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Analytical Methods

Highly sensitive electrochemical impedance spectroscopy immunosensor for the detection of AFB_1 in olive oil

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ABSTRACT

Aflatoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus* are commonly found in olive and its derivatives. Aflatoxin B_1 (AFB_1) is a predominant toxin detected abundantly and has been implicated in the etiology of human hepatocellular carcinoma. This study proposes a sensitive and convenient electrochemical impedance spectroscopy (EIS) method for determining AFB_1 by MWCNTs/RTIL composite films-based immunosensor. The calibration curve for AFB_1 was linear in the range of 0.1–10 ng mL^{-1} with the limit of detection (LOD) 0.03 ng mL^{-1}. The presence of MWCNTs warrant fast electron transfer, and the ionic liquid provides a benign microenvironment for antibody. The experimental parameters, such as pH and incubating time, have been investigated and optimized. Furthermore, the detection of AFB_1 is presented to test this method after extracted from olive oils. It can be anticipated that this method would be used for the detection of AFB_1 in various agriculture products and vegetable oils.

1. Introduction

Aflatoxins are a group of highly toxic secondary metabolites produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* on a variety of food products (Li et al., 2009). These toxins are known to be potent carcinogens, teratogens, mutagens, and immunosuppression and pose harmful threat to animal and human health. Naturally occurring aflatoxins are composed of B_1, B_2, G_1 and G_2. Among them, aflatoxin B_1 (AFB_1) is the most abundant and carcinogenic. The International Agency for Research on Cancer (IARC) has classified AFB_1 as a Group 1 carcinogenic to humans (Piermarini, Micheli, Ammida, Palleschi, & Moscone, 2007). The European Committee Regulations (ECR) has established the maximum permitted levels of 2 ng/g for aflatoxin B_1 and 4 ng/g for total aflatoxins (B_1, B_2, G_1 and G_2) in various products (Gilbert & Anklam, 2002).

The olive (*Olea europaea* L.) and its derivatives, in particular olive oil, are significant products in the Mediterranean and Middle East regions (Cavaliere et al., 2007). Several survey studies have shown that olives are often stored for weeks in conditions that promote the mold growth, and the possible presence of aflatoxins would transfer into olive oil (Ghitakou, Koutras, Kanellou, & Markaki, 2006). Since olive oils are widely consumed as diet in the Mediterranean, even low levels of contamination may cause severe health and safety problems.

Several methods and techniques for aflatoxin determination have been developed. High-performance liquid chromatography (HPLC) (Hu, Zheng, Zhang, & He, 2006), thin layer chromatography (TLC) (Lin, Zhang, Wang, Wang, & Chen, 1998), enzyme-linked immunosorbent assay (ELISA) (Rossi et al., 2012) and fluorescence methods have been used for the detection of aflatoxins. However, most of these techniques require well equipped laboratories, trained personnel, harmful solvents and are time-consuming. Thus, there is a need for developing a simple, rapid, and an effective analytical method for the determination of aflatoxins.

In comparison with abovementioned methods, the electrochemical impedance spectroscopy (EIS) technique is low-cost, sensitive and relatively easy to use (Ding, Du, Wu, & Ju, 2007). Impedance spectroscopy (EIS) is a non-destructive steady-state technique, and can be used as an ideal tool to detect the dynamics of bio-molecular interactions (Vig, Radoi, Muñoz-Berbel, Gyemant, & Marty, 2009).

In this work, a practical and sensitivity impedimetric immunosensor is proposed for the determination of aflatoxin-B_1 based on MWCNTs/RTIL/Ab-modified electrode. CNTs are highly electroconductive and characterized by a large surface area and excellent chemical and physical stabilities. Ionic liquids are ion conductive, and display a wide electrochemical potential window (Opallo &

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Lesniewski, 2011). Ionic liquids have also received much attention due to their unique structural and intermediate phase properties in nanometer-scale confinement (Chen et al., 2009). These properties are promising for electrode modifiers that are required to mediate electron-transfer reactions between electrodes and redox active species. The MWCNTs in the ILs exist as a three-dimensional (3D)-network structure of considerably untangled and much finer bundles, which are physically cross-linked due to cation–π and π–π interactions between the imidazolium cations and CNTs (Fujita, Murata, Masuda, Nakamura, & Ohno, 2012). The three-dimensional network will provide numerous conductive microcavities for the antibodies immobilization and small molecule hapten transfer (Li et al., 2012; Moghaddam, Løbersli, Gebhardt, Braunagel, & Marvik, 2001). The ionic liquid provides excellent biocompatible microenvironment to maintain activity of the antibody, and can be used as an excellent conductive binder to immobilize antibodies and MWCNTs onto the surface of electrode (Opallo & Lesniewski, 2011). Recombinant antibodies to low molecular weight haptens are of interest for diagnostic detection of food toxins, environmental pollutants and drugs of abuse. In present work, antibody against AFB_1 is used as probe for immunosensor construction. For practical application, the efficiency of the immunosensor has been evaluated by spiking blank olive oil with AFB_1.

2. Materials and methods

2.1. Materials

The aflatoxin B_1 (AFB_1) standard sample was obtained from Beijing Lianlixin BioTech Co., Ltd. Monoclonal antibody (Ab) was from Wuxi JieshengJiekang Bio-Tech Co., Ltd. Olive oil was purchased from a supermarket in Shanghai (China). Phosphate buffer solution (10 mM PBS, pH 7.5) contained 87 mM Na_2HPO_4, 14 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl. Aqueous solutions of $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1.0 mM, 1:1) mixture with 0.1 M KCl (as the supporting electrolyte) were prepared as electrochemical probes. 1-Butyl-3-methylimidazolium hexafluorophosphate ([BMIM]PF_6) was purchased from Hangzhou Kemer Chemical Co., Ltd. (China). Multi-wall carbon nanotubes (MWCNTs) (95% purity, diameter 10–20 nm, length 1–2 μm) were purchased from Shenzhen Nanotech Port Co., Ltd. Hexane (AR), methanol (AR), and chloroform (AR) were purchased from Shanghai Chemical Reagent Ltd. (China). Ultra-pure water (18.2 MO) was used throughout the experiments.

2.2. Apparatus

All of the electrochemical experiments were carried out with a model CHI660B electrochemical workstation (Shanghai Chenhua Instruments, Shanghai, China). Electrochemical measurements were performed with a conventional three-electrode system comprised of a platinum wire as the auxiliary electrode, saturated calomel electrode (SCE) as the reference and a modified GCE as the working electrode. All electrochemical measurements were carried out in PBS buffer (10 mM phosphate, pH 7.4) containing 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) and 0.1 M KCl as a redox couple. The electrochemical impedance spectra were recorded in the frequency range from 0.1 to 10^5 Hz, at the formal potential (0.20V versus SCE) of the redox couple and with a perturbation potential of 5 mV.

2.3. Functionalization of MWCNTs

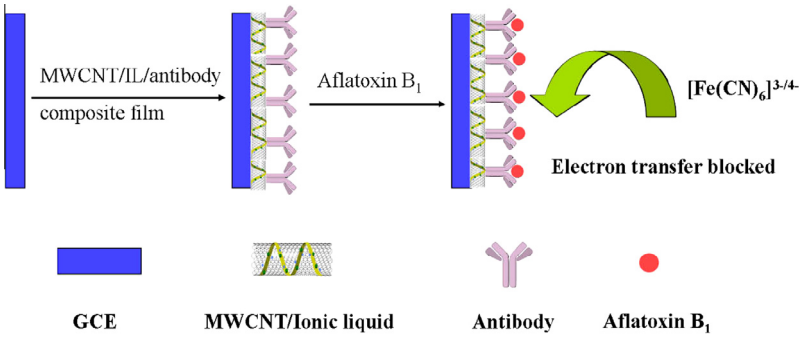
MWCNTs were suspended in a concentrated sulfuric acid/nitric acid mixture (3:1 v/v) and sonicated in a sonic bath for 16 h. After this treatment, the shortened carboxylate MWCNTs were obtained after filtration and were then thoroughly rinsed with water (Jia et al., 2010).

2.4. Electrodes preparation

Prior to modification, the glassy carbon electrodes (GCE, 3 mm in diameter) were polished with 0.3 μm and 0.05 μm alumina powder to form a mirror surface. The electrodes were successively sonicated in 1:1 (v/v) nitric acid/water solution, acetone and ultra-pure water, and then allowed to dry under a stream of nitrogen. Initially, a MWCNTs/ionic liquid mixture was prepared by adding 0.5 mg of the shortened carboxylate MWCNTs into 0.25 mL of the ionic liquid, and grinded them in an agate mortar for about 1 h. Then the suspensions were centrifuged at 15,000 rpm for about 30 min. The [bmim]PF_6/MWCNTs was obtained by removing the supernatant with a pipette. Following that, for preparing MWCNTs/RTILs/Ab modified electrode, 0.25 mL of the Ab (1.64 μg mL^{-1}) solution was dropped into the MWCNTs/RTILs mixture, and slightly stirred for 0.5 h. Then 3 μL of the above mixture was dropped on the electrode surface to fabricate electrochemical biosensing layers. The electrode was left in a fume cupboard to dry for 8 h at room temperature. Subsequently, for immunity of the sensor, the modified electrode was incubated in the 10 mL of PBS (pH 7.5) containing different concentrations of AFB_1 at 37 °C for 25 min. After this process, the MWCNTs/RTILs/Ab/AFB_1 electrode was rinsed with water and then with phosphate-buffered saline (pH 7.5) solution. As comparison, the MWCNTs/RTILs mixture modified electrodes were prepared in a similar way. The fabricated immunosensor was stored at 4 °C when not in use. The prepared process of MWCNTs/RTILs/Ab/AFB_1 immunosensor is schematically illustrated in Scheme 1.

2.5. Preparation of aflatoxin standards

Aflatoxin standard solutions were prepared as detailed in AOAC 971.22 (18th edition, 2005) (Horwitz & Jr., 2005). Using this



Scheme 1. Schematic representation of the fabrication of the immunosensor.

procedure, individual solutions of aflatoxins B_1 were prepared by dissolving approximately 0.1 mg of each aflatoxin with benzene/acetonitrile (98:2, v/v) in a 100 mL volumetric flask.

2.6. Spiking of olive oil samples

Oil samples were spiked with aflatoxins B_1 at 1, 10, 30, 50, 70, 100, 130, 150 ng mL^{-1} levels in triplicate. These spike levels represent values above and below the maximum allowable regulatory limits of the European Committee Regulations (ECR), which is 2 μg/kg. Oil samples (2 g) were spiked in triplicate with 100 μL of aflatoxin standard solution for each of the three aflatoxin concentrations. A blank spiked with the solvent only and no aflatoxin was also prepared for each oil sample.

2.7. Extraction

Aflatoxins were extracted from oil samples by using the procedure developed by Daradimos et al. during which the final clean-up step using Sep-Pak cartridge was omitted (Daradimos, Marcaki, & Koupparis, 2000). Briefly, spiked oil samples (20 g) and hexane (80 mL) were transferred into a 250 mL separating funnel and extracted with 120 mL methanol/water extraction solvent (60/40 v/v) containing 4% NaCl. Then, AFB_1 was extracted from the obtained aqueous/methanolic by chloroform (26 mL).

3. Results and discussion

3.1. Cyclic voltammetry characterization

The changes of interface properties on the modified electrode after each assembly step could be efficiently detected by CV. Fig. 1A shows the cyclic voltammetry measurements based on the MWCNTs/RTILs/Ab-based immunosensor incubated in PBS (at pH 7.5) containing different concentrations of AFB_1 (0.0, 1.0, 5.0 ng mL^{-1}) and 1.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$. A pair of well defined oxidation and reduction peaks is observed at the bare GCE (Fig. 1A, curve a) due to high electron-transfer between $[Fe(CN)_6]^{3-/4-}$ solution and the electrode. Immobilization of Ab-MWCNTs/RTILs on the GCE decreases the current of the redox couple (Fig. 1A, curve b). The reason is mainly attributed to the protein layer on the MWCNTs, which acts as an inert electron transfer blocking layer and hinders the diffusion of redox couple toward the electrode surface. After the binding of AFB_1 to the immobilized antibodies, the peak current of the redox couple was further reduced (Fig. 1A, curve c and d). It is reasonable that the Ab-AFB_1 complex layer blocked the electron communication between the redox couple $[Fe(CN)_6]^{3-/4-}$ and the electrode.

3.2. Impedance spectroscopy characterization

Electrochemical impedance spectroscopy is a highly effective technique to investigate the electron-transfer properties of the modified electrodes (Hu et al., 2012; Weng, Zhang, Sun, & Wang, 2011). The impedance spectra include a semicircle part at high frequencies and a linear part at lower frequencies. The semicircle portion corresponds to the electron transfer limited process and the linear portion results from the diffusion limiting step of the electrochemical process. The semicircle diameter in the impedance spectra infers the electron transfer resistance (R_{et}) (Cui, Huang, Yin, Gao, & Zhu, 2008). An equivalent circuit model (inset in Fig. 1B) was used to fit the impedance data into R_{et} values. As shown in Fig. 1B, at a bare GCE, the redox process of the probe showed an electron-transfer resistance of about 151.9 Ω (Fig. 1B, curve a), while the MWCNTs/RTILs/Ab-modified electrode showed a higher resistance (605.6 Ω, Fig. 1B, curve b). When AFB_1 was immobilized on MWCNTs/RTILs/Ab-modified electrode, the R_{et} increased to 1865 Ω (Fig. 1B, curve c) and 4185 Ω (Fig. 1B, curve d), which is proportional to the amount of AFB_1 (1.0 ng mL^{-1} and 5.0 ng mL^{-1}, respectively). The increase of R_{et} is caused by electrically insulating bioconjugates produced from specific interaction of AFB_1 and Ab, which will block the electron-transfer process of the redox probe. Therefore, the results of electrochemical impedance spectroscopy (EIS) assays were in correspondence with those of the above CV measurements.

3.3. Optimization of experimental conditions

At the fabricated immunosensor, the electrochemical signal was related to the preparation process of the modified film, containing pH of the solution and incubation time of antigen. It is well known that protein activity is highly pH dependent and the optimum pH for anti-AFB_1 antibody activity could be determined experimentally. It can be seen from Fig. 2A that the ΔR_{et} value increased with the increase of pH from 6.0 to 8.0. The immunosensor demonstrated the maximum response at pH 7.5. Consequently, an ideal pH of about 7.5 was selected for the test.

The incubation time was an important parameter for capturing AFB_1 antigens on the electrode surface. After increasing the incubation time with 7 ng mL^{-1} AFB_1, the impedance response at the MWCNTs/RTILs/Ab-modified electrode increased and tended to a steady value after 25 min (Fig. 2B), indicating that the bonding sites of anti-AFB_1 antibody tend to saturate. Longer incubation

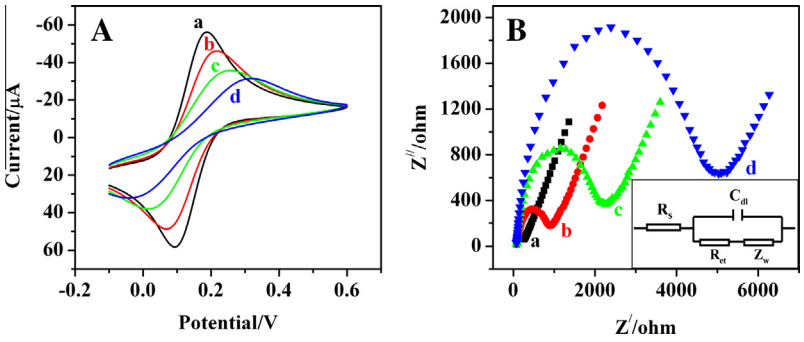


Fig. 1. Characterization of different electrodes by (A) cyclic voltammograms and (B) electrochemical impedance spectroscopy, (a) bare GCE and modified GCE fabricated based on the MWCNTs/RTIL composite films incubated in PBS (at pH 7.5) containing AFB_1 of (b) 0.0 ng mL^{-1}, (c) 1.0 ng mL^{-1} and (d) 5.0 ng mL^{-1} for 25 min in 1.0 m mol L^{-1} $[Fe(CN)_6]^{3-/4-}$. (Inset) An equivalent circuit representing the immunosensor-electrolyte solution interface. R_s is the solution-phase resistance; C_{dl} is the double-layer capacitance; R_{et} is the electron transfer resistance; Z_w is the Warburg impedance.

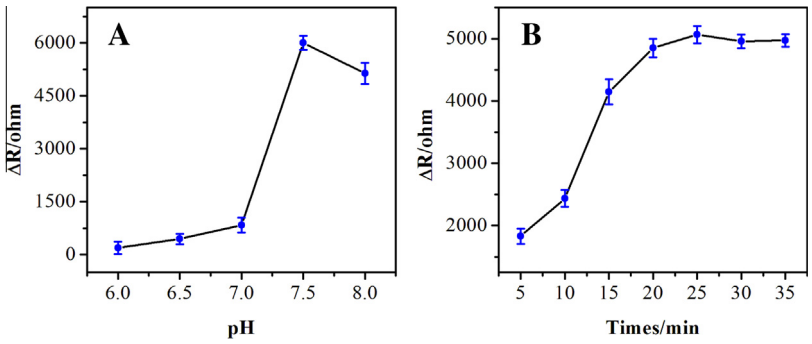


Fig. 2. Effect of (A) pH of PBS on the specific binding of AFB_1 and the Ab immobilized on the surface of the immunosensor. (B) Incubation time on the specific binding of AFB_1 and the antibody immobilized on the surface of the immunosensor.

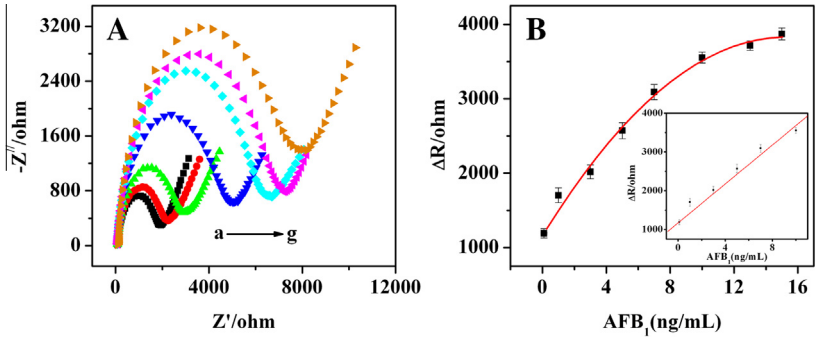


Fig. 3. (A) Nyquist diagrams of MWCNTs/RTIL/Ab/AFB_1 modified GCE after incubation at 37 °C with (a) 0.1 ng mL^{-1}, (b) 1 ng mL^{-1}, (c) 3 ng mL^{-1}, (d) 5 ng mL^{-1}, (e) 7 ng mL^{-1}, (f) 10 ng mL^{-1}, (g) 13 ng mL^{-1}, (h) 15 ng mL^{-1} of aflatoxin B_1 in pH 7.5 PBS containing 1.0 mM of $[Fe(CN)_6]^{3-/4-}$. (B) Linear relationship between electron transfer resistance and AFB_1 concentration in PBS.

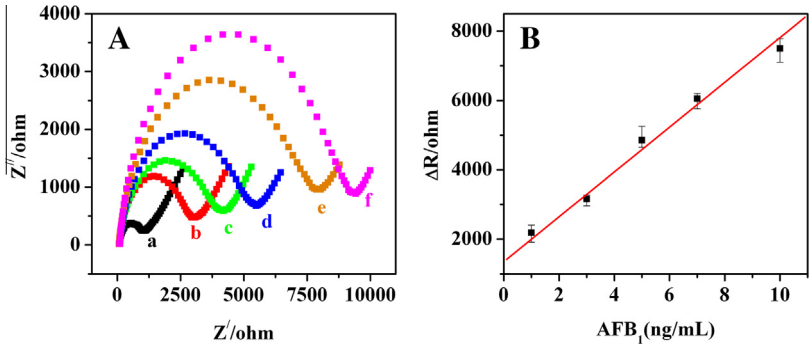


Fig. 4. (A) Bare GCE electrode after modified with MWCNTs/IL/Ab, and adhered with different concentrations of AFB_1 in olive oil extraction solution: (a) background and after respective incubation with (b) 1 ng mL^{-1}, (c) 3 ng mL^{-1}, (d) 5 ng mL^{-1}, (e) 7 ng mL^{-1}, (f) 10 ng mL^{-1}; (B) Linear regression of ΔR_{et} versus concentration of AFB_1 in olive oil extraction solution (1–10 ng mL^{-1}).

time would not enhance the response. Therefore, 25 min was chosen for the incubation of AFB_1.

3.4. Analytical characteristics

The electrochemical impedimetric immunosensor response to various AFB_1 concentrations was studied. The modified electrode was immersed in different concentrations of AFB_1 and incubated at 37 °C for 25 min. After rinsed thoroughly with PBS buffer (pH 7.5) to remove nonspecifically bound conjugations, the immunosensor was used for electrochemical detection of AFB_1. Measurements were carried out at conditions of frequency between 0.1 Hz to 10^5 Hz and an AC amplitude of 5 mV, and the representative Nyquist diagrams were shown in Fig. 3A, in which curve a–g represent the AFB_1 concentration of 0.1, 1, 3, 5, 7, 10, 13 and 15 ng mL^{-1}, respectively. It can be seen that the electron-transfer resistance (R_{et}) value increased obviously with the increasing concentration of AFB_1. This result demonstrated that the more AFB_1 bound on the immunosensor surface, the higher was the electron-transfer resistance of the immunosensors.

The calibration curve was obtained by plotting the relative resistance versus AFB_1 concentration (Fig. 3B). The increased R_{et} value was proportional to the logarithm of the AFB_1 concentration in the range of 0.1–10 ng mL^{-1}. The linear equation could be depicted as ΔR_{et} (Ω) = 268.4 C + 1217.6 (R = 0.995). The detection limit of 0.03 ng mL^{-1} could be estimated from the signal-to-noise characteristics of these measurement data (S/N = 3). The change of electron-transfer resistance tended to a relatively steady value when the concentration of AFB_1 exceeding 10 ng mL^{-1}.

Table 1
The results of spiked olive oil samples using an electrochemical biosensor for AFB_1 determination.

Samples	AFB_1 added (ng mL^{-1})	AFB_1 found (ng mL^{-1})	RSD (%)	Recovery (%)[a]
1	2	2.3	5.7	116
2	4	4.14	2.3	104
3	7	6.75	3.1	96

[a] Averages of three different samples.

3.5. Stability and reproducibility of the AFB_1 immunosensor

The long-term stability and reproducibility of the EIS biosensor was also tested. After two months' storage (4 °C), the impedance response was retained at 87% value of the initial response. This indicated that the developed AFB_1 biosensor possessed good storage stability. The relative standard deviation (RSD) of intra-assay was investigated to be 4.3% and 2.7% by measuring the immunosensor response at the AFB_1 concentrations of 3 ng mL^{-1} and 5 ng mL^{-1}, showing an acceptable precision. For inter-assay reproducibility testing, a series of six measurements based on six immunosensors resulted in an RSD of 5.6% at 5 ng mL^{-1} AFB_1. The results indicated that the preparation of the immunosensor had acceptable inter-assay reproducibility.

3.6. Measurement of AFB_1 in olive oils

To demonstrate the applicability of the proposed method, the immunosensor-based EIS assay was then applied to the detection of the AFB_1 in olive oils. The olive oils samples were collected and the extraction procedure was performed as described in preparation Section. The MWCNTs/RTILs/Ab-based immunosensor was incubated in olive oil extraction solutions containing different concentrations of AFB_1 (1–10 ng mL^{-1}) at 37 °C for 25 min, and then carried out a series of EIS measurements with $[Fe(CN)_6]^{3-/4-}$ as a redox probe (Fig. 4A). The corresponding calibration plot (Fig. 4B) of background-subtracted R_{et} value versus AFB_1 concentration was described by the follow equation: ΔR_{et} (Ω) = 600.56 C + 1651.9. Thus, the original concentration of AFB_1 in olive oil samples can be calculated from the calibration curve. For each concentration, five different samples were analyzed independently. Recovery studies were carried out with AFB_1 solution and AFB_1 spiked oil sample at different levels. Three olive oils samples were spiked to obtain final concentrations of AFB_1 equal to 2, 4 and 7 ng mL^{-1}, respectively. The recovery was found to be in the range of 96–116%. For the replicate measurements, the accuracy was determined by calculating the relative standard deviation (RSD) and the values are summarized in Table 1. The obtained good recovery data demonstrated the suitability of the proposed method for the detection of AFB_1 in olive oil.

Furthermore, the results obtained by this immunosensor-based method was also compared to a standard fluorescence-based approach. As displayed in Table S1, the relative deviation was lower than 6.5%, confirming our developed immunosensor has a potential application in detection AFB_1 with a good accuracy and precision.

4. Conclusion

In summary, this study has successfully developed a simple, reliable method for the determination of aflatoxins B_1 in olive oils after the simple and rapid extraction procedure. Due to the ionic liquid offers mild microenvironment and good conductibility, experimental results indicated that the electrochemical immunoassay exhibited high sensitivity, acceptable reproducibility and stability. The simplicity of the method should make it suitable for use in the detection of many other agriculture products and vegetable oils (such as cereals, nuts, peanuts, fruits, oilseeds and dried fruits). Our future study will use this method to prepare an electrochemical immunosensor for detecting aflatoxins in other products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.12.030.

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