Talanta 83 (2011) 966–973

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Citrinin (CIT) determination in rice samples using a micro fluidic electrochemical immunosensor

Fernando Javier Arévalo [a], Adrián Marcelo Granero [a], Héctor Fernández [a,*], Julio Raba [b], María Alicia Zón [a,**]

[a] Departamento de Química, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal No 3, (5800)-Río Cuarto, Argentina
[b] INQUISAL, Departamento de Química, Universidad Nacional de San Luis, CONICET, Chacabuco y Pedernera, D5700BWS, San Luis, Argentina

ARTICLE INFO

Article history:
Received 27 September 2010
Received in revised form 29 October 2010
Accepted 1 November 2010
Available online 10 November 2010

Keywords:
Enzyme immunoassays
Citrinin
Amperometric immunosensor
Flow injection analysis

ABSTRACT

The development of an electrochemical immunosensor incorporated in a micro fluidic cell for quantification of citrinin (CIT) mycotoxin in rice samples is described for the first time. Both CIT present in rice samples and immobilized on a gold surface electrodeposited on a glassy carbon (GC) electrode modified with a cysteamine self-assembled monolayer were allowed to compete for the monoclonal mouse anti-CIT IgG antibody (mAb-CIT) present in solution. Then, an excess of rabbit anti mouse IgG (H+L) labelled with the horseradish peroxidase (secAb-HRP) was added, which reacts with the mAb-CIT which is in the immuno-complex formed with the immobilized CIT on the electrode surface. The HPR, in the presence of hydrogen peroxide (H_2O_2) catalyzes the oxidation of catechol (H_2Q) whose back electrochemical reduction was detected on a GC electrode at −0.15 V *vs* Ag/AgCl by amperometric measurements. The current measured is proportional to the enzymatic activity and inversely proportional to the amount of CIT present in the rice samples. This immunosensor for CIT showed a range of work between 0.5 and 50 ng mL^{-1}. The detection (LOD) and the quantification (LOQ) limits were 0.1 and 0.5 ng mL^{-1}, respectively. The coefficients of variation intra- and inter-assays were less than 6%. The electrochemical detection could be done within 2 min and the assay total time was 45 min. The immunosensor was provided to undertake at least 80 determinations for different samples with a minimum previous pretreatment. Our electrochemical immunosensor showed a higher sensitivity and reduced analysis time compared to other analytical methods such as chromatographic methods. This methodology is fast, selective and very sensitive. Thus, the immunosensor showed to be a very useful tool to determine CIT in samples of cereals, mainly rice samples.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Mycotoxins are a structurally diverse group of secondary metabolites produced by fungi of different species. These toxic compounds may contaminate cereals, foodstuffs and their products worldwide. The ingestion of mycotoxins can be pathogenic to animals and humans causing serious health problems such as liver and kidney diseases, nervous system damage, immunosuppresion and carcinogenicity [1]. Thus, it is necessary to establish maximum permitted levels of these mycotoxins in different foods for the purpose of protecting public health [2].

Citrinin ($C_{13}H_{14}O_5$, or (3R,4S)-4,6-dihydro-8-hydroxy-3, 4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid, CIT) is a secondary metabolite produced by fungi of the *Aspergillus*, *Monascus* and *Penicillium* genera especially *P. citrinum*, *P. verruscosum and P. expansum*. CIT was one of the first mycotoxin isolated. However, its mechanism of toxicity is controversial so far. The International Agency for Cancer Research (IARC) classifies CIT in the Group III because there is little evidence of its toxicity in experiments conducted on animals and no evidence for human [3].

CIT may contaminate corn [4], wheat [5,6], rye and oat [6], barley [7], and rice [8] as well as other commodities. CIT contamination has been reported in a number of agricultural commodities, foods, feedstuffs as well as biological fluids from different geographical regions [9–13]. At the present, there is not a specific legislation on CIT permitted maximum levels in different foods. The main reason is the lack of an official analytical method [2] and its instability in food as a result of structural changes that CIT suffers in water at temperatures above 140 °C [14]. Various analytical methods have been described in the literature related to the determination of CIT, which have different sensitivity and accuracy. The methods used to determine CIT qualitatively and quantitatively are thin-layer chromatography (TLC) [2,12], high-performance liquid chromatography (HPLC) with UV or fluorescence detection (FD) [2,15], and

* Corresponding author.
** Corresponding author. Tel.: +54 358 467 6233; fax: +54 358 467 6233.
E-mail addresses: hfernandez@exa.unrc.edu.ar (H. Fernández), azon@exa.unrc.edu.ar, alicia_zon@hotmail.com (M.A. Zón).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.11.007

Table 1
Reference values reported in different countries for CIT in contaminated rice samples.

Commodity contaminated	Reporting country	CIT contents ($\mu g\,kg^{-1}$)	Reference
Rice	India	49–92	[8]
	Japan	700–1130	
	Taiwan – China	4200–251,000	[19]
	China	200–140,000	[21]

enzyme immunoassays (EIA) [16,17]. Recently, LC–MS and GC–MS [2,8,18] techniques have also become available for CIT determination. CIT values found in contaminated rice samples in different countries are shown in Table 1 [19].

In recent years, the immunosensors have attracted a great interest in the field of analytical chemistry due to their quick response, sensitivity and highly selective to the determination of a given substrate. The high selectivity and high affinity of antibodies to their specific antigen has been widely explored in the development of radio immunoassays (RIAs) and enzyme-linked immunosorbent assay (ELISA). However, these techniques require of highly training personnel, have a long tedious time of analysis and high cost equipment [20]. Anyway, the immunoassay methods are faster and simpler than to the conventional chromatographic methods. Thus, many immunosensors have been described in the literature. Some of them combine the ELISA classic format with chromatographic, photometric or amperometric methods. Therefore, the development of electrochemical immunosensors has gained great importance in recent years trying to improve statistical parameters such as sensibility, selectivity, reliability, facility of production and use and low cost. Thus, the use of electrochemical immunosensors as a very effective analytical tool is well known in the fields of clinical diagnosis and environmental and agro-alimentary control [21–24].

The amperometric immunosensors are based mainly on the ELISA format, measuring the enzymatic reactions products that are electrochemically active or using a redox mediator [25,26]. Therefore, amperometric immunosensors require, in most cases, that antigen or antibody must be labelled with an enzyme. In competitive assays generally the antigen and a labelled antigen compete for a limited number of binding sites to specific antibody. The amount of labelled antigen is inversely proportional to the amount of antigen in the sample. The conjugated enzyme is used to generate electrochemical signals that will determine the concentration of the antigen in the sample [27]. The immobilization of antigens or antibodies is vital in the construction of the immunosensor, as it plays a fundamental role in what concerns the stability, reproducibility and sensibility of the measured signal [28,29]. Immobilization processes found in the literature are based on the use of polymeric membranes [27,30–38], Langmuir–Blodgett films [39], pre-modification with the protein A [40–43], gold nanoparticles [32,42,44–46] and thiol self-assembled monolayers (SAM) [47–50]. The SAM technique offers some of the simplest way to generate reproducible, ultrafine and very ordered monolayers, which can then be modified with the antigen or antibody [51].

In this work we have developed a heterogeneous enzymatic immunoassay based on the CIT immobilization on an electrode surface. Moreover, the competition takes place between the specific antibody with the CIT from the sample in solution, and CIT immobilized.

It is necessary to use an enzyme-labelled antibody to reveal the immune response (antigen-antibody) at the stage of detection. The maximum signal is reached when the concentration of antigen in the sample is close to zero [26,52].

Heterogeneous enzymatic immunoassays, coupled to a flow injection (FI) system with amperometric detection, represent a powerful analytical tool to determine low levels of different substrates, such as hormones, antibodies, drugs, tumor markers, and viruses [53]. The amperometric detection offers a good sensitivity combined with a simple and low-cost instrumentation [54,55]. One approach used is the use of an enzymatic conjugated which generates an electrochemically active product [56–58]. To our knowledge, any electrochemical immunosensor for determining the CIT mycotoxin has been reported in the literature.

In this work, a micro fluidic electrochemical immunosensor system to detect and quantify CIT in rice samples is developed for the first time. CIT detection was performed using heterogeneous competitive immunoassays. A limited amount of monoclonal mouse anti-CIT IgG antibody (mAb-CIT) solution was added to CIT of the rice samples, which competed immunologically with CIT labelled with a chicken egg ovalbumin (OVA) (CIT-OVA) bonded at the GC electrode surface using glutaraldehyde. Previously, the GC electrode surface was modified by electrodeposition of gold and subsequent modification with a cysteamine SAM. The addition of an excess of rabbit anti mouse IgG (H + L) conjugated with horseradish peroxidase (IgG (H + L)-HRP) reacted with mAb-CIT, which is forming an immune-complex with CIT bonded to the gold disk electrode (Fig. 1.A). The horseradish peroxidase (HRP) catalyzes the oxidation of catechol (H_2Q) to benzoquinone (Q) in the presence of hydrogen peroxide (H_2O_2). Its back electrochemical reduction to catechol can be detected on the GC electrode surface at −0.15 V *vs* Ag/AgCl through amperometric measurements. The current obtained from the product of enzymatic reaction is proportional to the activity of the enzyme and inversely proportional to the amount of CIT in rice samples. Our micro fluidic immunosensor showed a very low sensitivity to determine trace levels of CIT in rice samples, compared to other conventional techniques.

2. Material and methods

2.1. Chemicals

Citrinin (CIT), chicken egg ovalbumin (OVA), rabbit anti mouse IgG (H + L) HRP conjugated (secAb-HRP), formaldehyde, dimethyl sulfoxide (DMSO), catechol (H_2Q), cysteamine and glutaraldehyde (25% aqueous solution) were obtained from Sigma Chemical Company. Chloroform and acetonitrile (ACN) were Sintorgan, HPLC grade. Monoclonal mouse anti-CIT IgG antibody (mAb-CIT) was given kindly by VICAM LP, Watertown (USA). Gold (III) chloride hydrate and potassium nitrate were obtained from Aldrich. 0.01 mol L^{-1} phosphate buffer solutions (PBS) (pH 7.20), 0.01 mol L^{-1} citrate-phosphate buffer solutions (pH 5.00), 0.20 M carbonate buffer solution (pH 10.00), 0.10 mol L^{-1} glycine–HCl (pH 2.00), 0.10 mol L^{-1} sodium acetate (pH 4.20), hydrogen peroxide (H_2O_2), sodium bicarbonate ($NaHCO_3$), hydrochloric acid (HCl) and potassium chloride (KCl) were from Merck (Darmstadt, Germany). Aqueous solutions were prepared using purified water from a Milli-Q system. All reagents were used as received. Rice was obtained from local supermarket. The Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto kindly collaborated with us to prepare the rice samples contaminated with CIT.

2.2. Sample preparation

Two rice samples were contaminated by inoculation of the fungus *Penicillium citrinum*. Water was added to contaminated rice samples in a 40:60 (w/w) ratio and allowed the fungus grows up in a shaker at 25 °C for 7 days. Half of each of these rice samples were

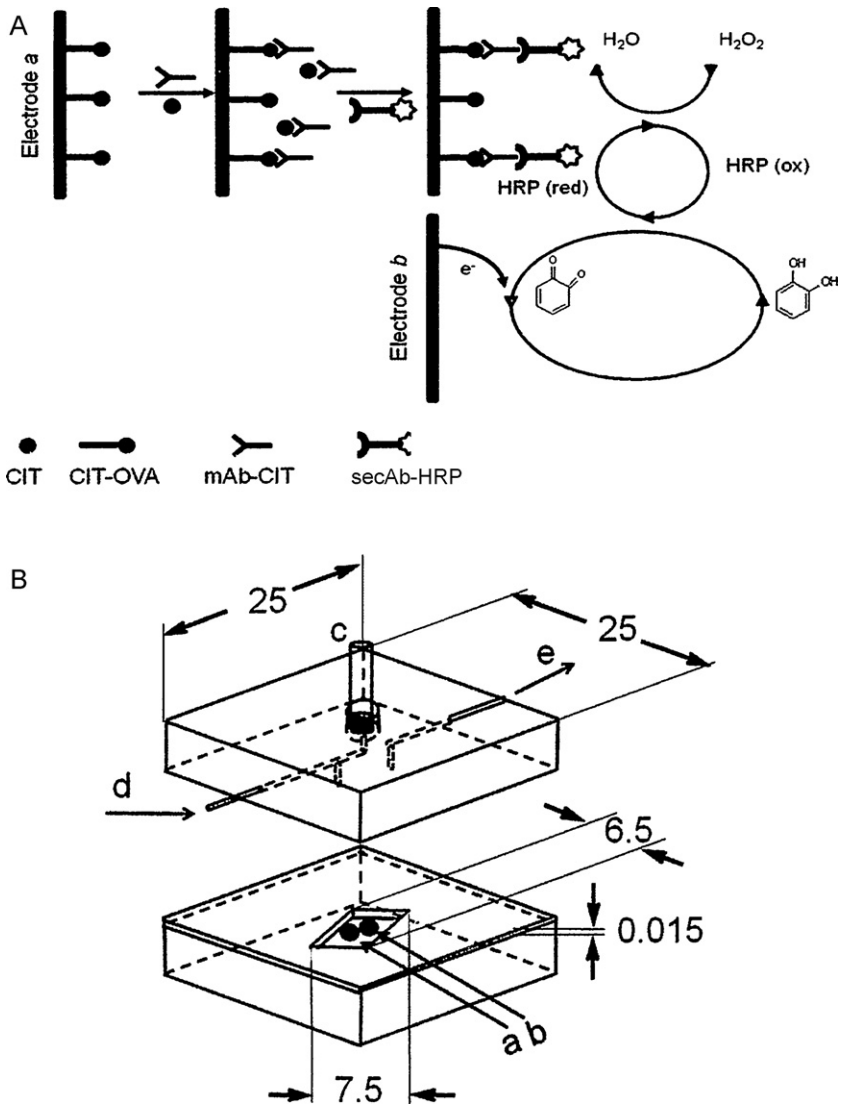

Fig. 1. (A) Schematic representation of the citrinin immunosensor based on electrochemical detection using competitive assays. CIT: citrinin; CIT-OVA: citrinin labelled with chicken egg ovalbumin; mAb-CIT: monoclonal mouse anti-CIT IgG antibody and secAb-HRP: rabbit anti mouse IgG (H + L)-HRP conjugated with horseradish peroxidase (HRP). (B) Schematic representation of the micro fluidic immunosensor cell. (*a* and *b*) glassy carbon electrodes, (*c*) reference electrode, (*d*) inflow, (*e*) outflow. Units of length are given in millimeters. Flow channels have an inner diameter (i.d). of 0.100 mm.

dissolved in 0.01 mol L^{-1} PBS (pH 7.20) and filtered to be used for electrochemical measurements. The other halves of the samples were used to perform fluorimetric measurements. A CIT extraction pretreatment was necessary to perform to these samples. The extraction of CIT from the contaminated rice samples was carried out following a procedure proposed by Pfohl-Leszkowicz et al. [59] with some modifications. Therefore, CIT was extracted from about 25 g of the contaminated rice samples with 125 mL ACN + 4% aqueous solution of KCl (9:1). The pH of the solution was adjusted to 2.00 using a diluted hydrochloric acid solution. Then, the solution was shaken for 20 min at room temperature and filtered using a Whatman No. 4 filter paper. The purification of the mycotoxin was performed by adding 125 mL of n-heptane to the filtrate, which was shaken for 10 min and, finally, the n-heptane was discarded. This extraction procedure was repeated for three times using 50 mL of n-heptane. Then, the lower phase was separated and was added 50 mL of distilled water and 50 mL of chloroform. This solution was shaken for 10 min and, then, the lower phase (chloroform) was collected. Moreover, the aqueous phase was re-extracted twice with 25 mL of chloroform using the same procedure described previously. The chloroform phase was extracted with 50 mL of 5% $NaHCO_3$ aqueous solution and then acidified to pH 2.00 with diluted hydrochloric acid. The acidified extract was re-extracted with 50 mL of chloroform. Finally, the chloroform was evaporated and CIT was dissolved in 200 mL of methanol.

2.3. Flow-through reactor/detector unit and apparatus

The electrochemical flow cell used was BAS, model CC-5, Bioanalytical Systems, Inc., USA (see Fig. 1.B). The cell consists of two bodies. The top body was made of stainless steel body and it was used as the counter electrode. The top body has internal holes that allow entry and exit of solution flow (*d* and *e*, respectively) as well as another channel that communicates with the reference electrode (*c*). The working electrodes were incorporated in the bottom body made of insulating material. They are two GC (*a* and *b*) disks of dia. 3 mm (BAS, model MR-3608, Bioanalytical Systems, Inc., USA). To separate the top stainless steel body from the working electrodes, two sheets of teflon of 0.075 cm of thickness were used, which possess an appropriated hole that permitted the solution passage. The *a* electrode was used to immobilize the CIT-OVA conjugated as indicated below (Section 2.5), while the *b* electrode was

used as detection unit. The cell volume was 7.50 μL. The reference electrode was a Ag/AgCl, 3.0 mol L^{-1} NaCl, (BAS, model MW-2030, Bioanalytical Systems, Inc, USA). Before using, the *a* and *b* GC electrodes were mechanically polished successively with wet alumina powder (0.3 and 0.05 mm, respectively, from Fischer), and washed ultrasonically in distilled water. The GC electrodes were dried under a nitrogen flow. The *a* GC electrode was immersed in a 0.1% $HAuCl_4$ solution containing 0.1 mol L^{-1} KNO_3 as supporting electrolyte, where the electrochemical gold deposition was performed at −0.200 V *vs* Ag/AgCl for 300 s applying a single step potential.

A Rheodyne injector, Dual Mode, model 7725 (i), Rheodyne LLC, USA, was used. The inner diameter (i.d.) of flow channels was 0.100 mm and the length of flow channels was 10 mm. A Pump (Baby Bee Syringe Pump, Bioanalytical System, West Lafayette, IN, USA) was used for pumping, sample introduction and stopping the flow.

The measuring system for performing cyclic voltammetry (CV) and amperommetry was constructed from an AUTOLAB PGSTAT30 potentiostat (Ecochemie, The Netherlands) and run with the GPES (version 4.9) electrochemical analysis software. The potential applied to the *b* electrode to carry out amperometric measurements was −0.15 V *vs* Ag/AgCl. A catalytic current was well established at this potential. The pH measurements were carried out with an HANNA instruments, Bench Meters, model pH 211, Romania. The buffers solutions were thermostatized to 37 °C using a stove NEO LINE thermostat, Argentina. Absorbance measurements were performed using Bio-Rad Benchmark micro plate readers (Japan) and a Hewlett–Packard spectrophotometer, Model 8452A, equipped with a temperature controller. Fluorescence spectra were measured at room temperature using a Spex Fluoromax Spectrofluorometer.

2.4. Conjugation of CIT with OVA

CIT conjugated with OVA was immobilized on the *a* electrode surface modified with Au/SAM. The conjugation of CIT was carried out through the reaction of *Mannich*. It consists of amino alkylation of an acidic proton close to a carbonyl functional group with formaldehyde and ammonia or any primary o secondary amine. The obtained product is a β-amine carbonyl compound, known as base of *Mannich* [16,60]. Briefly, 0.2 mg of CIT (dissolved in 40 (L of DMSO) and 10 mg of OVA (dissolved in 2 mL of 0.1 mol L^{-1} sodium acetate buffer, pH 4.20) were mixed and 100 (L of formaldehyde was added to the solution. The resulting solution was allowed to react for 24 h at room temperature under stirring. The molar ratio between CIT and OVA was 3.6. The reaction product (CIT-OVA conjugated) was then purified using a PD-10 column by means of gel filtration (packed with Sephadex™ 25) and then dialyzed with a cellulose membrane (cut-off molecular weight 12 kDa) in 0.01 mol L^{-1} PBS solution (pH 7.40) for 2 days. The reaction product was confirmed by UV–vis spectrophotometry given that CIT and OVA have absorption bands with maxima at 328 and 278 nm, respectively [17]. UV–vis spectra obtained for OVA (curve a), CIT (curve b) and CIT-OVA conjugated after purification (curve c) are shown in Fig. 2. The curve c in Fig. 2 shows both the absorption band of the protein and the absorption band of CIT. On the other hand, conventional ELISA experiments were carried out (data not shown). The results suggest that the union between OVA and CIT was carried out successfully. Finally, the CIT-OVA conjugated was stored at 4 °C.

2.5. Immobilization of CIT-OVA at the gold electrode surface

The surface of the *a* electrode, which has been modified by electrochemical deposition of gold was then modified by the formation of a cysteamine SAM. The clean *a* electrode was immersed in 0.01 mol L^{-1} cysteamine ethanolic solution at room temperature

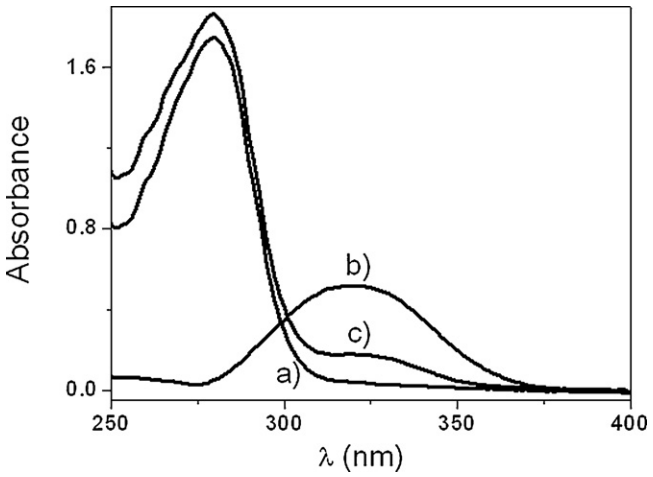

Fig. 2. UV–vis spectra of (a) OVA, (b) CIT and (c) CIT-OVA conjugated in 0.01 mol L^{-1} PBS solution (pH 7.40).

overnight and then washed sequentially with ethanol and distilled water to remove any cysteamine physically adsorbed. Then, the cysteamine modified gold electrode was allowed to react with an aqueous solution of glutaraldehyde 5% (w/w) in a 0.20 mol L^{-1} carbonate solution (pH 10.00) for 2 h at room temperature. An imine bond between the aldehyde and the terminal amine group of cysteamine was generated. Besides, after three steps of washing with purified water, 0.01 mol L^{-1} PBS (pH 7.20) and 30 μL of 10 μg mL^{-1} CIT-OVA were allowed to react with residual aldehyde groups of glutaraldehyde overnight at 4 °C, generating an imine bond with terminal amine groups present in the chains of lysine of the OVA. Then, 30 μL of 0.1 mol L^{-1} glycine solution was added to block the remaining reactive groups for 2 h at room temperature and then it was copiously rinsed with water. Then, aldehyde carbonyl groups were reduced with 30 μL of 0.5% $NaBH_4$ aqueous solution for 2 h at room temperature. Finally, the CIT-OVA conjugated was washed with 0.01 mol L^{-1} PBS (pH 7.20) and stored in the same buffer solution at 4 °C between measurements. The immobilized CIT-OVA conjugated was stable for at least 1 month.

2.6. Amperometric measurements of CIT in rice samples

The determination of CIT through competitive assays in different rice samples has been carried out through amperometric measurements. Non-specific bindings on the electrode surface were blocked by treatment with 3% low-fat milk in a 0.01 mol L^{-1} PBS (pH 7.20) at 37 °C for 10 min under stopped flow conditions. Finally, the electrode surface was washed with 0.01 mol L^{-1} PBS (pH 7.20) at a flow rate of 400 μL min^{-1} for 2 min. Solutions of CIT obtained from rice samples (see Section 2.2) and mAb-CIT in 30 μL 0.01 mol L^{-1} PBS (pH 7.20) were injected into the carrier stream and incubated at 37 °C under stopped flow conditions for 20 min (competitive assay). Then, the immunoreactor was washed using a 0.01 mol L^{-1} PBS (pH 7.20) for 2 min at a flow rate of 400 μL min^{-1} in order to eliminate any traces of unbound mAb-CIT. The secAb-HRP in 30 μL 0.01 mol L^{-1} PBS (pH 7.20) was injected into carrier stream and incubated at 37 °C under stopped flow conditions for 20 min. The immunoreactor was then washed using a 0.01 mol L^{-1} PBS (pH 7.20) for 2 min at a flow rate of 400 μL min^{-1} in order to eliminate any traces of unbound secAb-HRP. Finally, the flow line of the immunosensor was washed with 0.1 mol L^{-1} phosphate–citrate buffer (pH 5.00) at a flow rate of 400 μL min^{-1}. Then, 25 μL of substrate solution (1×10^{-3} mol L^{-1} H_2O_2 + 1×10^{-3} mol L^{-1} H_2Q in the same buffer solution) was injected at the flow rate previously indicated and the enzymatic reaction product (Q) was measured on

the *b* electrode surface at −0.15 V *vs* Ag/AgCl under continuous flow conditions for 2 min.

For the next assays, the immunoreactor was reconditioned by desorption using injections of buffer solution (0.1 mol L^{-1} glycine–HCl, pH 2.00) for 2 min and then washed with 0.01 mol L^{-1} PBS (pH 7.20). The desorption efficiency was checked by the absence of the reduction current of enzymatic reaction product at *b* electrode surface at −0.15 V *vs* Ag/AgCl after addition of $H_2O_2 + H_2Q$, which indicates the absence of secAb-HRP.

A calibration curve was constructed from amperometric measurements following the protocol previously described. The CIT calibration curve was linear from 0.5 to 50 ng mL^{-1}. Amperometric measurements were performed at 37 °C and the resulting anodic current was displayed on the *x*–*y* digital recorder. The stock solution of H_2Q was prepared freshly before the experiments and stored in the dark.

3. Results and discussion

The electrochemical behavior of H_2Q was examined by cyclic voltammetry. A cyclic voltammogram of 1×10^{-3} mol L^{-1} H_2Q in 0.1 mol L^{-1} phosphate–citrate buffer (pH 5.00) was obtained by scanning the potential from −0.25 to 0.60 V *vs* Ag/AgCl (results no shown). The cyclic voltammogram showed a well-defined anodic peak ($E_{p,a}$ = 0.239 V *vs* Ag/AgC) and their corresponding cathodic peak ($E_{p,c}$ = 0.141 V *vs* Ag/AgCl) when the scan rate was reversed. The oxidation of H_2Q to Q and the reduction of Q back to H_2Q is a quasi-reversible two-electron redox process [61]. Cyclic voltammograms were obtained under the same experimental conditions used in the amperometric immunoreactor, but using a conventional three-electrode cell. The potential applied for the amperometric detection was −0.15 V *vs* Ag/AgCl which ensures that all the produced Q is reduced back to H_2Q. This must be taken into account, considering that the shape of the cell and the distribution of electrodes in the immunoreactor are different with respect to the conventional three-electrode cell and the peak potential could be displaced.

3.1. Optimum conditions for the immune reactions and the determination of enzymatic products

Studies related to the variation of concentrations of CIT-OVA conjugated and mAb-CIT were carried out with the purpose to determine the best parameters for the immunoreaction using ELISA experiments. The ELISA experiments were performed in the concentration range from 1 to 10 μg mL^{-1} and 0.20–400 μg mL^{-1} for mAb-CIT and CIT-OVA conjugated, respectively. Absorbance *vs* CIT-OVA conjugated concentrations plots were obtained for three different mAb-CIT concentrations (1, 5 and 10 μg mL^{-1}). It was noted that the higher the concentration of mAb-CIT the greater the absorbance values (Fig. 3). The mAb-CIT concentration chosen was 10 μg mL^{-1} in order to achieve the best sensitivity. An increase of CIT-OVA conjugated concentration produced an increase in absorbance values for different mAb-CIT concentrations (Fig. 3). CIT-OVA conjugated concentrations higher than 220 μg mL^{-1} produced the saturation of mAb-CIT in solution considering that constant absorbance values were observed. Therefore, with the purpose that a true competition occurs between CIT of rice samples and immobilized CIT-OVA conjugated on the *a* electrode surface, a concentration of CIT-OVA conjugated bonded to the electrode surface of 200 μg mL^{-1} was chosen. The dilution factor of SecAb-HRP was 1:5000 which allows a maximum interaction with mAb-CIT (results not shown).

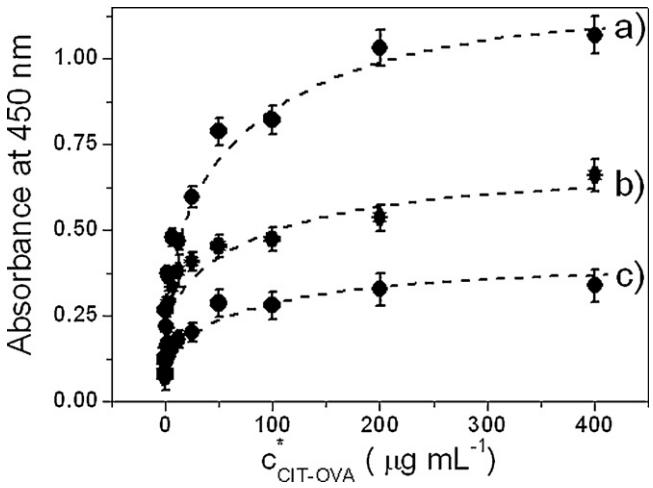

Fig. 3. Optimization of CIT-OVA conjugated and mAb-CIT concentrations. ELISA assays carried out for different mAb-CIT concentrations: (a) 10, (b) 5 and (c) 1 μg mL^{-1}. The secAb-HRP dilution factor was of 1:5000. $c^*_{CIT-OVA}$ is the CIT-OVA conjugated concentration.

3.2. Detection and competitive immunoassays on the immunoreactor

The enzymatic reaction rates under flow conditions as well as the H_2O_2 and H_2Q concentrations have been previously established [26,62]. Therefore, enzymatic reaction responses showed maximum values at pH 5.00 in 0.1 mol L^{-1} phosphate–citrate buffer. The effect of varying H_2O_2 concentration from 7.0×10^{-4} to 5.0×10^{-3} mol L^{-1} at a given H_2Q concentration (1.0×10^{-3} mol L^{-1}) as well as the effect of varying H_2Q concentration from 1.0×10^{-4} to 3.6×10^{-2} mol L^{-1} at a given H_2O_2 concentration (1.0×10^{-3} mol L^{-1}) was evaluated on immunosensor responses. The optimal H_2O_2 and H_2Q concentrations found were 1.0×10^{-3} mol L^{-1} and 1.0×10^{-3} mol L^{-1}, respectively (data not shown). On the other hand, it is well known that the optimal temperature of immunoreactions is about at 37 °C [63]. Thus, all measurements were performed at this temperature. The variation of current responses was studied varying the flow rate and the enzymatic substrate injection volume for a standard concentration of 10 ng mL^{-1} for CIT. It was found that the smaller is the flow rate during the amperometric measurement the greater the current response; however, the peak widens (Fig. 4.a). On the other hand, the peak obtained is wide and of low amplitude when working under conditions of stopped flow. In addition, the peak time of occurrence is higher (results not shown). This is due to that the arrival of Q from the *a* electrode to *b* electrode is carried out only by diffusion. Therefore, the purpose of obtaining greater sensitivity and fast responses a flow rate of 400 μL min^{-1} was chosen. On the other hand, the added volume of $H_2O_2 + H_2Q$ was optimized. It can be observed in Fig. 4.b the greater volume of $H_2O_2 + H_2Q$ was injected (injections for duplicate), the higher the current response. Volumes greater than 25 μL did not produce significant changes in current values. Therefore, 25 μL was injected in all experiments for the purpose of working with minimum consumption of reagents. In addition, a volume of 25 μL allowed to minimize the characteristic convective and dispersive effects in a FI system as well as to obtain a complete filling of the cell immunoreactor.

3.3. Quantitative assay for the determination of CIT in the micro fluidic immunosensor

The currents obtained for the reduction of Q in 0.1 mol L^{-1} phosphate–citrate buffer (pH 5.00) were indirectly proportional to

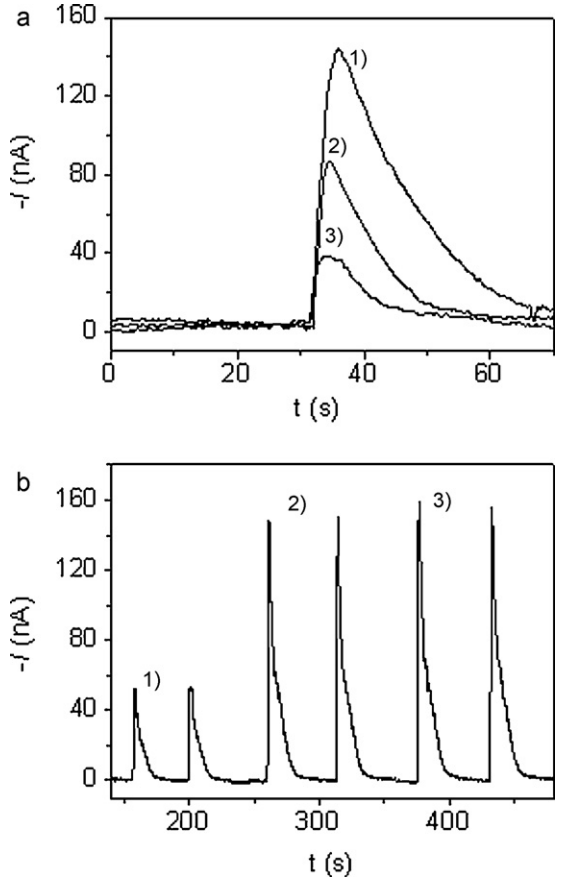

Fig. 4. (a) Effect of flow rate on the electrochemical responses. $c^*_{CIT} = 10\,ng\,mL^{-1}$, $c^*_{CIT\text{-}OVA} = 200\,\mu g\,mL^{-1}$ and $c^*_{mAb\text{-}CIT} = 10\,\mu g\,mL^{-1}$. Injected volume: 25 μL. Flow rates: (1) 400 μL min^{-1}, (2) 1000 μL min^{-1} and (3) 1600 μL min^{-1}. (b) Effect of injected volumes. Flow rate: 400 μL min^{-1}. Injected volumes: (1) 15 μL, (2) 25 μL and (3) 50 μL. Cell volume = 7.5 μL. The substrate enzymatic solution was 0.1 mol L^{-1} phosphate–citrate buffer, pH 5.00, 1.0×10^{-3} mol L^{-1} H_2O_2 and 1.0×10^{-3} mol L^{-1} H_2Q.

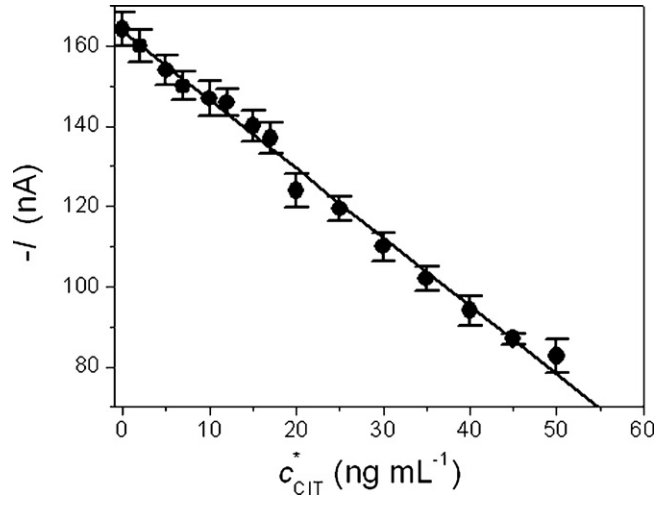

Fig. 5. Calibration curve obtained for CIT in rice samples. Each point was the average of five replicated measurements.

the amount of CIT in the sample, under the experimental conditions previously described. A linear calibration curve was found in the range from 0.5 to 50 ng mL^{-1} for CIT in the samples (Fig. 5). The

Table 2
Comparison between intra-assays accuracy (five measurements performed for the same control sample) and inter-assays accuracy (five measurements for each control sample, repeated for 3 consecutive days).

[a]CIT concentration	Intra-assays		Inter-assays	
	Mean	%VC	Mean	%VC
5	5.07	5.1	4.89	6.0
50	49.5	5.8	50.1	4.5

[a] ng mL^{-1}.

linear regression equation was:

$$-I\,[nA] = (163.61 \pm 1.05)\,[nA] - (1.71 \pm 0.04)\,[nA\,ng^{-1}\,mL] \times c^*_{CIT}\,[ng\,mL^{-1}] \quad (1)$$

with linear regression coefficient $r = -0.9950$. Each experimental point was the average of five replicated measurements. The percentage variation coefficient (%VC) for 5 ng mL^{-1} was 5.1%, showing a very good accuracy. The detection (LOD) and the quantification (LOQ) limits were 0.1 and 0.5 ng mL^{-1}, respectively. The LOD was determined as the lowest concentration experimentally measured for a signal to noise ratio of 3:1. The sensitivity was (1.71 ± 0.04) nA ng^{-1} mL in the range from 0.5 to 50 ng mL^{-1}. These values demonstrate that our micro fluidic immunosensor can be used to quantify CIT in rice samples.

The accuracy of the electrochemical assays was checked using standard solutions at concentrations of CITof 5 and 50 ng mL^{-1}. The intra-assay accuracy was tested through five consecutive measurements of the same sample. These measurement series were repeated for 3 consecutive days to estimate the inter-assay accuracy. The results obtained are summarized in Table 2. The CIT assays showed a good accuracy. The %VC intra-assays and inter-assays were 5.8% and 6%, respectively.

CIT has a planar conjugated chemical structure, and as a result, CIT has natural fluorescence. This makes possible the qualitative and quantitative determination of CIT using a fluorometer. A rapid semi-quantitative fluorimetric assay has been used for CIT testing in corn, barley, and peanuts [64]. The weak fluorescence of CIT can be greatly enhanced in acidic media. In recent years, the CIT fluorescence detection has been universally adopted considering that this technique shows a greater sensitivity than HPLC chromatographic technique with UV–vis detection. The fluorescence allows the analysis of a large number of samples using micro plates system. CIT has an absorption maximum at λ = 332 nm and an emission maximum at λ = 521 nm. It was built a calibration curve for CIT using the technique of fluorescence and the commercial reagent, which was dissolved in ACN + 1% acetic acid. The intensity of fluorescence was plotted as a function of CIT concentration in the range between 3 and 50 ng mL^{-1}. The linear regression equation can be expressed by:

$$I_f\,[A.U] = (1.61 \pm 0.02)10^6\,[A.U] + (3.22 \pm 0.09)10^4\,[A.U\,ng^{-1}\,mL] \times c^*_{CIT}\,[ng\,mL^{-1}] \quad (2)$$

where I_f is the fluorescence intensity and A.U are arbitrary units. The linear regression coefficient was $r = 0.9962$. The LOD and LOQ were 1.1 ng mL^{-1} and 3 ng mL^{-1}, respectively. The %VC was 4.5% at CIT concentration of 10 ng mL^{-1} for five replicated measurements. The results obtained with the technique of fluorescence were compared with those obtained by the amperometric immunosensor. We performed a plot of CIT concentrations obtained by both methods and found a linear correlation coefficient close to 1, indicating a good correspondence between methods (Fig. 6). Our electroanalytical method showed a LOD and LOQ less than those obtained from fluorimetric assays. In addition, the sensitivity obtained with the electrochemical immunosensor was high enough to allow deter-

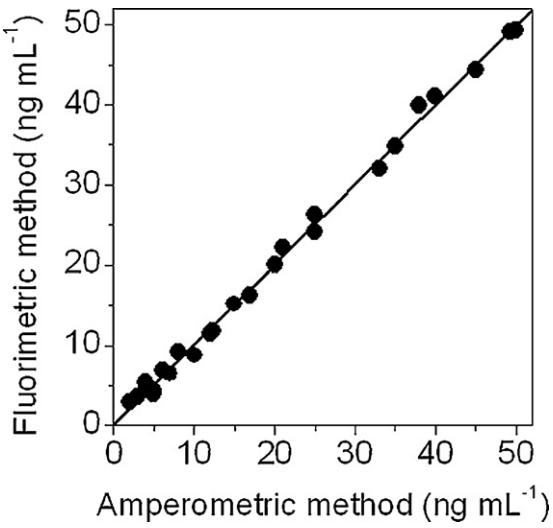

Fig. 6. Correlation between the CIT concentration values obtained from the electrochemical immunosensor and those obtained from the fluorimetric method. Slope = (0.998 ± 0.012), intercept = (0.18 ± 0.09), r = 0.9982.

mining CIT at trace levels in rice samples. Other advantages of our method are the use of small volumes of reagents and samples as well as a marked decrease in the time required for analysis. Moreover, the high selectivity of our immunoreactor allows working in complex matrices without pretreatment unlike of colorimetric methods that require separation steps using, for example, chromatographic techniques.

Two rice samples were analyzed using our electrochemical immunoreactor and the fluorimetric method. The rice samples were milled, homogenized and each one was divided in two parts. One of these parts was treated as previously described (see Section 2.2) and it was used to perform the fluorimetric assays. The other part was dissolved in 0.01 mol L^{-1} PBS (pH 7.20), filtered and used to carry out electrochemical immunoassays. Values obtained by both methods were similar (see Table 3). This behavior suggests that our electrochemical immunosensor can be used to determine CIT in rice samples. CIT concentration values determined from the fluorimetric method were lower than those obtained from our immunosensor. This behavior could be due to the loss of CIT in the different extraction steps.

The immunosensor stability was tested for nearly 25 days at a CIT constant concentration in the immunoreactor system. Current responses were practically constant during this period. The immunoreactor was regenerated by injection of a desorption buffer (0.1 mol L^{-1} glycine–HCl, pH 2.00) for 2 min and then washed with 0.01 mol L^{-1} in PBS (pH 7.20), which allowed us to use the immunoreactor to perform about of 80 determinations. CIT standard solutions as well as rice samples were employed alternatively for performing these measurements. The comparison with other methods reported in the literature show that our electrochemical immunosensor has lower LOD, i.e. 0.1 μg L^{-1} as compared to 1–10 μg kg^{-1} [2,8,18]. However, the advantages of the methodology here proposed are the use of smaller volumes of reagents and samples and the fact that it is not required any pretreatment of the samples.

Table 3
CIT mycotoxin determination in rice samples.

Method	Sample 1		Sample 2	
	[a]Concentration	%VC	[a]Concentration	%VC
Electrochemical immunoreactor	30.8	6.1	36.6	5.5
Fluorimetric method	28.2	4.0	33.4	4.3

[a] μg kg^{-1}.

4. Conclusions

We have developed for the first time a micro fluidic electrochemical immunosensor coupled with flow injection system that can be used for the rapid, sensitive and selective quantification of CIT at trace levels in rice samples using electrochemical detection. Determinations performed without any sample pre-treatment would indicate the high selectivity of the antibody. The immunoreactor developed can operate as a fast, selective, and sensitive detector when it is incorporated into a flow injection analysis system. The immunoreactor also minimizes the use of expensive antibodies and other reagents. It also shows physical and chemical stability, a wide working range and accuracy. It does not require highly skilled technicians or expensive and dedicated equipments. The electrochemical detection was carried out within 2 min and the total analysis time does not exceed 45 min. Immunosensor based on specific reactions between monoclonal antibody and CIT may make a significant contribution to faster, direct, and secure analysis of CIT in many areas such as clinical, environmental and food fields.

Acknowledgements

Financial supports from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Secretaría de Ciencia y Técnica (SECyT) from the Universidad Nacional de Río Cuarto are gratefully acknowledge. F.J. Arévalo and A.M. Granero acknowledge to CONICET for post-doctoral research fellowships. We thank to Dr. Gualberto Gonzalez-Sapienza from the Cátedra de Inmunología, Facultad de Química, Instituto de Higiene, UDELAR, Montevideo, who kindly provided us the sec-Ab-HRP.

References

[1] J.W. Bennett, M. Klich, Clin. Microbiol. Rev. (2003) 497.
[2] B. Xu, X. Jia, L. Gu, C. Sung, Food Control 17 (2006) 271.
[3] International Agency for Research on Cancer (IARC), 2006. Available in: http://www.iarc.fr.
[4] T.S. Nelson, L.K. Kirby, J.N. Beasley, Z.B. Johnson, A. Ciegler, Poult. Sci. 64 (1985) 464.
[5] B.G. Osborne, Food Cosmet. Toxicol. 18 (1980) 615.
[6] P.M. Scott, W. van Walbeek, B. Kennedy, B. Anyeti, J. Agric. Food. Chem. 20 (1972) 1103.
[7] E. Hökby, K. Hult, S. Gatenbeck, L. Rutqvist, Acta Agric. Scand. 29 (1979) 174.
[8] K. Tanaka, Y. Sago, Y. Zheng, H. Nakagawa, M. Kushiro, Int. J. Food Microb. 119 (2007) 59.
[9] D. Abramson, R. Hulasare, N.D.G. White, D.S. Jayas, R.R. Marquardt, J. Stored Prod. Res. 35 (1999) 297.
[10] J.D. Bailly, A. Querin, S. Le Bars-Bailly, G. Benard, P. Guerre, J. Food Prot. 65 (2002) 1317.
[11] R. Comerio, V.E. Fernández Pinto, G. Vaamonde, Int. J. Food Microbiol. 42 (1998) 219.
[12] A. Gimeno, M.L. Martins, J. Assoc. Offic. Anal. Chem. 66 (1983) 85.
[13] D. Heber, A. Lembertas, Y.Q. Lu, S. Bowerman, V.L.W. Go, J. Alternative Compl. Med. 7 (2001) 133.
[14] D. Flajs, M. Peraica, Arh Hig Rada Toksikol. 60 (2009) 457.
[15] C.M. Franco, C.A. Fente, B. Vazquez, A. Cepeda, L. Lallaoui, E. Prognon, G. Mahuzier, J. Chromatogr. A 723 (1996) 69.
[16] D. Abramson, E. Usleber, E. Märtlbauer, Appl. Environ. Microbiol. (1995) 2007.
[17] Z.-H. Duan, Z.-S. Lin, H.-R. Yao, Y.-H. Gao, K. Zhang, S.-Q. Zhao, Z.-Y. Zhu, Biomed. Environ. Sci. 22 (2009) 237.
[18] P.-Y. Shun, C.-H. Lin, Anal. Sci. 18 (2002) 283.
[19] G.R. Xu, C. Lu, X.Q. Mu, J.L. Chen, Y. Chen, Y.M. Gu, Y.P. Wu, F. Sheng, M.Y. Wu, Arch Lebensmittelhyg 50 (1999) 88.
[20] E. Mannaert, P. Daenens, Analyst 119 (1994) 2221.
[21] C.A. Vijayavardhara, B. Halsall, W. Heineman, in: A. Brajter-Toth, J.Q. Chambers (Eds.), Electroanalytical Methods for Biological Materials, Marcel Dekker, New York, 2002.
[22] D. Hernández-Santos, M.B. González-García, A. Costa-García, Electroanalysis 14 (2002) 1225.
[23] D.S. Hage, Anal. Chem. 71 (1999) 294R.

[24] P. Skladal, Electroanalysis 9 (1997) 737.
[25] L.X. Tiefenauer, S. Kossek, C. Padeste, P. Thiébaud, Biosens. Bioelectron. 12 (1997) 213.
[26] F.J. Arévalo, G.A. Messina, P.G. Molina, M.A. Zón, J. Raba, H. Fernández, Talanta 80 (2010) 1986.
[27] F. Darain, S.-U. Park, Y.-B. Shim, Biosens. Bioelectron. 18 (2003) 773.
[28] S. Storri, T. Santoni, M. Minunni, M. Mascini, Biosens. Bioelectron. 13 (1998) 347.
[29] S. Cosnier, Biosens. Bioelectron. 14 (1999) 443.
[30] Y.Y. Wong, S.P. Ng, M.H. Ng, S.H. Si, S.Z. Yao, Y.S. Fung, Biosens. Bioelectron. 17 (2002) 676.
[31] H. Korri-Youssoufi, C. Richard, A. Yassar, Mater. Sci. Eng. C 15 (2001) 307.
[32] G. Farace, G. Lillie, T. Hianik, P. Payne, P. Vadgama, Bioelectrochemistry 55 (2002) 1.
[33] A. Ramanavicius, A. Ramanaviciene, A. Malinauskas, Electrochim. Acta 51 (2006) 6025.
[34] H. Gao, J. Lu, Y. Cui, X.-X. Zhang, J. Electroanal. Chem. 592 (2006) 88.
[35] F. Darain, D.-S. Park, J.-S. Park, Y.-B. Shim, Biosens. Bioelectron. 19 (2004) 1245.
[36] J.J. Gooding, C. Wasiowych, D. Barnett, D. Brynn Hibbert, J.N. Barisci, G.G. Wallace, Biosens. Bioelectron. 20 (2004) 260.
[37] S. Grant, F. Davis, K.A. Law, A.C. Barton, S.D. Collyer, S.P.J. Higson, T.D. Gibson, Anal. Chim. Acta 537 (2005) 163.
[38] B.D. Malhotra, A. Chaubey, S.P. Singh, Anal. Chim. Acta 578 (2006) 59.
[39] S.T. Pathirana, J. Barbaree, B.A. Chin, M.G. Hartell, W.C. Neely, V. Vodyanoy, Biosens. Bioelectron. 15 (2000) 135.
[40] Y. Xu, S. Xia, C. Bian, S. Chen, Conference on Nano/Micro Engineered and Molecular Systems, January 18–21, 2006, Zhuhai, China.
[41] S. Babacan, P. Pivarnik, S. Letcher, A.G. Rand, Biosens. Bioelectron. 15 (2000) 615.
[42] D. Tang, R. Yuan, Y. Chai, Anal. Chem. 80 (2008) 1582.
[43] C.-C. Lin, L.-C. Chen, C.-H. Huang, S.-J. Dingd, C.-C. Chang, H.-C. Chang, J. Electroanal. Chem. 619–620 (2008) 39.
[44] E.M. Abad-Villar, M.T. Fernández-Abedul, A. Costa-García, Anal. Chim. Acta 409 (2000) 149.
[45] G. Giraudi, C. Giovannoli, C. Baggiani, L. Anfossi, C. Tozzi, Anal. Chim. Acta 417 (2000) 95.
[46] V. Carralero, A. González-Cortés, P. Yáñez-Sedeño, J.M. Pingarrón, Anal. Chim. Acta 596 (2007) 86.
[47] M. Murata, M. Nakayama, H. Irie, K. Yakabe, K. Fukuma, Y. Katayama, M. Maeda, Anal. Sci. 17 (2001) 387.
[48] V.M. Mirsky, Trends Anal. Chem. 21 (2002) 439.
[49] J.B. Jia, B.Q. Wang, A.G. Wu, G.J. Cheng, Z. Li, S. Dong, Anal. Chem. 74 (2002) 2217.
[50] A. Tlili, A. Abdenlghani, S. Ameur, N. Jaffrezic-Reanult, Mater. Sci. Eng. C 26 (2006) 546.
[51] X. Jia, Q. Xie, Y. Zhang, S. Yao, Anal. Sci. 23 (2007) 689.
[52] G.A. Messina, A.A.J. Torriero, I.E. De Vito, R.A. Olsina, J. Raba, Anal. Biochem. 337 (2005) 195.
[53] G. Gubitz, C. Shellum, Anal. Chim. Acta 283 (1993) 421.
[54] W.R. Heineman, H.B. Halsall, Anal. Chem. 57 (1985) 1321.
[55] D. Athey, C.J. McNeil, J. Immunol. Methods 176 (1994) 153.
[56] S.S. Babkina, E.P. Medyantseva, H.C. Budnikob, M.P. Tyshlek, Anal. Chem. 68 (1996) 3827.
[57] J. Parellada, A. Narvaes, M.A. Lopez, E. Domínguez, J.J. Fernández, J. Katakis, Anal. Chim. Acta 362 (1998) 47.
[58] T. Lim, Y. Komoda, N. Nakamura, T. Matsunaga, Anal. Chem. 71 (1999) 1298.
[59] M.T. Nguyen, M. Tozlovanu, T.L. Tran, A. Pfohl-Leszkowicz, Food Chem. 105 (2007) 42.
[60] R.T. Morrison, R.N. Boyd, Química Orgánica, 5th ed., Addison Wesley, Boston, Massachusetts, 1996.
[61] E.S. Forzani, G.A. Rivas, V.M. Solís, J. Electroanal. Chem. 435 (1997) 77.
[62] G.A. Messina, A.A.J. Torriero, I.E. De Vito, J. Raba, Talanta 64 (2004) 1009.
[63] S.S. Deshpande, Enzyme Immunoassays, from Concept to Product Development, Chapman & Hall, New York, 1996.
[64] A.L. Trantham, D.M. Wilson, J. AOAC 67 (1984) 37.