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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_2PO_4 25 mM at pH 7/acetonitrile (70:30). Aqueous solvent of the mobile phase was made by dissolving 5 g of NaH_2PO_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_2PO_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