Occurrence of erythromycin residues in sheep milk. Validation of an analytical method

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ABSTRACT

The paper describes a new and selective analytical sample treatment for quantitative extraction and preconcentration of erythromycin in presence of other macrolide antibiotics in sheep milk samples. The methodology is based on the use of a molecular imprinted polymer (MIP) employed as solid phase extraction sorbent (MISPE). The synthesized material by bulk polymerization using erythromycin (ERY) as template was evaluated as solid phase extraction sorbent, in a novel sample treatment technique that can be coupled to high-performance liquid chromatography with diode-array detector (HPLC-DAD). MIP selectivity was studied for other macrolide antibiotics with similar structures, such as tylosin (TYL), spiramycin (SPI), josamycin (JOS), roxithromycin (ROX) and ivermectin (IVER) getting recoveries for these interferents lower than 35%, for all cases except for ROX, which recoveries were around 85%. The variables affecting the molecularly imprinted solid-phase extraction (MISPE) procedure were optimized to select the best conditions of selectivity and sensitivity to determine ERY at concentration levels established by EU legislation in sheep milk. Under the selected experimental conditions, quantification limit was 24.1 µg kg^{-1}. Recoveries were higher than 98%, with RSDs between 0.7% and 2%. The proposed MISPE-HPLC method was validated and successfully applied to ERY analysis in sheep milk samples.

1. Introduction

Erythromycin (ERY) is a representative of macrolide antibiotics produced by *Saccharopolyspora erythrea*. It has an antimicrobial spectrum similar to or slightly wider than of penicillin and is often prescribed for people who have an allergy to it (Blasi, 2004; Mills et al., 2005). This macrocyclic antibiotic contains in the structure a 14-membered lactone ring with ten asymmetric centers and two sugars (L-cladinose and D-desosamine), making it a compound very difficult to produce via synthetic methods (Mazzei et al., 1993; Stephenson et al., 1994). Due to its biological effect against *Mycoplasma* and Gram-positive bacteria, ERY is extensively applied in veterinary practice, being, sometimes, administered as feed additives or via drinking water in order to prevent the outbreak of diseases and also in cases of disease, for dehydration or to prevent losses during transportation. Unfortunately, these applications of antibiotics and the lack of observance of the withdrawal time after treatment in animals have led to the presence of antibiotic residues in foods, which is potentially hazardous for human health (Forti

and Scortichini, 2009). The presence of ERY residues in foodstuff may cause toxic effects on consumers, especially in the most vulnerable risk groups, such as infants (Rodríguez et al., 2010). The use of this antibiotic and similar compounds may result to antibiotic resistance in treatment animal and humans, who can be affected by allergic reaction. For this reason, legislation regarding the control of antibiotic residues in live animals and animal products is given in Council Directive 96/23/EC (European Commission, 1996). In the context of this directive, details for methods and their performance criteria are described in Commission Decision 2002/657/EC (European Commission, 2002). The EU Council regulation 2377/90 provides the Community procedure for the establishment of maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin (European Commission, 1990). Due to limitations established by EU for ERY in milk, rapid sensitive and selective analytical procedures for ERY analysis are required. Nowadays, chromatography techniques, such as high-performance liquid chromatography coupled to diode-array (HPLC/DAD) (García et al., 2006; Xiachang et al., 2009) or mass spectrometry (HPLC/MS/MS) detectors (Liu et al., 2010; Wang and Leung, 2007), have been used to determine ERY levels in milk samples. However, due to the complex nature of this sample matrix and, in general of alimentary samples, the development of efficient pretreatments for clean-up and preconcentration is necessary in order to reduce the

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interferences and improve accuracy and precision in the analysis. In that regard, the application of molecular imprinting technology in the design of new, efficient and selective methods to achieve these objectives in food applications is constantly increasing (Beltrán et al., 2014; Granja et al., 2009). Much of the current research in the molecular imprinting field, for ERY determination, is concentrated on solid phase extraction (SPE) (Ezhova et al., 2011). In addition to the existing advantages of SPE, for example compared with liquid/liquid extraction (LLE) as low solvent consumption and the possibility of automation, molecularly imprinted-solid phase extraction (MISPE) technology offers predetermined selectivity for analytes of interest and the possibility of different MIP configurations, which is well suited for analytical chemistry. In this sense, different MIP formats to apply in SPE, such as nanoparticles (Kou et al., 2011, 2012), materials by sol-gel (Zhaohui et al., 2010) or polymer based on carbon nanotubes (Lian et al., 2012) have been developed for ERY determination.

The aim of this paper was the synthesis of a novel molecularly imprinted functional material and its application as SPE sorbent for the quantitative extraction of ERY in sheep milk samples. The selectivity of the ERY-polymer with respect to other macrolide antibiotics was evaluated. The determination of ERY was carried out in sheep milk samples by HPLC-DAD. This kind of milk sample comes from La Mancha region in Spain, and is used to produce the prestigious *Manchego cheese* which is granted Protected Designation of Origin (PDO) status by the European Union.

2. Experimental procedures

2.1. Reagents and solutions

Ethylene glycol dimethacrylate (EGDMA) and methacrylic acid (MAA) were purchased from Merck (Darmstadt, Germany), 2-2′-azobisisobutyronitrile (AIBN) from Fluka (Buchs, Switzerland). Commercial antibiotic standards (erythromycin, tylosin hemitartrate, spiramycin, josamycin, roxithromycin, ivermectin) and sodium phosphate monobasic were supplied by Sigma Aldrich (Madrid, Spain). Sodium hydroxide and n-hexane (purity > 99%) were obtained from Merck (Darmstadt, Germany). All reagents used were of analytical grade or better. Ultra-pure water (18.2 MΩ cm quality) was obtained using a Milli Q water system (Millipore Ibérica, Madrid, Spain).

Stock standards solutions of individual compounds at 1 g L^{-1} were prepared every 3 months by exact weighing of the powder and dissolved in 10 mL of methanol of HPLC-grade from Scharlab (Barcelona, Spain), which were then stored at 4 °C in the dark. A standard solution of each macrolide antibiotic (500 mg L^{-1}) were prepared by diluting the stock solution with acetonitrile (ACN) of HPLC-grade from Scharlab, and also stored at 4 °C in the dark. The working standard solution at adequate concentration of ERY was daily prepared by appropriate dilution of the mentioned solution with the dilution mixture NaH$_2$PO$_4$ 25 mM at pH 7/acetonitrile (70:30). Aqueous solvent of the mobile phase was made by dissolving 5 g of NaH$_2$PO$_4$ in 500 mL of Milli-Q water and sodium hydroxide was used to adjust the pH at 7. Subsequently, the solution was filtered through a 0.45 µm cellulosic membrane filter.

2.2. Instrumentation

HPLC analysis was performed on an Agilent Technologies model 1200 series liquid chromatographic equipped with an Agilent 1290 quaternary pump, auto sampler, and photo-diode array detector (Agilent Technologies, Germany). An ultraviolet lamp (Vilber Lourmat CN-6T) and a Digiterm 3000542 thermostat-controlled waterbath (Selecta, Barcelona, Spain) were used to provide the polymerization process. All pH readings were made with a Metrohm 654 pH meter. Template extraction was performed using a soxhlet extractor

system with cellulose extraction thimbles. Imprinted and control polymers were ground in a glass mortar (Aldrich, Madrid, Spain) and then passed through CISA standard sieves (200–355 µm) (Afora, Madrid, Spain). SPE was performed using a 20-Port Vacuum SPE manifold System (Supelco, Spain) with vacuum control-press pump (Selecta). Empty SPE cartridges (Supelco) of 3 mL of capacity with polyethylene frits were used to pack the solid phase.

2.3. Procedures

2.3.1. Milk samples pretreatment protocol

Several fresh morning milk samples from different sheep in the same stage of lactation were collected from CERSYRA, a Regional Centre of Animal Selection and Reproduction in Valdepeñas (Ciudad Real, Spain). The samples were transported to the laboratory and stored at –20 °C until use.

The protocol used for pretreatment of spiked milk samples is detailed below. Milk samples were allowed to thaw at room temperature and homogenized by heating at 35 °C for 5 min. An aliquot of 1 mL of homogenized milk was spiked with the desired amount of ERY, mixed by manual shaking and maintained at room temperature for 20 min to allow the equilibration of the macrolide with the milk matrix. Mixture of 4 mL NaH$_2$PO$_4$:ACN (3:2, pH 7) and 1 mL ACN were added simultaneously to precipitate the proteins. Sample was then centrifuged for 15 min at 1200 rpm and filtered with a fold filter before the application of extraction procedure.

2.3.2. Synthesis of molecularly imprinted polymer

For preparation of MIPs, 2×10^{-2} mmol of ERY was dissolved in 7 mL of ACN in a 25 mL glass tube and sonicated during 10 minutes. The functional monomer (MAA, 2.0 mmol), the cross-linker (EGDMA, 10.0 mmol) next, the radical initiator (AIBN, 5.1 mmol) were added. This mixture was purged with nitrogen for 10 min. The glass tube was then placed in a thermostat-controlled waterbath at 65 °C for 5 h, or under UV light at 235 nm at 5 °C for 3 h. The monolithic polymers obtained were crushed and sieved in a glass mortar to obtain particles with sizes of 200–355 µm, suitable for SPE evaluation. Finally, the template and non-polymerized compounds were removed by soxhlet extraction with MeOH (80 mL) for 25 h, until no ERY was detected by HPLC-DAD. Non-imprinted polymers (NIPs) were prepared under identical conditions except for the addition of the analyte.

2.3.3. Chromatographic conditions

Chromatographic separation of the analytes was performed on a Prontosil C18 (250 × 4.6 mm, 5 µm) HPLC column from Scharlab. A gradient program was used with the mobile phase, combining solvent A (25 mM phosphate buffer solution, pH 7) and solvent B (acetonitrile) as follows: 50% B (3 min) at 1 mL min^{-1}, 58% B (8 min) at 1.2 mL min^{-1} and 70% B (20 min) at 1.5 mL min^{-1}. The column temperature was kept at 60 °C. The injection volume was 20 µL, and all the compounds eluted within 30 min. The UV detector wavelengths were set at 210 nm (ERY, ROX), 231 nm (JOS, SPI), 254 nm (IVER) and at 287 nm (TYL). Quantification was performed using external calibration and peak area measurements.

2.3.4. MISPE procedure

An amount of 200 mg of dry imprinted and non-imprinted polymer was packed separately into empty SPE cartridges of 3 mL between two frits (length of 65 mm and i.d. 10 mm). MISPE steps were carried out at 0.7–1.0 mL min^{-1} on a vacuum manifold for 20 cartridges. MISPE cartridges were conditioned with 3 × 2 mL of MeOH and 3 × 2 mL of ACN prior to extraction. Then, 1 mL of milk sample previously pre-treated, following the procedure described in section 2.3.1 to erase the proteins, was loaded. When the sample loading was completed, prior to the elution step, the removal of fat was

carried out adding 6×1 mL of hexane. Then, the target analyte was eluted using 3×2 mL MeOH with 0.5% acetic acid. Elutes were evaporated to dryness under a gentle flow of nitrogen at 40 °C. The residue was re-dissolved with the adequate volume of mixture NaH$_2$PO$_4$ 25 mM (pH 7)/acetonitrile (70:30) and they were analyzed by HPLC using the method described above. Finally, the polymer was regenerated by passing 3×2 mL of MeOH and 3×2 mL of ACN for the next assay.

3. Results and discussion

3.1. Preparation and evaluation of molecularly imprinted polymers

Two non-covalent MIPs were synthesized by "bulk" polymerization using ERY as template to obtain a suitable MISPE for selective extraction of ERY. MIPs synthesis were carried out with the same ratios of template to monomer and cross-linker (1:100:500), but the polymerization process was allowed to proceed either under UV light (MIP1) or in a thermostat-controlled waterbath (MIP2). The corresponding NIPs were also synthesized.

A sufficient mechanical stability and a high degree of cross-linking of the polymer are certainly required for chromatography applications to obtain good separations. On that basis, a high molar ratio of template/cross-linker (1:500) was used in order to obtain a high degree of cross-linking and adequate rigidity in the polymer matrix to produce specific cavities of the correct size and shape and to preserve the imprinted memory. MAA was chosen as functional monomer due to its widely recognized ability to form hydrogen bonds, and because the binding and removal of the molecule template can be performed under mild conditions. The nature of the solvent polymerization or porogen is a very important factor to consider in the synthesis of MIPs. The porogen plays an important role in the final polymer morphology, being the one responsible of the pore diameter and influencing the recognition of the specific analyte. Usually, the non-covalent MIPs synthesis should be performed in aprotic and apolar solvents to avoid disabilities in the ionic interactions and hydrogen bonds between target and the functional monomer. For this reason, and taking into account that ERY is soluble in acetonitrile, this was the porogen selected for MIPs preparation. In regards to the polymerization method, it has influence in the morphology as well as the specific recognition characteristics of the polymer. Two different polymerization procedures were carried out: UV-initiated photopolymerization (235 nm) at 5 °C for 3 h (MIP1) and thermally initiated polymerization in a thermostat-controlled waterbath at 65 °C for 5 h (MIP2). The polymers obtained as bulk solids were ground with a glass mortar and sieved to obtain particles in the adequate size range, suitable for MISPE evaluation. Finally, polymers were extracted in a soxhlet apparatus to remove the template, and the washed MIP solutions were analyzed by HPLC to check the absence of ERY in MIP materials.

The corresponding blank or non-imprinted polymers were prepared under the same conditions without the use of template to act as control.

Binding studies in different solvents for both synthesized polymers (MIP1 and MIP2) were carried out in order to select the most suitable polymer to act as sorbent for a selective solid phase extraction for ERY determination. The MIP applicability was evaluated following the procedure described in Section 2.3.4. For that, different loading solvents were tested: chloroform, ACN and NaH$_2$PO$_4$:ACN (70:30) at pH 7, 1 mL of 50 mg L^{-1} ERY standard solution, prepared in each solvent mentioned above. Then, a washing step using ACN (3×2 mL) was carried out. The quantitative recovery of ERY was evaluated by the no bound analyte onto the MIP, and calculated as the difference between ERY total amount loaded onto the cartridge initially, and collected ERY in the fractions from the loading and washing steps. Table 1 summarizes the recoveries obtained for

Table 1
Comparison of MISPE recoveries obtained for ERY standard solution (1 mL, 50 mg L^{-1}) from binding studies in different loading solvents: chloroform, ACN and NaH$_2$PO$_4$:ACN (70:30, pH 7).

Polymer	ERY recoveries (%)		
	Chloroform	ACN	NaH$_2$PO$_4$:ACN (70:30, pH 7)
NIP1	10 ± 0.9	12 ± 1.7	16 ± 0.7
MIP1	18 ± 1.2	78 ± 1.5	24 ± 3.2
NIP2	9 ± 1.4	4 ± 0.7	9.5 ± 3.1
MIP2	29 ± 0.8	96 ± 1.1	38 ± 1.5

the different solvents assayed. In general, MIP2 showed better recognition properties than MIP1 in all solvents tested. When chloroform or NaH$_2$PO$_4$:ACN (70:30) at pH 7 were used, recoveries were lower than 40% for both, MIP1 and MIP2. Binding from ACN provided the best results, being 78% for MIP1 and 96% for MIP2. These results are according to the fact that generally the MIP exhibits better molecular recognition in the solvent used as a porogen during polymerization. The low recoveries obtained for NIPs (lower than 16%) indicated that the interactions between the template and MIPs were mainly specific. In case of NIP2, recovery in ACN was lower than 4%. Therefore, it can be concluded that ACN provides maximal interactions between ERY molecules and the binding sites in synthesized MIPs and also that, MIP2 exhibits a higher imprinting effect toward the analyte than MIP1. Then, MIP2 was selected as optimum sorbent for use in further experiments for the development of an off-line MISPE for ERY.

3.2. MISPE optimization

The functionality of ERY-MIP2 was confirmed in these previous binding studies and its performance was compared with NIP2 simultaneously under identical conditions to prove the nonspecific binding.

To obtain the best selectivity and recovery in the MISPE procedure, using MIP2, key factors influencing SPE efficiency, such as washing and elution steps were optimized. First washing step was tested using several solvents to wash off the non-specifically adsorbed compounds: H$_2$O and H$_2$O/ACN mixtures at increasing ACN proportions (50–100%) up to 6 mL (3×2 mL). Fig. 1 shows the results obtained when 1 mL of ERY standard solution at 50 mg L^{-1} in ACN was loaded onto the cartridge. The best results were obtained when the washing solvent used was ACN. As can be seen, losses of ERY were less than 1%, which imply that ERY was completely retained on the MIP. On the other hand, as ERY is highly soluble in water, when the proportion of water decreased in the washing solution, most of the ERY was retained in the cartridge. Therefore, a volume of 6 mL of ACN was used as optimum washing conditions. These results demonstrated that the MIP exhibited high binding affinity for ERY, and confirmed that the adsorption of this compound was due to imprinted binding sites and not to nonspecific binding.

To ensure a complete extraction of the adsorbed analyte from MISPE column is necessary to find out the appropriate eluent. It is a long-established principle that a mixture of methanol with different proportions of acetic acid is usually used to elute the analyte because acetic acid can compete with the analyte for the functionality of the monomer and impair the ionic bonds formed between them. Based on these premises, and taking into consideration that ERY is not stable at acid pH, the efficacy of various solvents for the elution of ERY was evaluated to optimize this technique for quantitative accuracy and to confirm the nature of specific interactions between the target molecule and the binding sites. The solutions examined were NaH$_2$PO$_4$:ACN, 70:30 at pH 7, MeOH and MeOH
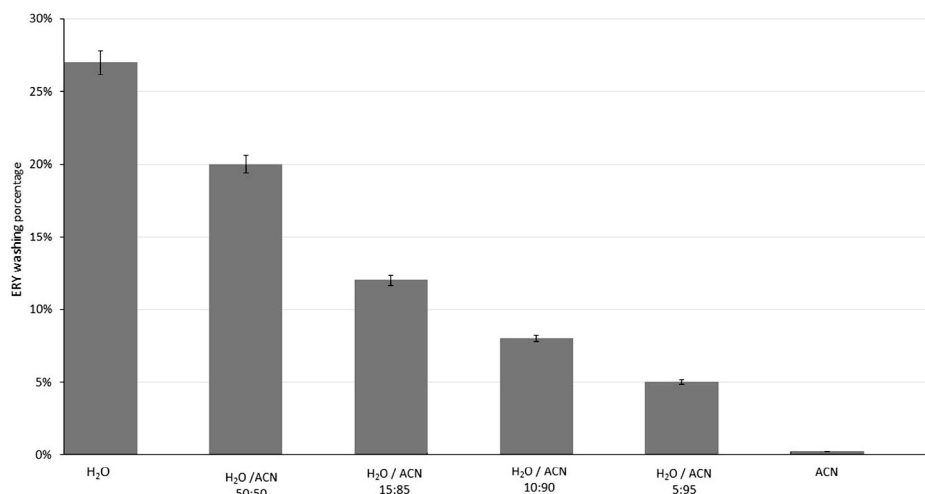
Fig. 1. Percentages of ERY obtained in all fractions collected from washing step using different washing solvents (H$_2$O, H$_2$O/ACN mixtures) when ERY standard solution was loaded onto the cartridge (1 mL of 50 mg L^{-1} in ACN).

containing different percentages of acetic acid (0.25, 0.5 and 1%), and different volumes (5, 6, and 7 mL) were assayed. Fig. 2 illustrates the effect of solvent polarity on elution efficacy in terms of quantitative recoveries of ERY in MIP. Recoveries were calculated from the fractions collected from elution step with respect to the total amount of ERY load onto the cartridges. As a general trend, an increase in the elution efficacy can be seen with increasing solvent polarity. With the use of less polar solvent (NaH$_2$PO$_4$:ACN, 70:30 at pH 7) a low elution of bound ERY (52%) was obtained.

The results showed that the presence of acetic acid in the elution solvent provided higher recoveries than pure MeOH and by increasing the content of acetic acid up to 0.5%, extraction recoveries increased slightly to reach a value of 98%. However, when a higher amount of acetic acid was used, elution efficacy decreased considerably. One percent acetic acid allowed extraction of only 63% of ERY. This observation can be explained due to the fact that ERY is degraded at increasingly acidic pH. These studies revealed that the optimum elution solvent was 0.5% acetic acid in MeOH, and a volume of 6 mL (2 × 3 mL) was necessary for the complete elution of ERY from the cartridge. The results suggest that ERY was bound to the binding sites through hydrogen bonds and ionic interactions which cannot be disrupted through apolar solvents or solvents with low polarity. It can be claimed that these ionic interactions may be affected by the alteration of the ionic strength of the solvent, and only polar solvents are able to compete with ERY for the binding sites allowing the rapid desorption of bound analyte.
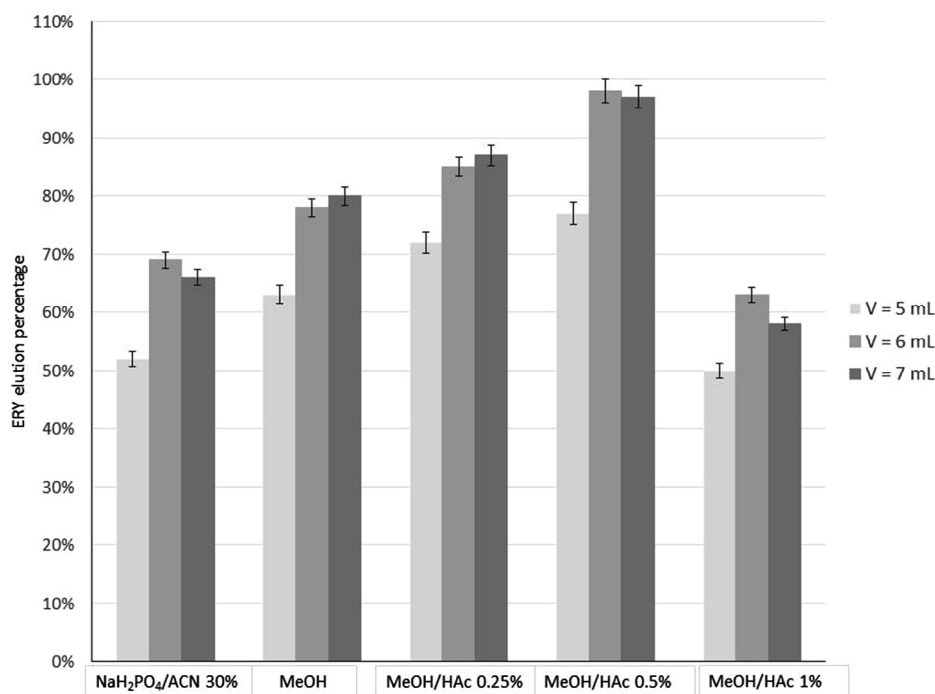

Fig. 2. Elution percentages of ERY in MISPE for different solutions: NaH$_2$PO$_4$:ACN (70:30, v/v), MeOH and MeOH at 0.25, 0.5, 1% in acetic acid. Several volumes of elution solvent (5–7 mL) were tested. The studies were developed with 1 mL of ERY standard solution at 50 mg L^{-1} in ACN.

3.3. Milk samples analysis

Milk is a highly complex matrix containing a great variety of compounds (proteins, sugars, lipids …) that can interfere in the preconcentration efficiency and recovery of ERY in SPE. Therefore, prior to MISPE procedure, a pre-treatment for proteins removal from the milk samples was required in order to avoid blockages in the MIP. For this study, several precipitating solvents or mixtures were studied: ACN, NaH_2PO_4:ACN (3:1, 3:2) modifying pH value (5, 6, 7). Different volumes of these solutions (3, 4, 5, 6 mL) were also tested. When the precipitation process was performed with ACN or NaH_2PO_4:ACN, it was observed that the samples remained with protein residues, offering the best results of the mixture 3:1 NaH_2PO_4:ACN, at pH 7. In any case, the precipitation process yield was better when 5 mL of solvent were added. Higher amounts of solvent did not involve an improvement in the process. These studies provided clear guidance to the choice of a suitable precipitation solvent for proteins. Then, precipitation proteins was produced more extensively when a mixture of NaH_2PO_4:ACN (4 mL, 3:2) at pH 7 plus 1 mL of ACN was used. After the proteins precipitation step, milk sample was ready to be analyzed by the above described MISPE procedure, including an additional step to remove the fat content and so avoid fat interferences in the determination.

Fat removal was carried out before the washing step, and was optimized taking into account a previous research (García et al., 2006). For that study two different solvents (n-hexane and 0.5 M NaOH) were examined varying the volume of them within the range 6–8 mL. When 0.5 M NaOH was used at all volumes tested, the complete removal of fat was not allowed. The samples after the complete MISPE procedure showed a fat residue which prevented their correct HPLC-DAD analysis. The use of n-hexane provided clean extracts, and ERY recoveries were higher than 95%. The optimum results were achieved with 6×1 mL of hexane. Increasing the volume of solvent until 8 mL did not show a significant yield improvement. It was observed that fat removal acted as washing step, being unnecessary an additional washing with ACN, allowing the elution step to be carried out immediately.

Optimal eluting performance, as it was reported in section 3.2, was achieved with MeOH:acetic acid mixture containing 0.5% of acetic acid (3×2 mL). After a MISPE procedure was completed, the eluents collected were taken to dryness and reconstituted in a volume of 1 mL in NaH_2PO_4 25 mM at pH 7.0/acetonitrile (70:30) mixture. Finally ERY in the milk extracts was detected by HPLC-DAD.

3.4. Analytical performance

Validation of the proposed method was performed to evaluate the accuracy and precision of the MISPE protocol, its linear range and limit of quantification (LOQ), by using spiked milk samples in all work performed. Results were summarized in Table 2. The accuracy was evaluated by calculating the recovery obtained for ERY in MISPE procedure of 1 mL of spiked milk samples at three concentration levels: low level (24.2 µg kg^{-1}), medium level (48.3 µg kg^{-1}) and high level (482.6 µg kg^{-1}). Obtained residues were analyzed by HPLC-DAD in triplicate. Recoveries were higher than 95% and their corresponding relative standard deviations (RSD) were less than 2%. The intra-day repeatability and inter-day reproducibility were

determined in triplicate assays at 48.3 µg kg^{-1} and 482.6 µg kg^{-1} spiking levels. Intra-day recoveries ranged from 91.2% to 99.7% with RSDs less than 2.2%; for the inter-day repeatability, recoveries ranged from 92.2% to 98.8% with RSD less than 5%. The linearity of the method with HPLC-DAD detection was tested in the range of 24–965 µg kg^{-1}. Calibration curve was obtained by preparing spiked milk samples in triplicate, containing concentrations of ERY in that working range. The calibration curve obtained ($y = 0.23\ C + 12.36$) showed linearity, with correlation coefficient $R^2 = 0.9994$. The limit of quantification, calculated as the lowest concentrations with RSD below 5%, was 24.1 µg kg^{-1}. The results demonstrated that the optimized MISPE-HPLC methodology proposed is easily and successful applied for determination of ERY in sheep milk at levels lower than the maximum residue limit established by the European Union (40 µg kg^{-1}).

3.5. Selectivity study

The molecular imprinting technique allows the designing of new artificial materials of specific recognition. After removal of template molecule, MIP shows a high selectivity to the imprinted molecules due to the arrangement of the functional groups of the monomer units around the print molecules. In order to verify the selectivity of MISPE toward ERY, other macrolide antibiotics with similar chemical structures as competitive molecules: josamycin (JOS), spiramycin (SPI), roxithromycin (ROX), ivermectin (IVER) and tylosin hemitartrate (TYL) were examined. For interference studies, recoveries of macrolide antibiotics at three concentration levels were tested by the MISPE procedure developed: low level (JOS, SPI, IVER, TYL at 10 mg L^{-1} and ERY, ROX at 100 mg L^{-1}), medium level (5:50 mg L^{-1}), high level (2.5:25 mg L^{-1}). Fig. 3 shows the chromatograms obtained in selectivity study at different wavelengths (a) for a standard mixture of the six macrolides studied and (b) for a milk sample spiked under the established MISPE method. Table 3 shows the results obtained in terms of percentages of recovery, calculated as the fractions collected from elution step with respect to the total amount of each compound load onto the cartridge. Recoveries obtained for JOS, SPI, IVER and TYL were between 15 and 35%, while for ERY and ROX were higher than 98 and 85%, respectively. The results were consistent according to the structures. The polymer showed higher adsorption capacity toward ERY than toward the other macrolide antibiotics studied due to this compound acting as molecule template during the synthesis. On the other hand, the polymer also exhibited specific selectivity for ROX which has a structure more similar to ERY compared to the other compounds assayed, being the most possible interfering compound that can significantly affect clean-up efficiency. Although MIP for ERY demonstrated to be also selective for ROX, no interference was produced during chromatographic determination due to the difference between the retention times of both compounds. In the binding process, many specific recognition sites with respect to the template were generated on the ERY-MIP surface, so the template was strongly bound by the imprinted polymer; all recoveries always were higher than 95%. The results demonstrated that the MIP-SPE column exhibited specific selectivity for ERY in the presence of other structurally related compounds.

Table 2
Analytical characteristics of the optimized MISPE-HPLC method for the ERY determination. All measures were average of three replicates.

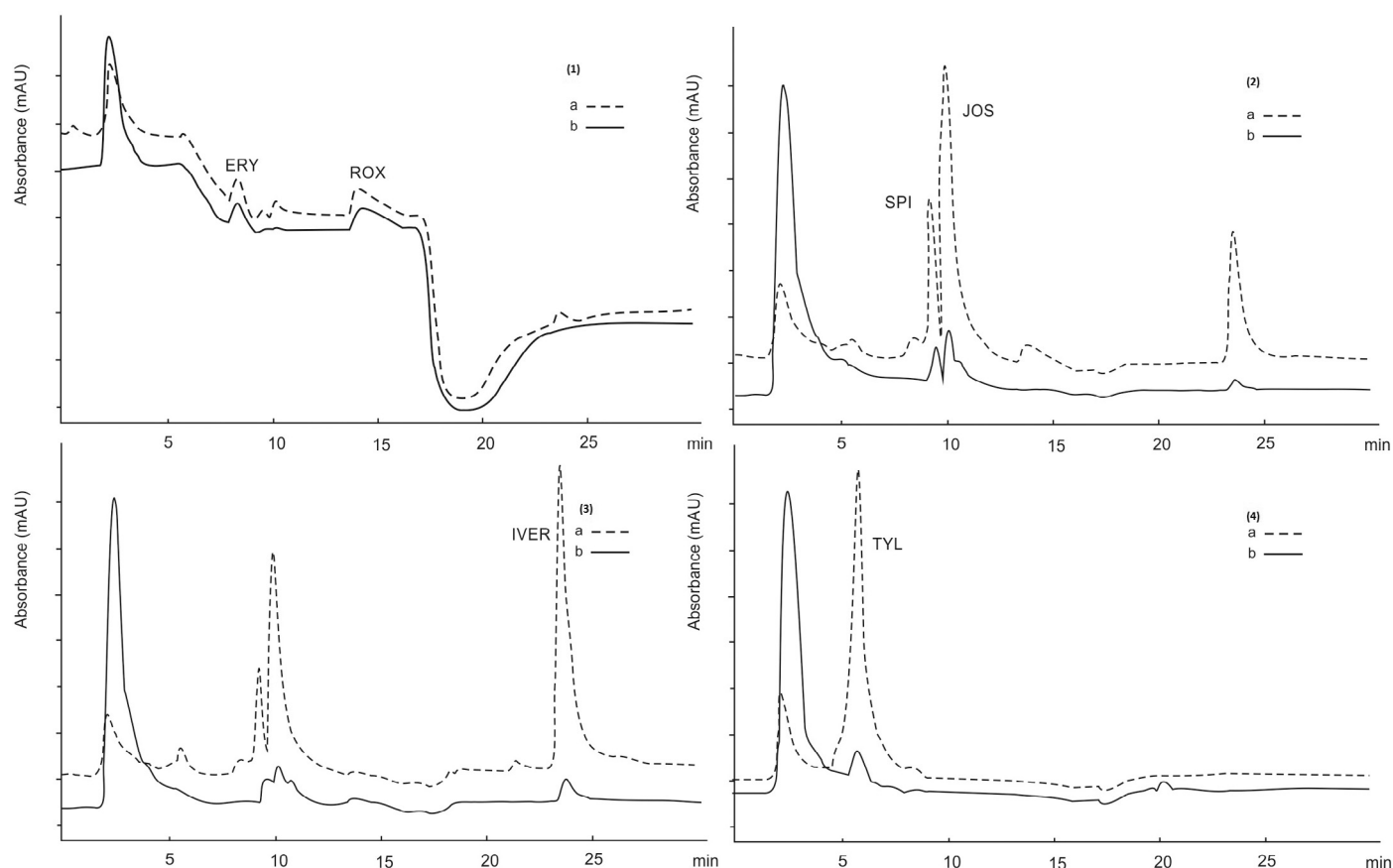| % Recovery			Intra-day recovery (%)		Intra-day recovery (%)		Regression equation	LOQ (µg kg^{-1})
Low level 24.2 µg kg^{-1}	Medium level 48.3 µg kg^{-1}	High level 482.6 µg kg^{-1}	Spiking level 48.3 µg kg^{-1}	482.6 µg kg^{-1}	Spiking level 48.3 µg kg^{-1}	482.6 µg kg^{-1}	Concentration working range (24–965 µg kg^{-1})	24.1
99.0 ± 1.7	98.9 ± 0.9	98.9 ± 0.7	98.8 ± 5.0	92.2 ± 3.4	99.7 ± 0.8	91.2 ± 2.2	$y = 0.23C+12.36$ $R^2 = 0.9994$	

Fig. 3. Chromatograms obtained for the six antibiotic macrolides studied at concentrations of 50 mg L^{-1} ERY, ROX and 5 mg L^{-1} JOS, SPI, IVER and TYL. (a) For an ACN standard mixture (b) MISPE extracted sheep milk. Chromatograms were obtained at the wavelength corresponding to each analyte studied: (1) ERY and ROX at 210 nm; (2) SPI and JOS at 231 nm; (3) IVER at 254 nm and (4) TYL at 287 nm.

Table 3
Macrolides recoveries at three concentration levels. Low level (JOS, SPI, IVER, TYL at 10 mg L^{-1} and ERY, ROX at 100 mg L^{-1}), medium level (5:50 mg L^{-1}), high level (2.5:25 mg L^{-1}), (n = 3).

Antibiotic	% Recovery		
	Low level	Medium level	High level
ERY	98.3 ± 1.8	98.6 ± 1.2	98.1 ± 0.9
ROX	88.3 ± 2.6	88.7 ± 5.5	84.7 ± 3.2
SPI	35.3 ± 7.4	30.3 ± 1.5	27.3 ± 0.6
JOS	34.3 ± 3.8	35.3 ± 2.5	32.3 ± 4.9
IVER	28.4 ± 1.2	21.0 ± 3.1	28.1 ± 1.2
TYL	14.2 ± 3.3	19.3 ± 5.1	24.3 ± 0.6

4. Conclusion

A reliable molecularly imprinted solid-phase extraction methodology for ERY antibiotic determination has been developed. MISPE selectivity was tested in the presence of the other antibiotics belonging to the family of macrolide antibiotics, which have similar structure to ERY, showing that the proposed method is thought to be highly selective. Cross-reactivity of these compounds was negligible. Synthesized polymer showed good selectivity and high adsorption capacity for ERY, also allowing the successful recovery of ROX without cross-reactivity. Moreover, polymer has been used for more than 200 assays without any degradation signals, which ensures high stability. The developed method involves a simplification, and time savings, of the sample treatment because the fat content of the milk samples can be removed on line during the MISPE procedure. HPLC combined with the MISPE procedure has led to a good sensitivity methodology that allows determination of ERY at levels below limits required by the UE, who establishes a maximum residue limit of 40 µg kg^{-1} of ERY in sheep milk. This work constitutes a significant advance in the simplicity and efficiency for the assessment of the potential risk of exposure to erythromycin residues in complex matrix such as sheep milk.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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