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Research Paper

Development of an electrochemical immunosensor to determine zearalenone in maize using carbon screen printed electrodes modified with multi-walled carbon nanotubes/polyethyleneimine dispersions

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ABSTRACT

An electrochemical immunosensor is developed to determine zearalenone (ZEA) mycotoxin in maize samples. It is based on the use of a composite, which was prepared from anti-ZEA poly-clonal antibody bonded to gold nanoparticles immobilized on multi-walled carbon nanotubes/polyethyleneimine dispersions. Carbon screen printed-electrodes (CSPE) were used in the electrochemical transduction stage. The immunoassay is based on a direct competitive assay between ZEA in maize samples and ZEA labeled with horseradish peroxidase enzyme (ZEA-HRP). ZEA determination was performed by amperometry, using an applied potential of −0.3 V. The H_2O_2, which was not consumed by HRP, was reduced at the electrochemical immunosensor surface. Thus, the reduction current was proportional to the amount of ZEA present in samples. All experimental variables involved in the construction of the electrochemical immunosensor were optimized. The linear concentration range is from 1×10^{-4} to 1×10^{-1} ng mL^{-1}. The limit of detection and SC_{50} were 0.15 pg mL^{-1} and 2 pg mL^{-1}, respectively. In addition, an acceptable accuracy, with a percentual coefficient of variation (%CV) less than 20%, and recovery percentages close to 105% were found. The electrochemical immunosensor has great advantages such as no pre-treatment of the sample is required, the sample volume is of 20 μL, the experiments require short times and a very low limit of detection is obtained. Results obtained with this electrochemical immunosensor were compared with those determined by HPLC-fluorescence detection, obtaining a very good correlation. The proposed immunosensor is a valuable alternative tool to determine ZEA in maize samples.

1. Introduction

Zearalenone (ZEA) is a mycotoxin produced by different filamentous fungi such as *Fusarium graminearum schwabe*, *Fusarium culmorum* and *Fusarium crookwellense* [1]. ZEA is found in cereals, mainly maize, and manufactured foods. It is one of the most widely distributed mycotoxins in the world, making ZEA study of great interest [2–34]. ZEA contamination can occur before and/or after harvest, and during grain processing. However, the main contamination whit ZEA occurs during storage, where moisture conditions allow the production of ZEA at high levels [5]. ZEA is a non-highly toxic mycotoxin; however, there are suspicions that it may be carcinogenic. Its lethal dose is between 4,000 and 10,000 mg kg^{-1} of corporal weight [5]. In addition, it is known that chronic exposure to ZEA can produce reproductive disorders, estrogenism, abortions and sterility [3,4,6,7], and hepatotoxic, immunotoxic and hematotoxic effects [8,9].

In order to prevent and improve the public health, national and international agencies adopted regulations for the control of ZEA contamination. Thus, the Food and Agriculture Organization of the United Nations (FAO), provides worldwide regulatory methods [10]. There are three official methods to determine ZEA in maize samples. They were established by the Association of Official Analytical Chemists (AOAC), and are based on thin layer chromatography (TLC) (976.22), HPLC with fluorescence detection (985.18), and a colorimetric immunoassay (994.01). In Argentina, the allowed maximum level of ZEA in maize is 200 μg kg^{-1} [11].

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The ZEA detection and quantification are mainly based on chromatographic techniques such as TLC [12,13], and HPLC [14,15] combined with mass spectrometry, uv-visible and/or fluorescence spectroscopies. Immunoaffinity columns (IC) are used if a pretreatment of samples is necessary [15,18,19]. In addition, ZEA quantification was also performed by reflectance lateral flow [21]. However, all these techniques have several disadvantages such as a high cost of equipment, and an excessive uses of solvents [22,23]. On the other hand, there are also immunological methods to determine ZEA, which have the advantage of being selective, accurate, sensitive, and with low limits of detections (LOD) [24]. Enzyme linked immunosorbent assay (ELISA) is the most used methodology. Thus, 994.01 AOAC method was developed to determine ZEA in maize, wheat, and feed samples [13]. In addition, ZEA was determined in maize, wheat, and milk samples by ELISA [13,14,25–28]. On the other hand, it is known that electroanalytical techniques are very sensitive [29]. Thus, ZEA electrochemical determination was developed, reaching low LOD [30,31].

Enzyme immunoassays with electrochemical detection, which combines the selectivity of antibodies with the sensitivity of electrochemical techniques, are particularly suitable for a rapid and direct detection of antibody–antigen interactions. The immobilization of antibodies at solid surfaces is the key stage, which determines the stability, reproducibility and sensibility of the measured signal [32]. Thus, the use of multi-walled carbon nanotubes (MWCNT) is an important option. It has been demonstrated that electrodes modified with MWCNT show an increment in the linear analytical response and lower LOD [33]. It is necessary to work with MWCNT homogeneous dispersions for obtaining reproducible results. Therefore, it is necessary to use soluble or hydrophilic polymers to promote homogeneous dispersions if aqueous solutions are used [34]. Polyethyleneimine (PEI) is a widely used polymer. It does not only allow to obtain homogeneous dispersions of MWCNT, but also allows to retein the biological activity of biomolecules such as enzymes, proteins and antibodies. PEI is a polycationic polymer, capable to interact with the carboxylate groups present on the proteins [35,36].

On the other hand, gold nanoparticles (AuNPs) have the ability to adsorb biological molecules without loss of their activity, and to promote electron transfer between electroactive molecules and electrodes [37]. In additions, AuNPs favor an adequate distribution and orientation of antibodies at the electrode surface [38].

In this paper, we report the development of an electrochemical immunosensor (EI) to quantify ZEA in maize whit a minimum pretreatment of samples. The EI was constructed by a layer-by-layer assembled at carbon screen printed-electrodes (CSPE). Therefore, MWCNT/PEI dispersions were deposited at the SPCE surface. Then, the modified CSPE was submerged in solution of AuNPs, which were adsorbed on amine groups present in the polymer. Finally, the anti-ZEA poly-clonal antibody (ZEA-pAb) was added. The ZEA determination was based on a direct competitive immunoassay. ZEA concentration was determined by amperometry through the H_2O_2 reduction currents, which was not consumed by the HRP conjugated to ZEA (ZEA-HRP) (Scheme 1). These currents were proportional to ZEA concentration in maize.

2. Experimental

2.1. Reagents

ZEA, ZEA-pAb, glycine, bovine serum albumin (BSA), HRP (type VI), N-(3-dimethylaminopropyl)-N-(ethylcarbodiimide)-hydrochloride) (EDAC), *ortho*-phenylenediamine, branched polyethyleneimine (PEI 25 K), MW of 25000 (25 K) and polyethyleneimine solution (PEI 750 K), MW of 750000 (750 K), with a purity grade of 99% were purchased from Sigma-Aldrich. ZEA was dissolved in acetonitrile (ACN) at a final concentration of 2.5 mg mL^{-1} and stored in the refrigerator at 4 °C.

The following buffer solutions were prepared from their salts (Merck, p.a.): 1×10^{-2} M phosphate buffer solutions, 0.137 M NaCl and 2.70×10^{-3} M KCl, pH 7.5 (PBS); 5×10^{-2} M citrate + 5×10^{-2} M phosphate buffer solution, pH 5.00 (CBS). ZEA dilutions were performed in 1 mM phosphate buffer solutions (PBS), pH 7.50. The ZEA-pAb solutions were prepared in 1 mM PBS at final concentrations as they are detailed for each experiment.

Pyridine, benzene, ethyl acetate, N,N'-dimethylformamide, methanol (MeOH), water and acetonitrile were Sintorgan, HPLC grade. MWCNT, 95% purity, 20 nm inner diameter and 1–5 μm length, were purchased from Nano-Lab.

Maize samples, sterilized by gamma irradiation (1200 krads), was provided by Dr. Sofía Chulze, Departamento de Microbiología e Immunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto.

2.2. Instrumentation

The CSPE (PalmSens BV, The Netherlands) was based on carbon working and counter electrodes, and a Ag/AgCl (0.137 M NaCl + 2.70×10^{-3} M KCl) reference electrode which is formed by contact between silver electrode and chloride ions of PBS. Before use, CSPE surface was electrochemically pre-treated by electro-oxidation in alkaline medium [39]. Amperometric and cyclic voltammetry (CV) measurements were performed with an Epsilon potentiostat (BASi Bioanalytical System, USA) coupled to a PC with electrochemical software incorporated.

2.3. Methods

2.3.1. Synthesis of HRP-labeled zearalenone (ZEA-HRP)

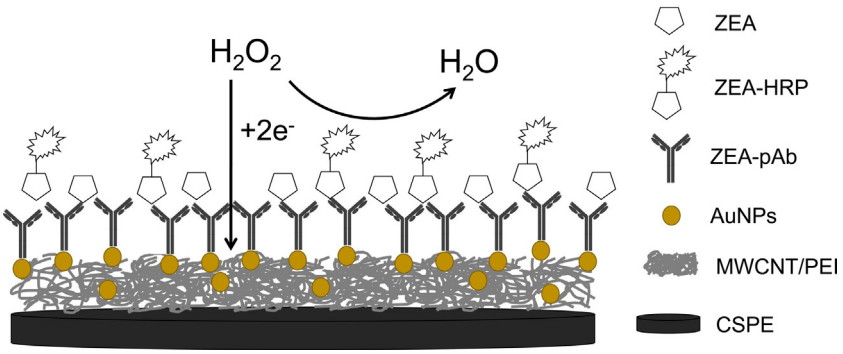
The ZEA-HRP conjugate was synthesized by the method developed by Thouvenot and Morfin [40], with some modifications. Thus, 0.5 mL of ZEA solution in ACN (2 mg mL^{-1}) was evaporated with argon, and then ZEA was solubilized in 200 μL of pyridine. Then, 3 mg of glycine was added to the ZEA solution, and allowed to react at room temperature with continuous stirring during 24 h. Then, the pH of the solution was adjusted to 8 by the addition of a 0.1 M NaOH aqueous solution. Three extractions were performed with benzene. The aqueous phase was acidified with HCl (pH 3), where a ZEA-glycine precipitate was observed. The solid was extracted with ethyl acetate, and then the organic solvent was evaporated. ZEA-glycine was solubilized in 100 μL *N,N'*-dimethylformamide.

On the other hand, a HRP solution (1 mg mL^{-1}) was prepared in PBS. The HRP and ZEA-glycine solutions were mixed with a HRP:ZEA-glycine ratio of 60:1. Then, 30 mg of EDAC was added, and the pH was adjusted to 6 with HCl. The reaction was performed at room temperature with continuous stirring during 24 h. Finally, 3 mg of EDAC was added and allowed reacting during 24 h. The product was purified by dialysis in PBS during 96 h using a dialysis membrane (10 kDa of cutoff). The ZEA-HRP conjugate was confirmed by uv-visible spectroscopy, and the ELISA test using sensitized wells with ZEA-pAb.

2.3.2. Preparation of multi-walled carbon nanotubes/polyethyleneimine dispersions

The preparation of (MWCNT)/PEI dispersions was studied and optimized varying the concentration of MWCNT and PEI, and the sonication time.

Thus, aqueous solutions of PEI 25 K and PEI 750 K at a concentration of 14 mg mL^{-1} each of them were prepared the previous day. A complete solubilization of the polymers was achieved. The optimum dispersion was that which showed the highest homo-



Scheme 1. Schematic representation of the electrochemical immunosensor to determine ZEA using the ZEA-pAb/AuNPs/MWCNT/PEI/CSPE.

geneity and stability. This was obtained by adding MWCNT to a solution of both polymers. Final concentrations were 1 mg mL^{-1} of MWCNT, 0.67 mg mL^{-1} of PEI 25 K and 2.30 mg mL^{-1} of PEI 750 K. Three cycles of five min each one, with manual agitation between each cycle were the optimal conditions for sonication. Resulting dispersions were stored in darkness at room temperature and were stable by two months. Then, they started to show aggregates.

2.3.3. Preparation of gold nanoparticles

The AuNPs were prepared following known methodologies [41,42]. Thus, 74 mL of 0.1 mg mL^{-1} $HAuCl_4$ solution was heated to boiling during fifteen min with continuous stirring. Then, 1.85 mL of 1% w/v aqueous sodium citrate solution was added. The mixture was boiled until the appearance of a red color, and then allowed to cool under continuous stirring. The size of AuNPs were estimated by uv-visible spectroscopy, according to the procedure described by Haiss et al. [43]. The diameter of AuNPs was of 31 nm. The AuNPs were stored in a dark glass bottle at 4 °C for further use.

2.3.4. Construction of the electrochemical immunosensor and description of immunoassays

The EI was constructed by modifications of the CSPE. Thus, 4 μL of MWCNT/PEI dispersions were deposited onto the CSPE surface and dried in an oven at 40 °C during 40 min, obtaining the MWCNT/PEI/CSPE. Then, 4 μL of the AuNPs dispersion was added on MWCNT/PEI/CSPE, and the modified electrode was dried in an oven at 40 °C during 40 min, obtaining the AuNPs/MWCNT/PEI/CSPE. Finally, 5 μL of ZEA-pAb solution (1:80 dilution) was added at the electrode surface and incubated during 24 h in a wet chamber to 4 °C. Thus, the ZEA-pAb/AuNPs/MWCNT/PEI/CSPE composite electrode was obtained.

A direct competitive immunoassay was used to determine ZEA using the EI (Scheme 1) [44]. ZEA and the ZEA-HRP compete for a limited amount of ZEA-pAb presents in ZEA-pAb/AuNPs/MWCNT/PEI/CSPE composite electrode. Immunoassays were performed as described below. First, 5 μL of 6% BSA in PBS was added on the immunosensor surface and incubated at 32 °C during 1 h in a wet chamber, to avoid non-specific adsorptions [45]. After incubation, the EI was washed with PBS to eliminate non-adsorbed BSA. Then, 2.5 μL of ZEA and 2.5 μL of ZEA-HRP solutions were added on the immunosensor surface and incubated during 1 h at 32 °C in wet chamber. Then, the immunosensor surface was washed with PBS, and 17 μL of CBS, pH 5.00 was added, at an applied potential of −0.3 V during 200 s. After 100 s, 3 μL of 6 μM H_2O_2 solution was added, and a steady state current (I_{ss}) was obtained, which was measured at a time of 100 s. It is well known that HRP reduces H_2O_2 to H_2O in first state of its catalytic cycle. The H_2O_2, which is not consumed by the HRP, was reduced at EI surface. I_{ss} obtained are inversely proportional to the amount of ZEA-HRP at the electrode surface, and proportional to the amount of ZEA in maize samples. I_{ss} obtained were normalized respect to the electrode electroactive area, obtaining steady state current densities (J_{ss}). The electrode electroactive area was determined by chronocoulometry using $K_3[Fe(CN)_6]$ [46].

2.3.5. Sample preparation

25 g of sterilized maize sample (free ZEA) was triturated and dried at 40 °C during 24 h. Then, 1.2 g of triturated sample was spiked with 1.2 μL of 1.17×10^{-3} M ZEA and mixed during 10 min. The final concentration of ZEA was 372 μg kg^{-1}. Then, 12 mL of a MeOH/ACN (50:50) mixture was added and mixed at 250 rpm. The solution was filtered and collected obtaining the ZEA final concentration of 47.07 ng mL^{-1}. Then, 1 mL of collected solution was evaporated by bubbling argon. Before performing EI measurements, the solid was solubilized in 1 mL PBS. On the other hand, extracts of maize samples in MeOH/ACN were treated to perform HPLC measurements using a procedure similar to that described previously by Urraca et al. [16], with some modifications. ZEA presents in collected solutions were purified by passing the extract through a column of reverse phase Strata-X 33 μ, using MeOH:ACN (90:10) as mobile phase. Collected extracts were introduced in the HPLC chromatograph.

A negative control (without ZEA added) was prepared as previously described.

3. Results and discussion

3.1. H_2O_2 reduction on AuNPs/MWCNT/PEI/CSPE electrode

Fig. 1 shows cyclic voltammograms recorded for the H_2O_2 reduction in CBS. A reduction peak is observed at a potential of about − 0.26 V during the cathodic sweep, which is attributed to the reduction of H_2O_2 to H_2O. The cathodic peak current ($I_{p,c}$) was proportional to the bulk H_2O_2 concentration, $c^*_{H_2O_2}$ up to $c^*_{H_2O_2} \leq 150$ μM. At $c^*_{H_2O_2} > 300$ μM, the current reaches constant values (inset Fig. 1). Based on these results, a potential of −0.3 V was chosen as the applied potential to perform amperometric measurements.

3.2. Determination of ZEA-pAb and ZEA-HRP concentrations

The optimization of ZEA-pAb and ZEA-HRP concentrations was performed by a colorimetric assay (ELISA), and the EI. Two-fold serial dilutions of ZEA-pAb starting at 1:20 dilution were applied to the wells of A–H for the ELISA experiments. After incubating during 24 h at 4 °C the wells were washed with PBS and blocked with 3% BSA in PBS (w/v). Then, the wells were washed with PBST (PBS containing 0.05% Tween 20). Two-fold serial dilutions of ZEA-HRP starting at 1:20 were added to columns 1–11 and incubated during 1 h at room temperature. Wells were washed with

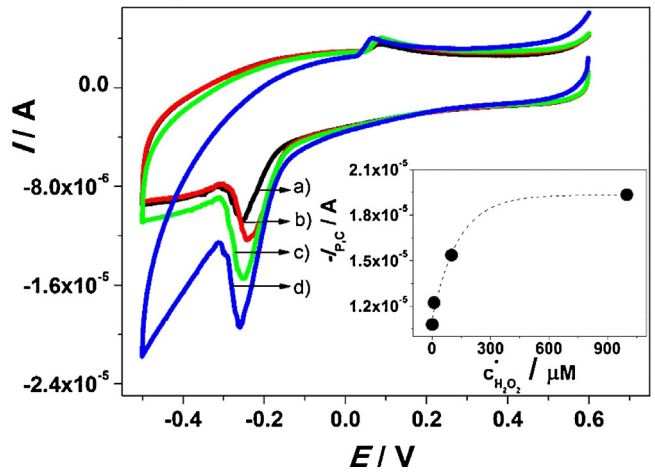


Fig. 1. Cyclic voltammograms recorded at AuNPs/MWCNT/PEI/CSPE in CBS. $c^*_{H_2}{}_{O_2}$: (a, —) 1; (b, —) 10; (c, —) 100 and (d, —) 1000 µM. Scan rate = 0.025 V s^{-1}. Inset: Plot of *I*p,c as a function of $c^*_{H_2}{}_{O_2}$. Each point is the average of three replicated measurements. The reference electrode used was Ag/AgCl (0.137 M NaCl + 2.70x10^{-3} M KCl).

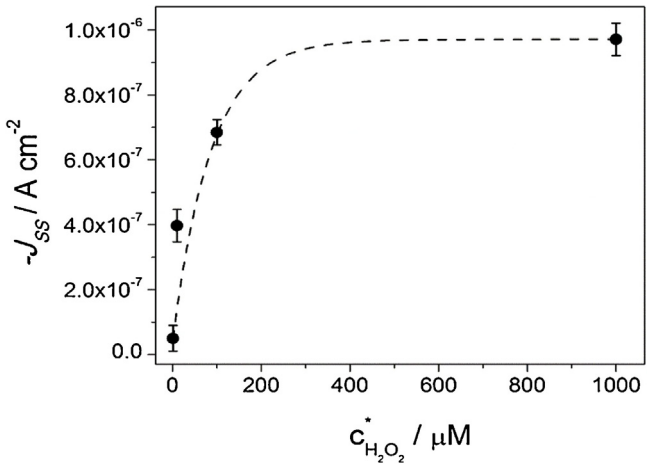


Fig. 2. Dependence of *J*ss with $c^*_{H_2}{}_{O_2}$. Dilution factors of ZEA-pAb and ZEA-HRP were 1:80 and 1:160, respectively. Cell volume = 35 µL. Applied potential = −0.3 V. Each point is the average of three replicated measurements.

Table 1
Density current values obtained for different factor dilution of ZEA-pAb and ZEA concentrations.

ZEA concentrations/ng mL^{-1}	Dilution factor of ZEA-pAb		
	1:80	1:160	1:320
	Density current/µA cm^{-2}		
10	0.68 ± 0.07	0.58 ± 0.07	0.38 ± 0.06
50	0.50 ± 0.05	0.46 ± 0.07	0.27 ± 0.03
100	0.20 ± 0.04	0.25 ± 0.05	0.20 ± 0.04

PBST. Then, 100 µL of the HRP substrate (2.5 mg mL^{-1} of *ortho*-phenylenediamine and 0.1 mL of 1% H_2O_2 in a total volume of 2.5 mL of CBS) was dispensed into each well. The enzymatic reaction was stopped after 15–20 min by adding 50 µL of 2 M H_2SO, and the absorbance was measured at 450 nm (corrected at 600 nm). The ZEA-pAb dilutions of 1:80; 1:160; and 1:320; and ZEA-HRP dilutions of 1:80 and 1:160 showed absorbance values of 50% of maximum absorbance (data not shown). The ZEA-pAb and ZEA-HRP dilutions above mentioned were tested for three ZEA concentrations (c^*_{ZEA}) (10, 50 and 100 ng mL^{-1}) using the EI, following the methodology described in Section 2.3.4. Therefore, when a ZEA-HRP dilution of 1:80 was used, a small difference in the I_{ss} was found for the three ZEA concentrations. On the other hand, the largest difference in I_{ss} was found when the ZEA-pAb and ZEA-HRP dilutions were 1:80 and 1:160, respectively (see Table 1). Each point is the average of three replicated measurements.

3.3. Study of the effects of H_2O_2 concentration and cell volume on the electrochemical responses

Different $c^*_{H_2O_2}$ were studied in the range from 1 to 1000 µM. At a constant c^*_{ZEA}, it was found an increase of current as the $c^*_{H_2O_2}$ was increased (Fig. 2). On the other hand, as the $c^*_{H_2O_2}$ increased, it was not found a marked difference between the magnitudes of I_{ss} for the different c^*_{ZEA}, which can be explained considering that there is a great excess of H_2O_2 respect to HRP. Thus, a compromise between the magnitude of I_{ss} and the possibility of differentiating very close mycotoxin concentrations was necessary. Based on these results, $ac^*_{H_2O_2}$ = 6 µM was chosen. In addition, the cell volume on CSPE was also studied. I_{ss} increased as the cell volume decreased (Fig. 3). The current values for different cell volume are show in Table 2. Therefore, a cell volume of 20 µL was chosen. In Figs. 2 and 3, each point is the average of three replicated measurements.

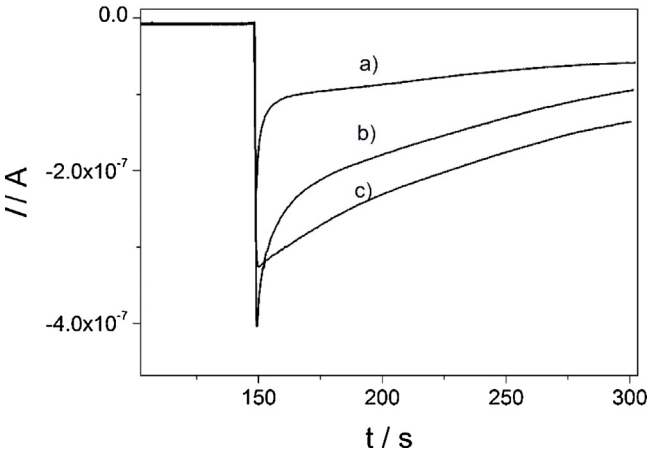


Fig. 3. Amperograms obtained for the following cell volumes: a) 30 µL; b) 25 µL and c) 20 µL. $c^*_{H_2}{}_{O_2}$ = 6 µM. Dilution factors of ZEA-pAb and ZEA-HRP were 1:80 and 1:160; respectively. Applied potential = −0.3 V.

Table 2
Current density obtained by different cell volume.

Cell volume/µL	-*J*ss/µA cm^{-2}
20	0.65 ± 0.01
25	0.48 ± 0.05
30	0.25 ± 0.04

3.4. Calibration curve for ZEA using the electrochemical immunosensor

A dose-response titration was carried out in the ZEA concentration range from 1 × 10^{-5} to 1 × 10^2 ng mL^{-1} using the EI (Fig. 4). The calibration curve was constructed using sterile extracts of a maize sample spiked with ZEA known amounts. The calibration curve was plotted as binding grade (B/B_0) vs.c^*_{ZEA}, using a logarithmic scale for ZEA concentration, where B is the corresponding J_{ss}, and B_0 is the J^0_{ss}

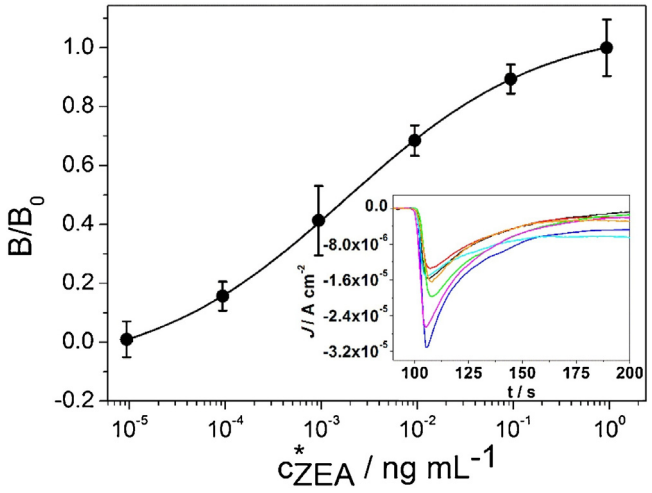


Fig. 4. Calibration curve obtained with the EI. Curve parameters for the competitive assay were as follows: SC_{50} = 2 pg mL^{-1}, LOD = 0.15 pg mL^{-1}, Hill Slope: 0.424, r = 0.9999 and χ^2 = 0.013. Each point is the average of four replicated measurements. Inset of the figure shows the amperograms recorded for different ZEA concentrations corresponding to the calibration curve. ZEA concentrations of inset curve: (—) blank, (—) 1x10^{-5}, (—) 1x10^{-4}, (—) 1x10^{-3}, (—) 1x10^{-2}, (—) 1x10^{-1} and (—) 1 ng mL^{-1}.

obtained at a ZEA saturation concentration. The calibration curve was fitted using the Eq. (1) [47].

$$y = A_1 + \frac{(A_2 - A_1)}{(1 + 10^{((\log SC_{50} - \log c^*_{ZEA})(\text{Hill Slope}))})} \quad (1)$$

where A_1 and A_2 are the maximum and minimum B/B_0 values, respectively, SC_{50} is the ZEA concentration which produces 50% of saturation signal and is a measure of the sensitivity of immunoassay, and Hill Slope is the slope at the midpoint of the sigmoid curve. The LOD of the assay was calculated as the concentration of ZEA that produces an increase in signal equal to three times the standard deviation of the blank [48]. The LOD was 0.15 pg mL^{-1}, the limit of quantification (LOQ) was 0.58 pg mL^{-1} for a 10:1 signal-to-noise ratio, and the SC_{50} = 2 pg mL^{-1}. The reproducibility of the EI was checked using ZEA standard solutions of 1×10^{-3} and 0.1 ng mL^{-1} in sterile extracts of the maize sample. Inter-assay parameters were checked by measuring each sample for three consecutive days. The EI showed a goodreproducibility as it is shown in Table 3.

Table 4 shows the LOD obtained for ZEA determination in samples of different nature using different methodologies. As it can be observed, the EI proposed by us shows the highest sensitivity. On the other hand, the pre-treatment of samples was minimal, which is a great advantage over the other methodologies. The EI was stable for 4 days when this was stored in a wet chamber at 4 °C. From day 5, the signal drops by 22%. At day 8, the signal is 45% of the signal from the electrochemical immunosensor recently prepared. 4950515253

Table 3
Reproducibility of electrochemical immunosensor for ZEA determination in maize sample.

ZEA concentration/ ng mL^{-1}	Inter-assay	
	Mean	%VC
1×10^{-3}	1.02×10^{-3}	20.6
0.1	9.7×10^{-2}	5.5

3.5. Analytical determination of ZEA in maize samples

ZEA concentration in maize samples was determined using EI and HPLC coupled to a fluorescence detector. The maize samples were prepared as described in Section 2.3.5.

3.5.1. Determination of ZEA by HPLC-fluorescence detection and electrochemical immunosensor

The results obtained with the EI were compared with those determined by HPLC-fluorescence detection using a methodology similar to that proposed by Urraca et al. [16]. Therefore, a reverse phase column C18 was used, and the mobile phase was MeOH:ACN (90:10). The method requires a previous stage of extraction, which was performed using a reverse phase column (Strata-X 33 μ). The excitation and emission wavelengths were 271 nm and 452 nm, respectively. The flow rate was 0.8 mL min^{-1}, and the ZEA chromatographic peak appears at 4.05 min. To minimize the matrix effect, the standard addition method was used to determine ZEA in maize samples by HPLC-fluorescence detection method. A standard addition plot (peak area vs.c^*_{ZEA}) was performed. A linear relationship was found in the concentration range from 5 to 500 ng mL^{-1}, which can be expressed by Eq. (2):

$$A[AU] = (1.18 \pm 0.08)10^3[AU] + (2.26 \pm 0.06)10^2 \frac{[AU]}{\text{ng mL}^{-1}} x c^*_{ZEA}[\text{ng mL}^{-1}] \quad (2)$$

with a linear correlation coefficient, r = 0.9987. The LOD (for a 3:1 signal-to-noise ratio), and the LOQ obtained using the HPLC-fluorescence detection method were 3.11 and 12.09 ng mL^{-1}, respectively.

Table 4
Different methods proposed to quantify ZEA.

Sample	LOD	Analytic Technique	Year	Reference
Maize	50 μg kg^{-1}	HPLC (AOAC 985.18) with fluorescence detection	1985	[14]
Maize, wheat and feeds	800 μg kg^{-1}	ELISA (AOAC 994.01) with UV–vis photometric detection	2002	[13]
Maize	3 μg kg^{-1}	Immunoaffinity column coupled to/HPLC with fluorescence detection	1998	[15]
Maize	6 μg kg^{-1}	HPLC with fluorescence detection	2004	[16]
Maize	0.9 μg kg^{-1}	Immunoaffinity column coupled to/HPLC coupled with mass spectroscopy	2005	[18]
Maize	30 μg kg^{-1}	Electroanalytical methods/Square wave volammetry	2005	[30]
Bread	0.4 μg kg^{-1}	HPLC coupled with mass spectroscopy	2005	[49]
Urine	1.4 ng mL^{-1}	LC/Amperometry	2008	[31]
Maize	31.56 μg kg^{-1}	Immunoaffinity column coupled to HPLC coupled fluorescence detection	2008	[19]
Cereals for breakfast	0.12 ng mL^{-1}	Electrochemical immunoassay	2009	[50]
Maize	0.77 μg kg^{-1}	Electrochemical immunoassay	2010	[51]
Feed	0.41 μg kg^{-1}	Electrochemical immunoassay	2011	[52]
Maize	5 μg kg^{-1}	HPLC coupled mass spectroscopy	2012	[20]
Buffer solution	3 pg mL^{-1}	Fluorescence Immunoassay	2013	[28]
Maize	15 μg kg^{-1}	Phage-based dot-immunoassay with UV–vis spectroscopy detection	2014	[26]
Milk	1.7 pg mL^{-1}	Electrochemical immunoassay	2014	[53]
Maize	0.15 pg mL^{-1}	Electrochemical immunoassay	This work	

Table 5
Recovery percentages to determine ZEA in maize samples using the EI and HPLC-Fluorescence.

ZEA concentration in maize sample	ZEA concentration determined	
47.05 ng mL^{-1}	HPLC-Fluorescence	Electrochemical Immunosensor
	47.23 ng mL^{-1}	49.22 ng mL^{-1}
	%R	
	100.4	104.6

A sterile maize sample was spiked with ZEA at a final concentration of 372 μg kg^{-1} and was treated as described in Section 2.3.5. The ZEA concentration in the extract was 47.05 ng mL^{-1}. Then, ZEA concentration was determined by both the EI and HPLC-fluorescence. Results are shown in Table 5. Very good recovery percentages (%R) were obtained. Thus, the ZEA concentration in the spiked maize sample obtained by both methodologies are in a satisfactory good agreement, indicating the very good performance of the EI. These results demonstrate that the EI has a high potential to determine ZEA in maize samples with a minimum pre-treatment of samples.

4. Conclusions

We have developed an electrochemical immunosensor, which is based on the use of a composite material obtained by anti-ZEA polyclonal antibody bonded to gold nanoparticles, which was immobilized on multi-walled carbon nanotubes/polyethyleneimines dispersions at carbon screen printed electrodes.

This electrochemical immunosensor allowed determining ZEA at trace levels in maize samples. All parameters of immune-affinity reaction, and electrochemical measurements were optimized. The immunosensor showed a very good analytical performance in terms of an excellent limit of detection (0.15 pg mL^{-1}) and sensitivity (SC_{50} = 2 pg mL^{-1}), good reproducibility and an acceptable accuracy. On the other hand, the immunosensor has several advantages over other methods to determine ZEA in maize, such as direct measurement with a minimum pre-treatment of the sample, and the use of small volumes (harmful solvents are avoided). The comparison between results obtained with the electrochemical immunosensor and those of HPLC-fluorescence detection chromatography demonstrates that the electrochemical immunosensor can be a reliable tool to determine ZEA. All these features make this device a very good alternative to quantify ZEA in maize samples.

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References

[1] J.M. Soriano del Castillo, Micotoxinas En Alimentos, Ediciones Díaz de Santos, España, 2007 (Director-Coordinador).
[2] Some Naturally Occurring Substances, Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 56, International Agency for Research on Cancer (IARC), UK, 1993.
[3] A. Bianchini, L.B. Bullerman, in: C.A. Batt, M.L. Tortorello (Eds.), Mycotoxins: Classification, Encyclopedia of Food Microbiology, vol. 2, Academic Press Oxford, 2014, pp. 854–861.
[4] A. Waśkiewicz, in: C.A. Batt, M.L. Tortorello (Eds.), Mycotoxins Natural Occurrence of Mycotoxins in Food, Encyclopedia of Food Microbiology, vol. 2, Academic Press, 2014, pp. 880–886.
[5] M. Weidenbörner, Encyclopedia of Food Mycotoxins, Springer, Berlin, 2001.
[6] Some Food Additives, Feed Additives and Naturally Occurring Substances, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 31, International Agency for Research on Cancer (IARC), France, 1983.
[7] S. Pepeljnjak, M. Šegvć, An Overview of Mycotoxins and Toxigenic Fungi in Croatia, An Overview on Toxigenic Fungi and Mycotoxins in Europe, in: A. Logrieco, A. Visconti (Eds.), Springer, The Netherlands, 2004, pp. 33–50.
[8] S. Bräse, F. Gläser, C.S. Kramer, S. Lindner, A.M. Linsenmeier, K.S. Masters, A.C. Meister, B.M. Ruff, S. Zhong, The Chemistry of Mycotoxins, Progress in the Chemistry of Organic Natural Products, vol. 97, Springer, 2013, pp. 97.
[9] J. Gil-Serna, C. Vázquez, M.T. González-Jaén, B. Patiño, Mycotoxins: Toxicology, Encyclopedia of Food Microbiology, in: C.A. Batt, M.L. Tortorello (Eds.), Academic Press Oxford, 2017.
[10] Worldwide regulations for mycotoxins in food and feedin 2003, FAO Food and Nutrition Paper, Food and Agriculture Organization of Unites Nations (FAO), Vol. 81, 2003, http://www.fao.org/publications/card/en/c/3c32b78f-9fc9-5b78-8e43-0a82a7bd772b/.
[11] Protocolo de Calidad para la Harina de Maíz o Sémola de Maíz de Cocción Rápida para Preparar Polenta, Res. SAGPyA N° 302/2007, Ministerio de Agroindustria, Secretaría de Agregado de Valor, Subsecretaría de Alimentos y Bebidas, Argentina, 2007.
[12] O.L. Shotwell, M.L. Goulden, G.A. Bennett, Determination of zearalenone in corn: collaborative study, J. Assoc. Off. Anal. Chem. 59 (1976) 666–670.
[13] J. Gilbert, E. Anklam, Validation of analytical methods for determining mycotoxins in foodstuffs, Trends Anal. Chem. 21 (2002) 468–486.
[14] G.A. Bennett, O.L. Shotwell, W.F. Kwolek, Liquid chromatographic determination of alpha-zearalenol and zearalenone in corn: collaborative study, J. Assoc. Off. Anal. Chem. 68 (1985) 958–961.
[15] A. Visconti, M. Pascale, Determination of zearalenone in corn by means of immunoaffinity clean-up and high-performance liquid chromatography with fluorescence detection, J. Chromatogr. A 815 (1998) 133–140.
[16] J.L. Urraca, M.D. Marazuela, M.C. Moreno-Bondi, Analysis for zearalenone and α-zearalenol in cereals and swine feed using accelerated solvent extraction and liquid chromatography with fluorescence detection, Anal. Chim. Acta 524 (2004) 175–183.
[17] S.J. MacDonald, S. Anderson, P. Brereton, R. Wood, A. Damant, Determination of zearalenone in barley, maize and wheat flour, polenta, and maize-based baby food by immunoaffinity column cleanup with liquid chromatography: interlaboratory study, J. AOAC Int. 88 (2005) 1733–1740.
[18] F. Berthiller, R. Schuhmacher, G. Buttinger, R. Krska, Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high performance liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 1062 (2005) 209–216.
[19] M. Marques, H.M. Martins, Comparison of the validation of two analytical methods for fungal toxins according to RELACRE and the European Decision 2002/657/EC, Rev. Port. Cienc. Vet. 103 (2008) 83–89.
[20] M. De Boevre, J.D. Di Mavungu, P. Maene, K. Audenaert, D. Deforce, G. Haesaert, M. Eeckhout, A. Callebaut, F. Berthiller, C. Van Peteghem, S. De Saeger, Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone T-2-toxin and some masked metabolites in different cereals and cereal-derived food, Food Addit. Contam. Part A 29 (2012) 819–835.
[21] J.S. Steven, T. Mark, Lateral Flow Quantitative Method for the Detection of Mycotoxins, Food Contaminants: Mycotoxins and Food Allergens, in: D.P. Siantar, M.W. Trucksess, P.M. Scott, E.M. Herman (Eds.), ACS Symposium Series, American Chemical Society Washington, 2008, pp. 314–319, 1001.
[22] A. Visconti, M. Pascale, G. Centonze, Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography, J. Chromatogr. A 864 (1999) 89–101.
[23] M. Solfrizzo, G. Panzarini, A. Visconti, Immunochemical Assays for Direct Sulfonamide Antibiotic Detection in Milk and Hair Samples Using Antibody Derivatized Magnetic Nanoparticles, J. Agric. Food Chem. 56 (2008) 11081–11086.
[24] D. Flajs, A.M. Domijan, D. Ivić, B. Cvjetković, M. Peraica, ELISA and HPLC analysis of ochratoxin A in red wines of Croatia, Food Control 20 (2009) 590–592.
[25] Y. Gao, M. Yang, C. Peng, X. Li, R. Cai, Y. Qi, Preparation of highly specific anti-zearalenone antibodies by using the cationic protein conjugate and development of an indirect competitive enzyme-linked immunosorbent assay, Analyst 137 (2012) 229–236.
[26] Q.H. He, Y. Xu, C.Z. Zhang, Y.P. Li, Z.B. Huang, Phage-borne peptidomimetics as immunochemical reagent in dot-immunoassay for mycotoxin zearalenone, Food Control 39 (2014) 56–61.
[27] N.V. Beloglazova, E.S. Speranskaya, S. De Saeger, Z. Hens, S. Abé, I.Y. Goryacheva, Quantum dot based rapid tests for zearalenone detection, Anal. Bioanal. Chem. 403 (2012) 3013–3024.
[28] W. Hu, X. Li, G. He, Z. Zhang, X. Zheng, P. Li, C.M. Li, Sensitive competitive immunoassay of multiple mycotoxins with non-fouling antigen microarray, 2013, Biosens. Bioelectron. 50 (2013) 338–344.
[29] J. Wang, Analytical Electrochemistry, 3rd ed., Wiley, New York, USA, 2006.
[30] E.A. Ramírez, P.G. Molina, M.A. Zon, H. Fernández, Development of an electroanalytical method for the quantification of Zearalenone (ZEA) in maize samples, Electroanalysis 17 (2005) 1635–1640.

[31] F. de Andrés, M. Zougagh, G. Castañeda, A. Ríos, Determination of zearalenone and its metabolites in urine samples by liquid chromatography with electrochemical detection using a carbon nanotube-modified electrode, J. Chromatogr. A 1212 (2008) 54–60.
[32] S. Cosnier, Biomolecule immobilization on electrode surfaces by entrapment or attachment to electrochemically polymerized films. A review, Biosens. Bioelectron. 14 (1999) 443–456.
[33] X.P. Wei, Y.L. Luo, F. Xu, Y.S. Chen, L.H. Yang, In-situ non-covalent dressing of multi-walled carbon nanotubes@titanium dioxides with carboxymethylchitosan nanocomposite electrochemical sensors for detection of pesticide residues, Mater. Des. 111 (2016) 445–452.
[34] F.A. Gutiérrez, M.D. Rubianes, G.A. Rivas, Electrochemical sensor for aminoacids and glucose based on glassy carbon electrodes modified with multi-walled carbon nanotubes and copper microparticles dispersed in polyethylenimine, J. Electroanal. Chem. 765 (2016) 16–21.
[35] Y. Bai, C.G. Koh, M. Boreman, Y.-J. Juang, I.-C. Tang, L.J. Lee, S.-T. Yang, Surface modification for enhancing antibody binding on polymer-based microfluidic device for enzyme-linked immunosorbent assay, Langmuir 22 (2006) 9458–9467.
[36] J. Horak, C. Dincer, E. Qelibari, H. Bakirci, G. Urban, Polymer-modified microfluidic immunochip for enhancedelectrochemical detection of troponin I, Sens. Actuators B: Chem. 209 (2015) 478–485.
[37] Y. Xiao, H.X. Ju, H.Y. Chen, Direct electrochemistry of horseradish peroxidase immobilized on a colloid/cysteamine-modified gold electrode, Anal. Biochem. 278 (2000) 22–28.
[38] T.O. Paiva, I. Almeida, J.T. Marquês, W. Liu, Y. Niu, G. Jin, A.S. Viana, Nanostructured interfaces with site-specific bioreceptors for immunosensing, Appl. Surf. Sci. 412 (2017) 455–463.
[39] D.M. Anjo, M. Kahr, M.M. Khodabakhsh, S. Nowinski, M. Wanger, Electrochemical activation of carbon electrodes in base: minimization of dopamine adsorption and electrode capacitance, Anal. Chem. 61 (1989) 2603–2608.
[40] D. Thouvenot, R.F. Morfin, Radioimmunoassay for Zearalenone and Zearalanol in human serum: production, properties, and use of porcine antibodies, Appl. Environ. Microbiol. 45 (1983) 16–23.
[41] J. Turkevich, P.C. Stevenson, J. Hillier, A study of the nucleation and growth processes in the synthesis of colloidal gold, Discuss. Faraday Soc. 11 (1951) 55–75.
[42] D.A. Handley, Colloidal Gold: Principles, Methods and Applications, in: M.A. Hayat (Ed.), Academic Press, New York, USA, 1989.
[43] W. Haiss, W.N.T.K. Thanh, J. Aveyard, D.G. Fernig, Determination of size and concentration of gold nanoparticles from UV–vis spectra, Anal. Chem. 79 (2007) 4215–4221.
[44] J.R. Crowther, ELISA. Theory and Practice Methods in Molecular Biology, vol. 42, Humana Press Totowa, New Jersey, USA, 1995.
[45] S.C.B. Gopinath, T.H. Tang, M. Citartan, Y. Chen, T. Lakshmipriya, Current aspects in immunosensors, Biosens. Bioelectron. 57 (2014) 292–302.
[46] A.W. Bott, W.R. Heineman, Chronocoulometry. Curr. Sep. 20 (2004) 121–126.
[47] D. Rodbard, Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays, Clin. Chem. 20 (1974) 1255–1270.
[48] D. MacDougall, W.B. Crummett, Guidelines for data acquisition and data quality evaluation in environmental chemistry, Anal. Chem. 52 (1980) 2242–2249.
[49] S. Biselli, C. Hummert, Development of a multicomponent method for Fusarium toxins using LC–MS/MS and its application during a survey for the content of T-2 toxin and deoxynivalenol in various feed and food samples, Food Addit. Contam. 22 (2005) 752–760.
[50] M. Hervás, M.Á. López, A. Escarpa, Electrochemical immunoassay using magnetic beads for the determination of zearalenone in baby food: an anticipated analytical tool for food safety, Anal. Chim. Acta 653 (2009) 167–172.
[51] N.V. Panini, F.A. Bertolino, E. Salinas, G.A. Messina, J. Raba, Zearalenone determination in corn silage samples using an immunosensor in a continuous-flow/stopped-flow systems, Biochem. Eng. J. 51 (2010) 7–13.
[52] N.V. Panini, E. Salinas, G.A. Messina, J. Raba, Modified paramagnetic beads in a microfluidic system for the determination of zearalenone in feedstuffs samples, Food Chem. 125 (2011) 791–796.
[53] L. Liu, Y. Chao, W. Cao, Y. Wang, C. Luo, X. Pang, D. Fan, Q. Wei, A label-free amperometric immunosensor for detection of zearalenone based on trimetallic Au-core/AgPt-shell nanorattles and mesoporous carbon, Anal. Chim. Acta-36. Figure Caption 847 (29) (2014).

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