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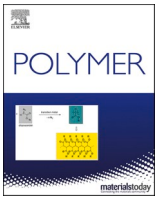

Development of a molecularly imprinted polymeric membrane for determination of macrolide antibiotics from cow milk

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ABSTRACT

A molecular imprinted membrane (MIM) was prepared for the selective binding of macrolide antibiotics by UV-initiated non-covalent imprinting approach. The membrane was modified by a UV-photographting technique in the presence of molecule templates of erythromycin (ERY) and spiramycin (SPI) with methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as cross-linker. The nanofunctionalized MIM obtained was characterized by a morphological study using scanning electron microscopy (SEM), as well as by a study of its adsorption capacity by online solid phase extraction (SPE) procedure. Variables affecting the MIM-SPE method were optimized to maximize the extraction of macrolide antibiotics of interest and a high-performance liquid chromatography (HPLC) method was used for the analysis. Good linearity and precision were obtained for ERY and SPI, with average recoveries up to 86.14% and 34.73%, respectively, with a relative standard deviation (RSD) lower than 6%. In addition, selectivity was studied for other macrolides with similar structure to the templates, such as roxithromycin (ROX), josamycin (JOS), ivermectin (IVER) and tylosin (TYL). The proposed MIM-SPE-HPLC methodology was effectively applied to ERY and SPI determination in commercial doped semi-skimmed cow's milk samples.

1. Introduction

Macrolide antibiotics are lipophilic molecules with a central lactone ring of 12–16 carbon atoms to which several amino and/or neutral sugars are bound [1,2]. They have been used since their discovery (in the 1950s), and inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit of prokaryotic organisms, causing a bacteriostatic effect [2,3]. Macrolides are widely used in the treatment of community-acquired infections and are an important alternative treatment for penicillin-allergic patients [4].

Erythromycin (ERY) and spiramycin (SPI) are the best known and most widely used members of macrolide antibiotics, as they are considered broad-spectrum antibiotics that act against Gram-positive and some Gram-negative bacteria [5,6].

In veterinary medicine, macrolide antibiotics are widely used worldwide in dairy cattle management for disease therapy and as growth promoting agents. Due to their biological effect against *Mycoplasma* and Gram-positive bacteria, ERY has sometimes been administered as a feed additive or via drinking water to prevent the outbreak of diseases and also in cases of illness, for dehydration or to prevent losses during transport due to the lack of space and hygienic conditions [7,8]. SPI was authorized in the European Union (EU) as feed additive to modulate the gut microbial flora, enhancing the growth rates in cattle animals until its ban in 1999 [9]. The most important health hazards of SPY to humans is the development of antimicrobial resistance and about transference of antibiotic resistance genes from animal to human microbiota, led to withdraw approval for antibiotics as growth promoters in the European Union [9]. The inappropriate use of antibiotics and lack of monitoring after withdrawal time in animals have led to the presence of antibiotic residues in human food, which is potentially hazardous for human health. The presence of antibiotics in milk and dairy products can be problematic because their residues can slowly destroy bacterial fermentation growth and cause allergic reactions in some hypersensitive people. Several studies indicate that low-level doses of antibiotics over prolonged periods could lead to bacteria resistance that could be transferred to humans, making it difficult to treat certain infections [8,10].

Milk is a complex matrix rich in proteins and lipids that needs to be pre-treated prior to proper analysis to reduce possible matrix effects for antibiotic detection [11,12]. The safety of milk is attracting much public

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attention nowadays due to its high worldwide consumption and the harmful effects that the presence of veterinary drug residues can cause in humans. The EU has established maximum residue limits (MRLs) in milk for some antibiotics through Regulation 2377/90 and its latest amendment reflected in Regulation 37/2010 [13,14] such as 40 μg kg^{-1} for erythromycin, 200 μg kg^{-1} for spiramycin, 10 μg kg^{-1} for ivermectin (IVER) and 100 μg kg^{-1} for tylosin (TYL). The veterinary use of antibiotics has been restricted by Regulation 1181/03 and their use as growth-promoting feed additives in livestock breeding has been banned [15]. These strict limits require the development of sensitive and specific methods to analyze antibiotic residues in milk. The most common methods for the extraction of macrolide antibiotics in milk samples are liquid-liquid extraction [8,16] or solid-phase extraction (SPE) [7,17,18] although matrix solid phase dispersion (MSPD) has also been used [19]. Subsequent determination has traditionally been carried out by microbiological and immunological methods [20–22]. However, these assays are too time consuming and lack the specificity and precision required by current legislation. The literature establishes different current methods for the determination of macrolides in biological and food samples, including milk, as thin-layer chromatography [23], liquid chromatography (LC) coupled with an ultraviolet (UV) detector [24], electrochemical [25], fluorescence [16,26] or liquid chromatography tandem mass spectrometry (LC-MS) [8,18,27–29].

The use of molecular imprinted technology (MIT) combined with SPE offers an interesting alternative that allows the formation of selective sites in a polymeric matrix with the memory of a template(s), allowing preconcentration of them, as well as the removal of the interfering compounds present in the sample matrix [30–32]. In previous works, a series of molecularly imprinted polymers (MIPs) complementary to macrolide antibiotics have been successfully synthesized for the clean-up and preconcentration of antibiotics in complex matrix such as sheep milk [33], dairy products [34], chicken muscle [35] or environmental water [36]. Recent advances in MIT to obtain molecularly imprinted membranes (MIMs) have generated numerous benefits and successful results [37,38]. MIMs maintain the advantages of molecular imprinted technology such as high molecular selectivity, high binding capacity and fast binding kinetics, as well as the simultaneously combination of the possibility of continuous operation, recyclability, easy scalability, high processability and cost-effective provided by membranes [37–39]. The successful applicability of MIMs for the selective extraction and detection of antibiotic residues such as cloxacillin [40], norfloxacin [41], sulfonamide [42], tetracycline [43] or vancomycin [44] from different biological matrices have been reported. However, no applications for multi-residue analyses of macrolides using this innovative technology based on molecularly imprinted polymeric membranes have been described so far. The widespread and efficient use of membranes promotes the search for increasingly selective membranes for the determination of chemical compounds in food matrices [45].

In this research, a novel nanofunctionalized molecularly imprinted membrane for ERY and SPI has been obtained by surface UV- photographting polymerization. The functionalized membrane was used to develop a simple and fast methodology for selective solid phase extraction (MIM-SPE) of macrolides from cow's milk samples. Physical characterization of the membrane was carried out by scanning electron microscopy (SEM) and variables affecting the online SPE procedure were optimized. In addition, the use of the non-molecularly imprinted membrane (NIM) was evaluated using the MIM-SPE procedure. The selectivity of the membrane was tested for structurally similar macrolides such as roxithromycin (ROX), josamycin (JOS), ivermectin (IVER) and tylosin (TYL). Analytical determination of the compounds was carried out by high performance liquid chromatography with UV diode-array detection (HPLC-DAD).

2. Experimental section

2.1. Chemicals and solutions

Erythromycin (ERY), spiramycin (SPI), roxithromycin (ROX), josamycin (JOS), ivermectin (IVER) and tylosin (TYL) were purchased from Sigma Aldrich (Madrid, Spain). Ethylene glycol dimethacrylate (EGDMA) and methacrylic acid (MAA) were obtained from Merck (Darmstadt, Germany), 4-(chloromethyl)-phenyltrimethoxysilane (CPTS) from Abcr Gmbh (Karlsruhe, Germany) and sodium diethyldithiocarbamate (Na-DTC) from Sigma Aldrich (Madrid, Spain). Sodium phosphate monobasic (NaH_2PO_4), sodium hydroxide (NaOH), acetic acid (HAc), methanol (MeOH), acetonitrile (ACN), n-hexane, tetrahydrofuran (THF) and silicone oil were obtained from Merck (Darmstadt, Germany). All reagents were of analytical grade and were used as received without further purification. Nitrogen gas was supplied by Carburos Metálicos (Barcelona, Spain) and ultra-pure water was provided from a Milli Q water system (Millipore Ibérica, Madrid, Spain).

Standard solutions (500 mg L^{-1}) of each macrolide antibiotic were prepared by dissolving the adequate amount of each substance in HPLC-grade acetonitrile and stored at 4 °C in amber glass vials. Working standard solutions at the appropriate concentration were prepared daily by appropriate dilution of the corresponding standard solution with the dilution mixture NaH_2PO_4 25 mM at pH 7.0/acetonitrile (70:30).

2.2. Preparation of the ERY-SPI-MIM

The polymeric membranes, molecularly imprinted and non-imprinted, have been prepared by bulk polymerization following a cold polymerization protocol with ultraviolet radiation, based, with certain modifications, on the molecularly imprinted polymer synthesis process developed by García Mayor et al. [33]. In this case, a concentration of 1 mmol of SPI and ERY as templates and Na-DTC as initiating agent were used for the synthesis of the MIM. For the UV-photographting polymerization of the hydrophilic fiberglass membrane (25 mm, 1 μm) (Symta, Madrid, Spain) was used a Digiterm 100 thermostat-controlled waterbath (Selecta, Barcelona, Spain) to provide constant temperature and an UV lamp (Vilber Lourmat CN-6T).

Specifically, to prepare the ERY-SPI-MIM, a first membrane activation step was carried out by a silanization process. An untreated fiberglass membrane was placed in a 25 mL glass tube and covered with a 5% solution of CPTS in dry toluene. It was then incubated in a silicone bath under stirring conditions at 328.15 K for 42 h. Afterwards, it was washed under vacuum filtration with toluene and acetone and dried at 313.15 K for 2 h in an oven (Selecta, Barcelona, Spain). Subsequently, free radicals binding of Na-DTC was performed on the surface of the silanized membrane. The membrane was incubated twice with 10 mL of 2% Na-DTC in tetrahydrofuran at 313.15 K for 4 h. Then, it was washed with tetrahydrofuran, water, methanol and acetone by vacuum filtration, and the membrane was dried at 313.15 K for 2 h.

To achieve the surface UV-photographting polymerization step, the pre-modified fiberglass microfiltration membrane was immersed in a sealed glass with the polymeric mixture of molecules template ERY ($1{\cdot}10^{-2}$ mmoL) and SPI ($1{\cdot}10^{-2}$ mmoL), 2 mmol of the functional monomer methacrylic acid (MAA) and 10 mmol of the cross-linker ethylene glycol dimethylacrylate (EGDMA) dissolved in 7 mL of ACN. The molar ratio of template/monomer/cross-linker was set to 1:2:10. The mixture was sonicated at room temperature (298.15 K) for 5 min and was purged with nitrogen for 10 min. Subsequently, the grafting and bulk polymerization by non-covalent imprinting process was carried out using UV light (365 nm) at 298.15 K for 6 h, in an oxygen-free atmosphere. A schematic illustration of the reaction process is shown in Fig. 1. The grafting of the MIP onto the MIM was repeated twice. Finally, to remove ERY and SPI from the membrane, the MIM was extracted with volumes of 10 mL of MeOH/HAc (99.5/0.5, v/v) using a Rotaterm orbital shaker from Selecta (Barcelona, Spain). Four successive washes

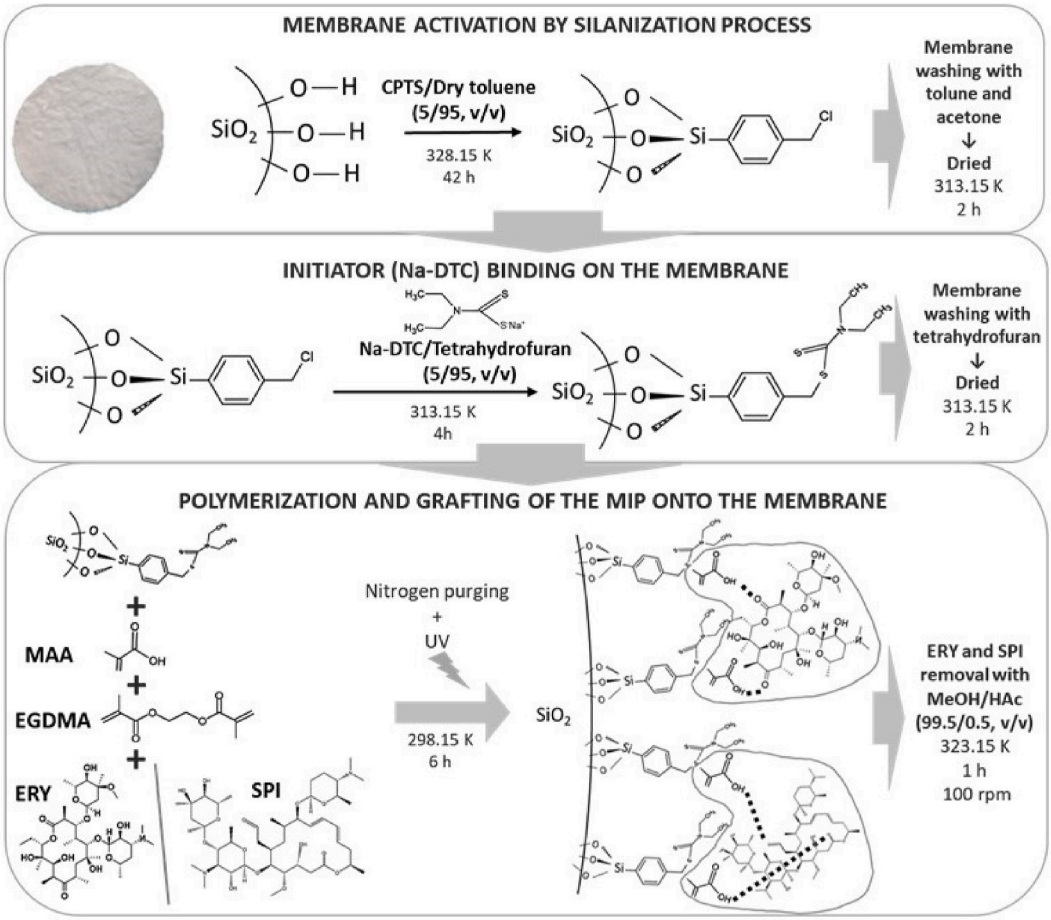

Fig. 1. Schematic illustration of the reaction steps in the formation of the ERY-SPI-MIM.

of 1 h each were performed at 323.15 K and 100 rpm. The collected eluates were analyzed by HPLC-DAD.

The corresponding non-imprinted membrane (NIM) was prepared following the same procedure, excluding both antibiotics from the polymeric mixture.

2.3. Morphological characterization of ERY-SPI-MIM

The morphology of the nanofunctionalized membrane was examined by scanning electron microscopy (SEM EM-30AX plus, Coxem, Daejeon, South Corea) and compared to untreated commercial membranes. Prior to SEM analysis, all samples were cut (to study both sides), deposited on a graphite adhesive conductive tape and vacuum-coated with a thin layer of Au (Sputter Coater SPT-20, Coxem, Daejeon, South Corea). A Jasco Attenuated total reflection (ATR)-Fourier transform infrared (FTIR) spectrometer was used to confirm the membrane functionalization. Spectra were collected from 7000 cm^{-1} to 300 cm^{-1} with a data interval de 1 cm^{-1} and the resolution was set at 4 cm^{-1}.

2.4. MIM-SPE procedure

The nanofunctionalized membrane obtained was used to develop a continuous solid phase extraction process (MIM-SPE). Thus, the MIM was placed in the central compartment of a 25 mm diameter swinnex polypropylene filter holder (Merck, Darmstadt, Germany) and connecting through a Tygon tube LMT-55 of 0.84 mm (Symta, Madrid, Spain) to a simple flow system with a peristaltic pump Minipuls 2 (Gilson, Madrid, Spain).

Under optimal conditions, the membrane was conditioned with 5 mL of ACN. Then, 5 mL of antibiotic solution (ERY and SPI) at appropriate concentrations in ACN/H_2O (90/10, v/v) were loaded onto the membrane at a flow rate of 2.07 mL min^{-1}. Non-specifically bound analytes were washed with 5 mL of hexane. Finally, the analytes of interest were eluted from the membrane by pumping 5 mL of MeOH. All collected fractions were evaporated to dryness under a stream of nitrogen and reconstituted in a suitable volume of NaH_2PO_4 25 mM (pH 7)/ACN (70/30, v/v). Samples were analyzed by high-performance liquid chromatography with UV diode-array detection using the method described below.

2.5. HPLC conditions

Chromatographic detection and quantification of the analytes was performed on an Agilent Technologies model 1200 series liquid chromatograph (Agilent Technologies, Germany) equipped with an Agilent 1290 quaternary pump, auto sampler, photo-diode array detector and a Prontosil C18 (250 × 4.6 mm, 5 μm) column (Scharlab, Spain). The mobile phase was a mixture of ACN (solvent A) and phosphate buffer solution 25 mM pH 7 (solvent B). The gradient program starts with 50% A for 3 min at 1 mL min^{-1} and then increase to 58% within 4 min. This composition is kept stable for 8 min at 1.2 mL min^{-1} and then increases to 70% of eluent A within 1 min. With the following equilibration time of 14 min at 1.5 mL min^{-1}, the resulting total run was 30 min. The samples were injected through the autosampler with 20 μL of injection volume. The column thermostat was set at 333.15 K. The detection wavelengths were 210 nm (ERY, ROX), 231 nm (SPI, JOS), 244 nm (IVER) and 287 nm (TYL). Quantification was performed by external calibration and peak area measurements. A heating module with an evaporating unit (Techne, Straffordshire, United Kingdom) was used to evaporate the samples to dryness using a nitrogen stream prior to HPLC-

DAD analysis.

2.6. Milk sample preparation

Commercial samples of semi-skimmed cow's milk from a local supermarket, supposedly free of antibiotics, were spiked with the desired amount of ERY and SPI. To precipitate the proteins, the spiked milk was pretreated with ACN in a 1:4 vol relation ratio, and the mixed solution was centrifuged (Didacen II Centrifuge, Ortoalresa, Spain) at 3000 rpm for 10 min. The supernatant solution obtained was filtered through a cellulose fold filter (Merck, Darmstadt, Germany) and used as loading solution in the developed MIM-SPE-HPLC procedure.

3. Results and discussion

3.1. ERY-SPI-MIM functionalization

The polymerization mixture of analyte/functional monomer/crosslinker was optimized in previous works [33,46], in which acetonitrile was chosen as porogen because it ensures good solubility of the templates and contributes to ionic interactions and hydrogen bonds between the targets and the functional monomer. Moreover, methacrylic acid was chosen as the functional monomer due to its widely recognized ability to form hydrogen bonds with the basic groups of the templates, and because binding and removal the molecule templates can be performed under mild conditions. A higher grade of crosslinker was used to obtain stability and adequate stiffness in the polymer matrix to produce specific cavities of the correct size and shape to preserve the imprinted memory.

3.2. Morphological characterization of ERY-SPI-MIM

Attenuated total reflection (ATR)-Fourier transform infrared (FTIR) spectroscopy was used to confirm the membrane functionalization. Fig. 2 highlights the band corresponding to the carbon-oxygen double bond of the monomer.

The morphology of the nanofunctionalized membrane has been examined, in comparison with untreated membranes, using a scanning electron microscope. Fig. 3 shows the micrographs of the membranes with asymmetric structure and glass fibre entanglement. Micrograph of the imprinted membrane (Fig. 3B) showed micro- and nano imprinted polymeric particles that have been distributed along the glass fiber after the polymerization process. Fig. 3A shows the SEM image of an untreated membrane.

3.3. MIM-SPE optimization

The usefulness of MIM as solid sorbent in the online SPE procedure was studied. A series of experiments were performed to optimize the experimental conditions of the loading, washing and elution steps, which affect the antibiotic recovery in the MIM-SPE procedure.

The molecularly imprinted polymeric membrane was conditioned with 5 mL of ACN, the same solvent used as polymerization medium and with lower polarity than the elution solvent to ensure the repeatability. A volume of 5 mL of a standard solution of ERY and SPI at concentrations of 200 and 20 mg L^{-1}, respectively, was then loaded onto the membrane. This standard solution was prepared in different solvents (ACN and ACN/H_2O mixtures in different ratios) to select the optimal loading solvent with the aim of maximizing the specific binding between the analytes and the specific cavities generated in the MIM. Fig. 4 shows the most satisfactory results obtained when using 5 mL of ACN/H_2O (90/10, v/v), reaching binding percentages up to 83.61 ± 5.02% for ERY. The specific binding capacity of the MIM to SPI is lower, obtaining percentages of 20.94 ± 1.35%. Then, 5 mL of ACN/H_2O (90/10, v/v) was used as loading solvent for the following assays.

Several solvents such as ACN, n-hexane or toluene were used to wash off the non-specifically bound compounds. The use of the hexane provided the best results, since it was capable of removing non-specific interferences and keeping intact the specific binding of the analytes on the membrane (no losses of ERY and SPI), which implies that the antibiotics they remain fully retained in the MIM. Therefore, 5 mL of n-hexane were used as washing solvent. In addition, the use of this solvent allowed a simplification, and time saving of the methodology developed because the fat content of the cow milk samples can be removed on line

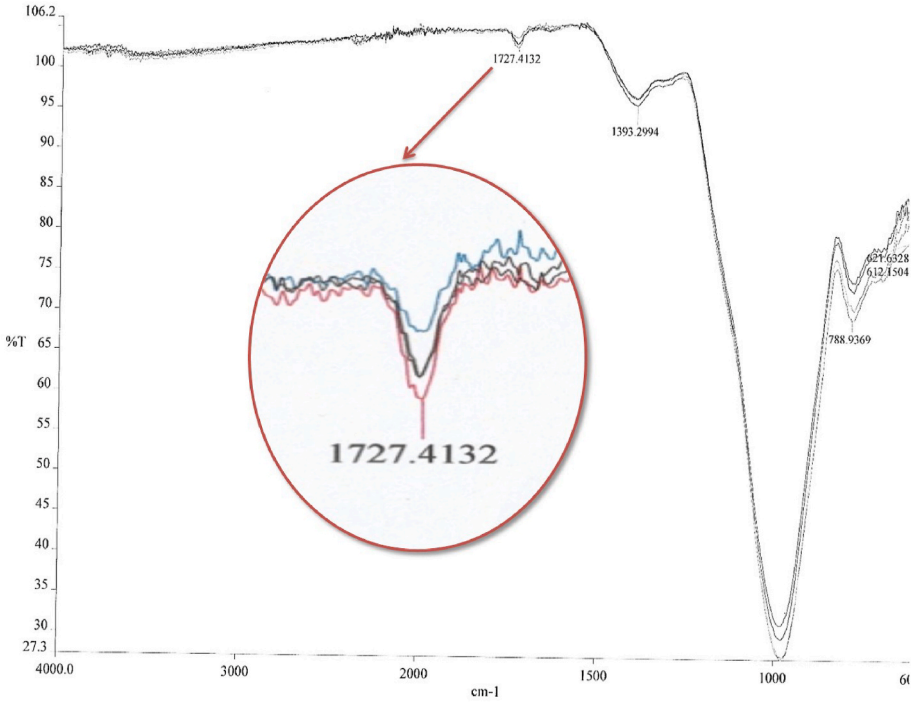

Fig. 2. Chemical characterization of the functionalized membrane by ATR-FTIR.

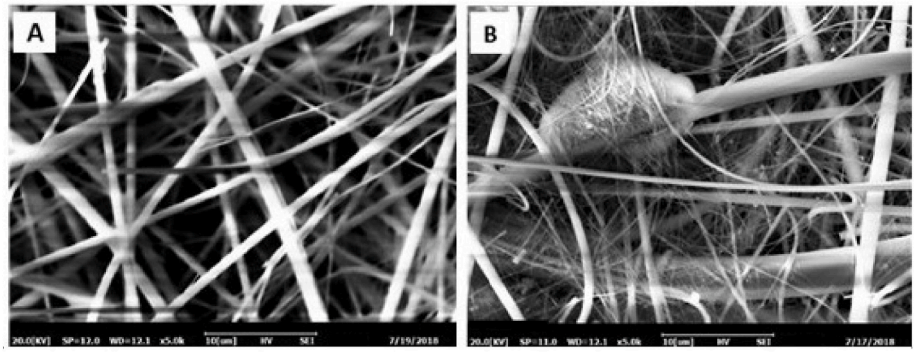

Fig. 3. (A) SEM images of untreated membrane (x5000 magnification, 20 kW) and (B) molecularly imprinted membrane (x5000 magnification, 20 kW).

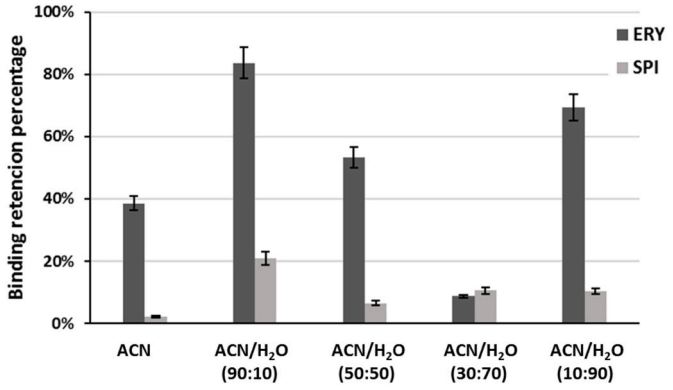

Fig. 4. Binding retention percentages of erythromycin (ERY) and spiramycin (SPI) collected from washing step using different loading solvents (ACN and ACN/H_2O mixtures in different ratios). All measures were the average of three replicates.

during the MIM-SPE procedure, avoiding the obstruction of the membrane.

The elution solvent plays a crucial role in MIM-SPE procedure to ensure an efficient extraction of the adsorbed analytes from the MIM. Different types of solvents were tested, including MeOH, MeOH/HAc (99.5/0.5, v/v) and MeOH/H_2O mixtures (Fig. 5). All assays were carried out using a volume of 5 mL. Antibiotic recovery rates were calculated as the percentage of recovery in the elution relative to the amount bounded in the membrane. The results showed that the optimal elution solvent was MeOH, with recoveries of 97.42 ± 4.77% in the case of ERY and 31.25 ± 1.89% for SPI. The polarity of this elution solvent was adequate to disrupt the chemical bonds established between the analytes and the MIM.

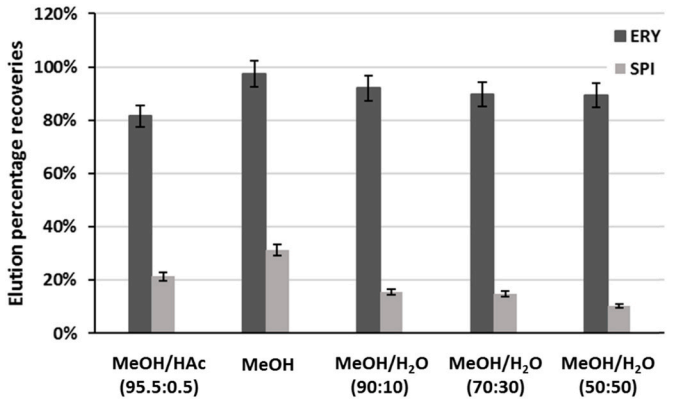

Fig. 5. Elution percentage recoveries of erythromycin (ERY) and spiramycin (SPI) for different solutions: MeOH, MeOH at 0.5% in acetic acid, MeOH/H_2O (90:10, v/v), MeOH/H_2O (70:30, v/v), MeOH/H_2O (50:50, v/v). All measures were the average of three replicates.

The extracts obtained were evaporated under a gentle stream of nitrogen to dryness and finally reconstructed in 100 μL in order to correctly quantify the analytes using the developed HPLC-DAD method. The complete sample treatment allows a preconcentration level of about fifty times.

The optimized procedure was also carried out using the non-molecularly imprinted membrane (NIM) to evaluate its binding capacity. Standard solutions of ERY and SPI at different concentrations were loaded onto the membrane. It was observed that none of the antibiotics was binding to the membrane at any concentration tested. The results confirmed that the retention of ERY and SPI by the MIM was due to the highly specific binding sites created in the functionalized membrane.

3.4. Method validation

The developed method was validated in terms of linearity, recovery, accuracy, precision and limits of detection (LOD) and quantification (LOQ) under optimal conditions using spiked cow's milk samples. The results were summarized in Table 1.

The linearity of the method was tested for the macrolide antibiotics studied. Calibration curves were constructed by the linear least-squares regression analyses of the peak absorbance area versus concentration,

Table 1
Method validation of the optimized MIM-SPE-HPLC method for the determination of ERY and SPI in terms of linearity, accuracy, precision and limits of quantification. All measures were the average of three replicates.

ERYTHROMYCIN						
Regression equation: y = 9.4101·[ERY]+ 4.1771, R^2 = 0.9982						
Concentration spiking level (range 0.04–10.00 mg L^{-1})						LOQ (mg L^{-1})
5.00 mg L^{-1}	3.50 mg L^{-1}	2.00 mg L^{-1}	5.00 mg L^{-1}	3.50 mg L^{-1}	2.00 mg L^{-1}	0.75
Intra-day recovery (%) ± RSD (%)			**Inter-day recovery (%) ± RSD (%)**			
84.54 ± 2.05	82.98 ± 2.62	81.43 ± 1.18	82.99 ± 2.52	82.76 ± 2.07	81.07 ± 1.90	
SPIRAMYCIN						
Regression equation: y = 79.113· [SPI] + 5.9474, R^2=0.9975						
Concentration spiking level (range 0.01–0.50 mg L^{-1})						**LOQ (mg L^{-1})**
0.50 mg L^{-1}	0.35 mg L^{-1}	0.20 mg L^{-1}	0.50 mg L^{-1}	0.35 mg L^{-1}	0.20 mg L^{-1}	0.16
Intra-day recovery (%) ± RSD (%)			**Inter-day recovery (%) ± RSD (%)**			
32.54 ± 3.46	32.45 ± 5.86	30.86 ± 5.26	32.09 ± 4.01	32.02 ± 5.27	30.10 ± 3.35	

preparing samples in triplicate in the concentration range of 0.04–10.00 mg L^{-1} for ERY and 0.01–0.50 mg L^{-1} for SPI. The calibration curves obtained (y = 9.4101 [ERY] + 4.1771 and y = 79.113 [SPI] + 5.9474) showed good linearity for both analytes, with correlation coefficients of $R^2 = 0.9982$ y $R^2 = 0.9975$, respectively.

To evaluate the precision of the proposed analytical method, the relative standard deviation (RSD, %) was calculated in terms of intra-day repeatability (n = 3) and inter-day reproducibility (three alternative days). It was determined by triplicate assays at three different spiking levels of ERY (5.00, 3.50 and 2.00 mg L^{-1}) and SPI (0.50, 0.35 and 0.20 mg L^1). Recoveries were up to 84.54% and 32.54% for ERY and SPI, respectively, with RSD lower than 5.86% for both analytes.

The LOD is defined as the smallest amount of an analyte that can be detected above the noise level. The LOQ is the value that corresponds to the smallest amount of analyte in a sample that can be determined quantitatively with acceptable accuracy and precision (RSD <7%) under the established experimental conditions. The obtained results calculated according to the FDA Guidance for Industry [47] at the lowest concentrations were LOD = 0.22 and 0.05 μg kg^{-1} and LOQ = 0.75 and 0.16 μg kg^{-1} for ERY and SPI, respectively, with RSD below 7%. The method MIM-SPE proposed allows the determination of SPI at lower levels than those required by the EU, which establishes a maximum residue limit (MRL) of 0.20 mg L^{-1} in bovine milk. However, for ERY with the optimal conditions obtained so far, a detection limit lower than the current MRL (0.04 mg L^{-1}) was not possible [14].

3.5. ERY/SPI competitiveness study

Taking into account the significant difference in the recovery percentages obtained for the two antibiotics tested, a competitiveness study was carried out by loading ERY and SPI at the same concentration, 30 mg L^{-1}, on the membrane. The recovery percentage obtained for ERY was around 86.14 ± 0.34%, higher than SPY recovery, which reaches around 34.73 ± 3.46%. The results of this study imply the existence of a possible competitiveness between the two antibiotics, which could be attributed to one of the effects that occur when multiple templates are used instead of a single template in a polymerization process. In a dual-template printing process, it may be the case that one of the template molecules dominates the interactions with the functional monomer, obtaining an adequate printing, while the printing effect for the other templates is reducing. It is also possible that printing efficiency deceases for all templates due to a reduced number of monomer molecules available to print each template molecule. Furthermore, it should be noted that there may be spatial differences in the formation of the different imprinted-template-polymer complexes, which may limit access to the binding sites of each. [48].

The above mentioned competition between different templates has also been observed in other studies such as the one developed by Zhang and coworkers in which a dual-template imprinted polymer with a metal ion as a pivot was successfully prepared and characterized for the detection of two non-steroidal anti-inflammatory drugs used as template molecules [49]. Furthermore, Ping Song and coworkers addressed the existence of possible competitiveness during the polymerization by developing a dual-template molecular impression polymer capable of simultaneously recognizing 8 fluoroquinolones and 8 sulfonamides in pig and chicken muscle [50].

3.6. Milk sample analysis

To test the suitability of the optimized MIM-SPE procedure, commercial samples of semi-skimmed cow's milk were analyzed. Spiked milk samples containing 100 mg L^{-1} of ERY and 10 mg L^{-1} of SPI were deproteinized following the procedure described in **section 2.6**, and then, loaded onto the nanofunctionalized membrane. Optimal washing, as indicated in **section 3.3**, was achieved with hexane, which allowed removal of non-specific interactions and removal of fat in a single step. Then, after 10 min drying at room temperature (298.15 K), the MIM-SPE procedure was completed with the elution step using 5 mL of MeOH. All collected fractions were taken to dryness and reconstituted in a volume of 100 μL in NaH_2PO_4 25 mM at pH 7.0/ACN (70:30) mixture. Finally, macrolides in the milk extracts were detected by HPLC-DAD. The assays were carried out in triplicate.

The recoveries rates of ERY and SPI from milk samples by the molecularly imprinted polymeric membrane were around 83.43 ± 0.65% and 24.57 ± 2.82%, respectively. Higher recovery percentages were obtained for ERY than for SPI, due to a possible competition between both antibiotics not only in the polymerization process, but also at the loading step in the MIM-SPE procedure. Fig. 6 shows the chromatograms obtained for the spiked milk sample after recovery of ERY and SPI by the MIM-SPE procedure (b), compared to a standard solution of the antibiotics at the same spiked concentration (a) and the chromatograms of a blank treated milk sample (c). It could be appreciated that the chromatograms of the samples are clear without matrix interferences.

3.7. Selectivity study of macrolide antibiotics

The selectivity of the MIM used as SPE sorbent in a continuous flow system was evaluated using other macrolide antibiotics (ROX, JOS, IVER and TYL) with similar chemical structures. In order not to saturate the membrane, a solution of macrolide antibiotics was loaded onto the membrane at 10 mg L^{-1} of SPI, JOS, IVER and TYL; and ERY and ROX at 30 mg L^{-1}. The percentages of analytes collected in the elution step were calculated in relation to the absorbance area values obtained for a standard solution in the HPLC-DAD. The highest recovery percentages were obtained for ERY and ROX. The results showed that ROX and JOS, which were structurally more closely related to the template molecules (ERY, SPI), presented similar recovery values. It was reflected that antibiotics of the family with a similar structure to those used as a template are retained in analogous percentages, having as expected a selectivity towards the same type of molecular structures used as a template in the membrane polymerization. Table 2 summarizes the recovery values obtained. Therefore, the MIM could be used as a suitable SPE methodology for the determination of macrolide antibiotics residues in cow's milk.

4. Conclusions

A new methodology for online solid phase extraction of macrolide antibiotics has been developed based on a novel nanofunctionalized membrane prepared by UV- photographting polymerization on a glass fiber ultrafiltration membrane. The imprinted polymer was grafted onto the membrane by polymerization of MAA as functional monomer, EGDMA as cross-linker, and Na-DTC as free-radical initiator in the presence of ERY and SPI as templates in acetonitrile solution. SEM characterization showed the formation of polymeric nanoparticle clusters amongst the membrane fibers after the polymerization process.

This research represents a relevant step in the development of new configurations within the molecular imprinting technology, offering a fast, reproducible, and simple online-SPE procedure. The optimized MIM-SPE procedure has been validated for the extraction of ERY and SPI, obtaining recoveries of 86.14% and 34.73%, respectively, with RSD below 6%. The results have shown specificity, good linearity, and precision, achieving a limit of quantification for SPI below the limit stablished by the EU legislation. Further studies focus on the composition of the polymeric mixture (concentration of SPI, functional monomer, crosslinker or initiating agent) would be necessary in order to improve the specific retention of spiramycin. HPLC-DAD combined with the MIM-SPE procedure ensures a good sensitive methodology for the determination of macrolide antibiotics. The selectivity of the MIM-SPE was tested in the presence of other antibiotics belonging to the macrolide antibiotic family, showing that the proposed method is thought to

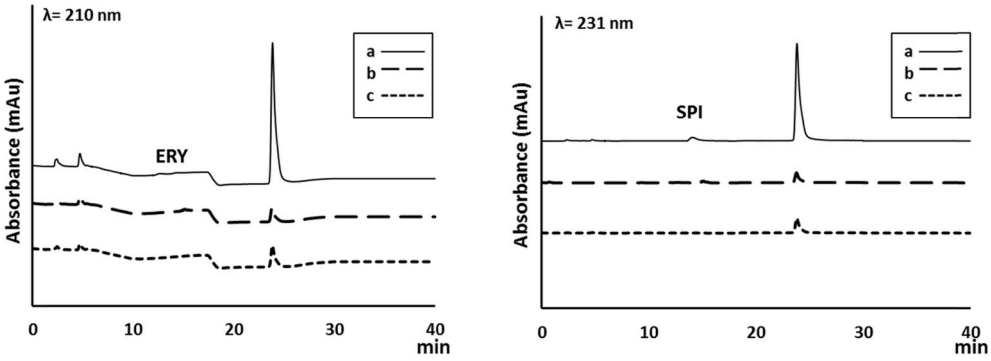

Fig. 6. Comparison of chromatograms obtained for antibiotic macrolides studied at different wavelengths: ERY (210 nm) and SPI (231 nm). (a) Standard antibiotic solution, (b) spiked milk sample after MIM-SPE treatment and c) blank milk sample after treatment. Concentrations: 100 mg L^{-1} (ERY) and 10 mg L^{-1} (SPI).

Table 2
Macrolide antibiotic recoveries in the selectivity study for erythromycin (ERY), roxithromycin (ROX) at 30 mg L^{-1} and spiramycin (SPI), josamycin (JOS), ivermectin (IVER), and tylosin (TYL) at 10 mg L^{-1}. All measures were the average of three replicates.

Antibiotic	Concentration (mg L^{-1})	Recovery (%) ± RSD (%)
ERY	30	86.02 ± 1.76
ROX	30	74.91 ± 4.33
SPI	10	27.32 ± 4.96
JOS	10	23.43 ± 6.91
IVER	10	5.01 ± 0.83
TYL	10	17.46 ± 3.86

be selective for the macrolide antibiotic templates and the similar molecular structures within this family. The developed method has been successfully applied for the simultaneous extraction and determination of ERY and SPY in cow's milk samples.

CRediT authorship contribution statement

R. Cañadas: Investigation, Funding acquisition, Data acquisition, Writing – original draft. **R.M. Garcinuño Martínez:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **G. Paniagua González:** Investigation, Writing – review & editing, Supervision. **P. Fernández Hernando:** Conceptualization, Writing – review & editing, Funding acquisition.

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.

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