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Molecularly imprinted solid-phase extraction of fumonisin B analogs in bell pepper, rice and corn flakes

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Abstract

A molecularly imprinted polymer (MIP) for the recognition of fumonisin B analogs (FB) using 2-(diethylamino) ethyl methacrylate (DEAEM) as functional monomer and trimethylolpropane trimethacrylate (TRIM) as cross-linker was prepared by bulk polymerization in acetonitrile. Fumonisin B₁ (FB₁) was used as template molecule. A molecularly imprinted solid-phase extraction (MISPE) procedure was developed for further application in the analysis of FB. The performance of the MIP throughout the clean-up of spiked bell pepper, rice and corn flake sample extracts was compared with the results obtained when using non-imprinted polymer, C₁₈, strong anion exchange and immunoaffinity sorbents. Extracts were analysed for FB with LC-MS/MS after clean-up. Depending on the food matrix and the concentration range of the fumonisins, recoveries after MISPE varied from 62 to 86%, 62 to 83% and 67 to 81% for fumonisin B₁ (FB₁), fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃) respectively. The selectivity of the synthesized MIP for mycotoxins belonging to the group of FB was confirmed by evaluating cross-reactivity from analogue structures and other mycotoxins. Analysis of 39 naturally contaminated samples (corn flakes) by liquid chromatography tandem mass spectrometry indicated that the synthesized MIP could be an excellent alternative for clean-up and pre-concentration of FB in food samples. Pearson correlations between immunoaffinity clean-up and MISPE were calculated and amounted to 0.923 for FB₁, 0.808 for FB₂ and 0.759 for FB₃. It was shown that the developed MIP could be re-used more than 50 times. The synthesis of a FB₁ imprinted polymer and its application in food analysis is reported for the first time.

Keywords: *Molecularly imprinted polymer, fumonisin B, solid-phase extraction, mycotoxins, molecular recognition, food*

Introduction

Fumonisin are secondary metabolites that are produced by *Fusarium* moulds, primarily *F. verticillioides* and *F. proliferatum* (Krska et al. 2007). Fumonisin are long-chain polyhydroxyl alkylamines containing two propane tricarboxylic acid moieties which are esterified to hydroxyl groups on adjacent carbon atoms (Paepens et al. 2005). At least 28 different fumonisins have been reported that can be classified into 4 main groups, identified as fumonisin A, B, C, P (Rheeder et al. 2002). Fumonisin B₁ (FB₁) is the main representative of the fumonisins, whereas fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃) are homologues lacking respectively the hydroxyl group at C-10 and the hydroxyl group at C-5 (Fig. 1) (Arranz et al. 2004). Fumonisin occur mainly in corn and corn products, rice and vegetables over the whole world (Seefelder et al. 2002; Zöllner and Mayer-Helm 2006; Tanaka et al. 2007; Roscoe et al. 2008; Monbaliu et al. 2008). Toxicological studies of FB₁ revealed that it could cause several animal diseases as pulmonary edema in swines, equine leukoencephalomalacia in horses and oesophageal cancer and hepatic cancer in horses and rats (Gelderblom et al. 1991; Harrison et al. 1990; Rheeder et al. 1992). The toxicity of fumonisins can be explained by their ability to alter the sphingolipid metabolism through inhibiting ceramide synthase (Desai et al. 2002). Furthermore the intake of FB₁ was correlated with neural tube defects (e.g. spina bifida) and a high rate of human oesophageal cancer in certain areas of South Africa and China (Rheeder et al. 1992; Yoshizawa et al. 1994; Missmer et al. 2006). Due to carcinogenic effects in animal experiments fumonisin B₁ is classified as potential carcinogen (group 2B carcinogen) by the International Agency for research on Cancer (IARC) (IARC 2002). Because of their potential toxicity the Food and Drug Administration (FDA) recommended maximum levels (2–4 ppm) for fumonisins (sum of FB₁, FB₂ and FB₃) in corn and corn products for human consumption, while the European Commission has set maximum levels (200-4000 µg/kg) for the sum of FB₁ and FB₂ since October 2007 (U.S. Food and Drug Administration 2001; European Commission 2007).

The analysis of fumonisins in complex food matrices requires selective and sensitive detection of these carcinogenic mycotoxins. Methods currently available to detect FB₁ and FB₂ include gas chromatography (GC), thin layer chromatography (TLC), enzyme linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) with fluorescence detection (FLD) and liquid chromatography-mass spectrometry (LC-MS) (Zöllner and Mayer-Helm 2006; Krska et al. 2007). The extraction of fumonisins from food

matrices has been generally accomplished using acetonitrile/water or methanol/water mixtures. Prior to analysis the extract must be purified to remove matrix impurities and enrich the fumonisins. Clean-up can be achieved by antibody-based immunoaffinity columns (IAC) as well as solid-phase extraction (SPE) using reversed-phase (C_{18}) or strong anion-exchange (SAX) cartridges (Hlywka et al. 1999; Hartl and Humpf 1999; Visconti et al. 2001; Dilkin et al. 2001; Meyer et al. 2003; Paepens et al. 2005). Except for IAC all these techniques rely on non-selective interactions causing remaining impurities in the sample extract after clean-up. Beside the lower binding capacity (1-2 $\mu\text{g FB}_1$) other disadvantages of IAC are the expensive and time-consuming isolation of antibodies and their instability in organic solvents, which limit the use of IAC clean-up (Jiang et al. 2006).

Similar to immunosorbents, molecularly imprinted polymers (MIPs) exhibit excellent target selectivity. Moreover MIPs do not suffer from instability or loss of activity, they are easily produced and they possess a high mechanical strength. Because of these advantages molecular imprinting is a useful technique to synthesise selective sorbents which can be applied in clean-up steps (Svenson and Nicholls 2001).

Molecular imprinting is a process in which functional and cross-linking monomers are polymerized in the presence of the target molecule (template). The technique relies on the formation of specific recognition sites in the synthesized polymer. First, the functional monomers interact with the template resulting in the formation of a pre-polymerization complex. Subsequently the polymerization is initiated through UV-light or heat. In the presence of a cross-linking monomer a dense polymer network is formed due to the fixation of the functional monomers around the target molecule. Finally, the template molecule is washed out of the imprinted polymer leaving binding sites behind, complementary in size and shape specific to the analyte. These recognition sites mimic the binding sites of antibodies and enzymes.

Depending on the type of interaction between the template molecules and the functional monomers two approaches are distinguished to prepare MIPs: the template can interact via reversible covalent binding to the monomer (covalent approach) or the template interacts through non-covalent interactions, e.g. hydrogen bonds, ionic bonds, hydrophobic interactions and van der Waals forces. Of the two approaches, non-covalent imprinting is mainly used because of a more simple synthesis of the pre-polymerization complex and because of a

greater variety of functionality in the imprinted binding sites (Pichon & Haupt 2006; Yan and Row 2006).

Notwithstanding the huge amount of publications on MIPs for pesticides (Baggiani et al. 1999; Pap et al. 2002; Sanchez-Barragan et al. 2007) and drugs (Caro et al. 2005; Dong et al. 2005; Turiel et al. 2007), only a small amount deal with imprinted polymers selective towards mycotoxins (ochratoxin A, zearalenone, deoxynivalenol and moniliformin) (Zhou et al. 2004; Urraca et al. 2005; Urraca et al. 2006; Weiss et al. 2003; Appell et al. 2007). No report has been published yet about the development of MIPs selective towards FB.

In this paper the development of a FB selective imprinted polymer and its application in the analysis of FB is described for the first time. The synthesized MIP was packed in cartridges and implemented in molecularly imprinted solid-phase extraction (MISPE) procedures for bell pepper, rice and corn flakes. Rice and bell pepper were selected as food matrices because recent papers demonstrated that FB could occur in these matrices. Tanaka et al. described that in the USA respectively 8, 6 and 5 of the 20 analysed rice samples were contaminated with FB₁, FB₂ and FB₃. Maximum levels of 4300 µg/kg for FB₁, 1200 µg/kg for FB₂ and 600 µg/kg for FB₃ were found. Recently it was discovered that internal fruit rot of sweet pepper can be caused by several *Fusarium* species. Because of the possible occurrence of *Fusarium* species in bell pepper, pure cultures of *Fusarium* spp. were isolated to inoculate bell peppers. The experiment revealed that bell peppers inoculated by *Fusarium proliferatum* contained FB₁, FB₂ and FB₃ (Monbaliu et al. 2008). Cross-reactivity from analogue structures and non-analogue mycotoxins was also evaluated using LC-MS/MS, as well as the Pearson correlations between immunoaffinity clean-up and MISPE.

Materials and Methods

Reagents

FB₁, FB₂ and FB₃ were purchased from Promec Unit (Tygerberg, South-Africa). Trimethylolpropane trimethacrylate (TRIM), 2-(diethylamino) ethyl methacrylate (DEAEM), deoxynivalenol (DON), aflatoxin B₂ (AFB₂), aflatoxin G₂ (AFG₂), citrinin (CIT), T-2 toxin (T-2), ochratoxin A (OTA), zearalenone (ZEN) and HT-2 toxin (HT-2) were from Sigma Chemical Co. (Bornem, Belgium). 2,2'-Azobisisobutyronitrile (AIBN), acetone and ethyl acetate were supplied by Acros Organics (Geel, Belgium). Chloroform (CHCl₃) was

purchased from Novolab (Geraardsbergen, Belgium). Acetonitrile (AcN) and methanol (MeOH) were bought from BDH (Poole, UK). Acetic acid (CH_3COOH) and sodium hydroxide (NaOH) were from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q Gradient system (Millipore, Brussels, Belgium). HPLC grade AcN was bought from Biosolve (Valkenswaard, the Netherlands). Bond Elut SAX (500 mg) and C_{18} cartridges (200 mg) were obtained from Varian (Sint-Katelijne-Waver, Belgium). Fumonitest[®] IAC was from VICAM (Cereal Tester, Fleurus, Belgium). Ederol filter paper (24 cm, quality 15) was supplied by VWR (Leuven, Belgium).

Synthesis of a fumonisin B₁ imprinted polymer

In a centrifuge tube 5 mg template (i.e. 0.0069 mmol FB_1), 9.3 μl functional monomer (0.055 mmol DEAEM) and 55.2 μl cross-linker (0.173 mmol TRIM) were dissolved in 0.5 ml AcN (porogen). DEAEM and TRIM were used without further purification. After dissolving the monomers and template, 2 mg AIBN (2 mol% of the reactive double bonds) was added to the solution as radical initiator. The reaction mixture was degassed by sonicating the solution during 15 minutes and purging nitrogen for 2 minutes before sealing the tube. The tube was placed under UV-light (365 nm) for 24h. A white solid bulk material was obtained which was crushed and ground with a steel spatula. The MIP particles were washed in MeOH, acetone and AcN to remove fine particles. To remove the template the MIP was washed subsequently with AcN, MeOH and a solution of MeOH: CH_3COOH (95:5) until no template was detected anymore. The imprinted polymer was dried for 48 h at a temperature of 50°C. After crushing and grinding 49 mg (73% yield) of MIP was obtained. A non-imprinted polymer (NIP) was prepared following the same synthesis procedure without the addition of template.

The morphology of the synthesized polymers was characterized using a FEI Quanta 200F scanning electron microscope (FEI, Hillsboro, Oregon, USA). The samples were sputter-coated with gold before characterization. The polymer particles were scanned at the desired magnification.

Safety note

Fumonisin have neurotoxic and nephrotoxic properties. Based on the available toxicological data the IARC has classified fumonisin B₁ as potential carcinogen for humans. Gloves were

worn when working with standards and samples. Standard and sample preparation was carried out in a fume hood.

LC-MS/MS analysis

LC analysis was carried out with a Waters Alliance 2695 XE HPLC system coupled to a Micromass Quatro Micro triple-quadrupole mass spectrometer (Waters, Milford, MA, USA). The analytical column was an Alltima C₁₈, 5 µm, 150 mm × 3.2 mm (Alltech Associates, Deerfield, IL, USA), the guard column was an Alltima C₁₈, 5 µm, 7.5 mm × 3.2 mm (Alltech Associates, Deerfield, IL, USA).

The chromatography was carried out under isocratic conditions at a flow rate of 0.3 ml/min. The mobile phase consisted of H₂O:AcN (60:40) containing 0.3% formic acid. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode using multiple reaction monitoring (MRM). The injection volume was 20 µl. Table 1 lists the precursor and product ions of FB₁, FB₂ and FB₃ as used in the MRM method. For each compound, the two most abundant product ions from the [MH]⁺ ions were selected to be monitored (Paepens et al. 2005).

Preparation of spiked samples

Blank yellow bell pepper was ground and homogenized, subsequently 1g of sample was weighed into a centrifugal tube and 6 ml of AcN:H₂O (84:16) were added. Extraction was performed by shaking the sample during 30 min using an overhead shaker. Afterwards the sample was centrifuged at 2670 g during 20 min. One ml of the supernatant was evaporated under nitrogen and spiked with varying amounts of FB₁, FB₂ and FB₃ for application to SPE and MISPE (table 2). The same protocol was followed using blank rice and blank corn flakes as matrix. Bell pepper, rice and corn flakes were bought in local stores.

MISPE for the analysis of bell pepper, rice and corn flakes samples

Twenty mg of the prepared (non-)imprinted polymers were dry packed in a SPE cartridge of 3 ml with two glass wool frits at each end. The polymers were first washed with 50 ml of a solution of MeOH:CH₃COOH (95:5), 50 ml MeOH and 50 ml AcN, consecutively, to wash out remaining template. At the end of each wash step an eluent fraction was collected, evaporated, redissolved in 100 µl of mobile phase and analysed via LC-MS/MS to confirm the absence of template bleeding.

First the MIP cartridge was conditioned with 2ml MeOH:CH₃COOH (95:5) and dried before percolating 6 ml of AcN. Sample extracts dissolved in 1 ml AcN:H₂O (90:10) were loaded onto the imprinted polymer. Subsequently the cartridge was washed with 1 ml H₂O and finally the analyte was eluted by 1 ml of MeOH: CH₃COOH (95:5). The flow rate during MISPE was in the range of 0.5 – 0.8 ml/min. The eluates were collected, dried, dissolved in 100 µl mobile phase and analysed with LC-MS/MS. The same protocol was carried out with the NIP to evaluate the selectivity of the MIP.

SAX and C₁₈ clean-up of bell pepper, rice and corn flakes samples

To clean-up sample extracts a method derived from Bennett and Richard (1994) is used. The SAX cartridge was conditioned with 2 ml MeOH and washed with 2 ml of MeOH:H₂O (75:25). After dissolving the sample extracts in 2 ml of MeOH:H₂O (75:25), the pH of the samples was adjusted to the range 5.8 – 6.5 using 0.1 M NaOH. After loading the extract, the column was washed with 2 ml MeOH:H₂O (75:25). The fumonisins were eluted with 2 ml MeOH:CH₃COOH (95:5). The eluates were evaporated under nitrogen, dissolved in 100 µl mobile phase and analysed with LC-MS/MS.

The C₁₈ column was conditioned with 2 ml MeOH and washed with 2 ml of MeOH:H₂O (20:80). Before loading onto the column the sample extracts were dissolved in 1 ml of MeOH:H₂O (20:80). After loading the extract, the column was washed with 1 ml acetone:ethylacetate (50:50). The fumonisins were eluted with 2 ml MeOH:CHCl₃:CH₃COOH (60:40:10). The eluates were evaporated under nitrogen, dissolved in 100 µl mobile phase and analysed with LC-MS/MS.

Immunoaffinity clean-up of naturally contaminated corn flakes samples

Clean-up of corn flakes extracts using IAC is described by Paepens et al (2005). The samples (20 g) were extracted with 50 ml MeOH:H₂O (70:30, pH = 4) by shaking for 30 min using an orbital shaker, centrifuged for 10 min at 2670 g and filtered. The remaining solid material was extracted again with 50 ml MeOH:H₂O (70:30, pH = 4), centrifuged and filtered. Ten millilitres of the combined extracts were mixed with 40 ml of 0.01 M PBS buffer. A volume of 15 ml was loaded onto the Fumonitest[®] immunoaffinity column. After washing the column with 15 ml H₂O, fumonisins were eluted with 2 ml MeOH. The eluates were collected, dried, dissolved in 100 µl mobile phase and analysed with LC-MS/MS.

Validation and correlation studies

In order to examine the advantages of the MIP over conventional sorbents, food sample extracts were loaded onto the MIP, NIP, SAX and C₁₈ cartridges. Recovery experiments were performed in triplicate. Precision was evaluated by computing the standard deviation for analyses (n = 3) repeated on three different days. Accuracy was determined by calculating the recovery. Quantitative determinations were based on the peak area ratios of the spiked sample extracts in comparison to that of extracts spiked after clean-up.

To determine the correlation of the MISPE protocol with immunoaffinity clean-up a total of 39 corn flakes samples collected in Belgian retail stores during 2003-2004 were resurveyed for the natural occurrence of FB₁, FB₂ and FB₃ using the MIP. These samples were analyzed in 2005 using an immunoaffinity clean-up and stored in the dark under dry conditions (Paepens et al. 2005). Extraction procedure was the same as mentioned for the spiked samples. The same samples were now analyzed applying the MIP sorbent to clean the extracts. The eluates were collected and analyzed with the same LC-MS/MS method as in 2005. To quantify the detected amount of FB₁, FB₂ and FB₃ in corn flakes a matrix assisted calibration curve was used. By comparison of the occurrence and levels of FB₁, FB₂ and FB₃, the Pearson correlation was calculated to evaluate if the obtained results were independent of the used clean-up method.

Cross-reactivity study

The selectivity of the MIP and conventional sorbents (i.e. C₁₈ and SAX) during the clean-up of bell pepper, rice and corn flakes extracts was examined by comparing the retention behaviour of FB₁ to that of analogue (FB₂ and FB₃) and non-analogue structures. The non-analogue structures used in this study were selected regarding the possible interactions that could occur with the imprinted polymer. CIT and OTA were chosen due to the presence of an ionized carboxyl group which might interact with the aminogroup of DEAEM. DON, ZEN, T-2 and HT-2 were selected due to their ability to form hydrogen bonds whereas the apolar mycotoxins AFB₂ and AFG₂ could interact via hydrophobic interactions. Using the previously described conditions 200 ng of each of the mycotoxins were percolated through the MIP, C₁₈ and SAX cartridge. These procedures were repeated three times.

Results and discussion

The objective of this work was to develop a MIP for the selective analysis of FB. FB possess diverse functional groups (amino, carboxyl and hydroxyl groups), enabling interaction with basic (DEAEM) monomers. Moreover, fumonisins contain a long hydrophobic alkyl backbone. Due to the low solubility of fumonisins in apolar solvents and the disrupting effect of protic solvents concerning the hydrogen bonds, AcN was selected as porogen. Besides the strength and number of interactions between analyte and functional monomer the amount of hydrophobic cross-linker is expected to play a significant role during the imprinting through the formation of a hydrophobic cavity around the template. Preliminary experiments using different functional monomers (methacrylic acid and DEAEM) and molar ratios were conducted (results not shown). DEAEM was selected as functional monomer and the chosen molar ratio of template:functional monomer:cross-linker to synthesize the MIP amounted to 1:8:25. After all when applying the non-covalent method an excess of functional monomer should be added to shift the equilibrium towards the formation of the pre-polymerisation complex. Keeping in mind that one molecule of FB carries four carboxyl groups (interaction points), it was decided that per molecule FB eight molecules of functional monomer should be present in the polymerization mixture.

The morphology of the developed polymer matrices is exemplified by scanning electron micrographs in Fig. 2. The polymers consisted of aggregates of small irregular polymer particles and as expected no spherical particles were obtained under the applied polymerization conditions. The MIPs and NIPs exhibited a rough and microporous structure.

To be competitive with conventional SPE sorbents, suitable MIPs have to exhibit both high affinity and selectivity towards the target analyte. Furthermore it is important to realize that MIPs are not intrinsically selective, but that the used MISPE procedure confers its selectivity. From this point of view the selection of solvents in the loading and washing steps was carefully considered in our research. In this paper we only present the final optimal solvents. During the sample loading the analyte should be adsorbed quantitatively and selectively. In our case a small amount of a polar modifier (H_2O) was added to the loading solvent to interrupt non-specific interactions. Interfering compounds however that were still retained by non-specific interactions should be removed during the washing step. To maximize the specific interactions between analyte and MIP, water was selected as washing solvent. At the end of the MISPE procedure the analyte must be eluted. Desorption of the

target molecules is achieved by percolating a solvent able to disrupt the specific interactions to recover them quantitatively. To get high enrichment factors, it is necessary to apply small volumes of solvent. Methanol acidified with acetic acid was chosen as elution solvent due to the ability to disrupt ionic interactions and hydrogen bonding. To elute the analyte quantitatively, MeOH:CH₃COOH (95:5) was selected as the elution solvent.

Keeping in mind that release of the template (template bleeding) from the MIP cartridge during extraction could cause false positive results, possible bleeding of the MIP was examined. With LC-MS/MS no detectable amount of FB₁ was observed in the eluates after percolating AcN, MeOH and MeOH:CH₃COOH (95:5) through the cartridge.

The binding properties of the imprinted polymer depended on the volume of extract percolated through the MIP column. Different volumes containing the same amount of FB₁ (i.e. 1 µg) were loaded onto the column (Fig. 3). Up to volumes of 2 ml little breakthrough was observed on the MIP. In order to reduce the breakthrough it was chosen to percolate an extract with a volume of 1 ml during the loading step although less non-specific interactions were observed when loading volume was set at 2 ml.

Validation study with spiked bell pepper, rice and corn flakes samples

Sample extracts were spiked just before clean-up to evaluate the efficacy of the clean-up procedure. Indeed, preliminary experiments showed that the used extraction solvent, AcN:H₂O (84:16), did not result in a significant loss of FB (94% extraction yield).

Results of the recovery experiments for bell pepper are shown in table 3. The data are the mean of three repeated experiments. Compared to the NIP good recoveries and precision during elution were obtained, demonstrating the potential for implementation of the developed MIP in the clean-up of FB contaminated food samples. Clean-up by the conventional C₁₈ and SAX columns resulted in varying recoveries in contrast to MISPE. Furthermore the precision of the SAX procedure was not as good as this obtained using the MIP. In contrast to the MIP and SAX cartridges, C₁₈ columns showed a lower recovery, especially when larger amounts of FB were percolated. Recoveries obtained after MISPE of yellow bell pepper extracts satisfy the criteria set by the European Commission (recovery > 60%) (EC 2006). The little varying recoveries on the MIP indicated an acceptable linearity for

calibration curves in matrix samples with a correlation coefficient of 0.9999 for FB₁, FB₂ and FB₃.

Clean-up of spiked rice extracts using the NIP and C₁₈ column resulted in low recoveries (Table 3). Although highest recoveries were achieved with SAX, good recoveries and precision were obtained using the MIP column demonstrating the possibility to clean-up rice extracts via the MISPE procedure. SPE of rice extracts using the imprinted polymer sorbent complied with the minimum recovery of 60% defined by the European Commission. To evaluate the linearity of the recoveries achieved with MISPE, the correlation coefficient was calculated. An acceptable linearity (correlation coefficient of > 0.9980 for FB₁ and FB₂) was obtained for calibration curves in matrix samples.

Results of the recovery experiments for corn flakes are shown in table 3. Applying the C₁₈ cartridge during SPE of spiked corn flakes extracts resulted in very low recoveries making this method inappropriate to analyse corn flakes. Although in comparison with the previous matrices smaller selectivity was observed for the MIP due to higher recoveries on the NIP, clean-up of corn flakes extracts using the blank polymer as sorbent led up to recoveries lower than the minimum limit set by the European Commission. Highest recoveries (71 – 111%) were achieved using the SAX cartridges, but also a worse precision was observed. MISPE of corn flakes samples however resulted in a satisfying recovery (> 60%) and a good precision indicating the possibility to implement the MIP in the analysis of corn flakes samples. The second advantage of MISPE is the linearity of the data (correlation coefficient above 0.999 for FB₁, FB₂ and FB₃).

Cross-reactivity study

Fig. 4 shows that the binding cavities created in the polymer network of the MIP are specific to mycotoxins belonging to the group of FB. Except for a small cross-reactivity for OTA, all the other mycotoxins were not retained on the MIP. Compared to FB₁ the other fumonisins belonging to group B only differ by one hydroxyl group resulting in high recovery percentages for FB₁ as well as FB₂ and FB₃. It seemed that a little decrease of the steric volume for substituents caused no decrease of the molecular recognition. As could be expected C₁₈ clean-up of bell pepper, rice and corn flakes samples showed no selectivity towards fumonisins at all in contrast to MISPE. Indeed the C₁₈ column only interacts through non-specific hydrophobic interactions with the matrix components. The recovery of all

mycotoxins in yellow bell pepper extracts, except citrinin and the fumonisins, ranged from 12 to 40%, while those for FB₁, FB₂ and FB₃ were respectively 86%, 81% and 80%. The recovery after C₁₈ clean-up of rice samples was in the range of 43 to 50% for the fumonisins and in the range of 13 to 36% for OTA, ZEN, DON, HT-2, T-2, AFB₂ and AFG₂. Also in the case of corn flakes no selectivity was seen using C₁₈ cartridges. Even a better retention was found for HT-2, T-2, AFB₂ and AFG₂ than for FB₂ and FB₃.

Because the SAX column interacts through non-specific ionic interactions it was hypothesized that mycotoxins possessing a carboxylic group (FB₁, FB₂, FB₃, OTA and CIT) would show a high retention on the SAX cartridge during the clean-up of food samples, while the other mycotoxins would not be retained due to the lack of an acid group. After all at a pH range of 5.8 - 6.5 the carboxylic group will be negatively charged and the functional group of the SAX column will be protonated. Fig. 4 shows that our hypothesis was partially correct. For instance in case of rice extracts a high retention was found for FB (93%, 85% and 84% for respectively FB₁, FB₂ and FB₃) and OTA (90%) as expected, but not for CIT (1%). More surprising was the significant retention of AFG₂ (40%), nevertheless this mycotoxin possesses no acidic functional group. The same conclusions could be made for SPE of spiked bell pepper and corn flakes extracts using SAX cartridges. All other mycotoxins did not interact with the SAX cartridge during the clean-up of rice, corn flakes and bell pepper extracts.

Contrary to SPE with C₁₈ or SAX columns, the imprinted polymer showed high selectivity for fumonisins in presence of other mycotoxins. Regarding the co-existence of different mycotoxins in the same food sample, high selectivity towards one mycotoxin without using IAC can only be accomplished by MISPE and not with conventional clean-up methods. One of the most important advantages of the MIP over the tested conventional sorbents (C₁₈ and SAX) is its reusability. During all the described series of analyses the performance of the MIP was tested by evaluating the recovery and selectivity and checking for template bleeding. No loss of recovery and selectivity was observed. Besides percolating blank samples through the MIP demonstrated that no FB₁ was retained on the MIP, revealing that the MIP did not lose its superior characteristics.

Naturally contaminated samples

The levels of fumonisins in the 39 corn flakes samples are demonstrated in Table 4. Approximately 60% of the samples were contaminated with fumonisins. FB₁ concentrations exceeded FB₂ and FB₃ concentrations. This follows the general pattern of fumonisin

contamination in maize and maize-based food. Occurrence data, mean, median, LOD and LOQ of respectively FB₁, FB₂ and FB₃ in corn flakes for each clean-up method are shown in Table 5. The corresponding concentration at 3 times the residual standard deviation of the y intercept divided through the slope of regression line equals the LOD. LOQ is equal to 6 times the residual standard deviation of the y intercept divided through the slope of regression line. The mean and median of the found contamination levels are approximately the same, especially for FB₁ and FB₂ using the MISPE or IAC method. Though a small difference was observed for corn flakes samples contaminated with FB₃. LOD and LOQ values obtained after immunoaffinity clean-up and MISPE were similar for FB₁ and FB₂ except for FB₃ for which LOD_{IAC} was about two times LOD_{MIP}, demonstrating the sensitivity of the MISPE procedure.

To calculate the correlation it was necessary to give values to samples in which no fumonisin was detected or only traces. Therefore the Pearson correlation was computed assuming that non detected samples contained half of the LOD, while samples with traces of FB contained half of the LOD and LOQ. Pearson correlations between immunoaffinity clean-up and MISPE amounted to 0.923 for FB₁, 0.808 for FB₂ and 0.759 for FB₃. A good correlation was present between analyses based on MISPE or immunoaffinity clean-up of samples for FB₁ and FB₂. As could be expected from the values of the mean and median a less correlation was observed for FB₃. One reason could be the difference in LOD and LOQ values causing errors in the calculation of the correlation due to the assumption that was made.

Conclusions

An imprinted polymer selective to FB was developed applying FB₁ as template. This is the first report of the successful synthesis of a FB₁ imprinted polymer. Use of a basic functional monomer and a hydrophobic cross-linker originated a MIP with specific recognition properties towards FB in miscible AcN-water mixtures, which permits to load directly (after dilution) the food extract onto the MIP, if desirable. Although no dummy molecule was used during the imprinting process, LC-MS/MS analysis of blank solutions showed no template bleeding.

The superior characteristics of the MIP over the conventional sorbents were expressed in several domains: selectivity towards FB, cleaner extracts after MISPE, good accuracy and precision. Moreover the MIP showed no false positive results or loss of binding capacity and selectivity after multiple use, demonstrating the reusability of the MIP cartridges contrary to

C₁₈, SAX and immunoaffinity sorbents. Throughout the whole series of previously described experiments the same imprinted polymer sorbent was applied. Furthermore low detection limits were achieved and a good correlation was shown between MISPE and immunoaffinity clean-up of corn flakes samples. The results indicate that the developed MIP could be an excellent alternative for selective immunoaffinity clean-up and pre-concentration of FB in bell pepper, rice and corn flakes.

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Figure captions

Fig. 1: Chemical structures of fumonisin B analogs (FB).

Fig. 2: Scanning electron micrographs of MIP (A) and NIP (B) (magnification: x30,000).

Fig. 3: Bound percentage of FB₁ (1 µg) vs. volume of bell pepper and rice extracts (AcN:H₂O (90:10)) percolated through the MIP and NIP. Data represent the mean (±1 S.D.) of three experiments.

Fig. 4: Recoveries obtained by percolating extracts of bell pepper (a), rice (b) and corn flakes (c) spiked with FB₁, FB₂, FB₃, CIT, DON, ZEN, T-2, HT-2, OTA, AFB₂ and AFG₂ through MIP, C₁₈ and SAX cartridges. Data represent the mean (±1 S.D.) of experiments performed in triplicate.

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Table 1: LC-MS/MS precursor and product ions.

Compound	Precursor ion (m/z)	Product ions (m/z)
FB ₁	722	352 ^a
		334
FB ₂	706	336 ^a
		318
FB ₃	706	688 ^a
		354

^a Most abundant ion

Table 2: Spiking levels of FB₁, FB₂ and FB₃ in blank bell pepper, rice and corn flakes samples.

Food matrix	Spiking levels (ng)		
	FB ₁	FB ₂	FB ₃
Bell pepper	50 - 1000	50 - 500	50 - 200
Rice	50 - 500	50 - 200	50
Corn flakes	50 - 1000	50 - 500	50 - 200

Table 3: Recovery of FB after clean-up of spiked yellow bell pepper (a), rice (b) and corn flakes (c) extracts using MIP (MISPE), NIP (NISPE), C₁₈ and SAX cartridges (n = 3).

A) bell pepper

Mycotoxin		Recovery (%)			
		MISPE	NISPE	C ₁₈	SAX
FB ₁ (ng)	50	84 ± 3	48 ± 6	82 ± 6	75 ± 18
	100	81 ± 5	48 ± 3	93 ± 5	93 ± 2
	200	86 ± 3	47 ± 7	77 ± 7	88 ± 11
	500	82 ± 1	46 ± 8	65 ± 4	98 ± 10
	1000	83 ± 3	42 ± 6	58 ± 2	106 ± 3
FB ₂ (ng)	50	80 ± 2	43 ± 4	82 ± 5	79 ± 18
	100	83 ± 3	49 ± 6	89 ± 3	87 ± 2
	200	81 ± 10	43 ± 3	76 ± 9	85 ± 8
	500	83 ± 2	46 ± 9	56 ± 3	92 ± 4
FB ₃ (ng)	50	81 ± 6	49 ± 9	78 ± 4	72 ± 16
	100	81 ± 4	51 ± 7	86 ± 3	93 ± 2
	200	80 ± 5	50 ± 8	74 ± 9	82 ± 8

B) rice

Mycotoxin		Recovery (%)			
		MISPE	NISPE	C ₁₈	SAX
FB ₁ (ng)	50	69 ± 1	25 ± 2	68 ± 3	97 ± 3
	100	66 ± 3	21 ± 4	67 ± 3	86 ± 11
	200	73 ± 5	31 ± 8	50 ± 12	93 ± 1
	500	89 ± 9	29 ± 3	60 ± 9	93 ± 5
FB ₂ (ng)	50	65 ± 4	28 ± 7	44 ± 4	75 ± 1
	100	62 ± 1	26 ± 3	53 ± 5	73 ± 10
	200	75 ± 8	35 ± 13	43 ± 2	85 ± 1
FB ₃ (ng)	50	67 ± 2	24 ± 6	43 ± 4	76 ± 3

C) corn flakes

Mycotoxin

Recovery (%)

		MISPE	NISPE	C ₁₈	SAX
FB ₁ (ng)	50	62 ± 7	56 ± 5	75 ± 4	80 ± 21
	100	64 ± 9	56 ± 6	61 ± 5	74 ± 9
	200	69 ± 2	61 ± 4	67 ± 4	89 ± 6
	500	70 ± 4	63 ± 3	22 ± 2	92 ± 8
	1000	72 ± 6	69 ± 8	37 ± 5	111 ± 1
FB ₂ (ng)	50	71 ± 5	62 ± 3	33 ± 2	77 ± 17
	100	74 ± 8	60 ± 12	24 ± 3	89 ± 3
	200	75 ± 2	64 ± 5	25 ± 1	82 ± 6
	500	73 ± 7	64 ± 11	25 ± 4	71 ± 13
FB ₃ (ng)	50	65 ± 3	57 ± 2	34 ± 2	69 ± 9
	100	66 ± 7	58 ± 8	28 ± 1	77 ± 12
	200	70 ± 3	59 ± 7	26 ± 1	85 ± 5

Table 4: Occurrence levels (µg/kg) of FB₁, FB₂ and FB₃ in corn flakes samples collected in Belgian stores. n.d. = not detected

Sample	FB ₁ (µg/kg)		FB ₂ (µg/kg)		FB ₃ (µg/kg)	
	IAC	MIP	IAC	MIP	IAC	MIP
1	48	104	20	19	<LOQ	12
2	66	74	<LOQ	13	<LOQ	15
3	292	254	18	<LOQ	28	n.d.
4	186	193	24	20	49	51
5	212	202	26	15	47	<LOQ
6	188	174	25	13	<LOQ	16
7	241	159	21	11	27	18
8	161	132	22	20	42	20
9	98	200	18	13	36	17
10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	53	48	<LOQ	<LOQ	<LOQ	<LOQ
12	84	71	17	<LOQ	<LOQ	n.d.
13	< LOQ	n.d.	<LOQ	10	<LOQ	n.d.
14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	53	44	<LOQ	14	<LOQ	18
19	91	88	<LOQ	12	<LOQ	21
20	267	260	17	21	38	38
21	88	60	<LOQ	9	<LOQ	<LOQ
22	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.
23	99	157	16	18	29	15
24	126	181	20	20	39	20
25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
27	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ
28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
29	< LOQ	81	<LOQ	<LOQ	<LOQ	<LOQ
30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
34	44	< LOQ	<LOQ	<LOQ	<LOQ	<LOQ
35	54	< LOQ	<LOQ	15	<LOQ	19
36	< LOQ	n.d.	<LOQ	11	<LOQ	n.d.
37	116	100	<LOQ	11	<LOQ	<LOQ
38	122	102	<LOQ	20	<LOQ	20
39	108	88	<LOQ	15	<LOQ	n.d.

LOD_{IAC} = 20, 7.5 and 12 µg/kg; LOQ_{IAC} = 40, 15 and 24 µg/kg (resp. FB₁, FB₂ and FB₃)
LOD_{MIP} = 22, 4.5 and 6 µg/kg; LOQ_{MIP} = 44, 9 and 12 µg/kg (resp. FB₁, FB₂ and FB₃)

Table 5: Occurrence data of FB₁, FB₂ and FB₃ in corn flakes collected on the Belgian market. Mean and median are calculated assuming that non detected samples contain half of the LOD, while samples with traces of FB contain half of the LOD and LOQ.

	IAC			MIP		
	FB ₁	FB ₂	FB ₃	FB ₁	FB ₂	FB ₃
Overall incidence (%)	64.1	64.1	64.1	61.5	64.1	53.8
Mean (µg/kg)	78	11	18	78	9	11
Median (µg/kg)	53	11	19	48	8	9
LOD (µg/kg)	20	7.5	12.5	22	4.5	6
LOQ (µg/kg)	40	15	25	44	9	12