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Enhancement of the isolation selectivity of isoflavonoid puerarin using oligo-β-cyclodextrin coupled polystyrene-based media

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Abstract

The isolation selectivity of the isoflavonoid puerarin, a well-known traditional Chinese medicine, was studied when native and oligo-β-cyclodextrin (CDP) coupled polystyrene-based macroporous resins were used as adsorbents by static tests. The research results indicated that the CDP coupled resin HPD-100-CDP offered the best adsorption and desorption capacity for puerarin than others and its equilibrium adsorption data at 25 °C fit best to the Freundlich isotherm. The performance of separation of puerarin on HPD-100-CDP column in one step was evaluated. Based on the above experimental data, a novel medium PS-CDP was synthesized and its chromatographic retention behaviors were also explored. ESI-MS/MS, ^{1}H NMR spectroscopy and UV absorption spectrum were used for the detection and characterization of puerarin in isolated fraction. Under the optimum mobile phase, methanol/acetic acid/water = 5.0/6.6/88.4 (v/v/v), the purity and recovery of puerarin were 95.3% and 86.7%, respectively, by HPLC analysis. In conclusion, the PS-CDP medium can enhance the isolated selectivity of puerarin and it can be applied in preparative scale operations.

Keywords: Oligo-β-cyclodextrin; Puerarin; Polystyrene-based media; Coupled; Chromatography; Isolation

1. Introduction

Radix puerariae, one of the most popular traditional Chinese herbal medicines, is the root of *Pueraria lobata* (Willd.) *Ohwi*. It was officially recorded in *Chinese Pharmacopoeia* under the monograph 'Gegen' (*Radix Puerariae*, RP) [1]. Extracts of RP are rich in isoflavonoids and have been employed to relieve fever and dysentery, promote the production of body fluid, facilitate eruption, lessen stiffness and pain of the nape, control alcoholism, and for the treatment of cardiovascular diseases, e.g. hypertension, myocardial infarction and arrhythmia [2–5]. Depending on its growing conditions, the total isoflavonoids in *Radix puerariae* root varies from 1.77 to 12.0%. RP contains significant amount of the isoflavonoid puerarin (daidzein 8-*C*-glucoside), which has been demonstrated to be the major efficient components of RP in pharmacology and clinical use [6]. Traditionally, the approaches to prepare the main component of RP, puerarin, include hydrolyzing, solvent extraction, precipitation and adsorption based on resin adsorbents [7–9]. A literature introduced a method for the separation and purification of puerarin from a crude extract of *Pueraria lobata* using HSCCC (high-speed counter-current chromatography) [10]. Nevertheless, these techniques, which are limited by excessive solvent usage or the lack of excellent selectivity towards puerarin results in low recovery and purity, consequently make the process impractical for scale-up. In recent years, the increasing requirement of puerarin on clinical use and pharmacological study necessitates the development of an efficient preparative separation method for this individual active herb component.

Due to the ability to form inclusion complexes (host–guest complexes), moreover the formation of the complexes may be influenced by the shape and size of the guest molecule, hydrogen bonding, and hydrophobic interaction, β-cyclodextrin (CD) and its derivatives are extensively used as ligand as well as stationary phases or mobile phase additives in liquid chromatography and capillary electrophoresis [11–14]. Compared to monomeric CD, immobilization of polymeric CD may be obtained the considerably higher local concentration of the CD group because the large soluble molecular aggregates were formed via the polymerization of the CD monomer before

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coupling. In our previous work [15,16], water-soluble CD oligomers (oligo-β-cyclodextrin or CDP) have been successfully coupled to allyl-substituted Sepharose HP. The experimental results indicated that high concentration of the CD group attributed to pendants of CDP is favourable to improving the separation efficiency of native matrix. The CDP-substituted Sepharose HP medium exhibited high selectivity of separation of puerarin from *Radix puerariae* flavones. But the agarose support medium is limited by its lack of mechanical rigidity, even in heavily cross-linked varieties, thus restricting its application in preparative scale operations.

Growing attention has been taken to separation and purification of pharmaceutical and natural products using polymeric resins because of their convenience, low cost, high chemically stability, easy regeneration and adjusted selectivity by modification of their surface chemistry and controlling their pore structure [17,18]. Although the wide variations in functionality, surface area, and porosity available for polymeric resins present the possibility of customizing resins for the separation of specific natural product species, the adsorption selectivity of these resins is generally low. This results from the sorption of sorbates on these resins driven by a single type of weak interaction. If CDP is anchored on polymeric supports capable of adsorbing for isoflavonoid puerarin, the selectivity of the media will be enhanced. We have successfully synthesized polyacrylate-based media D152-CDP for purification of puerarin in our laboratory [19], but the more efficient media should be manufactured in order to be employed on scale-up with high yield and purity. More recent studies in Chinese journals [20–24] depicted that polystyrene-based macroporous resins such as AB-8, HPD-100, HPD-300 and NKA-9 can be utilized to separate puerarin. Based on our previous works [15,19,25], the selectivity of these media for puerarin could be enhanced via CDP bonded on the bare supports. Moreover, the water wettability of these polystyrene-type adsorbents, which possess hydrophobic chemical moiety in molecular structure, can be improved by modifying the surface with the water-soluble CDP. Facile contact with aqueous solution will extend their broad application in the separation of natural product [26]. As far as we know, no observations of isolation of isoflavonoid puerarin have been reported for CDP coupled polystyrene-based media. The objectives of the present study, therefore, were to investigate the adsorption and desorption properties of puerarin on different macroporous resins before and after coupling CDP, and a one-step column chromatographic method for isolation of puerarin was performed with the optimal coupled resin. In accordance with the data of the above matrix, a polystyrene-type uniform porous microsphere medium was selected as support. Using this stationary phase, a simplified and efficient purification approach for puerarin from crude extract was established.

2. Experimental

2.1. Materials and reagents

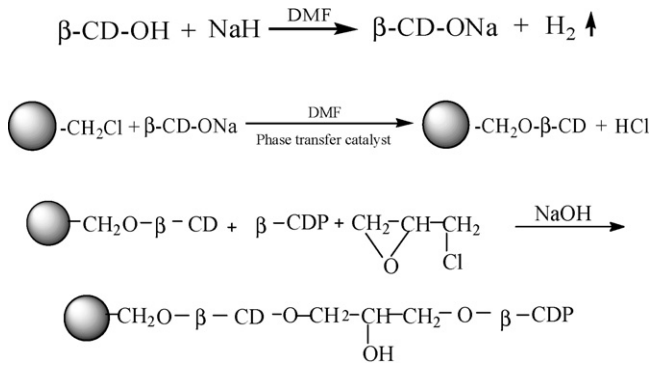
CD was purchased from the Nankai University Fine Chemicals Factory, Tianjin, China. It was recrystallized, vacuum dried at 110 °C for 24 h before use. Epichlorohydrin was obtained from Beijing Yili Fine Chemicals Factory, Beijing, China. Polystyrene-based macroporous resins including AB-8, S-8, NKA-9, NKA-II, HPD-100, HPD-450, HPD-600, YWD01B, YWD03F, YWD04, YWD06, D201 (chloromethylated polystyrene, content of chlorine: 16.5%) were purchased from Chemical plant of Nankai University, Cangzhou Baoen Chemical Ltd. and Cangzhou Yuanwei Chemical Ltd. Polystyrene-type uniform porous microsphere medium PS was the gift from Institute of Process Engineering Chinese Academy of Science. The physical properties of adsorbents are summarized in Table 1. The adsorbents were pre-treated by 1.0 M HCl and NaOH solutions successively to remove the monomers and porogenic agents trapped inside the pores during the synthesis process, then dried at 60 °C under vacuum. Prior to the immobilization experiments, an amount of adsorbents were soaked in ethanol and subsequently washed by deionized water thoroughly, dried under vacuum. A crude extract powder of *Radix puerariae* called "*Radix puerariae* flavone", and reference puerarin with a purity of >98% were bought from Luye Biology, Huainan, China. Sodium hydride (NaH) was purchased from Tianjin Huanwei Fine Chemicals Factory, Tianjin, China. Phase transfer catalyst, i.e. tetrabutyl ammonium bromide (TBAB) and Potassium iodidum (KI) were purchased from Shanghai Reagents Factory, Shanghai, China. Chloromethyl methylether was obtained from Langfang Chemical Co. Ltd. Langfang, China. Ethanol, methanol, acetic acid, acetone, acetonitrile, sodium hydroxide, zinc chloride, *N*,*N*-dimethylformamide (DMF), and others were of analytical grade and obtained from Beijing Chemicals Factory, Beijing, China.

Table 1
Physical properties of the adsorbents used for immobilization reaction

Name	Polarity	Particle diameter (mm)	Surface area ($m^2\ g^{-1}$)	Average pore diameter (nm)
AB-8	Weak-polar	0.3–1.25	480–520	13.0–14.0
S-8	Polar	0.3–1.25	100–120	28.0–30.0
NKA-9	Polar	0.3–1.25	250–290	15.5–16.5
NKA-II	Polar	0.3–1.25	160–200	14.5–15.5
HPD-100	Non-polar	0.3–1.25	600–630	10.0–12.0
HPD-450	Medium-polar	0.3–1.25	700–730	7.0–8.0
HPD-600	Polar	0.3–1.25	520–550	8.0–9.0
YWD01B	Non-polar	0.2–0.6	600–630	5.0–5.5
YWD03F	Weak-polar	0.2–0.6	500–530	12.0–13.0
YWD04	Medium-polar	0.2–0.6	500–530	9.0–10.0
YWD06	Polar	0.2–0.6	250–280	15.0–16.0
D201	Non-polar	0.3–1.25	220–250	14.5–15.5
PS	Non-polar	0.030	740.2	11.7

2.2. Preparation of the coupled media

Beads of adsorbents mentioned above in 2.1 were chloromethylated according to the previously reported procedure [18]. CD was swollen in DMF solution then reacted with NaH for 12 h. About 5.0 g chloromethylated resin beads and 9.0 g treated CD were, respectively, added into a three-necked round-bottomed flask equipped with a mechanical stirrer, a thermometer and a reflux condenser. In addition, about 3.0 g TBAB



Scheme 1. Immobilization of CDP onto polystyrene-based carrier.

and 2.0 g KI were put into as phase transfer catalyst. The mixture was stirred and heated to 70 °C for about 12 h. The reaction products P-CD (P, i.e. polymeric adsorbents) were filtered out, and washed with large amount of hot deionized water, methanol, and acetone. The CDP was synthesized according to reference [15] and its molecular weight distribution (MWD) is 5000–7000 by the determination of gel permeation chromatography (GPC). An amount of 5.0 g P-CD was transferred to a three-necked flask. After the addition of 50.0 mL 30% NaOH and 10.0 g CDP solution, the stirring started and the temperature was increased to 70 °C followed by drop-wise addition of 15.0 mL epichlorohydrin. Two hours later, the reaction was interrupted by addition of 10.0 mL 6.0 M HCl. The final immobilized matrices P-CDP were washed with large amount of hot deionized water, methanol and acetone then dried at 60 °C under vacuum. The main reactions are illustrated in Scheme 1.

The amount of CDP coupled on the polymeric adsorbents was measured according to the method described in the literatures [27,28]. Infrared spectra of the coupled media were recorded on Nicolet Nexus 670 (Thermo Nicolet, US) spectrometer using KBr disc.

2.3. Static adsorption and desorption tests

The static adsorption tests of *Radix puerariae* extracts on different macroporous resins before and after coupled by CDP were performed as follows: 2.0 g (dry weight) amounts of test resins were put into flasks with a lid. After added 20.0 mL of sample solutions of *Radix puerariae* extracts, the flasks were shaken (150 rpm) on oscillator for 12 h at 25 °C. The concentration of the puerarin remaining in the solution was measured by HPLC. The adsorption capacity of the adsorbents was calculated from the following equation:

$$Q_e = \frac{(C_o - C_e)V_i}{W} \quad (1)$$

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

where Q_e is the adsorption capacity at adsorption equilibrium (mg/g); E is the adsorption ratio (%). C_o and C_e are the initial and equilibrium concentrations of puerarin in the solutions, respectively (mg/mL); V_i is the volume of the initial sample solution (mL) and W is the weight of the dry resin (g).

The desorption processes were carried out as follows: after reaching adsorption equilibrium, the adsorbents were first washed by deionized water and then desorbed with 20.0 mL desorption solution. The optimum desorption solutions of different adsorbents were composed of varying concentrations of ethanol, methanol or acetic acid aqueous solution, respectively. The flasks were shaken (150 rpm) for 12 h at 25 °C. The desorption solutions were analyzed by HPLC. Desorption evaluation:

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

where D is the desorption ratio (%); C_d is the concentration of puerarin in the desorption solution (mg/mL); V_d is the volume of the desorption solution (mL).

The adsorption kinetic curves and the adsorption isotherms of puerarin on the selected coupled resins were studied according to the method described above. The respective concentrations of puerarin in the sample solutions were monitored at certain time intervals till equilibrium and the adsorption capacity Q_e can be calculated. The adsorption kinetic curves were obtained by plotting Q_e versus time. The tests for equilibrium adsorption isotherms on HPD-100-CDP and HPD-100 were conducted by changing the concentration of the sample solutions (concentrations of puerarin in the sample solution from 26.1 to 112.5 μg/mL), and then shaking for 12 h at a temperature of 25, 35 and 50 °C, respectively. The initial and equilibrium concentrations at different temperatures were determined by HPLC. Their degrees of fitness to Freundlich equations were appraised adopting least square method.

2.4. Chromatography isolation

HPD-100-CDP and PS-CDP were slurry packed in 250 mm × 4.6 mm i.d. stainless steel chromatographic columns, respectively. Isolation of puerarin from the crude *Radix puerariae* flavone extract was carried out on Shimadzu (Kyoto, Japan) HPLC apparatus consisting of a Shimadzu LC-20AT pump, a Shimadzu SPD-20A UV detector. Single solvent and binary mixed solvents were used as eluents. Before using, all mobile phases were prefiltrated to remove possible dust. For optimization of the separation conditions, the injected samples were prepared by an amount of 20.0 mg crude *Radix puerariae* flavone extract dissolved in 10.0 mL of various mobile phases. All samples were prefiltrated using a 0.45 μm syringe filter to remove dust before loaded onto the columns. The injected sample volume was 20 μL and eluted in isocratic chromatographic mode at a mobile phase flow velocity of 1.0 mL/min unless otherwise mentioned. The effluent was detected at UV 254 nm. The collected fraction of puerarin was evaporated with a rotary vacuum evaporator at 40–45 °C then for HPLC assay. Column chromatography isolation experiments were repeated three times and the average values are reported in this paper.

2.5. HPLC analysis

Alltech HPLC system equipped with reversed-phase C18 column (250 mm × 4.6 mm, 5 μm) from Beijing Analytical

Table 2
Results of adsorption and desorption ratios of different macroporous resins before and after immobilization

Name	Adsorption ratio (%)		Amount of β-CD immobilized (μmol/g)	Desorption ratio (%)	
	A[a]	B		A	B
AB-8	68.6	80.5	47.6	90.1	91.2
S-8	66.6	78.2	50.1	70.3	69.8
NKA-9	35.4	52.8	41.5	89.7	86.9
NKA-II	37.5	63.0	43.4	85.9	84.7
HPD-100	63.0	82.9	53.6	94.8	94.7
HPD-450	37.4	41.0	42.2	91.2	89.6
HPD-600	58.6	68.8	46.3	90.6	90.2
YWD01B	22.7	24.2	19.6	71.3	70.2
YWD03F	31.0	35.2	26.7	75.4	74.2
YWD04	26.3	30.2	29.5	70.1	72.4
YWD06	25.6	45.7	31.2	71.3	70.2
D201	24.4	56.4	55.2	70.3	71.7

[a] A and B meant macroporous resins before and after immobilization.

Instrument Apparatus Factory was used to analyze the crude sample, standard sample, desorption solutions and eluted peak fractions of the experiments. The column eluent was monitored by the UV detector at 254 nm. The mobile phase was composed of methanol/acetic acid/water = 27.0/0.4/72.6 (v/v/v), and the flow rate was 0.8 mL/min. The chromatographic peak of the puerarin was confirmed by comparing its retention time and UV spectrum with that of the reference standard. The working calibration curve based on puerarin standard solutions showed good linearity over the range of 0.5–200.0 μg/mL. The regression line was $Y = 60{,}102{,}351X + 94{,}843$, $R^2 = 0.9993$, where Y and X are the peak area and the concentration of puerarin (μg/mL), respectively.

2.6. MS and ^{1}H NMR identification

MS identification of the separated fraction was performed using a Waters Quattro Premier XE tandem quadrupole mass spectrometer (Waters). The instrument was set to collect data in multiple reactions monitoring (MRM) mode using electrospray ionization (ESI), switching between positive and negative-ion mode during the run. The ionization source parameters were: capillary voltage 3.5 kV; sample cone voltage 30 V; source temperature 100 °C; desolvation gas temperature 350 °C at a flow rate of 7.5 × 100 mL/min (N_2). Product ion spectra were obtained by selecting the protonated or deprotonated ions for collision. Data acquisition and processing were performed using MassLynx V4.1. ^{1}H NMR measurement was carried out on a Bruker AV600 spectrometer, operating at a 600 MHz ^{1}H frequency. The samples were dissolved with dimethyl sulfoxide-d_6 and the solution was measured with tetramethylsilane (TMS) as the internal reference.

3. Results and discussion

3.1. Adsorption capacities on coupled macroporous resins

The preliminary evaluation of the performance of the CDP coupled adsorbents was depended on their capacities of adsorption and desorption in static tests. The adsorption and desorption ratios of puerarin on macroporous resins before and after coupled by CDP were shown in Table 2. It can be seen that the CDP coupled adsorbents have the higher adsorption ratios for puerarin than the initial macroporous resins due to the specific inclusion interaction between CDP and the isoflavonoid molecules. From Table 2 it can also be discovered that the desorption ratios of coupled adsorbents were almost the same as that of their supports, i.e. macroporous resins, via choosing appropriate desorption solution. The amount of coupled CD correlates with the characteristic of the native macroporous resins. The resins with relatively larger surface areas and smaller particles, as well as pore diameters around 10 nm, such as AB-8, S-8, HPD-100, and HPD-600 were advantageous to the immobilization reaction and adsorption of puerarin molecule. Although the adsorption capacity of S-8-CDP was considerably high, the desorption capacity was rather low. Thus, coupled resins AB-8-CDP, HPD-100-CDP, and HPD-600-CDP were selected to further investigate their adsorption behavior towards puerarin.

3.2. Adsorption kinetics on selected media

Adsorption kinetics curves for puerarin on the selected media of AB-8-CDP, HPD-100-CDP and HPD-600-CDP were separately described in Fig. 1. It can be seen that the adsorption capacities for puerarin increased with the extension of adsorption time, reaching equilibrium for HPD-100-CDP and HPD-600-CDP at around 3 h, for AB-8-CDP at about 4 h and this value can be considered as the equilibrium time for puerarin. In the first 2 h the adsorption capacities increased rapidly, after 2 h the slopes which indicate the adsorption ratios varied little at different times. Compared the data exhibited in Fig. 1, at any time the adsorption capacity of HPD-100-CDP towards puerarin was higher than that of HPD-600-CDP and AB-8-CDP. In the comprehensive consideration of the adsorption and desorption ratio, as well as adsorption rate, HPD-100-CDP was selected as the most suitable medium for the following test.

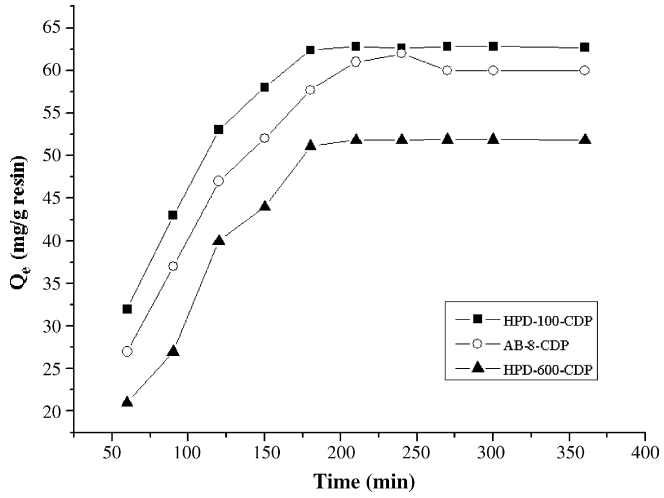


Fig. 1. Adsorption kinetics curves for puerarin on media.

3.3. Equilibrium adsorption

The equilibrium adsorption isotherms of puerarin on HPD-100-CDP and HPD-100 resins at 25, 35 and 50 °C were shown in Fig. 2. The adsorbing capacity for puerarin on the coupled resin HPD-100-CDP is higher than that on HPD-100, which can be contributed to the forming of inclusion complexes with the CDP ligand on the resin. Equilibrium data concerning the adsorption of the puerarin onto the two adsorbents HPD-100-CDP and HPD-100 at 25, 35, and 50 °C was further used to fit the Freundlich Eq. (4):

$$Q_e = KC_e^{1/n} \quad (4)$$

where K is the Freundlich constant that is an indicator of adsorption capacity, and $1/n$ is an empirical constant related to the magnitude of the adsorption driving force [29,30]. A linearized form of Eq. (4) can be written as:

$$\ln Q_e = \ln K + \frac{1}{n} \ln C_e \quad (5)$$

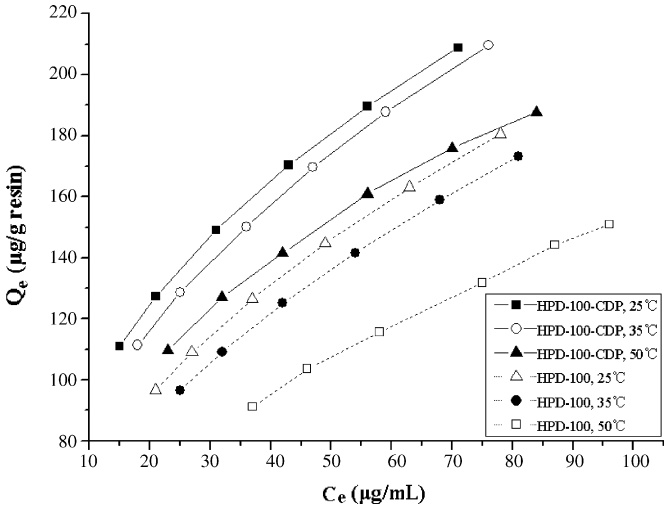


Fig. 2. Equilibrium adsorption isotherms of puerarin on HPD-100-CDP and HPD-100 at different temperatures.

Table 3
Freundlich equations of puerarin on HPD-100-CDP and HPD-100 at different temperatures

Temperature (°C)	Linearized form of Freundlich equation $\ln Q_e = \ln k + (1/n) \ln C_e$	K	n	R^2
HPD-100-CDP				
25	$\ln Q_e = 3.608 + 0.4067 \ln C_e$	36.90	2.459	0.9968
35	$\ln Q_e = 3.446 + 0.4386 \ln C_e$	31.36	2.280	0.9956
50	$\ln Q_e = 3.363 + 0.4253 \ln C_e$	28.88	2.351	0.9935
HPD-100				
25	$\ln Q_e = 3.125 + 0.4753 \ln C_e$	22.76	2.104	0.9951
35	$\ln Q_e = 2.971 + 0.4968 \ln C_e$	19.52	2.013	0.9943
50	$\ln Q_e = 2.629 + 0.5217 \ln C_e$	13.86	1.917	0.9918

Freundlich model is used to reveal the interaction of solutes with the adsorbents. The regression equations of Freundlich isotherm at different temperatures are summarized in Table 3. It is obvious that the correlation coefficients of Freundlich equations on HPD-100-CDP and HPD-100 for puerarin were rather high. That is to say the Freundlich law is applicable to the adsorption of puerarin on the above two resins. The correlation coefficients of Freundlich equation at 25 °C were the highest, which can better describe the adsorption behavior of puerarin on HPD-100-CDP and HPD-100. The correlative parameters of Freundlich adsorption isotherm equations for puerarin at different temperatures were also listed in Table 3. Based on the Freundlich theory, coefficient K is an indication of the adsorbing capacity. According to K in Table 3, the obviously higher adsorbing capacity toward puerarin on HPD-100-CDP can be expected. In general, the adsorption can take place easily when the $1/n$ value in the Freundlich equation is between 0.1 and 0.5, and it is not easy to happen if $1/n$ value is between 0.5 and 1.0, however, it is very difficult to occur if $1/n$ value exceeds 1.0 [31]. In Table 3, the $1/n$ values of HPD-100-CDP are all between 0.1 and 0.5, which illustrated the adsorption process of puerarin on HPD-100-CDP can take place easier than on HPD-100. Hence, the coupled medium is more appropriate for isolating puerarin.

3.4. Isolation of puerarin on HPD-100-CDP column

The one-step column chromatographic separation of puerarin content from the crude *Radix puerariae* flavone extract was investigated on HPD-100-CDP media using various mobile phases. It has been reported that the column resolution efficiency declined as the particle size of the stationary phase becomes larger because of the smaller contact area of the sample molecule with the surface of packing, longer flow paths, and larger diffusivity [32]. Thus, before slurry packed into columns, the coupled medium HPD-100-CDP was sieved and sedimentated suspended particles in acetone to obtain the final narrow fraction of microparticles in size of 40–74 μm. Fig. 3(A) shows the profile of chromatographic separation under the optimized mobile phase condition (methanol/acetic acid/water = 5.0/6.6/88.4 (v/v/v)). The peak marked 1 indicates the fraction corresponding to puerarin and was analyzed by HPLC in Fig. 3(B). The chromatograms indicated that the isoflavonoid components were not absolutely resolved on this

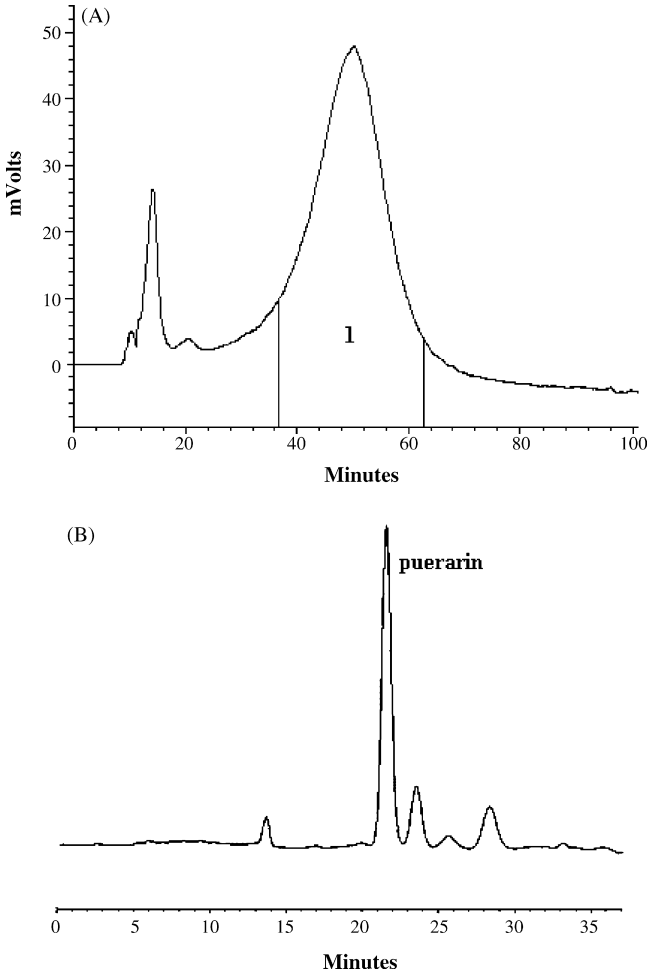


Fig. 3. (A) Chromatogram of separation *Radix puerariae* flavone on HPD-100-CDP matrix. Sample: puerariae flavone (2.0 mg/mL), flow rate: 1.0 mL/min, mobile phase: methanol/acetic acid/water = 5.0/6.6/88.4 (v/v/v). (B) HPLC analysis of the peak 1 in (A). Column: RPC18, mobile phase: methanol/acetic acid/water = 27.0/0.4/72.6 (v/v/v), flow rate: 0.8 mL/min.

matrix. The result in a single run showed that even on optimized condition, the desired product of puerarin from the HPD-100-CDP column was obtained at a purity of 75.8% with a recovery of 95.1%.

3.5. Chromatographic evaluation of PS-CDP column

The large particle size distribution and irregular shapes should be responsible for the existing of impurities. In order to improve the one-step isolation efficiency, uniform porous microsphere PS listed in Table 1, which was polymerized by combining microporous glass membrane emulsification technique and suspension polymerization process, was chosen as support for coupled by CDP. The selected foundation resulted from the static tests of macroporous resins: relatively smaller spherical particle, controlled pore size distribution and large specific surface area. The coupled medium slurry packed into columns, and the chromatographic retention behaviors of isolation puerarin were evaluated.

3.5.1. Effect of the mobile phase composition

The composition of the mobile phase is of extreme importance in the purification of the target product. The separation on a given packing material would be optimized by adjusting the polarity of the mobile phase. The optimum mobile phase compositions were experimentally determined on the basis of the purity and recovery of puerarin. Solvent systems of different concentration and composition were applied as mobile phases to validate an appropriate eluent. Solvent solutions of methanol–water, ethanol–water, acetic acid–water and their mixtures were used as mobile phases. The results demonstrated that the mixtures composed of methanol–acetic acid–water were superior to other single or binary solvents. The influence of the mobile phase composition on the recovery and purity of puerarin was shown in Fig. 4. The optimum mobile phase composition was confirmed as methanol/acetic acid/water = 5.0/6.6/88.4 (v/v/v), and the corresponding chromatographic profile was shown in Fig. 5(A) in which peak 1 contained the puerarin confirmed through compared with the sample solution of standard using HPLC and UV spectra analysis in Fig. 5(B). Comparison of the chromatograms, it is evident that under the above optimum mobile phase conditions there are no significant interferences to the puerarin peak by other endogenous components. The purity and recovery of puerarin in peak 1 of Fig. 5(A) cut were 95.3% and 86.7% by HPLC analysis. When applying a somewhat broader cut, a purity of about 90.3% was obtained with a recovery of about 95.5%. The reproducibility of the adsorption selectivity and separation efficiency of the coupled matrix was examined on PS-CDP columns after they used for 300 h in our laboratory without significant change in the purity and recovery of puerarin. It was testified that the oligo-β-CD coupled matrix PS-CDP exhibited highest puerarin adsorption selectivity and separation efficiency in optimal mobile phase solvents compared with previous polymeric adsorbents mentioned above.

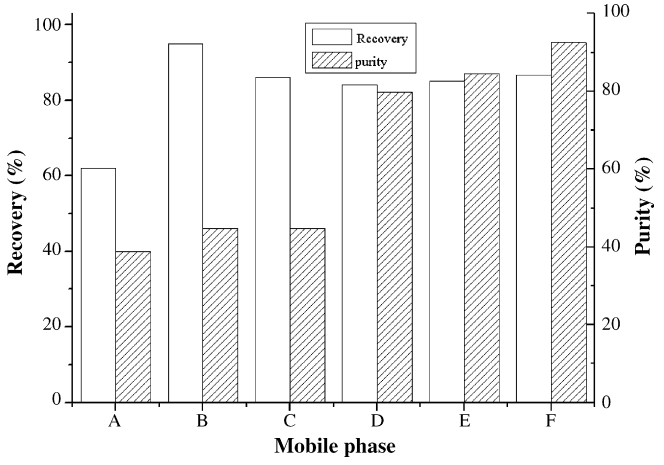


Fig. 4. The influence of the mobile phase composition on the recovery and purity of puerarin on PS-CDP column. Sample: puerariae flavone (2.0 mg/mL), flow rate: 1.0 mL/min. (A) acetic acid/water = 15.0/85.0 (v/v), (B) methanol/water = 30.0/70.0 (v/v), (C) ethanol/water = 50.0/50.0 (v/v), (D) methanol/acetic acid/water = 20.0/8.0/72.0 (v/v/v), (E) methanol/acetic acid/water = 10.0/4.5/85.5 (v/v/v), and (F) methanol/acetic acid/water = 5.0/6.6/88.4 (v/v/v).

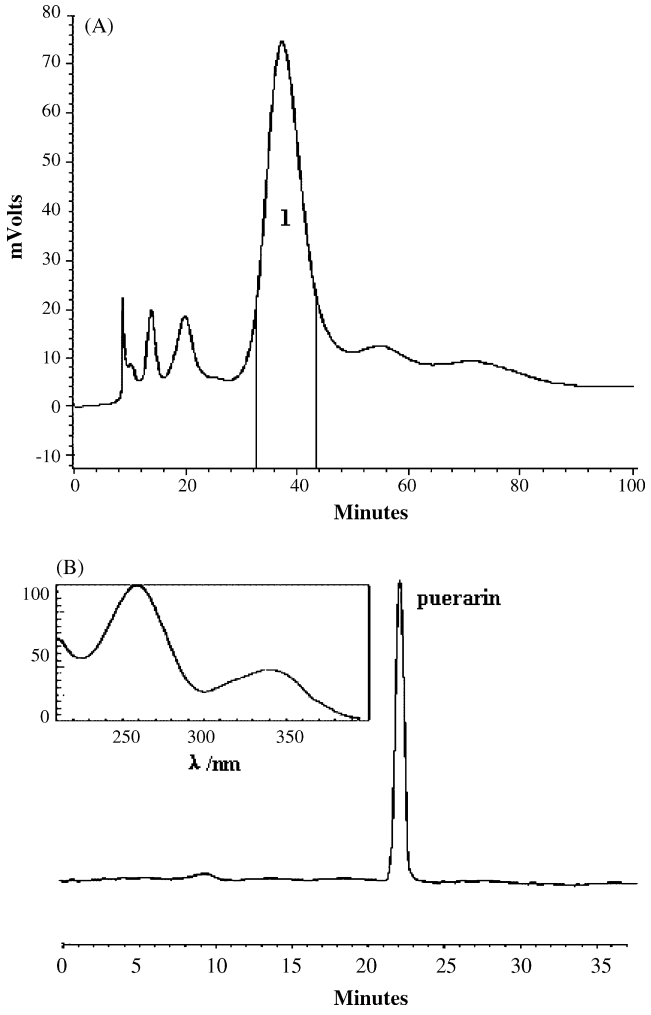


Fig. 5. (A) Chromatogram of separation *Radix puerariae* flavone on PS-CDP matrix. Sample: puerariae flavone (2.0 mg/mL), flow rate: 1.0 mL/min, mobile phase: methanol/acetic acid/water = 5.0/6.6/88.4 (v/v/v). (B) HPLC analysis and UV spectrum of the peak 1 in (A). Column: RPC18, mobile phase: methanol/acetic acid/water = 27.0/0.4/72.6 (v/v/v), flow rate: 0.8 mL/min.

3.5.2. *Identification of the isolated fraction*

The structure of the product was further confirmed by ESI-MS and ^{1}H NMR spectrometry. The spectral analyses of the separated fraction were carried out under the conditions stated in Section 2.6. The positive ion ESI mass spectrum Fig. 6(A) of the fraction corresponding to puerarin on the chromatogram peak 1 in Fig. 5(A) showed an abundant ion peak at *m*/*z* 417, which was the proton adduct of puerarin (mol. wt. 416 plus 1). The chemical structure of target compound is *C*-glycoside which has sugar substituent bonded to a carbon of the aglycon at positions *C*-8. Inspection of the product ion spectra as shown in Fig. 6(B), the MS–MS analysis of the *m*/*z* 417 peak as precursor ion gave rise to the major fragments of *m*/*z* 399, 381, 363, 351, 321, 297 and 267 (collision energy = 20 eV). The loss of 120 Da is indicative of *C*-glycosides [33]. A series of ions at *m*/*z* 399, 381, and 363 were obtained due to the successive neutral losses of water molecules. The ion peaks are consistent with those in the literature [34]. The 600 MHz ^{1}H NMR spectrum of the isolated fraction corresponding to puerarin was illustrated in Fig. 7. The

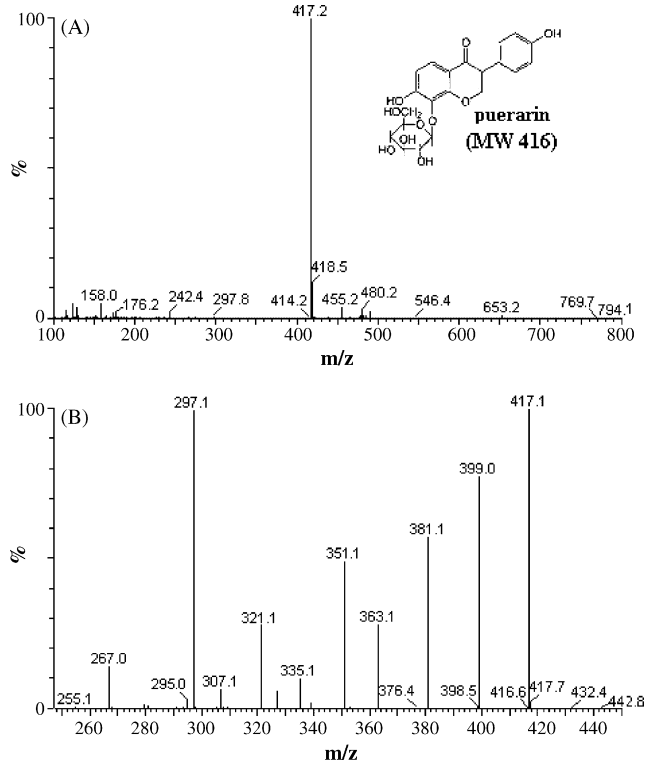


Fig. 6. Positive ion ESI mass spectra. (A) MS spectrum, and (B) MS–MS spectrum of puerarin isolated on PS-CDP column.

data were as follows: ^{1}H NMR (DMSO, 600 MHz) δ 9.49 (1H, s, 4′-OH), 8.34 (1H, s, 2-H), 7.94 (1H, d, J = 8.4 Hz, 5-H), 7.40 (2H, d, J = 9.0 Hz, 2′, 6′-H), 6.99 (1H, d, J = 8.4 Hz, 6-H), 6.80 (2H, d, J = 8.4 Hz, 3′, 5′-H), 4.81 (1H, d, J = 7.4 Hz, glc 1″-H), 4.01 (1H, s, glc 2″-H), 3.20–3.31 (3H, m, glc 3″, 4″, 5″-H). These data were in agreement to those reported in the literatures [35,36]. In addition, UV absorption spectrum of the separated fraction as shown in Fig. 5(B) was accordant with the reference standard [37]. Thus, the data obtained from these analyses make us confidently conclude that the separated target component is puerarin.

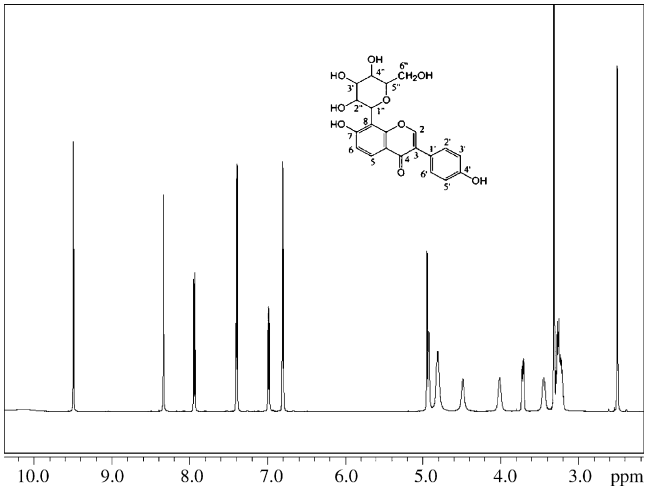


Fig. 7. ^{1}H NMR spectrum of puerarin isolated on PS-CDP column.

3.5.3. Elucidation of the retention mechanism

In the general case, retention of an analyte is the sum of different types of interaction with the stationary phase. Multi-interaction between stationary phase and adsorbates results in different retention which is conductive to the separation of the target product. The main driving force of retardation on the polystyrene packings is the formation of labile π-complexes between aromatic fragments of the stationary phase and π-systems of adsorbates [38]. The π–π interactions can be formed during chromatographic process of separation puerarin on polystyrene-based media because phenolic moieties were comprised in main isoflavonoid components of *Radix puerariae.* The contribution of π–π interactions "adsorbent–adsorbate", i.e. the formation of labile π–π complexes to the retention had been estimated in Ref. [39]. As mentioned above, the unique geometry of the coupled oligo-β-CD can grant β-CD moieties to form not only hydrogen bonding but also hydrophobic interaction with isoflavonoid components. It is textbook knowledge that urea and sodium dodecanesulphonate (SDS) at the high concentrations have the capacity to suppress hydrogen bond and hydrophobic interaction formation, respectively. The effect of urea and SDS on the retention of puerarin on PS-CDP column was presented in Fig. 8. From the chromatograms it can be seen, after adding 10% urea and 2% SDS into the optimum mobile phase, the retention time and the resolution of puerarin decreased a lot. These phenomena supported the tentative interpretation that multi-interaction governed the separation of puerarin on PS-CDP medium.

3.5.4. The synthesis of PS-CDP

The steric effect leads to decreased immobilization of large size reactants such as CD to be coupled onto the polymer support. Furthermore, extremely hydrophobic chemical structure of these polystyrene-based adsorbents results in their poor contact with aqueous solutions [37], and CD dissolves in several solvents such as water, DMF and pyridine but CDP just dissolves in water. The problem may be solved in one hand by linking a spacer –CH_2–Cl– on the reactive polymers which can connect with the hydroxyl groups of CD, in this way the CDP moiety can be coupled onto polystyrene-based adsorbents. On the other hand, in order to increase the water wettability of these carries, the hydrophobic polystyrene-based supports were firstly reacted with CD in DMF solution containing phase transfer catalyst. Then, the grafting CDP reaction carried out in aqueous sodium hydroxide solution. The detail is illustrated in Scheme 1.

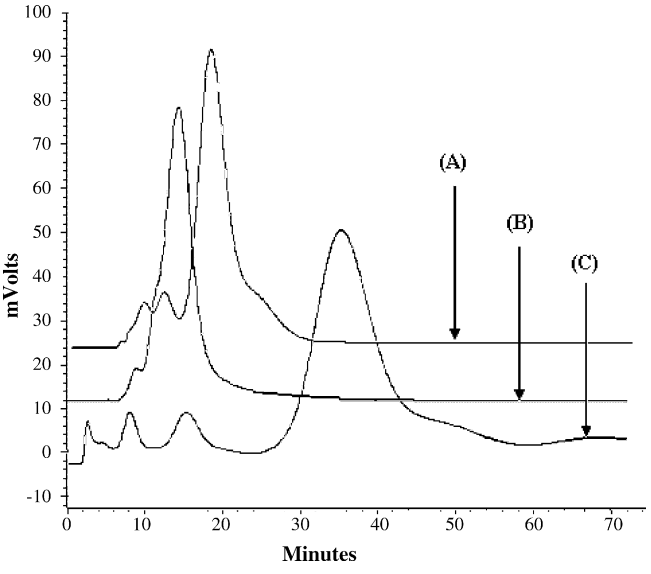


Fig. 8. Effect of urea and SDS on the retention of the puerarin peak on the PS-CDP column. (A) Chromatogram of 10% urea added in optimum mobile phase, (B) chromatogram of 2% SDS added in optimum mobile phase, and (C) chromatogram of optimum mobile phase.

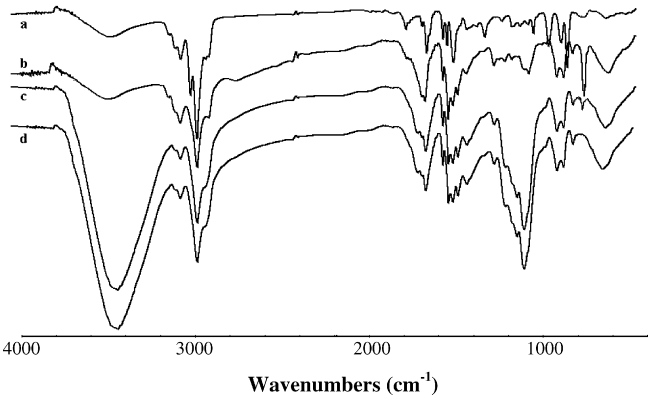


Fig. 9. Infrared spectra of (a) PS, (b) PS-Cl, (c) PS-CD, and (d) PS-CDP.

The infrared (IR) spectra of PS before and after the coupling reaction were illustrated in Fig. 9. It can be seen that the adsorption curves exhibited obvious differences between the IR spectroscopy of the PS (a), PS-Cl (b) and PS-CD (c), PS-CDP (d). After immobilization and grafting reaction, the strong absorption peaks at 3448 and 1680–1610 cm^{-1} in the spectra of (c) and (d) were ascribed to the transform vibration peaks of the –OH, and stretch vibration absorption peaks of C–O, C–O/C–C– synchronously appeared nearby 1170–1160 cm^{-1} and 1060–1020 cm^{-1}, which indicated the introduction of CD and CDP. Otherwise, in the spectra of (c) and (d), the obvious decrease and disappearance in the strength of the peak 680 cm^{-1}, respectively, which correspond with the stretch vibration of –CH_2–Cl–, illuminated the immobilization and grafting reaction successfully accomplished.

4. Conclusion

In the present study, the isolation selectivity of puerarin was studied when twelve native and coupled polystyrene-based macroporous resins were used as adsorbents by static tests, and an enhancement in selectivity was obtained when using coupled media. In term of representing the best adsorption and desorption capacity for puerarin than others, and its equilibrium adsorption data well fitting to the Freundlich isotherm which was used to describe the interactions between solutes and resin at different temperatures, the coupled resin HPD-100-CDP was selected as the most appropriate medium for the separation of puerarin. The performance for isolation of puerarin on HPD-100-CDP column in one step was evaluated sequentially. Based on the above experimental data, the CDP coupled

polystyrene-based matrix PS-CDP was developed to improve the separation efficiency of puerarin. The isolated fraction was identified by positive electrospray ionization tandem mass spectrometry (ESI-MS/MS), ^{1}H NMR and UV-absorbance spectra. The stationary phase was prepared under convenient method and was quite stable when using aqueous and organic mobile phases on the basis of reproducibility data. The infrared spectroscopy data indicated CDP was covalently immobilized on the polystyrene-based beads. The optimal mobile phase was methanol/acetic acid/water = 5.0/6.6/88.4 (v/v/v) in isocratic elution at a flow velocity of 1.0 mL/min on 250 mm × 4.6 mm column. The PS-CDP stationary phase can provide efficient separation of isoflavonoids from the *Radix puerariae* crude extract under optimum conditions. The purity and recovery ranging from 95.3–90.3 to 86.7–95.5% were, respectively, obtained for puerarin in a single PS-CDP column. The results in this work can be referenced for the preparative operation and the PS-CDP matrix can serve as a promising medium on scale-up to yield puerarin.

Acknowledgements

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