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Grafting of norfloxacin imprinted polymeric membranes on silica surface for the selective solid-phase extraction of fluoroquinolones in fish samples

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ABSTRACT

A high-density imprinted polymeric membrane was immobilized on a porous silica-gel support by polymerization of methacrylic acid with vinyl-silanized silica gel for the selective solid-phase extraction (SPE) of norfloxacin (NOR) in fish samples. The imprinted membranes showed high adsorption capacity (423.2 μmol g^{-1}), significant selectivity (k = 14.64, k' = 9.61) and good site accessibility (2 h) for NOR. The conditions of SPE were investigated, and water (pH = 6), methanol:water (1:1, v/v) and methanol–acetic acid–trifluoroacetic acid (90:9:1, v/v) were selected as the sample solvent, the washing solution and the eluting solution, respectively. Under the optimal SPE condition, three fluoroquinolone residues in fish were separated and detected by an off-line MIP–SPE–HPLC with better clean up and enrichment. The recoveries of norfloxacin, ofloxacin and ciprofloxacin were 89.3–94.8%, 69.3–102.8% and 85–90.5%, respectively, with relative standard deviations lower than 6.5%. The limits of detection (LOD) of the proposed method (S/N = 3) were in a range of 2.65–3.65 μg kg^{-1}, and the limits of quantification (LOQ, S/N = 10) were in a range of 8.82–12.16 μg kg^{-1}.

1. Introduction

Molecularly imprinted technique (MIT) is an increasingly developing technique for preparing polymers with desired and pre-determined selectivity, and provides specific binding sites or catalytic sites in molecularly imprinted polymers (MIPs) [1]. In the last few years, MIPs were widely used for the selective enrichment and pre-treatment of target compounds existing in complex matrix [2–4]. The conventional method is to synthesize the MIPs in bulk thermal- (or photo-) polymerization that produces a monolithic polymer that has to be grinded and sieved, which results in irregularly shaped materials with heterogeneous size and porosity. Moreover, the template and the binding sites are totally embedded in the polymer matrix. Although this technique has led to highly selective materials for a multitude of analytes, such materials are not well suited as packing materials for solid-phase extraction (SPE), owing to high backpressures and low mass transfer kinetics [2,5–8]. Surface imprinting technique can overcome these problems. Surface-imprinted materials have many advantages: the sites are more accessible; mass transfer is faster; the elution of the template is easier; and the binding kinetics is faster [6–16].

In order to improve the efficiency of molecular imprinting, the different imprinting methods were investigated. Sol–gel-based surface-imprinted polymers were prepared on the surface of silica gel particles by sol–gel process using alkyl silanoxides (such as 3-aminopropyltrimethoxysilane, trimethoxypropylsiane, phenyltrimethoxysilane) as a functional monomer [2,6–8]. With this procedure, the thickness of the polymer layer is difficult to control. This problems can be overcome by the grafting technique that the MIPs graft on preformed support materials of known morphology, such as the grafted initiator groups technique [9,10], the iniferter modified supports technique [11–13] and the grafted vinyl functional monomer technique [14–16]. These methods can produce the highly selective occurrence of polymerization on the surface of support. However, the grafting process involves in a complicated chemical procedure and a low grafting densities, which reduce the reproducibility of molecular imprinting process [14]. In the grafted vinyl functional monomer technique, the imprinting polymerization was directed by the end vinyl groups located at particle's surface to form the MIPs layer, and the process could be completed easily through simple free radical polymerization, but vinyl group grafting efficiency limits the synthesis of high-performance imprinting layer. Thus, the further development of the silica gel surface imprinting technique by a simple and reliable method for solid-phase extraction remains a challenge [14].

In the present paper, a norfloxacin-imprinted membrane on the surface of silica particles (MIP-Silica) was prepared by polymerization of methacrylic acid with vinyl-silanized silica gel. The proposed method was used to produce the MIP-Silica with optimal shell thickness, large capacity, high selectivity and fast kinetics of binding norfloxacin (NOR), and the MIP-Silica showed promising

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performances as sorbents for SPE. An off-line MIP–SPE–HPLC method was established for efficient separation and fast enrichment of NOR from fish samples.

2. Experimental

2.1. Reagents and chemicals

Silica gel (80–120 mesh, Qingdao Ocean Chemical Company, Qingdao, China) was used as the support to prepare the NOR-imprinted functionalized sorbent. Vinyltirethoxysilane (VTS) was purchased from Nanjing Lianye Chemical Co., Ltd. (Shanghai, China). 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 recrystalized from methanol before use. Ofloxacin (OFL), norfloxacin hydrochloride (NOR) and ciprofloxacin hydrochloride (CIP) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All the other chemicals were of the analytical or the HPLC grade and used without further purification. Doubly deionized water (DDW) was used throughout. Solutions for HPLC were filtered through a 0.45 μm membrane filter.

2.2. Instrumentation

The analytical HPLC system used throughout this study consisted of a LC-20AT pump and a SPD-20A UV–vis detector (Shimadzu, Japan). The analytes were separated on a Venusil XBP C18 column (150 mm × 4.6 mm, 5 μm) from Bonna-Agela Technologies (Tianjin, China). The mobile phase was acetonitrile (ACN)–13 mmol L^{-1} tetrabutylammonium bromide (TBAB) solution (6:94, v/v) and the flow rate was 0.8 mL min^{-1} at ambient temperature. Aliquots of 10 μL were injected into the column and the chromatograms were recorded at 272 nm.

2.3. Preparation and chemical modification of silica particles

Silica particles were first activated by reflux in hydrochloric acid for 4 h to remove any adsorbed metal ions, then filtered, washed repeatedly with de-ionized water simply removes the acid and dried in a nitrogen stream at 160 °C for 4 h.

To prepare the modified silica, 10 g of the dried silica particles was dispersed into 100 mL of dry toluene followed by dropwise addition of 5 mL VTS under nitrogen, and refluxed for 24 h at 110 °C. The modified silica particles were filtered and packed into the column and dried under a nitrogen stream at 160 °C for 2 h, and then washed successively with toluene and methanol, and dried in the vacuum at 60 °C for 4 h.

2.4. Imprinting of NOR molecules on the surface of the modified silica

Immobilization of the imprinted polymer on the modified silica was carried out according to Fig. 1. Norfloxacin (0.3193 g, 1 mmol) and MAA (0.34 mL, 4 mmol) were dissolved in 15 mL of chloroform while stirring. After stirring the mixture for 1 h, 2.16 g of the modified silica and 16.4 mg of AIBN were added to the mixture under nitrogen. The suspension solution was stirred at room temperature for 0.5 h, then the temperature gradually rose to 60 °C and remained at this temperature for an additional 24 h under stirring and nitrogen. The product was recovered by filtration and washed with acetic acid/ethanol (2:8, v/v) for 3 times to remove NOR templates, unreacted monomers and cross-linker. To ensure the complete removal of the template, the material was Soxhlet extracted with a solution

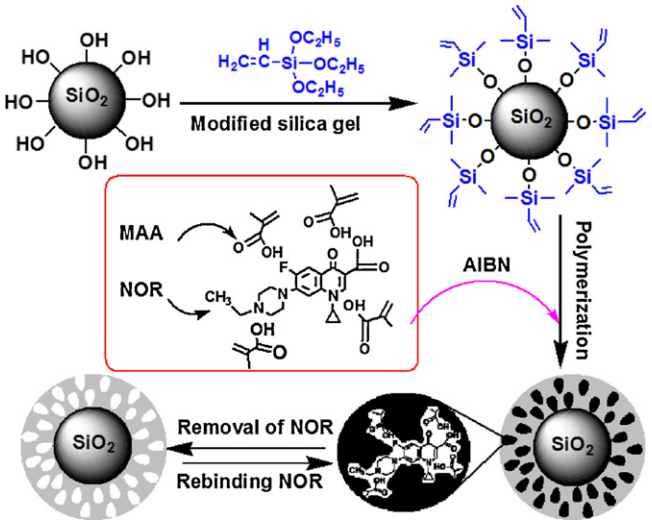

Fig. 1. Preparation protocol of the immobilization of the imprinted polymer on the silica-gel support.

of ethanol–acetic acid (4:1, v/v) for 24 h, and then washed with copious amount of methanol. The resultant NOR-imprinted silica particles (MIP-Silica) were finally dried at 60 °C in the vacuum for 24 h. A similar procedure without template was used to prepare non-imprinted modified silica (NIP) as control materials.

2.5. Static adsorption test

20 mg of MIP-Silica or NIP were mixed with 3.0 mL of water containing different concentrations of NOR (from 0.5 to 5 mmol L^{-1}). The mixtures were incubated for 2 h with continuous shaking in a horizontal shaker at room temperature. After incubation, the mixtures were filtered through 0.45 μm microporous membranes and the amount of unextracted NOR were determined by UV-vis Spectrophotometer.

Uptake kinetics of NOR by the imprinted silica sorbent was also examined. 20 mg of imprinted silica gel were added into 3 mL of water containing 1.5 mmol L^{-1} of NOR. The concentration of NOR at different time interval (1–12 h) was determined.

According to static adsorption procedure, adsorption and competitive recognition studies were performed with the mixture standard solution of NOR and structurally similar compounds OFL at the 1 g L^{-1} level.

2.6. MISPE procedure

A 50 mg of the MIP-Silica or NIP, respectively, was packed into 1 mL SPE syringe barrels, and capped with fritted polyethylene disks at the top and at the bottom. After the cartridge was conditioned with the following solvents (in order): 2 mL of methanol–acetic acid (4:1, v/v), 4 mL of methanol and 2 mL of water (pH 6), 5 mL of 1.5 mmol L^{-1} NOR solution was loaded onto the MISPE column and the NMIP–SPE column with the speed of 0.5 mL min^{-1}. Then the columns were washed with 2 mL methanol–water (1:1, v/v) and eluted with 2 mL of methanol (MeOH)–acetic acid (HAc)–trifluoroacetic acid (TFA) (90:9:1, v/v). The collected solutions were analyzed by HPLC–UV detection at 272 nm.

2.7. Determination of NOR in the fishes [17]

The fish samples were obtained from a Huiyou supermarket (Baoding). The samples were de-scaled, and the edible muscle

minced and homogenized using a kitchen blender. 5 g of fish edible tissue were weighed into a 50 mL centrifuge tube. After adding 25 mL of acetonitrile and 5 g of anhydrous sodium sulfate, the mixture was homogenized for 2 min using the homogenizer, then it was extracted by ultrasonication for 10 min and the tubes were centrifuged at 4000 rpm for 5 min. The residue was extracted twice with 15 mL acetonitrile. The supernatants obtained were combined and extracted by 15 mL hexane, and the hexane layer was abandoned. After solvent was removed with nitrogen in a hot water bath at 35 °C, the residues were dissolved in 2 mL of water and filtered through a 0.45 μm syringe filter. The filtrate was passed through the MIP-Silica cartridges. The above-mentioned MISPE procedure was used to determine NOR in fish samples in combination with HPLC. The fish extracts were spiked with fluoroquinolones at three concentration levels of 0.05, 0.1, 0.2 mg kg^{-1}. Experiments were repeated three times.

3. Results and discussion

3.1. Preparation of the NOR-imprinted silica gel sorbent

Silica gel is an amorphous inorganic polymer having siloxane groups (Si—O—Si) in the bulk and silanol groups (Si—OH) on its surface. The surface silanol groups facilitate the introduction of the organic groups that covalently bind to the silica surface. As the commercial silica gel contains a low concentration of surface silanol groups suitable for modification, the activation of silica gel surface is necessary. In this work, hydrochloric acid was used for the activation of silica gel.

After the introduction of VTS groups on the silica-gel surface, the immobilization of the polymer on the modified silica gel surface was carried out. The number of double bonds on the silica particles was determined through catalytic bromine addition [14]. The modified silica was dried with a stream of nitrogen at 160 °C to replace vacuum drying. The result was shown that the contents of double bonds at the silica gel surface significantly improved from 43 to 86 μmol g^{-1}. The complex was formed between NOR and MAA, then hydrolyzed and condensed with the activated silica gel without other cross-linker. Fig. 1 shows the possible preparation protocol of the NOR-imprinted silica sorbent. The template was removed leaving the tailor-made cavities, which own the geometrical and binding properties of the template in the MIP-Silica.

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

To ascertain the presence of VTS in the functionalized silica gel sorbents, FT-IR spectra were obtained from activated silica gel, the modified silica and NOR-imprinted silica sorbents. As shown in Fig. 2a, the observed peaks around 1108 cm^{-1} and 956 indicated Si—O—Si and Si—O—H stretching vibrations, respectively. OH vibration was reflected at 3425 and 1630 cm^{-1}. The bands around 795 and 473 cm^{-1} resulted from Si—O vibrations. Compared to activated silica gel, the double bond group of VTS-modified silica were confirmed by absorption peaks at 3014 and 1671 cm^{-1} for the =C—H and C=C stretching vibrations, and the infrared absorption intensity of silanol groups obviously decreased in Fig. 2b. These results suggest that —C=C be grafted onto the surface of activated silica gel after modification. A characteristic peak of the NOR-imprinted silica sorbents compared with activated silica gel is C=O band around 1729 cm^{-1} and saturated C—H band around 2923 cm^{-1} in Fig. 2c.

The thermal stabilities of the membranes were investigated by means of thermo-gravimetric analysis (TGA) in nitrogen atmosphere and the diagrams obtained are shown in Fig. 3. Fig. 3 shows a two-stage mass loss curve in the temperature range of 50–600 °C at a heating rate of 10 °C min^{-1}. The initial decomposition temperatures (IDT) below 150 °C (about 5% weight losses) were responsible for the removal of physically adsorbed water and solvent residues. The thermal degradation temperatures (T_d) are in the range of 340–430 °C, the steep decrease is attributed to the thermal decomposition of the imprinted layer and the modified silica. In addition, the loss weights (wt%) of the imprinted layer and the modified silica were about 10.2% and 2.1%, respectively.

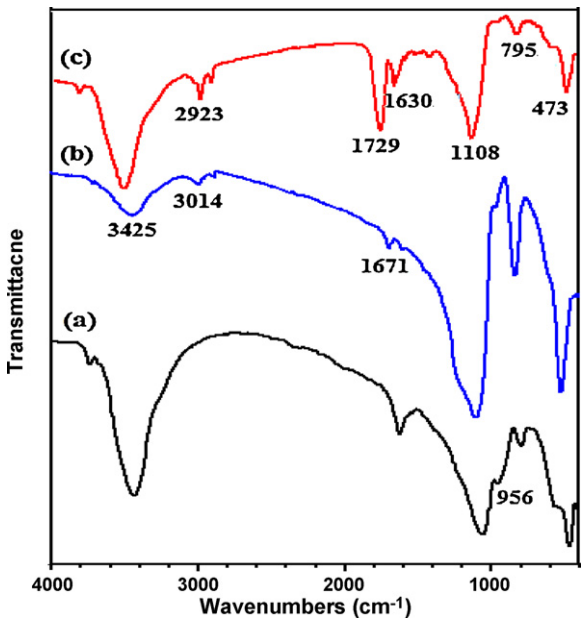

Fig. 2. FT-IR spectra of the activated silica gel (a), the modified silica (b) and the MIP-Silica (c).

3.3. Evaluation of the adsorption characteristic of NOR-imprinted silica sorbents

The static equilibrium adsorption experiments for the imprinted and non-imprinted polymers were carried out by varying the initial concentration of NOR in the range of 0.5–5 mmol L^{-1}. Adsorption saturated around 4 mmol L^{-1} (Fig. 4a). For NIP, the adsorption reached saturation when the concentration of NOR beyond 2.5 mmol L^{-1}. Obviously, the adsorption capacity of MIP-Silica was higher than that of NIP by the specific binding sites in cavities.

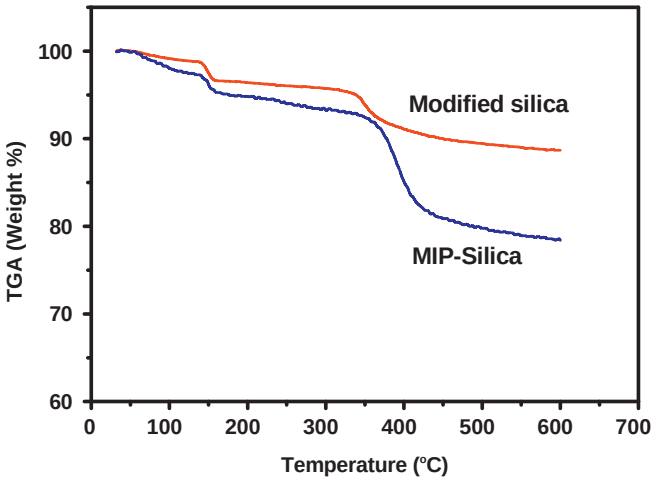

Fig. 3. Thermogravimetic weight loss curves of the modified silica and the MIP-Silica.

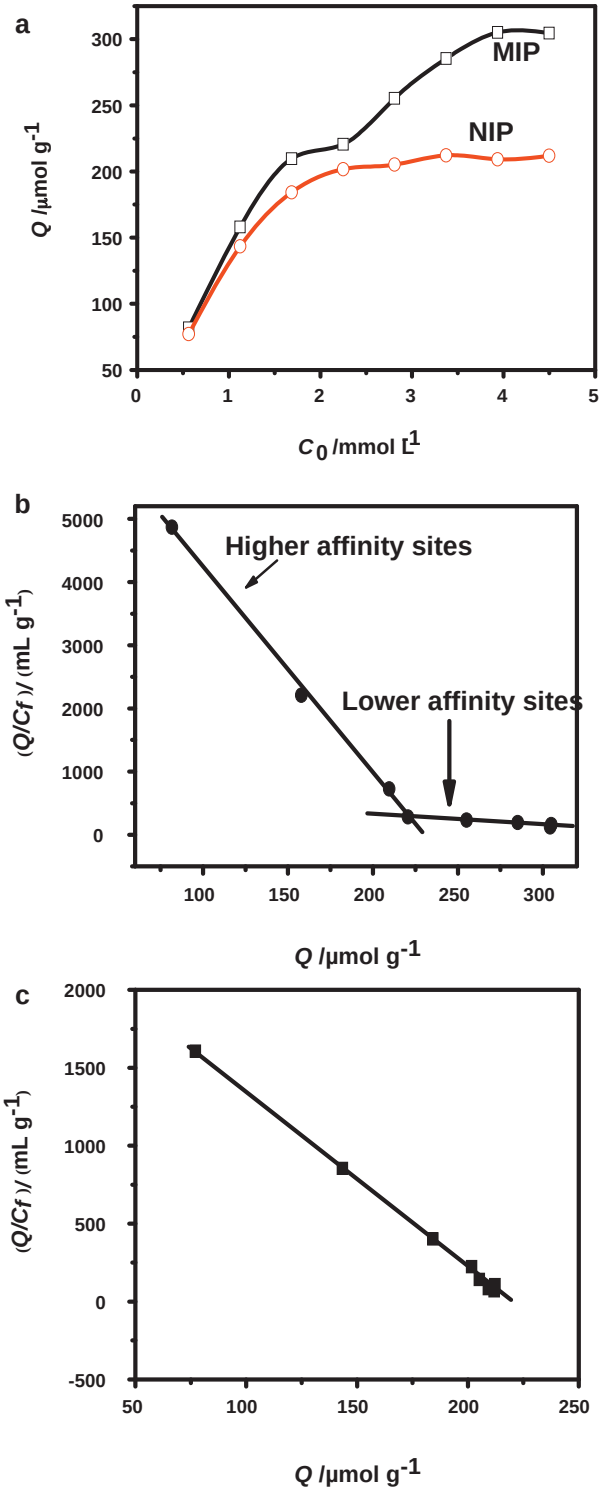

Fig. 4. Binding isotherm (a) and Scatchard plot analysis of the binding of NOR onto the MIP-Silica (b) and NIP (c).

In general, the Scatchard plot is used for the evaluation of adsorption parameters. The Scatchard equation [18] can be described as $Q/C_f = (Q_{max} - Q)/K_d$, where K_d is an equilibrium dissociation constant, C_f is the remaining free analytes concentration in the supernatant, Q is the binding capacity and Q_{max} is an apparent maximum binding capacity. When Q/C_f is plotted versus Q, K_d and Q_{max} can be estimated from the slope and the intercept, respectively. The average binding data of triplicate independent

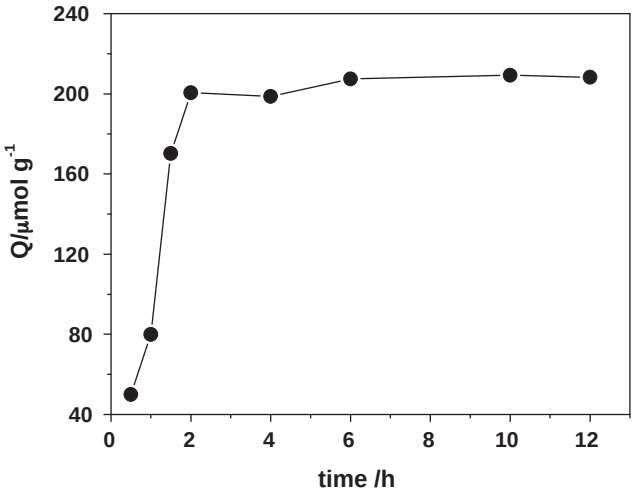

Fig. 5. The kinetic uptake of NOR molecules by NOR-imprinted silica sorbent. The amounts of rebinding NOR were measured by suspending 20 mg of imprinted sorbents in 3 mL water solution containing 1.5 mmol L^{-1} NOR.

results can be linearly transformed according to the Scatchard equation, and the result showed that there are double lines for the MIP-Silica (Fig. 4b), and only one line for NIPs (Fig. 4c). It indicated that there were two different binding sites in the MIP-Silica, and only one kind of binding site in the NIP. In Fig. 4b, the fitting liner equation: the steeper line measures the high affinity sites (specific binding sites) $Q/C_f = 7487.62 - 32.63Q$ (correlation coefficient $R = 0.9972$) and the flatter line measures the low affinity sites (non-specific binding sites) $Q/C_f = 584.1 - 1.38Q$ (correlation coefficient $R = 0.9814$). $K_{d1} = 3.065 \times 10^{-5}$ mol L^{-1}, $Q_{max1} = 273.3$ μmol g^{-1}, $K_{d2} = 7.246 \times 10^{-4}$ mol L^{-1}, $Q_{max2} = 423.2$ μmol g^{-1} can be calculated from the slope and the intercept of the linear equation. K_{d2} was about 10 times as much as K_{d1}. It showed that the MIP-Silica had high affinity and selectivity for NOR. Similarly, the equilibrium dissociation constant (8.834×10^{-5} mol L^{-1}) and the maximum binding capacity (219.1 μmol g^{-1}) were calculated from the fitting liner equation ($Q/C_f = 2480.4 - 11.32Q$, correlation coefficient $R = 0.9984$) for the NIP (Fig. 4c). It indicated that the non-imprinted silica gel has a lower binding capacity for NOR.

The kinetic uptake of norfloxacin by the MIP-Silica at different time intervals was investigated. As shown in Fig. 5, 95.82% uptake of NOR was achieved within 2 h. The silica surface imprinted sorbents required a shorter time, which is mainly attributed to more recognition sites that are situated at the surface or in the proximity of the surface, thus promoting the diffusion of targeted species into the recognition sites.

3.4. Selectivity of the imprinted sorbent

The structurally similar compound, ofloxacin, was chosen as the competitive species with NOR for the selective recognition

Table 1
Competitive loading of NOR and OFL by the imprinted and nonimprinted sorbents.

Sorbents	C_0/g L^{-1}		C_f/g L^{-1}		K_D[a]		k[b] (NOR/OFL)	k'[c]
	NOR	OFL	NOR	OFL	NOR	OFL		
MIP	1.0	1.0	0.32	0.87	0.32	0.02	14.64	9.61
NMIP	1.0	1.0	0.44	0.55	0.19	0.12	1.52	

[a] K_D, distribution coefficient; $K_D = \{(C_0 - C_f)/C_f\} \times \{$volume of solution [mL]/{mass of gel [g]}, where C_0 and C_f represent the initial and final concentrations, respectively.
[b] k, selectivity coefficient, $k = K_{D1}/K_{D2}$.
[c] k', relative selectivity coefficient, $k' = k_{MIP}/k_{NIP}$.

study. As can be seen in Table 1, distribution coefficient (K_D), selectivity coefficient of the sorbent (k) and the relative selectivity coefficient (k') was obtained in these competitive experiments. Distribution coefficient suggested the ratio of the binding capacity of sorbent to the free analytes concentration in the supernatant, $K_D = (C_0 - C_f)V/C_fW$, where C_0 and C_f represent the initial and free concentrations, respectively. The selectivity coefficient of the sorbent suggested the otherness of two substances adsorbed by one sorbent, $k = K_D(\text{NOR})/K_D(\text{OFL})$; the relative selectivity coefficient suggested the otherness of two sorbents, $k' = k_{MIP}/k_{NIP}$ [19].

NOR and OFL had the similar K_D on the NIP, but the MIP-Silica showed about five times adsorbed capacity to NOR than to OFL. The k value of the MIP-Silica (14.64) was larger than that of the NIP (1.52), which showed that the MIP-Silica had high selectivity for NOR over the structurally similar compounds OFL. The relative selectivity coefficient was 9.61, which shows the high selectivity of the MIP-Silica than the NIP.

3.5. Molecularly imprinted solid-phase extraction

Several parameters must be optimized in MISPE experiments in order to maximize the selective recognition of the analytes. In order to demonstrate the selectivity of the MIP-Silica for NOR, the structurally similar compounds such as OFL and CIP were also added to the sample. The cartridge was conditioned with the following solvents (in order): methanol–acetic acid (4:1, v/v), methanol and water (pH 6). Four different polarity solvents (*n*-hexane, chloroform, methanol, water) were utilized in loading step in order to find the best loading solvent. NOR was completely retained in the column when using chloroform and water (pH 6), and the higher column capacity (45.6 mg g^{-1}) were obtained when loading the analytes in water solution. Therefore, water (pH 6) was selected as loading solvent for further investigations.

In order to enhance the selectivity of MIP for NOR and decrease the cross-reactivity, a washing step in the MISPE procedure was investigated using toluene, chloroform, acetonitrile, methanol, water and acetate as potential washing solvents. The best results were obtained using 3 mL of MeOH—H_2O (1:1) as the washing solvent. The elution step was optimized when the sample was applied in water. Thus, MeOH, HAc, ACN and TFA were investigated as potential elution solvents. 2 mL of MeOH–HAc–TFA (90:9:1) was enough to completely remove the NOR from the cartridge. Therefore, MeOH:HAc:TFA = 90:9:1 was used as eluting solvent.

3.6. Application of the NOR imprinted sorbent to selective SPE–HPLC determination of NOR in fish samples

In this experiment, acetonitrile and hexane which have good ability for precipitating protein, lower solubility of lipids than other solvents and could penetrate meat after homogenization were selected to extract NOR from fish. After ultrasonication, centrifugation, the obtained solution was analyzed by HPLC. The chromatograms of the spiked fish and spiked fish after MISPE were shown in Fig. 6a and b, respectively. All fluoroquinolones were selectively extracted and no interferences from the fish matrix were observed after MISPE process, which demonstrates the high selectivity and better clean-up of the NOR-imprinted silica sorbents.

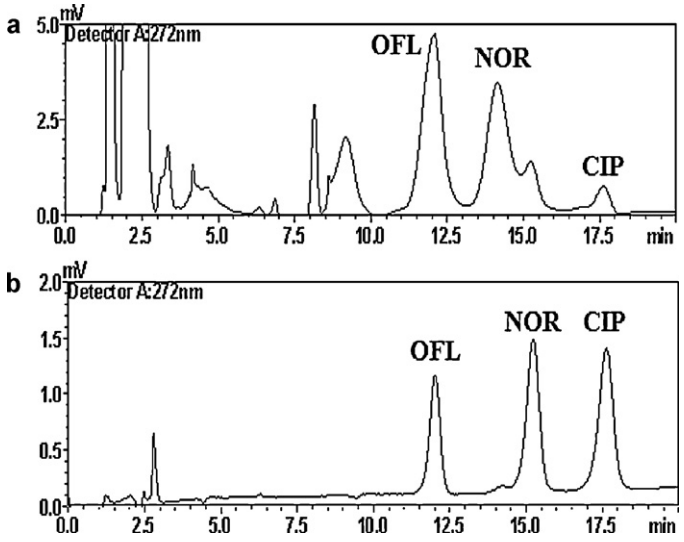

Fig. 6. Chromatogram of three fluoquinolones in fish samples. (a) Spiked fish; (b) spiked fish after MISPE.

The mean recoveries of NOR, OFL and CIP in fish evaluated by three spiked samples with concentrations (0.05, 0.1, 0.2 mg kg^{-1}) were 89.3–94.8%, 69.3–102.8% and 85–90.5%, respectively, with relative standard deviations (RSDs) lower than 6.5% (Table 2). The limits of detection (LOD) of the proposed method (S/N = 3) were in a range of 2.65–3.65 μg kg^{-1}, and the limits of quantitation (LOQ, S/N = 10) were in a range of 8.82–12.16 μg kg^{-1}. Comparison with the conventional approach, there were the lower LOQ values and high recoveries. Such as, recoveries (80–92%) and LOQ (15 μg kg^{-1}) for CIP and NOR were obtained by solid-phase extraction using Oasis HLB cartridges and HPLC with fluorescence detection [20].

4. Conclusions

A surface imprinting technique was developed by improving vinyl group grafting efficiency for the chemical stability and a controllable shell thickness of the grafting imprinting layer on the surface of silica particles. The surface imprinting technique cooperated with SPE–HPLC was confirmed to be a powerful tool for

Table 2
Precision, recoveries (R), limit of detection (LOD) and limit of quantitation (LOQ) of fluoroquinolones in fortified fish samples (n = 3).

Analytes	Spiked level (mg kg^{-1})	Detected (mg kg^{-1})	R (%)	RSD (%)	LOD[a] (μg kg^{-1})	LOQ[b] (μg kg^{-1})
OFL	0.05	0.05138	102.8	2.0		
	0.1	0.08506	85.1	4.4	3.65	12.16
	0.2	0.1385	69.3	6.5		
NOR	0.05	0.04738	94.8	3.2		
	0.1	0.0912	91.2	2.2	2.65	8.82
	0.2	0.1785	89.3	3.7		
CIP	0.05	0.04464	89.3	6.4		
	0.1	0.09054	90.5	5.5	3.04	10.14
	0.2	0.17	85.0	3.4		

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

efficient separation and fast enrichment of veterinary drug residues in fish samples.

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