The 16th International Electronic Conference on Synthetic Organic Chemistry
Section D. Polymer and Supramolecular Chemistry
1-30 November 2012

Development of molecularly imprinted polymers for the analysis of amphenicols in milk

Rocío Barreiro, Mónica Díaz-Bao, Patricia Regal*, José M. Miranda and Alberto Cepeda

Department of Analytical Chemistry, University of Santiago de Compostela, 27002 Lugo (Spain)

* Corresponding author: patricia.regal@usc.es

Abstract

Chloramphenicol (CAP) is a broad-spectrum bacteriostatic antibiotic commonly used in veterinary medicine. However, toxic effects in humans such as Grey syndrome, bone narrow suppression, and fatal aplastic anaemia have been described. As a consequence, the use of CAP in foodstuffs has been banned within European Union since 1994 and no maximum residue limit (MRL) has been established in animal-derived foods. On the other hand, thiamphenicol (TAP) and florfenicol (FLP) are allowed but different MRL have been set in foodstuff of animal origin. In this work, precipitation polymerisation has been used and different MIP sorbents were tested and optimized for the solid-phase extraction (MISPE) of the group of the three, structurally related amphenicols. Recoveries were calculated using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The applicability of these polymers for the extraction of amphenicols in spiked samples of bovine milk and baby formulas has been tested.

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

Introduction

Chloramphenicol (CAP) is a broad-spectrum bacteriostatic antibiotic commonly used in veterinary medicine and active against both Gram-positive and Gram-negative bacteria. However, toxic effects in humans such as Grey syndrome, bone narrow suppression, and fatal aplastic anaemia have been described. As a consequence, the use of CAP in foodstuffs has been banned within European Union since 1994 and no maximum residue limit (MRL) has been established in animal-derived foods [1]. CAP belongs to the amphenicol family that also includes thiamphenicol (TAP) and florfenicol (FLP); their structure is shown in Figure 1. For TAP and FLP, MRLs have been established in different matrices for all food producing species [2]. However, for CAP a zero tolerance level was established and only a minimum required performance limit (MRPL) was established at 0.3 μg kg^{-1} to reach a harmonized analytical performance of methods for monitoring CAP [3].

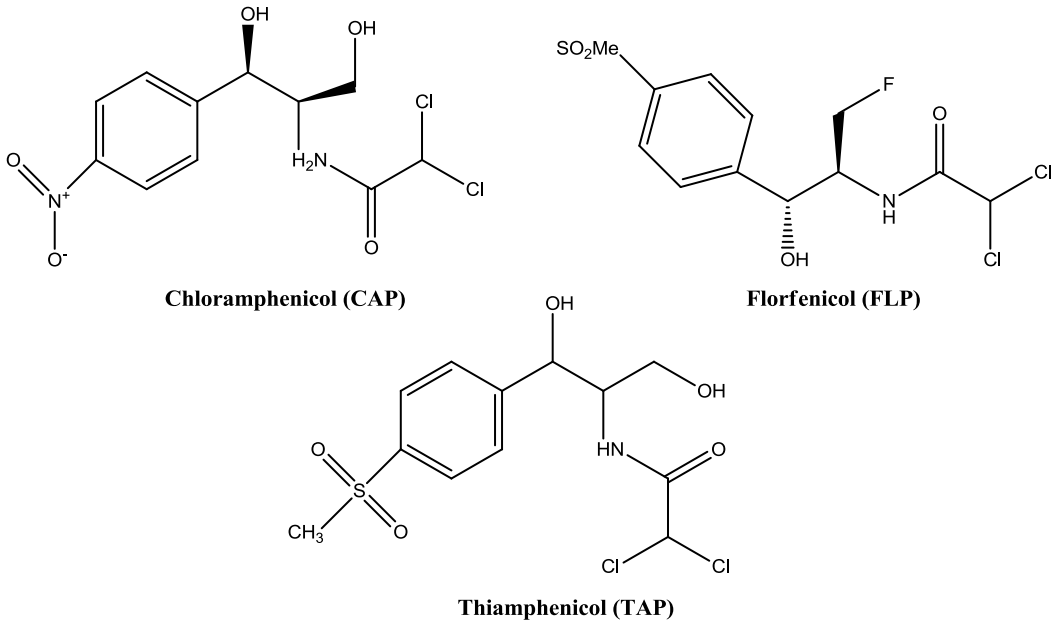

Figure 1. Chemical structures and abbreviations of amphenicol antibiotics.

Although LC–MS is the most widely used method for routine confirmation of amphenicols, the analysis of complex matrices such as milk usually implies previous clean-up steps, including common solid-phase extraction (SPE) procedures [4, 5]. The main drawbacks of this extraction technique are the lack of selectivity of the sorbents and the costs of commercial cartridges and solvents. Molecularly imprinted polymers (MIP) are synthetic materials with recognition sites that specifically bind target

molecules in mixtures with other compounds [6]. MIP sorbents, which imitate natural recognition, are capable of meeting the demands of SPE and they may be reused several times with optimum recoveries. The aim of the present work is to test the suitability of chloramphenicol as template molecule in the design of molecularly imprinted polymers for amphenicols extraction. Precipitation polymerisation has been used and different cross-linkers and porogens were tested for the design of sorbents for solid-phase extraction (MISPE) of the group of these three, structurally related amphenicols. Recoveries were calculated using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The applicability of these polymers for the extraction of amphenicols in bovine milk and baby formulas has been tested, within a European framework.

Experimental

Materials

Chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FLP) were obtained from Sigma- Aldrich (Madrid, Spain). The chemicals used for the polymers synthesis were methacrylic acid (MAA), divinylbenzene 80% (DVB-80), ethylene glycol dimethacrylate (EGDMA) and the initiator 2,2'-azobis-(2-methyl-butyronitril) (AIMN) from Sigma-Aldrich. MAA and EGDMA were freed from stabilizers by distillation under reduced pressure and AIMN was recrystallized from methanol prior to use. Additionally, 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

Recoveries were measured by LC-MS/MS. Separation was performed on 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 4 minutes with very aqueous mobile phase at 95% (B), binary gradient mixing was initiated as follows: (B) 95% to 30% for 2 min, 30% to 0% for 6 min and 0% to 95% for 13 min. A Q-Trap 2000

mass spectrometer with ESI Source from AB Sciex (Toronto, Canada) was used, working in negative mode. For quantification, the most intense MRM transition was monitored, along with a second transition for identity confirmation (Table 1).

Table 1. MRM transitions of each analyte and their respective collision energy (CE).

Compound	Precursor ion	Fragment ion	CE (volts) *
Chloramphenicol (CAP)	320.9	152.1	-26
		257.0	-12
Florfenicol (FLP)	356.1	185.0	-24
		336.0	-10
Thiamphenicol (TAP)	354.0	185.0	-30
		290.0	-12

***CE**: Collision energy in volts.

Preparation of polymers

The polymers were prepared by precipitation polymerization. Briefly, CAP molecule was used as template and MAA as functional monomer, and two different cross-linkers (EGDMA and DVB for MIP-1 and MIP-2, respectively) were tested, including different solvents in the polymerization mixture (Table 2). 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 vacuum 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.

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, ethyl acetate, toluene) and washing solutions (acetonitrile, methanol, toluene, 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 0.1% acetic acid) were evaporated under nitrogen stream and re-dissolved in mobile phase for recoveries calculation by HPLC-MS/MS.

Table 2. Composition of different MIP synthesized for extraction of amphenicols.

POLIMER COMPOSITION	MIP-1	MIP-2
Template	CAP (0.12 mmol)	CAP (0.19 mmol)
Monomer	MAA (0.75 mmol)	MAA (1.5 mmol)
Cross-linker	EGDMA (2.5 mmol)	DVB-80 (3.84 mmol)
Initiator	AIMN (3.2 mmol)	AIMN (0.27 mmol)
Porogen	MeOH/ACN TOL/DCM (12.5 mL)	ACN:TOL (3:1) (12.5 mL)

CAP: chloramphenicol; MAA: methacrylic acid; EGDMA: ethylene glycol dimethacrylate; DVB: divinyl bencene; AIMN: 2,2'-azobis-(2-methyl-butyronitril); MeOH: methanol; ACN: acetonitrile; TOL: toluene; DCM: dichloromethane

Milk and baby formulas: MISPE

Commercial bovine milk and baby formula samples (1.0 mL and 0.15 g) were spiked with a mixture of the assayed amphenicols in ethyl acetate at the level of interest (MRLs and MRPL). As for baby formulas, 0.15 g is the required amount to prepare 1 mL of liquid formula so this amount was used as it were 1 mL of milk. The performed MISPE protocol to clean-up milk and baby formula samples was the loading-washing-elution combination selected during MISPE optimization (MIP-2): toluene - toluene 5% acetonitrile - methanol 1% acetic acid. Thus, 2 mL of ethyl acetate containing CAP, TAP and FLP (0.3, 50 and 50 ng, respectively) were added to 1 mL of milk (or to 0.15 g of formula powder) to perform recovery experiments at the level of interest. Samples were vortexed for 1 min, centrifuged at 5,000 *g* for 10 min and the supernatant was transferred to an empty tube and evaporated under a nitrogen stream at 30 ºC. Dried residue was re-dissolved in 1 mL of toluene and loaded into the selected MIP-2

cartridge. The direct loading of ethyl acetate supernatant was also tested for milk and powder formula. After washing and eluting steps, the extract was dried under a nitrogen stream at 30 ºC and re-dissolved in 100 µL of mobile phase B. Thirty microliters were immediately injected into the chromatographic system and assayed with the developed HPLC-MS/MS method.

Results and Discussion

Preparation of polymers

CAP was selected as template molecule because it is the most commonly used amphenicol and it has the lowest level of interest (0.3 µg kg^{-1}). Using CAP as 'imprinting' molecule the selectivity of the obtained MIP should be higher for this compound than for the rest of the structurally related amphenicols. The selected polymerization technique was precipitation polymerization, which allows the formation of spherical polymer particles and avoid crushing and sieving steps. When using EGDMA as cross-linker monomer, it was impossible to obtain a complete dilution of CAP and MAA in toluene and dichloromethane. This latest fact is an unavoidable requirement for any MIP synthesis. Consequently, EGDMA polymers could only be achieved using acetonitrile and/or methanol as porogen solvents. Based on different polymerization mixtures, the reaction of polymerization yielded different amounts of MIP and NIP (see Table 3).

Table 3. Polymer recoveries obtained for different polymerization mixtures.

POLYMER		**POLYMERIZATION YIELD (%)**			
Cross-linker	Porogen	Initial [1]		Final [2]	
		MIP	NIP	MIP	NIP
DVB-80	ACN	66.56	70.12	64.52	68.05
EGDMA	ACN	100	100	98.21	96.36
EGDMA	MeOH	100	100	95.36	95.73

[1] After polymerization; [2] After template removal

The maximum yield was obtained using EGDMA. However, the imprinting effect was more evident in DVB-80 polymers and CAP-TAP-FLP recoveries were higher.

MISPE optimization

As described in experimental section, MISPE optimization protocol was performed with a mixture of amphenicols in the loading solvent, at a concentration of 1 μg mL^{-1}, using 1mL of different loading solvents (acetonitrile, ethyl acetate and toluene). It has been largely demonstrated that MIPs offer the highest selectivity when analytes are dissolved in the solvent previously used as porogen during the polymerization procedure. In this study, when loading in acetonitrile no imprinting effect was observed in MIP-1-ACN and MIP-1-MeOH cartridges and more than 50% of amphenicols eluted during the loading step. However, for MIP-2 cartridges only 17% would elute during the loading step with acetonitrile, plus a more evident imprinting effect was observed when comparing MIP-2 and its corresponding NIP (NIP elution: 50% during loading step). Aiming at reducing amphenicols lost during loading in MIP, toluene, a less polar solvent was also tested as loading solvent achieving better recovery results.

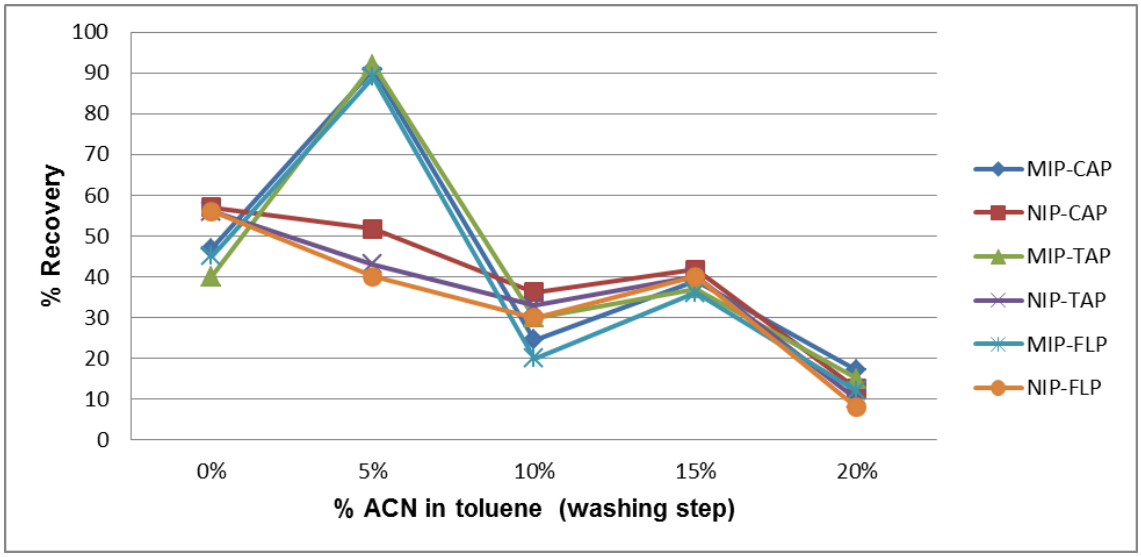

Figure 2. Recoveries obtained for CAP, TAP and FLP for MIP-DVB and its corresponding NIP as a function of a percentage of acetonitrile present in the washing toluene.

As for the washing step, acetonitrile and methanol were not useful solvents because they would cause the elution of analytes during this step instead of during the elution step. Consequently, toluene and toluene with small proportions of acetonitrile (5, 10, 15 and 20%) were tested as washing solvents. Figure 2 shows the variation of the recoveries obtained for amphenicols on MIP-2 and its NIP, as a function of the percentage of acetonitrile in toluene present in the washing solution. The higher

recoveries were obtained when using toluene 5% acetonitrile; however, strong non-specific interactions appear to be present in both MIP and NIP as the recoveries of analytes diminish in parallel for both polymers when the polarity of washing solution is increased. Summing up, loading in toluene, washing with toluene 5% ACN and eluting with methanol 1% acetic acid appeared as optimal steps for MISPE procedure to extract the selected corticosteroids from milk. Thus, MIP-2 was selected to be used as sorbent for amphenicols extraction.

Milk and baby formulas

To test the applicability of the selected MIP to real samples, both milk and baby formulas (1 mL and 0.15 g, respectively) were spiked with amphenicols at the level of interest. In the case of CAP, the MRPL established by the European legislation was used. For TAP, a MRL has been established (50 μg kg^{-1}) in different matrices for all food producing species, including bovine milk. However, there are no MRL established for FLP in milk. For this reason, the same MRL at 50 μg kg^{-1} was considered during this study for FLP determination in milk. For powdered baby formulas, these levels were accounted as they were liquid formulas, as it is the form they are consumed. A sample of 0.15 g of powder formula was analyzed because it is the amount necessary to obtain 1 mL of liquid formula. The analytical MISPE procedure only included a simple protein precipitation with ethyl acetate [8, 9], followed by evaporation and reconstitution in the loading solvent (toluene).

Table 4. Summary of the obtained results for real spiked samples as a function of the loading solvent.

SAMPLE	ANALYTE	Recovery (%)	
		Toluene	Ethyl acetate
MILK	CAP	80	10
	TAP	80	6
	FLP	100	11
BABY FORMULA	CAP	90	80
	TAP	100	100
	FLP	85	80

CAP: chloramphenicol; TAP: tiamphenicol; FLP: florfenicol

The direct loading of ethyl acetate supernatant into the MIP-2 cartridge, without any evaporation, was also tested for milk and powder formula. However, positive recovery results were only achieved for baby formulas. In the case of milk samples, recoveries

would strongly decrease when loading ethyl acetate extract so that toluene reconstitution could not be avoided. In table 4, a summary of the obtained results for real spiked samples is shown. Moreover, figure 3 shows a chromatogram of a spiked baby formula sample, containing 0.3, 50 and 50 µg kg^{-1} of CAP, TAP and FLP, respectively. No interfering peaks could be observed in the chromatogram.

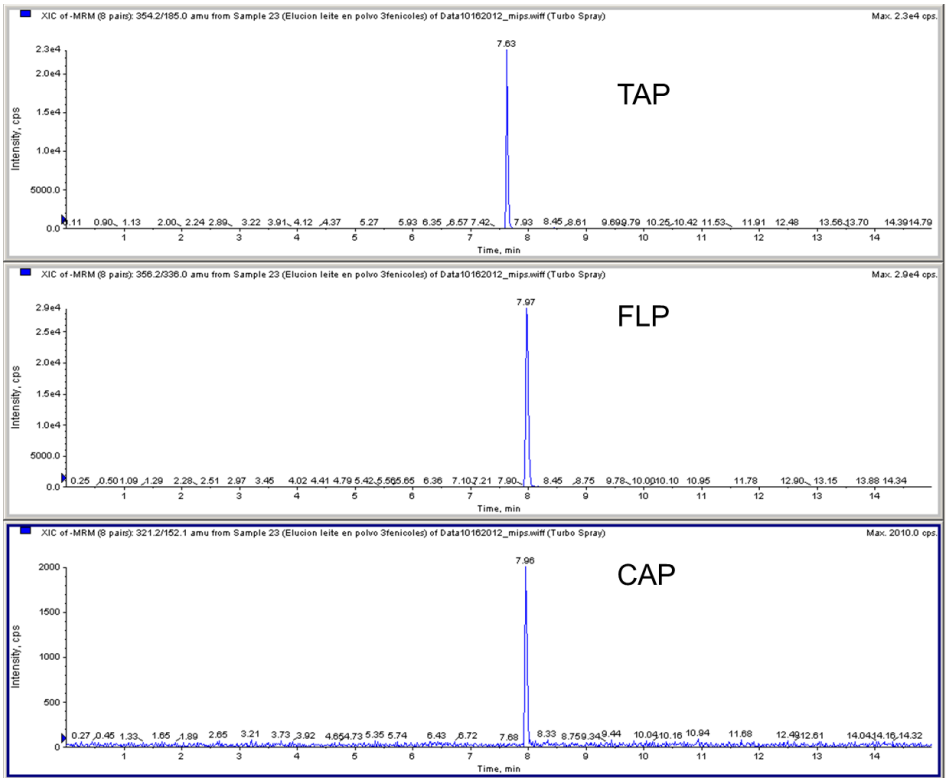

Figure 3. Chromatogram of a spiked baby formula sample, containing 0.3, 50 and 50 µg kg^{-1} of CAP, TAP and FLP, respectively.

When complex samples such as milk are monitored, LC-MS methodologies might suffer from significant enhancement or suppression of signal due to matrix effect [7]. Matrix effects must be considered and assessed when developing a method. In the present work, the application of MIP to the analysis of amphenicols in milk and milk powder allowed the separation of the analytes from the matrix-interfering compounds, thus allowing these complex samples to be analysed with any other clean-up step.

Conclusions

From the observed data it may be concluded that CAP is a good template for designing molecularly imprinting polymers to extract amphenicols in milk and baby formulas. A DVB-based MIP proved to be the more efficient sorbent for MISPE to extract CAP,

TAP and FLP from samples, using toluene as loading solvent and toluene containing 5% of acetonitrile as washing solvent. Additionally, the selected conditions provide the highest difference of recoveries between MIP-2 and its NIP. In the case of baby formulas, even ethyl acetate could be used to load the analytes into the MIP cartridge, obtaining enough retention of amphenicols at their level of interest using very few amount of sample.

Acknowledgements

This work was supported by the projects with reference AGL2009-14707 and IPT-060000-2010-14 from the Spanish Ministry of Science and Innovation. Authors wish to thank Javier Amat Casariego for his technical support.

References

1. Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in food animal origin (Amending Regulation No. 1430/94 of 22 June 1994) Off J Eur Commun L156/6.

2. Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, Off J Eur Union L 15/1.

3. Commission Decision of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin Off J Eur Union L71/17.

4. L. Guo, M. Guan, C. Zhao, H. Zhang (2008) Anal Bioanal Chem 392: 1431-1438.

5. M. Cronly, P. Behan, B. Foley, E. Malone, S. Martin, M. Doyle, L. Regan (2010) Food Add Contam 27: 1233-1246.

6. A. Martín-Esteban (2001) Fresenius J Anal Chem 370: 795-802.

7. M. Díaz-Bao, R. Barreiro, P. Regal, A. Cepeda, C. Fente (2012) Chromatographia 75: 223-231.

8. B. Boyd, H. Björk, J. Billing, O. Shimelis, S. Axelsson, M. Leonora, E. Yilmaz (2007) J Chromatogr A 1174: 63-71.

9. D.R. Rezende, N. Fleury Filho, G.L. Rocha (2012) Food Add Contam 29: 559-570.