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Magnetic molecularly imprinted polymer for the selective extraction of quercetagetin from *Calendula officinalis* extract

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ABSTRACT

A new magnetic molecularly imprinted polymers (MMIPs) for quercetagetin was prepared by surface molecular imprinting method using super paramagnetic core-shell nanoparticle as the supporter. Acrylamide as the functional monomer, ethyleneglycol dimethacrylate as the crosslinker and acetonitrile as the porogen were applied in the preparation process. Fourier transform infrared spectrometer (FT-IR), X-ray diffraction (XRD) and Vibrating sample magnetometer (VSM) were applied to characterize the MMIPs, and High performance liquid chromatography (HPLC) was utilized to analyze the target analytes. The selectivity of quercetagetin MMIPs was evaluated according to their recognition to template and its analogues. Excellent binding for quercetagetin was observed in MMIPs adsorption experiment, and the adsorption isotherm models analysis showed that the homogeneous binding sites were distributed on the surface of the MMIPs. The MMIPs were employed as adsorbents in solid phase extraction for the determination of quercetagetin in *Calendula officinalis* extracts. Furthermore, this method is fast, simple and could fulfill the determination and extraction of quercetagetin from herbal extract.

1. Introduction

As a gift of nature, the natural products extracted from the traditional Chinese medicine (TCM) containing flavonoids, terpenoids, polyphenols and thousands upon thousands compounds have cured diseases for people from five thousand years ago. These compounds not only built the foundation of TCM but also would become the dominate force of healing drugs for the future. Up to now, a great deal of extraction or methods [1–4] have been developed to realize the extraction of natural products from TCM, but the low abundance, complex composition, thermosensitive and easily hydrolyze of the natural products increased the difficult for their extraction. At the same time, most of these methods are always high-cost and time-consuming in the preparation process [5], and majority of these methods could not obtain selective recognition during the extraction. Thus a material, which has high selectivity for target analytes and can be prepared without much labor was expected.

Quercetagetin (3, 5, 6, 7, 3′, 4′-hexahydroxyflavone) [6], a characteristic flavonol compound with six additional phenolic hydroxyl groups based on the molecular structure of the flavones backbone, is widely distributed in the *Calendula officinalis* which was famous for its anti-inflammatory and anti-cancer [7]. Furthermore, quercetagetin exhibited a strong potential in the prevention or therapy of cancer and other chronic diseases [8], thus it could be used in the functional food and pharmaceutical industries. However, the analysis of quercetagetin was confined to the silica gel column chromatography [9] or other traditional methods [10] that has the disadvantages of unfriendly to environment, high labor intensity and low efficiency. Hence, exploring a new method for quick extraction and separation of quercetagetin was an urgent affair for analytical researchers.

Based on the above two considerations, molecularly imprinting polymers (MIPs), the new synthetic polymeric materials which were famous for their high selectivity toward template, excellent stability in organic solvents, ease for preparation and low cost, have attracted people's wide concern in recent years [11,12], and have been widely used in solid phase extraction [13], capillary electrophoresis [14], liquid chromatography [15], sensors [16] and many other areas for their outstanding advantages. In particulars, MIPs have shown strong advantages in the extraction and purification of TCM. Yu et al. [17] prepared the podophyllotoxin imprinted polymers which exhibited excellent adsorption ability and selectivity in the extraction of podophyllotoxin from the herb

Abbreviations: MMIPs, magnetic molecularly imprinted polymers; MNIPs, magnetic non-molecularly imprinted polymers; HPLC, high-performance liquid chromatography; HPLC-DAD, high-performance liquid chromatography diode array detection; TCM, traditional Chinese medicine

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sample. Our group also has been working in the extraction of natural products from TCM [18,19]. However, these MIPs were mostly synthesized by the bulk polymerization or precipitation polymerization and showed incomplete template removal, slow mass transfer and irregular shape [20], which seriously decreased the efficiency and accuracy of the extraction. Thus, more attention has been paid to surface imprinting [21], which is to immobilize all the imprinted sites on the surface of materials, providing the complete removal of template, good accessibility to the target molecules and low mass-transfer resistance [22]. Unfortunately, like many adsorbent processing, the time-consuming centrifugation and filtration steps have seriously restricted its development [23]. The generation of magnetic MIPs (MMIPs) [24] solved these problems well with high efficiency. In MMIPs, the molecularly imprinted polymers (MIPs) were coated on Fe_3O_4 or Fe_2O_3 so that the core-shell magnetic materials not only have magnetic characteristic but also gain high selectivity for the target molecule. The core-shell structure contributes to a mass of recognition sites to template molecular so that the MMIPs give easy accessibility and low mass transfer resistance to template molecules [25].

Therefore, in this work, we prepared quercetagetin imprinted magnetic polymers on the surface of Fe_3O_4@SiO_2 nanoparticles. Acrylamide (AM) was chosen to be the functional monomer, and ethylene glycol dimethacrylate (EGDMA) acted as the crosslinker. The characterization, adsorption capacity and selectivity of MMIPs and magnetic non-molecularly imprinted polymers (MNIPs) were investigated. The obtained quercetagetin MMIPs was applied to determine quercetagetin from *Calendula officinalis* extracts coupled with a high performance of liquid chromatographic (HPLC) technique. Also, the chromatographic analysis, and method validation such as linearity, limit of detection, repeatability and accuracy were targeted.

2. Experimental

2.1. Reagents and apparatus

Standard quercetagetin was prepared in our laboratory, while luteolin, apigenin8-C-glucoside, rutinum and resveratrol were provided by the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Ferrous chloride ($FeCl_2 \cdot 4H_2O$) and Ferric chloride ($FeCl_3 \cdot 6H_2O$) were obtained from Beijing Chemicals Corporation (Beijing, China). Acrylamide, 3-methacryloxypropyltrimethoxysilane (MPS), 2, 2′- azobisisobutyronitrile (AIBN), and Ethylene glycol dimethacrylamide (EGDMA) were supplied by Alfa Aesar (Tianjin, China). Toluene, acetic acid and ethanol were purchased from Lianlong Bohua Pharmaceutical Chemical Co., Ltd (Tianjin, China). Tetraethoxysilane (TEOS), isopropanol, ammonium hydroxide, anhydrous magnesium sulfate and acetonitrile were purchased from Tianjin Chemical Reagent Co. (Tianjin, China). Chromatographic grade methanol and acetonitrile was obtained from Merck Co. (Darmstadt, Germany). Deionized water (18 MΩ cm) was prepared by a water purification system (Shanghai, China). All the solutions used for HPLC were filtered through a 0.45 μm filter before use.

The MMIPs were characterized by Fourier-transform infrared spectrometry (Nexus 870, Nicolet, USA), and the wave numbers of FT-IR measurement range were controlled from 500 cm^{-1} to 4000 cm^{-1}. The magnetic properties were measured by a vibrating sample magnetometer (Lakeshore 7304, USA). The configurations of magnetic particles were characterized using a Cu-Ka1 radiation (PANalytical X'Pert, Holland).

2.2. HPLC analysis

The sample analysis was performed on an HPLC system (Agilent 1260, USA) with a diode array detector (DAD). The analytical column was a 150 mm × 4.6 mm, 5 μm C_{18} column (Agilent, USA). The mobile phase was consisted of acetonitrile and water containing 0.1% acetic acid-5 mmol/L ammonium acetate buffer at a ratio of 40:60 (v/v) with a flow rate of 1.0 mL/min. The detection wavelength was set at 210 nm for quercetagetin. The correlation coefficient, limit of detection (LOD), and limit of quantification (LOQ), and the calibration curve were applied to evaluate the proposed method and validate the analytical methodology in this work.

2.3. Preparation of MMIPs

The preparation protocol of quercetagetin imprinted MMIPs was shown in Fig. 1. Chemical co-precipitation method [26] was applied in the preparation of Fe_3O_4. According to the work of Zeng et al. [27], the Fe_3O_4 were modified with SiO_2. MPS was introduced to graft double bonds onto the surface of Fe_3O_4@SiO_2 to form polymerizable sites with EGDMA and AM in the next reaction process [28]. Briefly, 250 mg Fe_3O_4@SiO_2 nanoparticles were dispersed in 50 mL anhydrous toluene solution containing 5 mL of MPS in a three-necked flask under stirring, followed by 15 min of ultrasonic dispersion, the mixture reacted for 24 h at 70 °C with reflux in nitrogen atmosphere. After that, the products (Fe_3O_4@SiO_2-CH═CH_2)

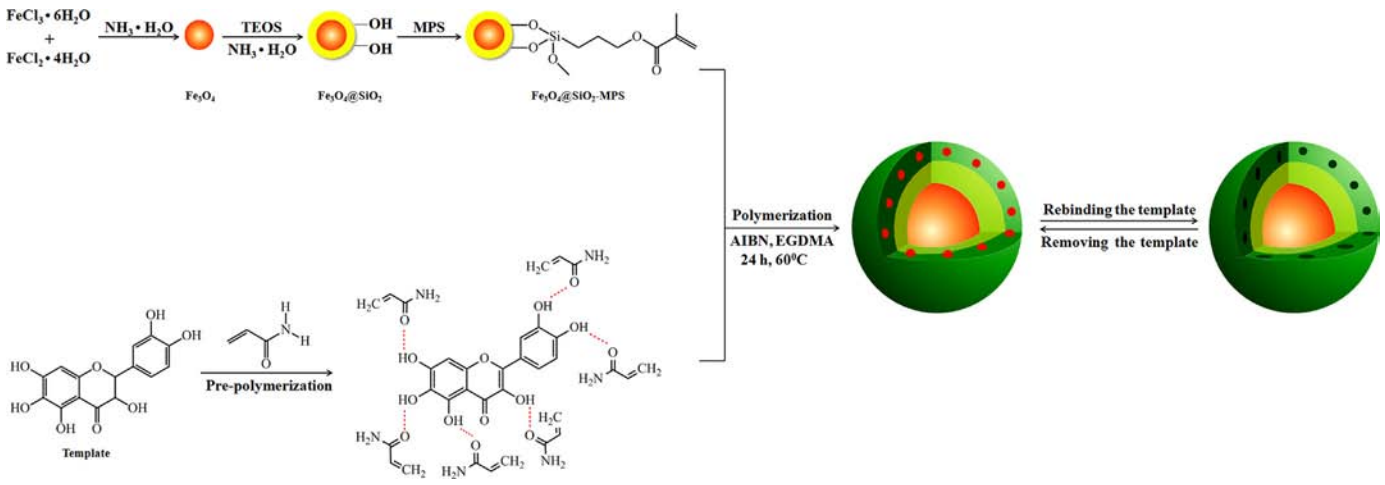

Fig. 1. Schematic representation of preparation for MMIPs.

were collected magnetically, then freeze-dried for subsequent using after washing by ethanol and deionized water.

Surface-imprinted polymerization was applied in this work. Using quercetagetin as the template, acrylamide as the monomer, EGDMA as the cross linking agent. As a famous aprotic solvent, acetonitrile acted as the reaction solvent and the porogen agent because of its excellent porogen ability [29]. AIBN was chosen to be the initiator and the functionalized Fe_3O_4@SiO_2 nanoparticles were used as the cores to support the surface imprinting. At the beginning, 16 mg of quercetagetin were dissolved in 20 mL of acetonitrile in a three-necked flask, 21 mg of AM were added into the solution and kept stirring for 5 h to form a pre-polymer [30], 100 mg dried Fe_3O_4@SiO_2–CH═CH_2 were dispersed in the above solution subsequently. Then 0.44 mL of EGDMA and 20 mg of AIBN were added for polymerization into the solution and degassed in an ultrasonic bath for 15 min to remove oxygen. After that, the system was prepared at 65 °C in an oil bath for 24 h under mechanical stirring. In the above process, nitrogen gas was purged continuously. At the same time, the MNIPs were prepared using the same procedure with the absence of quercetagetin. After synthesis, the MMIPs were collected by an external magnetic field and washed by a mixture of methanol/acetic acid (8:2, v/v) for many times until there was no template was detected by HPLC-DAD.

2.4. Adsorption equilibrium and selectivity evaluation

To evaluate the adsorption capacity of the MMIPs, the adsorption equilibrium experiment was performed. 20 mg of MMIPs or MNIPs were equilibrated with 1 mL of different concentrations (20 μg/mL-160 μg/mL) of quercetagetin dissolved in acetonitrile in a 5 mL centrifugal tube and shaken for 24 h at room temperature. Subsequently, the MMIPs and MNIPs were magnetically separated from the solution. Then, the supernatants were filtrated through a 0.45 μm micro-porous membrane before HPLC detection and the concentration of quercetagetin in the supernatants was measured by the UV-spectrometer at 210 nm detection wavelength. The equilibrium adsorption capacity Q (μmol/g) was calculated as,

$$Q = \frac{(C_i - C_f)V}{WM} \quad (1)$$

In this equation, C_i (μg/mL) means the initial concentration of quercetagetin solution. C_f (μg/mL) is the quercetagetin concentration of the supernatant solution after the adsorption. V (mL) equals the volume of the initial quercetagetin solution, M (g/mol) is the molar mass of quercetagetin, and W (g) is the weight of the MMIPs or MNIPs.

In order to evaluate the selectivity of the MMIPs, four potential interferents (luteolin, apigenin8-C-glucoside, rutinum and resveratrol) were investigated and their structures are shown in Fig. 2. Every 20 mg of MMIPs or MNIPs were mixed with 1 mL of 20 μg/mL solution of the studied compounds prepared in acetonitrile and then kept stirring for 24 h at room temperature. The particles were collected by a magnet. HPLC detections were applied in the supernatants of the studied solutions.

In this work, interrelated adsorbed coefficients which including distribution coefficient and selectivity coefficient were evaluated.

The distribution coefficient was determined by the following equations:

$$k_d = \frac{C_a}{C_f} \quad (2)$$

As the concentration of the adsorbed medium, $C_a = (C_i - C_f) \times V/W$, and C_f is the free concentrations of the solution. In this equation, C_i (μg/mL), C_f (μg/mL), V (mL), and W (g) were described preciously.

In order to compare the selectivity of the MMIPs between quercetagin and the potential interferents, the selectivity coefficient (k) was calculated according to the following formula:

The selectivity coefficient is calculated as follows:

$$k = \frac{k_{d1}}{k_{d2}} \quad (3)$$

k_{d1} stands for the distribution coefficient for quercetagin and k_{d2} stands for the distribution coefficients for the potential interferents.

2.5. Analysis of quercetagin in calendula officinalis extracts

1 mg of ethanol extract of *Calendula officinalis* was dissolved in 10 mL of acetonitrile. 50 mg of MMIPs were added into the obtained solution and kept stirring at room temperature for 25 min. After that, a magnet was applied to collect and separate the MMIPs from the solution and the MMIPs were washed by 1 mL of methanol/acetic acid (8:2) for 20 min. Then, 0.5 mL of supernatants were removed to evaporate to dryness and dissolved in 0.1 mL of acetonitrile for further HPLC-DAD analysis.

3. Results and discussions

3.1. Preparation of MMIPs

As the multistep procedure showed in Fig. 1, the synthesis of the MMIPs needed four steps. Firstly, the coprecipitation method was applied in this work to prepare super paramagnetic Fe_3O_4 nanoparticles. One thing calls for special attention was that the diameter of the Fe_3O_4 nanoparticles should be smaller than 25 nm in order to ensure the superparamagnetism of the MMIPs [31]. Secondly, the surface of the Fe_3O_4 nanoparticles was coated by silica to avoid oxidation. Furthermore, silica offered us a good platform which was bio-compatible, hydrophilic and easy to modify. Then, double bonds were grafted on the surface of the Fe_3O_4@SiO_2 through the introduction of MPS, which supplied the nanoparticles a mass of polymerizable sites for later polymerization. Lastly, the template, functional monomer and the cross-linker were polymerized to form a selective recognition surface to the template molecules.

The porogen, the type and amount of the functional monomer are the main factors which affect the molecular recognition capability of MMIPs [32]. In this work, acetonitrile was selected to be the porogen because both the template molecules and functional monomers could dissolve well in acetonitrile. In the other hand, although quercetagetin is a strong polar molecule, strong polar solvent could weaken the hydrogen-bond interaction and would have bad influence on the selectivity and affinity of MMIPs. For these reasons, acetonitrile, the weak polarity solvent was chosen as the solvent for non-covalent molecules recognition of MMIPs in this work. In MMIPs, the intermolecular interaction between the template molecule and functional groups in the polymers is the key factor which determines the molecular recognition of the template molecule and its structurally related compounds. In this study, three molar ratios of the function monomer to the template of 5:1, 6:1 and 7:1 were tested, and three molar ratios of cross-linkers to the function monomer 4:1, 5:1 and 6:1 were also investigated. The experimental results indicated that the optimum molar ratio of the template: function: cross-linker was 1:6:30 to prepare MMIPs for quercetagetin.

3.2. Characterization of MMIPs

The FT-IR spectra of Fe_3O_4, Fe_3O_4@SiO_2, Fe_3O_4@SiO_2-CH═CH_2, MMIPs and MNIPs were compared in Fig. 3. In Fig. 3a, a strong and

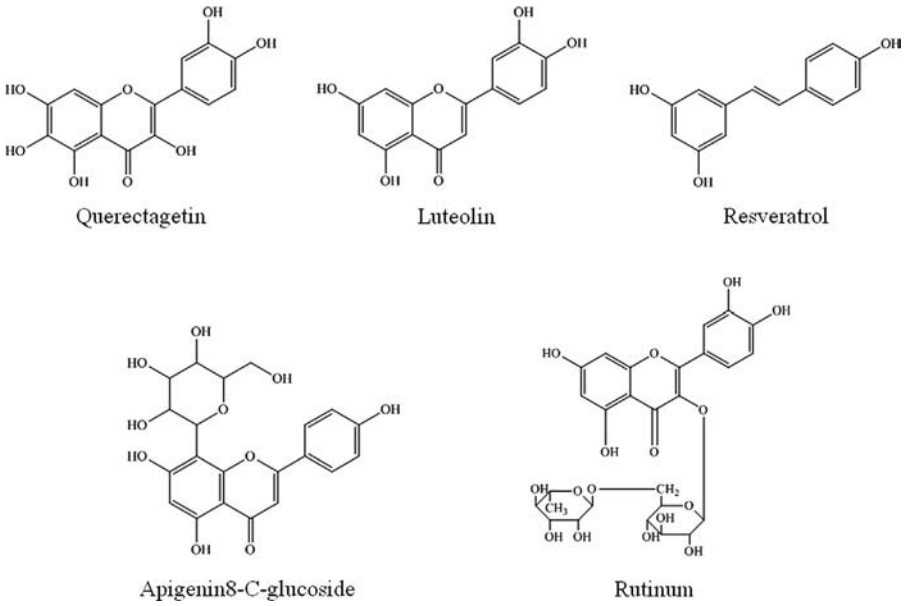

Fig. 2. Structures of resveratrol, quercetagetin and its analogues.

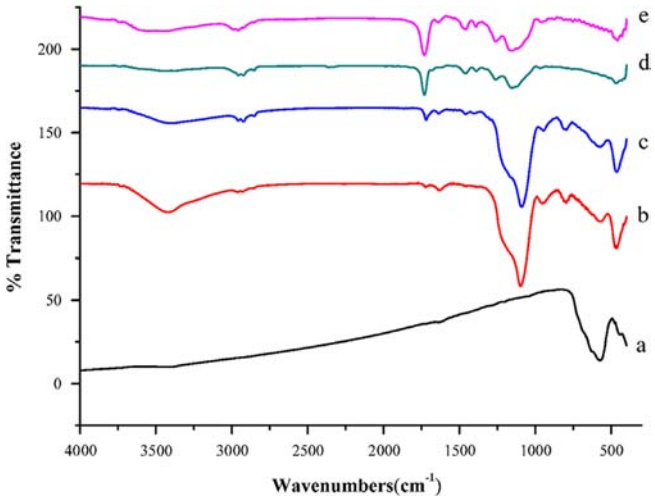

Fig. 3. FT-IR spectra of the Fe_3O_4 (a), Fe_3O_4@SiO_2 (b), Fe_3O_4@SiO_2-CH=CH_2 (c), MNIPs (d), MMIPs (e).

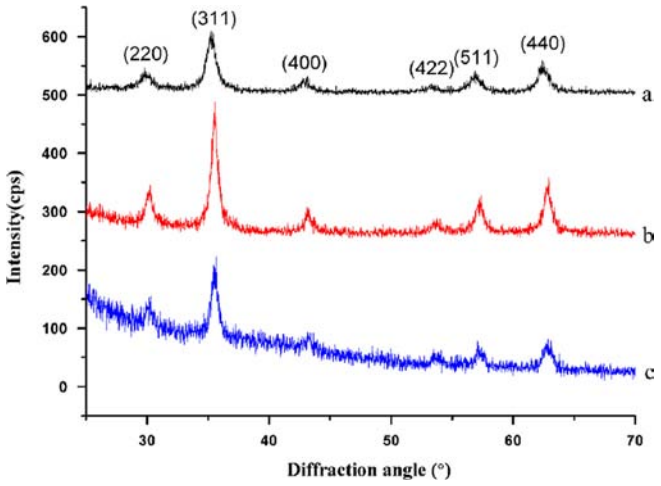

Fig. 4. XRD of Fe_3O_4 (a), Fe_3O_4@SiO_2 (b), and MMIPs (c).

sharp absorption peak at about 580 cm^{-1} is characteristic of the Fe-O vibration for Fe_3O_4 magnetic nanoparticles. In Fig. 3b, three strong peaks around 1100 cm^{-1} as a Si-O asymmetric stretching vibration, 800 cm^{-1} as a Si-O symmetric stretching vibration, 480 cm^{-1} as a Si-O bending vibration, respectively, mean SiO_2 is successfully encapsulated onto the surface of Fe_3O_4 magnetic nanoparticles. The broad absorption peak at about 3450 cm^{-1} corresponds to O-H bonding to hydroxyl group on the surface of Fe_3O_4. The peak at about 2900 cm^{-1} in Fig. 3c is assigned to the C-H stretching vibration in methylene group, which indicated that the double bonds are successfully grafted onto the surface of Fe_3O_4@SiO_2. In Fig. 3d and e, the FT-IR spectra of MMIPs and MNIPs are almost the same because of their similar chemical composition after removing the template molecule. And the peak around 1729 cm^{-1} represents the MMIP and MNIPs were synthesized by the polymerization of EGDMA and AM.

The XRD patterns of Fe_3O_4, Fe_3O_4@SiO_2 and MMIPs are shown in Fig. 4. Six characteristic diffraction peaks: (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0) are observed for all the three samples, which proves that the magnetic nanoparticles, Fe_3O_4@SiO_2 and MMIPs were composed of Fe_3O_4, and it further proves that the synthesized process did not change the crystalline phase of Fe_3O_4.

As an important feature of magnetic materials, sufficient magnetism ensures the quick separation of the materials from the liquid medium in practical application. Therefore, VSM was employed to study the magnetic properties of the materials at room temperature in this work. The magnetic hysteresis loops of Fe_3O_4 and MMIPs in Fig. 5 shows the saturation magnetization of MMIPs decreases in comparison with that of Fe_3O_4, which could be assigned to the shielding effect of the silica coating and the MIP shell layer on the surface of Fe_3O_4.

3.3. Adsorption isotherms

The characteristic adsorption isotherms of quercetagetin on MMIPs and MNIPs were studied at room temperature in Fig. 6. At the beginning, both the amounts of quercetagetin bounded to MMIPs and MNIPs increased along the increment of initial concentrations until they reached the saturation level and then

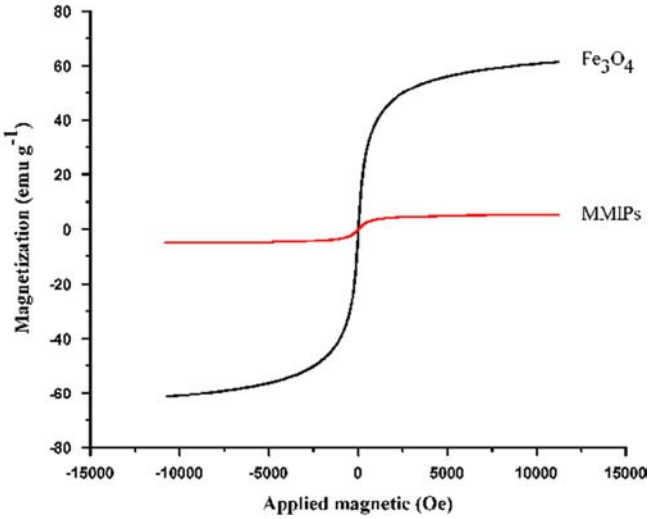

Fig. 5. Hysteresis loops of Fe_3O_4 nanoparticles and MMIPs.

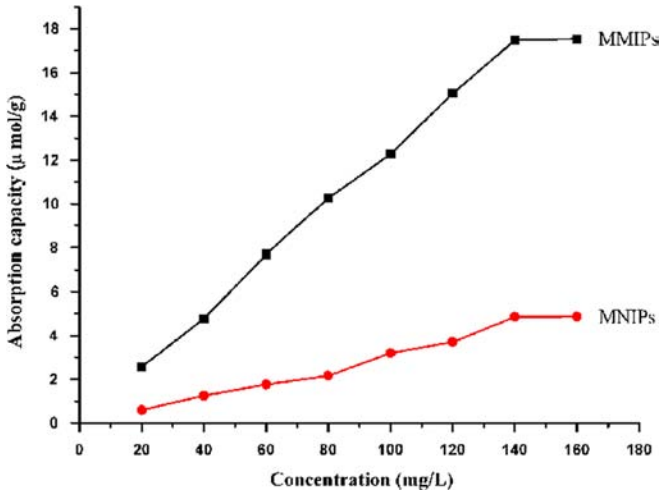

Fig. 6. The adsorption isotherms of quercetagetin on the MMIPs and MNIPs.

tended to be stable when the equilibrium concentration was equal or greater than 140 mg/L. It was obviously that the equilibrium adsorption amounts of quercetagetin on MMIPs were always higher than those on MNIPs, which could be associated with the existence of specific imprinting phenomenon of MMIPs.

Four adsorption isotherms-mathematical models, including the Langmuir, Scatchard, Freundlich, and Dubinin-Radushkevich isotherm models were used to interpret the nature of the template-polymer binding affinity and to study further on the binding properties of MMIPs and MNIPs. The results were shown in Fig. 7. Interestingly, both the Freundlich isotherm and Scatchard isotherm show the high linear correlation coefficients ($r_{Freundlich}$=0.9789, $r_{Scatchard}$=0.9797). But in Freundlich isotherm [33],

$$\log B = m \log F + \log a \quad (4)$$

Where B and F are the concentrations of bound and free analytes, respectively, a is the binding parameters, m indicates the intensity of the adsorption with heterogeneity index varies from 0 to 1. With the values turn from 0 to 1, the structure turns from heterogeneous to homogeneous. In this work, the value of m is 0.93502 which indicates that the surface structure inclines to be homogeneous and the result further illustrates the homogeneous recognition sites for template molecules are distributed on the MMIPs. But as it is known, the Freundlich isotherm is suitable for multilayer adsorption of heterogeneous system, so it should not be applied in this work. The Dubinin-Radushkevich isotherm exhibits a bad correlation coefficient value of 0.10438 in this work thus more discussion are useless here. In addition, the Langmuir isotherm which is basically used for monolayer adsorption onto a homogeneous surface was sidelined, because its correlation coefficient value of 0.9329 was smaller than the value of Scatchard isotherm (0.9921), an isotherm mainly used to explain the adsorption process of homogeneous surface, so the Scatchard might suits better in this work than other three isotherms. In Scatchard isotherm,

$$\frac{Q}{C} = \frac{(Q_{\max} - Q)}{k_d} \quad (5)$$

Where Q is the amount of quercetagetin bound to the polymers at equilibrium (mg/g), C is the free quercetagetin concentration at equilibrium (μg/mL), k_d is the dissociation constant (mg/L), Q_{max} is the apparent maximum binding amount (mg/L). Scatchard curves were obtained by taking Q as the x-coordinate and Q/C as the y-coordinate. As the results evaluated by Scatchard isotherm shown in Fig. 7a, k_d and Q_{max} were calculated to be 30.3214 mg/L and 146.5172 mg/L, respectively. The binding of quercetagetin to the MNIPs was also analyzed by Scatchard isotherm (Fig. 7b), and the k_d and Q_{max} were 20.18 μmol/L and 23.65 μmol/g. The results show that the homogeneous recognition sites for template molecules are formed in the MMIPs and MNIPs [34].

3.4. Binding specificity and selectivity of the MMIPs

Specificity tests were applied in this work to evaluate the selective recognition properties of MMIPs and MNIPs among different compounds and the results were displayed in Fig. 8. In this part, luteolin, apigenin8-C-glucoside, rutinum and resveratrol were selected as the potential interferents to investigate the selectivity of the imprinted nanoparticles based on their molecular weights and structures. The molecular recognition ability of MMIPs mainly depends on the binding ability which is closely related to the similarity between the template and the adsorbed molecules in functional groups, size and shape [35,36]. Obviously, the adsorption capacities of MMIPs to quercetagetin and other three analogical flavonoids were much higher than those of MNIPs, respectively.

As the structure of luteolin is showed in Fig. 2, luteolin almost have the same molecular skeleton compared with quercetagetin. The only different between them was the absent of two phenolic OH groups in luteolin, so that there was an infinitesimally small distinction in the adsorption ability between them. The adsorption capacity of MMIPs for quercetagetin was better than that of apigenin8-C-glucoside, which could be explained by the absent of the three phenolic OH groups and the substitution of one bulky glucose group on the structure of quercetagetin. For rutinum, the presence of the two glucose groups increased the volume of the molecule and decreased the opportunity for rutinum to enter the MMIPs cavity than quercetagetin. As a result, there was a serious decrease in the adsorption capacity of MMIPs for rutinum. There was no obvious adsorption difference between the MMIPs and MNIPs for resveratrol, which indicated that MMIPs and MNIPs did not have specific sites for resveratrol.

The selectivity of MMIPs for quercetagetin was evaluated using two parameters: k_d and k. Their values for the test compounds are shown in Table 1. In MMIPs, the value of k_d is 236.97 mL/g which shows a strong adsorption capability for target analytes. The parameter k also shows the high discrimination property of the MMIPs with high values. This result indicates that the prepared

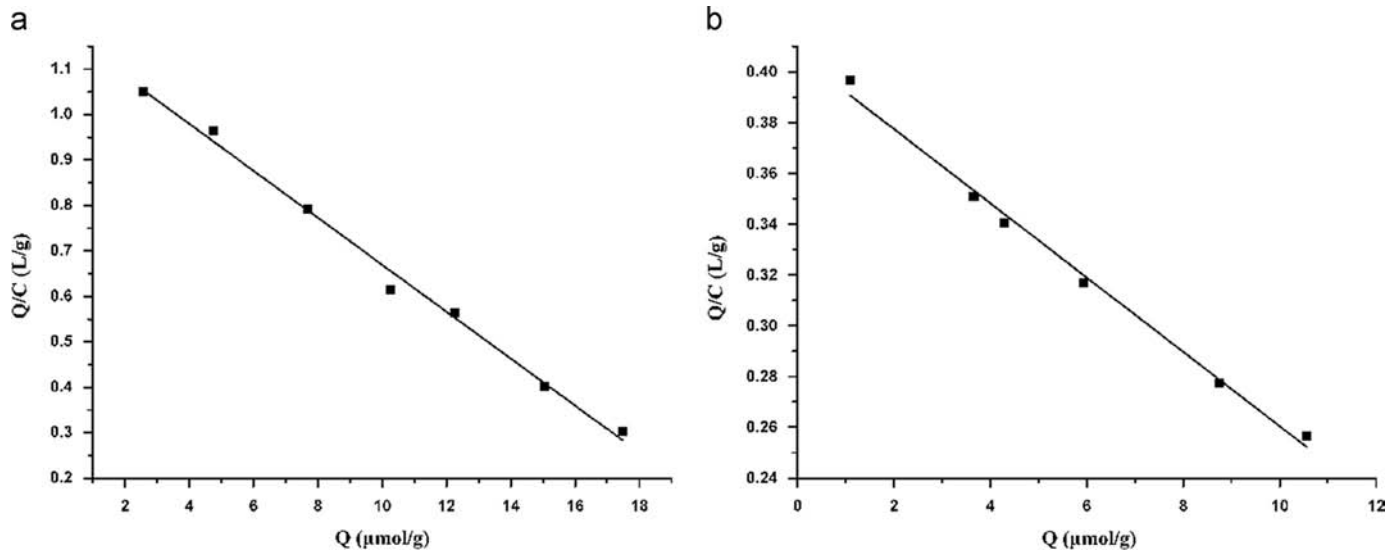

Fig. 7. Scatchard plot analysis of the binding of quercetagetin onto the MMIPs (a) and MNIPs (b).

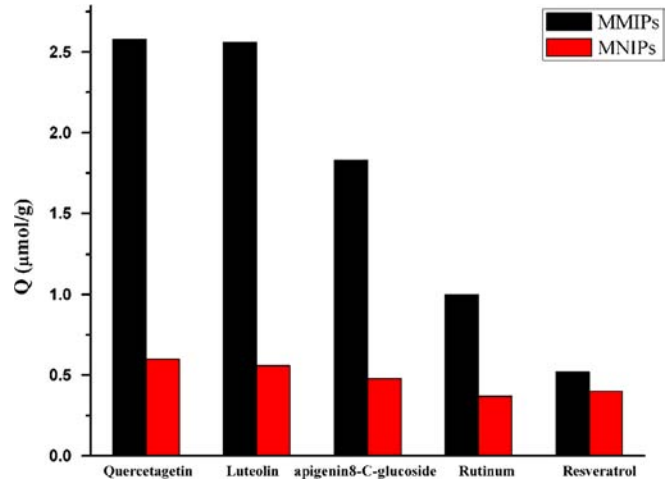

Fig. 8. The specific recognition capability of MMIPs and MNIPs for quercetagetin and the potential interferences.

Table 1
The selectivity parameters of the MMIPs and MNIPs.

	Analyte	k_d QUOTE QUOTE (mL/g)	k
MMIPs	Quercetagetin	236.97	–
	Luteolin	151.21	1.50
	Apigenin 8-C-glucoside	88.77	2.67
	Rutinum	50	4.74
	Resveratrol	50	4.74
MNIPs	Quercetagetin	11.92	–
	Luteolin	9.70	1.23
	Apigenin8-C-glucoside	8.00	1.49
	Rutinum	15.62	0.76
	Resveratrol	5.02	2.37

MMIPs exhibit almost the same degree of selectivity for quercetagetin and lutrolin, which is significantly higher than that of the other potential interferents.

3.5. Extraction and desorption time

The adsorption of templates needs enough time to achieve equilibrium, so optimizing the extraction condition is necessary to improve extraction process. In this work, acetonitrile was chosen as the extraction solvent and different amounts of MMIPs ranging from 5 to 100 mg in 1 mL of acetonitrile with 0.25 mg/L of quercetagetin which was close to the concentration in real sample. After that, 0.5 mL supernatants were removed and evaporated to dryness, then dissolved in 0.1 mL acetonitrile for further HPLC-DAD detection. The equilibrium adsorption amounts of quercetagetin were calculated by the Eq. (1), and the extraction time was investigated from 5 to 60 min. At the beginning, the quercetagetin bounding amounts increased along with the extraction time until it reached a saturation level at 25 min, and there are no obvious increases were observed at prolonged extraction time. Therefore, 25 min was chosen as the optimal extraction time for quercetagetin in the following experiments.

Desorption time was also optimized at the same time. In brief, different time intervals (i.e., 5, 10, 15, 20, 25, 30, 40 and 60 min) were evaluated, respectively. The results showed that the desorptioning amounts of the template molecular did not increased along with the time prolonged after 20 min. Thus, 20 min was set as the optimal desorption time in this work.

3.6. Analytical method validation

Chromatographic method containing a series of experiments about the linear range, correlation coefficient, limit of detection (LOD), and limit of quantification (LOQ) was applied to evaluate the proposed method and validate the analytical methodology in this work. The calibration curve was obtained by the linear regression method and peak areas were plotted versus concentrations. The linear range obtained for the determination of quercetagetin in *Calendula officinalis* ranged from 113 to 3840 μg/L, and the regression equation was $y = 29.619x - 46.618$ with correlation coefficient of 0.9991 for quercetagetin. The LOD and LOQ defined as 3 and 10 times of the signal to noise ratio were 32.66 μg/L and 117.82 μg/L, respectively.

Standard addition method combined with the HPLC-detection was used to evaluate the repeatability, accuracy and the recovery of the MMIPs-HPLC extraction process and the results are showed in Table 2. *Calendula officinalis* extracts was spiked with quercetagetin at three different concentration levels. The recoveries of the spiked *Calendula officinalis* samples for quercetagetin ranged from 82.75% to 95.41% with RSD % values ranging from 3.90% to 7.08%,

Table 2
Accuracy of the method for spiked at different levels ($n=3$).

Sample	Real content (μg/mL)	Added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD (%)
Calendula officinalis extracts	0.22	0.12	0.32	82.75	7.08
		0.2	0.38	88.12	5.66
		0.28	0.46	95.41	3.90

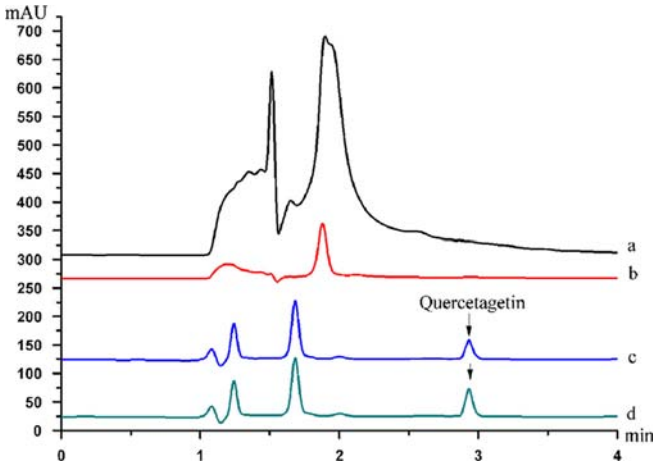

Fig. 9. Chromatography of Calendula officinalis extracts: initial sample (a), solution extracted by MNIPs (b), by MMIPs (c), spiked sample by MMIPs (d).

which demonstrated that the proposed method could be used for the determination of quercetagetin in natural products.

3.7. Analysis in real samples

In this study, we aimed at finding an excellent adsorption material to realize the extraction of quercetagetin from the *Calendula officinalis* with high selectivity. For this purpose, 50 mg MMIPs were used to increase the number of available binding sites to ensure all of the quercetagetin in the *Calendula officinalis* extracts being extracted. The chromatograms of the initial *Calendula officinalis* extracts, extracted by MMIPs and MNIPs, and standard addition in *Calendula officinalis* extracts extracted by MMIPs are shown in Fig. 9. Because of the complex interference of *Calendula officinalis* extracts, quercetagetin could not be determined directly by HPLC at the beginning (Fig. 9a). Quercetagetin peak could not be observed clearly as a result of nonspecific recognition of MNIPs (Fig. 9b), but it was clearly exhibited in Fig. 9c and d as a result of extracted by MMIPs. The content of quercetagetin is found to be 247 mg/kg in dry *Calendula officinalis* by this method. Compared with the traditional analytical method in the analysis of quercetagetin, such as HPLC-UV [37] and HPLC-MS [38], this method exhibited great superiority in selectivity and time-saving, due to the specific recognition sites and the magnetic force of the MMIPs. The results demonstrated that the quercetagetin imprinted magnetic polymers would be used in the determination and separation of quercetagetin in complex TCM samples.

4. Conclusion

In this study, magnetic molecular imprinted polymers were prepared to analyze quercetagetin in complex *Calendula officinalis* extracts samples. The obtained MMIPs were characterized through FT-IR, XRD, and VSM. The MMIPs exhibited excellent selectivity and adsorption capacity for template molecule. In the process of extraction, the selectivity was greatly increased because of the outstanding selectivity of MMIPs, and the separation was simplified due to the magnetic force of the MMIPs. Therefore, the extraction and separation efficiency was significantly enhanced because of the introducing of MMIPs, and the results showed a great potential in the recognition, extraction and separation of quercetagetin from TCM. It can be considered that this method is promising and can be a good alternative to the traditional method.

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