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Monoclonal antibody based electrochemical immunosensor for the determination of ochratoxin A in wheat

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Abstract

Competitive electrochemical enzyme-linked immunosorbent assays based on disposable screen-printed electrodes have been developed for quantitative determination of ochratoxin A (OTA). The assays were carried out using monoclonal antibodies in the direct and indirect format. OTA working range, I_{50} and detection limits were 0.05–2.5 and 0.1–7.5 μg L^{-1}, 0.35 (±0.04) μg L^{-1} and 0.9 (±0.1) μg L^{-1}, 60 and 100 μg L^{-1} in the direct and indirect assay format, respectively. The immunosensor in the direct format was selected for the determination of OTA in wheat. Samples were extracted with aqueous acetonitrile and the extract analyzed directly by the assay without clean-up. The I_{50} in real samples was 0.2 μg L^{-1} corresponding to 1.6 μg/kg in the wheat sample with a detection limit of 0.4 μg/kg (calculated as blank signal -3σ). Within- and between-assay variability were less than 5 and 10%, respectively. A good correlation ($r = 0.9992$) was found by comparative analysis of naturally contaminated wheat samples using this assay and an HPLC/immunoaffinity clean-up method based on the AOAC Official Method 2000.03 for the determination of OTA in barley.

Keywords: Immunosensors; ELISA; Ochratoxin A; Screen-printed electrodes; Wheat

1. Introduction

Ochratoxin A (OTA), 7-(L-β-phenylalanylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocumarin, is a mycotoxin produced by several *Aspergillus* and *Penicillium* species growing in different agricultural commodities in the field or during storage[1–2]. OTA has been shown to be nephrotoxic, teratogenic, carcinogenic and immunotoxic to several animal species. The International Agency for Research on Cancer (IARC) has classified OTA as possibly carcinogenic in humans (Group 2B) [3]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), after evaluation of OTA nephrotoxicity, proposed for this mycotoxin a provisional tolerable weekly intake (PTWI) of 0.1 μg/kg body mass (equivalent to 14 ng/kg body mass/day) [4].

OTA occurs in various foodstuffs such as cereals, coffee beans, nuts, cocoa and dried vine fruits, as well as processed foods derived from these products [3]. It has also been reported to occur in a number of associated beverages such as, wine, beer and grape juice. Recently, the European Commission fixed the maximum levels for OTA at 5 μg/kg in cereals (and derivative products) and roasted coffee, 3 μg/kg in all cereal products intended for direct human consumption, 10 μg/kg in dried vine fruits and soluble coffee and 2 μg/kg in wine, grape juice and must (Commission Regulation no. 472/2002 of 12 March 2002 and no. 123/2005 of 26 January 2005). Lower levels (0.5 μg/kg) have been established in foods for infants and young children (Commission Regulation No. 683/2004 of 13 April 2004).

Sensitive and accurate analytical methods are reported in the literature for the determination of several mycotoxins in differ-

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ent matrices, although only few methods have been validated by collaborative study [5]. Analytical aspects and challenges of the detection of OTA in foods has been recently reviewed by Monaci and Palmisano [5]. AOAC official methods are currently available for the determination of OTA in barley (AOAC Official Method 2000.03), roasted coffee (AOAC Official Method 2000.09), green coffee (AOAC Official Method 2004.10), beer and wine (AOAC Official Method 2001.01). All these methods are based on HPLC/immunoaffinity clean-up and allow to detect the analyte in such complex matrices with good selectivity, sensitivity and reproducibility.

Immunochemical methods exhibit high sensitivity and acceptable performances allowing the detection of OTA at concentrations equal or lower than the EC regulatory values. Several papers have been published about the development of traditional spectrophotometric enzyme-linked immunosorbent assays (ELISA) using polyclonal and/or monoclonal antibodies against OTA [6–10]. Generally immunoassays avoid the use of a clean-up/preconcentration step resulting in a rapid and semi-quantitative detection of the toxin ideal for screening purposes. However, this leads to a significant influence of the sample material and of the extraction procedure (for solid samples) on detection limits and sensitivity. The most sensitive procedures tested on real samples reported in the literature presents detection limits in the μg/kg range [11,12]. A flow-through membrane enzyme based immunoassay for the detection of OTA in wheat has been also described [13]. The system was based on visual assessment of the reaction and was able to detect in 15 min a wheat sample spiked with 4 μg/kg of OTA. An innovative approach is based on the use of a surface plasmon resonance based sensor for the detection of OTA [14]. A detection limit of 0.1 μg/kg is claimed for this sensor.

The availability of sensitive and fast methods of analysis that can be used in situ or for decentralised tests is highly desirable. In this perspective, electrochemical methods have shown important advantages compared to traditional methods currently in use because of cost effectiveness, ease of handling and sensitivity [15,16]. Moreover, the introduction of screen-printed electrochemical probes as complete electrochemical cells has led to further advantages in terms of biomolecules (antigen/antibody) immobilisation and sensitivity for toxins detection [17,18].

The present work describes the development of a rapid, selective and sensitive method for the determination of OTA in wheat joining the advantages of the immunochemical assay with those of the screen-printed technology. The optimisation of direct and indirect competitive ELISAs based on anti-OTA monoclonal antibody, the transfer of the assays to screen-printed carbon electrodes and the application of the assay in the direct format to the determination of OTA in naturally contaminated wheat samples are reported.

2. Materials and methods

2.1. Materials

OTA–BSA conjugate, alkaline phosphatase (AP: 10,000 units), 1-ethyl-3,3-dimethylamino-propyl-carbodiimide (EDPC) were purchased from Sigma–Aldrich Co. (St. Louis, USA). 1-Naphtylphosphate disodium salt and 4-nitrophenylphosphate disodium salt hexahydrate were purchased from Fluka BioChemica (Gallarate, Milan, Italy) and acetonitrile from Riedel-deHaën (Seelze, Germany). OTA was purchased from Alexis (Lausen, Switzerland), goat IgG (anti-mouse IgG) and goat IgG (anti-mouse IgG)-AP conjugated (SAb: secondary antibody) from Vector (Peterborough, England).

The monoclonal antibody used in this study has been produced according Gyöngyösi et al. [19]. As reported in the paper, the cross-reactivity with OTA and OTB (ochratoxin B) were 100 and 9.3%, respectively. No cross-reaction was detected with ochratoxin α, coumarin, or L-β-phenylalanine.

Microtitre plates were from Maxisorp F96 (Neerijse, Belgium). Dialysis tubes were purchased from Spectrum Laboratories Inc. [Spectral/Por® CE (cellulose Ester) Float A Lyzer™] (Breda, Netherlands), ultra filter (Amicom Ultra) and membrane filters (Millex®-GS, 0.22 μm) from Millipore (Bedford, USA).

Screen-printed electrodes were made in the Biosensor Laboratory of Prof. Mascini (University of Florence) using a model 245 screen-printer from DEK (Weimouth, England). The graphite based ink (Electrodag 421), silver ink (Electrodag SS SSRFU) and insulating ink (Electrodag 6018 SS) were from Acheson Italiana (Milan, Italy) and were printed on a polyester flexible film (Autostat HT5; from Autotype Italia, Milan, Italy). Pseudo-reference and counter electrode were silver based while working electrode was graphite based with a diameter of 3 mm. Details of the printing procedure have been already reported elsewhere [20,21].

Working buffers were the following: phosphate buffer saline pH 7.4 (PBS; 0.1 M phosphate buffer + 0.138 M NaCl + 0.0027 M KCl), phosphate buffer saline with Tween 20 (PBST; PBS 0.01 with 0.05% Tween 20), azide phosphate buffer pH 7.0 (A-PB; 0.1 M phosphate buffer with 0.1% sodium azide), 0.05 M carbonate buffer pH 9.6 (CB), diethanolamine buffer pH 9.5 (DEA; 0.97 M diethanolamine, 1 mM $MgCl_2$), polyvinylalcohol solution (PVA; 1% polyvinylalcohol in CB).

2.2. Equipment

(a) ELISA reader, Biorad Model 550 Micro plate reader. (b) Spectrometer, Unicam 8625 UV–vis. (c) Spectrofluorometer SPEX Fluoromax Fluorimeter (Edison, NJ, USA). (d) Electrochemical measurements were performed at room temperature, using an Autolab Electrochemical Analyser with the general purpose electrochemical software (GPSTAT30) operating system (Ecochemie, Utrecht, NL). (e) Screen Printed Electrodes (SPEs), consisting of graphite working electrode, an Ag pseudo-reference electrode and graphite counter electrode, were printed with a high performance multi-purpose precision screen printer DEK 245 (DEK, Weymouth, UK). Inks were from Acheson Italia (Milan, Italy). (f) HPLC apparatus with fluorescence detector set at excitation wavelength of 333 nm and emission wavelength of 460 nm (Agilent 1100 Series, Agilent Technology).

HPLC column, XTerra RP_{18} (150 mm × 4.6 mm, 5 μm, Waters, Milford, MA, USA) preceded by a 0.5 μm Rheodyne guard filter.

2.3. Preparation of OTA–alkaline phosphatase conjugate (OTA–AP)

OTA–Alkaline phosphatase conjugate (OTA–AP) was used as the labelled competitor in the direct ELISA. OTA–AP was prepared according to the modified water-soluble carbodiimide method of Chu et al., [22] to produce high activity conjugated antigen. Briefly, 2000 U of AP (50 μL of 3 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 30 mM triethanolamine solution), OTA solution (2 mg in 150 μL ethanol) and EDC (1.15 mg in 850 μL of 0.1 M phosphate buffer, pH 7.0) were mixed. The solution was stirred in the dark at room temperature for 24 h. After the reaction, the solution was dialyzed against 0.1 M phosphate buffer, pH 7.0, for 72 h at 4 °C. The conjugate was purified by filtration on a 1 cm × 18 cm Sephadex G25 column using A-PB like elution solvent.

The conjugated toxin–protein ratio was determined fluorometrically (excitation wavelength 333 nm, emission wavelength 447 nm) to determine toxin content. The AP concentration was determined at 570 nm via the BCA method (BCA Protein Assay Kit, purchased from Pierce, USA) using a calibration curve for albumin. The residual enzyme activity of AP conjugate was measured at 405 nm and was 4.45 U mL^{-1}.

2.4. ELISA procedures

2.4.1. Indirect ELISA

About 100 μL of OTA–BSA solution in a coating CB buffer were dispensed into the wells of a microplate and kept at 4 °C overnight. Then, 100 μL of 1% PVA were used to block the microwells (1 h at 37 °C), and 100 μL of mouse anti-OTA IgG solution (binding curve) or anti-OTA IgG + OTA standards (competition curve; anti-OTA/OTA volume ratio 1:1) were added and left to react for 1 h at room temperature. About 100 μL of secondary antibody (anti-mouse IgG–AP conjugate) was later incubated for 1 h at room temperature and 1 mg/mL solution of 4-nitronaphthyl phosphate in DEA buffer were used to detect the alkaline phosphatase reaction. Reading was performed at 405 nm with the microplate reader, after 30 min of incubation at room temperature. Between each step the microwells were subjected to a three-cycle washed procedure with PBST.

2.4.2. Direct ELISA

About 100 μL of goat IgG (anti-mouse IgG) in CB were dispensed into microplate wells and kept at 4 °C overnight, and then blocked with 1% PVA as for the indirect format. About 100 μL of OTA Ab were then added into each well and let to react for 1 h at room temperature. The OTA–AP conjugate (binding curve) or a mixture of (1:10 v:v) OTA–AP + OTA standard or sample (competition curve) was later added for 1 h at room temperature. AP activity detection and washing was carried out as in the indirect ELISA format.

2.5. Immunosensor assay procedures

2.5.1. Indirect format

About 6 μL of OTA–BSA in CB in CB were dispensed on the graphite-based screen-printed working electrodes and kept overnight at 4 °C. About 6 μL of 1% PVA solution was used to block the surface for 30 min at room temperature. This solution was already reported to be the best blocking solution for screen-printed based immunosensor [23] and was used also in the spectrophotometric ELISA assay for a better comparison of the analytical performances. Competition or binding was run with 6 μL of OTA–AP conjugate at the desired dilution or conjugate + standard for 30 min at room temperature. Washing was carried out as for the ELISA procedures. The activity of the label enzyme was measured electrochemically by addition of 50–100 μL of substrate solution (5 mg/mL, 1-naphthyl phosphate in DEA buffer; prepared daily), for 2 min at room temperature. The enzymatic product, 1-naphthol, was detected by Differential Pulse Voltammetry (DPV) under the following conditions: potential range 0–600 mV, pulse width 60 ms, pulse amplitude 50 mV and scan speed 50 mV/s. Electrochemical detection parameters have been previously optimised for other toxins [17].

2.5.2. Direct format

About 6 μL of goat IgG (anti-mouse IgG) were put on the working electrode and kept overnight at 4 °C. About 6 μL of OTA monoclonal antibody were then added to the electrode surface for 30 min at room temperature. All other procedures (competition and washing) were the same reported for the indirect format.

2.6. Wheat sample preparation and OTA determination

Samples contaminated with OTA in the range 0.4–13.1 μg/kg were prepared by mixing aliquots of finely ground fungal wheat culture contaminated with OTA at 808 μg/kg with not contaminated ("blank") wheat flour.

Culture material was prepared by inoculating 100 g of sterilized wheat kernels in 500 mL Erlenmeyer flask with a OTA producer strain of *Aspergillus ochraceus* (MPVP A 163), kindly supplied by Prof. P. Battilani, Università Cattolica, Piacenza, Italy. Wheat kernels were moistened up to 16% relative humidity, sterilized by autoclaving for 20 min at 120 °C and incubated in the dark for 15 days at 25 °C. Wheat culture was then dried at 40 °C, and finely ground with a laboratory mill (Cyclone Sample Mill, Model 3010-019, pbi International, Milan, Italy).

OTA was determined following the AOAC Official Method 2000.03 for the determination of OTA in barley [24], with minor modifications. Twenty-five grams of ground wheat was extracted with 100 ml extraction solvent (ACN:H_2O, 6:4 v/v) by shaking for 60 min. The mixture was filtered through filter paper (Whatman No. 4) to remove particulate matter. This filtered extract was used for the ELISA assay after dilution 1:1 (v/v) with working buffer. For the HPLC determination, 10 mL of filtered extract were diluted with 40 mL of distilled water, mixed and filtered through Whatman GF/A glass microfibre fil-

ter. Ten milliliters of the diluted extract was cleaned up through an OchraTest™ immunoaffinity column (Vicam, Watertown, USA) at a flow-rate of about one drop per second, followed by washing with 2 × 5 mL wash buffer (2.5% NaCl, 0.5% $NaHCO_3$, 0.01% Tween 20) and 10 mL distilled water at a flow-rate of 1–2 drops per second. OTA was then eluted with 1.5 mL methanol and collected in a clean vial. The eluted extract was evaporated under nitrogen stream at ca 50 °C and reconstituted with 500 μL of the HPLC mobile phase. Sample extracts were stored at +4 °C until HPLC analysis.

2.7. HPLC analysis of OTA

About 100 μL of reconstituted extract were injected into the chromatographic apparatus by full loop injection system. The mobile phase consisted of a mixture of acetonitrile:water:acetic acid (99:99:2) at a flow rate of 1.0 mL/min. Quantification of OTA was performed by measuring peak areas at OTA retention time and comparing them with the relevant calibration curve.

Recovery experiments were performed in quadruplicate by spiking "blank" wheat samples with OTA at levels of 1.0, 5.0 and 10.0 μg/kg. Average recoveries ranged from 90 to 97%, with relative standard deviations lesser than 10%. Detection limit was 0.1 μg/kg, based on a signal to noise ratio of 3:1.

3. Results and discussion

3.1. Optimisation of ELISAs

The first step in the development of a competitive ELISA is to determine the optimal dilution and concentration of the different reagents and the conditions of the assay as incubation times and temperatures. Particularly, the last two parameters have great importance because they influence the selectivity and sensitivity of the assay [25]. The monoclonal anti-OTA IgG has been initially characterised carrying out binding curves in indirect and direct competitive ELISA format using spectrophotometric detection. Optimal concentrations of mycotoxin–BSA, OTA–BSA, anti-OTA antibody, and secondary antibody–phosphatase alkaline conjugate for the indirect format and goat anti-mouse IgG, anti-OTA antibody and OTA–alkaline phosphatase conjugate (OTA–AP) for the direct format are reported in Table 1. These were selected from the binding curves obtained varying each of the parameters.

Table 1
Optimal dilution and concentration of the different reagents in the spectrophotometric (Sp) and electrochemical (SPEI) immunoassays

	Indirect format		Direct format	
	Sp	SPEI	Sp	SPEI
OTA–BSA (μg mL^{-1})	5	50	–	–
(Anti IgG) IgG (μg mL^{-1})	–	–	10	10
(Anti OTA) IgG (μg mL^{-1})	10	35	12	4
(Anti IgG) IgG–AP (v:v)	1:500	1:60	–	–
OTA–AP (v:v)	–	–	1:125	1:350

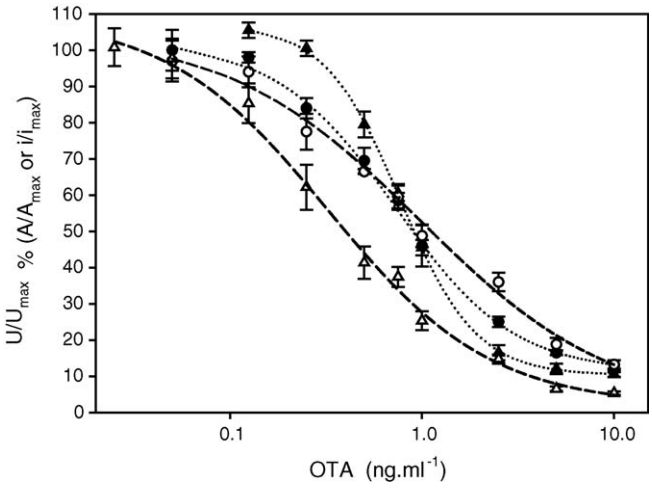

Fig. 1. Normalized standard curves for OTA standard solutions obtained by direct and indirect competitive ELISA (dc and ic) with spectrophotometric (Sp) and electrochemical (SPEI) detection. ic ELISA Sp (●); dc ELISA Sp (▲); ic ELISA SPEI (◯) and dc ELISA SPEI (△). Signals are in absorbance units for spectrophotometric-based assays and in nA for immunosensors.

In all the assays, the maximum signal was attained after 1 h incubation of the antigen at 37 °C. However, no significant loss in sensitivity in the immunosensor format was observed also after 30 min incubation at room temperature; thus, we used the latter conditions for the electrochemical assay. The other parameters are reported in Sections 2.4 and 2.5. In the direct assay a pre-coating of antimouse IgG was used for a more effective orientation of the anti-OTA antibody binding sites. A 10–20% increase of the signal was in fact observed comparing the binding curves obtained without anti-mouse IgG pre-coating.

The normalised standard curves for OTA generated using the conditions reported are shown in Fig. 1 for both formats and using spectrophotometric and electrochemical detection; each point is the mean of three measurements. For a better comparison of the competition the experimental data were fit using a non-linear four-parameters logistic calibration plot. The four parameters logistic is given by the equation:

$$f(x) = \frac{a - d}{[1 + (x/c)^b]} + d$$

where a and d are the asymptotic maximum and minimum values, c the inflection point (I_{50}) and b the slope. In Table 2 the data obtained are presented together with the regression equation obtained from the linear part of the curve (taken from seven measurements carried out in different days) and the limit of detection (calculated as the blank signal -3σ). In the indirect format the immunosensors gave analytical performances comparable with the corresponding spectrophotometric assay as evidenced by the detection limit, slope and I_{50} data. Better sensitivity of the electrochemical assay was obtained for the direct format. Sensors displayed wide current ranges, with average Δi of 5843 nA (ranging from 6019 to 176 nA), and 691 nA (ranging from 707 to 16 nA) for indirect and direct assays, respectively, whereas slopes were similar. The working range was 0.1–7.5 μg L^{-1} for the indirect format, and 0.06–2.5 μg L^{-1} in the direct format.

Table 2
Competition curve data of the spectrophotometric (Sp) and electrochemical (SPEI) immunoassays

Type of assay	LOD[a] (ng mL^{-1})	Competition curve parameters[b]				Linear regression[c]
		a (A or nA)[d]	b (nA ng mL^{-1})	c (I_{50}) (ng mL^{-1})	d (A or nA)[d]	
Indirect Sp	0.15	1.22 ± 0.05	1.4 ± 0.5	0.8 ± 0.2	0.13 ± 0.08	$f(x) = 49.3\ (\pm 0.8) - 57.5\ (\pm 0.1)\ x$ [$r = 0.991$]
Indirect c SPEI	0.10	$(602 \pm 1) \times 10^1$	0.9 ± 0.2	0.9 ± 0.1	$(18 \pm 3) \times 10^1$	$f(x) = 52.5\ (\pm 0.4) - 43.7\ (\pm 0.5)\ x$ [$r = 0.994$]
Direct c Sp	0.08	1.39 ± 0.06	2.2 ± 0.2	0.8 ± 0.1	0.13 ± 0.07	$f(x) = 47.4\ (\pm 0.7) - 86.0\ (\pm 0.3)\ x$ [$r = 0.993$]
Direct c SPEI	0.06	$(71 \pm 6) \times 10^1$	1.1 ± 0.1	0.35 ± 0.04	$(2 \pm 1) \times 10^1$	$f(x) = 34.9\ (\pm 0.6) - 52.2\ (\pm 0.9)\ x$ [$r = 0.992$]

[a] LOD = limit of detection (blank -3σ).
[b] By $f(x) = \{(a-d)/[1+(x/c)^b]\} + d$ fitting to the data point of Fig. 2.
[c] Linear regression of the normalized calibration curve (seven points).
[d] A = absorbance for Sp; nA = nanoAmpere for SPEI.

The within-day and inter-day coefficients of variations (CVs) for OTA standard concentration (0.01–10 μg L^{-1}) were between 2 and 10% with an average of 7%. It has also to be noted that, in the conditions reported, the assay time was considerably reduced using the electrochemical immunosensors with half of the time used for competition (30 versus 60 min) and only 2 min for detection (versus 30 min).

The immunosensor in the direct format exhibited superior sensitivity according to the I_{50} value (0.35 μg L^{-1}) and was then selected for the experimental work on real samples. This immunosensor-based method presents performances comparable to the more sensitive previously reported ELISAs run with standard OTA solutions. In fact, using the same monoclonal antibody and spectrophotometric detection (horseradish peroxidase as label enzyme) a I_{50} of 0.45 μg L^{-1} and LOD of 0.042 μg L^{-1} was found [19]. On the other hand, a comparison with a similar electrochemical assay using commercially available polyclonal antibodies (I_{50}: 6.1 μg L^{-1} and LOD: 0.18 μg L^{-1}) [25], demonstrate the excellent performance of the monoclonal antibody.

3.2. Measurement of OTA in wheat: solvent and matrix effect

A very important step for evaluation of any analytical procedure is the assessment of the matrix effect, which should be minimised by the sample preparation procedure. Many reports have been published concerning OTA extraction procedures from cereals. Chloroform, methanol, or dichloromethane based organic solvents added with acids such us chloridic, acetic, ascorbic, phosphoric and citric acid are frequently used as extraction solvents [26–29]. So far, no official method for the determination of OTA in wheat has been reported. In the present work OTA was extracted from the sample with aqueous acetonitrile (ACN:H_2O, 6:4 v/v) according to the AOAC Official Method 2000.03 for the determination of OTA in barley, as described in Section 2.6.

Due to the high amount of acetonitrile (ACN) to be used, the effect of the solvent on the immunological system was tested before matrix effect evaluation. The ACN effect on the alkaline phophatase activity was first evaluated. About 1 μg mL^{-1} of anti-mouse IgG–alkaline phosphatase conjugate was placed on the graphite screen-printed working electrode. The electrode was incubated in the same conditions of the competition assay (30 min at room temperature) with ACN–H_2O solutions at different ratios (from 1:9 to 9:1, v:v). The phosphatase alkaline activity resulted to be independent of the ACN amount (signal between 95 and 108% of the original activity). The solvent effect in the competition step was then evaluated. OTA competition tests at I_{50} concentration (0.35 μg L^{-1}) in 0.1 PBS 1:1 diluted with ACN:H_2O solutions were run. These tests showed an important effect of ACN in the competition step; in fact, a 20% decrease of the signal was observed for a 1:9 ACN-H_2O ratio, with maximum decrease of 40% for ratios higher than 2:8.

In Fig. 2 the competition curves carried out in 0.1 PBS and in the extraction mixture (ACN:H_2O, 6:4 v/v) are reported. Two important effects can be observed: a decrease of the slope of the calibration curve and the linear range displaced towards lowest OTA standard levels. The analytical data of the calibration curves are reported in Table 3, for comparison. Although the range of current is still acceptably wide, there is a clear decrease in the maximum signal in presence of ACN. The solvent clearly inhibits the antigen-antibody reaction and the decrease of the amount of conjugate available for the competition produces a decrease of the LOD and an increase of the working range of the assay. However, the I_{50} value is similar indicating that the immunosensor is still able to work in the presence of ACN

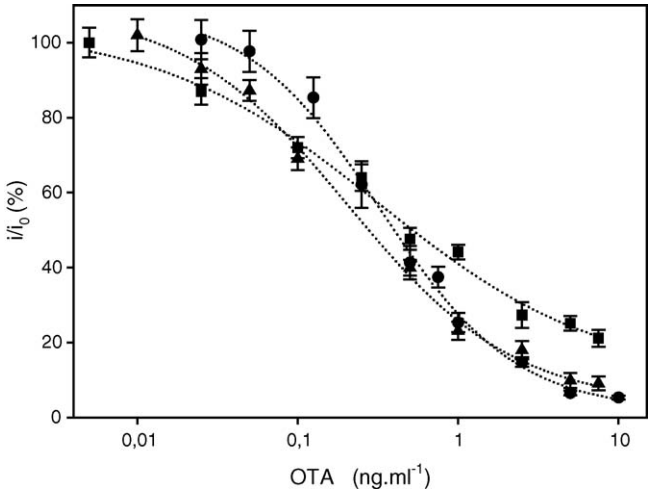

Fig. 2. Normalized standard curves for OTA standard solutions run by direct competitive ELISA with electrochemical detection in buffer PBS 0.1 (●), extraction solvent (ACN:H_2O, 6:4 v:v; ■) and wheat samples extract (▲).

Table 3
Competition curve data and analytical parameters of the electrochemical assay in PBS buffer, extraction solvent and wheat extract

Parameters	0.1 PBS buffer	ACN:H_2O (6:4)	Wheat extract
a (nA)	$(71 \pm 6) \times 10^1$	$(26 \pm 1) \times 10^1$	$(41 \pm 7) \times 10^1$
b (nA ng mL^{-1})	1.1 (±0.1)	0.62 (±0.03)	0.8 (±0.1)
c (ng mL^{-1})	0.35 (±0.04)	0.32 (±0.02)	0.20 (±0.03)
d (nA)	$(2 \pm 1) \times 10^1$	24 (±8)	$(1 \pm 1) \times 10^1$
w.r. (ng mL^{-1})	0.06–2.5	0.02–5.0	0.05–2.5
LOD (ng mL^{-1})	0.06	0.015	0.05
Linear regression	30.9 (±0.6)–52.2 (±0.9)*x*	42.3 (±0.3)–25.4 (±0.6)*x*	23.5 (±0.1)–41.1 (±0.5)*x*

at OTA values well below the EU regulatory limits fixed for cereals.

The competition curve was also carried out in wheat extract samples (ACN:H_2O 6:4, v/v) having an OTA concentration lower than 0.1 μg/kg (tested by HPLC) equivalent to an immunoassay concentration below the detection limit (lower than 0.013 μg L^{-1}). The curve run on five different extraction is reported in Fig. 2 and the relative data are reported in Table 3. The presence of the wheat extract seems to repress the inhibiting effect of ACN; the parameters reported are all in-between those found for PBS and ACN solutions. ACN affects the conformation of the OTA–AP conjugate decreasing the amount of the conjugate bound to the anti-OTA antibodies in the assay conditions. The presence of polyelectrolytes as proteins and starch in the sample decrease the "activity" of ACN on the conjugate allowing the binding of an higher amount of OTA–AP. Maximum signals were not significantly affected by the amount of wheat used in the assay (data not shown)

Working with these experimental conditions we have almost one order of magnitude improvement compared to the most sensitive immunoassay (ELISA) reported for OTA determination in wheat in term of working range (1–10 μg L^{-1}) and detection limits (0.5 μg/kg) [28].

It has to be noted that 0.2 μg L^{-1} (I_{50}) in the immunoassay corresponds to 1.6 μg/kg in the wheat sample. The minimum limit legally established is 3 μg kg^{-1} in all cereal products intended for direct human consumption corresponding to 0.375 μg L^{-1} in the immunoassay. This value is below the I_{50} for any of the calibration curves reported. An attempt to minimise the matrix effect was made using 1:4 and 1:8 ratio dilutions of extract:PBS in the competition step. No evident advantage was obtained. Within- and inter-day coefficients of variations

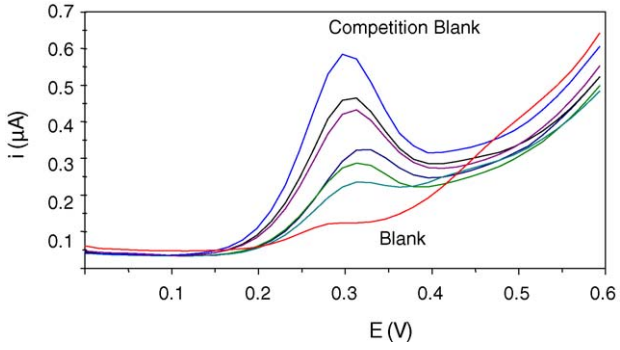

Fig. 3. Differential voltammograms obtained for the samples assayed in Table 4 (one representative voltammogram for each of the spiked levels, from 0.5 to 15 μg/kg). Competition blank signal is relative to OTA level = 0, blank signal is OTA-free wheat extract with no OTA–AP conjugate used in the assay.

(CVs) for OTA standard concentration (0.001–10 μg L^{-1}) were between 2 and 25% with mean between 10 and 15%.

Analyses of contaminated wheat samples were then carried out using normalised calibration curves of matrix extract (1:1 dilution). The wheat samples were spiked with OTA (ranging from 0.4 to 13.1 μg kg^{-1}) and assayed with the optimized electrochemical immunosensor assay and HPLC/immunoaffinity clean up method (see Section 2.6). For each concentration level, three different samples were independently analysed. Differential pulse voltammograms obtained for the samples in the optimised assay conditions are reported in Fig. 3. Only one voltammogram per sample is reported for clarity. Voltammograms generated also from a OTA-free sample (competition blank) and from the same sample assayed with no OTA–AP in solution are reported for a better comparison. A good correlation ($r = 0.9992$) was observed between results derived from the electrochemi-

Table 4
Comparison of wheat samples contaminated with OTA determined by electrochemical immunosensor and HPLC

Theoretical OTA level (μg/kg)	HPLC		Immunosensor	
	OTA ± S.D.[a] (μg/kg)	R.S.D.[b] (%)	OTA ± S.D. (μg kg^{-1})	R.S.D. (%)
0.5	0.44 ± 0.08	18.2	0.6 ± 0.1	16.7
1	0.9 ± 0.1	11.1	1.0 ± 0.1	10.0
4	3.8 ± 0.4	10.5	3.1 ± 0.4	12.9
5	4.5 ± 0.5	11.1	4.2 ± 0.6	14.3
15	13.1 ± 0.9	6.9	12 ± 1	8.3

[a] Mean value ± S.D. ($n = 3$, S.D. = standard deviation).
[b] R.S.D. = relative standard deviation.

cal immunosensor assay and the HPLC method. Precision was determined by calculating the RSD for the replicate measurements and accuracy (%) of electrochemical immunosensor assay was calculated by assessing the agreement between measured and nominal concentrations of the spiked samples determined by HPLC. Data are summarised in Table 4.

4. Conclusions

The present study shows for the first time the use of a competitive electrochemical enzyme-linked immunosorbent assay with monoclonal antibody for the determination of OTA in wheat samples. The assay combines the high selectivity of the immunoassay with the sensitivity of electrochemical screen-printed carbon electrodes. A one-step procedure of extraction with aqueous acetonitrile without any clean-up of extract combined with the electrochemical immunoassay provides good sensitivity, optimal working range and performances similar to robust HPLC methods for the determination of OTA in cereals.

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