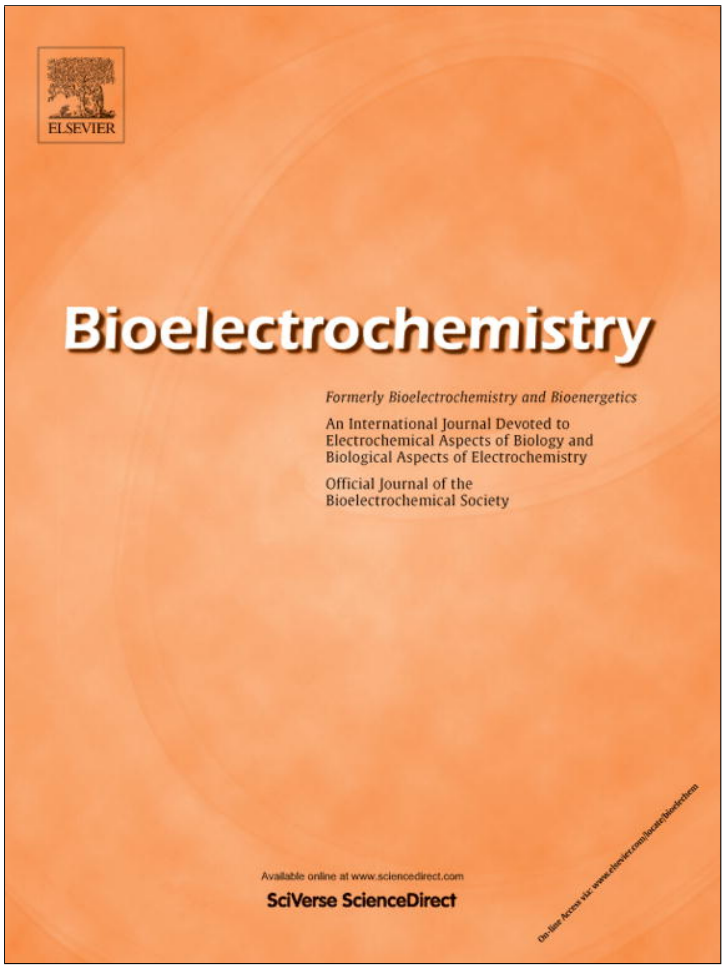

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Development of an amperometric biosensor based on peroxidases to quantify citrinin in rice samples

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ABSTRACT

An amperometric biosensor based on horseradish peroxidase (EC1.11.1.7,H_2O_2-oxide-reductases) to determine the content of citrinin mycotoxin in rice samples is proposed by the first time. The method uses carbon paste electrodes filled up with multi-walled carbon nanotubes embedded in a mineral oil, horseradish peroxidase, and ferrocene as a redox mediator. The biosensor is covered externally with a dialysis membrane, which is fixed to the body side of the electrode with a Teflon laboratory film, and an O-ring. The reproducibility and the repeatability were of 7.0% and 3.0%, respectively, showing a very good biosensor performance. The calibration curve was linear in a concentration range from 1 to 11.6 nM. The limits of detection and quantification were 0.25 nM and 0.75 nM, respectively. For comparison, the citrinin content in rice samples was also determined by fluorimetric measurements. A very good correlation was obtained between the electrochemical and spectrophotometric methods.

1. Introduction

Citrinin (CIT; Scheme 1) is a toxic secondary metabolite, which was first isolated from *Penicillium citrinum* [1].

CIT is also produced by other species of *Penicillium* and *Aspergillus* [2]. CIT is a kind of compound with highly toxic, mutagenic, teratogenic and carcinogenic properties, and it has been implicated as a causative agent of hepatic and extra-hepatic carcinogenesis in human [3]. CIT has often been found in crops, vegetables, and fruits. The International Agency for Research on Cancer (IARC) lists CIT in the Group 3 [4], because there is not enough evidence that it is carcinogenic to humans and cannot be included in the other Groups.

Rice is one of the most important food crops in China, produced mainly in Northeast and Southeast China [5]. CIT has also been isolated from cereals causing porcine nephropathy in Brazil [6]. It has been found as a contaminant in corn [7], wheat, rye, barley, oats [8], and yellow peanut kernels with damaged pods [9]. The natural occurrence of CIT has not yet been reported in Argentina, however, strains of *P. citrinum* frequently isolated from corn, wheat, soybeans, and rice have been reported as efficient producers of CIT [10–12].

To our knowledge, there is no specific legislation on maximum levels permitted for CIT in different foods, the main reason being the lack of an official analytical method for its determination [5].

The methods commonly used to determine qualitative and quantitative CIT are thin-layer chromatography (TLC), which exhibits relatively low sensitivities [13,14], high-performance liquid chromatography (HPLC) with UV or fluorescence detection (FD) [15,16], and enzyme immunoassays (EIA) [15]. Recently, LC–MS and GC–MS [17] techniques have also become available for CIT determination.

Another interesting proposal is the use of biosensors. According to the IUPAC definition, a biosensor is a device that uses specific biochemical reactions mediated by enzymes, immunosystems, tissues, organelles, or whole cells to detect chemical compounds, mainly using electrical, thermal, or optical signals. The biosensors have been proposed as an effective analytical tool for the determination of phenolic or polyphenolic compounds, exhibiting advantages such as a minimal preparation of the sample, good selectivity, sensitivity, and reproducibility, a rapid response time, and a simple application for continuous in-situ analysis. Recently, a micro fluidic electrochemical immunosensor has been developed for the first time to detect and quantify CIT in rice samples [18].

In addition, enzymatic amperometric biosensors are considered the most suitable for biochemical analysis because of their good selectivity, sensitivity, fast response, miniaturizable size, and reproducible results [19]. Ferrocene and its derivatives are substances well-known as redox mediators [20–23]. Carbon nanotubes (CNTs) [24] have attracted much attention in last years due to their high chemical stability, high surface area, unique electronic properties, and relatively high mechanical properties [25].

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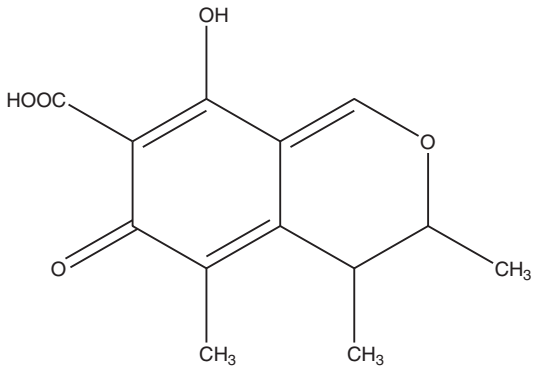


Scheme 1. Chemical structure of citrinin mycotoxin.

In this article, we propose the development of an amperometric biosensor based on peroxidases to determine the CIT content in rice samples. The method uses carbon paste electrodes filled up with multi-walled carbon nanotubes embedded with a mineral oil (MWCNT + MO), horseradish peroxidase (HRP), and ferrocene (Fc) at a given composition (MWCNT + MO–HRP–Fc). The composite electrode prepared, MWCNT + MO–HRP–Fc, has a high surface area, good mechanical stability, and good conductivity, which provides support to maintain the microenvironment of the immobilized enzyme, to increase the mediator loading, and, more important, to prevent the leakage of the mediator.

For comparison, the CIT content in rice samples was also determined by fluorimetric measurements.

2. Materials and methods

2.1. Reagents and materials

CIT, HRP (E.C:1.11.1.7,H_2O_2-oxide-reductases), Fc and o-dianisidine were purchased from SIGMA (USA). Phosphate buffer solutions (PBS) of pH 7 (20 °C; $Na_2HPO_4 + KH_2PO_4$, reference number 109439), hydrogen peroxide (H_2O_2), sodium bicarbonate ($NaHCO_3$), hydrochloric acid (HCl), potassium chloride (KCl), and n-heptane were purchased from Merck p.a. Chloroform and acetonitrile (ACN), were Sintorgan, HPLC grade. All reagents were used as received. All solutions were prepared using water purified by a Labconco WaterPro Mobile System, Model 90901–01 (HPLC grade water).

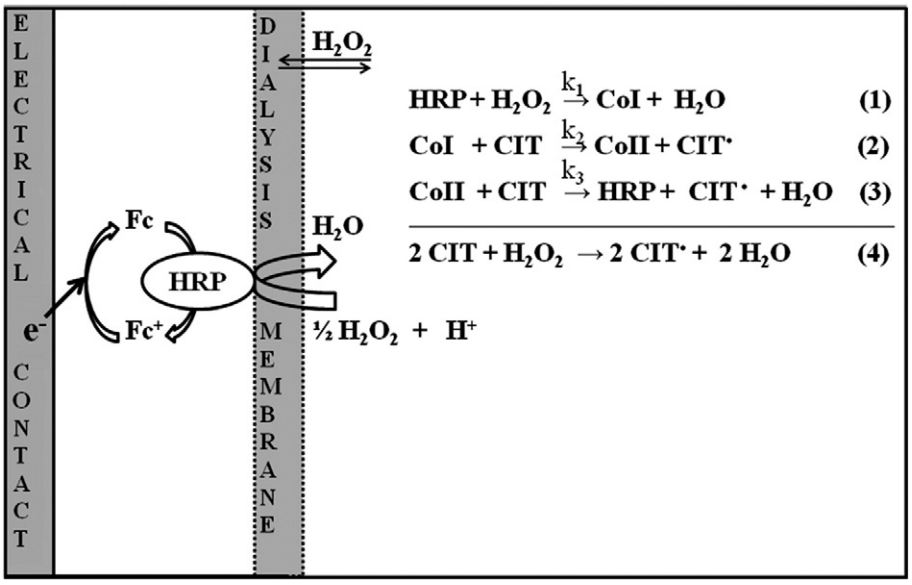
The concentrations of H_2O_2 and HRP were determined by UV–vis spectroscopy at $\lambda_{max} = 240$ nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$) [26], and at $\lambda_{max} = 403$ nm ($\varepsilon = 9 \times 10^4\ M^{-1}\ cm^{-1}$) [27], respectively. The peroxidase activity was determined with o-dianisidine as substrate [28] and expressed in international units (IU), which was defined as the amount of enzyme forming 1 μmol of product in 1 min under the experimental conditions used.

The MWCNT + MO composite was obtained by mixing different amounts of MWCNT (Sigma, inner diameter 110–170 nm, length 5–9 μm), purity 90% with a MO (Sigma) at a given composition (55% MWCNT + 45% MO), which was previously optimized by Granero et al. [29]. The biosensor was covered externally with a dialysis membrane (Scheme 2), which was fixed to the body side of the electrode with a Teflon laboratory film, and an O-ring. It is well known that phenolic and/or polyphenolic compounds can work as electron-donors for peroxidases in the catalytic reduction of H_2O_2 [30,31]. The most common reaction mechanism for peroxidases which is universally accepted is shown in Scheme 2 (Eqs. (1)–(4)) [32]. Peroxidase compounds I (CoI, Eq. (1)) and II (CoII, Eq. (2)) are formed in the presence of low H_2O_2 concentrations only. Compounds I and II are thus considered to be obligatory enzyme intermediates in overall peroxidase reaction regenerating the original ferriperoxidase.

This approach allows detecting the decrease in the H_2O_2 concentration in a solution after the oxidation of phenolic and/or polyphenolic compounds produced by the HRP in the presence of H_2O_2, given that HRP acts in cascade in the solution and at the electrode surface. The separation of the electrode surface from the solution by a semi-permeable membrane allows minimizing the electrical noises as well as the fouling of the electrode surface.

2.2. Sample preparation

The Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, kindly helped us to prepare the rice samples contaminated with the mycotoxin.



Scheme 2. Two-stage reaction scheme for detecting CIT using a carbon paste electrode filled up with multi-walled carbon nanotubes embedded in a mineral oil (MWCNT + MO), HRP, and Fc. CIT and CIT• are the reduced and the oxidized forms of CIT, respectively.

Five rice samples were contaminated by inoculation with *P. citrinum* fungi. Water was added to the contaminated rice samples in a 40:60 (w/w) ratio, and allowed fungi to grow up in a shaker at 25 °C for 7 days. The extraction of CIT from the contaminated rice samples was carried out following a procedure proposed by Nguyen et al. [3] with some modifications. Therefore, CIT was extracted from about 25 g of the contaminated rice samples with 125 mL of ACN + 4% KCl aqueous solution (9:1). The pH of the solution was adjusted to 2 using a HCl aqueous 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 carried out 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 50 mL of distilled water + 50 mL of chloroform were added. This solution was shaken for 10 min and, then, the lower phase (chloroform) was collected.

In addition, 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 with HCl aqueous solution. 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. Electrochemical instruments and experimental measurements

Cyclic voltammograms and amperometric measurements were performed using an Epsilon (Bioanalytical System, Inc.) potentiostat, controlled by the corresponding electrochemical analysis software. The electrochemical cell was a Pyrex cell of 2 mL. The working electrode was a carbon paste disk of 1.6 mm in diameter obtained from Bioanalytical System, Inc. The counter and reference electrodes were a platinum foil of large area ($A \approx 2\ cm^2$), and an aqueous Ag/AgCl, respectively. Amperometric measurements were performed at a potential of −0.1 V (vs. Ag/AgCl) in solutions stirred at 1600 rpm. We previously optimized this operational applied potential [29]. The Fc was used as a redox mediator. Biosensors were constructed by using the following procedure: MWCNT + MO, HRP, and Fc were mixed in different weight ratios in order to obtain the best biosensor composition. Then, the optimal composition was used to fill up carbon paste electrodes. Biosensors were stored at 4 °C in pH 7 PBS when they were not in use. Granero et al. [27] previously established an optimal pH value of 7 for peroxidase enzymes. In addition, measurements were carried out in non-deoxygenated solutions. UV–vis spectra were recorded immediately after the preparation of solutions by using a Hewlett–Packard Model 8452A spectrophotometer.

Fluorescence spectra were measured at room temperature using a Spex Fluoromax Spectrofluorometer.

3. Results and discussion

3.1. Responses of the MWCNT + MO–HRP–Fc biosensor to H_2O_2 concentrations

A cyclic voltammogram recorded in unstirred pH 7 PBS (supporting electrolyte solution) with the MWCNT + MO–HRP–Fc biosensor showed an increase in the anodic current at potentials higher than 0.2 V (vs. Ag/AgCl), which corresponds to the oxidation of Fc to Fc^+. A cathodic peak with a peak potential at about 0.18 V (vs. Ag/AgCl) was observed when the potential sweep direction was reversed at 0.8 V, which can be assigned to the reduction of Fc^+ to Fc at the electrode surface (Fig. 1). On the other hand, a cyclic voltammogram recorded when H_2O_2 was added to the reaction medium showed a cathodic current at potentials more negative than −0.3 V (vs. Ag/AgCl), which corresponds to the reduction of H_2O_2 at the biosensor surface. A similar behavior was previously found by Sljukic et al. [33] when studied the H_2O_2 electrochemical reduction at MWCNT modified electrodes. Therefore, the base current was practically zero between −0.3 and 0.15 V (vs. Ag/AgCl) in pH 7 PBS. This behavior was also previously found by us when a similar biosensor was employed to determine the t-resveratrol antioxidant using the commercial reagent [27] and for the determination of the total polyphenolic content in wine and tea samples [29].

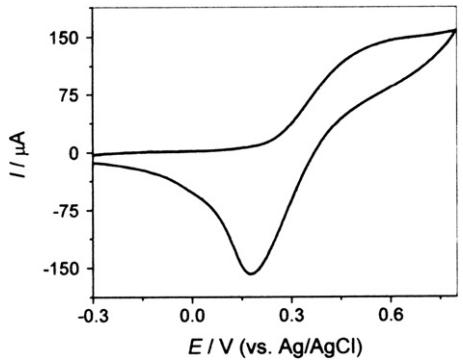


Fig. 1. Cyclic voltammogram recorded with the MWCNT + MO–HRP–Fc biosensor in unstirred pH 7 PBS. v = 0.050 V/s.

Studies were then conducted to find the best ratio between the different components of the biosensor in order to obtain its best composition. Therefore, five biosensors were constructed to optimize the composition of Fc in the biosensor, for which the proportion of the other two components remained constant, i.e., 22.5 mg (55% MWCNT + 45% MO), and 1.5 mg of HRP. These five biosensors were used to study their responses towards H_2O_2. Therefore, when only H_2O_2 was added to the stirred reaction medium, the enzymatic reaction took place between HRP on the electrode surface and H_2O_2 penetrated into the inner layer between the semi permeable membrane and the electrode surface. H_2O_2 was reduced to H_2O by HRP and the enzyme was reduced to its native form by Fc, which was oxidized to Fc^+. The Fc^+ was then immediately reduced to Fc at the electrode surface held at a potential of −0.1 V (vs. Ag/AgCl). Steady-state reduction currents ($I_{s,s}$) obtained after the addition of different aliquots of H_2O_2 are shown in Fig. 2a. The differences between the initial base current (I_o) and the corresponding $I_{s,s}$ ($\Delta I_{s,s} = I_{s,s} - I_o$) were proportional to the H_2O_2 bulk concentration, $[H_2O_2]$, showing a Michaelis–Menten type saturation (Fig. 2b). The Michaelis-Menten term for H_2O_2 can be expressed as:

$$\frac{\Delta I_{s,s}}{\Delta I_{max,H_2O_2}} = \frac{[H_2O_2]}{K_{A,H_2O_2} + [H_2O_2]} \tag{5}$$

where $\Delta I_{maxH_2O_2}$ is the H_2O_2 maximum current, and K_{A,H_2O_2} is the apparent Michaelis–Menten constant.

On the other hand, the Eadie–Hofstee equation can be expressed as:

$$\Delta I_{s,s} = \Delta I_{max,H_2O_2} - K_{A,H_2O_2} \frac{\Delta Is.s}{[H_2O_2]}. \tag{6}$$

Plots of $\Delta I_{s,s}$ vs. $\frac{\Delta I_{s,s}}{[H_2O_2]}$ (Eq. (6)) were linear. From these plots, kinetics parameters of the MWCNT + MO–HRP–Fc biosensor for H_2O_2 were calculated. Therefore, average values of $\Delta I_{max,H_2O_2}$, and K_{A,H_2O_2} were determined from the intercept and the slope, respectively, of Eadie–Hofstee plots for five replicated measurements. These values are shown in Table 1.

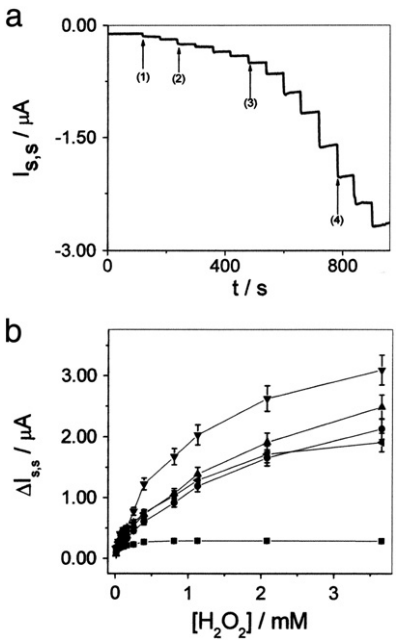


Fig. 2. (a) Steady-state current responses on the addition of different H_2O_2 concentrations at the stirred pH 7 PBS reaction medium measured with the MWCNT + MO–HRP–Fc (22.5–1.5–1.5) biosensor covered with a dialysis membrane. $[H_2O_2]$: (1) 0.005, (2) 0.044, (3) 0.743, and (4) 1.230 mM. (b) Differences between the base and the steady-state currents, $\Delta I_{s,s}$, as a function of $[H_2O_2]$ under the same experimental conditions as (a). The MWCNT + MO–HRP–Fc biosensor compositions were: -■-(22.5–1.5–0.5) -●-(22.5–1.5–1) -▲-(22.5–1.5–1.5) -▼-(22.5–1.5–2) - -(22.5–1.5–2.5).

From these results, we used those biosensors constructed using 22.5–1.5–2 mg of MWCNT + MO, HRP, and Fc, respectively, in next experiments, considering that this electrode composition gave the maximum current value. Then, similar experiments were performed to optimize the HRP amount. Thus, five new biosensors were constructed, where the amount of the other two components was remained constant, i.e., 22.5 mg MWCNT + MO (55% MWCNT + 45% MO), and 2 mg Fc. The $I_{s,s}$ values obtained after the addition of different aliquots of H_2O_2 were similar to those shown in Fig. 2a. The corresponding $\Delta I_{s,s}$ values were also proportional to $[H_2O_2]$, showing a Michaelis–Menten type saturation (Eq. (5), Fig. 3). From linear plots of $\Delta I_{s,s}$ vs. $\frac{\Delta I_{s,s}}{[H_2O_2]}$ (Eq. (6)), the kinetics parameters of the MWCNT + MO–HRP–Fc biosensor for H_2O_2 were calculated. Averages values of $\Delta I_{max,H_2O_2}$, and K_{A,H_2O_2} calculated from five replicated measurements are shown in Table 2. From these results, biosensors constructed using 22.5–2–2 mg of MWCNT + MO (55% MWCNT + 45% MO), HRP, and Fc, respectively, were used in next experiments, considering that this electrode composition gave the maximum current value.

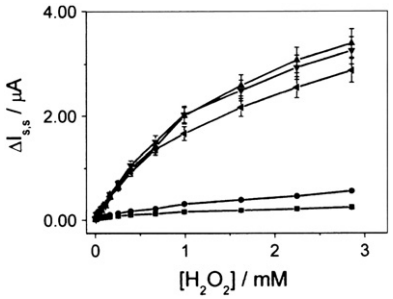


Fig. 3. Differences between the base and the steady-state currents, $\Delta I_{s,s}$, as a function of $[H_2O_2]$ measured with the MWCNT + MO–HRP–Fc (22.5–1.5-1.5) biosensor covered with a dialysis membrane after the addition of different H_2O_2 concentrations at the stirred pH 7 PBS reaction medium. The MWCNT + MO–HRP–Fc biosensor compositions were: -■- (22.5–0.5-2) mg -●- (22.5–1–2) - - (22.5–1.5–2) mg -▲- (22.5–2–2) mg -▼- (22.5–3–2) mg.

3.2. Responses of MWCNT + MO–HRP–Fc biosensors to CIT concentrations

When both HRP and CIT were added to the stirred reaction medium composed by pH 7 PBS at a given H_2O_2 bulk concentration, the enzymatic catalytic cycle also took place in the solution bulk (Eqs. (1)–(3) in Scheme 2). The oxidized HRP, produced as a result of its reaction with H_2O_2, is reduced back to its native state by CIT. The decrease of H_2O_2 in the solution bulk was detected as a decrease in the Fc^+ reduction current at the biosensor surface [27]. Thus, the addition of H_2O_2 to the pH 7 PBS + 56 IU/mL HRP reaction medium produced a steady state limiting current ($I_{s,lim}$) at −0.1 V (vs. Ag/AgCl) after 120 s, which corresponds to the reduction of Fc^+ generated by HRP catalyzed reduction of H_2O_2 to H_2O (Fig. 4a). The difference between $I_{s,lim}$ with and without the addition of CIT ($\Delta I_{s,lim}$) corresponds to the decrease of H_2O_2 concentration, which it is produced because the enzymatic reaction was also taking place in the bulk solution. The steady state currents obtained after the addition of different CIT aliquots were reached at about 60 s. Plots of $\Delta I_{s,lim}$ vs. the CIT bulk concentration, [CIT], also showed a Michaelis–Menten type saturation (Fig. 1b).

Studies were then conducted to optimize the concentrations of H_2O_2 and HRP in solution. Therefore, different experiments were performed in pH 7 PBS containing 56 IU/mL enzyme, and adding aliquots of the CIT solution at a given fixed concentration of H_2O_2. The values of maximum currents, $\Delta I_{max,CIT}$ and apparent Michaelis–Menten constants, $K_{B,CIT}$ for CIT obtained from Eadie–Hofstee plots at different H_2O_2 concentrations are shown in Table 3. An optimal value of 1200 μM for the H_2O_2 concentration was found. Therefore, we used in the next experiments a H_2O_2 concentration in solution of 1200 μM, considering that this H_2O_2 concentration gave the maximum current value (Table 3).

Then, similar experiments were performed to optimize the HRP bulk concentration at a fixed H_2O_2 concentration (1200 μM). The values of

Table 1
Values of the kinetics parameters I_{max,H_2O_2}, and K_{A,H_2O_2} obtained from biosensors of different compositions (showed in Fig. 2) after adding different aliquots of H_2O_2 to pH 7 PBS, with their corresponding standard deviations.

MWCNT + MO–HRP–Fc biosensor composition/mg	I_{max,H_2O_2}/μA	K_{A,H_2O_2}/mM
22.5–1.5–0.5	0.26 ± 0.01	0.25 ± 0.02
22.5–1.5–1	2.19 ± 0.08	0.71 ± 0.03
22.5–1.5–1.5	2.87 ± 0.08	0.77 ± 0.04
22.5–1.5–2	3.20 ± 0.09	0.30 ± 0.02
22.5–1.5–2.5	2.19 ± 0.07	0.33 ± 0.02

Table 2
Values of the kinetics parameters I_{max,H_2O_2}, and K_{A,H_2O_2} obtained from biosensors of different compositions (showed in Fig. 3) after adding different aliquots of H_2O_2 to pH 7 PBS, with their corresponding standard deviations.

MWCNT + MO–HRP–Fc biosensor composition/mg	I_{max,H_2O_2}/μA	K_{A,H_2O_2}/mM
22.5–0.5–2	0.23 ± 0.01	0.24 ± 0.02
22.5–1–2	0.53 ± 0.02	0.90 ± 0.05
22.5–1.5–2	3.20 ± 0.09	0.30 ± 0.02
22.5–2–2	3.39 ± 0.09	0.48 ± 0.03
22.5–3–2	3.27 ± 0.09	0.32 ± 0.02

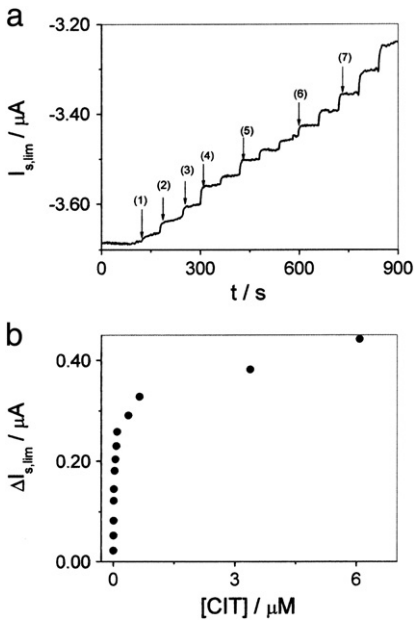


Fig. 4. (a) Steady-state current responses on the addition of different CIT concentrations in pH 7 PBS under stirring containing 56 IU/mL HRP + 875 μM H_2O_2 measured with the MWCNT + MO–HRP–Fc (22.5–2–2) biosensor covered with a dialysis membrane. [CIT]: (1) 1; (2) 3; (3) 6; (4) 8.7; (5) 33; (6) 95.5 and (7) 645 nM. (b) Differences between the base and the steady-state currents, $I_{s,lim}$, as a function of [CIT] under the same experimental conditions as in (a).

maximum currents, $\Delta I_{max,CIT}$ and apparent Michaelis–Menten constants, $K_{B,CIT}$ for CIT obtained from Eadie–Hofstee plots at different HRP concentrations are shown in Table 4. The optimum HRP concentration in solution was 56 IU/mL.

3.3. Biosensor statistical parameters

The reproducibility of the MWCNT + MO–HRP–Fc biosensor was tested by measuring the calibration curve slopes obtained after the addition of different CIT aliquots in a solution of pH 7 PBS + 56 IU/mL HRP + 1200 μM H_2O_2 using six different biosensors. A percent relative standard deviation (%RSD) of 7% was obtained. The repeatability assays were performed carrying out six consecutive amperometric measurements with the same biosensor. In this case, the %RSD of calibration curve slopes was 3%, when a MWCNT + MO–HRP–Fc (22.5–2–2) biosensor was used in pH 7 PBS + 56 IU/mL HRP + 1200 μM H_2O_2. The stability of MWCNT + MO–HRP–Fc biosensor was tested by using the same biosensor to determine the slopes from several calibration curves (n = 5) for CIT. The slopes obtained were practically constant in the order of the experimental error until about five days, showing a good stability of the biosensor. A noticeable decrease in the slope started from the fifth day and a decrease of about 35% was observed on the seventh day.

Table 3
Values of the kinetics parameters $I_{max,CIT}$, and $K_{B,CIT}$ obtained from the biosensor MWCNT + MO–HRP–Fc (22.5–2–2 mg) in response to the addition of different aliquots of CIT when different fixed concentrations of H_2O_2 were added to pH 7 PBS + 56 IU/mL HRP reaction medium, with their corresponding standard deviations.

$[H_2O_2]$/μM	$I_{max,CIT}$/μA	$K_{B,CIT}$/mM
300	0.08 ± 0.01	0.008 ± 0.001
600	0.18 ± 0.04	0.019 ± 0.002
875	0.88 ± 0.08	0.067 ± 0.009
1200	3.30 ± 0.09	0.13 ± 0.01
2400	2.12 ± 0.08	0.12 ± 0.01

Table 4
Values of the kinetics parameters $I_{max,CIT}$, and $K_{B,CIT}$ obtained from the biosensor MWCNT + MO–HRP–Fc (22.5–2–2) in response to the addition of different aliquots of CIT when different fixed concentrations of HRP were added to pH 7 PBS + 1200 μM H_2O_2 reaction medium, with their corresponding standard deviations.

[HRP]/IU/mL	$I_{max,CIT}$/μA	$K_{B,CIT}$/mM
27	0.53 ± 0.01	0.013 ± 0.003
37	2.15 ± 0.04	0.10 ± 0.02
56	3.30 ± 0.09	0.13 ± 0.01
146	2.80 ± 0.08	0.21 ± 0.02
243	1.40 ± 0.08	0.10 ± 0.02

The linear range between $\Delta I_{s,lim}$ and CIT bulk concentrations was from 1 to 11.6 nM. The calibration curve (Fig. 5) can be expressed by a least squares procedure as:

$$\Delta I_{s,lim}/\mu A = (0.004 \pm 0.001)\mu A + (0.0140 \pm 0.0003)\mu A/\text{nM} \times [\text{CIT}]$$

with a linear correlation coefficient, r = 0.9991.

The limits of detection (LOD) and quantification (LOQ) for the determination of CIT using MWCNT + MO–HRP–Fc biosensors were 0.25 nM and 0.75 nM, respectively. They were calculated as 3.28 S_0 and 10 S_0, respectively, where S_0 is the blank standard deviation [34].

3.4. Application of the MWCNT + MO–HRP–Fc biosensor for the determination of CIT in rice samples

The MWCNT + MO–HRP–Fc biosensor was then used to determine the CIT content in different rice samples. The results obtained through the electrochemical method were then compared with those obtained through a fluorimetric method. CIT values determined by both methods are shown in Table 5. There was a good agreement between the concentrations calculated by the two methods. Therefore, plots of CIT values determined by both methods were linear, with a slope of 0.97 ± 0.03 and r = 0.9968. These results demonstrate that the present electroanalytical method is useful for detecting CIT in real samples. In addition, our electrochemical method showed a LOD and LOQ less than those obtained from fluorimetric assays. Moreover, the LOD and LOQ were about one and half and three times lower, respectively, than those values obtained previously by us with the immunosensor [18]. Furthermore, the sensitivity obtained with the electrochemical biosensor was high enough to allow the determination of CIT in rice samples at trace levels.

4. Conclusions

The MWCNT + MO–HRP–Fc biosensor exhibited a very good performance, stability, repeatability, reproducibility, detection limit, and linear range for the quantification of CIT. This good analytical performance allowed us to determine CIT in samples of rice. The contents of CIT in samples of rice determined with our biosensor showed a good correlation with values obtained from the fluorimetric method. These results indicate

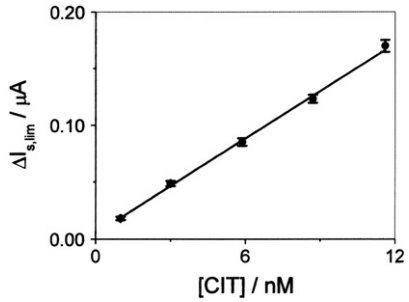


Fig. 5. Plots of the differences between the base and the steady-state currents, $\Delta I_{s,lim}$ with corresponding error bars, as a function of [CIT] in a reaction medium containing 56 IU/mL HRP + 1200 μM H_2O_2 in PBS pH 7 under stirring, obtained with the MWCNT + MO–HRP–Fc (22.5–2–2) biosensor covered with a dialysis membrane.

Table 5
Values determined for the CIT mycotoxin content in rice samples using fluorimetric and electroanalytical methods.

Sample	Fluorimetric method/μM	Biosensor/μM
Sample 1	22.7	22.9
Sample 2	29.8	27.8
Sample 3	32.6	33.9
Sample 4	45.2	45.3
Sample 5	70.0	68.5

that MWCNT + MO–HRP–Fc biosensors can be used as a useful tool for a rapid screening in the determination of CIT in samples rice.

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References

[1] A.C. Hetherington, H. Raistrick, On the production and chemical constitution of a new yellow colouring matter, citrinin, produced from glucose by *Penicillium citrinum* Thom, Philos. Trans. R. Soc. Lond. B. 220 (1931) 269–295.
[2] H. Kurata, Mycotoxins and mycotoxicoses. Overview, in: A.E. Pohland, V.R. Dowell, J.L. Richards (Eds.), Microbial Toxins in Foods and Feeds, Plenum Press, New York, 1990, pp. 249–259.
[3] M.T. Nguyen, M. Tozlovanu, T.L. Tran, A. Pfohl-Leszkowicz, Occurrence of aflatoxin B1, citrinin and ochratoxin A in rice in five provinces of the central region of Vietnam, Food Chem. 105 (2007) 42–47.
[4] International Agency for Research on Cancer (IARC), Available in: http://www.iarc.frMarch 2012.
[5] B. Xu, X. Jia, L. Gu, C. Sun, Review on the qualitative and quantitative analysis of the mycotoxin citrinin, Food Control 17 (2006) 271–285.
[6] C.A.R. Rosa, L.C.H. Cruz, W.A. Chagas, C.E.M.O. Veiga, Ocorrência natural de nefropatia micotóxica suína causada pela ingestão de cevada contaminada com citrinina, Rev. Bras. Med. Vet. 7 (1985) 87–90.
[7] T.S. Nelson, L.K. Kirby, J.N. Beasley, Z.B. Johnson, A. Ciegler, The effect of drying method and storage time on citrinin activity in corn, Poult. Sci. 64 (1985) 464–468.
[8] P.M. Scott, W. Van Walbeek, B. Kennedy, B. Anyeti, Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products, J. Agric. Food Chem. 20 (1972) 1103–1108.
[9] A.E. Pohland, G.E. Wood, Occurrence of mycotoxins in food, in: P. Krogh (Ed.), Mycotoxins in Food, Academic Press, London, 1987, pp. 35–64.
[10] G. Bresler, S.B. Brizzio, G. Vaamonde, Mycotoxin-producing potential of fungi isolated from amaranth seeds in Argentina, Int. J. Food Microbiol. 25 (1995) 101–108.
[11] H.H.L. González, S.L. Resnik, R.T. Boca, W.F.O. Marasas, Mycoflora of Argentinean corn harvested in the main production area in 1990, Mycopathologia 130 (1990) 29–36.
[12] S.A. Tonon, R.S. Marucci, G. Jerke, A. García, L. Vellon, J. Ferreras, Hongos contaminantes de arroz producido en la región central del Mercosur Aliment, Latinoamericana 214 (1996) 39–45.
[13] V. Betina, Chromatographic methods as tools in the field of mycotoxins, J. Chromatogr. 477 (1989) 187–233.
[14] P.M. Scott, Recent developments in methods of analysis for mycotoxins in foodstuffs, Trends Anal. Chem. 12 (1993) 382–386.
[15] D. Abramson, E. Usleber, E. Martlbauer, Rapid determination of citrinin in corn by fluorescence liquid chromatography and enzyme immunoassay, J. AOAC Int. 82 (1999) 1353–1356.
[16] B.I. Vazquez, C. Fente, C. Franco, A. Cepeda, P. Prognon, G. Mahuzier, Simultaneous high-performance liquid chromatographic determination of ochratoxin A and citrinin in cheese by time-resolved luminescence using terbium, J. Chromatogr. A 727 (1996) 185–193.
[17] P.Y. Shu, C.H. Lin, Simple and sensitive determination of citrinin in *Monascus* by GC–selected ion monitoring mass spectrometry, Anal. Sci. 18 (2002) 283–287.
[18] F.J. Arévalo, A.M. Granero, H. Fernández, J. Raba, M.A. Zon, Citrinin (CIT) determination in rice samples using a micro fluidic electrochemical immunosensor, Talanta 83 (2011) 966–973.
[19] N. Hamdi, J.J. Wang, E. Walker, N.T. Maidment, H.G. Monbouquette, An electroenzymatic L-glutamate microbiosensor selective against dopamine, J. Electroanal. Chem. 591 (2006) 33–40.
[20] A.E.G. Cass, G. Davis, G.D. Francis, H.A.O. Hill, W.J. Aston, I.J. Higgins, E.O. Plotkin, L.D.L. Scott, A.P.F. Turner, Ferrocene-mediated enzyme electrode for amperometric determination of glucose, Anal. Chem. 56 (1984) 667–671.
[21] A. Escorcia, A.A. Dhirani, Electrochemical properties of ferrocenylalkane dithiol-gold nanoparticle films prepared by layer-by-layer self-assembly, J. Electroanal. Chem. 601 (2007) 260–268.
[22] S.F. Wang, D. Du, Differential pulse voltammetry determination of ascorbic acid with ferrocene-L-cysteine self-assembled supramolecular film modified electrode, *Sens. Actuators, B* 97 (2004) 373–378.
[23] L. Fernández, H. Carrero, Electrochemical evaluation of ferrocene carboxylic acids confined on surfactant-clay modified glassy carbon electrodes: oxidation of ascorbic acid and uric acid, Electrochim. Acta 50 (2005) 1233–1240.
[24] S. Iijima, Helical microtubules of graphitic carbon, Nature 354 (1991) 56–58.
[25] V.G. Gavalas, S.A. Law, C. Ball, R. Andrews, L.G. Bachas, Carbon nanotube aqueous sol–gel composites: enzyme-friendly platforms for the development of stable biosensors, Anal. Biochem. 329 (2004) 247–252.
[26] E. Agostini, J. Hérnandez Ruíz, M.B. Arnao, S.R. Milrad, H.A. Tigier, M. Acosta, A peroxidase isoenzyme secreted by turnip (*Brassica napus*) hairy-root cultures: inactivation by hydrogen peroxide and application in diagnostic kits, Biotechnol. Appl. Biochem. 35 (2002) 1–7.
[27] A.M. Granero, H. Fernández, E. Agostini, M.A. Zón, An amperometric biosensor for trans-resveratrol determination in aqueous solutions by means of carbon paste electrodes modified with peroxidase basic isoenzymes from *Brassica napus*, Electroanalysis 20 (2008) 858–864.
[28] P.S. González, C.E. Capozucca, H.A. Tigier, S.R. Milrad, E. Agostini, Phytoremediation of phenol from wastewater by peroxidases of tomato hairy root cultures, Enzyme Microbiol. Technol. 39 (2006) 647–653.
[29] A.M. Granero, H. Fernández, E. Agostini, M.A. Zón, An amperometric biosensor based on peroxidases from *Brassica napus* for the determination of the total polyphenolic content in wine and tea samples, Talanta 83 (2010) 249–255.
[30] J. Kulys, U. Bilitewski, R.D. Schmid, The kinetics of simultaneous conversion of hydrogen peroxide and aromatic compounds at peroxidase electrodes, Bioelectrochem. Bioenerg. 26 (1991) 277–286.
[31] G. Marko-Varga, J. Emneus, L. Gorton, T. Ruzgas, Development of enzyme-based amperometric sensors for the determination of phenolic compounds, Trends Anal. Chem. 14 (1995) 319–328.
[32] H.B. Dunford, Heme Peroxidases, J. Wiley & Sons, New York, 1999.
[33] B. Sljukic, C.E. Banks, R.G. Compton, Nano Lett. 6 (2006) 1556–1558.
[34] J.N. Miller, J.C. Miller, Estadística y Quimiometría para Química Analítica, 4th ed. Prentice Hall, Madrid, España, 2002.

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