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Preparation of molecular imprinted polymers using bi-functional monomer and bi-crosslinker for solid-phase extraction of *rutin*

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ABSTRACT

Molecular imprinted polymers (MIPs) were prepared using *rutin* as the template, different reagents as the functional monomer and different reagents as the cross-linker by solution polymerization. Several parameters that would influence the performance of MIPs were investigated including the type of functional monomer (single or double) and cross-linker (single or double), and the molar ratio of the template, the functional monomer and the cross-linker. The optimum synthesis conditions of MIPs were found to be bi-monomers (acrylamide-co-2-vinyl pyridine, 3:1) and bi-crosslinker (ethylene glycol dimethacrylate-co-divinylbenzene, 3:1). The ratio of the template, the functional monomer and the cross-linker was found to be 1:6:20. MIPs synthesized under these conditions were filled into the cartridges as the adsorbents of solid-phase extraction (SPE). A competition test was conducted to authenticate the selectivity and the specificity of molecularly imprinted solid-phase extraction (MISPE) for *rutin* using the mixture solution of standard *rutin* and its structural analogs including quercetin, naringenin and kaempferol. Compared with purchased SPE including C_{18}, silica and PCX, MISPE showed better selectivity and enrichment property for *rutin* in the extracted solutions of Chinese medicinal plants than any others. The mean recoveries were 85.93% (RSD: 3.04%, $n=3$) for *Saururus chinensis* (Lour.) Bail and 88.61% (RSD: 3.36%, $n=3$) for Flos Sophorae, respectively, which indicated that the optimized *rutin*-MIPs possess the value of practical application.

1. Introduction

Molecular imprinting technique (MIT) is a new technology developed through simulating the interaction between antigen and antibody in the vivo, which forms selective sites in a polymer matrix with the memory of the template molecule due to shape recognition, hydrogen bonding and hydrophobic interactions [1,2]. In recent years, the technique has attracted wide attention of human and it has been successfully used for chromatographic separation [3], solid-phase extraction [4–6], antibody or receptor simulation [7], chemical biomimetic sensors [8–10], analog and catalytic synthesis of enzymes [11,12] and many other fields [13–18]. It has also shown good prospects applying to the separation of natural products [19].

Molecular imprinted polymer (MIP) is a receptor owning three-dimensional structure, which is synthesized based on the interaction among the template molecule that is the target analyte, the functional monomer and the cross-linking agent using the molecular imprinting technique [20]. MIP possesses a specific memory function, so that it has the performances of special affinity, high selectivity and excellent recognition ability to the template molecules [21,22]. Besides, MIPs have many other excellent characteristics, such as stable physical and chemical properties and mechanical properties, strong capability of standing high temperature and high pressure, strong resistance to acids, alkalis, high concentrations of ions and organic solvents, easy synthesis, long performance life, repeated and recycling use, and so on [23].

Although MIPs have so many advantages, they still have some shortages for the performance, for instance, unhomogeneous population of morphology and imprinting sites, slow transfer rate, poor reproducibility of binding sites, and low affinity [24], which needed to be improved further. People have explored many approaches for preparing molecular imprinted polymers to get uniform particles including suspension polymerization [25], emulsion polymerization [26], multi-step swelling and polymerization [27] and precipitation polymerization [28], all of which have improved the separation performance of MIPs to some degree. But using these methods mentioned above, it is still difficult to gain the MIPs with high affinity binding sites and their adsorption dynamics and the reproducibility of binding sites in MIPs are not obviously improved. Thus, it is of great urgency to synthesize the MIPs with high adsorption ability. To our best knowledge, the key to the manufacture of MIPs with good binding properties is the optimization of synthetic parameters [29]. Although the functional monomers (single [30,31]

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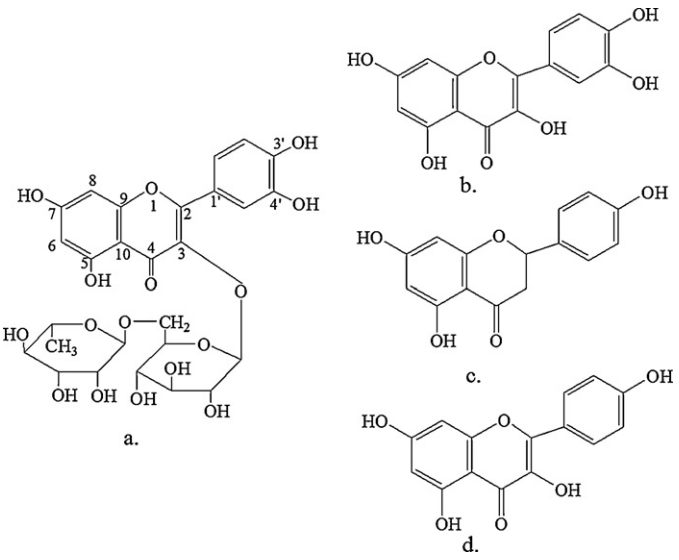

Fig. 1. The chemical structures of *rutin* (a) and its structural analogs including quercetin (b), naringenin (c) and kaempferol (d).

or double [32,33]), the cross-linkers (single [34,35] or double [36]) and porogenic agent nature [36] were investigated in many studies for many target molecules, there was not any researches combining bi-functional monomer with bi-cross-linker for preparing the MIPs with more excellent binding properties for the template.

Molecular imprinting of natural flavonoids [37,38] is of great interest for food, medicinal herb and pharmaceutical industry. *Rutin* (3′,4′,5,7-tetrahydroxyflavone-3-β-D-*rutino*side) is such a kind of natural flavonoids that has various biological activities, such as antioxidant, anti-virus, anti-tumor immune function and so on. The chemical structures of *rutin* and its structural analogs were shown in Fig. 1. As our previous experiment results, there is quite high content of *rutin* in Flos sophorae and in *Saururus chinensis* (Lour.) Bail. [39]. *Rutin* is so important for curing hypertension, angiocardiopathy, gastropathy, dermatosis, diabetes [40] that it is very significant to develop a quite effective, advisable and practicable enrichment materials or methods for its separation and purification from complex samples. *Rutin*-MIPs were synthesized using common bulk polymerization, common functional monomer and common crosslinker and studied in our previous experiment [41]. In present work, *rutin*-MIPs were synthesized using bi-functional monomer and bi-crosslinker.

Molecularly imprinted solid-phase extraction (MISPE) which uses MIPs as the adsorbents of SPE cartridges is just such a valuable and workable sample pre-treatment method exploited in the analysis of complex samples [42]. It has been widely applied in many areas, such as medicine [43,44], food [45,46], environment [47–49], commodity inspection [50], chemical industry [51] and so on. In respect of such analytical applications, it is clearly desirable to have a MISPE protocol where there is a high recovery of analyte [52], but also an approach which can purify the extracts before HPLC analysis.

Ionic liquid is a class of green solvents. In our previous study, [bmim]Br aqueous solution, one of ionic liquids was a good extraction solvent for extracting *rutin* from Chinese medicinal plants [39]. In this study, [bmim]Br aqueous solution was used as sampling solvent compared with methanol as sampling solvent.

The aim of this work is to find a much better combination of the template molecule, functional monomers and crosslinkers for solid-phase extraction of *rutin* from Chinese medicinal plants. Four different functional monomers and their combination, two kind of cross-linker reagents and their combination and different ratios of the template, functional monomer and cross-linker were performed to study the recognition properties of MIPs, respectively. And the optimal MIPs were used as the adsorbents of SPE. MISPE protocol was optimized for mixed standard solutions and extracts of real samples, the adsorption efficacies of which were also compared with those of purchased SPE (C_{18}, Silica and PCX) columns.

2. Experimental

2.1. Reagents and standards

Standard *rutin* and HPLC grade methanol used for mobile phase were bought from Sinopharm Chemical Reagent Co. (Shanghai, China). Standard naringenin and kaempferol were purchased from Chengdu Mansite Pharmacetical Co. Ltd. (Chengdu, China). Acetic acid and analytical grade methanol which were used to remove or wash the template were also obtained from Sinopharm Chemical Reagent Co. (Shanghai, China). Acrylic acid (AA, ≥99.5%) was purchased from Guangfu Fine Chemical Research Institute (Tianjin, China). Standard quercetin and methacrylic acid (MAA, ≥99%) was obtained from Sinopharm Chemical Reagent Co. (Shanghai, China). Acrylamide (AM, ≥99%) was bought from Shantou Guanghua Chemical Factory (Shantou, China). 2-vinyl pyridine (2-VP, ≥98%), divinylbenzene (DVB) and ethylene glycol dimethacrylate (EDMA, ≥98%) were got from Sigma–Aldrich (St. Louis, MO, USA). Azobisisobutyronitrile (AIBN) was purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). Tetrahydrofuran (THF, ≥99%) was obtained from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjin, China). Ultra pure water from a Purite Purification System was used to prepare mobile phase. [bmim]Br was synthesized by 1-bromobutane (≥98%) got from Sinopharm Chemical Reagent Co. (Shanghai, China) and 1-methylimidazole (≥99%) obtained from Aladdin Reagent (Shanghai, China) in our previous work.

2.2. Apparatus

HY-5A Cyclotron Oscillator (Jintan Fuhua Instrument Co. Ltd., Jiangsu, China) was applied for pre-polymerization and adsorption of MIPs or NIPs. HH-SD (DF-101S, Henan, China) was applied to synthesize MIPs. A rotary evaporator (RE52CS, Shanghai, China) was used to remove reaction solvents after the synthetic reaction of MIPs and NIPs. FT-IR spectra were registered on Spectrum One NTS (PerkinElmer, USA) equipped with a DTGS detector. A FEI Quanta 200 environmental scanning electron microscope (USA) was used to observe the morphology of MIPs. High-speed centrifugation (TG16, Shanghai, China) was employed to accelerate the phase separation process. A versatile plant pulverizer (FW-100, Beijing, China) was used to make the plant materials into powder. Pressure self-control microwave decomposition system (MDS-2002AT, Shanghai, China) was used for extraction. HPLC analysis was carried out on a Hitachi Series 2000 liquid chromatograph (Hitachi, Japan), equipped with a vacuum degasser, a quaternary pump (L2130), and a UV–vis detector system (L2420), connected to a reversed-phase column (MG C_{18} 5 μm 4.6 mm I.D. × 150 mm, Shiseido, Japan). Auto Science AS-3120 Ultrasonic Cleaner (Tianjin, China) was used for removing the template of *rutin*. 101 Electrothermal blowing dry oven bought from Beijing Yong-guangming Medical Instrument Factory (Beijing, China) was applied for drying glass apparatus and Chinese medicinal herbs.

2.3. Procedures

2.3.1. Synthesis and characterization of the molecularly imprinted polymers (MIPs)

Rutin-MIPs and nonmolecular imprinted polymers (NIPs) were prepared by a solution polymerization method. The synthesis

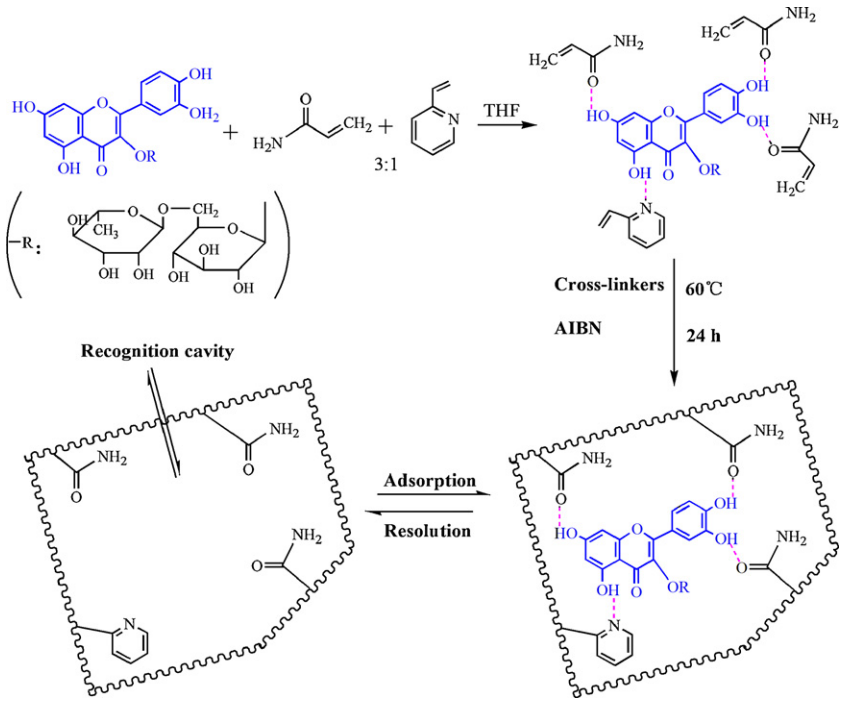

Fig. 2. Synthesis process of the optimal MIPs.

procedure of MIPs was shown in Fig. 2. For a general polymerizing procedure, 0.1 mmol template of *rutin* and 0.4 mmol functional monomer was diluted in 50 mL of tetrahydrofuran in a 100 mL glass bottle and pre-polymerized by rotating at 150 rpm for 6 h at ambient temperature. Cross-linker EDMA or/and DVB (2 mmol) and free-radical initiator AIBN (40 mg) were added into the mixture solution. After degassed in an ultrasonic bath for 20 min, the solution was spared with oxygen-free nitrogen for 15 min. Then the glass bottle was sealed and put in an oil bath which was set at the temperature 60 °C for 24 h. After synthesized, tetrahydrofuran was removed using a rotary evaporator. Then, the polymers were dried, ground into powders and sieved by a sieve with 19 μm of apertures. Then, the MIPs were treated by a ultrasonic cleaner in methanol–acetic acid 9:1 (v/v) for 1 h to remove the template *rutin*, which was repeated for 5 times. And then the MIPs were washed by methanol for 3 times to remove residual acetic acid and dried in vacuum overnight at ambient temperature. At the same time, NIPs were prepared under identical conditions without the presence of the template in the synthetic procedures and the omission of the template after synthesized.

A quantity of factors that influence the performance of MIPs were mainly investigated including the type of the functional monomer (single or double), the cross-linker (single or double) and the molar ratio of the template, the functional monomer and the cross-linker.

Morphology of the MIPs and NIPs was observed by a FEI Quanta 200 environmental scanning electron microscope (FEI, USA). Before SEM experiments, all of the dried specimen were coated with a thin layer of gold under vacuum.

FT-IR spectroscopic measurements were registered on Spectrum One NTS (PerkinElmer, USA) equipped with a DTGS detector with KBr pellet method, which was applied to characterizing the template molecule, the MIPs and the NIPs synthesized at optimum conditions with the wave numbers from 500 to 4000 cm^{-1}.

2.3.2. Static adsorption test

To evaluate the adsorption capacity of MIPs, 100 mg of MIPs or NIPs were equilibrated with 5 mL of different concentrations (0.01–0.15 mg mL^{-1}) of *rutin* dissolved in methanol in a 10 mL centrifugal tube. The mixtures were shaken for 6 h at room temperature in an oscillator and then separated centrifugally at 5000 rpm for 20 min by a high-speed centrifugation. Before measured by HPLC, the supernatants were filtrated through 0.45 μm micro-porous membranes. Every test was done for three times as parallel experiments and the experimental data was the mean of their results.

According to the difference amount of *rutin* before and after adsorption by MIPs or NIPs, the adsorption capacity (Q) which is the adsorption capacity per unit mass (mg) of the MIPs or NIPs was calculated from the following equation:

$$Q = \frac{(C_0 - C_s) \times 1000\,V}{m} \quad (\mu g\ g^{-1}) \tag{1}$$

in this equation, C_0 (mg mL^{-1}) is the initial concentration of *rutin* solution, C_s (mg mL^{-1}) is the *rutin* concentration of the supernatant solution after the adsorption, V (mL) is the volume of the initial *rutin* solution and m (g) is the mass of MIPs or NIPs.

2.3.3. Dynamic adsorption test

In order to further evaluate the adsorption capacity of MIPs, 100 mg of MIPs or NIPs were added with 5 mL of 0.05 mg mL^{-1} *rutin* methanol solution in a 10 mL centrifugal tube. The mixtures were shaken at 150 rpm for 0.5, 1, 2, 6, 12, 18 and 24 h, respectively, at room temperature in an oscillator and the following procedures were the same as the static adsorption test. Every test was done for three times as parallel experiments and the experimental data was the mean of their results.

2.3.4. Preparation of real samples

At first, all obtained samples were treated as follows: cleaned with water, dried at 60 °C, triturated by a pulverizer and passed through a stainless steel sieve. The milled samples of *S. chinensis* were degreased by Soxhlet extractor with petroleum ether until the eluates became colorless. It is no need to degrease the samples of Flos Sophorae, as they have little lipid which would not influence the detection of target analyte. After pre-treatment, all powders were stored in a closed desiccator.

The extracts of Chinese medicinal herbs were prepared using the optimum extraction method under the optimized extraction conditions as our previous work [39]. Briefly, 0.3 g of *S. chinensis* was put into a sealed vessel followed by adding 9 mL of 2.0 M [bmim]Br aqueous solution or methanol, and then the vessel was placed into the pressure self-control microwave decomposition system setting temperature at 60 °C for 12 min. While 0.2 g of Flos Sophorae was put into a sealed vessel followed by adding 6 mL of 2.0 M [bmim]Br aqueous solution, and then extracted by microwave at 70 °C for 10 min. The extract of *S. chinensis* was diluted to 25 mL. While that of Flos Sophorae was first diluted to 50 mL, 1 mL of which was then taken out and diluted to 10 mL. The extracts were all storied at 4 °C for the following experiments.

2.3.5. Molecularly imprinted solid-phase extraction (MISPE)

2.3.5.1. Preparation of SPE column. 100 mg of MIPs or NIPs synthesized under the optimal conditions were packed into an empty SPE cartridge (5 mL) which was capped at both ends with frits to prevent particles from leaking using wet-packing method with methanol. Then the SPE cartridge was preconditioned as the following sequence: 3.0 mL methanol and 3.0 mL water.

2.3.5.2. Extraction performance of the MISPE. 2 mL of the mixture solution composed of four standard solutions including *rutin* (0.05 mg mL^{-1}), naringenin (0.05 mg mL^{-1}), quercetin (0.05 mg mL^{-1}) and kaempferol (0.05 mg mL^{-1}) or the extracts of *S. chinensis* or Flos Sophorae were loaded onto the polymer, and then according to our previous work [41], washed with 2× 1 mL methanol. Subsequently, the target analyte was eluted with 2× 1 mL of a methanol/acetic acid (v/v = 9:1) mixture. And all solutions flown out of the columns were collected. 4 mL of methanol was immediately percolated through the cartridge to keep the polymer in this solvent before next analysis. Last, they were all analysed by HPLC–UV as specified in the section of HPLC analysis. All MISPE experiments were performed using same cartridges in this work.

2.3.5.3. Comparison with C_{18}, silica and PCX cartridges. In order to demonstrate that MISPE column has the ability of selectivity for the target compound, C_{18}, Silica and PCX cartridges were preconditioned as the following sequence: 3.0 mL methanol, 3.0 mL water. 2 mL of the extracts of *S. chinensis* or Flos Sophorae in [bmim]Br aqueous solution were loaded onto the three kind of purchased cartridges, and washed with 2× 1 mL methanol. Subsequently, the target analyte was eluted with 2× 1 mL of a methanol/acetic acid (v/v = 9:1) mixture. All solutions flown out of the cartridges were collected. Then they were all analyzed by HPLC–UV as specified in the section of HPLC analysis.

2.3.6. HPLC analysis

HPLC analysis was carried out on a Hitachi Series 2000 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump (L2130) and a UV–vis detector system (L2420), connected to a reversed-phase column (MG C_{18} 5 μm 4.6 mm I.D. × 150 mm, Shiseido, Japan).

The mobile phases were all composed of methanol (solvent A) and 0.05% (v/v) phosphoric acid aqueous solution (solvent B) for the following two kind of HPLC conditions.

HPLC 1: For the analysis of the mixture standard solutions, other conditions were as follows: The mobile phase of isocratic elution was composed of 60%A and 40%B with 0.6 mL min^{-1} of the flow rate; column temperature was ambient and injection volume was 10 μL; The UV detection wavelength applied here was 254 nm.

HPLC 2: For the analysis of real samples including *S. chinensis* and Flos Sophorae, other conditions were as follows: the mobile phase of isocratic elution was composed of 43%A and 57%B with 0.6 mL min^{-1} of the flow rate; Column temperature was ambient and injection volume was 10 μL; the UV detection wavelength applied here was 356 nm.

3. Results and discussion

3.1. Influence of polymerization conditions on recognition properties of MIPs

3.1.1. Functional monomer

As we all know, functional monomer plays an important role in the adsorption performance of MIPs to the template molecule. In this work, four different functional monomers including MAA, AA, AM and 2-VP and pair combinations between them at the different molar ratio were used to prepare MIPs and related NIPs for the rebinding study, and other synthesized conditions were as follows: the amount of *rutin* was 0.1 g, the molar ratio of the template molecule, functional monomer and crosslinker was 1:4:20, and the rebinding experiments of MIP and NIP synthesized with different functional monomers were shown in Table 1.

According to the structure of *rutin*, there are multiple hydroxyl groups that have certain acidities, while the oxygen atom in the pyrone ring contains lone pair electron which makes the *rutin* molecule performance weak alkaline. In theory, choosing basic functional monomers would be propitious to form stable host–guest complexes between the template and functional monomers. Thus, alkaline monomers including AM and 2-VP were better than acidic ones (MAA and AA), which accorded with the results of single functional monomer in Table 1. Moreover, the rebinding results obtained by AM-MIP and 2-VP-MIP were almost the same according to Table 1. Pair combination among them was also investigated. Based on the results of the rebinding experiments, it is strongly suggested that the combination of AM and 2-VP (3:1) should be opted as functional monomers for the preparation of *rutin*-MIPs, which would be related to the space structures of *rutin*, AM and 2-VP.

In the hydroxyl groups of non-glycosyl in rutin, due to the electron-withdrawing effect of the adjacent carbonyl, 5-OH is very easy to form hydrogen bonding with other groups owning surplus electronics. In addition, due to the molecular structures of 2-VP and rutin, 5-OH is more easy to form hydrogen bonds with 2-VP. Vice versa, the nitrogen element of pyridine ring which originates from 2-VP in the MIPs could easily form hydrogen bonds with 5-OH of rutin. While other three hydroxyl groups may easily form hydrogen bonds with AM. Thus, this combination of functional monomers can easily form complex compounds with the template, and further form MIPs. And this combination of functional monomers made the MIPs which had been removed the template molecule adsorb rutin easily from mixed solutions.

3.1.2. Cross-linker

The nature of cross-linker has impact on the morphology and the binding performance of MIPs. In this study, different cross-linkers and their combination were researched, and other synthesized conditions were as follows: the amount of *rutin* was 0.1 g, the molar ratio of the template molecule, functional monomer and crosslinker was 1:4:20. The rebinding results generated by different MIPs or NIPs prepared by different composition of cross-linkers were shown in Table 2. From the results we can see that the combination of EDMA and DVB with a molar ratio of 15:5 was best to synthesize MIPs for the adsorption of *rutin*.

3.1.3. The molar ratio of the template, functional monomer and cross-linker

In order to obtain the best molar ratio of the template, functional monomer and cross-linker for the adsorption of *rutin*, four different ratios were investigated and other conditions were as

Table 1
Rebinding experiments of MIP and NIP synthesized with different functional monomers ($n = 3$).

	Single functional monomers				Double functional monomers											
	AM	2-VP	AA	MAA	AM + 2-VP			AM + AA			AM + MAA			AA + MAA		
					1:3	2:2	3:1	1:3	2:2	3:1	1:3	2:2	3:1	1:3	2:2	3:1
C_0 (mg mL^{-1})	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
MIP																
C_S (mg mL^{-1})	0.0069	0.0094	0.0142	0.0125	0.0078	0.0065	0.0057	0.0118	0.0085	0.0071	0.0102	0.0087	0.0068	0.0096	0.0104	0.0135
Q (μg g^{-1})	862	812	716	750	844	870	886	764	830	858	796	826	864	808	792	730
NIP																
C_S (mg mL^{-1})	0.0424	0.0428	0.0430	0.0427	0.0431	0.0429	0.0428	0.0432	0.0430	0.0427	0.0428	0.0431	0.0429	0.0426	0.0428	0.0430
Q^a (μg g^{-1})	152	144	140	146	138	142	144	136	140	146	144	138	142	148	144	140
I^b	5.67	5.64	5.11	5.14	6.12	6.13	6.15	5.61	5.93	5.88	5.53	5.99	6.08	5.46	5.5	5.21

[a] $Q = (C_0 - C_s) \times 1000V/m$ (μg g^{-1}), m is the mass of MIP or NIP (0.25 g), V is the volume of the initial *rutin* solution (5 mL), C_0 (mg mL^{-1}) and C_s (mg mL^{-1}) are concentrations before and after adsorbed by MIP or NIP, respectively.
[b] $I = Q_{MIP}/Q_{NIP}$, I means the imprinting efficiency of MIP.

follows: the amount of *rutin* was 0.1 g, the combination of functional monomers was AM and 2-VP (molar ratio, 3:1) and the combination of cross-linkers was EDMA and DVB (molar ratio, 15:5). In the preparation of MIPs, the molar ratio of template molecule, functional monomer and cross-linker also affects the performance of MIPs. In general, for the ratio of the template molecule and functional monomer, increasing the proportion of the functional monomer could make the pre-assembled interaction between the template and functional monomer more complete. But it was not that the larger the proportion of the functional monomer is, the better the molar ratio is. On one hand, the greatly excessive of the functional monomer may cause non-selective binding sites increasing which is generated by the residues of non-assembled functional monomers. On the other hand, if the concentration of the functional monomer is overlarge, it may lead to the association by themselves which will result in the binding sites decreased.

Generally, the amount of cross-linker would impact the inflexibility of the polymers directly, and play an important part in the stabilization of the rebinding sites. If the amount of cross-linker used was too little and was not enough cross-linking degree, the synthesized MIP could not keep stable cavities and consequently led to the low capacity of the recognition. While, high concentration of the cross-linker would make the polymers the inflexibility of excessive larger and make it difficult to achieve the binding equilibrium between the MIP and the molecule of *rutin*. As a result, the imprinting effect was poor instead.

The rebinding experiments of MIPs and NIPs synthesized with different molar ratio of the template, functional monomer and cross-linker were shown in Table 3. From the results of Table 3, the optimal molar ratio of the template molecule, functional monomer and cross-linker was 1:6:20. The MIPs synthesized under this molar ratio had not only good inflexibility, but also excellent imprinting effect for *rutin*.

3.2. Characterization of the MIPs

The morphology of *rutin*-MIPs and NIPs prepared under the optimum conditions was observed by the scanning electron microscope (SEM) and the photos were shown in Fig. 3(A and B). From the two photos (A and B), we can know that the surface of MIPs was rougher than that of NIPs and there were many cavities in MIPs but few in NIPs, which indicated that MIPs had many imprinting cavities that were favorable for rebinding template molecules while NIPs had not. Thereby, the MIPs had stronger adsorption capacity to the template than the NIPs did.

The FT-IR spectra of *rutin*-MIPs before and after removing the template and NIPs which were all prepared under the optimal conditions were shown in Figs. S1(A–C) and S2(A and B). The absorption frequency of O—H stretching vibration (3412 cm^{-1}) from Fig. S1(A) was so strong which told us that there are so many hydroxyl groups in *rutin* molecules that they could form lots of hydrogen bonds with functional monomers. Comparing Fig. S1(B) and (C) with (A), the absorption frequencies of N—H stretching vibration (3464 cm^{-1} and 3180 cm^{-1}) indicated that AM was linked into the prepared polymers. In addition, the characteristic signals of MIPs removed *rutin* molecules including the absorption frequencies of C=O stretching vibration (1727 cm^{-1}), C=N stretching vibration (1638 cm^{-1}) and C—N stretching vibration (1150 cm^{-1}) are weaker than those of NIPs, which is probably due to the obstruction by *rutin* during the polymerization between functional monomers and cross-linkers when *rutin* was present, so that there were fewer functional monomers attached to the MIPs than to the NIPs.

Supplementary material related to this article found, in the online version, at doi:10.1016/j.talanta.2012.02.008.

Compared Fig. S2(A) with S2(B), it can be known that the absorption of C=O stretching vibration (1727 cm^{-1}), C=N stretching vibration (1638 cm^{-1}) and C—N stretching vibration (1150 cm^{-1}) were strengthened after the template molecules were removed

Table 2
Rebinding experiments of MIP and NIP synthesized with different cross-linkers ($n = 3$).

Cross-linkers		C_0 (mg mL^{-1})	C_s (mg mL^{-1})		Q^a (μg g^{-1})		I^b
			MIP	NIP	MIP	NIP	
EDMA + DVB	20:0	0.05	0.0041	0.0428	918	144	6.37
	15:5	0.05	0.0027	0.0431	946	138	6.86
	10:10	0.05	0.0058	0.0429	884	142	6.23
	5:15	0.05	0.0067	0.0426	866	148	5.85
	0:20	0.05	0.0073	0.0422	854	156	5.47

[a] $Q = (C_0 - C_s) \times 1000V/m$, m is the mass of MIP or NIP (0.25 g), V is the volume of the initial *rutin* solution (5 mL), C_0 (mg mL^{-1}) and C_s (mg mL^{-1}) are concentrations before and after adsorbed by MIP or NIP, respectively.
[b] $I = Q_{MIP}/Q_{NIP}$, I means the imprinting efficiency of MIP.

Table 3
Rebinding experiments of MIP and NIP synthesized with different molar ratio of the template, functional monomer and cross-linker ($n = 3$).

Molar ratio	C_0 (mg mL^{-1})	C_S (mg mL^{-1})		Q[a] (μg g^{-1})		I[b]
		MIP	NIP	MIP	NIP	
1:4:20	0.05	0.0052	0.0428	896	144	6.22
1:4:40	0.05	0.0065	0.0428	870	144	6.04
1:6:20	0.05	0.0028	0.0432	944	136	6.94
1:6:40	0.05	0.0109	0.0431	782	138	5.67

[a] $Q = (C_0 - C_s) \times 1000V/m$, m is the mass of MIP or NIP (0.25 g), V is the volume of the initial *rutin* solution (5 mL), C_0 (mg mL^{-1}) and C_s (mg mL^{-1}) are concentrations before and after adsorbed by MIP or NIP, respectively.
[b] $I = Q_{MIP}/Q_{NIP}$, I means the imprinting efficiency of MIP.

from the MIPs. That is because the carbonyl of AM and nitrogen atom of 2-VP were both released on account of the removal of *rutin*.

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The results of FT-IR spectra confirmed that there were functional groups in the MIPs which would interact with the template molecule.

3.3. Rebinding experiments of the optimal MIPs

3.3.1. Rebinding isotherm

The rebinding isotherm of *rutin* on MIPs and NIPs prepared under the optimal conditions in methanol was shown in Fig. 4(A), from which both specific and non-specific adsorption of *rutin* increased firstly with higher concentration of *rutin* solution, and then almost saturated after the concentration of *rutin* solution reached 0.05 mg mL^{-1}.

In order to further evaluate the binding properties of the MIPs, the obtained data were plotted by the Scatchard formula, which was expressed as follows:

$$\frac{Q}{C} = (Q_{max} - Q) \times K_d \quad (2)$$

Q (mg g^{-1}) was the equilibrium adsorption capacity of rutin bound to MIPs, C (mg mL^{-1}) was the equilibrium concentration of rutin in the solution, Q_{max} (mg g^{-1}) was the apparent maximum binding capacity of binding sites and K_d (mg mL^{-1}) was the dissociation constant. Scatchard curves were obtained by taking Q/C as the x-coordinate and Q as the y-coordinate. The results were shown in Fig. 4(B) and (C).

In Fig. 4(B), it is obvious that the plot of MIP contains two distinct linear sections formulated by $y = -494.2x + 484.6$ ($r^2 = 0.976$) and $y = -53.2x + 66.7$ ($r^2 = 0.897$), which suggests the presence of two distinct groups with different specific binding properties in the optimal MIPs. From the slope and the intercept of the Scatchard plots, K_d and Q_{max} for the higher affinity binding sites were calculated to 0.002 mg mL^{-1} and 0.969 mg g^{-1}, while K_d and Q_{max} for the lower affinity binding sites were 0.019 mg mL^{-1} and 1.267 mg g^{-1}. Contrary to that of the MIPs, the Scatchard plot of the NIPs shown in Fig. 4(C) exists of only one linear part, which indicate that the NIPs consisted of only one homogeneous binding site. And the K_d and Q_{max} were 0.014 mg mL^{-1} and 0.221 mg g^{-1}, which were calculated from the equation of $y = -72.1x + 15.8$ ($r^2 = 0.982$).

3.3.2. Rebinding dynamic

The rebinding dynamic of *rutin* on MIPs and NIPs synthesized with the optimum conditions in methanol was also investigated and the results were shown in Fig. 5. It plots that as time went on, the *rutin* rebound in MIPs and NIPs were increased at first, and it reached saturation for 6 h and 2 h, respectively. After that time, the rebound *rutin* would not increase.

3.3.3. Selective adsorption capability

Distribution coefficient (K_d), selectivity coefficient (k) and relative selectivity coefficient (k') were significant indicators for evaluating the adsorption performance of polymers. Distribution coefficient (K_d), which reflects migration and separation capacity of the solute in two phases, is the ratio of the concentrations or amounts of the compound in each of the two phases. Selectivity coefficient (k), which indicates the difference of two compounds adsorbed by the polymers, is defined as the ratio of the K_d values of two competitive compounds. Relative selectivity coefficient (k') is defined as the ratio of the k values of two competitive compounds adsorbed by different polymers. The rebinding constants of MIPs and NIPs were investigated to further evaluate selective recognition properties of MIPs and NIPs. The adsorption and selectivity

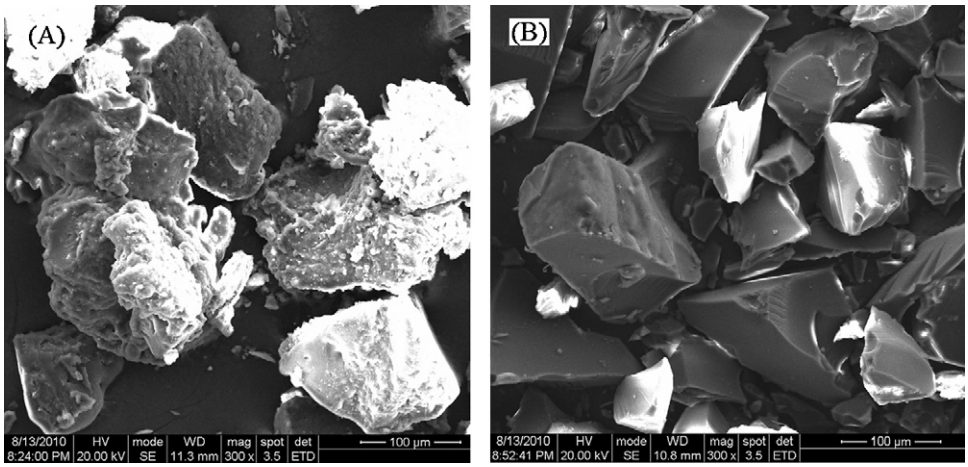

Fig. 3. Scanning electron micrographs of: (A) the optimal MIPs which were removed the template *rutin*; (B) NIPs prepared under identical conditions of the optimal MIPs with the absence of the template *rutin*.

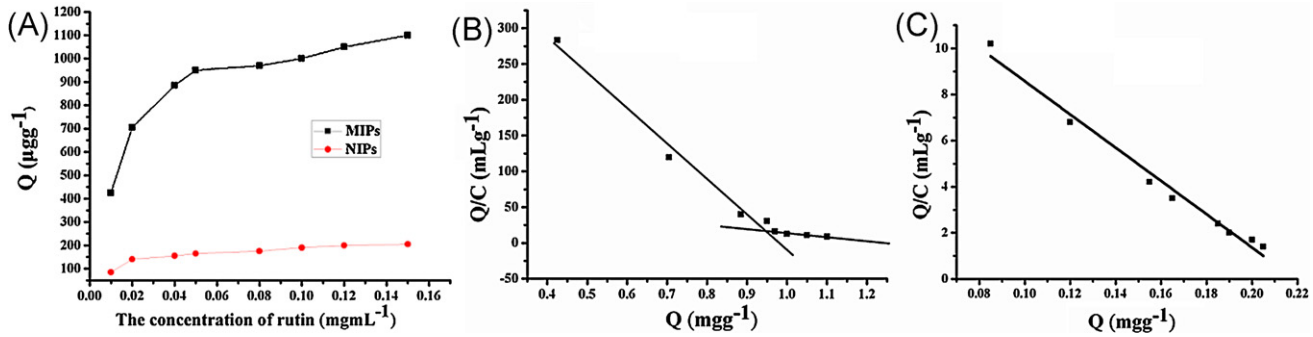

Fig. 4. The rebinding isotherm curves (A) of *rutin* on the optimal MIPs and NIPs, and Scatchard plots of MIPs (B) and NIPs (C).

Table 4
Adsorption and selectivity capability of MIP and NIP prepared under the optimal conditions for rutin and its structural analogs ($n = 3$).

No.	Compounds	C_0 (mg mL^{-1})		C_s (mg mL^{-1})		I	Q_{MIP} (μg g^{-1})	Q_{NIP} (μg g^{-1})	K_d [a]		k [b]		k' [c]
		MIP	NIP	MIP	NIP				MIP	NIP	MIP	NIP	
1	*Rutin*	0.05		0.031	0.047	950	150	6.3	0.613	0.064	/	/	/
2	Quercetin			0.042	0.047	400	150	2.7	0.190	0.064	3.23	1.00	3.23
3	Naringenin			0.045	0.048	250	100	2.5	0.111	0.042	5.52	1.53	3.60
4	Kaempferol			0.044	0.046	300	200	1.5	0.136	0.087	4.49	0.73	6.12

$Q = (C_0 - C_s) \times 1000V/m$, C_0 represents the concentration of the compound in the initial solution and C_s is the average of concentrations in the three final solutions; V is the volume of the solution (5 mL); m is the weight of the adsorbent (0.1 g); Q is the adsorption capability of the polymers); C_0 (mg mL^{-1}) and C_s (mg mL^{-1}) are concentrations before and after adsorbed by MIP or NIP, respectively. $I = Q_{MIP}/Q_{NIP}$, I means the imprinting efficiency of MIP.

[a] K_d, distribution coefficient: $K_d = (C_0 - C_s)/C_s$.

[b] k, selectivity coefficient, $k_i = K_{d1}/K_{di}$ ($i = 2, 3, 4$).

[c] k', relative selectivity coefficient, $k' = k_{MIP}/k_{NIP}$.

capability of MIP and NIP prepared under the optimal conditions for rutin and its structural analogs were shown in Table 4. It could be obviously found out from Table 4 that K_d of *rutin* is larger than that of others, which indicated that the separation capacity of MIPs to *rutin* is stronger than to the others. But K_d of the four substances were almost the same in the adsorption by NIPs. Moreover, the values of the relative selectivity coefficient (k') indicated that the selectivity coefficient of MIPs to *rutin* was bigger than that of the corresponding NIPs, which make sense for selective rebinding *rutin* from complex solutions depending upon the imprinted effect in the MIPs.

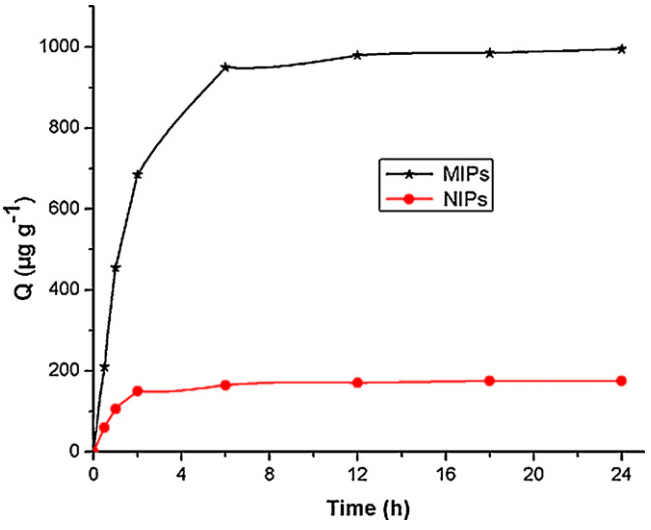

Fig. 5. The rebinding dynamic curves of *rutin* on the optimal MIPs and NIPs.

3.4. MISPE study

3.4.1. Selection of the sampling solvent

Methanol and [bmim]Br aqueous solution were studied as the sampling solvents for real samples on MISPE columns. The chromatograms of *S. chinensis* dealt with different sampling solvents were shown in Fig. S3. And the results exhibited in Fig. S3 were carried out by HPLC. There were fewer impurity compounds in the extracts of *S. chinensis* using [bmim]Br aqueous solution than that using methanol as extraction solvent. After sampling, fewer other compounds were adsorbed by the adsorbent of MISPE using [bmim]Br aqueous solution than methanol as the sampling solvent, which indicated that [bmim]Br aqueous solution was more beneficial to the adsorption of *rutin* on MISPE better than methanol when used as the sampling solvent for *S. chinensis*. Thereby, the extracts of real samples were all prepared by [bmim]Br aqueous solution.

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3.4.2. Binding selectivity of MISPE

Some structural analogs (quercetin, naringenin and kaempferol) of *rutin* were employed for the testing of the selectivity of MIPs when *rutin* was used as the template on SPE column. The rebinding situations of *rutin* and its structural analogs on the MISPE were shown in Table 5 and the chromatograms of the mixture solution composed of *rutin* and its structural analogs dealt with MISPE and NISPE were shown in Fig. S4. Obviously, the adsorption of *rutin* and its structural analogs in MIPs are all higher than that in NIPs from Table 5, indicating the higher specific bindings for the tested compounds in MIPs, which are due to the high similarity of the molecular structures between *rutin* and its structural analogs. In Table 5, the adsorption capability (Q_1) for the four substances was more or less the same, while the elution ratio for *rutin* was much

Table 5
Specific recognition properties of MISPE ($n = 3$) for rutin and its structural analogs.

Compounds	C_0 (mg mL^{-1})	C_1 (mg mL^{-1})	C_2 (mg mL^{-1})	C_3 (mg mL^{-1})	Q_1[a] (μg g^{-1})	Q_2[b] (μg g^{-1})	Q_3[c] (μg g^{-1})	Elution ratio (%)
Rutin	0.0500	0.0042	0.0034	0.0421	916	68	842	91.9
Quercetin	0.0500	0.0118	0.0353	0.0026	764	706	52	6.8
Naringenin	0.0500	0.0161	0.0304	0.0032	678	608	64	9.4
Kaempferol	0.0500	0.0140	0.0338	0.0019	720	676	38	5.3

Where C_0, C_1, C_2, C_3 represent the initial concentration, the concentration after adsorbed by the cartridge, the concentration after washed by methanol and the concentration after eluted by methanol–acetic acid of the compound, respectively.

[a] Q_1, adsorption capability, $Q_1 = 1000 \times (C_0 - C_1) \times$ (loading solution volume [mL]/adsorbent mass [g]).

[b] Q_2, rinsing capability, $Q_2 = 1000 \times C_2 \times$ (rinsed solution volume [mL]/adsorbent mass [g]).

[c] Q_3, elution capability, $Q_3 = 1000 \times C_3 \times$ (elution solution volume [mL]/adsorbent mass [g]).

Table 6
Analytical performance results.

Samples	Analytes	Linearity range (mg mL^{-1})	Quantification equations	Correlation coefficients (r^2)	Detection limits (μg L^{-1})
Mixture standard solutions	*Rutin*	0.001–0.1	$Y = 8{,}536{,}890X + 12417.463$	0.99934	8.3
	Quercetin	0.001–0.1	$Y = 12{,}130{,}600X + 5905.388$	0.99929	7.1
	Naringenin	0.001–0.1	$Y = 4{,}401{,}070X + 595.343$	0.99912	10.3
	Kaempferol	0.001–0.1	$Y = 16{,}906{,}900X + 3870.179$	0.99907	6.2
S. chinensis Flos Sophorae	*Rutin*	0.005 –0.1	$Y = 6441820X + 28398.690$	0.99920	6.7

higher than those for its structural analogs. That is because the spatial diameters of quercetin, naringenin and kaempferol are smaller than that of *rutin* since there is more than one glycosyl group in the *rutin* molecule, so quercetin, naringenin and kaempferol can easily enter into the cavities of the MIPs and form hydrogen bonds. At the same time, quercetin, naringenin and kaempferol are easily washed so that they would not influence the analysis of *rutin*.

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In addition, the comparison with our previous study was shown in Table 5, from which we can obtain that the adsorption capacity of the polymers synthesized using bi-functional monomer and bi-crosslinker in this study was much better than that of our previous study. Because our previous work was groping the synthesis of molecular imprinting polymer for the first time, and just focused on the optimization of the conditions of adsorption and desorption between MIP and the template molecule. While, this study focused on the optimization of synthetic conditions for *rutin*-MIPs. Obviously the specific recognition properties of MIPs as the adsorbent of SPE in this work was better than that of MIPs synthesized in our previous study.

3.4.3. Real sample analyses by MISPE

To certify the suitability, practicability and potential application of this sample pre-treatment method of MISPE in real samples, the extracts of *S. chinensis* and Flos Sophorae were processed using MISPE and all collection liquids after MISPE were detected by HPLC. The chromatograms of the extracts of *S. chinensis* and Flos Sophorae dealt with MISPE column were shown in Fig. 6(A) and (A′), respectively. It indicated that MISPE could enrich *rutin* selectively and efficiently by comparing Fig. 6A(I) with A(IV) and Fig. 6A′(I) with A′(IV), respectively.

The contents of *rutin* in the extracts of *S. chinensis* and Flos Sophorae were 4.763 and 169.27 mg g^{-1}, respectively. After MISPE, the contents of it were 4.519 and 158.60 mg g^{-1}, respectively. Although there were some loss of *rutin* by the rinsing procedure, all of other compounds in the complex samples were rinsed, which was beneficial for the detection of the target compound *rutin*.

3.4.4. Comparison with C_{18}, silica and PCX cartridges

To further validate the selectivity of MISPE, C_{18}, silica and PCX cartridges were purchased and experienced for the adsorption of rutin in the extracts of *S. chinensis* and Flos Sophorae in this study. And the results were exhibited in Fig. 6. The adsorption of silica cartridge was worse than other two purchased cartridges from the comparison of Fig. 6D(II) with B(II) and C(II) and Fig. 6D′(II) with B′(II) and C′(II). From the left chromatograms of Fig. 6, comparing the carves of (III) of *S. chinensis* after dealt by the four SPE, respectively, rutin was almost washed out by methanol using C_{18}, silica and PCX SPE columns, while it was not washed by methanol using MISPE column for the specific adsorption of MIPs to it. In addition, from all carves (IV) of *S. chinensis*, we could know that only the collected solution after eluted by methanol–acetic acid (v/v, 9:1) dealt with MISPE column was determined the present of rutin, which further demonstrated that only MISPE column has the specific adsorption to rutin and could separate the analyte of rutin from other interferents. The same results and conclusion of Flos Sophorae were obtained from the right chromatograms of Fig. 6.

In a word, from Fig. 6, it was obvious that MISPE column had specific recognition for *rutin* from *S. chinensis* and Flos Sophorae, which was outstanding in the analyses of real samples, while the purchased cartridges did not have this special function.

Table 7
Recoveries of *rutin* from S (*S. chinensis*) and F (Flos Sophorae) after dealt with MISPE ($n = 3$).

Herbs	No.	Sample (g)	Content (mg g^{-1})	Added (mg g^{-1})	Found (mg g^{-1})	Recovery (%)	Mean recovery (%)	RSD (%)
S	1	0.3002	4.67	5.0	8.373	86.59		
	2	0.3001	4.59	5.0	7.965	83.06	85.93	3.04
	3	0.3001	4.62	5.0	8.481	88.16		
F	1	0.2000	165.25	50.0	192.83	89.58		
	2	0.2002	166.48	50.0	196.94	90.97	88.61	3.36
	3	0.2001	164.82	50.0	183.16	85.26		

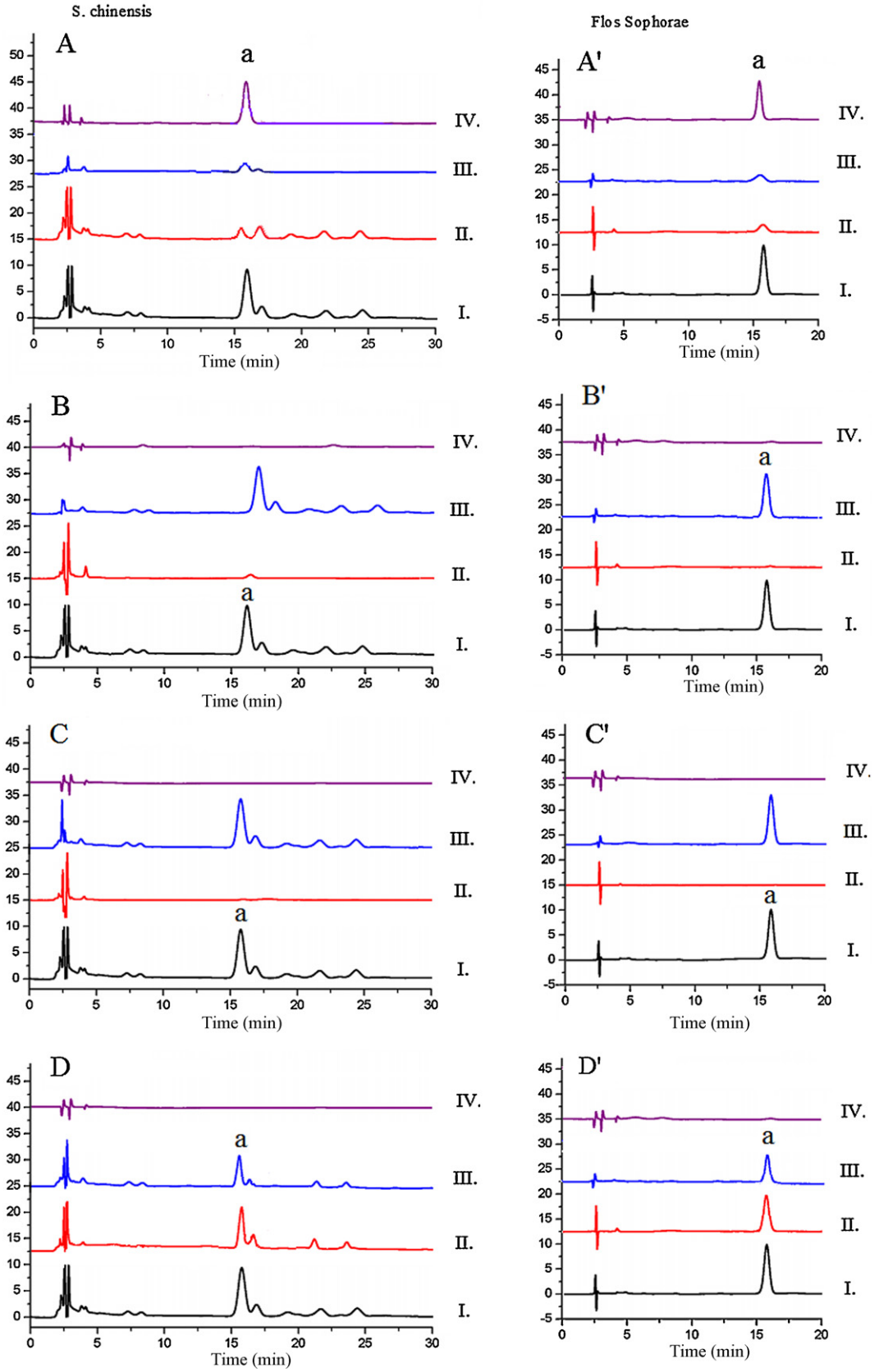

Fig. 6. The chromatograms of the extracts of *S. chinensis* and Flos Sophorae dealt with different columns: MISPE (A and A′), PCX (B and B′), C_{18} (C and C′) and silica (D and D′); I. before MISPE, II. after sampled, III. after washed by methanol, IV. after eluted by methanol–acetic acid (v/v, 9:1).

3.5. Method validation

In order to validate the method for the quantification of *rutin* in Chinese medicinal plants, the linearity, and the limit of detection (LOD) were calculated using the mixed standard solutions and the extracts of *S. chinensis* and Flos Sophorae. The Analytical performance results were shown in Table 6.

Using the optimum MISPE procedures, the recovery experiment was conducted and the results were shown in Table 7. 0.3 g powder of *S. chinensis* was added in 1.5 mg of *rutin* and 0.2 g of Flos Sophorae powder was added in 10 mg of *rutin*, respectively. And then they were dealt using the same procedure of "preparation of real samples" in the experiment part. At last, 2 mL diluted extracts of *S. chinensis* and Flos Sophorae was dealt with MISPE and all the collection solutions were analyzed by HPLC. And the mean recoveries of rutin in *S. chinensis* and Flos Sophorae were 85.93% and 88.61% with 3.04% and 3.36% of RSD ($n = 3$), respectively, which indicated that this pre-treatment approach was satisfactory.

4. Conclusion

By optimizing the polymerization conditions, such as functional monomer nature (single or double), cross-linker nature (single or double) and the molar ratio of the template, the functional monomer and the cross-linker, the optimal *rutin*-MIPs were synthesized by a free-radical solution polymerization using double functional monomers (AM-co-2-VP, 3:1), double cross-linkers (EDMA-co-DVB, 3:1) and tetrahydrofuran as the porogenic solvent. The optimal *rutin*-MIPs were characterized by SEM and FT-IR which depicted that there were many recognition cavities in them. The optimal *rutin*-MIPs as the adsorbents of SPE columns showed highly specific recognition for *rutin* from the mixed solution of *rutin* and its structural analogs compared to NIPs synthesized under the same conditions of *rutin*-MIPs as the adsorbents of SPE columns. In addition, the *rutin*-MIPs synthesized under optimum conditions used as the adsorbents of SPE columns were applied for the selective adsorption of *rutin* from the extracts of Chinese medicinal plants including *S. chinensis* and Flos sophorae, and the mean recoveries of them were 85.93% and 88.61% with 3.04% and 3.36% of RSD, respectively. Compared with those conventional adsorbents (C_{18}, silica and PCX) of SPE, the selective adsorption of MISPE was the best, which indicated that the optimal *rutin*-MIPs used as the adsorbents of SPE columns possess the value of practical application.

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