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Analysis of Zearalenone in Cereal and Swine Feed Samples Using an Automated Flow-Through Immunosensor

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The development of a sensitive flow-though immunosensor for the analysis of the mycotoxin zearalenone in cereal samples is described. The sensor was completely automated and was based on a direct competitive immunosorbent assay and fluorescence detection. The mycotoxin competes with a horseradish-peroxidase-labeled derivative for the binding sites of a rabbit polyclonal antibody. Control pore glass covalently bound to Prot A was used for the oriented immobilization of the antibody−antigen immunocomplexes. The immunosensor shows an IC_{50} value of 0.087 ng mL^{-1} (RSD = 2.8%, $n = 6$) and a dynamic range from 0.019 to 0.422 ng mL^{-1}. The limit of detection (90% of blank signal) of 0.007 ng mL^{-1} (RSD = 3.9%, $n = 3$) is lower than previously published methods. Corn, wheat, and swine feed samples have been analyzed with the device after extraction of the analyte using accelerated solvent extraction (ASE). The immunosensor has been validated using a corn certificate reference material and HPLC with fluorescence detection.

KEYWORDS: Zearalenone (ZON); immunosensor; food analysis; validation

INTRODUCTION

The *Fusarium* fungi are probably the most prevalent toxin-producing microorganisms found on cereals grown in the temperate regions of America, Europe, and Asia. *Fusarium* toxins have been shown to cause a variety of toxic effects in farm animals, and in some occasions, they have also been suspected to cause toxicity in humans (*1*).

Zearalenone (ZON) is a nonsteroidal estrogenic mycotoxin produced by several *Fusarium* species. It is found worldwide in a number of cereal crops such as maize, barley, oats, wheat, rice, and sorghum exposed to high moisture during storage (*2*, 3). ZON is a stable compound, both during storage/milling and processing/cooking of food, and it does not degrade at high temperatures (*4*). ZON was shown to be produced on corn by *Fusarium* isolates from the continents of Australia, Europe, and North America, as well as in New Zealand (*5*), and the wet milling of corn concentrates ZON in the gluten fraction. The occurrence of ZON in food and feed has also been demonstrated in South America, Africa, Taiwan, China, and the former U.S.S.R. (*6*).

This mycotoxin causes alterations in the reproductive tract of laboratory and domestic animals. In addition, various estrogenic effects such as decreased fertility, increased fetal resorptions, and changes in the weight of endocrine glands and serum hormone levels have been observed (*7*).

Studies of pharmacokinetics and metabolism have shown that ZON is rapidly absorbed following oral administration and can be metabolized by the mammals by reduction of the keto group at C-6′ to α- and β-zearalenol (α- and β-ZOL) (*8*). Other compounds structurally related are α- and β-zearalanol (α- and β-ZAL) (**Figure 1**), with estrogenic effects higher than that for ZON (*1*). The metabolite α-zearalanol (zeranol, ralgro) can also be produced synthetically from ZON and was applied as a growth promoter in animals, although its use has been banned in the European Union since 1985 (*9*−10). Among the various ZON derivatives that can be produced by *Fusarium* species, only α-ZOL has been found to occur naturally in cereal grains (*11*).

Some countries, namely, Austria, Brazil, France, Italy, Romania, Russia, and Uruguay, have specific regulations for ZON, ranging from 0.03 to 1 mg kg^1, that apply to either specific foodstuffs or to all food. However, no international harmonized maximum limit has been set for ZON (*7*, *11*).

Conventional methods for the extraction of zearalenone include liquid−liquid partitioning (L−L extraction) (*12*, *13*), solid-phase extraction (SPE), or immunoaffinity columns (IAC) (*12*, *14*, *15*). More recently other methods such as microwave extraction (*16*), ultrasonication (*17*), and accelerated solvent extraction (*18*, *19*) have also been successfully applied to the quantitative extraction of ZON from several matrixes.

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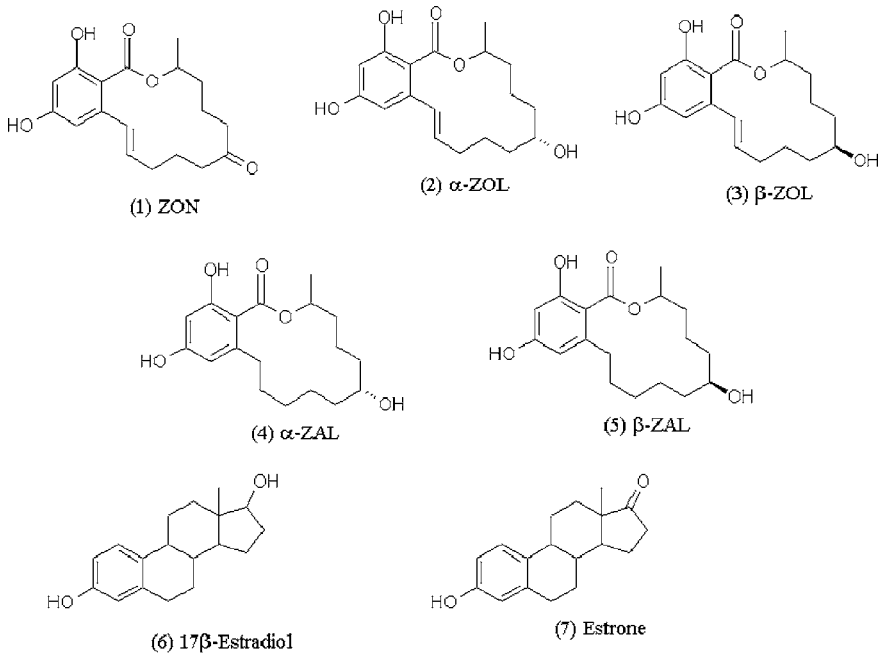

Figure 1. Chemical structures of (1) ZON, (2) α-ZOL, (3) *β*-ZOL, (4) α-ZAL, (5) *β*-ZAL, (6) 17-*β*-estradiol, and (7) estrone.

The main analytical techniques applied for the analysis of ZON in cereals include thin-layer chromatography (TLC) (*20*), gas chromatography (GC)−mass spectrometry (MS) (*21*), and high-performance liquid chromatography (HPLC) with fluorescence (*22*−*24*) or mass spectrometry detection (*25*). Immunochemical methods such as enzyme-linked immunosorbent assays (ELISA) (*26*−*29*) have also been applied as screening techniques with both official and laboratory purposes. An immunosensor based on a sandwich competitive assay has been described (*30*).

Immunoassays are usually performed on microtiter plates (*29*, *31*, *32*). This method, although very sensitive, requires extensive pipetting, washing, and incubation steps, thus, is time-consuming and cannot be easily applied to process control. These drawbacks can be solved with the aid of immunosensors that are based on inmunomethods allowing the determination of the analyte within short times (*33*, *34*). To this aim, the use of heterogeneous phase immunosensors and flow techniques allows the combination of the sensitivity and selectivity of the former with the accuracy and simplicity of the flow methods.

In direct flow-through immunosensors, the analyte and a constant amount of a labeled derivative compete for the antibody-binding sites. After competition, the immunocomplexes are retained on an immunosorbent packed in a reactor and the amount of labeled antigen bound to the antibody is measured, by different techniques, and can be related to the analyte concentration in the sample. Finally, the immunosorbent can be regenerated for a new use by the application of a solution (desorbent) that washes out the antigen−antibody complexes without affecting the characteristics of the support. The choice of the immobilization support is a key issue for the success of the assay. Some of the desired characteristics of these sorbents include (1) ease of regeneration to allow a good reproducibility and low cost per analysis, (2) the capability of antibody immobilization in an oriented manner to increase the sensitivity of the assay, (3) good hydrodynamic properties, and (4) compatibility with the use of organic solvents (*34*, *35*).

This work describes the optimization of a flow-through fluorimmunosensor for the determination of zearalenone in corn, wheat, and swine feed samples. The immunosensor is based on the competition of the mycotoxin with an enzyme-labeled derivative for the binding sites of a specific antibody (*28*). The assay is based on the oriented capture of the immunocomplexes formed in solution in an immunoreactor containing controlled pore glass (CPG) covalently bound to protein A (Prosep A). Several parameters affecting the immunosensor performance have been optimized such as the assay pH, the size of the reactor, the concentration of antibody and tracer, the flow rate, the nature of the immunosupport, the incubation time for the inmunological and enzymatic reactions, and the concentration of the enzyme substrates. The immunosensor has been applied to the analysis of zearalenone in different matrixes (corn, wheat, and swine feed) extracted using an accelerated solvent extraction (ASE) method, optimized previously (*19*), without further clean up.

EXPERIMENTAL PROCEDURES

Reagents and Materials. ZON, α-ZOL, *β*-ZOL, α-ZAL, and *β*-ZAL standards were purchased from Sigma (St Louis, MO). ZON and α-ZOL stock solutions were prepared in acetonitrile (5 mg mL^{-1}) and methanol (1 mg mL^{-1}), respectively, and were stored in the dark at −20 °C. ZON standard solutions for immunoassay calibration purposes were prepared daily upon dilution of the stock solutions in phosphate buffer (0.020 M at pH 8.0). Standard solutions for HPLC calibration or spiking purposes were prepared daily by diluting the stock solutions in methanol at a concentration of 10 μg mL^{-1}. Acetonitrile and methanol (HPLC grade) were supplied by SDS (Peypin, France). Water was purified with a Milli-Q system (Millipore, Bedford, MA). All other chemicals used were of analytical reagent grade. All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use. The wheat samples for the recovery experiments were purchased from a local store (Madrid, Spain). Swine feed samples were kindly supplied by Nutreco (Madrid, Spain). Corn reference material was provided by Prof. R. Krska from the Center for Analytical Chemistry in Tull, Austria.

Immunoreagents. ZON conjugated to horseradish peroxidase (HRP) (zearalenone−HRP, working dilution of 1:100 determined by ELISA with monoclonal antibody of anti-zearalenone) was supplied by the Agricultural Biotechnology Center (Gödöllö, Hungary). H_2O_2 and 3-(4-hydroxyphenyl)-propionic acid (HPPA), used as revealing agents for enzymatic reaction, were supplied by Aldrich (Steinheim, Germany). Zearalenone was converted to 6′-(carboxymethyl)zearalenone oxime and conjugated to bovine serum albumin for use as immunogen. Antiserum was prepared against it in rabbits, and antisera was purified

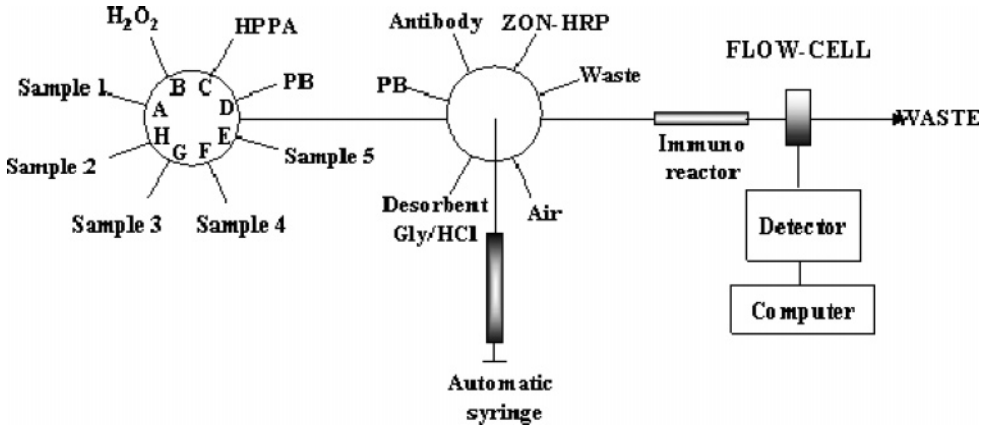

Figure 2. Scheme of the automated flow injection set up used for the measurements.

as described previously (*26*). The immunosorbent, Prosep A, and Ultralink immobilized protein A/G were purchased from Millipore (Bedford, MA) and Pierce (Madrid, Spain), respectively.

Measuring System. The automated flow injection manifold is depicted in **Figure 2** (*33*, *34*). An eight-way distribution valve (Kloehn, Las Vegas, NV) equipped with a 2.5 mL syringe pump is connected to another eight-way distribution valve. The whole system is controlled with the Winpump software provided by Kloehn (Las Vegas, NV). The output of the pump is connected to a homemade poly(methyl methacrylate) reactor (10 × 4 mm, length × diameter), thermostatized at 25 °C, and packed with CPG-bound protein A (Prosep A) applied to the immobilization of the antigen−antibody immunocomplexes. The output flow is driven to a flow-through cell (100 μL, Starna, Germany) placed in the fluorometer sample holder. Fluorescence intensity measurements (λ_{exc} = 320 nm; λ_{em} = 405 nm) were carried out in a Fluoromax 2 (Horiba Jobin Yvon, Longjumeau Cedex, France), and the instrumental parameters and data processing were controlled with the original software (Datamax). All of the solutions were thermostatized at 25 °C using a water bath, Precisterm JP (Selecta, Barcelona, Spain).

Assay Protocol. The measuring protocol is based on the principles of a competitive direct enzyme immunoassay. All of the solutions were prepared in 0.020 M phosphate buffer (PB) at pH 8.0 except the desorbent solution. Initially, the sample (0.7 mL) is mixed in the syringe with a constant amount (0.2 mL) of enzyme tracer (zearalenone−horseradish peroxidase conjugate, ZON−HRP) and of the antibody (0.2 mL). After 180 s, the solution (1 mL) is injected into the immunoreactor at a flow rate of 0.5 mL min^{-1} to allow the retention of the immunocomplexes onto the immunosorbent. The reactor is washed 4 times with 1.5 mL of PB to remove all of the unbound complexes. PB solutions of fluorogenic enzyme substrate HPPA (0.3 mL, 1.3 g L^{-1}) and H_2O_2 (0.2 mL, 0.025%) are mixed into the syringe, and 0.3 mL are injected into the reactor. After 180 s, the fluorescent product generated from the enzymatic reaction is driven through a flow-through cell placed in the fluorometer by injecting 2.5 mL of PB at 2 mL min^{-1}. After peak registration, the complex of antibody and hapten−enzyme conjugate or the analyte are dissociated from the immunosorbent by 2.5 mL of 0.1 M glycine/HCl at pH 2.0 and 0.5 mL min^{-1}. Before a new measurement, the reactor is washed with PB (2 × 2.5 mL of phosphate buffer at 0.5 mL min^{-1}). A complete cycle for the whole automated assay procedure required approximately 25 min including regeneration. The immunoreactor showed a great stability, and the system could be used for more than 150 measurements. Occasionally, the immunoreactor had to be back-flushed with washing buffer to maintain a constant flow rate. The reactor was stored at 4 °C when not in use.

Experimental signals were normalized using the following expression:

$$\text{normalized response} = (B - B_\infty)/(B_0 - B_\infty)$$

where B is the signal (intensity of fluorescence) measured in the presence of the increasing analyte concentrations, B_∞ is the background fluorescence obtained in the absence of the enzyme tracer, and B_0 is the signal in absence of zearalenone. The normalized response was plotted as a function of the analyte concentration (in logarithmic scale), and the experimental data were fitted to a four-parameter logistic equation (sigmoidal). The detection limit (LOD) was calculated as the analyte concentration for which the tracer binding to the antibody is inhibited by 10%, and the dynamic range (DR) of the method was evaluated as the analyte concentration that produce a normalized signal in the 20−80% range.

Table 1. Optimized ASE Operating Conditions for the Extraction of ZON from Cereal and Swine Feed Samples

extraction solvent	MeOH/AcN (50:50, v/v)
pressure (psi)	1500
temperature (°C)	50
heat-up time (min)	5
static time (min)	5
flush volume (%)	60
purge time (min)	1
number of cycles	1
cell volume (mL)	11
total extraction time (min)	12–14[a]
total solvent used (mL)	~16[a]

[a] Per sample.

Sample Analysis and Validation. Sample extraction was performed using an accelerated solvent extractor (ASE 200, Dionex) equipped with 11 mL stainless steel cells and 40 mL glass vials for the collection of the extracts, following a procedure optimized previously (*19*). Wheat, corn, and swine feed samples were ground in an Ika M20 mill (Ika-Werke, Staufen, Germany) and passed through a U.S. standard number 20 sieve (Filtra, Barcelona, Spain). Samples (50 g) were dried in an oven for 24 h at 40 °C. Subsamples (5 g) were weighed and transferred directly into the steel extraction cells from Dionex.

For validating purposes, ZON-free wheat and swine feed samples were directly spiked into the extraction cells to ensure a reproducible introduction of the mycotoxin with a stock solution of ZON in methanol (1.2 μg mL^{-1}) to a final concentration of 126 ng g^{-1}. The samples were allowed to equilibrate for at least 30 min before extraction. The corn sample was a certificate reference material and was introduced directly into the extraction cell. The selected ASE extraction conditions are summarized in **Table 1**. Extracts were made to a final volume of 20 mL (acetonitrile/methanol, 50%, v/v), filtered through a 0.25 μm PTFE membrane from Phenomenex (Torrance, CA), and previously tested for not retaining the target analytes. Samples were diluted 50-fold with PB (0.020 M at pH 8.0) and injected into the flow-through system. All measurements were carried out by triplicate.

For confirmation, samples were also analyzed by HPLC with fluorescence (FLD) detection. A 16 mL aliquot of the extract was transferred to a glass vial and rotary evaporated at 40 °C. The residue was redissolved in 1 mL of methanol with sonication and injected into the LC system without further clean up. Chromatographic analysis was carried out with an HPLC 1100 (Agilent) equipped with a quaternary

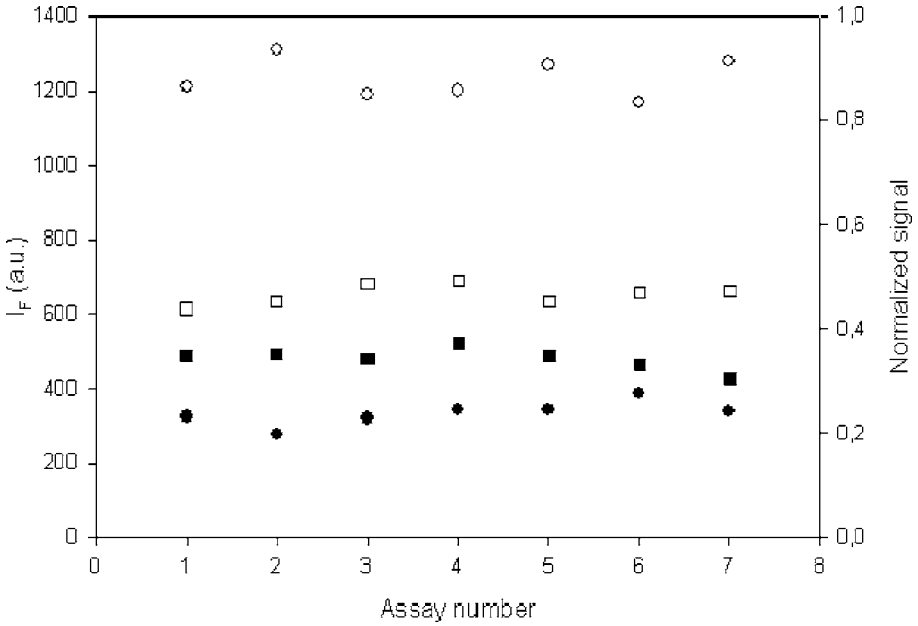

Figure 3. Effect of the immunosorbent nature on the long-term response obtained with the immunosensor to a blank sample (B_0) and to a 0.500 ng mL^{-1} ZON solution standard (Ab, 1:150). The signals correspond to (○) a blank sample (B_0) using Prosep A as the immunosorbent, (□) a blank sample (B_0) using Ultralink A/G as the immunosorbent, (●) normalized signal obtained with Prosep A in the presence of ZON, and (■) normalized signal obtained using Ultralink A/G in the presence of ZON.

pump, an auto-injector, and a fluorescence detector. The analytical column was a C_{18} reverse-phase Synergi Hydro (250 × 4.6 mm, 4 μm), protected by a RP18 guard column (4.0 × 3.0 mm, 5 μm), both from Phenomenex (Torrance, CA). The mobile phase consisted of a mixture of acetonitrile/methanol/water (15 mM ammonium acetate) 10:55:35 (v/v/v) at a flow rate of 1.0 mL min^{-1}. The injection volume was set at 8 μL. The column was kept at room temperature. The fluorescence detector was set at λ_{exc} = 271 nm and λ_{em} = 452 nm. Matrix-matched calibration standards were prepared by diluting adequate amounts of ZON with blank matrix solutions obtained by extracting blank wheat, corn, and swine feed matrixes under the same conditions applied for the samples. All measurements were carried out by triplicate.

RESULTS AND DISCUSSION

Immunoreagent Optimization. The detection of the enzyme tracer was carried out using H_2O_2 and HPPA as the fluorogenic substrate (*36*). To optimize detection, the zearalenone−horseradish peroxidase conjugate concentration was kept constant (dilution of 1:500) and the various concentrations of H_2O_2 and HPPA were assessed for maximal fluorescence intensity values. When HPPA concentrations were evaluated over a range of 0.5 to 3.0 mg mL^{-1}, the fluorescent signal increased with the concentration up to a value of 1.3 mg mL^{-1}, after which it remained constant. When concentrations of H_2O_2 were tested between 0.005 and 0.1%, the maximum signal was achieved for 0.025%. Higher concentrations yielded an important decrease in the fluorescence intensity, probably because of a loss of enzymatic activity. The effect of the pH of the buffer solution (0.020 M) on the sensitivity of the revealing reaction was tested in the 7.0−9.0 range. Fluorescence intensity decreased considerably at pH values below 8.0 (by 47% at pH 7); therefore, a pH 8.0 was selected for further measurements.

Nature of the Support, Size of the Reactor, and Reusability. The retention of immunocomplexes in the reactor is highly dependent on the nature of the immunosorbent and will have a large impact on the performance of the assay. Two different supports have been tested for the development of the immunosensor, protein A covalently bound to controlled pore glass (CPG protein A) (Prosep A) and protein A/G covalently bound to an azlactone-activated polymeric material (Ultralink A/G). The sorbents were packed in the reactor [10 × 4 mm, length × internal diameter (i.d.)], and the long-term response of both sorbents to a blank sample (B_0) and to a standard solution of ZON (0.500 ng mL^{-1}) were measured using a constant Ab concentration (1:150). Because a higher fluorescence signal as well as a slightly higher sensitivity to the mycotoxin was obtained when CPG protein A was used as the immunosorbent (**Figure 3**), this material was selected for the development of the immunosensor. Nevertheless, the reproducibility obtained with both sorbents was excellent, lower than 5.8% (n = 8).

Table 2. Effect of the Effective Reactor Size on the Response to a 500 ng L^{-1} ZON Solution and Relative Standard Deviation Corresponding to the Normalized Signals (n = 3)

length (mm)	internal diameter (mm)	B/B_0	RSD (%)
2	2	0.88	5.8
4	4	0.31	3.9
4	6	0.31	6.2
10	4	0.17	4.4
15	4	0.18	4.6

To evaluate the optimum amount of sorbent for the assay, several reactor sizes were tested (**Table 2**). The response and the reproducibility of the immunosensors to 0.50 ng mL^{-1} standard of ZON was better when using a 10 × 4 mm (inner length × inner i.d.) reactor packed with CPG protein A. The use of larger reactors did not increase the sensitivity of the assay while requiring larger volumes of immunosorbent, with the corresponding increment in the nonspecific binding, as well as in the assay price. The reactor could be reused for more than 150 assay cycles with no detectable loss of activity.

Immunosensor Optimization and Characterization. Conditions were established for optimal immunosensor operation. Decreasing the flow rate increased the amount of immunocomplexes bound to the sorbent and, correspondingly, the sensitivity but increased analysis time. When flow rates ranging from 0.25 to 2.00 mL min^{-1} were tested, 0.50 mL min^{-1} was selected as a compromise for optimal response and the minimum analysis

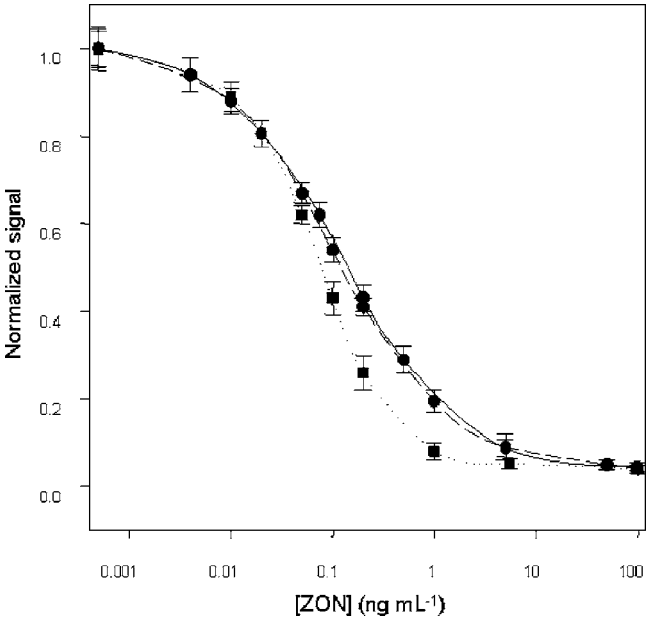

Figure 4. Competitive calibration curves obtained with the immunosensor for (■) ZON standard solutions in 0.020 M PB at pH 8, (●) ZON standard solutions prepared in a blank wheat extracted with the optimized ASE method (extraction conditions as in **Table 1**) diluted 50-fold with PB, and (gray circle) ZON standard solutions prepared in PB with 2% of acetonitrile/methanol (50:50, v/v). Each point was measured in two independent experiments in 3 different days ($n = 6$).

Table 3. Optimized Operating Conditions for the Analysis of ZON Using an Automated Immunosensor

parameter	optimized value
incubation time Ab + Ag/Ag* (min)	3
antibody dilution factor	1:150
enzyme tracer dilution factor	1:500
carrier	phosphate buffer pH 8.0, 0.020 M
HPPA concentration (mg mL^{-1})	1.3
H_2O_2 concentration (%)	0.025
injection rate HPPA-H_2O_2 (mL min^{-1})	2
incubation time HPPA-H_2O_2 (min)	3
regenerating solvent	Gly/HCl (pH 2)
regeneration rate (mL min^{-1})	0.5
reactor size (length × i.d., mm)	10 × 4
immunosupport	Prosep A
total analysis time (min per sample)	25

time. The incubation time of the enzyme substrate in the reactor also influenced the analytical signal. A contact time of 180 s was selected for the assay, because larger values did not render better sensitivities. In general, low antibody (Ab) and enzyme tracer (Ag*) concentrations were necessary to achieve high sensitivity but must be sufficient to yield a measurable signal. Optimal results were obtained for Ag* and Ab dilutions of 1:500 and 1:150, respectively. Overall, these conditions (**Table 3**) facilitated high assay sensitivity as well as a wide dynamic range for the immunosensor. The analysis time 25 min/cycle was half of that described in the literature for another ZON fluorescently based immunosensor (*30*).

When the assay was conducted with ZON standards at 0−100 ng mL^{-1} in phosphate buffer (**Figure 4**), the IC_{50} value was 0.087 ng mL^{-1} ZON (RSD = 2.8%, $n = 6$) and the limit of detection was 0.007 ng mL^{-1} (RSD = 3.9%, $n = 3$). The assay dynamic range, defined as the concentration of analyte that produces a normalized signal in the 20−80% range, was between 0.02 and 0.42 ng mL^{-1}. The sensitivity of the immunosensor was far superior to that reported for direct and indirect ELISA and radioimmunoassay methods (*26*).

Table 4. Cross-Reactivity for Some Zearalenone-Related Compounds

compound	50% inhibition (ng mL^{-1})	cross-reactivity (%)
zearalenone	0.087	100
α-zearalenol	0.091	96
β-zearalenol	0.381	21
α-zearalanol	0.343	24
β-zearalanol	1.692	5
17-β-estradiol	>1000	<0.01
estrone	>1000	<0.01

Reproducibility over 3 different days was excellent with RSD of the normalized signal ranging from 1.7 to 4.8%.

Cross-Reactivity. Cross-reactivity studies were carried out by measuring, in the optimized conditions, the competitive curves for different derivatives of ZON that can be present in food samples. Other estrogens such as estrone and 17-β-estradiol (**Figure 1**) with a similar structure to ZON were also included in the study. Cross-reactivity was calculated as the percentage between the IC_{50} value for ZON and the IC_{50} for the interfering compound (**Table 4**). The reactivity for α-zearalenol was 96%. This, in turn, can be an advantage because this compound is more estrogenic than ZON and it appears as a metabolite in cereal samples. The cross-reactivity of β-zearalenol and α-zearalanol is moderate (21−24%), whereas the cross-reactivity of β-zearalanol is much lower (5%). Consistent with these findings, Warner et al. (*26*) have previously shown using similarly prepared polyclonal antibody in ELISA that cross reactivities for α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalanol were 280, 35, 22, and 10%, respectively.

The estrogenic compounds, estrone and 17-β-estradiol, showed no affinity for the antibody.

Analysis of Wheat, Corn, and Swine Feed Samples. To apply the developed immunosensor to the analysis of ZON in cereal and swine food samples, calibration graphs were prepared by spiking blank matrix sample extracts, previously found to contain undetectable levels of the toxin, with different increasing concentrations of ZON in the range of 0.01−100 ng mL^{-1}. When the extract was diluted 50-fold with PB and compared to a ZON standard solution in PB buffer, a slight matrix effect was observed (**Figure 4**). To ascertain whether differences could be attributed to organic solvent (2%, acetonitrile/methanol, 50:50, v/v) used during the ASE extraction procedure, PB calibration standards were also prepared in the presence of the same amount of acetonitrile/methanol. The calibration curve in this media was equivalent to that obtained in the presence of the blank matrix extract, and the IC_{50} value [$IC_{50} = 0.121$ ng mL^{-1} (RSD = 2.3%, $n = 8$)] was slightly lower than that obtained in PB. Thus, to make the calibration as precise as possible, working standards were prepared in the blank extracts when available or in PB solutions with 2% acetonitrile/methanol (50:50, v/v).

The immunosensor was validated for corn analysis using a certified reference material (133 ng g^{-1}) that was extracted using the ASE procedure optimized previously (*19*). The results demonstrated the suitability of the developed system in combination with the extraction procedure for ZON analysis in this cereal (**Table 4**). When spiked wheat and swine feed samples (126 ng g^{-1}) were extracted with the ASE procedure and analyzed using the immunosensor, recovery values were excellent (>97%) (**Table 5**). ASE extracts were also analyzed using HPLC−FLD and compared to immunosensor data. No significant differences were observed between the two techniques.

Table 5. Recovery Studies with the Immunosensor and by HPLC of the ASE Extracts Corresponding to a Certificate Corn Sample (133 ng g^{-1}) and Wheat and Swine Feed Samples, Spiked with 126 ng g^{-1} ZON ($n = 3$)

sample	spike level (ng g^{-1})	immunoassay (ng g^{-1})[a]	HPLC–FLD (ng g^{-1})[a]
corn	133[b]	138 ± 11	130 ± 11
wheat	126	119 ± 10	132 ± 11
swine feed	126	133 ± 13	132 ± 15

[a] Confidence limit: $\pm ts/\sqrt{n}$ (95%). [b] Certificate reference material.

CONCLUSIONS

An automatic flow-though immunosensor for the analysis of ZON in cereal and swine feed samples was developed that allows the fast and sensitive detection of the mycotoxin in cereal and feed samples. Advantages over conventional ELISA included sensitive detection of the mycotoxin at pg g^{-1} levels and ease of operation as the system is fully automated. In addition, the immunosensor showed a high reusability (>150 cycles), excellent precision, and short analysis times that make it very suitable for routine analysis of ZON in food samples. CPG protein A was a better support for the capture of the immunocomplexes formed in bulk solution than protein A/G covalently bound to an azlactone-activated polymeric material. Among the ZON metabolites included in the cross-reactivity study, α-ZOL that can also be found in cereal samples shows appreciable cross-reactivity.

An accelerated solvent extraction procedure, optimized previously, has been used for the extraction of the mycotoxin from cereal samples. The extracts can be directly analyzed after buffer dilution (50-fold) using the immunosensor. The sensitivity slightly decreases in the presence of organic solvent (2% ACN/MeOH, 50:50, v/v) in the calibration samples.

The immunosensor has been validated for the analysis of corn samples using a certificate reference material. Excellent recoveries were also achieved for the analysis of spiked (spiking level of 126 ng g^{-1}) wheat and swine feed samples. The results calculated with the immunosensor were fully comparable to those obtained using HPLC−FLD as an alternative technique.

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