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Determination of Citrinin Using Molecularly Imprinted Solid Phase Extraction Purification, HPLC Separation, and Fluorescence Detection

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A liquid chromatography based method to detect citrinin in corn was developed using molecularly imprinted solid phase extraction (MISPE) sample clean-up. Molecularly imprinted polymers were synthesized using 1,4-dihydroxy-2-naphthoic acid as the template and an amine functional monomer. Density functional calculations suggest the mimic template interacts with the functional monomer in a similar manner as citrinin. Freundlich isotherm analysis indicated the template provided a significant imprinting effect for citrinin binding. A high performance liquid chromatography with fluorescence detection (HPLC-FD) method to detect citrinin in maize was developed utilizing the imprinted polymers for sample clean-up (excitation at 330 nm; emission at 500 nm). Recoveries of citrinin in spiked corn samples (0.03–3 µg g⁻¹) were between 82.3–91.5%. This study demonstrates that molecularly imprinted polymers are applicable in the solid phase extraction clean-up of corn samples for citrinin determination by HPLC-FD.

Keywords: food safety, HPLC, MIP, MISPE, molecularly imprinted polymer, mycotoxin

Introduction

Citrinin is a yellow colored nephrotoxic mycotoxin of increasing concern that is produced by Penicillium, Aspergillus, and Monascus spp. Monascus spp. are found in red yeast rice and these fungal species occasionally contaminate cereal grains, including corn.1,2 This toxin is associated with a variety of detrimental effects and advisory levels for this toxin have been set at 100 ppb in the EU and 200 ppb in Japan.3–5 Citrinin has become an increasing concern for food safety due to its association with the Endemic Balkan Nephropathy as well as liver and kidney damage.6 Furthermore, citrinin co-occurrence with ochratoxin A has been associated with DNA damage and cell death.7 In an effort to help reduce exposure to the deleterious effects of this toxin on humans and livestock, we have developed a HPLC-based analytical method to aid in the monitoring and assess exposure to this toxin using state of the art technology for sample clean-up.

In the past few years, several analytical methods have been developed to detect citrinin, and these are primarily validated for a limited set of commodities.8–13 A number of HPLC-based methods coupled with UV or fluorescence detection have been developed.8 Generally, fluorescence-based methods increase sensitivity by 100-fold over UV detection; however reversed phase HPLC methods with fluorescence detection are sensitive to matrix effects. Because of the low levels required to cause concern (100 ppb), clean-up and pre-concentration steps can aid in quantitative analysis.

Solid phase extraction is a common technique to develop analytical methods with lower detection limits, improve analyte isolation, and reduce interferences in analysis. Conventional solid phase extraction (SPE) lacks specificity and relies on loading/washing/eluting solvents to achieve limited selectivity in clean-up. However, the SPE clean-up technique is exceptionally powerful tool, when coupled to HPLC detection methods to enable extremely sensitive, accurate, and economical methods of determination. A recent platform for SPE is the use of molecularly imprinted polymers (MIP) and other synthetic receptors. Molecularly imprinted polymers are customizable synthetic receptor materials that possess high capacity, binding selectivity, and robust stability.14–16 The binding sites are formed during polymer synthesis around template molecules that interact with functional monomer polymer reagents in a pre-polymerization complex.17 Removal of the template leaves a polymer that includes binding sites with functional monomers in the appropriate geometry to interact with the desired analytes.18 Traditionally, the analyte is used as the template. In the case of toxins, we have found template mimics can provide a safer and more

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Hyperchem 8.0.10 and the PM3 semi-empirical method and calculations. Binding interaction complexes and components were fully density functional method was used to assess the binding interaction of the functional monomer with the tautomers of citrinin and the template. Density functional calculations were carried out to gain insight into the relative favorability of the interaction of the functional monomer with the tautomers of citrinin and the template with the tertiary amine functional monomer. The adsorption capacity and imprinting effect were evaluated in equilibrium binding assays. The polymer was applied in the MISPE format in a method to detect citrinin in corn using HPLC-FD detection.

**Experimental**

**Reagents and Materials**

Trimethylolpropane trimethacrylate (TRIM), 2-dimethylaminoethyl methacrylate, 2,2-azoisobutyronitrile (AIBN), tetrabutylammonium bisulfate, citrinin, acetic acid, trifluoroacetic acid, and 1,4-dihydoxy-2-naphthoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetone, ethanol, and acetonitrile were purchased from EMB (Gibbstown, NJ USA). Deionized water was used in the preparation of all reagents (Nanopure II, Sybron/Barnstead). Corn was purchased locally and did not have detectable levels of citrinin. Citrinin standards were prepared at concentrations of 1 mg mL⁻¹ in ethanol.

**Computational Chemistry**

A density functional theory computational chemistry study was carried out to gain insight into the relative favorability of the interaction of the functional monomer with the tautomers of citrinin and the template. Density functional calculations were carried out on PCs hardware and software using default settings as previously described. Structures were first built using Hyperchem 8.0.10 and the PM3 semi-empirical method and later optimized using density functional theory. The B3LYP density functional method was used to assess the binding interactions. Binding interaction complexes and components were fully geometry optimized at the 6-311 + +G** level of theory. Binding energies were calculated using the following relationship:

\[ E_{\text{Interaction}} = E_{\text{Complex}} - (E_{\text{Functional Monomer}} + E_{\text{Citrinin or template}}) \]

**Polymer Synthesis**

The molecularly imprinted polymer (MIP) and nonimprinted polymer (NIP) were prepared by thermolytic bulk polymerization. A glass vial (40 mL) containing the template 1,4-dihydoxy-2-naphthoic acid (408.4 mg, 2 mmol) was filled with 15 mL acetone/acetonitrile (1:3, v/v) and the functional monomer 2-dimethylaminoethyl methacrylate (1.35 mL, 8 mmol) followed by vortexing and sonication (15 min). The solution was stored at 0°C for 4 hr. Cross-linker TRIM (9.58 mL, 30 mmol) was added and MIP mixture was flushed with nitrogen for 5 min. Initiator, 2,2-azoisobutyronitrile (AIBN), (250 mg) was added, and the vials were vortexed, capped, and sealed. The reaction was allowed to proceed for 48 hr in a water bath at 50°C. Five vials were synthesized in parallel to obtain larger amounts of polymer in consistent batches and for safe handling of smaller reaction volumes. The synthesis produced a solid monolithic solid cylinder shaped polymer and approached quantitative yield. The MIP polymer monoliths were combined, washed, and sonicated (15 min) with the following: acetone (0.5 L x 3), ethanol (0.5 L x 3), and water (0.5 L x 3). The polymers were dried through vacuum filtration, ground, and sieved. The fraction between 38–75 μm was collected. The collected fractions were extracted by sonication (15 min) with 1% tetrabutylammonium bisulfate in water (0.5 L x 3), water (0.5 L x 3), 2% acetic acid in ethanol (0.5 L x 3), ethanol (0.5 L x 3), and water (0.5 L x 3). Washing solutions were removed by vacuum filtration. Nonimprinted polymer synthesis was conducted in parallel without the template.

**Polymer Characterization**

The surface area of the polymers was investigated by nitrogen isotherms collected at 77 K using a Quantachrome ASiQ (Quantachrome Instruments, Boynton Beach, FL, USA). Approximately 50 mg samples of the sorbents were degassed at 100°C for 18 hr prior to analysis. Surface areas were calculated using the multipoint Brunauer–Emmett–Teller (BET) method.

**Sorption Isotherm Analysis**

The imprinting effect was investigated by sorption isotherm analysis. The 1.8-mL vials containing 10 mg of MIP or NIP and 1 mL acetonitrile solution of a specific citrinin concentration 0.5 to 30 μg mL⁻¹ were placed on a wrist shaker for 16 hr with a shaker setting of 6. Following centrifugation, the supernatant was removed. Samples were syringe-filtered (0.20 μm Millipore PFA syringe filter) prior to LC-analysis. Higher concentration samples were analyzed by dilution with acetonitrile.

**Determination of Citrinin in Corn by MISPE Analysis**

A method was developed to analyze citrinin in maize between 100–3000 ppb. Citrinin spiked (10 ng g⁻¹ to 3 μg g⁻¹) ground corn (10 g) was placed in a 100-mL Pyrex bottle. A mixture of 70/30 methanol/water (50 mL) was added, and the citrinin was extracted by lab-line shaker with the setting of 10. After 1 hr, sonication was applied using a FS110H sonicator for 10 min. The contents were vacuum filtered over grade 54 hardened filter paper and the eluent was saved for analysis. MISPE clean-up was conducted with 8.0 mL reservoirs with polymer (300 mg) between two frits and flow was governed by a vacuum manifold. The columns were conditioned with 10 mL of methanol. Corn extract (1.0 mL) was loaded on the column and washed with water (1.0 mL). Finally, the column was eluted with 2% acetic acid in methanol (5.0 mL). The final eluent was collected, and reduced to dryness under a rotary evaporator with a 50°C water bath. The extract was resuspended in 0.5 mL of the LC mobile phase and the samples were syringe-filtered prior to analysis.

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**HPLC Analysis**

HPLC analysis with fluorescence detection was used to determine citrinin levels. The system consisted of a Shimadzu LC-20AT Quaternary Pump, RF-10AXL Fluorescence detector, and a CBM-20A Communication Bus Module. A Reodyne Injector with a 20-µL loop was used for injection and a Phenomenex Luna 5 µ C18 100A HPLC column (250 × 4.60 mm) provided separation. The mobile phase was a mixture of acetonitrile and water (50:50) with 0.03% trifluoroacetic acid. Citrinin levels were determined with excitation at 330 nm and emission recorded at 500 nm using peak area. (0.3–30 ng mL⁻¹ in LC mobile phase, $R^2 = 0.995$).

**Results and Discussion**

**Characterization of the Polymers**

The reagents used in polymer synthesis are shown in Figure 1. Citrinin exists in both a $p$-quinone and $o$-quinone form. Density functional calculations on the electronic structures can provide insight into favorability and orientation of the components’ pre-polymerization complexes. As shown in Figure 2 and Table 1, the template 1,4-dihydroxy-2-naphthoic acid forms a favorable interaction (−46.3 kJ mol⁻¹) with functional monomer 2-dimethylaminoethyl methacrylate (3). Importantly, the $p$-quinone form of citrinin (1a) forms a similar favorable (−42.5 kJ mol⁻¹) interaction with functional monomer. The $o$-quinone forms a slightly less favorable interaction (−41.3 kJ mol⁻¹). Also, the template and citrinin tautomeric forms have similar hydrogen bond schemes between the hydroxy groups with the carboxylic acid moiety. Furthermore, the 4-hydroxy of the template occupies a similar space as the ring carbonyl of the citrinin tautomeric forms. Overall, these results suggest that mimic template 1,4-dihydroxy-2-naphthoic acid interacts with the functional monomer in a similar manner as citrinin and is an inexpensive and safer alternative for imprinting polymers to bind citrinin.

The polymers were characterized by BET nitrogen sorption analysis to gauge the impact of imprinting on the surface area of the polymers. A significant decrease in surface area was observed in the imprinted polymer (MIP, 269 m²/g) as compared to the nonimprinted polymer (NIP, 385 m²/g). The template used in the synthesis of the imprinted polymer contains functional groups associated with anti-oxidant and free-radical scavenger properties. These properties may have reduced the extent of cross-linking during polymer synthesis of the imprinted polymer.

**Sorption Isotherms**

The binding properties and imprinting effects of the polymers were evaluated using equilibrium binding assays. Sorption isotherms and the associated Freundlich analysis parameters are given in Figure 3 and Table 2. The Freundlich isotherm is

<table>
<thead>
<tr>
<th>Parameter Complex</th>
<th>1a</th>
<th>2a</th>
<th>3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction energy (kJ mol⁻¹)</td>
<td>−42.5</td>
<td>−41.3</td>
<td>−46.3</td>
</tr>
<tr>
<td>O=C−OH···N (Å)</td>
<td>1.694</td>
<td>1.696</td>
<td>1.680</td>
</tr>
<tr>
<td>−OH···O=C (Å)</td>
<td>1.553</td>
<td>1.537</td>
<td>1.697</td>
</tr>
</tbody>
</table>

Fig. 1. Structures of citrinin (1a and 1b), mimic template 1,4-dihydroxy-2-naphthoic acid (2), functional monomer 2-dimethylaminoethyl methacrylate (3), and crosslinker trimethyloyl trimethacrylate (4).

Fig. 2. Pre-polymerization complexes of 2-dimethylaminoethyl methacrylate (3), the $p$-quinone citrinin (1a), $o$-quinone citrinin (1b), and 1,4-dihydroxy-2-naphthoic acid (2).
defined as:

\[ q_e = K_F C_e^{(1/n)} \]

where \( q_e \) is the amount of analyte bound per gram of polymer, \( K_F \) is the Freundlich binding constant, \( C_e \) is the concentration of analyte free at equilibrium, and \( n \) is the heterogeneity index (variability of adsorption force).\(^{[24–26]}\) Taking the log provides an opportunity for a linear relationship:

\[ \log q_e = \log K_F + (1/n) \log C_e \]

where linear fit gives the intercept that is equal to \( \log K_F \) and the slope is equal to \( (1/n) \). The Freundlich analysis indicates the MIP has a significant increase in affinity for citrinin.

**Citrinin Determination Using MISPE Clean-Up**

The imprinted polymer was investigated for its suitability as an analyte selective polymer for MISPE clean-up of citrinin from corn samples. Several extraction solvents have been reported in the literature to recover citrinin from food matrix.\(^{[8,12,13]}\) The extraction solvent of 70/30 methanol/water provided appropriate recoveries for development of a quantitative analytical method and as the loading solvent for MISPE clean-up. Water was found to be a suitable washing solvent; citrinin is poorly soluble in pure water and retained on the column during the water washing step. Liquid chromatography with fluorescence detection is a robust and selective platform to determine levels for analytes that exhibit fluorescence. The mobile phase in the HPLC-FD analysis includes trifluoroacetic acid to provide an acidic environment for which citrinin exhibits its greatest fluorescence intensity.

The results for the method developed are provided in Table 3 and a chromatogram in Figure 4. Significant recoveries of citrinin were obtained (82.3–91.5%) between 0.03–3 µg g\(^{-1}\). Higher concentrations of citrinin can be investigated by dilution of the sample. The limit of detection (LOD) was 0.01 µg g\(^{-1}\) and limit of quantitation (LOQ) was 0.03 µg g\(^{-1}\). Figure 4 provides a HPLC chromatogram of MISPE clean-up of citrinin from corn.

**Table 2. Citrinin sorption isotherm parameters**

<table>
<thead>
<tr>
<th>Polymer</th>
<th>( K_F (\text{mg g}^{-1})(\text{L mg}^{-1})^{1/n} )</th>
<th>( N )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIP</td>
<td>( 0.112 \pm 0.008 )</td>
<td>1.26</td>
<td>0.989</td>
</tr>
<tr>
<td>MIP</td>
<td>( 0.270 \pm 0.036 )</td>
<td>1.16</td>
<td>0.976</td>
</tr>
</tbody>
</table>

Experiments performed in triplicate.

**Table 3. Citrinin recovery and RSD**

<table>
<thead>
<tr>
<th>Citrinin concentration in corn</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 µg g(^{-1})</td>
<td>Trace</td>
<td>– –</td>
</tr>
<tr>
<td>0.03 µg g(^{-1})</td>
<td>87.1</td>
<td>7.2</td>
</tr>
<tr>
<td>0.10 µg g(^{-1})</td>
<td>89.7</td>
<td>4.8</td>
</tr>
<tr>
<td>0.30 µg g(^{-1})</td>
<td>91.5</td>
<td>4.1</td>
</tr>
<tr>
<td>1.00 µg g(^{-1})</td>
<td>89.3</td>
<td>3.7</td>
</tr>
<tr>
<td>2.00 µg g(^{-1})</td>
<td>87.6</td>
<td>6.7</td>
</tr>
<tr>
<td>3.00 µg g(^{-1})</td>
<td>82.3</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Experiments performed in triplicate.
Citrinin Determination in Corn

The citrinin peak elutes at 10.8 min and is clearly separated from the other components associated with the corn matrix. The entire analysis can be completed in less than 20 min. Reusability of MIP polymers can be affected by the challenge of removing all components of the corn matrix from the used polymers. In addition, polymer binding sites can change due to shearing of the polymers and swelling from changes/removal of solvent. The citrinin tailing effect seen in the chromatogram in Figure 4 is characteristic of HPLC and fluorescence detection of citrinin. The peak shape of citrinin has been reported to be associated with the amount of acetonitrile in the mobile phase and is the result of a compromise between sensitivity and resolution.[8]

Concluding Remarks

A mimic template was used to develop molecularly imprinted polymers for citrinin. The imprinted material exhibited significant increased affinity and capacity for citrinin compared to the nonimprinted material. Density functional theory calculations provided insight into the bound complex and indicated the 1,4-dihydroxy-2-naphthoic acid template possessed similar affinity for the functional monomer as the toxin citrinin. A selective HPLC-FD method to determine levels of citrinin in corn was developed based on MISPE clean-up, with the LOQ and LOD below the 100 ppb levels of concern. Overall, this research demonstrates that mimic templates are a promising approach to develop methods to detect toxins.

Acknowledgments

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