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Development of a highly sensitive xanthine oxidase-based biosensor for the determination of antioxidant capacity in Amazonian fruit samples

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ABSTRACT

The paper describes the development of an amperometric biosensor using Prussian Blue (PB) modified electrodes containing xanthine oxidase (XOD). The enzyme is immobilized by photo-polymerization into an azide-unit pendant water-soluble photopolymer (PVA-AWP). The parameters of the fabrication of the biosensor, XOD:PVA/AWP ratio, crosslinking irradiation time, and XOD charge, were optimized. Operational conditions for electrode preparation were defined as 1:2 ratio of XOD:PVA/AWP; exposure time to neon light of 30 min; pH = 7.5 at room temperature and enzymatic charge of 8 mU per electrode. The biosensors showed stable, fast, simple, selective, cost-effective and sensitive (-2.72E-8 A mol L^{-1}), with a good linear range (1.0–75 μmol L^{-1}), and respectively detection and quantification limits for antioxidants of 2.17, and 7.15 μmol L^{-1}. The applicability of this biosensor was demonstrated by in vitro analysis of gallic acid as standard antioxidant and Amazonian fruits as natural sources.

1. Introduction

Molecular oxygen is a central molecule in the cellular respiration of aerobic species [1]. During metabolism, the oxygen is reduced to water in sequential steps, generating a large number of short-lived intermediates called reactive oxygen species (ROS): superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$) [2].

ROS play an important role to protect the body against viruses and bacteria, to cell signalling, and to program cell death [3]. However, ROS can be over producted with the exposition to pollutants, tobacco, smoke, drugs, xenobiotics, radiation, and others. The excessive accumulation of ROS leads to oxidative stress which has been implicated in oxidative damage the structure of biomolecules of DNA, lipids, carbohydrates, and proteins, as well as other cellular components. Consequently, it causes numerous disorders, like mutagenesis, carcinogenesis, cardiovascular disturbances and ageing [4,5]. Antioxidants play a key role in preventing pathological levels of ROS, avoiding or slowing down the progression of these diseases [6,9].

Antioxidants are molecules with vary widely in chemical structure and mechanisms of action that can neutralize ROS forming relatively stable inactive products [7,8]. The human organism has an antioxidant defence system, formed by endogenous (enzymes, and nonenzymatic compounds) and exogenous (carotenoids, some minerals and vitamins, and polyphenols) sources [8,9]. However, the endogenous defence against antioxidants couldn't ensure rigorous control and complete protection to ROS. This fact explains the need for exogenous antioxidants from vegetables, fruit, herbs, spices, teas, nutritional supplements and pharmaceutical products [10].

In view of the protection provided by antioxidants, the use of these compounds in nutraceutical, medicinal, therapeutic, foods and cosmetic applications have been extensively reported [9,11–18]. Consequently, development of analytical tests for antioxidant capacity has received much attention. In general, spectrophotometric, electrochemical and chromatographic methods have been explored for this purpose [10,19]. In addition, in the last two decades, electrochemical biosensors have been considered an efficient alternative for measuring the antioxidant capacity in foods, presenting a higher performance to conventional assays. Among them, the biosensors based on the ability to scavenge the H_2O_2 and/or $O_2^{\bullet-}$ radicals have been developed [20]. However these methods have several disadvantages, such as high working potentials

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that allow electrochemical interferences, high enzyme amount which makes more expensive the total process, use of combined apparatus which leads to larger preparation time, non-application in real food samples and use of toxic heavy metals as electrochemical mediators [20–36].

This paper describes the development of an innovative XOD-based biosensor and the optimization of its bio-functionalization process by chemometric modelling. The developed biosensor was then applied to determine the antioxidant capacity of standards and real samples.

2. Materials and methods

2.1. Reagents and chemicals

Xanthine oxidase (XOD) of bovine milk 0.47 U protein mg^{-1} (EC 1.17.3.2), hypoxanthine (HX), and reference antioxidants were obtained from Sigma-Aldrich Corp (St. Quentin Fallavier, France). Azide-unit Pendant Water-soluble Photopolymer (PVA/AWP) was obtained from Toyo Kogyo Co. (Chiba, Japan). All reagents were of analytical grade and were used without further purification. All solutions were prepared by using 50 mM phosphate buffer pH 7.5 (K-PBS) with 10 mM KCl.

2.2. Apparatus

Amperometric measurements were performed by using a MicroAutolab III Type potentiostat (Metrohm, Netherlands) by applying -100 mV *vs*. a pseudo-reference Ag/AgCl electrode. Operational conditions: 10 mL glass cell under magnetically stirred condition (300 rpm), room temperature, in the dark, and the electro catalytic solution of 50 mM K-PBS buffer pH 7.5 containing 10 mM KCl.

2.3. Preparation of the biosensors

Screen-printed electrodes (SPE) were produced in our laboratory using a DEK 248 printing machine. The working electrode (WE) was a 4 mm graphite disk, the auxiliary electrode was a 16 mm × 1.5 mm curved line and the Ag/AgCl pseudo-reference electrode was a 5 mm × 1.5 mm straight line. The working electrode was modified with Prussian Blue (PB or ferric hexacyanoferrate).

2.3.1. Optimization of the enzyme immobilization

A homogeneous mixture containing XOD solution and PVA/AWP was prepared and 3 µL of this mixture was carefully spread on the WE surface. The electrodes were exposed to neon light at +4 °C to allow polymerization. The following parameters: XOD charge per electrode (C_{xod}), XOD:PVA/PWA ratio (R_{XP}), and polymerization time under UV exposition (T_p) were optimized for biosensor construction through Response Surface Methodology (RSM) on the Statistica software. Box-Behnken Design was used with 3 factors and 3 levels: C_{xod} (5, 8, and 10 mU/WE), R_{XP} (1:2, 1:1, 2:1), and T_p (48, 24, and 0.5 h). The statistic program constructed by 15 sets of fractional factorial experiments. .

2.4. Biosensor principle and amperometric measurements

All amperometric measurements were performed in a dark glass cell containing10 mL of K-PBS buffer magnetically stirred (300 rpm), at room temperature. The electrodes were previously tested in 10 mL of K-PBS buffer at a working potential of -100 mV *vs* Ag/AgCl, which corresponds to the reduction of H_2O_2. The initial current intensity was recorded after the current stabilization (baseline signal). Then, a 20 µL volume of 5 mM HX was successively added to the K-PBS buffer, in order to obtain increased substrate concentrations followed by the signal stabilization time of ~60 s before current record. Hence, calibration curves with increasing HX concentrations were constructed in the absence or presence of antioxidant (standards or samples), and the curve slopes (m_a) taken to further statistical evaluations.

The addition of antioxidants reduces the radical concentration (H_2O_2 and/or $O_2^{\bullet-}$), inducing the decrease of the intensity of the current and, consequently, a lower slope value is obtained (m_b). The antioxidant capacity, expressed by the inhibition of H_2O_2 and/or $O_2^{\bullet-}$ radicals, was determined by comparing the angular coefficients, according to the following equation:

$$\text{Antioxidant capacity (\%)} = 100*[1- (m_b/m_a)]$$

Antioxidant capacity can also be expressed as inhibition percent of H_2O_2 and/or $O_2^{\bullet-}$ for a given sample present in a known HX solution, as the mass equivalent of a reference antioxidant per gram of mass of the sample or extract, and even as the sample concentration necessary for 50 % signal inhibition (IC_{50}).

2.5. Analysis of real samples and reference substances

Samples of natural non-autochthon fruits (strawberry, orange, and passion fruit) and pulp of Amazonian fruits (graviola, bacuri, and murici) were purchased at the local supermarkets of São Luís city, State of Maranhão, at Northeast of Brazil. To frozen the pulps (1), nectars containing 10 % (v/v) of the depleted/chopped fruits were prepared in K-PBS buffer, whereas natural fruits (2) were transformed to a 25 % (v/v) refreshment, by mixing them to K-PBS in a 1:3 proportion of fruit/buffer.

The antioxidant capacity for each selected fruits was then determined according described in 2.4 section. Gallic acid (GA) was used as a reference antioxidant.

3. Results and discussion

3.1. Biochemical principle of the biosensor

The evaluation of the antioxidant capacity using the XOD-biosensor is based on the online monitoring of the H_2O_2 produced during oxidation process of the aqueous HX to uric acid in the presence of the enzyme XOD or by spontaneously dismutation of the $O_2^{\bullet-}$ radicals as shown in Fig. 1. The generated H_2O_2 is reduced on the polarized (-100mV *vs*. Ag/AgCl) WE surface, in presence of PB mediator, which has a well-known catalytic effect for the H_2O_2 reduction due to its peculiar chemistry structure [37]. In the presence of antioxidants, the $O_2^{\bullet-}$ radicals and/or H_2O_2 are scavenged with a decrease of the cathodic current allowing the antioxidant capacity quantification.

Electrochemical biosensors based on the ability to scavenge the H_2O_2 and/or $O_2^{\bullet-}$ radicals generated by the XOD oxidative catalyze of xanthine (XA) or HX have been developed with advantages and disadvantages inherent to its method. In general, these biosensors show differences in the antioxidant molecule interaction and electrode surface, different electrode materials and electroactive species, application in real samples, enzymatic system (bienzymatic system are commonly used with the combination of XOD and a second enzyme, generally cytochrome C/superoxide dismutase (SOD) to interact with $O_2^{\bullet-}$ radicals and produce H_2O_2), immobilization procedure and/or dissolved in solution, potential applied, number of steps, analytical performance, among others [20–36].

Campanella *et al.* [25] evaluated the antioxidant capacity to inhibit $O_2^{\bullet-}$ radicals of plant products and teas. The $O_2^{\bullet-}$ radicals were produced by XA/XOD in dissolved solution system, while the SOD enzyme was immobilized in a gel-like Kappa-carrageenan membrane in order to catalyze the $O_2^{\bullet-}$ dismutation to H_2O_2 which were oxidized in the platinum anode at +650 mV *vs* Ag/AgCl., generating an amperometric signal. Relative Standard Deviation (RSD) ≤/10 % and a Limit of detection (LOD) value about 0.1 for relative antioxidant capacity were obtained. Despite the short number of steps required and the good analytical performance, the high potential applied allowed the direct oxidation of phenolic compounds [38]. Besides that, a bi-enzymatic system was used (immobilized and in solution) which increased the

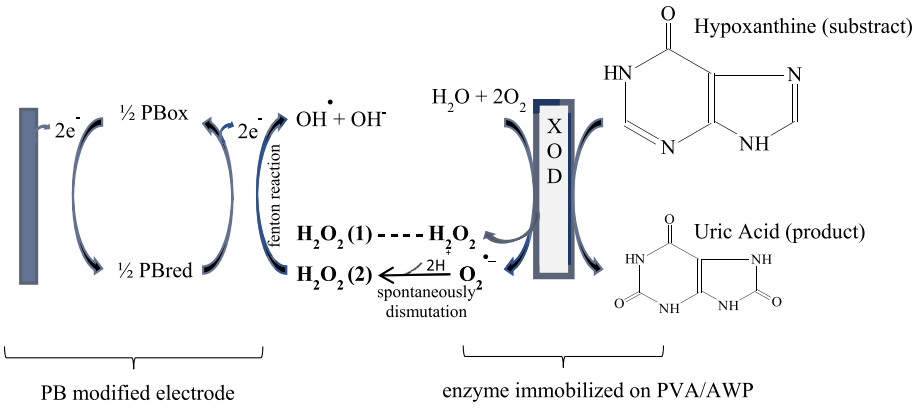

Fig. 1. Principle of detection of H_2O_2 generated by the HX/XOD enzymatic system, using the amperometric biosensor.

effective cost of its approach due to its price and the enzymes high amount necessary to dissolve in solution. In addition, the use of SOD for the generation of H_2O_2 is unnecessary once this occurs spontaneously at a fast rate and the suppression of SOD would simplify the construction and cost of the biosensor, as well as, the combined contribution measure of the antioxidant capacity against both $O_2^{\bullet-}$ and/or H_2O_2 species is more biologically interesting [39].

Lates *et al.* [20] determinate the ability of commercial beverages to scavenge both reactive species generated by the xanthine/XOD system using a bioreactor coupled with flow-through H_2O_2 amperometric biosensor at E = -100 mV *vs.* Ag/AgCl/KCl_{sat} and Os-wired horseradish peroxidase (HRP) modified WE. As immobilization procedure, the NH_2-CPG (controlled-pore glass) functionalized adsorbent coupled with glutaraldehyde crosslinking was used. LOD (2.2 mM), limit of measurement (7.5 mM), sensitivity (5 mA/M), and the linear range (up to 50 mM), were determinate. If on the one hand several advantages were showed such as the low potential applied that avoid electrochemical interferences, the simultaneous flow of antioxidant and XA subtract in the proposed bioreactor, the combined measure of the antioxidant capacity against both $O_2^{\bullet-}$ and/or H_2O_2 species, on the other hand, this approach suffers from some important shortcomings such as high cost due to the bi-enzymatic system used (XOD and HRP, to generate the oxidant species and to modify the WE, respectively), the limited binding of HRP to solid surfaces, the larger preparation steps required becoming more complicated, as well as, the expensive and toxic heavy metal used as electrochemical mediator.

3.2. Electrochemical characterization of the biosensor

Many works have already demonstrated the strong influence of modifying materials on the catalytic or electrical properties of the electrodes [40–42]. In fact, the improvement of electronic properties can lead to lower capacitive currents and better sensor sensitivity. This influence is highlighted by the electrochemical behaviour of the carbon SPE without and with PB mediator (Figs. S–1). The modification of the carbon sensor with the PB mediator significantly changed the electrical properties of the working electrode, with the cathodic peak potential of + 0.197 V *vs.* Ag/AgCl and the anodic peak of -0.073 V *vs.* Ag/AgCl

In this work, it is evident that the reduced form of PB mediator promotes a catalytic effect on the reduction of H_2O_2. and induces the reduction of the working potential to – 100 mV *vs* Ag/AgCl, a value appropriate to be applied in the subsequent electrochemical measurements. Wide linear ranges and better sensitivity are obtained in comparison with other working potentials evaluated (Fig. 2). A similar effect on linear range and sensitivity in amperometric biosensors using PB-modified SPE have been obtained at -100 mV after work potential optimization study [43,44]. However, a comparison of the performance of these is difficulted in view of the different target molecules and, therefore, the use of different substrates. It is worth noting that a

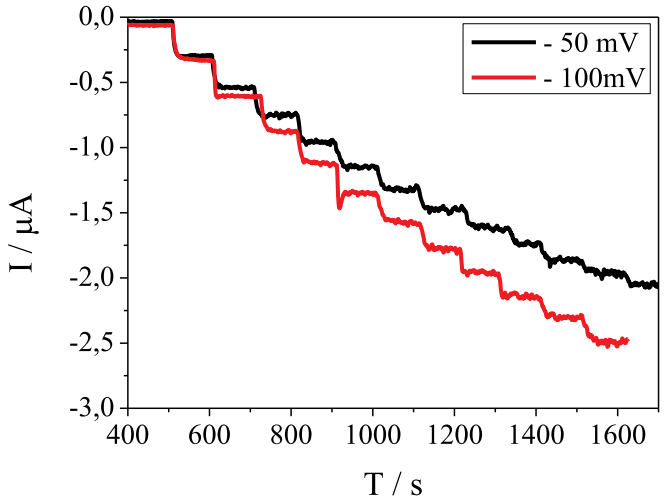

Fig. 2. Amperometric response of biosensor with the HX addition at different applied potential, and its respectively analytical performance.

potential range between 0 and -200 mV is desired, in view of the reduction of molecular oxygen (E < -200 mV) as well as the oxidation of antioxidant compounds, such as phenolic compounds and other electroactive substances which are frequently present in real sample, could also be oxidized to give interfering signals (E > 0 mV) [38].

Among the desirable effects of the Prussian Blue mediating, it is possible to include the zeolitic nature of PB with a cubic unit cell of 10.2 ˚A and with channel diameters of about 3.2 ˚A which allows the diffusion through the crystal of low molecular weight molecules (such as O_2 and H_2O_2) to its catalytic centre, excluding molecules with higher molecular weight [37,45,46]. Varvari and Popescu [47] who proposed a PB-modified amperometric sensor for antioxidant activity evaluation at -0.1 V vs. Ag/AgCl, KCl_{sat} confirmed this electrocatalytical effect for H_2O_2 reduction.

The PB selectivity and activity are comparable to those of a biological binding component but with all the advantages of an inorganic species (low cost, high stability at certain conditions, ease of electrode surface modification, no saturation effect for substrate) [37]. In general, the preparation of PB through, specially, adsorption and electrodeposition is simple, which, besides being cost-effective, is highly stable in acidic and neutral media [48–50].

3.3. Optimization of the enzyme immobilization procedure

The PVA-AWP is successfully used for the immobilization of enzymes onto the SPE surface offering several advantages such as better elasticity, low-toxicity, biocompatibility with enzymes, mechanical and

long-term stability, and biodegradability [51,52]. However, the thickness of the film, depending on the polymer concentration, and the degree of crosslinking, depending on the time of UV exposure, will affect the enzyme retention properties of the polymer and its permeability for the enzyme substrates and the products of the enzyme reaction [52].

We decided to optimize the fabrication of the biosensor in terms of the polymer concentration, irradiation time, and enzyme concentration using the chemometric method. RSM was applied to optimize C_{XOD}, R_{XP}, and T_P factors for enzyme immobilization onto PB modified WE. Box-Behnken design was used applying high, central, and low values for C_{XOD} as 5.0, 8.0, and 10.0 mU per electrode, for R_{XP} as 1:2 (0.33), 1:1 (0.50), and 2:1 (0.66) representing XOD-enzyme:PVA/AWP ratios, and for T_p as 48, 24, and 0.5 hour, respectively. 15 of the working set was generated by Statistica program and thus 15 electrodes were constructed. Each electrode was tested in 50 mM K-PBS buffer containing 10 mM KCl (pH 7.5) at a working potential of -100 mV *vs.* Ag/AgCl until steady state currents were obtained, then the net current values in 50 μM HX solution were measured.

For fitting of experimental data, linear (L) and quadratic (Q) models were tested. Significant **p**-value (< 0.05) was found in all factors to a linear model (Tables S–2), which showed 0.99887 predicted R-squared value in reasonable agreement with the adjusted R-squared value (0.99773). Also, the lack of fit **F** value of 1.792 implies the no significant value of lack of fit ($p > 0.05$) and represent validity of the linear model for explanation of experimental data of the present study which was used for further model construction.

Important results of the effects of simultaneous change of C_{XOD}, R_{XP}, and T_P are given by the Pareto Chart (Fig. 3) which shows the order and significance of each variable affecting on the current intensity response by the enzymatic reaction in the amperometric biosensor and in the RSM diagrams for depending on studied parameters (Fig. 4).

With respect to the main effect of each variable, two variables named T_P and R_{XP} affect negatively the current intensity response, where another variable named C_{XOD} affect positively the ROS production (Fig. 3). As shown in Fig. 4A, the R_{XP} and C_{XOD} variables determine the response surface. As the R_{XP} ratios decreased, the current values increased which reveal that with the same amount of enzyme higher amounts of PVA/PWA secure the crosslinking. A slight increment of C_{XOD} increases the signal. The current response increases when the T_P decreased (Fig. 4B), showing that the polymerization time significantly influences the enzyme activity. The decrease of the enzyme activity can be due to the long UV light exposition. Others T_P were tested (4.0 and 2.0 h) with similar results to 0.5 h ($p < 0.05$). For stationary T_P, the signal shows a slightly effect with the increased of C_{XOD} concentration. However, in 0.5 h there is no difference between the current signal at 8.0 and 10.0 mU XOD concentration ($p < 0.05$). As shown in Fig. 4C, as

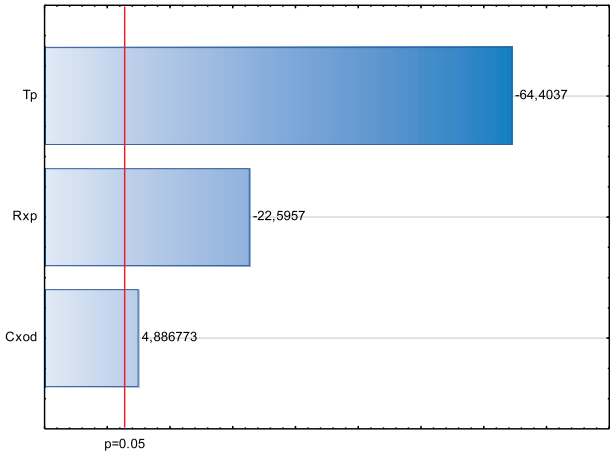

Fig. 3. Pareto chart representing the ANOVA analysis for linear components of the parameters studied. Results are significant for $p > 0.05$.

the R_{XP} ratios decreased, the current response values increase when the R_{XP} ratios decrease. In addition, as the T_P decreased the increased signal in net current values due to the maintenance of XOD enzyme activity.

The optimal enzyme immobilization conditions were chosen at low level 0.5 h and 0.33 respectively for T_P and R_{XP}, and C_{XOD} variable which exerted a slightly positive effect on ROS production was maintained at a central level (8 mU enzyme per WE) because there is no difference between a high level (10 mU). The XOD immobilization using PVA-AWP is an excellent strategy for SPE biofunctionalization in this biosensor construction and the RSM is a good method to determine the optimal conditions for enzyme immobilization.

3.4. Performance of the amperometric biosensor for antioxidant capacity screening

The performance of the XOD-PB-modified amperometric biosensor is shown in Tables S–3. The precision, in the absence of antioxidants, was evaluated with 10 μM HX for ten (10) successive assays [53]. RSD was 5.27 %, showing the good reproducibility of the tool proposed.

The detection and quantification limits were calculated by the ratio of the average of ten (10) determinations of the standard deviation of the 10 blanks measurements and the slope of the analytical curve (m), multiplied by factor 3 and 10, respectively [43].

Once prepared and kept in the conditions at 4°C and protected from light, the XOD-PB-modified electrodes shows a shelf life at least 2 days.

XOD-PB-modified electrodes presented operational stability for at least 6 hours if used in continue. Once swollen, the biosensor can be washed with K-PBS buffer and reuse, taking around of 8 min to current stabilize to new measurements. Fig. S-4 shows the range of HX concentrations for which the biosensor response changes linearly with the concentration (R^2: 0.997), but a non-linear adjustment can also be performed, thus increasing the range of analyses.

To achieve reliable results, quality control is required during all steps of the analysis including data processing and analysis. Fig. 5 shows such a control chart to 10 measurements of 10 μM HX in the antioxidant absence which is an effective method of quality control to ensure there are no changes to a process over time.

The biosensor exhibited good analytical performance for sensitivity, reproducibility, fast response time, low cost, good shelf life, signal stability, easy automation, the simplicity of operation and manufacturing.

Besides that, this biosensor presented advantages in relation to the biological relevance, once it measures the combined antioxidant capacity against the reactive oxygen species which are also produced by human body and not artificial radicals such as ABTS and DPPH used in conventional assays.

The influence of natural interferents (GA, ascorbic acid, and quercetin), as well as compounds involved in the detection scheme (HX) were evaluated. In this way, cyclic voltammetry measurements using the biosensor proposed was performed in absence or in presence of these compounds and the results showed that at the applied potential selected for amperometric detection (-100 mV *vs.* Ag/AgCl), no interferences were observed from neither in the substances usually found in food nor in those involved in the detection principle. To due the PB selectivity, the enzymatic oxidation of HX and the amperometric detection of H_2O_2 and/or $O_2^{\bullet-}$ at low potential practically assures the absence of interferences.

3.5. Determination of the GA antioxidant capacity

The developed biosensor was applied to the determination of the antioxidant capacity of GA, which is a strong reducing molecule found in food and herbs of great medical, pharmaceutical, and food technology interest. Elsewhere, GA is very often used as a reference. The addition of GA resulted in a decrease of the electrochemical signal which is proportional to its concentration. GA molecules and other

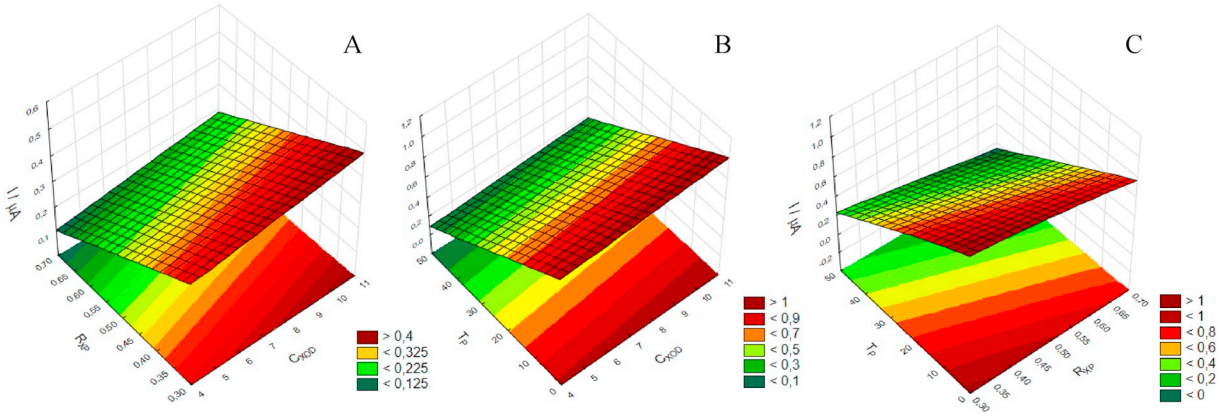

Fig. 4. Surface diagram for the current response depending on R_{XP} and C_{XOD} (A), T_P and C_{XOD} (B), T_P and R_{XP} (C).

antioxidant species react with $O_2^{\bullet-}$ radicals and/or H_2O_2, inducing a decrease in the amount of produced H_2O_2 and a subsequent decrease in the oxidation current, that allows the quantification of antioxidant capacity. Fig. 6 shows the calibration curve of the biosensor for GA in different concentration and its antioxidant capacity (%).

3.6. Application of the biosensor to the detection of the antioxidant capacity of real samples

We determined the antioxidant capacity of fresh and frozen fruit following the protocols detailed in Sections 2.4 and 2.5 (Fig. 7).

The graviola, bacuri, and murici nectar samples showed 55.9, 53.7, and 59.9 % of scavenging potential (Fig. 7B), which display a statistical similarity antioxidant capacity among those ($p < 0.05$). Bacuri and murici are Brazilian native fruit and murici is a cultivate fruit which is used by the local population as medical and food, being consumed in natura, as well as to produce juice, sweets, and ice cream.

The refreshment fruit samples results showed the strawberry as the highest antioxidant capacity refreshment fruit samples (93.0 %), followed by passion fruit (82.0 %), and orange (37.9 %), considering the inhibition of the observed slope (Fig. 7A). We have to recalculate the anti-oxidant capacity taking into account the method of preparation of the samples. The antioxidant capacities of refreshment fruit samples were respectively 37.2, 32.8 and 15.1 % for strawberry, passion fruit and orange (Fig. 7B). The highest anti-oxidant capacity was obtained for the Amazonian fruits. The lowest antioxidant capacity was obtained with the natura orange fruit.

4. Conclusion

In this paper, an enzymatic biosensor based on the XOD-PB-modified electrode was developed for the detection of the antioxidant capacity of natural or processed vegetal samples. The immobilization strategy of the XOD enzyme using PVA-AWP proved to be very efficient. The construction of the biosensor has been chemometrically optimized. The operational conditions lead to the reduction of electrochemical interferences and to a better sensitivity and precision, as well as reducing the time of analysis and quantities of reagents

The prototype also showed simplicity in construction and operation, low cost, stability of the generated signal, easy automation, good period of continuous operation and reuse, besides high capacity of portability.

A number of advantages is observed in our biosensor in comparison with previous biosensors for antioxidant activity, such as the low working potential that reduces electrochemical interference, the small amount of enzyme of the monoenzymatic system that reduces the total process and the use of PB as a mediator that provides selectivity and activity comparable to those of a biological binding component but with all the advantages of an inorganic species.

It is also worth noting the biological relevance of this tool in comparison to other tests, since, by using the biosensor, the antioxidant

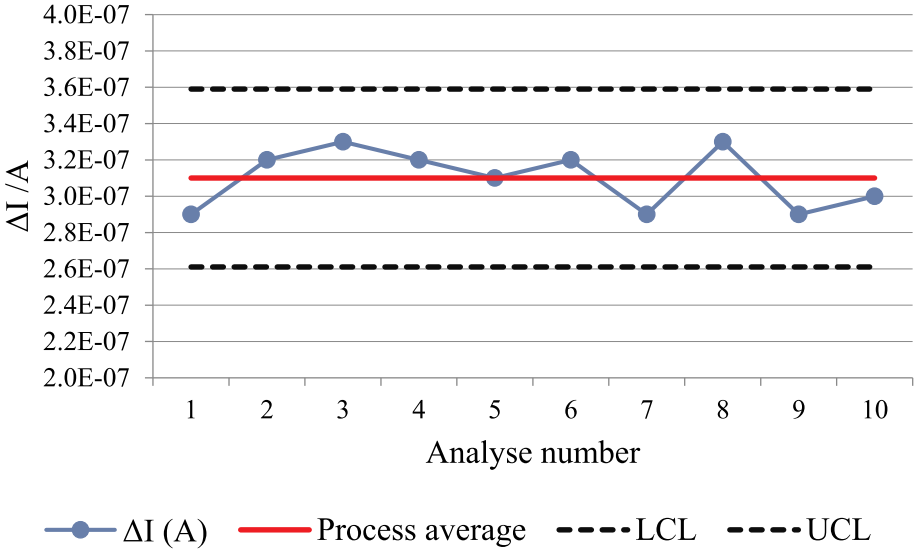

Fig. 5. In-house created control chart for H_2O_2 generation. The points are measurements and show that the sensor has not changed over many measurements. The dashed lines are 3 times the standard deviation to upper control limit (UCL) and lower control limit (LCL), and the solid line is the intensity current variation average obtained.

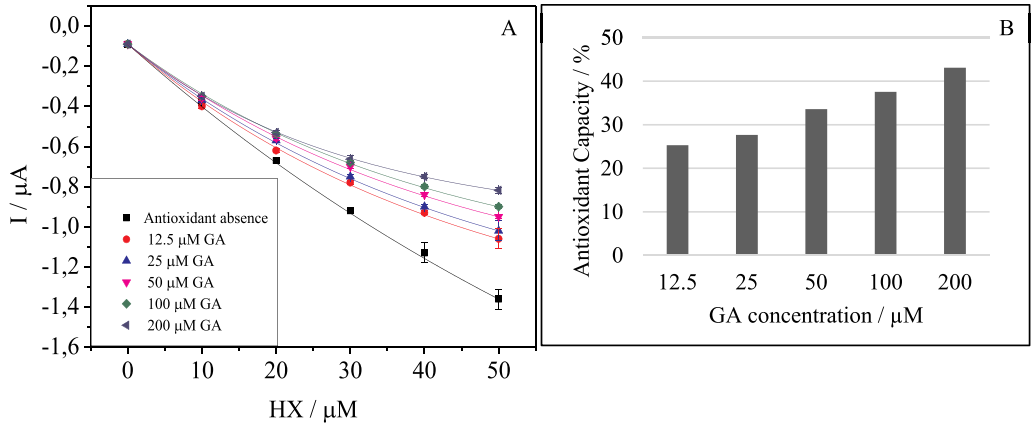

Fig. 6. Amperometric biosensor response in function of successive additions of 20 µL of 5 mM HX in the absence and presence of GA (A) and its antioxidant capacity correspondent, expressed in percentage (B).

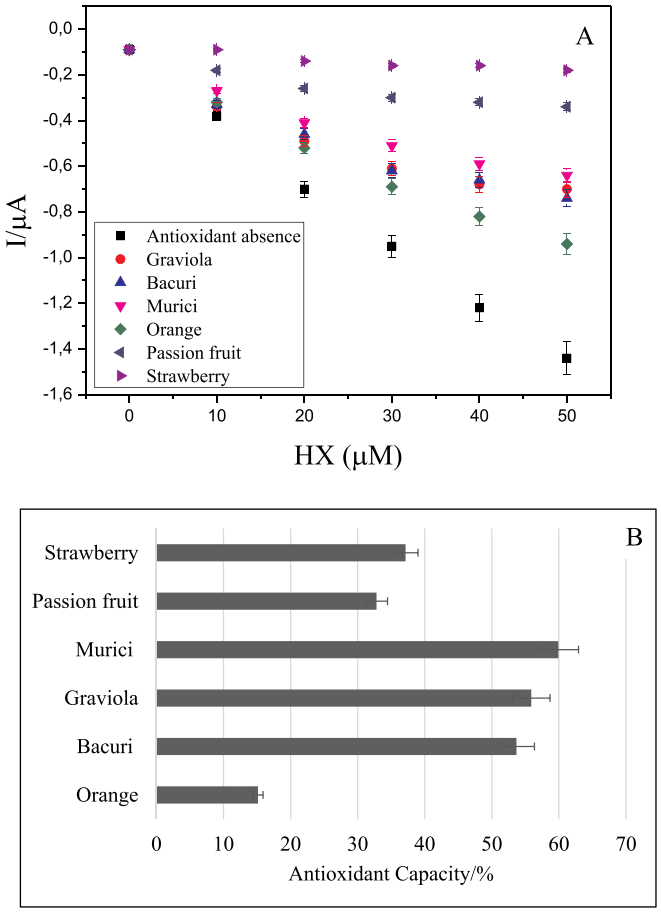

Fig. 7. Amperometric biosensor response in function of successive additions of 20 µL of 5 mM HX in the absence and presence of nectar and refreshment fruit (A) and its antioxidant capacity in percentage to 10 % v/v samples (B).

capacity of samples against the combined ROs ($O_2^{\bullet-}$ and/or H_2O_2) is determined, which are present in biological systems and exhibit cytotoxicity under conditions of oxidative stress. The bioelectroanalytical parameters, combined with the simplicity of its construction, make the proposed biosensor a promising tool for the determination of antioxidant capacity in natural or processed vegetable samples.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.06.002.

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