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Cost-effective electrochemical immunosensors for label-free quantification of AFB1 in cattle feed

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Abstract: In this work, a sensitive, brand new, and cost-effective method based on competitive *label-free* immunoassay for the quantitative detection of Aflatoxin B_1 (AFB_1) was presented. The main advantage of this label-free immunosensor is the strong limitation in terms of reagents and time-consuming procedures, usually needed in classic (labeled) analysis. The electrochemical immunosensor is realized by immobilizing of aflatoxin B_1-bovin serum albumin (AFB_1-BSA) conjugated on homemade screen-printed electrodes (SPE). In particular, after a competition step the electrochemical measurement for the determination of aflatoxin (in buffer or in sample) is carried out using $[Fe(CN)_6]^{3-/4-}$ as electroactive probes. The quantification step is realized using square wave voltammetry (SWV). Thus, an indirect proportionality to the aflatoxin amount in the standard or real sample matrix was assessed. An in-depth electrochemical and morphological characterization of the immunosensor was realized. The performances obtained using this immunosensor in fortified real samples matrix show satisfactory results, especially considering the simplicity of the technique applied and the reduction in number of reagents. The detection limit (3.78 $ngmL^{-1}$), the linear range (10-80 $ngmL^{-1}$) and the sensitivity (33.0 $ngmL^{-1}$) of this method are noteworthy and make this exploitable for real sample application. To validate the results obtained with the proposed device, the same solution of AFB_1 (in buffer or fortified samples) were analyzed with immunosensor based on competitive scheme where a secondary antibody labeled with enzyme was used and conventional chromatographic method.

Keywords: Aflatoxin B_1, label-free immnosensor, cattle feed, screen-printed electrodes.

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1. Introduction

Aflatoxins are genotoxic, carcinogenic and teratogen secondary metabolites mainly produced by two fungal species (*Aspergillus Flavus* and *Aspergillus Parasiticus*) in critical temperature and humidity conditions. All the aflatoxin family members are polycyclic lipophilic molecules whose presence in food and food derivatives is considered a real and effective risk to users for their extreme toxicity [1–3]. Crops contamination by aflatoxins is a worldwide food safety concern. It has been estimated that a quarter of the world's crops are infested to some extent with mycotoxins [4]. These toxins are classified as the most important non-infectious, chronic dietary risk factor, higher than pesticide residues, synthetic contaminants or food additives [5]. Aflatoxins were originally identified and

characterized after what is known as Turkey X disease [6-7], which caused the death and suppression of more than 100.000 poultries following the ingestion of fungi-contaminated feed. The most important aflatoxins are the aflatoxin B_1 and B_2, G_1 and G_2 and their mammalian-metabolic byproducts M_1 and M_2 [8].

Aflatoxin B_1 is considered the most harmful and is produced, together with aflatoxin B_2 by both *Aspergillus Flavus* and *Aspergillus Parasiticus*. Aflatoxin G_1 and G_2 are only produced by Aspergillus Parasiticus. Aflatoxin M_1 and M_2 are, finally, mammalian metabolite generated by the animal's liver as this tries to make these molecules more hydrophilic in order to be easily excreted from the body through the urine[9,10].Aflatoxin M_1 and M_2 can also be observed in children's excretion products if these latter are exposed to contaminated breast milk [10–12].

Aflatoxin B_1 is considered the most potent natural hepatocarcinogenic compound not produced by human activities but by a living organism, classified as Group I of Carcinogenic Substances by the International Agency for Research on Cancer (IARC) [13]. Many countries have tried to maintain exposure to aflatoxins as low as possible by applying law limits on commodities to be used as food and animal feed. Aflatoxins enter the food chain when toxigenic molds grow on food and feeds. Aflatoxins can be present in foods, such as maize, grapes, raisins and other dried foods, crude vegetable oils as a result of fungal contamination before and after the harvesting process [2,14-15]. The mold metabolic pathway and thus the aflatoxins biosynthesis is strongly influenced by environmental factors: as a result, the possibility of contamination depends on agricultural, agronomic practices and geographic location [16-17]. The most critical variables to keep under control during the storage process are - the moisture content of the substrate, insect damage and the relative humidity of the surroundings [2,14-15,18-19]. Aflatoxins have also been found in dairy products (milk and cheese) due to animal ingestion of toxin-contaminated feed and are a clear example of carry-over. The frequency and level of mycotoxin presence in the food chain are growing up in the last decades, probably due to the change in the environmental conditions, to a simpler goods exchange possibility between different countries and to the worldwide deployment of mold [19,20].

Long-lasting or chronic exposure to aflatoxins can cause many different diseases including liver cancer (the potency of aflatoxin B_1 to induce liver cancer is incredibly enhanced in the presence of infection with hepatitis B virus and immune suppression (thus reducing the resistance ability to infectious agents such as HIV or tuberculosis) [21–23]. Large doses of aflatoxins are responsible of acute poisoning (known as aflatoxicosis) that can be life threatening, usually through liver damage [24]. Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression and other "slow" and long-lasting pathological conditions. In particular, the consumption of food containing aflatoxin concentration equal or greater than 1 $mgkg^{-1}$ has been associated to aflatoxicosis [21-22,25-26]; based on the past outbreaks it has been established that, when consumed over a period of 1-3 weeks, an AFB1 dose of 20-120 µgkg-1 body-weight per day is heavily toxic and potentially lethal [23-24,26–28]. The aflatoxin toxicity in human is mediated by the cytochrome P450 enzyme that converts aflatoxins to the reactive 8,9-epoxide form (also known as aflatoxin-2,3 epoxide), which in turn binds to both DNA and proteins [29].

For all the reasons highlighted above, the exposure of human beings and animals to aflatoxin contaminated products should be kept as low as possible. The European Union (EU), in particular, introduced measures to minimize the presence of aflatoxins in different foodstuffs and raw materials. Maximum levels of aflatoxins in food have been discussed and updated in January 2007, the European Food Safety Authority (EFSA) scientific Panel on contaminants in the food chain concluded that increasing the current EU maximum levels of 4 μgkg^{-1} total aflatoxins in nuts to 8 to 10 μgkg^{-1} total aflatoxins would have had minor effects on the estimated dietary exposure, cancer risk and calculated margin of exposure. In June 2009 the European Commission asked EFSA to verify the effect on public health of an increase of the maximum level for total aflatoxins from 4 μgkg^{-1} to 10 µg kg^{-1}: the Panel concluded that public health would not be negatively affected by increasing

the levels of total aflatoxins to 4 μgkg^{-1} to 10 μgkg^{-1}; however, the Panel reiterated its previous conclusion regarding the importance of reducing the number of highly contaminated foods reaching the market every day [30].

Here, insert Table 1

In the end, even though desirable, it is not realistically possible to completely avoid aflatoxins contamination in foods since the mold colonization and thus their toxic byproducts are not under the all human control, due to many different biological, genetic and biochemical reasons. Numerous assay methods for detecting aflatoxins have been developed utilizing virtually all the common tools of analytical chemistry including TLC, HPLC, GC, MS [36-42], immunoassays, capillary electrophoresis and biosensors. Huge amount of electrochemical *label-free* biosensors has been developed during the last years including colorimetric, impedimetric or optical detection systems [32,35, 43-45]. All analytical procedures include three time-consuming steps: extraction, purification and determination. Moreover, because infesting agents (such as molds and aflatoxins) are not evenly distributed throughout the sample, appropriate sampling procedure is critical to identify in order to obtain a representative result. These techniques need to be reliable, easy to use and possibly cost-effective. Over the last decade, more and more researchers have focused their efforts on setting up a screen-printed based-sensor for the determination of aflatoxins. Here the most important works are reported (Table 1). Keeping in mind the extremely toxic behavior of aflatoxins, it is important to study, characterize and develop specific, useful and practical systems to monitor and easily quantify their concentration in foods.

Here, we report a cheap, reliable, low technology and transportable device (lab-on-chip) for aflatoxin B_1 monitoring, which may prove to be extremely useful in tackling the problem of a world-scale health-threatening contamination.

Being the specific binding of anti-AFB$_1$ to AFB$_1$ well established in our previous studies [38], during present work an attempt was made for in-situ immobilization of conjugated antigen onto a graphite screen printed electrode (SPE) surface employing Nafion. Nafion, a hydrophobic sulphonated tetrafluoroethylene polymer having short and regularly spaced perfluorovinyl branches has received much attention for chemical modification of electrode surface where analyte gets embedded in polymeric network results in much improved sensitivity of voltammetric assay [46,47]. Nafion polymeric network provides labile protons (-SO_3H) which facilitate covalent binding with the amine group [48] as evidenced by our previous report on soil potassium sensor [49]. Present study exploits the potentiality of Nafion modified SPE to fabricate label free biosensor strip for aflatoxin in a competitive immunoassay format. To validate the results of the proposed immunosensor, aflatoxin solution (in buffer or in samples) were analysed by the device developed by Ammida et al. [43] and using the conventional chromatographic method [43].

2. Materials and Methods

2.1 Materials

All chemicals from commercial sources were of analytical grade. Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2, B_1-BSA conjugate and anti-aflatoxin B_1 antibody were purchased from Alexis (Lausen, Switzerland). Potassium ferricyanide, Sodium bicarbonate, Potassium chloride, Nafion®117 (perfluorinated ion-exchange resin, 5% (vv^{-1}) solution in lower alcohols/water), isopropyl alcohol, polyvinyl alcohol (PVA) were purchased from Sigma-Aldrich (Steinheim, Germany). Ethyl alcohol was purchased from VWR Chemicals (Pennsylvania, United States). Buffer solutions used are: 0.1 M carbonate buffer, pH=9.6; 0.1 M carbonate buffer, 1% PVA (wv^{-1}), pH=9.6; 0.05 M phosphate buffer saline (PBS), 0.1 M KCl, pH=7.4; 0.05M phosphate buffer saline (PBS-T), 0.05% Tw20 (vv^{-1}); 0.01M acetate buffer solution, 0.1M KCl, pH=4.5.

2.2 Electrodes

Screen-printed electrodes (SPEs) were produced in house with a 245 DEK (High performance multi-purpose precision screen printer, Weymouth-UK) screen-printing machine. These devices are composed of three electrodes: a working (WE), a counter (CE) and a reference (RE) electrode, respectively. In particular, the WE (apparent geometric area of 0.07 cm^2) and CE, are deposited using a graphite-based ink (Elettrodag 421) from Acheson (Milan, Italy); whereas, the RE is produced using a silver ink (Acheson Elettrodag 4038 SS). The electrochemical cell (WE, CE, RE) is finely defined using an insulating layer (Argon Carbonflex 25.101S) [32].

2.3 Apparatus

Cyclic voltammetry (CV), chronoamperometry (CA) and square wave voltammetry (SWV) were performed using an Autolab electrochemical system (Eco Chemie, Utrecht, The Netherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands). SEM analyses were performed at Vega II Tescan (Brno, Czech Republic) microscope, endowed with a XFlash detector 5010 Bruker (Massachusetts, USA).

The HPLC system consisted of a modular CHROMQUEST spectra system from THERMOQUEST (San Jose, CA, USA), equipped with two LC'GA pumps, a Shimadzu UV-VIS spectrometer model (SPD-10AV), fluorescence (RF 10AXL) detectors, a vacuum SCM 1000 as degassing unit and an autosampler, AS 3500. A SN 4000 controller operated the HPLC system working under the control of software included in the CHROMQUEST module. The chromatographic separation was performed using a reverse phase C18 (VYDACTM, W.R. Grace &Co, cat. 210TP54) stainless steel column (5 µm spherical particle size, 150 x 4.6 mm I.D). The clean up procedure for AFB1 extracts was carried out using an immunoaffinity column, Afla BTM, Aflatoxin Testing System, which was obtained from VICAM (Watertown, USA).

2.4 Preparation of Electrochemical label-free Immunosensor for AFB1 detection

The preparation of the *label-free* electrochemical immunosensor follows the procedure below: prior to the immobilization step, screen-printed electrodes were pre-treated in a 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4 by applying an anodic potential of 1.7 V for 180 s. 6µL of AFB_1-BSA conjugate in 10 mM acetate buffer solution, pH 4.5 was drop onto the SPE working electrode (WE) via a drop-casting procedure. The electrode is stored overnight at 4°C temperature (*immobilization* process). The biomolecules were blocked with 6µL of 1% PVA solution (wv^{-1}) for 15 min at room temperature (*blocking* process). The *competition* process was subdivided in two different steps: in the first step the free antigen and antibody come in contact into a vial, in the second one competition proceeds on the WE's surface; the immunological chain (BSA-AFB_1/Ab present onto the WE) was monitored using potassium ferricyanide solution as electroactive probe (the electrochemical outcome was directly proportional to the amount of AFB_1 present in test solution or real matrix sample). After each process, a washing procedure has been carried out using PBS buffer.

2.5 Preparation of Nafion® 117-Electrochemical Immunosensor for AFB1 detection

The preparation of the *label-free*Nafion®117 electrochemical immunosensor follows the procedure below: prior to the immobilization step, screen-printed electrodes were pre-treated in a 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4 by applying an anodic potential

of 1.7 V for 180 s. On the WE was dropped 6 µL of Nafion®117 diluted in 1:1 10 mM acetate buffer solution, pH 4.5/ethanol mixture and left dry at room temperature for 60 min. 6 µL of AFB_1-BSA (0.5-1.0-1.5 μgmL^{-1} in 10 mM in acetate buffer, pH 4.5) was dropped on WE. The AFB_1-BSA modified electrodes were stored overnight at 4°C. The biomolecules were blocked with 6 µL of 1% PVA solution (wv^{-1}) for 15 min at room temperature (*blocking* process). The *competition* process is the same reported in section 2.4.1; finally the immunological chain (BSA-AFB_1/Ab present onto the WE) was monitored using potassium ferricyanide solution as electroactive probes, as described in section 2.4.1 After each process, a washing procedure has been carried out using PBS buffer.

2.6 Analytical parameters calculation

Standard curves were obtained using standard solutions of AFB_1 (0.5– 400 $ngmL^{-1}$) prepared in PBS for electrochemical measurements. The calibration curve was prepared by diluting the AFB_1 using cattle feed extract (preparation reported in sec 2.4.4) blank. This extract was prepared by applying the extraction procedure to grain samples that were not infected with *Aspergillus flavus*. For the electrochemical outputs, the standard curves were fitted using non-linear 4 parameter logistic calibration plots [50]. The four-parameter logistic function is represented in Equation 1:

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

where a and d are the asymptotic maximum and minimum values, c is the value of x at the inflection point and b is the slope. To compare different calibration curves, the current values obtained during experiments have been converted in percentage applying the following equation (Equation 2):

$$I\% = \left(\frac{I - I_{min}}{I_{max} - I_{min}}\right) \cdot 100 \quad \text{Equation 2}$$

where I corresponds to the faradic current value for a given aflatoxin concentration, I_{max} corresponds to the maximum current value observed during the experiment and I_{min} corresponds to the minimum current value observed during the analysis.

The limit of detection (LOD) is estimated from the analysis of ten different samples in which the analyte of interest is not present; thus, with the obtained current values, standard deviation (SD) is estimated. The result thus found has been included in the Equation 3:

$$LOD = I_{NC} - 3\sigma \quad \text{Equation 3}$$

in which σ and I_{NC} are the standard deviation and the current intensity of the no-competition point (no Ag), respectively.

In the cross-reactivity experiment, is possible to calculate the immunosensor's percentage response toward different tested aflatoxins. This can be realized applying the following formula (Equation 4):

$$\%response = \left(\frac{I_{AF_n}}{I_{AFB_1}}\right) \cdot 100 \quad \text{Equation 4}$$

In which I_{AFn} corresponds, respectively, to the different aflatoxins' faradic current value recorded in the cross-reactivity test (AFB_1, AFB_2, AFG_1 and AFG_2); I_{AFB1} corresponds to the AFB_1's faradic current value.

2.6 Sample preparation, extraction and study of matrix

Non-infected Raw cattle feed samples (mix of cottonseed, guar and barley) collected from local market, Jodhpur, India, were homogenized and 5 g weighed into a plastic vials.

Some of the vials were then fortified with different concentrations of AFB_1 under dry spiking methodology. Sample extraction was performed by adding 10mL of extraction solvent mixture (85% methanol: 15% PBS) to the real samples (blank and spiked samples). These samples solutions were sealed with parafilm and stirred in a horizontal shaker for 30 min at 100 rotations min^{-1} at room temperature. Samples were then centrifuged at 6000 rpm for 10 min. The supernatant was separated from the matrix pallet and used for AFB_1 detection by *label-free* methodology. Sample extraction for HPLC detection was performed as reported by Ammida and coworkers [43].

3. Results and Discussion

3.1. Determination of the ideal AFB1-BSA conjugate concentration

The paper aims is the realization of an electrochemical label-free immunosensor based on screen-printed electrodes (SPEs) for AFB_1 real-time monitoring and quantification, making use competitive) immunoassay. The electrochemical technique applied is the square wave voltammetry (SWV) and the electroactive species/probe is potassium ferricyanide (III).

As compared to classical analytical-immunoassay techniques, this innovative approach, presents several advantages such as inexpensiveness, shortness and transportability. More in detail, this kind of technique exploits the competition between the AFB_1-BSA conjugate, immobilized onto the WE's surface, and free-antigen (coming from standard or real spiked sample) toward the monoclonal antibodies (Mab) binding sites. Following the competition process, the amount of Mab, which reacted with the immobilized conjugate (immunocomplex formation), is evaluated by assessing the available electrodic-surface left free onto the WE. Potassium ferricyanide ($K_3[Fe(CN)_6]$) is applied in the quantification step: the lower the immunocomplex concentration formed on the electrodic surface, the higher the electrochemical response for a fixed amount of electroactive species.

Therefore, the electrochemical immunosensor measurements will be directly proportional to the analyte concentration in the tested sample. Matching the label-free and classical immunosensor approach, it is crucial to highlight the absence of marked-secondary antibody usually employed in the competitive ELISA, which helps reducing analysis costs and procedures.

In order to verify the proposed label-free technique, 6 µL of AFB_1-BSA-conjugate solution of different concentration (0.5,1.0 e 1.5 μgmL^{-1}) in 0.1 M carbonate buffer-pH 9.6 were immobilized on the WE and stored over-night at 4°C. Following the washing procedure, 6 µL of 1% PVA solution (wv^{-1}) in carbonate buffer were dropped onto the WE in order to minimize non-specific interactions and left reacting for 15 minutes at room temperature. Once completed the washing procedure, the electrodes have been analyzed using a 3mM potassium ferricyanide solution in PBS. The obtained results are reported below (Figure 1 and Table 1S). From Figure 1 it is easily possible to note that the higher the conjugate concentration (X axis) the lower the measured faradic current (Y axis) as a result of the decrease of the available active electrode surface. Good results are observed in terms of repeatability (RSD% equal to 4 and 2, respectively) in the case of poor bio-component concentration (0.5 and 1.0 μgmL^{-1}) while higher uncertainty can be noticed using the higher biomolecule concentration. These results, however, confirm the applicability of the suggested method and pave the way for further investigations.

(Here, Figure 1)

3.2. Determination of the ideal Nafion®117 concentration

The next step involved the study over the hypothetical use of Nafion®117 in order to obtain a more ordered structure to capture AFB_1-BSA and avoid the time-wasting blocking procedure. The fluoropolymer was applied following the procedure described in section 2.4 and to fully comprehend potentiality and behavior of this component, it has been utilized in different concentration (0.05 and 0.15%). Results obtained are reported below (Figure 2-Table 2s). Interesting is the fact that, compared to the bare electrode upon which the only pre-treatment is performed, a progressive increase of the recorded current values occurs by increasing the applied fluoropolymer concentration (this trend is confirmed for all the different ferricyanide concentrations). A possible explanation is that the polymer matrix, when dispersed in aqueous solution negatively charges itself. The charge properties of Nafion®117 derive from the incorporation of perfluorovinyl ether groups terminated with sulfonate groups onto a tetrafluoroethylene backbone. This structure significantly interacts with the positively charged potassium-ferricyanide couple (Fe^{2+}, Fe^{3+}) dissolved in solution. The attractive interaction between complementary charged structures could result in a diffusive processes reinforcement, which leads to an increase in measured faradic current. The optimal Nafion®117 concentration (equal to 0.15%) is chosen balancing high current values and limited standard error.

(Here, Figure 2)

3.3. Binding and competition curves

The following step concerned the addition of monoclonal antibody (Mab), which presents binding site complementary to AFB_1-BSA conjugate immobilized onto the working electrode's surface. In particular, three different immunosensor fabrication conditions were investigated: with (0.05 and 0.15% vv^{-1}) and without Nafion®117. For this purpose, after the fluoropolymer deposition step, a binding study (Figure 4a) was realized in order to determine the ideal Mab concentration (it ranges from 0 to 3 μgmL^{-1}) for the competition process. Increasing concentrations of Mab solutions were prepared as described in section2.4 and the obtained results are reported above. To fully understand the influence that Nafion®117 exerts in the binding process, different concentrations of fluoropolymer were tested and compared with the response of electrodes realized without the use of this inorganic matrix (bare electrode). As it can be seen from the curve above, by increasing the antibody concentration (moving from left to right on the x axis) a decrease in the recorded faradic current values is observed. This can be ascribed to the following mechanism: the higher the antibody concentration, the higher the molecular crowding on the electrode surface as a result of the immunocomplex AFB_1-BSA-Mab formation (the Mab reacts with the previously immobilized AFB_1-BSA conjugate due to molecular complementarity).This local, large biomolecule concentration leads to a massive decrease in avoidable electrodic surface able to oxidize potassium ferricyanide and a reduction of current values obtained. Moreover, as speculated, a lower Nafion®117 concentration leads to a better interaction among biocomponents and finally in a more suitable sigmoidal shape (clear indication of immunocomplex formation). In particular, using Nafion®117 concentration of 0.05% a positive effect on the recorded faradic current was found, due to the negatively charged fluoropolymer, as described in the previous section. While, by using fluoropolymer at a higher concentration (0.015%), a covering effect, which drastically reduces the current, has been established.

The antibody concentration, which has been applied in the next competition step, has been established considering the 70% of the maxim value of the association curves (corresponding with a linear trend) equal to 5µg/mL. Finally, it has been decided to proceed with both preparation methods (with or without Nafion®117) in order to obtain a comparison between different immunosensor performances.

Thus, the competition step was separated into two different phases: in the first, the pre-incubation step, antigen and antibody interact reciprocally within a centrifuge tube, in

order to accomplish the interaction of biomolecules, for 45 minutes at room temperature. In the second one, competition continues on the WE surface (upon which was previously immobilized AFB_1-BSA conjugated) for 30 minutes at room temperature. The electrochemical immunosensor's preparation method is summarized in Figure 3, reported above.

(Here, Figure 3)

The competition curves, obtained following this preparative methodology, are reported in Figure 4b. All the different aflatoxin concentrations used in the competition step are tested in triplicate and current % values are reported as a function of the concentrations. The decrease of standard aflatoxin concentration (200, 100, 50, 10, 1 and 0.5 $ngmL^{-1}$) leads to a reduction of the recorded current, as can be seen in Figure 4. In fact, the immuno-complex formation in solution, ascribable to the presence of aflatoxin, does not modify the active surface electrodic area (antibody binding site is already occupied when this reaches the WE surface). In the absence of toxin, however, the antibody will be available to interact with AFB_1-BSA conjugate, immobilized on the working electrode, decreasing the electrochemical response of the potassium hexacyanoferrate (III). Therefore, the higher the concentration of AFB_1, the greater the response in terms of current that should be observed (signal directly proportional to the AFB_1 concentration). Matching the results obtained using modified and unmodified platforms (with 0.05 and 0.15% or without Nafion®117, Figure 4b, respectively); the following values can be respectively highlighted: LOD equal to 2.3, 5.4 and 10.9 $ngmL^{-1}$ and linear range 5.5-84.5, 19-100 and 50.4-88.7 $ngmL^{-1}$ for Nafion 0.05%, 0.15% and bare SPEs-based immunosensor, respectively.

(Here, Figure 4)

3.4. Preliminary Real Samples application

The following section concerns the label-free electrochemical immunosensors application to real sample matrix. These are represented by different commercially available wheat flours mingled together to obtain a homogenous mixture. This matrix simulates feeds and food stocks usually employed in livestock industries. The analysis is previously performed using solvent extraction mixture (85% ethanol-15% PBS) alone, in order to evaluate the influence of the solvent on the label-free immunosensor. This mixture has been applied to the preparation of aflatoxin standards to be used in the competition step. The results are represented in Figure 5 and Table 2, respectively, for both bare and Nafion®117 (0.05%) modified SPEs based immunosensors. As can be seen below, the extraction solvent does not significantly alter the promising outputs observed above. Indeed, also in such case a sigmoidal-shaped curve is obtained with a directly proportional relationship between AFB_1 concentration and observed faradic current. Starting from these experiments, real matrix samples have been analyzed using the extraction procedure reported in section 2.6.

(Here, Figure 5 and Table 2)

3.5. Stability and cross-reactivity experiments

The stability of the label-free electrochemical immunosensors over time was evaluated using SPEs coated with AFB_1-BSA conjugate, blocked and stored in two different ways. Indeed, a parallel investigation was realized storing the SPEs at 4°C and at room temperature under vacuum. Assays were performed periodically, over a one-month period, using the assessed protocol. As highlighted in Figure 6a, the electrochemical outputs showed that both the electrodes could be used for up to one month after their

preparation. The subsistence of 90-100% of the SPE's activity indicated that the lifetime of the conjugate-immobilized electrodes could be even longer in either the storing methodology.

The last section of this work concerns cross-reactivity or specificity of the MAb toward its antigen. Since AFB_1 belongs to a varied family of toxins with similar structure, we decided to select other members of the same family in order to verify the Mab specificity. Different solutions of aflatoxin B_1, B_2 and G_1, G_2 were prepared and a competitive measure was carried out following the previously described procedure. Aflatoxins B_1 and B_2 differ each other exclusively by the presence of an unsaturation in the E ring; aflatoxins G_1 and G_2, on the other hand, have a larger A ring following the insertion of a heteroatom (an oxygen atom more than the previous structures) and differ from each other, also in this case, for the existence of a double bond in the E ring. The results of the specificity test are reported below (Figure 6b-Table 3S). Analyzing the data reported above, it is possible to conclude that the Mab chosen for the development of this immunosensor has a high specificity for AFB_1, with a 20, 18 and 19% cross-reactivity toward AFB_2, AFG_1 and AFG_2, respectively. In fact, Moreover, a low repeatability (high RSD% value) has been obtained for the interfering species analyzed, as reported in Table 3S.

(Here, Figure 6)

3.6. Immunosensor performance validation: comparison with HPLC analysis of contaminated wheat flour samples.

To better investigate our AFB_1 label-free immunosensor performances a comparative study using HPLC has been carried out. In particular, wheat flour samples contaminated with standard AFB_1 concentrations were tested with our voltammetric immunosensor and the results were compared with HPLC data. In figure 7a the linear regression ($y=263981x + 2061$, $r^2= 0.993$) obtained using seven standard concentrations of AFB_1 in flour extracted matrix are presented. The LOD and the LOQ calculated using these data are 0.6 $ngmL^{-1}$ and 2 $ngmL^{-1}$, respectively. Thus, the HPLC method is 3-fold more sensible than label-free immunosensor. However, sample preparation times, analysis times and costs are far longer than that of our electrochemical immunosensor, which also has a reproducibility roughly similar to the chromatographic method (RSD% 15% and 12%, respectively).

In Figure 7b, the correlation analysis of the data obtained analyzing wheat flour samples both using HPLC and the voltammetric immunosensore have been reported. In particular, the linear regression ($y=0.0025x + 0.967$, $r^2= 0.999$) obtained graphing the current result as a function of the HPLC's peak height shows a trend very similar to the ideal one, y=x, demonstrating the good agreement of the results obtained using two different detection methods.

(Here, Figure 7)

3.7. Morphological Characterization

In order to investigate the surface modification of working electrodes, a SEM analysis was carried out. A representative micrograph for each SPE is reported in Figure 8.Analyzing the surface of bare SPE, Figure 8a, graphite particles emerge from the polymer matrix used for printing purpose. The comparison with the surface of the Nafion®117-modified SPE (8c) highlights that the fluoropolymer retraced the underlying graphite particles and consequently increased neat arrangement of the working electrodes. The AFB_1-BSA conjugate adheres homogeneously to the bare SPE and to the fluoropolymer membrane: a thin layer of conjugate coats the graphite (bare SPE, Figure 8b) and Nafion®117structure (Figure 8d), clearly retracing the underlying structure.

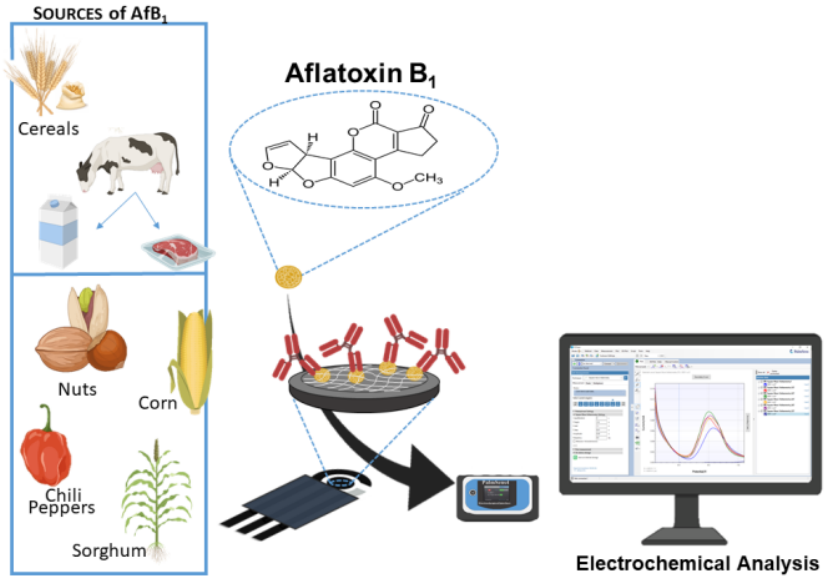
The homogeneous and thin coating of fluoropolymer is important to have a good sensing behavior: in fact, the highly regular surface area obtained by the deposition of Nafion®117allows uniform dispersion of biomolecules, enabling a more efficient antigen-antibody interaction during the comp[29]tion step. The conjugate thin coating guarantees the electrodic area does not decrease. In addition, Energy-Dispersive X-Ray Spectroscopy (EDX)analysis was carried out to investigate the ele[11]ntal composition of the modifiers (Table 3). This analytical technique is used for the elemental analysis or chemical characterization of the aforementioned samples. Its characterization capabilities are due in large part to the fundamental principle that each element has a unique atomic structure allowing an individual set of peaks on its electromagnetic emission spectrum. Starting from bare-SPEs, the immobilization procedure has been analyzed, verifying the elemental composition of the cast materials. Nafion®117 modification is confirmed by the increased weight percent (wt. %) o[23] F and S with respect to the unmodified platform. Nafion®117, in fact, comprises a polytetrafluoroethylene (PTFE) backbone with perfluorinated-vinyl-polyether side chains containing sulphonic acid end groups. The presence of the AFB_1-BSA conjugate instead, cannot be established for sure, but some indications can be assumed by the weight percent of O and N in the AFB_1-BSA modified platforms. In these latter, growth in O and N weight percent (from 2.92 and 1.22 to 4.43 and 3.38 for Bare SPE and Bare-SPE-AFB_1-BSA; from 18.85 and 5.98 to 21.87 and 7.32 for Nafion®117 SPE and Nafion®117-AFB_1-BSA, respectively) can be interpreted as indication of organic biomolecules-based modifications.

(Here, Figure 8 and Table 3)

4. Conclusions

[25]In this work, a disposable, simple, low-cost, label-free voltammetric immunosensor for the determi[14]on of Aflatoxin B_1 was successfully developed and characterized. The serigraphic sensor exhibited low [14]detection limit, good sensitivity, reproducibility, selectivity and storage stability, paving the way to its potential exploitation for the detection of A[10] in a wide range of real matrix samples. The proposed voltammetry-based sensor set-up was able to detect AFB_1 concentrations greater than 3 $ngmL^{-1}$ for standard solutions and 5 $ngmL^{-1}$ for spiked cattle feed samples, respectively. Furthermore, the proposed AF[10]label-free immunosensor exhibited a good selectivity against AFB_2, AFG_1, and AFG_2. Based on the European Commission reports regarding the control of the aflatoxin contamination in food staffs, the national action limit for AFB_1 is [10]gkg^{-1} to 10 μgkg^{-1}. Our sensor is able to detect this concentration, making it applicable in food safety and quality monitoring where the presence of mycotoxins is suspected.

Figures, Tables and Schemes



Graphical Abstract

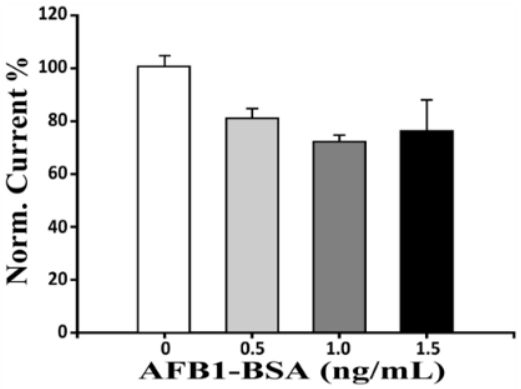


Figure 1. Decrease in recorded faradic current obtained analyzing AFB1-BSA-modified and bare electrodes. These results are obtained making use of SWV and 3mM potassium ferricyanide solution in 50 mM PBS + 10 mM KCl, pH 7.4 (Scan rate 30 mVs^{-1}).

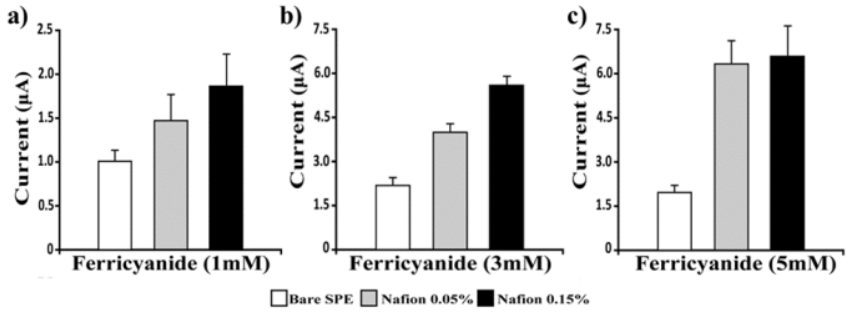


Figure 2. Histograms relative to the increment in faradic current of bare and Nafion®117 modified-SPEs (a, b, c). The results are obtained making use of SWV and 1,3 and 5 mM potassium ferricyanide solution in 50 mM PBS + 10 mM KCl, pH 7.4 (Scan rate 30 mVs^{-1}). 483 484 485

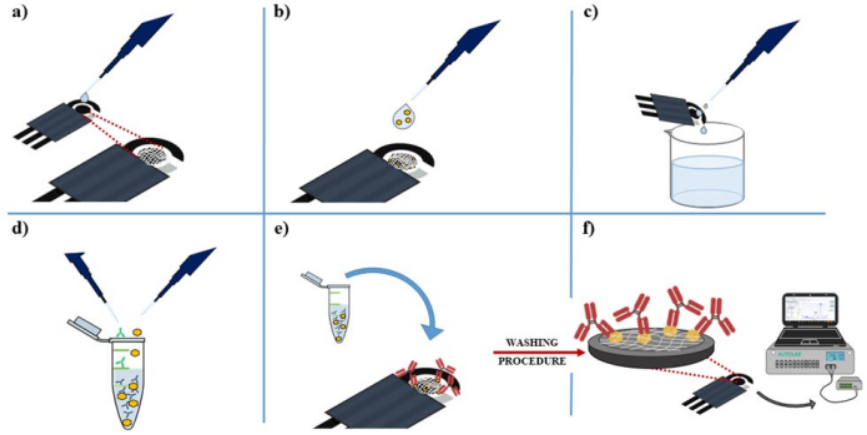


Figure 3. Schematization of AFB1-immunosensor layer-by-layer fabrication: 1) Nafion®117 polymerization on WE, b) AFB1-BSA immobilization, c) washing procedure, d) vial-competition or pre-incubation step, e) WE-competition step and f) analyte quantification using electroactive probe. 487 488 489

490

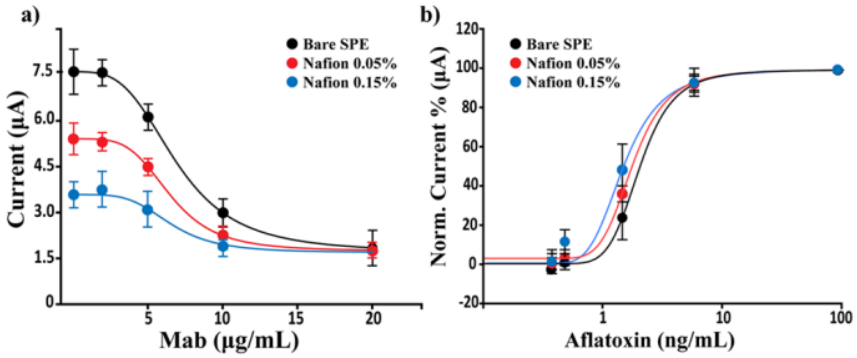


Figure 4. a) Binding curves (dilution of antibody solutions) and b) buffer competition curves obtained using Nafion®117 modified s and (0.05 and 0.15% vv^{-1}) and without fluoropolymer modification at room temperature. b) These results are obtained making use of SWV and 3 mM potassium ferricyanide solution in 50 mM PBS + 10 mM KCl, pH 7.4.

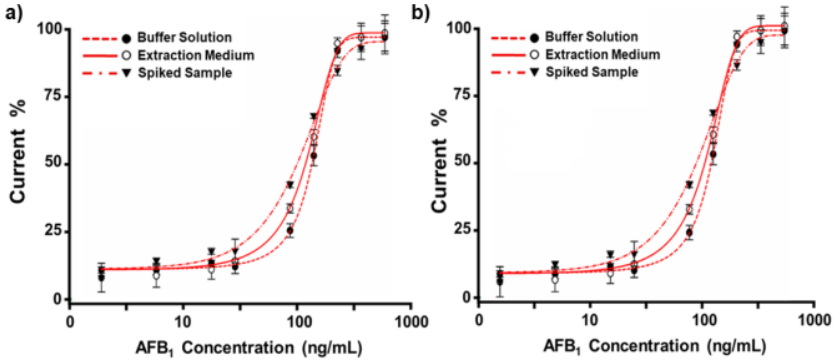


Figure 5. Competition curves realized a) with and without b) 0.05% Nafion®117 at room temperature. Solid lines represent real extraction medium, dashed lines buffer solution and dashed-dotted lines spiked sample measurements. These results are obtained making use of SWV and 5 mM potassium ferricyanide solution in 50 mM PBS + 10 mM KCl, pH 7.4

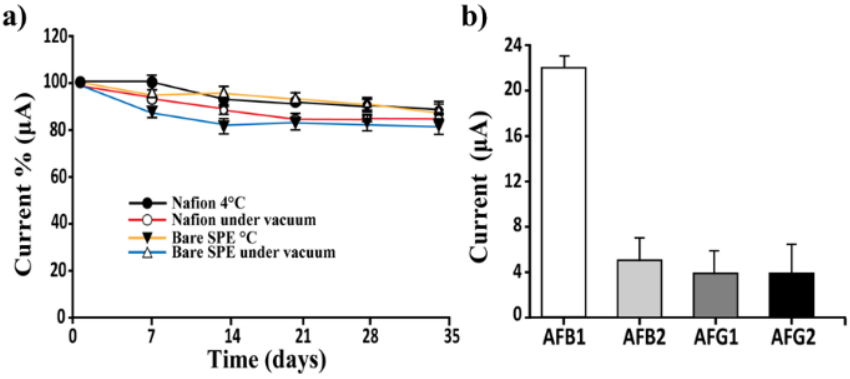


Figure 6. a) Stability of immunosensor, parallel investigation of bare and Nafion®117 (0.05%) modified electrodes stored under vacuum and at 4°C. b) Histograms relative to cross-reactivity experiments. All the measurements are realized in triplicate and the error measurements reported as data standard deviation. Aflatoxin concentration equal to 50 $ngmL^{-1}$.

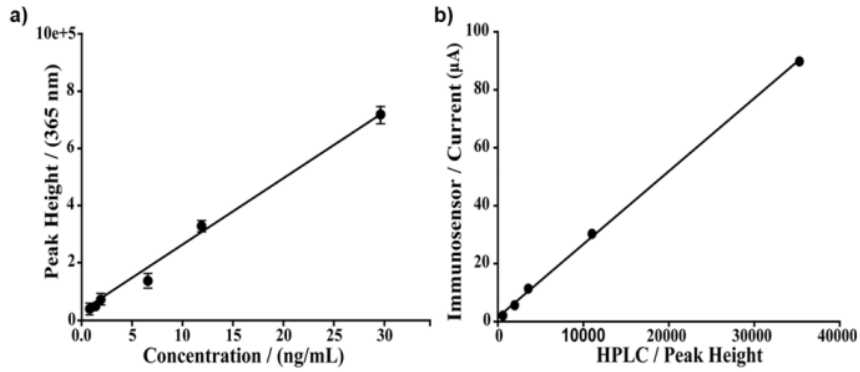


Figure 7. Immunosensor performance validation. a) Linear regression obtained using wheat flour samples contaminated with standard AFB1 concentrations tested with HPLC. b) Correlation plot of Voltammetric immunosensor and HPLC methods performances.

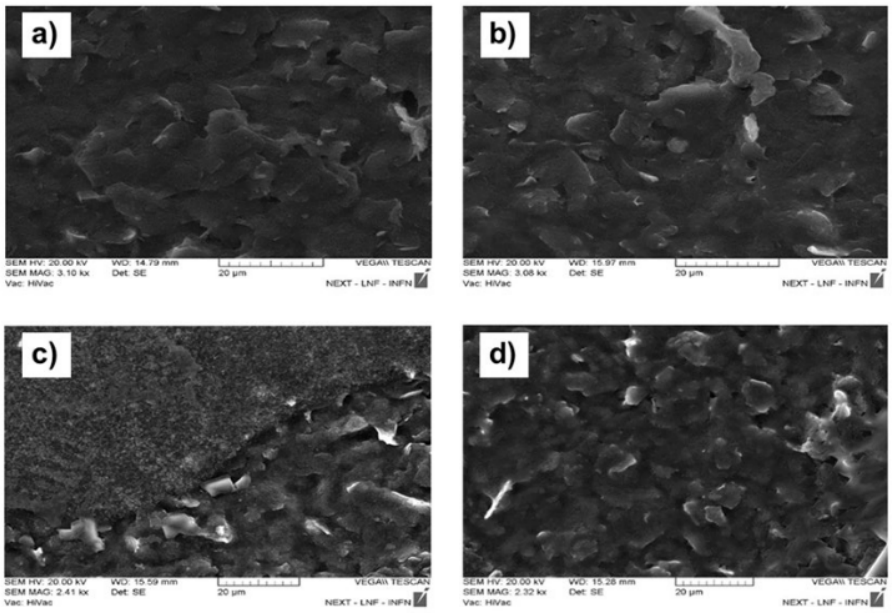


Figure 8. SEM characterization on SPEs A) bare and modified with B) AFB1-BSA conjugate, C) Nafion®117, D) Nafion®117-AFB1-BSA conjugate.

Table 1. Summary of the electrochemical devices based on screen-printed electrodes for determination of aflatoxin in food and feed.

Electrode*	Analyte	Analytical Methods	Linear Range	Detection Limit	Sample Matrix
SPCEs [31]	AFB_1	LSV	0.15-2.5 ng mL^{-1}	0.15 ng mL^{-1}	–
SPCEs [32]	AFM_1	chronoamperometry	30-160 ppt	25 ppt	Milk
SPEs [33]	AFB_1	linear voltammetry and impedance	1.0-50.0 nmol L^{-1}	0.47 nmol L^{-1}	Malted barley
SPEs [34]	AFM_1	CV	1–10^5 ppt	10 ppt	–
Au-SPEs [35]	AFB_1	chronoamperometry	0.16-2 pg mL^{-1}	0.159 pg mL^{-1}	Barley

SPE	Nafion ®117-present work	AFB_1	SWV	10-80 ng mL^{-1}	3.8 ng mL^{-1}	Cattle feed

Table 2. Analytical parameters summarization obtained analyzing real matrix samples using three different platforms: classical[38], label-free and Nafion 117 ®-modified label-free Immunosensors.

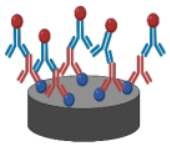
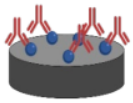
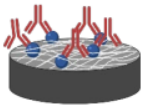
	 **Classical Immunosensor** [38]	 **Label-free Immunosensor**	 **Nafion®117 - Immunosensor**
LOD	0.09 ng mL^{-1}	9.81 ng mL^{-1}	3.78 ng mL^{-1}
Linear Range	0.1–10 ng mL^{-1}	36-165 ng mL^{-1}	10-80 ng mL^{-1}
RSD%	17%	20%	18%
Sensitivity	/	48 ngmL^{-1}	33 ngmL^{-1}

Table 3. EDX analysis on SPEs bare and modified with) AFB1-BSA conjugate, Nafion®117, and Nafion®117-AFB1-BSA conjugate.

	E1	**AN**	**C. norm (wt.%)**	**σ (wt.%)**
Bare-SPE	C	6	62.05	8.24
	Cl	17	12.53	0.45
	O	8	2.92	0.83
	N	7	1.22	0.78
	Si	14	0.19	0.04

	Total		100	
Bare-SPE-AFB1-BSA	C	6	58.97	6.79
	Cl	17	29.52	0.75
	O	8	4.43	0.89
	N	7	3.38	1.14
	Na	11	3.32	0.20
	Si	14	0.37	0.04
	Total		100	
Nafion®117-SPE	C	6	59.79	7.78
	O	8	18.85	5.58
	N	7	5.98	1.79
	F	9	5.55	1.31
	Si	14	4.62	0.24
	Na	11	2.02	0.19
	Al	13	1.68	0.12
	Cl	17	1.44	0.09
	S	16	0.07	0.03
	Total		100	
Nafion®-SPE-AFB1-BSA	C	6	52.53	0.79
	O	8	21.87	3.78
	N	7	7.32	3.54
	Na	11	7.49	0.49
	Cl	17	5.28	0.20
	Si	14	2.57	0.13
	F	9	2.27	0.60
	Al	13	0.88	0.07
	S	16	0.10	0.03
	Total		100	

Supplementary Materials:

Table 1S. Relative standard deviation (RSD%) and average current values obtained for different conjugate concentration.

AFB_1-BSA (μgmL^{-1})	Current (μA)	RSD%
0.5	4.33 ± 0.2	4
1.0	3.78 ± 0.1	2
1.5	3.94 ± 0.8	21
Bare Electrode	5.79 ± 0.1	2

Table 2S Relative standard deviation (RSD%) and average current values obtained for different conjugate concentration.

		Ferricyanide 1mM	Ferricyanide 3mM	Ferricyanide 5mM
Bare Electrode	Current [μA]	0.8 ± 0.2	1.8 ± 0.1	2.9 ± 0.2
	RSD%	3	5	8
Nafion®117-0.05%	Current [μA]	1.5 ± 0.3	4.3 ± 0.2	6 ± 1
	RSD%	22	5	29
Nafion®117-0.15%	Current [μA]	1.8 ± 0.4	4.7 ± 0.2	7 ± 2
	RSD%	22	5	32

Table 3S Average current and RSD percentage (RSD%) relative to cross-reactivity experiments.

Aflatoxin (ng/mL)	Current (μA)	RSD%	Response %
AFB_1	22.7 ± 4	17	100

AFB_2	4.5 ± 1.6	36	20
AFG_1	4 ± 3	75	18
AFG_2	4.4 ± 1.8	41	19

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