Journal of Chromatography A, 1218 (2011) 1122–1130

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Design of an imprinted clean-up method for mycophenolic acid in maize

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ARTICLE INFO

Article history:
Received 3 September 2010
Received in revised form
17 December 2010
Accepted 20 December 2010
Available online 28 December 2010

Keywords:
Molecularly imprinted polymer
Mycophenolic acid
Maize
Solid-phase extraction
Molecular recognition

ABSTRACT

In the present work, the development of imprinted polymers selective towards mycophenolic acid and their application in food analysis are reported for the first time. To synthesize the molecularly imprinted polymer (MIP) 4-vinylpyridine and ethyleneglycol dimethacrylate were applied as functional monomer and cross-linker, respectively. Besides the toxin itself, the implementation of structural analogues as templates was evaluated. A molecularly imprinted solid-phase extraction (MISPE) procedure was designed for the selective clean-up of maize extracts. Binding experiments and Scatchard analysis indicated the presence of specific binding sites in the imprinted polymers. The imprinting effect varied along with the selected template. The dissociation constant (K_D) of the higher affinity binding sites ranged from 0.8 μmol/l to 15.6 μmol/l, while the K_D of the lower affinity binding sites was in the range of 138.5–519.3 μmol/l. The performance of the MIPs throughout the clean-up of spiked maize sample extracts was evaluated and compared with the results obtained when applying a non-imprinted polymer. Depending on the polymers and the spiked concentration, recoveries after MISPE and non-imprinted solid-phase extraction varied respectively from 49% to 84% and from 28% to 31%. The imprinted polymers were superior regarding matrix effect, limit of detection (LOD) and limit of quantification (LOQ). LOD ranged from 0.17 μg/kg to 0.25 μg/kg and LOQ varied from 0.57 μg/kg to 0.82 μg/kg. Analysis of 15 maize samples by liquid chromatography tandem mass spectrometry revealed that the MIPs could be excellent sorbents for clean-up of contaminated food samples.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by fungi occurring on agricultural commodities. Worldwide approximately 25% of the crops is contaminated with these fungal toxins. Mycotoxins exhibit a broad range of toxic effects (carcinogenic, neurotoxic, nephrotoxic, immunosuppressive and estrogenic) implying a potential threat to human and animal health. About 400 of these secondary metabolites have been reported, all characterized by a structural and chemical diversity [1–3]. Despite many efforts to prevent the growth of mycotoxigenic fungi on crops and other agricultural products, mycotoxin contamination is still an emerging issue in food safety through the entire world.

Mycophenolic acid (6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid, MPA) is produced by several *Penicillium* species. *P. roqueforti* is one of the most important sources of MPA production [4]. MPA is a weak organic acid with antifungal, antibacterial and antiviral activities [5]. MPA is also the active metabolite of mycophenolate motefil, an immunosuppressant drug used to prevent rejection in organ transplantation [6]. Although exposure to MPA does not result in acute toxic effects, humans and animals exposed to high levels of MPA are at increased risk of infections. The immunosuppressive action causes animals not only to be more susceptible to infectious diseases, but also to be more sensitive to the presence of other mycotoxins [7]. Mycotoxins from *Penicillium* species can be a serious contamination problem in poorly stored food and feed. MPA is reported to be a suitable marker for food and feed infected by *Penicillium* species [8]. MPA occurs mainly in grain, silage, grass, bread, milk, cheese and fruit [7–13]. Notwithstanding average levels of MPA in fresh maize samples were 0.06 μg/kg, Sulyok et al. reported MPA levels up to 78,000 μg/kg [7,8]. Currently, no recommendation or regulatory levels for MPA were established by regulatory authorities in Belgium and the European Union.

The analysis of mycotoxins requires sensitive and selective detection as they occur in complex matrices at low levels. Currently high performance liquid chromatography–mass spectrometry (HPLC–MS) is generally applied to detect MPA in food and feed without previous clean-up step [8,12–16]. The absence of purification and preconcentration steps results in a high limit

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doi:10.1016/j.chroma.2010.12.085

of detection (LOD) and limit of quantification (LOQ) [10,14,15]. The implementation of intelligent polymers exhibiting a molecular memory towards an analyte, is a powerful technique in the analysis of mycotoxins. Clean-up can be achieved by molecularly imprinted polymers (MIPs), exhibiting excellent target selectivity. MIPs gain interest in mycotoxin analysis, especially as adsorbents in solid-phase extraction (SPE) [17–25].

Synthesis of MIPs is a straightforward and inexpensive procedure. Molecular imprinting is a process relying on the formation of specific recognition sites complementary in size and shape to the analyte. Because these binding sites mimic the binding sites of enzymes and antibodies, MIPs are also called artificial antibodies. The key step of the imprinting process is the interaction between the template (i.e. the target molecule or a structural analogue) and the functional monomers resulting in a pre-polymerization complex. Stabilizing this complex through polymerization gives a dense polymer network containing imprinted binding sites. Removing the template enables the polymer to rebind selectively the imprint molecule [26–28].

In this paper the synthesis of a MPA selective imprinted polymer and its application in mycotoxin analysis is presented. MIPs were prepared implementing MPA and structural analogues as template molecules. Via binding experiments and Scatchard analysis the specificity and affinity of the different MIPs were evaluated. The designed MIPs were implemented in the clean-up (molecularly imprinted solid-phase extraction, MISPE) of maize extracts. The efficacy of the clean-up was examined by determining the matrix effect and performing a cross-reactivity study.

2. Experimental

2.1. Reagents

MPA was purchased from Fermentek (Jeruzalem, Israel). 2,2′-Azobisisobutyronitrile (AIBN), dichloromethane (CH_2Cl_2), hexane and acetone were bought from Acros Organics (Geel, Belgium). Ethyleneglycol dimethacrylate (EGDMA), 2-vinylpyridine (2VP), 4-vinylpyridine (4VP), 2-methoxyphenol, 4-methoxyphenol, ethyl-5-bromovalerate, 5-indanol, sodium *tert*-butoxide (NatBuO), potassium *tert*-butoxide (KtBuO), triethylamine (TEA), T-2 toxin (T-2), HT-2 toxin (HT-2), deoxynivalenol (DON), nivalenol (NIV), citrinin (CIT), ochratoxin A (OTA), aflatoxin B_1 (AFB_1), aflatoxin G_2 (AFG_2) and fumonisin B_1 (FB_1) were supplied by Sigma Chemical Co. (Bornem, Belgium). Chloroform ($CHCl_3$) was purchased from Novolab (Geraardsbergen, Belgium). Methanol Hipersolv Chromanorm (MeOH) was bought from BDH (Poole, UK). Acetic acid (CH_3COOH), formic acid (FA), ethanol (EtOH), hydrochloric acid (HCl), sodium iodide, phosphoric acid, di-sodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, anhydrous magnesium sulphate ($MgSO_4$) and ammonium acetate were from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q Gradient system (Millipore, Brussels, Belgium). LC–MS grade MeOH was supplied by Biosolve (Valkenswaard, the Netherlands). Eppendorf tubes were purchased from Eppendorf (Oldenburg, Germany).

2.2. Synthesis of 5-(2-methoxy-phenoxy)-pentanoate ethyl ester

2-Methoxyphenol (5.0 g, 40.27 mmol) and NatBuO (4.65 g, 48.38 mmol) were dissolved in 40 ml EtOH. After stirring the mixture for 1 h under nitrogen atmosphere, ethyl-5-bromovalerate (6.45 ml, 40.27 mmol) and sodium iodide (0.16 g, 1.07 mmol) were added. The solution was stirred for 4 h at reflux temperature under nitrogen atmosphere. The reaction was quenched with 45 ml water and then extracted with CH_2Cl_2 (3× 20 ml). The organic phase was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. A yellow oil was obtained after recrystallization in MeOH with 30% of hexane. Yield: 2.31 g (23%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.83–6.95 (m, 4H), δ = 4.12 (q, 2H), δ = 4.03 (t, 2H), δ = 3.86 (s, 3H), δ = 2.40 (t, 2H), δ = 1.78–1.92 (m, 4H), δ = 1.24 (t, 3H).

2.3. Synthesis of 5-(4-methoxy-phenoxy)-pentanoate ethyl ester

The same procedure was followed as for the synthesis of 5-(2-methoxy-phenoxy)-pentanoate ethyl ester, except 4-methoxyphenol (5.0 g, 40.27 mmol) was used as reagent. The pure product was obtained after silica gel column chromatography using 5% EtOH in CH_2Cl_2 as eluent. Yield: 5.39 g (54%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.78–6.82 (m, 4H), δ = 4.04 (q, 2H), δ = 3.81 (t, 2H), δ = 3.67 (s, 3H), δ = 2.29 (t, 2H), δ = 1.64–1.78 (m, 4H), δ = 1.15 (t, 3H).

2.4. Synthesis of 5-(5-indanoxy)-pentanoate ethyl ester

5-Indanol (5.0 g, 37.27 mmol) and NatBuO (4.3 g, 44.74 mmol) were dissolved in 40 ml EtOH and the mixture was stirred for 1 h under nitrogen atmosphere. Ethyl-5-bromovalerate (7.08 ml, 44.72 mmol) and sodium iodide (0.15 g, 1.0 mmol) were added and the reaction was stirred at reflux temperature for 16 h. The mixture was poured into 45 ml water and then extracted with CH_2Cl_2 (3× 20 ml). The combined organic extracts were dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography using 5% EtOH in CH_2Cl_2 as eluent. Yield: 8.25 g (85%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.12 (d, 1H), δ = 6.81 (s, 1H), δ = 6.70 (d, 1H), δ = 4.16 (q, 2H), δ = 3.96 (t, 2H), δ = 2.82–2.93 (m, 4H), δ = 2.38 (t, 2H), δ = 2.11 (q, 2H), δ = 1.80–1.91 (m, 4H), δ = 1.24 (t, 3H).

2.5. Synthesis of 5-(2-methoxy-phenoxy)-pentanoic acid

5-(2-Methoxy-phenoxy)-pentanoate ethyl ester (1.0 g, 3.97 mmol) was transferred into 40 ml MeOH. After adding KtBuO (1.05 g, 9.388 mmol), dissolved in 15 ml water, the reaction mixture was stirred overnight at 50 °C. The pH of the solution was adjusted with 1 M HCl till pH amounted to 6. This solution was subsequently extracted with CH_2Cl_2 (3× 20 ml). Before evaporation, the combined organic extracts were dried over anhydrous $MgSO_4$. A white crystalline powder was obtained after recrystallization in hexane with 15% MeOH. Yield: 0.58 g (65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.79–6.90 (m, 4H), δ = 3.96 (t, 2H), δ = 3.79 (s, 3H), δ = 2.40 (t, 2H), δ = 1.72–1.90 (m, 4H).

2.6. Synthesis of 5-(4-methoxy-phenoxy)-pentanoic acid

The protocol to synthesize 5-(2-methoxy-phenoxy)-pentanoic acid was repeated, but 5-(4-methoxy-phenoxy)-pentanoate ethyl ester (1.0 g, 3.97 mmol) was applied as reagent. Yield: 0.51 g (57%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.84 (s, 4H), δ = 3.91 (t, 2H), δ = 3.77 (s, 3H), δ = 2.46 (t, 2H), δ = 1.80–1.92 (m, 4H).

2.7. Synthesis of 5-(5-indanoxy)-pentanoic acid

KtBuO (1.01 g, 9.033 mmol) was dissolved in 15 ml water and added to a solution of 5-(5-indanoxy)-pentanoate ethyl ester (1.00 g, 3.82 mmol) in 40 ml MeOH at 50 °C. The reaction mixture was stirred overnight at 50 °C. Adjusting the pH of the mixture to 6 by applying 1 M HCl resulted in the precipitation of the crude product. The white product was filtered and purified by washing with hexane. Yield: 0.79 g (88%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.04 (d, 1H), δ = 6.71 (s, 1H), δ = 6.59 (d, 1H), δ = 3.87 (t, 2H), δ = 2.72–2.83 (m, 4H), δ = 2.35 (t, 2H), δ = 1.99 (q, 2H), δ = 1.69–1.81 (m, 4H).

Table 1
Individual composition and yield of the designed polymers. 4-Vinylpyridine, ethyleneglycol dimethacrylate and chloroform were used as functional monomer, cross-linker and porogen, respectively.

	Template	Yield (%)
MIP4VP	Mycophenolic acid	68
DUMMY1	5-(4-Methoxy-phenoxy)-pentanoate ethyl ester	73
DUMMY2	5-(2-Methoxy-phenoxy)-pentanoate ethyl ester	51
DUMMY3	5-(5-Indanoxy)-pentanoate ethyl ester	48
DUMMY4	5-(4-Methoxy-phenoxy)-pentanoic acid	75
DUMMY5	5-(2-Methoxy-phenoxy)-pentanoic acid	55
DUMMY6	5-(5-Indanoxy)-pentanoic acid	43
NIP4VP	–	74

2.8. Synthesis of an imprinted polymer

In a centrifuge tube, template (0.0312 mmol), functional monomer and cross-linking monomer were dissolved in 1 ml $CHCl_3$. The compounds of the polymerization mixture are listed in Table 1. EGDMA was distilled before use. After dissolving the monomers and template, AIBN (2 mole% relative to the reactive double bonds) was added to the polymerization mixture. Before initiating the polymerization, oxygen was removed by sonicating the mixture during 15 min and purging nitrogen for 2 min. The solution was subsequently irradiated by UV-light (365 nm) for 4 h resulting in a yellow bulk material, which was crushed and ground. Fine particles and remaining monomer were removed by washing the polymer with acetone. Finally, template was eluted from the imprinted polymer with MeOH, MeOH/CH_3COOH (95/5, v/v) and MeOH/TEA (99/1, v/v) until no template was detected. The absence of template bleeding was confirmed by LC–MS/MS analysis. The MIP was dried for 48 h at a temperature of 50 °C. A non-imprinted polymer (NIP) was prepared according to the same synthesis procedure without the addition of template.

2.9. LC–MS/MS analysis

LC analysis was performed 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 a Symmetry C_{18}, 5 μm, 2.1 mm × 150 mm (Waters, Zellik, Belgium), the guard column was a Waters Sentry 3.5 μm, 2.1 mm × 10 mm (Waters, Zellik, Belgium). The mobile phase consisted of variable mixtures of mobile phase A (water/MeOH/CH_3COOH, 94/5/1, v/v/v and 5 mM ammonium acetate) and mobile phase B (water/MeOH/CH_3COOH, 2/97/1, v/v/v and 5 mM ammonium acetate). The compounds were eluted at a flow rate of 0.3 ml/min. The gradient elution started at 60% mobile phase A and decreased to 25% in 10 min. The next minute initial column conditions were reached. The mass spectrometer was operated in positive electrospray ionization (ESI+) mode using multiple reaction monitoring (MRM). The injection volume was 20 μl. Precursor ion of MPA was 321.3 *m*/*z* while product ions of MPA were 303.2 *m*/*z* and 207.2 *m*/*z*.

2.10. Equilibrium experiments

Five mg of MIP or NIP particles were transferred in Eppendorf tubes. Subsequently 1 ml of phosphate buffer (1/15 mol/l, pH = 9), spiked with varying concentrations of MPA in the range of 0.1–150 μg/ml (0.312–468 nmol/ml), was added. The tubes were shaken for 4 h on an orbital shaker prior to centrifugation (15 min at 14,000 × *g*). The supernatant was collected, evaporated under nitrogen and redissolved in 100 μl of mobile phase prior to LC–MS/MS analysis. The amount of MPA bound by the polymers was calculated by comparing the equilibrium concentration of free MPA to the initial concentration of MPA.

2.11. Liquid–liquid extraction

Maize samples were obtained from local suppliers. A 5 g portion of ground maize (absence of MPA was confirmed with LC–MS/MS) was weighed in a tube and 25 ml of extraction solvent (AcN/H_2O (90/10, v/v) + 0.1% FA) was added. The sample was tumbled for 30 min using an end-over-end tumbler. The extraction mixture was centrifuged at 2670 × *g* during 10 min. In case of MISPE or OASIS HLB® clean-up, 1 ml of the supernatant was evaporated and 10 or 1000 ng MPA was added before dissolving the dry residue prior to imprinted or OASIS HLB® clean-up. For liquid–liquid extraction (LLE), 10 ml of the supernatant was fortified with 100 or 10,000 ng MPA, prior to defatting the sample extract twice with 10 ml of hexane. Following the defatting step, 1 ml of the aqueous phase was evaporated under nitrogen and redissolved in 100 μl of mobile phase prior to LC–MS/MS analysis.

2.12. MISPE and NISPE for the analysis of maize samples

After wet-packing 150 mg of the (non-)imprinted polymers in an empty SPE cartridge between two glass wool frits, the sorbent was conditioned. The wet-packing protocol comprised transferring a slurry mixture (150 mg MIP or NIP in MeOH) in the cartridges. Possibly remaining impurities and analyte from a previous clean-up were eluted by passing 3 ml MeOH/CH_3COOH (95/5, v/v) through the column. Subsequently the column was dried by vacuum and conditioned with 3 ml H_2O. A sample, dissolved in 1 ml phosphate buffer (1/15 mol/l, pH = 9), was loaded onto the (non-)imprinted polymer. Next, the column was washed with 1 ml phosphate buffer (1/15 mol/l, pH = 3) and finally MPA was eluted with 3 ml MeOH/CH_3COOH (95/5, v/v). The eluent fractions were collected, evaporated under nitrogen and redissolved in 100 μl of mobile phase prior to LC–MS/MS analysis.

2.13. OASIS HLB® clean-up

OASIS HLB® clean-up protocol was derived from Garon et al. [29]. The cartridge was previously conditioned with 2 ml MeOH and equilibrated with 2 ml H_2O. A sample dissolved in 1 ml MeOH/H_2O (10/90, v/v) was percolated through the OASIS HLB® cartridge. The column was washed with 2 ml H_2O and finally MPA was eluted with 5 ml MeOH. The eluent fractions were collected, evaporated under nitrogen and redissolved in 100 μl of mobile phase prior to LC–MS/MS analysis.

2.14. Validation study

Recovery was examined by performing six experiments. Precision was obtained by computing the standard deviation ($n = 6$) for analyses repeated on different days. Accuracy was determined by calculating the recovery. LOD and LOQ were calculated as the concentration corresponding to a signal-noise ratio (S/N) of respectively 3 and 10.

To examine the implementation of the MIP in MPA analysis, the concentration of MPA present in naturally contaminated maize samples was determined with LC–MS/MS after MISPE. A matrix matched calibration curve was used to quantify the amount of MPA in the samples.

2.15. Cross-reactivity study

The selectivity of the MIP and NIP during the clean-up of maize samples was studied by comparing the retention behaviour of MPA to that of other mycotoxins. The mycotoxins in the cross-reactivity study were selected regarding their possible occurrence and the

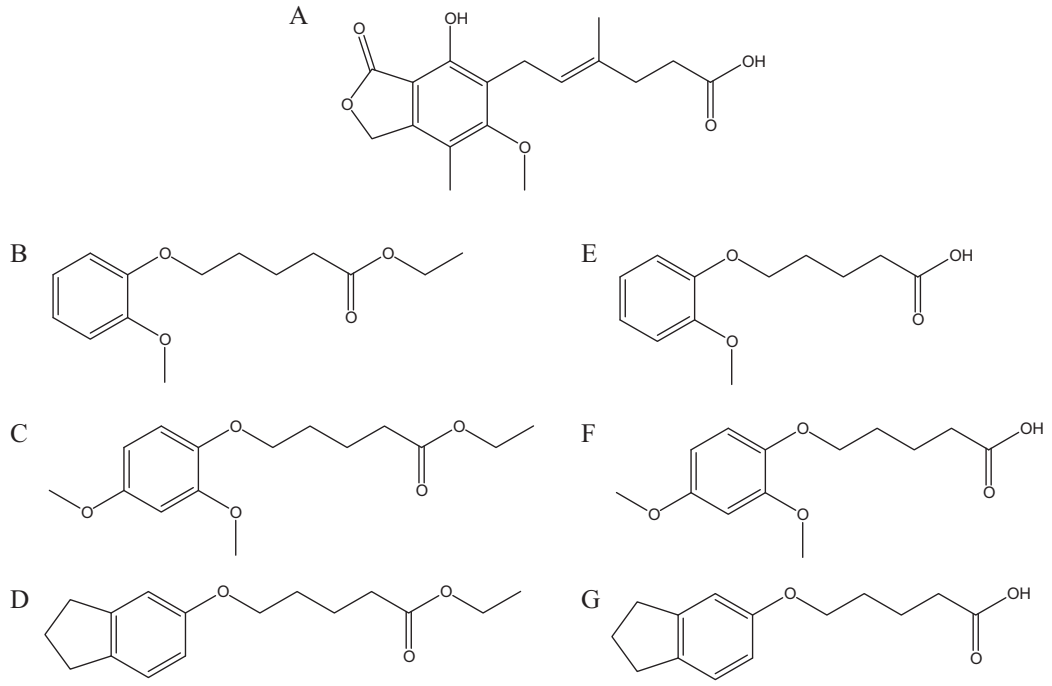

Fig. 1. Chemical structures of the examined templates: (A) MPA, (B) 5-(2-methoxy-phenoxy)-pentanoate ethyl ester, (C) 5-(4-methoxy-phenoxy)-pentanoate ethyl ester, (D) 5-(5-indanoxy)-pentanoate ethyl ester, (E) 5-(2-methoxy-phenoxy)-pentanoic acid, (F) 5-(4-methoxy-phenoxy)-pentanoic acid, and (G) 5-(5-indanoxy)-pentanoic acid.

potential non-specific interactions with the imprinted sorbent. Following the previously described methods, maize samples fortified with 200 ng of each of the mycotoxins (MPA, FB_1, DON, NIV, HT-2, CIT, T-2, OTA, AFB_1 and AFG_2) were implemented in MISPE, NISPE, LLE and OASIS HLB® clean-up. This procedure was repeated three times. The collected eluates were analysed with LC–MS/MS as described by Monbaliu et al. [30].

3. Results and discussion

Due to the absence of proper clean-up methods, most existing analytical methods for MPA lack sensitivity. The objective was to synthesize an imprinted polymer and optimize a MISPE procedure for the selective clean-up of maize samples contaminated with MPA. To design the MIP, the application of native MPA and six structural analogues as template was evaluated (Fig. 1). Dummy molecules were implemented in MIP synthesis because of the toxicity and the high cost price of MPA. The dummy templates were selected because these structures exhibited functionalities, size and shape similar to MPA. $CHCl_3$ was selected as porogen, because of the good solubility of MPA and the absence of disrupting interactions. The implementation of the functional monomers 2VP and 4VP was examined. It was expected that both monomers would assemble around the template via π–π stacking and ionic interactions. In addition, the amount of cross-linking monomer (EGDMA) is known to play a significant role in the formation of imprinted binding sites. Supported by data of preliminary experiments 4VP was chosen as functional monomer and the molar ratio of template:functional monomer:cross-linker was set to 1:4:20.

Before the imprinted polymer was implemented in the clean-up of maize extracts, the template was removed to obtain free imprinted binding sites. The final elution fractions were analysed with LC–MS/MS to verify that no MPA leached from MIP4VP (Fig. 2). Notwithstanding imprinted sorbents are characterized by their high affinity and selectivity towards the analyte, each step of the MISPE protocol must be optimized. Throughout the whole clean-up procedure the analyte should be adsorbed quantitatively contrary to matrix compounds. Percolating maize extracts, dissolved in $CHCl_3$, led to low retention (<25%). Because of the weak acidic properties of MPA and the application of alkaline functional monomer, buffer solutions were evaluated to load the sample and remove matrix interferences. The MISPE procedure was optimized by utilizing MIP4VP and NIP4VP as sorbents. The loading, washing and elution solvents were selected by examining the recovery of MPA, the reproducibility (standard deviation) and the percentage of specific interactions (comparison of recoveries obtained with MIP and NIP). To optimize the load and washing solutions, the pH values of the loading and washing conditions were screened. The optimized pH to load the maize extracts amounted to 9, while for eluting the non-specifically bound components a buffer with pH equal to 3 was preferred (Fig. 3). Finally 3 ml MeOH/CH_3COOH (95/5, v/v) was percolated through the column to elute the toxins quantitatively. Retention of MPA onto the MIPs is attributed to shape selectivity, π–π stacking and ionic interactions between 4-VP and MPA. Simon et al. demonstrated that MIPs prepared with templates with one or two template-functional monomer interactions rely heavily on shape selectivity [31]. Furthermore, contrary to 4VP, application of 2VP as functional monomer did not result in imprinting. While the vinylidene compound of the monomer 4VP is situated at the opposite side of the nitrogen atom in the aromatic structure, the vinylidene compound in 2VP is located next to the nitrogen atom and therefore sterically hindered the interaction of MPA with 2VP. The position of the nitrogen atom in the functional monomer determined the imprinting effect and therefore it was assumed that functional group pre-organization had an important role in molecular recognition. Since pH value affected the retention of MPA (Fig. 3) and the analyte was percolated through the MIP in aqueous media, π–π stacking and ionic interactions were occurring.

3.1. Scatchard analysis

Equilibrium experiments were completed to assess the binding performance of the (non-) imprinted particles towards MPA. The

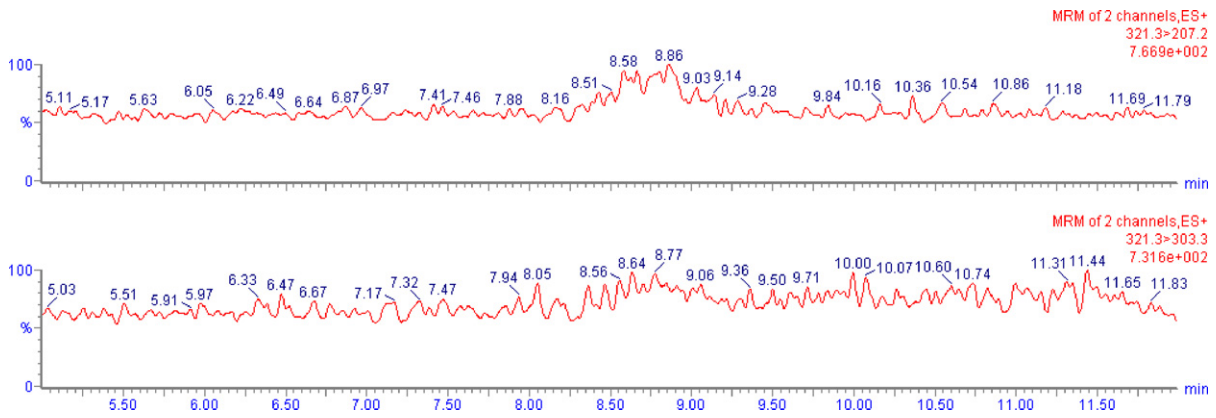

Fig. 2. LC–MS/MS chromatogram indicating the absence of template bleeding in case of MIP4VP (retention time MPA: 8.98 min).

amount of MPA bound to the polymers was studied in function of the initial concentration (Fig. 4). For all the imprinted polymers an increase in the amount of toxin bound at equilibrium was observed along with increasing initial concentration of MPA. Furthermore it was noticed that the amount of MPA bound to the MIP particles depended on the choice of template. Contrary to the MIPs, the binding isotherm of the NIP was characterized by a plateau, indicating the saturation of the binding sites at low concentration. The binding parameters of MIPs are generally determined by plotting the data according to the Scatchard equation:

$$\frac{Q}{[\mathrm{MPA}]} = \frac{Q_{\max} - Q}{K_D}$$

where Q represents the amount of MPA bound to the polymer at equilibrium, [MPA] the free analyte concentration at equilibrium, Q_{max} the apparent maximum number of binding sites and K_D the dissociation constant.

In the plots of the imprinted polymers two data groups, which could be linearly fitted, were distinguished. Meanwhile the Scatchard plot for the NIP existed of one linear part. The Scatchard analysis indicates that the binding sites in the MIP were heterogeneous, while the blank polymer consisted of homogeneous binding sites. From the linear regression equations K_D and Q_{max} were calculated (Table 2). Notwithstanding all the designed MIPs exhibited imprinting effect, the affinity and the number of binding sites differed with the selection of template. The data show that application of MPA as template during the synthesis resulted in an imprinted polymer (MIP4VP) with highest affinity towards the analyte, but a low number of binding sites. The high affinity of MIP4VP is explained by the implementation of MPA as template during the imprinting process. Therefore MIP4VP exhibited binding sites which were more complementary in shape, size and

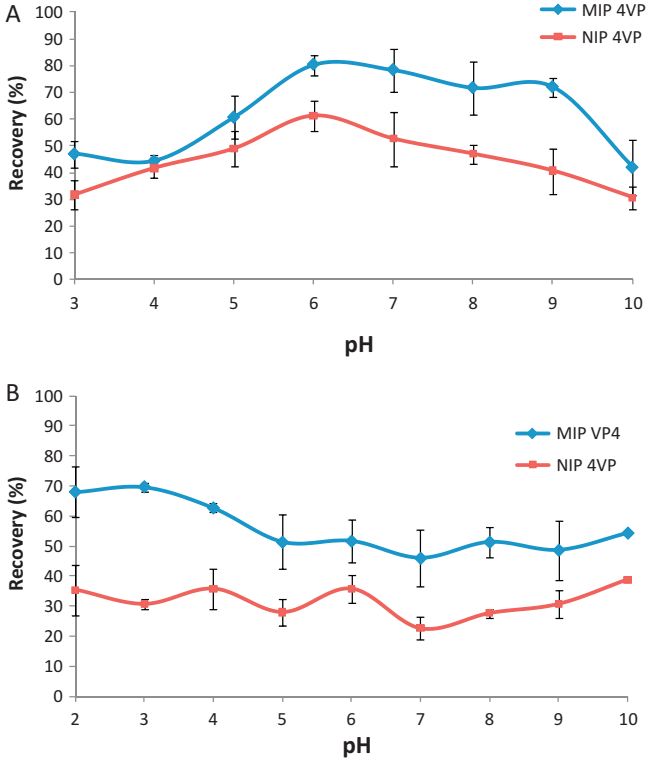

Fig. 3. (A) Representation of the recovery after loading maize extracts, dissolved in aqueous buffers with different pH. (B) Recovery after loading samples, dissolved in phosphate buffer (pH = 9), and washing the sorbent with aqueous buffers with different pH value.

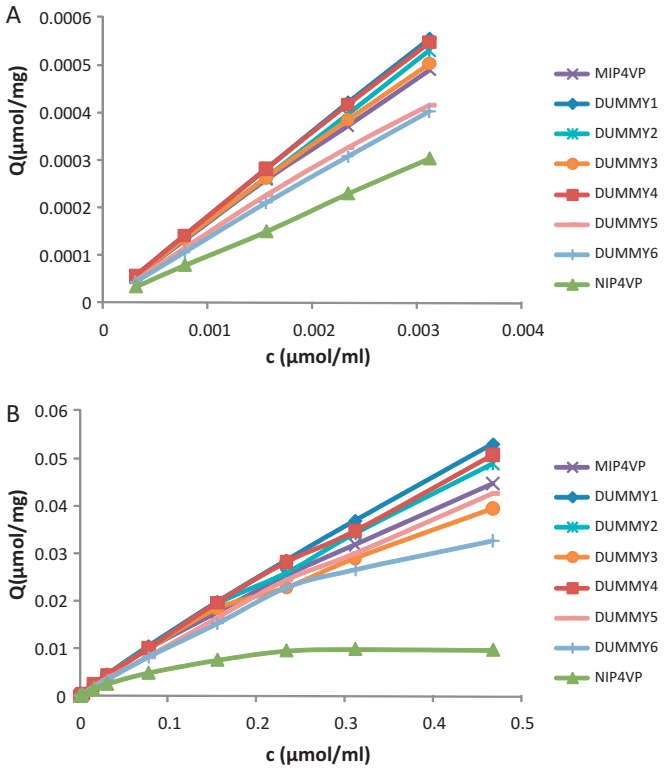

Fig. 4. Binding isotherms of MPA to MIPs and NIP at low (A) and high (B) concentrations.

Table 2
Binding sites' properties of MIPs and NIP.

	Low affinity		High affinity	
	K_D (μmol/l)	Q_{max} (μmol/g)	K_D (μmol/l)	Q_{max} (μmol/g)
MIP4VP	160.2	67.2	0.8	1.0
DUMMY1	289.9	123.8	3.2	6.0
DUMMY2	260.5	100.6	7.1	8.4
DUMMY3	138.5	55.7	6.4	6.9
DUMMY4	292.4	115.8	3.2	6.0
DUMMY5	519.3	130.4	15.7	8.0
DUMMY6	239.1	61.7	10.2	4.4
NIP4VP	58.9	11.5	/	/

functionality to MPA, compared to the other imprinted polymers. However, the lower reactivity of MPA towards 4VP resulted in a low number of high affinity binding sites. DUMMY1 and DUMMY4 demonstrated good affinity and a substantial number of binding sites clarifying the higher amount of bound MPA. Characteristic are the low percentages of specific binding sites present in the MIPs due to the incomplete template-functional monomer association. As a result of that, free functional groups occur in the MIP causing non-specific binding sites. So only a small percentage of the binding sites in the MIP are complementary in size and shape to the analyte.

3.2. Validation study with spiked maize samples

Recovery experiments were performed at two different spiking levels: 50 and 5000 μg/kg. Indeed several surveys demonstrated that high levels of MPA can be found in different types of food [7,8].

The results of the recovery experiments are shown in Table 3. The data are the mean of six experiments. The study revealed a satisfying precision and a significant difference in binding for all the imprinted polymers compared to the NIP. The imprinting effect of the DUMMY MIPs was also observed during Scatchard analysis and could be explained by similarities in functionalities, size and shape of the dummy templates and MPA. Since MPA is a structural analogue of the dummy templates, the specific binding sites in the DUMMY MIPs show a high affinity towards MPA. MPA exhibited best retention on DUMMY1 and DUMMY4, while lowest recovery was seen with DUMMY6. These recoveries correspond to the data of the equilibrium experiments. Results indicated that implementing structural analogues in MIP synthesis could lead to MIPs for which a higher recovery of MPA after clean-up was observed, than MIPs for which the analyte was applied as template. Relying on the data of the validation study and the Scatchard analysis it was decided to conduct further experiments (matrix effect, cross-reactivity and analysis of maize samples) with MIP4VP, DUMMY1 and DUMMY4. Furthermore, the fortified maize samples were also implemented in LLE or OASIS clean-up, since these procedures are frequently applied in mycotoxin analysis. SPE using OASIS HLB® columns resulted in low recoveries, while recoveries obtained after LLE were higher than 90%.

LOD and LOQ were lower than 1 μg/kg revealing the sensitivity of the imprinted clean-up method. Lowest LOD and LOQ were obtained with MIP4VP, respectively 0.17 μg/kg and 0.57 μg/kg. In case of DUMMY1 and DUMMY4 the LODs amounted to 0.25 μg/kg and 0.18 μg/kg, while the LOQs were 0.82 μg/kg and 0.60 μg/kg.

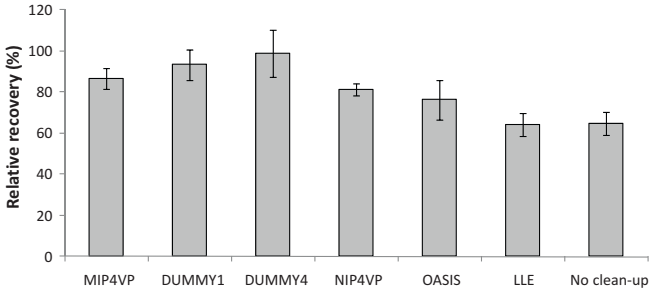

Fig. 5. Ion signal recovery of spiked sample extracts compared to standard solutions. Data represent the mean (±1SD) of experiments performed in triplicate.

Following OASIS HLB® clean-up or LLE, the LODs were 10 and 7.5 μg/kg, while the LOQs amounted to 30 μg/kg and 25 μg/kg, respectively. The results indicate that the MIPs are appropriate sorbents to detect and quantify low amounts of MPA. The imprinted particles could be reused in the course of the whole research (75 experiments) without loss of binding activity or elution of analyte remaining from a previous clean-up.

3.3. Study on matrix effects

To assess the efficacy of the MISPE-procedure the matrix effect was determined (Fig. 5). Matrix effect is the combined effect of all components of the sample other than MPA on the measurements. The matrix effect was set by conducting signal recovery studies in triplicate. The evaluation was carried out by comparing the instrument response of a standard solution with the same amount of MPA as added to the eluates of blank extracts after clean-up. The closer the relative signal recovery amounted to hundred percent the less matrix effect was present. Because MPA is often analysed without applying a clean-up step, a spiked extract was injected without previous purification resulting in a substantial decrease of the ion signal recovery. Injection of a sample after LLE caused a similar decrease of the ion signal recovery. MISPE of the maize extracts resulted in much less matrix effect, which emphasized the inevitability of an effective clean-up. Purification of the samples with the imprinted polymers (especially DUMMY1 and DUMMY4) showed less matrix effect than NISPE and OASIS HLB® clean-up. Furthermore a variation in matrix effect was observed for the different MIPs. Almost no decrease in ion signal recovery was seen for the DUMMY MIPs, while application of MIP4VP caused ion suppression. It seemed that along with the choice of template the matrix effect varied.

3.4. Cross-reactivity study

To evaluate the cross-reactivity of the designed polymers the retention of non-analogue mycotoxins, selected on behalf of their reported occurrence in maize or their ability to interact non-specifically with the MIP, was also examined (Fig. 6).

All examined polymers exhibited similar cross-reactivity except for AFB_1. AFB_1 retained well on MIP4VP and NIP4VP but not on the DUMMY MIPs, while for the structural analogue AFG_2 no significant difference in binding towards the polymers was noticed. Binding of

Table 3
Recovery of MPA after MISPE, NISPE, OASIS clean-up or LLA of spiked maize samples. Data represent the mean (±1SD) of experiments performed of 6 experiments.

Spiking level (μg/kg)	Recovery (%)									
	MIP4VP	NIP4VP	DUMMY1	DUMMY2	DUMMY3	DUMMY4	DUMMY5	DUMMY6	OASIS	LLE
50	78 ± 9	27 ± 5	84 ± 6	77 ± 9	68 ± 12	82 ± 6	66 ± 8	52 ± 9	54 ± 5	93 ± 7
5000	72 ± 8	27 ± 6	81 ± 5	78 ± 6	69 ± 7	80 ± 6	64 ± 4	51 ± 10	55 ± 5	94 ± 6

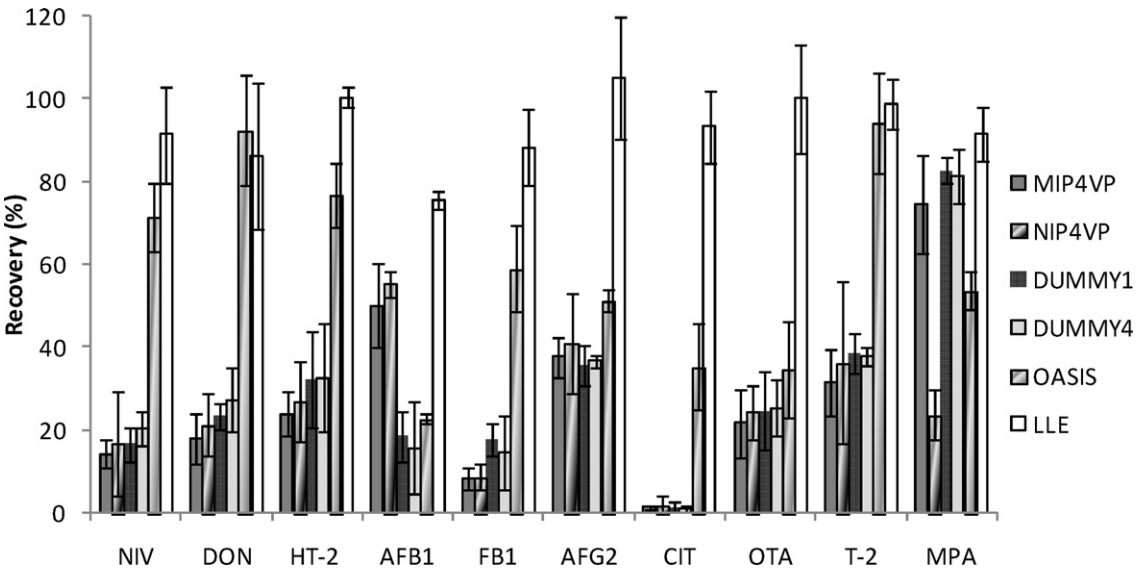

Fig. 6. Recoveries obtained by loading maize samples fortified with NIV, DON, HT-2, AFB_1, FB1, AFG_2, CIT, OTA, T-2 and MPA. Data represent the mean (±1SD) of experiments performed in triplicate.

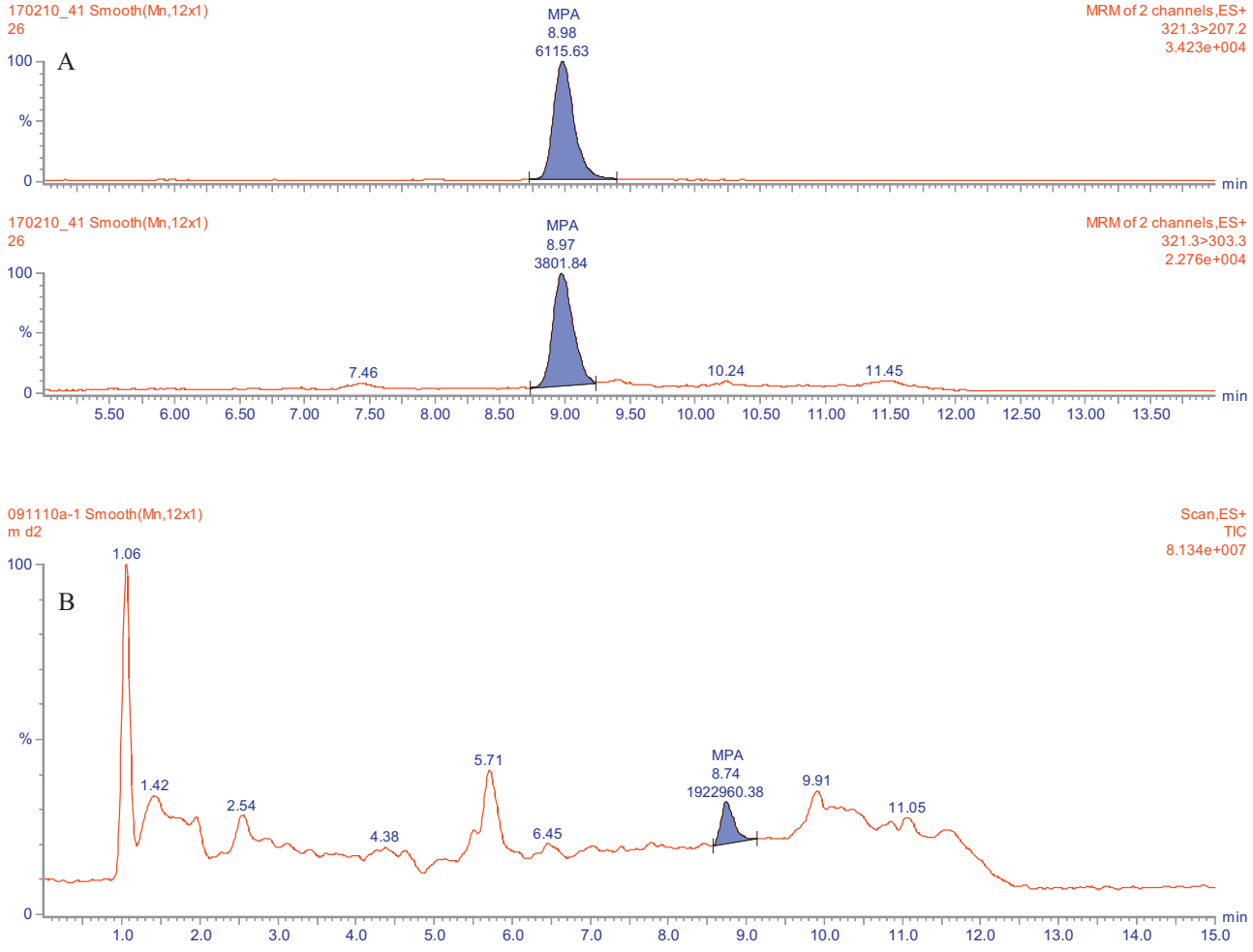

Fig. 7. (A) LC–MS/MS chromatogram in multiple reaction monitoring mode of a contaminated maize sample after MISPE. (B) Total ion chromatogram of a contaminated maize sample after MISPE.

Table 4
Occurrence levels (μg/kg) of MPA in maize. n.d. = not detected.

Sample	Concentration (μg/kg)				
	MIP4VP	DUMMY1	DUMMY4	OASIS	LLE
1	n.d.	n.d.	n.d.	n.d.	n.d.
2	7	9	6	n.d.	n.d.
3	5	7	6	n.d.	n.d.
4	25	36	31	<LOQ	30
5	68	81	85	78	86
6	n.d.	n.d.	n.d.	n.d.	n.d.
7	n.d.	n.d.	n.d.	n.d.	n.d.
8	n.d.	n.d.	n.d.	n.d.	n.d.
9	66	80	77	73	79
10	95	98	113	102	118
11	63	43	36	55	58
12	11	16	8	<LOQ	<LOQ
13	9	9	7	n.d.	<LOQ
14	n.d.	n.d.	n.d.	n.d.	n.d.
15	n.d.	n.d.	n.d.	n.d.	n.d.

the examined mycotoxins could be mainly ascribed to non-specific hydrophobic interactions. In general highest cross-reactivity was observed for the aflatoxins, while CIT did not retain onto the sorbents. Apart from the aflatoxins and CIT, cross-reactivity ranged from 14% to 38%. Cross-reactivity depended on the polarity of the mycotoxins: the more polar, the smaller the non-specific retention of the mycotoxins. This could be attributed to the affinity of the analyte towards the percolated solvents. Cross-reactivity did not influence MPA determination since no significant change in recovery of MPA and no interferences were observed (Fig. 6). The retention of non-analogue mycotoxins during LLE and OASIS HLB® clean-up was also evaluated. During LLE, it was observed that cross-reactivity varied from 76% to 105%. Moreover, OASIS HLB® clean-up exhibited also high cross-reactivity. Apart from AFB_1, retention of the mycotoxins ranged from 35% to 94%. Contrary to the conventional clean-up procedures, MISPE exhibited much higher selectivity for MPA in presence of other mycotoxins.

3.5. Analysis of naturally contaminated samples

The levels of MPA in 15 maize samples were determined with LC–MS/MS after MISPE, LLE or OASIS HLB® clean-up (Table 4). The samples were obtained from different sources. Out of 15 maize samples, 9 were contaminated with MPA. In Fig. 7 a LC–MS/MS chromatogram in MRM mode and a total ion chromatogram (TIC) of a naturally contaminated sample are shown. The TIC indicated that the washing step did not remove all matrix compounds, but that the retained compounds did not interfere during MPA analysis. Occurrence data, maximum level, mean and median concentration of MPA in maize are listed for the different applied MIPs in Table 5. To calculate the mean and median, the assumption was made that samples with traces of MPA contained half of the sum of the LOD and LOQ, while samples with no detectable levels of MPA contained half of the LOD. Analysis revealed that MPA is frequently found in maize but that concentration levels of MPA are not as high as in other types of food [8]. In order to detect low levels of MPA it is essential to apply selective sorbents. Indeed as demonstrated in Tables 4 and 5 the use of OASIS HLB® cartridges and LLE led to higher LOD and LOQ causing an underestimation of the percentage of contaminated samples. Implementation of imprinted polymers during the clean-up made it possible to detect low levels of MPA, while no MPA was detected in the same samples applying the OASIS HLB® cartridges and LLE.

Table 5
Occurrence data of MPA in maize.

	Maize				
	MIP4VP	DUMMY1	DUMMY4	OASIS	LLE
Overall incidence (%)	60	60	60	40	47
Maximum (μg/kg)	95	98	113	102	118
Mean (μg/kg)	23	25	25	31	33
Median (μg/kg)	7	9	6	5	4

Table 6
Pearson correlation between MISPE (MIP4VP, DUMMY1 and DUMMY4), LLE and OASIS HLB® clean-up.

	MIP4VP	DUMMY1	DUMMY4	OASIS	LLE
MIP4VP		0.97	0.96	0.99	0.99
DUMMY1			0.99	0.98	0.99
DUMMY4				0.98	0.99
OASIS					0.99

Mean and median concentrations of MPA in maize obtained after different clean-up procedures were similar. Pearson correlations were computed making the same assumptions as before with the calculation of the median and mean. The excellent correlations ($r \geq 0.96$) found between analyses based on MISPE, LLE and OASIS HLB® clean-up express the possibility to implement imprinted sorbents during the clean-up of maize samples (Table 6).

4. Conclusions

Imprinted polymers selective towards MPA, applying the toxin itself or structural analogues as template, were designed and evaluated. The target mimic must contain the functional features responsible for specific interactions plus a similar shape to achieve efficient recognition of the analyte. All the synthesized MIPs exhibited an imprinting effect depending on the choice of imprint molecule. Equilibrium experiments revealed that 5-(4-methoxy-phenoxy)-pentanoate ethyl ester and 5-(4-methoxy-phenoxy)-pentanoic acid were the most appropriate dummy molecules. Validation studies verified the excellent performance of DUMMY1 and DUMMY4 in terms of recovery and reproducibility. Despite the cross-reactivity with especially the hydrophobic mycotoxins, low matrix effect and detection limits were obtained, expressing the sensitivity of the MISPE procedure compared to LLE and OASIS HLB® clean-up. Analysis of naturally contaminated samples by LC–MS/MS demonstrated that imprinted clean-up is a suitable technique to detect MPA in maize. Additionally purification of maize samples using the developed imprinted polymers is not costly because of the inexpensive synthesis and the multiple use of one MIP cartridge.

Acknowledgement

Financial support by BOF (grant number 01J08409) of Ghent University is gratefully acknowledged.

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