

Water-compatible molecularly imprinted polymer for the selective recognition of fluoroquinolone antibiotics in biological samples

Elena Benito-Peña · Sofia Martins ·
Guillermo Orellana · María Cruz Moreno-Bondi

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Abstract A novel water-compatible molecularly imprinted polymer (MIP), prepared with enrofloxacin (ENR) as the template, has been optimised for the selective extraction of fluoroquinolone antibiotics in aqueous media. The results of a morphological characterisation and selectivity tests of the polymer material for ENR and related derivatives are reported. High affinity for the piperazine-based fluoroquinolones marbofloxacin, ciprofloxacin, norfloxacin and ofloxacin was observed, whereas no retention was found for nonrelated antibiotics. Various parameters affecting the extraction efficiency of the polymer have been optimised to achieve selective extraction of the antibiotics from real samples and to reduce nonspecific interactions. These findings resulted in a MISPE/HPLC-FLD method allowing direct extraction of the analytes from aqueous samples with a selective wash using just 50% (v/v) organic solvent. The method showed excellent recoveries and precision when buffered urine samples fortified at five concentration levels (25–250 ng mL⁻¹ each) of marbofloxacin, ciprofloxacin, norfloxacin, enrofloxacin and sarafloxacin were tested (53–88%, RSD 1–10%, *n*=3). Moreover, the biological matrix of the aqueous samples did not influence the preconcentra-

tion efficiency of the fluoroquinolones on the MIP cartridges; no significant differences were observed between the recovery rates of the antibiotics in buffer and urine samples. The detection limits of the whole process range between 1.9 and 34 ng mL⁻¹ when 5-mL urine samples are processed. The developed method has been successfully applied to preconcentration of norfloxacin in urine samples of a medicated patient, demonstrating the ability of the novel MIP for selective extraction of fluoroquinolones in urine samples.

Keywords Water-compatible molecularly imprinted polymers · Fluoroquinolones · Antibiotics · Solid-phase extraction · Urine samples

Abbreviations

MIP	molecularly imprinted polymer
NIP	nonimprinted polymer
MAA	methacrylic acid
HEMA	2-hydroxyethyl methacrylate
EDMA	ethylene glycol dimethacrylate
ABDV	2,2'-azobis(2,4-dimethylvaleronitrile)
FQs	fluoroquinolones
ENR	enrofloxacin
MAR	marbofloxacin
SAR	sarafloxacin
CIP	ciprofloxacin
NOR	norfloxacin
OFL	ofloxacin
AMX	amoxicillin
PEG	penicillin G
CLX	cloxacillin
CEP	cephapirin
DAP	dapsone
BZ	benzoic acid

E. Benito-Peña · S. Martins · M. C. Moreno-Bondi (✉)
Department of Analytical Chemistry, Faculty of Chemistry,
Universidad Complutense de Madrid,
28040 Madrid, Spain
e-mail: mcmbondi@quim.ucm.es

G. Orellana
Department of Organic Chemistry, Faculty of Chemistry,
Universidad Complutense de Madrid,
28040 Madrid, Spain

HPLC	high-performance liquid chromatography
FLD	fluorescence detector
DAD	diode array detector
LLE	liquid–liquid extraction
SFE	supercritical fluid extraction
PLE	pressurised liquid extraction
SPE	solid-phase extraction
MISPE	molecularly imprinted solid-phase extraction
MI-MSPD	molecularly imprinted matrix solid-phase dispersion

Introduction

Pharmaceuticals applied for human and veterinary use have been found in foodstuffs of animal origin [1, 2] and in different compartments of the environment. Thus, concerns regarding the toxicity of these contaminants [3] have emerged because strains of antibody-resistant bacteria have appeared and the effects of unintended human exposure are still unknown.

Fluoroquinolones (FQs) are synthetic antimicrobials that are widely used nowadays in veterinary and human medicine. The target proteins of fluoroquinolones are bacterial DNA gyrase and topoisomerase IV enzymes, essential for DNA replication and transcription. FQs have a common 4-oxo-1,4-dihydroquinoline skeleton, where the pharmacophore unit consists of a pyridine ring with a carboxyl group, a piperazinyl group and a fluorine atom placed at positions 3, 7 and 6, respectively [4].

Several analytical methods have been published for the analysis of fluoroquinolones in biological, food and environmental samples, such as milk [5], eggs [6], urine [7] or water and soil [8]. Most of the methods are based on chromatographic techniques, especially high-performance liquid chromatography (HPLC) with mass spectrometry (MS) [9], fluorescence (FLD) [10] or UV–visible detection [11].

The analysis of fluoroquinolones can be a difficult task in some matrices as they may bind to the lipoproteins in biological samples [12] or, for instance, FQs can be strongly adsorbed by soil samples [13], yielding low antibiotic recoveries or important matrix effects that make direct sample analysis difficult without further cleanup. Sample preparation methods for the analysis of FQs include liquid–liquid extraction (LLE) [14], dialysis [15], supercritical fluid extraction (SFE) [16], pressurised liquid extraction (PLE) [6] and conventional solid-phase extraction (SPE) [17]. Some of these afford low recoveries (50–60%) or require special instrumentation not available in every laboratory (e.g. microdialysis, PLE or SFE). Thus, simple extraction methods for the determination of FQs are required that can provide good recoveries and group selectivity, with low sample manipulation and solvent consumption.

Molecular imprinting is a template-directed polymerisation that allows the design and synthesis of well-defined artificial receptor sites for a large variety of target chemical species such as pharmaceuticals and pollutants [18–20]. Molecularly imprinted polymers (MIPs) allow one to obtain selective sorbents able to selectively bind antibiotics with good recoveries and low matrix effects; however, one of the main limitations for the broad application of MIPs and their use in replacing commercial sorbents for fluoroquinolone antibiotics analysis in biological and environmental samples is their limited recognition ability in aqueous media [21].

Molecularly imprinted solid-phase extraction (MISPE) has been applied to the extraction of different antibiotics from several matrices, such as environmental samples (river water [22] or soil [23]) and biofluids (urine, serum [21], milk [24]). In order to achieve the selective extraction of the analyte in aqueous samples, some authors include a cleanup step with an organic solvent in the MISPE procedure, prior to the elution step, to favour the specific interactions between the target and the imprinted sites and to minimise or suppress the nonspecific interactions. This cleanup step is more critical in MISPE procedures than in conventional SPE. In other cases, a two-step SPE procedure using a commercial precolumn has been applied to capture the analyte from the aqueous sample which is further eluted with an organic solvent that favours the specific interactions with the MIP. Any of these approaches increases the complexity and price of the procedure compared with commercially available SPE cartridges. So, a big effort has been focussed in recent years on the synthesis of aqueous-compatible MIPs.

Yan et al. [25] described the application of molecularly imprinted matrix solid-phase dispersion sorbents (MI-MSPD) and HPLC with fluorescence detection (FLD) to the analysis of enrofloxacin, ciprofloxacin (CIP), norfloxacin (NOR), ofloxacin (OFL) and perfloxacin (PER) in eggs and tissues samples with detection limits ranging from 0.05 to 0.09 ng g⁻¹ and recoveries of ca. 85–105% (RSD < 7%). Other water-compatible MIPs for fluoroquinolones have also been reported and used as SPE or liquid chromatography sorbents. However, these materials show a relatively high nonspecific retention in the nonimprinted polymer [26] and their cross-selectivity is not described or it is limited to only another fluoroquinolone [27].

In the present paper we describe the synthesis of a water-compatible MIP, imprinted with enrofloxacin, using methacrylic acid (MAA) and 2-hydroxyethyl methacrylate (HEMA) as monomers and ethylene glycol dimethacrylate (EDMA) as crosslinker. The synthesized material is a poly (MAA-co-HEMA-co-EDMA) polymer which exhibit good recognition properties in aqueous media. The MIP has been applied to the direct extraction of nanogram per millilitre levels of five fluoroquinolones (Fig. 1), marbofloxacin

(MAR), ciprofloxacin (CIP), norfloxacin (NOR), enrofloxacin (ENR) and sarafloxacin (SAR), from water and human urine samples. The method has been validated using spiked human urine samples and HPLC-FLD detection.

Experimental

Reagents

Methacrylic acid (MAA), 2-hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EDMA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The initiator 2,2'-azobis(2,4-dimethylvaleronitrile) (ABDV) was purchased from Wako (Neuss, Germany) and used as received. Enrofloxacin (ENR) was supplied from Fluka (Buchs, Switzerland), and ciprofloxacin (CIP), norfloxacin (NOR), lomefloxacin (LOM), ofloxacin (OFL), flumequine (FLU), oxolinic acid (OXO), penicillin G potassium salt (PEG), amoxicillin anhydrous (AMX), cephapirin sodium salt (CEP), cloxacillin salt monohydrate (CLX) and 4-

aminophenylsulphone (dapson, DAP) were from Sigma-Aldrich (St. Louis, MO, USA). Marbofloxacin (MAR) and sarafloxacin hydrochloride (SAR) were a gift from Fort Dodge veterinaria (Girona, Spain). Danofloxacin (DAN) was obtained from Riedel-de-Haën (Seelze, Germany), and benzoic acid (BZ) was supplied by Sigma-Aldrich (St. Louis, MO, USA). The chemical structures of the antibiotics are shown in Fig. 1.

Acetonitrile (ACN) and methanol (MeOH) (HPLC-grade) were provided by SDS (Peypin, France), and trifluoroacetic acid (TFA) (HPLC-grade, 99%) was from Fluka (Buchs, Switzerland). Analytical grade tetra-*n*-butylammonium hydrogen sulphate (TBA) (98%) was from Merck (Darmstadt, Germany).

Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The monomers were chromatographically purified, as required, immediately before use by using an inhibitor-remover from Aldrich (Milwaukee, WI, USA). All solutions prepared for HPLC were passed through a 0.45- μ m nylon filter before use. 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was supplied by

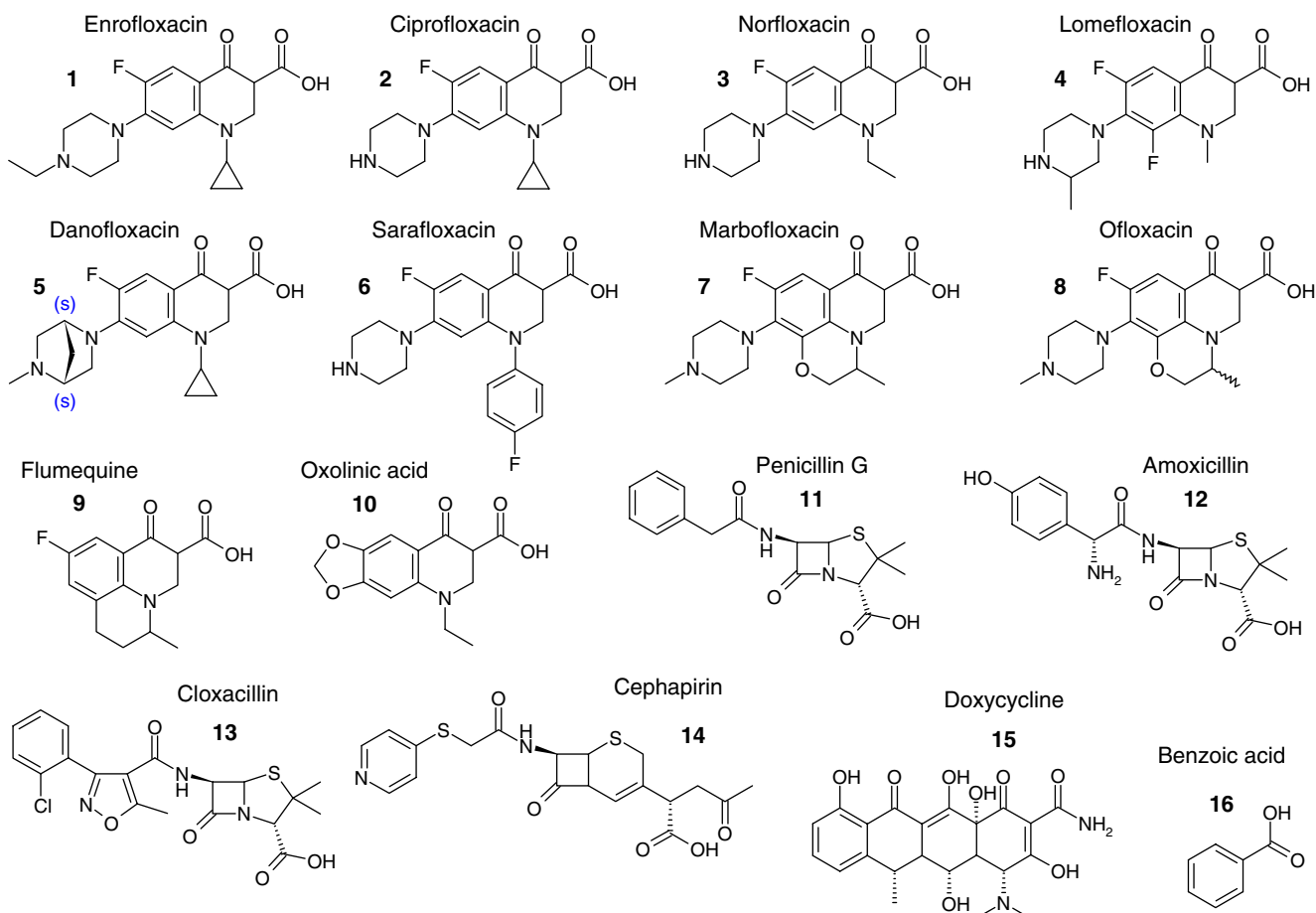


Fig. 1 Molecular structures of the antibiotics tested: **1** enrofloxacin, ENR; **2** ciprofloxacin, CIP; **3** norfloxacin, NOR; **4** lomefloxacin, LOM; **5** danofloxacin, DAN; **6** sarafloxacin, SAR; **7** marbofloxacin,

MAR; **8** ofloxacin, OFL; **9** flumequine, FLU; **10** oxolinic acid, OXO; **11** penicillin G, PEG; **12** amoxicillin, AMX; **13** cloxacillin, CLX; **14** cephapirin; (CEP); **15** doxycycline (DOX) and benzoic acid (BZ)

Aldrich (Steinheim, Germany), and a HEPES buffer solution, pH 7.5, was prepared by dissolving 23.83 g in 1 L of Milli-Q water (0.1 M).

Instrumentation

The pH of the buffer solutions and samples was adjusted with an Orion 710A pH/ISE meter (Beverly, MA, USA). Template extraction was carried out using a pressurised liquid extractor (PLE) (ASE 200, Dionex, Sunnyvale, CA, USA) equipped with 33-mL stainless steel cells. The extracts were collected in 60-mL glass vials. Imprinted and control polymers were ground in a MM-200 ball mill from Biometra (Madrid, Spain) and subsequently sieved through US standard 25- and 50- μm sieves (Filtru, Barcelona, Spain). A peristaltic pump Miniplus 3 (Gilson, Middleton, WI, USA) was used for sample preconcentration in the cartridges. Chromatographic analysis was carried out with an HP-1100 HPLC from Agilent Technologies (Palo Alto, CA, USA) equipped with a quaternary pump, an online degasser, an autosampler, an automatic injector and a column thermostat. A fluorescence detector (FLD) and a diode array detector (DAD) were used separately depending on the studies carried out. Scanning electron microscopic (SEM) measurements were performed with a JEOL JSM-6330F field emission scanning electron microscope at an acceleration voltage of 5 kV. The samples were coated with a thin gold film before analysis.

Chromatographic separation of the fluoroquinolones was performed on a LUNA C18 (2) (150 \times 4.6 mm, 5 μm) HPLC column protected by an RP18 guard column (4.0 \times 3.0 mm, 5 μm), both from Phenomenex (Torrance, CA).

A gradient program was used with a mobile phase comprising solvent A (Milli-Q water with 0.1% TFA), solvent B (ACN with 0.1% TFA) and solvent C (MeOH) as follows: 8% B and 10% C (12 min), 8–26.5% B and 10% C (7.5 min), 26.5–65% B and 10% C (9.5 min). Analyses were performed at a flow rate of 1.5 mL min⁻¹, and the column temperature was kept at 25 °C. The injection volume was 4 μL , and all the compounds eluted within 24 min. The fluorescence excitation/emission wavelengths were programmed at 280/515 nm for MAR, at 280/440 nm for CIP, NOR, ENR and SAR, and at 280/365 nm for OXO and FLU. Quantification was performed using external calibration peak area measurements. Linear calibration graphs were obtained in the 20–500 $\mu\text{g L}^{-1}$ range for all the antibiotics ($r^2 > 0.999$). For β -lactam antibiotics, the linear concentration range studied was 75–5,000 $\mu\text{g L}^{-1}$ ($r^2 > 0.999$), and the absorbance signal was recorded at 220 nm [28].

Polymer preparation

The template ENR (356.4 mg; 1 mmol) and the functional monomers MAA (350 μL ; 4 mmol) and HEMA (496 μL ;

4 mmol) were dissolved in 6.2 mL ACN and placed in a 25-mL glass vial. The mixture was left in contact for several minutes and then, after adding EDMA (3.77 mL; 20 mmol) and ABDV (49.28 mg), was cooled and purged with nitrogen for 15 min to remove dissolved oxygen. The glass tube was then sealed, and polymerisation was allowed to proceed thermally by placing the tube in a water bath set at 50 °C for 48 h. The resulting bulk polymer was broken into smaller fragments, and then the template was extracted by PLE. The washing solutions were combined and analysed by HPLC-DAD to check that template recovery was higher than 99.9% in all cases. Afterwards, the MIP was crushed and sieved, and particles in the size range 25–50 μm were collected for use in the chromatographic and SPE experiments. Prior to use, they were sedimented using MeOH/water (80:20, v/v) to remove fine particles. A nonimprinted polymer was prepared in the same way, but in the absence of the template molecule.

Polymer HPLC evaluation

The MIP and NIP polymers were slurry-packed into stainless HPLC columns (150 mm \times 4.6 mm) using slurry packer model 1666 (Alltech, UK). Fluoroquinolone individual stock solutions were prepared in water 0.02 M H₃PO₄ (0.01 M NaOH in the case of FLU and OXO) at a concentration of 200 $\mu\text{g mL}^{-1}$. These solutions were stored at 4 °C in the dark for not longer than 1 month.

The polymer binding affinity towards enrofloxacin (3 mM) was studied using HPLC-DAD. Different mobile phases ranging from 100% ACN to 100% aqueous HEPES buffer (0.1 M, pH 7.5) were tested. Analyses were performed at a flow rate of 1.0 mL min⁻¹, and the column temperature was kept at 25 °C. The injection volume was 20 μL , and the UV detector was set at 260 nm. The cross-selectivity tests were performed using the optimal mobile phase determined from the initial enrofloxacin binding studies, namely ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v). Analyte concentrations were 0.5 mM due the limited solubility of some antibiotics tested in the mobile phase. The retention factor (k) for each analyte was calculated as $k = (t - t_0)/t_0$, where t and t_0 are the retention times of the analyte and the void marker (methanol), respectively. Imprinting factors were evaluated as $\text{IF} = k_{\text{MIP}}/k_{\text{NIP}}$.

Determination of the binding capacity by frontal analysis

The binding capacity of the polymers has been evaluated by frontal analysis following a procedure described by Kim et al. [29]. Thus, nonimprinted and imprinted polymers were packed into stainless columns (50 \times 3 mm) and equilibrated with ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v) at a flow rate of 1 mL min⁻¹. Solutions of ENR (0.05–8.4 mM)

in ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v) were pumped at a flow rate of 1 mL min⁻¹ until a plateau in the absorbance signal was obtained. To ensure complete removal of the template, the columns were washed after each measurement with 20 mL MeOH containing 0.1% formic acid at 0.5 mL min⁻¹, followed by a re-equilibration step with 7.5 mL ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v) at a flow rate of 0.25 mL min⁻¹. The breakthrough volume was measured from the maximum numerical value of the first derivative of the frontal chromatogram. The breakthrough volume for a nonretained analyte was measured by eluting the columns with ACN containing 0.5% acetone.

Fluoroquinolone antibiotics extraction using MIP cartridges

Solid-phase extraction cartridges (Varian, Spain) with a 3-mL volume were packed with 150 mg of the imprinted or the corresponding nonimprinted polymers. The cartridges were equilibrated with 10 mL buffer (0.1 M HEPES, pH 7.5), and 5 mL of the antibiotic sample, dissolved in buffer (0.1 M HEPES, pH 7.5), was loaded at a constant flow rate of 0.75 mL min⁻¹, with the aid of a peristaltic pump.

The cartridges were washed with 25 mL ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v) to wash out the nonspecifically retained compounds. Finally, the antibiotics were eluted with 3 mL methanol containing 3% TFA. The cartridges were equilibrated with 10 mL buffer (0.1 M HEPES, pH 7.5) before a new application. The extract from MISPE column was injected into the HPLC system for analysis.

Human urine samples analysis

Human urine samples were collected from a healthy individual, not medicated with antibiotics for more than 6 months (control samples), and from other individual treated voluntarily with norfloxacin (400 mg norfloxacin per day for 6 days). Samples were stored at 4 °C in sterile containers to prevent bacterial growth.

For quantification purposes, human urine control samples were preconcentrated in the MIP cartridges and checked for lack of presence of the studied antibiotics at the method detection limits. Afterwards, 4-mL aliquots of the urine control samples were spiked with the stock solutions of the fluoroquinolones and the solutions were made up to a final volume of 5 mL with HEPES buffer (pH 7.5, final concentration 0.1 M). The final antibiotic concentrations in the urine samples were 20, 50, 80, 125 and 250 µg L⁻¹. The solution was filtered through a 0.45-µm nylon membrane, and thereafter the samples were preconcentrated using the MIP cartridges as described in [Fluoroquinolone antibiotics extraction using MIP cartridges](#). Urine samples of the medicated volunteer were adjusted to pH 7.5 and 0.1 M of

HEPES buffer prior to analysis as described above. All the analyses were carried out in triplicate.

Results and discussion

Synthesis and characterisation of the polymers

Our starting point for the preparation of an aqueous-compatible MIP selective for enrofloxacin was the well-characterised MIP consisting of poly(MAA-co-EDMA) modified with the hydrophilic co-monomer 2-hydroxyethyl methacrylate (HEMA), known to impart water compatibility in a high number of reported systems [30, 31]. Moreover, the monomer MAA was chosen because of its potential good ability to establish selective hydrogen or electrostatic binding interactions with functional groups of the ENR structure [21].

Chromatographic evaluation of the polymers

The polymers were first tested for their ability to retain the template molecule, ENR (Table 1). Based on our previous experience from handling PenG MIPs, we chose to evaluate the materials using mixtures of ACN and HEPES buffer (0.1 M, pH 7.5). This pH was chosen to ensure protonation of the piperazine moiety (zwitterion form) which is necessary for enhancing the electrostatic interaction with the carboxylate group of the MAA monomer.

Firstly, it should be noted that the high retention observed in MIP and NIP in water-poor mobile phase and water-rich systems is commonly described and is explained by a shift from an electrostatic retention mode to a hydrophobic mode, respectively. Thus, in pure ACN or buffer, ENR displays

Table 1 Retention behaviour of ENR in the imprinted and non-imprinted polymers ($n=3$)

Mobile phase ACN/water (v:v)	k_{MIP}	k_{NIP}	IF
100:0	NE	33.3	–
75:25	24.0	1.2	20.8
60:40	37.5	3.0	12.4
50:50	52.1	1.5	34.3
40:60	NE	3.08	–
25:75	NE	5.70	–
15:85	NE	NE	–
10:90	NE	NE	–
0:100	NE	NE	–

Mobile phase ACN/water (0.1 M HEPES, pH 7.5); sample volume 20 µL; ENR concentration 3 mM; flow rate 1 mL min⁻¹. The retention factor (k) and imprinting factor (IF) were calculated as $k=(t_r - t_0)/t_0$, where t_r is the retention time of the analyte, t_0 the retention time of methanol and $IF=k_{\text{MIP}}/k_{\text{NIP}}$

NE no elution observed after 140 min

dramatically higher affinity for the polymer (no elution within 140 min). Excellent imprinting factors ($IF > 12$) are obtained for mobile phases composed of ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v), (60:40, v/v) and (75:25, v/v), showing the success of the imprinting step and the good performance of the polymer in the hydroorganic media. The most interesting observations occur at 50% buffer where the difference between the recognition properties of the MIP and NIP is most remarkable ($IF > 34$).

We next decided to test the effect of pH on the recognition properties of the MIP and NIP towards ENR by preparing different mobile phases at 50% buffer and pH 3.0, 7.5 and 9.0. The results obtained are shown in Fig. 2.

The antibiotic ENR is strongly dependent of pH due to the carboxylic acid group at position 3 of the six-membered heterocyclic aromatic ring and the protonatable amino group located on the piperazine ring situated at position 7 of the first mentioned ring (except OXO and FLU). The retention behaviour of the analyte can be explained on the basis of its acid–base properties [4]. At low pH ($pH \ll pK_a$) and high pH ($pK_a \ll pH$) ENR shows low retention; however, it can be remarkable at pH 7.5 (zwitterionic form). The explanation could be supported by the cationic-exchange interaction mode that probably occurs between the carboxylic groups of the MIP sorbent (MAA) and the charged amino group at position 1 on the piperazine ring of ENR. In fact, these results confirm the hypothesis of an electrostatic retention mode of the polymer in water-rich mobile phases, and a pH of 7.5 was selected for further studies.

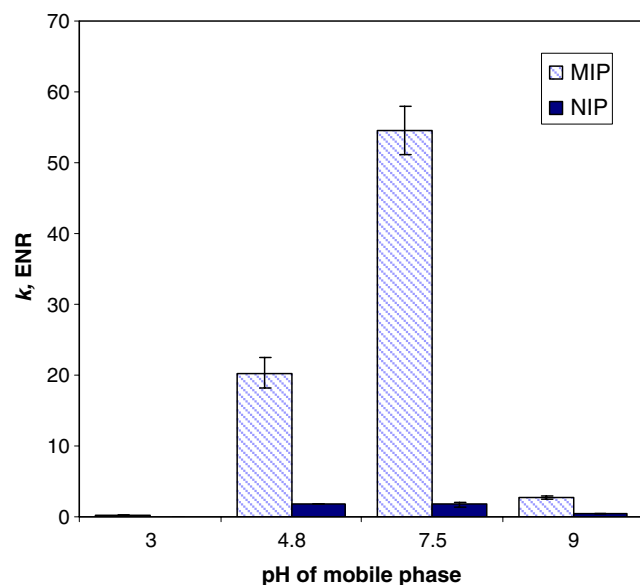


Fig. 2 Effect of pH on the retention factor (k) of ENR in the MIP and the NIP. Mobile phase ACN/water (0.1 M HEPES) (50:50, v/v), 3 mM ENR ($n=3$)

Evaluation of the MIP cross-selectivity

The specificity of the developed MIP aqueous-compatible polymer was evaluated by comparing the retention behaviour of other structurally related fluoroquinolones and nonrelated antibiotics to that of ENR. The mobile phase used for these experiments was the optimal one described above, i.e. ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v). The results are collected in Table 2.

The imprinted polymer showed a marked affinity for most of the fluoroquinolones antibiotics tested. CIP, NOR, DAN, SAR, OFL, MAR and LOM afforded the highest capacity factors ($k > 15$). This was not surprising because of the presence of the piperazine ring at position 7 of the heterocyclic aromatic ring that contains the abovementioned charged amino group at position 4 of the piperazine ring.

CIP, NOR, OFL and MAR have the most similar structures to the template ENR and contain a piperazine ring and an alkyl group at position 1 of the quinolone ring. Inspection of the large imprinting factors obtained supports the idea that the latter moiety on the drugs contributes significantly to the specific binding with these FQs. Moreover, replacement of the cyclopropyl ring of ENR by an ethyl group (i.e. in NOR) does not affect the planarity of the molecules and affords a remarkable IF (8.8) [32].

Substitutions can occur at several positions of the piperazine ring as seen in LOM and DAN. The IF obtained for both molecules does not dramatically change ($IF \geq 5.7$) compared with the IF of FQs studied before, demonstrating that their conformational structures are consistent with that of ENR. In the case of SAR, potential steric hindrance due to the larger fluorophenyl ring at position 1 seems to be weak, confirming that introduction of strong perturbation in the heteroaromatic ring moiety does not modify the specific recognition of the polymer. Other quinolones such as OXO or FLU which have no piperazine ring have an entirely different structure and are not recognised by the MIP.

Finally, no retention was observed for β -lactam antibiotics (AMX, PEG, CLX or CEP) and other antimicrobials (DAP) or structures (benzoic acid, BZ).

Morphological analysis

The SEM observations revealed no significant textural changes when the template takes part in the polymerisation process (Fig. 3). Both the MIP and NIP polymers show “type IV” nitrogen adsorption isotherms which are usually related to meso-macroporous materials. The BET area (S_{BET}) value for the MIP was $(217 \pm 3) \text{ m}^2 \text{ g}^{-1}$ with a total pore volume (V_t) of $0.70 \text{ cm}^3 \text{ g}^{-1}$. Similar values were obtained for the NIP ($S_{BET} = 213 \pm 3 \text{ m}^2 \text{ g}^{-1}$ and $V_t = 0.68 \text{ cm}^3 \text{ g}^{-1}$) but the chromatographic behaviour of these materials, prepared in the

Table 2 Evaluation of the cross-selectivity of the polymers in terms of retention factors (k) towards fluoroquinolones and other antibiotics (see Fig. 1)

Antibiotics		k_{MIP}	k_{NIP}	IF
Fluoroquinolones	ENR	52.1	1.5	34.3
	CIP	39.2	3.9	10.2
	NOR	40.1	4.5	8.8
	LOM	16.4	2.7	6.1
	DAN	31.2	5.5	5.7
	OFL	26.3	2.1	12.3
	MAR	17.5	1.6	11.2
	FLU	0.7	0.2	3.1
	SAR	26.1	4.0	6.6
	OXO	0.5	0.3	1.6
β -Lactams	AMX	0.0	0.0	0.0
	PEG	0.0	0.3	0.0
	CLX	0.0	0.0	0.0
Tetracycline	CEP	0.0	0.0	0.0
	DOX	0.6	0.6	0.9
Sulphone	DAP	0.0	0.0	0.0

Mobile phase ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v); sample volume 20 μL ; [analyte]=0.5 mM; flow rate 1 mL min^{-1} ; column 150 \times 4.6 mm; λ =260 nm for FQs; 220 nm for other antibiotics. Methanol was used as void volume marker. The retention factor (k) and imprinting factor (IF) were calculated as described in the **Experimental**. Results are the average of three separate analyte injections

presence and absence of the template molecule, were very different as will be shown in the next sections [33].

Determination of binding site distributions and affinities

The best MIP-based SPE material should display, in addition to a high affinity and selectivity, appreciable binding capacity for the analyte of interest. If all these requirements are fulfilled, SPE can be performed with small amounts of polymer, allowing the reduction of the absolute amount of nonselective analyte adsorption.

The binding features of ENR to polymers were calculated using discrete distribution models, such as Langmuir (LI) and bi-Langmuir (BLI) isotherms; continuous distribution models, such as Freundlich (FI), Jovanovic (JI) and bi-Jovanovic (BJI) isotherms; and hybrids models, such as Freundlich–Langmuir (FLI) and Freundlich–Jovanovic (FJI) isotherms [34]. The experimental binding data obtained with the MIP and the NIP displayed good adherence to the Jovanovic binary site model (Eq. (1)) and to the Freundlich (FI) isotherm model (Eq. (2)), respectively (Fig. 4).

$$\text{bi-Jovanovic model: } B = q_1(1 - e^{K_1 F}) + q_2(1 - e^{K_2 F}) \quad (1)$$

$$\text{Freundlich model: } B = aF^m \quad (2)$$

where B and F are the concentrations of bound and free analyte, respectively, q is the binding sites density and m is

the so-called heterogeneity index. The parameter n can take values from 1 to 0, increasing with decreasing heterogeneity of the material.

The parameters obtained for MIP in terms of binding constants (K_1, K_2) and binding capacity ($q_1 \times k_1$ and $q_2 \times k_2$) are collected in Table 3.

For imprinted polymer, a maximum affinity constant of $6 \pm 4 \text{ mM}^{-1}$ was obtained, and binding site densities of $q_1 = 17 \pm 4 \mu\text{mol g}^{-1}$ and $q_2 = 446 \pm 195 \mu\text{mol g}^{-1}$ were achieved.

In the case of NIP, the FI fitting revealed that a very low heterogeneity index ($m = 0.9 \pm 0.3$, where $m = 1$ corresponds to the minimum heterogeneity index) is obtained, demonstrating the absence of binding sites for the analyte in the material. The apparent binding sites density, q ($\mu\text{mol g}^{-1}$), and the apparent weighted average affinity, K (mM^{-1}), were (2.2 ± 0.4) $\mu\text{mol g}^{-1}$ and (0.9 ± 0.2) mM^{-1} , respectively [35]. These values are lower than those obtained with the imprinted polymer; therefore, the template molecule plays an important role in the heterogeneity of the prepared MIP.

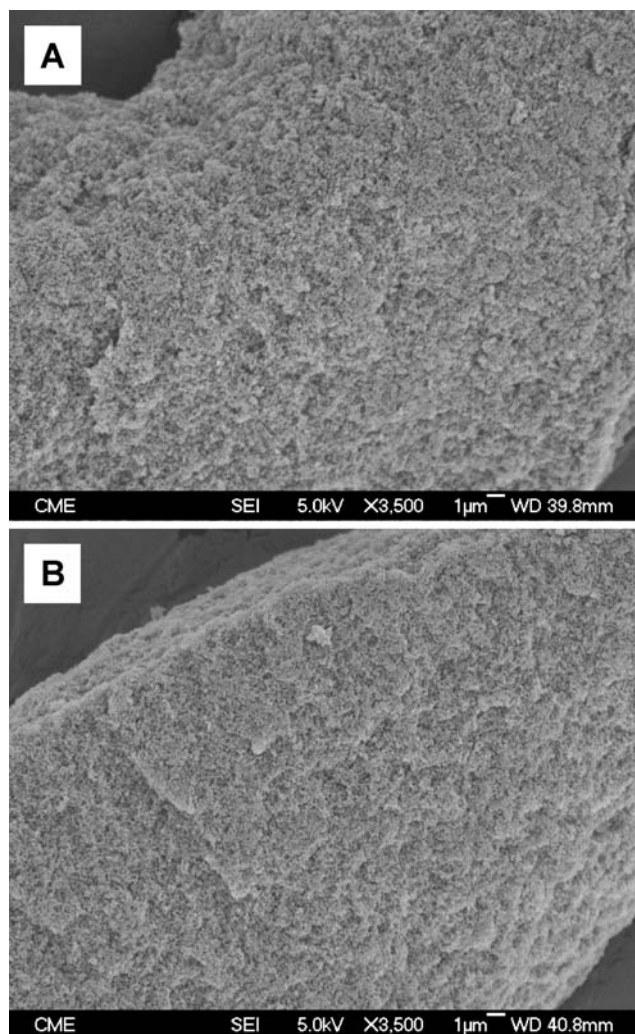


Fig. 3 Scanning electron micrographs of the **a** MIP and **b** NIP materials

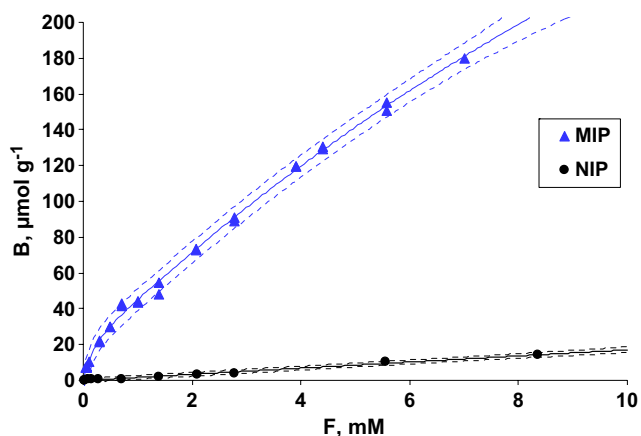


Fig. 4 Binding isotherms for the uptake of ENR by the MIP (triangles) and the NIP (circles) in ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v) obtained by frontal analysis. F (mM), concentration of the free solute; B ($\mu\text{mol g}^{-1}$), amount of bound solute per gram of polymer. The experimental data were fitted to a bi-Jovanovic (BJI) isotherm model in the case of MIP and to a Freundlich (FI) isotherm model for the NIP. Calculations are the mean of two replicate measurements with a coefficient of variation in the range of 0.3–5.0%

MISPE procedure

The factors evaluated to establish the optimum conditions for the SPE procedure include the study of the flow rate of the loading solution, the composition and volume of the eluting solvent and the composition of the washing solvent.

Sample loading flow rate

In order to evaluate the effect of the sample loading flow rate on the ENR recovery, 10 mL of a solution of the antibiotic ($300 \mu\text{g L}^{-1}$ in 0.1 M HEPES, pH 7.5) was loaded

Table 3 Adsorption isotherm models for binding of ENR to the imprinted and nonimprinted polymers and parameters obtained for the best fit of the results shown in Fig. 4 using the SSPS Tablecurve-2D software (v. 5.0)

	MIP ^a	NIP ^a
Isotherm model	Bi-Jovanovic	Freundlich
Affinity constant (K , mM^{-1})	6 ± 4	0.9 ± 0.2^b
	0.06 ± 0.04	
Binding site density (q , $\mu\text{mol g}^{-1}$)	17 ± 4	2.2 ± 0.4^b
	446 ± 195	
Heterogeneity parameter (n , $(\mu\text{mol g}^{-1}) (\text{M}^{-1})^m$)	–	0.9 ± 0.3
Binding capacity ($q \times K$, $\mu\text{mol g}^{-1} \text{L mmol}^{-1}$)	102 ± 6	1.7 ± 0.4
	27 ± 7	
r^2	0.997	0.992

^a \pm Standard error of regression coefficient

^b Values calculated according to Rampley et al. [32]. K (mM^{-1}) is the apparent weighted average affinity in the 0.12–72 mM^{-1} K range. Apparent binding site density, q ($\mu\text{mol g}^{-1}$), was also calculated in the interval 0.12–72 mM^{-1}

into the MIP/NIP cartridges at flow rates ranging from 0.25 to 4.0 mL min^{-1} . Recoveries close to 100% (RSD 2%, $n=3$) were obtained up to a flow rate of 0.8 mL min^{-1} . Higher loading rates yielded lower values due to the decrease of the interaction time between the analyte and the polymer binding sites. Therefore, a flow rate of 0.8 mL min^{-1} was selected for further experiments.

Elution solvent selection and optimisation

To optimise the elution solvent, 10-mL portions (each) of different samples containing 3 μg of ENR, dissolved in 0.1 M HEPES (pH 7.5), were percolated through the MIP/NIP cartridges, and 3 mL (1 + 1 + 1 mL) of different solvents were used to elute the retained antibiotic. The concentration of ENR was measured in each fraction. The eluting solvents tested were methanol; methanol/acetic acid (HAc) and methanol/trifluoroacetic acid (TFA). A methanolic solution of 0.05 M tetra-*n*-butylammonium hydrogen sulphate (TBA), an ion pairing reagent, was also evaluated owing to its known strong interaction with mixed mode polymeric sorbents [22, 28]. Unfortunately, a double peak was observed in the chromatogram, possibly due to the zwitterionic behaviour of the ENR in this media. The best recoveries were obtained

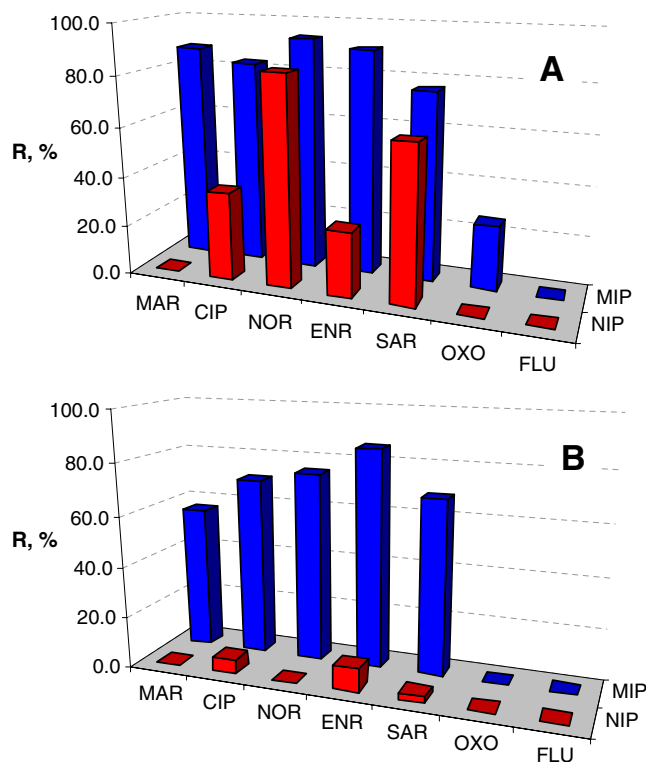


Fig. 5 Extraction recoveries (%) obtained with the MIP and the NIP cartridges for seven fluoroquinolones after percolation of 5 mL of a buffered human urine sample (0.1 M HEPES, pH 7.5) containing 50 $\mu\text{g L}^{-1}$ of each compound using a washing step with a 5 mL and b 25 mL of ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v)

with methanol/acid mixtures. The use of 3 mL MeOH (3% TFA) allowed the quantitative recovery of ENR (101%, RSD 2%, $n=3$).

In order to increase the sensitivity of the assay, the sample was evaporated under a nitrogen stream and reconstituted in 1 mL mobile phase prior to the injection into the HPLC-FLD. However the final recovery was lower than 89% (RSD 9%, $n=3$) so that the evaporation step was discarded in later analyses.

Analysis of biological samples

Washing solvent selection applied in urine samples

The nonspecific interaction between the fluoroquinolones included in the study and the imprinted polymer can be minimised in the presence of a mixture of ACN/water (0.1 M HEPES, pH 7.5) (50:50, v/v), as concluded from the chromatographic experiments ([Chromatographic evaluation of the polymers](#)). This mixture (50% (v/v) organic solvent) also allows one to wash out the less polar nonrelated

substances present in the matrix samples. Thus, this solvent was selected for the washing step.

To determine the optimum washing volume, 5 mL of a buffered human urine sample (0.1 M HEPES, pH 7.5) containing $50 \mu\text{g L}^{-1}$ MAR, CIP, NOR, ENR, SAR, OXO and FLU was loaded into the MIP/NIP cartridges, and different volumes (5, 10, 15 and 25 mL) of the hydro-organic mixture were applied in the washing step. Afterwards, the antibiotics were eluted with 3 mL MeOH (3% TFA) and the extract was analysed by HPLC-FLD. The results are collected in Fig. 5.

The use of 5 mL of the washing solvent (Fig. 5a) allowed recoveries higher than 80% (RSD 5.4–9.8, $n=3$) in the MIP cartridges for all the FQs tested, except OXO and FLU. However, the use of the NIP sorbent rendered recoveries in the range 36–84% (RSD 6.8–8.0%, $n=3$) for CIP, NOR, ENR and SAR. No recoveries are observed in the case of MAR, OXO and FLU.

When the washing volume is increased, the selectivity is better and excellent differences are showed between MIP/NIP when 25 mL of washing solvent is applied (Fig. 5b). In this

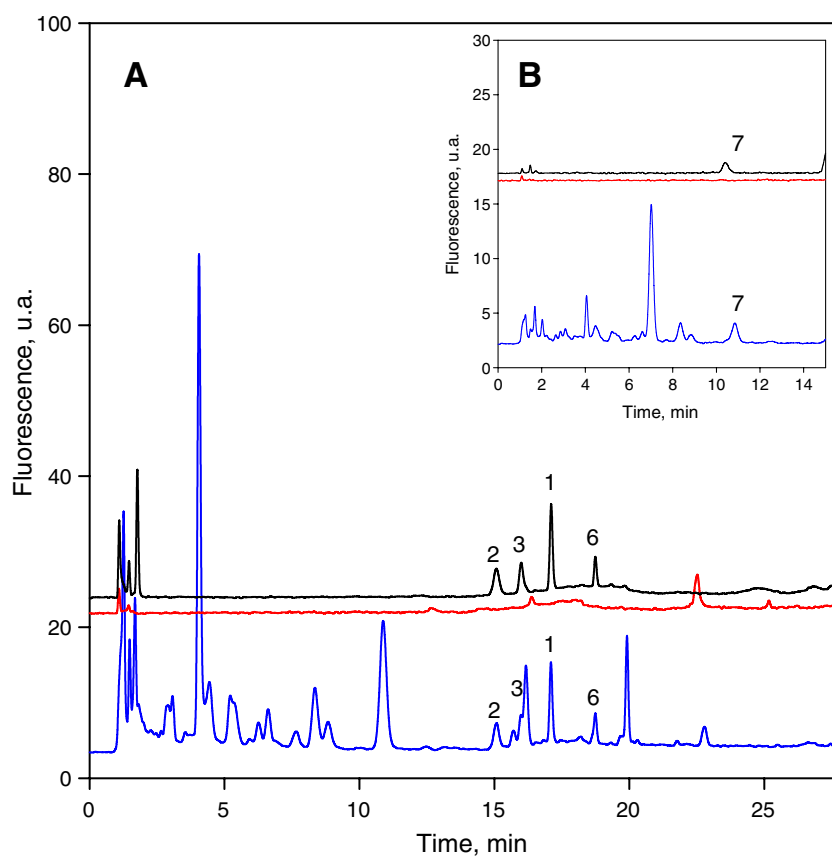


Fig. 6 Chromatogram obtained of a standard mixture of five fluoroquinolones after percolation of 5 mL of a buffered human urine sample (0.1 M HEPES, pH 7.5) containing $50 \mu\text{g L}^{-1}$ of ENR (1), CIP (2), NOR (3) and SAR (6) (MAR, $125 \mu\text{g L}^{-1}$) using a washing step with 25 mL of the solvent ACN/water (0.1 M HEPES, pH 7.5) (50:50,

v/v): *blue line* direct sample without MISPE step, *black line* urine sample after MIP cartridge percolation, *red line* urine sample after NIP cartridge percolation. Fluorescence excitation/emission wavelengths were **a** 280/440 nm for CIP, NOR, ENR and SAR; **b** 280/515 nm for MAR

Table 4 Average recoveries (*R*), relative standard deviations (RSDs, *n*=3) and limits of detection (LOD) and quantification (LOQ) obtained after solid-phase extraction of 5 mL buffer (0.1 M HEPES, pH 7.5) and urine spiked at 20, 50, 80, 125 and 250 µg L⁻¹ concentration levels

Analyte	Spiking level (ng mL ⁻¹)	Buffer (0.1 M HEPES, pH 7.5)			Urine			LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹)
		Level found (ng mL ⁻¹)	<i>R</i> (%)	RSD (%)	Level found (ng mL ⁻¹)	<i>R</i> (%)	RSD (%)		
MAR	20	ND	–	–	ND	–	–	34	113
	50	ND	–	–	ND	–	–		
	80	ND	–	–	ND	–	–		
	125	66.0	53	3.6	68.2	55	5.9		
	250	145.9	58	5.1	145.4	58	7.1		
CIP	20	14.0	70	3.4	14.1	71	5.5	4.9	16
	50	35.6	71	4.0	35.3	71	1.1		
	80	55.4	69	2.1	58.2	73	4.4		
	125	87.7	70	2.7	85.7	69	3.1		
	250	174.5	70	2.3	175.1	70	1.4		
NOR	20	14.5	73	2.2	14.9	74	3.4	6.1	20
	50	37.2	74	4.4	37.4	75	3.5		
	80	58.9	74	9.8	59.5	74	5.4		
	125	90.6	73	2.9	91.7	73	3.5		
	250	186.4	75	1.5	186.6	75	1.4		
ENR	20	17.5	87	5.6	17.0	85	2.2	1.9	6.3
	50	43.1	86	1.4	42.7	85	1.0		
	80	70.5	88	6.0	67.2	84	4.2		
	125	108.8	87	1.6	107.5	86	1.6		
	250	213.7	86	2.0	217.3	87	2.2		
SAR	20	14.0	69	1.5	13.7	68	1.9	6.0	20
	50	34.0	68	2.7	34.3	69	1.0		
	80	56.0	70	9.5	53.7	67	4.3		
	125	84.3	68	5.9	84.6	68	1.3		
	250	169.1	68	2.7	170.9	68	2.0		

ND not determined

case, the extraction recoveries in the NIP ranged between 0 and 10% (RSD 5.2–8.8%, *n* = 3) for all the antibiotics, whereas retention on the MIP was excellent for MAR, CIP, NOR, ENR and SAR (recoveries between 56–85%, *n*=3) and no retention is observed for OXO and FLU. These results are in agreement with those obtained in the HPLC studies when selectivity of the FQs in the MIP/NIP columns is performed. Moreover, the extracts eluted from MIP cartridge were very clean and thus matrix interferences were practically eliminated (Fig. 6).

In general these results are better than those reported by other authors who evaluate the same type of antibiotics in urine samples and using molecularly imprinted polymers selective for ENR. For example, Caro et al. [36] reported recoveries of 80% for CIP, 50% for NOR and 20% for ENR (RSD 10–18%, *n*=3). The same authors [21] reported recoveries of 80% for ENR, 35% for NOR and CIPR coeluted with an impurity. Moreover, neither article reports SPE results obtained with NIP cartridges, and for optimal results both studies required the use of a two-step MISPE approach combining a commercial SPE cartridge (Oasis HLB 60 mg) and the ENR-MIP cartridge.

Human urine sample analysis

To evaluate the applicability of the optimised MISPE procedure to real sample, trueness and precision of the method were determined using spiked blank samples at five concentration levels in buffer and urine matrix (three spiked samples for each level). The results, summarised in (Table 4), show the good accuracy of the method. Errors, expressed as relative standard deviation (RSD, %) are ≤ 10% for all the concentration levels tested. The limits of detection and quantification after the percolation of 5-mL urine samples through the MIP cartridges are shown in Table 4. The values obtained for CIP (4.9 ng mL⁻¹) or ENR

Table 5 Comparison between the results obtained by application of standard addition and an external standard method in a human urine sample obtained after 50 h post-administration of NOR 400 mg (*n*=3)

Analyte	Added (ng mL ⁻¹)	Found (ng mL ⁻¹)	RSD (%)
NOR	0	57	3
	50	104	3
	100	156	4

(1.8 ng mL⁻¹) are comparable or better than those reported by other authors for fluoroquinolone MISPE of urine samples [21, 27, 36]. Moreover, the results also compare favourably with those obtained using commercial cartridges for the same type of samples. For instance, Ballesteros et al. reported a LOD of 10 µg L⁻¹ for ENR, CIP and ofloxacin using 3M-Empore MPC extraction cartridges (Supelco, MO, USA).

The presence of the matrix components in urine samples does not affect significantly the preconcentration efficiency of antibiotics on SPE sorbent. The recoveries obtained for all the FQs except FLU and OXO were excellent and better than those reported in the literature [21, 36].

The proposed method was also applied to determine NOR in urine samples of a volunteer who was treated with 400 mg NOR once daily. Urine samples were collected at 50 h after the final dose and analysed with the optimised MISPE-HPLC/FLD method. Due to the complexity of the urine matrix and for validating purposes, a recovery study was performed by spiking 5 mL of the sample with increasing amounts (0.25 µg and 0.5 µg) of NOR. As shown in Table 5, excellent results are obtained with good accuracy and excellent precision (RSD ≤ 4%, *n* = 3).

Conclusions

This work demonstrates the applicability of a poly(MAA-co-HEMA-co-EDMA) molecularly imprinted polymer for the preconcentration of five fluoroquinolones in water and urine samples. The optimised method is based on a MISPE procedure followed by HPLC with fluorescence detection. Using the water-compatible MIP as a specific MISPE sorbent yields a method suitable to extract fluoroquinolones from biological samples and providing good recoveries and reproducibility. The cartridges can be reused for more than 90 assays without losing their concentration efficiency, which is promising for online preconcentration formats. Thus, this procedure is adequate for the analysis of the fluoroquinolones at the nanogram per millilitre level when processing urine samples without complex sample pretreatment.

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