

Synthesis and evaluation of molecularly imprinted polymers as sorbents of moniliformin

M. APPELL, D. F. KENDRA, E. K. KIM, & C. M. MARAGOS

Mycotoxin Research, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL 61604, USA

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Abstract

Moniliformin is a low molecular weight mycotoxin that has worldwide potential to contaminate cereal grains. Although several traditional methods have been developed to detect moniliformin, the lack of anti-moniliformin antibodies has created a need for materials that recognize moniliformin at the molecular level through a binding mechanism. To address this issue, the authors synthesized molecularly imprinted polymers that bind moniliformin. Imprinted and non-imprinted polymers were evaluated by equilibrium binding assays and moniliformin concentrations were measured by LC analysis using ultraviolet light detection. Successful polymers were imprinted with toxin analogues as the templates; non-imprinted polymers exhibited minimal binding in acetonitrile under the assay conditions. Selected imprinted polymers also bound moniliformin in ethanol, methanol and dimethyl formamide. Significant differences in moniliformin binding by the polymers were dependent on polymer composition, and these differences were highly dependent on the template used to imprint the polymer. Polymers were further evaluated as sorbents for molecularly imprinted solid-phase extraction (MISPE), and an imprinted polymer was used for preconcentration and clean-up of a moniliformin spiked corn extract.

Keywords: *Moniliformin, molecularly imprinted polymers, molecular recognition, mycotoxins*

Introduction

Moniliformin is a very important mycotoxin produced by a number of *Fusarium* species, including *F. proliferatum* and *F. subglutinans*, with worldwide potential to contaminate maize and other cereal grains. Contamination of cereal grains by moniliformin has been shown to cause death, reduced food intake and weight loss in animals *in vivo* (Čonková et al. 2003). One mode of action of moniliformin that has been identified is the suppression of cellular respiration through disruption of the Krebs cycle by covalent inhibition of the pyruvate dehydrogenase enzymatic system (Hofmeyr et al. 1979; Gathercole et al. 1986). The low molecular weight toxin is generally isolated as the sodium or potassium salt of 3-hydroxy-3-cyclobutene-1,2-dione, also known as semisquaric acid (Figure 1).

Several methods of the detection of moniliformin exist and additional methods are actively under development. These methods typically require a

clean-up step followed by a detection step. Extraction is generally carried out with acetonitrile/water and with an ion pair reagent, although a method using α -amylase has recently been reported (Chung et al. 2005). Clean-up steps may involve the use of SAX or C18 columns. Methods of detection have been developed using conventional analytical techniques including HPLC-UV and mass spectroscopy (Sharman et al. 1991; Sewram et al. 1999). An alternative method has been reported using capillary-zone electrophoresis-diode array detection (Maragos 2004). In addition, simple derivatization of moniliformin allows the desired crude products to be analysed by fluorescence detection or gas chromatography (Gilbert et al. 1986; Filek & Lindner 1996).

A method to detect moniliformin by selective recognition through a binding mechanism does not currently exist. Typically, antibodies are developed as a means to obtain a selective small-organic binding agent. In the case of moniliformin,

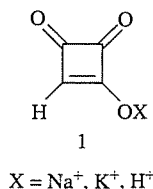


Figure 1. Moniliformin.

anti-moniliformin antibodies have not been reported, possibly due to the small size of moniliformin resulting in a limited arrangement of functional groups of moniliformin for interactions with an antibody binding site. One alternative to antibodies for selective binding materials is molecularly imprinted polymers (MIPs). Imprinted polymers possess binding sites with functional groups arranged in geometries capable of binding a substrate.

The binding sites of non-covalently imprinted MIPs are formed by the favourable interaction of a template with functional groups of reactive molecules (the functional monomers). The polymer is synthesized in solvent with a reactive cross-linker reacting with itself and the functional monomers to form a polymer embedded with binding sites. These binding sites are accessible by channels produced by solvent. In-depth description of molecular imprinting technologies has been detailed (Ramström et al. 2001; Sellergren 2001; Cormack & Elorza 2004) and MIPs will not be reviewed in further detail here.

From the above description of the traditional synthesis of MIPs, it can be seen that MIPs differ in the consistency and population of their binding sites than monoclonal antibodies. Monoclonal antibodies are developed to yield selective and unique binding sites reproducibly, while the types of binding sites in imprinted polymers are governed by the equilibrium of solution phase chemistry producing a population of heterogeneous binding sites. The binding sites of MIPs have the potential to have varying affinities (Umpleby et al. 2004).

Several MIPs have been reported for mycotoxins. Deoxynivalenol imprinted polymers and polymers imprinted with quercetin as a toxin analogue of zearalenone have been applied as a stationary phase in LC analysis (Weiss et al. 2003). The quercetin-imprinted polymers have successfully enhanced the detection of zearalenone in beer samples spiked with zearalenone (Krska et al. 2005). Aflatoxin B₁ imprinted polymers have been synthesized and characterized by solid-phase extraction columns (Subrahmanyam 2002). Ochratoxin A imprinted polymers have been applied as solid-phase extraction columns under aqueous conditions and utilized for the analysis of wheat extracts (Turner et al. 2004;

Zhou et al. 2004). Polymers imprinted with analogues of ochratoxin A have exhibited binding of ochratoxin A and seen application in solid-phase extraction columns (Baggiani et al. 2001; Jodlbauer et al. 2002; Maier et al. 2004). Ochratoxin A-imprinted polypyrrole materials have seen utilization as micro solid-phase extraction columns and as components of surface plasmon resonance sensors (Yu et al. 2005; Yu & Lai 2005). In addition, there is considerable interest for other types of non-imprinted sorbents that bind mycotoxins (Lemke et al. 2001; Bursian et al. 2004; Avantaggiato et al. 2005).

Antibodies exist for the previously published reports of the mycotoxin active imprinted polymers and these polymers have seen application as important alternatives to antibodies to enhance the detection of mycotoxins. To our knowledge this is the first report of this nature for an imprinted polymer that has been designed to bind a mycotoxin for which an antibody does not exist.

Materials and methods

Reagents

3,4-Dihydroxy-3-cyclobutene-1,2-dione, 3,4-diethoxy-3-cyclobutene-1,2-dione, 2-(dimethylamino)ethyl methacrylate, methacrylic acid, anhydrous dimethylformamide (DMF), trimethylolpropane trimethacrylate (TRIM), 2,2'-azobisisobutyronitrile (AIBN), tetrabutylammonium hydrogen sulfate, ethanol, acetone (HPLC grade) and acetonitrile (HPLC grade) were obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA). Inhibitors were removed by the appropriate inhibitor remover packed column (Sigma-Aldrich). Monobasic potassium phosphate was obtained from Taylor Scientific (St Louis, MO, USA). Moniliformin sodium salt was a generous gift from Ronald Plattner (USDA-ARS-NCAUR, Peoria, IL, USA).

Polymer synthesis

Imprinted and non-imprinted polymer synthesis was carried out in parallel. Templates (0.15 mmol) followed by the selected functional monomers (0.15–1.2 mmol) were dissolved by ultrasonication in anhydrous DMF (1 ml) in a 17 × 35 mm screw thread glass vial with a removable screw cap and a TFE/SIL seal. The solutions were placed in the dark for 4 h at room temperature. Following the incubation period, TRIM (6 mmol, 2.030 g) and AIBN (1.5% of the reactive bonds) in 0.875 ml anhydrous DMF were added. Reaction mixtures were purged with high-purity, dry nitrogen, and then incubated at 65°C in a MagniWhirl constant

temperature bath for 24 h. Non-imprinted polymers were synthesized in a similar manner, without template.

Each of the polymer monoliths was crushed and removed from the vials, then subjected to ultrasonication for 5 min in 200 ml methanol. The supernatant was decanted and discarded. Polymers were ground by mortar and pestle and wet sieved with acetone. The collected fractions (75–38 μm) were suspended in 40 ml methanol:acetic acid (9:1) in a 50 ml centrifuge tube, wrist shaken, ultrasonicated, sedimented, and the supernatant was decanted. This procedure was repeated for a total of three \times 40 ml methanol:acetic acid (9:1), three \times 40 ml distilled water, and three \times 40 ml acetonitrile. Following the last acetonitrile treatment, particles were separated from the solvent by vacuum filtration over filter paper (grade 54, Whatman).

Computational chemistry

All calculations reported were performed on a QS4-2200C-O64 QuantumCube by Parallel Quantum Solutions (PQS) using PQS software v3.2 (PQS, Fayetteville, AK, USA) using default settings. The geometry optimizations were carried using 6-31+G* and 6-311++G** basis sets and the B3LYP density functional. Hyperchem v7.52 was used to build the initial structures and calculate volumes (HyperCube, Gainesville, FL, USA).

LC analysis

Concentrations of moniliformin were measured by LC analysis using a Spectrasystem P4000 gradient pump, a manual injector, a C18 SecurityGuard HPLC Guard Cartridge System, and a Phenomenex, Luna 5 μm C18 100A HPLC column. Moniliformin was detected by ultraviolet detection at 229 nm on a Spectrasystems UV2000 ultraviolet detector and a Spectra Focus forward optical scanning detector. Results were recorded using AllChrom Plus Chromatography Data System on a PC with an AMD Athlon processor. Concentrations of moniliformin presented in the Freundlich isotherm and solid-phase extraction experiments were obtained on a Shimadzu HPLC equipped with a PDA detector (190–330 nm). Peak areas were calculated at 229 nm. Experiments were carried out at room temperature and solvents were flushed with helium before use. The solvent system consisted of 4:1 buffer/acetonitrile containing 1.14 mg ml^{-1} tetrabutyl ammonium hydrogen sulfate and 1.07 mg ml^{-1} potassium dihydrogen phosphate. Before mixing with acetonitrile, the pH of the buffer was adjusted to 4.0 using an aqueous solution of potassium hydroxide (1.0 and 0.1 N) measured by

an Accumet AP71 pH meter. Moniliformin and toxin analogue concentrations were calculated by peak area comparison with standard solutions.

Evaluation of polymers

Polymers were first evaluated by measuring their ability to bind moniliformin by equilibrium binding assays. Assays were conducted in 1.5 ml silanized screw cap vials. Polymers (10 mg) in a 1 ml solution of moniliformin in acetonitrile (10 $\mu\text{g ml}^{-1}$) were shaken on a Lab-line Multiwrist shaker set on speed 5 at room temperature for 1 h. The vials were centrifuged and the supernatant filtered through a Millex Syringe driven PTFE filter (0.2 μm). The samples were further diluted with an equal amount of nanopure water before analysis. Equilibrium binding studies for the ability of polymers to bind templates were carried out under similar conditions with absorbance measured at 254 nm. All binding experiments were performed in triplicate. For all results reported here, binding was determined through the comparison of a standard without polymer and samples run with polymers through the following relationship:

Percentage bound

$$= (1 - ([\text{MON}_{\text{polymer}}]/[\text{MON}_{\text{standard}}])) \times 100,$$

where $[\text{MON}_{\text{polymer}}]$ is the concentration (peak area) of moniliformin in the supernatant recovered from incubation with polymer, and $[\text{MON}_{\text{standard}}]$ is the concentration (peak area) of moniliformin recovered from incubation without polymer.

Selected polymers were evaluated as a sorbent in solid-phase extraction (SPE) columns. Polymer (25 mg) was packed between two frits in 1.5 ml solid-phase extraction reservoirs from Alltech (Deerfield, IL, USA). Freshly packed columns were washed with 10 ml water, methanol and acetonitrile before use. Columns were loaded with moniliformin (3 ml of 5 $\mu\text{g ml}^{-1}$ in 95% acetonitrile/water), washed with 95% acetonitrile/water (1 ml), and moniliformin was eluted with the LC mobile phase (1 ml). Application of the molecularly imprinted solid-phase extraction (MISPE) column was carried out on a ground corn extract. Ground corn (8 g) was extracted with 40 ml of 95% acetonitrile/water using a wristshaker for 30 min, then vacuum filtered. A portion of the filtrate (20 ml) was spiked with moniliformin for a final concentration of 0.5 of $\mu\text{g ml}^{-1}$. The MISPE column was loaded with the spiked corn extract (3 ml), washed with 95% acetonitrile/water (1 ml), and moniliformin was eluted from the column with the LC mobile phase (0.5 ml). Collected breakthrough and wash fractions were diluted 1:1 with elution buffer (LC mobile phase) before LC analysis for all

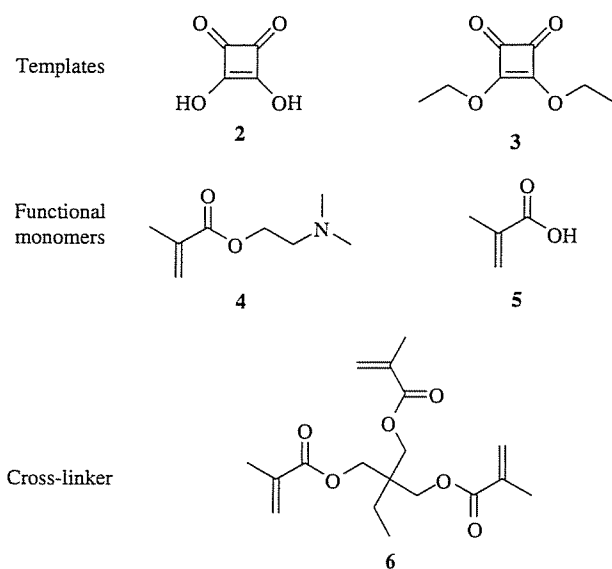


Figure 2. Reagents used to synthesize polymers.

MISPE experiments. Collected elution fractions were diluted 1:1 with 95% acetonitrile/water before analysis.

Results and discussion

The goal of the work presented herein is to evaluate the effectiveness of moniliformin analogues to imprint polymers and to investigate the effects of imprinting on the optimal polymer composition for moniliformin binding. Care was taken such that a synthetic chemist could synthesize and prepare these polymers using commercially available materials for further application without the need of specialized equipment. Our approach was iterative, based on polymer synthesis, and driven by maximizing the moniliformin binding of the resulting imprinted polymers compared with the non-imprinted polymers.

Reagents used to synthesize the polymers are shown in Figure 2. We investigated the use of both basic (2-(dimethylamino)ethyl methacrylate, 4) and acidic (methacrylic acid, 5) functional monomers. Although methacrylic acid is a very popular functional monomer, and is often described as acting as an acid and a base, we could not obtain detectable moniliformin binding for polymers with this functional monomer in the binding studies. Preliminary studies using divinyl benzene as a cross-linker produced polymers that were difficult to 'wet' under aqueous conditions, and it was decided not to pursue its use as a cross-linker any longer. Trimethylolpropane trimethacrylate, 6, was chosen as the cross-linker because of reported advantages

of cross-linkers with three connected polymerizable functional groups (Kempe and Mosbach 1995). All templates and reagents were soluble in anhydrous DMF, an aprotic solvent with a high dielectric constant commonly applied in synthesis. Moniliformin concentrations were measured by LC analysis to prevent potential interferences from components bleeding from the polymers. Selectivity was evaluated by comparing imprinted polymers with non-imprinted polymers. The non-imprinted polymers exhibited minimal activities under the binding assay conditions.

Binding interactions

The binding interactions involved in imprinted polymers are frequently similar to those of natural bound complexes with the most commonly considered are the favourable interactions of functional groups. In the case of moniliformin, the favourable interaction with the active functional monomer, 4, is through an ionic-strong hydrogen-bonding interaction, although other types of molecular interactions and bulk properties could influence binding (solvent effects). Furthermore, potential self-association of templates during polymer synthesis or substrates in the subsequent evaluation could complicate relating activities to a single bound complex. Such complicated associations have been observed in imprinted polymers (Svenson et al. 2004) and moniliformin is capable of forming self-complex interactions (Springer et al. 1974). However, early in this project it became apparent by real activities that successful moniliformin-binding polymers could be made by imprinting with a template similar in structure to moniliformin. This led to the investigation of template effects on moniliformin binding.

Template effects

Imprinted polymers reported herein were synthesized using commercially available moniliformin analogues 3,4-dihydroxy-3-cyclobutene-1,2-dione (2) and 3,4-diethoxy-3-cyclobutene-1,2-dione (3) (Figure 2). The use of toxin analogues offers several advantages over the use of toxins for imprinting MIPs, and there have been several previous reports of applying toxin analogues for the development of imprinted materials to bind mycotoxins (Baggiani et al. 2001; Jodlbauer et al. 2002; Weiss et al. 2003; Krška et al. 2005; Urraca et al. 2006).

Several factors were taken into consideration for the selection of the templates in this study, including safety, imprinting properties and cost. In MIP synthesis, milligram quantities of template will be embedded in gram quantities of polymers, creating a dangerous situation in the case of a very toxic

Table I. Moniliformin binding by polymers in acetonitrile.

Functional monomer	Mole ratio (template:functional monomer:crosslinker)	Bound moniliformin (%)
<i>Non-imprinted polymers</i>		
7a	(4) 0:8:40	6.1 ± 3.5
7b	(5) 0:8:40	n.a.
7c	(4) 0:4:40	n.a.
7d	(4) 0:2:40	n.a.
7e	(4) 0:1:40	n.a.
7f	none 0:0:40	n.a.
<i>3,4-Dihydroxy-3-cyclobutene-1,2-dione imprinted polymers (2)</i>		
8a	(4) 1:8:40	49.8 ± 1.2
8b	(5) 1:8:40	n.a.
8c	(4) 1:4:40	59.4 ± 0.4
8d	(4) 1:2:40	61.4 ± 2.9
8e	(4) 1:1:40	39.4 ± 1.8
8f	none 1:0:40	n.a.
<i>3,4-Diethoxy-3-cyclobutene-1,2-dione imprinted polymers (3)</i>		
9a	(4) 1:8:40	62.1 ± 1.6
9b	(5) 1:8:40	n.a.
9c	(4) 1:4:40	37.3 ± 3.7
9d	(4) 1:2:40	19.1 ± 6.1
9e	(4) 1:1:40	n.a.
9f	none 1:0:40	n.a.

All experiments were carried out at $10 \mu\text{g ml}^{-1}$ moniliformin and 10 mg ml^{-1} polymer. n.a., No activity (<3%).

All experiments were performed in triplicate. Results are reported as mean ± standard deviation.

template (a mycotoxin) for the synthetic chemist and the end user. In addition, toxin analogues can also be designed or selected for desirable properties. In our case, the toxin analogues used to make the polymers reported can possess an additional plane of symmetry, increasing the frequency for the substrate to be aligned properly in the binding site. An additional benefit of the toxin analogues selected is that analogues were less expensive allowing us to make a large number of polymers.

Results for binding of moniliformin by polymers in acetonitrile are shown in Table I. The non-imprinted polymers 7b–f lacked detectable binding of moniliformin under the evaluation conditions. Only polymer 7a, which contains eight equivalents of the basic functional monomer 4, possessed slight activity ($6.1 \pm 3.5\%$) under the conditions tested. We considered this type of binding for a non-imprinted polymer to be non-specific binding and thus did not pursue higher equivalents of functional monomer. Imprinting of polymers by templates 2 or 3 was necessary to obtain significant amounts of activity for the polymers tested. Polymers that did not have a functional monomer (7f, 8f and 9f) lacked activity as well.

The effects of the equivalents of functional monomer on the binding of moniliformin for each template and the non-imprinted polymers are shown

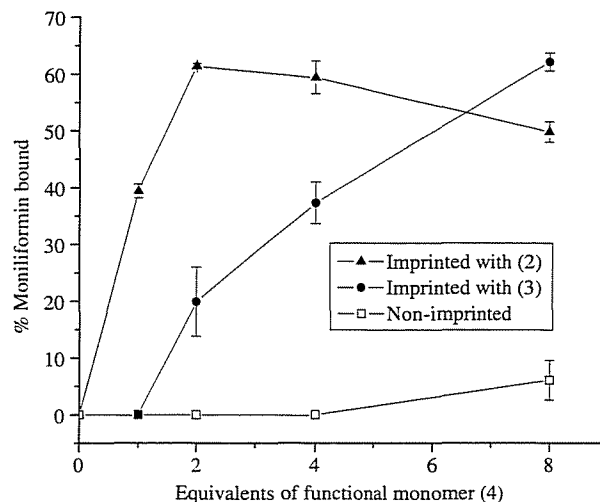


Figure 3. Moniliformin bound versus equivalents of functional monomer 4. All experiments were performed in triplicate. Error bars are standard deviations.

in Figure 3. Templates 2 and 3 produced polymers that were influenced differently by the amount of basic functional monomer present. Polymers imprinted with 2 possessed a complex relationship between equivalents of functional monomer and bound moniliformin (%) with maximal binding at a ratio of 1:2:40 under the conditions tested. Polymers imprinted with 3 exhibited an increase of moniliformin binding with an increase of the amount of functional monomer. Higher equivalents of the basic functional monomer 4 were not investigated since non-specific moniliformin binding became detectable for the non-imprinted polymer 7a with a ratio of 1:8:40.

The lack of binding of the non-imprinted polymer 7a is illustrated in the adsorption isotherm in Figure 4. Even higher concentrations of moniliformin lacked of detectable moniliformin binding for non-imprinted polymers 7a and 7d (data not shown). Polymers 8a and 8d (imprinted with template 2) produced complex adsorption isotherms with a lower percentage of moniliformin bound at lower concentrations of moniliformin and a higher percentage of moniliformin bound at higher concentrations.

The adsorption isotherm for polymer 9a is suitable for a Freundlich isotherm analysis (FI), which is defined by the following equation (Umpleby et al. 2001):

$$B = aF^m,$$

where a and m are fitting parameters. Parameter a is a measure of capacity and average affinity. The fitting parameter m is the heterogeneity index (0 to 1, where 1 is homogenous). FI analysis of the adsorption isotherm of 9a yields $a = 327 \pm 85$

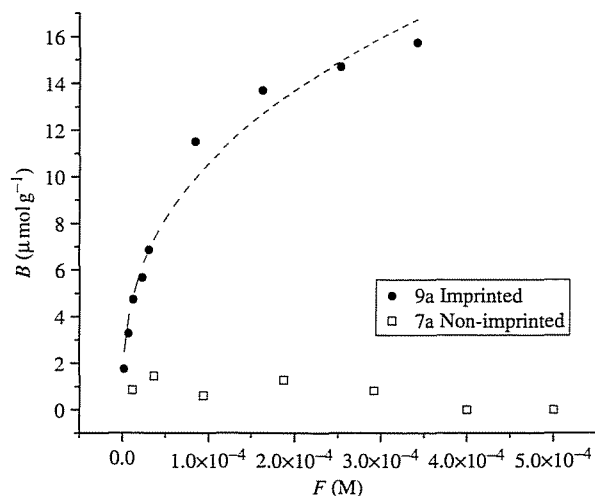


Figure 4. Adsorption isotherms for polymers 7a and 9a binding moniliformin. F is the concentration of the free moniliformin (M); B is the bound moniliformin (μmol) per g of polymer.

$(\mu\text{mol g}^{-1})(\text{l mol}^{-1})^m$ and a correlation coefficient of 0.97. The heterogeneity index was determined to be 0.37 ± 0.03 , indicating that the flexible template 3 imprinted a population of binding sites for moniliformin binding.

The binding assay results indicate the binding properties and the optimal composition of the polymers depend on the template used for imprinting. Templates 2 and 3 differ in their size and their interaction with the functional monomer. The Van der Waals volume of template 2 was calculated to be roughly approximately 80 \AA^3 , which is slightly larger than that of moniliformin of approximately 75 \AA^3 . Template 3 (approximately 150 \AA^3) is a larger, more flexible molecule that may form larger binding sites with more favourable access for moniliformin to interact with the active functional component of the polymer.

Another important comparison between the templates and moniliformin is the interaction with the functional monomer. Similar types of interactions have been investigated by calculations based on bound complexes of components of MIPs and these techniques have been successful in the design of imprinted polymers for which the substrate is the template (Piletsky et al. 2001; Diñeiro et al. 2005; Dong et al. 2005). The energy associated with the interaction of the stressed molecules of the geometry optimized complex of moniliformin free acid with the active functional monomer was calculated to be $-86.55 \text{ kJ mol}^{-1}$. It should be noted that moniliformin should never be interacting with the unreacted functional monomer in the work presented here, and the unreacted functional monomer is a representation of the reacted active moiety of the polymer in the calculation. In comparison, the

energy associated with the interaction of functional monomer 4 and template 2 in the bound complex is $-90.51 \text{ kJ mol}^{-1}$, whereas with template 3 is only $-10.52 \text{ kJ mol}^{-1}$. This analysis infers that template 2 forms a smaller, more geometrically defined binding site through the favourable interaction with the functional monomers. In contrast, template 3 forms a larger binding site that requires a larger number of functional monomer residues for favourable moniliformin binding. However, binding could be further complicated by template complexation during polymer synthesis.

Batch reproducibility

To test the reproducibility of the polymer synthesis and binding assay, three batches of polymers 8a and 9a were synthesized on different days and evaluated for their moniliformin binding properties. Table II lists the percentage moniliformin bound for three batches of polymers 8a and 9a. All batches of polymer 9a imprinted with template 3 consistently bound more moniliformin than any of batches of polymer 8a imprinted with template 2.

Template binding to synthesized polymers

Polymers 7a, 7b, 8a, 8b, 9a and 9b were evaluated for their ability to bind templates 2 and 3 under conditions similar to those used to evaluate moniliformin ($10 \mu\text{g ml}^{-1}$ substrate, 10 mg ml^{-1} polymer in acetonitrile). All binding studies using templates 2 and 3 as a substrate lacked a detectable benefit from imprinting for the polymers tested. However, the assay conditions and the composition of the polymers were developed for optimal moniliformin binding and MIP versus NIP selectivity in acetonitrile, without consideration for template activities. In addition, as with all results that are obtained by polymers imprinted with the substrate, these studies could be further complicated by template bleeding during the assay.

Polymers containing the basic functional monomer (7a, 8a and 9a) bound nearly all ($>82\%$) of 3,4-dihydroxy-3-cyclobutene-1,2-dione while polymers with acidic moieties bound a third of substrate 2. There was no detectable binding of template 3 for any of the polymers evaluated under the assay conditions. The results obtained for template binding correspond with the calculations that indicate template 3 interacts less favourably with the functional monomer 4 than template 2.

Activity of selected polymers in other solvents

Polymers 7a, 8a and 9a were evaluated for their ability to bind moniliformin in ethanol,

methanol and DMF (see Table III). The non-imprinted 7a polymer did not bind moniliformin in methanol, ethanol nor DMF. There was a significant loss in moniliformin binding for the polymer 8a for studies carried out in acetonitrile and those carried out in DMF and polar protic solvents methanol and ethanol. Polymer 9a maintained much of its moniliformin binding activity in the polar protic solvents evaluated. Polymer 9a bound 10% less moniliformin in DMF compared acetonitrile, despite use of DMF as the porogenic solvent. The loss of activity of both imprinted polymers in DMF may be attributed to differences in the solvent interactions of the templates and moniliformin. Polymer 9a bound more moniliformin than polymer 8a in the polar solvents considered.

Table II. Batch reproducibility of moniliformin binding polymers 8a and 9a in acetonitrile.

Polymer	Bound moniliformin (%)			Overall
	Batch 1	Batch 2	Batch 3	
8a	46.8 ± 5.3	49.8 ± 1.2	43.3 ± 3.4	46.6 ± 4.3
9a	69.8 ± 3.7	62.1 ± 1.6	66.7 ± 6.5	66.2 ± 5.1

All experiments were performed in triplicate. Results are reported as mean ± standard deviation.

SPE experiments

Imprinted polymers 8a, 8d and 9a and their non-imprinted counterparts 7a and 7d were evaluated as sorbents in solid-phase extraction columns to determine their usefulness for offline preconcentration of moniliformin for LC analysis. Polymers were loaded with 3 ml of a 5 µg ml⁻¹ solution of moniliformin in 95% acetonitrile/water, a reported moniliformin extraction mixture (Sharman et al. 1991). Columns were washed with 1 ml of 95% acetonitrile/water, and followed by 1 ml of elution buffer. The collected fractions were analysed by LC analysis, and the amounts of moniliformin in the breakthrough, wash and elution is listed in Table IV. The amount of moniliformin bound can be calculated by subtracting the moniliformin breakthrough from the amount loaded (15 µg).

All imprinted polymers evaluated by the MISPE method bound more moniliformin than their non-imprinted counterparts. The non-imprinted polymer 7d with two equivalents of functional monomer retained less moniliformin than the non-imprinted polymer with eight equivalents of functional monomer. However, as with the equilibrium binding assay results, the polymers imprinted with template 2 that had two equivalents of functional monomer (8d) exhibited more moniliformin binding (5.5 µg) than

Table III. Moniliformin binding by polymers in other solvents.

Polymer	Template	Mole ratio (template : functional monomer : crosslinker)	Bound moniliformin (%)		
			Methanol	Ethanol	DMF
7a	none	0:8:40	n.a.	n.a.	n.a.
8a	(2)	1:8:40	10.6 ± 0.9	14.8 ± 1.4	12.6 ± 2.2
9a	(3)	1:8:40	59.6 ± 1.5	59.5 ± 5.0	48.6 ± 3.9

All experiments were carried out at 10 µg ml⁻¹ moniliformin and 10 mg ml⁻¹ polymer. n.a., No activity (<5%). All experiments were performed in triplicate. Results are reported as mean ± standard deviation.

Table IV. Recoveries of moniliformin after solid-phase extraction through polymer packed columns.

Polymer	7a NIP	8a MIP	9a MIP	7d NIP	8d MIP
Template	none	(2)	(3)	none	(2)
Mole ratio (template : functional monomer : crosslinker)	0:8:40	1:8:40	1:8:40	0:2:40	1:2:40
Recoveries (µg moniliformin)					
Breakthrough (3 ml)	12.8 ± 0.5	10.1 ± 0.1	0.5 ± 0.4	13.5 ± 0.7	9.5 ± 0.9
Wash (1 ml)	1.0 ± 0.3	0.8 ± 0.1	0.2 ± 0.1	1.0 ± 0.3	0.9 ± 0.1
Elution (1 ml)	0.9 ± 0.2	2.9 ± 0.4	13.8 ± 1.2	0.5 ± 0.1	4.6 ± 0.9

All experiments were with 25 mg polymer in 1.5-ml reservoirs. Columns were loaded with 3 ml of a moniliformin standard (5 µg ml⁻¹ in 95% acetonitrile/water), then washed with 95% acetonitrile/water (1 ml), followed by elution with 1 ml of LC mobile phase.

the polymer imprinted with 2 and eight equivalence functional monomer 8b (4.9 μg). Polymer 9a, imprinted with 3, performed with significantly less moniliformin breakthrough and release during the wash, and subsequently, recovered the most moniliformin from the elution step.

The capacity of polymers 9a and 7a were measured by loading the 25 mg columns with 60 μg of moniliformin (5 $\mu\text{g ml}^{-1}$ in 95% acetonitrile/water) in 1 ml fractions. Non-imprinted polymer 7a had significant breakthrough with the first fraction collected (42.6% of 5 $\mu\text{g ml}^{-1}$) and results for imprinted polymer 9a are shown in Figure 5. In contrast to the non-imprinted polymer (immediate breakthrough), breakthrough for the MISPE column packed with 9a occurred at 20 μg moniliformin. The MISPE column packed with polymer 9a bound 30.3 μg of moniliformin compared with 3.1 μg of moniliformin the column packed with non-imprinted polymer 7a. Correcting for the non-specific binding of the non-imprinted polymer, the achieved capacity of imprinted polymer 9a was 1.09 mg moniliformin per g of polymer. The capacity is expected to vary depending on the matrix and load solvent. The wash (95% acetonitrile/water, 1 ml) released 2.2 μg of moniliformin from the MISPE column packed with 9a. Elution of the 9a packed column with the elution buffer released 27.2 μg of moniliformin in the first fraction (1 ml), and 1 μg with the second fraction (1 ml). A third elution fraction (1 ml) contained less than 0.2 μg moniliformin.

Application of polymer 9a for preconcentration of a moniliformin spiked corn extract is shown in Figure 6. The corn extract was spiked with

0.5 $\mu\text{g ml}^{-1}$ of moniliformin and 3 ml of this spiked extract was loaded on the MISPE column. The column was washed with 95% acetonitrile/water (1 ml), and eluted with 0.5 ml of elution buffer. The only major peak in the MISPE treated sample (B) is the preconcentrated moniliformin peak. In comparison the untreated spiked corn extract (A) is more complex.

Summary and conclusions

Imprinted polymers to bind moniliformin were synthesized using two different templates and the choice of template influenced the optimal polymer composition for moniliformin binding. Significant differences in moniliformin binding by the polymers

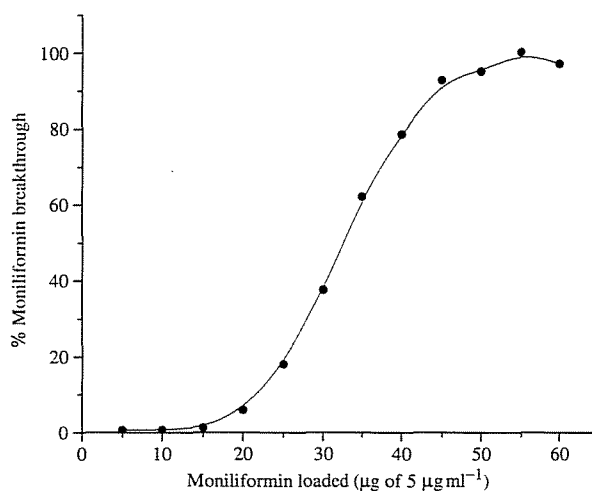


Figure 5. Capacity of MIP 9a (25 mg packed in an SPE column) for moniliformin (5 $\mu\text{g ml}^{-1}$ in 95% acetonitrile/water).

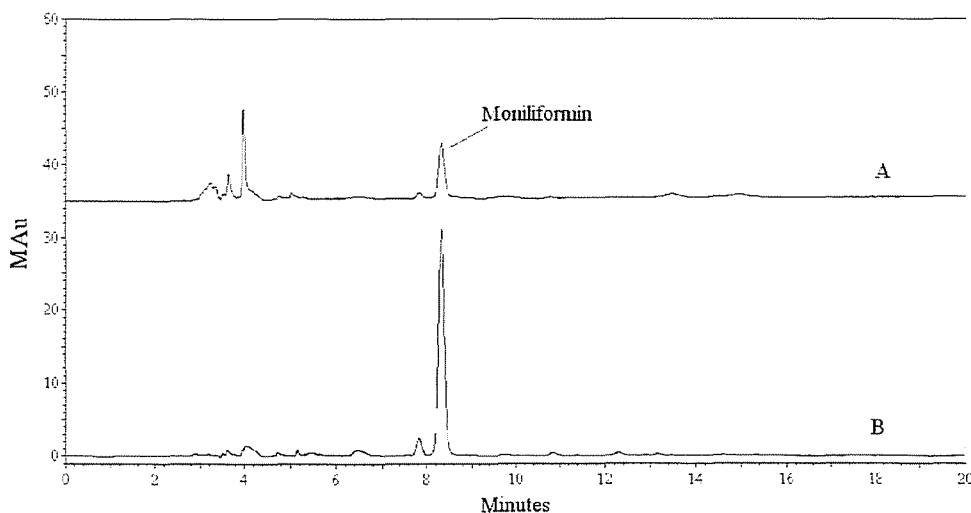


Figure 6. Chromatograms obtained of a corn extract spiked with 0.5 $\mu\text{g ml}^{-1}$ moniliformin before (A) and after offline preconcentration with MISPE 9a (B). Detection wavelength: 229 nm.

were dependent on polymer composition and the conditions the polymers were tested. Imprinted polymers were capable of binding greater amounts of moniliformin compared with non-imprinted polymers as sorbents in MISPE experiments, and an imprinted polymer was suitable for preconcentration of a moniliformin spiked corn extract.

Toxin analogues were a viable alternative to moniliformin for imprinting polymers that bind moniliformin. It was necessary to determine the optimal ratio of functional monomer to template for each toxin analogue used for imprinting. The small size of moniliformin, and the potential for complex interactions may complicate generalizing the results for these moniliformin binding polymers to all MIPs.

Molecular imprinting technology has brought about the development of materials designed for use in applications which rely on molecular recognition, including sorbents and sensors. This technology offers an additional approach to incorporate selectivity into methods of detection or removal of mycotoxins. The moniliformin binding polymers described here show potential as solid sorbents to remove moniliformin from liquids and as packing in MISPE columns.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. The authors wish to thank Mr. John Bobell, USDA-ARS-NCAUR, for outstanding technical assistance and Mr. Ronald D. Plattner, USDA-ARS-NCAUR, for graciously providing moniliformin.

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