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Development of a bio-electrochemical assay for AFB_1 detection in olive oil

Ines Ben Rejeb[a], Fabiana Arduini[b,c,*], Adina Arvinte[d], Aziz Amine[e], Mohamed Gargouri[a], Laura Micheli[b,c], Camelia Bala[d], Danila Moscone[b,c], Giuseppe Palleschi[b,c]

[a] Biocatalysis and Bioprocess Unit, National Institute of Applied Science and Technology (INSAT), B.P. 676, 1080 Tunis Cedex, Tunisia
[b] Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy
[c] Consorzio Interuniversitario Biostrutture e Biosistemi "INBB", Viale Medaglie d'Oro, 305 Roma, Italy
[d] Laboratory of Quality Control and Process Monitoring, Department of Analytical Chemistry, University of Bucharest, 4-12 Regina Elisabeta, 030018 Bucharest, Romania
[e] Faculté des Sciences et Techniques, B.P.146, Mohammadia, Morocco

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ABSTRACT

A novel biosensor assay format for aflatoxin based on acetylcholinesterase (AChE) inhibition by aflatoxin B_1 (AFB_1) is proposed. The AChE was present in solution and an amperometric choline oxidase biosensor was used for monitoring its residual activity. To create the biosensor, the choline oxidase was immobilized by cross-linking onto screen-printed electrodes modified with Prussian Blue (PB) and these were used to detect the H_2O_2 at low potential (−0.05 V versus a screen-printed internal silver pseudoreference electrode).

For the development of the AFB_1 assay, several parameters such as AChE and substrate concentration, the methanol effect, and pH were evaluated and optimized. The linear working range was assessed to be 10–60 ppb. Concentrations as low as 2 ppb, which correspond to the legal limit of AFB_1 in food for humans, were detected after a pre-concentration step. The suitability of the method was evaluated using commercial olive oil samples. A recovery equal to 78 ± 9% for 10 ppb of AFB_1 in olive oil samples was obtained.

1. Introduction

Aflatoxins (AFs) are a group of highly toxic and carcinogenic secondary metabolites which are produced by the molds *Aspergillus flavus* and *Aspergillus parasiticus* and may contaminate plants and plant products (Crisan, 1973; Roy et al., 1988). These toxins are potent carcinogens, teratogens, genotoxics and mutagens and pose severe hazards to animal and human health. Aflatoxins can affect a wide range of vegetable commodities such as cereals, nuts, peanuts, fruits, oilseeds and dried fruits in the field and during storage (Doradimos et al., 2000). Mycotoxin contamination is favoured by particular climatic conditions such that AFs occur mainly in hot and humid regions where high temperature and humidity are optimal for growth of the moulds and subsequent toxin production (Hussein and Brasel, 2001).

The Agency for Research on Cancer (IARC) has classified an AFB_1 as a group 1 human carcinogen and aflatoxins G_1, G_2 and B_2 in group 2, as possible human carcinogens. 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).

Given the demonstrated risk associated with the various AFs, the European commission has set current maximum levels for total AFB_1, AFB_2, AFG_1 and AFG_2 (4 μg kg^{-1}) and AFB_1 alone (2 μg kg^{-1}) in corn, groundnuts, nuts, dried fruit and cereals for human food (Gilbert and Anklam, 2002).

The olive and its derivatives, in particular olive oil, represent one of the most significant agricultural products in the Mediterranean. Still, compared with other agricultural commodities, the studies concerning mycotoxin contamination of olives and olive oil are few. Several authors have conducted investigations on an *Aspergillus* strain growth on olives (Doradimos et al., 2000; Le Tutour et al., 1983; Cavaliere et al., 2007; Leontopoulos et al., 2003; Ghitakou et al., 2006), and on AF contamination in olives and in their products. In fact, olives are often stored for weeks in conditions that promote the mould growth. Since olives and olive oil are a major and pervasive component in the Mediterranean diet, even low levels of contamination can pose a hazard to public health.

Because of AFs occurrence in a wide range of foods and their harmful effects on humans and animals, several methods and tech-

* Corresponding author at: Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy.
Tel.: +39 06 72594404; fax: +39 06 72594328.
E-mail address: fabiana.arduini@uniroma2.it (F. Arduini).

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niques for aflatoxin determination have been developed during the last few years. The current reference methods are primarily chromatographic, such as thin layer chromatography (TLC) (Park et al., 1994) and high-performance liquid chromatography (HPLC) (Doradimos et al., 2000; Cavaliere et al., 2007). A promising alternative approach involves the use of an enzyme-linked immunoassay (ELISA) developed by our group (Ammida et al., 2004; Micheli et al., 2005). These techniques, however, require specific antibodies and many steps (coating, incubation, washing, etc.), which increase the assay time. Consequently, there is a need for identifying techniques that are based on simpler accessible, and reliable analytical approaches in order to provide screening methods for the monitoring at all stages of food and feed production.

To respond to this issue, enzymatic methods have shown promise in some cases as an alternative to classical methods to achieve faster and simpler detection of some environmental pollutants such as pesticides and heavy metals (Amine et al., 2006). In the case of the aflatoxins, this approach pointed to either an enzyme that metabolizes AFB_1 into a detectable compound or an enzyme that can be inhibited by AFB_1. The inhibition by AFB_1 of acetylcholinesterase (AChE), a key enzyme in the transmission of nerve impulses has been investigated (Cometa et al., 2005). The AFB_1 inhibition towards AChE extracted from *mouse brain* was studied and the implications in terms of kinetic mechanism and toxicity discussed. In a recent study, a new analytical method for AFB_1 detection in barley was developed by our group based on acetylcholinesterase inhibition coupled with Ellman's spectrophotometric method (Arduini et al., 2007).

In addition, electrochemical sensors and biosensors have, in some cases, the advantage of rapidity and sensitivity over the traditional techniques. Amperometric biosensors based on cholinesterase inhibition are considered to be one of the best alternatives in the context of this strategy. The simplicity, the low cost of the equipment and the possibility of their miniaturization make them useful for analytical devices (Solè et al., 2003; Amine et al., 2006).

In this work, the AFB_1 determination is based on AChE inhibition and the AChE residual activity is determined using a choline oxidase amperometric biosensor coupled with AChE enzyme in solution, yielding hydrogen peroxide as final product of the enzymatic solution spiked with AFB_1 and analyzed.

2. Experimental

2.1. Materials

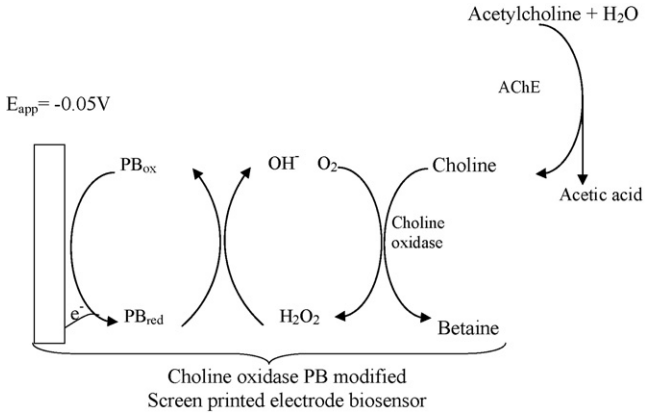
2.1.1. Reagents

All chemicals from commercial sources were of analytical grade.

Glutaraldehyde (GAD, 25%, v/v, aqueous solution), acetylcholine chloride and acetylcholinesterase from *electric eel* (EC 3.1.1.7) were from Sigma, St. Louis, MO, USA. Nafion® (perfluorinated ion-exchange resin, 5% (v/v) solution in lower alcohols/water), potassium ferricyanide, potassium chloride and choline oxidase from *Alcaligenes* sp. (11.6 U mg^{-1}) (E.C.1.1.3.17) were purchased from Fluka (St. Louis, USA). Bovine serum albumin (BSA), hydrogen peroxide (30%) and choline (grade > 99%) were supplied by Sigma. Chloropyrifos-methyl, dichlorvos, malathion and malaoxon were purchased from Riedel-de-Haen (Seelze, Germany).

Different concentrations of AFB_1 were obtained by suitable dilution of the stock solution in methanol.

Unless otherwise stated, all solutions were prepared with 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4. Standard solutions were prepared daily in the same buffer. For the pH test, a Britton-Robinson universal (4–12 pH) buffer + KCl 0.1 M was used.



Scheme 1. Schematic representation of the system adopted for AFB_1 determination, the final product is H_2O_2, which is measured at the PB-modified electrode at an applied potential of −0.05 V versus screen-printed internal silver pseudoreference electrode.

2.1.2. Electrodes

Screen-printed electrodes (SPEs) were home-produced with a 245 DEK (Weymouth, England) screen-printing machine. Graphite-based ink (Elettrodag 421) from Acheson (Milan, Italy) was used to print the working and counter electrode. The substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy).

The electrodes were produced in foils of 48 strips. Each sensor consists of three screen-printed elements: two carbon electrodes acting as working and counter, respectively and silver electrode acting as pseudoreference. The diameter of the working electrode was 0.3 cm. After the printing step, the foils were stored dry, at room temperature, in the dark.

2.2. Apparatus

Amperometric measurements were carried out using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected to an X-t recorder (L250E, Linseis, Selb, Germany).

2.3. Procedure

2.3.1. Choline oxidase biosensor

SPEs were modified with PB according to the procedure described by Ricci et al. (2003). PB-modified SPEs were used as support for enzyme immobilization carried out using a cross-linking method (Ricci et al., 2003). The biosensors were then kept in phosphate buffer solution 0.05 M + 0.1 M KCl, pH 7.4 at 4 °C until used.

2.3.2. Amperometric assay

Amperometric detection of AChE activity is based on the implementation of a second enzyme, ChOx, providing consecutive conversion of the native substrate (acetylcholine) to an electrochemically active product H_2O_2 (Scheme 1).

For the acetylcholine (ACh) measurement, the ChOx biosensor was placed in an electrochemical cell containing 3600 μl of stirred phosphate buffer solution (pH 7.4) and the potential of −0.050 V versus the silver pseudoreference electrode was applied. Then, a 200 μl aliquot of ACh solution (at different concentrations) and 200 μl of AChE (0.2 U/ml) to obtain a final enzyme concentration of 10 mU/ml were added to the batch solution and the response was measured after 10 min of reaction time. The current increases with time, according to the enzymatic kinetics of the AChE reaction in solution.

2.3.3. Measurement of the effect of methanol on the assay

Since methanol is often used to extract the AFB_1 from many contaminated agricultural samples (Gilbert and Anklam, 2002), it was chosen as a solvent to solubilize AFB_1. In the development of the experimental procedure, the effect of methanol on the assay was evaluated using the amperometric batch procedure. The experiments were conducted in two steps. Firstly, ACh was added to a stirred solution of phosphate buffer 0.05 M + KCl 0.1 M pH 7.4 and when a stable current background was reached, AChE was added to the solution and the formation of H_2O_2 was followed for 10 min. The resulting slope of the linear range was designated i_0.

In the second step, phosphate buffer containing 1% of methanol was employed and the same concentrations of ACh and AChE were added. The formation of hydrogen peroxide as a final product of the enzymatic reaction was measured for 10 min (i_i). Inactivation of enzyme by exposure to organic solvent was calculated by using Eq. (1):

$$I\% = \frac{i_0 - i_i}{i_0} \times 100 \quad (1)$$

where $I\%$ is percentage of enzyme inactivation due to methanol, i_0 is current obtained in buffered solution and i_i is the current obtained in buffered solution with methanol.

2.3.4. Measurement of AFB_1

Stock solutions of AFB_1 were prepared in methanol, this being the preferred solvent for AFB_1. The degree of inhibition was determined for different concentrations of AFB_1, always adding the same volume of aflatoxin solution, but varying its concentration. In this way, the percentage of methanol which interacts with the enzyme remains the same. The inhibition due to the toxin was calculated by using Eq. (1) where $I\%$ is % of inhibition, i_0 is current obtained by using methanol, and i_i is the current obtained by using this analyte in methanol.

A pre-concentration step was carried out by fortification of 40 ml of organic solvent with AFB_1. Then, the organic solvent was evaporated using a rotavapor system and the residue was solubilized in 4 ml of phosphate buffer with 1% methanol. Then, this solution was used for the electrochemical analysis.

2.3.5. Olive oil sample preparation and extraction of aflatoxins

Olive oil was purchased from the local market. For AFB_1 extraction from olive oil the procedure developed by Doradimos et al. (2000) was adopted without the final clean-up step using Sep-Pak cartridge.

To investigate the matrix effect, the inhibition was evaluated using Eq. (1) in which, i_0 reported the inhibition of AChE in the presence of the matrix and methanol, while i_1 was that measured in presence of matrix and AFB_1.

For recovery studies, aflatoxin-free oil samples were fortified with defined volumes of AFB_1 solution to obtain the concentration levels of 10 and 30 ng g^{-1} respectively. For this purpose, 10 ml of olive oil samples were spiked with 20 μl of AFB_1 5 and 15 ppm, respectively.

2.4. Safety conditions

Stock solutions of aflatoxins (highly toxic compounds) were prepared under appropriate safety conditions. In fact, in order to avoid contact with the powder or inhalation of vapor of aflatoxins, the operators were protected with lab dresses, gloves, mask, and glasses. Also, a dedicated hood has been used during sample preparation and analysis.

3. Results and discussion

3.1. PB-modified electrode performances

The response towards H_2O_2 of the PB-modified screen-printed electrodes was evaluated in batch mode by amperometric measurements.

The results showed a linear correlation: $y = 32.4x + 41.6$ where y is the current and x is the H_2O_2 concentration. The H_2O_2 sensors exhibited a sensitivity of 32 mA/M and a detection limit of 0.3 μM (for signal/noise = 3) with good linearity up to 85 μM.

Reproducibility was also calculated; the R.S.D. intra-electrode was up to 3% ($n = 3$ measurements) and inter-electrode R.S.D. was up to 4% performed with three different electrodes. The response time needed to reach 90% of the steady state response was 10 s. The results obtained are in agreement with our previous work (Ricci et al., 2003).

3.2. Choline biosensor

The PB-modified electrodes were then used as support for the oxidase enzyme immobilization. Choline biosensors were then tested in order to evaluate the detection limit, linearity range and reproducibility. The calibration curve for choline measurements showed a good linearity in the range between 2 and 200 μM, with a slope of 9.68 mA/M. A detection limit of 1.5 μM was calculated for a signal to noise ratio of 3.

The intra-electrode reproducibility of the biosensors obtained was quite good, being equal to 4% ($n = 3$ measurements). Three different biosensors were also tested, resulting in a R.S.D. value of 6% (inter-electrodes R.S.D. %). These results are in agreement with previous findings (Ricci et al., 2003).

3.3. Acetylcholinesterase activity measurement using the choline biosensor

In order to develop an effective assay for AFB_1 determination based on the decrease in AChE enzyme activity, the amperometric biosensor based on ChOx-PB-modified screen-printed electrodes was adopted for the determination of AChE activity. To obtain a low detection limit, some parameters such as substrate concentration, solvent, pH, etc. were investigated and optimized.

3.3.1. Effect of AChE and substrate concentration

In order to optimize the amperometric system for AFB_1 detection in olive oil, the dependence of the choline biosensor response on the amount of acetylcholinesterase in solution was studied. Fig. 1 shows that the response of the biosensor increases with the increase of AChE concentration; higher concentrations of the enzyme produce higher concentrations of choline, subsequently a higher response of choline biosensor. As can be seen in Fig. 1, for the first range of acetylcholinesterase concentration (1–10 mU/ml) the slope is large, probably because AChE was adsorbed on membrane of choline oxidase biosensor in agreement with the work reported by Mascini and Moscone (1986). A R.S.D. of 6% was observed for three replicates using the same choline biosensor (intra-electrode R.S.D. %). However, a washing step is required between measurements, probably due to the fact that AChE is likely to be adsorbed on the membrane surface of the choline biosensor, thus affecting successive measurements. To avoid this, a 2 min-washing step in a stirring buffer solution is required between the measurements.

As investigated in our previous work, the inhibition of acetylcholinesterase by AFB_1 is a reversible inhibition such that it is independent of the enzyme concentration and reaction time. However, the amperometric signal increases with increasing con-

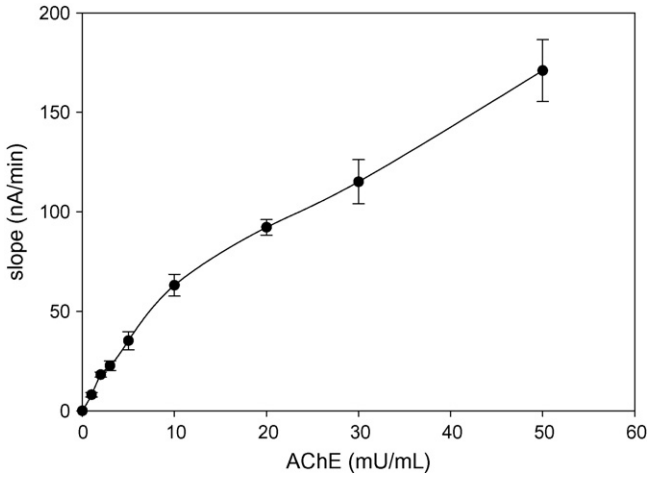


Fig. 1. Effect of acetylcholinesterase concentration. Acetylcholine concentration = 0.2 mM, phosphate buffer 0.05 M + KCl 0.1 M pH 7.4. All the values are the average of triplicate measurements.

centration of enzyme and time of reaction. As the best compromise in terms of time and sensitivity we choose 10 mU/ml of enzyme and 10 min as reaction time.

The study of the effect of substrate concentration is reported in Fig. 2. The calibration curve for different concentrations of substrate (i.e. ACh) could be described by the Michaelis–Menten equation. Using Michaelis–Menten theory it is possible to calculate the Michaelis–Menten constant (K_M), equal to 72 μM, that describes the affinity of the AChE for its natural substrate acetylcholine. This value appears to be in accordance with the value reported in the literature, equal to 95 μM (Keesey, 1987).

In order to choose the substrate concentration we have considered the mechanism of inhibition. As reported in our previous paper, since the inhibition is reversible with a mixed mechanism, the degree of inhibition is less dependent on substrate concentration. Thus, by using 0.2 mM as the substrate concentration, along with 10 mU/ml of enzyme and 10 min as reaction time, it is possible to obtain a good amperometric signal (around 500 nA). It should be noted that negligible signal (less than 50 nA) is observed due to the spontaneous hydrolysis of acetylcholine and thus was taken as background.

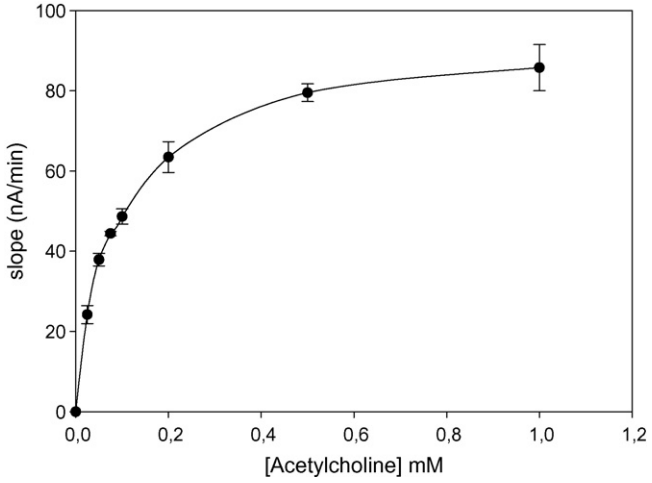


Fig. 2. Calibration plot of acetylcholine using AChE in solution and ChO biosensor. Experimental conditions: AChE = 10 mU/ml, phosphate buffer 0.05 M + KCl 0.1 M pH 7.4. All the values are the average of triplicate measurements.

3.3.2. pH study

It is well known that enzyme activity is highly pH dependent and the optimum pH for an enzymatic assay must be determined experimentally. As reported in the literature, pH 8 is the optimum pH for AChE activity and this pH is also the best for the detection of AFB_1 based on AChE inhibition using the spectrophotometric method (Arduini et al., 2005). In our electrochemical system, PB was used as redox mediator because it was also stable at alkaline pH (Ricci et al., 2003). The pH effect on the degree of AChE inhibition by AFB_1 was investigated (data not shown), showing that the best value of pH is equal to 7.4, taking into consideration the PB electrocatalytic activity and the bi-enzymatic (AChE-ChOx) system activity.

3.3.3. Study of the methanol effect

Since AFB_1 is insoluble in completely non-polar solvents and soluble in slightly polar solvents, AFB_1 is normally extracted using a mixture of organic solvents such as methanol, acetonitrile, chloroform or acetone. Methanol has previously been used to extract AFB_1 from many contaminated agricultural samples and was chosen in our experiments as the organic solvent for extraction of AFB_1. The effect of methanol on the analytical assay was then evaluated with the aim of developing a procedure for AFB_1 detection in olive oil. The results obtained have demonstrated (data not shown) that there is a slight decrease in the biosensor response with an increase in the amount of methanol; at 5% methanol (v/v) the biosensor response decreased by 15%.

Low methanol concentrations were found to be suitable for maintaining the stability of the assembled ChOx biosensor and did not show a high background current signal. Thus, to set up the inhibition assay system, 1% of methanol was used for the rest of the work.

3.4. Calibration curve in standard solutions

The degree of AChE inhibition by AFB_1 was then investigated using the choline oxidase amperometric biosensor coupled with AChE enzyme in solution. The inhibition of AChE activity, proportional to AFB_1 concentration, could be measured given that there was a decrease in the formation rate of hydrogen peroxide, which was reflected in turn in a decrease in the current generated by the biosensor as reported in the original recordings (Fig. 3).

It was found that the response for the optimized assay was linear in the range between 10 and 60 ppb. Successive calibration curves were generated and the results showed a linear correlation expressed with the equation $y = 0.604x + 6.93$ where y is the degree of inhibition and x is AFB_1 concentration in ppb; R^2 was equal to 0.974 (Fig. 4). The calibration curve was produced using an incubation time of 1 min. The lower limit of the linear range, defined as the concentration giving an inhibition of 20%, was 10 ppb of AFB_1. This value does not match the legal limit of the European Community regulation for human food, which is 2 ppb (European Community Regulation, 2003). To detect AFB_1 concentrations lower than 10 ppb, a pre-concentration step was required as reported in Section 2.3.5. In this case, the solution was concentrated five times and 2 ppb were measured giving a degree of inhibition equal to 28 ± 5%.

3.5. Interference study

Phenolic compounds, usually present in olive oil samples, have a significant effect on olive oil flavour. Hydroxytyrosol, tyrosol, caffeic acid, gallic acid, coumaric acid, and *p*-hydroxybenzoic acid have a strongly influence on the sensory characteristics of olive oil. Olive oils contain on average 200 ppm of total phenolic compounds (Bonoli et al., 2003) which can be possible electrochemical

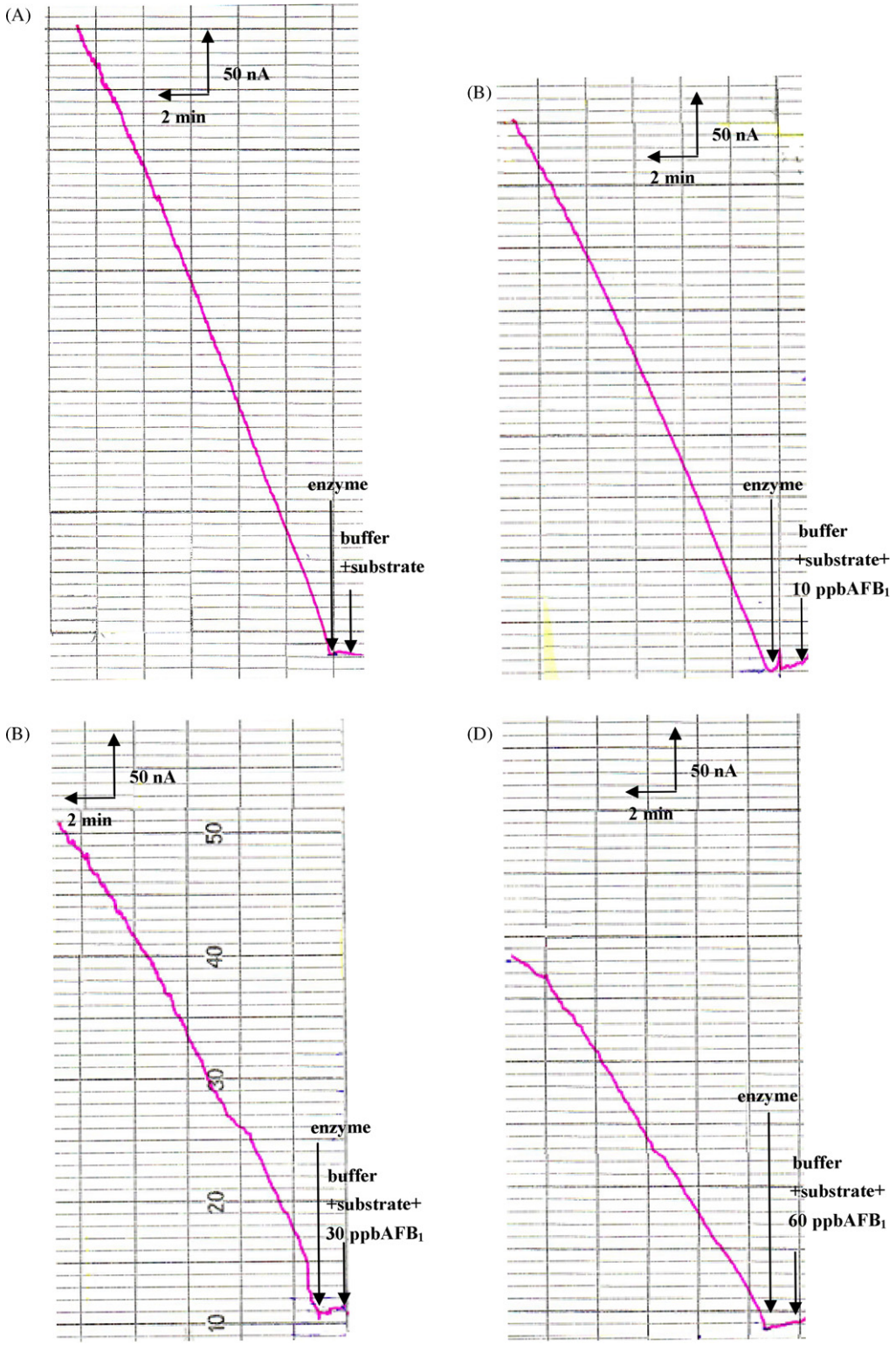


Fig. 3. Original recording in absence of AFB_1 (A), with 10 ppb (B), 30 ppb (C) and 60 ppb of AFB_1 (D). Experimental conditions: applied potential = −0.05 V versus screen-printed internal silver pseudoreference electrode. [Acetylcholine] = 0.2 mM, [AChE] = 10 mU/ml, percentage of methanol = 1%, phosphate buffer 0.05 M + KCl 0.1 M pH 7.4.

interferences. Caffeic acid was tested as target phenolic compound at 200 ppm concentration. In our case a slight response equal to 70 nA was observed, as expected since it is well known that the phenol compounds usually are detected at higher positive potentials than that used here. However, this interference can be avoided by adding the olive oil extract before the enzyme.

AChE is also inhibited by other aflatoxins. Both AFB_1 and AFB_2 provoked considerable inhibition of AChE, while AFM_1, AFG_1 and AFG_2 showed limited capacity to inhibit the enzyme (Arduini et al., 2007). It is important to stress at this point, that the method proposed is not a selective method for AFB_1, but represents a method for the determination of total aflatoxin content. However, it should

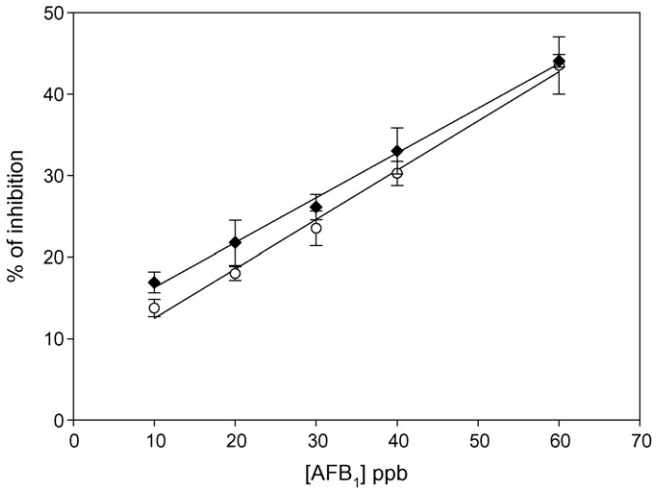


Fig. 4. Calibration plot for inhibition by AFB_1 in buffer (○) and in matrix (♦). Experimental conditions: applied potential = −0.05 V versus screen-printed internal silver pseudoreference electrode. [Acetylcholine] = 0.2 mM, [AChE] = 10 mU/ml, percentage of methanol = 1%, phosphate buffer 0.05 M + KCl 0.1 M pH 7.4. All values are the average of triplicate measurements.

be noted that olive oils are usually contaminated by AFB_1 among the aflatoxins as reported in the literature (Doradimos et al., 2000; Le Tutour et al., 1983; Cavaliere et al., 2007; Leontopoulos et al., 2003; Ghitakou et al., 2006).

Among the other inhibitors, other possible interferents in the detection of AFB_1 using an assay based on AChE inhibition can be due to the organophosphorous and carbammic pesticides, that are irreversible inhibitors of the enzyme. The pesticides can be present in olive oils, and often at ppb concentrations. In order to evaluate the effect of this potential interferent, chlorpyrifos-methyl, malathion, malaoxon and dichlorvos were analyzed as target pesticides. 50 pbb of standard solutions of the target pesticide were tested and no inhibition was observed for chlorpyrifos-methyl and malathion while a degree of inhibition equal to 55% and 100% was obtained for dichlorvos and malaoxon, respectively. These results are in agreement with the studies reported in the literature (Arduini et al., 2006) in which it was demonstrated that the organothiophosphates have a lower potency to inhibit the acetylcholinesterase enzyme than the organophosphate oxo pesticides.

The aflatoxins are reversible inhibitors and the degree of inhibition is independent of both enzyme loading and incubation time (Arduini et al., 2007). By contrast, the organophosphorous and carbammic pesticides are irreversible inhibitors and require a long incubation time and a low concentration of the enzyme to reach high degrees of inhibition. Thus, avoiding any incubation time, decreasing the reaction time to 1 min, and increasing the enzyme concentration to 40 mU/ml, no inhibition was observed with dichlorovos and 65% inhibition for a standard solution of malaoxon. It should be kept in mind that a protocol for the AFB_1 extraction from olive oil samples would eliminate malaoxon during the steps of extraction. Indeed, no inhibition was observed when samples of olive oil were contaminated with 50 ppb of malaoxon. The results obtained demonstrate that selection of experimental conditions for sample treatment and measurement should be taken into consideration to avoid interference from the presence of the pesticides in the sample.

3.6. Determination of AFB_1 in olive oil

To demonstrate the suitability of the proposed method, we have measured the AFB_1 in olive oils to evaluate, firstly, the matrix effect and then the recovery studies. The resulting calibration curve for the extract of a commercial extra virgin olive oil (Carapelli) was described by the follow equation: $y = 0.549x + 10.8$ with $R^2 = 0.974$ where y is the degree of inhibition and x is AFB_1 concentration in ppb. The ratio between the slope produced in the matrix and that in the standard is 0.91 indicating that the matrix effect is quite negligible (Fig. 4). This means that the simple pre-treatment that we adopted is sufficient for our purpose.

Table 1
Recovery studies of spiked olive oil samples using an electrochemical biosensor for AFB_1 determination based AChE inhibition (concentration in olive oil). The data reported in the table represent the average of three measurements.

AFB_1 added (ppb)	AFB_1 found (ppb)	% Recovery ± S.D.
10	7.8	78 ± 9
30	21	76 ± 7

To evaluate the suitability of the proposed method, recovery studies were carried out by spiking blank olive oil with AFB_1 before the extraction. Two samples of olive oil were spiked to obtain final concentrations of AFB_1 equal to 10 and 30 ppb, respectively. Each sample was analyzed in triplicate and the average is reported in Table 1. On the basis of the calibration curve performed with enzyme in extract solution (Fig. 4), it was possible to calculate the extraction efficiency of the analyte. The good recovery data obtained demonstrated the suitability of the proposed method for routine screening of AFB_1 in olive oil.

4. Conclusion

A novel electrochemical method for AFB_1 detection based on acetylcholinesterase (AChE) inhibition was developed. The residual enzymatic activity was measured using a choline oxidase amperometric biosensor (choline oxidase immobilized onto SPEs modified with PB) coupled with the AChE enzyme reaction in solution.

The analytical conditions were optimized achieving a lower limit of the linear range (defined as the concentration giving an inhibition of 20%) equal to 10 ppb of AFB_1. In order to detect 2 ppb of AFB_1 using the proposed method, a simple pre-concentration step was introduced.

An advantage of this method is that it is much simpler and faster than HPLC or it uses less expensive reagents than the specific antibodies adopted in ELISA. In addition the electrochemical measurement of acetylcholinesterase activity allows measurement of low concentrations of AFB_1 because it avoids the dilution step that is required for the spectrophotometric method due to the presence of the coloured extract.

The ease of performance and cost effectiveness of the proposed system for AFB_1 detection and the good results obtained in terms of recovery in real samples demonstrate the high potential of this assay as a screening procedure for AFB_1 detection in food.

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References

Amine, A., Mohammadi, H., Bourais, I., Palleschi, G., 2006. Biosens. Bioelectron. 21, 1405–1423.
Ammida, N.H.S., Micheli, L., Palleschi, G., 2004. Anal. Chim. Acta 520, 159–164.

Arduini, F., Errico, I., Amine, A., Micheli, L., Palleschi, G., Moscone, D., 2007. Anal. Chem. 79, 3409–3415.
Arduini, F., Ricci, F., Tuta, C.S., Moscone, D., Amine, A., Palleschi, G., 2006. Anal. Chim. Acta 580, 155–162.
Arduini, F., Ricci, F., Bourais, I., Amine, A., Moscone, D., Palleschi, G., 2005. Anal. Lett. 38, 1703–1719.
Bonoli, M., Montanucci, M., Gallina Toschi, T., Lercker, G., 2003. J. Chromatogr. A 1011, 163–172.
Cavaliere, C., Foglia, P., Guarino, C., Nazzari, M., Samperi, R., Lagana, A., 2007. Anal. Chim. Acta 596, 141–148.
Crisan, E.V., 1973. Appl. Microbiol. 26, 991–1000.
Cometa, M.F., Lorenzini, P., Fortuna, S., Volpe, M.T., Meneguz, A., Palmery, M., 2005. Toxicology 206, 125–135.
Doradimos, E., Marcaki, P., Koupparis, M., 2000. Food Addit. Contam. 17, 65–73.
European Community Regulation, 2003/100 (31/10/2003).
Ghitakou, S., Koutras, K., Kanellou, E., Markaki, P., 2006. Food Microbiol. 23, 612–621.
Gilbert, J., Anklam, E., 2002. Trends Anal. Chem. 21, 468–486.
Hussein, H.S., Brasel, J.M., 2001. Toxicology 167, 101–134.
Keesey, J., 1987. Biochemica Information, 1st ed. Boheringer Mannheim Biochemicals, Indianapolis.
Leontopoulos, D., Siafaka, A., Markaki, P., 2003. Food Microbiol. 20, 119–126.
Le Tutour, B., Tantaoui-Elaraki, A., Ihlal, L., 1983. J. Am. Oil Chem. Soc. 60, 835–837.
Mascini, M., Moscone, D., 1986. Anal. Chim. Acta 179, 439–444.
Micheli, L., Greco, 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.
Park, D.L., Trucksess, M.W., Nesheim, S., Stack, M., Newell, R.F., 1994. J. AOAC Int. 77, 637–646.
Ricci, F., Amine, A., Palleschi, G., Moscone, D., 2003. Biosens. Bioelectron. 18, 165–174.
Roy, A.K., Sinha, K.K., Chourasia, H.K., 1988. Appl. Environ. Microbiol. 54, 842–843.
Solè, S., Merkoci, A., Alegret, S., 2003. Crit. Rev. Anal. Chem. 33, 89–126.