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Development of a Laccase Biosensor for Determination of Phenolic Micropollutants in Surface Waters

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Abstract: Laccase is a poliphenoloxidase enzyme that catalyzes the oxidation of phenolic compounds in the corresponding quinones. The current obtained in this redox process can be used for quantitative analysis. In this work, a carbon paste biosensor modified gluteraldehyde functionalized silica and an enzymatic extract of the Pycnoporus sanguineus fungi as a lacase source is proposed for phenol determination. The effect of carbon paste and electrolyte composition, pH from 3.0 to 8.0, start potential from 0.55 to 0.25 mV, scan rate from 5 to 25 mV s^{-1} and potential pulse amplitude from 10 to 60 mV on the differential pulse voltammetric response was investigated. A linear correlation of $r^2 = 0.9946$ was obtained for the phenol content (catechol) in the concentration range from 50 to 500 nmol L^{-1}, with a detection limit of 30 nmol L^{-1}. This biosensor was used for the determination of different kinds of phenolic compounds, presenting a better response for catechol.

Keywords: Biosensor; laccase; phenolic micropollutants; catechol; *Pycnoporus sanguineus* fungi; functionalised silica

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Introduction

Each year, huge volumes of pollutants are released into the environment, negatively impacting on aquatic ecosystems [1]. The variety of pollutants is very diverse, all resulting from various anthropogenic activities, but phenolic pollutants deserve a special mention, since it is an

ubiquitous class of industrial contaminants, which can be persistent even at low concentrations and still cause damage to the aquatic biosphere [2,3]. Brazilian legislation, CONAMA Resolution 357 [11], establishes limits for phenolic compounds of different classes in surface waters. For river class 2, which integrates most of the surface waters used for domestic supply and in industrial use, the threshold for these compounds are: 0.1 μg L^{-1} of 2-chlorophenol, 0,3 μg L^{-1} of 2,4-dichlorophenol 0.003 mg L^{-1} of total phenols (substances that react with 4-aminoantipyrine), 0.009 mg L^{-1} of pentachlorophenol and 0.01 mg L^{-1} 2,4,6-trichlorophenol. For treated water, the Ordinance 2914 (Brazil) [12] provides the limits and regulations for the above compounds, for which different threshold values apply.

The effective monitoring of phenolic pollutants requires the development of sensitive and selective methods. It is known that phenolic compounds are separated by chromatography before detection and this takes time and requires pre-concentration, including expensive equipment that is also not portable. In this context, electrochemical methods are highlighted by low cost, high sensitivity, convenience and easy adaptation to miniaturized systems and applications in the field [3-5].

The phenolic compounds may be oxidized at potentials that vary depending on the substitution pattern, at about 0.2 to 1.2 V vs. Ag/AgCl, pH = 7 [3,6,7]. However, when phenols are oxidized, especially mono- or di-hydroxy substituted in the meta position examples, whose anode potential is very high, they can undergo a number of side reactions, particularly polymerization, leading to passivation of the electrode. For this reason careful consideration is needed in deciding which potential to apply [3,6,7].

Thus, electrochemical biosensors emerge as promising analytical tools, especially since detection is also possible at low micro-molar range (sub ppb range) and minimum sample preparation is required [8-12]. These devices combine the sensitivity of electrochemical transducers, *i.e.* amperometric or potentiometric selectivity of biological recognition agents, *i.e.* enzymes, plant tissues, etc. [3-5].

In this study the focus was also to construct a different sensor platform for laccase biosensor analysis, using functionalized silica. An electrochemical matrix was obtained by silica gel surface organofunctionalization. This process is based on the reactivation of the active hydrogen atom of the silanol groups that is dispersed on the silica gel surface, which has the ability of reacting with organosilyl groups to give some organic nature to the inorganic carrier. The immobilization of this desired reactive atom group gives versatility to this surface; consequently, developing various functions that will interact with the active site of the phenolic compound under investigation [15].

Among the most promising systems, amperometric biosensors based on oxidase enzymes can be highlighted for practicality, good selectivity and high sensitivity. These biosensors can measure the current generated by the reaction biocatalyzed oxidation or reduction of electro-active species at the electrode surface, which in general will be processed at a potential of about 0 mV (vs. Ag/AgCl). This potential offers the possibility of minimizing, both the contribution of interfering species (easily oxidized or reduced) and the possibility of minimizing side reactions [3,5].

Extracellular oxydases are distinguished by the ease with which they are produced or isolated and purified, therefore their application in this study. Laccases are polyphenol oxidases produced by various fungi, plants and bacteria. The enzyme is a glycoprotein, which contains copper in its active site and catalyzes the oxidation of phenolic substrates, being molecular O_2 as an agent for regeneration of the active form of the enzyme. The oxidation of the substrate occurs by abstraction of an electron from the phenolic compound generating a radical phenolate ion as can be seen in the following Eqns. (1) and (2) [3,5,8].

$$2\ Cu^{2+}\text{ - Laccase} + \text{phenol} \rightarrow 2\ Cu^{+}\text{ - Laccase} + \text{phenolate radical} + 2\ H^{+} \quad (1)$$

$$2\ Cu^{+}\text{ - Laccase} + O_2 + 2\ H^{+} \rightarrow 2\ Cu^{2+}\text{ - Laccase} + H_2O \quad (2)$$

The radicals formed can be converted into quinones by a second stage enzyme catalyzed reactions or by spontaneous non-enzymatic disproportionation. Quinones and free radicals that are capable of electrochemical reduction, a reaction that can be monitored electrochemically [8]. Besides the optimum pH, the efficiency of enzymatic biosensors also depends on factors such as the method of immobilization, which besides conferring biosensor stability, can also lead to increased activity [3,5,9].

The immobilization technique emplyed during enzyme incorporation and biosensor construction, vary according to the mechanism employed and simplicity of biosensor construction. Among the simplest methods, the one involving the immobilization for occlusion paste and carbon adsorption, are most popular. However, these methods are based on physical mechanisms of immobilization (using hydrophobic and Van der Waals interaction), which may give the biomolecule present in return greater leaching capabaility. An alternative approach that promotes greater stability is the immobilization and incorporating of a nanocomposite functionalized with groups able to establish covalent bonds, in order to reduce loss of biological material. Emphasizing applications using functionalized silicas with amide groups, *i.e.* glutaraldehyde [33].

Thus, this study aimed to evaluate the use of functionalized silica with gluteraldehyde for immobilization of the enzyme laccase (*Pycnoporus sanguineus*) and the development of a biosensor matrix for phenolic compounds. The functioning of the biosensor was further optimized (enzyme concentration, stability, sensitivity and response time) for the determination of phenolic micropollutants in samples of surface municipal and treated water collected in the vicinity of the city of Trindade, Goiás (Brazil).

Experimental

Chemicals, Reagents, Stock, and Standard Solutions

The reagents chlorophenol, phenol, catechol, resorcinol, cresol and nitrophenol were purchased from Sigma-Aldrich (St. Louis, USA). All standard solutions were prepared using analytical grade reagents and purified water (conductivity $\leq$ 0.1 mS cm^{-1}) using a Millipore Milli-Q system (Millipore SA, Molsheim, France). The buffers employed as supporting electrolyte were prepared according to established procedures [10]. The analyzed sample was treated water, obtained in a Water Treatment Station (WTS).

Laccase Preparation

Bottles of 250 mL, plus a conical flask containing 50 mL of culture medium were inoculated with 5 disks (6 mm) mycelia *Pycnosporus sanguineus* (CCT-4518) and incubated at 28 °C under constant agitation of 150 rpm. After 24 hrs, two microliters were added 2,5-xylidine each flask and the flasks were again placed on agitation (150 rpm at 28 °C). After 48 hours, the contents of the flasks were vacuum filtered with filter paper and frozen. The medium was 1.25 % (w/v) malt extract and 0.0005 % (w/v) copper sulfate.

Silica Organofunctionalization

A sample of 30.0 g of activated silica gel was suspended in 50.0 mL of dry xylene and subjected to mechanical agitation under reflux (140 ° C) and nitrogen atmosphere for 1 hr. To this suspension were added 10.0 mL of 3-aminopropyl triethoxysilane (APTES) dropwise while stirred. The mixture was refluxed for another 72 hrs period and the solid was then filtered and washed with water and ethanol. This immobilized surface (so called $SiNH_2$) was vacuum dried at room temperature for 12 hrs.

In this step 10.0 g of modified silica ($SiNH_2$) were suspended in 50.0 mL of xylene and 5.0 mL of gluteraldehyde. The reaction system was maintained under mechanical stirring and nitrogen atmosphere at 80 °C for 24 hrs. The solid formed was called (SiGlu). After cooling the suspension, the solid was separated (SiGlu) by vacuum filtration. The collected solid material was washed with xylene and ethanol, respectively, and finally dried for 12 hrs at 80 °C followed by another 24 hrs under vacuum.

Characterization of Modified Silica

The content of gluteraldehyde on the silica gel surface was determined by elemental analysis. The surface area of SiGlu material was determined by using a Perkin Elmer Instrument with combustion temperature of 965 ºC and reduction on 640 ºC. The ^{29}Si and ^{13}C NMR spectra of the solid samples were determined at 59.61 Hz using a Varian Mercury Plus 300 spectrometer at room temperature. For each determination, approx. 1 g of modified silica was compacted into a 7 mm silicon nitrite rotor, employing a magic angle spinning speed of 3 kHz.

Electrochemical Tests

The voltammetric tests were conducted on a Potentiostat/galvanostat μAutolab III® software integrated with GPES 4.9®. The electrochemical cell used has a volume of 10 mL and a system of three electrodes. The laccase biosensor coupled to the support was the working electrode, the saturated calomel electrode DropSens® (Oviedo, Spain) was used as reference electrode and platinum electrode as a counter or auxiliary electrode. Electroanalytical parameters used for differential pulse voltammetry (DPV) were: equilibration time 60 s; modulation time 0.05 s, interval time 0.5 s; measure the potential 4.95 mV, modulation amplitude 50 mV, initial potential 0.35 V and final potential -0.1 V vs. SCE. All measurements were performed at room temperature.

Biosensor Preparation

The modified carbon paste silica organofunctionalized biosensors were prepared from physical mixtures of different proportions of graphite, mineral oil and silica support. The better signal and less response time biosensor was obtained with a mixture of 85 mg graphite, 15 mg SiGlu and 500 μl of crude extract of purified laccase. The mixture was homogenized in a mortar with pestle for 15 minutes and drying at room temperature, then 35 mg of mineral oil were added to obtain homogeneous paste. This biosensor developed from modified carbon paste with the SiGlu *Laccase* was designated B-SGL.

Stability, Sensitivity, and Response Time

The studies of stability, sensitivity and response time of the B-SGL sensor were tested against 0.1 mmol L^{-1} solution of catechol in phosphate buffer 0.1 mol L^{-1}, pH = 5.0. For comparison purposes a carbon paste biosensor prepared in similar proportions, but without the incorporation of SiGlu, herein designated PCL was also evaluated in these studies. The performances of each of the B-SGL and PCL biosensors were evaluated and compared for baseline studies.

Electrochemical Treatment of Data

The data obtained in GPES 4.9® software were treated with the purpose of improving the visualization and identification of peaks without introducing any artifacts. Although, in some cases it was observed a reduction of the intensity of peak (< 10 %) occurred, when compared to the voltammogram obtained in the untreated data. The current peaks presented in the figures of the voltammograms were evaluated to that of the so-called untreated voltammograms. The plot of the voltammetric curves for final presentation in this study was drawn using Origin Pro 8® software.

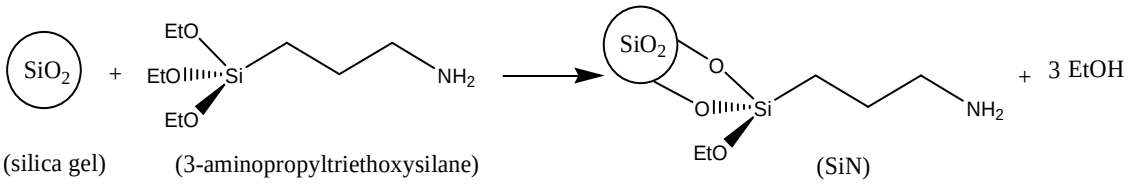
Surface Municipal Water and Treated Water Supply

The water used in the study was collected in the Arrozal stream, which is a spring that is responsible for the public water supply of the city of Trindade, Goiás (Brazil). The collection procedure was recommended by CETESB [13]. The treated water was collected at the WTS exit of the stream. These waters were characterized by means of physical, chemical and bacteriological according to Standard Methods [14].

Results and discussion

Synthesis and Characterization of Modified Silica

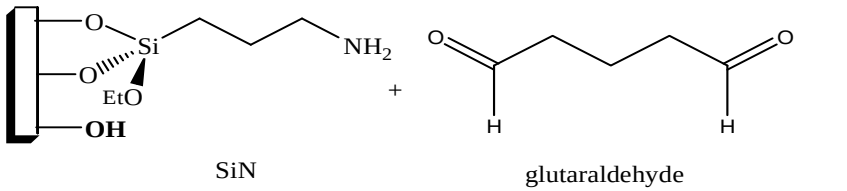
The synthetic procedure utilised to produce the sensor SiGlu was based on the modification of silica gel with 3-aminopropyltriethoxysilane to obtain the precursor surface (SiN), as according to Eqn. (3)**.**



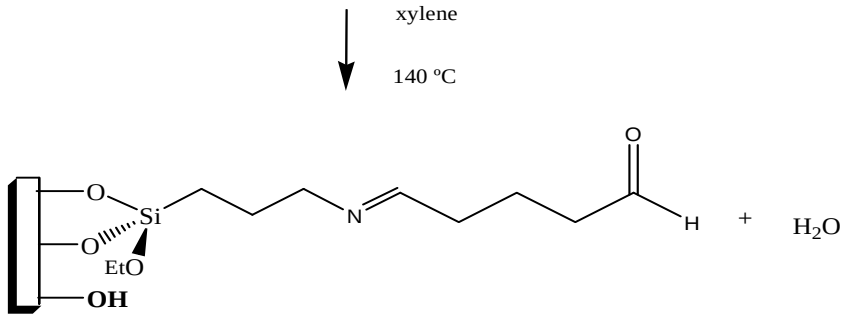
(silica gel) (3-aminopropyltriethoxysilane) (SiN)

(3)

The precursor (SiN) reacted with solution 25 % of gluteraldehyde to form the product SiGlu, as according to Eqn. (4):



SiN glutaraldehyde



SiGlu

(4)

The amount of gluteraldehyde anchored onto silica gel surface was determined by elemental analyses using a PE-2400 elemental analyzer (Perkin-Elmer, USA), and the obtained value was 1.57 mmol of gluteraldehyde per gram of silica.

SiGlu nitrogen adsorption–desorption isotherms are shown in Fig. 1. These isotherms show that the adsorption–desorption process is not reversible. This is a consequence of the hysteresis loops caused by capillary condensation. The isotherm is reversible up to a relative pressure of about 0.50 units. Irreversibility is observed between 0.43 and 0.85 units, whereas the condensation in primary mesopores takes place slightly above the latter pressure limit, but the adsorption and desorption branches are not parallel to each other, resulting in the hysteresis loop. A specific area of 174 $m^2 g^{-1}$ was determined by a BET method.

Solid-state NMR of the Si-29 spectrum of SiD presented four typical peaks as shown in Fig.2. The first one at -51 ppm was assigned to the silicon atom of the silylating agent, which was bounded to one hydroxyl group [$RSi(OSi)_2(OH)$], denominated T^3 signal.

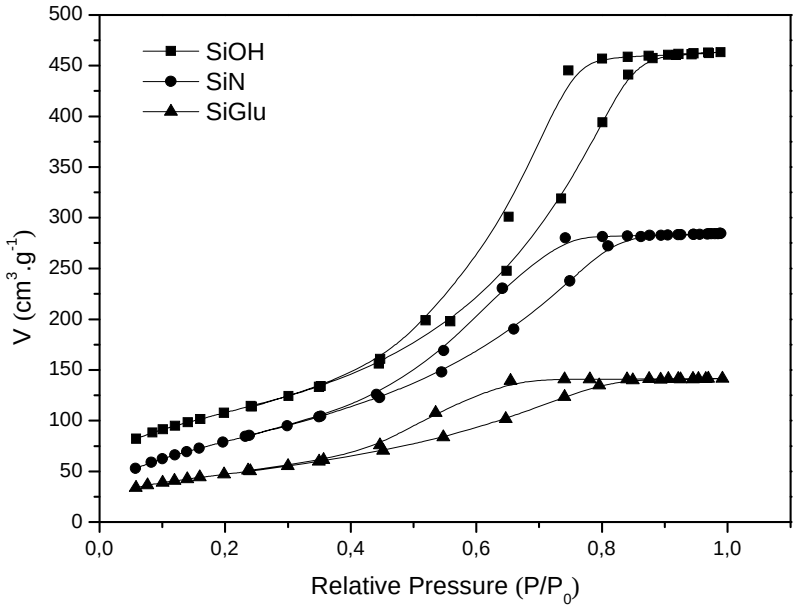


Fig.1: *Nitrogen adsorption isotherms from silica* (SiOH) *and modified silicas* (SiN *and* SiGlu).

The second peak at -59 ppm was related to $RSi(OSi)_3$ (T^4 signal). The T^3 and T^4 signals confirm that the organic groups were covalently bonded to the silica matrix. Other two peaks, at -95 and -105 ppm, were related to pure surface signals $Si(OSi)_3OH$, named Q^3 signal, and $Si(OSi)_4$, corresponding to the Q^4 signal, respectively [16, 17].

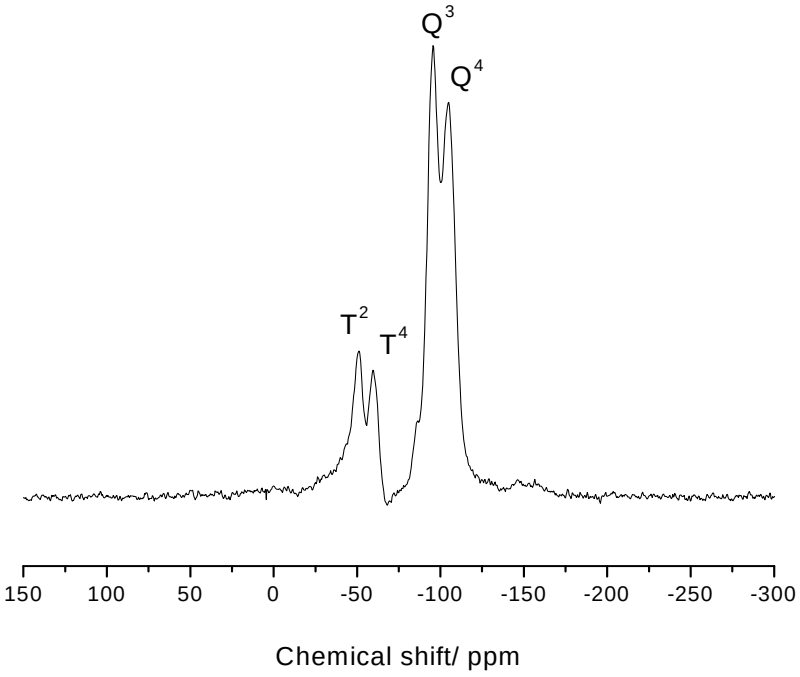


Fig. 2: *[29]Si NMR spectra of the solid material SiGlu.*

The ^{13}C NMR spectra of the solid product of the reaction of the silica with gluteraldehyde (SiGlu), because they are in the same chemical environment , some of the peaks of the spectrum of this material could not be visualized , but is possible to see clearly the presence of some characteristic peaks: on 9 and 23 ppm, arising from carbons (-CH_2-) propyl grouping of the APTES , and the second peak was almost concealed by a peak in the same region assigned to the CH_2 fragment of gluteraldehyde chemically bonded to the support, a peak in 41 ppm referring α-carbonyl carbon, a peak of low intensity at 65 ppm assigned to the carbon (-CH_2-) attached to the imine nitrogen, and finally a peak at 148 ppm attributed to the unsaturated carbon (-CH=) connected in the nitrogen of the imine. Due to the change in chemical environments which causes the displacement of the overlapping peaks, some groups and peaks could not be characterized [18-21].

Stability, Sensitivity, and Response Time

The peak current responses obtained for quinone reduction of catechol (Figure 3) was about two times higher to that obtained for the biosensor PCL [5], showing that incorporation of SiGlu leads to increased sensitivity when compared to the B-SGL biosensor.

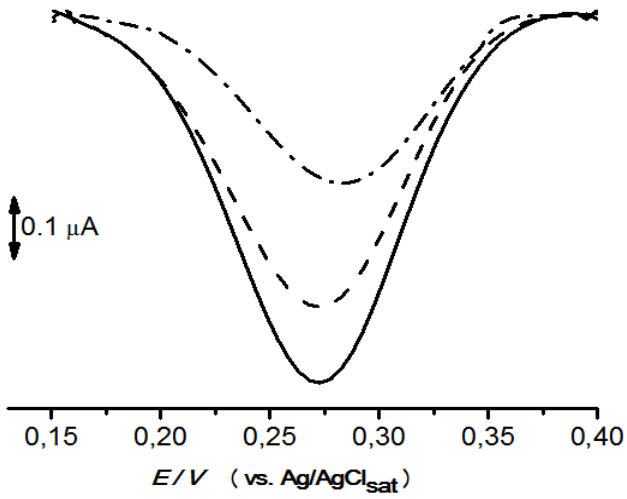


Fig. 3: *Reduction of the quinone catechol biosensors*: B-SGL (——), PCL (– – – –), and PC (— · —). Phosphate buffer 0.1 mol L^{-1}, pH = 5.0 solution.

The stability of the constructed biosensor was investigated by evaluating both the stability during storage and operation of the biosensor. Although the lifespan expressed by the maintenance of relative stability has been shown to be equivalent for both biosensor systems,

i.e to at least 20 days of storage, the B-SGL biosensor showed better stability data that was significantly higher during continuous use, compared to the PCL biosensor. These results are consistent with stability offered by the enzyme being covalently immobilized during biosensor construction, which further reduces leaching of the enzyme. On the contrary, the B-SGL biosensor showed no significant differences regarding the response time when compared to the PCL biosensor.

Affinity

Assays to investigate the affinity to different equimolar solutions obtained with different phenolic compounds were also investigated. The sensitivity of catechol and hydroquinone proved superior to that observed for mono-phenols, whose relative sensitivity ranged from 10 to 40% of that observed for catechol.

Experimental Parameters

The instrumental parameters for applied pulse amplitude and scan rate were rigorously evaluated in previous studies and confirmed during optimization of the assay in this study. The pulse amplitudes found to be working best during the experiment was in the order from 20 to 60 mV. The results obtained have shown that larger values for the pulse amplitude give greater sensitivity, but loss of resolution was observed. Thus the value of 50 mV adopted proved to be quite satisfactory. In turn, considering that the resolution test is usually compromised in DPV scan speeds greater than 15 mV s^{-1}; the value being set at 10 mV s^{-1}.

In order to evaluate the electrolytic conditions, the concentrations from 0.05 to 0.5 mmol L^{-1} are usually employed. Lower concentrations can lead to ohmic drop and require greater overpotentials for the redox process execution. Thus, a concentration of 0.1 mmol L^{-1} was adopted after successful evaluation.

Calibration Curve

Figure 4 shows the results obtained for the detection of phenol content (with the highest sensitivity for catechol obtained) under optimal conditions. It was observed that a linear correlation with $r^2 = 0.99456$ in the concentration range of 0.05 to 0.5 µmol L^{-1} with a detection limit (LOD) of 30 nmol L^{-1} is attainable.

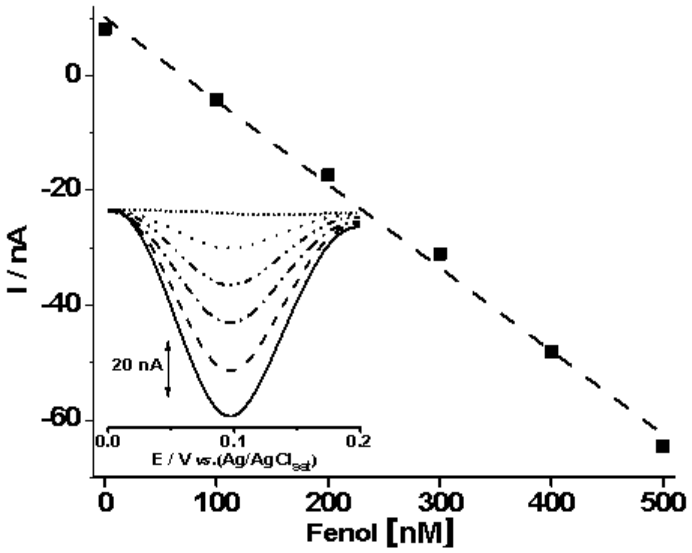


Fig. 4: *Calibration curve and differential pulse voltammograms obtained for different concentrations of total (catechol) in phosphate buffer (0.1 mol L^{-1}, pH = 5.0).*

The performance of the B-SGL biosensor was superior to that observed in the literature for other laccase biosensors [3,5]. These results were further outstanding, emphasizing the low cost of production of laccase enzyme extracts and excellent stability observed for the enzyme to maintain its sufficient activity, even after long periods of storage under refrigeration. Furthermore, when immobilized on SiGlu, the biosensor retained up to 90 % of its activity for more than 20 repetitions.

Characterization of Surface Municipal Water and Biosensor Application

The characterization of the raw and treated water samples was also performed, in order to showcase the application of the constructed biosensor to real samples analysis. Table 1 shows the results obtained in the determination of percentage recoveries from catechol added to water samples from the fountain public water supply of the Trindade City, Goiás (Brazil).

The recovery values obtained ranged between 95-103 % of the value added, further indicating that the recoveries were higher when the phenol concentration added was equal or less than 100 nmol L^{-1} (sample A). When higher phenol concentrations were added (more than 100 nmol L^{-1}), the recoveries were better and slightly less than 100 %. Similar trends were observed in the evaluation of sample B.

Table I: *Evaluation of the recovery of phenol from water from the public municipal water supply of the Trindade City and standard addition*.

Samples	Phenol added (nmol L^{-1})	Recovery (%)
A	50	103
	100	101
	200	99
	300	98
	400	95
	500	97
B	50	102
	100	102
	200	99
	300	98
	400	99
	500	101

It should also be noted that the analysis by the standard addition method in any of the samples detected the presence of phenolic compounds, and background subtraction was employed to overcome this limitation.

Conclusions

Given the results expressed in this study, it could be concluded that the modification of laccase biosensors using silica functionalized with gluteraldehyde improved stability, sensitivity and efficiency for the determination of phenolic micropollutants in municipal waters for public supply. For the constructed biosensor it was determined that specific parameters are important in the optimization of the B-SGL biosensor, which included the amount of carbon paste and electrolyte composition, pH from 3.0 to 8.0, start potential from 0.55 to 0.25 mV, scan rate from 5 to 25 mV s^{-1} and potential pulse amplitude from 10 to 60 mV on the differential pulse voltammetric responses investigated.

The B-SGL biosensor operation also delivered a linear correlation of $r^2 = 0.9946$ that was obtained for the phenol content (catechol) in the concentration range from 50 to 500 nmol L^{-1}, with a detection limit of 30 nmol L^{-1}. Application of the B-SGL biosensor to recovery studies, using municipal water samples, have shown that spiking the water with phenol concentrations higher than 200 nmol L^{-1}, delivered better recoveries compared to when the phenol concentrations was equal to or smaller than 100 nmol L^{-1}.

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