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Development of molecularly imprinted polymers and its magnetic version for the extraction of fluoroquinolones from milk☆

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ABSTRACT

The aim of the present study is to develop selective extracting materials applicable to a diversity of fluoroquinolones. A series of Molecularly Imprinted Materials (MIPs) were prepared in order to choose the nature of the monomer and that of the porogen together with its ratio with the cross-linker. A non-regulated quinolone, Levofloxacin, was used as a template to avoid false positive results in its application. The resulting MIPs were evaluated in MISPE experiments for Enrofloxacin. The polymer prepared using methyl acrylate (MA) as a monomer and MeOH/$CHCl_3$ (1:1, v/v) as a porogen provided the desired selectivity. Concretely, 100 μL (1.1 mmol) of MA as a monomer, 1.30 mL (9.8 mmol) of EGDMA (ethyleneglycol dimethacrylate) as a cross-linker, 31 mg (0.19 mmol) of AIBN (2,2′-azobis(2-methylpropionitrile)) as an initiator, were mixed with 85 mg (0.24 mmol) of levofloxacin as a template and 4 mL of MeOH/$CHCl_3$ (1:1, v/v) as a porogen.

The obtained optimized composition was upgraded to magnetic molecularly imprinted polymers (MMIPs) to be used as a dispersive-SPE extracting materials in the analysis of fluoroquinolones in milk. The selectivity of the resulting material for several fluoroquinolones (enrofloxacin, ciprofloxacin, sarafloxacin and norfloxacin) was studied. Finally, the MMIP was tested against real quinolone positive milk samples to evaluate its applicability.

1. Introduction

The use of quinolones for the treatment of bacterial infections, in human as well as in veterinary contexts, has increased in recent years. Additionally, although since 2006 the European Union (EU) legislation has forbidden it, these drugs are used as prophylactics or growth promoters in food-producing animals [1]. Thus, the UE Regulation 37/2010 [2] established the Maximum Residue Limit (MRL) for different classes of antibiotics in animal products destined to human consumption. In milk, the MRL range for quinolones has been established between 30 for danfloxacin and 100 μg kg^{-1} for enrofloxacin. These limits permit to ensure the safety of food for human consumption considering that if animals are slaughtered before the natural metabolization/elimination of antibiotics, the latter could enter the food chain in humans. This may represent a risk for the consumer health from the point of view of toxicity, allergy and bacterial resistance.

In this context, it is of great importance to have robust, accurate and sensitive analytical methods for the determination of these compounds in highly complex matrices such as food products of animal origin. A high variety of sample preparation methods and materials have been used for this purpose. Among them, liquid–liquid extraction (LLE), solid phase extraction (SPE), stir bar sorptive extraction (SBSE), dispersive liquid–liquid microextraction (DLLM), and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Save), are the most commonly applied [3–9]. Also, Magnetic Covalent Organic Frameworks (MCOFs) have been used for this purpose [10,11]. Although, all these methods implement advantages over the previous ones, the diversity and the lack of a broadly accepted technology, indicates the absence of a remarkable

☆ This article is dedicated to the memory of Professor Alberto Navalón of the Universidad de Granada (Spain).

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advantage for one of them.

For instance, traditional extraction methods, such as LLE [3] and SPE [4,5], used in complex matrices, such as milk, are not selective enough to extract quinolones. Other disadvantages associated to LLE and SPE are their cost and their time-consuming and environmentally unfriendly character.

To improve the selectiveness to quinolones, other techniques such as the use of molecular imprinted polymers (MIPs) as sorbent in SPE (MISPE) were developed. MIPs are synthesized to selectively bind the molecule of interest usually through weak interactions between the target and the polymer matrix. The advantage of this kind of polymers in sample treatment is their selectivity for the target molecule, thus allowing the elimination of any interfering species prior analysis [12].

Usually, the molecule chosen to act as a template is the same as the analyte to be dosed in samples. By proceeding in this way, a theoretical maximal selectivity for the considered compound is achieved. Thus, most of the examples in the literature follow this approach by using compounds regulated by the EU. Enrofloxacin (ENRO), ciprofloxacin (CIPRO) and sarafloxacin (SARA) are among the most used [13–15]. However, one of the inconveniences of MIPs consists in ensuring the perfect removal of the template from MIP before its use in sample preparation [16]. Otherwise, the possibility to obtain a "false positive" result is not negligible. The use as a template of a non-regulated compound could allow us to discard univocally the event of "false positive" results. Nevertheless, the selectivity for the target molecule of the obtained MIP may be compromised.

The existence of a cross selectivity for structurally related compounds in MIPs is a known fact often considered in a number of studies [9,13,17]. If the selectivity for a group of molecules is maintained, this characteristic of MIPs may result advantageous as it may allow the simultaneous analysis of several analytes belonging to the same structural family.

In order to overcome the tedious procedure of MISPE, magnetic nanoparticles (MNPs) have been developed [18,19]. In this case, the extractant material has a core composed by magnetite, which allow the magnetic separation from the sample by applying an external magnet. The technology improves extraction efficiency and avoids the time–consuming steps of MISPE, such as column-packing, sample-loading and filtration/centrifugation separation, being the sample treatment as easy and quick as QuEChERS methodology. The preparation of MNPs involves the previous obtaining of the magnetite nucleus followed by activation/silanization of the particle surface. Finally, an affinity inducing agent is bonded to this surface. This method permits to bond chemically the magnetic particle to the corresponding affinity agent. If MIPs are considered as affinity agents [20], these can be bond to the silanized magnetic particles by polymerization onto their surface. Alternatively, surfactants can be used to directly polymerize MIPs onto the non-silanized magnetic particle, which makes easier the preparation of MMIPs.

Regarding the analysis of fluoroquinolones in milk, the MMIP technology has been applied. Using an alternative preparation method for MMIPs, authors use commercially available MIPs for quinolones, which are combined with magnetic NP to obtain the final extracting material [21]. More recently, the application of MMIPs to the extraction of norfloxacin from milk using silanized magnetic NPs in the preparation of the extracting imprinted material has been described [16].

This study is undertaken with the aim to obtain a polymer provided with a selectivity broader enough to be applicable to a several compounds members of the same structural family, fluoroquinolones, while keeping the known selectivity of MIPs. Additionally, considering the diversity of methods described [16,20], it is our aim to keep it as simple as possible. For this reason, the polymer composition has been optimized by modifying several factors such as monomer, porogen and cross–linker ratio. In order to avoid false positive results, Levofloxacin (LEVO), a non-regulated quinolone, was used as a template. The selectivity of the resulting material for several quinolones was studied. The obtained optimized conditions will be applied to the preparation of a MMIP. The later was prepared and tested to be used as a dispersive-SPE extracting material in the analysis of fluoroquinolones in milk. Dispersive-SPE procedures are considered advantageous over other similar sample preparation procedures given the easy manipulation. Finally, the resulting MMIP was applied to real quinolone positive milk samples to evaluate its applicability.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Ciprofloxacin (CIPRO, ≥98 %), enrofloxacin (ENRO, 99.8 %), levofloxacin (LEVO, ≥98 %), norfloxacin (NOR, ≥98 %), sarafloxacin (SARA, ≥98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents such as iron(II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$, >99 %), iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$, >98 %), methyl acrylate (MA, 99 %), 2-(diethylamino)ethyl methacrylate (DAM, 99 %), 4-vinylpyridine (VP, 95 %), oleic acid (90 %), ethyleneglycol dimethacrylate (EGDMA, 98 %), 2,2′-azobis(2-methylpropionitrile) (AIBN, 98 %), polyvinylpyrrolidone (PVP) and polyvinylalcohol (PVA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH, 98 %) and methanol (MeOH, 99.9 %) was obtained from Panreac Quimica (Castellar del Vallès, Barcelona, Spain). Formic acid (HCOOH, >98 %), acetic acid (AcOH, 99.8 %), ammonium hydroxide (NH_4OH 25 %) and chloroform ($CHCl_3$) were supplied by Scharlab (Sentmenat, Barcelona, Spain). Sodium dodecyl sulfate (SDS) was obtained from Merck (Darmstadt, Germany) and acetonitrile (MeCN, 99.9 %) was purchased from VWR Chemicals (Fontenay-sous-bois, France).

2.2. Standards and stock solutions

Quinolones stock solutions were prepared at a concentration of 50 $\mu g{\cdot}mL^{-1}$ in $50{\cdot}10^{-3}$ $mol{\cdot}L^{-1}$acetic acid (AcOH) aqueous solution. The individual working solutions were prepared by dilution in water at a concentration of 10 $\mu g{\cdot}mL^{-1}$, used in MIP studies, or 5 $\mu g{\cdot}mL^{-1}$, used in MMIP studies. A quinolone mixture (CIPRO, ENRO, NOR and SARA) stock solution was prepared at a concentration of 50 $\mu g{\cdot}mL^{-1}$, for each one, in 50 mM acetic acid (AcOH) aqueous solution. The corresponding working solutions were prepared by dilution in water at a concentration of 5 $\mu g{\cdot}mL^{-1}$ for each quinolone, used in MIP studies, or at 0.5 $\mu g{\cdot}mL^{-1}$, used in MMIP studies. All solutions were stored in dark glass bottles to prevent degradation.

2.3. Instrumentation

2.3.1. Chromatographic system

An Agilent 1100 LC system (Agilent Technologies, CA, USA), equipped with a vacuum degasser, a quaternary pump, an auto-sampler and a diode array detector (DAD 1260 infinity series). A Symmetry C8 column (50 × 2.1 mm, 5 μm) and a Symmetry C8 (20 mm) precolumn, both supplied by Waters (Milford, MA, USA), were used in the chromatographic separation. The mobile phase was composed of 0.2 % HCOOH in Milli-Q water (A) and 0.2 % HCOOH in MeCN (B). The flow rate was 300 μL min^{-1} and the injection volume was 10 μL. Gradient conditions were as follows: initial mobile phase, 94 % (A), held for 4 min, then decreased to 85 % (A) in 3 min, running to 70 % (A) in 2 min and held for 1 min, and so increased to 94 % (A) in 2 min and finally held for 5 min. Total run time was 17 min. The wavelength of observation of the FQs was 280 nm.

2.3.2. Other instrumentation

MIPs were characterized by scanning electron microscopy (SEM). The instrument used was a Jerol 7100F working at 10 kV and the samples were coated with a thin gold film before observation. Nitrogen

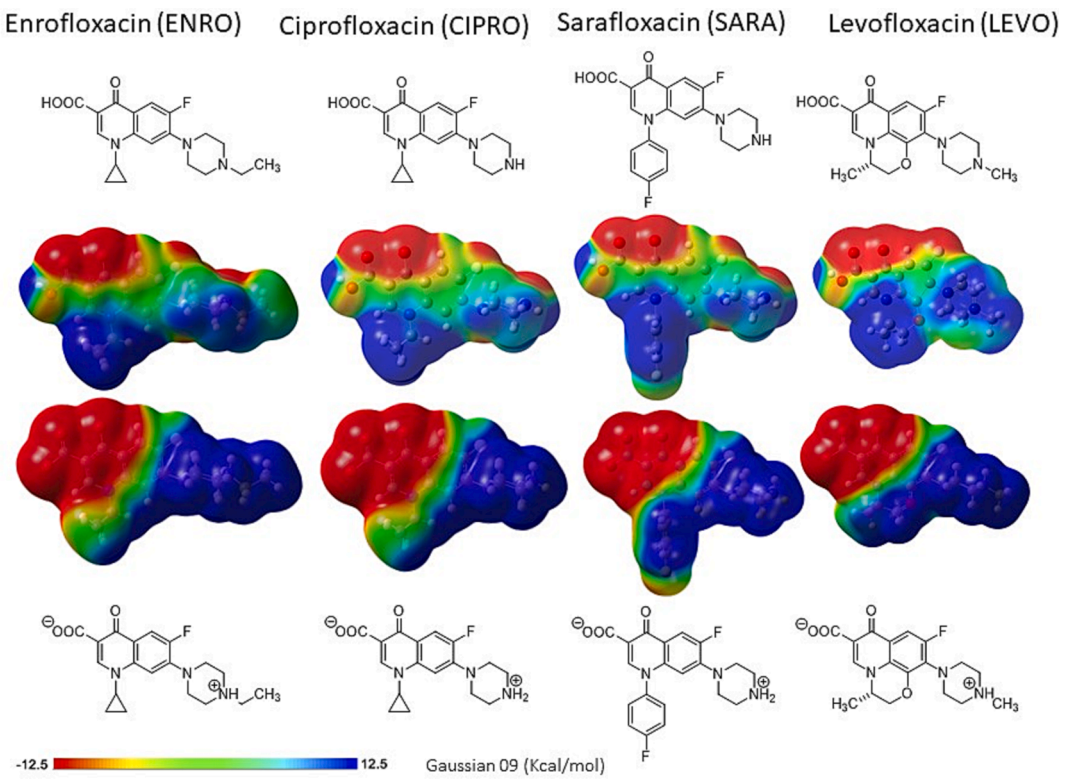

Fig. 1. Electronic densities of the fluoroquinolones used in the study. Upper line: neutral structures. Lower line: Zwitterion structures. Calculated using Gaussian 09.

adsorption/desorption isotherms were measured using a LS Particle Size Analyser using 120 mg of each polymer. The Brunauer-Emmett-Teller (BET) method and the Barret-Joyner-Halenda (BJH) theory were used to calculate the specific surface area, the pore volume and the pore size distribution.

Centrifuges used were Sigma 2-16KL (Osterode, Germany) and Hettich Zentrifugen MIKRO 220R (Tuttlingen, Germany). The evaporation system was a MiVac Genevac (Ipswich, England). Milli-Q Water was purified using an Evoqua Water Technologies LaboStar (Warrendale, PA, USA). The stoves used were a J.P. Selecta Hotcold-S (Abrera, Spain) and a Memmert UF 110 (Schwabach, Germany).

2.4. Synthesis of MIPs

The polymer [22] was prepared mixing 85 mg of LEVO as a template, 100 μL of MA as the functional monomer in 2.5 mL MeOH/$CHCl_3$ (1:1, v/v). Then, 1.3 mL EGDMA as cross-linker and 31 mg of AIBN was added as a radical initiator. The resulting solution was allowed to polymerize in a stove at 60 °C for 24 h. The resulting monolith polymer was ground to obtain fine particles. In order to remove the template, the obtained solid was washed as follows: 1 g of polymer was disposed in a tube with 25 mL of MeOH/HCOOH (95:5, v/v) and the suspension was vigorously shaken for 2 min. The tubes were centrifuged during 10 min at 10000 rpm and the supernatant eliminated. This step was repeated six times. In order to verify the absence of template (LEVO), the last washing supernatant was injected into the LC system. The washed polymer was rinsed on a filter with 3x150 mL of H_2O Milli-Q, to remove the acid remains, and then 50 mL MeOH. The solid was allowed to dry under vacuum. Afterwards, the MIP was sieved, and particles in the size range 50–150 μm were collected to be used in MISPE experiments. The same procedure was followed to prepare NIPs but template was not added in this case.

2.5. Synthesis of MMIPs

Magnetite, Fe_3O_4, was obtained by co-precipitation in basic conditions. 3.98 g of $FeCl_2{\cdot}4H_2O$ and 10.81 g of $FeCl_3{\cdot}6H_2O$ were added to 200 mL H_2O under stirring. An excess of NaOH 2 M (100 mL) was slowly added [23]. The obtained black solid (magnetite) was washed with H_2O prior to use. A 250 mg amount of magnetite was added to 250 μL of oleic acid and the mixture vortexed. To this mixture, a solution of 85 mg of LEVO, in 2.5 mL of H_2O containing 75 μL of HCOOH and 180 μL of MA, was added. To this mixture 943 μL of EGDMA as cross-linker and 25 mL of MeOH. Finally, 0.25 g of initiator (AIBN) were added. The mixture was allowed to polymerise at 60 °C during 24 h with constant stirring [23]. The obtained polymer was collected by filtration and washed with MeOH.

In order to remove the template 15 mL of MeOH/HCOOH (95:5, v/v) as cleaning mixture was added to 1 g of MMIP. The suspension was centrifuged 10 min at 10000 rpm and the supernatant was removed. This procedure was repeated six times. The first, third and sixth supernatants were analysed by HPLC in order to ensure the quality of the cleaning and the absence of template (LEVO) in the final polymer. Finally, the solid was treated with 5x10 mL of H_2O and 3x10 mL of MeOH. The magnetic polymer was collected using a magnet in order to discard possible non-magnetic particles and dried under vacuum.

2.6. SPE using MIPs

An amount of 100 mg of MIP was packed into a SPE cartridge, using upper and lower frits to prevent sorbent loss. The cartridge was conditioned with 1 mL of MeOH and 2 mL of H_2O.

A 10 $\mu g{\cdot}mL^{-1}$ working solution of ENRO in H_2O (0.5 mL) was loaded and collected as pass sample. Then 2 mL of milli-Q H_2O was passed as "clean-up" step and collected as wash sample; finally 1 mL of MeOH/NH_4OH (98:2) was used to elute ENRO and collected as basic elute sample. To enhance analyte collection a second step elution was applied using MeOH/HCOOH (95:5, v/v) and collected as acid elute sample. All the solutions were analysed in the LC, evaluating the area of ENRO peak after each step of the MISPE procedure.

Table 1
Composition of the diverse MIPs prepared.

Polymer	Template (mg)	Monomer (μL)	Cross-linker (mL)[a]	Porogen[b]
P1	LEVO (85)	DAM (240)	EGDMA (1.30)	MeOH/$CHCl_3$ (1:1)
P2	LEVO (85)	DAM (240)	EGDMA (1.30)	MeCN/$CHCl_3$ (1:1)
P3	LEVO (85)	DAM (240)	EGDMA (1.30)	$CHCl_3$
P4	LEVO (85)	DAM (110)	EGDMA (1.43)	MeOH/$CHCl_3$ (1:1)
P5	LEVO (85)	VP (130)	EGDMA (1.30)	MeOH/$CHCl_3$ (1:1)
P6	LEVO (85)	VP (130)	EGDMA (1.30)	MeCN/$CHCl_3$ (1:1)
P7	LEVO (85)	VP (130)	EGDMA (1.30)	$CHCl_3$
P8	LEVO (85)	MA (100)	EGDMA (1.30)	MeOH/$CHCl_3$ (1:1)
P9	LEVO (85)	MA (100)	EGDMA (1.30)	MeCN/$CHCl_3$ (1:1)
P10	LEVO (85)	MA (100)	EGDMA (1.30)	$CHCl_3$
P11	LEVO (50)	MA (100)	EGDMA (1.30)	MeOH/$CHCl_3$ (1:1)
P12	LEVO (30)	MA (100)	EGDMA (1.30)	MeOH/$CHCl_3$ (1:1)
P13	–	DAM (240)	EGDMA (1.30)	MeOH/$CHCl_3$ (1:1)
P14	–	VP (130)	EGDMA (1.30)	MeOH/$CHCl_3$ (1:1)
P15	–	MA (100)	EGDMA (1.30)	MeOH/$CHCl_3$ (1:1)

[a] Initiator: AIBN (31 mg); [b]Porogen: 4 mL.

2.7. Dispersive-SPE using MMIPs

A 100 mg amount of MMIP was placed in a tube at which 200 μL of MeOH were added. To this mixture 2 mL of an ENRO 5 $\mu g \cdot mL^{-1}$ standard solution were added. The mixture was vortexed and after 2 h in contact, the supernatant was separated.

To desorb the quinolone from the MMIP surface, 1 mL of a basic mixture clean of MeOH/NH_4OH (98:2, v/v) was added and was left in contact during 2 h. The supernatant was separated and the solid treated with 1 mL of an acidic mixture (MeOH/HCOOH (95:5, v/v)). The three supernatant solutions were analyzed. The test was performed by quintuplicate.

2.8. Application to milk samples

Diverse samples of milk (2 g) from animals medicated with ENRO, but also from non-treated animals, supplied by the farm Granja La Saireta S.C.P. (Vallfogona de Balaguer, Lleida), were used for evaluate the applicability of the polymer.

Initially 2 g of milk were diluted using 5 mL of H_2O and the procedure described in 3.7 was followed. In this case, supernatants acidic and basic were collected in the same tube and evaporated. The residue was reconstituted with 0.2 mL of H_2O/HCOOH (95:5, v/v) and analysed using the HPLC method previously described.

3. Results and discussion

3.1. Composition optimization and characterization of MIPs

The preparation of conventional MIP polymers was undertaken as a first step in the preparation of the MMIP materials considered the final aim of the study. There are several procedures to synthesize MIPs. Perhaps, one of the most popular is bulk polymerization, which consists in mixing together template and monomer, with initiator, cross-linker and porogen. While there is a high not written consensus about the nature of initiator (AIBN) and cross-linker (EGDMA), this is not the case for monomer and porogen. For the former it may depend among others on the nature and functionality of the template.

In order to choose a template useful, not only for a particular quinolone, but for several of the members of the series, the electronic density of some of the family members was modelled. Most quinolones are amino acids. Therefore, it is reasonable to consider the ionized form that will be the most likely form existing in an aqueous solution such as milk. As shown in Fig. 1, the electronic density of several of the members of the family is similar, even more when the ionised form is considered. In this modelization the regulated quinolones ENRO, CIPRO and SARA were included together with the non-regulated LEVO. At this point LEVO was chosen to be used as a template considering its similarity with the regulated quinolones. Thus, the putative presence of template in the analysis will not originate false positive results.

A series of polymers were prepared by varying the nature of monomer (DAM, VP and MA) and the proportions of this component and that of cross-linker (Table 1). Also, several solvents were considered as porogen (MeOH/$CHCl_3$ (1:1, v/v); MeCN/$CHCl_3$ (1:1, v/v) and $CHCl_3$). The amount of template was also taken into account.

A general test of retention for a standard solution of ENRO, using SPE experiments, was established to evaluate the diverse polymers prepared. The peaks of the chromatograms obtained after each step of the SPE procedure were integrated and the areas were used to calculate the percentage of retention (%Rt) of ENRO selectively retained.

$$\%Rt = \frac{A_{std} - (A_p + A_c)}{A_{std}} 100$$

where A_{std} corresponds to the area of the initial ENRO solution. A_p is the area corresponding to the solution that goes through the SPE cartridge without interacting with the material. A_c corresponds to the eluate of the clean-up step (section 2.6 in Methods).

Two different ratios of monomer (DAM) and cross-linker (EGDMA)

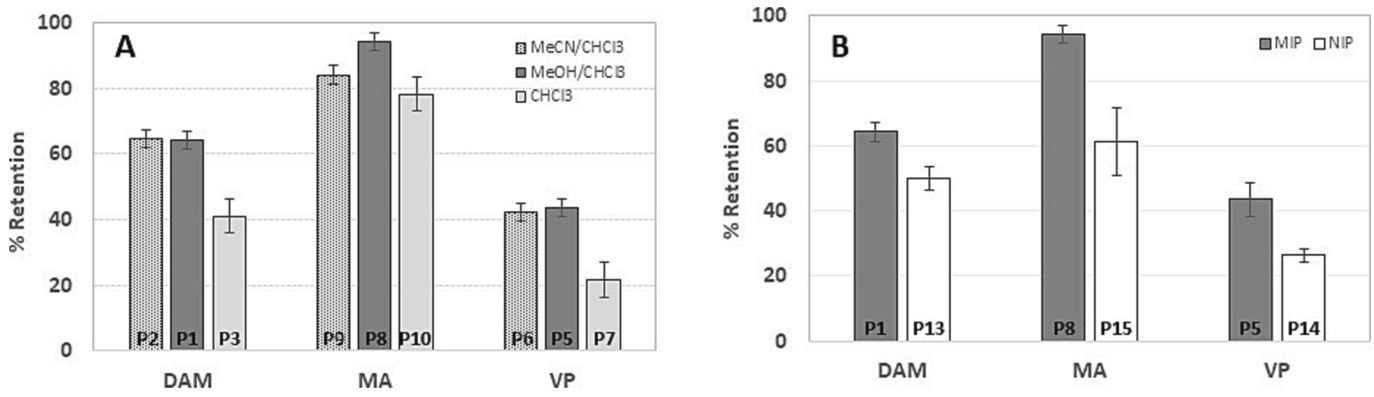

Fig. 2. A) Effect of monomer and porogen on retention of ENRO. B) Comparison of ENRO retention for MIPs and NMIPs prepared on the same conditions.

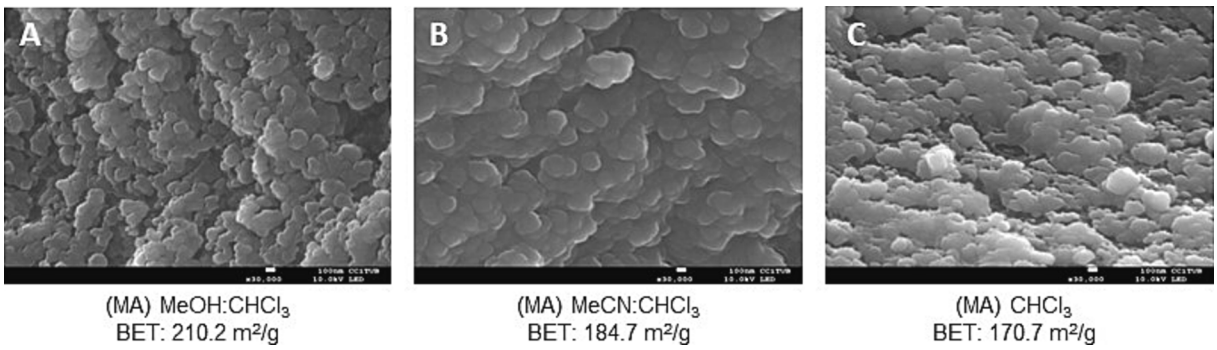

Fig. 3. SEM images for polymers **P8** (A), **P9** (B) and **P10** (C).

were studied. Polymer **P1** contained a ratio of 1:5.4 (DAM/EGDMA, v/v) while the ratio for these two components was 1:13 (v/v) for **P4**. In the two cases, the total volume DAM + EGDMA was maintained. When tested polymer **P4** was much less permeable than **P1**, which makes elution and the realization of the retention test difficult. Therefore, the ratio 1:5.4 (v/v) between monomer and cross-linker was maintained in all other compositions tested.

In the next step, diverse monomers (DAM, VP and MA) using three compositions of porogen were tested for the retention of ENRO (Fig. 2A) (**P1-P3, P5-P10**). The percentages of retention were between a minimum of 21.6 %, obtained with VP as monomer and chloroform as porogen (**P7**), to a maximum of 94.5 % for the polymer prepared with MA as monomer and MeOH/$CHCl_3$ (1:1, v/v) as porogen (**P8**). In all cases, the use of MeOH/$CHCl_3$ (1:1, v/v) as a porogen yielded the best results. As far as monomers are concerned, the order of efficacy in retaining ENRO was MA > DAM > VP. The optimal results, expressed in terms of retention of ENRO, were obtained when MA was used as monomer and MeOH/$CHCl_3$ (1:1, v/v) as a porogen (**P8**). At this point, the difficulties encountered in absolutely removing the template from the prepared polymers drive us to attempt the decrease of the amount of template used (**P11**, **P12**). However, the retention of ENRO in the resulting polymers was always lower than in **P8**. Therefore, considering that template was chosen in particular to not interfere in further analyses, the original amount of LEVO used was maintained. In addition, the actual effectiveness of imprinting (imprinting factor) was checked by comparison with the corresponding NIPs (Fig. 2B) (**P13-P15**). Again, the polymer prepared with MA as a monomer was the one showing the highest difference (33 %) in retention between MIP and NIP, which indicates the preference for a specific retention over the unspecific retention of the analyte.

The obtained polymers were physically characterized, in terms of porosity. Polymers prepared using different porogens (MeOH/$CHCl_3$ (1:1, v/v), MeCN/$CHCl_3$ (1:1, v/v) and $CHCl_3$) were analysed through Scanning Electron Microscopy (SEM). In Fig. 3 microscopic images corresponding to polymers **P8**, **P9** and **P10** (Table 1) are shown.

The images show a similar morphology characterized by rounding shape agglomerates of polymer. The morphologic differences observed were considered non conclusive. Therefore, nitrogen adsorption/desorption isotherms were registered for these polymers. Specific surface areas of 210 m^2g^{-1},185 m^2g^{-1} and 171 m^2g^{-1} were calculated using Brunauer-Emmet-Teller (BET) method for polymers **P8**, **P9** and **P10**, respectively. A direct relationship between surface area and retention of ENRO was observed. Thus, the highest surface area the highest retention (Fig. 2A).

Considering all the results obtained up to this point, the polymer prepared using MA as a monomer and MeOH/$CHCl_3$ (1:1, v/v) as a porogen (**P8** in Table 1) showed the desired selectivity. Nevertheless, difficulties were encountered when trying to exhaustively remove the template. This drawback was attributed to a possible slow mass-transfer which may affect the reproducibility of results in the everyday routinely use. At this point, we decided to explore the magnetic MIPs format, as a dispersive-SPE material, in order to improve the control of contact time and reproducibility in the analysis.

3.2. *Upgrade to the preparation of MMIPs and their application*

The conditions previously determined to yield MIPs with the desired selectivity, type of monomer and amount of template, were applied. Nevertheless, instead of a bulk polymerization, which is not an appropriate methodology in this context, an emulsion polymerization was performed. This technology involves the following steps. In brief, magnetite plus oleic acid, and the prepolymerization mixture, constituted by the template and the monomer, is added to the mixture of porogen/cross-linker and emulsifier at which the initiator is incorporated. The mixture requires stirring to favour the polymerization of the polymer around magnetite particles.

This change in methodology forces to modify the ratio monomer/cross-linker in favour of the former and to increase the amount of porogen. The influence and role that the addition of an emulsifying agent [23] have in the reaction mixture was studied considering removal of template (LEVO) and retention of ENRO. Several polymers were prepared using three emulsifiers polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA) and sodium dodecyl sulphate (SDS). In addition, a polymer was prepared **W**ithout using any **E**mulsifier (WE).

No significant differences were detected during preparation. However, when SDS was used as emulsifier, the template was significantly more difficult to remove from the polymer. This fact could be the consequence of a major occupation of the recognition sites what will result in a lower efficacy in the recognition of other quinolones. The behaviour of the remaining three polymers was similar regarding the ability to be cleaned. Additionally, the retention of ENRO for these polymers was similar (PVP Rt: 39 ± 12 %; PVA Rt: 40 ± 6 %; WE Rt: 54 ± 11 %). Given the similarity of results, we decided to synthesize the MMIP without adding any emulsifier. It is likely that the oleic acid used in the mixture plays already this role during polymerisation. An ash test permitted to determine a content of 91 % (RSD = 1 % n = 3) of polymer for the prepared MMIP.

A competitivity test was performed with a mixture of quinolones in order to measure the relative affinity of these compounds for the prepared polymer. Thus a mixture of ENRO, CIPRO, SARA, and NOR containing 0.5 $mg{\cdot}L^{-1}$ for each one was kept in contact with the MMIP for 2 h. The retained quinolones were quantified. Thus, a retention of 79 ± 2 % was obtained for CIPRO, 72 ± 3 % for SARA and 57 ± 5 % for ENRO. Simultaneously and unexpectedly, a peak corresponding to LEVO was observed. The presence of LEVO was attribute to the association of the other quinolones that will compete for the same recognition sites on the polymer. Unfortunately, this peak was overlapping with that of NOR which prevented the quantification of the latter.

The difference in retention for CIPRO and ENRO, whose structures only differ in an ethyl group on the piperazine ring, seems to point out

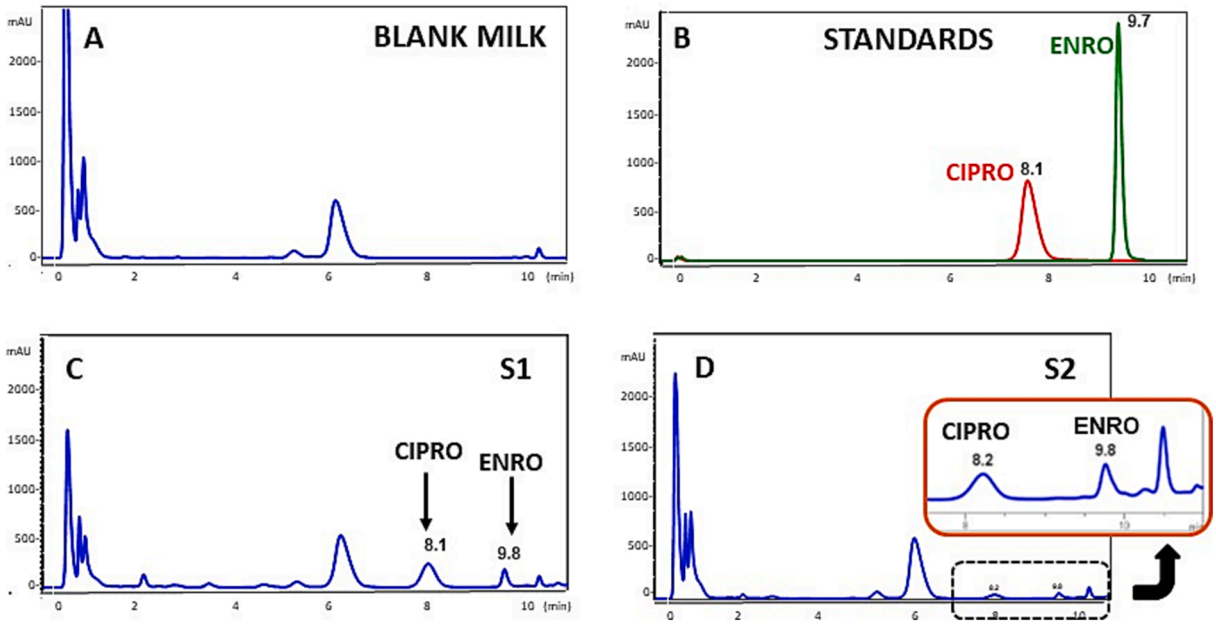

Fig. 4. HPLC-UV chromatograms (λ = 280 nm). A) Blank milk sample, B) Standards for CIPRO and ENRO (0.5 μg·mL^{-1} each quinolone), C and D) Positive Samples S1 and S2 (see the text for further information).

the significance of this ring in the interaction with the polymer. This hypothesis can be reinforced by the retention shown by SARA. In spite of the bulky group in the quinolone skeleton, SARA, which is not substituted in the piperazine ring, shows a retention similar to CIPRO.

A qualitative analysis by HPLC-UV was performed to test the ability of the polymer to retain quinolones present in real milk samples (Fig. 4). A blank sample was used in order to discard matrix elements (Fig. 4A). The obtained HPLC-UV chromatogram for a standard prepared with ENRO and CIPRO is shown in Fig. 4B. Two ENRO positive samples were analysed by HPLC-UV using the obtained MMIP for the extraction of quinolone residues. The first one (S1), corresponds to the third day of ENRO administration to cow (Fig. 4C), while the second sample (S2) (Fig. 4D) corresponds to the fourth day of the pharmacological process, when the treatment with ENRO is already stopped. Both ENRO and CIPRO were detected because CIPRO is the metabolite of ENRO. The chromatograms (HPLC-UV) show the decrease in the content of quinolones as a result of cow metabolism. These are real samples used in our lab in the context of a preceding study in which the amount of ENRO was quantified by an alternative procedure [8]. The content of the drug was determined to be 21.9 ± 1.7 μg·kg^{-1} (S1) and 6.7 ± 2.2 μg·kg^{-1} (S2), respectively. These values permit to demonstrate the applicability of the polymer at concentrations 5–15 times lower than the regulated MLR values (100 μg·kg^{-1}) for ENRO in a complex matrix such as milk.

4. Conclusions

With the aim to develop a selective extracting material applicable to a diversity of quinolones, a series of polymers were prepared. The nature of monomer (DAM, VP and MA) and that of the porogen component and its ratio with the cross-linker were considered. The polymer that provided a better combination between retention and selectivity was obtained using MA as a monomer and MeOH/$CHCl_3$ (1:1, v/v) as a porogen. A non-regulated quinolone (LEVO) was used in all instances as a template. Nevertheless, the selectivity for ENRO was demonstrated for the resulting polymer when applied in a MISPE procedure.

In order to overcome the experimental variability associated to the manual home-made preparation of MISPE, magnetic MIPs have been developed to be applied as a dispersive-SPE material. The MMIP was prepared using an emulsion polymerization procedure on a magnetite core, which involves the modification of the ratio monomer/cross-linker in favour of the monomer and to increase the amount of porogen. It is not needed any silanization reaction which made simple the preparation.

In spite of being LEVO the template used in the polymerization, the resulting material showed good retention values for CIPRO, SARA and ENRO. The use of LEVO as a template permits to avoid false positive results while simultaneously keeping the selectivity for other quinolone structures. Then the MMIP was applied to real quinolone positive milk samples to evaluate its applicability. The resulting material showed enough selectivity to detect ENRO and CIPRO in samples of considerable complexity such as milk when using HPLC-UV.

CRediT authorship contribution statement

Elena Megias-Pérez: Investigation, Writing – original draft. **Javier Giménez-López:** Investigation. **Arena Lascorz:** Investigation. **Barbara Benedetti:** Investigation. **Cristina Minguillón:** Conceptualization, Supervision, Writing – review & editing. **Dolores Barrón:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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