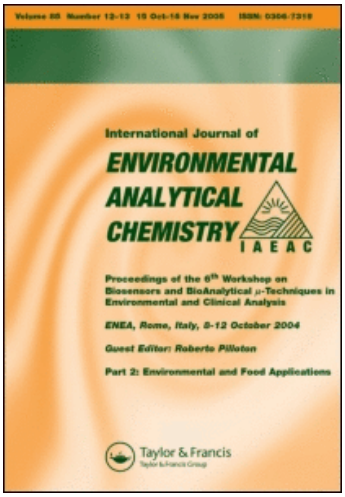


International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Characterization of graphite electrodes modified with laccases from *Trametes hirsuta* and *Cerrena unicolor* and their use for flow injection amperometric determination of some phenolic compounds

Behzad Haghighi[a]; Anna Jarosz-Wilkołazka[b]; Tautgirdas Ruzgas[c]; Lo Gorton[c]; Andrzej Leonowicz[b]
[a] Department of Chemistry, Institute for Advanced Studies in Basic Sciences, Gava Zang, Zanjan, Iran [b] Department of Biochemistry, Maria Curie-Skłodowska University, 20-031 Lublin, Poland [c] Department of Analytical Chemistry, University of Lund, S-221 00 Lund, Sweden

To cite this Article Haghighi, Behzad , Jarosz-Wilkołazka, Anna , Ruzgas, Tautgirdas , Gorton, Lo and Leonowicz, Andrzej(2005) 'Characterization of graphite electrodes modified with laccases from *Trametes hirsuta* and *Cerrena unicolor* and their use for flow injection amperometric determination of some phenolic compounds', International Journal of Environmental Analytical Chemistry, 85: 9, 753 — 770
To link to this Article: DOI: 10.1080/03067310500153710
URL: http://dx.doi.org/10.1080/03067310500153710

Intern. J. Environ. Anal. Chem.
Vol. 85, Nos. 9–11, 10 August–15 September 2005, 753–770

Taylor & Francis
Taylor & Francis Group

Characterization of graphite electrodes modified with laccases from *Trametes hirsuta* and *Cerrena unicolor* and their use for flow injection amperometric determination of some phenolic compounds

BEHZAD HAGHIGHI*†, ANNA JAROSZ-WILKOŁAZKA‡, TAUTGIRDAS RUZGAS§, LO GORTON§ and ANDRZEJ LEONOWICZ‡

†Department of Chemistry, Institute for Advanced Studies in Basic Sciences, PO Box 451951159, Gava Zang, Zanjan, Iran
‡Department of Biochemistry, Maria Curie–Skłodowska University, Skłodowska Square 3, 20-031 Lublin, Poland
§Department of Analytical Chemistry, University of Lund, PO Box 124, S-221 00 Lund, Sweden

(*Received 14 September 2004; in final form 10 December 2004*)

Fungal laccases from *Trametes hirsuta* and *Cerrena unicolor* were immobilized on spectroscopic graphite electrodes using physical absorption. The laccase-modified graphite electrodes were mounted as the working electrodes into a wall jet flow through an electrochemical cell and then used for flow injection amperometric determination of different phenolic compounds of relevance both for environmental and clinical analysis. At optimum conditions for determination of catechol serving as a model compound, the output response signals of the laccase-modified electrodes were recorded as the result of injections of 50 μL of solutions with different phenolic substrates into the carrier citrate buffer solution with a working potential of −50 mV *vs.* Ag|AgCl. Statistical aspects were applied and the Michaelis–Menten constants evaluated were correlated to the chemical structure of the investigated phenolic compounds.

Keywords: Biosensors; Flow injection; Amperometry; Laccase; Phenols

1. Introduction

The blue multicopper oxidases constitute a class of enzymes that can be defined by their spectroscopy, sequence homology and reactivity [1]. A combination of detailed spectroscopic studies and X-ray crystallography has revealed that all contain at least one blue copper (T1 site) and a T2/T3 trinuclear cluster as the minimal functional unit. The currently well-defined multicopper oxidases are laccase (Lc), ascorbate oxidase (AO), ceruloplasmine (Cp) and bilirubin oxidase (BO). These enzymes catalyse the reduction

*Corresponding author. Fax: +98 211-4249023. Email: haghighi@iasbs.ac.ir

International Journal of Environmental Analytical Chemistry
ISSN 0306-7319 print: ISSN 1029-0397 online © 2005 Taylor & Francis
DOI: 10.1080/03067310500153710

of molecular oxygen by various organic compounds [2] directly to water without intermediate formation of hydrogen peroxide. Functionally, all blue multicopper oxidases couple the four-electron reduction of dioxygen to water with the oxidation of a substrate (an electron donor). In these well-characterized enzymes (Lc, AO, Cp, BO) the substrate is oxidized through a one-electron reaction mechanism.

Lcs (*p*-diphenol: dioxygen oxidoreductase, EC 1.10.3.2) are widely distributed in fungi [3], higher plants [4] and in some bacteria [5]. The first plant Lc was obtained from the juice of the Japanese lacquer tree *Rhus vernicifera*, but it has also been purified to homogeneity and characterized from *Rhus succedanea* and other sources [1].

A notable number of fungi are known to produce Lc. The most studied fungal Lcs appear to be from *Trametes* (*Polyporus*, *Coriolus*) *versicolor*, *Trametes hirsuta* (*Coriolus hirsutus*), *Agaricus bisporus*, *Podospora anserina*, *Rhizoctonia practicola*, *Pholiota aegerita*, *Pleurotus ostreatus*, and *Neurospora crassa* [1, 6]. All fungal Lcs are monomers or homodimers with 10–30% glycosolation, with the exception of Lc from *Podospora anserine*, which appears to be a homotetramer.

There are essentially three possible functions which have been ascribed to fungal Lcs: pigment formation, lignin degradation and detoxification [1]. Lc is capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water through its multicopper system. The molecular reaction models of Latour [7] and Yaropolov *et al*. [6] can be simplified to the reaction sequence:

$$2Cu^{2+} + \text{benzenediol} \rightarrow 2Cu^{+} + \text{quinone} + 2H^{+} \quad (1)$$

$$2Cu^{+} + \tfrac{1}{2}O_2 + 2H^{+} \rightarrow 2Cu^{2+} + H_2O \quad (2)$$

The reaction products formed as a result of oxidation of monophenols by Lc are not known in detail but it seems as though the radical products formed are almost electrochemically inactive, in contrast to those formed from diphenols, and are prone to form inactive reaction degradation products.

In most cases, the oxidation of the phenolic substrates by Lc finally leads to polymerization reactions of the products through oxidative coupling. This reaction is generally seen as detoxification of phenolic concomitants. The application of (immobilized) Lc for the continuous elimination of phenolic pollutants [8, 9], fermentation inhibitors [10] and in bio-fuel cells [11–13] could be of practical interest as besides the substrate, only molecular oxygen as an electron acceptor is necessary for the enzymatic reaction.

The determination of phenols and their derivatives are of environmental importance since these species are released into the environment by a large number of industries, e.g. the manufacture of plastic, dyes, drugs, antioxidants and waste water from pulp and paper production [14–17]. In recent years, many papers have been published in which simple and inexpensive methods for the analysis of phenolic compounds are proposed based on the use of enzyme biosensors. These biosensors can be categorized into two groups, viz. those that are based on a phenol oxidizing enzyme, e.g. tyrosinase [16, 18–20], Lc [14, 21–25], and peroxidase [26–28], where the (di)phenol acts as an electron donor to the oxidized enzyme and the phenoxy radical formed is in turn electrochemically re-reduced to form a (di)phenol (see reaction scheme (3)), and those that are based on a quinone reducing enzyme, e.g., oligosaccharide dehydrogenase (ODH) [29], glucose (PQQ) dehydrogenase (GDH) [30] or cellobiose dehydrogenase (CDH) [31, 32], in which the reaction sequence starts with an initial

electrochemical oxidation of the (di)phenol transforming it into a compound (quinone) that can act as an electron acceptor to the reduced enzyme thus reforming the (di)phenol again (see reaction scheme (4))

$$\text{phenol} \xrightarrow{\text{Phenol oxidizing enzymes e.g. Laccase}} \text{phenoxy radical} \xrightarrow{\text{Electrochemical reduction}} \text{phenol} \quad (3)$$

$$\text{phenol} \xrightarrow{\text{Electrochemical oxidation}} \text{quinone} \xrightarrow{\text{Quinone reducing enzymes e.g. GDH}} \text{phenol} \quad (4)$$

Analysing both reaction schemes reveals the presence of an amplification reaction cycle. Hence, for all these biosensors, higher sensitivities and lower detection limits can be obtained compared with the 'ordinary' enzyme-based amperometric biosensors or with direct electrochemical conversion of the analyte. The reactivity and therefore the selectivity of the various enzyme-based biosensors for phenols/quinones can vary drastically because of thermodynamic and structural reasons. For phenol detection all the enzymes mentioned additionally need another substrate, i.e., Lc and tyrosinase need molecular oxygen, peroxidase needs hydrogen peroxide, and ODH, GDH and CDH need a sugar. Lc and tyrosinase are the two enzymes that offer the simplest detection system, as molecular oxygen in sufficient concentrations is present in ordinary buffers, whereas the other enzyme substrates need to be added. In order to increase the number of analytes that can be detected or to increase the amplification cycle number, more than one enzyme can be immobilized on the electrode surface. Examples of such systems are co-immobilization of Lc and tyrosinase [14], Lc and GDH [33, 34]. A variety of immobilization methods (e.g. physical adsorption, cross-linking, covalent coupling and physical entrapment) and different electrode materials including surface modification of solid electrodes as well as bulk modification of carbon composite electrodes have been used [14].

In the present work a comprehensive study on the immobilization of *Trametes hirsuta* Lc through adsorption on a solid graphite electrode was performed. The response of the biosensor for catechol was examined in terms of immobilization and stabilization parameters, pH, applied potential and carrier flow rate in a flow injection system. Finally, comprehensive kinetic studies were performed with the investigated *T. hirsutua* Lc-modified electrode and additionally with *C. unicolor* Lc-modified graphite electrode as the biosensing elements for flow injection amperometric determination of a variety of phenolic compounds, some of relevance for clinical, some for environmental monitoring, and some expected as lignin degradation products, trying to correlate the sensor responses to the chemical structures of the phenolic compounds.

2. Experimental

2.1 *Chemicals*

Fungal laccase (EC 1.10.3.2) from *Trametes hirsuta* was kindly provided by Prof. A.I. Yaropolov (Laboratory of Analytical Biotechnology, Bakh Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia). The enzyme was used without further purification. According to the provider, the enzyme had been purified

to a homogeneous state with HPLC, through sulfate ammonium precipitation, isoelectric focusing in the range between pH 3 and 6, and finally with ion exchange chromatography on DEAE-Toyopearl 650. The molecular weight and the carbohydrate moiety of the Lc were 55 kDa and 12%, respectively. The concentration of the *T. hirsuta* Lc stock solution was 78 mM containing 4290 $\mu g\,mL^{-1}$ of Lc. Fungal Lc from the white rot *C. unicolor* (from the Fungal Collection (FCL) at the Department of Biochemistry, UMCS, Lublin, Poland) was purified using a previously described procedure [3]. Working Lc solution was prepared by thawing a Lc stock solution stored in a freezer below $-18^{\circ}C$ and diluting it with citrate buffer solution.

Citrate buffers (0.1 M) of various pHs were prepared by mixing a stock citrate acid monohydrate solution (pro-analysis, Merck, Darmstadt, Germany) with appropriate amounts of sodium hydroxide solution. Buffer solutions were equilibrated with air overnight at room temperature before use to prevent microbubbles appearing in the flow system.

Phenol, 4-methoxyphenol, 4-chlorophenol, hydroquinone, resorcinol, vanillin (4-hydroxy-3-methoxy-benzaldehyde), guaiacol, cinnamaldehyde, *p*-cresol, *o*-cresol and 4-hydroxybenzoic acid were obtained from Merck. Cinnamic acid, DL-noradrenaline and *o*-aminophenol were obtained from Fluka (Buchs, Switzerland). Syringic acid, coniferyl alcohol, ferulic acid, adrenaline, 3,4-dihydroxyphenylacetic acid (DOPAC), L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine, 2,6-dimethoxyphenol, acetovanillone, acetosyringone, caffeic acid, 2,2′-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS) and syringaldazine were obtained from Sigma (St. Louis, MO, USA). Coniferylaldehyde, 3,4-dihydroxybenzylamine (DHBA), *p*-aminophenol, 3,4-dihydroxybenzoic acid and 3,4-dihydroxybenzaldehyde were obtained from Aldrich (Steinheim, Germany). Catechol and vanillic acid were obtained from ICN Biomedical Inc. (Aurora, OH, USA) and 4-hydroxybenzaldehyde was obtained from Acros (Morris Plains, NJ, USA). All chemicals were of analytical grade and used without further purification. Stock substrate solutions (10 or 100 mM) were prepared by dissolving the appropriate amount of analyte in a methanol/water (1 : 4) mixture. Working substrate solutions were prepared daily by stepwise dilution of stock substrate solutions with citrate buffer solution. Water purified with a Milli-Q system (Millipore, Milford, CT, USA) was used.

2.2 *Apparatus*

A single line flow injection system with a three-electrode wall-jet flow-through cell [35] was used for the amperometric measurements. A peristaltic pump (Minipulse 2, Gilson, Villier-le-Bel, France) propelled the citrate buffer (0.1 M at the various pHs) as the carrier into the flow line using Tygon tubing (0.89 mm i.d.). A 50 µL sample solution containing substrate was injected into the carrier stream via a LabPRO six-port injection valve (PR700-100-01, Rheodyne, CA, USA). The flow line was made from Teflon tubing (0.5 mm i.d.). A Lc-modified graphite electrode, an Ag|AgCl (0.1 M KCl) electrode and a platinum wire were used as the working, reference and auxiliary electrodes, respectively. The working potential was applied by a three-electrode potentiostat (Zäta Electronics, Lund, Sweden) and the output signal was recorded by a strip chart recorder (Kipp and Zonen, type BD111, Delft, The Netherlands). All measurements were performed at room temperature.

2.3 *Preparation of* T. hirsuta *laccase-modified graphite electrode*

The end of a solid spectrographic graphite rod (Ringsdorff Werke GmbH, Bonn, Germany, type RW001) with an outer diameter of 3.05 mm was polished on wet fine emery paper (Turfbak Durite, P1200). The polished electrode was then carefully rinsed with deionized water and dried with tissue paper. To find the optimal concentration of Lc from *T. hirsuta*, an aliquot of 6 μL of a working *T. hirsuta* Lc solution containing different amounts of Lc was placed on the polished surface of the graphite electrode. Also, to find the optimal adsorption time, the electrodes after application of Lc were kept at 4°C for various times in water-saturated air to prevent immediate drying. Then the non-adsorbed enzyme was removed by repeated washing with deionized water. The *T. hirsuta* Lc-modified graphite electrodes were then stabilized by storing at 4°C for various times in 0.1 M citrate buffer at the same pH as that of the carrier solution. The electrodes were washed with deionized water before use.

2.4 *Preparation of* C. unicolor *laccase-modified graphite electrode*

A 10-μL aliquot of the *C. unicolor* Lc solution was placed on the cleaned, polished surface of the spectrographic graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05-mm diameter) and the electrodes were then kept at 4°C for 15 h in a glass beaker covered with sealing film, to prevent rapid evaporation of the enzyme solution. The Lc-modified electrodes were then carefully rinsed with deionized water and were stored at 4°C for 5 h in 0.1 M citrate buffer pH 5.0 at 4°C. The procedure was the same as previously reported [36].

3. Results and discussion

An Lc-modified electrode in the flow injection system at an appropriate applied potential generates a steady state (background) current. The observed current is due to the bioelectrocatalytic reduction of dissolved molecular oxygen to water through a direct electron transfer (DET) mechanism between the graphite electrode and the T1 copper site of the immobilized Lc [37] (figure 1a). Direct electron transfer between redox enzymes and electrodes has been thoroughly discussed by Frew *et al.* [38], Ghindilis *et al.* [39], Gorton *et al.* [40], Habermüller *et al.* [41], Armstrong *et al.* [42] and by Kano and Ikeda [43]. If the sample solution containing a phenolic substrate is injected into the flow line, an additional current peak is observed on top of the steady-state current. In this case, at the electrode surface the phenolic substrate will act as an electron donor competing with DET and will form the oxidized phenolic compound, which in turn will be electrochemically re-reduced at the electrode surface in a mediated electron transfer (MET) step. So, in the MET mechanism the electrons are shuttled between the electrode and the enzyme (e.g. Lc) via the mediator (e.g. phenolic compounds) and an electrochemical response due to the mediated electron transfer [14, 44] is observed as a peak on top of the steady-state current (figure 1b).

A comprehensive investigation was therefore undertaken in order to optimize the immobilization procedure of the Lc from *T. hirsuta* on the graphite surface by physical adsorption to suppress the high and drifting steady-state (background) current and at the same time to obtain as sensitively as possible a response (peak intensity)

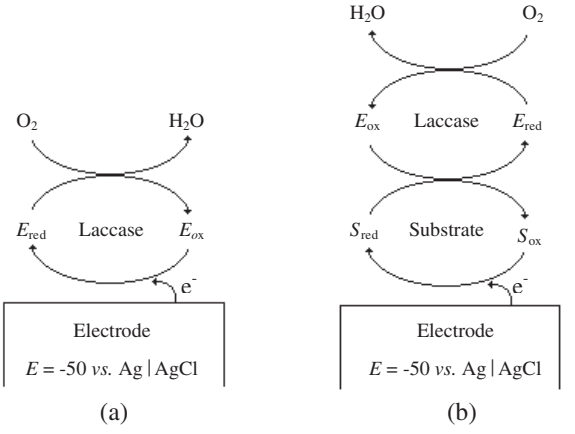


Figure 1. (a) Direct electron transfer (DET) and (b) mediated electron transfer (MET) mechanisms on the Lc biosensor. S: substrate; E: enzyme; red and ox: reduced and oxidized forms.

for the injected phenolic substrate. After establishing the best procedure for *T. hirsuta* Lc immobilization (see below), the response of the biosensor for catechol solution was investigated in terms of pH, applied potential and carrier flow rate. Finally, the analytical features were determined both for *T. hirsuta* and *C. unicolor* Lc-modified graphite electrodes at the optimum conditions for catechol for some phenolic compounds in the flow injection mode.

3.1 *Immobilization and stabilization of* T. hirsuta *laccase*

Six microlitres of working *T. hirsuta* Lc solution of different concentrations (4290, 429, 42.9 and 4.29 $\mu g\,mL^{-1}$) were placed on the polished surface of the graphite electrodes and were allowed to adsorb for 15 min (adsorption time) at 4°C in a water-saturated atmosphere. The Lc-modified electrodes were then washed several times with water and buffer and placed immediately in the amperometric flow-through cell. The initial investigations were carried out by propelling the citrate buffer (0.1 M at pH 5.0) as the carrier stream with a flow rate of 0.51 $mL\,min^{-1}$ and at a potential of −50 mV *versus* Ag|AgCl.

Decreasing the concentration of the working *T. hirsuta* Lc solution from 4290 to 4.29 $\mu g\,mL^{-1}$ reduced the intensity of the background current. At the same time the highest peak current intensity for 10 μM catechol was obtained for electrodes modified with 6 μL of 429 $\mu g\,mL^{-1}$ Lc solution. All enzyme electrodes investigated with different concentrations of *T. hirsuta* Lc suffer to some extent from a large background current and especially a large drift (see table 1). These two undesirable features constitute important limiting factors of the sensor. However, due to the large drift and large background current obtained for electrodes modified with 429 $\mu g\,mL^{-1}$ Lc solution we selected instead electrodes modified with 42.9 $\mu g\,mL^{-1}$ Lc solution for further studies as those showed a smaller background current with a smaller drift and a good peak current intensity.

Further studies were performed in order to stabilize the drifting background current through the extent of time for adsorption, and the time lap of storage of the enzyme-modified electrode in buffer solution before starting the measurement, denoted

Table 1. Bioelectrochemical characteristics of *T. hirsuta* laccase-modified electrodes, obtained from different concentrations of working *T. hirsuta* laccase solution and different adsorption and stabilization times.

Concentration of working laccase solution ($\mu g\,mL^{-1}$)	Background current (nA)	Peak current[a] (nA)	Drift ($nA\,min^{-1}$)	Adsorption time (min)	Stabilization time (min)
4290	7000	250	20	15	[b]
429	5000	620	12	15	[b]
42.9	4000	280	6	15	[b]
4.29	2500	12	2.5	15	[b]
42.9	4000	280	6	15	[b]
42.9	4000	420	9	60	[b]
42.9	3000	340	2.5	> 720	[b]
42.9	3000	180	1.5	60	> 720[c]
42.9	3000	300	1.5	> 720	> 720[c]

[a]Due to injection of a 10 μM catechol solution.
[b]Washing with water and citrate buffer solution several times.
[c]Washing carefully with water and keeping at 4°C in the citrate buffer solution (0.1 M, pH = 5.0).

as adsorption time and stabilization time, respectively. As shown in table 1, both the adsorption and stabilization times are critical to obtain a high peak current and at the same time a low background current with a small drift. Improved responses were obtained using prolonged adsorption and stabilization times, i.e., at least 12 h (overnight) for each step. At the selected conditions the relative standard deviation of the biosensor for catechol (10 μM, $n = 11$) was 1.2%. The reproducibility between 10 *T. hirsuta* Lc-modified graphite electrodes prepared and used on different days was 9.6%.

3.2 *Effect of pH*

The effect of the pH of the citrate buffer (0.1 M) on the amperometric responses of the *T. hirsuta* Lc-modified electrode to a series of catechol solutions was investigated in the pH range between 3 and 7. As shown in figure 2(a), the *T. hirsuta* Lc-modified electrodes, which have been prepared and operated with citrate buffer and different pHs ranging from 3 to 7 show a linear response behaviour to catechol in the concentration range of 1–10 μM with a maximum response at about pH 5. However, as revealed in figure 2(b), the linearity of the biosensor response for catechol in the concentration range of 1–100 μM at pH 4.5 is better than at pH 5, and also the sensitivity of the enzyme electrode for catechol with concentrations higher than 50 μM at pH 4.5 is better than at pH 5. A pH of 4.5 was therefore selected for further studies.

3.3 *Effect of applied potential*

The dependence of the response of the *T. hirsuta* Lc-modified electrode for catechol was studied as a function of the applied potential. As is shown in figure 3, the response of the biosensor for catechol (10 μM) is independent on the applied potential in a wide potential range from +100 mV down to −200 mV *versus* Ag|AgCl. The slopes of the calibration curves for catechol in the concentration range of 1–10 μM were linear in the entire working potentials range (starting from +225 down to −200 mV *vs.* Ag|AgCl). The sensitivity of the biosensor increased as the working potential shifts from +225 down to +100 mV *versus* Ag|AgCl and becomes constant from +100 mV

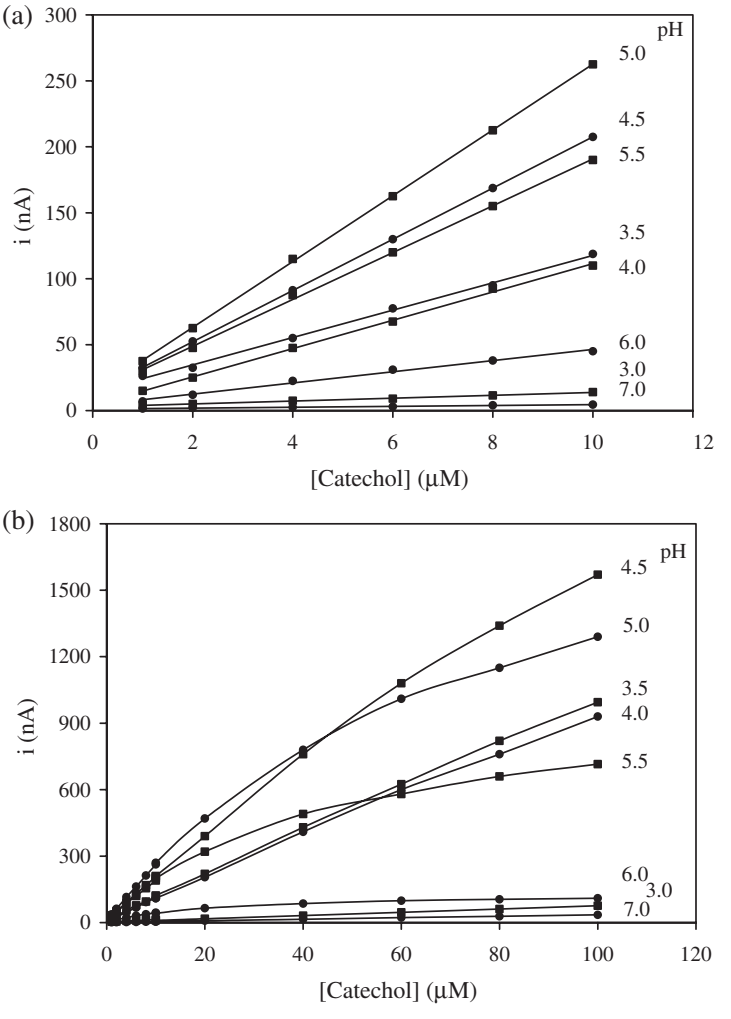


Figure 2. Bioelectrochemical response of a *T. hirsuta* Lc-modified electrode for catechol in the concentration range of (a) 1–10 μM, (b) 1–100 μM as a function of the pH of the carrier citrate buffer (0.1 M). Experimental conditions: carrier, 0.1 M citrate buffer with a flow rate of 0.51 mL min^{-1}, applied potential: −50 mV *vs.* Ag|AgCl.

towards more negative values. The working potential was fixed at −50 mV *versus* Ag|AgCl for further studies, because at this potential the biosensor showed a good sensitivity and it is expected, for practical applications, to be less exposed to interference from several other compounds, mainly in complex matrices.

3.4 *Effect of carrier flow rate*

The influence of the flow rate on the amperometric response of the *T. hirsuta* Lc biosensor for catechol was investigated both in the flow injection mode, when 50 μL of catechol solution (1–100 μM) was injected into the carrier flow, and in the steady state mode when a concentration of 10 μM of catechol was dissolved in the carrier

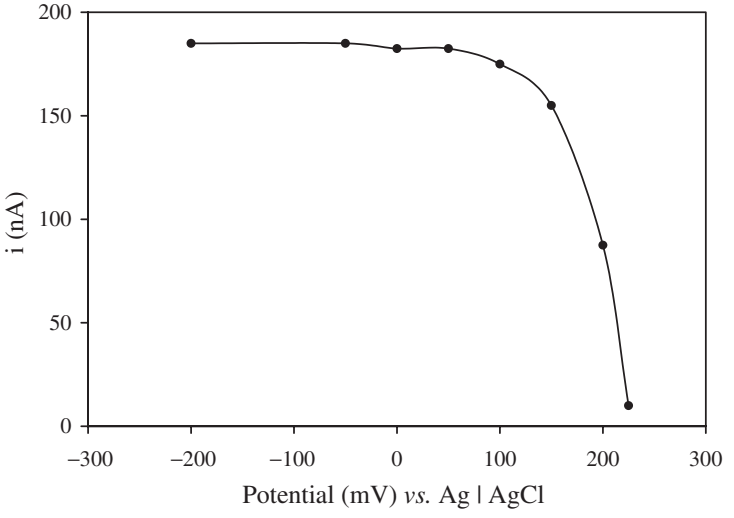


Figure 3. Amperometric response of a *T. hirsuta* Lc-modified electrode for 10 μM catechol as a function of the applied potential. Experimental conditions: carrier, 0.1 M citrate buffer (pH = 4.5) with a flow rate of 0.51 mL min^{-1}.

solution. The results showed that the peak and steady-state currents in both flow injection and steady-state modes are independent of the carrier flow rate ranging from 0.14 to 0.67 mL min^{-1}. The results also showed that the calibration curves in the flow injection mode for catechol in the concentration range of 1–10 μM and at the different flow rates are linear and the slopes of the calibration curves are similar. So, it can be concluded that the electrode response is highly kinetically controlled and the electron transfer between catechol and Lc is the rate-limiting step of the overall redox reaction. It was also observed that for flow rates higher than 0.43 mL min^{-1}, air bubbles appeared in the system after about 10 min disturbing the flow profile at the electrode surface with irreproducible response peaks as the result. Further studies on the applicability of the *T. hirsuta* Lc-modified electrode were therefore carried out at 0.43 mL min^{-1}, as at this flow rate no air bubbles appeared up to 90 min, the biosensor response for catechol was still good (20.79 nA μM^{-1}) and allowing a reasonably good sample throughput (78 samples h^{-1}). The results of the extensive investigations to find the optimum conditions for making and operating *T. hirsuta* Lc-modified electrodes are applicable when using another Lc [36, 45] and was confirmed also for *C. unicolor* Lc (results not shown).

3.5 *Bioelectrochemical behavior of laccase-modified electrode for some phenolic compounds*

The amperometric responses of the *T. hirsuta* and *C. unicolor* Lc-modified electrodes for some phenolic compounds were recorded in the flow injection mode using the parameters found optimal for catechol detection in this work and previously reported [36, 45], respectively. The sensitivities, apparent Michaelis–Menten constants and maximum currents have been calculated by fitting the data, i.e. peak current *versus*

concentration of the phenolic compound, to the electrochemical Michaelis–Menten equation [46]:

$$I = \frac{I_{max}[S]}{[S] + K_m^{app}} \quad (5)$$

where [S] is the substrate concentration, I_{max} the maximum current and K_m^{app} the apparent Michaelis–Menten constant. The calculated values of K_m^{app} and sensitivities are presented together with detection limits, linear dynamic ranges and sample throughput in table 2.

Figure 4 shows the sensitivity and the chemical structure of the investigated phenolic compounds. Lcs are classified as *p*-diphenol oxidases, but are generally considered to be remarkably non-specific to their substrates, being able to oxidize *in vitro* a range of aromatic substances such as polyphenols, substituted phenols, synthetic amines, thiols, dyes, etc. [2, 47, 48]. Since the catalysed reaction involves withdrawal of one electron from the substrate, it is conceivable that the electron density at the level of the oxidizing group plays an important role in determining the rates of oxidation of the substrates. Garzillo *et al.* [48] indicate that the polar effect of non-bulky substituent groups on the aromatic ring is the most relevant factor for determining the rates of substrate oxidation by Lc from *Trametes trogii*. In a comparison of a series of *ortho*- and *para*-substituted phenols presented by Xu [2], it is indicated that the presence of electron-withdrawing substituents decreased the activity of phenol towards recombinant Lc from *Polyporus pinsitus*. These substituents reduce the electron density at the phenoxy group, thus making it more difficult to be oxidized, less reactive in surrendering an electron to the T1 copper of Lc and less basic.

Based on the results obtained in this work, as seen in figure 3 for *ortho*-substituted phenols, the sensitivity of both Lc-modified electrodes increases in the following substitution order –H, $–CH_3$, –OH, $–NH_2$ or $–OCH_3$. The sensitivity of both Lc-modified electrodes increases with an additional *ortho*-OH group in *para*-substituted phenols when compared with their respective monophenols. Substituents with lone pairs (e.g. $–OCH_3$, $–NH_2$, –OH) on the atoms adjacent to the π system are electron-donating groups and activate the aromatic ring by increasing the electron density of the ring through a resonance donating effect. The resonance effect only allows electron density to be located at the *ortho*- and *para*-positions. Hence these sites are more nucleophilic. Alkyl substituents (e.g. $–CH_3$) are also electron-donating groups and activate the aromatic ring by increasing the electron density of the ring through an inductive donating effect. Their overall effect is similar to that described above but weaker. Further substituting *ortho*-diphenols in the *para*-position with $–CH_2–$ or –CH=CH– groups (DOPAC, caffeic acid) or *ortho*-methoxyphenols in the *para*-position with a –CH=CH– group (coniferyl alcohol, ferulic acid and coniferylaldehyde) causes the sensitivities of both biosensors modified with Lc to increase for those phenolic compounds. Substituents with C=C (e.g. -vinyl or -aryl) are also electron-donating groups and activate the aromatic ring by a resonance donating effect. This is a similar effect to that for lone pairs except that the electrons are from a bonded pair not a lone pair. *Para*-substitution of –COOH and –CHO groups in all of the studied *ortho*-diphenols and *ortho*-methoxyphenols decreases the sensitivity of both Lc-modified electrodes. Substituents with π bonds to electronegative atoms

Table 2. Results evaluated from fitting of amperometric signals of the *T. hirsuta* (plain numbers) and *C. unicolor* (italic numbers) laccase-modified electrodes to the Michaelis–Menten equation for some phenolic compounds.

Substrate	I_{max} (nA)	Error (nA)	K_m^{app} (μM)	Error (μM)	r	I_{max}/K_m^{app} (nA μM^{-1})	Detection limit (μM)	Linear dynamic range (μM)	Sample throughput (samples h^{-1})
Syringic acid	3941	88.90	31.63	1.71	0.9991	124.6	0.17	1.2–12	36
	ND	ND	*96.9*	*11.0*	*1*	*44.65*	*1.27*	*1–40*	ND
Guaiacol	4559	55.35	76.64	1.71	0.9999	59.48	0.18	1.1–11	28
	ND	ND	*51.0*	*2.2*	*0.999*	*22.38*	*0.90*	*1–20*	ND
Hydroquinone	3890	98.75	66.66	3.14	0.9997	58.36	0.07	0.1–8	38
	ND	ND	*19.0*	*1.2*	*0.999*	*50.99*	*0.58*	*1–10*	ND
Ferulic acid	3605	84.02	64.71	2.94	0.9997	55.70	0.30	1–10	85
	ND	ND	*94.8*	*14.0*	*0.999*	*69.63*	*1.56*	*1–40*	ND
Coniferyl alcohol	1800	29.95	33.57	0.91	0.9999	53.63	0.33	1.1–11	69
	ND	ND	*7.83*	*0.45*	*0.999*	*98.70*	*0.35*	*0.2–6*	ND
2-Aminophenol	4355	134.3	82.50	4.39	0.9997	52.79	0.12	1–10	88
	ND	ND	*7.59*	*0.82*	*0.995*	*53.96*	*0.95*	*1–8*	ND
Catechol	4232	97.77	160.6	5.23	0.9999	26.35	0.83	1–40	30
	ND	ND	*246*	*23.0*	*0.999*	*24.40*	*0.89*	*1–20*	ND
DOPAC	1811	20.49	90.15	1.99	0.9999	20.09	0.43	1–20	78
	ND	ND	*12.7*	*0.58*	*0.999*	*52.11*	*0.23*	*1–10*	ND
Coniferylaldehyde	8319	86.34	464.3	9.74	0.9999	17.92	0.77	1–100	98
	ND	ND	*46.7*	*6.0*	*0.999*	*14.03*	*0.68*	*1–20*	ND
3,4-Dihydroxybenzoic acid	3742	49.88	223.8	7.18	0.9996	16.72	0.13	1–100	100
	ND	ND	*55.3*	*4.2*	*0.998*	*14.70*	*3.80*	*1–40*	ND
4-Aminophenol	3837	69.65	235.1	8.99	0.9996	16.32	0.39	1–100	77
	ND	ND	*13.5*	*1.6*	*0.999*	*32.4*	*0.61*	*1–10*	ND
3,4-Dihydroxybenzaldehyde	3580	59.27	405.6	14.07	0.9997	8.83	0.58	1–100	75
	ND	ND	*167*	*71.0*	*0.999*	*5.76*	*5.18*	*10–150*	ND
Dopamine	1517	26.61	210.1	5.15	0.9999	7.22	0.52	1–10	91
	ND	ND	*61.1*	*4.3*	*0.999*	*11.08*	*1.51*	*1–60*	ND
DL-Noradrenaline	2259	21.51	431.3	7.77	0.9999	5.24	0.80	1–130	88
	ND	ND	*125*	*15.0*	*1*	*6.26*	*1.90*	*1–80*	ND

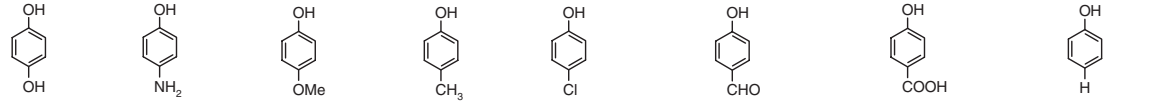
(*Continued*)

Table 2. Continued.

Substrate	I_{max} (nA)	Error (nA)	K_m^{app} (μM)	Error (μM)	r	I_{max}/K_m^{app} (nA μM^{-1})	Detection limit (μM)	Linear dynamic range (μM)	Sample throughput (samples h^{-1})
Adrenaline	1995	44.74	477.8	19.39	0.9997	4.17	0.75	1–80	90
	ND	ND	*302*	*30.2*	*0.998*	*3.16*	*10.03*	*10–150*	ND
L-DOPA	1427	21.07	349.7	8.86	0.9998	4.08	0.67	1–200	80
	ND	ND	*146*	*5.0*	*0.999*	*5.13*	*0.49*	*1–40*	ND
Vanillic acid	2449	101.6	690.0	52.57	0.9992	3.55	7.94	10–200	60
	ND	ND	*203*	*13.0*	*0.997*	*3.45*	*9.48*	*10–100*	ND
p-Cresol	2988	197.2	3401	270.4	0.9999	0.8785	6.55	10–400	78
	ND	ND	*2160*	*66.0*	*0.999*	*0.44*	*39.34*	*10–1000*	ND
Vanillin	1626	47.21	4355	249.1	0.9993	0.3733	11.51	10–1000	63
	ND	ND	*3570*	*813*	*0.998*	*0.251*	*30.86*	*10–400*	ND
o-Cresol	1671	60.29	5191	356.1	0.9992	0.3220	23.22	100–1000	68
	ND	ND	*3690*	*149*	*0.999*	*0.289*	*53.84*	*10–1000*	ND
4-Hydroxybenzaldehyde	326.1	15.38	13295	1348	0.9962	0.0245	717	1000–10000	91
	ND	ND	*10800*	*1110*	*0.998*	*0.018*	*782*	*1000–10000*	ND
4-Hydroxybenzoic acid	287.0	18.37	16778	2156	0.9941	0.0171	525	1000–10000	83
	ND	ND	*16780*	*2160*	ND	ND	ND	ND	ND
Phenol	547.5	22.78	35587	*2339*	0.9992	0.0154	628	1000–10000	80
	ND	ND	*43400*	*2030*	*1*	*0.011*	*296*	*1000–10000*	ND
DHBA	1882	51.17	1525	59.57	0.9999	1.23	0.48	1–200	91
Resorcinol	1352	60.02	13247	1020	0.9989	0.1021	1049	1000–10000	94
2,6-Dimethoxyphenol	ND	ND	*6.54*	*0.71*	*0.999*	*202.09*	*0.091*	*0.1–2*	ND
Caffeic acid	ND	ND	*27.4*	*2.7*	*0.999*	*57.92*	*0.56*	*1–10*	ND
ABTS	ND	ND	ND	ND	*0.999*	*38.63*	*0.58*	*1–10*	ND
Acetosyringone	ND	ND	*22.4*	*1.6*	*0.999*	*29.0*	*0.40*	*1–10*	ND
4-Methoxyphenol	ND	ND	*88.3*	*6.1*	*0.998*	*9.09*	*7.88*	*20–100*	ND
Syringaldazine	ND	ND	*280*	*55.0*	*1*	*2.5*	*3.09*	*1–100*	ND
Acetovanillone	ND	ND	*714*	*32.0*	*0.999*	*0.42*	*6.06*	*10–100*	ND
4-Chlorophenol	ND	ND	*22400*	*1120*	*1*	*0.02*	ND	*1000–10000*	ND

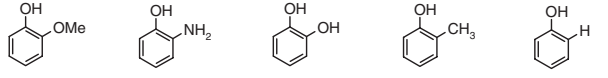
r, Correlation coefficient; ND, not determined.
Experimental conditions for *T. hirsuta* Lc-modified electrode; carrier: 0.1 M citrate buffer (pH = 4.5), applied potential: −50 mV *vs.* Ag|AgCl, flow rate: 0.43 mL min^{-1}.
Experimental conditions for *C. unicolor* Lc-modified electrode; carrier: 0.1 M citrate buffer (pH = 5.5), applied potential: −50 mV *vs.* Ag|AgCl, flow rate: 0.60 mL min^{-1}.

para substituted phenols:



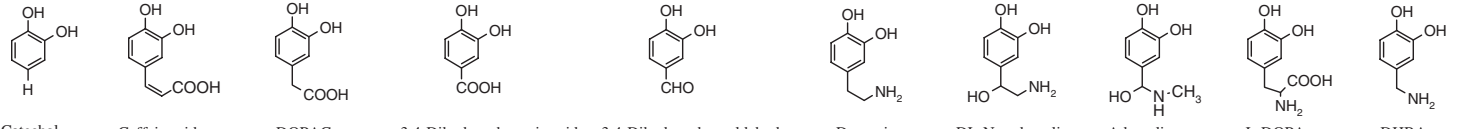
Hydroquinone (58.36, 66.66) *(50.99, 19.0)*; 4-Aminophenol (16.32, 235.1) *(32.40, 13.5)*; 4-Methoxyphenol *(9.09, 88.3)*; p-Cresol (0.878, 3401) *(0.44, 2160)*; 4-Chlorophenol *(0.02, 22400)*; 4-Hydroxybenzaldehyde (0.0245, 13295) *(0.018, 10800)*; 4-Hydroxybenzoic acid (0.0171, 16778) (, 16780); Phenol (0.0154, 35587) *(0.011, 43400)*

ortho substituted phenols:



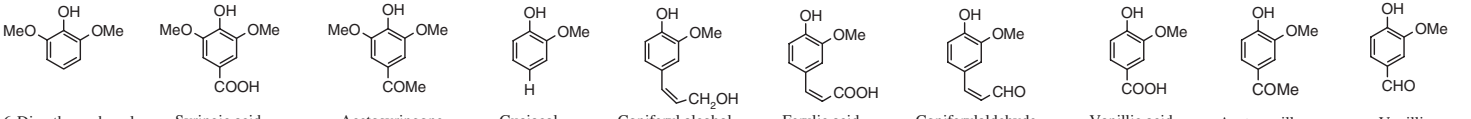
Guaiacol (59.48, 76.64) *(22.38, 51.0)*; 2-Aminophenol (52.79, 82.50) *(53.96, 7.59)*; Catechol (26.35, 160.6) *(24.40, 246)*; o-Cresol (0.322, 5191) *(0.289, 3690)*; Phenol (0.0154, 35587) *(0.011, 43400)*

para substituted ortho-diphenols:



Catechol (26.35, 160.6) *(24.40, 246)*; Caffeic acid *(57.92, 27.4)*; DOPAC (20.09, 90.15) *(52.11, 12.7)*; 3,4-Dihydroxybenzoic acid (16.72, 223.8) *(14.70, 55.3)*; 3,4-Dihydroxybenzaldehyde (8.83, 405.6) *(5.76, 167)*; Dopamine (7.22, 210.1) *(11.08, 61.1)*; DL-Noradrenaline (5.24, 431.3) *(6.26, 125)*; Adrenaline (4.17, 477.8) *(3.16, 302)*; L-DOPA (4.08, 349.7) *(5.13, 146)*; DHBA (1.23, 1525)

para substituted ortho-methoxy phenols:



2.6-Dimethoxyphenol *(202.09, 6.54)*; Syringic acid (124.6, 31.63) *(44.65, 96.9)*; Acetosyringone *(29.0, 22.4)*; Guaiacol (59.48, 75.64) *(22.38, 51.0)*; Coniferyl alcohol (53.63, 33.57) *(98.70, 7.83)*; Ferulic acid (55.70, 64.71) *(69.63, 94.8)*; Coniferylaldehyde (17.92, 464.3) *(14.03, 46.7)*; Vanillic acid (3.55, 690) *(3.45, 203)*; Acetovanillone *(0.42, 714)*; Vanillin (0.373, 4355) *(0.251, 3570)*

Figure 4. Chemical structures of the studied phenolic compounds. The numbers in parentheses indicate the sensitivity (nA μM^{-1}) and K_m^{app} (μM) of the *T. hirsuta* (plain numbers) and *C. unicolor* (italic numbers) Lc-modified electrodes with respect to phenolic compound, respectively.

(e.g. –C=O, $–NO_2$) adjacent to the π system are electron-withdrawing groups and deactivate the aromatic ring by decreasing the electron density on the ring through a resonance withdrawing effect. The resonance *decreases* the electron density at the *ortho*- and *para*-positions. Hence these sites are less nucleophilic. In the case of *para*-substituted *ortho*-diphenols the enzyme oxidizes more preferentially substances with a –COOH and an –RCOOH group (DOPAC, caffeic acid, 3,4-dihydroxybenzoic acid) than compounds with an $–RNH_2$ group in the *para*-position (dopamine, DL-noradrenaline, adrenaline, L-DOPA and DHBA).

An increase in the sensitivity of the biosensor indicates that the enzymatic oxidation products (i.e. phenoxy radicals) are more rapidly produced and re-reduced at the electrode, viz. an increase in the amplification reaction cycle. This would indicate that electron-donating substituents tend to increase the electron density on the hydroxyl group, leading to a higher oxidation rate and electron-withdrawing substituents tend to reduce the electron density on the hydroxyl group, leading to a lower oxidation rate.

Within the group of *ortho*-methoxyphenols it is observed that the presence of –COOH and –CHO groups in the *para*-position causes a decrease in the sensitivities of both Lc electrodes, and in *para*-substituted *ortho*-methoxyphenols it is also observed that the presence of an additional $–OCH_3$ group allows Lc to oxidize dimethoxyphenols more effectively. As shown in figure 5 these trends agree with the magnitude of Hammett's σ values of the functional groups (i.e. the electronic nature of the substituents) attached to a benzene ring [49, 50] but the trend in *para*-substituted phenols does not agree with Hammett's σ values. From this it follows that the electronic nature of the substituents is one of the factors that influence the sensitivity of both Lc-modified electrodes.

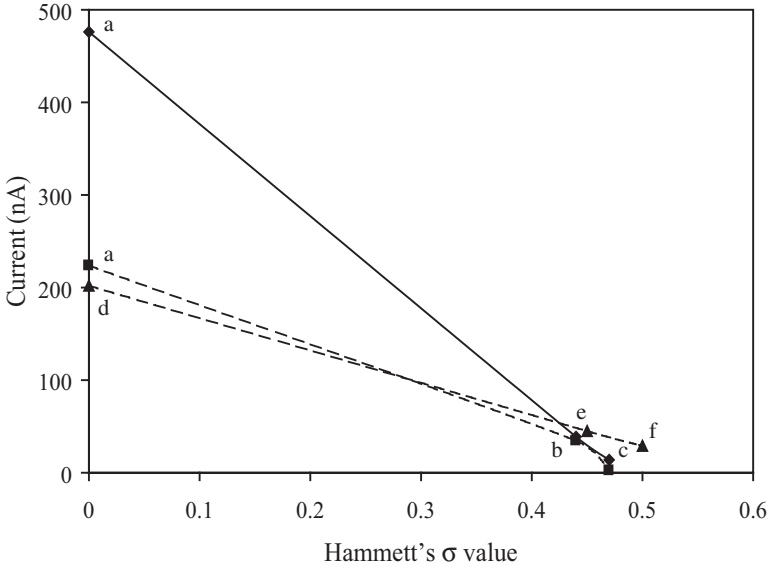


Figure 5. Correlation of flow injection responses of *T. hirsuta* (solid line) and *C. unicolor* (dashed line) Lc-modified electrodes with Hammett's σ values for 10 µM guaiacol (a), vanillic acid (b), vanilline (c), 2,6-dimethoxyphenol (d), syringic acid (e) and acetosyringone (f).

Solutions containing either cinnamic acid or cinnamaldehyde were also examined using *T. hirsuta* Lc-modified electrodes. Cinnamic acid produced a couple of small response peaks for each injection. An initial anodic peak followed by a second very weak cathodic peak. However, injections of a cinnamaldehyde solution produced only one peak per injection only in the anodic direction. The intensities of the biosensor response for these two aromatic and non-phenolic compounds were weaker than the intensity of the response for phenol at the same concentration.

Table 3 presents a comparison of the rates of oxidation of the selected phenolic compounds by native Lcs from different sources in solution with data obtained in this work using Lcs from *T. hirsuta* and *C. unicolor* adsorbed on graphite electrodes. It is necessary to take into consideration the fact that the electrochemical reactions take place on the boundary between two phases with electronic (electrode) and ionic (electrolyte) conductivity, where the exchange of electrons between the electrode and the reactants in the solution takes place [51]. In liquid solutions using phenolic substrates and native enzymes, the exchange of electrons takes place in one phase. Our data obtained using immobilized Lc are comparable to some data obtained with Lcs from other white rot fungi [52–55]. When comparing results with *C. unicolor* Lc immobilized on graphite (this work) with those of the enzyme in solution reported by Rogalski *et al.* [56], it can be seen that in the case of guaiacol a 2-fold higher K_m^{app} value was given when using soluble *C. unicolor*. Lc immobilized on the electrode surface is more sensitive in the reactions with caffeic acid, guaiacol, syringic acid, and vanillic acid in comparison with reactions with using Lc from *Phlebia radiata* in its soluble form.

4. Conclusions

The present study on fungal Lc (EC 1.10.3.2) from *T. hirsuta* showed that *T. hirsuta* Lc is successfully adsorbed and stabilized on top of a graphite electrode by allowing at least 12 h for adsorption as well as an additional 12 h for electrode stabilization at 4°C. The *T. hirsuta* Lc-modified electrode was optimized for catechol determination in the flow injection mode. The biosensor response was maximum at a pH of about 4.5 to 5.0. The working potential and carrier flow rate could be selected from a wide range, where the electrode response was independent on the applied potential (from +100 to −200 mV *vs.* Ag|AgCl) and carrier flow rate (from 0.14 to 0.67 mL min^{-1}). The results of the optimum conditions for making and operating *T. hirsuta* Lc-modified electrodes were applicable when using another Lc (from *C. unicolor*). At the selected conditions for catechol the electroanalytical behaviours of the *T. hirsuta* and *C. unicolor* Lc-modified electrodes for some phenolic compounds were studied. The results showed that the sensitivity of both biosensors for simple phenolic compounds increased with substituting $–CH_3$, –OH, $–NH_2$ and $–OCH_3$ groups in the *ortho*-position of the phenol, i.e. the sensitivity increases in the order phenol $\ll$ *o*-cresol $\lll$ catechol < 2-aminophenol and guaiacol < syringic acid (two $–OCH_3$ groups in *ortho*-position). *Para*-substitution of –COOH and –CHO groups in *ortho*-diphenols and *ortho*-methoxyphenols decreases the sensitivity of both Lc-modified electrodes, i.e. the sensitivity increases in the order 3,4-dihydroxybenzaldehyde < 3,4-dihydroxybenzoic acid < catechol and vanillin < vanillic acid < guaiacol. Thus it can be concluded that electron-donating substituents promote the rate of oxidation (increase in the

Table 3. K_m^{app} values (mM) for some phenolic compounds and laccases from different sources.

Substrate	*C. unicolor*[a]	*T. hirsuta*[a]	*C. unicolor* [56]	*Cerrena maxima* [52, 53]	*T. hirsuta* [52, 54]	*Coriolus zonatus* [52]	*Phlebia radiata* [55]
2,6-Dimethoxyphenol	0.00654	ND	0.0078	ND	0.053	ND	ND
ABTS	0.0166	ND	ND	ND	0.0567	ND	ND
Hydroquinone	0.0190	0.0667	ND	0.095	ND	0.086	ND
Acetosyringone	0.0224	ND	ND	ND	0.0605	ND	ND
Caffeic acid	0.0274	ND	0.0364	ND	ND	ND	0.34
Guaiacol	0.051	0.0756	0.116	0.255	0.0109/0.065	0.091	1.76
Ferulic acid	0.0948	0.0647	0.0887	0.034	0.0168/0.031	0.025	ND
Syringic acid	0.0969	0.0316	0.08	ND	ND	ND	0.21
Vanillic acid	0.203	0.690	0.172	ND	ND	0.165	0.68
Catechol	0.246	0.160	ND	0.122	0.0399	0.197	ND
Syringaldazine	0.280	ND	ND	ND	0.1427	ND	ND

[a]This work; ND, not determined.

amplification reaction cycle) and electron-withdrawing substituents reduce the oxidation rate.

Acknowledgements

The authors thank the Swedish Research Council (VR), the Swedish International Development Cooperation Agency (SIDA, 348-2003-4947) and the European Community (ICA2-CT-2000-10050) for financial support. The Swedish Institute (SI) is acknowledged for the support of a postdoctoral fellowship for B.H.

References

[1] E.I. Solomon, U.M. Sundaram, T.E. Machonkin. *Chem. Rev. (Washington, DC)*, **96**, 2563 (1996).
[2] F. Xu. *Biochemistry*, **35**, 7608 (1996).
[3] J. Luterek, L. Gianfreda, M. Wojtas-Wasilewska, J. Rogalski, M. Jaszek, E. Malarczyk, A. Dawidowicz, M. Finksboots, G. Ginalska, A. Leonowicz. *Acta Microbiol. Pol.*, **46**, 297 (1997).
[4] G. Gramss, K.D. Voigt, B. Kirsche. *Chemosphere*, **38**, 1481 (1999).
[5] G. Alexandre, I.B. Zhulin. *Trends Biotechnol.*, **18**, 41 (2000).
[6] A.I. Yaropolov, O.V. Skorobogat'ko, S.S. Vartanov, S.D. Varfolomeyev. *Appl. Biochem. Biotechnol.*, **49**, 257 (1994).
[7] J.M. Latour. *Bull. Soc. Chim. Fr.*, **3**, 508 (1988).
[8] G. Hublik, F. Schinner. *Enzyme Microb. Technol.*, **27**, 330 (2000).
[9] P. Piacquadio, G. De Stefano, M. Sammartino, V. Sciancalepore. *Biotechnol. Tech.*, **11**, 515 (1997).
[10] S. Larsson, P. Cassland, L.J. Jönsson. *Appl. Environ. Microbiol.*, **67**, 1163 (2001).
[11] P. Atanassov. *Abstracts of Papers, 223rd ACS National Meeting*, Orlando, FL, United States, April 7–11, 2002, COLL-378 (2002).
[12] F. Barriere, Y. Ferry, D. Rochefort, D. Leech. *Electrochem. Commun.*, **6**, 237 (2004).
[13] V. Soukharev, N. Mano, A. Heller. *J. Amer. Chem. Soc.*, **126**, 8368 (2004).
[14] A.I. Yaropolov, A.N. Kharybin, J. Emnéus, G. Marko-Varga, L. Gorton. *Anal. Chim. Acta*, **308**, 137 (1995).
[15] S. Canofeni, S. Di Sario, J. Mela, R. Pilloton. *Anal. Lett.*, **27**, 1659 (1994).
[16] C. Nistor, J. Emnéus, L. Gorton, A. Ciucu. *Anal. Chim. Acta*, **387**, 309 (1999).
[17] R. Sanchez Freire, R. Pelegrini, L.T. Kubota, N. Duran, P. Peralta-Zamora. *Quim. Nova*, **23**, 504 (2000).
[18] J. Svitel, S. Miertus. *Environ. Sci. Technol.*, **32**, 828 (1998).
[19] I.M. Russell, S.G. Burton. *Anal. Chim. Acta*, **389**, 161 (1999).
[20] A. Lindgren, T. Ruzgas, J. Emnéus, E. Csoeregi, L. Gorton, G. Marko-Varga. *Anal. Lett.*, **29**, 1055 (1996).
[21] F.F. Bier, E. Ehrentreich-Foerster, F.W. Scheller, A. Makower, A. Eremenko, U. Wollenberger, C.G. Bauer, D. Pfeiffer, N. Michael. *Sens. Actuators, B*, **B33**, 5 (1996).
[22] R. Rella, D. Ferrara, G. Barison, L. Doretti, S. Lora. *Biotechnol. Appl. Biochem.*, **24**, 83 (1996).
[23] R.S. Freire, N. Duran, L.T. Kubota. *Talanta*, **54**, 681 (2001).
[24] L. Gianfreda, F. Xu, J.-M. Bollag. *Biorem. J.*, **3**, 1 (1999).
[25] N. Duran, E. Esposito. *Appl. Catal., B*, **28**, 83 (2000).
[26] T. Ruzgas, E. Csoeregi, J. Emnéus, L. Gorton, G. Marko-Varga. *Anal. Chim. Acta*, **330**, 123 (1996).
[27] S.S. Rosatto, L.T. Kubota, G. de Oliveira Neto. *Anal. Chim. Acta*, **390**, 65 (1999).
[28] F.-D. Munteanu, A. Lindgren, J. Emneus, L. Gorton, T. Ruzgas, E. Csoeregi, A. Ciucu, R.B. van Huystee, I.G. Gazaryan, L.M. Lagrimini. *Anal. Chem.*, **70**, 2596 (1998).
[29] F.F. Bier, E. Ehrentreich-Förster, A. Makower, F.W. Scheller. *Anal. Chim. Acta*, **328**, 27 (1996).
[30] F. Lisdat, U. Wollenberger, A. Makower, H. Hortnagl, D. Pfeiffer, F.W. Scheller. *Biosens. Bioelectron.*, **12**, 1199 (1997).
[31] A. Lindgren, T. Ruzgas, L. Gorton, L. Stoica, A. Ciucu. *Analyst (Cambridge, UK)*, **124**, 527 (1999).
[32] L. Stoica, A. Lindgren-Sjölander, T. Ruzgas, L. Gorton. *Anal. Chem.*, **76**, 4690 (2004).
[33] R.S. Freire, S. Thongngamdee, N. Duran, J. Wang, L.T. Kubota. *Analyst (Cambridge, UK)*, **127**, 258 (2002).
[34] J. Szeponik, B. Moller, D. Pfeiffer, F. Lisdat, U. Wollenberger, A. Makower, F.W. Scheller. *Biosens. Bioelectron.*, **12**, 947 (1997).

[35] R. Appelqvist, G. Marko-Varga, L. Gorton, A. Torstensson, G. Johansson. *Anal. Chim. Acta*, **169**, 237 (1985).
[36] A. Jarosz-Wilkolazka, G. Janusz, T. Ruzgas, L. Gorton, E. Malarczyk, A. Leonowicz. *Anal. Lett.*, **37**, 1497 (2004).
[37] A. Christenson, N. Dimcheva, E.E. Ferapontova, L. Gorton, T. Ruzgas, L. Stoica, S. Shleev, A.I. Yaropolov, D. Haltrich, R.N.F. Thorneley, S.D. Aust. *Electroanalysis*, **16**, 1074 (2004).
[38] J.E. Frew, H.A.O. Hill. *Eur. J. Biochem.*, **172**, 261 (1988).
[39] A.L. Ghindilis, R. Krishnan, P. Atanasov, E. Wilkins. *Biosens. Bioelectron.*, **12**, 415 (1997).
[40] L. Gorton, A. Lindgren, T. Larsson, F.D. Munteanu, T. Ruzgas, I. Gazaryan. *Anal. Chim. Acta*, **400**, 91 (1999).
[41] K. Habermüller, M. Mosbach, W. Schuhmann. *Fresenius J. Anal. Chem.*, **366**, 560 (2000).
[42] F.A. Armstrong, G.S. Wilson. *Electrochim. Acta*, **45**, 2623 (2000).
[43] K. Kano, T. Ikeda. *Anal. Sci.*, **16**, 1013 (2000).
[44] G. Marko-Varga, J. Emnéus, L. Gorton, T. Ruzgas. *Trends Anal. Chem.*, **14**, 319 (1995).
[45] B. Haghighi, L. Gorton, T. Ruzgas, L.J. Jönsson. *Anal. Chim. Acta*, **487**, 3 (2003).
[46] F.R. Shu, G.S. Wilson. *Anal. Chem.*, **48**, 1679 (1976).
[47] C.F. Thurston. *Microbiology (Reading, UK)*, **140**, 19 (1994).
[48] A.M.V. Garzillo, M.C. Colao, C. Caruso, C. Caporale, D. Celletti, V. Buonocore. *App. Microbiol. Biotechnol.*, **49**, 545 (1998).
[49] C. Hansch, A. Leo, R.W. Taft. *Chem. Rev. (Washington, DC, USA)*, **91**, 165 (1991).
[50] T.G. Strein, B.J. Ximba, A.H. Hamad. *Electroanalysis*, **11**, 37 (1999).
[51] A.I. Yaropolov, A.N. Kharybin, J. Emnéus, G. Marko-Varga, L. Gorton. *Bioelectrochem. Bioenerg.*, **40**, 49 (1996).
[52] S.A. Smirnov, O.V. Koroleva, V.P. Gavrilova, A.B. Belova, N.L. Klyachko. *Biochemistry (Moscow, Russian Federation)* (Translation of Biokhimiya (Moscow, Russian Federation)), **66**, 774 (2001).
[53] O.V. Koroleva, I.S. Yavmetdinov, S.V. Shleev, E.V. Stepanova, V.P. Gavrilova. *Biochemistry (Moscow, Russian Federation)* (Translation of Biokhimiya (Moscow, Russian Federation)), **66**, 618 (2001).
[54] K.S. Shin, Y.J. Lee. *Arch. Biochem. Biophys.*, **384**, 109 (2000).
[55] J. Rogalski, E. Jozwik, A. Hatakka, A. Leonowicz. *J. Mol. Catal. A: Chem.*, **95**, 99 (1995).
[56] J. Rogalski, A. Dawidowicz, E. Jozwik, A. Leonowicz. *J. Mol. Catal. B: Enzym.*, **6**, 29 (1999).