chemosensors

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Review

Electrochemical Biosensors for the Determination of Toxic Substances Related to Food Safety Developed in South America: Mycotoxins and Herbicides

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Abstract: The goal of achieving food safety and quality has become increasingly important in relevant areas. The achievement of this objective includes a significant effort in different areas related to the production of raw materials, storage, transportation, etc. One of the central areas in the verification of food safety and food quality control is related to the analysis of food components and, in particular, possible toxic substances that they may contain. Therefore, the demand for appropriate methods for the determination of these substances is increasingly demanding. Thus, not only is accuracy and precision sought in the results of the analysis, but also the speed, simplicity and lowering of costs. In this way, electrochemical techniques and, particularly, electrochemical biosensors have emerged in recent times as good candidates to satisfy such requirements. This review summarizes the advances made in research and development centers located in South American countries related to the development of electrochemical biosensors for the determination of toxic substances present in foods, particularly mycotoxins and herbicides.

Keywords: food; safety; electrochemical biosensors; mycotoxins; herbicides

1. Introduction

Science and technology have made significant progress in recent years in the search for new methods and alternatives for the analysis of many substances in the most diverse materials, particularly those related to food safety that make up the everyday life of people [1,2]. Thus, it is possible to find in the literature different proposals, within which it can be asserted that the chromatographic methods have achieved a position of relevance [3]. In recent years, the demand for the determination of toxic substances at trace levels has gained great importance [3]. Official regulations tend to be increasingly rigorous, aiming at achieving quality in food to ensure the good health of the inhabitants of our planet [4]. Therefore, the great challenge in the development of new methods for the determination of toxic substances is aimed at achieving methodologies that, besides having high precision, accuracy, low limits of detection, etc., should be of simple construction and of low cost. A line of study that emerges as a good alternative to satisfy the requirements previously defined is that of biosensors.

Biosensors are devices that combine a biological recognition element, called the bio-receptor, and a suitable transducer, which measures the effect produced by the interaction between the substrate and the bio-receptor and transforms it into an electric signal [5]. Bio-receptor materials include enzymes, monoclonal and polyclonal antibodies, artificial binding proteins, nucleic acids, cells, tissues, etc. According to their transduction mechanism, the biosensors can be classified as optical, thermal, piezoelectric, electrochemical, etc. [6]. Thus, the literature shows a large number of proposals of the most varied designs and construction materials, including nanomaterials [7]. Biosensors based on electrochemical measurements emerge as good alternatives to the proposal previously described, with applications in different areas, which include the analysis of various substrates in foods, including toxic substances [8]. Thus, special emphasis has been placed on the development of electrochemical biosensors, given their high potential as measurement devices, for the determination of substances related to food safety and quality control [1,9–17].

Mycotoxins and herbicides are among the toxic substances of food, due to which their determination by rapid methods has attracted much attention in the last few years [3,18–24]. Mycotoxins are toxic substances that often contaminate food and raw materials. They are usually produced by fungi that are generated under certain unsuitable conditions of production, storage, transportation, etc. The presence of mycotoxins in food has severe implications for the health of humans and animals. Therefore, these substances have been the object of studies from different perspectives, and the development of rapid analytical techniques for their determination has concentrated the interest of several protagonists, among them state and private research and development centers, academia, etc.

Among mycotoxins, at present, there is no doubt that aflatoxins (AFB, AFM) are the ones that have attracted more attention by the different actors involved in the issue of food safety and food quality control. It is not a coincidence that precisely aflatoxins are the only ones that have been proven to be carcinogenic to humans [25]. For this reason, the enormous number of articles that have been published related to the development of analytical methods for the determination of AFB [26–33] and AFM1 [34–37] at trace levels is understandable. However, another important set of mycotoxins may be mentioned, regardless if their carcinogenic effect has not been categorically established for humans at present, such as ochratoxin, citrinin, sterigmatocystin and *Fusarium* mycotoxins [3]. Some recent reports related to the use of biosensors for the determination of mycotoxins that deserve sustained attention can be cited, such as those for aflatoxins [26–37], citrinin [38,39], sterigmatocystin [40–42], *Fusarium* mycotoxins [43] and ochratoxin A [44–48]. Undoubtedly, specialized agencies in developed countries have been at the forefront of these challenges. However, some attempts have also been made in centers located in emerging countries [49]. The huge number of papers related to this subject that appear daily in the world literature obscures some attempts that are made in places more unknown and far from the great centers of technology and research.

Thus, this review aims to report on results related to the development of electrochemical biosensors for the determination of mycotoxins and herbicides achieved in research centers located in South America, all in the context of food safety.

2. Mycotoxins

The mycotoxins that have been the focus of research in South America, from the point of view of the development of electrochemical biosensors to be used in food safety and quality control, are ochratoxin A, citrinin, zearalenone, zeranol, α-zearalanol and sterigmatocystin [50–54]. The results of the search in South America show that the development of electrochemical biosensors for the determination of mycotoxins focuses on two research groups in Argentina, at the Universidad Nacional de San Luis and, mainly, at the Universidad Nacional de Río Cuarto.

2.1. Ochratoxin A

Ochratoxin A (OTA) is a mycotoxin produced mainly by several species of fungi of the genera *Aspergillus* and *Penicillium*. Its molecular structure is shown in Figure 1:

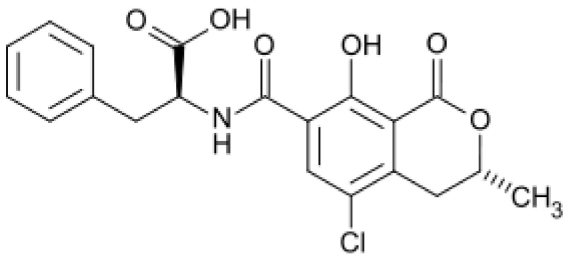


Figure 1. Molecular structure of OTA.

OTA is toxic to humans and animals through contamination of food and feed and beverages. The International Agency for Research on Cancer (IARC) has classified OTA as a Group 2B carcinogen (carcinogenic to animals and possible carcinogenic to humans) possibly by induction of oxidative DNA damage. The Commission of European Union, in the Regulation No. 1881, established that the OTA maximum permitted level in wines and fruits is 2.0 $\mu g \cdot L^{-1}$ and 0.5 $\mu g \cdot L^{-1}$ for cereal-based processed food for infants and young children. Therefore, the development of relatively fast analytical methods for OTA determination in naturally-contaminated samples is a very important challenge, when sensitivity, selectivity, precision and accuracy are required [55,56].

Researchers at the Universidad Nacional de San Luis (UNSL) (Argentina) have developed an electrochemical method using potential pulse techniques to determine OTA in wines. "An electrochemical method using square wave voltammetry (SWV) combined with modified magnetic nanoparticles (MNPs) has shown a sensitive and rapid determination of OTA in wine grapes' (Cabernet Sauvignon, Malbec and Syrah) post-harvest tissues. The wine grapes were inoculated with *Aspergillus ochraceus* to obtain OTA in artificially-infected samples. OTA was directly quantified using SWV. The current obtained is directly proportional to the concentration of OTA present in the samples. This method has been used for OTA determination in wine grapes, and it was validated against a commercial ELISA test kit. The limits of detection (LOD) obtained for electrochemical detection and the ELISA were 0.02 $g \cdot kg^{-1}$ and 1.9 $g \cdot kg^{-1}$, respectively, and the percentual coefficients of variation for accuracy and precision were below 5.5%. This method promises to be suitable for the detection and quantification of OTA in apparently healthy fruits post-harvest for assuring safety and quality of food, as well as consumers health" [57].

The literature shows several analytical methodologies for the determination of polyphenolic compounds. Among them, the first, second and third generation amperometric enzymatic biosensors represent an efficient analytical tool [5]. The biosensors can be based on the electroactivity of the bio-receptor substrate or product (first generation biosensors), utilization of redox mediators, either free in solution or immobilized with the biomolecule (second generation biosensors), or direct electron transfer between the redox-active biomolecule and the electrode surface (third generation biosensors) (Figure 2).

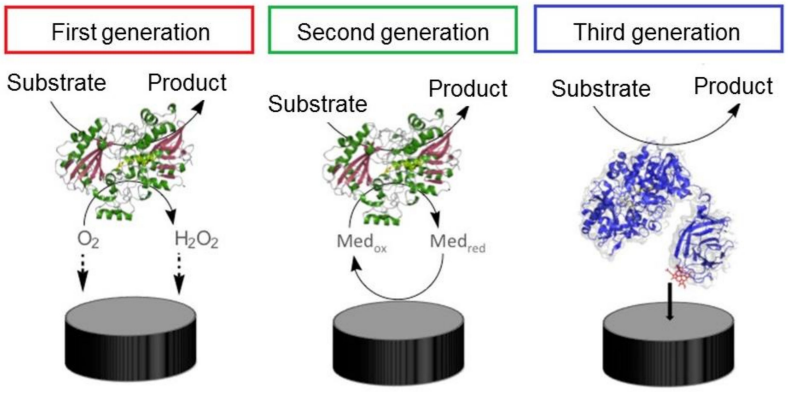
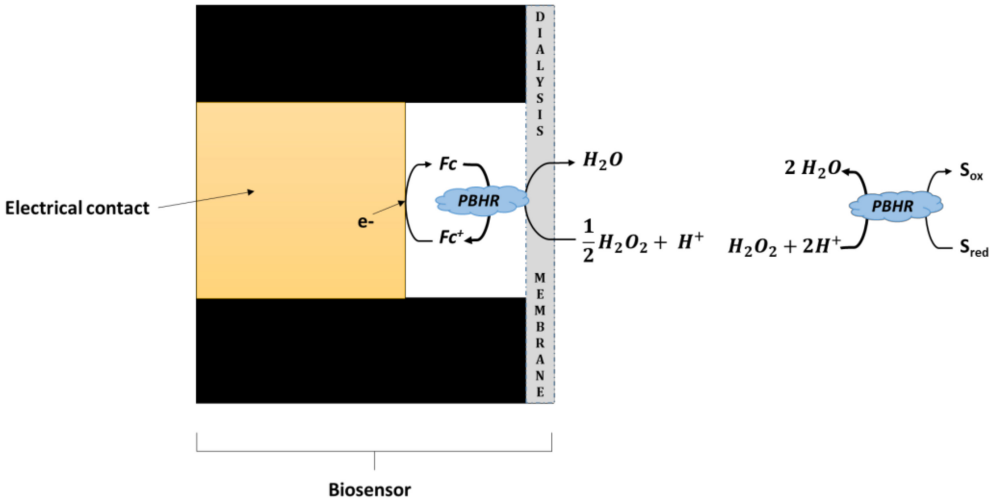


Figure 2. First, second and third generation amperometric enzymatic biosensors.

Most are based on carbon paste composites (CPC) containing some given enzyme according to the analyte to be quantified and in some cases a redox mediator. Carbon nanotubes (CNTs) of single or multiple walls, among other materials, are usually added to the carbon paste to increase, in many cases, the catalytic properties of the electrode. CNTs are suitable for the modification of different electrodes due to their high electronic conductivity and their high electrochemical stability in both non-aqueous and aqueous solutions [58–61]. In addition, the development of efficient electrochemical biosensors using CNTs-modified electrodes is very promising considering that they promote electron-transfer reactions in several molecules of biological importance [62–64]. The CPCs have been shown to serve as a basis for the construction of several electrochemical biosensors for the determination of different types of analytes [65]. A basic scheme of an electrode of this nature is shown in Scheme 1.



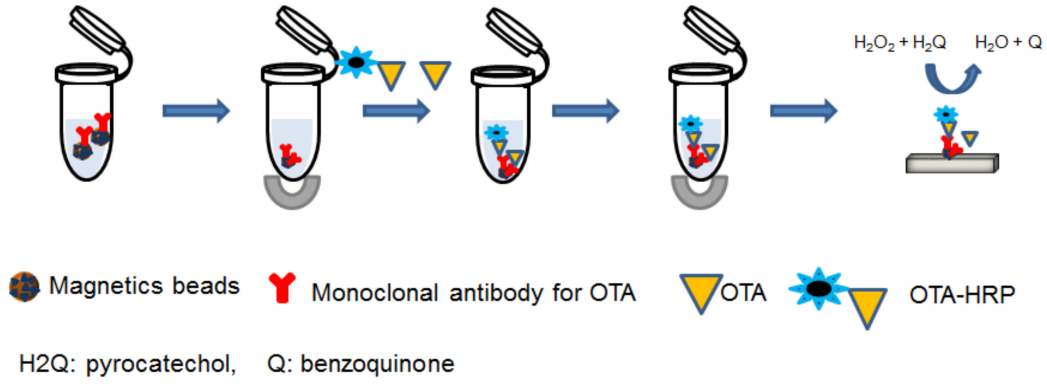
Scheme 1. General scheme of carbon paste composite (CPC) electrochemical enzymatic biosensors used to determine ochratoxin A (OTA) and citrinin (CIT) in real samples (see below). Fc: ferrocene, redox mediator. PBHR: enzyme peroxidase of *Brassica napus* hairy roots. $S_{ox/red}$: substrate.

Thus, Ramírez et al., at the Universidad Nacional de Río Cuarto (UNRC) (Argentina), proposed an amperometric biosensor based on *Brassica napus* hairy roots peroxidases (PBHR) to determine OTA. "The enzymatic reaction rate was studied under different experimental conditions, and the kinetics parameters were determined. The method employs a dialysis membrane-covered, peroxidase-entrapped and ferrocene-embedded carbon paste electrode (PBHR-Fc-CP) and is based on the decrease in the amount of H_2O_2 produced by the action of PBHR being proportional to the concentration of oxidized OTA in solution. PBHR-Fc-CP biosensors showed a good stability during at least five days. The reproducibility and the repeatability were 7.7% and 5.4%, respectively, showing a good biosensor performance. Comparison between intra- and inter-assays showed a good accuracy. The calibration curve was linear in the OTA concentration range from 1×10^{-8} to 1.4×10^{-4} mol·L^{-1}. The lowest concentration experimentally measured for a signal to noise ratio of 3:1 was 5.7×10^{-9} mol·L^{-1}" [66,67].

On the other hand, it is well known that antibodies show an excellent affinity by a specific target. The affinity constant values are very high ($\approx 1 \times 10^9$ M^{-1}) [68]. The use of antibodies is very common in clinical analysis. In the mycotoxins world, there are structural similarities between different mycotoxin families; therefore, the development of tools for selective analysis is necessary. From this point of view, antibodies appear as excellent candidates. Thus, an immunoassay methodology has been developed by Fernández-Baldo et al., characterized and applied for OTA determination. It is "comprised of magnetic nanoparticles (MNPs) as a platform for immobilizing bioactive materials incorporated into a microfluidic system for rapid and sensitive quantification of OTA in apples (Red Delicious) contaminated with *Aspergillus ochraceus*. The sensor has the potential for automation, and the detection of OTA was carried out using a competitive indirect immunoassay method based on

the use of anti-OTA monoclonal antibody immobilized on 3-aminopropyl-modified MNPs. The total assay time in the microfluidic competitive immunosensor was 16 min, and the calculated LOD was 0.05 mg·kg^{-1}. Moreover, the intra- and inter-assay percentual coefficients of variation were below 6.5%. The proposed method can be a very promising analytical tool for the determination of OTA in apparently healthy fruits post-harvest and for its application in the agricultural industry" [69].

In addition, the important interest in the development of alternative methods for the determination of OTA in matrices of importance in food safety led to the proposal of new electrochemical immunosensors for the quantification of OTA in matrices as complicated as that of red wine. Thus, investigators at the Universidad Nacional de Río Cuarto (UNRC) (Argentina) have developed an electrochemical immunosensor for the determination of OTA in red wine samples. "This immunosensor was based on protein G-functionalized magnetic beads (MBs) as the solid phase for affinity reaction between OTA and OTA monoclonal antibody (mAbOTA). The electrochemical immunoassay for OTA determination was based on a heterogeneous direct competitive immunoassay. Thus, a wine sample containing OTA with a known concentration of enzyme-labeled antigen (OTA-HRP) was used. The MBs were used as the solid support to bind the mAbOTA. OTA and OTA-HRP compete for a limited amount of OTA monoclonal antibody (mAbOTA). The HRP, in the presence of hydrogen peroxide, catalyzes the oxidation of catechol to benzoquinone, whose back electrochemical reduction was detected on a carbon screen printed-electrode (CSPE) by square wave voltammetry. A CSPE system was used as the electrochemical transduction element" (Scheme 2).



Scheme 2. Schematic representation of the electrochemical immunosensor to determine OTA.

"The experimental variables involved in the immunosensor response to OTA were evaluated. The performance obtained for the electrochemical immunosensor was an analytical range of 0.01 to 20 ppb, LOD of 0.008 ppb and IC_{50} = (0.272 ± 0.081) ppb (IC_{50}: concentration of substrate that produce 50% of the maximum inhibition). In addition, an acceptable accuracy with a coefficient of variation of 5.56% and very good recoveries (92% to 110%) was found. This work shows the potential of the electrochemical immunosensor for the direct measurement of OTA in red wine samples combining square wave voltammetry as the electroanalytical technique with the MBs and CSPE. This electrochemical immunosensor has great advantages as direct measurement of red wine samples without a pretreatment, the small volume of the sample, the short time consumption of experiments and LOD well below those established by the Regulatory Commission of the European Community (2 μg·kg^{-1})" [70,71].

2.2. Citrinin

Citrinin (CIT) is a mycotoxin isolated from filamentous fungus *Penicillium, Aspergillus* and *Monascus.* Its molecular structure is shown in Figure 3:

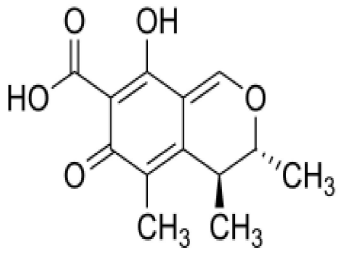
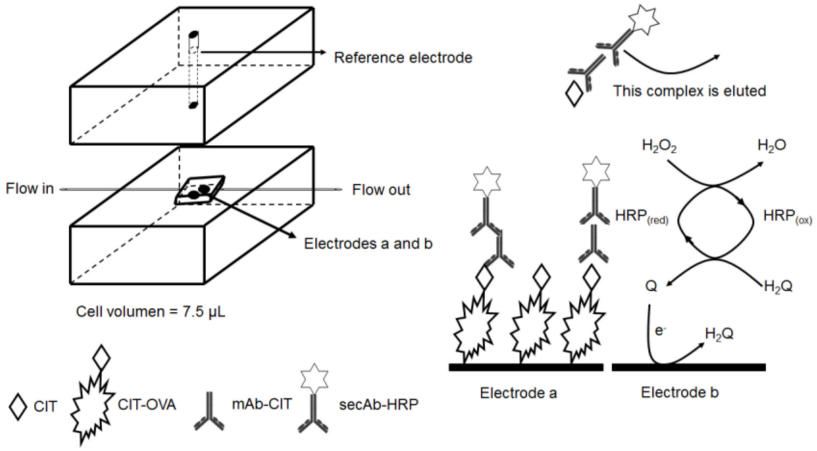


Figure 3. Molecular structure of CIT.

CIT presents antibiotic, antifungal, bacteriostatic and antiprotozoal properties. At the same time, CIT is known as a hepato-nephrotoxin in a wide range of species, and also, some studies carried out "in vitro" have demonstrated that CIT produced multiple effects on renal mitochondrial function [72]. In addition, CIT occurs frequently in foodstuffs, such as cereals, fruits, meat and cheese, and acts synergistically. The presence of OTA, CIT and aflatoxin B1 in rice samples collected from five provinces of the central region in Vietnam has recently been studied using high-performance liquid chromatography (HPLC) with fluorometric detection [72,73]. There is no particular legislation for CIT all over the world to date. The International Agency for Research on Cancer (IARC) classifies CIT in Group 3 because there is little evidence of its toxicity in experiments conducted on animals and no evidence for humans [74]. Either way, the development of new analytical devices for quantifying CIT at trace levels in food is particularly important. Recently, an immunosensor for CIT quantification has been proposed by Arévalo et al. "The development of an electrochemical immunosensor incorporated in a micro fluidic cell for quantification of CIT in rice samples was 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 HRP (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, catalyzes the oxidation of catechol, whose back electrochemical reduction was detected on a GC electrode at −0.15 V vs. Ag/AgCl by amperometric measurements (Scheme 3). 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 ng·mL^{-1} and 50 ng·mL^{-1}. The LOD and the quantification limit (LOQ) were 0.1 ng·mL^{-1} and 0.5 ng·mL^{-1}, respectively. The coefficients of variation in the 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 pretreatment" [75]. This point is very important because the electrochemical immunosensor proposed can be regenerated and re-used, which is not common in the immunosensor family. The reusability is due to the fact that the antibody anti-CIT is not directly immobilized on the electrode surface, but CIT is covalently bound onto the electrode surface via ovalbumin. "This 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 was shown to be a very useful tool to determine CIT in samples of cereals, mainly rice samples" [75].

On the other hand, an enzymatic electrochemical sensor for the determination of CIT in rice has been developed by the "Grupo de Electroanalítica" GEANA (Argentina) based on the enzyme horseradish peroxidase. "An amperometric biosensor based on horseradish peroxidase (EC1.11.1.7, H_2O_2-oxide-reductases) to determine the content of CIT in rice samples was also proposed. 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 7.0% and 3.0%, respectively, showing a very good performance. The calibration curve was linear in a concentration range from 1 nM to 11.6 nM. The LOD and LOQ were 0.25 nM and 0.75 nM, respectively. The CIT content in rice samples was also determined by fluorometric measurements. A very good correlation was obtained between the electrochemical and spectrophotometric methods" [63,76].



Scheme 3. Schematic representation of the electrochemical immunosensor to determine CIT.

2.3. Zearalenone

Zearalenone (ZEA) is a mycotoxin produced by different filamentous fungi, such as *Fusarium graminearum* Schwabe, *Fusarium culmorum* and *Fusarium crookwellense* [25]. Its molecular structure is shown in Figure 4:

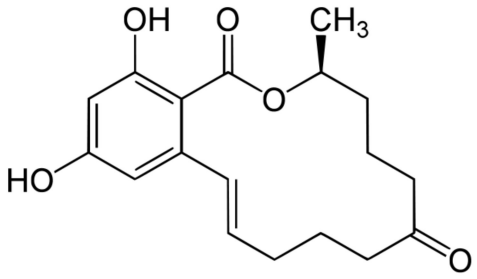


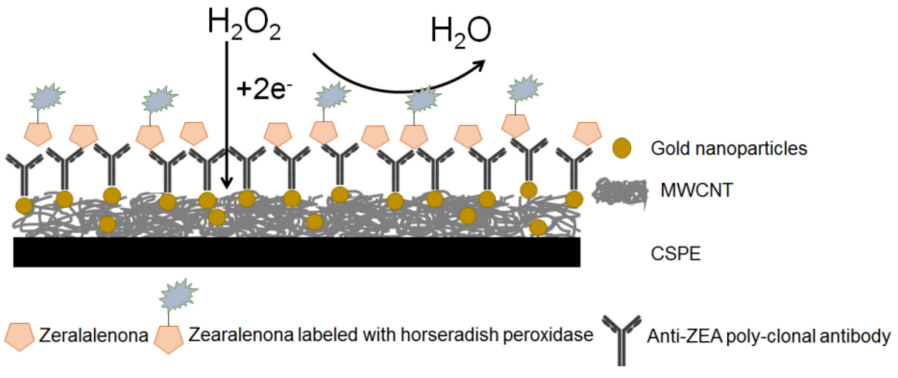
Figure 4. Molecular structure of ZEA.

ZEA is found in cereals and manufactured foods. It is one of the most distributed mycotoxins in the world, which makes ZEA study of great interest [77]. Chronic exposure to ZEA can produce reproductive disorders, estrogenism, abortions and sterility [77,78], as well as hepatotoxic, immunotoxic and hematotoxic effects [79]. Thus, the Food and Agriculture Organization of the United Nations (FAO) provides worldwide regulatory methods [80]. In Argentina, the allowed maximum limit of ZEA in maize is 200 µg kg^{-1} [81].

Panini et al. has described the design of an electrochemical immunosensor constructed from a GC electrode modified with multiwall CNT integrated with a continuous-flow system for the quantification of ZEA in corn silage samples. "Mouse monoclonal anti-ZEA antibodies were immobilized on a rotating disk. ZEA in the corn sample was allowed to compete immunologically with ZEA bound to HRP for the immobilized antibodies. HRP in the presence of hydrogen peroxide catalyzes the oxidation of 4-tert-butylcatechol, whose back electrochemical reduction was detected at −0.15 V.

The total assay time was 15 min. The electrochemical immunosensor showed higher sensitivity and lower LOD than the standard ELISA method, which shows potential for detecting ZEA in foods and feeds diagnosis" [82]. Besides, the same authors also developed a microfluidic immunoassay method for the quantification of ZEA in feedstuffs samples. "The detection of ZEA was carried out using a competitive direct immunoassay method based on the use of anti-ZEA monoclonal antibodies immobilized on magnetic microspheres 3-aminopropyl-modified manipulated for external remobilization magnets. ZEA in the feedstuffs sample was allowed to compete with ZEA HRP conjugated for the immobilized anti-ZEA antibody. The HRP, in the presence of hydrogen peroxide, catalyzes the oxidation of 4-tert-butylcatechol, whose back electrochemical reduction was detected on the gold electrode at 0.0 V. The calculated LODs for the electrochemical detection and ELISA procedure were 0.41 and 2.56 μg kg^{-1} respectively. The intra- and inter-assay percentual coefficients of variation were below 6.5%, and the total assay time was 30 min. The microfluidic immunosensor showed higher sensitivity and lower LOD than the standard ELISA method, which shows potential for detecting ZEA in foods and feeds' diagnosis" [83].

Very recently, a new electrochemical immunosensor was developed by the GEANA group (UNRC) to determine ZEA in maize samples. The electrochemical immunosensor is based on a composite material, prepared from anti-ZEA poly-clonal antibody bonded to gold nanoparticles, which was immobilized on multi-walled carbon nanotube/polyethyleneimine dispersions. CSPE was used in the electrochemical transduction stage. The immunoassay is based on a direct competitive assay between ZEA in maize samples and ZEA labeled with HRP (ZEA-HRP). ZEA determination was performed by amperometry, using an applied potential of −0.3 V. The hydrogen peroxide, which was not consumed by HRP, was reduced at the electrochemical immunosensor surface. Thus, the reduction current was found proportional to the amount of ZEA present in samples (Scheme 4). All experimental variables involved in the construction of the electrochemical immunosensor were optimized. The linear concentration range was from 1×10^{-4} ng·mL^{-1} to 1×10^{-1} ng·mL^{-1}. The LOD and IC_{50} were 1.5×10^{-4} ng·mL^{-1} and 2×10^{-3} ng·mL^{-1}, respectively. In addition, an acceptable accuracy with percentual variation coefficients between 5% and 20% and recovery percentages close to 105% were found. The electrochemical immunosensor has great advantages such as no pre-treatment of the sample being required, the sample volume is small, the experiments require short times and a very low LOD was obtained. On the other hand, the electrochemical immunosensor is disposable. Results obtained with the immunosensor were compared with those determined by HPLC-fluorescence detection, obtaining a very good correlation. The proposed immunosensor is proposed as an alternative tool to determine ZEA in maize samples [84,85].



Scheme 4. Schematic representation of the electrochemical immunosensor to determine zearalenone (ZEA). CSPE, a carbon screen printed-electrode.

2.4. Zeranol

Zeranol (ZER) is a resorcylic acid lactone derived from ZEA [86]. Its molecular structure is shown in Figure 5 [87]:

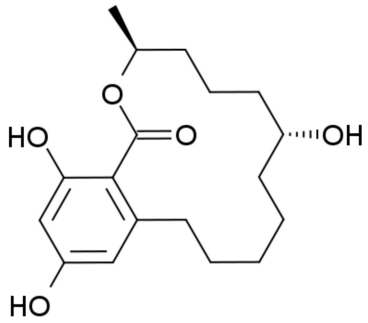


Figure 5. Molecular structure of ZER.

It has been cited that ZER is one of the compounds commonly used as a growth promoter to increase feed efficiency in livestock production. [88]. "The use of this anabolic agent in animals may leave harmful residues with a long half-life and stability in tissues intended for human consumption. These residues could present a potential risk for human health if they remain in the meat products" [89]. "Moreover, ZER is considered as an endocrine-disrupting compound due to its high estrogen receptor binding affinity and estrogenic potency" [90].

An accurate and sensitive strategy for ZER determination in bovine urine samples has been reported by Regiart et al. "ZER determination was accomplished using an electrochemical system in which bimetallic Au-Pt nanoparticles (Au–PtNPs) were electro-synthesized on a screen printed carbon electrode (SPCE). The obtained Au-PtNP platform was immunofunctionalized using specific anti-ZER antibodies as a strategy to avoid potential interference. ZER was directly oxidized and detected by square wave voltammetry after biorecognition. The Au-PtNP surface was characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD) and cyclic voltammetry (CV). The LOD calculated was 0.01 ng mL^{-1} with a wide linear range from 0.03 ng·mL^{-1} to 30 ng·mL^{-1}. This method promises to be suitable for ZER quantification in bovine urine samples ensuring food quality and safety, as well as consumers' health [91]. The same research group has recently proposed another strategy to determine ZER (also called α-zearalanol) [92]. "The sensing scheme is based on a nanocomposite consisting of gold nanoparticles electrodeposited on multi-walled carbon nanotubes that were modified with poly(vinylpyridine) through "in-situ" polymerization. The electrodeposition of the gold nanoparticles enlarges the surface available to immobilize antibodies against α-zearalanol. The nanocomposite film was characterized by scanning electron microscopy, energy dispersive X-ray spectroscopy and cyclic voltammetry. The calibration plot had a linear response in the concentration range from 0.05 ng·mL^{-1} to 50 ng·mL^{-1}, and the LOD was 16 pg·mL^{-1}. The time required for analysis was 12 min only, which compares quite favorably with the time (90 min) required by the conventional ELISA. As mentioned by the authors, the method exhibits good selectivity, stability and reproducibility for detecting ZER in the livestock production" [92].

2.5. Sterigmatocystin

Sterigmatocystin (STM) is a precursor of aflatoxin B1 in biological transformation [93]. The molecular structure of STM is shown in Figure 6:

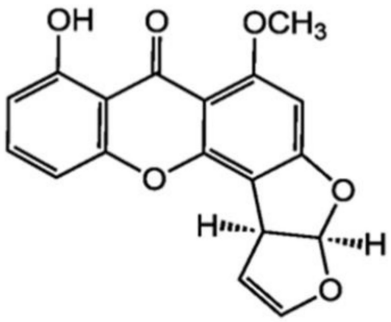


Figure 6. Molecular structure of STM.

STM is classified as a 2B carcinogen by the IARC [74,94,95]. STM is also a carcinogenic compound, which has been shown to affect several species of experimental animals [96,97]. STM causes skin tumors in experimental animals after 70 days of cutaneous application [98]. No country has legislation related to STM levels permitted in food. However, some countries set STM maximum levels allowed in some food [96].

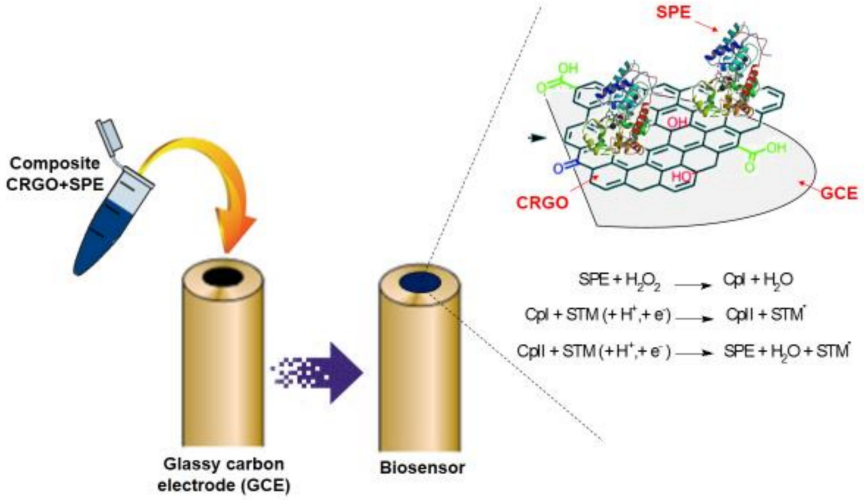
Díaz Nieto et al. have very recently developed a third generation enzymatic electrochemical biosensor to determine hydrogen peroxide [99]. This biosensor encompass the first step in the development of an enzymatic electrochemical biosensor for the determination of STM. The biosensor is based on the use of a composite obtained from soybean peroxidase enzyme (SPE) and chemically-reduced graphene oxide (CRGO) deposited on GC electrodes. Experiments were carried out in 0.1 M phosphate buffer solutions, pH 7.0. Cyclic voltammograms of the biosensor show two reduction peaks centered at about 0.15 V and −0.4 V, respectively. A quasi-reversible redox couple is defined in the region of potentials of the first peak, which is assigned to the reduction/oxidation of compound II involved in the peroxidase catalytic cycle. The surface concentration of the electroactive enzyme was $(1.1 \pm 0.2) \times 10^{-10}$ mol·cm^{-2}, representing only 7.5% of the total enzyme deposited. Values of 0.33 s^{-1} and 0.04 s^{-1} were determined for the cathodic charge transfer coefficient and the heterogeneous electron transfer rate constant, respectively. Electrochemical impedance spectroscopy was used to characterize the various stages of the electrode surface modification. Amperometric measurements were performed at a potential of −0.090 V vs. Ag/AgCl. Current responses were linear in the concentration range from 2.5×10^{-7} M to 3.0×10^{-6} M. The LOD, LOQ, reproducibility and repeatability were 5×10^{-8} mol·L^{-1}, 1.5×10^{-7} mol·L^{-1}, 9% and 4%, respectively. The biosensor was stable during five days. The Michaelis–Menten apparent constant was 1.6×10^{-6} M. The presence of uric acid, ascorbic acid, glucose and dopamine does not interfere in the determination of H_2O_2 [99,100].

On the other hand, the catalytic properties of SPE towards STM were demonstrated for the first time by UV-visible spectroscopic measurements by the GEANA group. The kinetics parameters were determined [101,102]. On the other side, the apparent acidity constant of STM was determined recording the UV-visible absorption spectra at different pHs and its value has been reported for the first time [102].

Very recently, a third-generation enzymatic biosensor was developed by Díaz Nieto et al. for the quantification of STM (Scheme 5).

It uses a composite of SPE and CRGO. The optimal conditions were determined through an experimental design of response surfaces (composite central design). The experimental measurements were performed in 0.1 mol·L^{-1} phosphate buffer solutions, pH 5. Amperometric measurements were carried out at a potential of −0.09 V vs. Ag/AgCl (3 mol·L^{-1} NaCl). The biosensor shows a linear response in the concentration range from 1.25×10^{-7} mol·L^{-1} to 5.0×10^{-7} mol·L^{-1}, and the LOD was 2×10^{-8} mol·L^{-1}. The apparent Michaelis–Menten constant ($K_{M,app}$) for the SPE adsorbed on the electrode surface was 2.2×10^{-7} mol·L^{-1} for STM. Then, STM was analyzed in maize samples contaminated "ex professo", with averages recoveries of 96%. The STM concentration determined

using the biosensor was in very good agreement with the value determined by HPLC, which shows the good analytical performance of the biosensor. The biosensor was also used to determine STM in maize samples contaminated with *Aspergillus flavus* fungus. STM biosynthesis began the first day of incubation, with a maximum production on the third day. STM production decreased in the next few days of incubation and started to increase production of aflatoxin B1 [101,102].



Scheme 5. Scheme of the preparation of a third generation enzymatic electrochemical biosensor for sterigmatocystin (STM) determination using a composite of SPE and chemically-reduced graphene oxide (CRGO) in the transduction stage.

3. Herbicides

Herbicides are widely used in agriculture and industry worldwide due to their high insecticidal activity. The presence of herbicide residues and metabolites in food, water and soil is currently one of the main problems of environmental chemistry and food safety. Herbicides, among others, are one of the most important environmental pollutants due to their increasing use in agriculture [103]. The results of the search in South America show that the development of electrochemical biosensors for the determination of herbicides is mainly carried out by researchers from Argentina, Brazil and Uruguay.

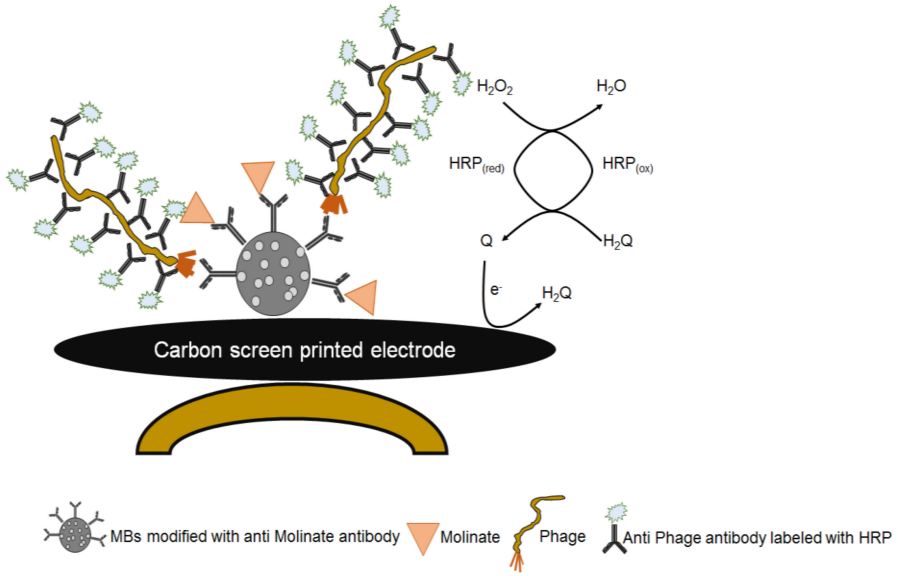
3.1. Diclofop

Franca et al. have proposed a method for the design of enzyme nanobiosensors based on a molecular modeling technique. They used this technique for the design of nanobiosensors to determine herbicides. The authors showed that "nanobiosensors can be built via functionalization of atomic force microscopy (AFM) tips with biomolecules capable of interacting with the analyte on a substrate and the detection being performed by measuring the force between the immobilized biomolecule and the analyte. It has been found that the theoretical calculations were validated through the measurements, displaying high selectivity for the family of the diclofop herbicides" [104].

3.2. Molinate

Immunosensors for small analytes have been a great contribution to the set of analytical tools due to their high analytical range and high sensitivity. The way of detecting the analyte in these systems is based on its competence with a tracer compound to bind to the detecting antibody. Recently, the use of phage particles having peptides that mimic the analyte of interest as surrogates for conventional tracers has been introduced [105]. On the other hand, the phage particles are very big, and they provide a large surface to react with labeled antibodies. This fact allows increasing the sensibility of the immunoassay. This concept has been used by Arévalo et al. for developing a magneto-electrochemical

immunosensor for the herbicide molinate (MO) [105]. A schematic representation of the immunoassay is shown in Scheme 6. "Using the same anti-molinate antibody and phage particles bearing an MO peptidomimetic, the magneto-electrochemical immunosensor performed with an IC_{50} of 0.15 ng·mL^{-1} (linear range from 0.73 × 10^{-2} ng·mL^{-1} to 10 ng·mL^{-1}) and an LOD 1400-fold better than that of the conventional ELISA. The electrochemical immunosensor (EI) produced consistent measurements and could be successfully used to assay river water samples with excellent recoveries. By using the same magneto-electrochemical immunosensor with a conventional tracer, it has been found that an important contribution to the gain in sensitivity is due to the filamentous structure of the phage (9 × 1000 nm), which works as a multi-enzymatic tracer, amplifying the competitive reaction. Since phage-borne peptidomimetics can be selected from phage display libraries in a straightforward systematic manner and their production is simple and inexpensive, they can contribute to facilitating the development of ultrasensitive biosensors" [105].



Scheme 6. Schematic representation of the electrochemical immunosensor to determine molinate (MO). MB, magnetic bead.

On the other hand, Oliveira et al. developed a biosensor by using glutathione-S-transferase (GST) to quantify molinate in environmental water. "The biosensor construction was based on GST immobilization onto a GC electrode via aminosilane-glutaraldehyde covalent attachment. The principle supporting the use of this biosensor consists of the GST inhibition process promoted by molinate. Differential pulse voltammetry was used to obtain a calibration curve for molinate concentration, ranging from 0.19 mg·L^{-1} to 7.9 mg·L^{-1} and presenting an LOD of 0.064 mg·L^{-1}. The developed biosensor is stable and reusable during 15 days. The GST-based biosensor was successfully applied to quantify molinate in rice paddy field floodwater samples. The results achieved with the developed biosensor were in accordance with those obtained by HPLC. The proposed device is suitable for screening environmental water analysis, and since no sample preparation is required, it can be used "in situ" and in real-time measurements" [106].

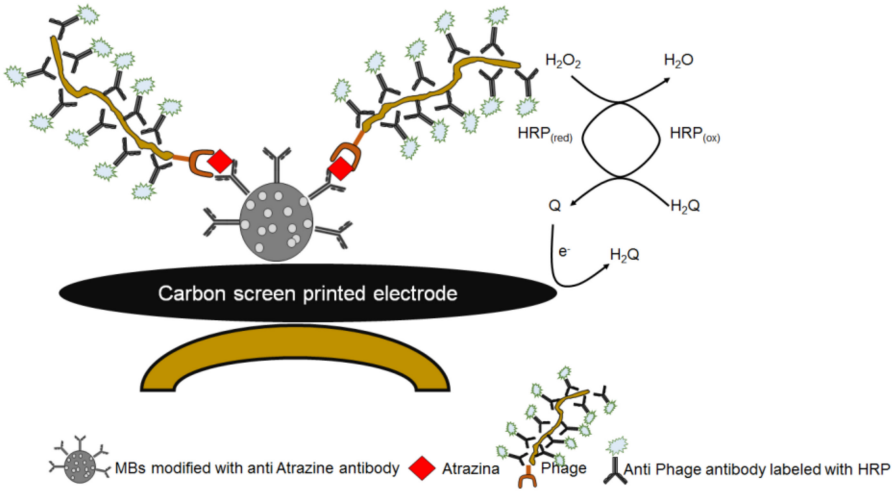
3.3. Glyphosate

Glyphosate (*N*-(phosphonomethyl)glycine) is a post-emergent herbicide used worldwide. It belongs to the chemical group of substituted glycines. It possess systemic action, and it is classified as non-selective. An excellent control of weeds is due to the broad spectrum of the action of glyphosate. Oliveira et al. proposed a biosensor based on atemoya peroxidase immobilized on modified nano clay for the determination of glyphosate by the enzyme inhibition method. "The inhibitor effect

on the biocide results in a decrease in the current response of the hydroquinone that was used as a phenolic substrate to obtain the base signal. The biosensor was constructed using graphite powder, multiwalled carbon nanotubes, peroxidase immobilized on nano clay and mineral oil. Square wave voltammetry was used for the optimization and application of the biosensor, and several parameters were investigated to determine the optimum experimental conditions. The best performance was obtained using a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0), 1.9 × 10^{-4} mol L^{-1} hydrogen peroxide, a frequency of 30 Hz, a pulse amplitude of 50 mV and a scan increment of 4 mV. The glyphosate concentration response was linear between 0.10 mg L^{-1} and 4.55 mg L^{-1} with an LOD of 30 µg L^{-1}. The average recovery of glyphosate from spiked water samples ranged from 94.9% to 108.9%. The biosensor remained stable for a period of eight weeks" [107].

3.4. Atrazine

Small compounds cannot be simultaneously recognized by two antibodies, impeding their detection by noncompetitive two-site immunoassays. It is well known that noncompetitive immunoassays are superior to competitive ones in terms of kinetics, sensitivity and working range. Recently, González-Techera et al. have proposed an interesting biosensor design that combines the advantages of magneto-electrochemical immunosensors with the improved sensitivity and direct proportional signal of noncompetitive immunoassays to develop a new phage anti-immunocomplex electrochemical immunosensor (PhAIEI) for the detection of the herbicide atrazine (AT). AT is an herbicide that is used to stop pre- and post-emergent broadleaf and grassy weeds in a variety of crops. "The noncompetitive assay is based on the use of recombinant M13 phage particles bearing a peptide that specifically recognizes the immunocomplex of AT with an anti-atrazine monoclonal antibody (Scheme 7). The PhAIEI performed with an LOD of 0.2 pg mL^{-1}, which is 900-fold better than the LOD obtained using the same antibody in an optimized conventional competitive ELISA, with a large increase in working range. The developed PhAIEI was successfully used to assay undiluted river water samples with any pretreatment and excellent recoveries. Apart from the first demonstration of the benefits of integrating phage anti-immunocomplex particles into electrochemical immunosensors, the extremely low and environmentally-relevant LOD of AT attained with the PhAEIS may have direct applicability to fast and sensitive detection of this herbicide in the environment" [108].



Scheme 7. Schematic representation of the electrochemical immunosensor to determine atrazine (AT).

On the other hand, given that anti-immunocomplex peptides can be isolated in a straightforward manner from phage display libraries resulting in non-competitive assays with improved sensitivity, the development of this type of immunoassay stands as a relevant option to boost the sensitivity of current electrochemical competitive immunosensors for the detection of small molecules.

4. Conclusions

Electrochemical immunosensors, as analytical tools, have the great advantage that they can be used in a vast repertoire of chemical substances. Antibodies can be produced for toxins such as mycotoxins and herbicides, as well as for non-toxic substances, such as proteins and other small molecules, as long as they are foreign to the immunizing species. The high affinity of the antibodies for their targets allowed the development of very sensitive and specific immunosensors. This property constitutes the great advantage that has allowed the development and potential use of these devices. However, a disadvantage shown by electrochemical immunosensors is that they are not sufficiently stable over an extended period of time, although in recent times, numerous efforts are being made with respect to ways of immobilizing the antibody on the surface of the sensor to make it more stable for reasonable periods of time. This unresolved problem has led, as a reasonable option, to the development of disposable immunosensors. Either way, achieving stability conditions is one of the current challenges on which significant efforts are being made. The goal of future investigations is the development of stable electrochemical immunosensors for long time periods.

On the other hand, electrochemical enzymatic biosensors based on plant peroxidases are also a good alternative to determine toxic substances (mycotoxins, etc.). They are easy and quick to construct, and at a low cost. With regard to enzymatic electrochemical biosensors for the determination of mycotoxins and other substances of interest in food safety, a clear challenge is to develop modified electrodes that allow one to anchor the enzyme effectively on the surface of the electrode. An interesting current option in the preparation of modified electrodes is the use of chemically-reduced graphene oxide, thus allowing the development of third generation biosensors, which avoid, on the other hand, the use of redox mediators.

It is fair to recognize that the research with a projection for the development of electrochemical biosensors for food safety application is scarce in the countries of the Southern Cone in comparison to the developed countries; humble, but significant efforts are being carried out by the present researchers of countries like Argentina, Uruguay and Brazil to make contributions that may be useful in an area of significant importance as food safety.

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