Electroanalytical determination of catechol by a biosensor based on laccase from *Aspergillus oryzae* immobilized on gold screen-printed electrodes

S J Caballero¹, M A Guerrero¹, L Y Vargas¹, C C Ortiz¹, J J Castillo¹, J A Gutiérrez² and S Blanco¹

¹Universidad Industrial de Santander, Bucaramanga Cra. 27 Cl. 9. Colombia.
²Universidad del Quindío. Armenia Cra. 15 Cl. 12N. Colombia
Email: ²siblanco@uis.edu.co

Abstract. In the present work, the detection of catechol (CC) was studied by using a biosensor of gold screen-printed electrode functionalized with laccase and cysteine as a linker. Cyclic voltammetry (CVs) studies were performed in order to determine the stability range of the biosensor and the redox processes present on the surface of the modified screen-printed gold electrodes (SPGE). CVs studies showed a variation in the response of the electrodes, finding one oxidation signal at 400 mV in presence of catechol, corresponding to the bio-electrocatalytic oxidation of catechol. The chronocoulometric response of the sensor to different concentration of catechol was measured applying 500 mV, obtaining a linear response of the current in function of the catechol concentration. The linear range of the detection was 0.02 – 2.4 mM, with a sensibility of 3.93 μA μM⁻¹ cm⁻² and a detection limit of 1.2 μM with a response time lower than 5 s. The results obtained in this work open new possibilities for the development of portable electrochemical biosensors for quick and efficient detection of catechol in industrial wastewater.

1. Introduction
The increase and lack of control in the amount of toxic waste founded in the environment has motivated scientists to find new methods of detection and quantification of these polluting substances. Specifically, a considerable number of organic pollutants with phenolic structures are found in industrial wastewater, mainly due to the fact that are related to the petrochemical, agrochemical and textile processes [1, 2]. Catechol (CC) is a highly corrosive and toxic phenolic compound with carcinogenic effects, which also causes endocrine disruptions that affect human development and reproduction [1, 3]. The conventional methods for the determination of catechol are based on analytical techniques such as high performance liquid chromatography, mass spectrometry, fluorescence, among others [4, 5]. All these techniques require long sample treatments (conservation, pre-concentration, etc.), high cost equipment, specialized operators and extensive analysis times. Electrochemical sensors emerge as an alternative analytical method for the detection of compounds in wastewater, which offer a quick, in situ, and sensitive response with low cost equipment and easy handling. Specifically, electrochemical sensors activated with biomolecules allow the selective determination of different compounds [6].

In recent years, the use of biomolecules such as enzymes have generated an impact in industries due to their bio-electrocatalytic properties and selectivity. The laccases are enzymes that are found in certain
plants and fungi of nature with ancient enzymatic systems, which lead to an extensive study of their catalytic mechanism [7, 8]. The laccase is a multi-copper protein, composed of a group of four copper atoms (copper type I, copper type II and two copper atoms type III) that form the active site of the enzyme using molecular oxygen to oxidize several aromatic and non-aromatic compounds, accompanied by the reduction of four electrons from oxygen to water. Therefore, laccase biosensors directly catalyze the oxidation of catechol (hydroquinone) to p-quinone without solvent and allow the quantitative determination of hydroquinone [9]. In this work, the determination of catechol was evaluated using a laccase-cysteine/cysteine/gold screen printed electrode by chronoamperometric methods, furthermore, the possible interference of other common contaminant substances present in the industrial wastewater was analyzed.

2. Experimental procedure

2.1. Materials
Laccase (from Aspergillus sp., Novozym 51003), L-cysteine (Cys), Catechol ≥99%, 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich. All other chemicals were of analytical grade. Screen-printed Gold Electrodes (SPGE) DRP-250BT, the working (4 mm diameter) electrode is made of gold, counter electrode is made of gold, reference electrode is made of Ag and electric contacts are made of silver was purchased DropSens.

2.2. Enzymatic activity of laccase
The enzymatic activity was determined as the oxidation substrate of 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). For the assay was mixed 1 mL ABTS 0.50 mM and 1 mL of acetate buffer 0.10 M (pH 5) with 10 µL of laccase dissolved in buffer (from Aspergillus oryzae) 1:10 (dilution factor). This mixture was analyzed immediately using a UV-vis UV-1800 spectrophotometer (λ = 420 nm, ε_{420} = 3.6x10^4 mol / L cm^{-1}) for 30 s.

2.3. Immobilization of laccase biosensor
Gold electrodes were modified by deposition of cysteine solution (10 mM) for cross-linking reaction between the laccase and the surface of the electrode. Then, 5 µL of a mixture Cys-laccase (1:1) was deposited on the cysteine modified electrode. The electrodes were kept refrigerated at 4° C for a maximum time of 15 h before any electrochemical test. All measurements of cyclic voltammetry and chronoamperometry were with a potentiostat / galvanostat PGSTAT101.

3. Results and Discussion

3.1. Cyclic voltammetry studies
The electrochemical response of the SPGE at different concentrations of catechol in acetate buffer pH 5 is shown in figure 1. The electrode response in buffer solution without catechol showed one oxidation peak at 720 mV followed by the oxygen reaction. In the back scan two reduction peaks appears at 263 and -507 mV. These peaks are related to the oxidation and reduction of the species present in the buffer solution. In the presence of 1 mM of catechol two new peaks appears corresponding to the oxidation and reduction of catechol at the surface electrode. The first one appears at 76 mV and is related to the oxidation of hydroquinone to generate quinone. The second one appears at 0 mV and correspond to the quinone reduction. After new additions of catechol, the oxidation current increases as can be seen in the insert, a the linear relation between the oxidation peaks current with the catechol concentration with an adjusted coefficient of determination (r^2) of 0.97.
Figure 1. Cyclic voltammetry response of the gold electrode in different concentrations of catechol (CC). Scan rate 50 mV s⁻¹.

The gold electrode in the presence of catechol has good signal current, but without the selectivity to our specific analyte. In this way, it is well known that cysteine act as linker promoting the electron transfer between organic molecules and metallic electrodes. To ensure this activity it is necessary the stabilization in the metallic electrode surface. Therefore, the electrode surface was modified with amino acids such as cysteine by chemical absorption. The figure 2(a) shows the response of gold/cysteine electrode for different catechol concentrations. The variation in the electrochemical response of the electrode are related to the formation of a full coverage self-assembled monolayer of cysteine on the surface, confirmed by the intensity decreases in the redox peak current intensity of the catechol reaction on gold surface. Two new peaks appear at 717 and -850 mV but without a clear linear relation with the catechol concentration (r² = 0.33). Figure 2(b) shows the electrochemical response of the laccase/cysteine/gold SPE, as noted, some new peaks appear related to the redox process of the catechol on the laccase. But it is important to note that before this experiment we evaluated the response on laccase/gold SPE (results do not show), on this configuration of the electrodes, and the enzyme showed poor stability, for that reason and to improve the electron transference we use the cysteine as a cross-linker.

In order to increase the stability of the enzyme in the gold electrode, the last layer of enzyme was replaced by one of cysteine-enzyme, additionally the electronic transference to the lower layers was improved. The results are shown in figure 3(a), in this case we change the potential window used to perform the experiment and to avoid the water hydrolysis reactions (hydrogen reduction and oxygen oxidation), additionally we reduce the scan rate to 20 mV s⁻¹ to obtain a better appreciation of the surface electrode reactions. The cyclic voltammograms shows one redox process, related to the autocatalytic reaction of the catechol with the enzyme (figure 3(b)), without interference of secondary reactions. Catechol is a compound known as ortho-dihydroxybenzene or hydroquinone from the phenol family. The laccase enzyme uses its catalytic activity, starting with copper type I that is found in the active site where the substrate oxidation (catechol) takes place, extracting an electron, which is internally transferred through a cysteine and a histidine to the trinuclear site which contains copper type II and III, and as consequence to produce the reduction of molecular oxygen in water.
As noted in the insert of figure 2(a) the anodic current peak has a good linearity ($r^2 = 0.993$) in function of catechol concentration in the evaluated range. It is of standing out that the experiments where performed using one electrode for several test showing high stability after many cycles, confirming the expected function of cysteine.

3.2. Chronoamperometric response of laccase biosensor
In the chronoamperometric measurements, an oxidation potential was established at low catechol concentrations around 500 mV. The chronoamperometric response of the sensor was evaluated adding consecutive doses of 14 mM catechol each 60 s. The results are shown in figure 4, where it seen an increment in the current for each added dose, with a response time $\leq 5$ seconds. The insert shows the linear relationship between the average current for each addition and the catechol concentration. Where
the linear range is 0.02 - 2.4 mM, with a sensitivity corresponding to the slope of the graph (ΔI / ΔC A) of 3.93 μA μM⁻¹ cm⁻² and a detection limit the 1.2 μM.

Figure 4. Biosensor chronoamperometric response in the presence of catechol, E = 500 mV. Insert is the linear plot for the average current response vs. [CC] μM.

To evaluate the laccase biosensor selectivity, interference measurements were made using other petrochemical industrial residues such as toluene and phenol. Figure 5 shows the chronoamperometric response for catechol (substrate of interest) additions, followed by consecutive additions of toluene and phenol, showing no response or current changes. The consecutive addition of the three compounds was repeated four times, showing no variation of the current response in comparation with the first dose, it implies that the biosensor evaluated in this work keep high stability and selectivity after several additions of other contaminants.

Figure 5. Biosensor amperometric response in presence of catechol, toluene and phenol, E = 500 mV.
4. Concluding remarks

An electrochemical biosensor for the determination of catechol has been developed by the bioconjugation of laccase and cysteine on the gold electrode surface. The laccase-cysteine/cysteine/gold SPE sensor shows a sensitivity of 3.93 μA μM⁻¹ cm², a detection limit of 1.2 μM, and a response time of less than 5 s. Furthermore, the analysis of the interference with toluene and phenol confirm the stability and selectivity of the biosensor, which suggests its possible use as a good alternative for the detection of catechol in industrial wastewater, in addition to having short response times, simple and low-cost procedures compared with conventional methods. Finally, this study opens doors for new research in the development and control of environmental contaminants that represents a big problem in the world.

Acknowledgments

We are grateful to Industrial University of Santander, School of Chemistry, Investigation Group in Biochemistry and Microbiology (GIBIM), Vicerrectoría de Investigación y Extensión (VIE-UIS) for the financial support of the internal project code: 2317 and for Estancias Postdoctorales Program.

References

[1] Zhou Y, Tang L, Zeng G, Chen J, Cai Y, Zhang Y, and Tang W 2014 Biosens. Bioelectron. Mesoporous carbon nitride based biosensor for highly sensitive and selective analysis of phenol and catechol in compost bioremediation 61 519-25.
[2] U.S. National Library of Medicine, Toxicology Data Network
[3] Marrubini G, Calleri E, Coccini T, Castoldi A and Manzo L 2005 Chromatographia Direct Analysis of Phenol, Catechol and Hydroquinone in Human Urine by Coupled-Column HPLC with Fluorimetric Detection 62 25-31.
[4] Bhanger M, Niaz A, Shah A, and Rauf, A 2007 Talanta Ultra-trace level determination of hydroquinone in waste photographic solutions by UV–vis spectrophotometry 72 (2) 546-53.
[5] Lonergan G, Mew E, Schliephake K and Baker W L 1997 FEMS microbiology letters Phenolic substrates for fluorometric detection of laccase activity 153 (2) 485-90.
[6] Shokker R, Sehgal S, Kamthania M and Kumar A 2011 Enzyme research 217861 Laccase: microbial sources, production, purification, and potential biotechnological applications 2011 11.
[7] Mayer A M and Staples R C 2002 Phytochemistry Laccase: new functions for an old enzyme 60 (6) 551-65.
[8] Qu J, Lou T, Kang S and Du X 2014 Analytical Letters Laccase biosensor based on graphene–chitosan composite film for determination of hydroquinone 47 (9) 1564-78.
[9] Rodriguez-Delgado M M, Alemán-Nava G S, Rodriguez-Delgado J M, Dieck-Assad G, Martinez-Chapa S O, Barceló D and Parra R 2015 TrAC Trends in Analytical Chemistry Laccase-based biosensors for detection of phenolic compounds 74 21-45.