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Molecularly imprinted polymers and HPLC-MS/MS for determining penicillins in infant formulas

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Abstract

The dairy cattle may suffer from different infections relatively often, but the inflammation of the mammary gland is very important to the farmer. Mastitis is an animal welfare problem and a big economic problem because it deteriorates the milk quality. These infections are frequently treated with penicillins. However, their use may result in the presence of residues in milk and dairy products, such as milk powder and infant formulas, and it represents a potential risk for consumers. To avoid health risks for the consumer, the EU has defined safe maximum residue limits (MRL) through Commission Regulation (EU) No. 37/2010. Although LC–MS is a trustful option for confirmation and quantification of antibiotics, the analysis of real samples with complex matrices usually implies previous clean-up steps using common solid-phase extraction procedures. In this work, precipitation polymerization has been used and different MIP sorbents were tested and optimized for the solid-phase extraction (MISPE) of eight common penicillins (ampicillin, amoxicillin, oxacillin, penicillin G, penicillin V, cloxacillin, dicloxacillin and nafcillin). The detection was performed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and the applicability of these polymers as sorbents for the extraction of penicillins at MRL levels in infant formulas was proved.

Keywords: MIP, MISPE, penicillins, milk, LC-MS/MS

Introduction

The term antibiotic refers to a very diverse range of chemical substances produced from bacteria or fungi in a natural, semisynthetic or synthetic way that possess antibacterial activity, by killing or inhibiting the growth of microorganisms. β-lactam antibiotics (BLAs) constitute one of the most widely used anti-microbial drug in veterinary medicine, especially to treat and prevent bacterial infections (respiratory, urinary or skin infections) of dairy cattle. This group of antibiotics can be classified into several groups (penicillins, cephalosporins, and more recently, carbapenems) according to their structural characteristics, but their unique structural feature is the presence of the four-membered BLAs (2-azetidinone) ring [1].

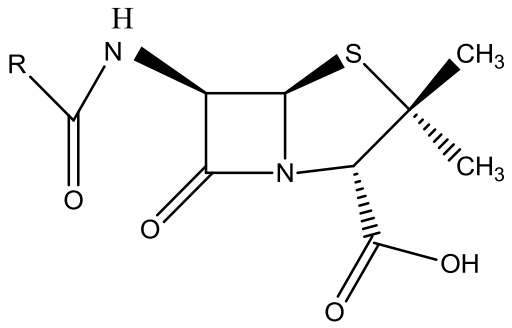

Figure 1. General structure of penicillins (R = lateral amino chain)

In dairy cattle, in addition to digestive and respiratory diseases, the inflammation of the mammary gland is very problematic for the farmer. Mastitis, although an animal welfare problem, is a big economic problem because it increases the somatic cell count, a milk quality indicator [2]. These infections are frequently treated with penicillins, one of the most applied drugs in dairy cattle (see Figure 1). However, the incorrect use of these antibiotics may lead to residues in food products of animal origin, such as, milk, muscle, meat and kidney. It is well known that the improper use may have adverse effects on consumer health, including bacterial resistance to these drugs in humans, and also problems in dairy industry [3]. Bovine milk is a rich source of many nutrients that are necessary for the neonate and plays an important role during humans´ adult life, helping them to meet their nutritional requirements. The presence of penicillins in milk may represent a risk to consumer health, such as allergic reactions and anaphylactic shock in sensitive individuals [4]. To avoid health risks for the consumer due to

residues, the EU has defined safe maximum residue limits (MRL) through Commission Regulation (EU) No. 37/2010. The limits implemented are shown in Table 1.

Table 1. MRLs established by Commission Regulation (EU) No. 37/2010 for penicillins residues in milk.

ANALYTE	Regulation 37/2010
Amoxicillin	4 $\mu g.kg^{-1}$
Oxacillin	30 $\mu g.kg^{-1}$
Cloxacillin	30 $\mu g.kg^{-1}$
Penicillin G	4 $\mu g.kg^{-1}$
Penicillin V	4 $\mu g.kg^{-1}$
Ampicillin	4 $\mu g.kg^{-1}$
Dicloxacillin	30 $\mu g.kg^{-1}$
Nafcillin	30 $\mu g.kg^{-1}$

Milk samples are usually analyzed by rapid screening methods that only indicate whether some antibiotics are present or not and also by immunoassays to determine the type of antibiotics. Because of their low MRL levels, it is necessary to use hightly sensitive and selective methods for confirmation and quantification of penicillins in milk. High performance liquid chromatography coupled to mass spectrometry (HPLC/MS) is a trustful option with complex matrices, usually implies previous clean-up steps using common solid-phase extraction (SPE) procedures. The clean-up of the samples plays indeed a key role in determining the detection capability of the instrumental techniques because of its ability to reduce matrix interferences [5]. The main drawback of SPE techniques is the lack of selectivity of the sorbents. Molecularly imprinted polymers (MIP) are synthetic materials with recognition sites that specifically bind target molecules in mixtures with other compounds. MIP sorbents, which imitate natural recognition, are capable of meeting the demands of SPE and they may be reused several times with optimum recoveries [6]. The applicability of MIP as selective sorbents for the extraction of some components of BLA group has already been reported [7]. However, it would be interesting to develop polymers that allow simultaneous detection of various analytes of the same group. The aim of the present work is to test the suitability of various MIP, synthesized using different templates and cross-linker monomers, for the simultaneous extraction of eight structurally related penicillins (ampicillin, amoxicillin, oxacillin, penicillin G, penicillin V, cloxacillin, dicloxacillin

and nafcillin). The detection was performed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and the applicability of these polymers as sorbents for the extraction of penicillins at the level of interest (MRLs) in infant formulas was proved.

Experimental

Materials

Ampicillin (AMP), amoxicillin (AMX), oxacillin (OXA), penicillin G (PEN G), penicillin V (PEN V), cloxacillin (CLOX), dicloxacillin (DICLOX) and nafcillin (NAFC) were obtained from Sigma-Aldrich (Madrid, Spain). The chemicals used for the polymers synthesis were methacrylic acid (MAA), divinylbencene 80% (DVB-80), ethylene glycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TRIM) and the initiator 2,2`- azobis-(2-methyl-butyronitril) (AIMN) from Sigma-Aldrich. MAA, EGDMA and TRIM were freed from stabilizers by distillation under reduced pressure and AIMN was recrystallized from methanol prior to use. Aditionally, DVB-80 was freed from stabilizers by passing through a small column packed with neutral alumina (Aldrich). HPLC grade solvents were supplied by Merck (Madrid, Spain).

Apparatus

The recoveries of different polymers were calculated using LC-MS/MS. Separation was performed in an 1100 series HPLC system from Agilent Technologies (Minnesota, USA). A Synergi 2.5 µm MAX-RP 100A (100 x 2 mmm) column from Phenomenex (Torrance, CA, USA) was used. The mobile phase was acetonitrile (A) mixed on a gradient mode with 0.2% aqueous formic acid (B) at a flow rate of 300 µL min^{-1}. After the first 2 minutes with very aqueous mobile phase at 90% (B), binary gradient mixing was initiated as follows: (B) 90% to 0% for 16 min, and 0% to 90% again for 3 min, at this point the gradient was kept isocratic for 4 min. A Q-Trap 2000 mass spectrometer with ESI Source from AB Sciex (Toronto, Canada) was used, working in positive mode. For quantification, the most intense MRM transition was monitored along with a second transition for indentify confirmation (Table 2).

Table 2. MRM transitions of each analyte and their respective collision energy (CE).

Compound	Mw	Precursor ion	Fragment ion	CE (volts) *
Amoxicillin	365.4	366	349	13
			114	25
Oxacillin	401.4	402	144	33
			186	21
Cloxacillin	435.9	436	178	31
			220	21
Penicillin G	334.4	335	217	17
			202	31
Penicillin V	350.4	351	229	19
			257	17
Ampicillin	349.4	350	106	21
			160	9
Dicloxacillin	470.3	470	212	33
			254	23
Nafcillin	414.4	415	199	17
			171	51

***CE:** Collition energy in volts.

Preparation of polymers

The polymers were prepared by precipitation polymerization. OXA, AMOX and NAFC were used as template and MAA as functional monomer. As cross-linkers DVB, EGDMA and TRIM were tested, including different solvents in the polymerization mixture. The different combinations of template, monomer, cross-linker, initiator and porogen were as indicated in the table 3.

Table 3. Composition of different MIP synthesized for the simultaneous extraction of penicillin drugs.

TEMPLATE	Functional monomer	Cross-linkers	Porogens	Initiator	Polymerisation problems	Conditions
Oxacillin	MAA	DVB	ACN/TOL	AIMN	Not dissolved	–
		EGDMA	MeOH	AIMN		L: TOL; W: TOL10%ACN
Amoxicillin		DVB	ACN/TOL	AIMN	Not dissolved	–
		EGDMA	MeOH	AIMN		L: TOL; W: TOL10%ACN
Nafcillin		DVB	ACN/TOL	AIMN		L: ACN; W: ACN
		TRIM	ACN	AIMN		L: ACN; W: ACN
		TRIM	MeOH	AIMN		L: ACN; W: ACN
		EGDMA	**ACN**	**AIMN**		**L: ACN; W: ACN**
		EGDMA	MeOH	AIMN		L: ACN; W: ACN

MAA: methacrylic acid; EGDMA: ethylene glycol dimethacrylate; DVB: divinyl bencene; AIMN: 2,2`-azobis-(2-methyl-butyronitril); TRIM: trimethylolpropane trimethacrylate;MeOH: methanol; ACN: acetonitrile; TOL: toluene; L: loading sample; W: washing.

The different polymerization mixtures were simultaneously introduced into a temperature controllable incubator equipped with a low-profile roller at 24 r.p.m. and 60ºC for 24 hours. The polymer particles were separated and cleaned by vaccum filtration through a nylon membrane filter of 0.45 µm of pore diameter, using 50 mL of acetonitrile and 50 mL of methanol. Then the imprint molecule was removed by Soxhlet extraction for 8 h using a methanol/acetic acid mixture (1:1). In each case, non-imprinted polymers (NIP) were prepared in the same way but without the addition of template.

Optimization of molecularly imprinted solid-phase extraction (MISPE)

Molecularly imprinted and non-imprinted polymers (0.05 g) were placed in empty SPE glass cartridges. The cartridges were coupled to an SPE manifold and several experiments were carried out using different loading (acetonitrile, toluene) and washing solutions (acetonitrile, methanol, toluene with different % of acetonitrile), by loading 1 µg of each analyte per cartridge. In parallel, the same experiments were carried out on NIP cartridges in order to prove the existence of template-specific imprinted sites into the MIP. The obtained elutions (methanol 1% acetic acid) were evaporated under nitrogen stream and re-dissolved in mobile phase for recoveries calculation by HPLC-MS/MS. Optimized MISPE protocol (different loading and washing steps, elution was always methanol 1% acetic acid) for maximum recoveries for all the selected antibiotics when using each polymer are shown in table 3.

MISPE of infant formulas

After MISPE optimization, infant formulas (0.3 g) spiked at the level of interest (MRL, see table 1) with a mixture of the selected penicillins in acetonitrile were extracted using the selected polymer (NAFC-MAA-EGDMA-CAN-AIMN). The performed MISPE protocol was the loading-washing-elution combination selected during MISPE optimization: acetonitrile-acetonitrile-methanol 1% acetic acid. Samples were extracted with 1 mL of acetonitrile, vortexed for 1 min, centrifugated at 5,000 g for 10 min and the supernatant was directly loaded into the selected MIP cartridge. After washing and eluting steps, the extract was dried under a nitrogen stream at 30ºC and re-dissolved in 100 µL of mobile phase B. Thirty microliters of extract were immediately injected into the chromatographic system and assayed with the developed HPLC-MS/MS method.

Results and discussion

Preparation of polymers and MISPE optimization

The selected polymerization technique was precipitation polymerization, which have an increased rebinding capacity and a more homogeneous distribution binding sites with microspherical shapes of uniform sizes. For penicillins, NAFC resulted on an adequate template in combination with MAA and EGDMA, all dissolved in ACN, to obtain MIP for the simultaneous extraction of all the selected analytes. Acetonitrile 100% as loading and washing solution provided the higher differences between MIP and NIP cartridges in terms of recovery, with good recoveries in MIP for all penicillins (> 70%). In general, recoveries with other penicillin-based MIP were lower than with this NAFC-based polymer. For example, the polymers prepared using OXA and/or AMOX provided good retention of penicillins during the loading step (approx. 90% of retention of most penicillins) but not enough to achieve good recoveries during elution step. With the OXA-based and/or AMOX-based polymers, a high portion of analytes would elute during washing (using acetonitrile or methanol) obtaining no more than 20% of recovery during elution steps. Additional experiments were performed with toluene, in both loading and washing steps. However, the elution was not homogeneous for all penicillins, getting good recoveries for some of them (OXA and PEN G) but not for the whole group of antimicrobials. High recovery for all penicillins was preferred due to the low MRL of these analytes in milk.

Also, some polymerization problems were found when using OXA and/or AMOX as templates in combination with DVB-80 in acetonitrile/toluene porogen, mainly due to the low solubility of these drugs in non-polar solvents (the mixture ACN/TOL presents the lower polarity of all the tested porogens). In this case, no polymerization was achieved, because it was not possible to dissolve the template in the polymerization mixture, which is a key factor in MIP synthesis. When using DVB as cross-linker, the combination of acetonitrile and toluene is required to obtain polymers with good performance and with the production of monodisperse, imprinted-polymer beads with well-developed, permanent pore structures [7]. Summing up, NAFC-MAA-EGDMA-ACN was selected as SPE polymer because it provided the higher retention capacity for eight penicillins studied (ampicillin, amoxicillin, oxacillin, penicillin G, penicillin V,

cloxacillin, dicloxacillin and nafcillin), enabling the simultaneous extraction of these drugs at the level of interest in milk.

MISPE of infant formulas

Applicability of NAFC-based MIP in real samples was tested using infant formulas as analytical matrix. The matrix was selected based on the importance that food safety, and more precisely antimicrobials residues, has for vulnerable population, as babies and children. Powdered infant formulas were sampled as they were liquid formulas because there is no MRL specifically established for powdered formulas. Thus, a sample of 0.3 g of powder formula was analysed because it is the amount necessary to obtain 2 mL of liquid formula. The sample size was also selected based on the instrumental analysis limits. The analytical MISPE procedure only included a simple protein precipitation with acetonitrile, followed by the direct loading of the extract into the NAFC-MAA-EGDMA-ACN polymeric SPE cartridge. The selected polymer was chosen basing on the previous recovery experiments and aiming at a reasonable recovery for all the analytes. Figure 2 shows a chromatogram of a spiked infant formula sample, containing 4 µg of AMOX, PEN G, PEN V and AMPI and 30 µg of OXA, CLOXA, DICLOX and NAFC per liter of sample (150 g of formula). The analytes were successfully detected and quantified using calibration curves. Besides, no interfering peaks could be observed in the chromatogram.

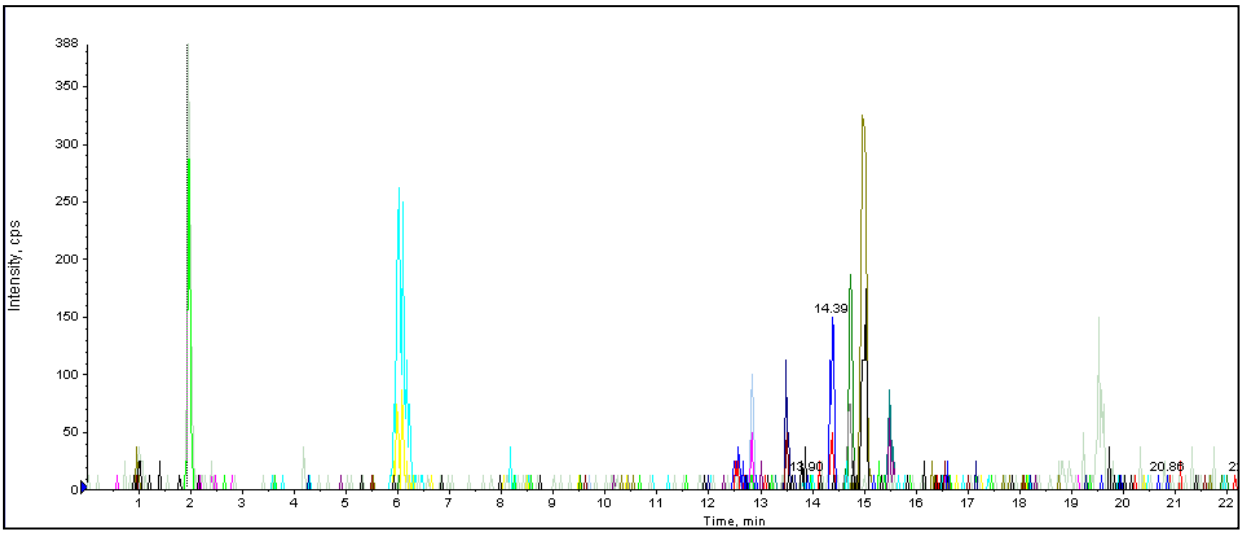

Figure 2: Chromatogram of an infant formula spiked at the MRL level for ampicillin, amoxicillin, oxacillin, penicillin G, penicillin V, cloxacillin, dicloxacillin and nafcillin

Conclusions

From the observed data it may be concluded that MIP sorbents are good solutions for SPE of different veterinary drugs. In the case of real samples, the developed MISPE protocols provided enough recovery for various penicillins at their level of interest using small amount of sample and a very simple clean-up protocol. Different mixtures were tried, but only one of them resulted in an equilibrated recovery for all the analytes when simultaneously extracted. The latest fact proves that various assays are preferable when designing MIP for solid phase extraction, especially in the case of a group of structurally related analytes.

Acknowledgements

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