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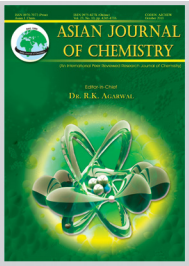

Effect of Solvents on Selective Molecularly Imprinted Solid-Phase Extraction of Enrofloxacin from Fish Samples

YUN-KAI LV*, LI-MIN WANG, MI-GE ZHAO, JING-QI ZHANG and HAN-WEN SUN

College of Chemistry and Environmental Science, Hebei University, Key Laboratory of Analytical Science and Technology of Hebei Province, Baoding 071002, P.R. China

*Corresponding author: Fax: +86 312 5079628; Tel: +86 312 5079359; E-mail: lvyunkai@hbu.edu.com

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A sample preparation method of solid-phase extraction using a molecularly imprinted polymer as selective adsorbent for the enrofloxacin has been developed. The conditioning, loading, washing and eluting solvents were investigated and evaluated by the recovery and retention of enrofloxacin to obtain the optimum conditions of the molecularly imprinted solid-phase extraction (MISPE). Under the optimal solid phase extraction condition, three fluoroquinolones residues in fish were separated and detected by an off-line MIP-SPE-HPLC with better clean-up and enrichment.

Key Words: Solvents, Molecularly imprinted polymers, Enrofloxacin.

INTRODUCTION

Enrofloxacin (ENR) is a high bactericidal activity against major pathogenic bacteria found in diseased animals[1,2]. However, the residues of fluoroquinolones in edible animal tissues and food produced from animal are potentially hazardous to human health. To ensure that consumers are not exposed to residues at potentially harmful concentrations, 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 fluoroquinolone antibiotics in food[3-5]. In order to control the amount of fluoroquinolone residues in harmful concentrations, many methods have been proposed for the analysis of the drugs in biological matrices. However, a method development for the selective extraction and determination of these compounds from the complex matrix is necessary.

Molecular imprinting technique (MIT) is an increasingly developing technique for preparing polymers with desired and predetermined selectivity and provides specific binding sites or catalytic sites in molecularly imprinted polymers (MIPs). Molecularly imprinted polymers had been attracted extensive attention and have been used extensively in sensors, immunoassay-type binding assays in place of antibodies and the separation techniques, which involve HPLC, affinity chromatography, capillary electrochromatography and thin layer chromatography[6,7]. A particularly promising application of molecularly imprinted polymers is as selective sorbents in solid phase extraction (SPE) for the clean-up and preconcentration of compounds from low concentrations or complex matrices[7,8]. The key of molecularly imprinted solid-phase extraction (MISPE) development is preparations of the molecular imprinting sorbents and optimization of solid phase extraction conditions. It was reported that the solvents play a crucial role in the adsorption capacity as well as the selectivity of the molecularly imprinted polymers by affecting significantly the interaction between the monomer and the template molecule in the molecularly imprinted solid-phase extraction procedure[9-11].

Therefore, the preparation and application of molecularly imprinted solid-phase extraction, especially in veterinary drugs, has aroused great interest. Moreover, enrofloxacin-imprinted solid-phase extraction sorbent was also prepared and applied to clean-up food samples, such as milk, fish, urine eggs and tissue[12-17]. However, there are few studied to design of sample pretreatment using molecularly imprinted solid-phase extraction from complex biological samples. In the present study, the conditions of molecularly imprinted solid-phase extraction were investigated and the binding selectivity was discussed. The proposed method was successfully applied to preconcentration and separation of the fluoroquinolone residues in the fish samples.

EXPERIMENTAL

Enrofloxacin (ENR), ofloxacin (OFL) and ciprofloxacin (CIP) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The

structure of these molecules are shown in Fig. 1. Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA) were purchased from Tianjin Chemical Reagent Research Institute (Tianjin, China), Trading Co. Ltd. (Shanghai, China) respectively and were freshly distilled to removing inhibitors prior to use. 2,2-Azobisisobutyronitrile (AIBN) was purchased from Beijing Chemical Reagent Company (Beijing, China), and recrystallized from methanol before use. All the other chemicals were of the analytical or the HPLC grade and used without further disposal.

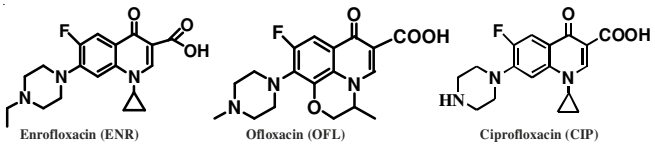

Fig. 1. Molecular structures of enrofloxacin, ofloxacin and ciprofloxacin

HPLC analysis was performed using a liquid chromatography system containing a LC-20AT pump and a SPD-20A UV-VIS detector and RF-10AXL (Shimadzu, Japan). The analytes were separated on a Venusil XBP C18 column (250 × 4.6 mm, 5 mm) from Bonna-Agela Technologies (Tianjin, China). The mobile phase was acetonitrile/13 mM tetrabutyl ammonium bromide solution (6:94, v/v) and the flow rate was 1.0 mL min^{-1} at 25 ºC. Aliquots of 20 µL were injected into the column and the chromatograms were recorded at 278 nm.

Solid phase extraction was performed with a 12-ports vacuum solid phase extraction 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 278 nm.

Preparation of the imprinted polymer: For the preparation of the enrofloxacin-imprinted polymer, the template (ENR, 0.52 g, 1.46 mmol) was dissolved in 6.66 mL of acetonitrile (ACN) in a 25 mL thick-walled glass tube. The functional monomer (MAA, 0.40 g, 4.64 mmol), the cross-linker (EDMA, 4.60 g, 23.20 mmol) and the initiator (AIBN, 0.08 g, 0.51 mmol) were then added. The resultant solution was sonicated for 5 min and purged with nitrogen gas for 10 min. The polymerization was allowed to proceed at 50 ºC for 24 h in a water bath. After this period, the glass tube was broken and the monolith obtained was crushed, ground and sieved to obtain regularly sized particles between 38.5 and 63 µm suitable for the chromatographic and solid phase extraction evaluations and application. A non-imprinted polymer (NIP) was prepared and treated in an identical manner to the molecularly imprinted polymer without the template molecular.

The molecularly imprinted solid-phase extraction procedure: The polymers (50 mg) were packed into empty solid phase extraction cartridges and capped with fritted polyethylene disks at the top and bottom. After the cartridge was conditioned with distillated water/acetic acid (80:20,v/v), MeOH and dichloromethane, 3 mL of enrofloxacin dichloro-methane solution (0.5 mg mL^{-1}) was passed through the cartridges. After being dried for 10 min, the cartridges were washed with 2 mL distillated water and eluted with 3 mL MeOH/acetic acid (AcOH) /trifluoroacetic acid (TFA) (90:9:1, v/v). The adsorption capacity was measured by T6 UV-VIS spectrophotometer.

Sample preparation[17]: Fresh fish sample (5 g) was crushed up and extracted twice with 25 mL of acetonitrile and 5 g of sodium sulfate. Afterwards, all extraction was extracted again with another 15.0 mL of *n*-hexane. The combined acetonitrile solvent was evaporated to dryness in a rotary evaporator and the residue was dissolved in 5 mL of dichloromethane and filtrated through a 0.45 µm syringe filter. 3 mL of the filtrate was passed through the molecularly imprinted polymer cartridges. The above-mentioned molecularly imprinted solid-phase extraction procedure was used to separate and detected enrofloxacin, ofloxacin and ciprofloxacin in fish. The fish samples were spiked with fluoroquinolones antibiotics at three concentration levels of 0.2, 0.5 and 1 mg kg^{-1} and experiments were repeated three times. The collected solutions were dried using a gentle stream of nitrogen. The residues were redissolved in the mobile phase and analyzed by HPLC-UV at 278 nm.

RESULTS AND DISCUSSION

IR spectra: The obtained particles were characterized by IR spectroscopy. Fig. 2 shows the IR spectra of non-imprinted polymer, extracted MIP (remove the template) and unextracted MIP (without remove the template). It can be seen that the shape and position of all peaks in IR spectra are exhibited similarly. The peaks of 2950 cm^{-1} and 1719 cm^{-1} correspond to absorption bands of -CH_2- or -CH_3 and -C=O stretching vibration, respectively[18]. The symmetric and dissymmetry stretching vibration of -C-O-C of ester appeared near 1264 cm^{-1} and 1146 cm^{-1}. The peak band of 1588 cm^{-1} in corresponds to absorption of C-N, which is clearly observed in Fig. 2b. The disappearance of 1588 cm^{-1} band in Fig. 2c suggests that the majority of the template molecules have been successfully extracted from the imprinted polymer[19].

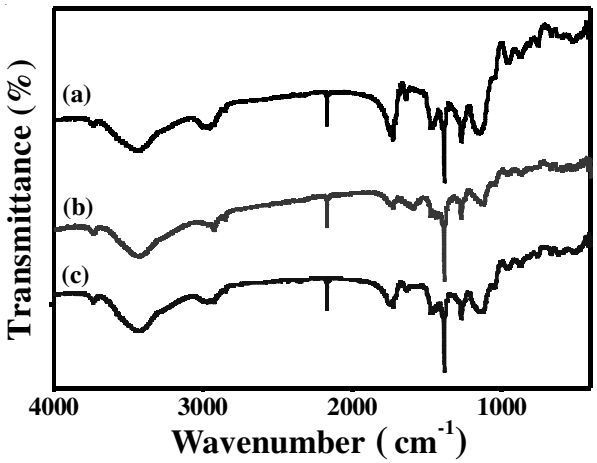

Fig. 2. FT-IR spectra of NIP (a) and MIP (b) and MIP containing the template molecular (c)

Optimization of the molecularly imprinted solid-phase extraction procedure: To achieve good adsorption for the analyte, the conditioning, loading, washing and elution steps had to be optimized. In the conditioning step, the binding sites must be activated by acid solution. Meanwhile, the favourable

conditions were produced for the formation of the hydrogen bonds between enrofloxacin and the selective cavities of the molecularly imprinted polymer by non-polar and weak polar solution[20]. So the cartridge was conditioned with the following solvents (in order): water/AcOH (80:20,v/v), MeOH and dichloromethane before the loading step.

It has widely demonstrated that the rebinding of the template takes place in polymers using the solvent of similar polarity as during polymerization. Consequently, analyte retention decreases when the polarity of the solvent used increases[9]. 3.0 mL of different loading solvents, including water, MeOH, dichloromethane and acetonitrile were passed through cartridges. The result showed that the higher adsorption capacity (27.69 mg g^{-1}) were obtained when loading the analytes in dichloromethane (Fig. 3). Therefore, dichloromethane was selected as loading solvent for further investigations.

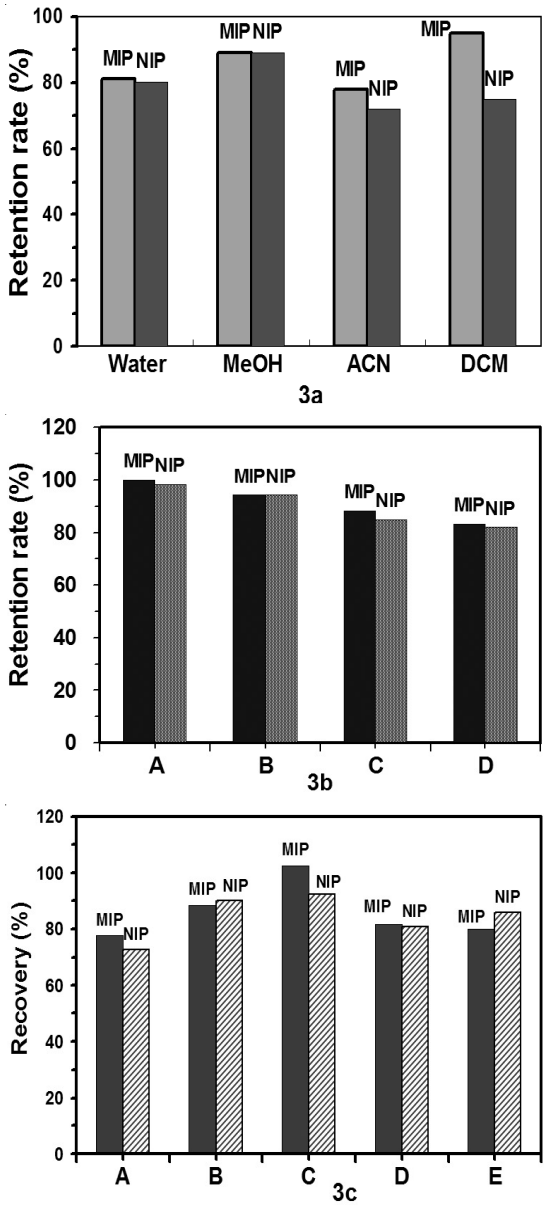

Fig. 3. Effect of different the loading solvents (3a), the washing solvents (3b) and the elution solvents (3c) on the retention rates or the recoveries of ENR. (3b): A, water (pH = 6.0); B, chloroform; C, MeOH; D, ACN. (3c): A, MeOH; B, MeOH/HAc (90:10); C, MeOH/HAc/TFA (90:9:1); D, ACN/TFA (99.5:0.5); E ACN/HAc (90:10)

To enhance the selectivity of molecularly imprinted polymer, the washing step was optimized. 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[20]. The wash procedure was assessed for obtaining the minimum removed percentage of the analytes using a variety solvent, including water, chloroform, MeOH and acetonitrile. The results (Fig. 4) were shown that washing with 2 mL of water (pH = 6.0) only had slightly effect on the retention of enrofloxacin on both MIP and non-imprinted polymer cartridges.

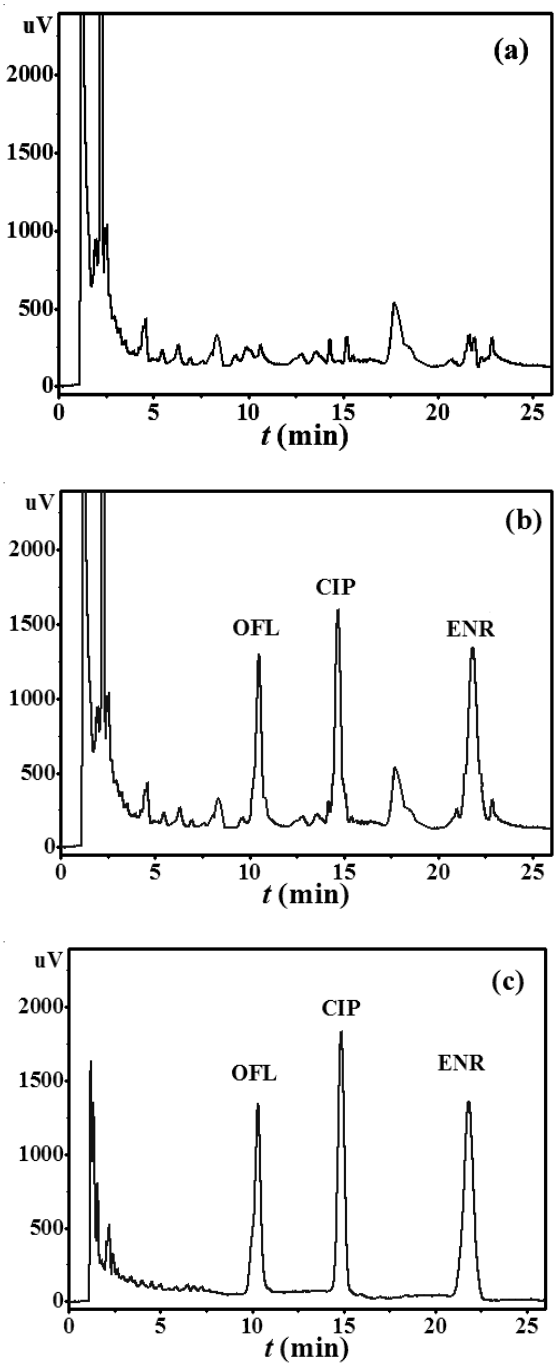

Fig. 4. Chromatograms obtained from the extraction of ENR, OFL and ENR from the fish samples. (a) blank milk (non-spiked); (b) spiked fish; (c) spiked fish with a clean-up of MISPE; Mobile phase: acetonitrile -13 mM tetrabutylammonium bromide solution (6:94, v/v); Flow rate: 1.0 mL min^{-1}; Samples spiked concentration: 0.1 mg kg^{-1}; Injection volume: 20 µL; Detection: 278 nm

TABLE-1
AVERAGE RECOVERIES (R), RELATIVE STANDARD DEVIATIONS (RSD, *n*=3), LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ) OF THREE FLUOROQUINOLONES OBTAINED AFTER MISPE OF SPIKED FISH SAMPLES

Analyte	Spiked level (mg kg^{-1})	Detected (mg kg^{-1})	R (%)	RSD (%)	LOD[a] (μg kg^{-1})	LOQ[b] (μg kg^{-1})
ENR	0.2	0.516	108.0	3.0	11	37
	0.5	0.79	98.0	3.4		
	1	1.33	103.0	6.6		
OFL	0.2	0.294	82.0	2.6	13	43
	0.5	0.576	89.2	3.6		
	1	1.02	89.0	3.9		
CIP	0.2	0.382	86.0	5.8	21	70
	0.5	0.643	86.6	3.3		
	1	1.02	81.0	5.9		

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

The analytes are usually eluted with polar and porogenic solvents, including some acids. In this way, the template-monomer interactions based on hydrogen bonding are disrupted and the analytes are released from the MIP[9]. MeOH and acetonitrile were initially selected for this purpose, since it had been noted that they were the most efficient solvent at removing the enrofloxacin from the MIP during the washing step. To increase the recovery, both AcOH and TFA were added. As shown in Fig. 4, the retained enrofloxacin was completely recovered when 3 mL of MeOH/AcOH/TFA (90:9:1, v/v) was used in the elution step.

Analysis of the fish samples: To demonstrate the clean-up ability and selectivity of the MIP from the complex matrix, the MIP was applied to solid phase extraction of the fish samples and the spiked samples (Fig. 4). Compared with the blank milk sample (Fig. 4a) and the spiked milk (Fig. 4b), the spiked samples after molecularly imprinted solid-phase extraction (Fig. 4c) showed the matrix interferences were eliminated. It confirmed that satisfactory sample clean-up was achieved by the molecularly imprinted solid-phase extraction when applied to a complex matrix. To demonstrate the feasibility of using molecularly imprinted solid-phase extraction to extract the fluoroquinolone residues from the biological samples at trace levels. The recoveries was 98.0-108.0 % for enrofloxacin, 82.0-89.2 % for ofloxacin and 81.0-86.6 % for ciprofloxacin (Table-1), respectively, with relative standard deviations (RSD) lower than 6.6 %. The limits of detection (LOD, S/N = 3) and the limits of quantitation (LOQ, S/N = 10) of the proposed method were 11 and 37 μg kg^{-1} for enrofloxacin, 13 and 43 μg kg^{-1} for ofloxacin, 21 and 70 μg kg^{-1} for ciprofloxacin, respectively.

Conclusion

It has been shown that a molecularly imprinted polymer prepared with enrofloxacin (ENR) as template, methacrylic acid as functional monomer, ethylene glycol dimethacrylate as cross-linker and dichloromethane as porogen exhibited high affinity for enrofloxacin. It was demonstrated that solvents play a crucial role in deciding the adsorption capacity in molecularly imprinted solid-phase extraction. The results obtained the interactions between the MIP and the template enrofloxacin performed differently in various organic conditioning, loading, washing and eluting solvents. The molecularly imprinted solid-phase extraction technique was confirmed to be a powerful tool for efficient separation and fast enrichment of veterinary drug residues in fish samples.

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