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One-step rapid determination and purification of puerarin from *Radix puerariae* by *n*-octylamine-modified poly(methacrylate-*co*-ethylene dimethacrylate) monolith

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ABSTRACT

n-Octylamine-modified poly(methacrylate-*co*-ethylene dimethacrylate) monoliths were prepared for rapid screening, determination and one-step purification of puerarin from *Radix puerariae* (a crude extract of the root of *Pueraria lobata*). The modified monolith showed a specific surface area of 17.8 $m^2 g^{-1}$, an average pore size of 0.76 μm and a total porosity of 60.8%. Fast separation of *R. puerariae* crude extract was achieved within 5 min at a flow velocity of 722 cm h^{-1} resulting in a puerarin purity of 97%, with a recovery of 85%. This demonstrates the potential of *n*-octylamine-modified poly(methacrylate-*co*-ethylene dimethacrylate) monolith for the rapid analysis and separation of isoflavonoids. Preparative scale sample loading (12 mg in 2 mL) resulted in a purity of 95%, and a recovery of about 69%. HPLC, FTIR, MS and ^{1}H NMR spectroscopy were used for the characterization and quantification of puerarin in isolated fraction.

1. Introduction

Traditional Chinese Medicines (TCMs) have been extensively used to prevent and cure many diseases in China, thanks to low toxicity and rare complications. The roots of *Pueraria lobata* (Wild.) Ohwi and *Pueraria thomsonii* Benth (Fabaceae family), two commonly used Chinese herbal medicines, were officially included in the Chinese Pharmacopoeia (CP) until the year 2000 edition under the same name 'Gegen' (*Radix puerariae*, RP) [1]. Isoflavonoids, such as puerarin, daidzin, genistin, genistein and daidzein have been responsible for the broad therapeutic effects of RP connected to its estrogenic activity (phytoestrogens) [2–4]. Puerarin (7,4′-dihydroxyisoflavone-8β-glucopyranoside) with a concentration of approximate 4% in RP has many pharmacological effects, such as anti-hypertension, anti-arteriosclerosis, dilating coronary arteries, decreasing myocardial oxygen consumption and improving microcirculation in both animals and humans suffering from cardiovascular disease [5]. However, most of the TCMs have complicated and structurally related components. Therefore, a rapid, simple and effective method is required for the separation and purification of these substances.

β-Cyclodextrin (β-CD) coupled to different media [2,6–9], high-speed counter-current chromatography [10] and extraction [11] were shown useful for the separation and purification of puerarin. Micellar electrokinetic capillary chromatography with electrochemical detection [12], non-aqueous capillary electrophoresis [13] and HPLC [14–18] were also used for the separation of puerarin, however, primarily for analytical purposes and not for preparative chromatography due to their very low binding capacities. Monolithic stationary phases, directly cast in tubes by radical polymerization were developed by several groups [19–21]. They have high permeability and good mass transfer, leading to improved resolution at high linear flow rates. Monoliths based on poly(methacrylate-*co*-ethylene dimethacrylate) were usually modified to ion-exchangers [22,23], reversed-phase chromatography media [24,25] and affinity chromatography media [26]. However, since the components of *R. puerariae* extracts contain aromatic rings and phenolic groups, it is reasonable to assume that hydrophobic interaction and hydrogen bonds may contribute to the separation process. In this paper, monoliths based on *n*-octylamine-modified poly(methacrylate-*co*-ethylene dimethacrylate) were shown to be efficient for rapid separation and purification of puerarin from crude extracts of *R. puerariae* in one step. This work suggests a new approach for the rapid separation of puerarin and introduces a new application of monolithic columns for fast separation of natural products. The dynamic binding capacity, adsorption characteristics and fast separation of puerarin were investigated. Compared with published literatures, *n*-octylamine-modified

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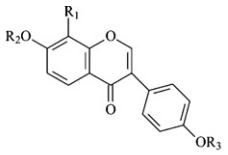

Compound	Group		
	R_1	R_2	R_3
Daidzein	H	H	H
Daidzin	H	Glucose	H
Puerarin	Glucose	H	H
Daidzein-4',7-diglucoside	H	Glucose	Glucose

Fig. 1. The molecular structures of *Radix puerariae* flavonoids.

monolith could attain a very fast separation of puerarin for analytical purposes as well as for preparative chromatography with high efficiency in one step. Poly-β-CD-substituted Sepharose HP [2,7], Superose 12 HR 10/30 [6], oligo-β-CD-coupled polyacrylate [8] and polystyrene beads [9] were perfect media for the preparation of puerarin from crude extracts of *R. puerariae*. Comparatively speaking, monolith saved more time, work and mobile phase, thanks to good mass transfer property.

2. Materials and methods

2.1. Materials

Glydiyl methacrylate (GMA, 99%) and ethylene dimethacrylate (EDMA, 99%) were purchased from Acros (Morris Plains, NJ, USA). 2,2-Azobis-(2-methylpropionitrile) (AIBN) was bought from Beijing Chemical Reagent Plant (Beijing, China) and crystallized with ethanol before use. A crude extract powder of *R. puerariae* called "*Radix puerariae* flavone" (content of flavones >49%), and reference standard puerarin with a purity of >98% were bought from Luye Biology Limited Co. (Huainan, China). The isoflavone content of *Radix puerariae* flavone is 10–14%, mainly composed of daidzein, daidzin, puerarin and daidzein-4′-7-diglucoside, whose structures were shown in Fig. 1 [6]. The content of puerarin is approximately 4%.

The pueraria flavones solution was prepared by dissolving 6.0 g of pueraria flavones powder in 100 mL 20% (v/v) ethanol followed by centrifugation. The resulting supernatant solution was diluted fourfold with distilled water and filtered through a 0.45 μm membrane before injection to the preparative column.

2.2. Preparation and modification of monolithic columns

The stationary phase was prepared by in situ polymerization in a 100 mm × 4.6 mm i.d. stainless steel chromatographic column tube. The porogenic solvent was a mixture of cyclohexanol and dodecanol with an 87:13 volume ratio. Functional and cross-linking monomers were GMA and EDMA with a 5.76 molar ratio. The porogenic solvent and the monomers were thoroughly mixed in a 6:4 volume ratio to prepare a uniform solution in which AIBN (1% (w/v) with respect to monomers) was dissolved. The column was sealed with a polystyrene cap in one end, filled with 1.7 mL of the monomer solution and degassed by ultrasonication for 15 min followed by sealing the other end of the tube with another cap. The polymerization was allowed to proceed at 60 °C for 12 h followed by replacing the polystyrene caps with column end fittings. The column was connected to a HPLC system. The porogenic solvent and other soluble compounds present in the formed monolith were removed by pumping tetrahydrofuran (50 mL) through the column. Subsequently, the column was washed with ethanol (20 mL) and filled with 50% (v/v) *n*-octylamine ethanol solution. The modification was performed in a water bath at 55 °C for 12 h followed by washing with ethanol and then with 1% (v/v) acetic acid solution.

2.3. Characterization of the monolithic column

After the chromatographic experiments, the monolith rod was pumped out of the tube, cut into small pieces and dried at 70 °C for 12 h. The morphology of the polymer was studied by scanning electron microscopy (SEM) of Sterescan 250 MK3 (Cambridge, UK). The pore size and specific surface area of the dried monolith were determined using a mercury intrusion porosimeter of Pascal 240, Thermo Electron (Waltham, MA, USA).

2.4. Separation of Radix puerariae flavone by monolithic chromatography

The chromatographic experiments were carried out using a chromatography system from Alltech Company. Crude *Radix puerariae* flavone was dissolved in 10 mL of various mobile phases at a concentration of 1 mg mL^{-1}. Before injection to the column, all samples were pre-filtrated using a 0.45 μm syringe filter to remove non-dissolved particles. For analytical applications, the injected sample volume was 20 μL. For preparative applications, the injected sample volume varied from 0.5 mL to 2 mL. The effluent was monitored with a UV detector at 250 nm and peak fractions were collected according to the elution profile. The isoflavonoids in *R. puerariae* were separated on the monolith using 1% (v/v) acetic acid solution as the mobile phase at different linear flow velocities.

2.5. Analysis of puerarin by HPLC

Crude samples, standard sample and eluted peak fractions were analyzed on an Alltech HPLC system equipped with a reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μm) from Beijing Analytical Instrument Apparatus Factory (Beijing, China). The column effluent was monitored using a UV detector at 250 nm. The mobile phase was composed of methanol–water (27:73, v/v, 0.5% HAc), and the flow rate was 1.0 mL min^{-1}.

2.6. Determination of dynamic binding capacity and column efficiency

To determine the dynamic binding capacity of *n*-octylamine monolith, frontal analysis of the column was carried out with 1 mg mL^{-1} puerarin prepared in the mobile phase of 1% (v/v) acetic acid solution. The binding capacity (Q) was calculated by the following equation:

$$Q = \frac{(V_{HB} - V_0)c}{m} \quad (1)$$

where V_{HB} (mL) is the half breakthrough volume of puerarin, V_0 the dead volume the column, c (mg mL^{-1}) the puerarin concentration in the mobile phase and m (g) is the dry weight of monolithic media.

The column efficiency was investigated according to literature [27]. Peak broadening caused by the *n*-octylamine-modified monolith was determined by first and second peak moments using toluene as a probe. Twenty microlitres of 0.1 mg mL^{-1} toluene prepared in the mobile phase was loaded into the monolith and eluted isocratically using methanol/water (75/25, v/v) as the mobile phase at velocities varying from 361 cm h^{-1} to 1445 cm h^{-1}. The theoretical plate of toluene under isocratic elution conditions was

expressed as

$$\mathrm{HETP} = L\frac{\sigma^2}{\mu^2} \quad (2)$$

where μ is the first moment and σ the second moment, and L is the length of the column.

2.7. Determination of the ligand density

n-Octylamine was used as modifier at a concentration of 50% (v/v) resulting in a relatively high ligand density. This is why acid–base titration was considered accurate enough for the determination of the ligand density. The titration was performed as follows: about 0.2 g media was weighed and 5 mL 0.05 M hydrochloric acid was added. The mixture was stirred for 2 h and then filtrated. The filtrate was titrated with 0.05 M NaOH. The ligand density (Q, mmol g^{-1}) of the media was calculated according to the following equation:

$$Q = \frac{0.05V_{\mathrm{HCl}} - 0.05V_{\mathrm{NaOH}}}{m} \quad (3)$$

where V_{HCl} (mL) was the volume of hydrochloric acid, V_{NaOH} (mL) the volume of sodium hydroxide and m (g) was the dry weight of the media.

2.8. FTIR, MS and NMR analysis of puerarin

The collected puerarin fractions and puerarin standard dissolved in 20% (v/v) ethanol were analyzed by Fourier transform infrared spectroscopy-attenuated total reflection (FTIR-ATR). The spectra of all solutions were measured using a Nicolet/FTIR NEXUS spectrometer in the 4200–700 cm^{-1} region with a resolution of 4 cm^{-1}. The spectrometer was equipped with an ATR attachment with a ZnSe crystal detector. The spectrum of each solution was obtained by averaging 32 consecutive scans.

To identify the separated components, selected fractions were submitted to MS using a Waters Quattro Premier XE tandem quadrupole mass spectrometer. 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 as follows: 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^{-1} (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.

The collected puerarin fractions were also analyzed by ^1H NMR (nuclear magnetic resonance). The NMR experiments were performed on a BRUKER AV-600 spectrometer operating at 600.13 MHz. Chemical shifts were referenced to the solvent reference signal. The samples were dissolved with dimethyl sulfoxide-d_6 (DMSO) and the solution was measured with tetramethylsilane (TMS) as the internal reference. Samples were placed in a 5 mm i.d. sample tube. The chemical shifts are given in parts per million (ppm) at 300 K. The spectra were recorded at 300 K using a sweep width of 6009 Hz, 32 FID's of 16 K data points, giving a digital resolution of 0.37 Hz. The recycle delay was 2 s.

3. Results and discussion

3.1. Morphology of the monolithic polymer

The morphology of the *n*-octylamine-modified monolith was studied by scanning electron microscopy as shown in Fig. 2. Like

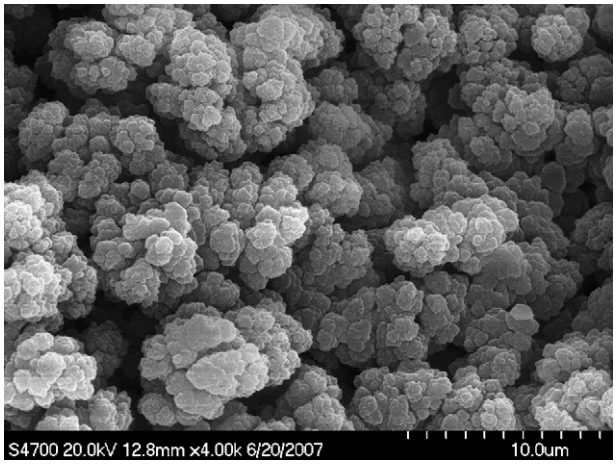

Fig. 2. Scanning electron micrograph of the monolith based on a porogenic solvent mixture of cyclohexanol–dodecanol (87:13, v/v).

in previous studies [22], the monolithic polymers were found to consist of aggregates of irregular micro-globules, with the interspaces between the aggregates forming channels allowing flow of the mobile phase. The pores in the polymer skeletons are highly interconnected, forming a network of channels. The monolith skeleton in the dry state was characterized by mercury intrusion porosimeter, and showed a specific surface area of 17.8 $m^2\,g^{-1}$, average pore size of 0.76 μm, and a total porosity of 60.8%. The large pores present in this type of stationary phase, allow the mobile phase to pass through the column with very low flow resistance.

The obtained flow rate/back pressure relationship is shown in Fig. 3. It can be seen that the monolith gave rise to a back pressure of approximate 3.7 MPa at a linear flow rate of 1445 cm h^{-1}. The linearity relationship confirms that the bed is incompressible.

3.2. Dynamic adsorption properties and column efficiency

An *n*-octylamine ligand density of 2.3 mmol g^{-1} was determined according to the procedure described in Section 2.7.

The monolith could allow chromatographic performance at very fast velocity without losing resolution for the convective mass

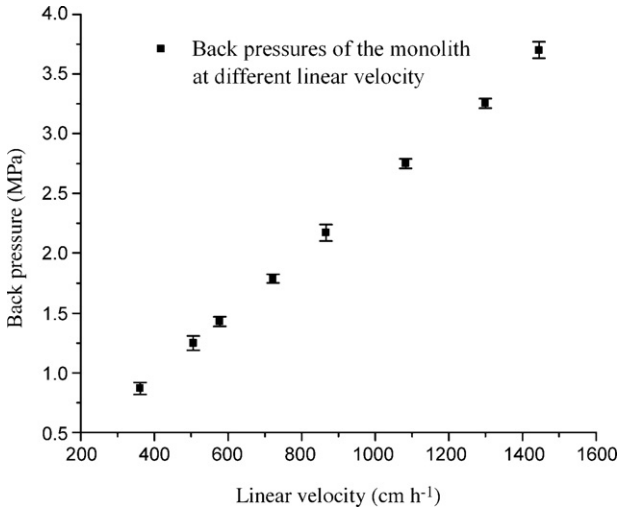

Fig. 3. Effect of liquid flow rate on the back pressure of the monolith with 1% acetic acid–water solution as the mobile phase ($n = 3$).

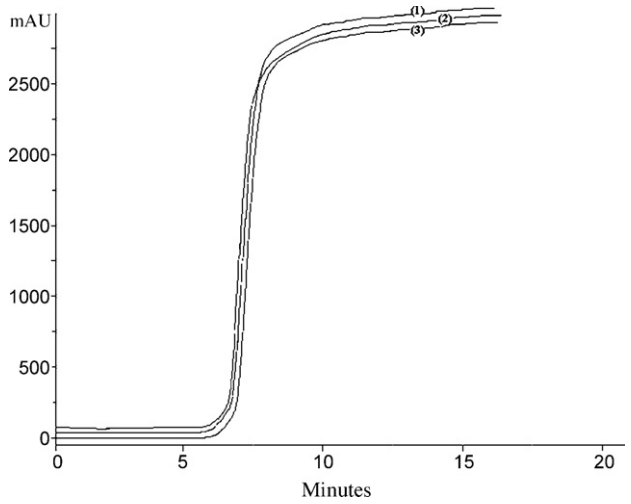

Fig. 4. The breakthrough curves of puerarin on *n*-octylamine-modified monolith at different linear velocities of (1) 361 cm h^{-1}, (2) 722 cm h^{-1} and (3) 1445 cm h^{-1}. A puerarin concentration of 1 mg mL^{-1} in 1% acetic acid–water solution was employed for the breakthrough analysis ($n = 3$, relative standard deviation (R.S.D.) for (1), (2), (3) were 3.6%, 2.7% and 4.5%, respectively).

transfer between mobile phase and liquid phase. This can be demonstrated by super-imposing breakthrough curves developed at different flow rates. As indicated in Fig. 4, the breakthrough curves provided from frontal analysis for puerarin exhibited the similar shape, demonstrating that the mass transfer of analytes in *n*-octylamine-modified monolith was almost kept the same within a range of linear velocity from 361 cm h^{-1} to 1445 cm h^{-1}. The highest binding capacity of the monolith for puerarin was approximately 15 mg g^{-1} calculated according to Eq. (1) in Section 2.6.

Isocratic elution profiles of toluene at varying flow velocities were also investigated according to Eq. (2) to determine the column efficiency of the monolith. Since isocratic elution was applied, it was appropriate to determine the height equivalent to a theoretical plate (HETP) value of the column. The raw data were fitted by PeakFit and the pseudo-HETP under isocratic elution conditions is summarized in Fig. 5. The HETP values decreased slightly and reached a plateau around 0.75 mm with increasing linear flow velocities.

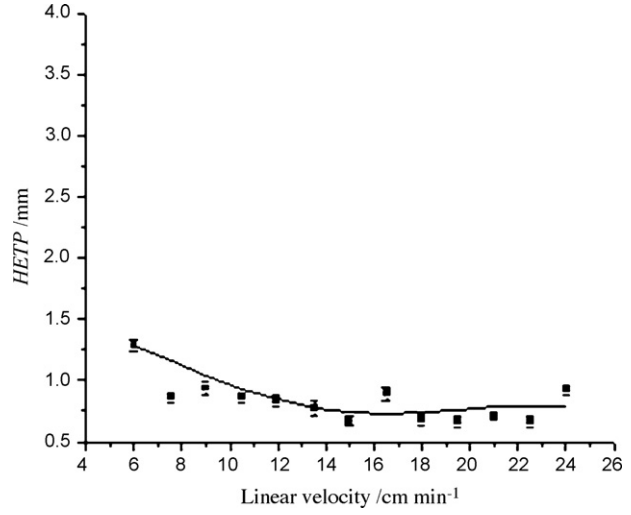

Fig. 5. The HETP of toluene on *n*-octylamine modified monolith vs. the linear flow rate of mobile phase under isocratic elution with a methanol–water (75: 25, v/v) mixture ($n = 3$).

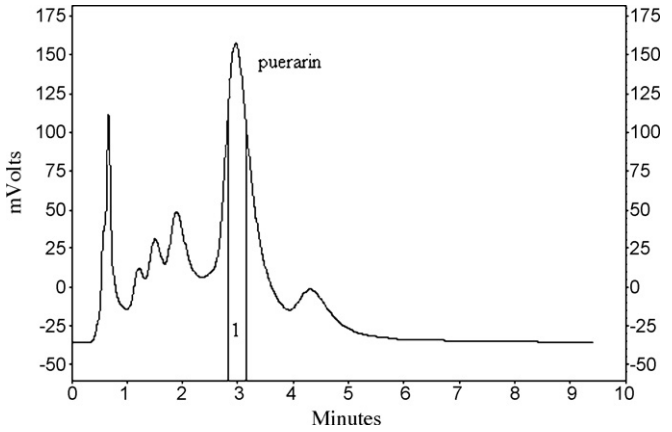

Fig. 6. Chromatogram of *Radix puerariae* flavone crude extract on a monolith prepared using a porogenic solvent mixture of cyclohexanol–dodecanol (87:13, v/v). Sample: puerariae flavone, 0.6 mg mL^{-1}; injection volume, 20 μL; flow rate, 722 cm h^{-1}; mobile phase, 1% acetic acid.

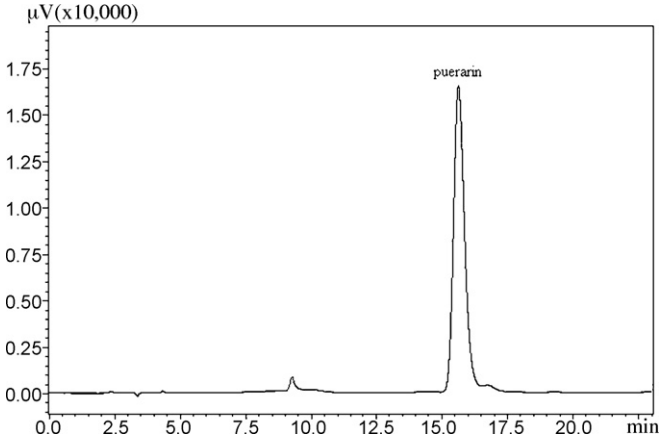

Fig. 7. HPLC analysis of puerarin fraction separated by *n*-octylamine-modified poly(methacrylate-*co*-ethylene dimethacrylate) monolith.

3.3. Fast separation and determination of puerarin

During the mobile phase screening, it became clear that most organic solvents tested did not work well for this separation,

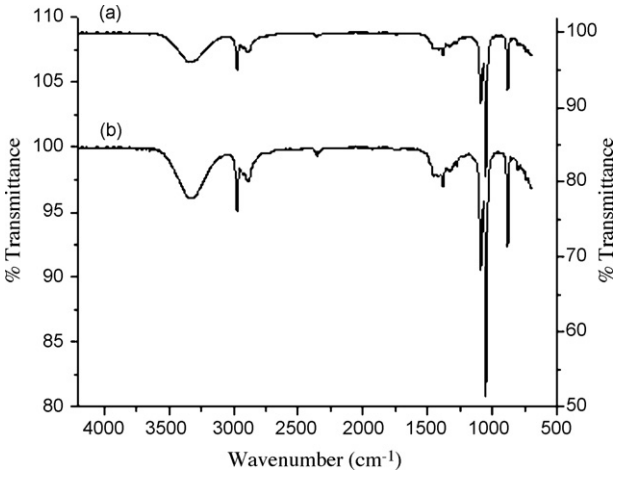

Fig. 8. FTIR-ATR spectra of (a) puerarin peak fraction collected from the separation of crude *Radix puerariae* flavone on *n*-octylamine-modified poly(methacrylate-*co*-ethylene dimethacrylate) monolith; (b) puerarin reference standard dissolved in 20% ethanol–water solution (0.2 mol L^{-1}).

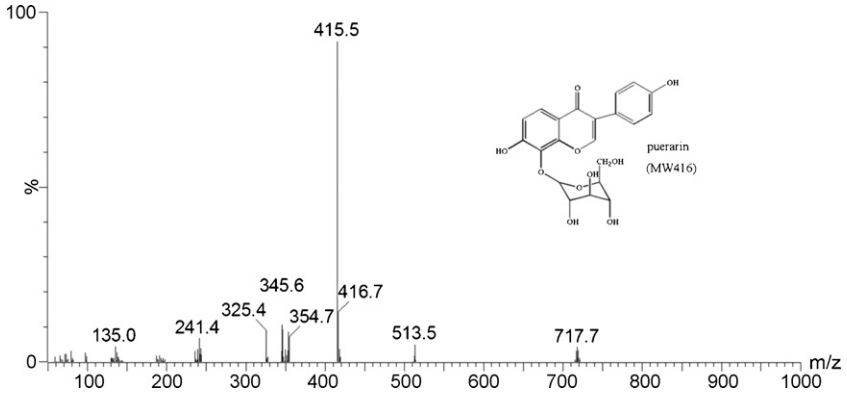

Fig. 9. Positive-ion ESI mass spectra of puerarin isolated on *n*-octylamine-modified monolith.

including those organic solvents that were tried as co-solvents with water (data not shown). Only 1% (v/v) acetic acid gave the desired result indicating that hydrophobic interaction (aromatic ring adsorption) and hydrogen bond formation played a major role for the separation. So for the separation of puerarin from *Radix puerariae* flavone crude extract, the optimized mobile phase was 1% (v/v) acetic acid in water.

Fig. 6 shows the separation of *Radix puerariae* flavone crude extract on a monolith prepared using a porogenic solvent mixture of cyclohexanol–dodecanol (87:13, v/v). Fast separation was achieved within 5 min at a velocity of 722 cm h^{-1}, showing a great potential for the separation and determination of puerarin compared with published data obtained by HPLC [14–18].

The monolith showed good functional stability after storage in 1% acetic acid solution for several weeks. Pressure drop and column efficiency did not change with the accumulation of the number of injections and cleaning cycles.

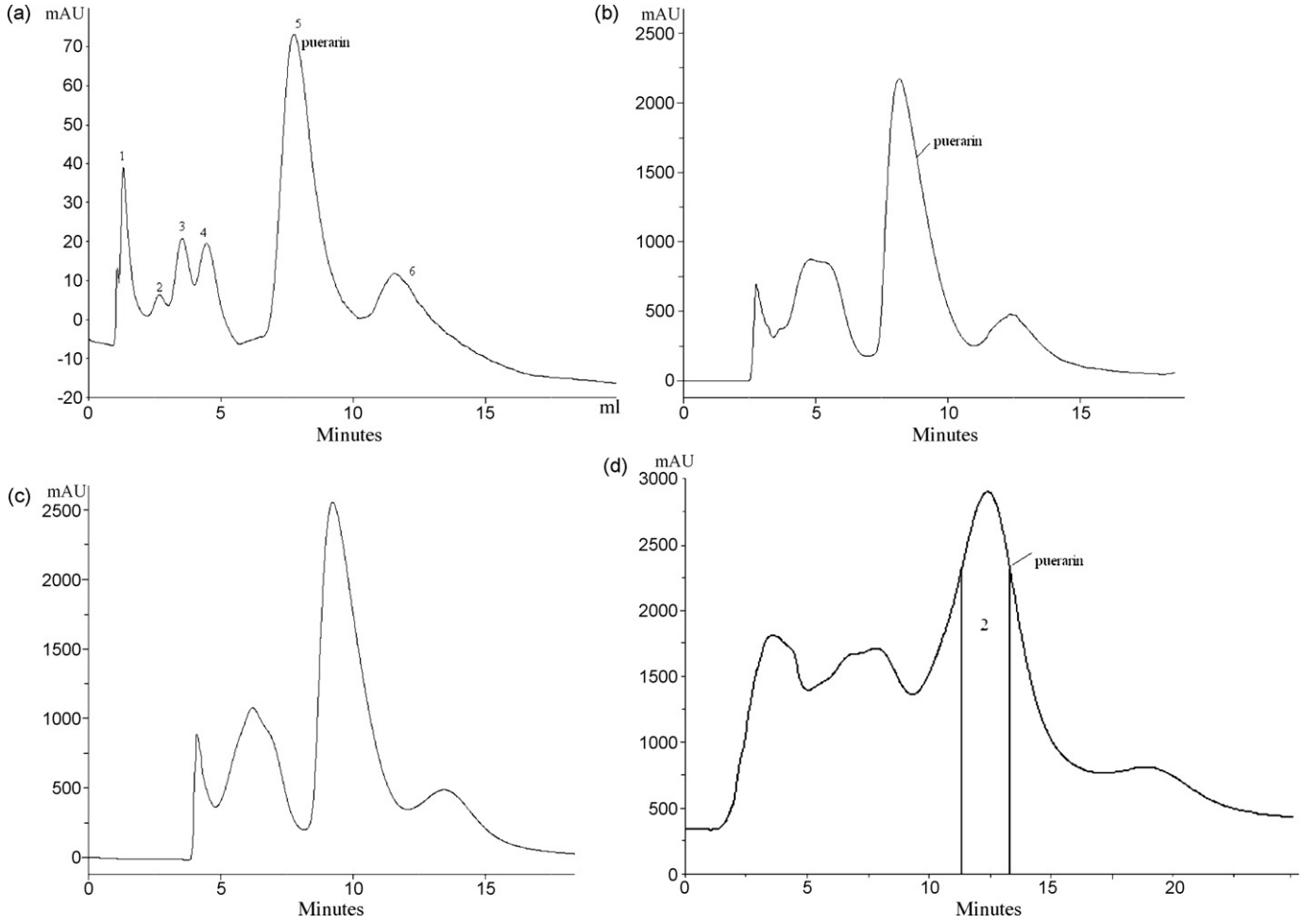

Fig. 10. Isocratic adsorption chromatograms of puerarin on *n*-octylamine-modified poly(GMA–EDMA) monolith at different sample loading (a) 1.2 mg in 2 mL, (b) 3.6 mg in 2 mL, (c) 6 mg in 2 mL and (d) 12 mg in 2 mL. Linear flow rate, 361 cm h^{-1}; mobile phase, 1% acetic acid–water solution; UV signal recorded at 250 nm.

3.4. Identification of the isolated fraction

The peak fraction in Fig. 6 marked as 1 was collected for HPLC analysis. The result is shown in Fig. 7 and is confirmed by comparison with a reference standard of puerarin. For further confirmation, the peak fraction was mixed with a solution of puerarin reference standard and reinjected resulting in one well-defined peak (data not shown). The collected peak fraction was also analyzed by FTIR spectroscopy, MS and ^{1}H NMR spectrometry under the conditions stated in Section 2.8. In Fig. 8, the puerarin peak fraction and reference standard had the same functional groups. The positive-ion ESI mass spectrum (Fig. 9) of the puerarin collected fraction showed an abundant ion peak at m/z 415.5, which was the proton adduct of puerarin (mol. wt. 416 minus 0.5). The chemical structure of target compound is *C*-glycoside which has sugar substituent bonded to a carbon of the aglycon at positions C-8. The 600 MHz ^{1}H NMR spectrum data of the isolated fraction corresponding to puerarin were as followed: ^{1}H NMR (DMSO, 600 MHz): δ 9.42 (1H, s, 4′-OH), 8.36 (1H, s, 2-H), 7.97 (1H, d, J = 8.4 Hz, 5-H), 7.32 (2H, d, J = 9.0 Hz, 2′,6′-H), 6.94 (1H, d, J = 8.4 Hz, 6-H), 6.76 (2H, d, J = 8.4 Hz, 3′,5′-H), 4.76 (1H, d, J = 7.4 Hz, glc 1″-H), 3.98 (1H, s, glc 2″-H), 3.18–3.25 (3H, m, glc 3″,4″,5″-H). These data were in agreement to those reported in the literatures [9,28,29]. Thus it is safe to conclude that a *n*-octylamine-modified monolith can rapidly screen and separate puerarin resulting in a purity of up to 97% and with a recovery of about 85%. The degree of purity and recovery is related to the extent of peak cutting after the chromatographic separation.

3.5. Sample load and purification of puerarin

Fig. 10 shows the results of different sample load to the *n*-octylamine-modified monolith. The sample loading capacity studies were performed with 1.2–12 mg *Radix puerariae* flavone powder dissolved in 2 mL 20% (v/v) ethanol–water mixture.

The data in Fig. 10 indicate that with increasing sample loading, the resolution of puerarin is adequate up to about 12 mg in 2 mL. The peak fraction marked 2 was collected and analyzed for puerarin purity and recovery by HPLC with methanol–water (27:73, v/v, 0.5% HAc) as the mobile phase. The purity of puerarin was 95%, and the recovery was 69%. Bad separation efficiency arises from further increasing of sample loading (data not shown). After scaling-up, the *n*-octylamine-modified monolith has a potential for one-step rapid preparative purification of puerarin.

4. Conclusions

One-step rapid screening, determination and purification of puerarin from *R. puerariae* (a crude extract of the root of *P. lobata*) was achieved on *n*-octylamine-modified poly(methacrylate-*co*-ethylene dimethacrylate) monoliths with 1% (v/v) acetic acid–water solution as the mobile phase. The monoliths had the potential for analytical purposes as well as preparative chromatography in the separation of natural products. Preparative separation with sample loads of up to 12 mg crude extracts *R. puerariae* (100 mm × 4.6 mm i.d. monolith) gave satisfactory results.

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