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Detection of Aflatoxin B_1 in Barley: Comparative Study of Immunosensor and HPLC

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Abstract: In the present work, an indirect competitive electrochemical enzyme linked immunosorbent assay (ELISA) has been used for determination of aflatoxin B_1 (AFB_1) in barley. The method involves the use of disposable screen-printed carbon electrodes (SPCEs) and anti-aflatoxin B_1 monoclonal antibodies (MAb) for immunosensor development.

The specificity of the assay was assessed by studying the cross-reactivity of the MAb relative to AFB_1. The results indicated that the MAb could readily distinguish AFB_1 from other toxins, with the exception of AFG_1.

The stability of the coating reagents was evaluated using SPCEs coated with AFB_1-bovine serum albumin (BSA) conjugate. The results showed that the coated electrodes could be used for up to one month after their preparation and storage at 4°C.

Prior to evaluating the performance of the electrochemical immunosensor for AFB_1 with spiked samples, the effect of barley extract on assay performance was tested. Using this calibration method, the limit of detection (LOD) was found to be 90 pg mL^{-1}.

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The linear range was 0.1–10 ng mL^{-1}, and recoveries ranged from 100%–125%. The results obtained were confirmed by high performance liquid chromatography (HPLC) coupled with fluorescence detection. These results demonstrated the suitability of the proposed method for routine screening of AFB_1 in barley.

Keywords: Aflatoxin B_1, electrochemical immunosensor, ELISA, HPLC, barley

1. INTRODUCTION

The aflatoxins are the main toxins produced by the *Aspergillus flavus, A. parasiticus*, and *A. nomius* groups of fungi that can be found in corn, cottonseed, peanuts, and other nuts, grains, and species (Anklam et al. 2002; Gilbert and Anklam 2002). The aflatoxins commonly found are: AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1 (Fig. 1). They have received worldwide attention due to their deleterious effects on human and animal health as well as their importance in international trade. The International Agency for Research on Cancer (IARC 1993) has classified AFB_1, AFB_2, AFG_1, and AFG_2 as group I carcinogens. Among these, AFB_1, the most potently carcinogenic, mutagenic, teratogenic, and immunosuppressive agent, is generally found in the highest concentration in food and animal feed (Stroka and Anklam 2002).

Aflatoxins cannot be entirely avoided or eliminated from foods and feeds even by current agronomic and manufacturing processes, thus they are considered unavoidable contaminants (Wood 1989). However, European Community legislation establishes that 4 μg of total aflatoxins and 2 μg of

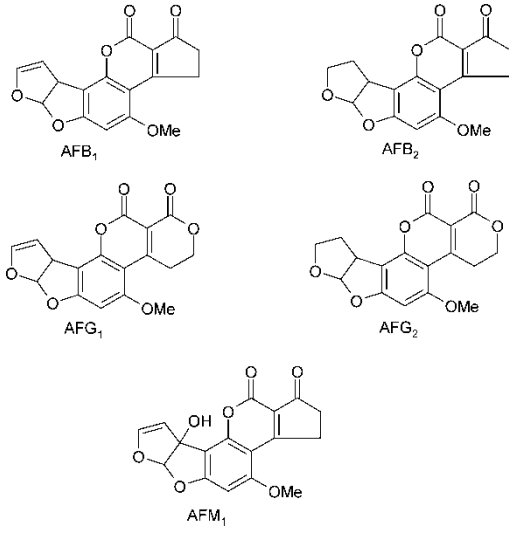

Figure 1. Chemical structure of aflatoxins.

AFB_1 alone are the maximum amounts permitted per kg in nuts, nut products, figs, fig products, and cereals (Anklam and Battaglia 2001).

Due to the significant health risks associated with the presence of aflatoxins in foods, and also to satisfy the stringent legal requirements, it is important to have efficient techniques for their detection. Traditional analytical methods for the determination of aflatoxins employ column chromatography, liquid-liquid partition, or chemical adsorption methods for removing interfering compounds. Subsequent quantification is often performed by either thin-layer chromatography (TLC) (Lin et al. 1998) or HPLC with various detection systems (Chiavaro et al. 2001; Jaimez et al. 2000; Elizalde-Gonzales et al. 1998; Papp et al. 2002; Blesa et al. 2003; Ventura et al. 2004).

A TLC analysis is a relatively economical method requiring little equipment but can be tedious and is time and labor consuming. The HPLC analysis requires an extensive clean up procedure and derivatization to improve the detection sensitivity, it also requires specially trained personnel to perform the analyses.

The aflatest immunoaffinity column, coupled with either solution fluorimetry or liquid chromatography with derivatization has been adopted as the official method by the association of official analytical chemists (AOAC, 1995) for the determination of aflatoxin in corn, raw peanuts, and peanut butter at a total aflatoxin concentration of $\geq 10\ \mu g\ kg^{-1}$.

However, the immunochemically based assays that are used for detecting aflatoxins and aflatoxin metabolites (including radioimmunoassay and ELISA), have seen rapid development over the past two decades for their simplicity, adaptability, sensitivity, and selectivity. A number of kits for immunoenzymatic determination of AFB_1 are now commercially available (Ram et al. 1986; Chu et al. 1987; Ramakrishnan et al. 1990; Tsci and Yu 1999; Pesavento et al. 1997).

Sequential injection immunoassay has also been used for AFB_1 determination with both fluorometric (Carlson et al. 2000) and colorimetric (Garden and Strachan 2001) detection. Nasir and Jolley (2002) proposed a rapid fluorescence polarization immunoassay (FPIA) for aflatoxin determination in grains. Xiulan et al. (2005) have prepared an antibody colloidal gold probe (conjugate) specific for AFB_1 and its use in developing a rapid AFB_1 diagnostic method has been demonstrated.

Moreover, spectrophotometric ELISA and electrochemical immunosensors, using screen-printed carbon electrodes (SPCEs), were developed by our group for preliminary determination of AFB_1 in barley (Ammida et al. 2004).

In the present work, a disposable electrochemical immunosensor based on the indirect competitive enzyme linked immunosorbent assay (ELISA), for simple and fast measurements of aflatoxin B_1 in barley was developed using differential pulse voltammetry (Del Carlo et al. 1997). The stability and the specificity of electrochemical immunosensors were studied. Finally after studying the matrix effect, the immunosensor was used to detect AFB_1

in spiked barley samples. In addition, HPLC coupled with fluorescence detection, and precolumn derivatization with trifluoroacetic acid (TFA), was used as a confirmatory method for the results obtained (AOAC 1995).

2. EXPERIMENTAL

2.1 Reagents

Rabbit anti-aflatoxin B_1 (MAb, cod. A8679), aflatoxin B_1-BSA conjugate (AFB_1-BSA), N,N-dimethylformamide (DMF), polyvinyl alcohol (PVA), trifluoroacetic acid (TFA), and polyoxyethylene sorbitan monolaurate "Tween 20" (Tw20) were from Sigma-Aldrich Co. (St. Louis, MO, USA). Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1) and ochratoxin A were purchased from Alexis (Lausen, Switzerland). 1-Naphthylphosphate-disodium salt (1-NPP), sodium chloride, potassium chloride, magnesium chloride, and diethanolamine (DEA) were purchased from Fluka Chemie (Sigma-Aldrich, Milan, Italy). The affinity purified goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (Ab_2-AP) was from Bio-Rad Labs (Hercules, CA, USA). Methanol and acetonitrile for HPLC were obtained from Riedel-de Haën (Sigma-Aldrich Laborchemikalien GmbH).

2.2 Buffer Solutions

1 M Diethanolamine buffer (DEA), pH 9.6, containing 1 mM $MgCl_2$ and 15 mM KCl, was used as the enzymatic substrate buffer for the electrochemical measurement. 0.1 M carbonate buffer, pH 9.6, was used for the immobilization of the AFB_1-BSA on graphite working electrodes (coating step). Polyvinyl alcohol (PVA) solution 1% (v/v) in carbonate buffer was used as blocking reagent. 15 mM Phosphate buffered saline (PBS), pH 7.4, was used for the competition step and also for the dilution of Ab_2-AP. The washing solutions, used after each assay step, were prepared by adding 0.05% (v/v) Tw20 to the PBS (PBS-T).

2.3 Instrumentation

Electrochemical measurements were performed at room temperature, using a computer-controlled system, AUTOLAB model GPSTAT-12 with GPES software, (Ecochemie, Utrecht, Netherlands). The SPCEs were purchased from EcoBioservice and Research (Florence, Italy). The SPCEs were produced in sheets of 80 strips. Each SPCE consisted of three printed electrodes, two carbon electrodes acting as working electrode and counter electrode, and a silver electrode acting as pseudo reference. The diameter of the

working electrode was 0.3 cm, which resulted in an apparent geometric area of 0.07 cm^2.

The SPCEs were pretreated in a 0.05 M phosphate buffer containing 0.1 M KCl, pH 7.4, by applying an anodic potential of 1.7 V for 3 min. They were then stored dry at room temperature in the dark (Ricci et al. 2003).

The HPLC system consisted of a modular CHROMQUEST spectra system from THERMOQUEST (San Jose, CA, USA), equipped with two LC'GA pumps, a Shimadzu UV-VIS spectrometer model (SPD-10AV), fluorescence (RF 10AXL) detectors, a vacuum SCM 1000 as degassing unit, and an autosampler, AS 3500. A SN 4000 controller operated the HPLC system working under the control of software included in the CHROMQUEST module.

The chromatographic separation was performed using a reverse phase C18 ($VYDAC_{TM}$, W.R. Grace &Co, cat. 210TP54) stainless steel column (5 μm spherical particle size, 150 x 4.6 mm I.D). The clean up procedure for AFB_1 extracts was carried out using an immunoaffinity column, Afla B^{TM}, Aflatoxin Testing System, which was obtained from VICAM (Watertown, USA).

2.4 ELISA Protocol for Electrochemical Measurements

The ELISA protocol for electrochemical measurement was similar to that used in our previous work (Ammida et al. 2004) with the exception of the 1000-fold dilution of secondary antibody.

Briefly, 6 μL of AFB_1-BSA (1 μg mL^{-1}) in carbonate buffer was dispensed onto the working electrode of the pretreated graphite-based SPCEs and left moist overnight at 4°C. The washed electrodes were blocked with 6 μL 1% PVA solution for 15 min at room temperature. To perform the competition step, AFB_1 standard (or diluted sample extract) was mixed with an equal volume of MAb solution (diluted 1000-fold in PBS). Six μL of this mixture was added onto the working electrode surface and allowed to react with the coated AFB_1-BSA for 30 min at room temperature. Unbound MAb was removed by washing the electrodes. Next, 6 μL of Ab_2-AP diluted (1000-fold in PBS) were added and the electrodes were incubated 15 min. Between each step (coating, blocking, competition, and labeling), the electrodes were subjected to the 3-cycle washing procedure, involving two washes with PBS-T and one with PBS. Finally, the activity of the enzyme label was measured electrochemically by adding 100 μL of 1-NPP substrate solution (2 mg mL^{-1} of 1-NPP in DEA buffer, prepared daily) and allowed to react for 2 min at room temperature. The enzymatic product, 1-naphthol, was detected by DPV under the following conditions: potential range 0–600 mV, pulse width of 50 ms, pulse amplitude of 70 mV, and scan speed of 300 mV s^{-1}.

2.5 Calibration Graphs

Standard curves were obtained using standard solutions of AFB_1 (0.05–100 ng mL^{-1}) prepared in PBS for electrochemical ELISA, and (4–24 ng mL^{-1}) for the HPLC method. The calibration curve was prepared by diluting the AFB_1 using a barley extract blank. This extract was prepared by applying the extraction procedure to barley samples that were not infected with *Aspergillus*. For the ELISA, the standard curves were fitted using "non-linear 4 parameter logistic calibration plots" (Warwick 1996). The four parameter logistic function is: $f(x) = [(1 - a)/(1 + (x/c)^b) - d]$, where a and d are the asymptotic maximum and minimum values, c is the value of x at the inflection point and b is the slope.

2.6 Sample Preparation and Extraction

One kilogram of noninfected barley was collected and ground to powder using a blender. In all the spiking studies a dry spiking method was used, in which 5 g of noninfected barley sample was weighed into a centrifuge tube and then spiked with AFB_1 in methanol, to reach three different levels (1, 2, and 4 ng g^{-1}). These samples were then thoroughly mixed with a vortex mixer for 1 min before extraction for ELISA and HPLC analysis.

Sample extraction for ELISA detection was performed by adding 10 mL of extraction solvent (85% methanol: 15% PBS) to the spiked samples. These samples were sealed with parafilm and agitated in a horizontal shaker for 30 min at 100 rotations min^{-1} at room temperature. Samples were then centrifuged at 6000 rpm for 10 min. one milliliter of supernatant was then diluted two times (1:1 v/v) with PBS and used for AFB_1 detection by ELISA protocol. The concentration of AFB_1 in diluted sample extracts was determined from the calibration curve and used to calculate the concentration in the original sample according to Eq. (1):

$$\begin{aligned} AFB_1(\text{ng } g^{-1}) = \{&([AFB_1(\text{ng } mL^{-1}) \text{ in sample extract}] \\ &[\text{solvent extract volume}])/\text{sample weight}\} \\ &\times \text{dilution factor} \end{aligned} \quad (1)$$

where the volume of solvent extract is 10 mL, sample weight is 5 g, and the dilution factor is 2. By substituting these values in Eq. (1), the concentration of AFB_1 in ng g^{-1} can be directly calculated by multiplying the concentration of AFB_1 (ng mL^{-1}) by 4 as in Eq. (2):

$$AFB_1(\text{ng } g^{-1}) = AFB_1(\text{ng } mL^{-1}) \times 4 \quad (2)$$

The HPLC analysis was performed using the official AOAC method with some modifications. As amount of 10 mL of methanol: water (80:20 v/v)

mixture was added to the spiked barley sample and then the mixture was agitated by a horizontal shaker for 30 min. After centrifugation (6000 rpm for 10 min), an aliquot of the supernatant (5 mL) was diluted with water to 25 mL and thoroughly mixed. The solution obtained was passed slowly through the Afla B immunoaffinity column, which was conditioned with 10 mL of water. After washing with 20 mL of water, the AFB_1 was eluted with 2 mL methanol. The methanol was evaporated using a water bath, and 100 μL of TFA was added to the residue from the sample extract (or AFB_1 standard). The solution was allowed to stand at room temperature for 15 min in the dark. A 400 μL amount of acetonitrile:water (1:9 v/v) solution was added to the AFB_1-TFA derivative solution. A 20 μL portion of sample or standard solution was then applied to the HPLC column. The HPLC analysis was carried out using an acetonitrile:methanol:water (8:27:65 v/v) mobile phase at a flow rate of 0.7 mL min^{-1}. The AFB_1-TFA derivative was detected using a fluorescence detector with the excitation and emission wavelengths set at 365 and 450 nm, respectively.

3. RESULTS AND DISCUSSION

The various ELISA parameters (such as dilution of MAb and Ab_2-AP) affecting the assay response were quantitatively investigated and optimized in PBS using standard solutions of AFB_1. The best conditions for carrying out the electrochemical indirect competition ELISA were as follows: 1 μg mL^{-1} of AFB_1-BSA conjugate, overnight incubation at 4°C; MAb dilution of 1:2000 (v/v) for 30 min incubation, while a dilution of 1:1000 (v/v) with an incubation time of 15 min was used for Ab_2-AP.

3.1 Cross Reactivity of MAb

The specificity of the assay was evaluated by examining the cross-reactivity of the MAb against aflatoxin congeners (AFB_1, AFB_2, AFG_1 and AFG_2—Fig. 1) as well as another known metabolite (AFM_1) and other mycotoxins (ochratoxin A), which might be found in the same food commodities.

The protocol used was similar to that used for AFB_1 assay, only substituting this latter with the interfering toxin mixed with a constant amount of diluted MAb (1:1000 v/v) and then 6 μL of this mixture was added to a SCPE coated with AFB_1-BSA conjugate. The following steps were analogous to those described earlier in the general procedure for the electrochemical ELISA protocol. Finally, cross-reactivity (Table 1) was calculated using the formula: $x/y \times 100$, where x is the amount of the AFB_1 and y is the amount of interfering mycotoxins required to produce 50% inhibition of the binding between MAb and AFB_1-BSA (Law and Biddlecombe 1996).

Table 1. Cross-reactivity % of MAb for aflatoxins obtained with immunosensors

Compound	% of response
AFB_1	100
AFB_2	21
AFG_1	112
AFG_2	8
AFM_1	8
Ochratoxin A[a]	—

[a]No inhibition of current response was observed for ochratoxin A.

The results obtained are reported in Table 1 and indicate that the MAb was relatively specific for AFB_1 and AFG_1. The AFB_2 cross-reacted to the extent of 21% relative to AFB_1, while the cross-reactions for both AFM_1 and AFG_2 were 8%. No cross-reaction was observed with ochratoxin A. It can be concluded that the antibody's affinity was mainly directed toward the dihydrodifuran moiety. The strong binding affinity toward dihydrodifuran ring was evident in the cross-reaction observed with AFG_1 and not with the other compounds having a modified dihyrodifuran moiety. For example the hydroxyl group on the dihydrodifuran ring in AFM_1 significantly reduced the MAb binding, giving only 8% cross-reaction relative to AFB_1. The cross-reaction for AFB_1 and AFB_2 suggested that the MAb was also exhibiting relatively weak affinity toward the cyclopentanone ring.

3.2 Stability of Immunosensor

The stability of coating reagents was evaluated using SPCEs coated with AFB_1-BSA conjugate, blocked, and stored at 4°C. A parallel investigation was done by treating the AFB_1-BSA conjugate–coated electrodes with 5 μL of ProClin 200 (Supelco) for 30 min at room temperature after the blocking step. The electrodes were then washed and stored at 4°C. The ProClin preservative is known to be a highly effective biocidal agent for inhibiting the growth of microorganisms in biological media. It is also compatible with most enzyme systems and does not inhibit antibody binding. Assays were performed periodically, over a one month period, using the assessed protocol.

As illustrated in Fig. 2, the results showed that the coated electrodes could be used for up to one month after their preparation. The maintenance of 100% of the activity indicated that the lifetime of the coated electrodes could be even longer; moreover, the ProClin preservative had no effect in improving the immunosensor stability over one month.

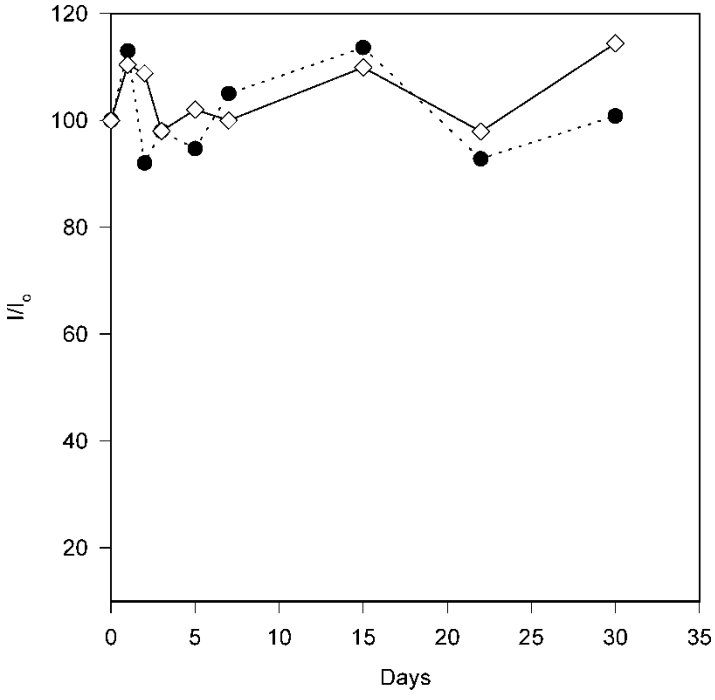

Figure 2. Stability of immunosensor, parallel investigation of electrodes treated with ProClin preservative (◇), and nontreated electrodes (●).

3.3 Comparison between Immunosensor and HPLC Results

The ELISA procedure requires water-soluble reagents for mixing the sample extract with the diluted MAb in PBS; however, aflatoxins are traditionally efficiently extracted with a mixture of water and polar organic solvent. Barley samples were spiked with 10, 50, 250, and 500 ng AFB_1 g^{-1} then the samples were extracted with 85% methanol.

Prior to evaluating the ELISA for AFB_1 on spiked barley samples, the effect of barley extract on assay performance was tested by adding known amounts of AFB_1 (0, 0.05, 0.25, 0.5, 1, 2, 5, 10, and 100 ng mL^{-1}) to an extract made from noninfected barley samples. For the electrochemical ELISA, the comparison of the standard curve in PBS:CH_3OH (60:40), in order to check the interference of methanol on the electrochemical measurement, with that in the extract of non-infected barley diluted two times with PBS (Fig. 3) demonstrated that there was a clear matrix effect. As the dilution of the extract is a commonly used strategy to reduce interference, a standard curve for AFB_1 was also prepared using noninfected barley extract diluted four times (1:3 v/v) with PBS. The comparison of these three curves indicated that there was still a detectable matrix effect even with the largest

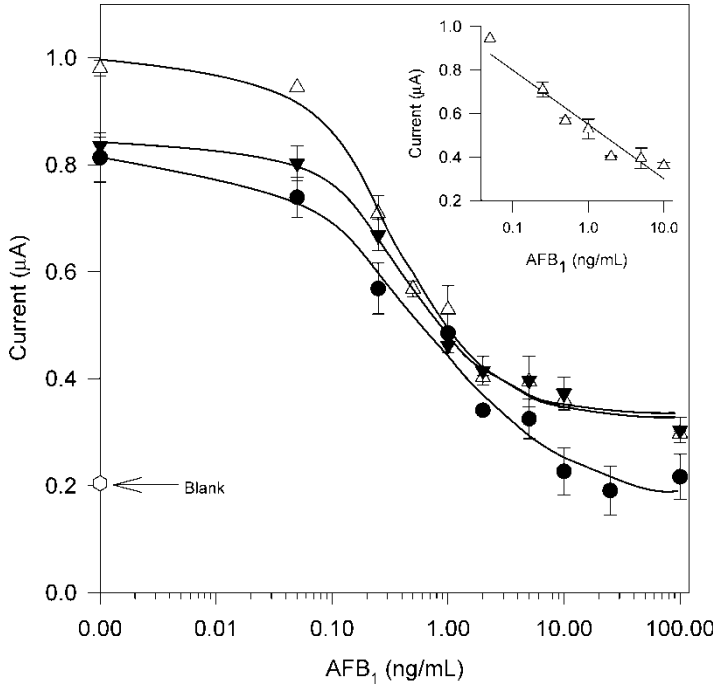

Figure 3. Effect of barley extract on standard curves of AFB_1 detected by electrochemical immunosensors. Standard curve prepared in noninfected barley extracts diluted two times (△) and four times (▼) with PBS. Standard curve of AFB_1 in PBS (●). The standard curve of AFB_1 using noninfected barley extract diluted two times with PBS (△), shows a LOD of 90 pg mL^{-1} and a working range between 0.1 and 10 ng mL^{-1}. The linear regression in the working range is: $f(X) = 0.40\ (\pm 0.12) - 0.32\ (\pm 0.10)X$, the correlation coefficient $r^2 = 0.94$ ($n = 4$).

dilution used. In addition, the background is similar for the standard curves prepared in both diluted extracts, while it is higher than that for the standard curve prepared in PBS. This background difference could indicate a non-specific absorption of interferents on the graphite of SPCE, an effect that is not affected by matrix dilution.

In any case, the results indicated that matrix effects had to be taken into consideration, so a calibration curve made in matrix extract should be carried out. As the two-fold dilution of extract allowed us to detect the half, double, and regulatory limit (2 ng g^{-1}), this dilution was used for the calibration and the validation of the assay parameters.

Using this calibration method, the LOD was found to be 90 pg mL^{-1}. Taking into consideration the sample extraction procedure, this limit corresponded to 360 ng AFB_1 per kg of barley. It should be noted that this value is about 5 times lower than the maximum residual limit regulated by the EC (2 ng g^{-1}). The linear range was 0.1–10 ng mL^{-1}.

Table 2. Precision (RSD%), accuracy (RE%), and recovery for AFB_1 in barley samples determined by electrochemical immunosensor and HPLC

Electrochemical immunosensor					HPLC			
AFB_1 added (ng g^{-1})	AFB_1 found (mean $\pm$ sd) (ng g^{-1})	RSD (%)	RE (%)	Recovery (%)	AFB_1 found (mean $\pm$ sd) (ng g^{-1})	RSD (%)	RE (%)	ELISA/HPLC (RE%)
1.0	1.1 ± 0.2	18	10	110	1.10 ± 0.08	7	10	0
2.0	2.3 ± 0.5	22	15	115	2.00 ± 0.09	4	0	15
4.0	4.1 ± 0.8	20	2	103	4.3 ± 0.3	7	8	-5

Each value is the mean of 8 samples (two per day for 4 days).

To study the recovery of AFB_1 during extraction, noninfected barley samples were spiked with AFB_1 at three levels (1, 2, and 4 ng g^{-1}). These samples were extracted, diluted two times in assay buffer, and quantified by use of the electrochemical immunosensor. The recovery of AFB_1 was calculated from the concentration of AFB_1 in the sample, obtained from the ELISA method, relative to the expected (spiked) value. The recovery was found to be in the range of 103%–115% (Table 2).

In order to evaluate the repeatability and accuracy of the method, two replicates of blank samples fortified with AFB_1 at concentrations of 1, 2, and 4 ng g^{-1} were prepared, extracted, and analyzed on each of four days for each concentration ($n = 24$, 8 for each level).

Precision was determined by calculating the relative standard deviation (RSD%) for the replicate measurements, accuracy (RE%) was calculated by assessing the agreement between the measured and the nominal concentration of the fortified samples.

Finally, results obtained using the electrochemical immunosensor were confirmed by analyzing the same samples using a validated HPLC-fluorescence method. These values are reported in Table 2 together with the accuracy of the electrochemical immunosensor vs. HPLC.

4. CONCLUSION

Sensitive immunosensors, using an indirect competitive ELISA format, have been used for the determination of AFB_1 in barley. This disposable immunosensor combines the high selectivity and simplicity of an immunoassay with high sensitivity, rapidity, and low cost of electrochemical measurements. The method also involved the application of a simple extraction procedure of AFB_1 from barley. The immunosensor (coated SPCE) can be stored for up one month to be ready for in situ determination. The immunosensor exhibited a linearity range that is comparable to that for conventional methods (HPLC) and had also a detection limit suitable for on-site monitoring.

The simplicity of the method should also make it suitable for use in the detection of many other toxins and environmental pollutants.

The MAb used in this study has the same specificity for both AFB_1 and AFG_1, while our future study will use this antibody to prepare an electrochemical immunosensor for total aflatoxins.

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