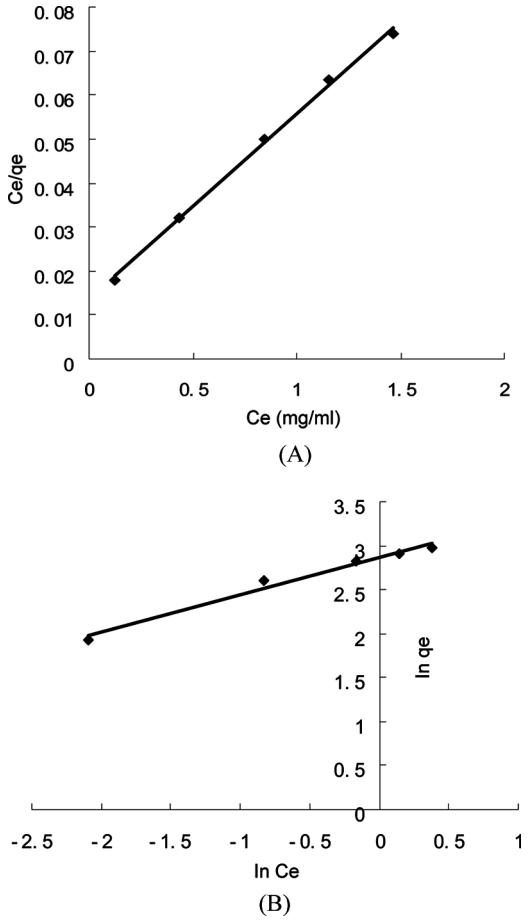

FIG. 1. Adsorption isotherm of Ginkgo flavonoid on polyamide resin. (A) Langmuir equation isotherm; (B) Freundlich equation isotherm. Adsorption temperature was 25°C.

The results (Table 1) of the static adsortption and desorption showed that the static desorption capacity of the polyamide resin was not so high. It might be due to the eluent characteristics such as the volume and the proportion of the ethanol in the eluent (5,21).

Adsorption Kinetic on the Polyamide Resin

The mere use of static adsorption capacity and desorption ratio was not enough for assessing the performance of the resin. The adsorption capacity increased with the extension of adsorption time. The adsorption capacity showed rapid increases in the early 20 minutes, and little changes after 45 minutes. Data fitting to the Langmuir adsorption rate equation were shown in Fig. 2 and the equation was $\ln\frac{q_e}{q_e-q_t} = 0.0638t + 0.563$ ($R^2 = 0.995$). The equation indicated that the adsorption kinetic was in good agreement with the Langmuir adsorption rate equation.

TABLE 2
Langmuir and Freundlich parameters of total flavonoid of EGb on polyamide resin at 25°C

	Langmuir equation			Freundlich equation		
Adsorbate	q_{max}	k	R^2	a	1/n	R^2
Tatal flavonoid	23.53	3.17	0.998	17.60	0.42	0.980
Equation	$C_e/q_e = 0.0425C_e + 0.0314$			$q_e = 17.60Ce^{0.42}$		

The equilibrium adsorption rate constant k was 0.0638/min. The resin reached adsorption equilibrium at 45 min, and it was much faster as compared to the general macroporous resin which almost reached equilibrium at about 100 min (5,12,22).

Dynamic Leakage Curve

The dynamic leakage curve was obtained based on the volume of the effluent and the flavonoid concentration (determined using the colorimetric method) of the effluent (Fig. 3).

The adsorption effect of the polyamide resin was determined mainly by hydrogen bonding interaction (23). When the adsorption reached the breakthrough point, the adsorption affinity decreased, even disappeared, and the solutes flowed out from the resin. So it was important to set up the leakage curve in order to calculate the processing volume of the sample solution. The breakthrough point generally referred to the circumstances under which the adsorbate concentration in the eluate reached 5% of that in the feeding sample solution (concentration was 1.41 mg/mL) (12). In the present case, the breakthrough point appeared at a processing volume of sample solution around 50 mL (approximately 1.7 BV), when 70.5 mg total flavonoids, equivalent to 82.9% of the corresponding static saturation adsorption capacity were adsorbed.

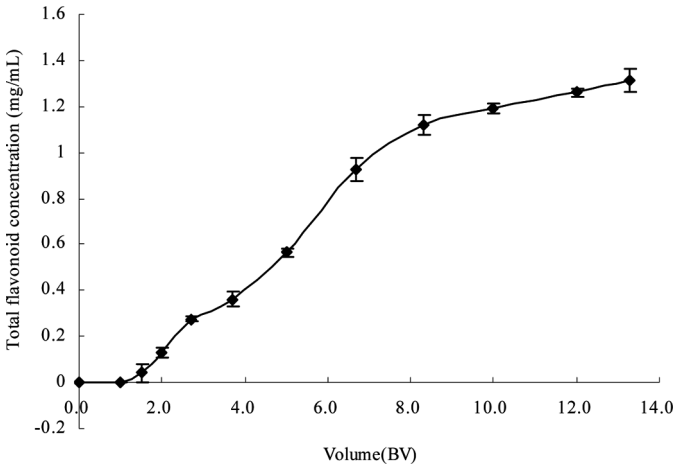

FIG. 3. Dynamic leakage curve of flavonoid on column packed with polyamide resin. Results are mean ± SD of three parallel measurements.

Dynamic Desorption Curve

The dynamic desorption curve was obtained based on the volume and the flavonoid concentration of the eluate (Fig. 4). As can be seen from Fig. 4, total flavonoids could be desorbed from the resin with approximately 10 BV (300 mL) of 30% ethanol solution. The desorption solution

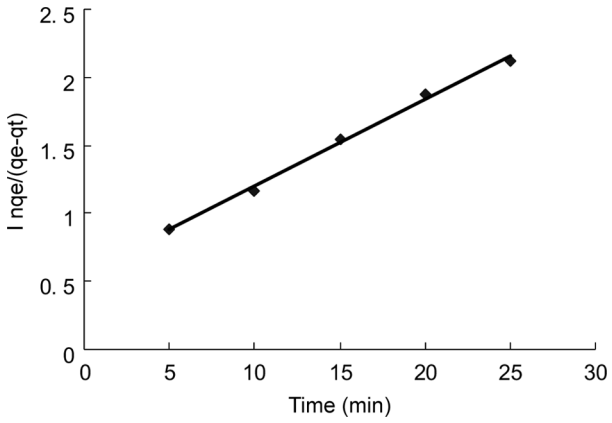

FIG. 2. Adsorption kinetic curve with Langmuir adsorption equation.

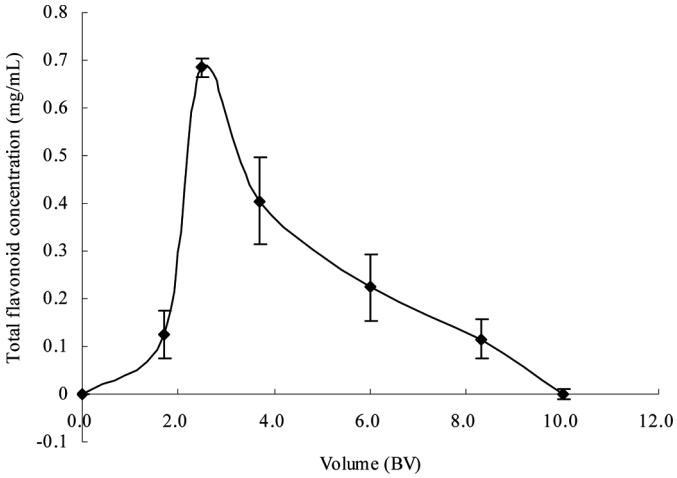

FIG. 4. Dyamic desorption curve of flavonoid on column packed with polyamide resin. Results are mean ± SD of three parallel measurements.

was concentrated and dried under vacuum. The dried product was weighed and the total flavonoids content was calculated. After treatment with polyamide resin column chromatography procedure, the content of total flavonoids reached 55.0% in the product, which was 2.3-fold higher than that of the original extract and the recovery yield of flavonoid was 42.2%. The polyamide resin was regenerated by flowing 100 mL of sodium hydroxide solution (0.5 M) through the glass column at a flow rate of 2 mL/min, and subsequently washed thoroughly by pure water before its reuse. The polyamide resin exhibited excellent reusability property, and no remarkable change was observed on the separation performance of Ginkgo flavonoid during 5 successive separation cycles.

The optimum parameters for the purification of total flavonoids of EGb with polyamide resin were confirmed as follows: flavonoid concentration in the sample solution 1.41 mg/mL, sample volume 1.7 bed volumes (BV), temperature 25°C (for adsorption), eluent 30% ethanol solution, 10 BV, flow rate 1.8 mL/min (desorption).

The HPLC profiles of the samples before and after polyamide resin chromatography method are shown in Fig. 5. It can be seen that some impurities were removed in the purified product.

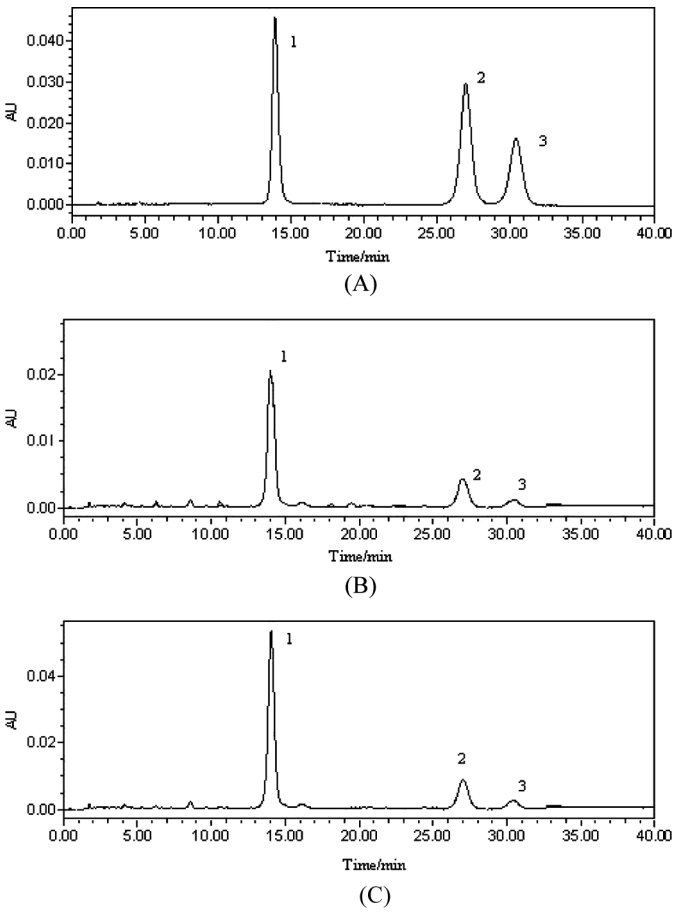

FIG. 5. The HPLC chromatograms of the analysis of standard reference materials and the samples. The chromatograms at 360 nm are as follows: (A) standards; (B) the EGb before purification; (C) the EGb after purification. Identified compounds of peaks 1–3 are quercetin, kaempferol, and isorhamnetin respectively.

CONCLUSIONS

In this study, the preparative separation of total flavonoids from EGb on polyamide resin was achieved and the results showed that polyamide resin can be a good adsorbent for the separation and purification of Ginkgo flavonoid in the column chromatography procedure. The equilibrium adsorption experiment on polyamide resin was fitted to the Langmuir isotherms. Several important parameters in the separation process, such as the concentration and volume of the feeding sample, the volume of the eluent, were optimized for most effective enrichment and preparative separation. After one run of adsorption and desorption procedure, the total flavonoids content of the product was increased from 24% to 55%. Compared to the conventional methods, this method can improve the total flavonoids content in the Ginkgo extract effectively. On further scaling up, this method can serve as the basis for the preparative separation of total flavonoids from Ginkgo and other plant extracts.

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