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Development, optimization and validation of an electrochemical immunosensor for determination of total aflatoxins in pistachio

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ABSTRACT

Aflatoxins (AFs) can be produced by fungi in nuts such as pistachios, groundnuts, and cashews. As ingestion of even low amounts of AFs can lead to adverse effects for humans, their levels in food are strictly regulated. Electrochemical immunosensing, providing sensitive, simple, and cost-effective instrumentation, has emerged as an advantageous alternative to standard AFs analysis, which is typically based on liquid chromatography with fluorescence or mass spectrometry detection.

Approaches for AFB1 detection, the most common in food and the most potent genotoxic and carcinogenic aflatoxin, have been widely reported. However, total AFs (sum of AFB1, AFB2, AFG1 and AFG2) determination, also required by the legislation, is scarcely reported with electrochemical immunosensors.

We present here a novel sensor to detect traces of total AFs in pistachio and its cross-validation using a confirmatory method. The technology consists in an immunosensor on a screen-printed carbon electrode, featuring a competitive assay, followed by electrochemical detection. The optimized sensor covered a linear range of 0.01–2 μg L^{-1} with good reproducibility (RSD: 2%). The limit of detection was estimated at 0.017 μg L^{-1} and 0.066 μg kg^{-1} in PBS buffer and in pistachio matrix respectively, the latter being well below the maximum level for total AFs in pistachio set by the European legislation. The system exhibited excellent selectivity against Zearalenone and Ochratoxin and other possible interferences (glucose, Na^+, K^+), as well as a long-term stability of at least 30 days at room temperature. The methodology comprised extraction of AFs from pistachio using immunoaffinity columns and showed good recovery (87–106%), in addition to being validated with a LC-MS/MS method. Overall, the developed immunosensor is simpler, cheaper and more rapid, and exhibits sensitivity comparable to ELISA and LC-MS/MS, making our approach a promising tool for total AFs screening at the point-of-use.

1. Introduction

Aflatoxins (AFs) are mycotoxins produced mainly by filamentous fungi such as *Aspergillus flavus* and *Aspergillus parasiticus*. AFs are secondary toxic metabolites, which are classified as carcinogenic to humans (Group 1), according to the International Agency for Research on Cancer (IARC) (Gacem & Ould El Hadj-Khelil, 2016; IARC, 2012). Moreover, they are considered to be teratogens, mutagens and immunosuppressants. Metabolites of these types of mycotoxins are able to bind to nucleic acids of RNA and DNA or to proteins, forming products that accumulate over years (Tirmenstein & Mangipudy, 2014). The determination of AFs is of great relevance in the agri-food field since they are natural contaminants in a wide variety of agricultural products, such as nuts, cereals and milk.

The most toxic AFs, and strictly regulated for food control, are: AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2. AFM1 and AFM2 are secondary metabolites produced after digestion of food/feed contaminated with AFB1 and AFB2. Abdominal pain, vomiting, pulmonary edema, liver damage, jaundice and even death are the short-term consequences of the consumption of food contaminated with AFs. Long-term consequences

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are associated to an increased probability to develop liver cancer (NTP (National Toxicology Program), 2021).

Maximum levels (ML) for AFB1 and for total AFs (the sum of AFB1, AFB2, AFG1 and AFG2) in foodstuffs are laid down in the European Commission Regulation (EC) No 1881/2006 a,b. The maximum levels in pistachio are 8 μg kg^{-1} for AFB1 and 10 μg kg^{-1} for the sum of AFB1, AFB2, AFG1 and AFG2, respectively, so trace detection methods for total AFs determination are necessary.

A possible strategy to control compliance with legislation on aflatoxins is to combine screening methods with existing confirmatory, laboratory-based methods.

Confirmatory analytical techniques for quantitative trace determination of mycotoxins (Turner et al., 2015) are typically based on high-resolution liquid chromatography with fluorescence detection (HPLC-FLD) (Martínez-Miranda, Taborda-Ocampo, & Rosero-Moreano, 2015) and high-resolution liquid chromatography combined with mass spectrometry (HPLC-MS). These techniques require skilled operators, extensive sample pretreatments, expensive instruments and time-consuming procedures, thus being less applicable i) "in the field", at the point of need/use and ii) in countries with low infrastructure and limited analytical resources.

To be able to apply smart sampling and rapidly and cost-effectively screen a high number of samples for non-compliances with a low rate of false negatives (5%), methods based on antigen-antibody recognition have emerged as screening methods for testing for aflatoxins.

Enzyme-linked immunosorbent assays (ELISA), based on the specific binding between toxin target antigen and its antibody, is one example of a method used for screening of mycotoxins (Li et al., 2009). ELISA has been successfully employed for years in the screening of mycotoxins (Azer & Cooper, 1991; Leszczyńska, Masłowska, Owczarek, & Kucharska, 2018), however, it suffers from important limitations, such as the consumption of high volumes of solvents and long analysis time, in addition to the need of specialized laboratory equipment for optical detection and signal transduction.

Therefore, to focus limited resources (in terms of staff and equipment) and to provide analytical laboratories with sensitive, simple, cost-effective and low-cost instrumentation, immunosensing methods, particularly those coupled to electrochemical detection, have emerged as advantageous alternative for aflatoxins detection (Liu et al., 2020). These systems benefit from the inherent advantages of the electrochemical techniques in terms of sensitivity, simplicity and low cost of instrumentation (Wen, Yan, Zhu, Du, & Lin, 2017).

Electrochemical approaches for mycotoxin analysis, including those based in immunosensors have been recently reviewed in the bibliography (Atul Sharma, Goud, Hayat, Bhand, & Marty, 2017; Chauhan, Singh, Sachdev, Basu, & Malhotra, 2016; Goud et al., 2018; Liu et al., 2020; Pérez-Fernández & de la Escosura-Muñiz, 2022; Reverté, Prieto-Simón, & Campàs, 2016; Wang, Niessner, Tang, & Knopp, 2016; Zhou & Tang, 2020). Traditional electrodes like those made of glassy carbon (GCE) (Arati Sharma, Kumar, & Khan, 2018; Shu, Qiu, Wei, Zhuang, & Tang, 2015; Zhang et al., 2016) and indium tin oxide (ITO) (Singh et al., 2013; Solanki, Singh, Rupavali, Tiwari, & Malhotra, 2017), modified with different nanomaterials have been proposed for AFB1 immunosensing. However, such electrodes require relatively high sample volumes and quite complicated cell set-up, which makes them not particularly suitable for "in field" applications. On the opposite, screen-printed electrodes (SPEs) have emerged as an outstanding alternative, overcoming the limitations of traditional ones. Screen printing is a well-developed method technology used to fabricate disposable and economical electrochemical sensors, satisfying the need for highly reproducible, sensitive and cost-effective detection, that requires minimal sample volume (Yamanaka, Vestergaard, & Tamiya, 2016). Their adaptability and ease of modification are of great importance and allow for detection of specific targets, such as pesticides (Pérez-Fernández et al., 2019a, 2019b, 2020), drug residues (Honeychurch et al., 2013) and heavy metal ions (Metters, Kadara, & Banks, 2012), among others.

Competitive electrochemical immunosensors for mycotoxins like AFB1 (Pemberton, Pittson, Biddle, Drago, & Hart, 2006), AFM1 (Karczmarczyk, Baeumner, & Feller, 2017; Micheli, Grecco, Badea, Moscone, & Palleschi, 2005; Parker & Tothill, 2009; Vig, Radoi, Muñoz-Berbel, Gyemant, & Marty, 2009), Ochratoxin A (OTA) (Alarcón, Micheli, Palleschi, & Compagnone, 2004, 2006; Karczmarczyk et al., 2017; Vidal, Bonel, Duato, & Castillo, 2011) and Zearalenone (ZEN) (Goud et al., 2019; Hsieh, Chen, Chang, She, & Chou, 2013) detection using unmodified screen-printed carbon electrodes (SPCEs) can be found in the literature. With the aim of improving the system performance, SPEs modification with different nanomaterials has also been proposed for the detection of AFB1 (Azri, Selamat, & Sukor, 2017, 2018), AFM1 (Abera et al., 2019; Tang et al., 2022), OTA (Cancelliere et al., 2021; Jodra, Hervás, López, & Escarpa, 2015; Malvano, Albanese, Crescitelli, Pilloton, & Esposito, 2016; Malvano, Albanese, Pilloton, & Di Matteo, 2016; Perrotta, Arévalo, Vettorazzi, Zón, & Fernández, 2012; Radi, Muñoz-Berbel, Lates, & Marty, 2009; Vidal, Bonel, Ezquerra, Duato, & Castillo, 2012) and ZEN (Hervás, López, & Escarpa, 2010; Riberi, Tarditto, Zon, Arévalo, & Fernández, 2018).

Although detection of AFB1 is important from a toxicological perspective, as AFB1 is the most common in food and among the most potent genotoxic and carcinogenic of the aflatoxins, total AFs (sum of AFB1, AFB2, AFG1 and AFG2) determination is also required by the legislation on food safety control, and scarcely reported with electrochemical immunosensors.

In this context, the objective of this work is the development of an immunosensor based on SPCEs for the detection of total AFs (sum of AFB1, AFB2, AFG1 and AFG2) in pistachio samples at levels lower than 10 μg kg^{-1} levels (maximum level of total AFs in pistachio in the EU legislation), and the cross validation of the sensor performance using a validated sample preparation method and a confirmatory approach using LC-MS/MS. The importance of this study also lies in the provision of a general methodology and know-how to analysts from least developed countries, that can adopt the procedure to prepare their own cost-efficient testing devices, independently from commercial ready-to-use analytical tools.

2. Experimental section

2.1. Materials

Aflatoxin Mix 4 solution (AF-MIX) (certified reference material containing aflatoxin B1, B2, G1 and G2; Ref. 33415); zearalenone (ZEN; Ref. CRM46916); ochratoxin A (OTA; Ref. 494128); bovine serum albumin fraction V (BSA) (Ref. A3059); 3,3′,5,5′-Tetramethylbenzidine (TMB) (Ref. T0440); phosphate buffered saline solution (PBS) 10 mmol L^{-1}, pH 7.4; sodium chloride (Ref. 746398); were purchased from Sigma-Aldrich (Germany). Aflatoxin-BSA conjugate (BSA-AF) (Ref. MBS5307971) and mouse monoclonal antibody specific to aflatoxin B1, B2, G1 and G2 (mAb-AF) (Ref. MBS569063) were purchased by MyBioSource (USA). Polyclonal rabbit anti-mouse IgG-HRP (anti-IgG-HRP) (Ref. P026002-2) was purchased by Drako, Agilent (Germany). Glucose (Ref. 108337), potassium chloride (Ref. 104933) and methanol (Ref. I536909-021) were purchased from Merck (Spain). All chemicals employed were of analytical reagent grade. 0.45 μm syringe filters (PTFE) (Ref. 514–0069) were purchased by VWR International (Austria). 0.2 μm syringe filters (PTFE) (Ref. 10463881) were purchased from Whatman (Austria). Immunoaffinity columns AflaTest WB (IAC) (Ref. G1024) were acquired from VICAM (USA).

Ultrapure water was obtained with an EMD MilliporeTM Direct-Q5TM purification system from Millipore Ibérica SA (Spain) and from MilliQ Integral 3 from Millipore (Austria).

Working solutions of BSA-AF, mAb-AF, anti-IgG-HRP and BSA were prepared daily in 10 mmol L^{-1} pH 7.4 PBS buffer.

Organic pistachios were bought in a local supermarket and processed using an automatic pestle and mortar mill RM200 (Retsch, Germany). A

vortex mixer (IKA, Germany) and a Sigma 3–30 KS centrifuge, (VWR International, Austria), were used for the sample preparation.

Chronoamperometric measurements were performed with an EmStat3 Blue potentiostat purchased from PalmSens (The Netherlands) controlled by a Smartphone interface. All measurements were carried out at room temperature.

Screen-printed carbon electrodes (SPCEs) (carbon working and auxiliary electrodes and a silver/silver chloride quasi-reference electrode) (Ref. DRP-110) were purchased from Dropsens (Spain). Details of the electrodes and the electrochemical set-up are shown at Fig. 1.

For the cross-validation of the method, the pistachio extracts were analyzed using ultra high-performance liquid chromatography (Shimadzu Nexera X2 Series, Kyoto, Japan) equipped with LD-30AD solvent delivery unit, SIL-30AC autosampler, CBM-20A communications bus module and CTO-20AC prominence column oven, coupled to a Shimadzu 8060 triple quadrupole mass spectrometer (Kyoto, Japan). An Acquity UPLC® HSS T3, 1.8 μm, 2.1 × 100 mm, column was used for chromatographic separation.

Conditions of LC-MS/MS analysis: Mobile phases: A, 10 mmol L^{-1} ammonium acetate in water; B, methanol. Gradient program: 5% B to 100% of B over 4.5 min, held at 100% B until 9.5 min then decreased to 5% B. Total run time: 12 min with a flow rate of 0.4 mL min^{-1}. Oven temperature: 40 °C. Injection volume: 4 μL. The MS parameters and the collision energies were optimized to achieve the highest responses for all analytes. Nebulizing gas flow: 3 L min^{-1}, heating gas flow rate: 10 L min^{-1} and drying gas flow rate: 10 L min^{-1}. Interface temperature: 400 °C, desolvation line temperature: 250 °C, heat block temperature: 400 °C. Electrospray ionization (ESI) was employed in positive mode. Interface voltage: 4.5 kV. The multiple reaction monitoring (MRM) transitions and collision energies were selected and optimized by flow injection analysis. The aflatoxins, the optimized transitions and the collision energies chosen are summarized in Table S1 at the Supplementary Material.

2.2. Methods

2.2.1. Preparation of blank pistachio sample

Organic pistachio samples were dehulled and subsequently subjected to homogenization in an automatic pestle and mortar mill using liquid nitrogen. The resulting homogenized pistachio "paste" was stored in a freezer (−20 °C) until analysis. The pistachio paste was analyzed by LC-MS/MS to verify the absence of response at the target retention time of the aflatoxins in MRM mode.

2.2.2. Preparation of fortified pistachio samples

Individual portions of 2.5 g of homogenized blank pistachio were fortified at 0.25, 0.50, 1, 2 and 4 μg kg^{-1} (μg of total AFs per kg) with AF-MIX solution. For the electrochemical detection, every measurement on SPCE was repeated in triplicate. For the method validation studies using LC-MS/MS, the fortification of the blank samples was carried out at 3 concentration levels, 2, 4 and 10 μg kg^{-1} with AF-MIX, generating 5 replicate analytical portions at each level, and the study was repeated on 3 different days.

2.2.3. Extraction of aflatoxins using immunoaffinity columns

The treatment of the blank and fortified pistachio samples consists in an extraction and purification protocol using immunoaffinity columns (IAC) (Diella et al., 2018; Nilüfer & Boyacioglu, 2002; VICAM, n.d.) (Fig. 2a). 2.5 g of homogenized pistachio sample were placed in a 50 mL centrifuge tube to which 0.5 g of NaCl and 10 mL of MeOH were added. The tube was shaken vigorously for 4 min at max speed at room temperature in a vortex mixer. Then, the samples were centrifuged for 5 min at 12581 g at 20 °C, and the organic phase was subsequently filtered using a 0.45 μm syringe filter (PTFE). 5 mL of the supernatant were dissolved in 14.5 mL of ultrapure water and mixed 5 s in a vortex. Subsequently, 10 mL of the final solution was added to an empty IAC. The IAC washing step was carried out by passing 20 mL of ultrapure water through the column. Finally, the concentrated sample extract was collected in a vial by passing 1 mL of MeOH in the IAC to elute the aflatoxins. The eluate was filtered with a 0.2 μm syringe filter (PTFE). The methanol extract was directly injected into the LC-MS/MS.

The extract to be applied on the SPCE was first dried with a gentle stream of N_2 in a water bath set at 42 °C and reconstituted with 1 mL of measurement buffer (10 mmol L^{-1} PBS pH 7.4; 0.5% BSA), before being analyzed by the immunosensor.

2.2.4. Competitive immunoassay for total aflatoxins determination

10 μL of 4 μg mL^{-1} BSA-AF conjugate in 10 mmol L^{-1} PBS pH 7.4 were deposited on the working electrode and incubated overnight at 4 °C (see Fig. 2b). After washing with ultrapure water, 40 μL of 1% BSA in 10 mmol L^{-1} PBS pH 7.4 were placed and incubated for 30 min to block the electrode surface and avoid possible unspecific absorptions. After washing, 20 μL of either a solution of AFB1, AFB2, AFG1 and AFG2 in 10 mmol L^{-1} PBS pH 7.4 with 0.5% BSA (at different concentrations: 0.005–500 μg L^{-1}) or the fortified extracts and 2 μg mL^{-1} of monoclonal antibody (mAb-AF) in 10 mmol L^{-1} PBS pH 7.4 with 0.5% BSA (previously mixed incubated 30 min under shaking at 400 rpm, 20 °C), were placed on the electrode and let to react for 60 min at room temperature. After washing, 20 μL of 7.5 μg mL^{-1} anti-IgG-HRP in 10 mmol L^{-1} PBS pH 7.4 with 0.5% BSA, were placed on the electrode and incubated at room temperature for 60 min.

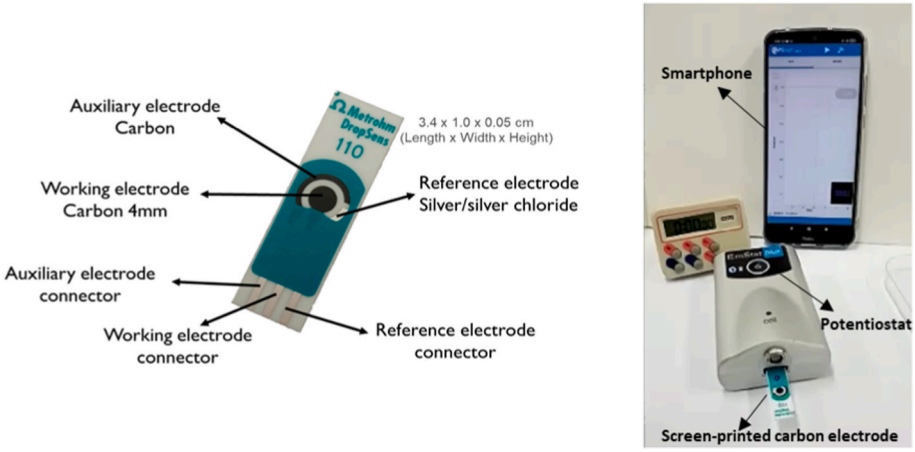

Fig. 1. (Left) Detail of the screen-printed carbon electrode (SPCE) used as sensing platform and transducer; (Right) Picture of the set-up used for the electrochemical measurements.

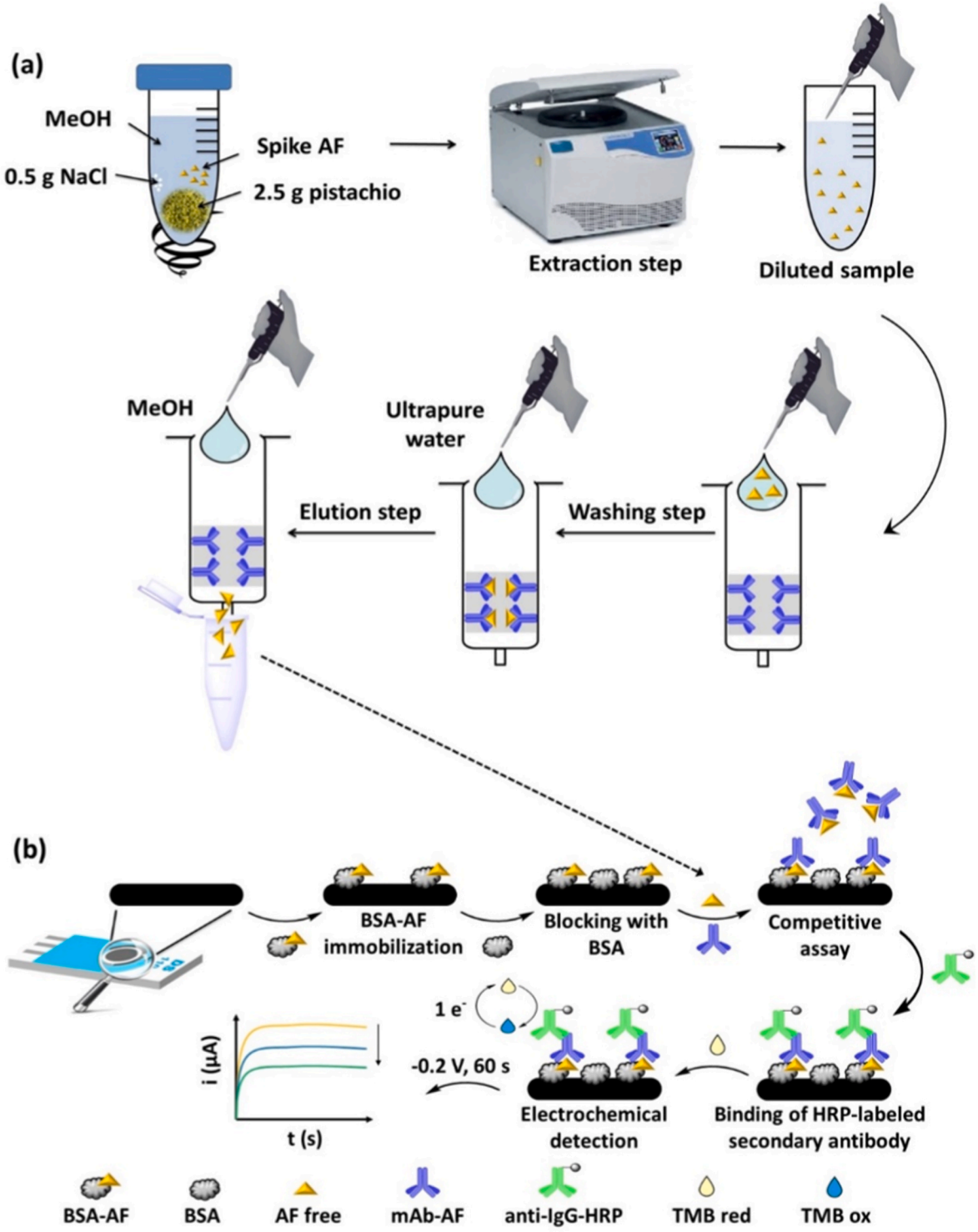

Fig. 2. Schematic representation of: (a) Pistachio sample preparation for AFs determination using immunoaffinity columns (IAC); (b) Competitive immunoassay for total AFs on screen-printed carbon electrodes (SPCEs) using monoclonal antibodies, enzymatic reaction and electrochemical detection.

2.2.5. Enzymatic reaction and electrochemical detection

After washing with ultrapure water, the enzymatic reaction was performed by adding 40 μL of TMB solution and incubating in the dark for 2 min. Chronoamperometric detection was performed applying a potential of −0.2 V for 60 s, being the recorded current proportional to the amount of TMB reduced on the electrode. The analytical signal corresponds to the value of the current recorded at 60 s. All analysis were performed in triplicate.

Videos showing the experimental procedure followed for both the 'Sensors preparation and calibration plot' and the 'Analysis of pistachio samples' are provided at the Supplementary Material (S5 and S6, respectively).

3. Results and discussion

3.1. Development and optimization of a screening sensor

3.1.1. Sensing principle: competitive immunoassay using monoclonal antibodies

The scheme of the competitive immunoassay for total AFs detection on screen-printed carbon electrodes (SPCEs) using monoclonal antibodies is depicted in Fig. 2b. The BSA-labelled AF (BSA-AF) immobilized on the electrode surface and the free AF analyte compete for the specific monoclonal antibody (mAb-AF). The HRP enzyme tag is then specifically linked to the electrode by anti-IgG-HRP secondary antibodies. The HRP is responsible for the TMB substrate oxidation. Oxidized TMB is then electrochemically reduced by applying a potential of −0.2 V for 60 s. The absolute value of the catalytic current recorded at 60 s, which is proportional to the AF amount, is chosen as the analytical signal.

3.1.2. Optimization of the main parameters affecting the analytical signal

The different experimental parameters affecting the analytical signal were evaluated: electrode blocking step, enzymatic reaction time, reagents (BSA-AF, mAb-AF and anti-IgG-HRP) concentration and incubation times (Table S2 summary in the Supplementary material).

3.1.2.1. BSA concentration and incubation time for electrode blocking to avoid unspecific adsorptions. Bovine serum albumin (BSA) was used as blocking agent of the electrode surface, to avoid unspecific absorptions. Concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% were evaluated, fixing the following parameters; BSA incubation time: 30 min; BSA-AF concentration: 5 μg mL^{-1} (in 10 mmol L^{-1} PBS pH 7.4); BSA-AF incubation time: overnight (at 4 °C); mAb-AF concentration: 8 μg mL^{-1} (in 10 mmol L^{-1} PBS pH 7.4; 0.5% BSA); mAb-AF incubation time: 60 min at room temperature; anti-IgG-HRP concentration: 10 μg mL^{-1} (in 10 mmol L^{-1} PBS pH 7.4; 0.5% BSA); anti-IgG-HRP incubation time: 60 min at room temperature; enzymatic reaction time: 10 min. Fig. 3a shows the intensity values obtained for the different concentrations of blocking agent for assays performed in the absence of monoclonal antibody and with a 8 μg mL^{-1} antibody concentration. The concentration at which the absolute difference between the signal with antibodies (specific signal) and the signal obtained without antibodies (unspecific signal) is maximum was chosen as the optimal concentration of BSA. This corresponds to a value of 1% of BSA.

In Fig. 3b is shown the optimization of the incubation time for 1% BSA. Times of 20, 30, 40, 50 and 60 min were studied, selecting as the optimum time 30 min, since the ratio between unspecific/specific signal is maximum, together with a better reproducibility.

3.1.2.2. Enzymatic reaction time. Enzymatic reaction times of 0.5, 1, 2, 5, 10, 15 and 20 min were evaluated, fixing the following parameters: BSA concentration: 1%; BSA incubation time: 30 min; BSA-AF concentration: 5 μg mL^{-1} (in 10 mmol L^{-1} PBS pH 7.4); BSA-AF incubation time: overnight (at 4 °C)); mAb-AF concentration: 8 μg mL^{-1} (in 10 mmol L^{-1} PBS pH 7.4; 0.5% BSA); mAb-AF incubation time: 60 min at room temperature; anti-IgG-HRP concentration: 10 μg mL^{-1} (in 10 mmol L^{-1} PBS pH 7.4; 0.5% BSA); anti-IgG-HRP incubation time: 60 min at room temperature. As shown in Fig. 3c, a maximum signal with also a better reproducibility was recorded for 2 min, noticing then a saturation/decrease in the signal. For this reason, 2 min was chosen as optimum enzymatic reaction time.

3.1.2.3. Aflatoxin-BSA conjugate (BSA-AF) concentration and incubation time. Different concentrations of the immobilized BSA-AF conjugate, in the range 0.6–14 μg mL^{-1}, were evaluated, fixing the following parameters: BSA concentration: 1%; BSA incubation time: 30 min; BSA-AF incubation time: overnight (at 4 °C); mAb-AF concentration: 8 μg mL^{-1} (in 10 mmol L^{-1} PBS pH 7.4; 0.5% BSA); mAb-AF incubation time: 60 min at room temperature; anti-IgG-HRP concentration: 10 μg mL^{-1} (in

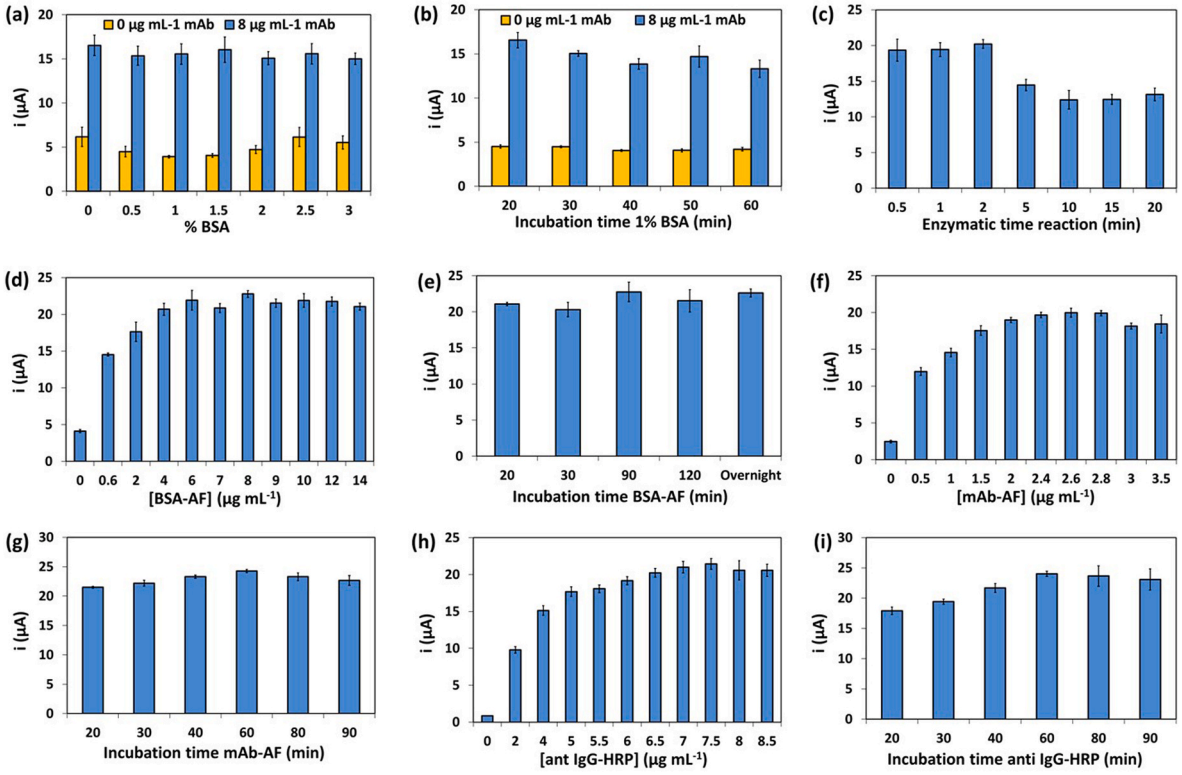

Fig. 3. Optimization of (a) BSA concentration; (b) BSA incubation time; (c) Enzymatic reaction time; (d) Aflatoxin-BSA conjugate (BSA-AF) concentration; (e) Aflatoxin-BSA conjugate (BSA-AF) incubation time; (f) Monoclonal antibody (mAb-AF) concentration; (g) Monoclonal antibody (mAb-AF) immunoreaction time; (h) Polyclonal secondary antibody (anti IgG-HRP) concentration; (i) Polyclonal secondary antibody (anti IgG-HRP) immunoreaction time.

10 mmol L^{-1} PBS pH 7.4; 0.5% BSA); anti-IgG-HRP incubation time: 60 min at room temperature; enzymatic reaction time: 2 min. In Fig. 3d is observed how the analytical signal increases when the concentration of immobilized antigen increases up to 6 μg mL^{-1}, reaching then a plateau. 4 μg mL^{-1} was selected as the optimal BSA-AF concentration, since a compromise between performance, reproducibility and reagent consumption is obtained for this concentration. The same criteria were followed for selecting as optimum an incubation time of 12 h, which may correspond to an overnight BSA-AF incubation (Fig. 3e).

3.1.2.4. Monoclonal antibody (mAb-AF) concentration and immunoreaction time. A critical parameter in a competitive immunoassay is the monoclonal antibody concentration. Concentrations in the range 0.5–3.5 μg mL^{-1} were evaluated, fixing the immunoreaction time at 60 min and the rest of optimum parameters as detailed in the previous sections (BSA: 1%, BSA incubation time: 30 min, enzymatic reaction time: 2 min, BSA-AF concentration: 4 μg mL^{-1}, BSA-AF incubation time: overnight, anti IgG-HRP concentration: 10 μg mL^{-1}, anti IgG-HRP incubation time: 60 min). As shown in Fig. 3f, current intensity increases with increasing antibody concentration up to 2.6 μg mL^{-1}, reaching then a plateau. From these results, a 2 μg mL^{-1} concentration was selected as optimum, so as to assure that small amounts of analyte in the competitive assay will lead to a substantial decrease in the analytical signal. Regarding the BSA-AF – mAb-AF immunoreaction time, it was noticed a gradual increase in the signal in the range 20–90 min (Fig. 3g), reaching then a saturation. For this reason, 60 min was chosen as the optimum immunoreaction time.

3.1.2.5. Polyclonal secondary antibody (anti IgG-HRP) concentration and immunoreaction time. Finally, the concentration of polyclonal secondary antibody was evaluated in a range from 2 to 8.5 μg mL^{-1}, fixing an immunoreaction time of 60 min and the rest of parameters at the previously detailed optimum conditions. Fig. 3h shows how current intensity increases with the concentration of anti-IgG-HRP up to 7.5 μg mL^{-1}, suggesting that this concentration is the optimum for the biosensor development. 60 min of immunoreaction with the mAb-AF antibody were the optimum, as shown in the study summarized at Fig. 3i.

3.1.3. Total aflatoxins determination using the immunosensor

Aflatoxins solution with concentrations of 0.005–500 μg total AFs per L, prepared from the AF-MIX mixture were evaluated in the competitive immunoassay under the optimized conditions. Chronoamperograms displayed in Fig. 4a show a decrease in the absolute value of the cathodic current corresponding to the TMB reduction process, for increasing concentrations of the total AFs solution. The absolute value of the current generated at 60 s (analytical signal) was used for the quantification of AFs. As shown in Fig. 4b, a proportional decrease of the analytical signal was observed with the corresponding increase in the concentration of the total AFs in the range 0.005–500 μg L^{-1}. Such decrease was adjusted to the following linear relationship (correlation coefficient: 0.999) in the acceptable accuracy range 0.01–2 μg L^{-1} (inset of Fig. 4b):

$$i\ (\mu A) = -6.59\ [Total\ AFs]\left(\mu g\ L^{-1}\right) + 17.98$$

The limit of detection (LOD) and the limit of quantification (LOQ) as defined in (Miller & Miller, 2010) was calculated using the buffer calibration as 3*Sb/m* and 10*Sb/m*, where *Sb* is the standard deviation of the buffer (blank) and *m* is the slope of the calibration line. The LOD and LOQ in PBS buffer were estimated at 0.017 μg L^{-1} and 0.056 μg L^{-1} respectively.

The reproducibility of the immunosensor was estimated from the calibration slopes for the assays performed in different days (see Table S3 at the Supplementary Material). The relative standard deviation (RSD) was 2%, and the inter-electrode precision was estimated at 1%.

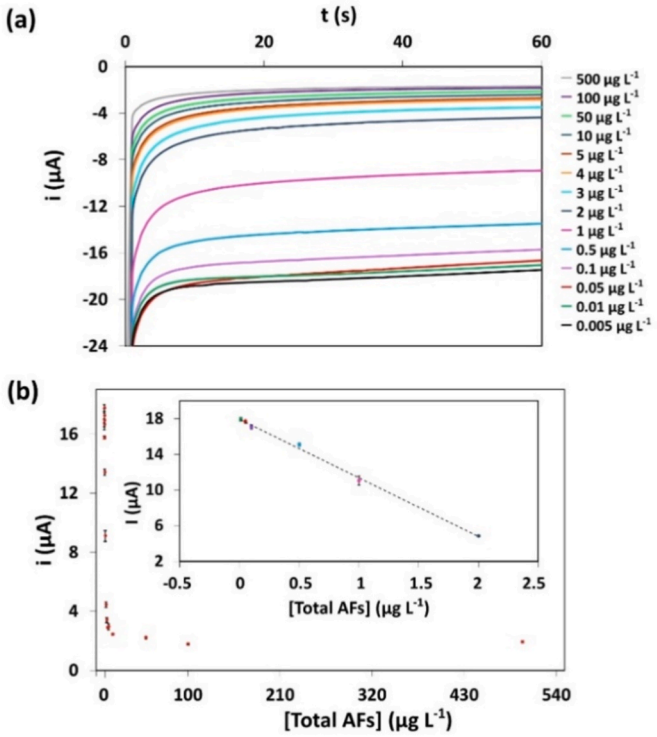

Fig. 4. (a) Chronoamperograms recorded by applying a potential of −0.2 V for 60 s, after performing the competitive immunoassay for solutions of total AFs of increasing concentrations. (b) Relationship between the analytical signal (absolute value of current recorded at 60 s) and the AF concentration. The linear range of response is shown in the inset graph.

As summarized in Table 1, the developed and optimized biosensor exhibits a wider linear range and a lower limit of detection than most of those previously reported for the determination of individual aflatoxins using electrochemical immunosensors, without the need of SPCE modification.

3.1.4. Immunosensor selectivity and stability

The selectivity of the immunosensor against other mycotoxins that may be present in a real sample, such as ZEN and OTA (at a concentration of 2 μg L^{-1} each), individually and mixed (MIX-3) was evaluated. Other possible interferences such as glucose, Na^+ and K^+ (at 1 mg L^{-1} each) individually and mixed (MIX-4), and all interferences mixed (MIX-5) were also studied. As shown in Fig. 5a, no significant changes in the analytical signal were noticed for any compound, neither individually nor mixed, demonstrating the selectivity of the immunosensor. Moreover, the presence of such compounds in mixtures at a concentration of 2 μg L^{-1} of total AFs (MIX-1 and MIX-2) did not show any effect on the specific signal from the AFs.

The long-term stability of the immunosensor was evaluated by storing at room temperature a set of electrodes prepared with BSA-AF immobilized on the SPCE. Immunoassays for a total AFs mixture with concentration 1 μg L^{-1} were performed in different days for several weeks. As shown in Fig. 5b the response of the immunosensor was stable and reproducible for at least 30 days.

3.2. Method validation for aflatoxins in pistachio by LC-MS/MS

Uncontaminated pistachio samples were used as blank materials, and analytical portions of 2.5 g of homogenized pistachio blanks were individually fortified for method validation studies.

A blank matrix and a reagent blank were prepared and injected in

Table 1
Analytical performance of different electrochemical immunosensors reported for mycotoxins detection.

Mycotoxin	Electrode	Linear range (μg L^{-1})	LOD (μg L^{-1})	Ref.
AFB1	AuNPs/ PEDOT-GO/ GCE	0.5–20 20–60	0.109	(Arati Sharma et al., 2018)
AFB1	SWNTs-CS/ GCE	0.01–100	0.0035	Zhang et al. (2016)
AFB1	PtNPs/ CoTPP/ rGO/ AuNPs/GCE	0.005–5	0.0015	Shu et al. (2015)
AFB1	nBi_2O_3/ITO	0.01–0.7	0.087	Solanki et al. (2017)
AFB1	c-MWCNTs/ ITO	0.25–1.37	0.08	Singh et al. (2013)
AFB1	SPCE	0.15–2.5	0.15	Pemberton et al. (2006)
AFM1	SPCE	Up to 1	0.039	Parker and Tothill (2009)
AFM1	AuSPE	0.01–1000	0.015	Karczmarczyk et al. (2017)
AFM1	SPGE	0.03–0.160	0.025	Micheli et al. (2005)
AFM1	SPGE	15–1000	15	Vig et al. (2009)
OTA	AuSPE	0.01–1000	0.024	Karczmarczyk et al. (2017)
OTA	SPCE	0.35–8.06	0.30	Vidal et al. (2011)
OTA	SPGE	0.25–250	0.18	Alarcón et al. (2004)
OTA	SPGE direct SPGE indirect	0.06–2.5 0.10–7.5	0.06 0.10	Alarcón et al. (2006)
ZEN	SPCE	0.25–256	0.25	Goud et al. (2019)
ZEN	SPCE	50–25000	25	Hsieh et al. (2013)
AFB1	MWCNTs/ CS/SPCE	0.0001–10	0.0003	Azri et al. (2018)
AFB1	MWCNTs/ CS/SPCE	0.0001–10	0.0001	Azri et al. (2017)
AFM1	SWCNTs/ SPE	0.01–1	0.02	Abera et al. (2019)
AFM1	EDC/NHS/ 4-ABA/ SPCE EDC/NHS/ 4-AP/SPCE	0.25–5	0.09	Tang et al. (2022)
OTA	EDC/NHS/ 4-CP/AuSPE	1–20	0.5	Radi et al. (2009)
OTA	Cys/AuNPs/ SPCE	0.3–20	0.25	Malvano, Albanese, Crescitelli, Pilloton, and Esposito (2016)
OTA	MBs/SPCE	0.26–8.87	0.134	Vidal et al. (2012)
OTA	MBs/SPCE	0.01–20	0.008	Perrotta et al. (2012)
OTA	MBs/SPCE	1.3–153.8	0.32	Jodra et al. (2015)
OTA	EDC/NHS/ AuSPE	0.01–5	0.005	Malvano, Albanese, Pilloton, and Di Matteo (2016)
OTA	EDC/NHS/ AuSPE	0.37–2.86	0.19	Cancelliere et al. (2021)
ZEN	MBs/SPCE	–	0.007	Hervás et al. (2010)
ZEN	AuNPs/ MWCNTs/ PEI/SPCE	0.00001–100	0.00015	Riberi et al. (2018)
Total aflatoxins (B1, B2, G1, G2)	**SPCE**	**0.01–2**	**0.017**	**This work**

AuNP: gold nanoparticles; PEDOT: poly (3,4-ethylendioxythiophene); GO: graphene oxide; GCE: glassy carbon electrode; SWNT: single-walled carbon nanotubes; CS: chitosan; PtNPs: platinum nanoparticles; CoTPP: 5,10,15,20-tetraphenyl-21H,23H-porphine cobalt; rGO: reduced graphene oxide; nBi_2O_3: bismuth oxide nanorods; ITO: indium-tin-oxide; c-MWCNT: carboxylated multiwalled carbon nanotubes; SPCE: screen-printed carbon electrode; AuSPE: screen-printed gold electrode; SPGE: screen-printed graphite electrode; OTA: ochratoxin A; ZEN: zearalenone; SPE: screen-printed electrode; EDC: N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; NHS: N-hydroxysuccinimide; 4-ABA: 4-aminobenzoic acid; 4-AP: 4-aminophenyl; 4-CP: 4-carboxyphenyl; Cys: cysteamine; MB: paramagnetic microbeads; PEI: polyethyleneimine.

each batch of analysis to demonstrate that there was no cross-contamination and interference during the analysis. The parameters tested for validation included analytical range, linearity, limit of quantification (LOQ), trueness, within laboratory repeatability and reproducibility, and matrix effects, and estimated as described earlier (Maestroni et al., 2018). The analytical calibration was carried out in each batch of analysis using matrix-matched standards, covering the range of 0.16–31 μg kg^{-1} (0.1−20 μg L^{-1}) and using the bracketing calibration modality. The results of method validation studies are included in Supplementary Material S4 (Fig. S4 and Table S4).

3.3. Total aflatoxins analysis in pistachio samples: evaluation of matrix effects

Despite IAC cleanup, residual matrix is still present in the sample extracts, as demonstrated by the signal obtained for the blank samples. Matrix effects studies were conducted using matrix matched standards at 0, 0.25, 0.50, 1, 2 and 4 μg kg^{-1} (0, 0.16, 0.32, 0.64, 1.28 and 2.56 μg L^{-1} respectively). The sample extracts were prepared by fortification of blank samples and extraction/clean up as described above. The signals from the extracts were compared with those obtained for AF solutions prepared in PBS buffer to establish the matrix effects.

Fig. 6 shows that the pistachio matrix has an effect on the performance of the immunosensor in comparison to the AF standards in buffer solution, evidenced by a decrease in the calibration slope. Since matrix effects cannot be eliminated but only compensated for, the recommendation is to prepare matrix matched calibrators for the analytical determination of AFs in pistachio samples.

Table 2 shows the recovery, which is the ratio between the measured amount (in μg L^{-1}) and the nominal concentration in the extract (μg L^{-1}) using the immunosensor. The recovery varies between 87 and 106%, with respect to the analytical signal obtained from the matrix calibration curve $(i\,(\mu A) = -3.49\,[Total\ AFs](\mu g\,L^{-1}) + 10.88 \quad r = 0.985)$. The LOD and the LOQ in pistachio matrix from using the immunosensor was estimated at 0.066 μg kg^{-1} and 0.221 μg kg^{-1}, respectively, using the formulas given above.

The satisfactory levels of recovery, as well as the method LOD estimated in pistachio matrix, which is low enough compared to the regulatry limit of 10 μg kg^{-1}, provide further confirmation that the developed and optimized immunosensor is fit for purpose for the detection of the total content of AFs in pistachio samples.

3.4. Cross-validation of immunosensor performance with LC-MS/MS

In order to validate the performance of the immunosensor, a comparison was made with a validated reference method using LC-MS/MS for the detection of aflatoxins in pistachio. For this, a matrix matched calibration was constructed, and the same sample extracts were analyzed with both systems: the immunosensor and the LC-MS/MS. As shown in Fig. 7, there is a good correlation (r = 0.997) between both methods. This indicates that there are no systematic errors, concluding that the biosensor performance has been validated by a reference method.

It is important to note that the limit of quantification (LOQ) of the LC-MS/MS method is set at 2 μg kg^{-1} level, corresponding to 1.28 ng mL^{-1}. This corresponds to the lowest concentration assessed during validation that meets method performance criteria according to the Codex guideline CXG 90–2017 (FAO, 2017).

The electrochemical immunosensor for total AFs has an LOQ that is

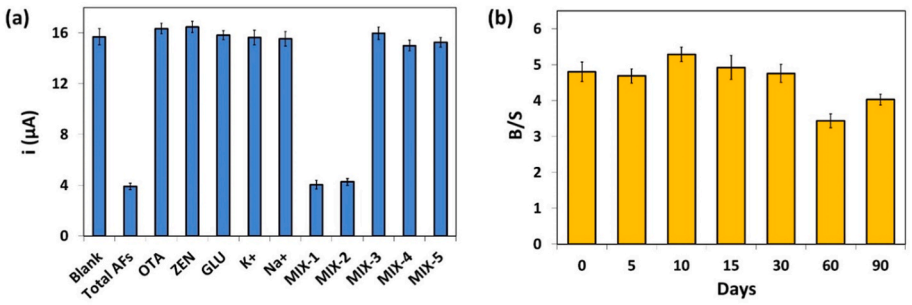

Fig. 5. (a) Analytical signals obtained for different interference compounds (ZEN, OTA, Glu, Na^+, K^+) individually and in mixtures (MIX-1: total AFs mixture, OTA, ZEN; MIX-2: total AFs mixture, OTA, ZEN, Glu, Na^+, K^+; MIX-3: OTA, ZEN; MIX-4: OTA, ZEN, Glu, Na^+, K^+; MIX-5: Glu, Na^+, K^+). (b) Long-term stability study: B/S (blank/signal) ratio obtained for immunoassays (Mixture of total aflatoxins with concentration 1 μg L^{-1}) performed in different days after the BSA-AF immobilization. Electrodes stored at room temperature.

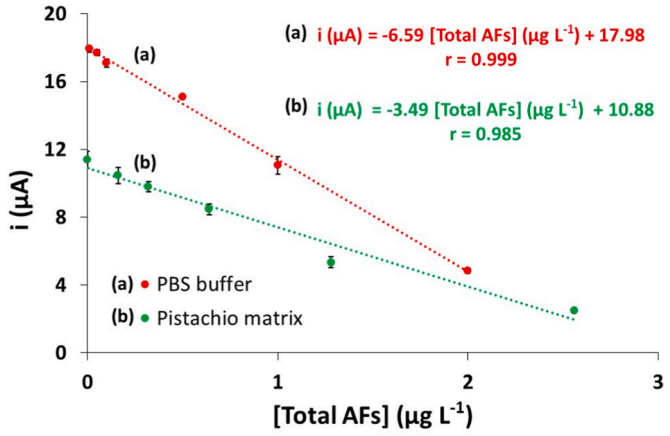

Fig. 6. Study of the matrix effect. Relationship between current intensity and total aflatoxin concentration in (a) buffer solution (10 mmol L^{-1} PBS pH 7.4; 0.5% BSA) and (b) pistachio matrix.

significantly lower than the LOQ of the confirmatory method, making it a perfect solution for a semi-quantitative screening method.

4. Conclusion

A competitive immunosensor on screen-printed carbon electrodes, using specific monoclonal antibodies, has been successfully developed and optimized for the analysis of total aflatoxins (sum of AFB1, AFG1, AFB2 and AFG2) via electrochemical detection. All of the aspect of method optimization and validation have been described in detail making the methodology fully available to analytical chemist in less developed countries, and 2 videos, available as Supplementary Material, have been recorded for information transfer. The know-how is readily available for scientist wishing to build their screening technology using the immunosensing technique.

The immunosensor exhibits an excellent analytical performance in terms of dynamic range of response, limit of detection, reproducibility, selectivity and long-term stability. A simple protocol involving the use of immunoaffinity columns allows to extract the aflatoxins from pistachio samples, while the use of matrix matched calibrators minimizes matrix effects, as evidenced with the high recoveries obtained.

Importantly, the immunosensor performance has been cross-validated with a reference method (LC-MS/MS), showing an excellent correlation. For such validation, the blank homogenized pistachio samples were fortified with a standard solution. It is important to mention that fortification of sample prior to extraction and clean up represents the worst-case scenario, which provides assurance that the method is really effective to analyze the analytes in the sample. This is especially true in the case of mycotoxin contamination, as the detection of naturally contaminated samples has a potential likelihood of being unsuccessful if one doesn't invest in a proper sampling, with many incremental samples to build a reliable laboratory sample, and a reliable homogenization and a particle size verification. In addition, the efficacy of the biosensor was demonstrated at different fortification levels, which is important to consider for the validation, as the contamination levels may naturally be very different depending on the geographical areas. The biosensor was essentially validated to cover the aflatoxin levels that

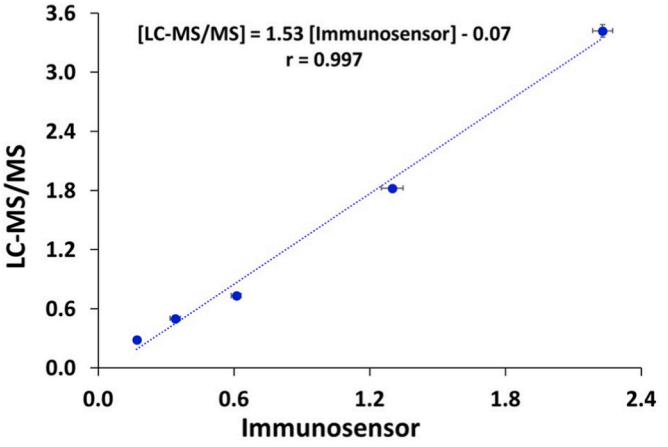

Fig. 7. Correlation between the developed electrochemical immunosensor and LC-MS/MS.

Table 2
Evaluation of the recovery values. The study was carried out by analyzing extracts fortified at 0.25, 0.50, 1, 2 and 4 μg kg^{-1} and comparing the analytical signals with the matrix matched calibration (average of measurements of 3 different days, n = 4).

Sample	Fortification level (μg kg^{-1})	Nominal concentration [a] (μg L^{-1})	Current matrix [b] (μA)	Current in the sample extract (μA)	Measured amount (μg L^{-1}) ± CV %	Recovery
Pistachio	0.25	0.16	10.45 ± 0.46	10.27 ± 0.02	0.17 ± 4%	106%
	0.50	0.32	9.81 ± 0.30	9.69 ± 0.06	0.34 ± 5%	106%
	1.00	0.64	8.46 ± 0.33	8.76 ± 0.06	0.61 ± 3%	95%
	2.00	1.28	5.34 ± 0.33	6.33 ± 0.12	1.30 ± 3%	102%
	4.00	2.56	2.50 ± 0.16	3.09 ± 0.11	2.23 ± 1%	87%

[a] Nominal concentration of AFs in extract diluted in 1 mL PBS buffer.
[b] Matrix matched calibration signal.

are regulated to provide consumer protection even at low levels of contamination.

Overall, the developed immunosensor is simpler, cheaper and more rapid, equally sensitive than alternative standard analytical methods based on LC-MS/MS and ELISA. The excellent performance observed in pistachio samples together with the low detection limit, that is far below the maximum levels of total AFs allowed by current legislation, make the immunosensor approach a promising tool for the rapid screening of AFs at the point-of-need. Potential application of the immunosensor might include analysis of AFs in i.e. baby foods, where very low levels of AFs may be detrimental to child growth and even lethal.

CRediT authorship contribution statement

Beatriz Pérez-Fernández: Formal analysis, Writing – original draft. **Britt Marianna Maestroni:** Conceptualization, Data curation, Supervision, Writing – review & editing, Funding acquisition. **Shuichi Nakaya:** Formal analysis. **Sofia Bussalino:** Formal analysis. **Christina Vlachou:** Conceptualization, Data curation, Supervision, Writing – review & editing, Funding acquisition. **Alfredo de la Escosura-Muñiz:** Conceptualization, Data curation, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2023.109859.

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