

Magnetic molecularly imprinted stirring bar for isolation of patulin using grafting technique

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Abstract

Mycotoxins are low-molecular-weight natural products with great structural diversity produced as secondary metabolites by fungi. One of the principal toxic fungal metabolites is patulin, produced by over 30 genera of mold including species as *Penicillium expansum* or *Penicillium griseofulvum*, and normally related to vegetable-based products and fruit, mainly apple. These molds grow easily in damaged fruit or in derived products as juices if storage conditions are deficient. Some of the most serious effects of patulin ingestion are agitation, convulsions, edema, ulceration intestinal, inflammation and vomiting. Thus, European Regulation 1881/2006 established a maximum content of 10 ppb in infant fruit juices, 50 ppb for fruit juices in adults and 25 ppb in fruit-derived products. Nowadays, the official analytical method adopted by AOAC International is HPLC with UV detection, using clean-up with ethyl acetate and sodium carbonate. However, the diverse drawbacks of this method (poor stability of patulin under alkaline extraction, poor resolution between patulin and co-extracted hydroxymethylfurfural) have originated interest in alternative options, such as LC methods coupled to mass spectrometry. In the last years, purification with molecularly imprinted polymers started to be used and they are becoming promising materials in analytical chemistry. In the present work, a selective magnetic molecularly imprinted stir-bar has been developed for the rapid isolation of patulin. A structural analogue, 2-oxindole, was used as dummy template. The polymer was grafted to the silanized glass surface of the stir-bar.

Keywords: patulin; MMIP; stir-bar; grafting; HPLC-MS/MS; apple

Introduction

Mycotoxins are low-molecular-weight natural products with great structural diversity produced as secondary metabolites by fungi. Patulin (PAT) is an important toxic metabolite or mycotoxin produced by over 30 genera of mold such as *Penicillium*, *Aspergillus*, and *Byssoschlamys*. Particularly, *Penicillium expansum* is known as the main source of PAT and it is commonly associated with apple rot (Desmarchelier *et al.*, 2011). Although, PAT is mainly detected in apples and derived products, it is also found in barley, wheat, corn, silage, pears, grapes and peaches (Smet *et al.*, 2011). These molds grow easily in damaged fruit or in derived products as juices, if storage conditions are deficient. Some of the most serious health effects of PAT ingestion in humans are agitation, convulsions, edema, intestinal ulceration, inflammation and vomiting (Silva *et al.*, 2007). The toxicity of these molecules has led to the setup of strict regulations in many countries for their control in food and feed, and the consequent establishment of official legislation. The establishment of maximum limits in some food products resulted in an increasing demand of sensitive, selective and effective analytical methods (Pires *et al.*, 2012). Thus, the European Commission Regulation (EC) 1881/2006 established a maximum content for PAT of 10 ppb in infant fruit juices, 50 ppb for fruit juices in adults and 25 ppb in fruit-derived products (EC, 2006).

For the determination of PAT, thin layer chromatography was firstly used. Nowadays, the official analytical method adopted by the Association of Official Analytical Chemists (AOAC) for the analysis of PAT in food is HPLC with UV detection, using an extraction with ethyl acetate and clean-up with sodium carbonate (Brause *et al.*, 1996). The main drawback of UV detection is the poor resolution between PAT and other co-extracted compounds such as hydroxymethylfurfural. As an additional problem, PAT is vulnerable to the alkaline conditions of the previously mentioned extraction method. Purification by solid-phase extraction (SPE) has been frequently applied as an alternative procedure. More recently, LC methods coupled to mass spectrometers are preferred in routine because of their great selectivity and sensitivity (Moake *et al.*, 2005). In the last years, molecularly imprinted polymers (MIPs) started to be used, and are becoming promising materials for extracting different analytes present in food (Díaz-Bao *et al.*, 2015; Regal *et al.*, 2012). Currently, SPE using MIPs (MISPE) is extensively used for the extraction of various biologically active compounds. However, mycotoxins are usually too toxic or too expensive to be used as template molecules in MIP preparation. Template “bleeding” may be an additional problem of these polymers, especially when dealing with very low detection levels. In the present work, a rapid and selective method based on an in-house designed magnetic molecularly imprinted stir-bar (MMISB)

has been developed for the isolation of PAT. For MIP synthesis, a structural analogue of PAT, 2-oxindole, was used as dummy template (Figure 1) (Khorrami & Taherkhani, 2011; Smet *et al.*, 2011). The molecularly imprinted polymer was grafted on the silanized surface of a glass-covered stir-bar using an adaptation of typical protocols used in grafting techniques. The applicability of this novel stirring bar for the extraction of PAT has been tested in spiked of apples using HPLC-MS/MS for detection.

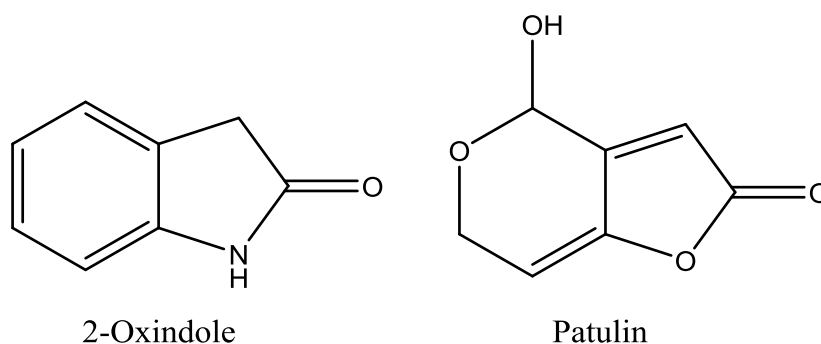


Figure 1: Chemical structures of dummy template and patulin.

Experimental

Materials

The standard for PAT was purchased from Sigma-Aldrich Chemical Company (Madrid, Spain). PAT was suspended in methanol, resulting in the desired concentration for the analysis. The chemicals used for the polymers synthesis were 2-oxindole as dummy template, 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. DVB-80 was freed from stabilizers by passing through a small column packed with neutral alumina (Aldrich). The 3-(methacryloxy) propyltrimethoxysilane was purchased from Sigma-Aldrich Chemical Company. HPLC grade solvents were supplied by Merck (Madrid, Spain).

Apparatus

These polymerization mixtures were simultaneously introduced into a temperature controllable incubator (Stuart Scientific, Redhill, Surrey, UK) at 60 °C for 12 hours. The analyte recoveries were

calculated using HPLC-MS/MS. Separation was performed in an 1100 series HPLC system from Agilent Technologies (Minnesota, USA). A Luna 3 μm C18 (150 x 2 mm) column from Phenomenex (Torrance, CA, USA) was used. The mobile phase was water and methanol with 0.1% formic acid, mixed in isocratic mode at 70% and 30%, respectively; the analytical run lasted for 10 min at 250 $\mu\text{L}/\text{min}$. A Q-Trap 2000 mass spectrometer with ESI Source from AB Sciex (Toronto, Canada) was used, working in negative mode for PAT and in positive mode for 2-oxindole. For quantification of PAT, the most intense MRM transition was monitored along with a second transition for identity confirmation (Table 1).

Table 1. MRM transitions monitored for PAT detection in ESI negative mode, using HPLC-MS/MS.

MS/MS transition (m/z)	Used for
153>109	Quantitation
153>81	Confirmation

Design of molecularly imprinted stir-bars (MMISB)

To achieve a stir-bar grafted with molecularly imprinted polymer on its surface, a chemical coating protocol adapted from the work of Turiel and Martín-Esteban was used (Turiel & Martín-Esteban, 2012). First, a commercial glass-covered magnetic stir-bar remained in a combination of methanol and hydrochloric acid (1:1, v/v), stirring for 30 min for clean-up of the glass surface. Next, the surface was silanized with 2% 3-(methacryloxy) propyltrimethoxysilane in toluene for 1 hour (Cras *et al.*, 1999). Finally, the stir-bar was rinsed methanol and let dry under N_2 stream.

Once the silanized glass surface was ready, the chemical coating process took place as follows. In a glass tube of 4 mL, the bar was placed and covered with the pre-polymerization mixture (Table 2). The stirring bar immersed in the monomeric recipe was allowed to polymerize at 60 $^{\circ}\text{C}$ for 12 hours, or until the appearance of the polymer was completely white. After (bulk) polymerization, the glass tube was broken to release the polymer-coated stir bar (Figure 2). Then, the bars were placed in a beaker and covered with water and methanol (50/50, v/v) to test the adhesion to the surface and the resistance of the polymer, using a magnetic stirrer. Next, the template molecule was removed by Soxhlet extraction for 12 hours with methanol/acetic acid (1/1) solution. To optimize the conditions of use of this MMISB for the extraction of PAT, different loading, washing and

elution solution were tested. A magnetic non-imprinted stir bar (MNISB) was prepared in parallel, without the addition of template.

Table 2. Pre-polymerization mixture used to elaborate molecularly imprinted stir-bars and non-imprinted bars (no template addition) for PAT extraction.

PRE-POLYMERIZATION MIXTURE		
Function	Component	Ratio
Dummy template	2-oxindole	1
Functional monomer	MAA	4
Cross-linker	EGDMA	20
Function	Component	Proportion
Porogen	Toluene/Methanol (90:10)	40% wt.
Initiator	AIMN	2% wt.



Figure 2: Magnetic molecularly imprinted stir bar (MMISB) developed for the extraction of patulin; the screw cap was placed below the stir-bar to illustrate its size.

Application to real samples

The optimized MMISB extraction protocol started adding water to cover 3 g of diced apple in a 50 mL tube. Apple samples were spiked with water containing PAT at 50 ppb (MRL to for fruit juices in adults) and introduced into an ultrasound bath for 30 min. Samples were shaken for 10 min,

centrifuged at 5,000 g for 10 min and the supernatant was transferred to a baker with the stir bar. A conventional SPE protocol was applied, consisting of loading, washing and elution steps with different solvents. The analyte was loaded within the pores (active sites) of the MMISB under stirring for 30 min. After this sorptive extraction, water was removed with the aid of external magnet and stir-bar was washed with water for 5 min. Then, to elute the retained patulin in MMISB, 5 mL methanol/acetic acid (75/25) were added and kept stirring for 30 min. Finally, the eluate was evaporated under nitrogen stream at 30 °C and re-dissolved in 100 µL of mobile phase. Twenty microliters were immediately injected into the chromatographic system for analysis. Recoveries were calculated using HPLC-MS/MS.

Results and discussion

A structural analogue of patulin, 2-oxindole, was used as a dummy template to synthesize a MIP capable of selectively binding the molecule of PAT (Khorrami & Taherkhani, 2011). The obtained cavities in the prepared MIP are supposed to have a complementary shape and electronic affinity to the template. This dummy-template approach was preferred to minimize the manipulation of patulin, because this type of analytes can be hazardous to the personnel working in the laboratory. The selected polymerization technique was bulk polymerization, which it does not require sophisticated instrumentation and because the reaction conditions can be easily controlled. Furthermore, it is the most widely used method for the preparation of polymers (Regal *et al.*, 2012).

In the design of any pre-polymerization mixture, the selection of cross-linker and porogen solvent are two key factors. These two components would determine the aspect, strength and even the color and porosity of the final polymer. DVB-80 and EGDMA, two of the most common cross-linkers used for MIP synthesis, were tested in combination with various combinations of toluene with methanol and/or acetonitrile as porogenic solvent. The different combinations of monomer-solvent were introduced in an injection vial and let polymerize under bulk conditions for 24 hours at 60 °C. After polymerization, the glass vials were broken and the resulting material was tested visually and by touching. With the use of DVB-80 as cross-linker, it was impossible to obtain a strong and tough polymer. The resulting polymer would be a plastic, yellowish, cracked and fragile material, not suitable for resisting the required stirring conditions. Thus, adequate divinylbenzene polymers could not be achieved using the different porogens solvents (acetonitrile or methanol), even changing the toluene percentage (from 0 to 90 % toluene). As for EGDMA, the polymers were in every case stronger and wither than those obtained with DVB, showing more resistance to compressive strength and even more when using methanol (combined with toluene) instead of acetonitrile. The

mixture of methanol and toluene (90:10) with EGDMA proved to be useful, resulting in a hard and white polymer. Consequently, this combination was used for MMISB preparation.

Then, the efficiency of the obtained polymer was evaluated with a very simple extraction protocol, for the isolation of PAT from apple samples prior to analysis. The protocol consisted simply on pouring-in, stirring and pouring-out solvents from a beaker. To carry out the extraction process, it was only necessary to have a magnetic stirrer and an external magnet. Satisfactory recoveries of PAT were obtained using this molecularly imprinted glass-covered stir bar, with 60-70% of recovery in a minimum loading time of 45 min under stirring. Besides, the deterioration of the polymeric coat was imperceptible after several hours of use, indicating that the adhesion of the polymer was strong and stable. The glass layer as cohesion agent between the bar and the polymeric coat can be assumed. NIP stir bars showed a mean recovery difference of 20% less.

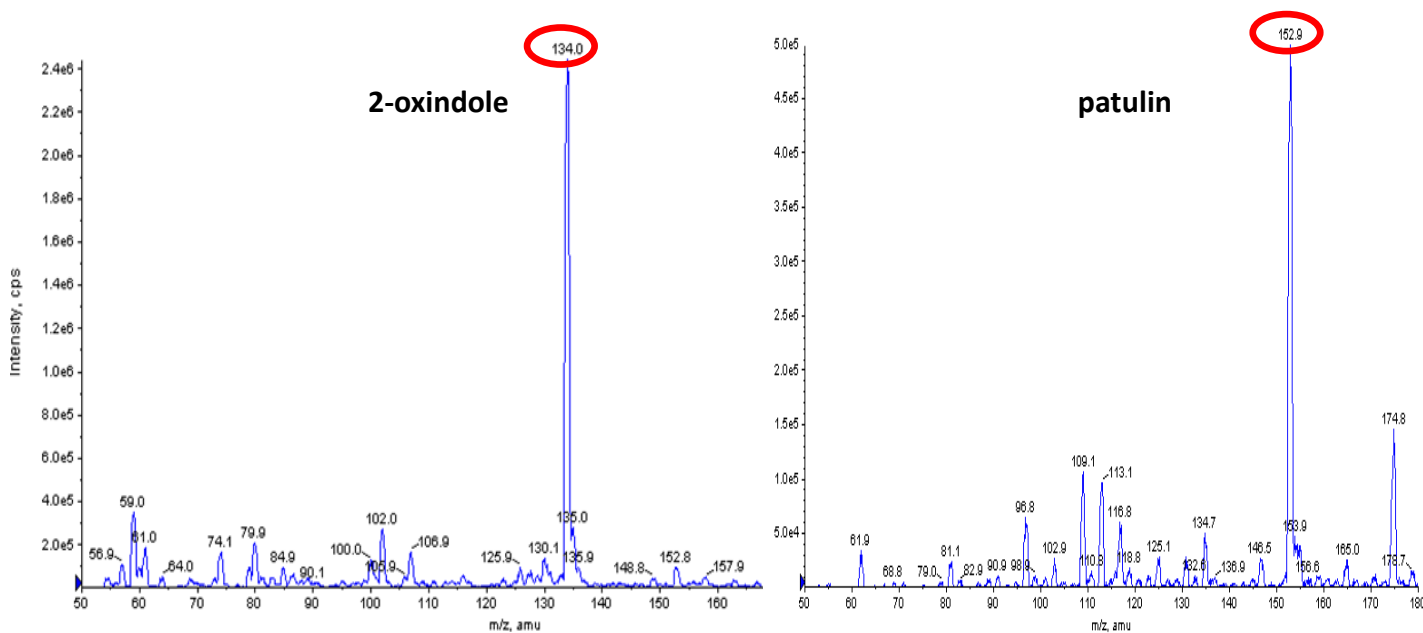


Figure 3. MS spectrum obtained from a standard solution of PAT infused in the MS using ESI negative mode, and 2-oxindole using ESI positive mode, showing the structural similitudes.

Conclusions

The proposed magnetic extraction has demonstrated to be useful for the isolation of PAT in apple at 50 ppb. The stir-bars are easy to use and the extraction protocol was reduced to simply add and remove solvents from a beaker, with the aid of a magnetic stirrer and an external magnet. MIPs offer a broad range of cheap, versatile and selective pretreatment solutions for food analysis. The polymer coated on the surface of magnetic stir-bars was prepared in a non-covalent approach, using

a dummy template to avoid “template bleeding” and to limit the exposure of laboratory staff to mycotoxins. In this case we selected 2-oxindole, structurally related to PAT molecule (Figure 3), as template for the design of a MIP selective towards PAT.

Acknowledgements

This research was supported by the Project for Emerging Researchers EM 2012/153 from Consellería de Cultura, Educación e Ordenación Universitaria (Xunta de Galicia).

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