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Development of a novel molecularly imprinted stir-bar for isolation of aflatoxins

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Abstract

Mycotoxins are natural compounds produced as secondary metabolites by a wide variety of different species of filamentous fungi. One of the most important groups in terms of occurrence and toxicity is the group of aflatoxins (AFs). The major members of this group are aflatoxin B1, B2, G1, G2, M1 and M2. Exposure to AFs can cause chronic and acute toxic effects, as they can be carcinogenic, mutagenic, teratogenic and immunosuppressive. AFB1 is considered as the strongest carcinogen of natural origin and it is normally predominant in crops and their derived products. Aflatoxin M1 is the main metabolite of aflatoxin B1 in humans and animals and as such it may be present in milk from animals fed with contaminated feed. Although the toxicity of M1 is about ten times lower than for B1, it also has hepatotoxic and carcinogenic effects. For the analysis of mycotoxins in cereals and/or milk, IAC in combination with HPLC are increasingly used as reference method due to their high selectivity and good elimination of matrix interferences. However, the cost of analysis using IAC is usually high. In recent years, molecularly imprinted polymers (MIP) have become very popular and promising materials for extracting different analytes in several matrices. AFs are too toxic to be used in MIP preparation, and template bleeding may be an additional problem, especially when dealing with very low levels. For this reason, in this study the use of a dummy template was preferred for MIP synthesis. A rapid, affordable and selective extraction method based on magnetic MIPs (MMIP) has been developed for the isolation of aflatoxins (B1, B2, G1, G2, M1), using a combination of imprinted polymers and magnetite. The successful MMIP stirring "cake" (used as a stir-bar) has been combined with HPLC-MS/MS for the determination of AFM1 in milk powder (infant formulas) to demonstrate its applicability to real samples.

Keywords: aflatoxin; MMIP; stir-bar; HPLC-MS/MS; milk powder.

Introduction

Humans are exposed to numerous chemicals during their life and through the food chain, including carcinogenic substances. Mycotoxins are natural substances produced as secondary metabolites by a wide variety of different species filamentous fungi such as *Aspergillus, Penicillum* and *Fusarium* (Pereira *et al.*, 2014). These fungi proliferate and may produce mycotoxins, plant pathogens which have been found in foods and feeds, characterized by high and varied toxicity for humans and animals (Szumski *et al.*, 2014). The ability of these molds to produce mycotoxins is greatly influenced by environmental factors, being the most important temperature, relative humidity, insect damage, drought and inadequate storage conditions (Rodríguez-Carrasco *et al.*, 2013). They commonly enter the food chain through contaminated crops, mainly cereals. Hundreds of classes of mycotoxins have already been identified but one of the most important ones, regarding their occurrence and toxicity, is the group of aflatoxins (AFs). AFs are difuranocoumarin derivatives produced by a polyketide pathway by some *Aspergillus* species, namely *A. flavus* and *A. parasiticus* (Turner *et al.*, 2009). Also, the rare *A. nomius* can contaminate a wide range of agricultural products (Beizaei *et al.*, 2015). Although more than 20 AFs have been identified, only aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) are classified as human's carcinogens (Beizaei *et al.*, 2015).

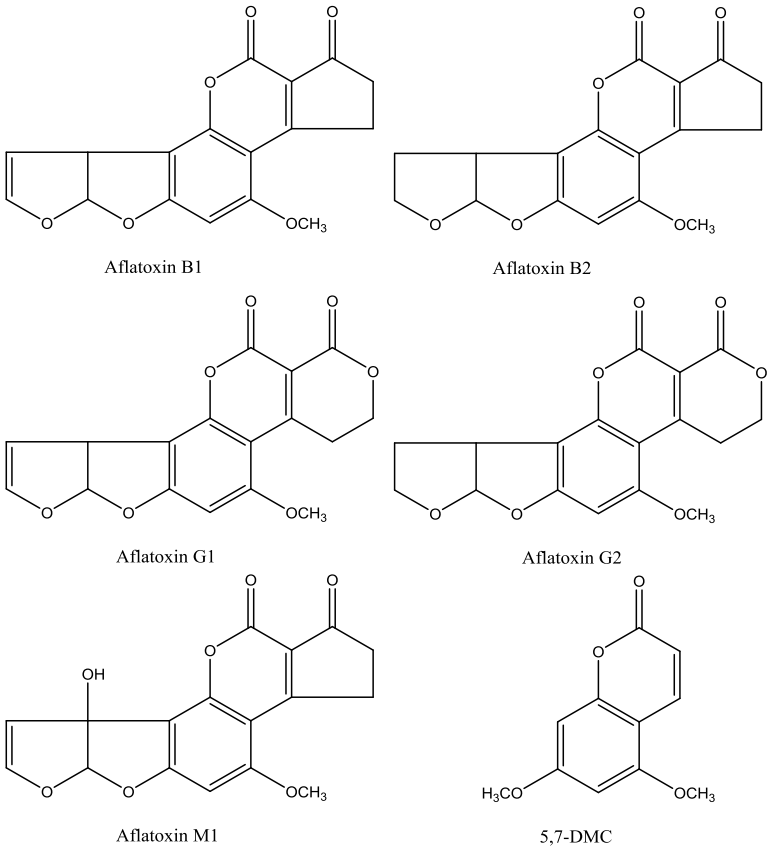

Figure 1: Chemical structures of selected mycotoxins (aflatoxins) and dummy template for MIP synthesis.

In humans, exposure to AFs can cause chronic and acute toxic effects (carcinogenic, mutagenic, teratogenic and immunosuppressive) or even death. Aflatoxin M1 is a major metabolite of AFB1 in humans and animals, and as such it may be present in milk from animals fed with B1 contaminated feed. The toxicity is about ten times lower than B1, but it also has hepatotoxic and carcinogenic effects. The presence of AFM1 in milk and dairy products would have negative health implications for consumers, particularly for infants and children because they are considered vulnerable population, more susceptible to mycotoxin exposure than adults (Juan *et al.*, 2014). The potential health risk of any contaminant in foods for infants is three times higher than for adults. As a consequence, the European Commission has set the maximum levels of AFB1 for processed cereal-based foods and baby foods for infants and young children at 0.1 µg Kg^{-1} (EC, 2010). The limit of AFM1 in milk and infant formulas and follow-on formulas, including infant milk and follow-on milk, was set at 0.05 µg Kg^{-1} and 0.025 µg Kg^{-1}, respectively (EC, 2010).

Many sensitive methods for the analysis of aflatoxins in foods and feed have been developed, using for instance TLC, HPLC or immunoassays (Xiulan *et al.*, 2005). The most popular technique for determining aflatoxins is HPLC with fluorescence or mass spectrometric detection (Turner *et al.*, 2009; Jaimez *et al.*, 2000). Solid-phase extraction (SPE), clean-up with organic solvents or immunoaffinity columns (IAC) have been frequently applied to isolate aflatoxins from cereals and/or milk (Turner *et al.*, 2009). In recent decades, molecularly imprinted polymers (MIP) have become very popular and promising materials for extracting different analytes that are present in food (Regal *et al.*, 2012; Díaz-Bao *et al.*, 2015). However, aflatoxins are too toxic to be used as templates in MIP preparation. Template bleeding may be an additional problem, especially when dealing with very low levels. In this work, a rapid and selective extraction method based on magnetic molecularly imprinted polymers (MMIP) has been developed for the isolation of aflatoxins (B1, B2, G1, G2, M1) using a dummy template. The successful MMIP stirring "cake" has been combined with HPLC-MS/MS for the determination of AFM1 in milk powder (infant formulas) to demonstrate its applicability.

Experimental

Materials

The standards for aflatoxins B1, B2, G1, G2, and M1 were purchased from Sigma-Aldrich Chemical Company (Madrid, Spain). They were suspended in methanol, resulting in the desired concentrations for each analysis. The chemicals to prepare magnetite $FeCl_2$, $FeCl_3$ and $NH_3 \cdot H_2O$ (25%) were purchased from Sigma-Aldrich Chemical Company (Madrid, Spain). The chemicals used for the polymers synthesis were 5,7-dimethoxycoumarin (DMC) as dummy template, methacrylic acid (MAA), 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. HPLC grade solvents were supplied by Merck (Madrid, Spain).

Apparatus

The recoveries were calculated using LC-MS/MS. Separation was performed in an 1100 series HPLC system from Agilent Technologies (Minnesota, USA). A Luna 3 µm C18 (150 x 2 mmm) column from Phenomenex (Torrance, CA, USA) was used. The mobile phase was water with 0.2% formic acid (A) mixed on a gradient mode with methanol (B) at a flow rate of 250 µL min^{-1}. After the first 3 minutes with very aqueous mobile phase at 95% (A), binary gradient mixing was initiated as follows: (A) from 95% to 0% in 2 min, isocratic at 0% for 5 min, and from 0% to 95% again in 3 min, at this point the gradient was kept isocratic for 2 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 identity confirmation (Table 1).

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

Compound	Mw	Precursor ion	Fragment ion	CE *
AFB1	312.06	312	240	51
			284	23
AFB2	314.07	314	258	45
			287	41
AFG1	328.06	328	242	43
			199	57
AFG2	330.07	330	312	23
			189	59
AFM1	328.00	328	273	25
			229	55
DMC	206.19	206	192	25
			121	55

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

Magnetite preparation

The Fe_3O_4 magnetite was synthesized by the coprecipitation method: 0.01 mol of $FeCl_2 \cdot 4H_2O$ and 0.02 mol of $FeCl_3 \cdot 6H_2O$ were dissolved in 80 mL of water in a three-necked reactor (250 mL). The mixture was stirred and when the temperature increased to 80ºC, 10 mL of $NH_3 \cdot H_2O$ (25%) were added to the mixture. This reaction lasted 1 h and remained at 80ºC. When the temperature dropped

to room temperature, the obtained magnetic precipitates were isolated from the solution by an external magnetic field and washed with deionized water several times until it was neutral. Magnetite was let dry overnight.

Polymerization mixture

The template (DMC, 0.1 mmol) was dissolved in a combination of toluene with methanol (90:10, v/v), 0.4 mmol of MAA were added and the solution was stirred for 30 min for the preparation of the pre-assembly solution. Then 2 mmol EGDMA and 0.25 mmol AIMN were added to the previous solution. Immediately, the pre-polymerization mixture was combined with magnetite to prepare the MMIPs stirring cake, using bulk polymerization.

Table 2. Combination of template (*dummy template), functional monomer, cross-linking monomer, initiator and porogen used to obtain a magnetic molecularly imprinted stir-bar, the so-called "stirring-cake", to isolate aflatoxins from food.

ROLE	COMPOUND	AMOUNT
Template*	5,7-Dimethoxycoumarin (DMC)	0.1 mmol
Monomer	Methacrylic acid (MAA)	0.4 mmol
Cross-linker	Ethylene glycol dimethacrylate (EGDMA)	2 mmol
Initiator	2,2'- azobis-(2-methyl-butyronitril) (AIMN)	0.25 mmol
Porogen	Toluene/Methanol (90:10)	1 mL
Magnetic component	Magnetite	0.7 g

Preparation of MMIPs

A total amount of 0.7 g of freshly prepared magnetite was placed in an injection vial (1.5 mL) and half of the total volume of the vial was completed with the pre-polymerization mixture previously described (Table 2). The combination was introduced into a temperature controllable incubator (Stuart Scientific, Redhill, Surrey, UK), horizontally, with the vial lying down along its longitudinal axis, at 60 ºC for 24 h. After (bulk) polymerization, the glass vial was broken to release the polymer. The MMIP had the aspect of a 'cake', where the polymer gives shape to a semi-cylinder filled with magnetite (Figure 2). In parallel, a magnetic non-imprinted polymeric (MNIP) stirring 'cake' was prepared using the same pre-polymerization mixture and processed similarly, but without the addition of template. The obtained magnetic polymers were washed with methanol and water. Then, they were placed in a beaker and covered with water and methanol (50/50, v/v), and their stirring capacity was

tested using a magnetic stirrer, demonstrating that these MMIP/MNIP would stir liquid samples effectively. The template molecule was removed from the polymeric matrix by Soxhlet extraction with MeOH/HAc (1:1) during 12 hours, or until the template molecule could not be detected by LC-MS/MS.

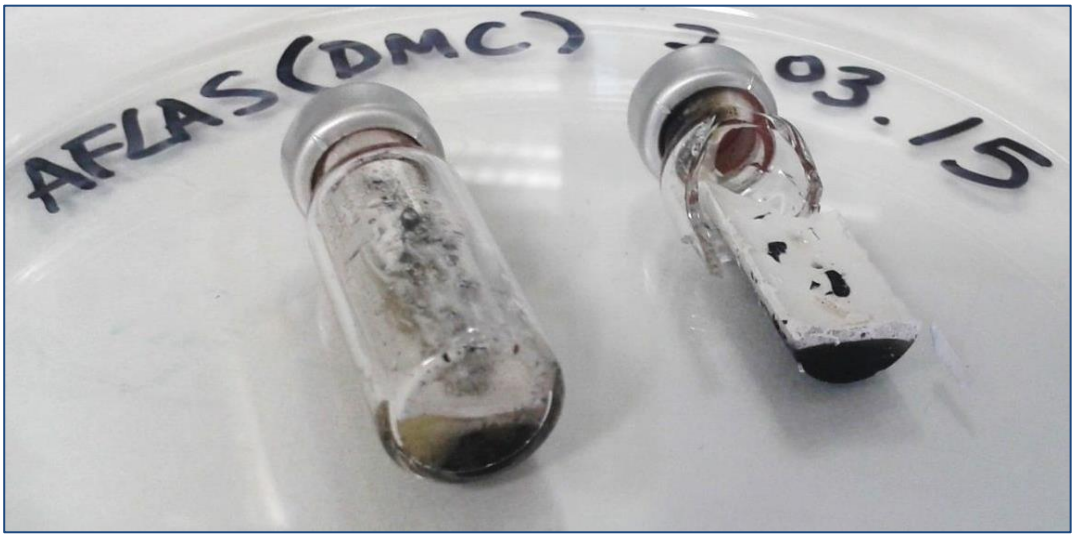

Figure 2: Magnetic molecularly imprinted polymer (MMIP) and magnetic non imprinted polymer (MNIP) with stirring ability, developed for the extraction of aflatoxins using dimethoxycoumarin as dummy template.

MMIP solid-phase extraction of milk samples

Several experiments, using different loading, washing and elution solvents, were performed in order to optimize the extraction protocol for aflatoxins in milk (Table 3). A standard solution of DMC was used for these experiments.

Milk power samples were extracted with the selected MMIP protocol, as detailed below. Infant formulas were reconstituted according to the manufacturer's instructions. Instead of water, 1% formic acid (aq.) was used as solvent. Thus, 1 g of powdered milk was dissolved in 3 mL of 1% formic acid and spiked with AFM1 at 1 ng kg^{-1} of powder. The reconstituted formula was shaken vigorously by hand for 30 seconds and placed in an ultrasonic water bath for 20 min. After centrifugation at 5000 r.p.m for 10 min, the upper cream layer was discarded. Two extractions were performed with chloroform (3 mL) and chloroform was evaporated under nitrogen stream to subsequently redissolve the extract in 1 mL of Milli-Q water. In a second clean-up step, the aqueous extract was placed in a baker containing the developed MMIP stirring ‘cake’, plus 3 mL of water to cover the bar. The sample was stirred for 45 minutes with the MMIP bar, as if it were a conventional magnetic stir-bar. With the aid of an external magnet, the remaining sample was removed by simply emptying the baker. Afterwards, 3 mL of water were added to wash the bar for 10 min under stirring. After

removing the washing solution, the analyte was eluted from the polymers using 3 mL of 75/25 MeOH/AcH (v/v) and stirring 45 minutes. The elution was evaporated under nitrogen stream and redissolved in 80 µL of mobile phase for injection. Sample was passed through a (PTFE 0.22 µm, Waters) filter to remove any impurities, and 30 µL were analyzed.

Results and discussion

A novel stir-bar was developed using a combination of magnetite and molecularly imprinted polymers, polymerized inside an LC injection vial and under bulk polymerization conditions. The resulted bulk semi-cylindrical polymer would have small pieces of magnetite inside, as if it were a nuts 'cake', resulting in magnetic stirring properties (Figure 2). The DMC was used as structural analogue of aflatoxins for MIP design (template molecule), based on previously published results (Szumski *et al.*, 2014). During the optimization of the MMISPE protocol, DMC was also used as target analyte to seek for the higher recoveries of analyte. This approach was preferred to minimize the manipulation of mycotoxins, because these analytes could be hazardous to the personnel working in the laboratory. Several experiments were carried out using different solvents and/or loading times (Table 3). The elution times were always the same as for loading.

Table 3. Experiments performed using the MMIP stirring cake and DMC in order to optimize the extraction protocol for AFs determination in food matrices.

MMISPE STEP	Exp.1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8	Exp. 9*
Loading	MeOH	Tol/MeOH (90:10)	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
Washing	MeOH	Tol/MeOH (90:10)	H_2O/MeOH (5%)	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
Elution	MeOH/AcH (0.5%)	MeOH/AcH (1%)	MeOH:AcH (75:25)	MeOH/AcH (10%)	MeOH:AcH (50:50)	MeOH:AcH (75:25)	MeOH:AcH (75:25)	MeOH:AcH (75:25)	MeOH:AcH (75:25)
Time	10´	5´	10´	10´	10´	15´	10´	30´	45´
Recovery (%)	4	0	43	32	61	43	28	68	97

* Selected MMIP solid-phase extraction protocol

A minimum loading time of 45 minutes was required to obtain maximum recoveries of DMC. This fact was already expected, as it is usually difficult to reach the active sites/pores of bulk polymers. The use of water as loading and washing solvent gave better results than methanol and/or toluene (or combinations) and it was also preferred because of its lack of toxicity. A 25% of acetic acid in methanol was enough to elute the analyte from the bulk polymer.

Aflatoxin M1 was selected as the main aflatoxin of interest in milk and as such, further experiments were performed with the MMIP bar in milk powder spiked with AFM1. Milk powder samples were extracted first with chloroform, and second with a MMIP solid-phase protocol (MMISPE), to obtain a final extract as clean as possible. Instead of water, 1% formic acid (aq.) was used to dissolve the milk powder. This acidic water permitted eliminating most fat from the sample. After extracting twice with chloroform, the selected MMIP protocol (exp. 9 in Table 3) was applied to milk samples spiked with AFM1 in order to demonstrate the applicability of this novel polymeric stirring cake. The successful extraction obtained with AFM1 also proved the adequacy of the selected dummy template as structural analogue of AFs. Figure 3 shows a chromatogram obtained from a milk powder sample extracted with the above mentioned protocol (A) and a chromatogram of a standard solution of aflatoxins.

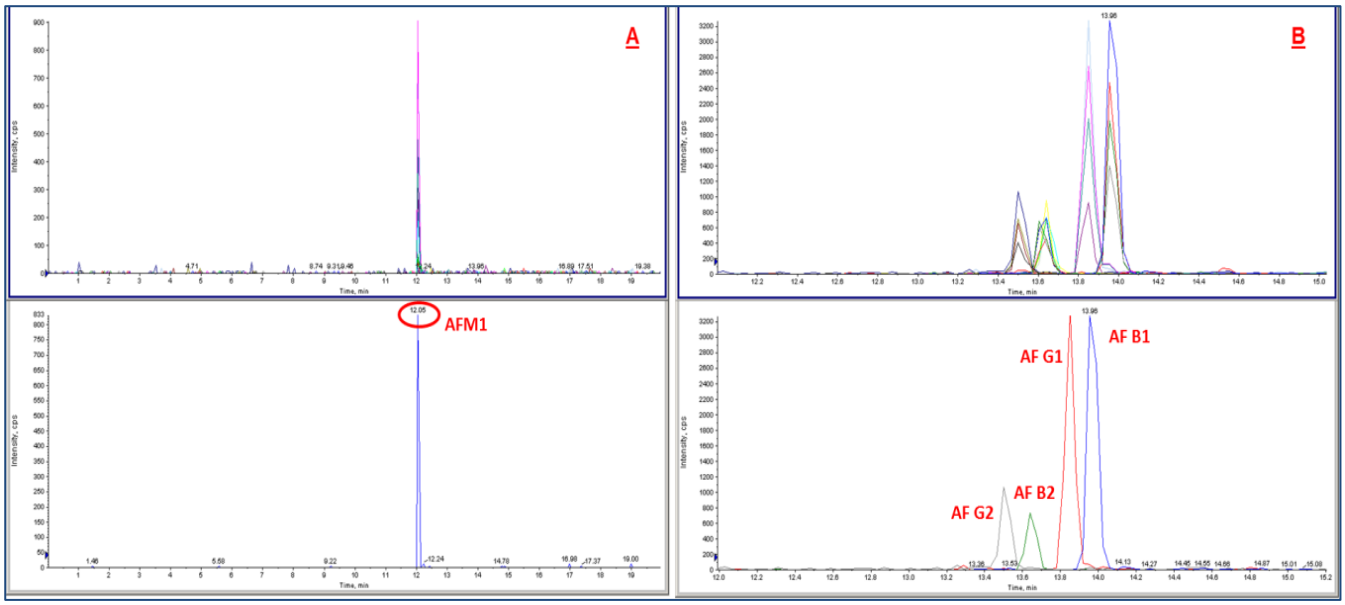

Figure 3. A) Chromatogram of AFM1 extracted from milk powder (1 ppb) using the MMIP stirring cake. B) Chromatogram showing a standard solution of AF B1, G1 (100 ppb) and B2, G2 at (30 ppb).

Conclusions

The extraction efficiency of these novel polymeric and magnetic stirring "cakes" was confirmed at a level of 1 ppb for M1 in infant formulas. The required legal limit for aflatoxin M1 in infant formulas is 0.025 ppb. To reach this low level further studies are required, including for example the use of more initial sample amount or even additional pre-concentration steps. In the future, these polymeric devices will be also tested for determining AF B1, B2, G1 and G2 in cereal-based products for babies, in order to extend the study.

Acknowledgements

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