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Quercetin molecularly imprinted polymers: Preparation, recognition characteristics and properties as sorbent for solid-phase extraction

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ABSTRACT

Molecular imprinted polymers (MIPs) were prepared through thermal polymerization by using quercetin as the template molecule, acrylamide (AA) as the functional monomer and ethylene glycol dimethacrylate (EDMA) as the cross-linker in the porogen of tetrahydrofuran (THF). The synthesized MIPs were identified by both Fourier transform infrared (FTIR) and scanning electron microscope (SEM). Systematic investigations of the influences of key synthetic conditions, including functional monomers, porogens and cross-linkers, on the recognition properties of the MIPs were conducted. Scatchard analysis revealed that the homogeneous binding sites were formed in the polymers. Besides quercetin, two structurally similar compounds of rutin and catechol were employed for molecular recognition specificity tests of MIPs. It was observed that the MIPs exhibited the highest selective rebinding to quercetin. Accordingly, the MIPs were used as a solid-phase extraction (SPE) sorbent for the extraction and enrichment of quercetin in cacumen platycladi samples, followed by HPLC-UV analysis. The application of MIPs with high affinity and excellent stereo-selectivity toward quercetin in SPE might offer a novel method for the enrichment and determination of flavonoid compounds in the natural products.

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1. Introduction

Molecular imprinting technique (MIT) is an attractive synthetic approach to mimic natural molecular recognition [1–4], which exhibits a high affinity and selectivity toward a particular target compound (template molecule). The MIT utilizes template molecules to assemble their own recognition sites by interacting with complementary functional groups of appropriate monomers. The technique is schematically depicted in Fig. 1. In the process, the selected template molecules (quercetin) are first allowed to establish binding interactions with functional monomers (acrylamide, AA), in which the polymerizable functional monomers are pre-arranged around a template molecule in porogenic solvent. The resulting complexes are then copolymerized with an excess of cross-linkers (ethylene glycol dimethacrylate, EDMA) in the presence of a free radical initiator (2,2'-Azobisisobutyronitrile, AIBN) by thermal polymerization. Subsequent removal of the template molecules creates specific binding sites with the precise spatial arrangement of the functional groups in the polymer network together with the shape [5]. Thus,

reversible re-binding and high selective recognition of the target molecules are realized. Since it was invented by Wuff and Sarhan [6], MIT has been exploited extensively in many fields including chiral resolution [7], biosensor [8], biochemical separation [9–11], antibody simulation [12], enzyme catalysis simulation [13], etc. So, molecularly imprinted polymers (MIPs) display excellent application prospects. In particular, MIPs used as adsorbents of solid-phase extraction (SPE) are being widely involved in the concentration and determination of drug molecules, and the extraction of active components from natural products [2,14–16].

Quercetin (3,3',4,5,7-penta-hydroxy flavone) is the most active compound in flavone family, widely occurring in leaves, fruits, and flowers of many plants [17]. Quercetin has now become the topic of increasing interest based on its various bioactivities such as inoxidability, antiviral property, antitumor and adjusting the immune function [18], as well as its chemical applications, e.g., quercetin is immobilized on silica gel and used as a solid-phase reagent for tin (IV) determination [19]. The determination of quercetin is typically performed with HPLC-UV [15,20,21], besides electrogenerated chemiluminescence (ECL) [22] and capillary electrophoresis (CE) [23]. Because of the existence of quercetins in nature at low concentrations, the complexity of the sample matrices and the structural similarity to other flavonols, selective sample preparation methods are necessary

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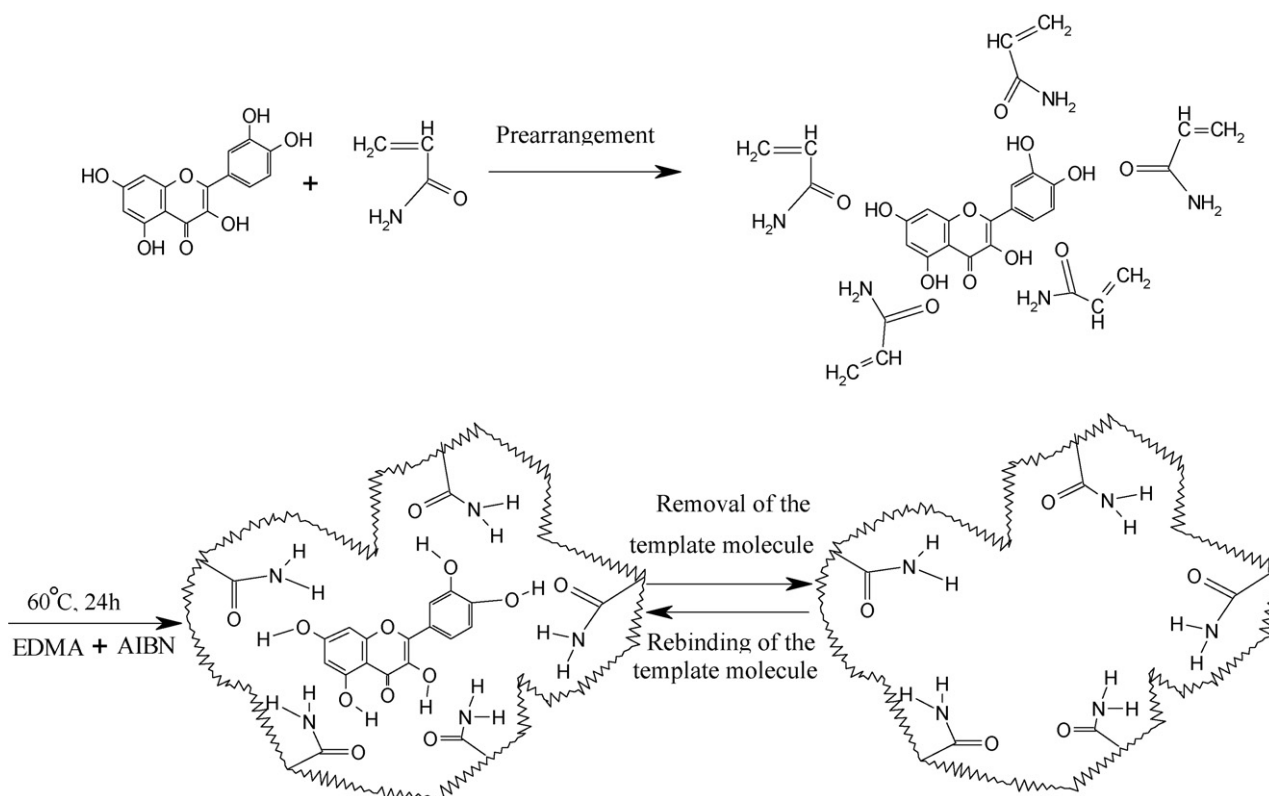


Fig. 1. Schematic representation of MIPs for quercetin.

prior to chromatographic analysis. Preconcentration and/or separation of the compounds are frequently conducted by using SPE [20,21], in which new selective materials employing molecular recognition mechanisms might guarantee selective analyte separation from complex real world matrices. This mechanism can improve significantly the method specificity and sensitivity. Related works on quercetin determination and separation by using MIT have been reported [5,24,25]. Weiss et al. reported that MIPs for quercetin was prepared by using quercetin as template molecule, 4-vinylpyridine as functional monomer and EDMA as cross-linker [5]. The prepared MIP showed excellent performance on quercetin separation. Yan et al. also prepared effective MIPs for quercetin by using AA as a functional monomer [24]. He and Deng reported the effectiveness of MIPs prepared by using different functional monomers including methacrylic acid (MAA), AA and 2-vinylpyridine, and found the properties of MIPs for quercetin were varying in different functional monomers [25].

In this work, the feasibility of preparing quercetin imprinted polymers and the utilization of the selective membranes as sorbent for SPE were demonstrated. The influences of porogenic solvents, functional monomers and cross-linkers on the adsorption specificity for quercetin of the MIPs were investigated and the stabilization energy calculated by computational quantum chemical analysis (CQCA) was adopted to support the analysis. The synthesized MIPs were characterized by Fourier transform infrared (FTIR) and scanning electron microscope (SEM). HPLC-UV and Scatchard method were used to evaluate the adsorption properties and recognition mechanism of the MIPs. Structurally similar compounds including rutin and catechol were utilized for verifying the molecular selectivity and characterizing the recognition capability of the MIPs. The MIP-SPE was testified to be applicable for the preconcentration and determination of cacumen platycladi samples.

2. Experimental

2.1. Chemicals

Quercetin, rutin and catechol were purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd. Fig. 2 shows their chem-

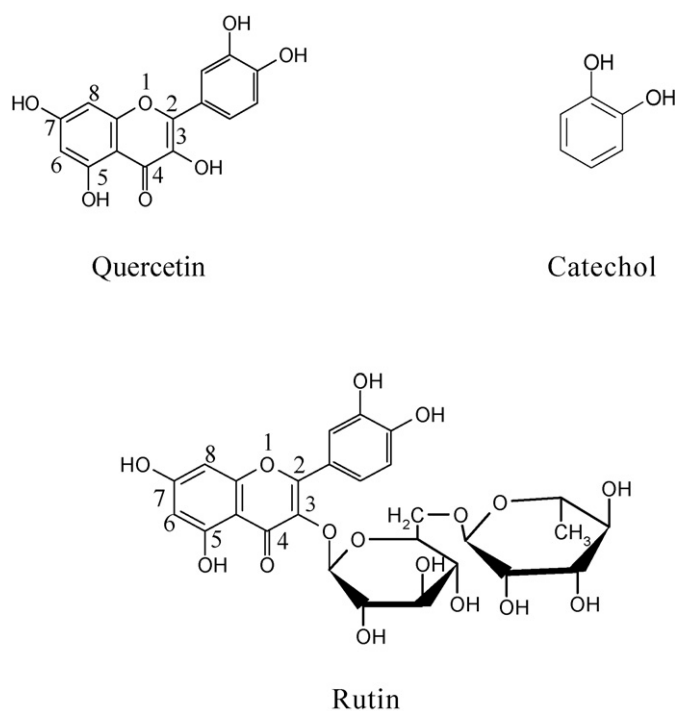


Fig. 2. Chemical structures of quercetin, catechol and rutin.

ical structures. AA was supplied from Tianjin Chemical Research Institute. EDMA, divinylbenzene (DVB), pentaerythritol triacrylate (PTA) and N,N'-methylenebisacrylamide (BisAA) were purchased from Sigma–Aldrich (Taufkirchen, Germany). AIBN was obtained from Shanghai Chemical Reagent Company. HPLC-grade tetrahydrofuran (THF), acetone, acetonitrile (ACN), 1,4-dioxane, methanol (MeOH) and anhydrous acetic acid were all from Merck (Darmstadt, Germany).

2.2. Preparation of the MIPs and NIPs

The MIPs were prepared with a slight modification according to the published procedures [24]. Briefly, 0.13 g (0.40 mmol) of template molecules (quercetins) and 0.20 g (2.8 mmol) of functional monomers (AA) were dissolved in 5 mL different porogenic solvents including 1,4-dioxane, THF, acetone and ACN in four corked test tubes, respectively. After oscillating for 30 min, 3.5 mL (18.5 mmol) of cross-linkers (EDMA) and 0.01 g initiators (AIBN) were added. The solutions were degassed in a sonicating bath for 15 min and filled with nitrogen for 15 min and sealed. The test tubes were immersed into isothermal water bath and the mixtures were thermally polymerized at 60 °C for 24 h. The resultant polymers were then grinded and sieved through a 105 μm sieve. The polymer particles were extracted with a mixture of MeOH/acetic acid (9:1, v/v) in Soxhlex extractor for 24 h to remove the template molecules (quercetins) and then were rinsed with MeOH. After removing acetic acid, the MIPs were dried at 60 °C under vacuum for 24 h and stored in a desiccator for further use. As references, the non-imprinted polymers (NIPs) in the absence of template molecules were prepared and treated by using the above same method. The compositions of MIPs (M1–M4) and NIPs (N1–N4) are shown in Table 1.

2.3. FTIR measurements

FTIR spectroscopic measurements were performed on model Avatar 370 FTIR Spectrometer (Thermo Nicolet Corporation, USA) with KBr pellet method. The wave numbers of FTIR measurement range were controlled from 500 to 4000 cm^{-1} , and collected at one data point per 2 cm^{-1} with sample scanning for 32 times.

2.4. Adsorption determination of MIPs and NIPs for quercetins

The adsorption experiments were carried out to confirm the rebinding performance and the subsequent Scatchard analysis of the polymers as follows.

The MIPs and NIPs (Table 1) of 100 mg were added into eight 50 mL conical flasks, respectively, and were mixed with 25 mL of solution containing a known concentration of 20 $\mu\text{g mL}^{-1}$ quercetins in MeOH. Then, these mixtures were shaken at 20 °C by using SHA-B Water Bath Isothermal Shaker (Jiangsu Jintan Medical Instrument Factory) and centrifuged using a high-speed centrifuge. 20 μL of the supernatant was taken out for HPLC analysis to measure the free concentration of quercetin and determine

the adsorption quantity of quercetin in different time intervals.

In the other hand, the M2 and N2 (Table 1) of 100 mg, respectively, were added into 50 mL conical flasks, and were mixed with 25 mL of a known initial concentration of quercetin (0.01–0.1 $\mu\text{mol mL}^{-1}$) in MeOH solution. The mixtures were oscillated by using a SHA-B Water Bath Isothermal Shaker at 20 °C for 24 h, and then were centrifuged for 10 min. The concentration of quercetin in the solution was analyzed using HPLC–UV. And thus, the saturated extents of adsorption of MIPs and NIPs for quercetins were obtained. The selectivity of MIPs was investigated by using quercetin adsorption on the MIPs and NIPs.

2.5. Computational quantum chemical analysis (CQCA)

Molecular simulation as a promising technology of computational chemistry is also a useful tool in the studies of molecular imprinting technology, so the stabilization energy by solvent was often used to evaluate the interaction strength of molecular template with solvent, monomer or cross-linker with solvent. In this study according to calculation method of Gaussian 98, B3LYP/6-31+G**, the stabilization energy of quercetin and AA with different solvents was obtained through calculating the interaction energy between gas and solvent. The simulation of solvent effect is based on the Onsager self-consistent reaction field (SCRF) technique [26], which supposes that the solute molecule is embedded into a spherical cavity with radius a_0 surrounded, and the solvent is characterized by a given dielectric constant. The gas-phase geometry of quercetin and functional monomer or cross-linker is used as its initial guess to start the full optimizations at the same level of B3LYP/6-31+G** with different dielectric constants of porogenic solvents. The radius a_0 automatically resorted from quantum mechanical procedures. After stationary points were located, vibrational frequencies were calculated in order to ascertain that each structure was characterized to be the stable structure (no imaginary frequencies). All calculations were carried out with the Gaussian 98 program on a Pentium IV computer using the default convergence criteria. The stabilization energy of solvents is calculated as $E_{\text{solvation}} = E_{\text{in solvent}} - E_{\text{in gas}}$. The $E_{\text{solvation}}$ is the energy stabilized by a solvent, namely the relative energy difference of a complex in the solvent and that in a gas phase.

2.6. MIP–SPE procedures

A PTFE frit (10 μm in porosity size) was placed on the bottom of an empty polypropylene SPE cartridge (5 mL in total volume and 8 mm in diameter) purchased from Agilent Technologies (USA), which was employed as the MIP–SPE column, packed with 500 mg of quercetin–MIPs in 5 mL MeOH solution using a wet packing method. Then another frit was placed on the top of the cartridge. Prior to loading the sample, the cartridge was washed with a mixture of MeOH/acetic acid (9:1, v/v) until quercetin could not be detected by the HPLC analysis in the filtrate. Then the cartridge was conditioned with 10 mL of MeOH.

Considering the solubility of quercetin and its structurally similar compounds (rutin or catechol), a mixture containing quercetin and its analogues in MeOH was prepared. Then, 2–4 mL of the different concentration mixtures were loaded onto the SPE cartridge at 1 mL min^{-1} . Subsequently, the cartridge was consecutively rinsed with 3 mL of MeOH and 3 mL of the mixed solution of MeOH/acetic acid (v/v, 9:1). The collected column-solution, rinsed solution and eluates were evaporated to dryness under nitrogen and the residues were redissolved in 1 mL of MeOH, respectively, for HPLC–UV analysis.

In this work, the enrichment factor (EF) was introduced and it was defined as the ratio of the analyte concentration in the con-

Table 1
Compositions of MIPs and NIPs.

Polymers	Quercetin (g)	AA (g)	EDMA (mL)	AIBN (g)	Porogenic solvents
M1	0.13	0.2	3.5	0.01	1,4-Dioxane
M2	0.13	0.2	3.5	0.01	THF
M3	0.13	0.2	3.5	0.01	Acetone
M4	0.13	0.2	3.5	0.01	ACN
N1	–	0.2	3.5	0.01	1,4-Dioxane
N2	–	0.2	3.5	0.01	THF
N3	–	0.2	3.5	0.01	Acetone
N4	–	0.2	3.5	0.01	ACN

centrated phase (C_{con} , $\mu\text{mol mL}^{-1}$) and the initial concentration of analyte within the sample (C_0 , $\mu\text{mol mL}^{-1}$):

$$EF = \frac{C_{con}}{C_0}$$

The C_{con} was obtained from the HPLC analysis for the analyte in eluates after being redissolved in 1 mL of MeOH.

2.7. Preparation of cacumen platycladi

The preparation of cacumen platycladi was consulted to previous work [27,28]. In brief, 1.0 g of cacumen platycladi was placed into a 100 mL of conical flask, followed by 50 mL of 50% MeOH, stewing for 6 h; subsequently the flask was marinated into a water bath at 80 °C for 6 h. The resultant leaching liquors were filtrated with a 0.45 μm membrane filter, and then evaporated to dryness under low fire. The extracts were dissolved in doubly distilled deionized (D.D.I.) water and extracted by 25 mL of saturated ethyl acetate for three times. The three aliquots of extraction solutions were pooled and dried with anhydrous sodium sulfate, and then they were transferred to a 250 mL of round flask for reduced pressure distillation. The residues were dissolved with anhydrous MeOH for use.

2.8. HPLC-UV analysis

The HPLC-UV analysis was performed using EasySepTM-1010 HPLC (Unimicro Technologies Co., Ltd., Shanghai, China) with a 1010 LC liquid delivery pump and a 1010 UV ultraviolet/visible variable wavelength detector with a spectral range from 190 to 700 nm. Column was C-18 column (2.1 mm \times 250 mm). The analytical procedure was conducted according to reported work [29,30] with necessary modification. Briefly, the column was rinsed with a mobile phase until a stable baseline was obtained, which consisted of ACN and 0.1% (w/v) H_3PO_4 aqueous solution (36:64, v/v). The detection was carried out with wavelength at 365 nm. Column oven temperature was controlled at 25 °C. The flow-rate was maintained at 0.5 mL min^{-1} and 20 μL of sample was injected.

3. Results and discussion

3.1. Preparation and characterization of the quercetin-MIPs

Quercetin was dissolved in THF at the usual imprinting concentration, and AA was selected as the functional monomer because it was favorable for hydrogen-bonding interaction in the solvent [24]. Thus, a stable donor–receptor complex between template and functional monomer is formed in the imprinting process shown in Fig. 1. The existence of such a complex leads to the formation of well-defined specific binding sites in the MIPs. Then a thermal polymerization was followed, in which AIBN was used as free radical initiator and its half-life at 60 °C was around 10 h and proper for polymerization. The cross-linker, EDMA, formed a “frozen” spatial structure. The quercetin molecule contains five hydroxyl groups and one carbonyl group, which can form hydrogen bonds with functional groups such as hydroxyl, amino and carbonyl groups. From the structure of quercetin shown in Fig. 2, both the γ -OH (No. 3) and ε -OH (no. 5) of the quercetin molecule can form strong intra-molecular hydrogen bonds with the δ -carbonyl group (no. 4), and therefore the inter-molecular interaction between the template and monomer is weakened. The remained three hydroxyl groups as main imprinting functional groups supply strong inter-molecular hydrogen bonds with the polymer and control the precise imprinting sites. So, there are less five apparent selective binding sites in the quercetin molecule. After removal of the template molecule,

the specific imprinting sites will be maintained. These sites will selectively adsorb quercetin molecules.

To further ensure that the quercetin-MIPs had been made, we performed additional FTIR analysis (see Fig. 3). Fig. 3a shows the FTIR spectrum of quercetin. A strong and broad stretching vibration absorbance peak of hydroxyl groups was found at 3299 cm^{-1} , suggesting there are a few hydroxyl groups in the quercetin molecule, which might form the intra- and/or inter-molecular hydrogen bonds. A stretching vibration peak of carbonyl group was found at 1656 cm^{-1} [31], moving toward the low wave number region in the conjugated system. In addition, stretching vibration peaks of cyclobenzene skeleton in the quercetin molecule was found at 1615, 1512 and 1432 cm^{-1} [32]. Fig. 3b shows the FTIR spectrum of AA (monomer). The main absorption bands of AA situating around 3356 (and 3188), 1673, and 1613 cm^{-1} were assigned to the following vibrations: N–H stretching, being characteristic absorption peaks of primary acylamide, C=O stretching and C=C stretching, respectively.

For MIPs, shown in Fig. 3c, the peak at 1732 cm^{-1} was attributed to C=O stretching vibration absorbance, because the MIPs were synthesized by the polymerization of EDMA and AA, and the cross-linked polymers must carry the repeated cross-linking units of EDMA. At 1637 cm^{-1} , the weak absorbance peak of C=C suggests that most AA were cross-linked and only a few ones were remained. The broad peak at 3441 cm^{-1} was assigned to the asymmetric stretching vibration of N–H in acylamide. Moreover, the stretching vibration peak of methyl or methylene was found at 2990 cm^{-1} , and C–O stretching vibration of asymmetric ester was found at 1154.37 cm^{-1} . For NIPs shown in Fig. 3d, the characteristic signals of FTIR were almost the same, except for the intensity of the

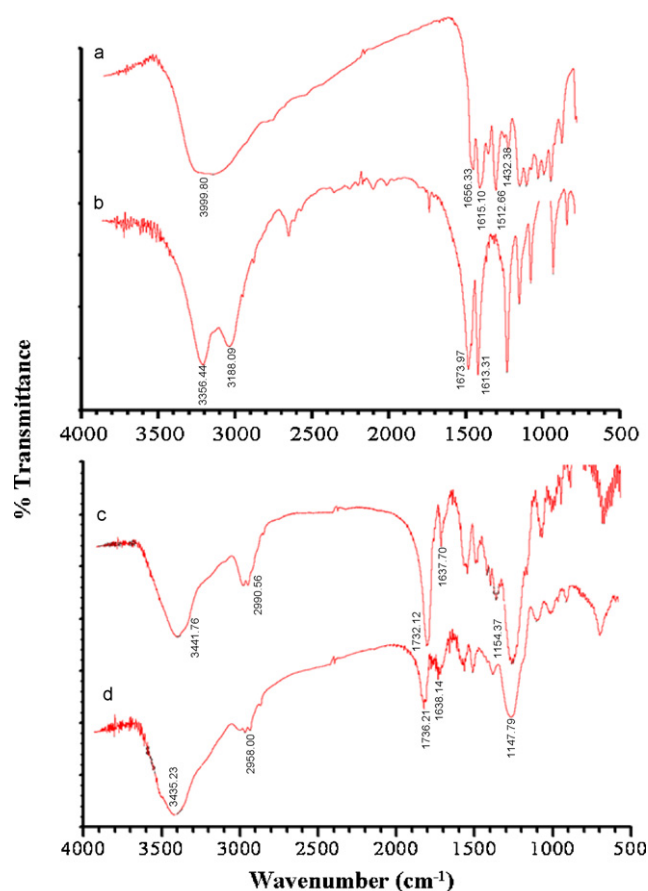


Fig. 3. FTIR spectra of quercetin (a), AA (b), MIPs removal of the template molecule (c), and NIPs (d).

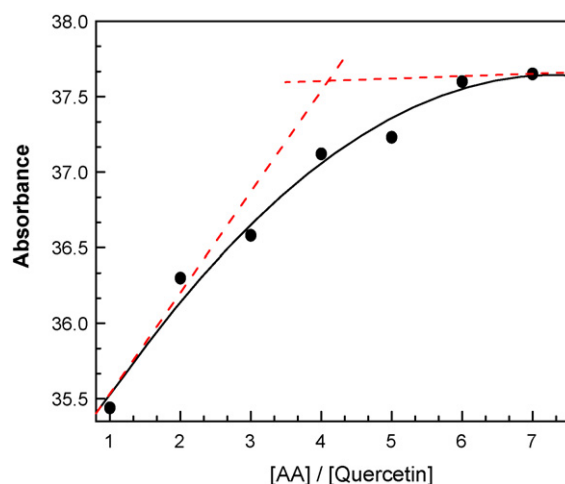


Fig. 4. Absorbance curve in different ratios of [AA]/[Quercetin].

stretching vibration peak of C=O (1736 cm^{-1}), distinctly lower than that for MIPs. The possible reason was that the template molecules which were assembled with monomer via the hydrogen-bonded interaction in preparing MIPs, helpful for the co-polymerization of monomer and cross-linker, and thus stable imprinting cavities was formed by ordered distribution of functional groups containing carbonyl group. The result of FTIR confirmed that obviously there were functional groups in the MIPs which would interact with the template molecule, namely acylamide group in the quercetin-MIPs.

3.2. Influence of polymerization conditions on adsorption properties of MIPs

3.2.1. Functional monomers

For non-covalent MIPs, the complementary inter-molecular reactions between the template and the functional monomer are crucial for the subsequent affinity and selectivity of the MIPs [33]. It is therefore important to search for a suitable functional monomer that can strongly interact with the template and form specific “donor–receptor” or “antibody–antigen” complexes prior to polymerization. Due to the higher specific binding but the lower non-specific adsorption of the resultant MIPs, AA was thus selected as the functional monomer for preparing the MIPs [24].

The molar ratio of functional monomer to template is important in terms of its interaction strength, which will have an effect on the number and property of MIP recognition sites [34]. The intensity and ratio range of the MIPs were estimated by absorption measurement. As shown in Fig. 4, the equilibrium absorbance of the complex increases with the ratio of AA to template (M/T) increasing, suggesting that the quercetin molecules form super-molecular structure with AA via non-covalent inter-cavity mode in the THF solution. At 7:1, the plot begins to level off, indicating that almost all the template molecules had been bound with the functional monomers. The coordination number was expected 4 (Fig. 4); from the quercetin structure (Fig. 2), it is clear that the number of the apparent selective binding sites was less 5. The molar ratio should be higher than 4:1. At lower content monomer, it is difficult to form stable imprinting sites. An excess of functional monomers can cause more non-selective adsorption sites with random distribution in the MIPs and result in self-association of the monomers, which will reduce the selective adsorption sites and increase the adsorption mass transfer resistance. So, the optimum ratio of M/T at 7:1 was chosen for preparing the maximum specific-selective MIPs in the following investigations.

3.2.2. Porogens

It is essential for the adsorption properties of MIPs that the functional monomer and imprinting molecules can form stable complexes. Strong interaction between imprinting molecules and functional monomers produces excellent stability of the resultant complex, which therefore results in high recognition specificity of the MIPs. On the other hand, weak interaction often causes inefficiency of MIPs. So, it is important to investigate the influence of porogens. The porogens generally act as dispersion media and pore forming agents in the polymerization process. The solvent is very important for providing the synthesis environment of MIPs. The polarity of solvents will affect the interaction between template molecule and functional monomer, and thereby the adsorption properties of MIPs.

As shown in Table 2, the interactions between solvent and quercetin or AA increased with increasing polarity of solvents, while the specific recognition capacity of the MIPs decreased. Higher polar solvents competed more efficiently with template molecules for binding the recognition sites of the functional monomers and thus weakened the interaction between the latter two compounds. This leads to forming fewer imprinting sites in MIPs and reduces its adsorption effectiveness for quercetin. In addition, quercetin was not easily dissolved and its floccule was easily observed in ACN, likely causing phase separation. This resulted in unfavorable MIPs with inhomogeneous pores and reduced internal surface area. For less polar solvent having less interaction with either template molecule or functional monomer, in which the interaction of template molecule and functional monomer is strong, however, the formed MIPs are easily precipitated due to less solubility in the less polar solvent. Only when the solvent has medium polarity, does it have proper interactions with quercetin or AA. And for the medium polar THF, it also offers better dissolving capacity for the quercetin-template and the initial polymers, which maintains the pore dimension, specific surface area and mass transfer rate, and therefore facilitates synthesizing the MIPs with homogeneous pores and high specific surface area. This assists to form uniform imprinting sites in MIPs. So, THF was selected as the porogenic solvent to produce better MIPs.

Meanwhile, the surface morphologies of MIPs prepared in different solvents were studied by using SEM (Fig. 5). Although all have rough surfaces, the MIPs from THF and acetone seemed more dense and homogenous with more and larger-dimension pores (Fig. 5a and b) than that from ACN and 1,4-dioxane (Fig. 5c and d). It can be evaluated that the surface of the MIPs in THF exhibits bigger dimension of the pore and bigger volume rough structure than that of the MIPs in acetone. The uniform and more open structure is obviously favorable for the embedding of the template molecules and mass transfer.

3.2.3. Cross-linkers

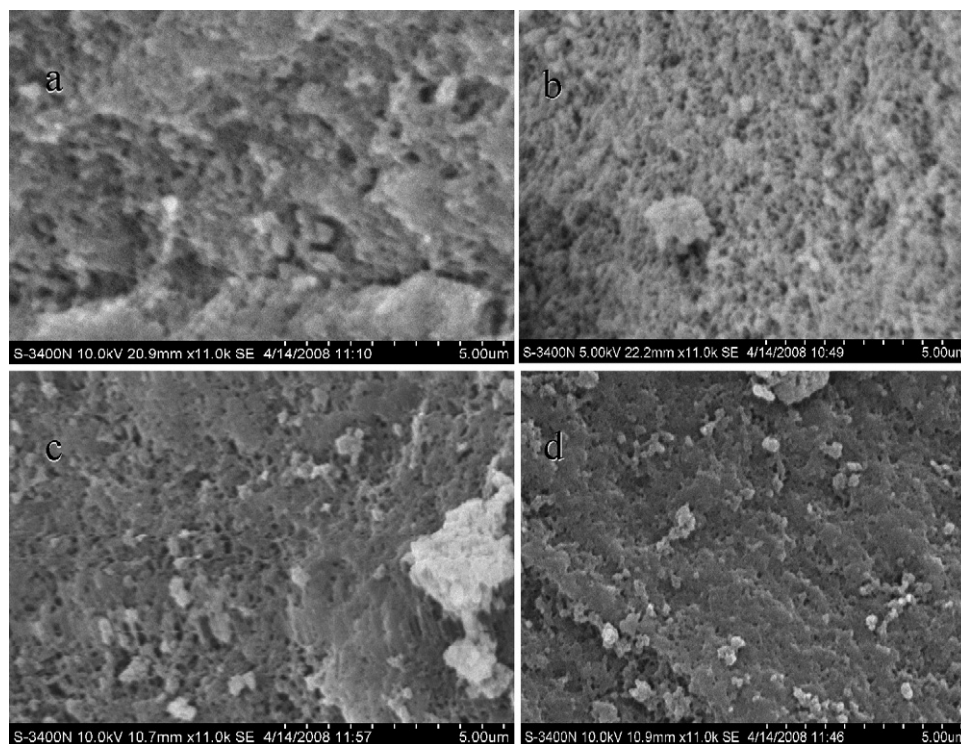
The basic structures of the polymers synthesized via cross-linkers and function monomers play a key role for preparing MIPs. Because the specificity of the template cavity is not controlled by the template molecules but is mainly dependent on the network spatial array of MIPs, the structural optimization is vital for the selectivity of MIPs. In the polymerization, cross-linkers are employed to control matrix stability and solid shapes of imprinting binding sites after removal of template molecules. The cross-linkers of DVB, EDMA, PTA and BisAA were introduced to investigate their influences on the adsorption properties of MIPs.

Fig. 6 shows the effects of solvation energy of cross-linkers on the imprinting factors of the four MIPs. DVB and EDMA are both better cross-linkers in the polymerization. The molecular chains of MIPs linked by EDMA are suitably feasible while the polymers containing DVB are of considerable rigidity but sometime they do not facilitate the rebinding of template molecules with MIPs.

Table 2

Specific adsorption properties of MIPs in different porogenic solvents.

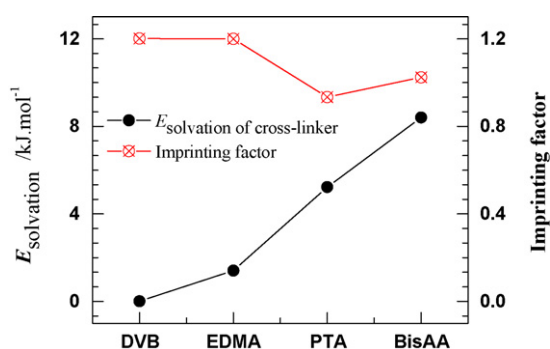
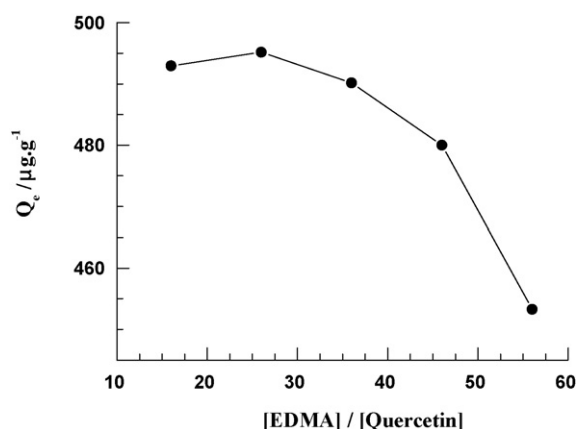
Solvents	ϵ	$E_{\text{solvation, AA}}$ (kJ mol ⁻¹)	$E_{\text{solvation, quercetin}}$ (kJ mol ⁻¹)	Q_{MIP} (μg g ⁻¹)	Q_{control} (μg g ⁻¹)	Imprinting factor (α)
1,4-Dioxane	2.25	3.23	4.78	372	354	1.05
THF	7.58	4.00	6.90	480	400	1.20
Acetone	20.7	7.25	9.78	409	381	1.07
ACN	37.5	9.18	11.2	309	300	1.03

**Fig. 5.** SEM microphotographs of MIPs prepared in different porogens. (a) THF, (b) acetone, (c) ACN and (d) 1,4-dioxane.

Enhancement of the interaction between cross-linkers and template molecules will benefit the formation of selective template cavity. Increasing interaction between cross-linkers and solvents will increase the competition and therefore weaken the interaction between cross-linkers and template molecules. This is adverse to immobilize the binding sites and the mobility of sites will bring down the recognition specificity of MIPs.

In order to improve the recognition performance of the MIPs prepared by using EDMA, a series of polymers have been synthesized by increasing the molar percentage ratio of EDMA to quercetin (C/T) in the polymerization mixture. As shown in Fig. 7, the adsorption properties of MIPs are remarkably affected by the content

change of cross-linkers, where Q_e (μg g⁻¹) is the equilibrium adsorption amount at each concentration. Increasing cross-linkers will increase the rigidity of MIPs and decrease the swelling in solvents, and thus will reduce the change of the spatial structure and the binding groups in the polymerization and recognition, which will facilitate forming stable binding sites and therefore improve the selectivity of MIPs. It is observed that for the MIPs, at the ratios of 10:1 to 25:1, the specific adsorption amounts were rapidly increased and the MIPs exhibited the better selectivity and

**Fig. 6.** Effects of the solvation energy of cross-linkers on the specific adsorption of MIPs.**Fig. 7.** Effects of the amounts of cross-linkers on the equilibrium adsorption amounts of MIPs.

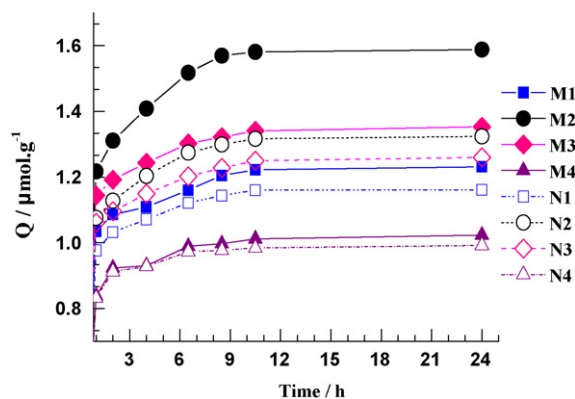


Fig. 8. Dynamic curves for the adsorption of quercetins onto MIPs and NIPs.

recognition capacity. The high cross-linking degree will contribute to stabilize the recognition sites of MIPs. While the ratio of C/T is increased especially to 40:1, the specific adsorption and specific binding capacity of the resultant MIPs rapidly decay. At high concentrations of cross-linkers, the binding sites in MIPs are too mobile, which will reduce the orderliness of matrix arrangement of MIPs and increase non-specific cavity, resulting in high non-specific binding and low affinity.

3.3. Adsorption analysis and recognition mechanism

Fig. 8 shows the dynamic curves for the adsorption of quercetin onto the MIPs and NIPs in four different solvents, where Q is the amounts of quercetins adsorbed at any time. It is observed that the amounts of quercetins both adsorbed onto MIPs and bound to NIPs increased with time and nearly reached saturation state within 10 h. For the adsorption equilibrium, the adsorption time was set for 24 h in the following experiments. It is assumed that the high adsorption rate within 6 h might result from the preferential and rapid adsorption of template molecules onto the recognition sites in the surface of MIPs. When these imprinted sites in the surface were occupied, it became difficult for quercetin to implant into the MIPs. This would cause the adsorption to slow down. The various contributions of polymers on the template molecular recognition would be attributed to the hydrogen bonds between template and monomer, and the comprehensive functions of resultant microenvironment and complementary cavity to the template molecules [35]. It is also observed that both M2 and N2 have the largest adsorption amounts in THF among the MIPs and NIPs, respectively. Although NIPs show similar trend to MIPs on the quercetin adsorption (Fig. 8), their adsorption capacities are much lower. This might be likely because of the non-specific adsorption of NIPs caused by van der Waals force, due to the absence of imprinting procedure and thereafter lacking suitable recognition sites and imprinting cavities in the NIPs. However, there were numerous and precise imprinting sites in the MIPs, resulting in specific adsorption, and thus they produced higher adsorption capacity. So, the presence of complementary cavities to the template molecules in the MIPs is vital for the specific adsorption.

Fig. 9 shows the adsorption isotherms of quercetins onto the MIPs and NIPs using THF as recognition medium. The curves were obtained by plotting the saturated adsorption amounts with equilibrium concentrations of the quercetins in MeOH solution. In other words, the adsorption capacity (Q) of quercetin increased quickly with the increase of initial concentration. At higher equilibrium concentration than $6.0 \mu\text{g mL}^{-1}$, adsorption of MIPs became stable and its recognition sites were almost saturated. NIPs had the same trend as MIPs with lower saturated adsorption amounts. Thus,

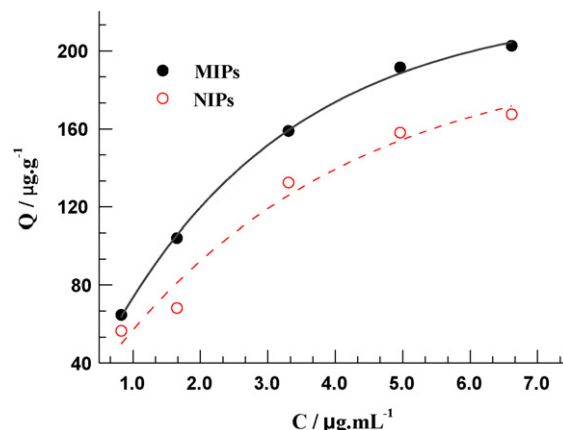


Fig. 9. Adsorption isotherms of polymers using THF as solvent.

adsorption isotherms are important for describing how adsorbates interact with adsorbent [36].

Scatchard analysis was employed to further analyze the binding isotherms, which is an approximate model commonly used to estimate the binding parameters of MIPs [2,26]. The Scatchard equation can be expressed as, $Q/C = (Q_{\text{max}} - Q)/K_d$, where K_d ($\mu\text{mol mL}^{-1}$) is defined as the equilibrium dissociation constant of the binding sites, Q ($\mu\text{mol g}^{-1}$) is the amount of quercetin bound to the polymer, Q_{max} ($\mu\text{mol g}^{-1}$) is the maximum adsorption amounts of quercetin on the polymer, and C ($\mu\text{mol mL}^{-1}$) is equilibrium concentration of quercetin in the solution. Fig. 10 shows the Scatchard plots of the binding of quercetin to MIPs and NIPs, respectively. It is clear that the Scatchard plot for MIPs is a single straight line. The linear regression equation was, $Q/C = -32.32Q + 98.30$ ($R^2 = 0.9903$), suggesting that the homogeneous recognition sites for quercetins were formed in the MIPs, namely the adsorption interaction was nearly equivalent for the four binding sites. From the slope ($-32.32 (1/K_d)$) and intercept ($98.30 (Q_{\text{max}}/K_d)$), K_d and Q_{max} for the affinity binding sites were calculated to be $0.03 \mu\text{mol mL}^{-1}$ and $3.04 \mu\text{mol g}^{-1}$, respectively. For NIPs, the non-linearity indicated that there were not selective adsorption sites for quercetins on them. From the Scatchard analysis, it could be assumed that the rebinding sites in the MIPs are mainly dependent on hydrogen bonding, while the interaction between NIPs and quercetins was mainly from non-specific adsorption such as van der Waals.

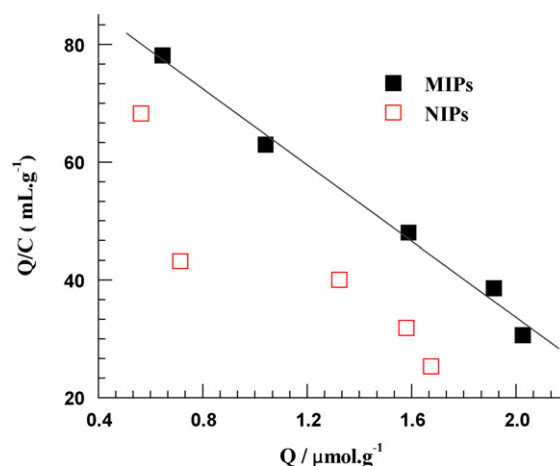


Fig. 10. Scatchard plots of MIPs and NIPs.

Table 3

Distribution coefficient (K_d) and ratio (β) of distribution coefficients between two tested substrates.

Sorbents	Initial conc. ($\mu\text{g g}^{-1}$)		Final conc. ($\mu\text{g mL}^{-1}$)		K_d (mL g^{-1})		β
	Quercetin	Rutin	Quercetin	Rutin	Quercetin	Rutin	
MIPs	20	20	16.9	18.2	46.1	25.1	1.84
NIPs	20	20	19.0	19.1	13.2	12.5	1.06

3.4. Molecular selectivity of MIPs

The selective adsorption of MIPs was evaluated by using quercetin and its structurally similar compounds, rutin and catechol, as substrates in THF. Rutin and catechol were chosen as references for investigating the static adsorption specific selectivity and MIP-SPE specific selectivity, respectively. The static adsorption distribution coefficient K_d was utilized to evaluate the molecular selectivity of polymers [24]. K_d is defined as follows, $K_d = C_p/C_s$, where C_p ($\mu\text{g g}^{-1}$) is the concentration of the tested substrates adsorbed on the MIPs and C_s ($\mu\text{g mL}^{-1}$) is the equilibrium concentration of substrates in solution. Table 3 shows the K_d and the separation factor, β , namely the ratio of K_d of the two tested substrates. It is seen that the adsorption capacities of NIPs are very close for both rutin and quercetin because there are not selective recognition sites in the NIPs and the adsorption for substrates are non-selective. On the other hand, obviously, the MIPs offer better selectivity for quercetins since the MIPs present the β value about 1.7 times of that for the corresponding NIPs. The molecular recognition of MIPs mainly depends on two factors, molecular dimension of template and matching degree of the bonding sites [25]. For the rutin molecule, it has a large substitution group in γ -OH position of quercetin. Different molecular dimensions cause the rutin not complementary to the imprinting cavities of the quercetin-MIPs and therefore weak interactions. As a result, the MIPs gave a lower selectivity for rutin than that for quercetin.

Table 4 shows the comparisons of MIP-SPE properties between quercetin and catechol. As seen, the MIPs exhibit much stronger binding affinity for quercetin even if the concentration of catechol is 15 times of quercetin, while both have the same adsorption trend in different solvents, namely strongest in THF and weakest in ACN. Since the molecular dimension of catechol is smaller than that of quercetin, the molecule maybe easily enters the MIP cavity. Also, because it contains two phenolic hydroxyl groups, the molecules could form two hydrogen bonds with the amino groups of AA in the MIPs. However, the smaller structure of catechol makes it poorly match the cavities induced by quercetin, resulting in weaker binding interaction and lower selectivity.

3.5. SPE specificity of the MIPs for quercetin

The prepared quercetin-polymer was employed as the sorbent of SPE column for the pretreatment of quercetin and structurally similar rutin. Fig. 11 shows the HPLC-UV chromatograms of the standard mixtures. Good baseline separation was obtained for them without SPE procedure and rutin was eluted first (Fig. 11a). Not any apparent peak is observed in Fig. 11b for determining

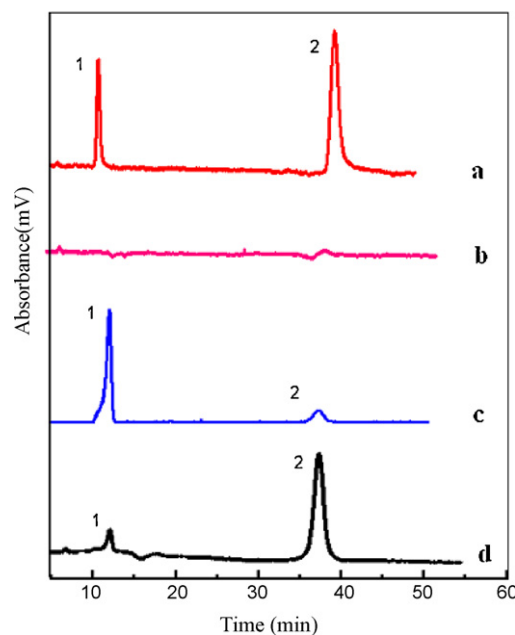


Fig. 11. Chromatograms of the standard mixture. (a) Initial solutions before MIP-SPE, (b) solutions after MIP-SPE, (c) solutions after rinsing with MeOH, and (d) eluates after washing with MeOH/acetic acid (9:1, v/v). (1) Rutin and (2) Quercetin.

the solutions after the MIP-SPE, which indicates quercetin and rutin were almost completely adsorbed onto the MIPs. Fig. 11c shows that almost all the rutins were eluted with only minor quercetins by rinsing the MIP-SPE column with MeOH; quercetin was not easily eluted with MeOH. Then using the more polar eluting solutions, namely MeOH/acetic acid (9:1, v/v), quercetins were nearly all eluted, shown in Fig. 11d. The linking of a rutinose group (α -L-mannopyranosyl- β -D-glucopyranosyl) in rutin causes a larger space volume than that of quercetin, and it is therefore most difficult for the rutin to be embedded into the cavity of the quercetin-MIPs, so it was easily eluted with MeOH. However, the quercetins adsorbed onto the MIPs through the specific recognition effects could be destroyed only by the strongly polar solvents containing acetic acid. Thus the MIPs are highly structural-selective toward quercetins.

In addition, quercetins in two eluates were analyzed after the above same MIP-SPE procedure for the standard mixture of quercetin and rutin of 2 mL at $20 \mu\text{g mL}^{-1}$ and 5 mL at $4 \mu\text{g mL}^{-1}$, respectively. The molar ratios of quercetin to rutin were both 2 to 1 before SPE, and then they became 8 to 1 and 143 to 1, respectively, for the two aliquots. The EF of quercetin compared to rutin was improved by at least 4–71.5 times. Therefore, under the suitable conditions of loading, rinsing and eluting, the MIPs with high selectivity can be employed not only to separate some structurally similar compounds, but also to excellently concentrate and clean up the trace analytes.

Fig. 12 shows the chromatograms of the solutions from *cacumen platycladis*. The peaks of quercetin and rutin were identified with the comparison of chromatographic retention times with those of the spiked standards shown in Fig. 11. It is supposed that querceins mainly exist in the *cacumen platycladis*. Also, a relatively large peak appeared between ca. 16–20 min shown in Fig. 12a, which is not surprising in the extracts before the MIP-SPE, and they were not identified. On the other hand, by the same MIP-SPE pretreatment as that for Fig. 11d, quercetin is predominantly present and there is only a minor of impurities and rutin is hardly observed (Fig. 12b). The MIP-SPE is not only high selective for quercetin but also offers cleanup and enrichment effects for the real samples. The obtained unique profile demonstrates

Table 4

Comparisons of two analogues before and after MIP-SPE.

Solvents	Before SPE ($\mu\text{g mL}^{-1}$)		After SPE ($\mu\text{g mL}^{-1}$)		K_d (mL g^{-1})	
	Quercetin	Catechol	Quercetin	Catechol	Quercetin	Catechol
1,4-Dioxane	2	30	0.56	15.0	17.1	6.67
THF	2	30	0.28	12.3	41.0	9.59
Acetone	2	30	0.40	12.9	26.7	8.84
ACN	2	30	0.74	15.6	11.4	6.15

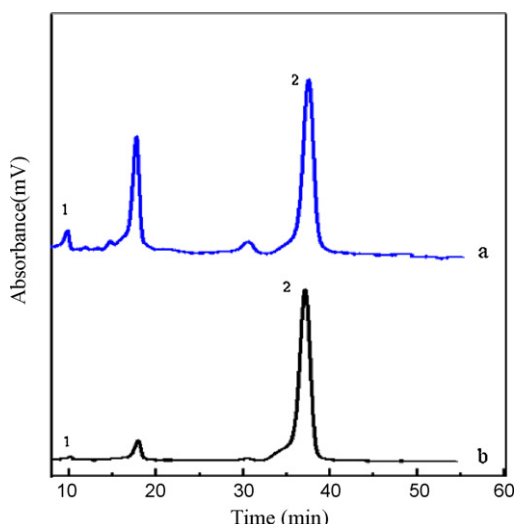


Fig. 12. Chromatograms of the solutions from cacumen platycladis. (a) Solutions before MIP-SPE and (b) eluates after MIP-SPE. (1) Rutin and (2) Quercetin.

the endogenous quercetins could be efficiently separated from the extracts of cacumen platycladis. It provides an effective method for the separation and determination of active ingredients from cacumen platycladis and further for flavonoid compounds in the traditional Chinese medicine. Besides for the SPE application, the quercetin-MIPs have been also employed in diverse fields. To take two examples, quercetin-MIPs immobilized silica gel could be used as a solid-phase reagent for the tin determination by using sorption-spectroscopic method [19] and quercetin-MIPs could be developed into sensors by self-assembling monolayer/mediator system for analyzing biomolecules and drug molecules [37]. Thus, further analysis and applications of quercetin-MIPs are under on the way.

4. Conclusions

The prepared quercetin-MIPs were confirmed by both FTIR and SEM, characterized by HPLC-UV, and applied for the SPE of a natural product (cacumen platycladi). Optimal imprinting parameters for enhanced recognition properties of MIPs toward quercetin were attained, which were very important for the successful preparation and application of the MIPs. The resultant MIPs displayed fast adsorption dynamics and high adsorption capacity. And they could contain single selective adsorption sites for quercetin. The molecular selectivity of the MIPs was also evaluated by using rutin and catechol as references. The high specific binding could be attributed to a large population of sites of high affinity for the templates. Selective recognition for the isolation of quercetin from cacumen platycladi samples was achieved, and thus the MIPs would powerfully be applied for the preconcentration of traditional Chinese medicines. Further research will focus on developing new imprinting materials for the separation and determination of

quercetins from natural products, and extending to more flavonoid compounds, as well as using the promising quercetin-MIPs as an analytical tool for various potential applications.

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