Anal Bioanal Chem (2016) 408:1843–1854
DOI 10.1007/s00216-015-8941-6

RESEARCH PAPER

Molecularly imprinted polymer beads for clean-up and preconcentration of β-lactamase-resistant penicillins in milk

Javier L. Urraca[1,2] · Raquel Chamorro-Mendiluce[3] · Guillermo Orellana[3] · Maria C. Moreno-Bondi[1]

Received: 7 June 2015 / Revised: 21 July 2015 / Accepted: 25 July 2015 / Published online: 5 September 2015

Abstract This work describes the development and application of class-selective molecularly imprinted polymers (MIPs) for the analysis of beta-lactamase-resistant penicillins, namely cloxacillin (CLOXA), oxacillin (OXA), and dicloxacillin (DICLOXA), in milk samples. Our method is based on molecularly imprinted solid-phase extraction (MISPE) coupled to high-performance liquid chromatography (HPLC) with diode-array detection (DAD). 2-Biphenylylpenicillin (2BPEN), a surrogate with a close resemblance to beta-lactamase-resistant penicillins in terms of size, shape, hydrophobicity, and functionality, was synthesized and used as the template for the polymer synthesis. A MIP library was prepared and screened to select the optimum functional monomer, *N*-(2-aminoethyl)methacrylamide, and cross-linker, trimethylolpropane trimethacrylate, that provided the best recognition for the target antibiotics. For the MISPE application, the MIPs were prepared in the form of microspheres, using porous silica beads (40–75 μm) as sacrificial scaffolds. The developed MISPE method enables efficient extraction from aqueous samples and analysis of the antimicrobials, when followed by a selective washing with 2 mL acetonitrile–water (20:80 *v/v*) and elution with 1 mL 0.05 mol L^{-1} tetrabutylammonium in methanol. The analytical method was validated according to EU guideline 2002/657/EC. The limits of quantification (S/N=10) were in the 5.3–6.3 μg kg^{-1} range, well below the maximum residue limits (MRLs) currently established. Inter-day mean recoveries were in the range 99–102 % with RSDs below 9 %, improving on the performance of previously reported MISPE methods for the analysis of CLOXA, OXA, or DICLOXA in milk samples.

Keywords Penicillin · Molecularly imprinted polymers · Surrogate template · MISPE · Milk · Method validation

Published in the topical collection *Analytical Applications of Biomimetic Recognition Elements* with guest editors Maria C. Moreno-Bondi and Elena Benito-Peña.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-8941-6) contains supplementary material, which is available to authorized users.

✉ Javier L. Urraca
jurracar@quim.ucm.es

✉ Maria C. Moreno-Bondi
mcmbondi@quim.ucm.es

[1] Department of Analytical Chemistry, Faculty of Chemistry, Universidad Complutense de Madrid, 28040 Madrid, Spain

[2] CEI Campus Moncloa, UCM-UPM, Avda Complutense s/n, 28040 Madrid, Spain

[3] Department of Organic Chemistry, Faculty of Chemistry, Universidad Complutense de Madrid, 28040 Madrid, Spain

Introduction

Penicillins are among the most commonly prescribed antimicrobials for food-producing animals [1]. These pharmaceuticals, belonging to the beta-lactam group of antibiotics, are active against a wide variety of aerobic and anaerobic bacteria [2]. The chemical structure of all penicillins includes a thiazolidine moiety attached to a fused beta-lactam ring and a side chain, and their antibacterial effect results from inhibition of the bacterial cell-wall synthesis. According to a recent report, these antimicrobials are the second most-sold veterinary antimicrobial agents (22 %) after tetracyclines (37 %) in 26 EU countries [1] and, together with cephalosporins, they constitute the group of antibiotics most frequently used in veterinary medicine for treatment of bacterial infections [3].

Penicillins (Fig. 1) can be classified in several groups [1, 3] as:

1. natural penicillins, including penicillin G (PENG) and penicillin V (PENV), which were the first introduced into clinical use but, because of the increasing resistance of microbes, are being replaced by other antibiotics;
2. beta-lactamase-resistant penicillins, including nafcillin (NAFCI), oxacillin (OXA), cloxacillin (CLOXA), and dicloxacillin (DICLOXA), which owe their increased effectiveness to the addition of a bulky side chain to the penicillin structure; and
3. penicillins with extended spectrum of action, including ampicillin (AMPI), amoxicillin (AMOX), and metampicillin.

The number of microbe strains resistant to antibiotics prescribed to treat human and animal infections alike has increased substantially in the past years, and there is evidence that globalization and migration may favor spread of the antimicrobial-resistance problem worldwide [4, 5]. Moreover, antimicrobial residues in food can induce serious allergic reactions in consumers. Several international authorities, including the USA Food and Drug Administration (FDA) [6], the European Union (EU) [7], and the Codex Alimentarius [8], have established maximum residue levels (MRLs) for veterinary drugs in foods; these are the levels that could safely remain in the tissue or in food products derived from a food-producing animal treated with a veterinary drug [9]. The EU banned the use of these pharmaceuticals as growth promoters in feed in 2006, in an effort to reduce antibiotic-resistant strains in the microbial flora of farm animals [10].

The most common therapeutic indication of penicillins for cattle is the treatment of bovine mastitis, a disease which causes substantial economic losses. Therefore, milk is the animal product most frequently analyzed for penicillins [11]. A variety of analytical techniques have been used over the years in the dairy industry to detect the presence of these antimicrobials [12, 13]. Chromatographic methods coupled to MS or to other types of detection (UV, FLD) [14–18] are the choice for identification and quantification of penicillin residues in milk. Other techniques, including capillary electrophoresis, microbiological inhibition tests, lateral-flow assays, immunoassays, or biosensors with different transducers, have also been used [13, 19–21]. Sample treatment and pre-concentration, along

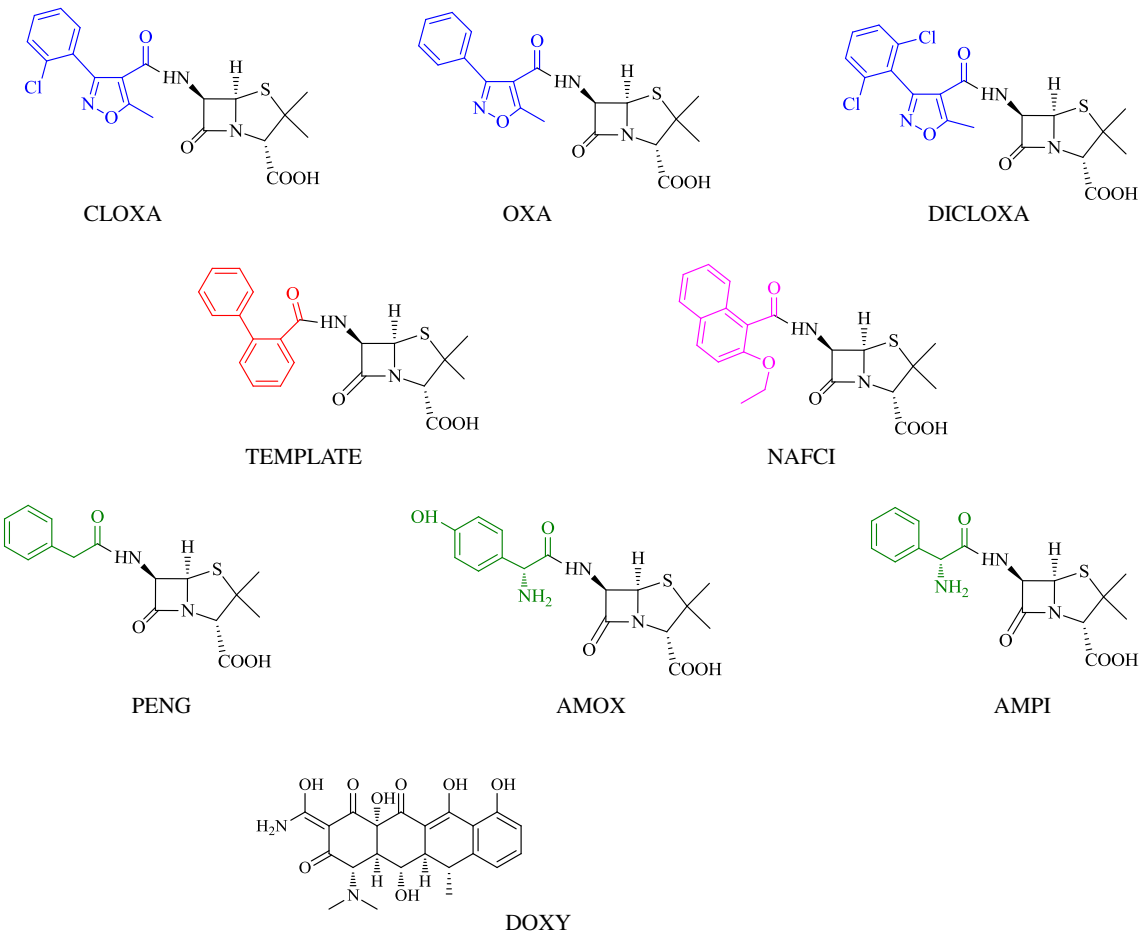

Fig. 1 Chemical structure of the penicillins included in the study and of the surrogate template used for the MIP syntheses

with the limited stability of penicillins under different conditions, are usually the main challenges for their routine analysis in milk samples [20, 22]. Molecularly imprinted polymers (MIPs) have revealed to be a useful alternative to immunoaffinity columns and other commercially available solid-phase-extraction (SPE) cartridges for sample clean-up and selective pre-concentration of different analytes in complex matrices, as the so-called MISPE (molecularly imprinted solid-phase extraction) [23]. Their features are especially useful for the analysis of milk samples which contain a large number of matrix components that may co-elute with the antibiotics and invalidate a quantitative analysis [19]. MIPs can be modified to recognize a single compound or a family of structurally-related analogues, a feature of special interest for multiresidue analysis in food samples [24]. Several MISPE methods have been reported for determination of penicillins in milk [25–30] (Table S1, Electronic Supplementary Material, ESM). Most MIPs use methacrylic acid as functional monomer and PENG or PENV as template molecules. However, the size and shape of these molecules and, especially, their hydrophobicity (evaluated as log*P*) are significantly different from that of beta-lactamase-resistant penicillins including CLOXA or DICLOXA, which are frequently used for the treatment of mastitis in dairy cows [31]. Moreover, the use of analyte mimics as templates for MIP generation is advantageous when they are targeted for trace analysis, because this avoids interference as a result of slow leaching from an incompletely washed MIP [32].

In this work, MIP beads were developed to determine CLOXA, OXA, and DICLOXA in a MISPE method by HPLC with diode-array (DAD) detection. These three beta-lactamase-resistant penicillins have MRLs of 30 μg kg^{-1} in milk [7]. A mimic that resembles the target antibiotics in terms of size, shape, and polarity was prepared and used as template for the polymer preparation using a non-covalent approach. A MIP–NIP library (NIP=non-imprinted polymer) was obtained in this way (see Table 1 for details) by using three different functional monomers, namely *N*-(2-aminoethyl)methacrylamide (EAMA), *N,N*-diethylaminoethyl methacrylate (DAEM), and 1-(4-vinylphenyl)-3-(3,5-bis(trifluromethyl)phenyl)-urea (VPU), in combination with the cross-linkers divinylbenzene (DVB), ethylene glycol dimethacrylate (EDMA), or trimethylolpropane trimethacrylate (*TRIM*) to select the composition leading to the polymer with best recognition of the target antibiotics. For the MISPE application, the polymer networks were prepared in the form of microspheres using porous silica beads (40–75 μm) as sacrificial scaffolds. The MISPE–HPLC–DAD method was optimized for determination of CLOXA, OXA, and DICLOXA in aqueous samples. Validation of the proposed method was performed according to the European Commission Decision 657/2002/EC [33] for analysis of the selected penicillins in raw milk samples.

Table 1 Amount of antibiotic re-bound to the polymers (B,%) after 24 h incubation in AcN or HEPES buffer (0.1 mol L^{-1}, pH 7.5). Incubation conditions: [CLOXA]=1 mg L^{-1}; 20 mg polymer; solvent volume=1 mL (n=2)

	VPU		DAEM		EAMA	
	AcN[a]	HEPES[b]	AcN[a]	HEPES[b]	AcN[a]	HEPES[b]
NIP TRIM	5	100	40	100	100	99
NIP EDMA	19	100	61	98	96	96
NIP DVB	25	100	32	100	100	100
MIP TRIM	8	100	45	98	96	99
MIP EDMA	24	99	56	99	97	99
MIP DVB	26	100	45	99	99	100

[a] RSD<7.8 %; [b] RSD<8.3 %

Experimental

Chemicals and materials

N-(2-Aminoethyl)methacrylamide (EAMA) hydrochloride was purchased from Polysciences (Eppelheim, Germany). *N, N*-diethylaminoethyl methacrylate (DAEM), ethylene glycol dimethacrylate (EDMA), divinylbenzene (DVB), trimethylolpropane trimethacrylate (*TRIM*), doxycycline (DOXY), and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were from Sigma–Aldrich (St. Louis, MO, USA). The urea-based functional monomer, 1-(4-vinylphenyl)-3-(3,5-bis(triflurometh)phenyl)-urea (VPU), was prepared as described elsewhere [34]. The initiator *N,N*'-azo-bis(2,4-dimethyl)valeronitrile (ABDV) was purchased from Wako Chemicals (Neuss, Germany).

Penicillin G (PENG) potassium salt, penicillin V (PENV) potassium salt, amoxicillin (AMOX), nafcillin (NAFCI) sodium salt, cloxacillin (CLOXA) sodium salt, dicloxacillin (DICLOXA) sodium salt, oxacillin (OXA), and ampicillin (AMPI) were supplied by Bayer (Leverkusen, Germany) and used as received. Trifluoroacetic acid (TFA) (HPLC grade, 99 %) was from Fluka (Buchs, Switzerland) and 15-crown-5 ether from Acros (Geel, Belgium). Tetra-*n*-butylammonium hydrogensulfate (TBA) (98 %) and sodium chloride were from Merck (Darmstadt, Germany).

HPLC-grade acetonitrile (AcN), dimethylsulfoxide (DMSO), and methanol (MeOH) were purchased from SDS (Peypin, France), and HPLC water was purified with a Milli-Q system (Millipore, Bedford, MA). All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use.

Apparatus

^{1}H NMR spectra at 300 MHz were recorded at UCM NMR Central Instrumentation Facilities (CIF) on a Bruker Avance

DPX 300MHz-BACS60. NMR chemical shifts are expressed relative to the signals of the partially or non-deuterated traces of the solvent (DMSO-d_6) at 2.54 ppm. Mass spectra were recorded by MALDI TOF at UCM MS-CIF from methanol solutions with a Bruker Ultraflex.

The pH of the buffer solutions was adjusted with an ORION 710A pH/ISE meter (Beverly, MA, USA). Chromatographic analyses were performed with a 1200 Series Rapid Resolution LC system from Agilent Technologies (Palo Alto, CA, USA) equipped with a binary pump, on-line degasser, autosampler, automatic injector, column thermostat, and a diode-array-detection system (DAD).

Chromatographic separation of OXA, CLOXA, and DICLOXA was performed on an ACE Excel 2 C18-PFP (2) (100×2.1 mm, 2 μm) HPLC column from ACE (Aberdeen, Scotland). A gradient program was used with the mobile phase, combining solvent A (0.1 % TFA in water) and solvent B (0.1 % TFA in AcN) as follows: 68 % A (10 min); 68–10 % A, 32–90 % B (10→15 min); 10–68 % A, 90–32 % B (15→20 min). Analyses were performed at a flow of 0.4 mL min^{-1} while the column temperature was kept at 40 °C; the injection volume was 100 μL and all compounds eluted within 11 min. The diode-array-detector wavelength was set at 220 nm. For quantification purposes, matrix-matched calibration standards were prepared by diluting adequate amounts of OXA, CLOXA, and DICLOXA with blank solutions obtained by extracting milk samples not containing the antimicrobials at the method detection limit, under the same conditions used for the samples. All measurements were performed in triplicate. Linear calibration plots were obtained in the 15−2500 μg L^{-1} range for all antibiotics tested (r^2> 0.9998).

Synthesis of the surrogate of the antibiotic

To a solution of 2-phenylbenzoic acid (11.36 mmol, Aldrich) in anhydrous CH_2Cl_2 (7.1 mL, dried over molecular sieves) at 0 °C under argon atmosphere, oxalyl chloride (36.8 mmol, Acros Organics) and a catalytic amount of anhydrous *N,N*-dimethylformamide (0.36 mmol, dried over molecular sieves) are added. The mixture is stirred for 2 h under those conditions. Then, the reaction mixture is treated with a 4 % sodium hydrogencarbonate solution at pH 8. The organic layer is collected, washed with water, dried over $MgSO_4$, filtered, and then concentrated under vacuum in a rotary evaporator at room temperature. The expected acid chloride is obtained in 89 % yield as a clear yellow oil, which is used directly in the following step [35].

The 2-biphenylylpenicillin (2BPEN) surrogate template (Fig. 1) was prepared by the Hoover et al. method [36]. A solution of 10.11 mmol 2-phenylbenzoic acid chloride in 20 mL acetone is added dropwise to a solution of 11.14 mmol 6-aminopenicillanic acid (Aldrich) in 83 mL 3 % aqueous sodium bicarbonate and 55 mL acetone at −20 °C, and stirred at −20 °C for 1 h and for a further 3 h at room temperature. The reaction mixture is extracted with 80 mL ethyl ether in three portions and the organic phase is discarded. The aqueous layer is covered with diethyl ether previously cooled to 4 °C, stirred, and acidified to pH 2 with 1 mol L^{-1} hydrochloric acid. The mixture is shaken and the layers allowed to separate; then the aqueous layer is extracted immediately with 60 mL ether in three portions. The combined ethereal extracts are washed with the same volume of water in two portions, dried over $MgSO_4$, and filtered. The solvent is evaporated immediately under vacuum in the rotary evaporator without heating to yield a colorless syrup of 2-biphenylylpenicillin (as the acid form). Finally, 4.64 mmol of the product is dissolved in 8 mL methanol, previously cooled to 4 °C, and 0.5 mol L^{-1} methanolic sodium methoxide (4.41 mmol, Aldrich) is added to the clear solution with stirring. The sodium salt of the 2-biphenylylpenicillin is precipitated by adding several volumes of diethyl ether. To isolate the solid product, the ether suspension of the precipitate is centrifuged at 10,800 rpm at 0 °C.

^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.45 (s,3H), 1.53 (s,3H), 3.92 (s,1H), 5.32–5.38 (m,2H), 7.32–7.56 (m, 9H), 9.1 (d, J= 6.9 Hz, 1H). MS (MALDI TOF): *m/z* calculated for $C_{21}H_{19}N_2NaO_4S$ [$M^{\cdot+}$]: 418.10; found: 418.10. Elemental analysis: calculated for $C_{21}H_{19}N_2NaO_4S$ 60.28 % C, 4.58 % H, 6.69 % N, 7.66 % S; found: 59.97 % C, 5.05 % H, 6.73 % N, 7.16 % S.

Combinatorial MIP library

The functional monomers and cross-linkers used for the preparation of the MIP–NIP library are shown in Fig. S1 and Table S2 in the ESM. For the MIP synthesis, the template molecule (25 μmol) was mixed with 50 μmol 15-crown-5 ether, 100 μmol functional monomer, 500 μmol cross-linker, and 4.8 μmol radical initiator (ABDV). The necessary amount of porogen solvent (AcN–DMSO, 60:40, *v/v*) was calculated as that resulting from a ratio of porogen volume to the overall porogen plus monomers volume of ~0.57 [37]. The prepolymerization mixtures were transferred into 96-well PTFE microtiter plates and N_2-purged for 5 min [24]. NIPs were prepared in the same way as MIPs but in the absence of template. The plates were sealed with a PTFE-coated lid (Radleys, Essex, UK). Then, the polymerization was initiated thermally at 50 °C, and, after 24 h, the polymers were then cured at 60 °C for a further 24 h. Finally, the plates were left in a vacuum oven (0.1 Torr) at 50 °C for 24 h to allow evaporation of the porogen. The resulting polymers were weighed, ground, and transferred into a 96-well 0.45 μm Captiva filter plate (Biotage, Uppsala, Sweden) and sequentially washed with MeOH, MeOH with TFA (1 %), and water for thorough template removal.

Before the rebinding experiments, the MIP–NIP library was equilibrated with HEPES buffer (0.1 mol L^{-1}, pH 7.5). Thereafter, a solution of CLOXA (1000 μL, 1 mg L^{-1}) in HEPES buffer (100 mmol L^{-1}, pH 7.5) was incubated for 24 h with 20 mg of each polymer at room temperature. After filtration, the concentration of free CLOXA in the supernatant was determined by HPLC–DAD, using AcN–water (0.1 % TFA) (35:65 *v/v*) as mobile phase.

The amount of antibiotic bound to the polymeric particles (B, %) was calculated from Eq. 1:

$$B = (C_0 - C) \Big/ C_0 \times 100 \quad (1)$$

where C_0 (mmol L^{-1}) is the initial CLOXA concentration and C (mmol L^{-1}) is the final antibiotic concentration in the supernatant. Incubations were repeated using AcN as solvent to evaluate the amount of CLOXA re-bound to the MIP or NIPs under such conditions.

After the first binding experiment, the 96-well plate was washed with MeOH–TFA (99:1, *v/v*) until the analyte could no longer be detected by HPLC–DAD in the washing solution. The plates were reused in subsequent binding experiments. All the experiments were performed in duplicate.

Polymerization after pore filling of the silica beads

A pre-polymerization mixture was prepared by mixing 0.25 mmol of the 2BPEN template, 0.50 mmol 15-crown-5 ether, and 1 mmol EAMA dissolved in 0.70 mL porogenic solvent (AcN–DMSO, 60:40, *v/v*). The solution was mixed with 5 mmol TRIM and ABDV (2 % by weight of the final mixture). Then, 6.0 g silica (Si-500, 40–75 μm, Silicycle, Québec, Canada) was placed in a 100-mL glass vial and mixed by stirring with 2.5 mL of the pre-polymerization mixture until the silica beads were freely flowing. The vial was sealed with a rubber septum and purged with N_2 for 5 min. Then the mixture was placed in the oven at 50 °C for 24 h to undergo polymerization.

For the silica etching, the resulting composite particles were shaken for 8 h with 150 mL 3 mol L^{-1} aqueous solution of ammonium hydrogen difluoride. This procedure was repeated three times. The polymer obtained was washed with water (until pH~7), 1 L methanol–TFA (99:1, *v/v*), and 0.5 L MeOH. Then the solid was dried in a vacuum oven (0.1 Torr) at 50 °C for 24 h. Before their first use, the polymers were suspended in MeOH–water (80:20, *v/v*) to remove fine particles. The non-imprinted polymer was prepared in the same way but in the absence of the template molecule.

Chromatographic evaluation of the polymers

The MIP and NIP polymers were slurry-packed in methanol into stainless-steel HPLC columns (150×2.1 mm I.D.), using MeOH–water (80:20, *v/v*) as the pushing solvent. Retention of the antimicrobials and non-related compounds in the MIP and NIP columns was evaluated using two mobile phases based on a binary mixture of AcN–water (20:80 or 25:75, *v/v*). For these experiments, the following conditions were used: 1 mL min^{-1} flow; 1 mmol L^{-1} antimicrobial; 10 μL sample volume. The DAD detection wavelength was set at 220 nm and each elution was repeated three times. Methanol was used as a void volume marker. 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. The imprinting factor (IF) was calculated as k_{MIP}/k_{NIP}, i.e. the ratio of the retention factor of each analyte in the MIP column to that in the NIP column.

Equilibrium re-binding experiments

MIP beads (20 mg) were mixed with 2 mL water–AcN mixtures (80:20 *v/v*) containing increasing concentrations of CLOXA (0.01–4.00 mmol L^{-1}), and incubated for 24 h at room temperature. Next, the supernatant was collected and injected into the HPLC using the gradient program described in the *Apparatus* section. The amount of analyte bound to the polymer (B) was calculated by subtracting the free amount (F) from the initial CLOXA concentration in the mixture. Fourteen experimental points per plot (n=2) were analyzed to calculate the binding isotherms.

Optimized extraction procedure of the antibiotics in the MIP cartridge

Solid-phase 1-mL extraction cartridges (Varian, Palo Alto, CA) were packed with 20 mg of the MIPs or the corresponding NIPs. The cartridges were first equilibrated with 10 mL MeOH and 10 mL buffer (0.1 mol L^{-1} HEPES, pH 7.5). Then 10 mL of the antibiotic mixture dissolved in buffer (0.1 mol L^{-1} HEPES, pH 7.5) was percolated at a constant flow of 0.75 mL min^{-1} with the aid of a peristaltic pump (4-channel Gilson Minipuls 3, Villiers-le-Bel, France). The cartridges were rinsed with 3 mL water-AcN (80:20 *v/v*) to wash out the non-specifically-retained compounds. Then, the antibiotics were eluted with 1 mL 0.05 mol L^{-1} TBA in methanol. The cartridges were equilibrated with 10 mL MeOH and buffer (0.1 mol L^{-1} HEPES, pH 7.5) before each new application. The eluates from the MISPE column (400 μL) were diluted with 600 μL water, and 100 μL aliquots were injected into the HPLC system for analysis. The experiments were performed in triplicate, and the analyte recovery was calculated.

MISPE–HPLC–DAD method validation in raw milk samples

Validation of the performance of the MISPE–HPLC–DAD method in raw milk samples is based on the criteria described in EU Commission Decision 2002/657/EG. The raw milk samples were removed from the freezer and allowed to thaw in a water bath at 25 °C. Thirty grams of each were weighed into 60-mL polypropylene centrifuge tubes with a screw cap and spiked with CLOXA, OXA, and DICLOXA at half the MRL, the MRL, and 1.5 times the MRL concentrations for each β-lactam antibiotic, and centrifuged at 11,000*g* at 35 °C for 30 min. Then, 6.7 g of the defatted milk (milk collected from the intermediate fraction) was mixed with 20 mL AcN using a vortex mixer and filtered through a 7–9 μm pore cellulose filter (Millipore), and 20 mL of the mixture was transferred to a round-bottom flask. The supernatant was evaporated to dryness using a rotary evaporator at 37 °C (*ca.*15 min), and the final extract was dissolved in HEPES buffer (0.1 mol L^{-1}, pH 7.5) to a final volume of 10 mL. The resulting solution was pre-concentrated as described above (*Optimized extraction procedure of the antibiotics in the MIP cartridge*). The experiments were performed in quintuplicate and the recovery was expressed (as a percentage) as the amount of antibiotic obtained after the MISPE step relative to the amount spiked into the milk samples.

Results and discussion

Selection of the polymer composition

A MIP library was prepared using 2BPEN, a synthetic surrogate of our target beta-lactamase-resistant penicillins, as template molecule. This compound has a chemical structure and molecular size similar to that of CLOXA, OXA, and DICLOXA, and has a similar polarity (log*P*(2BPEN)=2.1± 0.2 [38]; log*P*(OXA)=2.38; log*P*(CLOXA)=2.43; log*P*-(DICLOXA)=2.91 [39]). Therefore, it should enable generation of binding sites with better recognition properties than those of MIPs prepared with penicillin G [25–27, 29, 40] or 6-aminopenicillanic acid [30] templates, two molecules that only bear the structural moiety common to all penicillins.

Three functional monomers (Fig. S1 in the ESM), namely EAMA, DAEM, and VPU [41, 42], targeting recognition by the carboxylic acid or carboxylate group on the antimicrobial backbone were combined with three cross-linkers (ESM Fig. S1), ranging from moderately polar (TRIM, EDMA) to non-polar (DVB), to investigate the effect of the matrix polarity on the MIP affinity for the target. The polymer library obtained thereof included all possible combinations of the functional monomers and the cross-linkers, resulting in nine MIPs and their corresponding NIPs (ESM Table S1).

The MIPs were synthesized with a fixed 1:4:20 template–functional monomer–cross-linker mole ratio, except for the VPU-based polymers (1:2:20 ratio). The template molecule was dissolved in the porogenic solvent mixture, AcN–DMSO (60:40 *v/v*), in the presence of 15-crown-5 ether (1:2 T–CE mole ratio) to achieve quantitative formation of the carboxylate form for further interaction by hydrogen bonding (VPU) or electrostatic attraction (DAEM, EAMA) with the functional monomers.

After template extraction from each MIP, the polymer library (20 mg each) was incubated for 24 h with 1 mL 1 mg L^{-1} CLOXA in HEPES buffer (0.1 mol L^{-1}, pH 7.5). The amount of CLOXA in the supernatant was determined by HPLC–DAD, and the amount of antimicrobial bound to the polymer (B,%) was calculated as described in the Experimental section (*Combinatorial MIP library*). The experiments were repeated using AcN as incubation solvent instead of buffer.

As shown in Table 1, the MIP and NIP polymers obtaining the highest re-binding of CLOXA, both in HEPES buffer and in AcN, were prepared with EAMA functional monomer, whereas those prepared with VPU achieved the lowest retention of the antimicrobial in the organic solvent. To further investigate the performance of the EAMA-based polymers as MISPE sorbents, they were packed (20 mg) in SPE cartridges. Then, 1 mL CLOXA (1.0 mg L^{-1}) in HEPES buffer (pH 7.5) was loaded and the antibiotic was eluted with 1 mL 0.05 mol L^{-1} TBA in methanol. As shown in Fig. S2 (ESM), the use of TRIM as cross-linker enabled quantitative recovery (*R*) of CLOXA from the MIP when the antibiotic was loaded in HEPES buffer (R_{MIP}=101 %, RSD<6.9 %, n=3) and led to some selectivity compared with the NIP (R_{NIP}=74 %, RSD< 5.7 %, n=3). Thus, a combination of EAMA as functional monomer and TRIM as cross-linker was finally selected for the synthesis of polymer beads using silica gel as sacrificial scaffold for MISPE method development.

Chromatographic evaluation of the MIP beads and cross-selectivity

The polymer beads were packed in chromatographic columns (100×4.6 mm) and tested for their ability to retain CLOXA using mobile phases based on binary mixtures of H_2O and AcN ranging from 100 % H_2O to 100 % AcN. In pure AcN, the retention of the antibiotic in the MIP (t_{MIP}=5.2 min, k_{MIP}= 4.14) was higher than in the NIP (t_{NIP}=2.8 min, k_{NIP}=1.77) revealing an imprinting effect (IF=2.3). This behavior may be explained by considering that the ionization constant of carboxylic acids is significantly lower in non-aqueous solvents than in water [43]. Therefore, the carboxylic acid group of the antimicrobial will be protonated in pure acetonitrile and the ionic interactions between the antibiotic and polymer are suppressed. Increasing the water content in the mobile phase to

75 % causes a significant rise of the retention time of CLOXA in the MIP (k_{MIP}=17.78) but not in the NIP (k_{NIP}=0.22). This observation can be explained by considering that in (partially) aqueous media the amine group of EAMA is positively charged (pK_a~9.2 [44]) and CLOXA is deprotonated (pK_a= 2.78 [38]). Therefore, in that medium, ionic interactions are responsible for the selective recognition of the antimicrobial by the MIP binding cavities. A further increase of the water content in the mobile phase (water–AcN 80:20, *v/v*) resulted in a slight rise in the retention times in the NIP and no elution from the MIP after 20 min.

Finally, no elution of the antimicrobial was observed with 100 % H_2O as the mobile phase, either from the MIP or from the NIP (t_r>20 min). This retention behavior is commonly observed for MIP and has been attributed to the contribution of both specific and nonspecific interactions to the overall retention mechanism, brought about by the shift from a predominantly electrostatic retention mode in water-poor mobile phases to a mainly hydrophobic interaction mode in water-rich systems [41].

The cross-selectivity of the polymers to other penicillins was evaluated in terms of the retention factors (k) for the MIP and the NIP and of the corresponding imprinting factors (IF), using water–AcN (75:25 or 80:20, *v/v*) as mobile phase. The results are summarized in Table 2. All the penicillins tested were weakly retained in the NIP but strongly retained in the MIP, especially the NAFCI, OXA, and DICLOXA (k_{MIP}>17.21), which bear a closer resemblance to the template molecule (Fig. 1). The lack of correlation between the retention factors in the MIP and the log$P$ values shown in Table 2 indicates that the enhanced retention observed in the MIP is not caused by nonspecific hydrophobic interactions but rather by selective interplay with the binding sites that are capable of accommodating the bulkier substituents of these antimicrobials, leading to the success of the imprinting process. The retention of the more hydrophilic penicillins, AMPI and, particularly, AMOX, in the MIP was weaker, probably because of their zwitterionic character resulting from the presence of carboxylic and amino groups in the molecules. DOXY, a tetracycline antibiotic with a structure totally unrelated to that of penicillins and significantly hydrophilic, was the least retained of all the compounds (k_{MIP}=1.85; k_{NIP}=0.32).

Determination of the binding-site distribution and affinity

The binding properties and the heterogeneity of the binding sites of the EAMA–TRIM polymer were assessed by equilibrium analysis in water–AcN (80:20 *v/v*). Selection of this solvent mixture was on the basis of the higher retention factors obtained for CLOXA in the chromatographic evaluation and of the results of the MISPE experiments.

Binding of CLOXA to both the MIP and the NIP was adequately modeled with the Freundlich isotherm (Fig. 2). Table 3 summarizes the fitting coefficients for the binding, the apparent weighted average affinity ($\overline{K}_{K1-K2}$), and the apparent number of binding sites ($\overline{N}_{K1-K2}$) calculated from the Freundlich isotherm-affinity distribution method reported by Rampey et al. [45].

The apparent weighted average affinity was higher for the MIP than for the NIP (K_{MIP}:34±2 mmol^{-1} L; K_{NIP}:13± 1 mmol^{-1} L) in the measured concentration range

Table 2 Effect of the composition of the mobile phase on the retention of penicillins in the imprinted and non-imprinted polymers. Sample volume= 10 μL; [antimicrobial]=1 mmol L^{-1}; λ(DAD)=220 nm; temperature=25 °C; flow=1.0 mL min^{-1} (n=3)

Analyte	Water–AcN (80:20, *v/v*)					Water–AcN (75:25, *v/v*)					
	t_R NIP (min)	t_R MIP (min)[a]	k_{NIP}	k_{MIP}	IF	t_R NIP (min)	t_R MIP (min)	k_{NIP}	k_{MIP}	IF	Log$P$[b]
AMOX	1.1	3.5	0.11	2.39	>20	1.1	2.2	0.09	1.27	14.2	−1.99
AMPI	1.1	5.5	0.16	4.13	>20	1.1	6.8	0.11	2.76	25.1	−1.13
DOXY	2.1	ne	1.09	3.21	2.9	1.3	2.8	0.32	1.85	5.8	−1–(−0.01)[c]
PENG	1.3	ne	0.34	>20	>20	1.1	11.6	0.14	10.85	77.5	1.83
NAFCI	1.7	ne	0.71	>20	>20	1.2	19.3	0.23	18.70	81.3	3.79[d]
OXA	1.5	ne	0.54	>20	>20	1.2	17.8	0.19	17.21	90.5	2.38
CLOXA	1.7	ne	0.67	>20	>20	1.2	18.4	0.22	17.78	80.8	2.44
DICLOXA	2.0	ne	0.95	>20	>20	1.2	20.8	0.27	20.21	74.9	2.91

[a] ne: no elution after 20 min

[b] Ref. [40] unless otherwise stated

[c] Bottle CY, Dubar F, McFadden Geoffrey I, Marlechal E, Biot C (2012) Chem Rev 112:1269–83

[d] Díaz-Cruz S, López de Alda MJ, Barceló D (2006) J Chromatog A, 1130:72–82

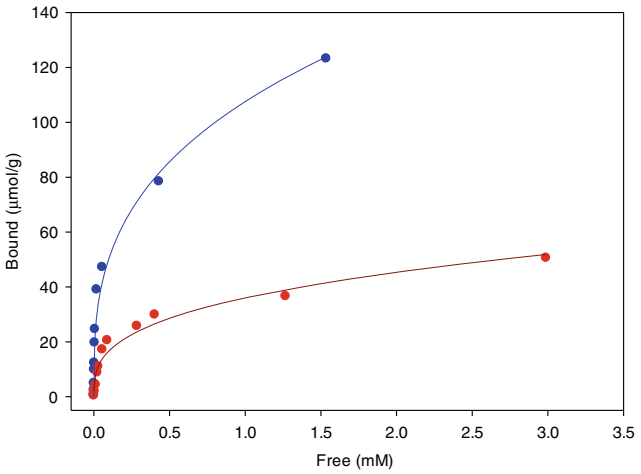

Fig. 2 Equilibrium binding isotherms for the uptake of CLOXA by the MIP (*blue*) and NIP (*red*) in water–AcN (80:20, *v/v*). *Free*, concentration of CLOXA in solution; *Bound*, concentration of CLOXA bound to the polymer. The experimental data were fitted to a Freundlich isotherm (FI) model (see text for details)

([CLOXA]:0.01–4.00 mmol L^{-1}). The total number of binding sites for CLOXA was also significantly higher in the MIP ($\overline{N}_{MIP}$:124±4 µmol g^{-1}) than in the NIP ($\overline{N}_{NIP}$:31±2 µmol g^{-1}). However, the heterogeneity index was not statistically different for the two polymers (m_{MIP}:0.32±0.02; m_{NIP}:0.34±0.03). In agreement with Rampey et al. [45], polymers with a sizeable imprinting effect are consistently more heterogeneous, so that MIP usually have a higher degree of heterogeneity (i.e., a lower heterogeneity index,*m*) than the corresponding NIP controls. However, we have observed that NIP beads prepared with the sacrificial imprinting approach usually have (lower) heterogeneity indexes similar to those found for the MIP, compared with those measured for polymers prepared from bulk or precipitation polymerization [46].

Optimization of the MISPE procedure

Several factors were evaluated to select the optimum conditions for the MISPE method, including composition and volume of the eluting solvent, composition of the washing solvent, loading flow rate, and breakthrough volume (i.e., maximum sample volume that can be loaded with quantitative recovery of analyte).

Selection of the elution solvent and effect of the flow-rate of the loading solution

The composition of the elution solvent was first optimized. To this end, 1 mL samples containing 1 µg CLOXA dissolved in 0.1 mol L^{-1} HEPES (pH 7.5) were loaded into the cartridge solid support and eluted with 1 mL 0.05 mol L^{-1} TBA in MeOH. As we have revealed elsewhere [42, 47], because of formation of ion pairs with the analyte, TBA can be used to increase the elutropic strength of MeOH when penicillins are pre-concentrated with mixed-mode polymeric SPE sorbents. Moreover, all the penicillins included in this study were confirmed to be stable for up to 18 h in 0.01–0.2 mol L^{-1} TBA–MeOH [47]. The application of 1 mL 0.05 mol L^{-1} TBA solution in MeOH enabled quantitative recovery of the antibiotic (R_{MIP}=101.3 %; RSD=3.1 %; n=3), so this solution was chosen for further experiments. A dilution of the 400 µL eluate of the MISPE cartridges with 600 µL water was used as non-eluting focusing solvent to avoid peak distortion in the chromatographic separation, as reported elsewhere [47].

The effect of the flow rate of the loading solution on the recovery of CLOXA was evaluated in the 0.5 to 1.5 mL min^{-1} range. A solution (1 mL) of CLOXA (1 mg L^{-1}, in 0.1 mol L^{-1} HEPES, pH 7.5) was loaded into the cartridge and eluted with 1 mL 0.05 mol L^{-1} TBA in MeOH, followed by HPLC–DAD analysis. An average extraction recovery of 98 % (RSD=3 %, n=3) was obtained at flows≤ 0.75 mL min^{-1}. The use of higher values yielded decreased extraction recoveries because of the reduced contact time between the antimicrobial and the MISPE sorbent. Therefore, a loading rate of 0.75 mL min^{-1} was adopted for further experiments.

Washing-solvent selection

As described above (*Chromatographic evaluation of the polymers*), the non-specific interactions of the penicillins with the imprinted polymer can be minimized using water–AcN mixtures as mobile phase. To evaluate the applicability of these solutions as washing solvents for the MISPE method, a 1 mL sample of CLOXA (1 mg L^{-1} in 0.1 HEPES, pH 7.5) was percolated through the MIP or the NIP cartridges and

Table 3 Freundlich fitting parameters, weighted average affinity ($\overline{K}_{K1-K2}$), and number of sites ($\overline{N}_{K1-K2}$) obtained with the experimental binding data of CLOXA towards the 2BPEN-imprinted and non-imprinted polymers [45]

Isotherm model	Weighted average affinity, $\overline{K}_{K1-K2}$ ($mmol^{-1}$ L)	Total number of binding sites $\overline{N}_{K1-K2}$ (µmol g^{-1})	K_{range} ($mmol^{-1}$ L)	Heterogeneity index, *m*	Binding capacity, *a* (µmol g^{-1} ($mmol^{-1}$ L)^m)	Regression coefficient r^2
Freundlich MIP	34±2	124±4	0.8–901	0.32±0.02	107±4	0.99
Freundlich NIP	13±1	31±2	0.6–275	0.34±0.03	36±1	0.98

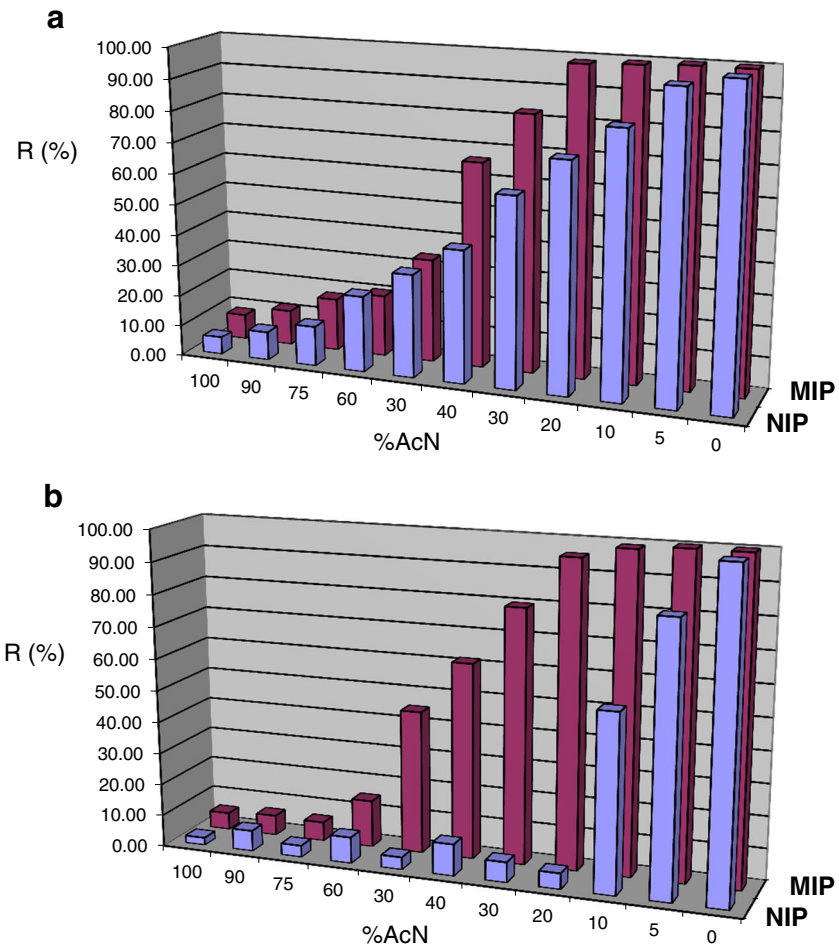

Fig.3 Extraction recoveries (%) obtained on the MIP and the NIP, after the percolation of 1 mL buffer (HEPES 0.1 mol L^{-1}, pH 7.5) spiked with 1 μg CLOXA using a washing step with (**a**) 2 mL and (**b**) 3 mL water–AcN mixtures of different concentrations (from 0 to 100 %, *v/v*). (n=3; RSD_{MIP}: 1.1–7.2 %; RSD_{NIP}: 1.2–8.0 %)

different volumes (2 or 3 mL) water–AcN (0 to 100 % *v/v*) were used for the washing step. Next, the antimicrobial was eluted with 1 mL 0.05 mol L^{-1} TBA in MeOH and the extracts were analyzed by HPLC–DAD. The results are summarized in Figs. 3a,b.

In agreement with the chromatographic experiments, higher recoveries, both in the MIP and the NIP, were obtained with higher water concentrations in the washing solution. However, the use of 3 mL water–AcN (80:20, *v/v*) as washing solvent significantly decreased the non-specific interactions of CLOXA with the imprinted polymers. Under these conditions, retention in the NIP was much reduced (R_{NIP}=4.9 %; RSD= 1.8 %; n=3), whereas recoveries in the MIP were close to 100 % (R_{MIP}=95.3 %, RSD=2.3 %), confirming the high affinity of the prepared MIP for the antimicrobial. Therefore, 3 mL water–AcN (80:20, *v/v*) was adopted for the washing step in the MISPE method.

Breakthrough volume

The cartridge breakthrough volume (i.e. the largest volume of a sample that can be percolated through the cartridge without significant loss of the analyte, the recovery of which, after elution for all sample volumes less than the breakthrough volume, will be 100 % [48]) was evaluated by percolating

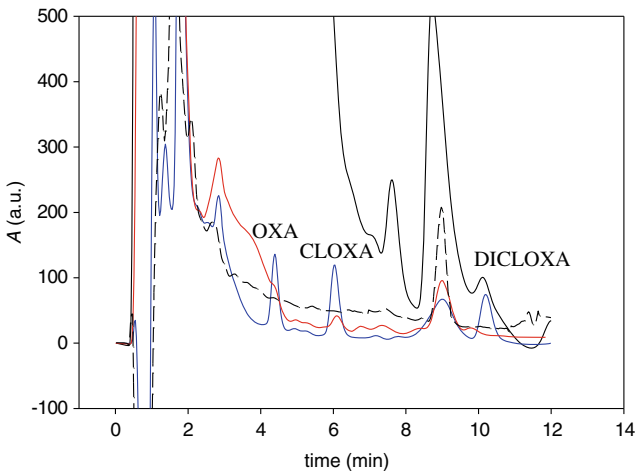

Fig. 4 HPLC–DAD chromatograms of blank milk extract: after MISPE clean-up (*dashed line*); before (*solid black line*) and after (*solid blue line*) application of the MISPE–LC–DAD method to a milk sample fortified with the target penicillins at the MRL (30 μg kg^{-1}); and after NIP clean-up (*solid red line*)

Table 4 MISPE–HPLC–DAD method accuracy and precision in raw milk samples

	Analyte		
	OXA	CLOXA	DICLOXA
Repeatability (intra-day)			
Spiking level (μg kg^{-1})	15, 30, 45		
Recovery (%)	95–101	96–100	94–99
RSD (%) (n=6)	3–6	2–5	2–6
Reproducibility (inter-day)			
Spiking level (μg kg^{-1})	15, 30, 45		
Recovery (%)	92–101	93–101	93–102
RSD (%) (n=18)	4–9	3–6	3–8

increasingly high volumes (0.5, 1, 2, 5, 10, and 50 mL) of a sample containing 0.1 mg L^{-1} CLOXA in 0.1 mol L^{-1} HEPES, pH 7.5. The cartridges were washed with 3 mL water–AcN (80:20, *v/v*) and the antimicrobial was eluted with 1 mL 0.05 mol L^{-1} TBA in MeOH, followed by chromatographic analysis.

As can be observed from Fig. S3 (ESM), recoveries higher than 93 % (RSD>3 %, n=3) were obtained with percolated volumes below 25 mL, leading to a 25-fold pre-concentration factor. Slightly lower recovery yields (R=81.2 %, RSD= 3.5 %, n=3) were obtained after percolation of 50 mL samples, because the breakthrough volume for the binding sites was reached.

Raw-milk-sample analysis and method validation

The MISPE–HPLC–DAD method was validated for the analysis of CLOXA, OXA, and DICLOXA in milk samples according to EU guideline 2002/657/EC in terms of specificity, linearity, accuracy, precision, repeatability, within-laboratory reproducibility, and method sensitivity, by calculating both the decision limit (CC_α) and detection capability (CC_β).

The specificity of the proposed method was evaluated by analyzing a representative number (n=20) of non-spiked milk samples. The absence of any chromatographic peaks at the same retention time as the target antibiotics indicated that the proposed method is free of matrix interferences. Figure 4 shows the chromatograms of a blank milk extract after MISPE clean-up, before and after the application of the MISPE–LC–DAD method to a milk sample fortified with the target penicillins at the MRL (30 μg kg^{-1}), and after NIP clean-up.

Linearity was evaluated using six-point matrix-matched standards prepared by spiking blank milk extracts (previously found to contain undetectable levels of the antibiotics at the method detection limits) with CLOXA, OXA, and DICLOXA in the 7.5 to 480 μg kg^{-1} range. Calibration curves were built by plotting the peak areas against the concentrations of the analytes. Good linearity (r^2>0.9998) was observed within the concentration range for all the antimicrobials.

The novel analytical method was validated in terms of precision (repeatability and laboratory reproducibility) and accuracy. Raw milk samples were spiked with the antibiotics at three concentration levels (n=6), corresponding to 0.5, 1, and 1.5 times their respective MRL (30 μg kg^{-1}). The samples were extracted and analyzed in triplicate on three different days to determine the inter-day reproducibility. The results of these experiments are summarized in Table 4. Intra-day and inter-day mean recoveries were in the range 95–101 % (RSDs<6 %) and in the range 99–102 %, (RSDs<9 %), respectively, confirming the excellent reproducibility of the developed method in comparison to other MISPE procedures described in the literature for the analysis of CLOXA, OXA, or DICLOXA in milk samples [27, 28, 30].

Following guideline 2002/657/EC [33], the decision limit ($CC\alpha$) can be calculated by analyzing at least 20 blank samples fortified with the analyte(s) at the MRL, using Eq. 2:

$$CC_a = MRL + 1.64 s_{MRL} \quad (2)$$

where MRL represents the concentration at the permitted limit for each analyte and s_{MRL} is the corresponding standard deviation.

Determination of the detection capability was performed by analyzing 20 blank samples fortified with the analyte(s) at the decision limit, using Eq. 3:

$$CC_\beta = CC_\alpha + 1.64 s_{CC\alpha} \quad (3)$$

where CC_β is the concentration at the CC_α plus 1.64 times the within-laboratory standard deviation at the CC_α level.

The results for the MISPE–HPLC–DAD method are summarized in Table 5. The limits of detection (LODs) and quantification (LOQs) for each antibiotic in milk samples are expressed as the concentration of the target producing a S/N ratio of 3:1 and 10:1, respectively. The obtained LODs, in the

Table 5 LOD, LOQ, CC_α, and CC_β values obtained for OXA, CLOXA, and DICLOXA in raw milk samples by use of MISPE–HPLC–DAD

Analyte	LMR (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	CC_α (μg kg^{-1})	CC_β (μg kg^{-1})
OXA	30	1.9	6.3	33	36
CLOXA	30	1.6	5.3	34	37
DICLOXA	30	1.9	6.2	34	38

1.6–1.9 μg kg^{-1} range, were comparable with [27] or significantly better than [28] those reported using MISPE–HPLC–MS–MS methods. Finally, the cartridges were reused for 30 assays (in milk samples) without losing their preconcentration features.

Conclusions

The results described in this work confirm the applicability of MIP extraction sorbents for clean-up and preconcentration of beta-lactamase-resistant penicillins in milk matrices. The use of a surrogate molecule with a molecular structure close to such antimicrobials yielded class-selective MIP with better recognition characteristics for the less hydrophobic beta-lactams than those reported elsewhere using the natural antibiotic PENG or 6-APA as template molecules for the polymer synthesis. Antimicrobial residues can be detected at a level 15 times lower than the MRL in such samples using HPLC–DAD detection. The optimized method was validated in milk samples according to the European Commission regulations, thus confirming the selectivity and suitability of the MISPE procedure as a promising alternative to commercially available SPE sorbents for the determination of beta-lactamase-resistant penicillin residues in complex matrices including milk.

Acknowledgments This work was supported by MICINN (INNPACTO IPT-060000-2010-14, MIPFOOD project) and MINECO (CTQ2012-37573-C02). The authors thank Priégola Cattle Farm (Madrid, Spain) for providing the milk samples. J.L. Urraca thanks the CEI-Moncloa for a post-doctoral contract.

Conflict of interest The authors have no conflict of interest to declare.

References

1. Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2012, European Surveillance of Veterinary Antimicrobial Consumption (ESVAC). http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/document_listing/document_listing_000302.jsp. Online document
2. Gorbach SL, Bartlett JG, Blacklow NR (2004) Infectious diseases, 3rd edn. Lippincott Williams & Wilkins, Philadelphia
3. De Briyne N, Atkinson J, Pokludová L, Borriello SP (2014) Antibiotics used most commonly to treat animals in Europe. Vet Rec 175:325–332
4. Annual antimicrobial resistance surveillance reports (2013) European Centre for Disease Prevention and Control. http://ecdc.europa.eu/en/publications/surveillance_reports/arhai/Pages/annual-antimicrobial-resistance-surveillance-report.aspx. Online document
5. Barton MD (2000) Antibiotic use in animal feed and its impact on human health. Nutr Res Rev 13:279–299
6. http://www.fas.usda.gov/maximum-residue-limits-mrl-database. Accessed 14 May 2015
7. Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. (2010) Off. J. Eur. Union, L15:1–72
8. http://www.codexalimentarius.net/mrls/vetdrugs/jsp/vetd_q-s.jsp. Accessed 14 May 2015
9. Botsoglou NA, Fletouris DJ (eds) (2000) Drugs residues in foods: pharmacology, food safety and analysis. Marcel Dekker Inc., New York
10. Cogliani C, Goossens H, Greko C (2011) Restricting antimicrobial use in food animals: lessons from Europe. Microbe 6:274–279
11. Pyorala S, Taponen J, Katila T (2014) Use of antimicrobials in the treatment of reproductive diseases in cattle and horses. Reprod Dom Anim 49:16–26
12. Kantiani L, Farre M, Barcelo D (2009) Analytical methodologies for the detection of β-lactam antibiotics in milk and feed samples. Trends Anal Chem 28:729–743
13. Lara FJ, del Olmo-Iruela M, Cruces-Blanco C, Quesada-Molina C, García-Campaña AM (2012) Advances in the determination of β-lactam antibiotics by liquid chromatography. Trends Anal Chem 38:52–66
14. Junza A, Dorival-García N, Zafra-Gómez A, Barróna D, Ballesteros O, Barbosa J, Navaló A (2014) Multiclass method for the determination of quinolones and β-lactams, in raw cow milk using dispersive liquid-liquid microextraction and ultra high performance liquid chromatography-tandem mass spectrometry. J Chromatogr A 1356:10–22
15. Msagati TAM (2007) Nindi MM (2007) Determination of β-lactam residues in foodstuffs of animal origin using supported liquid membrane extraction and liquid chromatography-mass spectrometry. Food Chem 100:836–844
16. Junza A, Montane A, Barbosa J, Minguillon C, Barron D (2014) High resolution mass spectrometry in the identification of transformation products and metabolites from β-lactam antibiotics in thermally treated milk. J Chromatogr A 1368:89–99
17. Pereira RV, Siler JD, Bicalho RC, Warnick LD (2014) Multiresidue screening of milk withheld for sale at dairy farms in central New York State. J Dairy Sci 97:1513–1519
18. Karageorgou EG, Samanidou VF (2012) Development and validation according to European Union Decision 2002/657/EC of an HPLC-DAD method for milk multi-residue analysis of penicillins and amphenicols based on dispersive extraction by QuEChERS in MSPD format. J Separ Sci 34:1893–1901
19. Piñero MI, Bauza R, Arce L, Valcarcel M (2014) Determination of penicillins in milk of animal origin by capillary electrophoresis: is sample treatment the bottleneck for routine laboratories? Talanta 119:75–82
20. Babington R, Matas S, Marco MP, Galve R (2012) Current bioanalytical methods for detection of penicillins. Anal Bioanal Chem 403:1549–1566
21. Beltrán MC, Borràs M, Nagel O, Althaus RL, Molina MP (2014) Validation of receptor-binding assays to detect antibiotics in goat's milk. J Food Protect 77:308–313
22. Gross M, Rodríguez-Mozaza S, Barceló D (2013) Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-highperformance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J Chromatogr A 1292:173–188
23. Haupt K (ed) (2012) Molecular Imprinting. Springer, Heidelberg, Germany
24. Urraca JL, Schillinger E, Esselmann H, Wiltfang J, Sellergren B (2011) Polymeric complements to the Alzheimer's disease biomarker β-amyloid isoforms Aβ1–40 and Aβ1–42 for blood serum analysis under denaturing conditions. J Amer Chem Soc 133:9220–9223
25. Zhang J, Wangy H, Liuy W, Bai L, Ma N, Lu J (2008) Synthesis of molecularly imprinted polymer for sensitive penicillin determination in milk. Anal Lett 4:3411–3419

26. Royen GV, Dubruel P, Daeseleire E (2014) Development and evaluation of a molecularly imprinted polymer for the detection and cleanup of benzylpenicillin in milk. J Agric Food Chem 62:8814–8821
27. Zhang X, Chen L, Xua Y, Wang H, Zeng Q, Zhao Q, Ren N, Ding L (2010) Determination of β-lactam antibiotics in milk based on magnetic molecularly imprinted polymer extraction coupled with liquid chromatography–tandem mass spectrometry. J Chromatogr B 878: 3421–3426
28. Díaz-Bao M, Barreiro R, Miranda JM, Cepeda A, Regal P (2015) Fast HPLC-MS/MS method for determining penicillin antibiotics in infant formulas using molecularly imprinted solid-phase extraction. J Anal Meth Chem 959675/1–8
29. Du W, Zhou H, Luo Z, Zheng P, Guo P, Chang R, Chang C, Fu Q (2014) Selective determination of penicillin G from tap water and milk samples using surface molecularly imprinted polymers as solid-phase extraction sorbent. Mol Impr 2:18–29
30. Giovannoli C, Anfossi L, Biagioli F, PassiniC BC (2013) Solid phase extraction of penicillins from milk by using sacrificial silica beads as a support for a molecular imprint. Microchim Acta 180: 1371–1377
31. http://www.sanidadanimal. Bayerandina.com. Accessed 14 May 2015
32. Urraca JL, Marazuela MD, Merino ER, Orellana G, Moreno-Bondi MC (2006) Molecularly imprinted polymers with a streamlined mimic for zearalenone analysis. J Chromat A 1116:127–134
33. Commission Decision 2002/657/EC of 12 August implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of the results. (2002) Off. J. Eur. Union L, 221:8–36
34. Hall AJ, Manesiotis P, Emgenbroich M, Quaglia M, de Lorenzi E, Sellergren B (2005) Urea host monomers for stoichiometric molecular imprinting of oxyanions. J Org Chem 70:1732–1736
35. Branca M, Pena S, Guillot R, Gori D, Alezra V, Kouklovsky C (2009) Memory of chirality of tertiary aromatic amides: a simple and efficient method for the enantioselective synthesis of quaternary α-amino acids. J Am Chem Soc 131:10711–10718
36. Hoover JRE, Chow AW, Stedman RJ, Hall NS, Greenberg HS, Dolan MM, Ferlauto RJ (1964) Semisynthetic penicillins. I. 2-biphenylylpenicillins. J Med Chem 7:245–251
37. Sellergren B, Shea KJ (1993) Influence of polymer morphology on the ability of imprinted network polymers to resolve enantiomers. J Chromatogr 637:31–49
38. Calculated using Advanced Chemistry Development (ACD/Labs) software V11.02
39. Zerara M, Brickmann J, Kretschmer R, Exner TE (2009) Parameterization of an empirical model for the prediction of *n*-octanol, alkane and cyclohexane/water as well as brain/blood partition coefficients. J Comput Aided Mol Des 23:105–111
40. Benito-Peña E, Moreno-Bondi MC, Aparicio S, Orellana G, Cedefur J, Kempe M (2006) Molecular engineering of fluorescent penicillins for molecularly imprinted polymer assays. Anal Chem 78:2019–2027
41. Urraca JL, Moreno MC, Hall A, Sellergren B (2006) A stoichiometric molecularly imprinted polymer for the class-selective recognition of antibiotics in aqueous media. Angew Chem Int Ed 45: 5158–5161
42. Urraca JL, Moreno MC, Hall A, Sellergren B (2007) Direct extraction of penicillin G and derivatives from aqueous samples using a stoichiometrically imprinted polymer. Anal Chem 76:695–701
43. Sucha L, Kotryl S (1972) Solution equilibria in analytical chemistry. Van Nostrans Reinhold, New York
44. Calculated using Marvin Sketch software V5.12 from Chem Axon (www.chemaxon.com)
45. Rampey AM, Umpleby RJ, Rushton GT, Iseman JC, Shah RN, Shimizu KD (2004) Characterization of the imprint effect and the influence of imprinting conditions on affinity, capacity, and heterogeneity in molecularly imprinted polymers using the Freundlich isotherm-affinity distribution analysis. Anal Chem 786:1123–1133
46. Wulandari M, Urraca JL, Descalzo AB, Amran MB, Moreno-Bondi MC (2015) Molecularly imprinted polymers for cleanup and selective extraction of curcuminoids in medicinal herbal extracts. Anal Bioanal Chem 407:803–812
47. Benito-Peña E, Partal-Rodera AI, León-González ME, Moreno-Bondi MC (2006) Evaluation of mixed mode solid phase extraction cartridges for the preconcentration of beta-lactam antibiotics in wastewater using liquid chromatography with UV-DAD detection. Anal Chim Acta 556:415–422
48. Poole CF, Poole SK (2002) In: Simpson NJK (ed) In: Theory meets practice. Solid phase extraction: principles, techniques and applications. Marcel Dekker Inc, New York, pp 183–226