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Detection of Mycoestrogen Zearalenone by a Molecularly Imprinted Polypyrrole-Based Surface Plasmon Resonance (SPR) Sensor

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The aim of the present work was to investigate the feasibility of employing the molecular imprinting polymer technique for detecting the mycotoxin zearalenone using a surface plasmon resonance (SPR) transducer. The molecularly imprinted polypyrrole (MIPPy) film was prepared by electropolymerization of pyrrole onto the bare Au chip in the presence of a template zearalenone molecule. The MIPPy-SPR sensor exhibited a linear response in the range of 0.3−3000 ng/mL (R (*2*) = 0.993) for detection of zearalenone. The selectivity efficiencies of zearalenone and other structurally related analogues were 1.0 and 0.15−0.27, respectively. The limit of detection and average recovery of blank corn matrix spiked with 30 ng/g zearalenone were 0.3 ng/g and 89%, respectively, and these were found to be comparable to those obtained by enzyme-linked immunosorbent assay. These results suggest that a combination of SPR sensing with MIPPy film is a promising alternative method for the detection of zearalenone.

KEYWORDS: Zearalenone; polypyrrole; molecular imprinting polymer; surface plasmon resonance; enzyme-linked immunosorbent assay

INTRODUCTION

Zearalenone, 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid lactone, is a secondary metabolite produced by several species of the genus *Fusarium*, mainly *F. graminearum* and *F. culmorum* (*1*). Zearalenone is found in a number of cereal crops, such as maize, barley, oats, wheat, rice, and sorghum (*2*), and is known to act as an endocrine disruptor (*3*). This estrogenic activity leads to the dysfunction of the reproductive system in humans and animals, which has the potential to cause substantial economic impacts (*4*). Tolerance levels have been established in many countries of the world and range from 30 to 1000 μg/kg in grains (*5*). While these limits are set to protect the food supply as well as trade in agricultural products, especially grains, they necessitate the development of rapid, reliable, and sensitive analytical methods for the quantitative measurement of these compounds in complex matrices such as grains.

Current analytical methods for the detection of zearalenone include thin-layer chromatography (TLC) (*6, 7*), gas−liquid chromatography (*7, 8*), gas chromatography−mass spectrometry (*9, 10*), HPLC (*11*), and HPLC−mass spectrometry (*12*). Although several of these methods achieve low levels of detection, in practice they consume large amounts of time and solvent, and require one or more cleanup steps involving liquid−liquid partition or solid-phase extraction (*13*). Immunological methods, including line immunoblot (*14*), dipstick immunoassay (*15*), and enzyme-linked immunosorbent assay (ELISA) (*16, 17*), have also been used for the determination of zearalenone. Nevertheless, these methods are labile to physical and chemical conditions (*18*).

Applications of natural receptor molecules, such as antibodies, are limited because they are costly and unstable, which makes them less suitable for chip coating (*19*). These limitations have generated the need to investigate potential artificial recognition sites. Among artificial receptors, molecular imprinting polymers (MIPs) have proven their potential as synthetic receptors in numerous applications ranging from liquid chromatography to sensor technology (*20, 21*).

Recently, biochip-based immunosensors, which transduce antigen−antibody interactions directly into physical signals, have been studied to detect food contaminants with high sensitivity and rapidity (*22*). Transducers of immunosensors are based on various techniques, such as optics, electronics, and mechanics. Among these transducers, it is well-known that surface plasmon resonance (SPR) has better signal reliability and stability than other transducers (*23, 24*). In addition, SPR is highly sensitive, even for the detection of subnanomolar concentrations due to

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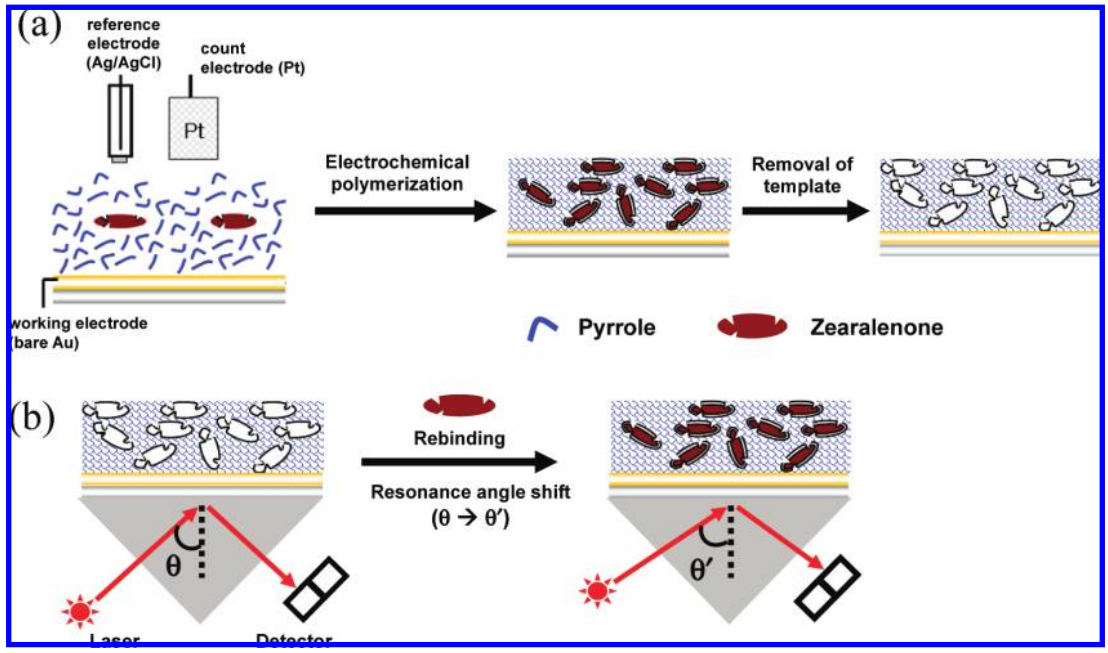

Figure 1. Schematic diagrams of the setup for electropolymerization and SPR for detecting zearalenone. (**a**) Preparation of molecularly imprinted polypyrrole on bare Au using a three-electrode electrochemical system. (**b**) The shift in resonance angle of the SPR sensor resulting from the rebinding of zearalenone to the MIPPy film.

the change in refractive index on the metallic surface that can be measured. Thus, since the end of 1990, SPR has been rapidly developed as a nonlabeling detection method in biosensors and has evolved as a good alternative to ELISAs.

The objective of our present work was to investigate the feasibility of employing the MIP technique as a recognition element for detecting zearalenone using an SPR transducer. The polymer was prepared by electropolymerization of pyrrole onto the bare Au in the presence of a template zearalenone molecule. A molecularly imprinted polypyrrole (MIPPy) film was characterized by SPR, attenuated total reflection Fourier transformation infrared, and atomic force microscopy in terms of the thickness, structural properties, and surface morphology. Further, the analytical performances of the MIPPy-based SPR sensor were evaluated with respect to sensitivity, linearity, and selectivity.

MATERIALS AND METHODS

Chemicals. Pyrrole, tetraethylammonium tetrafluoroborate, and acetonitrile were obtained from Aldrich (St. Louis, MO). Pyrrole was purified by distillation and then stored at 4 °C in the dark. Tetraethylammonium tetrafluoroborate (99%) and acetonitrile (anhydrous grade, 99.8%) were used without further purification. Zearalenone, α-zearalenol, β-zearalenol, zearalanone, and α-zearalanol were purchased from Sigma (St. Louis, MO). Other chemical reagents were of certified analytical grade and were used without further purification.

Apparatus. Electropolymerization of pyrrole was carried out using a BAS-100B electrochemical analyzer (Bioanalytical Systems Inc., West Lafayette, IN). The MIP film was characterized using attenuated total reflection (ATR) FTIR with a Magma-IRTM 550 (Nicolet Instrument Corp., Madison, WI) and atomic force microscopy (AFM) using an XE-150 (Park Systems Corp., Suwon, Korea). The reactions between zearalenone and MIP were measured with a homemade SPR, which consisted of a light source, an integrated flow cell, an interface controller, and application software (*25, 26*).

Preparation of MIPPy and Non-MIPPy. Electropolymerization of pyrrole was carried out using a three-electrode electrochemical system. Au film (50 nm thickness) was deposited on a glass slide (18 × 18 mm^2) by electron beam evaporation and served as a cathode after making contact with the aluminum foil in the Teflon-made electrochemical cell, using Ag/AgCl as a reference electrode and a platinum grid as a counter electrode, as shown in **Figure 1A**. For MIPPy preparation, 1 mL of acetonitrile solution containing 0.5 M pyrrole as a functional monomer, 0.2 M tetraethylammonium tetrafluoroborate as electrolyte, and 3.0 mM template zearalenone was placed on the gold surface to cover the counting and reference electrodes with a constant potential of 0.9 V and a current density of 5 mC/cm^2 at room temperature. After the synthesis, the films were subjected to a successive washing procedure in acetonitrile, methanol, and chloroform to remove the zearalenone and electrolytes entrapped in the polymeric matrix. Following this, the films were dried in N_2 gas after overnight storage in methanol. Non-MIPPy was prepared following the same procedure described above without the presence of the template molecule.

Characterization of the MIPPy Film. The MIPPy film was characterized using ATR-FTIR and AFM. A Magma-IRTM 550 spectrometer purged with N_2 gas and containing a liquid nitrogen-cooled mercury cadmium tellurium detector was used to obtain the ATR-FTIR spectrum. For evaluating the changes in surface morphology and film thickness, AFM images produced with a XE-150 were acquired in noncontact mode with 910-NCHR silicon cantilevers (Nanosensors, Neuchatel, Switzerland) at a resonance frequency of between 300 and 340 kHz. The scanning parameters were adjusted to provide clear images. Most of the images were acquired in the 10 × 10 μm^2 range with 512 × 512 pixels. The reaction between zearalenone and MIPPy was measured using a homemade SPR. Au-coated glass substrates were index-matched with a BK7 prism ($n = 1.515$, Sigma). Optical contact between the prism and the Au substrate was achieved with a refractive index matching fluid (nD = 1.515−1.517) (Merck, San Diego, CA). A *p*-polarized He−Ne laser set at 670 nm was used as the probe beam. The intensity of the beam reflected through the prism was measured with a photodiode detector. The incident angle of the prism was varied using a D80 motorized rotary stage and its controller (Suruga Seiki, Shizuoka, Japan) with a minimum resolution of 0.004°.

SPR Measurements. To obtain SPR measurements, MIPPy-coated Au chips were mounted on the SPR cell as shown in **Figure 1B**. An evaluation of the optical parameters of MIPPy was carried out under the following conditions. The incident angle of the laser varied from 35 to 55° with a resolution of 0.04° in air. The reflected intensities of the SPR for investigating the recognition between zearalenone and MIPPy (or Non-MIPPy) were measured according to the incident angle of the laser from 68 to 88° with a resolution of 0.012° and intervals of 10 min at room temperature. The resonance angle of the SPR, which

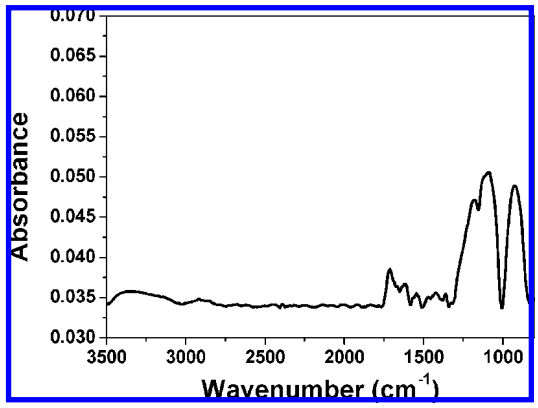

Figure 2. ATR-FTIR spectrum of the MIPPy film on a bare Au chip.

was determined at the minimum intensity of the SPR curve, was compared [0.1% ethanol solution without zearalenone compared with 0.1% ethanol standard solution of zearalenone (0.3−3000 ng/mL)] and plotted vs time of measurement. To evaluate the cross-selectivity of the MIPPy film, several metabolites were injected onto the MIPPy film, and the shifts in their resonance angles were compared with that induced by zearalenone at a concentration of 3000 ng/mL.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was performed according to the procedure suggested in the AgraQuant Zearalenone kit manual (Romer Laboratories, Union, MO). Briefly, a 10-g ground corn sample was extracted with 50 mL of 70% (v/v) methanol and 4% (v/w) NaCl by shaking the mixture for 3 min. The sample solution was filtered through a Whatman No. 1 filter, and the filtrate was then diluted five times. Standard solutions of zearalenone or corn extracts were mixed with zearalenone−horseradish peroxidase conjugate, and 100 μL of this mixture was incubated over the antibody solid phase for 10 min at room temperature. The 96-well microtiter plate was washed, and substrate was added to each well of the plate. Following incubation of the plate for 5 min at room temperature, the absorbance of each well at 450 nm was read with an ELISA reader (Molecular Devices, Sunnyvale, CA). The limit of detection was determined using the average values of 10 zearalenone-free samples plus two standard deviations. Recovery was evaluated by spiking blank corn samples with 30 ng/g zearalenone.

RESULTS AND DISCUSSION

Characterization of the MIPPy Film. A typical FTIR spectrum of a MIPPy film on a bare Au chip before removal of zearalenone templates is shown in **Figure 2**. The bands at 926, 1095, and 1617 cm^{-1} might arise from C−H in-plane bending, polypyrrole ring deformation, and C=C stretching of the ring, respectively. Zearalenone in the MIPPy film showed two bands at 1713 and 3300 cm^{-1}, representing the stretching vibration mode of the C=O group and the O−H group, respectively. The IR spectrum of our polypyrrole was similar to those of previous reports in the literature (*27*, *28*). Thus, FTIR spectroscopy confirms that the MIPPy film was successfully synthesized on the bare Au chip.

The MIPPy film formed should be of an adequate thickness to detect zearalenone using SPR. In the preliminary study, broadening of the SPR curves around the resonance angle was observed due to the increase in reflectance associated with an increment in the thickness of the MIPPy film, and these in turn resulted in a decrease in sensitivity (data not shown). Thus, as shown in **Figure 3A**, we fabricated the MIPPy film with an optimum thickness, which did not affect the reflectance of the SPR curves. Following the synthesis of the MIPPy film on the bare Au chip, a shift in the resonance angle of 1.341° to the right from 44.835° to 46.176° was observed. In order to estimate the thickness of the MIPPy film, the theoretical reflectance of a four-layer system, comprising the prism (1st layer), Au (2nd layer), MIPPy (3rd layer), and external dielectric environment (4th layer), is described as follows from the Fresnel equations (*29*):

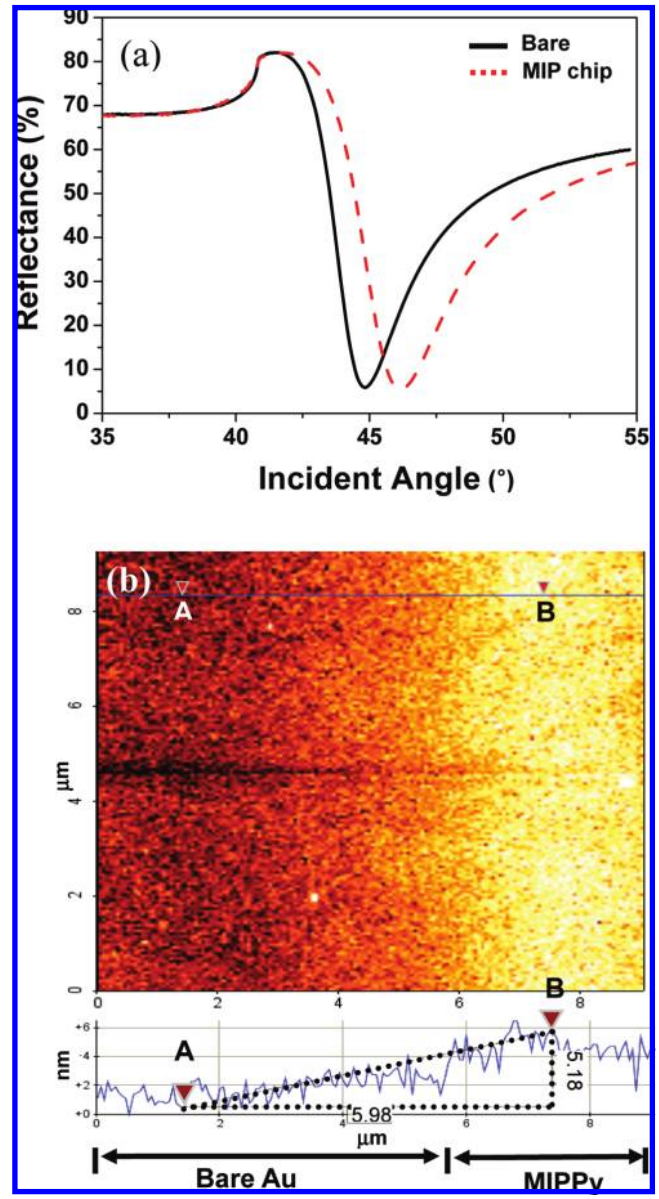

Figure 3. Characterization of a MIPPy film using (**A**) SPR and (**B**) AFM.

$$R = \left| \frac{r_{12} + r_{23}S_1^2 + r_{34}S_1^2S_2^2 + r_{12}r_{23}r_{34}S_2^2}{1 + r_{12}r_{23}S_1^2 + r_{23}r_{34}S_2^2 + r_{12}r_{34}S_1^2S_2^2} \right|^2 \qquad (1)$$

where

$$S_i = \exp(i\delta_{xi}d_i), \quad r_{ij} = \frac{\varepsilon_i\delta_{xj} - \varepsilon_j\delta_{xi}}{\varepsilon_i\delta_{xj} + \varepsilon_j\delta_{xi}}, \quad \varepsilon = (n + ik)^{1/2},$$

$$\delta_{xi} = \frac{2\pi}{\lambda}(\varepsilon_i - \varepsilon_o \sin^2\theta)^{1/2}$$

Here, ε_i is the dielectric constant of the ith layer, d_i is the thickness of the ith layer, θ is the incident angle at the prism−Au interface, and λ is the wavelength of the light source. The dielectric constant can be defined as a complex index of refraction, which is relative to a refractive index (n) and an extinction coefficient (k). From eq 1, depending on the incident angles, the thickness (d) of the MIPPy layer can be determined

Table 1. Optical and Morphologic Parameters[a] Measured by SPR and AFM

	SPR			AFM	
layer	*n*	*k*	*d* (nm)	roughness	*d* (nm)
Au	0.362	3.435	47	0.8	
MIPPy	1.731	0.065	5	1.1	4−6

[a] *n*, refractive index; *k*, extinction coefficient; *d*, thickness.

and the best-fit values are shown in **Table 1**. The *n*, *k*, and *d* values for the bare Au chip were 0.362, 3.435, and 47 nm, respectively. The thickness of the bare Au chip determined from the fit of these results was only slightly less than the expected thickness (50 nm) of the Au deposit on the glass substrate. In a similar manner, we obtained the best-fit values for the MIPPy film from the values for the bare Au chip. As a result, the thickness of the MIPPy film was estimated to be around 5 nm.

Figure 3B shows the AFM scan image of 10 × 10 μm^2 area with noncontact mode. In order to confirm the thickness of the MIPPy film, we measured the change in thickness around the boundary of the MIPPy and the bare Au using AFM.

The line profile represents a surface height aspect of the film section between point A (bare Au region) and B (MIPPy region) in the AFM image. The height difference of point A and B was 5.18 nm. The root-mean-square (rms) of the roughness of the bare Au (point A) and the MIPPy (point B) region were 0.8 and 1.1 nm, respectively (**Table 1**). Thus, the thickness of the MIPPy film on the bare Au was estimated to be 4−6 nm. This result was in good agreement with the value of the thickness derived from the best-fit of the SPR results.

Binding Properties of Zearalenone to the MIPPy Film. The performance of the MIPPy film prepared on the bare Au chip has been assessed with SPR measurements, for the investigation of the recognition behavior of the film in relation to the concentration of zearalenone. We have shown the time- and concentration-dependent shifts in resonance angle in **Figure 4A**. An increase in the resonance angle was observed upon the interaction of the MIPPy with zearalenone standard solutions relative to a blank solution without zearalenone. The resonance angle shifted from 85.702° to 85.917° when 3000 ng/mL of zearalenone was injected into the SPR cell, and saturation was observed after 40 min. In order to investigate the specific recognition between the binding site of MIPPy and zearalenone, the MIPPy film was rinsed three times with water and a 2-mL blank solution; the resonance angle then decreased to 85.816°. However, the resonance angle increased by 0.114° after an injection of zearalenone. This increase in the resonance angle indicates an increase in the surface reflective index on SPR and can be attributed to the rebinding of zearalenone to the MIPPy film. The resonance angle of nonimprinted polypyrrole (non-MIPPy) without zearalenone binding sites shifted by as little as 0.001°, and this minimal shift resulted from weak and nonspecific adsorption due to the random arrangement of the functional groups in the non-MIPPy. This result is similar to that reported for a previous study in which caffeine was detected using a non-MIPPy quartz crystal microbalance (QCM) (*30*). The calibration curve shown in **Figure 4B** was obtained by plotting the differences between the resonance angles of MIP and non-MIPPy after rinsing with 0.1% ethanol, and was observed to have a very high linearity (R^2 = 0.993).

To evaluate the cross-selectivity of the MIPPy film, the changes in the resonance angle of zearalenone were compared to those of other structurally related analogues (**Figure 5**), such

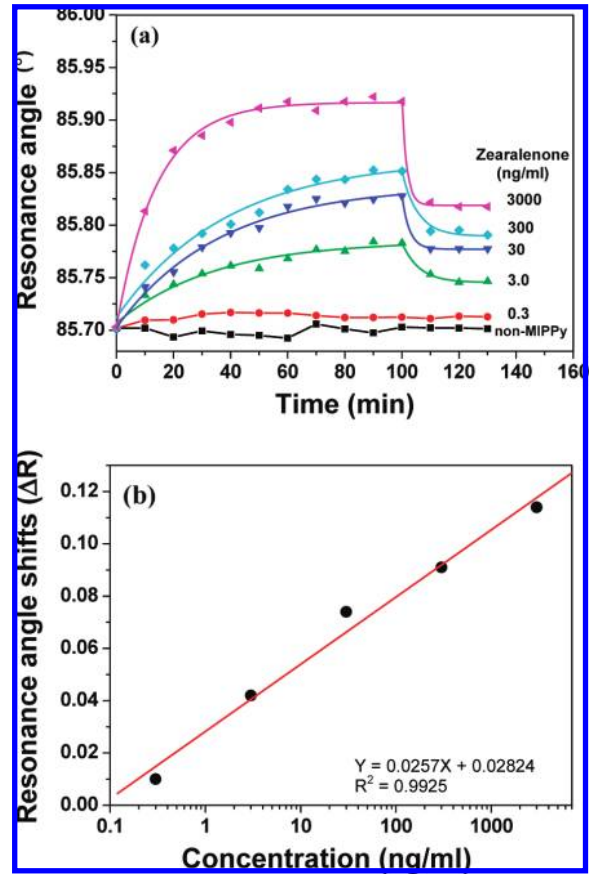

Figure 4. Resonance angle shifts according to zearalenone concentration and reaction time between zearalenone and its analogues and MIP chips: (**A**) resonance angle shifts according to zearalenone concentration and reaction time; (**B**) calibration curve obtained from the resonance angle shifts in part **A**. The non-MIP curve was obtained using a nonmolecular imprinted polymer and a 3000 ng/mL concentration of zearalenone.

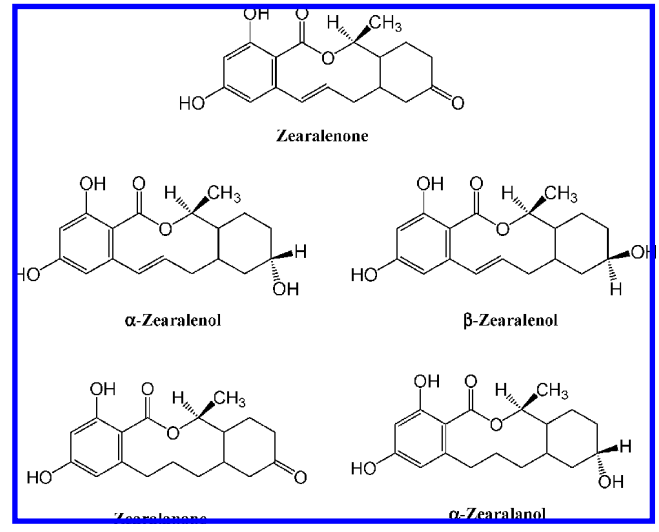

Figure 5. Structures of zearalenone and its analogues.

as α-zearalenol, β-zearalenol, zearalanone, and α-zearalanone. The selectivity efficiency was obtained from the following equation:

$$\text{selectivity efficiency} = \frac{\Delta R_{\text{analogues}}}{\Delta R_{\text{Zearalenone}}} \times 100 \qquad (3)$$

Here, ΔR is the difference in SPR resonance angles before and after adding the zearalenone analogues. As shown in **Figure**

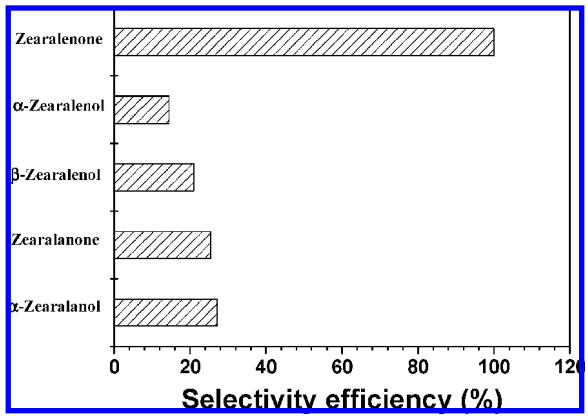

Figure 6. Selectivity efficiency of MIPPy film with respect to several structurally similar zearalenone analogues. Selectivity efficiency was obtained as follows: selectivity efficiency $= \Delta R_{\text{zearalenone analogues}}/\Delta R_{\text{zearalenone}}$, where ΔR is the difference in SPR resonance angle before and after the addition of zearalenone analogues.

6, the selectivity efficiencies of zearalenone, α-zearalenol, *β*-zearalenol, zearalanone, and α-zearalanone were 100, 15, 21, 25, and 27%, respectively. These results imply that MIPPy film has a strong affinity for zearalenone. The imprinting of zearalenone in the Ppy matrix is facilitated by the formation of a hydrogen bond between the functional groups of zearalenone and the imine hydrogen atoms in the pyrrole molecule (*31*, *32*). It is well-known that zearalenone in polypyrrole matrix has two reactive OH groups on the aromatic ring and less reactive carbonyl groups on the macrocyclic ring. From the cross-reactivity result, selectivity appears to be attributable to the structural differences in the macrocyclic ring of the zearalenone template. In addition, the selectivity efficiencies of zearalanone and α-zearalanone, which lack the double bond in the lateral chain of the macrocyclic ring, were 25 and 27%, respectively. This implies that the double bond in the lateral chain of zearalenone may be a probable recognition point for the functional monomer, pyrrole. These selectivity values differed from those reported by Urraca et al. (*33*). In their approach, they used the cyclododecanoyl ester of resorcilic acid as a mimic template for zearalenone and allylpiperazine as the functional monomer. The resulting imprinted polymer recognized not only zearalenone but also its analogues, α-zearalenol, *β*-zearalenol, zearalanone, and α-zearalanone. We do not have an explanation for this finding, but differences in the method of polymerization, functional monomer, and template might be related to the differences in selectivity.

Evaluation of the MIPPy-SPR Sensor. Corn sample analysis was used to compare a MIPPy-SPR sensor and a commercially available ELISA kit for zearalenone detection, as zearalenone is frequently found in corn, and the results are shown in **Table 2**. The limit of detection (LOD) for the ELISA method, expressed as the average values of 10 zearalenone-free samples plus two standard deviations, was found to be 20.8 ng/g. In contrast, the LOD for the MIPPy-SPR sensor, calculated as the zearalenone concentration corresponding to the average deviation of the resonance angle (0.009°) for blank sample solutions, was 0.3 ng/g, suggesting that this method has greater sensitivity than the ELISA. These values are much lower than the maximum levels allowed by the European Union for zearalenone in unprocessed cereals (*5*). Using samples spiked with zearalenone at a concentration of 30 ng/g, the level of detection and recoveries were 33 ng/g and 110% for the ELISA method, and 26 ng/g and 89% for the MIPPy-SPR method, respectively. These results demonstrate a novel combination of a SPR device and the MIP technique in terms of sensitivity and recovery. It suggests that the high concentration of acidic matrix components (such as tartaric acid, malic acid, and lactic acid) in wine extracts might bind with the MIPPy, thus causing interference in the assay to detect ochratoxins (*34*). However, since other sample matrices apart from corn matrix have not yet been tested, further studies on these samples will be required. In addition, this mass production of MIPPy specific to zearalenone for routine analyses may be challenging due to the high price and toxicity of this compound. To overcome these difficulties, a template mimic approach will be needed.

Table 2. LOD and Recovery in Zearalenone Determination Using Both ELISA and MIPPy-SPR Methods in Corn Sample Spiked with 30 ng/g Zearalenone

detect method	LOD (ng/g)	corn sample spiked with 30 ng/g zearalenone	recovery (%)
ELISA	20.8[a]	33	110
MIPPy-SPR	0.3[b]	26	89

[a] Average values of 10 zearalenone-free samples plus 2 standard deviations. [b] Calculated value of the zearalenone concentration corresponding to the average deviation of the resonance angle in blank sample solutions.

In this study, the MIP technique, which functions as a recognition element, was combined with a SPR transducer to detect the mycotoxin zearalenone. The polymer was prepared by electropolymerization of pyrrole onto a bare Au SPR chip in the presence of a template zearalenone molecule. The successful synthesis of the MIPPy film on bare Au was demonstrated by determining the characteristics of the film (thickness, structural properties, and surface morphology) using SPR, ATR-FTIR, and AFM. The MIPPy-SPR sensor exhibited a linear response in the range of 0.3−3000 ng/mL ($R^2 = 0.993$) for the detection of zearalenone. The selectivity efficiencies of zearalenone and other structurally related analogues (α-zearalenol, *β*-zearalenol, zearalanone, α-zearalanone) were 100 and 15−27%, respectively, indicating the strong binding affinity for zearalenone. The limit of detection and average recovery of blank corn matrix spiked with 30 ng/g zearalenone were 0.3 ng/g and 89%, respectively, and these were found to be comparable to the values obtained by ELISA. These results suggest that a combination of SPR sensing with MIPPy film is a promising alternative method for the detection of zearalenone. However, it will be necessary to develop methods for the mass production of MIPPy specific to zearalenone for use with matrices other than corn.

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