Bioelectrochemistry 135 (2020) 107543

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Covalent attachment of laccase to carboxymethyl-botryosphaeran in aqueous solution for the construction of a voltammetric biosensor to quantify quercetin

Adriana Gomes [a], Gabriel J. Mattos [a], Bruna Coldibeli [a], Robert F.H. Dekker [b], Aneli M. Barbosa Dekker [a], Elen R. Sartori [a,*]

[a] Universidade Estadual de Londrina, Centro de Ciências Exatas, Departamento de Química, CEP: 86057-970, Londrina, PR, Brazil
[b] Universidade Tecnológica Federal do Paraná, Programa de Pós-Graduação em Engenharia Ambiental, Câmpus Londrina, CEP: 86036-370, Londrina, PR, Brazil

ARTICLE INFO

Article history:
Received 28 March 2020
Received in revised form 22 April 2020
Accepted 29 April 2020
Available online 15 May 2020

Keywords:
Fungal laccase
β-glucan
Electrochemical transduction
Voltammetry
Biopolymer
Flavonoids

ABSTRACT

Laccase from *Botryosphaeria rhodina* MAMB-05 was covalently immobilized on carboxymethyl-botryosphaeran by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC/NHS) in aqueous solution. This approach was employed to fabricate a novel laccase-based biosensor to electrochemically quantify quercetin (QCT), using a simple carbon black paste electrode as a transducer. The proposed biosensor was characterized by electrochemical impedance spectroscopy and Nyquist plots were used to evaluate the immobilization of the enzyme. For determining QCT, variables were optimized, that included experimental conditions for laccase immobilization, pH of the supporting electrolyte, and instrumental parameters of the electroanalytical technique. From square-wave-voltammograms, a linear dependence between the cathodic current peak and QCT concentration was observed within the range 4.98–50.0 $\times$ 10^{-8} mol L^{-1}, with a theoretical detection limit of 2.6 $\times$ 10^{-8} mol L^{-1}. The proposed method was successfully applied to determine QCT in beverages, pharmaceuticals, and biological samples. The proposed biosensor device presented good selectivity in the presence of uric acid, various inorganic ions, as well as other phenolic compounds, demonstrating the potential application of this biosensing platform in chemically complex solutions. Operational and analytical stability of the laccase-biosensor were evaluated, and good intra-day (SD = 1.23%) and inter-day (SD = 2.32%) repeatability, and long storage stability (SD = 3.47%) are presented.

1. Introduction

Natural biopolymers have received much attention in fundamental science, industrial biotechnology and applied research in the biomedical and pharmacological fields. Considering their remarkable structural and functional properties, polysaccharides, such as chitosan and carboxymethyl-cellulose, are some of the most applicable in the field of advanced sensor and biosensor technology [1,2]. Recently, our research group reported the application of botryosphaeran as a matrix for enzymatic immobilization and the construction of new bioanalytical devices [3,4]. This exocellular carbohydrate is produced by the ascomyceteous fungus *Botryosphaeria rhodina* MAMB-05, and its chemical structure consists of a backbone chain bound by β-(1 → 3)-glucosidic linkages with 22% side-branches of β-(1 → 6)–linked glucose and gentiobiose units [5].

The carboxymethylated derivative of botryosphaeran (carboxymethyl-botryosphaeran, CMB) was recently used in the construction of a novel electrochemical sensor, as described by Eisele et al. [6]. According to the authors, the electroanalytical studies indicated the formation of a homogenous, adherent and stable film of CMB on the surface of a glassy carbon electrode (GCE), generating a reproducible and sensitive electrochemical response for organic molecules. Carboxymethylation of botryosphaeran occurs primarily at the carbon-6 position, and the degree of substitution can vary according to the experimental conditions of carboxymethylation. The carboxylic groups inserted in the polysaccharide chain act as immobilization sites for proteins/enzymes, and the covalent bond linking the enzyme to CMB can be promoted through the action of activation/cross-linking agents.

* Corresponding author at: Universidade Estadual de Londrina, Centro de Ciências Exatas, Departamento de Química, Rodovia Celso Garcia Cid, PR 445, Km 380, Cx.P. 10.011, CEP: 86057-970, Londrina, PR, Brazil.
E-mail address: elensartori@uel.br (E.R. Sartori).

https://doi.org/10.1016/j.bioelechem.2020.107543
1567-5394/

Carboxyl-reactive chemical groups in biomolecular probes for labeling and crosslinking carboxylic acids to primary amines include the carbodiimide compound 1-ethyl-3-[3-dimethylamino propyl]carbodiimide hydrochloride (EDC) [7]. N-hydroxysuccinimide (NHS) is often included in EDC-coupling protocols to improve efficiency and create stable intermediates. The combined system, EDC/NHS, is one of the most attractive approaches for the covalent attachment of enzymes onto carboxyl- terminated surfaces in electrochemical biosensors, because of its high efficiency in coupling reactions and biocompatibility. These coupling reagents only slightly affect enzymatic activity [8]. Wang et al. [9] and Janegitz et al. [10] used EDC/NHS for enzyme immobilization on functionalized carbon nanotubes (CNTs) for bioelectroanalytical applications. However, the use of CMB as a support matrix for covalent immobilization of an enzyme using carbodiimide cross-linkers has not been described. Besides, this type of procedure for immobilizing the enzyme laccase onto functional groups of polysaccharides from the same biological source (*B. rhodina* MAMB-05) is not described in the literature, showing a novelty feature on the work we report.

In general, considering previous works reported in the literature, the preparation of electrochemical devices for biosensing applications involves several steps, all performed directly on the electrode surface [11–13]. However, this method has some drawbacks, such as time-consuming procedures, continuous enzyme leakage due to variable distribution on the electrode, and large quantities of reagents that are required. Exploring the immobilization of enzyme on a suitable support in aqueous solution with the activating agents EDC/NHS [9,10] minimizes time of the coupling reaction, the reagents used, and the biosensor device can be prepared in several units at a time, modifying the electrode only once with the proposed enzyme.

B. rhodina MAMB-05 besides producing the β-glucan, botryosphaeran, also produces an extracellular laccase (EC 1.10.3.2) constitutively, but which can be induced at higher enzyme activity levels by the inducer veratryl alcohol [14]. Laccases are glycoproteins within the class of multicopper oxidases that contain a cluster of copper atoms (T1/T2/T3) at the enzyme's active center [15]. This enzyme acts selectively in the oxidation of phenolic compounds in the reduced form that occurs at the T1 copper center, while simultaneously reducing molecular oxygen to water, which takes place at the T2 and T3 copper sites.

Laccase (LCE) is a strong candidate to act as a biorecognition molecule for the monitoring of phenolic compounds (analytes), providing some advantages over other enzymes considering its ability to catalyze electron-transfer reactions without additional cofactors or mediators [16]. Several enzyme-based biosensor topics of research have been conducted on determining phenolic compounds based on the laccase-catalyzed reaction for applications in environmental, medical and food analyses [17–19]. LCE has also been used in the design of bioanalytical devices for the detection of flavonoids, a subclass of polyphenols [20–22]. These compounds represent a diverse group of plant natural products that are ubiquitously distributed in nature, and are present in a variety of plant-based foods common to the human diet [23].

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one; QCT) is a flavonoid and a natural substrate for laccases. There are beneficial associations between QCT intake and human health, including the prevalence or incidence of conditions such as stroke, cardiovascular diseases, cognitive dysfunction, and cancer [24]. In order to evaluate the potential impact of consuming foods of high flavonoid content, and/or extract-based dietary supplements, an appropriate level of food characterization based upon analytical determinations is required. In this context, electrochemical biosensors act as promising bioanalytical tools, as these devices are chemically selective, have relatively low construction costs, and are applicable in routine chemical analyses.

According to the aforementioned, this study reports for the first time the construction of a laccase-biosensor based upon the covalent attachment of the enzyme laccase on CMB as support matrix via activation by EDC/NHS in aqueous solution, employing a simple carbon black paste electrode (CBPE) as the transducer. The proposed biosensor, named CBPE/CMB-LCE, was characterized electrochemically employing the redox pair $[Fe(CN)_6]^{3-/4-}$ by electrochemical impedance spectroscopy (EIS). The analytical performance of this novel electrochemical biosensor is described and discussed, as well as its comparison to other biosensors reported in the literature. Furthermore, the developed method using this bioanalytical device was applied for the determination of QCT in beverages, pharmaceutical and biological samples.

2. Materials and methods

2.1. Reagents and solutions

N-Hydroxysuccinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), QCT and H_2SO_4 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Botryosphaeran and laccase were obtained as previously described [25,26]. Carboxymethylation of botryosphaeran was performed with some modification as described by Xu et al. [27]. Carbon black VXC72R/LOT-3945501 was kindly provided by Cabot Brasil Indústria e Comércio Ltda., São Paulo-SP, Brazil. Uric acid and $K_4[Fe(CN)_6]$ were obtained from Synth (São Paulo-SP, Brazil). All reagents were of analytical grade and used as received. Commercial beverages and pharmaceutical samples were acquired from a local market and drugstore, respectively, in Londrina, Brazil.

All solutions were prepared using ultra-purified water supplied by a Milli-Q system (Millipore®) with resistivity greater than 18 MΩ cm. Phosphate buffer solutions (PBS) (0.1 mol L^{-1}), that were used as the supporting electrolyte throughout the electroanalytical experiments, were prepared from potassium monobasic phosphate and potassium dibasic phosphate (pH adjusted to pH 6.0). A stock solution of 10 mmol L^{-1} QCT was freshly prepared daily in a solution of 0.1 mol L^{-1} PBS (pH 6.0). Standard solutions of QCT were prepared from the stock solutions by appropriate dilutions.

2.2. Apparatus

Square-wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) measurements using the fabricated biosensor were performed on a FRA2 μAutoLab Type III potentiostat/galvanostat (Metrohm Autolab B.V., The Netherlands), and controlled with NOVA 2.1 software (www.metrohm-autolab.com/Products/Echem/Software). A conventional three-electrode single-compartment glass cell was used at room temperature (25.0 ± 0.5 °C) for all experiments, and comprised the biosensor as the working electrode, an Ag/AgCl (3.0 mol L^{-1} KCl) reference electrode, and a platinum plate as the auxiliary electrode. To ensure QCT was totally solubilized within the PBS contained in the electrochemical cell, the contents were stirred for 30 s prior to the measurements.

SW-voltammograms were baseline-corrected using the moving average method (peak width: 0.001). The EIS experiments were performed in the range of 10 mHz to 100 kHz (10 points per decade), and with a 10 mV (r.m.s.) ac perturbation for a 1 mmol L^{-1} $K_4[Fe(CN)_6]$ in 0.1 mol L^{-1} KCl solution.

All pH measurements were made at 25.0 ± 1.0 °C using a combined glass electrode with an Ag/AgCl (3.0 mol L^{-1} KCl) external

reference electrode connected to a pH meter, model W3B (BEL, SP, Brazil).

High-Performance Liquid Chromatography (HPLC) determination of QCT was carried out using a LC Shimadzu (Shimadzu, SP, Brazil) coupled to a system with a LC-20AT pump, SIL-20 AC automatic injector and SPD-M20A PDA detector, and an ACE 5 C-18 column (250 mm × 4.6 mm i.d., particle size: 5 μm) at 25.0 ± 1.0 °C. The mobile phase was composed of methanol and water (65:35). UV absorbance was monitored from 200 to 400 nm.

2.3. Preparation of the CBPE-CMB/LCE biosensor

CBPE was prepared by mixing carbon black and mineral oil (Nujol®) in a ratio of 30:70% (w/w). We observed that the paste prepared could be used over a five-month period without any variation in the values of the current obtained in the electrochemical analyses. The CB paste was carefully packed in the cavity of a Teflon® tube supported with a copper plate, and the contents compacted to produce a smooth surface.

Two methods were investigated to immobilize laccase onto CMB. In the first, the enzyme was immobilized onto CMB in solution, and the prepared immobilized enzyme soluble complex was then layered onto the surface of the CBPE electrode (designated CBPE/CMB-LCE/1; see Schematic 1), while in the second procedure, laccase was immobilized in a sequential manner directly onto the surface of the electrode (designated CBPE/CMB-LCE/2); see Schematic SM1 (SM – Supplementary Material).

The carboxyl coupling reaction (method 1) was performed by mixing an aliquot of 0.5 mL of PBS (pH 6.0) containing EDC (2 mmol L^{-1}) with a solution of 0.5 mL CMB (6 mg mL^{-1} in PBS, pH 6.0) and left stirring for 1 h at room temperature. Thereafter, the same procedure was used with the NHS solution (2 mmol L^{-1} in PBS, pH 6.0), as shown in Schematic 1. To covalently couple laccase to the activated carboxylic groups on CMB, 0.5 mL of LCE (activity 27.5 U mL^{-1}) was added to the above solution and thoroughly stirred for 1 h. Following the coupling step of laccase onto CMB, an aliquot sample (10 μL) was layered onto the surface of the prepared CB paste electrode, and the electrode cured for 1 h at 16 °C to evaporate the solution. The developed biosensor was stored at 4 °C (refrigerator) when not in use. It is important to mention that the final solution (2 mL) can be used to produce 200 bioanalytical devices.

For comparative purposes, a biosensor was prepared by directly immobilizing the enzyme on the CBPE surface (method 2). The electrode was modified by first dropping 10 μL of the CMB solution (1.5 mg mL^{-1} in PBS, pH 6.0) using a micropipette on the electrode surface and allowing it to dry for 1 h at room temperature. After this step, 10 μL of EDC solution (0.5 mmol L^{-1} in PBS, pH 6.0) was dropped on the modified surface of CBPE containing CMB and the electrode allowed to dry (1 h, room temperature). This was followed by applying 10 μL of NHS solution (0.5 mmol L^{-1} in PBS, pH 6.0) onto the electrode surface, and then allowed to dry for 1 h. Finally, 10 μL of LCE (activity 6.88 U mL^{-1}) was dropped onto the top of the activated modified electrode and allowed to couple and dry for 1 h at 16 °C. During the preparation of the electrode (CBPE/CMB-LCE/2), it was rinsed carefully with PBS (pH 6.0) after the deposition of each component. Schematic SM1 shows a schematic view of the mechanisms carried out in each step in the preparation of this biosensor.

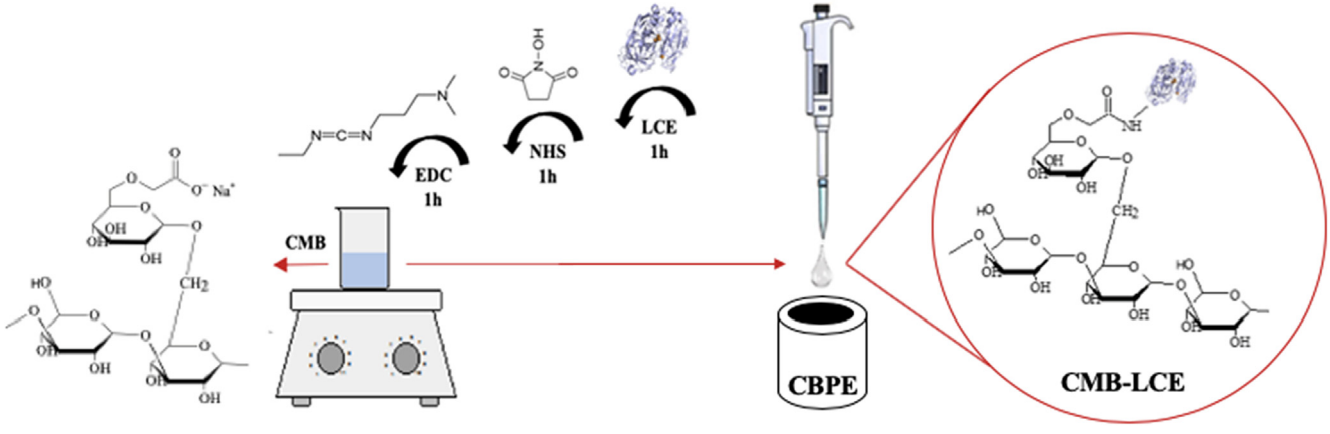
2.4. Measurement procedures

Square-wave voltammograms were obtained for the different sensors constructed (CBPE, CBPE/CMB, and CBPE/CMB-LCE), and in the presence of QCT to evaluate the electrochemical contribution of each modified procedure of the electrode architecture developed. The voltammetric measurements were performed using an electrochemical cell containing 10 mL of PBS (pH 6.0) and scanning the potential from 0.5 to −0.5 V. Measurements were carried out in triplicate to ensure the reproducibility of the results.

The calibration curve for QCT (4.98 to 50.0 × 10^{-8} mol L^{-1}) was obtained by adding small volumes of a standard solution (1.0 × 10^{-5} mol L^{-1}) in the electrochemical cell containing 10 mL of PBS (pH 6.0), and measurements were performed in triplicate. SW voltammograms of QCT were recorded scanning the potential from 0.5 and −0.5 V, after successive additions. The limit of detection (LOD) was calculated from the formula 3*S*/*M*, where *S* is the standard deviation of ten measurements of the blank solution, and *M* is the slope of the calibration curve [28].

2.5. Preparation and analysis of pharmaceutical samples

Ten capsules of a commercial pharmaceutical sample (250 mg capsule^{-1}) containing QCT were weighed and ground to a fine powder. A mass corresponding to the average weight of one capsule was transferred to a volumetric flask (100 mL) and made up to volume with PBS (pH 6.0). The solution was shaken for one minute to ensure the dissolution of QCT. Then, 60 μL of this solution was transferred to a 5.0 mL volumetric flask and the volume completed with PBS (pH 6.0). An aliquot was directly transferred to the electrochemical cell containing 10 mL of PBS (pH 6.0), without filtration of the undissolved excipients. The current was measured in



Schematic 1. Scheme showing the steps involved in the procedure for the immobilization (in solution) of laccase onto the activated carboxylic groups of carboxymethylbotryosphaeran (CMB).

triplicate and QCT concentration was determined by interpolation from the obtained analytical curve.

2.6. Preparation and analysis of commercial beverage samples (wine, apple juice, lemon juice and green tea) for quercetin

The beverage samples (red wine (merlot), apple juice, lemon juice and green tea) were added directly in the electrochemical cell, without any pretreatment step. The quantitative analysis of QCT in these samples was conducted in triplicate using the standard QCT calibration curve. Standard addition analysis was accomplished by measuring the current intensity on 200 μL of tea and wine samples, and 100 μL of apple/lemon juices, before and after the addition of precise aliquots of a known standard solution of QCT (1.0×10^{-5} mol L^{-1}).

In order to check the accuracy of the proposed method employing the laccase-biosensor, chromatographic determination (HPLC) of QCT was carried out on green tea, red wine, apple juice, and lemon juice as described by Price et al. [29], Dias et al. [30], Kahle et al. [31], and Gattuso et al. [32], respectively.

2.7. Preparation and analysis of a biological sample

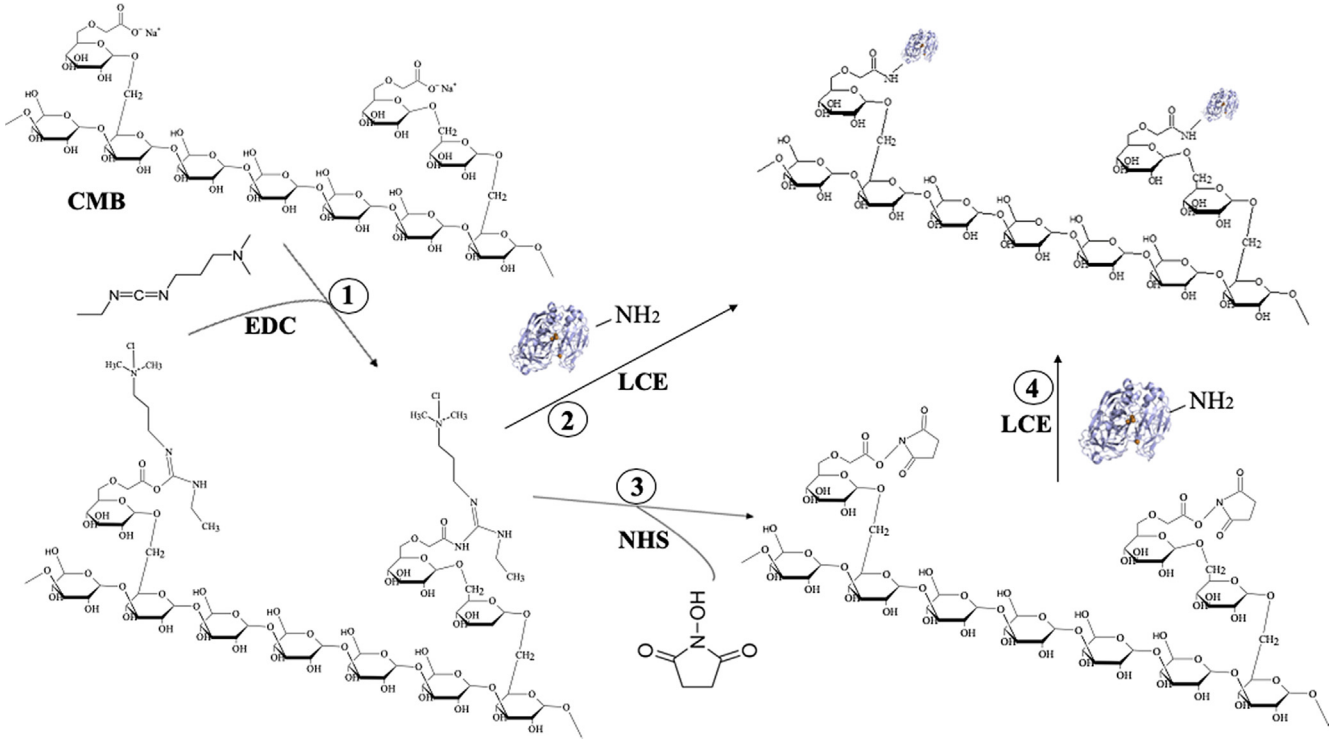
As an example of a biological sample, urine was selected to determine whether concomitant substances interfered with the analysis of QCT. A urine sample was acquired from a healthy adult male volunteer (male, 21 years old, non-smoker) immediately prior to the experimental measurements. For analysis, 0.1 mL of fresh urine was placed in the electrochemical cell containing 10 mL of PBS (pH 6.0). This solution was then spiked with a standard solution of QCT (1.0×10^{-5} mol L^{-1}) to achieve the required concentration. Addition and recovery tests were performed on the urine sample to verify the accuracy of the method on this biological specimen.

3. Results and discussion

3.1. Covalent attachment of LCE on CMB by EDC/NHS activating agents

EDC reacts with carboxylic acid groups on the carboxymethylbotryosphaeran chain to form an active *O*-acylisourea intermediate (Schematic 2, step 1) that is easily displaced by nucleophilic attack from primary amino groups present on laccase. The primary amine forms an amide bond with the original carboxyl group, according to the representation of the chemical reactions in Schematic 2 (step 2) [33]. NHS, or its water-soluble analog (sulfo-NHS), is often included in EDC-coupling reactions to improve efficiency [34]. EDC couples NHS to carboxyl groups forming an NHS ester (step 3) that is considerably more stable than the *O*-acylisourea intermediate while allowing for an efficient conjugation to the primary amines (step 4).

EDC/NHS crosslinking is most efficient under pH conditions in the range from 4.5 to 7.2, and phosphate buffer solutions with pH less than 7.2 are compatible with the reaction as well [35]. In this study, the process was conducted in PBS (pH 6.0), as the laccase from *B. rhodina* MAMB-05 remains stable under these conditions [25]. Covalent immobilization provides the strongest enzyme/support interaction, and ensures low protein leakage during catalysis, besides increasing the resistance to changes under conditions such as pH or temperature. This approach is the most effective in the field of bioelectrochemical devices since the enzyme maintains its catalytic activity, and the products are easily separated from the active site, allowing the device to be reused [36]. In this case, the CMB-LCE film on the CBPE surface was stable, reproducible and sensitive, providing excellent analytical perfor-



Schematic 2. Reaction scheme showing the proposed covalent coupling of laccase onto CM-botryosphaeran via activation by EDC and NHS.

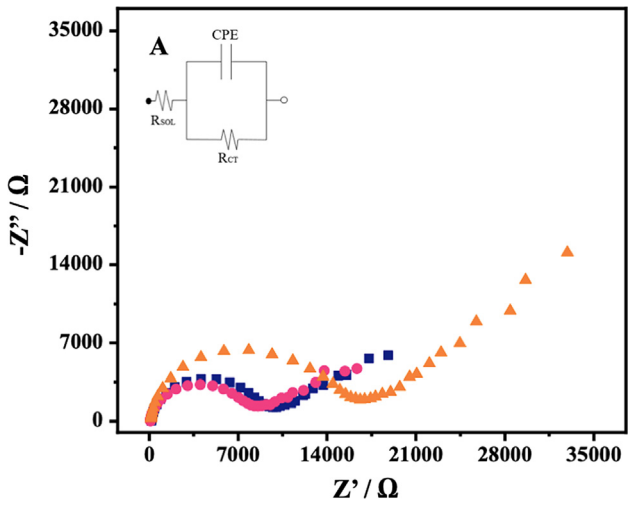


Fig. 1. Electrochemical impedance spectra for CBPE (●), CBPE/CMB (■) and CBPE/CMB-LCE/1 (▲) in 0.1 mol L^{-1} KCl solution containing 1.0 mmol L $[Fe(CN)_6]^{4-}$, open circuit mode (Inset A circuit model), 10 mV amplitude and frequency range of 10 mHz to 100 kHz.

mance for monitoring flavonoids, as we discuss throughout the text.

3.2. Electrochemical characterization of the CBPE-CMB/LCE biosensor

The physical-chemical characterization of bioelectrochemical devices can be performed based on impedance measurements associated with the electronic transfer kinetics for the surface phenomena. The electrochemical characterization of the following fabricated electrodes: CBPE, CBPE/CMB, and CBPE/CMB-LCE/1 was performed by EIS using the classical electrochemical probe $[Fe(CN)_6]^{3-/4-}$. The Randles circuit is one of the most common models used for impedance measurements and electrochemical characterization. This system includes only solution resistance (R_{SOL}), a parallel combination of a double-layer capacitor (CPE – constant phase element) and a charge transfer or polarization resistance (R_{CT}), as represented in the circuit model shown in Fig. 1 (inset **A**) [37]. The Nyquist plot of a Randles cell can be used to evaluate the effect of different modifications on the electrochemical surface. The diameter of the semi-circle at high frequencies represents the charge transfer resistance, and the straight line at an angle of 45° to the real axis at lower frequencies represents the diffusion process (mass transfer control).

As shown in Fig. 1, the layer of CMB caused an electrochemical alteration at the electrode/solution interface, increasing the semi-circle ratio (R_{CT} 11.9 kΩ) compared to the unmodified CBPE (R_{CT} 8.3 kΩ). This result suggests that the negative charges from the carboxyl groups on the carboxymethyl-botryosphaeran structure repel the ferrocyanide group, decreasing the diffusion of this inorganic electroactive species on the electrode surface. When the CBPE was layered with the complex CMB-LCE, the device reached the highest resistance value (R_{CT} 16.3 kΩ). According to studies reported in the literature, laccases commonly present an isoelectric point between 3.0 and 5.0 [38]. In this case, the impedance experiment was performed at pH 7.0, above the laccase isoelectric point. In this condition, the enzyme presents a negative charge, and the electric repulsion causes the increase in the resistance. In addition, previous work in the literature described that the immobilized proteins seriously inhibited the electron transfer of the electroactive probe with the electrode [39–41]. Therefore, our result ensures the efficient and stable immobilization of LCE on the CMB structure.

3.3. Electrochemical behavior of the quercetin molecule on the CBPE-CMB/LCE biosensor surface

As previously mentioned, laccases catalyze the oxidation of polyphenolic compounds, such as QCT, in the presence of molecular oxygen as the final acceptor of electrons. In this study, the electrochemical monitoring was based on the oxidation of QCT to the corresponding *o*-quinone by laccase, followed by the electrochemical reduction at the surface of the proposed biosensor at 0.23 V *vs.* Ag/AgCl, according to the mechanism shown in Fig. 2. The measured cathodic peak current from the electrochemical reduction of the *o*-quinone on the biosensor surface was directly proportional to the concentration of QCT in the electrochemical cell, allowing the determination of this flavonoid in real samples.

Fig. SM1 (SM – Supplementary Material) presents the electrochemical behavior of 99.0 μmol L^{-1} QCT studied by cyclic voltammetry in PBS (pH 6.0) at a scan rate of 100 mV s^{-1}. As can be seen, using the CBPE/CMB-LCE/1 device resulted in a decrease in the oxidation peak and a remarkable increase in the reduction peak compared to the non-enzymatic electrodes (CBPE and CBPE/CMB). As discussed above, laccase catalyzes the oxidation of QCT to *o*-quinone. Therefore, the *o*-quinone is reduced electrochemically (reverse scan), regenerating the QCT at the electrode surface and increasing the voltammetric signal.

The electrochemical behavior of the *o*-quinone was evaluated by SWV on the surface of the four different working electrodes: CBPE, CBPE/CMB, CBPE/CMB-LCE/1 (laccase immobilized in solution), and CBPE/CMB-LCE/2 (enzyme immobilized directly on the CBPE surface). Fig. 2 shows the square-wave voltammograms obtained in PBS (pH 6.0) resulting from the electrochemical reduction of *o*-quinone at an initial quercetin concentration of 99.0 μmol L^{-1}. Based on the presented behavior, the significant increase in current magnitude for QCT *o*-quinone reduction on the CBPE/CMB surface, compared to unmodified CBPE, suggests probable hydrogen bonding interaction between the carboxyl groups on the structure of CMB and the hydroxyl groups on *o*-quinone.

The best performance in terms of analytical signal intensity was obtained for the proposed biosensor (CBPE/CMB-LCE/1, immobilized in solution) using laccase as a biocatalyst. As can be seen, there is no significant difference between the two methods of

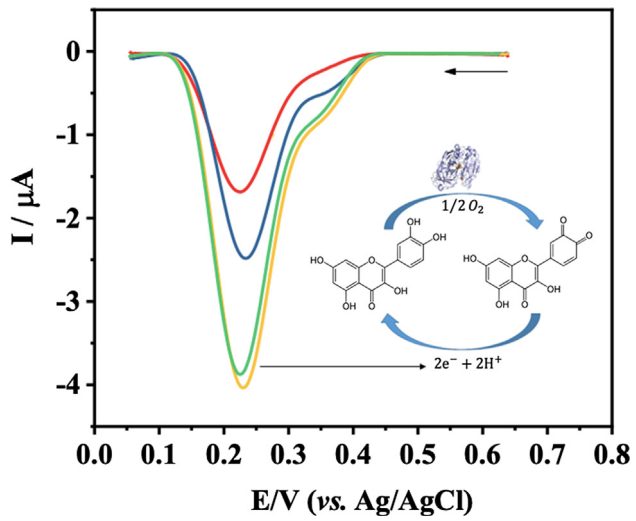


Fig. 2. SW voltammograms obtained using the electrodes: CBPE (▬), CBPE/CMB (▬), CBPE/CMB-LCE/1 (immobilization in solution) (▬) and CBPE/CMB-LCE/2 (immobilization directly on the CBPE) (▬) in PBS (pH 6.0) containing 90.0 μmol L^{-1} QCT. SWV conditions: a = 50 mV, ΔE_s = 2 mV and f = 30 Hz. *Inset*: proposed mechanism for the enzymatic oxidation of QCT and the subsequent electrochemical reduction of *o*-quinone.

immobilizing laccase. However, performing the covalent attachment in aqueous solution allows the large-scale production of the immobilized enzyme for constructing the proposed biosensor. The enzymatic activity based on a stereospecific reaction confers high selectivity on the proposed bioanalytical device and promotes the rapid conversion of QCT to *o*-quinone (oxidized structure) on the biosensor surface, which upon reduction by the electrochemical electrode results in a greater signal of cathodic current.

The stability and preservation of laccase activity of the proposed biosensor (CBPE/CMB-LCE) were tested by evaluating the electrochemical responses after a long period of storage. The response was monitored in QCT solution (99.0 μmol L^{-1}) by repetitive measurements over several days. After each measurement, the biosensor was rinsed and stored at 4.0 °C (refrigerator). After 30 days of these sequential experiments, cathodic current signals showed a standard deviation (SD) of 3.24%, providing 300 stable and reproducible measurements taken without surface renovation, as determined by electrochemical responses obtained from the analysis of QCT by the bioanalytical device. The stable maintenance of laccase activity on the electrode is attributed to the preservation of the three-dimensional structure of the enzyme's polypeptide chain at all structural levels (primary, secondary, tertiary and quaternary) due to the efficient covalent immobilization onto the CMB structure via EDC/NHS. Thus, this carboxymethylated derivative of the exopolysaccharide botryosphaeran shows itself as a biological matrix suitable for the immobilization of laccase and fabrication of a biosensor device, maintaining enzyme activity, ensuring stability and a long lifetime for the constructed biosensor.

3.4. Selection of the experimental/instrumental parameters for the best analytical performance of the proposed laccase biosensor

The electrochemical behavior of organic molecules on the interfacial phenomena is usually dependent upon the pH of the supporting electrolyte. Since these compounds have different chemical groups that act as weak acids or bases, the charge is variable according to the pKa for each molecule, and this affects the electroactive profile. In the field of biosensor technology, electroanalytical measurements are dependent upon enzyme activity. Enzymes have a primary structure made up of L-amino acids that present weak acidic and basic groups, which are affected by pH variations resulting in different levels of enzymatic activity.

The influence of pH of PBS (ranging from 4.0 to 8.0) on the reduction of the current peak of *o*-quinone was evaluated for the response of the biosensor. As shown in Fig. 3A, the highest voltammetric response for the reduction of *o*-quinone was registered at pH 6.0. The internal transfer of electrons from the T1 Cu ion site to the trinuclear copper cluster of the active center of laccase is affected by the binding of OH^- ions to T2 Cu and T3 Cu sites at higher pH's, as can be seen for PBS at pH 7.0 and 8.0 [42]. The interaction between hydroxide and the copper ions hinders the catalytic stability of the laccase resulting in lower levels of *o*-quinone, and consequently, less cathodic current is monitored. Therefore, PBS of pH 6.0 was used in further studies in order to obtain the best reproducible intensity of the analytical signals. Fig. 3B shows the linear variation between the peak potential and the pH of PBS with a slope of −54.8 mV/pH (r^2 = 0.998); a value closer to that expected for a Nernstian system with two electrons and two protons involved, as described above for the inserted mechanism in Fig. 2 [43].

Prior to the construction of the analytical curve, the SWV operating parameters were optimized in order to obtain a higher current signal and a defined peak for the determination of QCT. The parameter and ranges investigated were: 10–100 mV, for the pulse amplitude (*a*); 10–100 Hz, for the square wave frequency (*f*) and 1–6 mV, for the scan increment (Δ*E*s). The selected values for

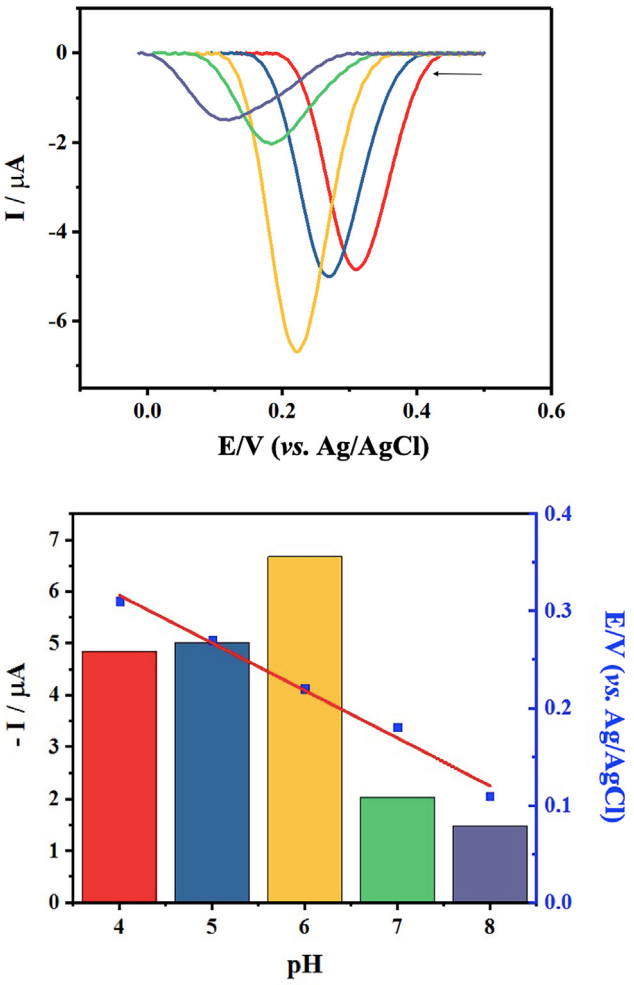


Fig. 3. (A) SW voltammograms obtained using the CBPE/CMB-LCE/1 biosensor with 99.0 μmol L^{-1} QCT in PBS at different pH values: 4.0 to 8.0 and (B) Influence of pH on the cathodic peak current (bars) and the peak potential (red line) of the proposed biosensor. The conditions of SWV were the same as those indicated in Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

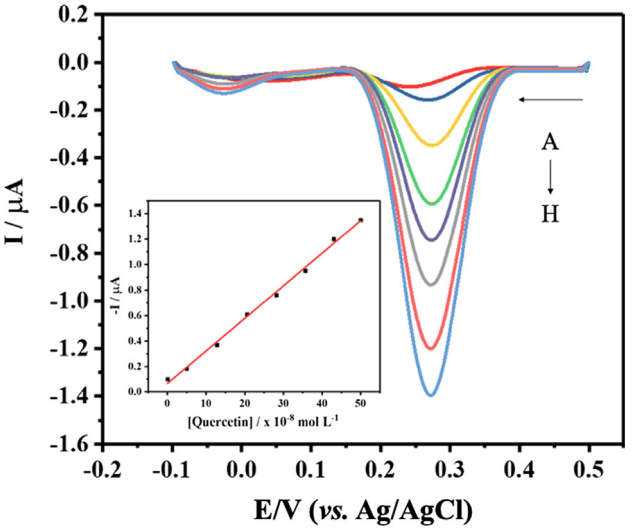


Fig. 4. SW voltammograms obtained in PBS (pH 6.0) employing the CBPE/CMB-LCE/1 biosensor as working electrode for the following concentrations of QCT: (B – H): 4.98 – 50.0 × 10^{-8} mol L^{-1}. SWV conditions: *a* = 50 mV, *f* = 50 Hz, and Δ*E*s = 3 mV *Inset*: Corresponding analytical curve.

Table 1
Comparison of the analytical parameters and method features presented by the proposed biosensor with other electrodes for determining quercetin.

Technique/Electrode	Linear range (μM)	LOD (μM)	Method features	Reference
SWV / GCE-MWCN-monosuccinyl β-cyclodextrin	1.5–7.5	–	- time-consuming for electrode preparation; selectivity problems.	[44]
SWV / GCE-MWCN-Nafion	0.02–1.7	–	- rapid determination; selectivity problems.	[45]
DPV / MWCN-Nujol	0.1–1.0	0.03	- rapid determination; but is not selective.	[46]
SWV / Lac/Ag-BMI.Tf_2N	0.499–7.407	0.037	- time-consuming for electrode preparation; selectivity.	[43]
SWV / CBPE/CMB-LCE/1	0.0498–0.794	0.026	- lowest LOD; efficient method for enzyme immobilization; selectivity.	This work

GCE – glassy carbon electrode; MWCN – multi-walled carbon nanotubes; Lac and LCE – laccase; Ag-BMI.Tf2N – silver nanoparticles in 1-butyl-3-methylimidazolium bis (trifluoromethylsulfonyl)imide; Nafion – name for a sulfonated tetrafluoroethylene based fluoropolymer- copolymer, an ionomer.

Table 2
Quercetin content of commercial beverage samples determined using the proposed laccase-biosensor and compared to HPLC analysis.

Beverage sample	QCT amount (mg L^{-1})[a]			
	LCE-biosensor	HPLC	E (%)[b]	F_{calc}[c]
Red wine	9.8 ± 0.2	10.2 ± 0.3	−3.9	2.25
Green tea	7.6 ± 0.3	7.8 ± 0.1	−2.6	0.11
Apple juice	6.7 ± 0.4	6.5 ± 0.2	3.1	0.25
Lemon juice	6.5 ± 0.8	6.8 ± 0.5	−4.4	0.39

[a] Average of three measurements.
[b] 100 × [(LCE-biosensor − HPLC method)/HPLC method].
[c] Critical F-value = 19.0 (95% confidence level).

further voltammetric measurements were: a = 50 mV, f = 50 Hz, and ΔEs = 3 mV. These parameters were used to record the analytical curve using aliquots of a standard solution of QCT in PBS (pH 6.0).

The analytical curve was obtained from the resultant cathodic peak current recorded in the square-wave voltammograms, as a function of the concentration of QCT (Fig. 4). A linear relationship was observed between the current (μA) and QCT concentrations ranging from 4.98 to 50.0 × 10^{-8} mol L^{-1} (Fig. 4, *Inset*), using the linear regression equation: I_{CP} (μA) = 0.07 + 0.025 [c (×10^{-8} mol L^{-1})] (r^2 = 0.998) at pH 6.0 where I_{CP} is the cathodic peak current, and c the QCT concentration. The theoretical LOD was 2.64 × 10^{-8} mol L^{-1}.

The analytical performance of the proposed biosensor was compared with those previously reported in the literature for modified electrodes or biosensors used for the determination of QCT, and this information is presented in Table 1. The comparative data confirms that the proposed biosensor presents excellent performance, in terms of LOD and the linear range examined, with analytical parameters better than those reported in the literature. In addition, using CMB as a matrix for covalent immobilization of enzyme guaranteed a longer lifetime for laccase, maintaining its catalytic activity and protecting the enzyme against inactivation by external factors. Therefore, the biosensor constructed, as well as the proposed immobilization method of laccase, was found to be promising in developing an electroanalytical method for the determination of QCT in real samples.

The repeatability of the electroanalytical measurements performed using the proposed biosensor was evaluated by monitoring the cathodic current by SWV in PBS (pH 6.0) using 15.0 μmol L^{-1} QCT. After ten successive measurements, the current signals presented a SD of 1.23% on the same device, without any necessary electrode surface treatment or renovation. The results confirmed the stability of the laccase immobilized, and that this biosensor can be used in repetitive analyses providing accurate results.

In order to evaluate the reproducibility level of the biosensor assembly, the cathodic currents of three biosensor constructed using the same immobilization procedure (in solution) were monitored using 15.0 μmol L^{-1} QCT in PBS (pH 6.0). The SD between measurements of the three devices at the same concentration was 2.32%, indicating excellent reproducibility for the biosensor constructed.

3.5. Selectivity and analytical application of the proposed CBPE/CMB-LCE biosensor for the determination of quercetin

In order to assess the selectivity of the proposed biosensor, the effect of different chemical species on the electrochemical reduction of *o*-quinone was evaluated by SWV. This procedure was performed to determine possible interference in measuring QCT using a standard solution of 99.0 μmol L^{-1}, and evaluated at a ratio concentration of QCT-to-other chemical species of 1:1 and 1:10. Substrate specificity was evaluated in the presence of related phenol-model compounds: epinephrine, dopamine, paracetamol, guaiacol and catechol. No significant interference was observed in the cathodic current signal corresponding to QCT (SD < 2%) since the reduction signals for each phenolic compound analyzed did not overlap with the QCT peak. In the presence of uric acid and some inorganic ions commonly found as complexes (Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, SO_4^{2-} and NO^{3-}), the proposed biosensor also presented a stable and reproducible response to QCT (RSD < 1%).

In order to evaluate the applicability of the proposed biosensor in real and chemically complex samples, QCT was determined in commercial flavonoid-containing beverages, such as green tea, red wine, and apple/lemon juice. Fig. SM2 shows the SW-voltammograms obtained by the standard addition analysis for (A) green tea, (B) red wine, (C) apple juice and (D) lemon juice. A linear signal response for all the samples was observed in the concentration range of 0.0 to 35.7 × 10^{-8} mol L^{-1}, following equations: (A) I_{CP} (μA) = 0.171 + 0.003 [c (×10^{-8} mol L^{-1})] (r^2 = 0.998); (B) I_{CP} (μA) = 0.208 + 0.005 [c (×10^{-8} mol L^{-1})] (r^2 = 0.996); (C) I_{CP} (μA) = 0.147 + 0.003 [c (×10^{-8} mol L^{-1})] (r^2 = 0.998) and (D) I_{CP} (μA) = 0.100 + 0.005 [c (×10^{-8} mol L^{-1})] (r^2 = 0.999).

The results of the analysis of all the samples are summarized in Table 2. No significant differences were observed between the values found for the contents of QCT in the commercial samples using the developed bioanalytical device, and the validated HPLC analyses [29–32]. According to the *F*-test [47], the two methods provide

an equivalent level of precision at a 95% confidence level. All calculated values were lower than the standard value ($F_{critical}$ = 19.0). Besides, considering that the paired *t*-test [47] was applied to these results and the calculated *t* value (t = 1.35) is smaller than the critical value (3.18, α = 0.05), the results obtained with either method are statistically equivalents, at a 95% confidence level.

In the pharmaceutical samples analyzed, the results obtained for QCT in one sample employing the laccase-biosensor (249 ± 2 mg capsule^{-1}) was very similar to that obtained by Shaikh and Jain (2018) using the HPLC technique (251 ± 3 mg capsule^{-1}) [48]. In addition, good recovery was obtained for the commercial sample investigated, ranging from 98.4% to 102%, indicating there was no interference by the excipients in the preparation.

The proposed biosensor was also employed to determine QCT when added to a biological specimen; e.g., human urine. The samples were doped with two different concentrations of QCT: 2.0 and 6.0 mg L^{-1}, and excellent recovery resulted in values close to 100% (98 and 102%, respectively). Based upon these results, the proposed bioanalytical device ensured a high degree of accuracy in the quantitative analysis of this flavonoid.

4. Conclusion

The proposed electrochemical biosensor (CBPE/CMB-LCE/1) was fabricated with a fungal laccase immobilized onto carboxymethyl-botryosphaeran in aqueous solution; a procedure that minimized the number of steps in preparing this analytical device. This novel biosensing platform was successfully applied to the detection and quantitation of QCT in commercial beverages and pharmaceutical preparations, and a biological sample, human urine. The excellent analytical performance of this biosensing device was attributed to the stable covalent coupling of the enzyme on the CMB structure in aqueous solution through the use of the activating agents EDC/NHS. The laccase biosensor maintained its catalytic activity at the electrode/solution interface, providing selectivity and stability of the proposed analytical method. In view of this, carboxymethyl-botryosphaeran was demonstrated to be a promising biomaterial for the construction of bioanalytical sensor devices. The determination of QCT in chemically complex samples (beverages and biological fluids) did not require *a priori* treatment. Based upon the addition and recovery studies for human urine, no interference from other components in these samples was observed, confirming the biosensor was highly selective. The results for the quantitation of QCT in beverages and commercial pharmaceutical preparations were in agreement with HPLC determinations at 95% confidence level. Furthermore, the proposed biosensing platform presented in this study appears to have wide applications in bioanalytical determinations, with fast response times, chemical selectivity, in a cost-effective manner and non-polluting; recognition elements for commercial production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge the financial support and scholarships from the Brazilian funding agencies CNPq (grants no. 408591/2018-8 and 305320/2019-0; E. R. Sartori), Fundação Araucária do Paraná and CAPES. Dr A. M. Barbosa-Dekker is grateful to CNPq (Proc. 309717/2015-9) and Fundação Araucária for a senior scholarship.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2020.107543.

References

[1] N. Hernández-Ibáñez, L. García-Cruz, V. Montiel, C.W. Foster, C.E. Banks, J. Iniesta, Electrochemical lactate biosensor based upon chitosan/carbon nanotubes modified screen-printed graphite electrodes for the determination of lactate in embryonic cell cultures, Biosens. Bioelectron. 77 (2016) 1168–1174, https://doi.org/10.1016/J.BIOS.2015.11.005.

[2] J. Chang, W. Xiao, P. Liu, X. Liao, Y. Wen, L. Bai, L. Li, M. li, Carboxymethyl cellulose assisted preparation of water-processable halloysite nanotubular composites with carboxyl-functionalized multi-carbon nanotubes for simultaneous voltammetric detection of uric acid, guanine and adenine in biological samples, J. Electroanal. Chem. 780 (2016) 103–113.

[3] G.J. Mattos, J.T. Moraes, E.C.M. Barbosa, P.H.C. Camargo, R.F.H. Dekker, A.M.B. Dekker, E.R. Sartori, Laccase stabilized on β-D-glucan films on the surface of carbon black/gold nanoparticles: a new platform for electrochemical biosensing, Bioelectrochemistry (2019), https://doi.org/10.1016/J.BIOELECHEM.2019.05.002.

[4] J.H. Coelho, A.P.P. Eisele, C.F. Valezi, G.J. Mattos, J.G. Schirmann, R.F.H. Dekker, A.M. Barbosa-Dekker, E.R. Sartori, Exploring the exocellular fungal biopolymer botryosphaeran for laccase-biosensor architecture and application to determine dopamine and spironolactone, Talanta 204 (2019) 475–483, https://doi.org/10.1016/J.TALANTA.2019.06.033.

[5] R.F.H. Dekker, E.A.I.F. Queiroz, M.A.A. Cunha, A.M. Barbosa-Dekker, Botryosphaeran – A Fungal Exopolysaccharide of the (1{\textrightarrow}3)(1{\textrightarrow}6)-$β$-D-Glucan Kind: Structure and Biological Functions, in: E. Cohen, H. Merzendorfer (Eds.), Extracell. Sugar-Based Biopolym. Matrices, Springer International Publishing, Cham, 2019: Ch. 11, pp. 433–484. https://doi.org/10.1007/978-3-030-12919-4_11.

[6] A.P.P. Eisele, C.F. Valezi, T. Mazziero, R.F.H. Dekker, A.M. Barbosa-Dekker, E.R. Sartori, Layering of a film of carboxymethyl-botryosphaeran onto carbon black as a novel sensitive electrochemical platform on glassy carbon electrodes for the improvement in the simultaneous determination of phenolic compounds, Sensors Actuators B Chem. 287 (2019) 18–26, https://doi.org/10.1016/J.SNB.2019.02.004.

[7] L.-S. Jang, H.-K. Keng, Modified fabrication process of protein chips using a short-chain self-assembled monolayer, Biomed. Microdevices. 10 (2008) 203–211, https://doi.org/10.1007/s10544-007-9126-7.

[8] J.V. Staros, N-hydroxysulfosuccinimide active esters: bis(N-hydroxysulfosuccinimide) esters of two dicarboxylic acids are hydrophilic, membrane-impermeant, protein cross-linkers, Biochemistry 21 (1982) 3950–3955, https://doi.org/10.1021/bi00260a008.

[9] J. Wang, M. Musameh, Y. Lin, Solubilization of carbon nanotubes by Nafion toward the preparation of amperometric biosensors, J. Am. Chem. Soc. 125 (2003) 2408–2409, https://doi.org/10.1021/ja028951v.

[10] B.C. Janegitz, R. Pauliukaite, M.E. Ghica, C.M.A. Brett, O. Fatibello-Filho, Direct electron transfer of glucose oxidase at glassy carbon electrode modified with functionalized carbon nanotubes within a dihexadecylphosphate film, Sensors Actuators B Chem. 158 (2011) 411–417.

[11] E.R. Sartori, F.C. Vicentini, O. Fatibello-Filho, Indirect determination of sulfite using a polyphenol oxidase biosensor based on a glassy carbon electrode modified with multi-walled carbon nanotubes and gold nanoparticles within a poly(allylamine hydrochloride) film, Talanta 87 (2011) 235–242, https://doi.org/10.1016/J.TALANTA.2011.10.003.

[12] D. Quan, Y. Kim, W. Shin, Characterization of an amperometric laccase electrode covalently immobilized on platinum surface, J. Electroanal. Chem. 561 (2004) 181–189, https://doi.org/10.1016/J.JELECHEM.2003.08.003.

[13] M.L. Mena, V. Carralero, A. González-Cortés, P. Yáñez-Sedeño, J.M. Pingarrón, Laccase biosensor based on N-succinimidyl-3-thiopropionate-functionalized gold electrodes, Electroanalysis 17 (2005) 2147–2155, https://doi.org/10.1002/elan.200503345.

[14] A.F.D. Vasconcelos, A.M. Barbosa, R.F.H. Dekker, I.S. Scarminio, M.I. Rezende, Optimization of laccase production by Botryosphaeria sp. in the presence of veratryl alcohol by the response-surface method, Process Biochem. 35 (2000) 1131–1138, https://doi.org/10.1016/S0032-9592(00)00149-7.

[15] M.D. Cannatelli, A.J. Ragauskas, Two decades of laccases: advancing sustainability in the chemical industry, Chem. Rec. 17 (2017) 122–140, https://doi.org/10.1002/tcr.201600033.

[16] M.M. Rodriguez-Delgado, G.S. Alemán-Nava, J.M. Rodríguez-Delgado, G. Dieck-Assad, S.O. Martínez-Chapa, D. Barceló, R. Parra, Laccase-based biosensors for detection of phenolic compounds, TrAC - Trends Anal. Chem. 74 (2015) 21–45, https://doi.org/10.1016/j.trac.2015.05.008.

[17] L.P. Mei, J.J. Feng, L. Wu, J.Y. Zhou, J.R. Chen, A.J. Wang, Novel phenol biosensor based on laccase immobilized on reduced graphene oxide supported palladium-copper alloyed nanocages, Biosens. Bioelectron. 74 (2015) 347–352, https://doi.org/10.1016/j.bios.2015.06.060.

[18] I. Vasilescu, S.A.V. Eremia, M. Kusko, A. Radoi, E. Vasile, G.-L. Radu, Molybdenum disulphide and graphene quantum dots as electrode modifiers for laccase biosensor, Biosens. Bioelectron. 75 (2016) 232–237, https://doi.org/10.1016/j.bios.2015.08.051.

[19] S. Palanisamy, S.K. Ramaraj, S.M. Chen, T.C.K. Yang, Y.F. Pan, T.W. Chen, V. Velusamy, S. Selvam, A novel laccase biosensor based on laccase immobilized graphene-cellulose microfiber composite modified screen-printed carbon electrode for sensitive determination of catechol, Sci. Rep. 7 (2017) 1–12, https://doi.org/10.1038/srep41214.
[20] S.C. Fernandes, I.R.W.Z. de Oliveira, O. Fatibello-Filho, A. Spinelli, I.C. Vieira, Biosensor based on laccase immobilized on microspheres of chitosan crosslinked with tripolyphosphate, Sensors Actuators B Chem. 133 (2008) 202–207, https://doi.org/10.1016/J.SNB.2008.02.023.
[21] A. Jarosz-Wilkołazka, T. Ruzgas, L. Gorton, Use of laccase-modified electrode for amperometric detection of plant flavonoids, Enzyme Microb. Technol. 35 (2004) 238–241, https://doi.org/10.1016/J.ENZMICTEC.2004.04.016.
[22] A.C. Franzoi, P. Migowski, J. Dupont, I.C. Vieira, Development of biosensors containing laccase and imidazolium bis(trifluoromethylsulfonyl)imide ionic liquid for the determination of rutin, Anal. Chim. Acta 639 (2009) 90–95, https://doi.org/10.1016/J.ACA.2009.03.012.
[23] P. Bonaccorsi, C. Caristi, C. Gargiulli, U. Leuzzi, Flavonol glucoside profile of Southern Italian red onion (Allium cepa L.), J. Agric. Food Chem. 53 (2005) 2733–2740, https://doi.org/10.1021/jf048152r.
[24] S.P. Wong, L.P. Leong, J.H. William Koh, Antioxidant activities of aqueous extracts of selected plants, Food Chem. 99 (2006) 775–783, https://doi.org/10.1016/J.FOODCHEM.2005.07.058.
[25] R.F. Dekker, A.M. Barbosa, The effects of aeration and veratryl alcohol on the production of two laccases by the ascomycete Botryosphaeria sp, Enzyme Microb. Technol. 28 (2001) 81–88, https://doi.org/10.1016/S0141-0229(00)00274-X.
[26] A.M. Barbosa, R.M. Steluti, R.F.H. Dekker, M.S. Cardoso, M.L.C. Silva, Structural characterization of Botryosphaeran: a (1→3;1→6)-β-d-glucan produced by the ascomyceteous fungus, Botryosphaeria sp, Carbohydr. Res. 338 (2003) 1691–1698.
[27] J. Xu, W. Liu, W. Yao, X. Pang, D. Yin, X. Gao, Carboxymethylation of a polysaccharide extracted from Ganoderma lucidum enhances its antioxidant activities in vitro, Carbohydr. Polym. 78 (2009) 227–234.
[28] G.L. Long, J.D. Winefordner, Limit of detection. A closer look at the IUPAC definition, Anal. Chem. 55 (1983) 712A–724A. https://doi.org/10.1021/ac00258a001.
[29] K.R. Price, M.J.C. Rhodes, K.A. Barnes, Flavonol glycoside content and composition of tea infusions made from commercially available teas and tea products, J. Agric. Food Chem. 46 (1998) 2517–2522, https://doi.org/10.1021/jf9800211.
[30] F.S. De Dias, J.M. David, J.P. David, Determination of phenolic acids and quercetin in Brazilian red wines from vale do São Francisco region using liquid-liquid ultrasound-assisted extraction and HPLC-DAD-MS, J. Braz. Chem. Soc. 27 (2016) 1055–1059, https://doi.org/10.5935/0103-5053.20150363.
[31] K. Kahle, M. Kraus, E. Richling, Polyphenol profiles of apple juices, Mol. Nutr. Food Res. 49 (2005) 797–806, https://doi.org/10.1002/mnfr.200500064.
[32] G. Gattuso, D. Barreca, C. Gargiulli, U. Leuzzi, C. Caristi, Flavonoid composition of citrus juices, Molecules 12 (2007) 1641–1673, https://doi.org/10.3390/12081641.
[33] M.J.E. Fischer, Amine Coupling Through EDC/NHS: A Practical Approach, in: N.J. Mol, M.J.E. Fischer (Eds.), Surf. Plasmon Reson. Methods Protoc., Humana Press, Totowa, NJ, 2010, pp. 55–73. https://doi.org/10.1007/978-1-60761-670-2_3.
[34] Thermo Fisher, Chemical reactivity of crosslinkers and modification reagents, Crosslink. Technol. React. Chem. Appl. Struct. Ref. (2012) 3–4.
[35] Z. Grabarek, J. Gergely, Zero-length crosslinking procedure with the use of active esters, Anal. Biochem. 185 (1990) 131–135, https://doi.org/10.1016/0003-2697(90)90267-D.
[36] S.J. Novick, J.D. Rozzell, Immobilization of Enzymes by Covalent Attachment, in: J.L. Barredo (Ed.), Microb. Enzym. Biotransformations, Humana Press, Totowa, NJ, 2005, pp. 247–271. https://doi.org/10.1385/1-59259-846-3:247.
[37] V.F. Lvovich, Wiley InterScience (Online service), Impedance spectroscopy: applications to electrochemical and dielectric phenomena, Wiley, 2012.
[38] B. Viswanath, B. Rajesh, A. Janardhan, A.P. Kumar, G. Narasimha, Fungal laccases and their applications in bioremediation, Enzyme Res. 2014 (2014) 163242. https://doi.org/10.1155/2014/163242.
[39] Y. Chen, P.X. Yuan, A.J. Wang, X. Luo, Y. Xue, L. Zhang, J.J. Feng, A novel electrochemical immunosensor for highly sensitive detection of prostate-specific antigen using 3D open-structured PtCu nanoframes for signal amplification, Biosens. Bioelectron. 126 (2019) 187–192, https://doi.org/10.1016/j.bios.2018.10.057.
[40] Y. Chen, A.J. Wang, P.X. Yuan, X. Luo, Y. Xue, J.J. Feng, Three dimensional sea-urchin-like PdAuCu nanocrystals/ferrocene-grafted-polylysine as an efficient probe to amplify the electrochemical signals for ultrasensitive immunoassay of carcinoembryonic antigen, Biosens. Bioelectron. 132 (2019) 294–301, https://doi.org/10.1016/j.bios.2019.02.057.
[41] Y. Chen, L.P. Mei, J.J. Feng, P.X. Yuan, X. Luo, A.J. Wang, Simple one-pot aqueous synthesis of 3D superstructured PtCoCuPd alloyed tripods with hierarchical branches for ultrasensitive immunoassay of cardiac troponin I, Biosens. Bioelectron. 145 (2019), https://doi.org/10.1016/j.bios.2019.111638 111638.
[42] F. Xu, Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases, J. Biol. Chem. 272 (1997) 924–928, https://doi.org/10.1074/JBC.272.2.924.
[43] A.C. Franzoi, I.C. Vieira, C.W. Scheeren, J. Dupont, Development of quercetin biosensor through immobilizing laccase in a modified p-cyclodextrin matrix containing ag nanoparticles in ionic liquid, Electroanalysis. 22 (2010) 1376–1392, https://doi.org/10.1002/elan.200900591.
[44] J.H. Jin, H. Kim, S. Jung, Electrochemical selectivity enhancement by using monosuccinyl β-cyclodextrin as a dopant for multi-wall carbon nanotube-modified glassy carbon electrode in simultaneous determination of quercetin and rutin, Biotechnol. Lett. 31 (2009) 1739–1744, https://doi.org/10.1007/s10529-009-0066-6.
[45] G.R. Xu, S. Kim, Selective determination of quercetin using carbon nanotube-modified electrodes, Electroanalysis. 18 (2006) 1786–1792, https://doi.org/10.1002/elan.200603587.
[46] J.B. He, X.Q. Lin, J. Pan, Multi-wall carbon nanotube paste electrode for adsorptive stripping determination of quercetin: a comparison with graphite paste electrode via voltammetry and chronopotentiometry, Electroanalysis. 17 (2005) 1681–1686, https://doi.org/10.1002/elan.200503274.
[47] R.L. Anderson, Practical Statistics for Analytical Chemists, Van Nostrand Reinhold, New York, 1987.
[48] S. Shaikh, V. Jain, Development and validation of a RP-HPLC method for the simultaneous determination of quercetin, ellagic acid and rutin in hydroalcoholic extract of triphala churna, Int. J. Appl. Pharm. 10 (2018) 169–174, https://doi.org/10.22159/ijap.2018v10i3.25860.