ORIGINAL ARTICLE

Amperometric detection of triclosan with screen-printed carbon nanotube electrodes modified with Guinea Grass (*Panicum maximum*) peroxidase

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Abstract

Triclosan is a compound with antimicrobial activity broadly used in consumer products. Because of its well-documented toxicity, the amount of triclosan present in different products needs to be tightly controlled. This paper outlines a new amperometric sensor for triclosan detection consisting of a screen-printed carbon nanotube electrode (SPCNE) modified with Guinea grass peroxidase (GGP). The GGP-modified SPCNE was able to detect an enhanced electrochemical response of triclosan, unlike the bare SPCNE. The cyclic voltammograms of the GGP-modified SPCNE in a solution of potassium ferrocyanide showed an increase in the current values and linearity between scan rates and oxidation peak currents, suggesting a surface-controlled process. The GGP-modified SPCNE showed an excellent electrocatalytic activity to triclosan oxidation, at a redox potential of 370 mV, in the presence of hydrogen peroxide, exhibiting a linear response between 20 mM to 80 mM and a detection limit of 3 μM. This new amperometry system, based on carbon nanotubes integrated with GGP, becomes a potential tool for environmental analysis and food quality control.

Keywords: amperometric biosensor; carbon nanotubes; guinea grass peroxidase; screen printed electrodes; triclosan.

Introduction

Triclosan (TCS) is an antibacterial and antifungal molecule present in consumer products, such as toothpaste, soap, detergents, and surgical cleaners (Lenz *et al*., 2017). TCS has been reported to be a precursor of substances such as dioxins, which are extremely toxic. TCS role as an endocrine disruptor has been revealed (Ozaki *et al*., 2017). Because of accumulation and biomagnification of TCS in water, this compound can ultimately be consumed by humans. Due to its harmful effects, TCS has been considered as a high priority pollutant and its presence in household products should not exceed 0.3 % (w/w).

Different methods have been reported to detect TCS, including liquid chromatography (Guo *et al.*, 2009), chemiluminescence (Song *et al.*, 2007), and gas chromatography coupled to mass spectrometry (Shi *et al.*, 2013). Although the effectiveness and robustness of these methods are indisputable, they are expensive, complex, and time-consuming. Therefore, developing novel, straightforward, and sensitive methods for TCS detection will expedite routine chemical analyses.

Electrochemical methods are attractive (due to their simplicity, rapidity, and convenience) for the development of enzymatic biosensors to detect environmental and biomedical analytes (Rotariu *et al.*, 2016). Recently, a novel technology based on screen-printed electrodes (SPE) has been implemented in many bioanalytical applications. For example, an SPE was developed to detect TCS by cyclic voltammetry (Yang *et al.*, 2009). SPE technology offers some advantages over traditional electrodes regarding cost, solution volume, and operation temperatures.

TCS molecules contain an electrochemically and easily oxidizable phenolic group, as shown in **Fig. 1**. This phenolic group can be more readily oxidized in the presence of peroxidases (EC 1.11.1.7). Peroxidases are enzymes able to catalyze the oxidation of different amines and phenols using H_2O_2 (Sakharov *et al.*, 2003, Besharati *et al.*, 2018, Alemzadeh *et al.*, 2009). Due to its broad affinity for phenolic compounds, horseradish peroxidase (HRP) is the most commonly employed redox enzyme in the fabrication of amperometric biosensors (Wang *et al.*, 2012). However, a recent study showed that HRP exhibited a low activity on aniline oxidation, and that it is unstable at pH < 4.5 during the synthesis of polyaniline (Sakharov *et al.*, 2003). Therefore, new plant peroxidases are needed that do not have the limitations of HRP. The
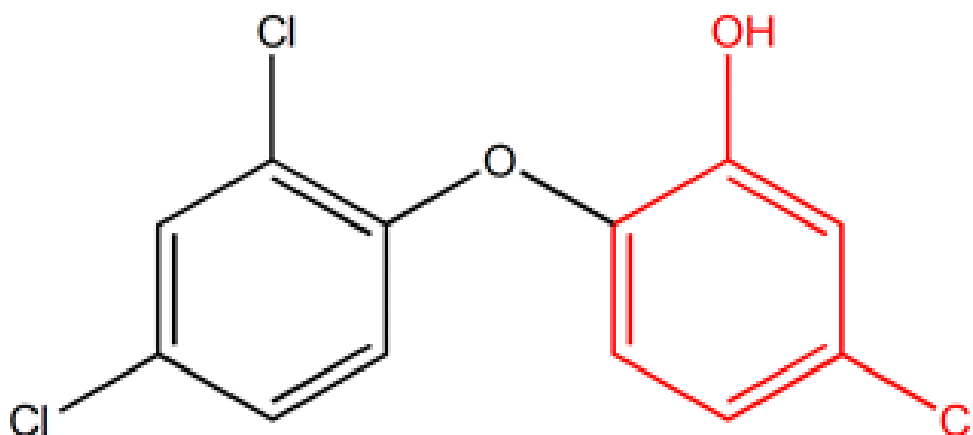

Figure 1. Molecular structure of triclosan, emphasizing its phenolic group (in red).

guinea grass (Panicum maximum) peroxidase (GGP) was recently semi-purified and characterized (Centeno *et al*., 2017). Remarkably, the GGP exhibited greater resistance to high concentrations of hydrogen peroxide and was less pH- and temperature-sensitive than commercial HRP.

Different authors have reported the use of electrochemical sensors for the determination of TCS. Screen-printed carbon electrodes have been prepared using different electroanalytical techniques and nanomaterials (Li *et al*., 2014). Dai *et al*., (2012) employed glassy carbon electrodes modified with carbon nanodots and chitosan to construct a TCS sensor, and Yang *et al*., (2009) used multiwall carbon nanotube films to fabricate an electrochemical sensor for rapid TCS detection. Recently, Wu *et al*., (2017) prepared a hybrid between palladium and graphene nanoparticles to develop an electrochemical TCS detector. An analytical method based on the effect of sodium dodecyl sulfate (SDS) on the voltammetric behavior of TCS was developed to determine TCS

traces with promising results (Fotouhi *et al*., 2010). Notwithstanding, none of these previous studies has explored the possibility of determining TCS using enzymatic sensors, nor have they considered the use of screen-printed carbon nanotube electrodes (SPCNE) modified with GGP.

Aiming at creating an analytical tool for the rapid determination of TCS in samples of environmental importance, in this work we describe the construction of an amperometric biosensor based on the modification of SPCNE with GGP using N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide (EDC) as a linking agent. We employed cyclic voltammetry and chronoamperometric methods to characterize the biosensor's electrodes, and we tested the modified SPCNE detecting H_2O_2 and TCS. Finally, we determined the biosensor's analytical parameters such as its limit of detection (LOD), linear range, and sensibility.

Materials and methods

Extraction and purification of guinea grass peroxidase

Leaves of Guinea grass (*Panicum maximum*) were collected from pastures in the region of Santander, Colombia. First, all collected leaves were cut in small pieces (5 cm) and washed in 30 mM of phosphate buffered saline (PBS), pH 8.0. The extracts obtained were treated with polyethylene glycol and ammonium sulphate ($(NH_4)_2SO_4$) in order to make a two-phase system; then, the aqueous phase containing the GGP was semi-purified by gel permeation chromatography with a Sephadex G-50 column calibrated with

a TRIS buffer 3 mM, pH 7.5. Under these conditions, the GGP extract was adsorbed on the Sephadex G-50 column and eluted with TRIS buffer. Finally, the elution containing the peroxidase was collected and concentrated by ultracentrifugation.

Enzyme activity

GGP activity was measured by UV-vis spectroscopy, as follows: 10 μL of the enzyme was added to 2.5 mL of 30 mM PBS of pH 8.0 containing 10 mM guaiacol and 4.4 mM H_2O_2 as substrates (Centeno *et al.*, 2017). The increase in absorbance values was monitored at 25 °C (guaiacol = 5 200 M $\cdot$ cm^{-1}). Activity (U) was defined as the amount of GGP that produced the modification of 1 μmole of guaiacol in one minute. The specific activity is expressed as units of activity per mg of protein. The amount of protein was determined by the Bradford method (Bradford *et al.*, 1976) as described in Centeno *et al.*, (2017).

Modification of the screen-printed carbon nanotube electrode

To modify the SPCNE, 4 μL of a 25 mM EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) solution were placed onto the electrode's surface. EDC is a carbodiimide that enables the conjugation of proteins containing carboxyl groups with amines. In the present study, EDC was used as a coupling agent to mediate the formation of links between the carboxyl group of the electrode's carbon nanotubes and the amine groups of the GGP. Next, 6 μL of GGP were added onto the EDC-modified SPCNE surface. After incubation at 4 °C for 4 hours, the modified electrode was washed with phosphate buffer to eliminate any excess of EDC or GGP. A schematic of the SPCNE modification with GGP and the electrochemical TCS detection is shown in **Suppl. 1.**

Electrochemical procedures

Electro-analytical measurements were performed with an Autolab PGSTAT101 device (Echo Chemie, Utrecht, the Netherlands) run by the NOVA 1.10.1.9 software (Metrohm, Filderstadt, Germany). Screen-printed carbon nanotube electrodes modified with carboxyl-functionalized single-walled carbon nanotubes (SPCNE, 110SWCNT) were obtained from DropSens (Oviedo, Spain). Carboxyl functional groups on the surface of the electrodes were modified by the enzyme's amine groups. These screen-printed electrodes (consistingconsisting of a working electrode of 4 mm in diameter, a silver rod pseudoreference electrode, and an auxiliary carbon electrode) were composed of single-walled carbon nanotubes. The GGP-modified SPCNE was

fitted into a methacrylate electrochemical cell of 5 mL of volume (DropSens, Oviedo, Spain). Cyclic voltammetry experiments were carried out at 27 °C in different solutions. Before each cyclic voltammetry experiment, the solutions were freed of oxygen bubbles through degasification with N_2 and magnetic stirring for 30 s.

For the chronoamperometric experiments, the potential was set at 370 mV (vs Ag) and the solution was magnet-stirred before and during the addition of 10 μL of a 5 mM TCS-H_2O_2 solution. All the applied potentials used in this study are referred to the SPCNE's internal silver pseudoreference electrode. Using the GGP-modified electrode, the effects of potentially interfering compounds, namely o-nitrophenol, ascorbic acid, and glucose, were evaluated under optimized experimental conditions. Possible changes in the current signal of two TCS test solutions (1 and 2 mM) during the sequential addition of each interfering compound were evidenced by comparing the resulting chronoamperometric response to that obtained in a TCS solution without the addition of any of these compounds.

Analysis of TCS dissolved in tap water

Three different GGP-modified SPCNEs were employed to detect TCS in different concentrations (5 μ, 12 μ, and 22 μM) in tap water samples. The potential was set at 370 mV (vs Ag), as previously determined by the cyclic voltammetry experiments.

Results and Discussion

GGP partial purification results are summarized in **Table 1**. The obtained guinea grass extract was rich in pigments that were efficiently removed by extraction in an aqueous bi-phasic system formed by PEG-$(NH_4)_2SO_4$. Subsequent Sephadex-based elution and concentration steps yielded a partially purified GGP with a specific activity of 609 U mg^{-1} of protein.

The cyclic voltammograms obtained from unmodified SPCNEs and GGP-modified SPCNEs in a potassium ferrocyanide ($K_3Fe(CN)_6$) solution are shown in **Fig. 2A**. The Cyclic voltammogram of the unmodified electrode revealed a redox process of the ferri/ferrocyanide couple. Whereas, the cyclic voltammogram plot of the GGP-modified electrode showed an increase in both anodic and cathodic current peaks (Fig 2A), suggesting that the added peroxidase is important in the electronic communication between redox species and the electrode's surface.

voltammetry analysis of the electrochemical responses of the GGP-modified SPCNE in three solutions: H_2O_2, TCS, and H_2O_2 + TCS are shown in Fig. 2B. The cyclic voltammetry plot of the GGP-modified SPCNE in the

Table 1. Partial purification of the peroxidase from guinea grass leave extract.

Step	Volume (mL)	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Initial extraction	130	60	11765	198	100	1
Pigment extraction	59	26	11660	444	98	2.2
Gel permeation chromatography	18	11	2822	249	24	1.2
Ultracentrifugation	9	4	2452	609	20	3

H_2O_2 solution (Fig. 2B, red curve) exhibited a cathodic current signal around -791 mV due to the bio-electrocatalytic reduction of H_2O_2 mediated by the peroxidase (Centeno *et al.*, 2017). The cyclic voltammetry response of the GGP-modified SPCNE in the TCS solution (Fig. 2B, black curve) revealed an oxidation current at 370 mV. This may be due to the GGP's heme group onto the electrode's surface electrocatalyzing the phenoxy group of the TCS molecules (Brusova *et al.*, 2005). For the H_2O_2 + TCS solution, the
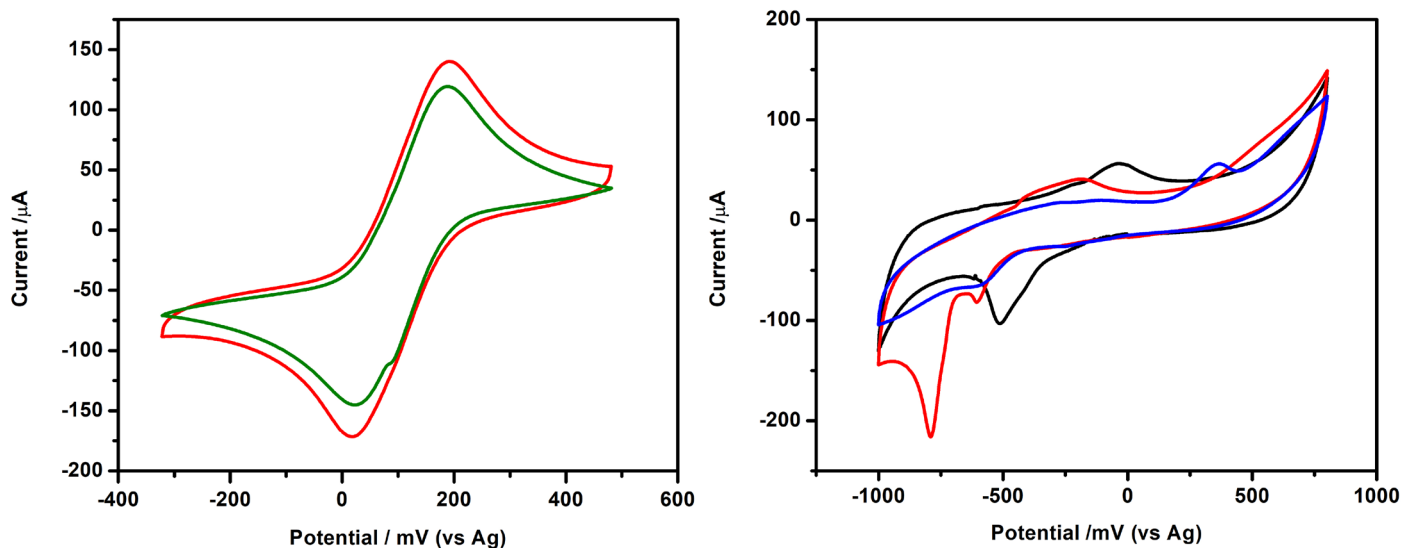

Figure 2. (**A**) Cyclic voltammograms of a bare SPCN electrode (green) and a GGP-modified SPCN electrode (red) in the presence of 10 Mm $K_3Fe(CN)_6$, scan rate 100 mVs^{-1}. (**B**) Cyclic voltammograms of a GGP-modified SPCN electrode in three different solutions: H_2O_2 5 mM (red line), TCS 1 mM (black line), and H_2O_2 5 mM + TCS 1 mM (blue line).

GGP-modified SPCNE revealed a well-defined redox peak around -31 and -427 mV (Fig, 2B; blue curve) attributed to the iron present in the GGP's heme group (Brusova *et al.*, 2005, Gaspar *et al.*, 2000). Similar studies have reported oxidation potentials close to the value obtained in this work (Wu *et al.*, 2017, Fotohui *et al.*, 2010). Based on this result, we can suggest that the carbon nanotube electrode modified with the GGP can be used as a potential bioanalytical device to detect TCS.

When the GGP-modified SPCNE was tested with 0.03 M PBS (pH 8.0) at scan rates, ranging from 40 to 150 mVs-1, both anodic and cathodic current peaks increased linear to the scan rate root, producing diffusion of redox species to the electrode (**Fig. 3**). This behavior is related with the kinetics of the reactions, suggesting a diffusion controlled and quasi-reversible process (Huang *et al.*, 2009).

pH influenced the bio electrooxidation current of TCS (**Fig. 4**). At pH < 6, the relative current decreased in approximately 60 % suggesting that the peroxidase is inactivated either by the stability of charges or by loss of its tertiary structure. In the pH range between 6 and 8, the bioelectrocatalytic activity of the GGP in the presence of TCS increased more than 50 % of the relative current, and in the pH range of 8 to 10, the increase in GGP bioelectrocatalytic activity was less pronounced. Altogether, in the pH range from 6 to 10, the enzyme exhibited a better electrocatalytic response, reaching its maximum at pH 10.
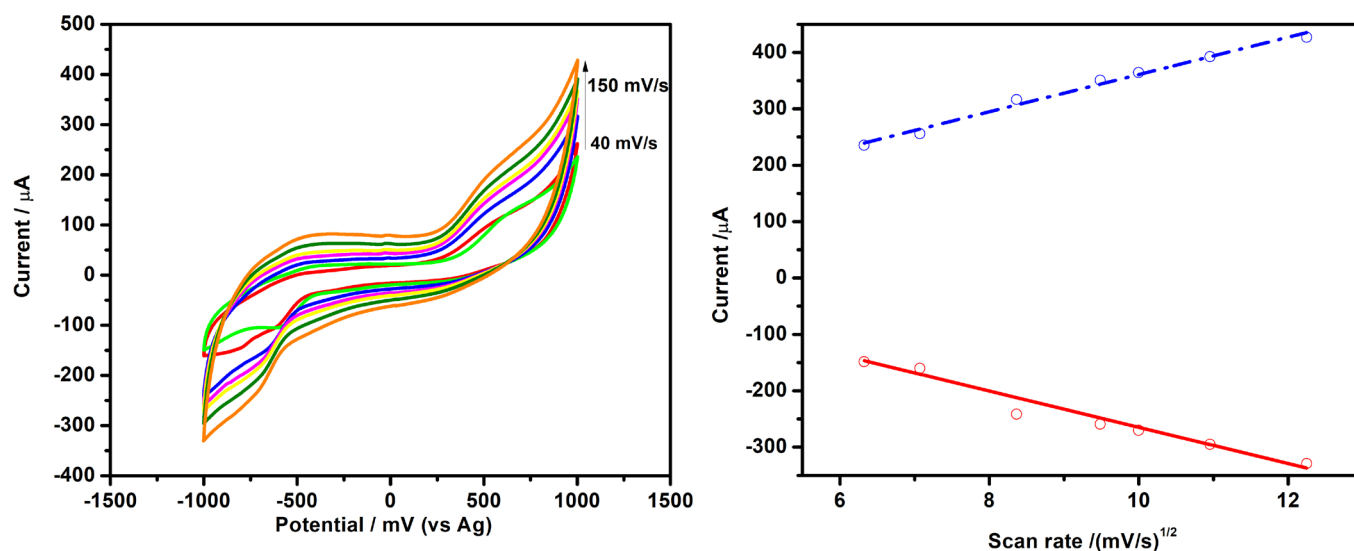

Figure 3. (A) Cyclic voltammograms of modified SPCNE at different scan rates from 40 to 150 mVs^{-1}. (B) Anodic (red dashed line) and cathodic current (blue dashed line).
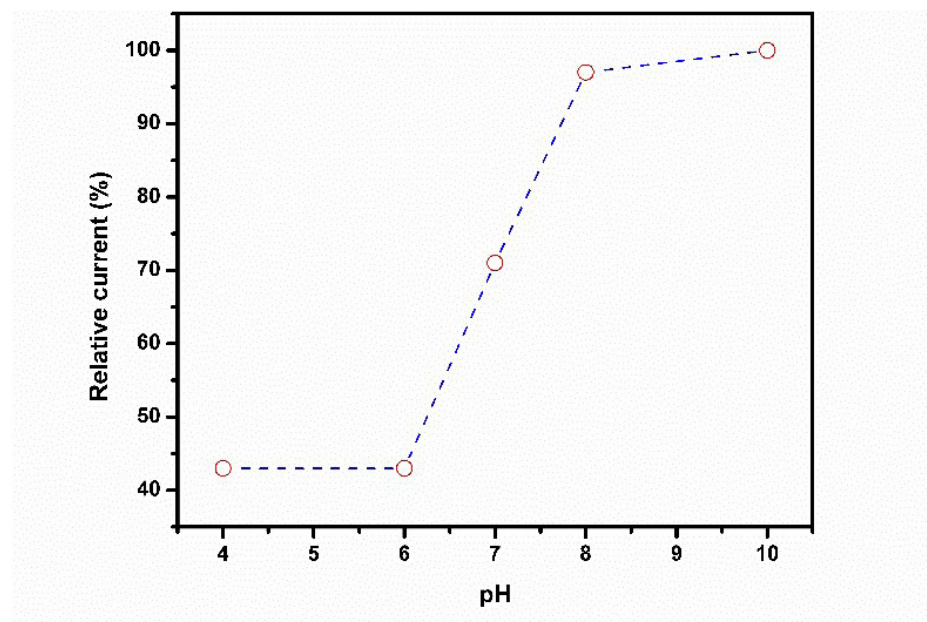
Figure 4. Effect of the pH value on the oxidation current signal of triclosan.

Peroxidases contain heme groups that mediate the single electron oxidation of phenolic compounds in the presence of hydrogen peroxide. The electron generated can migrate to the electrode's surface producing a current signal that can be used to detect TCS.

To date, no previous studies have investigated the role of peroxidases, let alone a guinea grass peroxidase, to detect TCS molecules in the presence of H_2O_2. In this study, we also carried out chronoamperometric measurements to monitor the current reduction signal of our GGP-modified electrode with different H_2O_2 concentrations. We obtained a current-time plot for our GGP biosensor after successive additions of 0.3 mM H_2O_2 to 7 mL of PBS (pH 8.0) at an applied potential of -791 mV (**Fig. 5A**). The GGP at the electrode's surface showed an electrocatalytic reduction with stable values upon peroxide addition to the PBS solution, revealing a fast electron transfer communication between the redox center of the GGP and the carbon nanotube electrode surface. Furthermore, as shown in Fig. 5B, we observed a linear response of the biosensor when the concentration of the H_2O_2 solution varied from 0.1 mM to 3.3 mM. The bottom detection limit was 99 μM (signal to noise ratio S/N = 3) with a sensitivity of 34.5 nA/M. None of the control experiments, with the electrode without GGP, at -791 mV (vs Ag) exhibited signals after the addition of H_2O_2 (Fig. 5A, red line).

The chronoamperometric measurements for the GGP-modified electrode at an applied potential of 370 mV, after successive additions of 10 μM TCS to 7 mL PBS (pH 8.0) with 5 mM H_2O_2, revealed that the oxidation current
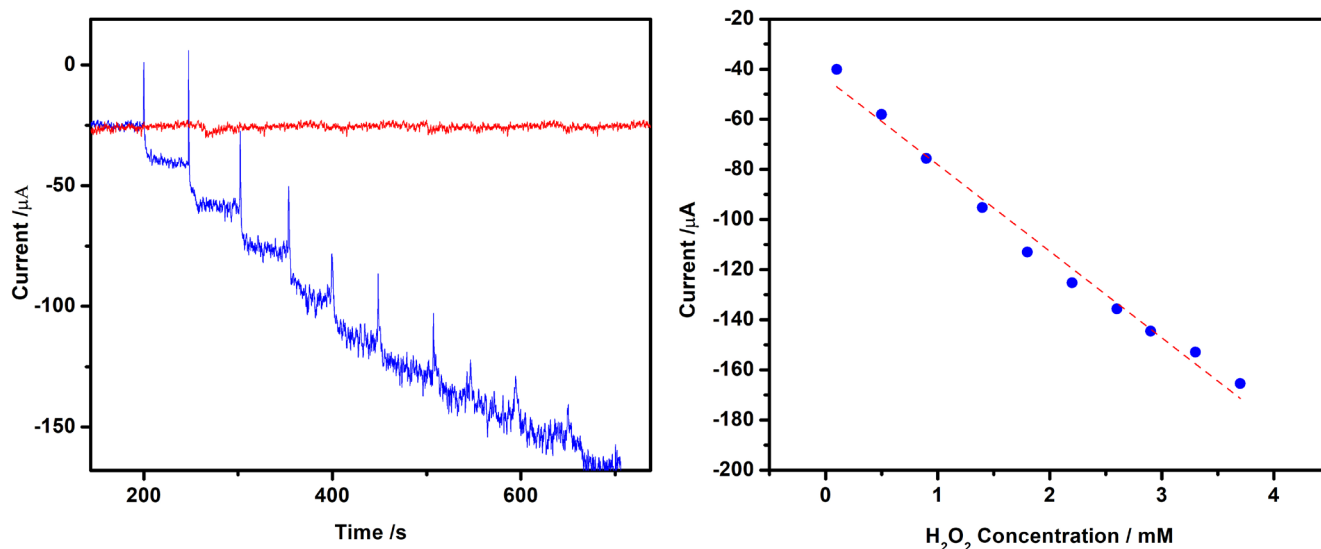
Figure 5. (**A**) Amperometric response of SPCN (red) and GGP-modified SPCN (blue) electrodes to sequential additions of 0.3 mM H_2O_2 to a 10 mM PBS pH 8.0 solution, E = -791 mV vs Ag. (**B**) Average current response of SPCNE/GGP electrode as a function of H_2O_2 concentration.

signal increased in proportion to the concentration of TCS (**Fig. 6A**). A linear behavior was obtained for the TCS concentration range of 20 μM to 80 μM, and the resulting linear regression equation was I μA) = 0.0249 (μM) + 8.43 (R^2 = 0.995) with a detection limit of 3 μM at S/N= 3 (Fig. 6B).

The stability of the GGP-modified SPCNE was evaluated with 13 continuous cyclic scans. No appreciable variation was observed in the voltammetric waves (**Suppl. 2**.). Furthermore, after storing the electrodes at 4 °C for three weeks, the GGP-modified electrode retained its bioelectrocatalytic activity, as evidenced by the values in the amperometric current signal being close to the initial current response. This long-term stability could be attributed to the strong interaction between the carbon nanotubes and the amino and carboxylic groups of the GGP.

After the sequential addition of *o*-nitrophenol, ascorbic acid, and glucose (1 mM each), no outstanding interference signals were observed on the TCS detection performance of the GGP-modified SPCNTE (**Fig. 7**). This result suggests that the tested biosensor has a high selectivity and specificity for TCS.

We evaluated our analytical application with environmental samples, immersing our GGP-modified SPCNE in three different concentrations of TCS in tap water, revealing a linear trend in current response as TCS concertation increased (Fig. 8). These results showed that satisfactory recovery
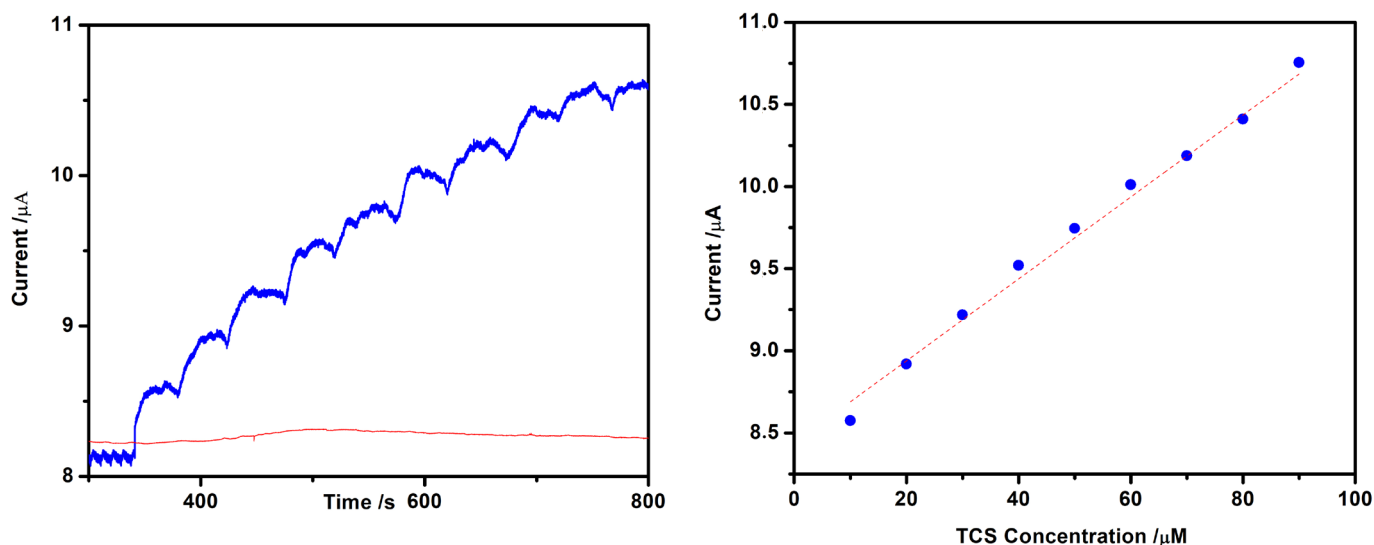
Figure 6. (A) Amperometric response of the SPCN (red) and the GGP-modified SPCN (blue) electrodes to subsequent additions of 10 μM triclosan to a 10 mM PBS (pH 8.0) + 5 mM H$_2$O$_2$ solution. E = 370 mV vs Ag. **(B)** Average current response of the GGP-modified SPCNE as a function of the TCS concentration.

of TCS could be obtained using our GGP-modified SPCNE indicating that our sensor could be used as an interesting bioanalytical tool for sensing TCS and phenol related compounds in water samples.
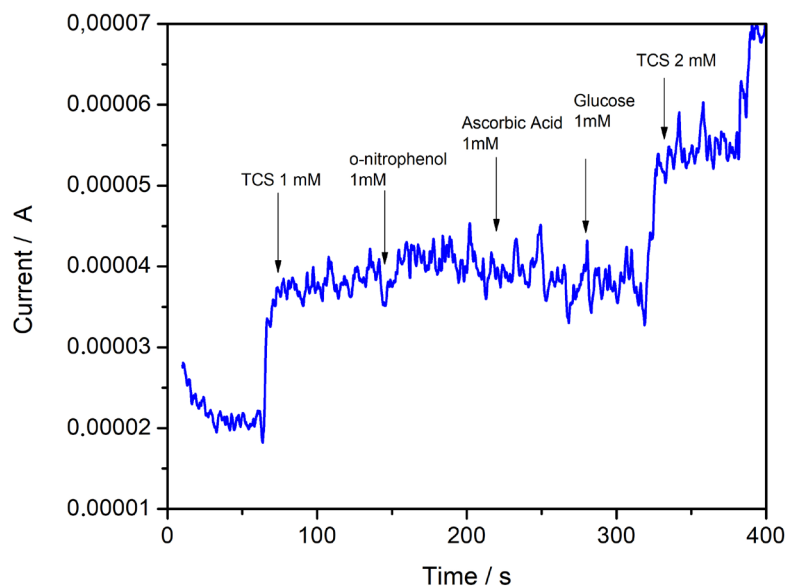

Figure 7. Chronoamperometric response of the GGP-modified SPCNE, at 370 mV, in the presence of different concentration of TCS, o-nitrophenol, ascorbic acid, and glucose.

Table 2. Electrochemical detection of TCS in tap water samples (n = 3 electrodes)

Sample No.	Current (μA)	TCS added (μM)	TCS detected (μM)	Recovery (%)
1	8.46	3	5	60
2	8.78	9	12	75
3	8.95	19	22	86

To the best of our knowledge, this is the first study that involves a plant peroxidase integrated in a carbon nanotube screen-printed electrode to detect TCS. Consequently, this GGP-modified SPCNTE is a potential bioanalytical platform for environmental samples.
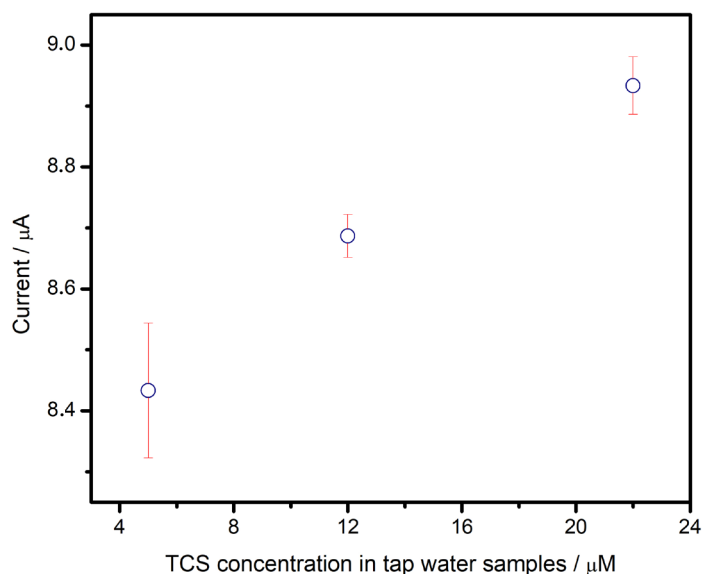
Figure 8. Current response of SPCNE/GGP electrode at 370 mV as a function of TCS concentration in tap water samples.

Conclusions

A new amperometric biosensor was developed through the modification of the electrode surface of a screen-printed carbon nanotube electrode with a highly stable peroxidase from guinea grass leaves in order to detect triclosan.

The resulting biosensor showed a swift electronic communication and a good performance towards hydrogen peroxide reduction and triclosan oxidation. This GGP-modified electrode features wide linearity, high sensitivity, and a redox potential of -791 and 370 mV. Thus, this new amperometric detection system becomes a promising and potential bioanalytical tool for the *in situ* analysis of samples of environmental interest.

Acknowledgements

Conflict of interest

The authors have no conflict of interest to declare.

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Detección amperométrica de triclosan con electrodos de nanotubos de carbono impresos en pantalla modificados con peroxidasa de Pasto Guinea (Panicum maximum)

Resumen: El triclosan es un compuesto con actividad antimicrobiana, ampliamente usado en productos para el consumidor. Por su bien documentada toxicidad, la cantidad de triclosan presente en diferentes productos debe ser estrictamente controlada. Este artículo describe un nuevo sensor amperométrico para la detección de triclosan, que consiste en un electrodo de nanotubos de carbón serigrafiado (Screen-printed carbon nanotube electrode-SPCNE) modificado con peroxidasa de pasto Guinea (GGP). A diferencia del SPCNE no modificado, el SPCNE modificado con GGP fue capaz de detectar una respuesta electroquímica mejorada del triclosan. Los voltamogramas cíclicos del SPCNE modificado con GGP en una solución de ferrocianuro de potasio mostraron un aumento en los valores de corriente y linealidad entre las velocidades de barrido y las corrientes de pico (máximas) de oxidación, lo cual sugiere un proceso de superficie controlada. El SPCNE modificado con GGP mostró una excelente actividad electrocatalítica para la oxidación del triclosan, a un potencial redox de 370 mV, en presencia de peróxido de hidrógeno, exhibiendo una respuesta lineal entre 20 y 80 mM y un límite de detección de 3 µM. Este nuevo sistema amperométrico basado en nanotubos de carbón integrados con GGP se convierte en una herramienta potencial para el análisis ambiental y el control de calidad de alimentos.

Palabras clave: Biosensor amperométrico; nanotubos de carbón; peroxidasa de pasto Guinea; electrodos impresos en pantalla; triclosan.

Resumo

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