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Development of a computationally-designed polymeric adsorbent specific for mycotoxin patulin

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Patulin is a toxic compound which is found predominantly in apples affected by mould rot. Since apples and apple-containing products are a popular food for the elderly, children and babies, the monitoring of the toxin is crucial. This paper describes a development of a computationally-designed polymeric adsorbent for the solid-phase extraction of patulin, which provides an effective clean-up of the food samples and allows the detection and accurate quantification of patulin levels present in apple juice using conventional chromatography methods. The developed bespoke polymer demonstrates a quantitative binding towards the patulin present in undiluted apple juice. The polymer is inexpensive and easy to mass-produce. The contributing factors to the function of the adsorbent is a combination of acidic and basic functional monomers producing a zwitterionic complex in the solution that formed stronger binding complexes with the patulin molecule. The protocols described in this paper provides a blueprint for the development of polymeric adsorbents for other toxins or different food matrices.

Introduction

Patulin, 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one, is a secondary metabolite and mycotoxin produced by groups of fungal species such as Aspergillus (*A. amstelodami)*, Penicillium (*P. aurantiogriseum*, *P. chrysogenum*, *P. expansum*, *P. roqueforti*) and Byssochlamys genera.[1,2] Patulin is largely found in the tissues of rotting apple where it can even penetrate approximately one centimetre into the proximate healthy tissue. On an industrial scale this inconvenience presents a serious risk of contamination of apple-based products with patulin.[3] The most common product that is contaminated by patulin is apple juice. Patulin is soluble in water, which is the main component of apple juice.[4] Patulin is also stable in acidic conditions and heat.[5,6] Thus, patulin is not destroyed during the manufacturing process of apple juice.[6]

Humans are mainly exposed to patulin via consumption of contaminated food products. In addition to apples, patulin can be found in barley, wheat, corn, pears, peaches and products derived from them. Nevertheless, apples and apple juice are among the fruits and fruit-based products most regularly consumed by largest consumer group which ranges from children to elderly.[7] Patulin has been found to be an acutely toxic, genotoxic, teratogenic and possibly immunotoxic compound.[8] Though the studies carried out have been solely animal based, there is great concern that the symptoms observed in animals may be similar those of humans if there is regular or excess intake of patulin. The International Agency for the Research on Cancer (IARC) has reviewed suggestions that patulin may be a carcinogen even at low intake levels.[9] Due to the health risk posed by dietary exposure, a provisional maximum tolerable daily intake (PMTDI) of patulin is set as 0.1 μg kg^{-1} body weight/day for children and 0.2 μg kg^{-1} body weight/day for adult by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA).[10] The European Union has regulated that patulin level in fruit juices, concentrated fruit juices and fruit nectars not surpass a limit of 50 μg kg^{-1}. The legal patulin level in apple juice and other apple-based products for infants and young children is limited to 10 μg kg^{-1}.[11]

Due to the varied levels of patulin found in apple-based products its detection and quantification are very important. The most predominantly used method of patulin quantification is a high-performance liquid chromatography (HPLC) set-up equipped either with a UV-vis detector at a wavelength of 276 nm or with a mass-spectrometry detector. However, due to the complexity and variability of the food samples tested, the precision and limit of detection of the measurement heavily depends on availability of clean-up materials and the effectiveness of the purification protocols[12].

Immuno-affinity columns (IACs) are currently considered as essential tools in the purification of all mycotoxins, except patulin. Due to the high toxicity of patulin, production of patulin-specific antibodies is problematic; therefore, no commercial IACs for purification of patulin are available. Generally, there are only a few commercial materials that are

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specifically designed to isolate patulin from food samples using a solid-phase extraction setup. One of them is the AFFINIMIP™ cartridge produced by Polyintell Ltd. (France) which has reported recoveries of 90.6% and 80% when using samples spiked with 40 ng mL^{-1} of patulin (testing by POLYINTELL and Gilson, Inc. respectively).[13,14] However, since the conventional method of producing MIPs includes the presence of a template molecule, there is a high chance that some trace amounts of the template are left inside the polymeric network even after washing. In addition to the fact that the binding sites within the polymer are occupied by unremoved template molecules and therefore cannot be involved in the re-binding during extraction, the greater problem is that the template may "bleed" into the filtrates and eluents used for analysis.[15] Other potential drawbacks of MIP production include the high cost of the resin, mainly due to the cost of the template, the necessity to have an intensive washing cycle and the high price of disposal of toxin-contaminated manufacturing waste.

In order to overcome this problem, we have developed a protocol for designing an adsorbent possessing a high binding affinity towards patulin under the required conditions but does not involve the use of the toxic template for its manufacturing. Computational modelling is used to select the functional monomers possessing natural affinity towards the compound of interest in combination with quick screening of a limited number of polymers under the required extraction conditions, allowing the rapid production of an effective and inexpensive material suitable for patulin purification and analysis in complex food matrices. Since a template is not used in the synthesis of the polymers there is no any occurrence of false-positive results due to the bleeding of the template. This paper also describes the optimisation of the purification protocols which could be used in combination with the developed specific adsorbent in order to detect and quantify the amount of patulin present in apple juice.

Experimental

Materials

Patulin, 2-acrylamido-2-methylpropane sulfonic acid (AMPSA), 2-vinylpyridine (2-VP), diethylaminoethyl methacrylate (DEAEM), hydroxyethyl methacrylate (HEM), ethylene glycol methacrylate phosphate (EGMP), divinylbenzene (DVB) and ethylene glycol dimethylacrylate (EGDMA), and initiator 1,1'-azobis(cyclohexanocarbonitrile) (ABCN) were purchased from Sigma-Aldrich (Gillingham, UK). Methanol and dimethylformamide (DMF) were purchased from Thermo Fischer Scientific (Rugby, UK). 1-mL empty PE cartridges and polyethylene frits were from Supelco, UK.

Molecular design of the patulin-specific polymers

Monomer-template interactions were analysed using the SYBYL 7.3 (Tripos Inc. ST. Louis, MO, USA) molecular modelling software package in conjunction with the SPECTRE operating system at the University of Leicester on a HP Compaq 8200 elite ultra slim desktop computer. A 2-dimensional model of patulin was downloaded from the PubChem database and SYBYL was used to minimise the energy of the molecule to obtain the 3-dimensional characteristics of the lowest energy conformation. The energy minimisation was conducted until it reached the minimum value of 0.01 kcal mol^{-1}. The selection of the functional monomers has been performed using a protocol developed by the Leicester Biotechnology Group.[16,17]

The Leapfrog algorithm was applied to screen a virtual library of 25 commercially-available functional monomers against the patulin molecule in order to investigate interactions with functional monomers and calculate their corresponding binding energies. In order to examine fully the interactions with the template and selected functional monomers, 60,000 iterations were performed. The functional monomers which demonstrated the most negative values of binding energies were expected to have the strongest interactions with patulin and were selected for polymers preparation and further investigation.

Polymers preparation

The polymers were prepared by solubilising 1.26 g of the functional monomer (AMPSA, EGMP, DEAEM, 2-VP, acrylamide, HEM, DVB) with 23.8 g of cross-linker (EGDMA or DVB) and 25 g of the DMF. 25 mg of the initiator was added to the monomeric mixture. The solution was purged with nitrogen for 5 min and heated at 80 °C for 24 hours in the glass bottle tightly sealed to avoid loss of a solvent.

The polymers containing two functional monomers, so-called "copolymers", were prepared using the same procedure as the other polymers however the percentage of reagents differed. The compositions of the copolymers are shown in the Table 1.

Table 1. Compositions of copolymers.

Polymer	Functional monomers, g		Initiator, g	Solvent, g	Cross-linker, g	
	AMPSA	2-VP	ABCN	DMF	DVB	EGDMA
AVD	0.3	0.7	0.25	10	9	
AVE	0.3	0.7	0.25	10		9

The polymers were ground using an Ultracentrifuge Mill ZM200 (Retsch, UK) which was set to rotate at 10,000 rpm. Polymers particles with diameter 63-125 µm were collected using corresponding sieves (Retsch, UK) and dried in an oven at 80 °C. In order to purify the polymer by removing the unreacted monomers and fine particles, the polymer fraction was then placed in a cellulose thimble (Fisher, UK) and washed with methanol using Soxhlet apparatus for 24 h. The polymer was then dried in the oven at 80 °C for 4 hours and stored under room temperature.

Screening of the polymers using model solution of patulin

Each 1-mL SPE tube was packed with 100±5 mg of the polymers between two polyethylene frits and placed on the

SPE manifold (Phenomenex, UK) connected to a vacuum pump. All cartridges were conditioned with deionised water at a flow rate of 0.5 mL min^{-1}. A loading was conducted by filtering 1 mL of 60% methanol spiked with 10 µg mL^{-1} of patulin through each cartridge. The filtrate was collected and analysed using a spectrophotometer. The polymers demonstrating the highest adsorption of patulin from the model solution were selected for the testing with food samples.

For the extraction of patulin from apple juice, after filtering 1 mL of the sample consisting of undiluted apple juice spiked with 10 µg mL^{-1} of patulin, polymer cartridges were washed with 2 mL of deionised water in order to remove any weakly bound interfering compounds of the food matrix.

The elution of patulin from the polymer has been optimised by testing several aqueous eluents which contained increasing concentration of methanol up to 50%. The optimisation objectives were to find such balance between the washing and elution conditions which would ensure the largest recovery and highest purity of the toxin. All fractions eluted from the tested polymers were analysed using HPLC protocol which allowed the determination of patulin concentration and monitoring of purity by measuring total peak area of interfering compounds.

Chemical and physical characterisation of the polymers

UV-vis analysis of all patulin standard solutions, filtrates and eluents was performed using a PC-controlled UV1800 spectrophotometer executing the UVProbe v.2.33 software (Shimadzu, Japan). All absorption spectra were recorded between 190-500 nm using a 1-mL quartz cuvette. The polymer with the highest binding affinity towards patulin was selected for extraction of patulin from the food matrices.

The surface area of the developed polymer was characterised using a Nova 1000e series high-speed gas sorption analyser (Quantachrome, U.K.)

HPLC protocols

HPLC separations were performed using Shimadzu Prominence HPLC equipped with a UV-vis detector. A reversed-phase column (C18, 30 × 5 mm, Shimadzu, Japan) used as the stationary phase was kept at 20 °C in the column oven. The isocratic separation was performed using 20% methanol in water containing 1% (v/v) of acetic acid as a mobile phase. All HPLC measurements were done at wavelength of 276 nm. The running time for each analysis was 15 minutes and an injection volume of 50 µL. The flow rate was set to 1.5 mL min^{-1}. The column was washed for a total of 10 minutes after each analysis of apple juice-derived samples in order to ensure complete regeneration.

Calibration curves were built for the absorbance of standard patulin solutions in water and 60% methanol (v/v) in the concentration range between 0.625 and 10 µg mL^{-1}.

Results and Discussion

Molecular modelling

The molecular structure of patulin was drawn and minimised using Sybyl 7.3 software as previously described (Figure 1).

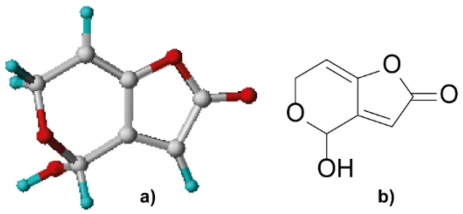

Figure 1. 3D molecular model of patulin (atoms: oxygen - red, carbon - grey and H - blue) a) 2D chemical structure of patulin.

In molecular modelling a lower binding score indicates the formation of strong interactions between the functional monomer and the patulin molecule. The functional monomers used for polymer preparation and testing are shown in Table 2.

Table 2. Binding energies (kcal mol^{-1}) observed between patulin and selected functional monomers and an experimental binding of patulin by corresponding polymer.

Functional monomers	Binding energy (kcal mol^{-1})	Patulin binding, %
-AMPSA	-44.17	12.1
-EGMP	-29.14	27.1
+DEAEM	-26.30	11.5
Acrylamide	-23.31	18.7
HEM	-15.60	13.3
2-VP	-15.41	21.7
DVB	-5.13	25.1

The polymers containing the computationally-selected functional monomers have been prepared and screened using model solutions of patulin. It was found that the majority of rationally-selected functional monomers had free amino, amine or sulphur groups. It correlated with mechanisms of molecular interaction between patulin and its biological receptors, supporting the idea of computational modelling mimicking biological interactions.[12]

Laboratory screening of the polymer library

Each prepared polymer was packed in SPE cartridges and tested for binding of patulin with the 10 µg mL^{-1} solution of patulin in 60% methanol. It was found that AMPSA, which showed the strongest binding during virtual screening (-44.17 kcal mol^{-1}), demonstrated a low binding of 12.1% (Table 2) despite containing a strong sulfonic acid group and an amide group. 2-VP had the second highest binding percentage of 21.7% despite demonstrating weaker binding energy of -15.41 kcal mol^{-1} (Table 2). It is possible to suggest that the bonding that occurs between 2-VP and patulin most likely involves some π-π interactions, which are also present during the

binding between patulin and DVB (binding of 25%). An attempt to improve the AMPSA-polymer binding was made by mixing it with 2-VP polymer. The mixture of two individually prepared APMSA- and 2-VP-based polymers (1:2, w/w) were packed in the SPE cartridge and tested, resulting in the highest binding of patulin of 38.5%. However, the tested different functional monomers were individual components rather than copolymers in solution so further investigation was done by examining the effects of the copolymerised functional monomers.

The EGMP-based polymer, which was prepared with EGDMA as a cross-linker, demonstrated the highest patulin binding of 27%, which correlated with a low binding energy of -29.14 kcal mol^{-1} demonstrated by EGMP during modelling. However, the EGMP-based polymer prepared using DVB as a cross-linker has not shown any binding to patulin. This indicates that the choice of cross-linker is very important as it affects the ability of the resulting polymer to bind to patulin.

Acrylamide had a binding energy of -23.31 kcal mol^{-1} whereas the binding energy of MBAA was -27.77 kcal mol^{-1}. This suggests that MBAA should have a higher binding affinity. However, MBAA showed no successful binding to patulin with the DVB cross-linker whereas AA with an EGDMA cross-linker gave a binding of 18.7%. The HEM monomer was not one of the lowest computationally calculated binding energies and so its poor performance was not surprising.

Another promising monomer/cross-linker was DVB which demonstrated a relatively good binding (25%) despite not being among the top-scorers in the Leapfrog table. This is possibly because the benzene rings of DVB closely pack when polymerised without the presence of a copolymer. The binding of DVB to patulin is thought to occur via hydrogen bonding between the O-H group on the patulin and the slightly polarised C-H bond on the DVB. The polarisation is caused by the electron withdrawing aromatic ring removing electron density from the C-H bond. An alternative possibility is hydrogen bonding between the hydrogen atoms on the benzyl substituent and the epoxy or carbonyl oxygen atoms on patulin. The various ratios between AMPSA and 2-VP functional monomers have been selected for preparation of co-polymer with EGDMA and DVB cross-linkers.

AMPSA/2-VP copolymer testing

Two copolymers consisting of AMPSA and 2-VP were synthesised using different cross-linkers, DVB (AVD polymer) and EGDMA (AVE polymer) to investigate the results from the previous experiments which suggest that polymers formed using the EDGMA cross-linker have superior binding to patulin when compared with polymers produced using the DVB cross-linker.

Two copolymers were then tested in their ability to bind to patulin in different solvents to examine their effectiveness in different matrices. The initial tests were carried out using 10 µg mL^{-1} patulin in 60% methanol followed by testing using the same initial concentration of patulin but with water as the solvent.

The use of different cross-linkers and the different solvents caused drastic variations in the amount of patulin that bound to the copolymers (Table 3). The percentage of bound patulin more than doubled in AVD when the methanol solution was used as the solvent compared to water (36% to 15%) however in AVE there was more binding in water than in methanol (94% to 26%). However, when comparing polymeric adsorbents AVD to AVE, where only the cross-linker was varied, they show a clear difference in their affinity to bind to patulin, so the cross-linker must be responsible for the variations in binding percentages.

Table 3. UV-vis analysis of patulin binding by copolymers AVD and AVE.

Polymeric adsorbent	Loading solvent	Filtrate		Binding, %
		Wavelength, nm	Absorbance, a.u.	
AVD	60% methanol	276.2	0.49	36
AVD	H_2O	275.6	0.90	15
AVE	60% methanol	270.0	0.57	26
AVE	H_2O	270.2	0.06	94

The patulin eluted from AVD and AVE copolymers had different absorption wavelengths (276 nm and 270 nm respectively) and this is thought to be caused by free patulin reacting with the adsorbent to form a protonated species as 2-VP is a weak base with a pKa value of 4.98 compared to AMPSA which has a pKa of 0.7 so it is a strong acid. The suggested mode of action for the AVE copolymer is the protonation of the basic 2-VP by the acidic AMPSA (Figure 2). This would lead to the formation of a zwitterionic species with a larger capacity for hydrogen bonding to patulin.

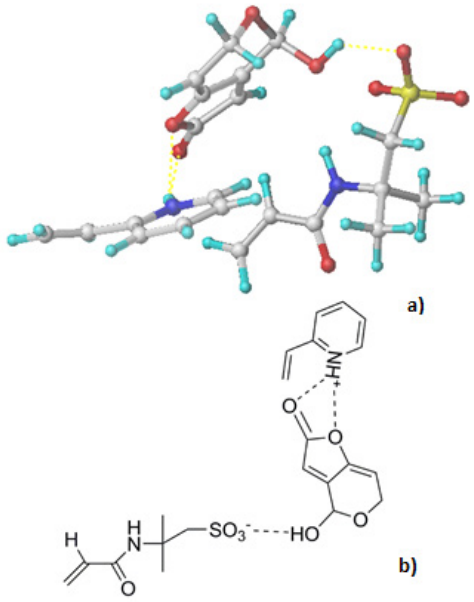

Figure 2. The molecular complex between the charged AMPSA and 2-VP functional monomers and patulin; 3D structure (a) and 2D structure (b). The hydrogen bonds are depicted as dotted lines.

AVE has demonstrated much higher binding to patulin from the aqueous solution as compared to all other tested polymers. The AVE polymer was characterised (the surface area of 146.4 m^2 g^{-1}, total pore volume of 0.03 cm^3 g^{-1} and average pore radius was 18.11 Å) and selected for the purification of patulin from the food extracts.

Eluent optimisation

For accurate detection and quantification of the extracted patulin it was necessary to optimise the eluent to ensure that other contaminants which were extracted from complex matrices, such as apple juice, were not eluted out alongside the patulin. This is of particular importance when using UV-vis as the SPE may extract compounds which are also UV active which could interfere with the patulin peak.

Table 4. HPLC analysis of patulin recovery from SPE cartridge with different eluents.

Eluent (% MeOH)	Elution, (% recovery)
0	24
10	33
20	54
30	76
40	83
50	93

The results show that as the concentration of MeOH increased, so did the percentage of patulin recovery and HPLC analysis indicated that even at 50% MeOH there was little contaminant elution but the majority of the patulin was eluted allowing for accurate quantification (Table 4). Therefore, 50% MeOH was selected as a suitable eluent for the removal of patulin from the apple juice matrix.

Extraction of patulin from natural matrices

The final testing stage involved extracting patulin from spiked food matrices such as pressed apple juice and corn extract using the AVE copolymer. In order to quantify the amount of patulin bound by the AVE polymer, and hence indicate the amount of patulin present in the sample, HPLC was used to avoid potential interference from other UV-active compounds in food matrices adsorbed by the AVE. The examples of the interfering compounds present in the apple juice include ascorbic acid and fructose which have UV-vis absorbance peaks in the wavelength range between 260 and 280 nm, similar to patulin, making UV-vis analysis of the untreated samples unreliable.

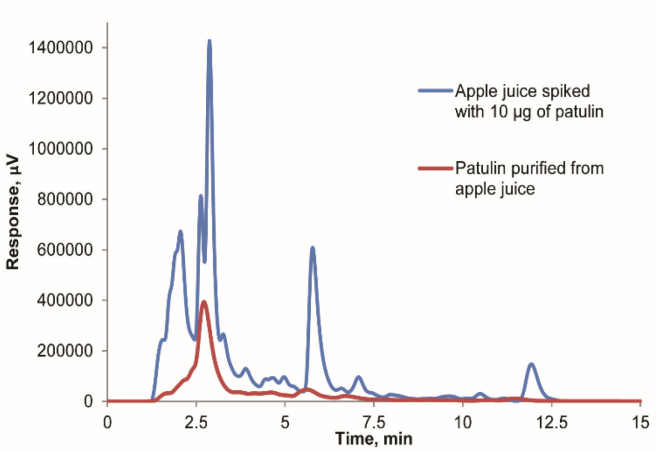

Figure 3. Typical chromatogram for apple juice spiked with patulin (in blue) and purified patulin using copolymer (in red, dilution factor x2).

Table 5. HPLC analysis: patulin binding and recovery in different matrices.

Food matrix	Binding, %	Recovery, %
Water	100	82
Apple Juice	99	93
Corn extract in 60% methanol	28	26

The results have demonstrated that the AVE copolymer was a suitable material for the extraction of patulin from apple juice and water (Figure 3). The similarity of the chromatography profiles of the patulin purified from model water sample and from undiluted apple juice confirms the selectivity of the developed polymer towards patulin. However, the low percentage of binding from corn extract in 60% methanol suggests that the extraction protocol should be further optimised to recover the patulin from other relevant food matrices (Table 5).

Conclusions

It was demonstrated that computational molecular modelling can be a useful guide in the development of polymeric adsorbents specific for a particular analyte. We have been able to show that in conjunction with UV-vis spectroscopy and HPLC the polymeric adsorbents are important tools that can be used to improve the quality of the detection and quantification of the harmful mycotoxins in commonly consumed food matrices.

This technology could be implemented to ensure that food products meet current and future safety regulations. The methods used in this paper provide a reliable and accurate protocol for the development of the polymeric adsorbents in the testing and quantification of mycotoxins, as polymeric adsorbent will need to be optimised for specific food matrices.

Whilst this paper has primarily investigated polymeric adsorbent in a detection and quantification capacity there is

further scope to investigate them as a tool for the removal of patulin and other contaminants in food products.

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