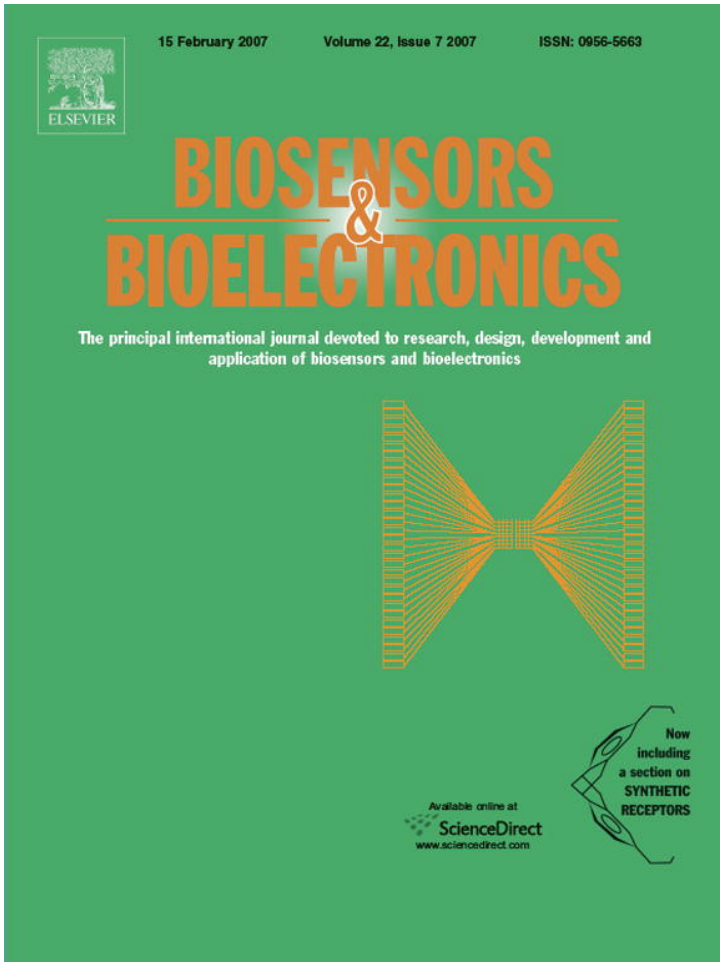


Available online at www.sciencedirect.com
ScienceDirect
Biosensors and Bioelectronics 22 (2007) 1434–1440
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Electrochemical immunosensor array using a 96-well screen-printed microplate for aflatoxin B_1 detection

S. Piermarini [a], L. Micheli [a,*], N.H.S. Ammida [a,b], G. Palleschi [a], D. Moscone [a]

[a] *Dipartimento di Scienze e Tecnologie Chimiche, Università "Tor Vergata", via della Ricerca Scientifica, 00133 Rome, Italy*
[b] *Department of Chemistry, Garyounis University, Benghazi, Libya*

Received 13 April 2006; received in revised form 6 June 2006; accepted 15 June 2006
Available online 8 August 2006

Abstract

A novel analytical immunosensor array, based on a microtiter plate coupled to a multichannel electrochemical detection (MED) system using the intermittent pulse amperometry (IPA) technique, is proposed for the detection of aflatoxin B_1 (AFB_1). In the present work, the electrochemical behaviour and electroanalytical performance of the thick-film carbon sensors (also designated as screen-printed electrodes) incorporated in the multichannel electrochemical plate were first evaluated. Then the 96-well screen-printed microplate was modified in accord with a competitive indirect enzyme-linked immunoassay (ELISA) format for aflatoxin B_1 detection. The measurements were performed using both spectrophotometric and electrochemical procedures and the results of the calibration curves, detection limit (LOD), sensitivity and reproducibility of the respective assay systems were evaluated.

The immunoassay was then applied for analysis of corn samples spiked with AFB_1 before and after the extraction treatment, in order to study the extraction efficiency and the matrix effect, respectively. These studies have shown that using this system, AFB_1 can be measured at a level of 30 pg/mL and with a working range between 0.05 and 2 ng/mL. Good recoveries ($103 \pm 8\%$) were obtained, demonstrating the suitability of the proposed assay for accurate determination of the AFB_1 concentration in corn samples.

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

Keywords: Aflatoxin B_1; 96-Well screen-printed microplate; Intermittent pulse amperometry; ELISA; Corn

1. Introduction

Immunoassays have been demonstrated to be potentially useful tools for the analysis of many toxic substances now subject to stringent EU regulations as to their permissible levels in food and feed (Edward, 1997). While providing good sensitivity and selectivity, the immunoassays are often too time consuming and complicated to be suitable for regulatory monitoring. In the present work, we have taken advantage of the commercial availability of an electrochemical array sensor system to develop a significantly better enzyme-linked immunoassay (ELISA).

The disposable sensor array employed is a device manufactured using the screen-printing technology. It consists of a 96-well plate whose bottom has been modified with an array of 96 screen-printed sensors, each of which is constituted of a carbon working electrode and an Ag/AgCl pseudoreference electrode. Such a multichannel electrochemical plate has been designed and developed for simultaneous and independent measurements of nucleic acids, incorporating horseradish peroxidase (HRP) enzyme as a marker (Wojciechowski et al., 1999). In particular, the device was designed to operate using intermittent pulse amperometry (IPA), an electrochemical method, which involves a series of millisecond pulses of the same potential applied to the working electrode and separated by longer periods when the electrode is disconnected from the potentiostatic circuit.

Thus far, this device has been applied only to DNA analysis, and the provider, which also furnishes a dedicated small instrument for data acquisition, has optimised the IPA electrochemical detection only for the measurement of the HRP enzyme activity intended as DNA-label (Wojciechowski et al., 1999). At the same time, for the other most commonly used label enzyme alkaline phosphatase (AP), there have been neither reports of this

* Corresponding author. Tel.: +39 0672594420; fax: +39 06 72594328.
E-mail address: laura.micheli@uniroma2.it (L. Micheli).

0956-5663/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2006.06.029

electrochemical immunosensing array nor applications based on the measurement of its activity. Avidin-biotin based electrochemical immunoassay for thyrotropin was developed by Athey et al. (1993). The product 1-naphthol was detected at disposable printed carbon eight-electrode combs using specially designed instrumentation. Tang et al. (2002) have proposed an immunoassay using a microtiter plate incorporating a multichannel electrochemical detection (MED) system consisting of sets of eight Pt electrodes which can be immersed in a row of spectrophotometric microtiter wells to measure RIgG.

A disposable multichannel immunochemical sensor, based on the array of eight working electrode (gold) and a silver reference electrode printed on a ceramic substrate, was developed for determination of 2,4-dichlorophenoxyacetic acid (Skládal and Kaláb, 1995).

In the present work, the disposable electrochemical plate was used for the first time as a support for the assembly of an immunoassay and then the system was optimised for detection of AFB_1 in corn. Aflatoxin B_1 (AFB_1) was chosen as an exemplar of a group of highly toxic difuranocoumarin derivatives that are produced by many strains of *Aspergillus flavus* and *A. parasiticus* and that may contaminate a wide range of foods and animal feedstuffs stored under temperature and humidity conditions favourable to mould growth (Cole and Cox, 1981). They are of particular analytical interest because of recent regulatory decisions. The four major aflatoxins have been designated B_1, B_2, G_1 and G_2 based on their fluorescence under UV light and their relative chromatographic mobility during thin-layer chromatography. The International Agency for Research on Cancer (IARC) has classified aflatoxin B_1 as a group 1 human carcinogen and aflatoxins G_1, G_2, and B_2 in group 2, as possible human carcinogens (IARC, 1993). These toxins exhibit carcinogenic, teratogenic and mutagenic properties and have now been isolated from a wide variety of agricultural products (Moss, 2002). AFB_1 can enter the food chain mainly by ingestion via the dietary route in humans and animals; the intake of AFB_1 over a long period of time, even at very low concentration, may be highly dangerous (Miraglia et al., 1996).

Due to the demonstrated risk associated with aflatoxins, the current maximum levels for aflatoxins set by the European Commission are 2 ng/g for AFB_1 and 4 ng/g for total aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in corn, groundnuts, nuts, dried fruit and cereals (European Commission, 1998; Gilbert and Anklam, 2002).

Chemical methods such as thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and overpressure-layer chromatography have been those most commonly used for the analysis of aflatoxins in foods and feed (Gilbert, 1999). Several of these methods have been approved by the Association of Analytical Communities (AOAC, 2004; Horwitz, 2000).

Among immunoassay methods, the enzyme-linked immunosorbent method (ELISA) is undoubtedly the most frequently applied. During the past two decades spectrophotometric ELISAs specific for AFB_1 (Dutta and Das, 2000; Kolosova et al., 2006), total aflatoxins (Ayciek et al., 2005; Zheng et al., 2005) and AFM_1 (Yaroglu et al., 2005; Rastogi et al., 2004) have been developed and their simplicity, adaptability and sensitivity have been demonstrated.

To achieve higher sensitivity and move to the use of disposable probes, electrochemical immunosensors based on single screen-printed electrodes have also been recently proposed by our group for the detection of AFB_1 in barley (Ammida et al., 2004) and for AFM_1 in milk (Micheli et al., 2005).

In this paper, a newly available electrochemical immunoplate with multichannel read-out is used in an indirect competitive ELISA format in which a competition between the AFB_1–BSA conjugate (immobilised on the surface of the electrode present into each well of the plate) and the free AFB_1 (standard or sample) occurs for the binding sites of the anti-aflatoxin B_1 antibody (PAb). The amount of PAb bound to the immobilised AFB_1–BSA was signalled using a secondary antibody labelled with alkaline phosphatase (Ab_2–AP). The detection of this marker was in turn accomplished by use of the IPA technique, here optimised with AP together 1-naphthylphosphate as substrate through a voltammetry of 1-naphthol. Finally, an optimisation of all other analytical parameters was performed.

2. Experimental

2.1. Materials and reagents

Polystyrene microtitre plates, MaxiSorp, were purchased from NUNC (Roskilde, Denmark). Anti-aflatoxin B_1 antibody (PAb), aflatoxin B_1–BSA conjugate (AFB_1–BSA), aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), polyvinylalcohol (PVA) and polyoxyethylene sorbitan monolaurate "Tween 20" (Tw20) were from Sigma–Aldrich Co. (St. Louis, MO, USA).

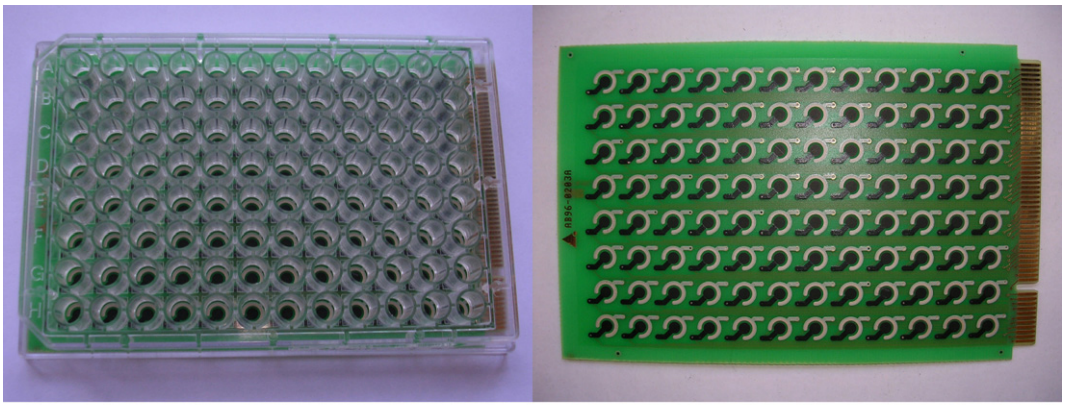
1-Naphthylphosphate-disodium salt (1-NPP), 1-naphthol (1-NP), 4-nitrophenylphosphate (4-NPP), sodium chloride, potassium chloride, magnesium chloride and diethanolamine (DEA) were purchased from Fluka Chemie (Sigma–Aldrich, Milan, Italy). Affinity-purified goat anti-mouse IgG (H + L) alkaline phosphatase conjugate (Ab_2–AP) was from Bio-Rad Laboratories (Hercules, CA, USA). Methanol and *n*-hexane were obtained from Riedel-dehaen (Sigma–Aldrich Laborchemikalien). Corn samples were purchased from local supermarkets.

The 96-well screen-printed microplates were obtained from Alderon Bioscience Incorporated (Durham, USA). Working graphite electrodes (Ø 3 mm) with silver reference electrode, screen-printed on a 0.5 mm plastic substrate, formed the two-electrode system used (Fig. 1a). The plate is connected to the electrochemical reader through a 56 dual positions card edge connector (Fig. 1b).

2.2. Apparatus

A model 550-Microplate Reader (Bio-Rad Labs.) was used to read the absorbance on ELISA plates at 405 nm.

The electrochemical 96-well microplate reader (AndCare 9600) operates using intermittent pulse amperometry. IPA measurement on the AndCare 9600 sensor reader instrument involves a series of millisecond pulses of the same potential



(a) (b)

Fig. 1. Picture of a complete 96-well microplate (a) and of the support where the 96 sensors are printed (carbon and Ag/AgCl) (b) showing also the comb-type connections (on the right site).

applied individually to each of the 96 sensing electrodes. All IPA measurements were carried out at an applied potential of +400 mV with a pulse width of 1 ms and a selected frequency of 50 Hz. The results were recorded on a PC using dedicated software.

Further laboratory equipment includes an Autovortex SA6 (Stuart Scientific, UK), Laboratory blender (Waring Commercial®, USA), a horizontal shaker (Instruments s.r.l, Milan, Italy) and a centrifuge Mod PK 120 (ALC(R)-Tecnochimica Moderna s.r.l., Italy).

2.3. Buffer solutions

The 1 M DEA buffer, pH 9.6, containing 1 mM $MgCl_2$ and 15 mM KCl, was used as the enzymatic substrate buffer for the electrochemical and spectrophotometric measurements.

The 0.1 M carbonate buffer, pH 9.6, was used for the immobilisation of the AFB_1–BSA on the support (coating step). A PVA solution 1% (w/v) in carbonate buffer was used as blocking reagent. A 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 solution, used after each assay step, was prepared by adding 0.05% Tween20 (v/v) to the PBS (PBS-T).

The extraction solvent was prepared by adding methanol (85%) to the PBS (15%).

The AFB_1, AFB_2, AFG_1, AFG_2 stock solutions (1 mg/mL) were prepared in methanol and stored at −20 °C in tightly capped and dark bottles.

2.4. Procedure

2.4.1. Pretreatment procedure

As an initial phase in the assembly of a disposable immunoplate for aflatoxin detection, a pretreatment procedure was tested on the electrochemical plates for its capacity to improve the reproducibility of the electrochemical signal and the amount of the reagents to be immobilised on the surface of the screen-printed electrodes. It consisted in a washing step using different solutions (distilled water, phosphate saline buffer, phosphate saline buffer and Tween20) followed by an evaluation of the IPA results obtained using 1 mM of 1-naphthol. Each measurement was repeated using an entire row of the immunoplate (8 replicates).

2.4.2. Choice of the applied potential

In order to establish the most suitable working potential for the determination of the enzymatic product of alkaline phosphatase, 1-naphthol, we carried out current measurements in the potential range between 0 and +600 mV. The hydrodynamic experiments were performed by adding 100 μL of 1-naphthol solutions (1 and 0.01 mM) onto the screen-printed electrode present on the bottom of each microplate well. The same test was performed using a solution of the enzymatic substrate alone, α-napthylphosphate, in order to test for its non-electroactivity. Each measurement was repeated in triplicate.

2.4.3. Calibration plot for 1-naphthol

With the optimised conditions, a calibration plot for 1-naphthol was obtained in the concentration range 0.01–600 μM. Following the addition of 100 μL of the enzymatic product solution, the potential (+400 mV) was applied and an oxidation current was recorder within a few seconds (not more than 15 s) for the all microplate. All measurements were repeated in triplicate.

2.4.4. Procedure for spectrophotometric ELISA

Prior to the analysis by multichannel electrochemical immunoplate (MEI), all the work was also characterised by competitive enzyme immunoassays performed in conventional microplates with spectrophotometric detection during the development phase. The indirect ELISA was performed according to the procedure described in Ammida et al. (2004), with minor modifications such as in the characteristic of specific antibody used during the competition step. The plate was coated with AFB_1–BSA conjugate (1 μg/mL, 50 μL/well) in CB, pH 9.6, overnight at 4 °C, then blocked with 1% PVA (50 μL/well) for 1 h at room temperature. Finally, 50 μL of a mixture of aflatoxin solution (prepared in PBS) and an equal volume of PAb

(1:2000, v/v, in PBS) were added to each well. The competition was then performed for 30 min at room temperature. After that, 50 μL of Ab_2–AP (1:1000, v/v) in PBS were added to the wells and incubated for 1 h. Between each step, a three-cycle washing procedure, twice with 200 μL of PBS-T and once with 200 μL of PBS, was adopted.

Finally, the activity of the enzyme label was determined using 50 μL of 4-NPP substrate solution (2 mg/mL of *p*-NPP in DEA buffer) after a 30 min reaction at room temperature with the absorbance being measured at 405 nm.

2.4.5. Procedure for 96-well electrochemical immunoplate (MEI)

The screen-printed wells were pre-wetted with 200 μL of deionised water prior to the coating deposition. The surfaces of the screen-printed electrodes were then coated with 80 μL of AFB_1–BSA (1 μg/mL) and incubated overnight at 4 °C. The well surfaces were then blocked by adding 80 μL of PVA (1%) and incubated for 30 min at room temperature. To perform the competition step, AFB_1 standards were mixed with an equal volume of PAb solution (1:2000, v/v). The 80 μL of this mixture was added into each well of the microplate and allowed to react with the coated AFB_1–BSA for 15 min at room temperature. Next, 80 μL of Ab_2–AP (1:1000, v/v) was added and the wells were incubated for 15 min at room temperature. After each step (coating, blocking, competition and labelling) three washing steps were carried out, twice with PBS-T and once with only PBS. Finally, 80 μL of 1-NPP substrate (1 mg/mL of 1-NPP in DEA buffer) was added into each well and allowed to react for 1 min at room temperature. The amount of enzymatic product (1-naphthol) formed was detected by IPA.

2.5. Calibration graphs

Standard curves were obtained using standard solutions of AFB_1 (0–100 ng/mL) prepared in PBS. The standard curves were fitted using "non-linear 4 parameter logistic calibration plots" (Warwick, 1996). The four parameter logistic function is:

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

where a and d are the asymptotic maximum and minimum values, respectively; c the value at the inflection point (IC_{50}) and b is the slope.

To allow the direct comparison of calibration curves, current values were converted into their corresponding test inhibition values (I/I_0 %) as follow:

$$\frac{I}{I_0}(\%) = \frac{I - I_{sat}}{I_0 - I_{sat}} \times 100$$

where I is the current response at certain concentration of analyte, I_{sat} and I_0 are the current values corresponding to the saturating and the non-competition concentration of analyte, respectively (Crowther, 1995).

The detection limit (LOD) was defined as the decrease of the maximum signal equal to three times the value of the standard deviations ($I_0 - 3$S.D.), measured in the absence of AFB_1 (Law and Biddlecombe, 1996). The working range was evaluated as the toxin concentration that gave test inhibition values of 90 and 10% of I/I_0 (Giraudi et al., 1999).

Cross-reactivity (%CR) for different aflatoxins was determined by performing competitive assays and comparing the analyte concentration that resulted in half-maximum inhibition (IC_{50}, ng/mL), and calculated as:

$$\%CR = \left(\frac{IC_{50} \text{ for } AFB_1}{IC_{50} \text{ for analyte}}\right) \times 100$$

2.6. Corn sample preparation and extraction

Non-infected corn kernels (from local markets) were first ground in a household blender at high speed for 1 min. A 5 g ground corn sample was spiked with 100 μL of AFB_1 standard solution in order to have fortified samples with the following concentrations: 2.5, 12.5 and 50 ng/g. The spiked samples were fully mixed using an auto-vortex for 1 min and extracted with 25 mL of extraction solvent (85% methanol in PBS) by shaking for 45 min. Then the extract was separated from the insoluble materials by centrifugation for 10 min at 6000 rpm. A 2 mL of the extract was diluted five times with PBS. The suspension, defatted one time with 5 mL *n*-hexane, was then hand shaken for 5 min. After the separation of the two layers, the aqueous layer was used for AFB_1 detection by the MEI procedure.

The concentration of AFB_1 in diluted sample extracts was determined from the ELISA calibration curve and used to calculate the concentration in the original sample according to the following equation:

$$AFB_1(ng/g) = \left\{\frac{[AFB_1 \text{ (ng/mL) in sample extract}][\text{Solvent extract volume}]}{\text{Sample weight}}\right\} \times \text{Dilution factor}$$

where the volume of solvent extract is 25 mL, sample weight 5 g and the dilution factor is 5.

3. Results and discussion

3.1. MEI parameters

While the 96-well carbon sensor microplate device has been designed for use with HRP, it was here used for an immunoassay using alkaline phosphatase as marker enzyme because, from the electrochemical point of view, the latter gives a better signal than HRP (Khatkhatay and Desai, 1999). Before running the study to optimise the immunoassay parameters, amperometric experiments were carried out in order to evaluate the optimal conditions for measurement of the enzymatic activity of AP. These studies used both α-naphthylphosphate (1-NPP) and 1-naphthol (1-NP), substrate and product of the enzymatic reaction, respectively.

It was found that the reproducibility of the 1-naphthol electrochemical measurements could be improved by washing procedures to remove unwanted substances remaining on the plates. Different washing procedures of the multichannel plate have been investigated. Results showed that when the electrodes were washed with distilled water, the reproducibility (2%) improved compared with no washing step (10%). Other washing solutions (water-T, PBS, PBS-T) were also tested in this study, but without satisfactory results.

In order to determine the best conditions for electrochemical detection of 1-naphthol, the current measurements at different potentials (0 to +600 mV) were performed to find the most suitable applied potential value. On the basis of this series, +400 mV versus Ag pseudo-reference electrode was chosen as a working potential for IPA measurements of 1-naphthol. In fact, Fig. 2 shows results obtained for different concentrations of 1-NP and 1-NPP, and the oxidation current obtained by applying the selected potentials to the electrodes. It can be noted that 1-NPP is not electroactive over the entire applied potential range, while little or no oxidation current is evident for 1-NP if the applied potential is lower than +200 mV, these voltages being insufficient to oxidize 1-NP. At potentials higher than +200 mV, an increase in the oxidation current is evident for each of the 1-NP concentrations used (1 and 0.01 mM), and currents increased with the increase in applied potential up to +400 mV. Because no further current increase occurs at higher potentials, this latter was selected as the optimal potential chosen for further analysis.

Next, the calibration plot for 1-NP was obtained (Fig. 3) using sequential dilutions (0.01–600 μM) of the enzymatic product. The linear range was found to be between 0.5 and 200 μM and the calibration curve ($y = 0.014x + 0.134$, $r^2 = 0.991$) showed a detection limit of 0.1 μM of 1-NP and a reproducibilty of 3%. These results are comparable with those obtained using different electrochemical detection techniques such as the differential pulse voltammetry (DPV), which in any case requires more complicated instrumentation (Del Carlo et al., 1997). Moreover, the

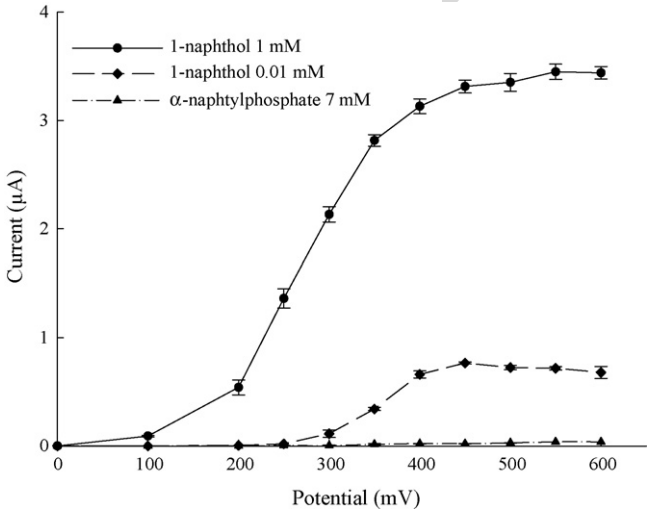


Fig. 2. Choice of the applied potential using 1-naphthol solutions 1 mM (●), 10 μM (♦) and 1-naphtylphosphate 7 mM (▲) in DEA pH 9.6, voltage range 0 to +600 mV.

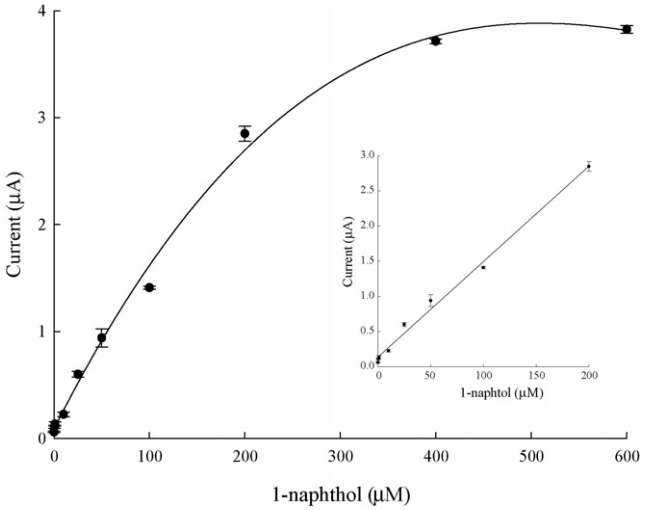


Fig. 3. Calibration curve of 1-naphthol between 0 and 600 μM in DEA pH 9.6 using IPA. Linear range: 0.5–200 μM, calculated detection limit 0.1 μM. Applied potential +400 mV vs. int. ref.

IPA measurement is very rapid with the reading of the entire plate (96 sensors) requiring no more than 15 s.

3.2. Optimisation of MEI for AFB_1 detection

To evaluate the effect of the concentration of PAb on the assay performance, standard competition curves for AFB_1 have been generated using four different dilutions of PAb (1:500, v/v; 1:1000, v/v; 1:2000, v/v and 1/3000 v/v).

The best conditions for the running of an indirect competition assay were found to be as follows: a concentration of AFB_1–BSA 1 μg/mL for overnight incubation time at 4 °C, a dilution of PAb 1:2000, v/v for a 15 min incubation time at room temperature, a dilution of Ab_2–AP 1:1000, v/v for 15 min at room temperature and a concentration of the 1-NPP as substrate of 1 mg/mL for a 50 s incubation time on the microplate.

The standard MEI curve using these optimised conditions indicated a high sensitivity; it was possible to detect a concentration range of 0.05–2 ng/mL with a detection limit of 0.03 ng/mL. The intra-electrode reproducibility (expressed as %R.S.D.) was 5% (for 10 replicates), while the inter-assay repeatability of the MEI was 6% (three replicates of each point of the calibration curve performed in four different days).

A standard spectrophotometric calibration curve for AFB_1 in buffer was also generated, and had a working range of 0.25–2 ng/mL with a detection limit of 0.15 ng/mL. Table 1 summarizes the analytical characteristics of the spectrophotometric and electrochemical calibration curves for AFB_1. The results show that the MEI format provides a significant improvement by achieving higher sensitivity, as well a shorter analysis time compared to the spectrophotometric ELISA, while the features are comparable to those reported in our previous working using single SPEs. However, it has to be emphasized that the 96-well plate has advantages relative to the same assay using an individual SPE since it is more of user-friendly and provides the possibility of carrying out many experiments in parallel, greatly

Table 1
Analytical parameters of the spectrophotometric ELISA and multichannel electrochemical immunoassay (MEI) for aflatoxin B_1 detection

Analytical parameters	Spectrophotometric detection	MEI
Detection limit (ng/mL)	0.15	0.03
Dynamic range (ng/mL)	0.25–2.0	0.05–2.0
IC_{50} (ng/mL)	0.77	0.26
R.S.D., intra-assay (%)[a]	4	5
R.S.D., inter-assay (%)[b]	6	6
Assay time (h)	3	1

[a] $n = 8$.
[b] 4 days ($n = 3$), see text for details.

reducing the time necessary to develop new procedures, the total time for a complete assay (calibration and sample analysis) is much shorter and, in addition, one can make use of various automatic devices (for pipetting, washing, etc.) that simplify and reduce the labour involved.

3.3. Cross-reactivity of PAb to aflatoxins

The cross-reactivity of this immunoassay format was studied using other mycotoxins such as AFB_2, AFG_1 and AFG_2. In this study, the immunoplate was used as an array, with addition of the different aflatoxins in different rows of the same immunoplate, and carrying out the calibration curves for all the cross-reactants simultaneously. For each cross-reactant the corresponding IC_{50} was calculated, and the relative cross-reactivity of the tested toxins is presented in Table 2, taking the IC_{50} value for AFB_1 as reference. The results of the study showed that PAb was relatively specific for AFB_1 with cross-reactivity for AFG_1 of 50%, while this parameter was only about 10% for AFG_2 and AFB_2. Spectrophotometric studies of PAb cross-reactivity were also carried out and gave comparable results (Table 2). The results reported here indicate the possibility of using the same assay for the determination of AFB_1 and AFG_1 simultaneously.

3.4. Stability of immunosensor plate

The stability of the coating reagent was evaluated using the 96-well multichannel immunoplate coated with AFB_1–BSA conjugate, blocked and then stored at 4 °C. A parallel investigation was done by treating the modified plate (coating and blocking) for 1 h at room temperature with 80 μL of ProClin 200 preservative (Supelco). The microplate was 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 the antibody binding. Assays were performed periodically over 1 month period, using the assessed protocol.

Results (not shown) demonstrated that the coated electrodes could be used for up to 1 month after their preparation; the maintenance of 100% of the activity indicated that the lifetime of the coated electrodes could be even longer; while the ProClin preservative had no effect on the immunosensor stability over 1 month period. Similar results have been obtained by Micheli et al. (2004), using disposable screen-printed electrodes.

Table 2
Cross-reactivity % of polyclonal antibody (PAb) to different aflatoxins obtained with multichannel electrochemical immunoassay (MEI) and spectrophotometric ELISA

	% of response	
Aflatoxins	MEI	Spectrophotometric detection
AFB_1	100	100
AFB_2	10	11
AFG_1	56	54
AFG_2	9	10

3.5. Measurements of AFB_1 in corn

The electrochemical immunoplate was then applied to the detection of AFB_1 in corn to test its performance in a real matrix. Corn samples were collected and the extraction procedure was performed as described in the experimental section to evaluate the matrix effect and the extraction efficiency.

The indirect competitive assay required water-soluble reagents for mixing sample extracts with the PAb. Methanol, a water miscible solvent, has been previously used to extract AFB_1 from contaminated agricultural samples (Ram et al., 1986; Chu et al., 1987). In this work, the extraction solvent consisted in a mixture of 85% methanol and 15% PBS.

The matrix effect was evaluated using non-infected (toxin free) corn samples. In Fig. 4, the standard curves of AFB_1 in PBS (●) were compared with the calibration curve, obtained by adding different concentrations of AFB_1 (0–100 ng/mL) to 1:5 (v/v) diluted blank corn extract, after the extraction procedure (■) and to extraction solvent alone (▲). The results indicated

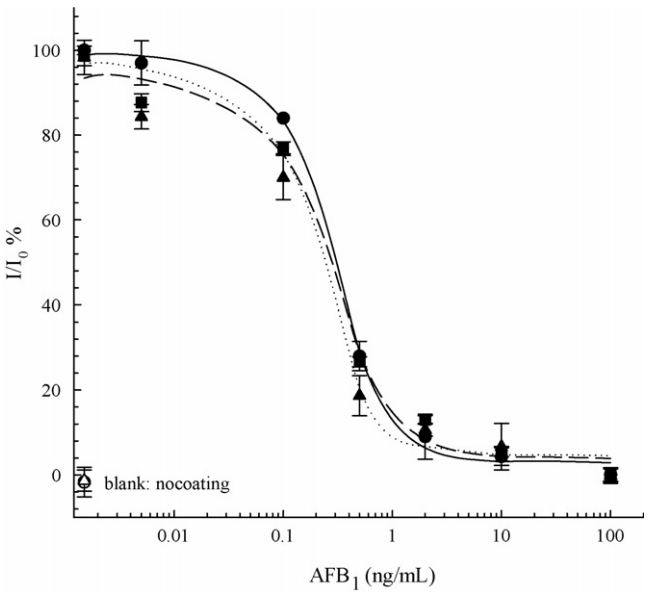


Fig. 4. Effect of corn extract on the standard curve of AFB_1 detected by MEI. Standard solution of AFB_1 prepared in PBS (●), in extraction solvent (▲) and in non-infected corn extracted (■) diluted 1:5 (v/v) with PBS. The concentration of AFB_1–BSA was 1 μg/mL, the dilutions of PAb and Ab_2–AP were 1:2000 and 1:1000 (v/v), respectively. The current response detected by IPA (applied potential +400 mV vs. int. ref.), using 1-NPP (1 mg/mL) as substrate for Ab_2–AP.

Table 3
Extraction efficiency obtained for the MEI using three fortified samples

AFB_1 added (ng/g)	AFB_1 found (ng/g)	R.S.D. (%)	Recovery (%)
2.5	2.8	7	110
12.5	11.0	10	88
50.0	55.5	8	111

Each value is the mean of 10 measurements (5 replicates of spiked samples, each replicate analysed twice using two different electrochemical wells).

little matrix effect, with the working range being comparable to the one obtained in buffer.

After assessing the matrix effect, the recovery study was performed by spiking non-infected ground corn samples with AFB_1 standard at three different levels (2.5, 12.5 and 50 ng/g), that after sample treatment (as reported in Section 2.6) giving rise to overall AFB_1 concentrations 0.1, 0.5 and 2 ng/mL, respectively. For each concentration, five different samples were independently processed and analysed using 10 different microtitre wells. On the basis of the calibration curves prepared in extracted corn, it was possible to calculate the recovery of the analyte, which ranged from 88 to 111% of AFB_1, with an average of (103 ± 8) %. The precision was determined by calculating the relative standard deviation (%R.S.D.) for the replicate measurements. The results are summarized in Table 3.

4. Conclusion

In this work, it was demonstrated that newly available device utilising a 96-well screen-printed microplate, could be successfully used for an indirect competitive electrochemical ELISA for the detection of aflatoxin B_1 in corn with significant gains in overall performance. Using the plates with IPA, as the read-out technique, the system was adapted for the detection of 1-naphthol, the enzymatic product of alkaline phosphatase used as the antibody label.

This study shows that the electrochemical immunoplate is a potentially very useful device, given that 96 electrochemical sensors form the bottom of a 96-well plate and the multichannel read-out can be performed virtually simultaneously. This offers the unique possibility to combine the high sensitivity of electrochemical SPE-based immunosensors with the favourable characteristics of high throughput ELISA procedures. These advantages consist in rapidity of analysis and the possibility to carry out the calibration and the analysis of several unknown samples (in replicate) at the same time. In this way it becomes possible to overcome the most significant drawback of electrochemical disposable immunosensors, which attaches to the fact that they have to be handled singly, thus increasing the time of the overall analysis. Given the costs of the currently available materials, the MEI array also results in a cost/electrode 1/5 that for individual SPE produced by thick film technology.

Moreover, the MEI format allows the creation of a multianalyte array, by immobilising in different rows or columns the various antibodies for different analytes.

Acknowledgements

The authors wish to thank the MIPAF project 'AFLARID' and EUROLAB S.r.l. for financial support.

We gratefully thank Dr. Nancy Downer for her suggestions and skilful collaboration in revising the English manuscript.

References

Ammida, N.H., Micheli, L., Palleschi, G., 2004. Anal. Chim. Acta 520, 159–164.
AOAC International Performance Tested Methods, 2004. Toxin tests kits (www.aoac.org).
Athey, D., Ball, M., McNeil, C., 1993. Ann. Clin. Biochem. 30 (6), 570–577.
Ayciek, H., Aksoy, A., Saygi, S., 2005. Food Control 16, 263–266.
Chu, F., Ian, T., Zhang, G.-S., Xu, Y.C., Susan, F., McMahon, P., 1987. J. Assoc. Off. Anal. Chem. 70, 854–857.
Cole, R.J., Cox, R.H., 1981. Handbook of Toxic Fungal Metabolietes. Academic Press, New York.
Crowther, J.R., 1995. Theory and Practice. Humana Press, Totowa, New Jersy, USA.
Del Carlo, M., Lionti, I., Taccini, M., Cagnini, A., Mascini, M., 1997. Anal. Chim. Acta 342, 189–197.
Dutta, T.K., Das, P., 2000. Mycopathologia 15, 29–33.
Edward, R., 1997. In: Price, C.P., Newman, D.J. (Eds.), Principles and Practice of Immunoassay. Macmillan Regerence Ltd., New York, pp. 325–348.
European Commission, Commission Regulation (98/53/EC) 1998. Official Journal of European Communities 1998 L201/93.
Gilbert, J., 1999. Nat. Toxins 7, 347–352.
Gilbert, J., Anklam, E., 2002. Trends Anal. Chem. 21, 468–486.
Giraudi, G., Rosso, I., Baggiani, C., Giovanoli, C., Vanni, A., Grassi, G., 1999. Anal. Chim. Acta 392, 85–94.
Horwitz, W., 2000. Official Method of Analysis of AOAC International, 17th ed. AOAC International, Gaithershurg, MD.
International Agency for Research on Cancer, 1993. IARC Monographs on the Evaluations of Carcinogenic Risks to Human, vol. 56. IARC, Lyon, pp. 489–521.
Khatkhatay, M.I., Desai, M., 1999. J. Immunoassay 20 (3), 151–183.
Kolosova, A.Y., Shim, W.B., Yang, Z.Y., Eremin, S.A., Chung, D.H., 2006. Anal. Bioanal. Chem. 384, 286–294.
Law, B., Biddlecombe, R., 1996. In: Law, B. (Ed.), Immunoassay A Practical Guide. Taylor and Francis, London, pp. 171–183.
Micheli, L., Radoi, A., Guarina, R., Massaud, R., Bala, C., Moscone, D., Palleschi, G., 2004. Biosens. Bioelectron. 20, 190–196.
Micheli, L., Grecco, R., Badea, M., Moscone, D., Palleschi, G., 2005. Biosens. Bioelectron. 21, 588–596.
Miraglia, M., Brera, C., Colatosti, M., 1996. Microchem. J. 54, 472–477.
Moss, O.M., 2002. Int. Biodeteior. Biodegrad. 50, 137–142.
Ram, B., Hart, P., Shotwell, O., Pestka, J., 1986. J. Assoc. Off. Anal. Chem. 69, 904–906.
Rastogi, S., Divedi, P.D., Khanna, S.K., Das, M., 2004. Food Control 15, 287–290.
Skládal, P., Kaláb, T., 1995. Anal. Chim. Acta 316, 73–78.
Tang, T.C., Deng, A., Huang, H.J., 2002. Anal. Chem. 74, 2617–2621.
Warwick, M.J., 1996. In: Brian, L. (Ed.), Immunoassay, A Practical Guide. Taylor & Francis Ltd., London, UK, pp. 150–170.
Wojciechowski, M., Sundseth, R., Moreno, M., Henkens, R., 1999. Clin. Chem. 45 (9), 1690–1693.
Yaroglu, T., Oruc, H.H., Tayar, M., 2005. Food Control 16, 883–885.
Zheng, Z., Humphney, C.W., King, R.S., Richarad, J.L., 2005. Mycopathologia 159, 1–9.