Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number: FOODCHEM-D-16-00228R1

Title: SYNTHESIS AND CHARACTERIZATION OF A MOLECULARLY IMPRINTED POLYMER FOR THE DETERMINATION OF SPIRAMYCIN IN SHEEP MILK

Article Type: Research Article (max 7,500 words)

Keywords: Molecularly imprinted polymer; Solid phase extraction; Spiramycin; Sheep milk

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Abstract: A series of molecularly imprinted polymers (MIPs) against to macrolide antibiotic spiramycin (SPI) by noncovalent bulk polymerization technique were synthetized. Different polymerization process and their recognition efficiency were evaluated in binding studies. MIP was morphologically characterized and evaluated as a sorbent for extraction and preconcentration of SPI from aqueous and sheep milk samples, and an off-line MISPE method followed by high-performance liquid chromatography with UV diode-array detection was established. Good linearity were obtained for SPI in a range of 24-965 µg kg-1 and the average recoveries at three spiked levels in milk samples were higher than 90% (RSD < 5%). Limit of quantification was 24.1µg kg-1. Cross-reactivity studies from other macrolides with similar structure were tested. The optimum imprinted polymer showed good selectivity and affinity for SPI, demonstrating the potential of the proposed MISPE for rapid, sensitive and effective sample pretreatment for selective determination of SPI in sheep milk.

***Highlights (for review)**

- Synthesis of molecularly imprinted polymers by non-covalent bulk polymerization technique.
- Characterization morphological and evaluation of binding capability of SPI-MIPs.
- Imprinted solid phase extraction methodology for the analysis of spiramycin in milk samples.
- Determination of spiramycin in sheep milk by liquid chromatography-UV diode-array detection.

SYNTHESIS AND CHARACTERIZATION OF A MOLECULARLY IMPRINTED POLYMER FOR THE DETERMINATION OF SPIRAMYCIN IN SHEEP MILK

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Abstract

A series of molecularly imprinted polymers (MIPs) comprising reactionary sites which are complementary to macrolide antibiotic spiramycin (SPI) were synthetized by noncovalent bulk polymerization technique. MIPs were synthesized under different polymerization process and their recognition efficiency was evaluated in binding studies in comparison with non-imprinted polymers. The best MIP was morphologically characterized and equilibrium assays were carried out. The MIP was evaluated as a sorbent for extraction and preconcentration of SPI from aqueous and sheep milk samples, and an off-line MISPE method followed by high-performance liquid chromatography with UV diode-array detection was established. Good linearity were obtained for SPI in a range of 24-965 μg kg^{-1} and the average recoveries at three spiked levels in milk samples were higher than 90% (RSD < 5%). Limit of quantification was 24.1 μg kg^{-1}. Cross-reactivity studies from other macrolides with similar structure were tested. The optimum imprinted polymer showed a good selectivity and affinity for SPI, demonstrating the potential of the proposed MISPE for rapid, sensitive and effective sample pretreatment for selective determination of SPI in sheep milk samples.

Keywords: Molecularly imprinted polymer; Solid phase extraction; Spiramycin; Sheep milk

1. Introduction

Spiramycin (SPI) is a 16-member macrolide antibiotic with a wide range of veterinary uses. This macrolide was authorized in the past, as feeds in order to modulate gut microbial flora, thus enhancing the growth rates performances in calves, cattle, pigs and poultry. Nowadays, their application is restricted only to veterinary therapeutic practice. The systematic administration of these compounds at sub-therapeutic doses may leave residues in edible tissues or in food animal origin, such as milk, egg, and meat (Chung et al., 2009). The presence of antibiotic residues in foodstuff may cause different diseases or disorders in consumers (Pascal Demoly, & Romano, 2005). Also, it may cause problems in the milk industry, due to modify or inhibit the fermentation processes performed in dairy products such as cheese and yoghurt (Berruga et al., 2009). Therefore, to protect consumer health and to ensure high quality of produced milk, the European Union and the Swiss regulation authorities have established maximum residues limits (MRLs) for these drugs residues in milk (ECC No. 2377/90, 1990; RS817.021.23). Specifically, the limit for spiramycin in milk is 200 μg kg^{-1}.

To ensure the safety of milk, reliable analytical methods need to be developed that are capable of measuring residues of macrolides at these levels. Macrolides in milk have been commonly analyzed by bioassays (Nagel et al, 2013), but these tests using microorganisms are not sensitive enough to detect many of these antibiotics employed to treat livestock; e.g., spiramycin, and lincomycin (Linage et al., 2007). At the present, analytical techniques such as liquid chromatography coupled to diode-array (LC/DAD) (Dubois et al., 2001), ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) (Romero et al., 2011), liquid chromatography/tandem mass spectrometry (LC-MS/MS) (Juan, & Mañes, 2010) or

liquid chromatography with fluorescence detection (LC/UV) (Gomis et al., 2004) have been used for the determination of spiramycin in milk samples.

On the other hand, milk is an aqueous sample that contains proteins and fat, components that may hinder development of the analysis. Due to the complexity of milk, previous sample preparation method is very often required. Some developed methods concerning the sample preparation for determination of SPI in milk samples using a combination of liquid-liquid extraction (LLE) and solid-phase extraction (SPE) as extraction and purification techniques, respectively (Turnipseed et al., 2008) and matrix –assisted solid-phase dispersion (MSPD) (García et al., 2012) have been published. SPE has a low-cost and is easily automated to pretreat food samples and it can be coupled to both liquid and gas chromatography. The main drawback of these sample preparation techniques is the lack of selectivity of the sorbents. These are being replaced more and more by polymeric sorbents as molecular imprinted polymers (MIPs). In contrast to classical SPE sorbents, MIPs exhibit high affinity and selectivity towards a target compound or class of structurally related compounds. These materials have demonstrated binding to trace levels of target analytes, and display high selectivity in presence of other compounds that have similar physic-chemical properties, as well as are extremely stable (Cameron et al., 2006).

The objective of this work was to achieve the synthesis of a SPI-specific MIP for the determination of SPI in sheep milk samples. For that purpose, a series of molecularly imprinted polymers were synthetized by noncovalent bulk polymerization using different synthesis conditions. MIPs obtained were then evaluated by binding studies to screen out the appropriate MIP for its application as solid phase extraction sorbent. In order to demonstrate the clean-up and preconcentration capability of the MIP selected,

the analysis of SPI in sheep milk samples by HPLC-with photo diode array detector was applied. The cross-reactivity for others macrolide antibiotics were tested.

2. Experimental

2.1. *Chemicals*

Spiramycin (SPI), tylosin hemitartrate (TYL), erythromycin (ERY), josamycin (JOS) and ivermectin (IVER) were purchased by Sigma Aldrich (Madrid, Spain). Ethylene glycol dimethacrylate (EGDMA) and methacrylic acid (MAA) were obtained from Merck (Darmstadt, Germany), 2-2´-azobisisobutyronitrile (AIBN) from Fluka (Buchs, Switzerland). Sodium phosphate monobasic, sodium hydroxide and n-hexane (purity> 99%) were obtained from Merck (Darmstadt, Germany). All reagents used were of analytical grade. Ultra-pure water was obtained from a Milli Q water system (Millipore Ibérica, Madrid, Spain).

The stock standards solutions (500 mg L^{-1}) of all compounds were prepared by dissolving the adequate amount of substances in methanol of HPLC-grade from Scharlab (Barcelona, Spain) and stored at 4 ºC. Standard solutions of each macrolide antibiotic (50 mg L^{-1}) were prepared by diluting the stock solution with acetonitrile (ACN) of HPLC-grade from Scharlab and also stored at 4 ºC. Working standard solutions at adequate concentration were daily prepared by appropriate dilution of the mentioned solution with the dilution mixture NaH_2PO_4 25 mM at pH 7/ acetonitrile (70:30).

121 *2.2. Apparatus and material*

122 A Digiterm 3000542 thermostat-controlled waterbath (Selecta, Barcelona, Spain)
123 was used to provide constant polymerisation temperature. An ultraviolet lamp (Vilber
124 Lourmat CN-6T) was employed for UV-initiated photopolymerization. pH readings
125 were made with a Metrohm 654 pH meter. Imprinted and non-imprinted polymers were
126 ground in a glass mortar (Aldrich, Madrid, Spain) and then passed through CISA
127 standard sieves (200-355 µm) (Afora, Madrid, Spain). Template extraction was
128 performed using a Soxhlet extractor system with cellulose extraction thimbles. SPE was
129 carried out using a 20-Port Vacuum SPE manifold System (Supelco, Spain) with
130 vacuum control-press pump (Selecta, Spain). Empty SPE cartridges (Supelco, Spain) of
131 3 mL of capacity with polyethylene frits (20 µm) were used to pack the solid phase.
132
133 *2.3.Chromatographic analysis*
134 HPLC analyses were performed using an Agilent Technologies chromatograph
135 model 1200 series equipped with an Agilent 1290 quaternary pump, auto sampler, and
136 photo-diode array detector (Agilent Technolies, Germany). Data acquisition was
137 performed with LC-DAD Chemstation Software (Agilent technologies).The analytical
138 column was a Prontosil Hypersorb ODS (5.0 µm, 250×4.6 mm) from Scharlab
139 Company (Barcelona, Spain). The column thermostat was set at 60ºC. The mobile phase
140 was a mixture of acetonitrile-phosphate buffer.
141 Chromatographic analyses were carried out following a previous method developed
142 for our research group (García et al., 2006). The elution gradient used was phosphate
143 buffer solution (25 mM, pH 7) as component A (was made by dissolving 5 g of
144 NaH_2PO_4 in 500 mL of Milli-Q water, and sodium hydroxide was used to adjust the pH
145 at 7) and acetonitrile as component B. The gradient started with 50% B for 3 min at 1

mL min^{-1} and then increased to 58% within 4 min. This composition was stable for 8 min at 1.2 mL min^{-1}, then increased to 70% of eluent B within 1 min. With the following equilibration time of 20 min at 1.5 mL min^{-1}, the resulting total run was 30 min. The injection volume was 20 µL. The detection wavelengths were 231 nm (SPI, JOS), 210 nm (ERY), 254 nm (IVER) and at 287 nm (TYL). Quantification was performed using peak area measurements and external calibration.

2.4.Preparation of SPI-imprinted polymers

To prepare the SPI-MIPs, the template molecule (2×10^{-2} mmol) and the functional monomer MAA (2 mmol) were dissolved in acetonitrile as polymerization media (7 mL) into a 25-mL glass tube. The mixture was sonicated at room temperature for 5 min. Subsequently, the cross-linker EGDMA (10 mmol) and the radical initiator AIBN (5.1 mmol) were added, following sonication for 10 min. The solution was degassed with a stream of oxygen-free nitrogen for 7 min, and then the glass tube with the mixture was placed in a thermostat-controlled waterbath at 65 ºC for 4 h (MIP1), or under UV light at 365 nm at 5ºC for 6 h (MIP2) to carried out the polymerization processes. The resulting bulk polymers were crushed in a glass mortar and wet-sieved by methanol to obtain particles with sizes between 200 and 355 µm. Finally, the template and non-polymerized compounds were extracted in a Soxhlet apparatus with methanol (80 mL) for 20 h, until no SPI could be detected by HPLC-DAD. The non-imprinted polymers (NIPs) as control polymers were also prepared and treated using an identical procedure without adding SPI.

171 *2.5. Morphological characterization of the SPI-imprinted polymer*

172 The textural characterization of polymers was made using a Micromeritics
173 ASAP 2010 equipment (Norcross, USA). A N_2 adsorption/desorption experiment by
174 nitrogen porosimetry was used for the determination of the specific area (S), the specific
175 pore volume (Vp) and the average pore diameter (Dp) of the polymers. 1g of dry
176 polymer was degassed at 70ºC under nitrogen flow for 4 h prior to measurement to
177 remove the absorbed gases and moisture. Nitrogen adsorption/desorption data were then
178 recorded at liquid-nitrogen temperature of -196 ºC. Specific surface area was calculated
179 from the nitrogen adsorption data using the Brunauer-Emmett-teller (BET) equation
180 (Bruneauer et al., 1983). The external surface area (Sext) and the micropore volume
181 (V1) were calculated by the t-plot method (Horvath, & Kawazoe, 1983), and the pore
182 size distributions and total pore volume of the MIP (Vp) by the Functional Theory of
183 Densities model (DFT) (Oliver, 1995).

184

185 *2.6. Batch binding analysis*

186 To select the optimum synthetized polymer, preliminary studies of the rebinding
187 capability of MIPs and NIPs were evaluated by batch binding experiments. For this
188 study, 43 mg of each SPI-MIP or NIP was added to 4 mL of SPI solution at 100 mg L^{-1}
189 in ACN, and the mixture was incubated over night at room temperature. The resultant
190 mixture was centrifuged at 1200 rpm for 5 min. The supernatant containing the non-
191 binding SPI was analyzed by HPLC-DAD at 231 nm. The concentration of SPI in
192 solution was determined by reference to calibration curve previously plotted. The
193 amount of bound SPI was calculated from the difference between the concentration
194 added initially and the SPI content of the supernatant. Binding ability of MIPs was
195 determined by the partition coefficient k= Cp /Cs (Cai, & Gupta, 2004), and it was

196 calculated as the ratio between the amount of SPI binding to the MIP (Cp) and the
197 concentration of SPI in the solution (Cs). The imprinting factor (α), representing the
198 degree of imprinting achieved, was calculated as the ratio of MIP-bound SPI to NIP-
199 bound SPI.

200 To evaluate the adsorptive properties of the optimum polymer selected, the
201 adsorption isotherms were carried out. 20 mg of MIP or NIP were mixed with 2.5 mL of
202 SPI solutions in ACN at concentrations ranging from 0 to 50 mg L^{-1} and incubated at
203 20ºC for 24 h. The resultant mixtures were centrifuged at 1200 rpm for 7 min and an
204 aliquot of supernatants was used to analyze the amount of SPI not bound to polymers.
205 From the difference in concentration of SPI solution before and after incubation, the
206 amount of SPI bound by the polymers was evaluated. All the solutions were subjected
207 to HPLC-DAD analysis.

208

209 *2.7. MISPE conditions*

210 Appropriate amounts of the dry polymer particles of MIPs or NIPs (200 mg)
211 were packed into the SPE cartridges of 3 mL with two polyethylene frits (length of 65
212 mm and i.d. 10 mm) between the polymer particles. The prepared columns were
213 conditioned with 6 mL of MeOH (3×2 mL) and 6 mL of ACN (3×2 mL) to remove any
214 possible contaminant. Then, 1 mL of SPI solution in ACN at adequate concentration
215 was loaded onto the SPE column with a flow rate of 0.2 mL min^{-1}. The non-specific
216 bound analyte was washed in a single washing step using 6 mL (3×2 mL) of ACN. The
217 analyte was desorbed with 3×2 mL MeOH: acetic acid (0.5%, v/v) solution. The
218 obtained fractions were evaporated to dryness under a gentle flow of nitrogen at 40 ºC
219 and redissolved in 1 mL of mixture NaH_2PO_4 25 mM (pH 7)/acetonitrile (70:30).
220 Quantification of SPI from the obtained reconstituted samples was carried out 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.

2.8. Milk sample preparation

Sheep milk samples were collected from different sheep in the same stage of lactation by CERSYRA, a Regional Centre of Animal Selection and Reproduction in Valdepeñas (Ciudad Real, Spain). The samples were stored at -20 ºC until use. To validate the MISPE-HPLC developed method sheep milk samples were spiked with SPI. The procedure used for pretreatment of spiked milk samples is detailed below. Milk samples were allowed to thaw at room temperature and homogenized by heating (30 ºC for 5 min). A volume of 1 mL of homogenized milk was spiked with the desired amount of SPI into a 10 mL conical flask and mixed by manual shaking. To allow the equilibration of the analyte with the milk matrix, the spiked sample was maintained at room temperature for 20 min. The spiked milk was pretreated using 4 mL of NaH_2PO_4:ACN (3:2, pH 7) and 1 mL of ACN simultaneously to precipitate the proteins. Subsequently, the mixed solution was centrifuged (1200 rpm for 15 min) and the obtained supernatant solution was filtered with a fold filter. Finally, 1 mL of the deproteinized milk samples were passed through the MISPE column, and then was washed and eluted following the extraction procedure described above. Finally, the elution fractions were collected for subsequent HPLC analysis.

3. Results and discussion

3.1. Synthesis of SPI-MIPs

Two molecular imprinted polymers for SPI (MIP1 and MIP2) were prepared according to non-covalent bulk polymerisation method. Rational selection of the

functional monomer and the proper molecular ratios of the polymerization reagents can be produce better extraction performance, because this will determine the stability of the formed complex before and during the polymerization process and the subsequent ability of the MIP to interact selectively with the target molecule (Beltran et al., 2010). In this work, MIPs were synthetized using MAA as functional monomer due to the carboxyl group of the acid functionality could form hydrogen bonds with the hydroxyl and ionic bond with the basic groups of the template. SPI has three hydroxyl groups, an amine group, and a tertiary amine on one of the sugar units, which can form a hydrogen bond and an ionic bond with the corresponding functionalities, respectively.

During the polymerization process, cross-linker fulfils some major functions: was employed to provide mechanical stability to the polymeric matrix, control the morphology of the polymer and stabilize the molecular recognition sites. EDGMA was chosen in this work due to it is the most widely used cross-linker. An excess of functional monomer versus the template was indispensable, since it improves the stability of the pre-polymerisation complex by shifting the association-dissociation equilibrium towards complex formation. Then, the molar ratio of template/monomer/cross-linker was fixed to 1:100:500.

In the synthesis of MIPs, the porogen plays an important role in formation of the porous structure of the polymer. Moreover, the selection of porogen is one of the determining factors in the effective molecular recognition of the template because the accuracy of the assembly between the template and the monomer is related to the physical and chemical characteristics of the solvent. Accordingly, acetonitrile was selected as the porogen solvent because this organic media ensures good solubility of the template and contributes to the formation of polar interactions such as hydrogen bond and electrostatics interactions between template and functional monomer.

The imprinted polymers MIP1 and MIP2 were synthetized according to the bulk polymerisation strategy due to its simplicity, and because the non-regular shape of the particles obtained are not a real limitation for off-line SPE application. Both MIPs were synthetized using the same composition by two different polymerisation procedures: thermally iniated polymerisation in a thermostatic-controlled waterbath at 60 ºC for 4 h (MIP1) and UV-initiated photopolymerisation at 235 nm for 6 h (MIP2). The bulk polymer monoliths were then crushed, ground and wet-sieved using methanol to obtain particles mainly in the 200-355 µm size range. The template was removed by Soxtlet extraction with 80 mL of methanol for 20 h. Non-imprinted polymers (NIPs), were in both cases prepared in the same way but without SPI, as control polymers.

3.2. MIP´s characterization

Recognition efficiency of MIPs. The molecular recognition of MIPs depends two factors mainly, three-dimensional spatial configuration of molecule template and matching degree of the bonding sites. In this study, two MIPs were synthetized at the same ratio of template to monomer and using ACN as porogen, but different polymerization conditions were assayed that could affect the imprinting result and so the specificity recognition.

To determine the recognition capacity of MIPs, specific binding, partition coefficient and imprinting factor were studied and calculated by batch binding assays in triplicate (Table 1). Specific binding is refers to the amount of SPI bound to the MIPs and NIPs calculated as the percentage of rebinding to the MIP minus the percentage for the NIP. The specific binding for MIP1 (53.1%) was higher than for MIP2 (37.7%). The low amounts of analyte bound by the NIPs in both cases (< 25%) indicated that the presence of template during the imprinting process imparts recognition capacity. The partition

coefficient values were higher for MIPs than for NIPs and the highest value was achieved when MIP 1 was assayed. The difference between MIPs and NIPs indicates that the imprinting procedure has created highly specific cavities designed for the antibiotic SPI in the MIP. The imprinting factor (α) was higher for MIP1 than for MIP2, indicating a major degree of imprinting in MIP1. According to these obtained results, MIP1 was selected as optimum sorbent to be applied in solid phase extraction procedure for SPI determination. The obtained results showed that the polymerization conditions are a key factor for recognition characteristics of MIP.

Adsorption isotherm study. The adsorption capacity is an important factor that reflected the efficiency and affinity of the polymers towards the analyte. Equilibrium binding experiments were carried out to obtain polymer adsorption isotherms and to investigate the adsorption behavior of MIP1 and NIP1. The adsorption isotherm model bi-Langmuir equation was used to fit the data. Langmuir isotherm model describes monolayer adsorption based on the assumption that all the adsorption sites have equal template affinity and that adsorption at one site does not affect adsorption at an adjacent site. The adsorption isotherms showed in Fig.1A describes the saturation adsorption of MIP1 and NIP1 bound with different concentrations of SPI solution. The adsorption of MIP1 was not linear with respect to the increase of the initial SPI concentration. At each SPI concentration tested, MIP1 could bind much more SPI than the NIP1, and the binding amount increased with the increase of the initial SPI concentration, ultimately reaching a stable plateau.

To estimate the binding parameters of MIP1, the binding data in Fig. 1A were plotted according to the following Eq. (1) (Feldman, 1972; Nörby et al., 1980):

$$\frac{B}{F} = \frac{B_{max1}}{K_{d1} + F} + \frac{B_{max2}}{K_{d2} + F} \quad (1)$$

where B is the amount of SPI bound to MIP at equilibrium, F is the free SPI concentration, B_{max1} and B_{max2} are the maximum numbers of the higher and lower-affinity binding sites, and K_{d1} and K_{d2} are two equilibrium dissociation constants related to the affinity of the adsorption sites.

The data of the MIP binding characteristic was used for the Scatchard analysis by the Eq. (2):

$$\frac{B}{F} = \frac{(B_{max} - B)}{K_d} \quad (2)$$

where Kd and B are the equilibrium dissociation constant and the apparent maximum number of binding sites, respectively. F is the free concentration of SPI in binding solution. The Scatchard plot obtained by the method is presented in Fig.1B and allowed to estimate the binding nature of MIP1. As it is shown, the Scatchard was not linear and composed of two straight lines, which suggested that the binding sites of MIP1 for SPI were heterogeneous and rather two kinds of binding sites existed in the polymer. Apparently, highly selectivity of binding sites for SPI can be explained as result from the carboxyl group of the acid functionality of the monomer which cooperatively bound with the hydroxyl and amine groups of the SPI. In our system, the coefficients of Eq. (1) were calculated: for high affinity binding sites, K_{d1}= 0.08706 mg L^{-1} and B_{max1}= 1.27 mg g^{-1}; and for low affinity binding sites, K_{d2}= 0.08705 mg L^{-1} and B_{max2}= 3.56 mg g^{-1}. It is suggested that the dual-site Langmuir binding model might describe the SPI rebinding on molecularly imprinted polymer surface.

Morphological characterization of the SPI-imprinted polymer. In order to characterize the structure and porous nature of MIP1 the BET analysis was carried out. The textural characterisation of the SPI-MIP was accomplished by nitrogen gas adsorption at -196 ^{o}C. The specific surface area (BET) was 296 m^2 g^{-1}. The total volume of pores was

found to be 0.429 cm^3 g^{-1}; which a micropores volume of 0.043 cm^3 g^{-1}, and a mesopores volume of 0.306 cm^3 g^{-1}. The average pore size (DFT) of MIP1 was 5.8 nm.

3.3. Optimization of MISPE procedure

After the evaluation of the binding capacity of the synthetized MIPs, the applicability of MIP1 as solid sorbent in the SPE procedure (MISPE) was studied. MISPE is based on conventional solid-phase extraction procedure, therefore, typical loading, washing and elution steps are carried out as a matter of routine. In this study, a series of experiments was performed to optimize the experimental conditions affecting the SPI recognition by MIP1 in a MISPE procedure including composition and amount of washing and eluting solvents. An amount of 200 mg of MIP1 was sufficient for being used as sorbent to develop an off-line MISPE for SPI due to the high affinity of this polymer. Firstly, the prepared MISPE column was conditioned with 6 mL of MeOH (3×2 mL) and 6 mL of ACN (3×2 mL). To achieve a selective extraction, a clean-up step with a suitable solvent was used prior to the analyte elution from the column. This washing solvent is one of the crucial factors in MISPE procedure to maximize the specific interactions between the analyte and binding sites, and simultaneously destroy non-specific interactions to discard matrix components from the cartridge. In this study, different washing solutions such as H_2O, ACN and H_2O/ACN mixtures at different proportions (50-95% ACN) were investigated. Volumes up to 6 mL were assessed. As presented in Fig. 2, when the washing solution was H_2O more than 70% of the loaded SPI was recovered in the fractions collected from washing steps. However, the amount of analyte washing from the cartridge was less than 10% when ACN was used. Therefore, 6 mL of ACN was selected as optimum washing solvent.

The elution solvent plays an important role in MISPE procedure since the target analyte should be efficiently desorbed from the cartridge. Usually, for the recovery of strongly bounded analyte, a small amount (1-10%) of modifier, such as water or weak acids is added to help the breaking of the hydrogen-bonding. The effect of elution solvents and volume on extraction efficiency of SPI were tested for different types of solvents including NaH_2PO_4:ACN (70:30, v/v) at pH 7, MeOH and MeOH containing acetic acid at different percentages (0.25, 0.5 and 1%). The studies were developed with 1 mL of SPI standard solution at 50 mg L^{-1} in ACN. Results showed that the presence of acetic acid in solvent elution provided higher recoveries with respect to the use of 100% MeOH (Figure 3). The use of MeOH:acetic acid (99.5:0.5, v/v) the best elution efficiency (95 %). Recovery did not improve when MeOH:acetic acid (99:1, v/v) was used. Due to SPI is stable at pH between 4-5, percentages of acetic acid higher than 1% were not tested. Different volumes of elution solvent ranged from 4 to 7 mL were assayed in order to optimize the elution volume. Volumes higher than 6 mL of MeOH:acetic acid (99:1, v/v) hardly had any benefit to the recoveries of SPI. Consequently, SPI was quantitatively eluted from the sorbent with 6 mL (3×2) of MeOH containing 0.5% acetic acid. The polarity of this elution solvent was enough to disrupt the interaction ionic and hydrogen bonds established between analyte and polymer.

3.4. Application in real milk samples

To evaluate the performance of the proposed MISPE-HPLC method for the sample clean up and determination of SPI, real sheep milk samples were analysed under the optimal conditions. Milk samples are complicated matrix, over 100.000 different molecular species have been identified in it. Although milk contains approximately 90%

391 water, it can be described as an *oil-in-water* emulsion with the fat globules dispersed in
392 the continuous serum phase, or as a *colloid suspension* of casein micelles, or as a
393 *solution* of lactose, soluble proteins, minerals, vitamins and other components
394 (Samanidou, & Karageorgou, 2011). Moreover, milk composition can be affected by
395 many factors, e.g. breed variations, herd-to-herd variations chiefly attributed to feed
396 considerations, seasonal and geographic aspects. Due to its complexity, pretreatment of
397 milk matrix is usually needed before analysis. In this work, previously the MISPE
398 procedure, the samples were pretreated according to the procedure mentioned in *Section*
399 *2.8* for proteins removal. Otherwise, the impurities, such as protein or saturated fat
400 would block the cartridge and decrease the recovery. After the proteins precipitation
401 step, milk sample was analysed by the above described MISPE procedure. Then, 1mL
402 of deproteinized milk sample was loaded onto SPE cartridge previously conditioning at
403 a rate of 0.2 mL min^{-1}.

404 The removal of fat content in milk, that could produce interferences in the
405 determination of SPI, was also required. Triglycerides account for around 98% of milk
406 fat. Other classes of lipids include phospholipids (<1%), which are mainly associated
407 with the fat globule membrane, and cholesterol (<5%), which is mostly located in the fat
408 globule core. Based on a previous works (Garcia et al., 2012), fat removing step was
409 optimized. For this study, two solvents (n-hexane and NaOH at different concentration
410 between 0-2 M) by passing a volume of 6 mL through the SPE column after the sample
411 was loading were tested. Recoveries of SPI higher than 90% were obtained when n-
412 hexane was used. However, when NaOH 0.5 M was assayed at all concentrations tested,
413 the complete removal of fat was not allowed. Different volumes of n-hexane were tested
414 and the obtained results showed that the use of more than 6 mL did not improve
415 considerably SPI recovery. Therefore, 6 mL (6×1 mL) of n-hexane was chosen as

optimum solvent conditions for this purpose. It is remarkable that the use of n-hexane was enough to wash off the content of fat and interferences in milk samples in the same step, avoiding washing step with ACN. This is leading to significant time saving and simplify the procedure. In all cases, elution was carried out with 3×2 mL of MeOH containing 0.5% of acetic acid. Fractions collected were taken to dryness and reconstituted in a volume of 1 mL in NaH_2PO_4 25 mM/ACN (70:30) mixture at pH 7. Finally, SPI was detected by HPLC-DAD.

3.5. Validation method

The developed method was validated for linearity, recovery, accuracy, precision (inter- and intra-assay), and detection limit under the optimum conditions for sheep milk matrix. Calibration curve was obtained by least-squares linear regression analysis of the peak area versus SPI concentration, preparing spiked milk samples in triplicate in the concentration range of 24-965 µg kg^{-1}. Good linearity (R^2= 0.9998) was established throughout the studied concentration range for SPI. The limit of quantification (LOQ), estimated as the lowest concentrations with RSD below 5%, was 24.1 µg kg^{-1}. The accuracy was assessed by calculating the recovery obtained for SPI in MISPE procedure of 1 mL of spiked milk samples at three concentrations levels: low level (48.3 µg kg^{-1}), medium level (482.6 µg kg^{-1}) and high level (965.2 µg kg^{-1}). Residues were analysed by HPLC-DAD in triplicate. The obtained results are shown in Table 2. Recoveries were higher than 90% with RSDs lower than 5%. The repeatability of MISPE method was assessed by injection a solution of SPI (three times in one day) at two different concentrations (241.3 and 965.2 µg kg^{-1}). Precision was tested in terms of reproducibility and repeatability at two concentration levels (241.3 µg kg^{-1}, 965.2 µg kg^{-1}). The reproducibility (day-to-day variability) between three different days was

441 checked and recoveries ranged from 97.9 to 99.4% with RSDs less than 2% were
442 obtained. Reproducibility (day-to-day variability) values ranged from 86.4-88.3% with
443 RSD lower than 9% (Table 2).
444
445 *3.6. Selectivity*
446 Once the MISPE procedure for SPI was optimized, cross-reactivity studies were
447 carried out in order to evaluate the selectivity of the MIP used as a solid-phase
448 extraction sorbent. MIPs are not intrinsically selective. Their selectivity results from the
449 combination of a polymerization procedure that gives rise to specific cavities for the
450 target analytes together with the association of an extraction procedure involving
451 solvents able to develop interactions that should only take place into the cavities. For
452 this study, other macrolide antibiotics such as JOS, IVER, ERY and TYL were chosen
453 as competitive molecules because of their similar structures. To evaluate the effect of
454 coexisting substances on the recovery of SPI by the MIP, macrolides mixture solution at
455 a spiramycin:interferent ratio of 2.5:25 mg L^{-1} (low level), 5:50 mgL^{-1} (medium level)
456 and 10:100 mg L^{-1} (high level) were tested. Fig. 4 showed the chromatograms obtained
457 at different wavelengths for macrolides extraction by MISPE procedure. Recoveries for
458 macrolide antibiotics under study were calculated as the difference between the total
459 amount of each compound load into the cartridge and the fractions collected from
460 elution step. Table 3 summarizes the obtained results. Recoveries for SPI were higher
461 than 90%, while recoveries for the rest of antibiotics tested were less than 30%. The
462 results revealed a significantly higher selectivity of the MIP1 for SPI in comparison
463 with other structurally related macrolide antibiotics.
464
465

4. Conclusions

The developed extraction method provided satisfactory recoveries and RSD values, and LOD lower than levels established by current legislation. An unique washing step was necessary in MISPE procedure to remove the fat content of the milk samples and to reduce the non-specific interactions of SPI with MIP, keeping the analyte specifically retained on the MIP. This makes it easier the milk treatment and supposes a very important saving in solvents and the analysis time. The optimized MISPE indicated that the MIP can recognize SPI without cross-reactivity to other macrolides studied. So, it has been demonstrated that methacrylic based SPI-MIP obtained has a great potential for utilization as specific SPE sorbent for SPI clean-up and preconcentration in complex mixtures such as sheep milk, offering an rapid, sensitive and cost-effective alternative tool to the existing sorbents for analyzing SPI in milk samples.

Acknowledgements

This research work was supported by Comunidad Autónoma of Madrid (Spain) and European funding from FEDER program (project S2013/ABI-3028, AVANSECAL). The authors thank the Regional Centre of Animal Selection and Reproduction (CERSYRA) for supplying sheep milk samples.

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573 **Figure captions**

574 Fig.1 - (A) The saturation adsorption of SPI onto the MIP1 and NIP1 at different SPI
575 concentrations. (B) Scatchard analysis to estimate the binding nature of SPI to the
576 MIP1.

577

578 Fig. 2 - Recoveries of SPI in MISPE column after washing for 1 mL of 50 mg L^{-1} of SPI
579 standard solution.

580

581 Fig. 3 - Recoveries of SPI after eluting with different solutions.

582

583 Fig. 4 - Comparison of chromatograms obtained for antibiotic macrolides studied: (a)
584 standard mixture solution in ACN (b) spiked sheep milk sample with MISPE treatment.
585 Concentrations: 5 mg L^{-1} (SPI, JOS, IVER, TYL), 50 mg L^{-1} (ERY). Detection
586 wavelengths: SPI, JOS (231nm); ERY (210 nm); IVER (254 mn); TYL (287 nm).

587

588

589 **Tables**

590 Table 1. Specific binding, partition coefficients and imprinting factors for MIPs and
591 NIPs in ACN at 20ºC.

592 Table 2. Analytical characteristics of the optimized MISPE-HPLC method.

593

594 Table 3. Macrolides recoveries at three concentration levels.

595

596

597

Table(1)

TABLE 1

Absorbent	% Specific binding*			Partition coefficient (k)*		Imprinting factor (α)*
	MIP	NIP	Specific	MIP	NIP	
Polymer 1	75.3±1.2	22.2±2.0	53.1±1.1	3.0±0.1	0.29±0.02	3.4±0.4
Polymer 2	62.2±0.7	24.5±1.1	37.7±0.9	1.6±0.1	0.32±0.04	2.5±0.5

*Mean value ± Standard deviation

Table(2)

TABLE 2

% Recovery			Inter-day recovery (%)		Intra-day recovery (%)	
			Spiking level (μg kg^{-1})		Spiking level (μg kg^{-1})	
Low level (48.3 μg kg^{-1})	Medium level (482.6 μg kg^{-1})	High level (965.2 μg kg^{-1})	241.3	965.2	241.3	965.2
99.8 ± 0.2	90.3±2.4	91.8±4.3	88.3±7.6	86.4±8.8	99.4±1.1	97.9±1.6

Table(3)

TABLE 3

Antibiotic	% Recovery Low level	% Recovery Medium level	% Recovery Maximum level
ERY	-	-	-
SPI	99.8 ± 0.2	90.3 ± 2.4	91.8 ± 4.3
JOS	26.9 ± 2.8	25.0 ± 5.7	20.5 ± 6.9
IVER	27.0 ± 1.1	22.3 ± 5.3	23.9 ± 7.4
TYL	21.8 ± 5.3	16.9 ± 0.4	18.4 ± 3.9

Figure(1)

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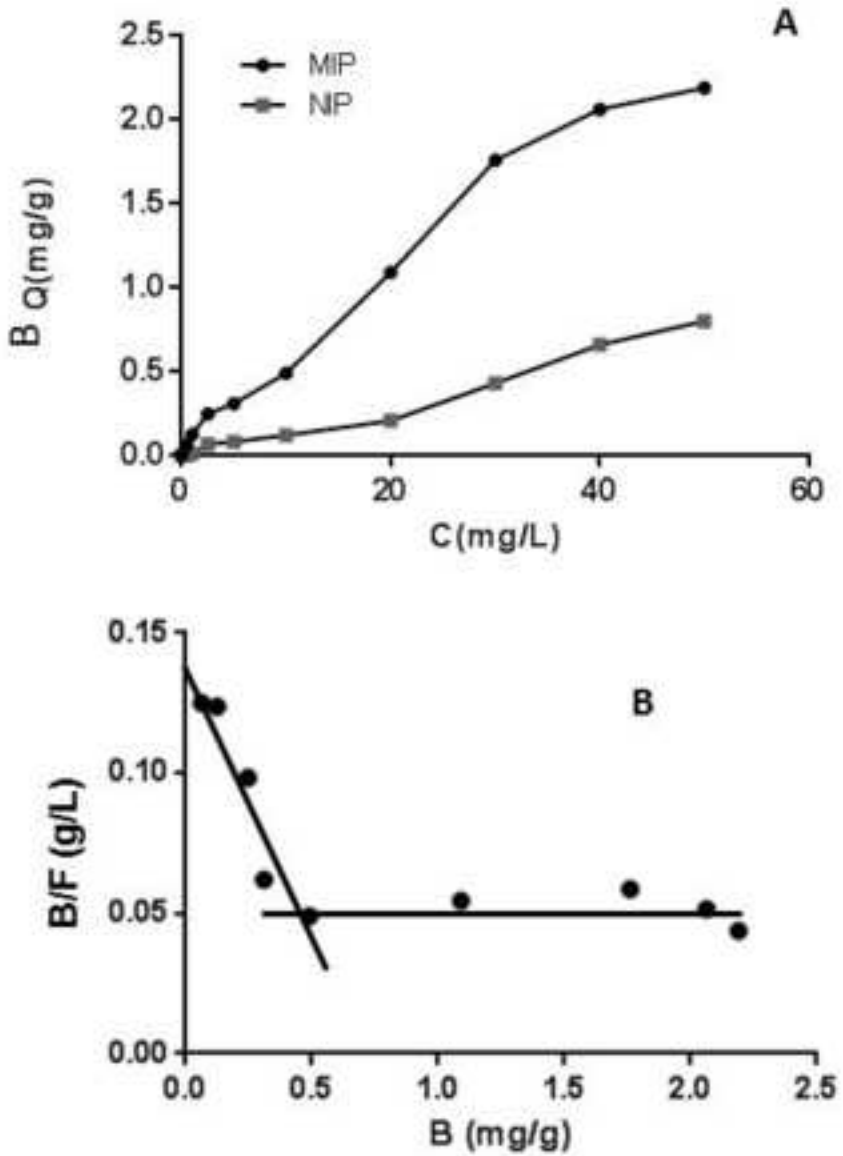

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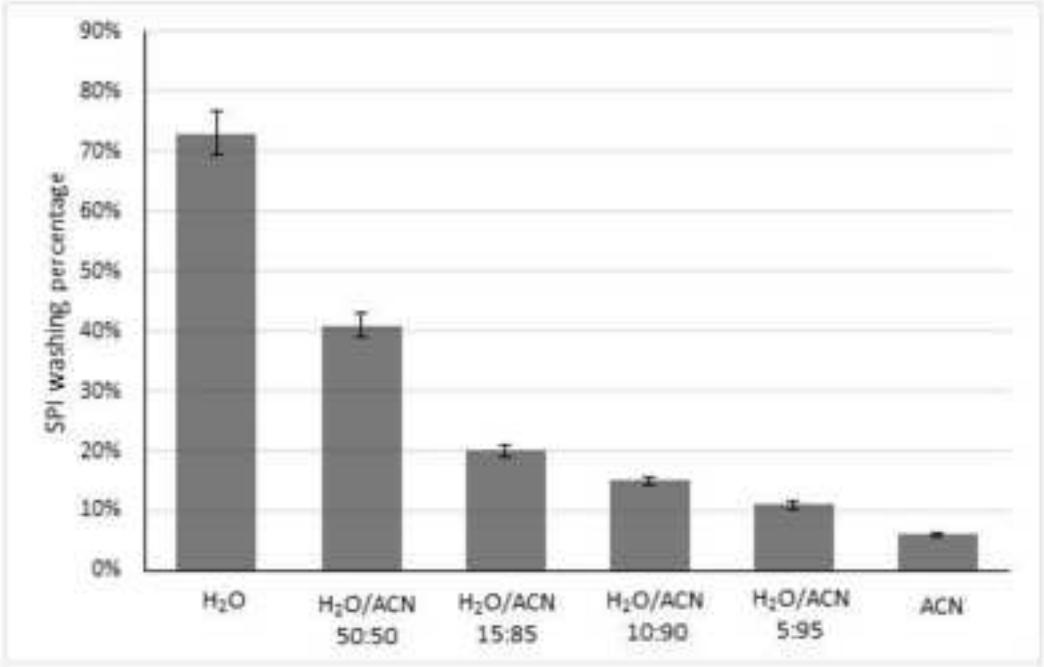

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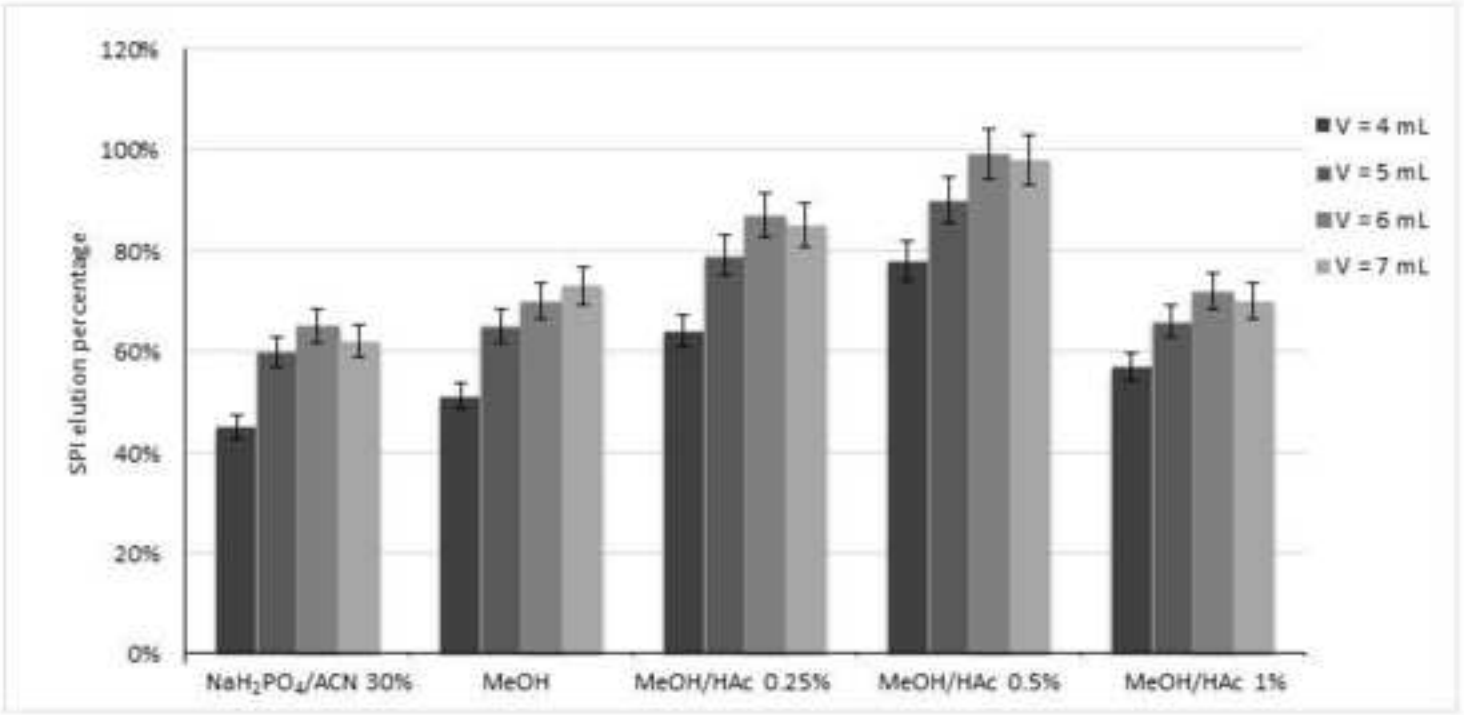

Supplementary Material (fig 4.PDF)

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