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Synthesis and application of molecularly imprinted poly(methacrylic acid)–silica hybrid composite material for selective solid-phase extraction and high-performance liquid chromatography determination of oxytetracycline residues in milk

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ABSTRACT

A novel molecularly imprinted organic–inorganic hybrid composite material (MIP-HCM) was developed based on molecular imprinting technique in combination with hybrid composite synthesis and sol–gel technology for selective solid-phase extraction (SPE) of tetracyclines residues in milk. The MIP-HCM was prepared using oxytetracycline as the template, methacrylic acid as organic functional monomer, tetraethoxysilane as inorganic precursor and methacryloxypropyltrimethoxysilane as the coupling agent. Synthesis conditions are optimized by changing some factors to obtain sorbent with the controllable adsorption capacity, selectivity, hardness and toughness. Binding study demonstrated that the imprinted hybrid composites showed excellent affinity and high selectivity to oxytetracycline. An enrichment factor of 18.8 along with a good sample clean-up was obtained under the optimized SPE conditions. The average recoveries of three tetracyclines antibiotics spiked milk at 0.1, 0.2 and 0.5 mg kg^{-1} were in the range of 80.9–104.3% with the precision of 1.5–5.0%. The limits of detection and quantitation of the proposed method were in a range of 4.8–12.7 μg kg^{-1} and 16.0–42.3 μg kg^{-1}, respectively.

1. Introduction

Tetracyclines antibiotics (TCs) are widely used in veterinary practice to control disease, promote mass and prolong milk freshness. All these cases raise the possibility that antibiotic residues may remain in edible animal tissues and milk intended for human consumption, and in some cases, these can cause a serious threat to human health, such as allergies, toxic effects and bacterial resistances [1,2]. Therefore, US Food and Drug Administration (FDA), European Union (EU) and Chinese Ministry of Agriculture have established a maximum residue limit (MRL) of 0.1 mg kg^{-1} for tetracycline antibiotics in milk [3–5]. These limits require the development of sensitive and selective methods for antibiotic residues in food to guarantee the safety of food.

For quantification of tetracyclines antibiotics residues in milk, the main analytical methods are based on HPLC with fluorescence [6], ultraviolet [7] or mass spectrometric detection [8,9]. Most of these methods involve a preliminary extraction step followed by a second clean-up and preconcentration step with liquid–liquid extraction (LLE) and solid-phase extraction (SPE) [10]. SPE has appeared as an alternative to LLE owing to its simplicity, low cost and easy automation, coupled to both HPLC and GC [11]. However, the commercially available sorbents, such as alkyl-silica, copolymers and graphitized carbon, usually appear as low selectivity for analytes in the SPE procedure. Consequently, the development of new SPE sorbents with high selectivity and fast kinetics becomes mandatory.

Molecular imprinting technique (MIT) is one of the most promising techniques for sample clean-up and concentration. Molecularly imprinted polymers (MIPs) with high selectivity and affinity to the target molecule have attracted considerable attention in analytical chemistry. Organic polymer-based MIPs are extensively applied due to their excellent pH stability and the easy availability of various monomers [10–15], but they may shrink or swell when exposed to different organic solvent, and thus considerably cause the deformation of the MIPs receptors and decrease the recognition ability towards the template. Molecularly imprinted organic–inorganic hybrid materials (hybrid-based MIPs) have been extensively studied, as the inorganic matrix can offer excellent mechanical strength and good solvent resistance. However, hybrid-based MIPs are prepared using organic metal alkoxide as a functional monomer by conventional hydrolytic sol–gel process, which often requires curing and aging at the higher temperature, and inevitably results in the poor properties due to the cracking and shrinkage of the hybrid-based MIPs [12,16–19].

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As the hybrid composite material (HCM) possesses the advantages of both organic polymers and inorganic ceramics, which combine the superior thermal stability, high hardness and refractive index, low thermal expansion coefficient, and wide range of permittivity of ceramics with the tremendous toughness, ductility, process ability, and crack-deflection properties of polymers [20], an alternative approach to prepare molecularly imprinted organic–inorganic hybrid composite material (MIP-HCM) was developed by combining a molecular imprinting technique with a hybrid composite material synthetic method in order to obtain desired sorbent which is applicable to solid phase extraction. The MIP-HCM is fabricated from a sol–gel hydrolysis process and a pre-polymerization reaction through covalent bonds to yield organic–inorganic hybrid phase that further happen co-polymerization and incorporation of the template molecules into a three-dimensional cross-linked network structure. After removal of the template, molecular cavities with distinct pore size, shape and chemical functionality were remained in the cross-linked host.

Therefore, we attempted to develop a novel MIP-HCM synthetic method and explore the preparation feasibility of a controllable adsorbent with desired adsorption capacity, selectivity, hardness and toughness for applying to molecularly imprinted solid-phase extraction (MISPE). For these purposes, we choose oxytetracycline as the template, methacrylic acid as organic functional monomer, tetraethoxysilane as inorganic precursor, and methacryloxypropyltrimethoxysilane as the coupling agent, which was used to form the covalent bonding between organic and inorganic phases. An oxytetracycline imprinted poly(methacrylic acid)–silica hybrid composite material (MIP-HCM) was synthesized and applied to selective solid-phase extraction for efficient separation and fast enrichment of tetracycline antibiotics residues from milk samples.

2. Experimental

2.1. Reagents

Oxytetracycline hydrochloride (OTC), metacycline (MTC) and doxycycline (DOTC) were purchased from Fluka (Buchs, Switzerland). Fig. 1 shows their chemical structures. Methacrylic acid (MAA) was purchased from Tianjin Chemical Reagent Research Institute (Tianjin, China) and cleaned to remove the inhibitor prior to polymerization. 2,2-Azobisisobutyronitrile (AIBN) was purchased from Beijing Chemical Reagent Company (Beijing, China) and recrystallized from methanol before use. Methacryloxypropyltrimethoxysilane (KH570) and tetraethoxysilane (TEOS) were purchased from Nanjing Lianye Chemical Co., Ltd. (Shanghai, China). All the other chemicals were of the analytical or the HPLC grade and used without further disposal. Doubly deionized water (DDW) was used throughout. Samples for HPLC were filtered through a 0.45 μm membrane filter.

The stock solutions of tetracycline antibiotics were prepared at a concentration of 200 mg L^{-1} in methanol, and diluted to the final concentration with HPLC mobile phase when used. All solutions were stored at −18 °C in a refrigerator and re-prepared every month.

A Na_2EDTA–McIlvaine buffer solution (0.1 M) was prepared by mixing 1000 mL of 0.1 M citric acid with 625 mL of 0.2 M disodium hydrogen phosphate (pH adjusted to 4.0 ± 0.05 with NaOH or HCl as needed), and then 60.5 g of $Na_2EDTA{\cdot}2H_2O$ was added into the above mixture [21].

2.2. Instrumentation and analytical conditions

HPLC analysis was performed using a liquid chromatography system containing a LC-20AT pump, a SPD-20A UV-vis detector and RF-10AXL (Shimadzu, Japan). The analytes were separated in a Venusil XBP C18 column (250 mm × 4.6 mm, 5 μm) from Bonna-Agela Technologies (Tianjin, China). The mobile phase was methanol/acetonitrile/10 mM oxalic acid solution (5:25:70, v/v) and the flow rate was 1.0 mL min^{-1} at 25 °C. Aliquots of 10 μL were injected into the column and the chromatograms were recorded at 350 nm.

SPE was performed in a 12-Ports Vacuum SPE Manifold System (Beijing Peaksharp Analytical Instrument Co., Ltd., China) with vacuum control valve and poly (tetrafluoroethylene) cartridge adapters. The adsorption capacity was measured by T6 UV-vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China) at 350 nm.

2.3. Preparation of the OTC-imprinted poly(methacrylic acid)–silica hybrid composite materials

To prepare the OTC-imprinted poly(methacrylic acid)–silica hybrid composite material, 0.160 g of OTC (as template) dissolved in 1.125 mL of acetonitrile, and mixed with 0.207 mL of MAA. The mixture was incubated for 1 h at room temperature, and then 0.554 mL of KH570 and 0.068 g of AIBN were added following sonication for 2 min. After stirring the mixture under a continuous flow of nitrogen gas for 3 h at 53.8 °C, TEOS (2.73 mL of TEOS was dissolved in 5.60 mL of ethanol and stirred for 1 h) and 0.10 mL of HCl were added. The reaction was allowed to proceed under magnetic stirring at 60.0 °C for 3 h. The resultant was adjusted to pH 6 with sodium hydroxide solution before drying at 65.0 °C. The obtained material was crushed, grounded, and sieved to obtain regularly sized particles between 38.5 and 63.0 μm that were suitable for the evaluation and application of SPE. To ensure the complete removal of the templates, the materials were Soxhlet extracted with a mixture of methanol/acetic acids (4:1, v/v) for 24 h, and then washed with copious methanol. The MIP-HCM was finally dried at 60.0 °C in the vacuum oven for 24 h. A similar procedure without template was used to prepare the non-imprinted polymer (NIP) as control material.

2.4. Rebinding experiment

In order to investigate the binding property of the MIP-HCM, static absorption experiment and Scatchard analysis were employed in this work. 50 mg of the dry polymer (MIP-HCM or NIP) was placed in 10 mL glass flask, and 5 mL of the solution of OTC (concentration varying from 0.2 to 2.8 mg mL^{-1}) was added. All of the solutions were prepared in dichloromethane/methanol (92:8, v/v). The flasks were properly sealed and the mixture was incubated under agitation in a horizontal shaker for 6 h. After that, aliquots of the supernatant were collected after centrifugation, and the OTC was quantified by UV-vis Spectrophotometer. The equilibrium adsorption capacity (Q) was calculated according to Eq. (1):

$$Q = \frac{V(C_0 - C_e)}{m}, \quad (1)$$

where V is the volume of solution (mL); m is the mass of the MIP-HCM; C_0 and C_e are the initial and the equilibrium concentration of OTC in dichloromethane/methanol (92:8, v/v) solution, respectively.

2.5. Molecularly imprinted solid-phase extraction procedure

50 mg of the MIP-HCM and NIP were packed into empty SPE cartridges which are 5 mm in diameter, respectively, and capped with fritted polyethylene disks at the top and bottom. After condition with the following solvents (in order): 3 mL of water/acetic

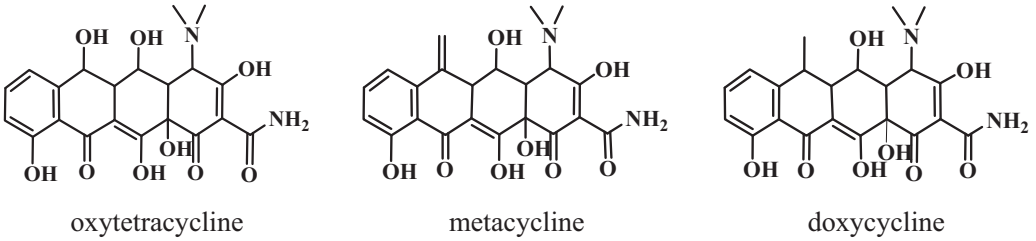

Fig. 1. Chemical structures of oxytetracycline, metacycline and doxycycline.

acid 90:10 (v/v), 3 mL of water, 3 mL of methanol and 3 mL of cyclohexane. 4.0 mL of OTC solution or standard mixture solution in dichloromethane/methanol (92:8, v/v) was passed through the cartridges at a flow rate of 0.2 mL min^{-1}. Then the cartridges were washed with 1 mL of toluene/methanol (85:15, v/v). The OTC was eluted with 2 mL of methanol/acetic acid (60:40, v/v). The collected solution was dried using a gentle stream of nitrogen. The residues were redissolved in the mobile phase and analyzed by HPLC–UV at 350 nm. The cartridges were regenerated with 10 mL methanol/acetic acid (80:20, v/v), dried, and reused in subsequent studies.

2.6. Analysis of OTC in spiked samples

The milk samples were obtained from a local supermarket. 5.0 g of the milk was accurately weighed and placed in a 50 mL centrifuge tube, 20 mL of Na_2EDTA–McIlvaine buffer was added into the sample and thoroughly mixed. Subsequently, ultrasound-assisted extraction was carried out at room temperature for 5 min and the tubes were centrifuged at 4000 rpm for 10 min. The residue was extracted again with 15 mL Na_2EDTA–McIlvaine buffer solution. The supernatants obtained were combined and evaporated at 45 °C by rotary evaporators [21]. The residues dissolved with dichloromethane/methanol (92:8, v/v) to 5 mL and filtration through a 0.45 μm syringe filter, 4 mL of the filtrate was passed through the MIP-HCM cartridges. The above-mentioned MISPE procedure was used to separate and detect TCs in milk. The milk samples were spiked with tetracycline antibiotics at three concentration levels of 0.1, 0.2, 0.5 mg kg^{-1}, and experiments were repeated three times [10,21,22].

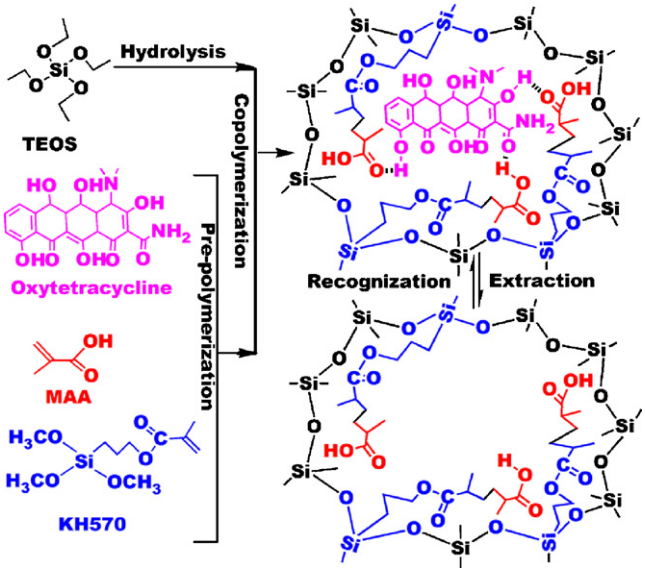

Fig. 2. Synthesis protocol of OTC-imprinted poly(methacrylic acid)–silica hybrid composite materials and recognition mechanism.

3. Results and discussion

3.1. Optimization of preparation conditions and studies of recognition mechanism

The selectivity of the MIP-HCM depends on various factors. The preparation method of the MIP-HCM was optimized by changing some of the preparation factors, including the proportion of organic functional monomer (MAA), inorganic precursor (TEOS), the coupling agent (KH570) to the template; the variety of porogen solvents; the initiator (AIBN) molar amount in the total moles of monomer; the pre-polymerization conditions of MAA and KH570; the hydrolysis conditions of TEOS; aging/drying conditions; the template molecular extracted conditions from the matrix. The possible preparation protocol of the MIP-HCM and recognition mechanism was shown in Fig. 2.

To prepare the MIP-HCM, OTC was dissolved in acetonitrile, and MAA was selected as the functional monomer, which was favorable to hydrogen-bonding interaction during in the solvent. Thus, a stable donor–receptor complex between the template molecule and the functional monomer is formed in the prepolymerization process, which will lead to the formation of the specific binding sites in the polymers [19]. A chain structure pre-polymer was formed by copolymerization of MAA and KH570 in the presence of an initiator and the template molecule. When the pre-polymer mixed with the TEOS hydrolysis solution, a MIP-HCM was prepared. After removal of the template molecule, the specific imprinting sites will be maintained in three-dimensional network structure.

When the ratio of OTC/MAA was 1:4, the MIP-HCM exhibited no selectivity for OTC; when the OTC/MAA was 1:16, the MIP-HCM showed lower adsorption capacity and flow rate in SPE procedure; only when the OTC/MAA was 1:8, did the MIP-HCM have both recognition ability and suitable column pressure. The change of the molar ratio of inorganic precursor and organic functional monomer will cause a remarkable alteration of adsorption capacity. The inorganic precursor is employed to improve the structural order and mechanical strength of MIP-HCM while the organic functional monomer controlled active group of the template cavity. When the molar of MAA/TEOS was 1:4, the polymer exhibited high uptake capacities (64.80 mg g^{-1}) for OTC. The optimum ratio of OTC/MAA/KH570/TEOS at 1:8:8:32 was chosen for preparing the MIP-HCM in the following investigations.

3.2. Characteristic of the FT-IR spectra and TGA analysis

To ascertain the presence of organic–inorganic hybrid phase in the MIP-HCM, FT-IR was employed to characterize the MIP-HCM (Fig. 3b) in comparison with silica gel (Fig. 3a) and (Fig. 3c) polymethacrylic acid (PMAA). As shown in Fig. 3a and b, the observed feature around 1133 cm^{-1} indicated Si—O—Si stretching vibrations; OH vibration was reflected at 2960 and 1642 cm^{-1}; The bands around 795 and 457 cm^{-1} resulted from Si—O vibrations. The observed features around 1719 and 2960 cm^{-1} of C=O and C—H stretching vibrations, respectively, introduced by PMAA also

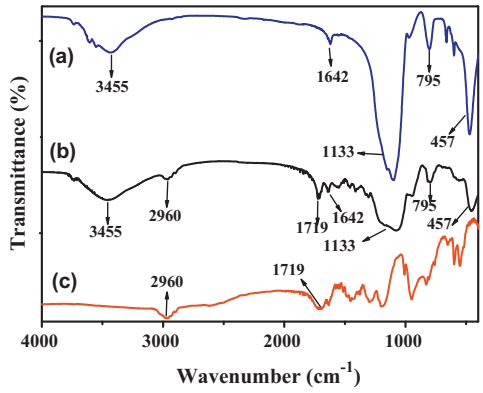

Fig. 3. FT-IR spectra of silica (a), MIP-HCM (b) and PMAA (c).

appeared in the spectrum of the MIP-HCM. These results suggest that the organic phase was covalently bonded to the silica matrix.

Fig. 4 shows the results of differential thermo-gravimetric analysis of PMAA and MIP-HCM. The MIP-HCM appears a slight decrease at 300 °C due to the removal of the adsorbed water and solvent. And a major weight loss of the polymer at onset temperature of about 350 °C, which is attributed to the decomposition of the polymers, while the PMAA present abrupt decrease in weight at approximately 300 °C. The weight of residue at 470 °C is the inorganic silica content in the MIP-HCM, and the weight retention is about 70%, which exhibits good heat resistance. The thermal stabilities of the hybrid polymers was better than that of PMAA, since the ordered alignment of polymer chain was melded by the cross-linking between inorganic precursor and organic functional monomer which delayed and prevent the thermal decomposition of the polymers [23,24]. The morphological characteristics of the MIP-HCM were investigated by the nitrogen adsorption experiment in different synthesis solvents (acetonitrile and ethanol) and the SEM experiment in comparison with MIPs, the results showed no significant difference.

3.3. Binding study and Scatchard analysis

The static equilibrium adsorption experiments for the MIP-HCM and NIP were carried out by varying the initial concentrations of OTC in the range of 0.2–2.8 mg mL^{-1}. The binding isotherm of OTC onto MIP-HCM was shown in Fig. 5a. The adsorption of MIP-HCM increased with increasing of the initial concentration. In general, the Scatchard plot is used for the evaluation of adsorption parameters. Furthermore, the Scatchard plot can indicate how many kinds of binding sites exist in the MIP-HCM. The average binding data of triplicate independent results can be linearly transformed

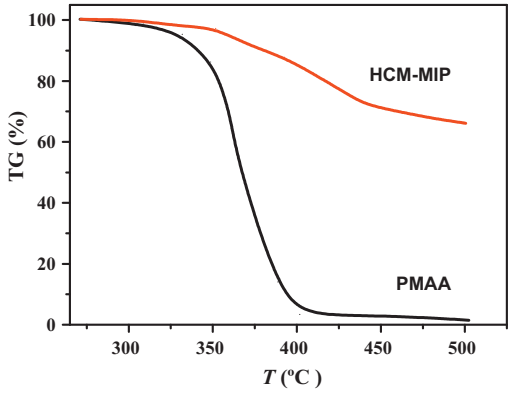

Fig. 4. TG curves of the MIP-HCM and PMAA.

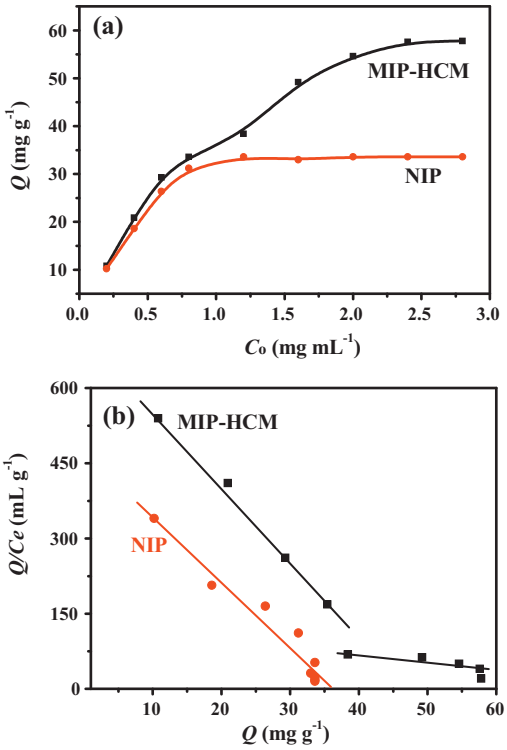

Fig. 5. Binding isotherm (a) and Scatchard analysis of the binding of OTC onto the MIP-HCM (b).

according to the Scatchard equation ($Q/C_e = (Q_{max} - Q)/K_d$). The results indicated that there were two different binding sites in the MIP-HCM and only one kind of binding site in the NIP (Fig. 5b). In Fig. 5b, the fitting liner equation for the MIP-HCM: $Q/C_e = 714.23 - 15.31Q$ and $Q/C_e = 299.37 - 4.62Q$, the equilibrium dissociation constants (K_d) of 6.5×10^{-2} and 2.2×10^{-1} mg mL^{-1}, and the apparent maximum binding capacities (Q_{max}) of 46.65 and 64.80 mg g^{-1} could be calculated from the slope and the intercept of the linear equation. Similarly, K_d (7.8×10^{-2} mg mL^{-1}) and Q_{max} (36.49 mg g^{-1}) were calculated from the fitting liner equation ($Q/C_e = 470.78 - 12.90Q$) for the NIP (Fig. 5b). It indicated that the specific affinity and the binding capacity of the MIP-HCM are significantly larger in comparison with the NIP.

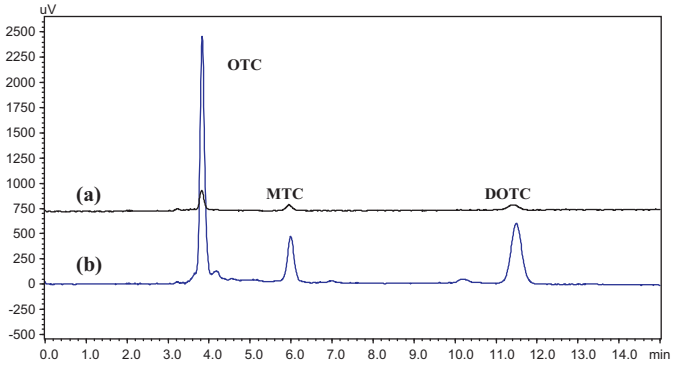

Fig. 6. Chromatograms of (a) the mixture standard solution (0.1 mg L^{-1}) and (b) the eluate of 20 mL of the mixture standard solution through the MIP-HCM cartridge. Mobile phase: methanol/acetonitrile/10 mM oxalic acid solution (5:25:70, v/v). Flow rate: 1.0 mL min^{-1}. Samples spiked concentration: 0.1 mg kg^{-1}. Injection volume: 10 μL.

Table 1
The linear regression equations, correlation coefficient (r), limit of detection (LOD) and enrichment factors (EF) of three TCs.

	Analyte	Linear equation	r	Linear range (mg L^{-1})	EF	LOD (μg L^{-1})
Standard solution	OTC	$Y = -48 + 1.55 \times 10^4 C$	0.9998	0.075–1.0		17
	MTC	$Y = -233 + 7.58 \times 10^3 C$	0.9989			50
	DOTC	$Y = -245 + 1.09 \times 10^4 C$	0.9986			58
Enrichment of MISPE	OTC	$Y = -5241 + 2.92 \times 10^5 C$	0.9716		18.8	1.4
	MTC	$Y = -4244 + 1.21 \times 10^5 C$	0.9409		16.0	7.6
	DOTC	$Y = -2676 + 1.15 \times 10^5 C$	0.9527		16.1	5.6

3.4. Molecularly imprinted solid-phase extraction

In order to optimize the selectivity of MISPE, conditioning, loading, washing and elution steps were evaluated and optimized. After pre-conditioning, the loading step was optimized. 4.0 mL of different loading solvents including methanol (MeOH), water, dichloromethane (DCM)/MeOH, chloroform/MeOH and ACN/MeOH was passed through cartridges packed with 50 mg of the MIP-HCM. It was shown that OTC was retained on the MISPE column (loading concentration is less than 0.4 mg mL^{-1} of OTC) and 40.6 mg g^{-1} of the column capacity (0.8 mg mL^{-1} of OTC) were obtained when DCM/MeOH (92:8, v/v) was used as loading solution, whereas the column capacities were 19.3, 15.5, 0.36 and 11.2 mg g^{-1}, respectively with MeOH, water, chloroform/MeOH and ACN/MeOH as loading solution, and nonspecific binding on the NISPE ranged from 80.2% to 88.5%. The amount of MeOH and DCM in the loading solution was investigated. The results show that the adsorption capacity increased as the amount of methanol decreased. However, when the methanol content was less than 8%, the OTC in DCM/MeOH solution was not stable, which would emerge crystalline particles. The results showed that the selectivity of the MIP-HCM is obtained mainly by hydrogen bonding interaction (specific interaction) and the electrostatic interactions is the mainly interactions (non-specific interaction).

In order to enhance the selectivity of MIP and decrease the cross-reactivity, the washing step was optimized. It is well known that the template could be retained on the MIP-HCM by selective and nonspecific interactions. Thus, a washing solution with moderate elution strength was used to damage the nonspecific interactions and to let the target analyte be retained by specific interactions [10]. After loading the cartridge with 4.0 mL of 0.4 mg mL^{-1} of OTC in DCM/MeOH (92:8, v/v), several solvents such as MeOH, DCM, chloroform and toluene were investigated as washing solvents, and the percentage of OTC removed from MIP-HCM were 48%, 10%, 12% and 0%, respectively. Consequently, different proportions and volumes of toluene and methanol were investigated. The better result was obtained using 1 mL of toluene/methanol (85:15, v/v) as the washing solvents.

The eluting step was optimized based on the principle of elution that the analytes could be eluted completely by a small volume (1–2 mL) of strong solvent, while stronger than the analytes as much as possible to retain the impurities remain in the MISPE column. A series of elution solutions, water, MeOH and ACN with HAc in different proportions were used to optimize the eluting conditions. The best recovery was obtained by using 3 mL of methanol/acetic acid (60:40, v/v) as eluting solution.

3.5. Preconcentration and clean-up

The MIP-HCM can also recognize other tetracycline antibiotics on account of their similar structure. So a mixture standard solution of the tetracycline antibiotics (OTC, MTC and DOTC) with the MRL (0.1 mg L^{-1}) concentration was used as the loading solution to evaluate the enrichment ability of MISPE. 20 mL of 0.1 mg L^{-1} mixture standard solution in dichloromethane/methanol (92:8, v/v) was loaded onto the MISPE column and extracted in accordance with Section 2.5. The residues were redissolved in 1 mL of the mobile phase and analyzed by HPLC–UV at 350 nm. The enrichment factors (EF) obtained by comparing the slopes of the linear portion of the calibration curves before and after the MISPE was 18.8, 16.0 and 16.1 (Table 1) for OTC, MTC and DOTC, respectively, and enrichment chromatograms of MISPE were shown in Fig. 6. It demonstrates that a real contribution of selectivity and enrichment for target analytes is brought by the MIP-HCM.

In order to investigate the potential of the MIP-HCM for the selective entrapment of target analyte from complex milk samples, satisfactory sample clean-up was achieved by the MISPE. Fig. 7 shows the chromatograms obtained for a blank milk sample (Fig. 7a), a spiked milk (Fig. 7b) and a spiked milk with a clean-up of MISPE (Fig. 7c). As it can be observed in the chromatograms, after MISPE pretreatment, the concentration of the tetracycline antibiotics was high enough to be quantitatively analyzed while it was too low to be quantitated without MISPE. Moreover, the baselines' noises in Fig. 7c were much less than those in Fig. 7a and b. The clean-up effect of the MIPs (Fig. 7d) and HLB Oasis cartridge [25] are similar to the MIP-HCM, but the swelling and hardness of MIPs

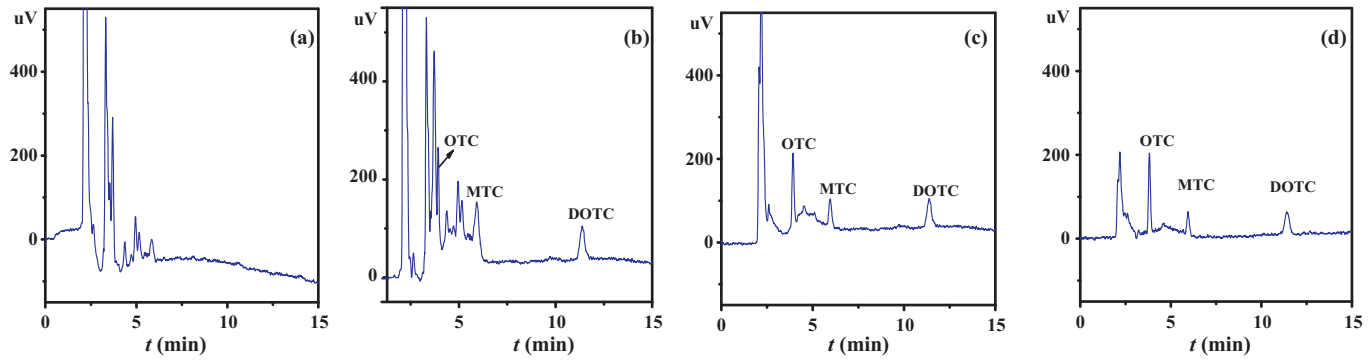

Fig. 7. Chromatograms obtained from the extraction of oxytetracycline, metacycline and doxycycline from the milk samples: (a) blank milk (non-spiked); (b) spiked milk; (c) spiked milk with a clean-up of MISPE (MIP-HCM) and (d) spiked milk with a clean-up of MISPE (MIPs). Mobile phase: methanol/acetonitrile/10 mM oxalic acid solution (5:25:70, v/v). Flow rate: 1.0 mL min^{-1}. Samples spiked concentration: 0.1 mg kg^{-1}. Injection volume: 10 μL.

Table 2
Average recoveries (R), relative standard deviations (RSDs, $n = 3$), limit of detection (LOD) and limit of quantitation (LOQ) of three TCs obtained after MISPE of the spiked milk samples ($n = 5$).

Analyte	Spiked level (mg kg^{-1})	Detected (mg kg^{-1})	$R \pm$ SD (%)	RSD (%)	LOD[a] (μg kg^{-1})	LOQ[b] (μg kg^{-1})
OTC	0.1	0.104	104.3 ± 4.1	2.0	4.8	16.0
	0.2	0.190	95.0 ± 1.6	1.5		
	0.5	0.491	98.3 ± 1.9	1.7		
MTC	0.1	0.082	81.7 ± 3.2	4.2	12.7	42.3
	0.2	0.163	81.5 ± 2.2	4.7		
	0.5	0.493	98.6 ± 2.1	3.6		
DOTC	0.1	0.081	80.9 ± 4.2	4.9	12.7	42.3
	0.2	0.172	85.8 ± 2.3	4.3		
	0.5	0.430	86.1 ± 2.6	5.0		

[a] LOD calculated as 3 times the signal-to-noise ratio.
[b] LOQ calculated as 10 times the signal-to-noise ratio.

limit the flow rate of SPE and the reuse. The impurity which co-elutes with the TCs is nearly completely removed after the clean-up step as can be observed in Fig. 7c. It confirmed that satisfactory sample clean-up was achieved by the MISPE when applied to a complex matrix.

3.6. Determination of tetracyclines antibiotics by MISPE-HPLC

To demonstrate the feasibility of using MISPE to extract TCs from the milk samples at MRL levels (0.1 mg L^{-1}), 5 g of the milk samples was treated using the protocol described in the experimental section [21]. The mean recoveries of OTC, MTC, and DOTC in milk evaluated by three spiking samples with different concentrations (0.1, 0.2 and 0.5 mg kg^{-1}) were 95.0–104.3%, 81.5–98.6% and 80.9–86.1%, respectively, with relative standard deviations (RSDs) of 1.5–5.0% (Table 2). The limits of detection (LOD, S/N = 3) and the limits of quantitation (LOQ, S/N = 10) of the proposed method were 4.8 and 16.0 μg kg^{-1} for OTC, 12.7 and 42.3 μg kg^{-1} for MTC, 12.7 and 42.3 μg kg^{-1} for DOTC, respectively.

4. Conclusions

We have demonstrated successful fabrication of new molecularly imprinted organic–inorganic hybrid composite material for selective solid-phase extraction (SPE) of tetracycline antibiotics residues in milk. By coupling molecular imprinting technique to hybrid composites synthesis technology and sol–gel technology, this method provides the flexible reaction conditions, which can improve adsorption capacity, selectivity, hardness and toughness by changing some factors, such as the molar ratio of inorganic precursor and organic functional monomer. We expect that synthesis and application protocol of the molecularly imprinted organic–inorganic hybrid composite materials is promising as a general strategy for the fabrication of high selective imprinted hybrid composite materials and solid-phase extraction of veterinary drug residues in food.

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