

Artigo

Development of Electrochemical HRP-MWCNT-Based Screen-Printed Biosensor for the Determination of Phenolic Compounds in Effluent from Washing Coffee Beans

Duarte, R. R.; Giarola, J. F.; da Silva, D.N.; Saczk, A. A.; Tarley, C. R. T.; Ribeiro, E. S.; Pereira, A. C.*

Rev. Virtual Quim., 2021, 13 (1), no prelo. Data de publicação na Web: 10 de Novembro de 2020<http://rvq.sbq.org.br>**Desenvolvimento de um Biossensor Eletroquímico Baseado em HRP-MWCNT para Determinação de Compostos Fenólicos em Efluentes da Lavagem de Grãos de Café**

Resumo: O presente estudo demonstra o desenvolvimento de um biossensor descartável impresso à base de HRP (peroxidase de rábano silvestre) – MWCNT (nanotubo de carbono de paredes múltiplas) para a determinação de compostos fenólicos em efluentes da lavagem de grãos de café. A imobilização enzimática na superfície do MWCNT foi realizada por adição covalente de glutaraldeído reticulado com BSA (albumina sérica bovina) e o desempenho do método eletroquímico foi comparado com o método de referência (Folin-Denis). O nanocompósito HRP-MWCNT foi caracterizado por Microscopia Eletrônica de Varredura (MEV) e Espectroscopia de Infravermelho por Transformada de Fourier (FT-IR). Parâmetros experimentais incluindo pH, concentração de tampão, concentração de H₂O₂, quantidade de enzima, BSA e glutaraldeído foram otimizados. As melhores condições para a preparação do biossensor descartável à base de HRP foram: 25 µL de solução de HRP (53 U mg⁻¹ sólido em 100 µL de água), 1.0 mg de BSA e 5 µL de glutaraldeído (5% v/v). O método proposto apresentou sensibilidade de 0,283 A L mol⁻¹ com uma faixa de resposta linear de 20 a 32,5 µmol L⁻¹, limites de detecção e quantificação de 0,16 µmol L⁻¹ e 0,47 µmol L⁻¹, respectivamente, usando a voltametria de onda quadrada. Os resultados revelaram que o biossensor apresentou um bom desempenho na determinação de compostos fenólicos, quando comparado a outros métodos descritos na literatura. O biossensor descartável proposto foi aplicado com sucesso na determinação de compostos fenólicos em amostras de águas residuais da lavagem de grãos de café, mostrando potencial aplicação no controle de qualidade de efluentes.

Palavras-chave: Biossensor impresso; compostos fenólicos; nanotubo de carbono de paredes múltiplas; peroxidase.

Abstract

The present study demonstrates the development of an electrochemical HRP (horseradish peroxidase) - MWCNT (multiwalled carbon nanotube) based screen-printed disposable biosensor for the determination of phenolic compounds in effluent from washing coffee beans. The enzyme immobilization onto the surface of MWCNT was performed by covalent addition of glutaraldehyde cross-linked with BSA (bovine serum albumin) the performance of electrochemical method was compared with the reference method (Folin-Denis). The nanocomposite HRP-MWCNT was characterized by Scanning Electron Microscopy (SEM) and Spectroscopy Fourier Transform Infrared (FT-IR). Experimental parameters including pH, buffer concentration, H₂O₂ concentration, amount of enzyme, BSA and glutaraldehyde have been optimized. The best conditions for the preparation of the disposable biosensor based HRP were: 25 µL solution of HRP (53 U mg⁻¹ solid in 100 µL of water), 1.0 mg of BSA and 5 µL of glutaraldehyde (5% v/v). The proposed method had a sensitivity of 0.283 A L mol⁻¹ with a linear response range from 20 to 32.5 µmol L⁻¹, limits of detection and quantification of 0.16 µmol L⁻¹ and 0.47 µmol L⁻¹, respectively, using the square wave voltammetry. The results revealed that the biosensor had a good performance for the determination of phenolic compounds, when compared to other methods described in the literature. The proposed disposable biosensor was successfully applied for the determination of phenolic compounds in samples of wastewater from washing coffee beans, showing potential application in quality control of effluents.

Keywords: Screen-printed biosensor; phenolic compounds; multiwalled carbon nanotube; peroxidase.

* Universidade Federal de São João del Rei, Campus Dom Bosco, Departamento de Ciências Naturais, Praça Dom Helvécio 74, Fábricas, CEP 36301-160, São João del Rei-MG, Brasil.

Development of Electrochemical HRP-MWCNT-Based Screen-Printed Biosensor for the Determination of Phenolic Compounds in Effluent from Washing Coffee Beans

Raquel Reis Duarte,^a Juliana de Fátima Giarola,^a Daniela Nunes da Silva,^a Adelir Aparecida Saczk,^b César Ricardo Teixeira Tarley,^c Emerson Schwingel Ribeiro,^d Arnaldo César Pereira^{a,*} 

^a Universidade Federal de São João del Rei, Campus Dom Bosco, Departamento de Ciências Naturais, Praça Dom Helvécio 74, Fábricas, CEP 36301-160, São João del Rei-MG, Brasil.

^b Universidade Federal de Lavras, Campus Universitário, Departamento de Química, Centro, CEP 37200-000, Lavras-MG, Brasil.

^c Universidade Estadual de Londrina, Centro de Ciências Exatas, Departamento de Química, Rodovia Celso Garcia Cid PR 445 Km 380, CEP 86050-482, Londrina-PR, Brasil.

^d Universidade Federal do Rio de Janeiro, Instituto de Química, Departamento de Química Inorgânica, CEP 21941-909, Rio de Janeiro-RJ, Brasil.

*arnaldocsp@yahoo.com.br

Recebido em 13 de Maio de 2020. Aceito para publicação em 7 de Outubro de 2020.

1. Introduction

2. Experimental

2.1. Reagents and solution

2.2. Instrumentation and electrochemical characterization

2.3. Enzyme immobilization onto the surface of multi-walled carbon nanotube by covalent crosslinking

2.4. Preparation of the biosensor based on the disposable electrode

2.5. Evaluation of experimental parameters

2.6. Evaluation of the operational parameters of electrochemical techniques employing disposable electrode

2.7. Determination of total phenolic compounds

2.8. Application in samples

3. Results and Discussions

3.1. Composite characterization

3.2. Optimization of the biosensor for catechol

3.3. Evaluation of the experimental parameters

3.4. Evaluation of the operating parameters of different electroanalytical techniques employing the disposable electrode

3.5. Application of the method in a sample of environmental interest using the disposable biosensor

4. Conclusions

1. Introduction

Monitoring of phenolic compounds in the food industry and in environmental and biomedical analyses, by using portable, cost-effective devices, has become an area of growing interest over the past decade. This because some phenols are ubiquitous pollutants that come to natural waters from the effluents of chemical industrial activities, such as coal refineries, pharmaceuticals, production of resins, paints, textiles, petrochemicals, and pulp, including the manufacturing of phenol.^{1,2}

Consequently, aquatic organisms, including fish, are subjected to these pollutants, and, due to their toxicity, some phenolic compounds are subject to regulation as water pollutants. Thus, both the European Commission (EC) and the US Environmental Protection Agency (US EPA) have created lists and classifications to prioritize hazardous substances for their monitoring in drinking or natural waters, and, among them, special attention has been devoted to phenolic compounds (e.g., polycyclic aromatic hydrocarbons (PAHs) and organophosphates). Of particular concern are emerging pollutants, mainly those occurring in phenolic compounds, with endocrine-disrupting activity, and those represented by chlorophenols and their derivatives.³

In this context of generation of emerging contaminants due to several activities, such as agricultural, the Brazil faces serious problems with regard to the final destination of the various types of residues generated in wet processing of coffee fruits, such as liquids (washing water, pulp and demucilage) and solids (wet pulp and bark).⁴ One of the residues generated in large amounts during wet processing of coffee, the bark, contains compounds such as tannins, caffeine and phenolic compounds which limit its application, characterizing a serious disposal problem in coffee producing countries.^{5,6}

In the work described by Bolanões *et al.* using this type of matrix (wastewater) were found high content of total phenolic compounds (0.5 mg L^{-1}), which exceed the limits established by CONAMA (Brazilian organ responsible for adopting measures regarding the national environment system) and may affect the activity of the microorganisms in a biological treatment, thus justifying the need for analytical methods capable of quantifying these compounds quickly and accurately.⁷

Although spectrophotometric and chromatographic techniques are the most common methods for the determination of phenolic compounds, capable of identifying and quantifying them with great accuracy, a wide variety of pollutants exist at trace levels, and the most recent research in monitoring techniques is mainly focused on analytical tools, such as sensors and biosensors, which offer advantages over classical analytical techniques in terms of selectivity, sensitivity, short assay times, and reduced cost of analysis.⁸⁻¹¹ The use of enzymatic biosensors has increased over time, due to their specific and peculiar properties. Some biosensor research has been carried out on the detection of phenolic compounds based on enzymes, such as tyrosinase and horseradish peroxidase (HRP). The HRP is a important redox mediator for the reduction of H_2O_2 as electrons relay due to the intrinsic selectivity and intensification of the enzymatic reaction.¹²

The immobilization of enzymes on electrodic materials surface provides greater stability and protection from the environment different from that behavior observed in bulk solution. The literature present immobilization methods commom such as cross-link bonding, covalent attachment, polymer inclusion and sample adsorption.¹³ The immobilization of an enzyme entails interactions the enzyme and the carrier. Therefore, carriers with high surface area and chemically and mechanically stable are preferred to allow a higher enzyme loading and a better efficiency of immobilization.¹⁴

Carbon nanotubes (CNTs) paste enzyme electrodes have been widely prepared by mixing CNTs with mineral oil. CNTs provide fast electron transfer between biorecognition elements and transducers due to their great electrical and mechanical properties. Because the CNTs have electrochemical stability and high surface area, they are very employed in sensors and biosensors.^{15,16}

Screen printed electrodes (SPEs) have also been widely employed in sensors for their especially low cost, fast response and large-scale production.¹⁷ Regardless of these advantages, signal conductivity remains a weak point of SPE electrode. To solve this problem, various materials such as carbon nanomaterials,¹⁸ metal nanoparticles¹⁹ and polymers²⁰ have been used to modify SPEs. In this sense, the modification of the electrode surface with MWCNT (multiwalled carbon nanotube) could increase the conductance of the electrode, thus is

possible to increase the electron transfer between enzyme and the electrode.

According to aforementioned the main aim of this study was development of a new electrochemical HRP (horseradish peroxidase)-MWCNT (multiwalled carbon nanotube)-based screen-printed disposable biosensor for monitoring phenolic compounds in effluent from washing coffee. Until at moment there are no reports from the present study.

2. Experimental

2.1. Reagents and solution

All chemical reagents used in the development of this work had analytical purity. Sodium acetate, sodium phosphate monobasic, Hepes, Pipes, Tris, hydrogen peroxide, mineral oil, bovine serum albumin (BSA), horseradish peroxidase (HRP) 53 U mg⁻¹, all from Sigma-Aldrich brand; Hydrochloric acid and sodium hydroxide (Vetec) and glutaraldehyde (Dinâmica). The multiwalled carbon nanotube (MWCNT) was acquired by the company Nanocyl and has the following specifications: purity of 99%, diameter between 6 and 13 nm, length between 2.5 and 20 μm; the graphite powder with 98% purity was purchased from Synty brand. All solutions were prepared with milli-Q purified water by the Millipore® system with resistivity of 18 MΩ cm⁻¹.

2.2. Instrumentation and electrochemical characterization

The electrochemical measurements were performed using a potentiostat/galvanostat from Autolab model PGSTAT12, interfaced to a

microcomputer equipped with GPES 4.9 software, to acquire the experimental data.

A conventional electrochemical cell system was used in the electrochemical measurements and it was consisted of three electrodes: a reference electrode Ag/AgCl (KCl 3.0 mol L⁻¹), the counter electrode, a platinum wire spiral and the proposed biosensor as working electrode.

Two working electrodes were used. The first electrode consisted of a Pt plate located at the upper end of a 15 cm long glass tube, with a lower cavity with 4 mm in internal diameter, depth of 1 mm and area of 0.12 cm². A Pt plate was connected to a nickel/chromium wire that was responsible for the electrical contact of the electrode and the modified carbon paste with nanotube was deposited under the surface of the plate. The second working electrode used was a disposable electrode purchased commercially from Metrohm Pensalab, where the system is constituted as follows: printed working electrode with carbon nanotube multi-walled, silver reference electrode and auxiliary carbon. Their dimensions were 3.4x1.0x0.05 cm and electrical silver contacts were used. Figure 1 illustrates a disposable electrode.

To prepare the different solutions, the reagents were weighed out on a Shimadzu analytical balance, model AUW2200. The solutions pH values were adjusted by means of a properly calibrated Tecnal pHmeter.

The prepared electrodes were characterized by scanning electron microscopy (SEM, Jeol®, Model JSM 300, Tokyo, Japan) and infrared spectroscopy. Fourier transform infrared (FT-IR) absorbance spectrum was recorded on a FT-IR 8300 (Shimadzu®, Kyoto, Japan) (spectra recorded in the region of 4000-500 cm⁻¹) spectrometer equipped with a DTGS detector and the KBr pellet technique was used to characterize the electrode.

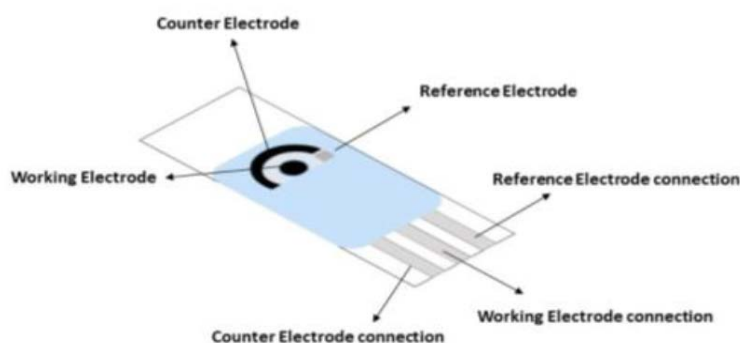


Figure 1. Schematic of a disposable electrode

These analyzes were performed at the Center of Exact Sciences in the Department of Chemistry, State University of Londrina (UEL) in collaboration with Professor César Ricardo Teixeira Tarley.

2.3. Enzyme immobilization onto the surface of multi-walled carbon nanotube by covalent crosslinking

The obtaining of the biosensor for the determination of phenolic compounds was performed by immobilizing the enzyme HRP by covalent crosslinking using glutaraldehyde, which acts by reacting with the amino groups of the enzyme, providing a strong chemical bond between the biomolecules. That is, the crosslinked did not relate to immobilization bioelement the surface of a support, but the bond between the enzymes forms a large complex three-dimensional structure.²¹

The biological material has been immobilized by homogenizing 10.0 mg of carbon nanotube, 25 μL of a solution of HRP (53 U mg^{-1} solid in 100 μL of water), 1 mg of BSA, 5 μL of glutaraldehyde (5% v/v) for 15 minutes. This mixture was allowed to stand in the refrigerator for 15 hours. After this step, 1 mg of powdered graphite and 20 μL of mineral oil were added to the mixture. Then, obtained paste was introduced into a cavity in the working electrode.

2.4. Preparation of the biosensor based on the disposable electrode

The disposable biosensor was obtained after the surface modification of the commercial disposable electrode. So, a solution was prepared from 1 mg of HRP (53 U mg^{-1} solid), 4 mg of BSA and 20 μL of glutaraldehyde (5% v/v) in 100 μL of water. Then, 25 μL of this solution were added in the contact of the working electrode, and it was allowed to stand for 2 hours at room temperature.

2.5. Evaluation of experimental parameters

To evaluate the sensibility of the biosensor for the determination of phenolic compound, a standard solution of catechol with a concentration of 3.0 mmol L^{-1} was used and an analytical curve was constructed in the range of 30 to 300 $\mu\text{mol L}^{-1}$ of catechol, using the cyclic voltammetry technique. All linear correlation coefficients obtained were greater than 0.99.

First, the amount of HRP added to the carbon paste (0.24, 0.48, 0.72, 0.96, 1.20, 1.45, 1.69, 1.93 U mg^{-1} of carbonaceous material) was varied, then, the type of buffer system (phosphate, trizma, pipes, hepes), concentration of phosphate buffer solution (0.05, 0.2, 0.25, 0.3, 0.5 mol L^{-1}) and the pH of the phosphate buffer (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) were evaluated. Finally, the concentration of hydrogen peroxide (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 $\mu\text{mol L}^{-1}$) was varied. These are the parameters that are aimed at obtaining greater sensibility of the biosensor for the determination of phenol compounds.

The reagents that influenced the immobilization of the enzyme were also optimized. The amount of glutaraldehyde solution 5% v/v was varies between 0 to 7 μL . According to several studies reported in the literature,²²⁻²⁴ in this study, a concentration of 5% v/v of glutaraldehyde was used. This concentration of glutaraldehyde was maintained for studying the optimal amount of BSA. The evaluated values were: 1.0; 1.5; 2.0; 2.5; 3.0; 3.5 mg.

2.6. Evaluation of the operational parameters of electrochemical techniques employing disposable electrode

The choice of the electrochemical technique which permitted a better response in the determination of phenolic compounds was performed from the comparison of different techniques (cyclic voltammetry, differential pulse voltammetry and square wave voltammetry), after optimization of the parameters related to the technical.

The cyclic voltammetry technique was used to study the electrochemical behavior of catechol and for the optimization of experimental parameters. The measurements were performed in a potential range of -0.2 to 0.5 V vs. Ag/AgCl using biosensor based on carbon nanotube (disposable electrode) as working electrode.

In differential pulse voltammetry two parameters were evaluated: scan rate (v) and the pulse amplitude (A_p) in the potential range of -0.2 to 0.5 V vs. Ag/AgCl using the proposed biosensor working electrode. In the square wave voltammetry, the frequency (f) and A_p (pulse amplitude) applied the same potential range were evaluated.

After the optimization of the parameters for each electrochemical technique, analytical curves were constructed using each of the techniques

mentioned, and the calibration curve for the determination of phenolic compounds in real samples was obtained using the most suitable technique for the determination of the analyte using the disposable biosensor.

2.7. Determination of total phenolic compounds

To be credible on the quantification of phenolic compounds by the method developed, a comparison between the results obtained was done, with an already reliable method from the addition of standards. The comparison of methods consists on the comparative evaluation of the results obtained using the proposed method and the results obtained using a reference method, assessing the degree of proximity between the results obtained by the two methods, that is, the degree of accuracy of the tested method in respect to the reference. This approach assumes that the uncertainty of the reference method is known.²⁵

The reference method of Folin-Denis was used for the determination of total phenolic compounds in environmental samples in order to compare the results obtained through the disposable biosensor modified with HRP. The statistical *t*-test is used for statistical analysis between the two methods.

To perform this test it is necessary to make a comparison between the limits of reliability for the average of the data, using the value of t_{critic} .

The standard addition method consists in the addition of known amounts of the substance of interest that is being analyzed to known quantities of the sample prior to its preparation. An analytical curve correlating the quantity of the substance added to the sample is built and the extrapolation of the linear region on the x-axis defines the concentration of the substance in the sample.

2.8. Application in samples

To evaluate the performance of the disposable biosensor developed, the determination of the phenolic compounds in environmental samples (wastewater from coffee washing) was done. Aliquots of the samples were added to the electrochemical system without any pretreatment for performing the analysis.

Wastewater from wet processing of coffee fruits were collected at São Carlos farm, located in Bom Sucesso, Minas Gerais, Brazil, at 1006 m altitude, 21° 01' 31.95'' latitude and 44° 53' 14.5'' W longitude.

Four collection points of wastewater were used: Point 1: water from the washer water; Point 2: water from the peeler; Point 3: water from the mucilage remover and Point 4: water from the storage tank, where all the processing effluent is mixed. The samples were vacuum pre-filtered with Whatman filter paper nº 15 to remove any interfering impurity and they were stored in amber bottles at 4 °C.

3. Results and Discussions

3.1. Composite characterization

The Fourier transform infrared spectra recorded in the region of 4000-500 cm^{-1} for the CNT, for the HRP enzyme and for the composite formed by the method of immobilization of enzyme in the carbon nanotube by covalent crosslinking were evaluated in this work, with the aim of showing the different functional groups present in the studied materials.

Figure 2a shows the FT-IR spectra obtained for the sample of the carbon nanotube. It was observed an absorption peak at 3449 cm^{-1} attributed to the stretching of the O-H bond.²⁴ The small oscillations appearing in 2925 and 2853 cm^{-1} may be associated with symmetric and asymmetric stretches of CH_2 and indicate the presence of fractures on the sides of the tubes.²⁴ The absorption peak at 1688 cm^{-1} is related to the presence of carbonyl group.²⁶

In Figure 2b, the region between 1637-1676 cm^{-1} is attributed to alpha helical structure at 1650 cm^{-1} . One can see in Figure 2c that the bands showed a shift in regions of lower wavelengths of 1688 cm^{-1} (carbon nanotube) to 1650 cm^{-1} . The spectra show only variations in the intensity of the bands. The intensity can decrease due to the coupling HRP immobilized of with glutaraldehyde.²⁷

The morphological characteristics of these same materials were evaluated employing scanning electron microscopy. In Figure 3a it was possible to observe the SEM images for carbon nanotubes where a medley of nanotubes was noticed. Figure 3b illustrates the SEM images of the enzyme HRP in nature. It is observed that the enzyme is composed of a very thin plate with a smooth surface. This fact contributes to the increased number of active sites on the electrode surface, allowing a homogeneous crosslinking to occur with glutaraldehyde, favoring the process of immobilization by crosslinking.²⁴

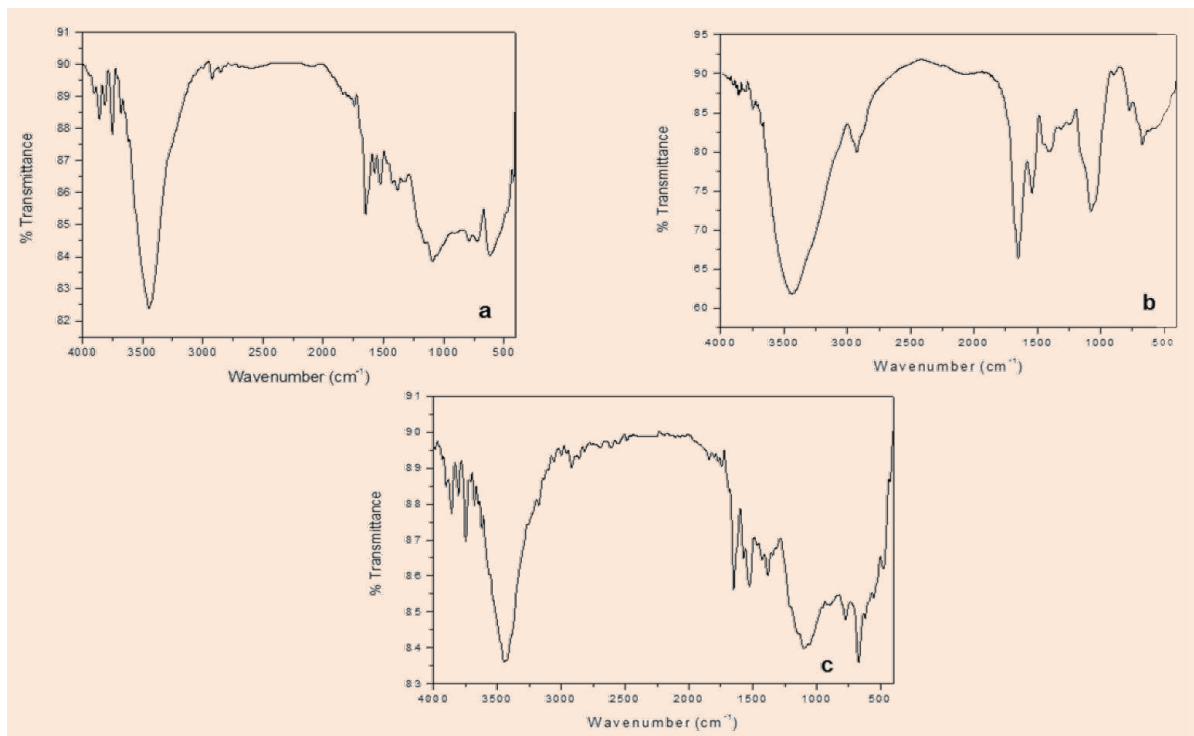


Figure 2. FT-IR spectra of the (a) carbon nanotube, (b) HRP enzyme and (c) composite obtained by the immobilization of HRP enzyme in the carbon nanotube by covalent crosslinking

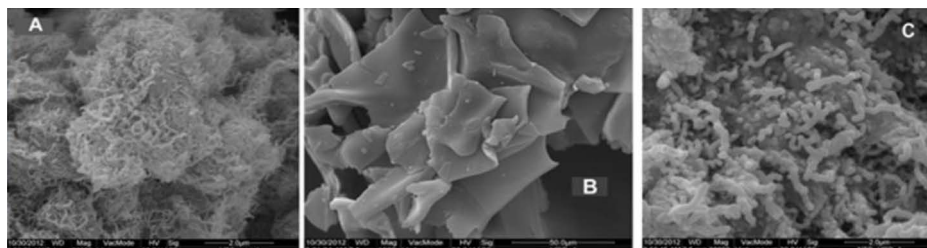


Figure 3. SEM images of the multi-walled carbon nanotube with magnification of 30000x (A), SEM images of HRP enzyme with magnification of 1600x (B) and SEM images of the composite formed with magnification of 30000x (C)

Figure 3c shows the SEM images of the composite obtained from the immobilized enzyme by covalent crosslinking. In this figure, a fill between filamentous structures CNT stands out, i.e., the homogeneous filling of the crosslinking between glutaraldehyde and the enzyme. In this image, the carbon nanotubes are well dispersed throughout the matrix, indicating that the procedure was successfully employed.

3.2. Optimization of the biosensor for catechol

The electrochemical behavior of the biosensor obtained by immobilizing the enzyme HRP by the method of covalent crosslinking was evaluated by cyclic voltammetry and compared to the electrode without modification.

Comparing the voltammograms in the presence of catechol in the modified electrode and the electrode unmodified, note voltammograms obtained with the modified electrode, the presence of minimum and maximum points of current peaks correspond to the reduction and oxidation of catechol, which respectively occur at values near +0.10 and +0.30 V vs. Ag/AgCl. The reduction of catechol in modified electrode generated a cathodic peak current greater than that produced by the electrode unmodified, probably due to the catalytic activity of the enzyme when it was immobilized.

The voltammograms in Figures 4 (a) and (b) in the absence of catechol, present no faradaic process, characterized by the formation of the electrical double layer between the electrode surface and the

electrocatalytic solution. The absence of cathodic and anodic peaks in the modified electrode may be related to a larger blockade in the electron transfer between the electrode and the enzyme, in the redox couple Fe^{2+}/Fe^{3+} present in the heme group of HRP, preventing the direct transfer between the electrode and the HRP enzyme.^{28, 29}

3.3. Evaluation of the experimental parameters

The main problem during the process of immobilization of an enzyme is a partial or complete loss of activity, which can be attributed to several factors, such as the properties of the enzyme, of the support, of the reagents used and the experimental conditions.^{30,31} Thus, were studied the influences of all reagents used

in the enzyme immobilization procedure and electrochemical systems, as well as all operational parameters of the analytical technique chosen to obtain the maximal response of the biosensor.

Figure 5 shows the cyclic voltammograms with successive additions of catechol from a stock solution with a concentration of 1.0 mmol L^{-1} . The choice of the optimal value for each experimental parameter studied was performed by comparing the sensibility of the biosensor obtained through the slope of the analytical curve of cyclic voltammetry.

3.3.1. Evaluating the amount of biological material

The sensibility of the modified electrode depends on the amount of enzyme incorporated into the carbon paste, as shown in Table 1.

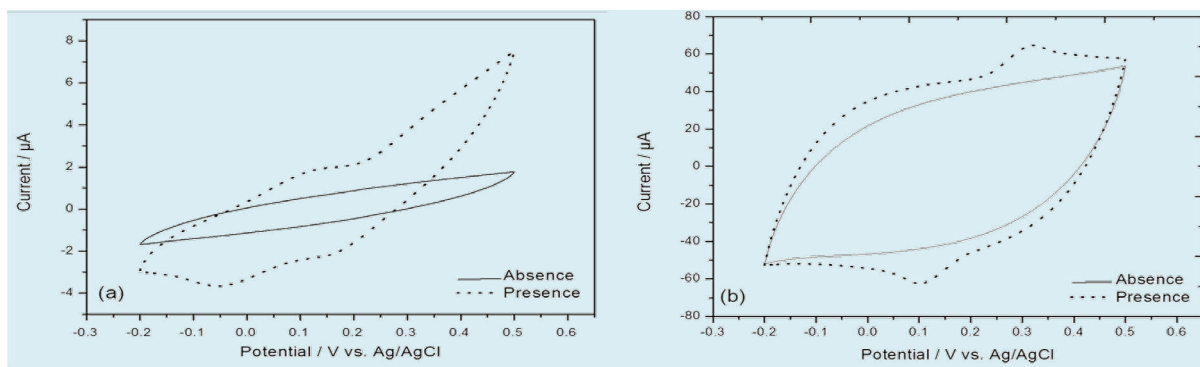


Figure 4. (a) Cyclic voltammograms to the CNT unmodified, in the absence and presence of catechol. (b) Cyclic voltammograms to the CNT modified, HRP immobilized by covalent crosslinking method, in the absence and presence of catechol. Measurements performed in PBS 0.1 mol L^{-1} pH 6.5, at a scan rate (ν) of 30 mV s^{-1} , at a potential range of -0.2 to $0.5 \text{ V vs. Ag/AgCl}$ in the absence and presence of $150 \text{ } \mu\text{mol L}^{-1}$ catechol

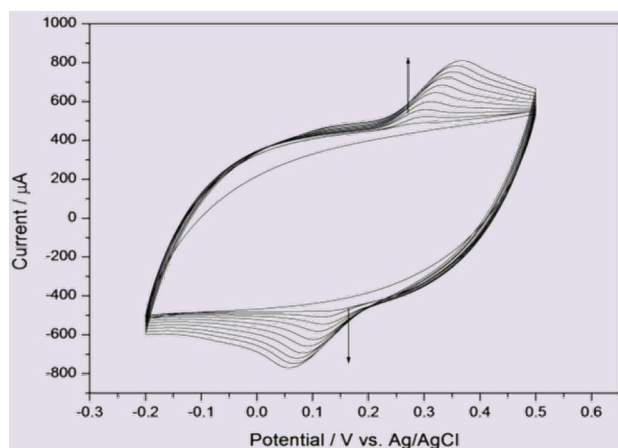


Figure 5. Cyclic voltammograms obtained from different concentrations of catechol (30 to $300 \text{ } \mu\text{mol L}^{-1}$) in increments of $30 \text{ } \mu\text{mol L}^{-1}$ and $\nu = 30 \text{ mV s}^{-1}$. Measurements performed in PBS 0.1 mol L^{-1} pH 7.0, containing $20 \text{ } \mu\text{mol L}^{-1}$ of H_2O_2

Table 1. Response biosensor for catechol obtained with different amounts of HRP

Amount of HRP (U mg ⁻¹)	Sensitivity (A L mol ⁻¹)
0.24	14.08
0.48	30.71
0.72	42.71
0.96	72.74
1.20	123.5
1.45	96.16
1.69	95.11
1.93	42.11

From the data presented in Table 1, it was possible to observe an increase in the sensibility of the biosensor to 1.20 U mg⁻¹ of enzyme, whereas for larger values there was a decrease in the response, because the high amount of enzyme caused diffusional limitations.³² Therefore, all new experiments were performed using 1.20 U mg⁻¹ of HRP.

3.3.2. Effect of buffer system

The choice of the electrolyte is important to improve the sensibility of the biosensor in determining catechol. Several buffer systems were prepared in the same concentration and pH in order to determine the effect of these parameters on the biosensor response.

Table 2 shows that the PBS had greater sensibility to the method. This result may be related to the size of the ions present in the electrolyte, allowing greater ease of diffuse through the electrode compared with the ions of the other studied electrolytes.³³

3.3.3. Study of the concentration of phosphate buffer solution

According to the results, shown in Figure 6, the PBS concentration that showed better sensibility

in electrochemical response of the biosensor developed for the determination of catechol was 0.1 mol L⁻¹, indicating that the increased concentration of ions existing in the solution causes a greater transport of charges.

The reduced sensibility at concentrations higher than 0.1 mol L⁻¹ can probably be explained by the fact that increasing the amount of phosphate ions, the diffusion process may still be impaired, due to decreased ionic mobility of this ion at high concentrations. Thus, the concentration chosen to be used in subsequence experiments was 0.1 mol L⁻¹.

3.3.4. Effect of pH

Another factor that greatly influences the response of the HRP-based biosensor is the pH of the buffer solution, since the charges of the amino and carboxylic groups will vary with pH, and enzymes exhibit a characteristic pH where they have a maximum activity. Table 3 presents the evaluation of the response of the biosensor in PBS 0.1 mol L⁻¹ in the range of pH from 5.0 to 8.0.

According to literature,³⁴ the optimum pH of peroxidase varies between 6.0 and 6.5. As the results presented in Table 3 it is observed that the best response of the biosensor was at pH 6.5 within the range of optimal pH of the enzyme, showing that the immobilization process did not

Table 2. Response of the biosensor for catechol obtained in different buffer solutions, concentrations 0.1 mol L⁻¹, pH 7

Buffer Solution	Sensitivity (A L mol ⁻¹)
Trizma	0.719
Pipes	0.349
Hepes	0.156
Phosphate	0.803

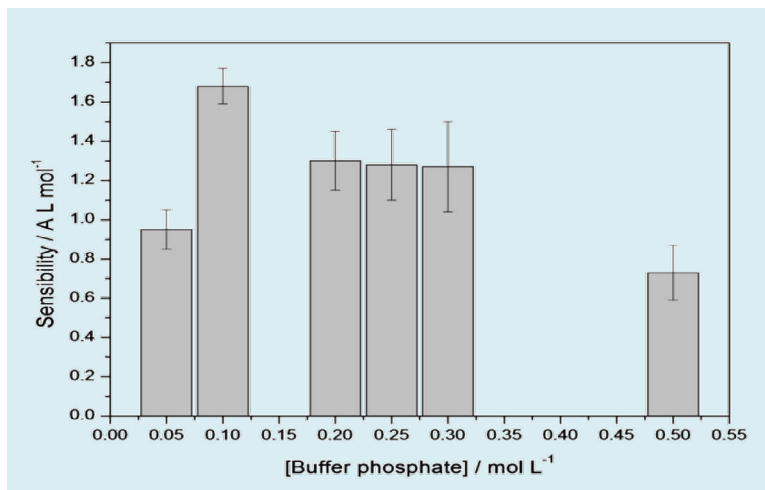


Figure 6. Influence of the PBS concentration in the sensitivity of the biosensor to catechol. Measurements obtained at different concentrations of PBS, pH 7.0, $v = 30 \text{ mV s}^{-1}$ and the absence of the analyte containing $20 \mu\text{mol L}^{-1}$ of H_2O_2 in the work cell

Table 3. Response of the biosensor to catechol at different pH values

pH	Sensibility (A L mol ⁻¹)
5.0	0.52
5.5	0.53
6.0	0.58
6.5	1.02
7.0	0.89
7.5	0.75
8.0	0.64

affect the catalytic activity of the immobilized biological material. The better sensitivity at pH 6.5 can also be related to the better immobilization of the enzyme on the electrode surface in this condition. pH values above and below 6.5 resulted in less sensitivity, probably the immobilization was not as effective, so pH 6.5 was selected as the optimal value for the proposed sensor.

3.3.5. Effect of hydrogen peroxide

The influence of hydrogen peroxide concentration on the response of the proposed biosensor for the detection of catechol was investigated from cyclic voltammetry at different concentrations of hydrogen peroxide. Hydrogen peroxide is the natural substrate of the peroxidase enzyme. However, at very high concentrations of hydrogen peroxide, inactivation of the enzyme can occur, which is an important factor to be optimized.³⁵

Table 4 shows the dependence of the concentration of hydrogen peroxide on the

sensibility of the biosensor. The results show that the response of the device increases up to a concentration of $30 \mu\text{mol L}^{-1}$ of hydrogen peroxide, decreasing for higher concentrations. This observation is a consequence of the decrease in the formation of inactive enzyme, due to high concentrations of peroxide. H_2O_2 concentration was fixed at $30 \mu\text{mol L}^{-1}$ for further studies, as it was in this condition that the biosensor showed the best sensibility.

3.3.6. Study of the amount of glutaraldehyde

Glutaraldehyde is a bifunctional reagent used for immobilizing the enzyme (organic material) on the surface of the carbonaceous where an aldehyde group at one end of the molecule attached itself to the electrode and the other group attached to the enzyme. The immobilization may cause a decrease in the catalytic activity of the enzyme at certain concentrations due to the distortion in the conformational structure of the enzyme.

Table 4. Dependency of concentration of H_2O_2 on the response of the biosensor

$[\text{H}_2\text{O}_2]$ ($\mu\text{mol L}^{-1}$)	Sensibility (A L mol^{-1})
10	0.75
20	1.15
30	1.18
40	1.16
50	0.61
60	0.59
70	0.92
80	0.96
90	0.96
100	0.97

Consequently, the optimization of the amount of glutaraldehyde must be carried out to verify the viability of this method of immobilization for the determination of phenolic compounds.³⁶

Figure 7 shows the responses obtained by a biosensor for the determination of phenolic compounds using different concentrations of glutaraldehyde. The highest sensibility was found when 5.0 μL of a solution of 5% (v/v) glutaraldehyde was used, and then this concentration was chosen for further studies.

The increase in the amount of glutaraldehyde resulted in an increase in the sensibility of the biosensor, since the immobilized enzyme was more effective, avoiding loss of enzymatic material by leaching. The decrease in the sensibility of the biosensor when it was added a volume of 5 μL can be explained by the distortion of the

conformational structure of HRP caused by the excess of glutaraldehyde.

3.3.7. Evaluation of the influence of the amount of BSA

Generally, the immobilization of the enzyme through covalent crosslinking is done with the use of BSA, which aims to change the environment of the enzyme to the closest to nature, enabling better sensibility and stability.³⁷ Since glutaraldehyde can decrease the enzyme activity, the function of the BSA is to increase the number of crosslinks in order to obtain a greater physical strength of the immobilized layer, reducing the disadvantage of the use of glutaraldehyde. As can be seen in Figure 8, the best response of the proposed biosensor for the determination of catechol was obtained when a

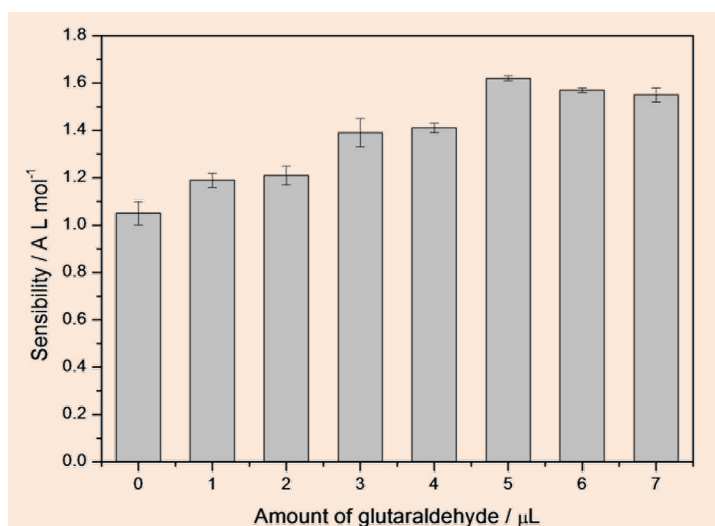


Figure 7. Influence of the amount of glutaraldehyde on the sensitivity of the biosensor to catechol. Measurements obtained in PBS 0.1 mol L^{-1} , pH 6.5, $v = 30 \text{ mV s}^{-1}$ and the absence of the analyte containing 30 $\mu\text{mol L}^{-1}$ of H_2O_2 in the work cell

mass of 1.0 mg of BSA was used, and therefore this value was set for subsequent studies. The decrease in the responses to values larger than 1.0 mg of BSA mass can be related to the increase in diffusional resistance, rigid fixation of the enzyme or enzyme replacement for BSA in the surface of the biosensor.³⁷ Probably below 1.0 mg of BSA, the sensitivity will be low, because the amount of BSA is not enough to cause a favorable effect on enzyme immobilization.

3.4. Evaluation of the operating parameters of different electroanalytical techniques employing the disposable electrode

The disposable electrode used is a device containing carbon nanotubes printed on an inert material. After optimization of the experimental parameters that influenced the response of the bench scale biosensor, the electrochemical behavior of the modified disposable electrode was evaluated by optimizing the operating parameters of different electroanalytical techniques for the determination of catechol phenolic compound, so the technique that showed the best sensitivity was chosen for this determination.

3.4.1. Cyclic Voltammetry

Figure 9a illustrates cyclic voltammograms obtained from the successive addition of catechol to the electrochemical cell. Under optimized experimental conditions, in order to evaluate the effect of the immobilization of HRP enzyme on the surface of the disposable electrode, a calibration

curve was constructed, and it showed a linear response in the range from 20 to 32.5 $\mu\text{mol L}^{-1}$ (Figure 9b), with a correlation coefficient, $r = 0.9921$ ($n = 6$). The analytical curve was adjusted by Equation 1 showing sensitivity of $0.05 \mu\text{A L } \mu\text{mol}^{-1}$.

$$I (\mu\text{A}) = 0.05 [\text{catechol}] (\mu\text{mol L}^{-1}) - 0.484 \quad (1)$$

Cyclic voltammetry (CV) is a technique to obtain qualitative information about electrochemical processes, but it is not a very sensitive technique for quantitative applications.³⁸ To obtain voltammograms with an improved relationship between resolution and sensibility for the determination of phenolic compounds, the differential pulse voltammetric (DPV) and square wave voltammetric (SWV) techniques were used.

3.4.2. Differential pulse voltammetry (DPV)

With the optimization of operational parameters of the Differential Pulse Voltammetry (DPV) – Scan rate 5 mV s^{-1} , Amplitude 30 mV – analytical curves were constructed with successive additions of catechol.

The differential pulse voltammograms, shown in Figure 10(a) were obtained with successive additions of catechol in the electrochemical cell under optimized conditions. As shown in Figure 10b, the analytical curve obtained by differential pulse voltammetry to determine catechol showed a sensitivity equal to $0.121 \mu\text{A L } \mu\text{mol}^{-1}$. The linear response range was between 20 to 32.5 $\mu\text{mol L}^{-1}$ with a correlation coefficient, $r = 0.9915$ for $n = 6$, (Equation 2).

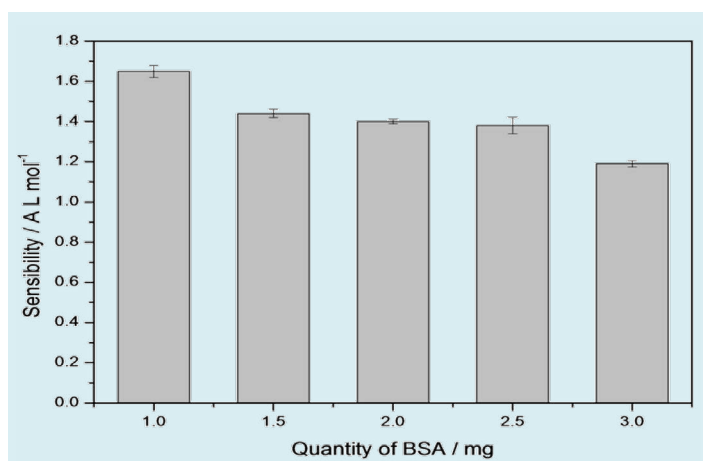


Figure 8. Influence of the quantity of BSA in the sensitivity of the biosensor to catechol. Measurements obtained in PBS 0.1 mol L^{-1} , pH 6.5, $v = 30 \text{ mV s}^{-1}$ and the absence of the analyte containing $30 \mu\text{mol L}^{-1}$ of H_2O_2 in the work cell

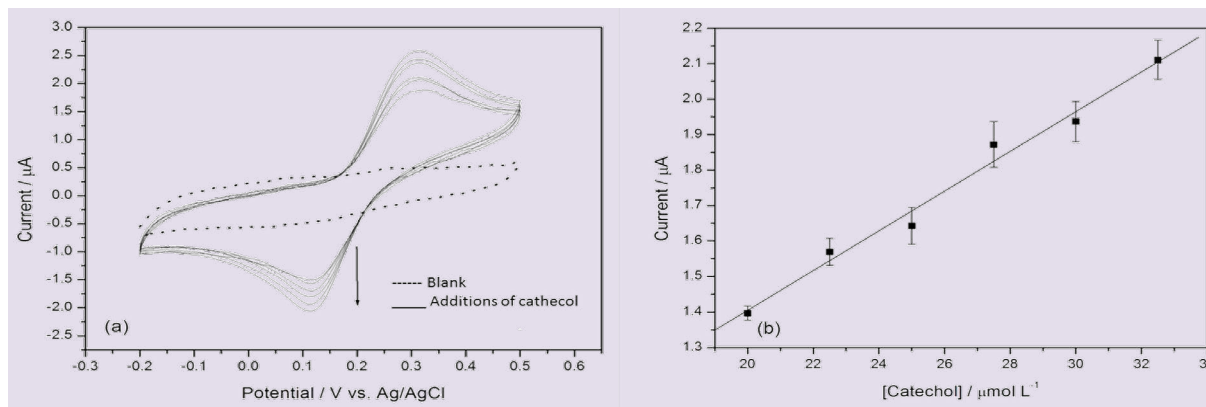


Figure 9. (a) Cyclic voltammograms obtained using the disposable biosensor, with PBS 0.1 mol L⁻¹, pH 6.5, $\nu = 30 \text{ mV s}^{-1}$ in presence of $30 \mu\text{mol L}^{-1}$ of H_2O_2 , for successive additions of catechol. (b) Analytical curve using the disposable biosensor, developed from the cyclic voltammograms

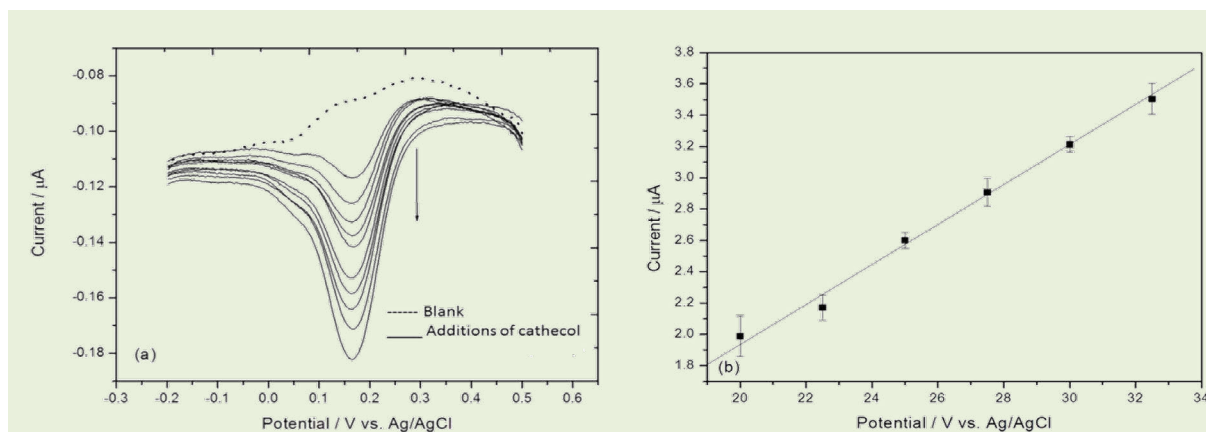


Figure 10. (a) Differential pulse voltammograms obtained using the disposable biosensor after successive additions of catechol, with PBS 0.1 mol L⁻¹, pH 6.5, in presence of $30 \mu\text{mol L}^{-1}$ of H_2O_2 , Scan rate = 0.005 V s^{-1} and 0.03 V of amplitude. (b) Analytical curve for catechol using the disposable biosensor from the differential pulse voltammograms

$$I (\mu\text{A}) = 0.121 [\text{catechol}] (\mu\text{mol L}^{-1}) - 0.439 \quad (2)$$

The voltammograms obtained by DPV, after optimization of the operating parameters, showed a good resolution and a greater sensibility than the one obtained by CV.

3.4.3. Square wave voltammetry (SWV)

After optimization of operational parameters for Square Wave Voltammetry (SWV) – Amplitude 25 mV, Frequency 15 Hz – analytical curves were constructed with successive additions of catechol standard.

Figure 11a illustrates square wave voltammograms obtained from successive

additions of catechol. Figure 11b shows the analytical curve obtained from the data of the cathodic current intensity generated in the redox process of the catechol over the biosensor developed. The curve has a linear region between 20 and $32.5 \mu\text{mol L}^{-1}$ with $r = 0.9963$ for $n = 6$ and sensitivity of $0.2827 \mu\text{A L } \mu\text{mol}^{-1}$. The calibration curve for the determination of the phenolic compound, catechol, was adjusted by Equation 3.

$$I (\mu\text{A}) = 0.282 [\text{catechol}] (\mu\text{mol L}^{-1}) - 0.117 \quad (3)$$

The analytical curves were constructed in order to evaluate which of the electroanalytical techniques showed a better sensitivity to catechol, aiming at the application of the disposable biosensor proposed in determining the target

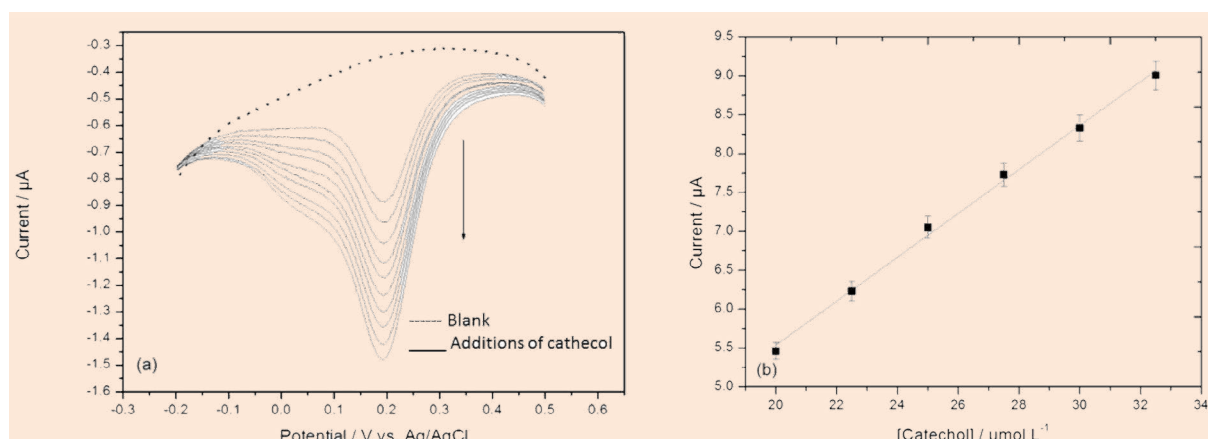


Figure 11 (a) Square wave voltammograms obtained with the disposable electrode modified with 1.20 U mg⁻¹ of HRP in 0.1 mol L⁻¹ PBS, pH 6.5, in the presence of 30 μmol L⁻¹ of H₂O₂. (b) Analytical curve for catechol

analyte in real samples. In Table 5 it can be seen that the square wave voltammetry was the most sensitive. This result was expected because in the square wave voltammetry, the measurements of electric currents are made at the end of the direct and reverse pulses and the signal is obtained as a resultant differential current, which usually ensures high analytical sensitivity compared to other voltammetric techniques .³³

After choosing the technique and optimizing the operational parameters, the LOD and LOQ were estimated using the equations $LOD = 3 \times s/a$ and $LOQ = 10 \times s/a$, where s represents the standard deviation of the peak currents obtained in blank solution (in the absence of catechol) ($n = 10$) and a refers to the slope of the calibration curve. The LOD and LOQ were equal to 0.16 and 0.47 μmol L⁻¹, respectively.³⁹

The present method was based on univariate calibration, analyzing only the analytical response of the catechol. Phenolic compounds can be easily analyzed by electrochemical detection, due to the fact that they can generate electroactive products in the presence of the HRP enzyme. Oxidation products can be reduced electrochemically in the electrode surface and therefore an electrochemical signal directly proportional to the concentration of the phenolic compound is obtained when H₂O₂ is present in excess.⁴⁰

Based on these results, it was possible to determine phenolic compounds using electroanalytical method developed. As the disposable biosensor allow good sensitivity and high-speed scanning, it was employed to determine phenolic compounds in wastewater from coffee washing (environmental sample), using the voltammetry square wave technique.

3.5. Application of the method in a sample of environmental interest using the disposable biosensor

It is required the quantification of phenolic compounds present in wastewater from coffee washing, prior to subsequent treatment and disposal, in comply with the legislation; or for appropriate reuse in order to minimize environmental impact in terms of coffee post-harvest. Therefore, the disposable biosensor based on HRP was tested for the determination of the amount of total phenolic compounds in samples from different points of coffee washing.

Table 6 presents the results obtained by applying the proposed method and the standard Folin-Denis method at different points of the wastewater sample from coffee washing. Point 1, point 2, point 3 and point 4 are related, respectively, to water from the washer, water peeler, water desmucilador/pulper and the water of the ditch.

Table 5. Response of the biosensor for catechol obtained from different methods of analysis. The experiments were performed with 0.1 mol L⁻¹ PBS pH 6.5 using a disposable biosensor

Technical Electroanalytical	Sensibility (A L mol ⁻¹)
Square wave voltammetry	0.28
Differential pulse voltammetry	0.12
Cyclic voltammetry	0.05

The statistical *t*-test was used to determine the difference between the proposed method and a standard method. The paired *t*-test uses the difference between the values obtained from the two methods. Equation 4 determines the value of the test: ⁴¹

$$t = \frac{\bar{d}}{sd/\sqrt{N}} \quad (4)$$

$$\bar{d} = \frac{\sum d_i}{N} \quad (5)$$

$$sd = \sqrt{\frac{\sum d_i^2 - (\sum d_i)^2/N}{N-1}} \quad (6)$$

where **d** corresponds to the average difference (Equation 5), **s_d** is the standard deviation of the difference (Equation 6), and **N** is the number of samples.

The calculated value of the *t* test, used to compare the Folin-Denis method and the analytical method proposed in this work, is *t* = 3.49. Therefore, for a confidence level of 99%, $t_{\text{calculated}} < t_{\text{critical}}$. Thus, statistically, the two methods provide equivalent results in a confidence level of 99% and 3 degrees of liberty.

Table 7 compares the data obtained between the developed biosensor and biosensors and sensors described in the literature. The proposed biosensor is characterized by low cost and manufacturing simplicity with good analytical performance, so it represents a viable option in comparison to other available electrodes in literature. Due to the sensitivity presented by the developed method, the proposed biosensor can be used in the detection of phenolic compounds in a large number of real samples.

Table 6. Determination of the amount of total phenolic compounds by Folin Denis method and by the biosensor developed

Wastewater	Folin-Denis method(mg L ⁻¹)	Electroanalytical method developed (mg L ⁻¹)	Relative error ^[a] (%)
Point 1	11.41	15.33	34.36
Point 2	119.07	95.27	-24.98
Point 3	168.83	154.59	-9.21
Point 4	115.27	138.59	16.83

[a] Relative Error = [(Proposed method – Standard method) / (Standard method)] × 100

Table 7. Analytical characteristics of biosensors and sensors described in the literature in order to compare with the developed biosensor

Electrode	Method of Immobilization	Analyte	Sensibility (mA L mol ⁻¹)	LOD (μmolL ⁻¹)	Reference
Disposable graphite electrode modified with "ferrocene"	Sol-gel	Catechol	3.0	10	42
		Caffeic acid	6.2	6	
Disposable graphite electrode	Encapsulation	Hydroquinone	9.4	1.1	43
Biosensor-based field effect transistors	Nanostructured films	Polyphenols	1.54	4.82	44
Carbon paste electrode	Adsorption	Guaiacol	-	1.89*	45
Carbon paste electrode	Crosslinking	Catechol	0.36**	4.5	46
Electrodes of graphite rod	Adsorption	Phenol	0.26**	-	47
Electrode paste CNT	Covalent crosslinking	Hydroquinone	8.0	6.42	48
CRGO modified GCE ^[a]	-	Hydroquinone (HQ), dopamine (DP), catechol (CT)	0.0659** (HQ), 0.1452 (DP), 0.0866 (CT)	0.052 (HQ), 0.012 (DP), 0.055 (CT)	49
Boron doped diamond (BDD) electrode	-	Catechol	3.97	0.69	50
Disposable carbon nanotube electrode	Covalent crosslinking	Catechol	0.28**	0.16	This work

LOD = Limit of Detection. * Detection limit at mmol L⁻¹. **Sensibility at μA/μmolL⁻¹. ^[a] Glassy carbon electrode (GCE) modified with chemically (CRGO) and electrochemically reduced graphene oxide (ERGO)

4. Conclusions

This work showed that the disposable biosensor modified with HRP enzyme is a viable alternative for the analytical determination of total phenolic compounds in different environmental samples, like, wastewater from coffee washing, by square wave voltammetry. In addition, it has many advantages such as ease of enzyme immobilization by the covalent crosslinking method and quickness of analysis, electroanalytical method developed proved to be very simple and sensitive to the analyte.

The studies showed that the carbon nanotubes matrix is a great environment for immobilization of HRP. The process for immobilization of the enzyme to the substrate held by the reagent glutaraldehyde has a great importance in the sensibility of the biosensor. The covalent bonds created during the crosslinking reaction of the enzyme were stable, since the use of BSA is intended to become the closest to the natural environment of the enzyme, allowing a better enzyme activity. The images of SEM and FT-IR spectra obtained for characterizing the material used for the modification of the electrode showed that there was an effective immobilization of HRP by the covalent crosslinking method. The electrochemical characterization system proposed for the determination of the catechol shows that the formed composite is stable, and that the proposal relating to the development of the device for the determination of phenolic compounds was successful.

The result of the comparison of the proposed method with the Folin-Denis standard method showed the reliability of the biosensor, thus it can be concluded that the method is suitable for the detection of phenolic compounds, showing the possibility of using this biosensor without previous treatment of the samples.

Acknowledgements

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Araucária do Paraná, INCT de Bioanalítica and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial

support and for granting research fellowships. To Priscila Maria Batista Chagas for the Folin-Dennis Method analyzes.

References

- ¹ Gamella, M.; Campuzano, S.; Reviejo, A. J.; Pingarrón, J. M. Electrochemical Estimation of the Polyphenol Index in Wines Using a Laccase Biosensor. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 7960. [CrossRef]
- ² Baldrian, P. Fungal laccases – occurrence and properties. *FEMS Microbiology Reviews* **2006**, *30*, 215. [CrossRef]
- ³ Chang, H.-S.; Choo, K.-H.; Lee, B.; Choi, S.-J. The methods of identification, analysis, and removal of endocrine disrupting compounds (EDCs) in water. *Journal of Hazardous Materials* **2009**, *172*, 1. [CrossRef]
- ⁴ Carbanellas, C. F. G. *Tese de Doutorado*, Universidade Federal de Viçosa, 2004. [Link]
- ⁵ Pandey, A.; Soccol, C. R.; Nigam, P.; Brand, D.; Mohan, R.; Roussos, S. Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochemical Engineering Journal* **2000**, *6*, 153. [CrossRef]
- ⁶ Soares, M.; Christen, P.; Pandey, A.; Soccol, C. R. Fruity flavour production by *Ceratocystis fimbriata* grown on coffee husk in solid-state fermentation. *Process Biochemistry* **2000**, *35*, 857. [CrossRef]
- ⁷ Bolaños, M. L. R.; Varesche, M. B. A.; Zaiat, M.; Foresti, E. Phenol degradation in horizontal-flow anaerobic immobilized biomass (HAIB) reactor under mesophilic conditions. *Water science and technology* **2001**, *44*, 167. [CrossRef]
- ⁸ María-Pilar, M.; Damià, B. Environmental applications of analytical biosensors. *Measurement Science and Technology* **1996**, *7*, 1547. [CrossRef]
- ⁹ Zheng, H.; Yan, Z.; Wang, M.; Chen, J.; Zhang, X. Biosensor based on polyaniline-polyacrylonitrile-graphene hybrid assemblies for the determination of phenolic compounds in water samples. *Journal of Hazardous Materials* **2019**, *378*, 120717. [CrossRef]
- ¹⁰ Abbas, W.; Akhtar, N.; Liu, Q.; Li, T.; Zada, I.; Yao, L.; Naz, R.; Zhang, W.; Mazhar, M. E.; Zhang, D.; Ma, D.; Gu, J. (2018). Facile green synthesis of 3D nano-pyramids Cu/Carbon hybrid sensor electrode materials for simultaneous monitoring of phenolic compounds. *Sensors and Actuators B: Chemical* **2019**, *282*, 617. [CrossRef]

- ¹¹ Gan, T.; Wang, Z.; Gao, J.; Sun, J.; Wu, K.; Wang, H.; Liu, Y. (2018). Morphology-dependent electrochemical activity of Cu₂O polyhedrons and construction of sensor for simultaneous determination of phenolic compounds with graphene oxide as reinforcement. *Sensors and Actuators B: Chemical* **2019**, 282, 549. [CrossRef]
- ¹² Alim, S.; Kafi, A. K. M.; Rajan, J.; Yusoff, M. M. Application of polymerized multiporous nanofiber of SnO₂ for designing a bienzyme glucose biosensor based on HRP/GOx. *International Journal of Biological Macromolecules* **2018**, 123, 1028. [CrossRef]
- ¹³ Yotova, L.; Yaneva, S.; Marinkova D. Biomimetic nanosensors for determination of toxic compounds in food and agricultural products (Review). *Journal of Chemical Technology and Metallurgy* **2013**, 48, 215. [Link]
- ¹⁴ Avnir, D.; Coradin, T.; Lev, O.; Livage, J. Recent bio-applications of sol-gel materials. *Journal of Materials Chemistry* **2006**, 16, 1013. [CrossRef]
- ¹⁵ Iijima, S. Helical microtubules of graphitic carbon. *Nature* **1991**, 354, 56. [CrossRef]
- ¹⁶ Aybuke Bekmezci, S.; Soylemez, S.; Yilmaz, G.; Udum, Y. A.; Yagci, Y.; Toppare, L. A New ethanol biosensor based on polyfluorene-g-poly(ethylene glycol) and multiwalled carbon nanotubes. *European Polymer Journal* **2019**, 122, 109300. [CrossRef]
- ¹⁷ Michopoulos, A.; Kouloumpis, A.; Gournis, D.; Prodromidis, M. I. Performance of layer-by-layer deposited low dimensional building blocks of graphene-prussian blue onto graphite screen-printed electrodes as sensors for hydrogen peroxide. *Electrochimica Acta* **2014**, 146, 477. [CrossRef]
- ¹⁸ Gomez, F. J. V.; Martín, A.; Silva, M. F.; Escarpa, A. Screen-printed electrodes modified with carbon nanotubes or graphene for simultaneous determination of melatonin and serotonin. *Microchimica Acta* **2015**, 182, 1925. [CrossRef]
- ¹⁹ Niu, P.; Fernández-Sánchez, C.; Gich, M.; Navarro-Hernández, C.; Fanjul-Bolado, P.; Roig, A. Screen-printed electrodes made of a bismuth nanoparticle porous carbon nanocomposite applied to the determination of heavy metal ions. *Microchimica Acta* **2016**, 183, 617. [CrossRef]
- ²⁰ Sahin, M.; Ayranci, E. Electrooxidation of NADH on Modified Screen-Printed Electrodes: Effects of Conducting Polymer and Nanomaterials. *Electrochimica Acta* **2015**, 166, 261. [CrossRef]
- ²¹ Lin, J.; Fan, L.; Miao, R.; Le, X.; Chen, S.; Zhou, X. Enhancing catalytic performance of laccase via immobilization on chitosan/CeO₂ microspheres. *International Journal of Biological Macromolecules* **2015**, 78, 1. [CrossRef]
- ²² Brondani, D. Vieira, I. C.; Piovezan, C.; Ramos da Silva; J. M.; Neves, A.; Dupont, J.; Scheeren, C. W. Sensor for fisetin based on gold nanoparticles in ionic liquid and binuclear nickel complex immobilized in silica. *Analyst* **2010**, 135, 1015. [CrossRef]
- ²³ Gil, E. S.; Muller, L.; Santiago, M. F.; Garcia, T. A. Biosensor Based on Brut Extract from Laccase (*Pycnoporus sanguineus*) for Environmental Analysis of Phenolic Compounds. *Portugaliae Electrochimica Acta* **2009a**, 27, 215. [CrossRef]
- ²⁴ Silva, V. P. A. d.; Alves, C. R.; Dutra, R. F.; Oliveira, J. E. d.; Rondina, D.; Furtado, R. F. Biosensor amperométrico para determinação de peróxido de hidrogênio em leite. *Eclética Química* **2011**, 36, 143. [CrossRef]
- ²⁵ Sin, D. W. M.; Wong, Y. C.; Mak, C. Y.; Sze, S. T.; Yao, W. Y. Determination of five phenolic antioxidants in edible oils: Method validation and estimation of measurement uncertainty. *Journal of Food Composition and Analysis* **2006**, 19, 784. [CrossRef]
- ²⁶ Silverstein, R. M.; Webster, F. X. *Identificação espectrométrica de compostos orgânicos*, 7 ed., LTC: Rio de Janeiro, 2007.
- ²⁷ Costa N. C.; *Análise Orgânica: Métodos e Procedimentos para Caracterização de Organoquímicos*, volume 1, editora UFRJ: Rio de Janeiro, 2004.
- ²⁸ Li, J.; Wang, J. J.; Guo, X.; Zheng, Q.; Peng, J.; Tang, H.; Yao, S. Z. Carbon Nanotubes Labeled with Aptamer and Horseradish Peroxidase as a Probe for Highly Sensitive Protein Biosensing by Postelectropolymerization of Insoluble Precipitates on Electrodes. *Analytical Chemistry* **2015**, 87, 7610. [CrossRef]
- ²⁹ Santos, A. S.; Costa, V. C.; Felício, R. C. Comparative Study of Nanostructured Matrices Employed in the Development of Biosensors Based on HRP Enzyme for Determination of Phenolic Compounds. *Electroanalysis* **2015**, 27, 1572. [CrossRef]
- ³⁰ Dominguez, R. B.; Alonso, G. A.; Muñoz, R.; Hayat, A.; Marty, J.-L. Design of a novel magnetic particles based electrochemical biosensor for organophosphate insecticide detection in flow injection analysis. *Sensors and Actuators B: Chemical* **2015**, 208, 491. [CrossRef]
- ³¹ Oliveira, I. R. W. Z.; Vieira, I. C. Construção e aplicação de biosensores usando diferentes procedimentos de imobilização da peroxidase de vegetal em matriz de quitosana. *Química Nova* **2006**, 29, 932. [CrossRef]

- ³² Yang, S., Chen, Z., Jin, X., Lin, X. HRP biosensor based on sugar-lectin biospecific interactions for the determination of phenolic compounds. *Electrochimica Acta* **2006**, 52, 200. [[CrossRef](#)]
- ³³ Cao, X.; Luo, L.; Ding, Y.; Zou, X.; Bian, R. Electrochemical methods for simultaneous determination of dopamine and ascorbic acid using cetylpyridine bromide/chitosan composite film-modified glassy carbon electrode. *Sensors and Actuators B: Chemical* **2008**, 129, 941. [[CrossRef](#)]
- ³⁴ Chattopadhyay, K.; Mazumdar, S. Structural and Conformational Stability of Horseradish Peroxidase: Effect of Temperature and pH. *Biochemistry* **2000**, 39, 263. [[CrossRef](#)]
- ³⁵ Mohamed, S. A.; Aly, A. S.; Mohamed, T. M.; Salah, H. A. Immobilization of Horseradish Peroxidase on Nonwoven Polyester Fabric Coated with Chitosan. *Applied Biochemistry and Biotechnology* **2008**, 144, 169. [[CrossRef](#)]
- ³⁶ Silva, V. P. A.; Alves, C. R.; Dutra, R. F.; Oliveira, J. E.; Rondina, D.; Furtado, R. Ferro. Biossensor amperométrico para determinação de peróxido de hidrogênio em leite. *Eclética Química* **2011**, 36, 143. [[CrossRef](#)]
- ³⁷ Palod, P. A.; Singh, V. Improvement in glucose biosensing response of electrochemically grown polypyrrole nanotubes by incorporating crosslinked glucose oxidase. *Materials Science and Engineering: C* **2015**, 55, 420. [[CrossRef](#)]
- ³⁸ Wang, J. *Analytical Electrochemistry*, 3 ed., WILEY-VCH: New Jersey, 2006.
- ³⁹ ICH, ICH validation of analytical procedures: methodology, technical requirements for the registration of pharmaceuticals for human use. In *International Conference on Harmonization*, Geneva, Switzerland, 1995. [[Link](#)]
- ⁴⁰ Hernandez, S. R.; Kergaravat, S. V.; Pividori, M. I. Enzymatic electrochemical detection coupled to multivariate calibration for the determination of phenolic compounds in environmental samples. *Talanta* **2013**, 106, 399. [[CrossRef](#)]
- ⁴¹ Skoog, D. A.; Holler, F. J.; Nieman, T. *Princípios de Análise Instrumental*, 5 ed.; Editora Bookman: São Paulo, 2009.
- ⁴² Montereali, M. R.; Della Seta, L.; Vastarella, W.; Pilloton, R. A disposable Laccase-Tyrosinase based biosensor for amperometric detection of phenolic compounds in must and wine. *Journal of Molecular Catalysis B-Enzymatic* **2010**, 64, 189. [[CrossRef](#)]
- ⁴³ Ibarra-Escutia, P.; Gómez, J. J.; Calas-Blanchard, C.; Marty, J. L.; Ramírez-Silva, M. T. Amperometric biosensor based on a high resolution photopolymer deposited onto a screen-printed electrode for phenolic compounds monitoring in tea infusions. *Talanta* **2010**, 81, 1636. [[CrossRef](#)]
- ⁴⁴ Guivar, J. A. R.; Fernandes, E. G. R.; Zucolotto, V. A peroxidase biomimetic system based on Fe₃O₄ nanoparticles in non-enzymatic sensors. *Talanta* **2015**, 141, 307. [[CrossRef](#)]
- ⁴⁵ Vieira, S. N. *Tese de Doutorado*, Universidade Federal de Uberlândia, Uberlândia, 2011. [[Link](#)]
- ⁴⁶ Gil, E. S.; Muller, L.; Santiago, M. F.; Garcia, T. A. Biosensor Based on Brut Extract from Laccase (*Pycnoporus sanguineus*) for Environmental Analysis of Phenolic Compounds. *Portugaliae Electrochimica Acta* **2009a**, 27, 215. [[CrossRef](#)]
- ⁴⁷ Silva, J. S. *Dissertação de Mestrado*, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2010. [[Link](#)]
- ⁴⁸ Ozoner, S. K.; Erhan, E.; Yilmaz, F. Enzyme based phenol biosensors, *Environmental Biosensors*, Vernon Somerset, IntechOpen, **2011**. [[CrossRef](#)]
- ⁴⁹ Rocha, D. P.; Dornellas, R. M.; Cardoso, R. M.; Narciso, L. C. D.; Silva, M. N. T.; Nossol, E.; Richter, E. M.; Munoz, R. A. A. Chemically versus electrochemically reduced graphene oxide: Improved amperometric and voltammetric sensors of phenolic compounds on higher roughness surfaces. *Sensors and Actuators B: Chemical* **2018**, 254, 701. [[CrossRef](#)]
- ⁵⁰ Salvo-Comino, C.; Rassas, I.; Minot, S.; Bessueille, F.; Rodriguez-Mendez, M. L.; Errachid, A.; Jaffrezic-Renault, N. Voltammetric sensor based on electrodeposited molecularly imprinted chitosan film on BDD electrodes for catechol detection in buffer and in wine samples. *Materials Science and Engineering: C* **2020**, 110, 110667. [[CrossRef](#)]