RAPID SCREENING ELECTROCHEMICAL METHODS FOR AFLATOXIN B₁ AND TYPE-A TRICHOTHECENES: A PRELIMINARY STUDY.


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Rapid screening for AFB1, T-2 and HT-2

ABSTRACT

In this work are presented methods for detection of Aflatoxin B1 and type-A trichothecenes, based on the use of indirect competitive ELISA format coupled with a 96-well screen-printed microplate.

Electrochemical immunoassays for AFB1, T-2 and HT-2 were performed and the activity of the alkaline phosphatase or horseradish peroxidase labelled enzymes were measured using Intermittent Pulse Amperometry as electrochemical technique.

Using standard solutions of the target analyte the LOD of the assays were 0.3 and 0.2 ng mL^{-1} for T-2 and AFB1 respectively, while the sensitivity was 1.2 ng mL^{-1} for both.

For Aflatoxin B1, a stability study of electrochemical plate was also carried out. Moreover, the matrix effect was evaluated using two different extraction treatments from corn.

Keywords: Aflatoxin B1, Type-A trichothecene, 96-well screen-printed microplate, competitive ELISA.
1. INTRODUCTION

Mycotoxins are low-molecular-weight natural products produced as secondary metabolites by filamentous fungi. Aflatoxins, trichothecenes, zearalenone, fumonisins, and patulin, are the most important for their public health and agroeconomic significance (Bennett and Klich 2003).

Aflatoxin B$_1$ (AFB$_1$), the most intensively studied among the mycotoxins (because of its carcinogenic effects), is a metabolite mainly produced by the fungi *Aspergillus flavus* and *A. parasiticus*. For this toxin, the European Commission set the maximum level for AFB$_1$ in food to 2 ng g$^{-1}$ (European Commission 1998).

Mainly produced by *Fusarium* fungi, which infect cereals in the field, Trichothecenes have been associated with outbreaks of diseases both in humans and in farm animals. They are often classified as Group A and Group B compounds depending on whether they present a side chain on the C7 atom. Most important Trichothecenes are T-2 and HT-2 for group A and DON and NIV for group B. For type A trichotheccene the maximum levels in food have not been still established, but the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has concluded that the toxic effects of T-2 toxin and its metabolite HT-2 toxin could not be differentiated, and that the toxicity of T-2 ‘in vivo’ might be due at least partly to toxic effects of HT-2. Hence, HT-2 was included in the provisional maximum tolerable daily intake (PMTDI), fixed at 60 ng Kg$^{-1}$ body weight per day of T-2 and HT-2 toxins, alone or in combination (Joint FAO/WHO Expert Committee on Food Additives, 2001).
Contamination of cereals and related products with mycotoxins causes food- and feed-borne intoxication and carcinogenic effects in humans and farm animals (Krska et al. 2001).

Commonly used methods for Trichothecenes and Aflatoxin detection include separating techniques coupled to different detectors such as GC/FID, GC/ECD, GC/MS, HPLC/UV, HPLC/FL, HPLC/MS (Schothorst and Jekel 2003; Milanez et al. 2006; Klotzel et al. 2006; Jaimenez et al. 2000; Stroka and Anklam 2002). However, these methods require specialized personnel and are very expensive. Several alternative methods have been recently proposed for a rapid detection of these contaminants including thin layer chromatography (Gilbert 1999; Snyder 1986), enzyme linked immunosorbent assay (Piermarini et al. 2006; Ammida et al. 2004; Lee et al. 2004; Kawamura et al. 1990; Ridascreen) and flow through immunoassays (Sibanda et al. 2000) (only for trichotecenes).

‘Rapid tests’ for mycotoxin analysis have been increasingly demanded by analytical community, especially during the last decade.

This paper is focused on the development of Multichannel Electrochemical Immunosensors (MEI), using a 96-well screen-printed microplate, for AFB\textsubscript{1}, T-2 and HT-2 detection. Preliminary studies are presented. A competitive indirect scheme for detection of the target analytes was performed using monoclonal antibodies. At the end of all immunological reactions, the enzymatic substrates (TMB or 1-NP), used
Rapid screening for AFB₁, T-2 and HT-2

for signal generation, were added in each well and their conversion to electroactive products was measured using Intermittent Pulse Amperometry (IPA) technique.

2. EXPERIMENTAL

2.1 Reagents and materials

Polystyrene microtitre plates, MaxiSorp, were purchased from NUNC (Roskilde, Denmark). Mouse monoclonal antibody against aflatoxin B₁ (MAb) 5.4 mg mL⁻¹ was from HyTest Ltd (Finland). aflatoxin B₁, aflatoxin B₁-Bovine Serum Albumine conjugate (AFB₁-BSA), polyvinylalcohol (PVA), polyoxyethylene sorbitan monolaurate “Tween 20” (Tw20), 3,3’,5,5’-tetramethylbenzidine were from Sigma-Aldrich Co. (St. Louis, MO, USA). T-2-Keyhole Limpet Hemocyanin (T-2-KLH, 0.5 mg mL⁻¹), mouse monoclonal antibody against T-2 toxin (clones: T-2C6 0.39 mg mL⁻¹, T-2B2 0.5 mg mL⁻¹, T-2B1 0.7 mg mL⁻¹), were kindly supplied by partners of the BIOCOP project, while T-2, HT-2, DON and NIV were purchased from Biopure (Tulin, Austria).

1-naphthylphosphate-disodium salt (1-NP), sodium chloride, potassium chloride, magnesium chloride and diethanolamine (DEA) were provided by Fluka Chemie (Sigma-Aldrich, Milan, Italy). Affinity-purified goat anti-mouse IgG (H+L) alkaline phosphatase conjugate (Ab₂-AP) and nonfat dry milk blotting grade were from Bio-Rad Laboratories (Hercules, CA, USA). Peroxidase anti-mouse IgG (H+L) affinity-purified (Ab₂-HRP, 1 mg mL⁻¹) was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Methanol and n-hexane were obtained from Riedel-dehaen (Sigma-Aldrich
Lavorchemikalien). Corn samples were purchased from local supermarkets. Glass microfiber filter (pore size 1.0 \(\mu\)m) from Whatman International (Ltd Maidston England) and syringe filter glass fibre Acrodisc (1.0 \(\mu\)m, 37 mm), Pall Life Science, from Sigma Aldrich.

### 2.2 Apparatus

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

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.

The electrochemical 96-well microplate reader (AndCare 9600) operates using Intermittent Pulse Amperometry. IPA measurement on the AndCare 9600 sensor reader (Fig. 1b) instrument involves a series of millisecond pulses of the same potential applied individually to each of the 96 sensing electrodes.

By using Ab2-AP and 1-NP (as enzymatic substrate), 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. By using Ab2-HRP and TMB (as enzymatic substrate), the applied potential was –100 mV, pulse width and frequency were 10 ms and 5 Hz, respectively.

The results were recorded on a PC using dedicated software.
Rapid screening for AFB₁, T-2 and HT-2

Further laboratory equipment includes an Autovortex SA6 (Stuart Scientific, UK), a high-speed blender Osterizer (Sunbeam), 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 Procedures

2.3.1 T-2, HT-2: procedure for spectrophotometric ELISA

The microtitre plate wells were coated overnight by incubation at 4 °C with 200 µL of conjugate T-2-KLH solution (2 µg mL⁻¹ for T-2B1 clone, 5 µg mL⁻¹ for T-2B2 and T-2C6 clones) in pH 9.6 carbonate buffer solution, and then blocked (1 h at RT=room temperature) with a solution of 3% dry milk.

After blocking, a one step competition was performed as following: 100 µL of standard solutions were added in triplicate to the wells, followed by 100 µL of MAb anti-T-2 diluted 1:1000 v/v, 1:1000 v/v, 1:100 v/v for T-2B1, T-2B2, T-2C6 clones, respectively. The competition was performed for 2 h at RT. The antibody-toxin-KLH complex was revealed using affinity purified peroxidase-labelled goat anti-mouse IgG (200 µL/well) diluted 1:1000 v/v (1 h at RT). Between each step (coating, blocking and competition) a three-cycle washing procedure using 0.05% Tween 20 in PBS (PBS-T) was adopted.

Each solution, except the coating and blocking ones, was prepared in PBS. Finally, a stock solution of TMB (20 mM) in DMSO was prepared and 200 µL of substrate solution (3 x 10⁻⁴ M TMB + 10⁻³ M H₂O₂ in 0.05 M citrate phosphate buffer + 0.1 M
KCl, pH = 5.0) were added to each well; the enzymatic reaction was stopped after 5 min by adding 50 µL of 2 M H$_2$SO$_4$, and the absorbance was read at 405 nm.

2.3.2 T-2: MEI procedure

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 T-2-KLH (2 µg mL$^{-1}$ in carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The well surfaces were then blocked by adding 80 µL of 1% dry milk (prepared in carbonate buffer) and incubated for 15 min at RT.

A one step competition was performed, using only the clone T-2B2 (selected in the spectrophotometric assay), as following:

40 µL of anti-T-2 MAb (1:1250 v/v in PBS) and T-2 standard solutions (prepared in PBS) were added into each well and the competition, between free and coated analyte, was performed for 30 min at RT. The antibody-toxin-KLH complex was revealed using Ab$_2$-HRP (80 µL/well) diluted 1:500 v/v (30 min at RT). Between the coating, blocking, competition and labelling, three washing steps were carried out, twice with PBS-T and once with only PBS.

Each solution, except the coating and blocking ones, was prepared in PBS. Finally, 80 µL of a substrate solution, prepared as reported in the spectrophotometric procedure, was added to each well and the current was recorded after 2 min at RT.

The amount of the enzymatic product (TMB$_{red}$) formed was detected by IPA, at an applied potential of −100 mV.
2.3.3 Aflatoxin B₁: MEI procedure

Also in this case, the screen-printed wells were pre-wetted. The surfaces of the screen-printed electrodes were then coated with 80 µL of AFB₁-BSA (1 µg mL⁻¹ in carbonate buffer, pH 9.6) and incubated overnight at 4°C. The well surfaces were then blocked by adding 80 µL of 1% PVA (prepared in carbonate buffer) and incubated for 30 min at RT.

A two-step competition was performed as following:

In the first step, equal volumes of anti-AFB₁ MAb diluted 1:13500 v/v in PBS (which corresponds to a concentration of 0.4 µg mL⁻¹) and standard AFB₁ solutions were mixed and allowed to react at RT for 10 min. In the second step, 80 µL of this mixture were added into each well of the microplate and the competition, between free and coated analyte, was performed for 15 min at RT. The labelling step was carried out by adding 80 µL of Ab₂-AP (1:100 v/v in PBS) and incubating for 5 min at RT. After each step, three washings were carried out such as in T-2 MEI procedure. Finally, 80 µL of 1-NP substrate (5 mg mL⁻¹ in 0.97 M DEA buffer + 1 mM MgCl₂ + 0.15 M KCl, pH 9.8) were added into each well and allowed to react for 1 min at RT. The amount of enzymatic product (1-naphthol) formed was detected by IPA, at an applied potential of + 400 mV.

2.4 Calibration graphs

The standard curves were fitted using “non-linear 4 parameter logistic calibration plots” (Warwick, 1996). The four parameter logistic function is:
Rapid screening for AFB\textsubscript{1}, T-2 and HT-2

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

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

The detection limit (LOD) was defined as the concentration corresponding to the \(f(x)\) value obtained by subtracting three standard deviations of zero point (no analyte) from the mean of the zero point measurements (mean value-3sd) (Law and Biddlecombe, 1996).

Cross-reactivity of the antibodies (anti-AFB\textsubscript{1} and anti-T-2) against other mycotoxins was used to determine the specificity of the assays. Cross-reactivity was defined as 100 \(x/y\), where \(x\) is the amount of the target analyte and \(y\) is the amount of interfering mycotoxins required to produce 50\% inhibition of the binding between MAb and coated-toxin (AFB\textsubscript{1}-BSA, T-2-KLH).

2.5 Corn sample treatment

Two different corn sample treatments were carried out and only the matrix effect on the performance of the MEI system was evaluated.

Non-infected corn kernels were first ground in a household blender at high speed for 1 min.

The first procedure employed for sample treatment (S. Piermarini et al. 2006) is the following:

- weight 10 g of corn powder;
Rapid screening for AFB₁, T-2 and HT-2

• add 50 mL of extraction solvent (85% methanol in PBS) and mix for 45 min in a horizontal shaker;
• centrifuge at 6000 rpm for 10 min;
• dilute 2 mL of the supernatant with 8 mL of PBS;
• defatted for 5 min with 5 mL n-hexane;
• after separation of the two layers, the aqueous layer was recovered.

The second procedure, employed for sample treatment, was a modification of that reported by the kit “I’screen AflaB₁ gold” (Tecna, Trieste, Italy):

• weight 50 g of corn powder;
• add 5 g of NaCl;
• add 100 mL of a solution of methanol 80% in distilled water and mix thoroughly for 3 min a high-speed blender;
• filter (glass microfiber filter pore size 1.0 µm);
• mix 2 mL of filtrate with 8 mL of PBS;
• filter (syringe filter glass fibre Acrodisc, 1.0 µm).

In order to evaluate the matrix effect, at the end of both procedures, standard solutions of AFB₁ were added to sample extracts and analysed by the MEI system.
3. RESULTS AND DISCUSSION

3.1 Type-A Trichothecenes detection.

3.1.1 Spectrophotometric assay

Because the JECFA has fixed a provisional maximum tolerable daily intake of 60 ng Kg$^{-1}$ body weight per day for T-2 and HT-2 toxins, alone or in combination, it is important to develop a system for the simultaneous detection of both toxins.

For this reason, a spectrophotometric test using different monoclonal antibodies anti-T-2 toxin (produced by three different clones) has been accomplished in order to select those with the greatest cross-reactivity for HT-2 toxin.

For each clone, binding curves using different concentrations (1, 2 and 5 µg mL$^{-1}$) of the coated molecule (T-2-KLH) and serial dilutions of MAb (1:100 – 1:50000 v/v) were constructed in order to fix the best conditions for the competitive ELISA. These experiments were performed as reported in the section 2.3.1 but, in this case, only 200 µL of MAb in PBS (instead of 100 µL of MAb and 100 µL of free analyte) were added in triplicate to the wells. From the binding curves (data not shown) it was established that 2 µg mL$^{-1}$ (for T-2B1 clone) and 5 µg mL$^{-1}$ (for T-2B2 and T-2C6 clones) of T-2-KLH gave the best absorbance signals; while for MAb anti-T-2 dilutions of 1:2000 v/v, 1:2000 v/v, 1:200 v/v (for T-2B1, T-2B2, T-2C6 clones, respectively), corresponding to the inflection points of the three binding curves, were selected.
Typical sigmoid calibration curves for T-2 toxin, obtained with the three clones, are reported in Fig. 2.

The detection limit was calculated to be 0.7, 0.6 and 0.3 ng mL\(^{-1}\) for T-2B1, T-2B2 and T-2C6 respectively. Sensitivities, calculated as the amount of T-2 toxin to produce a 50% decrease in the signal were 1.8, 2.3 and 1.0 ng mL\(^{-1}\) for T-2B1, T-2B2 and T-2C6, respectively. The analysis time was about 4.5 h.

The cross-reactivity of the three MAb clones for HT-2 and the most common type-B trichothecenes (DON and NIV) was also evaluated, carrying out the calibration curves for all toxins. For each cross-reactant the corresponding amount required to produce 50% inhibition of the binding was calculated, and the relative cross-reactivity of the tested toxins is presented in Table 1. As shown the T-2B2 clone gave the highest cross-reactivity (62%) towards HT-2 toxin while no appreciable interaction with type-B trichothecenes was observed. For this reason, T-2B2 clone was selected to develop the MEI system.

3.1.2 MEI assay

Also in this case binding curves were constructed using different dilutions of the selected MAb (T-2B2 clone) and two different concentrations of the coated molecule (T-2-KLH) in order to establish the best conditions for the competitive indirect ELISA.

These experiments were performed as reported in section 2.3.2 but, in this case, 80 µL of MAb in PBS (instead of 40 µL of MAb and 40 µL of free analyte) were added
in triplicate to the wells. Also, to evaluate the non-specific adsorption of the monoclonal antibody to the electrode surface, the same MAb dilutions were added to the blocked screen-printed-wells but without T-2-KLH. As shown in Figure 3, the best conditions were obtained using a dilution of 1:2500 v/v for MAb and a concentration of 2 \( \mu \text{g mL}^{-1} \) for T-2-KLH. Non-specific adsorptions was not observed. The dilutions chosen above, for MAb and conjugate, were used for further competitive assay. The standard curve generated using these optimized conditions is shown in Figure 4. The detection limit and the sensitivity were 0.3 and 1.2 ng mL\(^{-1}\) respectively, while the analysis time was about 1.5 h.

Although this system is very rapid and easy to use for the analysis of T-2 and HT-2 trichothecene, it is still under study because we are screening new monoclonal antibodies and we have obtained promising results with a MAb clone produced against HT-2 toxin, that appears to give a 97% of cross-reactivity with T-2 toxin. Only in this way it will be possible to obtain an accurate quantification of the total amount of T-2 and HT-2 toxins.

### 3.2 Aflatoxin B\(_1\): MEI assay

This part of the work represents a continuation and improvement of our previous studies using a multichannel electrochemical 96-wells plate coupled with polyclonal antibody for aflatoxin B\(_1\) determination in corn (Piermarini et al. 2006). In these previous studies the cross-reactivity of the PAb towards different aflatoxins has been evaluated, showing a value of 56% for AFG\(_1\), while in another paper (Ammida et al.
2006) the same antibody gave a value of 112% for AFG1. These experiments showed that there was considerable variation between different lots of polyclonal antibody, so all further experiments, presented in this paper, were carried out using monoclonal antibodies, produced from a single clone against AFB1. Also in this case, the cross-reactivity of the MAb, toward other aflatoxins, was evaluated showing a high specificity for AFB1.

Such as for trichothecene assay, binding curves using different concentrations of AFB1-BSA and serial dilutions of MAb were performed. Figure 5 reports the chosen titration curve obtained with 1 µg mL⁻¹ of the coated molecule. We select a dilution of 1:27000 v/v (corresponding to a concentration of 0.2 µg mL⁻¹), which represents the inflection point of the curve. By using the procedure reported in section 2.3.3, a calibration curve for AFB1 (labelled with closed circles in Figure 7) was carried out. LOD and sensitivity were calculated to be 0.2 and 1.2 ng mL⁻¹ respectively, with an analysis time of about 1 h.

The stability of the coated and blocked plate (dry stored at 4° C) was evaluated by comparing calibration curves performed periodically during one month. As shown in Figure 6, the immunoplate can be used until 30 days of its preparation without lost of sensitivity. Moreover, considering the possibility to prepare and store the coated and blocked plates, the analysis time can be reduced at 30 min.

For future applications of this system to real samples, the matrix effect was evaluated using two different extraction treatments from corn, as reported in section 2.5.
In Figure 7 are reported calibration curve in PBS compared with those obtained by adding the same concentrations of AFB$_1$ (0-100 ng mL$^{-1}$) to 1:5 v/v diluted blank corn extracts. The results indicated a negligible matrix effect, with the advantages that the second procedure is more simple and rapid than the first one.

4. CONCLUSIONS

In this paper preliminary studies about the development of two immuno-electrochemical systems, based on the use of a 96-well screen-printed plate, for detection of AFB$_1$, T-2 and HT-2 toxins are presented. Both tests were found to be simple, rapid, specific for the target analyte, and could allow simultaneous analysis of numerous samples. Only after a complete characterization and application of these systems to a large number of cereal samples, experimentally and naturally contaminated, they could be considered a future trend in the screening analysis of mycotoxins.

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Rapid screening for AFB₁, T-2 and HT-2


Rapid screening for AFB₁, T-2 and HT-2


Rapid screening for AFB$_1$, T-2 and HT-2

Legend of figures

**Fig. 1**: a) 96-well screen-printed microplate; b) AndCare 9600 sensor reader.

**Fig. 2**: spectrophotometric competition curves for T-2 toxin using different MAb clones: T-2C6 (▼), T-2B2 (○) and T-2B1 (●).

**Fig. 3**: binding curves obtained by MEI system using different dilutions of MAb (T-2B2) and two concentrations of coated molecule (T-2-KLH): 2 µg mL$^{-1}$ (●) and 0.5 µg mL$^{-1}$ (▲). Blanks (○) carried out in the same experimental conditions but without immobilization of T-2KLH.

**Fig. 4**: electrochemical standard curve for T-2 toxin.

**Fig. 5**: selected binding curve obtained by MEI using different dilutions of MAb and a concentration of the coated molecule AFB$_1$-BSA = 1 µg mL$^{-1}$. No coating (○) carried out in the same experimental conditions, using only MAb diluted 1:5400 v/v.

**Fig. 6**: stability studies of the coated and blocked plate performed periodically, during 30 days.

**Fig. 7**: Effect of corn extracts on the standard curve of AFB$_1$ detected by MEI. Standard solution of AFB$_1$ prepared in PBS (●), in non-infected corn extracted by the first procedure (◇) and by the second procedure (Δ), both diluted 1:5 v/v with PBS before the analysis.
Table 1. Cross-reactivity % of three clones of monoclonal antibodies to different trichothecenes.

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<th>Trichothecene</th>
<th>Cross-reactivity (%)</th>
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<tr>
<td>NIV</td>
<td>0</td>
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<tr>
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Rapid screening for AFB₁, T-2 and HT-2

Figure 1.
Rapid screening for AFB1, T-2 and HT-2

Figure 2.
Rapid screening for AFB₁, T-2 and HT-2

![Graph showing the relationship between Mab T-2B2 dilutions and current (µA).](image)

**Figure 3.**
Rapid screening for AFB$_1$, T-2 and HT-2

Figure 4.
Rapid screening for AFB1, T-2 and HT-2

**Figura 5.**

![Graph showing the relationship between MAb AFB1 dilutions and current (µA)](image-url)
Figure 6.
Rapid screening for AFB₁, T-2 and HT-2

Figure 7.