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ORIGINAL PAPER

Diazonium-functionalized tyrosinase-based biosensor for the detection of tea polyphenols

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Abstract A tyrosinase-based biosensor was constructed by immobilizing the enzyme on diazonium-functionalized screen-printed gold electrodes. Under optimized conditions, the biosensor exhibited rapid response to the changes in the concentration of all the tested phenolic compounds (catechol, catechin, caffeic acid and gallic acid). Sensitivity, linear range and limit of detection (LOD) were determined, and catechol was found to display the highest sensitivity (36.3 $mA M^{-1}$) and the lowest LOD (0.1 $\mu mol L^{-1}$). The biosensor was successfully applied to the detection of polyphenols in tea samples.

Keywords Biosensor · Tyrosinase · Diazonium salt · Phenolic compounds · Tea · Screen-printed gold electrodes

Introduction

Polyphenolics are a broad group of compounds present in plant-derived foods including wine, tea, cacao or fruits [1]. Last years, some authors have associated the consumption of polyphenol-rich products with a reduction in the incidence of cardiovascular diseases, certain cancers and other diseases related to aging [2, 3]. This is related to the fact that their chemical structure enables polyphenols to act as antioxidant and anti-inflammatory compounds, scavenging and neutralizing free radicals, and inhibiting lipoprotein oxidation [4, 5].

The determination of both the content and the antioxidant activity of polyphenols in natural samples is extremely difficult. The tedious and complicated extraction processes, the complex polyphenols composition and the presence of interferences in the extracted samples impede a precise determination of the "total phenolic content". Traditionally, the "total phenol content" has been determined by using spectrophotometric methods based on the reaction of phenolic compounds with a colorimetric reagent and the measurement of the developed color in the visible region [6]. However, these methods tend to overestimate the real content of phenolic compounds [7]. More accurate methods have been used nowadays, such as HPLC [8–10], capillary electrophoresis [11, 12] or chemiluminescence [13].These highly sensitive standard methods are expensive, time consuming and require formal training that considerably limits their wide spread use.

Amperometric biosensors are postulated as an attractive alternative for their rapid response, cost-effectiveness, simplicity of operation and manufacturing, minimal involved sample pretreatment and solvent requirements [14]. Most of biosensors currently used for the evaluation of the polyphenol content are based on polyphenol oxidases, tyrosinase [15–18] or laccase [19–21], or on peroxidase [22–24]. From all of them, tyrosinase biosensors are the most commonly applied to determine phenolic compounds in food samples. In the presence of oxygen, tyrosinase catalyses the oxidation of monophenols and *o*-diphenols to quinones. This enzymatic mechanism involved the emergence of two measurement approaches: (1) the amperometric detection of the oxygen consumption using a Clark-type electrode [17] and (2) the reversible electrochemical

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reduction of the *o*-quinone formed from phenols in the enzymatic reaction [25]. In both cases, the measured signal is proportional to the polyphenol concentration in solution. Classically, enzymatic immobilization protocols were based on either the physical entrapment or the covalent cross-linking of the enzyme with glutaraldehyde [17, 25, 26]. Last years, monolayer strategies have become increasingly popular because they provide controlled and oriented recognition interfaces [27, 28]. From early 1990s when Pinson and co-workers introduced the use of aryl diazonium salts for electrode modification [29], this immobilization scheme has been applied to a range of biosensing applications [30–37]. Advantages of this approach are a highly stable surface, ease of preparation and the ability to synthesize diazonium salts with a wide range of functional groups [38].

In this paper, a diazonium-functionalized tyrosinase-based biosensor for the detection of polyphenols in real samples of tea is presented. Screen-printed gold electrodes (SPGEs) were grafted with a p-nitrophenyl (p-NP) film by electrochemically reducing the in-situ generated p-nitrophenyl diazonium salt (p-NPDS), without isolation of the diazonium organic salt, followed by electro-reduction of the nitro groups to amino groups. The amino-modified electrodes were further immobilized with tyrosinase using glutaraldehyde as a cross-linker, binding the enzyme amino groups to the electrode-surface amine. Diazonium-functionalized tyrosinase-based biosensors showed good response to phenolic compounds and were successfully applied to the detection of polyphenols in tea samples.

Experimental

Reagents

Tyrosinase from mushroom (EC 1.14.18.1, 5370 U per mg solid), catechol, catechin, caffeic acid, gallic acid, 4-nitroaniline, potassium chloride, sodium nitrite and Tween 20 were purchased from Sigma (www.sigmaaldrich.com). Hydrochloric acid and 25% glutaraldehyde were supplied from Prolabo (fr.vwr.com) and Aldrich (www.sigmaaldrich.com), respectively. All reagents were of analytical grade and were used without further purification. All solutions were prepared using Milli-Q water.

Preparation of the tea samples

Three different brands of black teas were purchased from a local market. Extraction of polyphenols was performed as follows: the tea bags were infused in 25 mL boiling water and kept at 90 °C for 5 min. The infusion was rapidly filtered through a Minisart syringe filter (pore size 5 μm; Sartorius, www.sartorius.com).

Equipment

All electrochemical measurements were performed with an Autolab PGSTAT 100 (Metrohm, www.metrohm.com). Cyclic voltammetry (CV) was employed to assess the electrochemical behavior of the modified electrodes whereas performance characteristics of the diazonium-functionalized tyrosinase-based biosensor were evaluated using chronoamperometry (CA). A three-electrode configuration was used for all the electrochemical measurements.

SPGEs, purchased from DropSens (www.dropsens.com), include three electrodes printed on a ceramic substrate (3.4×1.0 cm) and subjected to low-temperature curing. In terms of configuration, a gold disk-shaped (12.6 mm^2) working electrode (WE) was surrounded by a gold counter electrode (CE) to ensure near-homogeneous polarisation of the WE [39]. A silver serigraphic electrode was included and used as pseudo-reference (pRE). All potential values were referred to the screen-printed silver pRE.

Biosensor fabrication

Prior to modification, electrodes were electrochemically cleaned by cycling the potential between 0 and +1.4 V at 100 mVs^{-1} in 0.1 $molL^{-1}$ H_2SO_4 until the characteristic cyclic voltammogram for a clean gold electrode was obtained.

The diazonium organic salt synthesis and the subsequent modification of the SPGE was performed as detailed in the literature [30]. The modification protocol scheme is represented in Fig. 1.

Briefly, the diazonium cations were synthesized in situ by reaction of 1.0 $mmolL^{-1}$ sodium nitrite and 1.0 mmol L^{-1} 4-nitroaniline in 0.5 $molL^{-1}$ HCl solution for 5 min (step 1). The diazonium salt was electrodeposited on the WE of SPGEs after one cycle from 0.4 to −0.4 V at 100 mVs^{-1} (step 2). SPGEs were then subjected to 5 potential scans between 0.4 and −1.25 V at 100 mVs^{-1} in 0.1 $molL^{-1}$ KCl for reduction of the p-NP film to p-aminophenyl (p-AP) (step 3). The amine on electrode surface was activated by incubation with 2.5% glutaraldehyde in 0.2 $molL^{-1}$ phosphate buffer pH 7.0 for 30 min (step 4). After washing with distilled water, 50 μL of tyrosinase solution (2.6 μg in 1.0 mL of 50 $mmolL^{-1}$ phosphate buffer pH 7.0) were spread onto the WE surface and incubated for 60 min at room temperature (step 5). Finally, electrodes were washed with phosphate buffered solution + 0.1% Tween 20 (3×100 μL), 1 $molL^{-1}$ KCl (3×100 μL) and 0.1 $molL^{-1}$ phosphate buffer pH 7.0 (3×100 μL). Biosensors were kept dry at 4 °C until used.

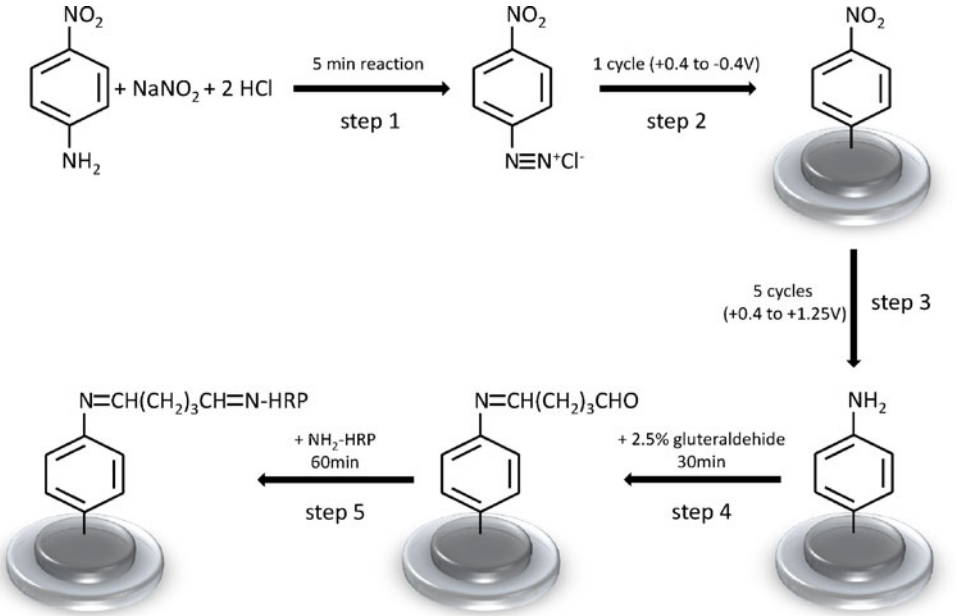


Fig. 1 Schematic representation of the tyrosinase-based biosensor fabrication protocol

Measurement protocol

Experiments were carried out in an electrochemical cell containing 5 mL of 0.1 molL^{-1} phosphate buffer pH 7.0 by sequential addition of aliquots of either stock phenols solutions of known concentration or tea samples. After each addition, the sample was stirred for 30 s to ensure homogeneity before measuring. Chronoamperometric data were obtained by applying −200 mV for 120 s. Each calibration curve was derived from the average of three independent biosensors (n=3).

Results and discussion

Evaluation of the SPGEs grafting via electrochemical reduction of diazonium salts

Diazonium-functionalized tyrosinase-based biosensors were prepared following the protocol described above. Figure 2a shows cyclic voltammograms recorded for the SPGEs in the diazotation mixture of 1 mmolL^{-1} $NaNO_2$ and 1.0 mmolL^{-1} p-nitroaniline in 0.5 molL^{-1} HCl (step 2, Fig. 1). An irreversible cathodic peak at −0.168 V/Ag pRE is observed. This peak was assigned to the reduction of diazonium species to aryl radicals, via one electron process. The aryl radicals coupled to the WE surface leading to irreversible grafting of a strongly bonded organic layer of p-NP. Very low currents were observed during the second and third voltammetric cycles, evidencing surface saturation and suggesting that a monolayer of fixed molecules was achieved.

Figure 2b plots the first and the last cyclic voltammogram of the p-NP to p-AP reduction process (step 3, Fig. 1). The voltammograms give rise to a split wave which corresponds to the irreversible reduction of grafted nitro groups in a multielectron and multiproton pathway, first to hydroxylamine derivatives ($4e^-/4H^+$) and further to the corresponding amines ($2e^-/2H^+$). The reduction of the wave indicates that nearly all the electroactive nitro groups are reduced.

The surface coverage of the attached p-NP was evaluated from the area under the reduction peak [40]. Indeed, a full surface coverage, Γ was attained from one cyclic voltammogram and a value of 6.90×10^{-10} molcm^{-2} was found. This was consistent with the coverage found for the formation of close pack monolayer of grafted p-NP groups on glassy carbon electrode [41].

Optimization of the enzymatic biosensor: tyrosinase concentration and pH

In the optimization of the tyrosinase concentration, five biosensors were prepared following the protocol described in the experimental section by incubating each electrode with 2.0, 3.9, 6.9, 13.8 and 27.6 activity units of tyrosinase per mL (UmL^{-1}), respectively. CA measurements were carried out in 15 μmolL^{-1} catechol. The current response of the sensor with the concentration of tyrosinase was plotted and it was found that the current response initially increased when increasing the concentration of tyrosinase until a maximum was reached at 13.8 U tyrosinase mL^{-1} (data not shown). Thus, from this point, all biosensors were prepared by incubating the WE with 13.8 U tyrosinase mL^{-1}.

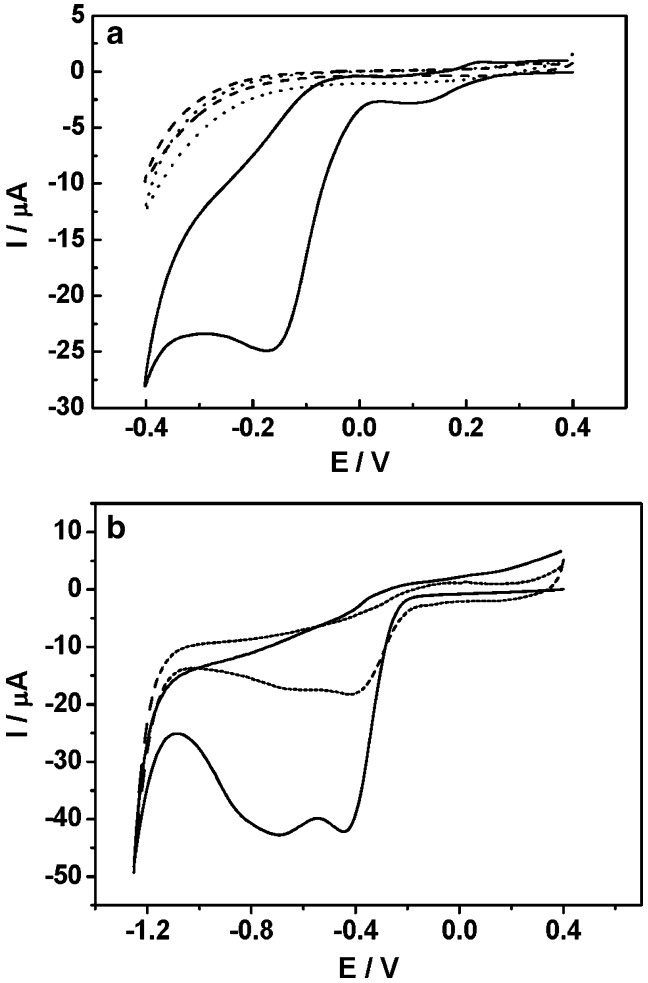


Fig. 2 **a** Cyclic voltammograms for in-situ-generated p-NPDS in the diazotation mixture (1.0 $mmolL^{-1}$ $NaNO_2$ +1.0 $mmolL^{-1}$ 4-nitroaniline in 0.5 $molL^{-1}$ HCl) at SPGEs; scan rate 100 mVs^{-1}. **b** First (solid line) and last (dashed line) cyclic voltammograms of the p-NP-modified SPGE in 0.1 $molL^{-1}$ KCl; scan rate 100 mVs^{-1}

The enzyme activity is known to be seriously affected by the pH value of the buffer solution. The effect of the pH value was investigated in the range from 5.5 to 9.0 (Fig. 3). In these experiments, CA measurements were made using 15 $\mu molL^{-1}$ catechol in phosphate buffer at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 or 9. The maximum current response was obtained at the pH 7.0. Thus, in order to obtain the maximum sensitivity and bioactivity, a buffer of pH 7.0 was used throughout the research.

Repeatability, reproducibility and stability of the sensor

Repeatability, immobilization reproducibility and operational stability of the tyrosinase-based biosensors developed in the present work were evaluated.

In the repeatability analysis, biosensors were measured using CA in phosphate buffer containing 15 $\mu molL^{-1}$ catechol.

After 15 successive measurements, the repeatability of each electrode was analyzed in terms of the relative standard deviation (RSD). Tyrosinase-based biosensors showed good repeatability since in all cases the RSD never exceeded a magnitude of 5%.

The reproducibility was evaluated comparing the response of 5 biosensors prepared as previously detailed and measured using CA in phosphate buffer containing 15 $\mu molL^{-1}$ catechol. Reproducibility was again analyzed in terms of RSD and an accurate value of 5.8% was found.

The operational stability of the biosensor was analyzed with sensors prepared as before and successively measured during 8 h at 30 min intervals using CA in phosphate buffer containing 15 $\mu molL^{-1}$ catechol. Between measurements, the electrochemical cell was washed with buffer to eliminate catechol residues and biosensors were stored in buffer solution. After 15 successive substrate injections, a RSD of 8% was obtained. Long-term storage experiments showed high decreases in the sensor response. Considering the easy and rapid preparation protocol of the sensor (below 2 h), it is extremely recommended to prepare them every day.

Analysis of the response of the tyrosinase-based biosensor to phenolic compounds

Sensors were prepared as previously detailed. The performance of the biosensor to phenolic compounds, precisely catechol, catechin, caffeic acid and gallic acid, was investigated by means of CA. Regarding Fig. 4a, the current response given by the electrochemical reduction of the *o*-quinone resulting from the enzyme reaction was detected at −200 mV/Ag pRE. Experimentally, 25, 25, 50, 50, 75, 75, 100, 100 and 100 μL of each phenolic

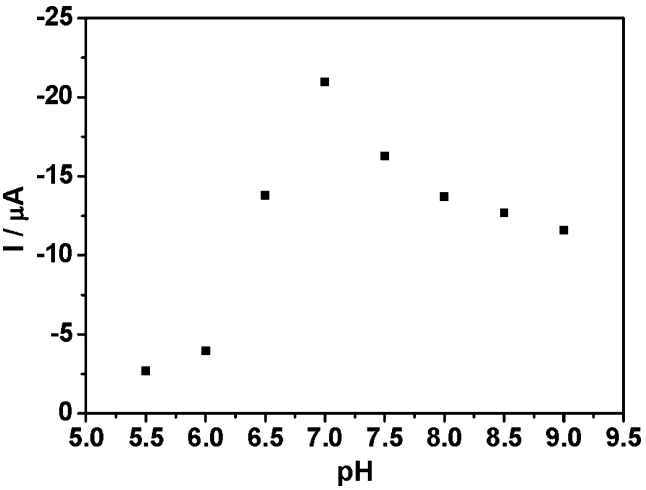
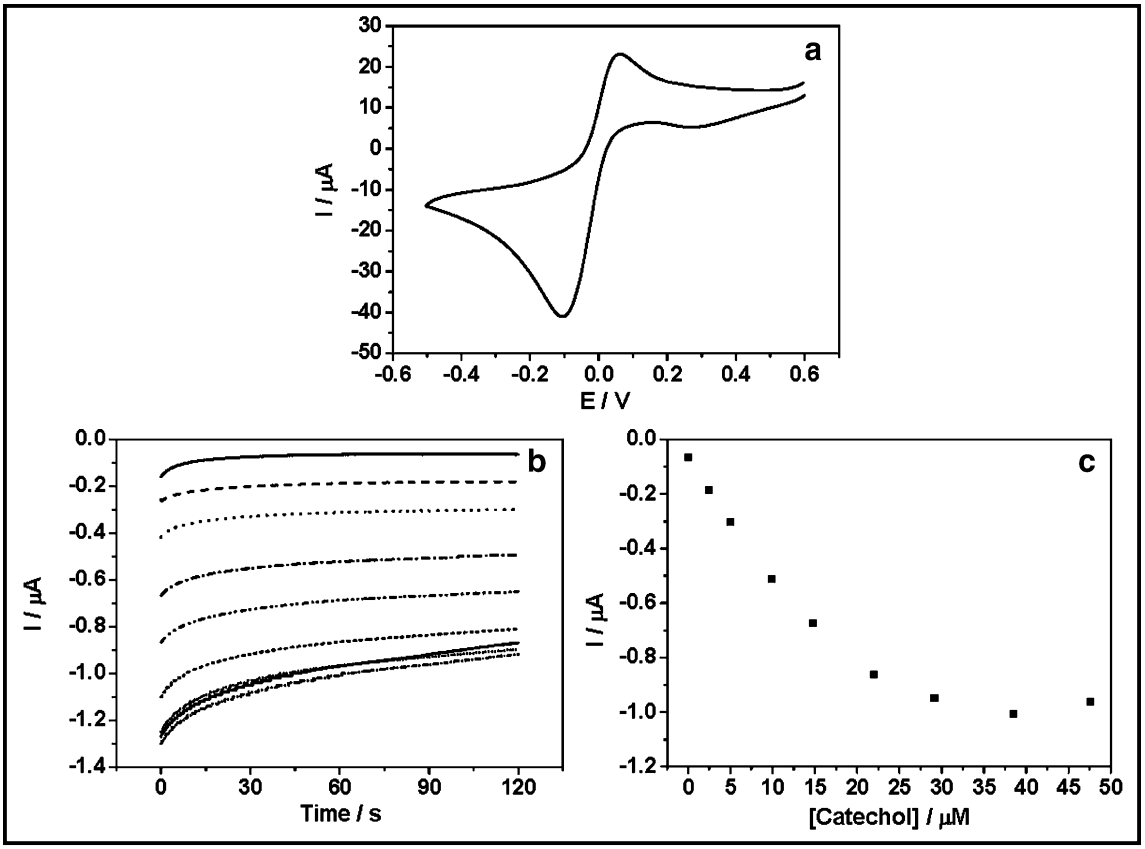


Fig. 3 Representation of the dependence of the CA reduction current on the pH using the tyrosinase-based biosensor. Current values were obtained in 15 $\mu molL^{-1}$ catechol



	Sensitivity / mA M^{-1}	Linear range / μmol L^{-1}	r	LOD / μmol L^{-1}
Catechol	36.3 ± 0.7	0.1 – 22	0.986	0.1
Catechin	3.8 ± 0.2	2.8 – 29	0.988	2.8
Caffeic acid	2.9 ± 0.2	0.3 – 83	0.998	0.2
Gallic acid	2.9 ± 0.1	2.5 – 65	0.983	2.1

Fig. 4 **a** Cyclic voltammogram of the *o*-quinone resulting of the action of the tyrosinase-based biosensors in 1 mmolL^{-1} catechol; scan rate 50 mVs^{-1}. **b** CA data for consecutives additions of 15 μmolL^{-1} catechol by applying −200 mV/Ag pRE for 120 s. **c** Dependence of the CA reduction current on the concentration of catechol for the tyrosinase-based biosensor. Inset: Analytical performances of the tyrosinase-based biosensor. Average values were obtained with n=3 biosensors

compound (1 mmolL^{-1} in all cases) were sequentially introduced into 5 mL of phosphate buffer (pH 7) until no change was registered in the current response magnitude. After each addition, the solution was homogenized by stirring for 30 s before recording the current response. The CA data for catechol is plotted in Fig. 4b.

In each CA, the average of response intensities corresponding to the last 10 s of the measurement were taken as the intensity magnitude for the corresponding phenolic compound at that concentration. The representation of the obtained intensity magnitudes against the phenolic compound concentration was used as calibration curve (Fig. 4c, for catechol). The calibration curves of the phenolic compounds under study were used to evaluate the response of the tyrosinase-based biosensor to each molecule (Fig. 4, table inset). Calibration curves were analyzed in terms of sensitivity, linear range and limit of detection (LOD). The LOD was calculated according to the $3s_b/m$ criterion, where s_b and m are the standard deviation of the background current and the slope of the calibration plot,

respectively. Huge differences in terms of sensitivity and LOD were observed when analyzing different phenolic substrates. Precisely, the sensitivity obtained with catechol was about 10 times bigger than the one obtained with the other compounds, which agrees with data reported by Zhang et al. [42], since catechol is the natural substrate of tyrosinase [43]. Moreover, the LOD of the biosensor to catechol is also the lowest and thus, catechol was taken as a reference and the "total phenol concentration" was always expressed as mgL^{-1} of equivalent catechol. It is worth to point out that in general very low LODs were obtained.

Characterization of the response of the biosensor to real samples of tea

The practical usefulness of the tyrosinase-based biosensor was evaluated by estimating the total phenolic content in teas. Three different brands of tea (samples designed as T1, T2 and T3) were purchased from a local supermarket. 100 μL of the tea decoction filtrate were injected to the electrochemical cell (final volume 5 mL) and CA measurements were performed. In the polyphenols determination, catechol was used as standard compound. The estimated concentration of total polyphenols in the tested teas were 0.77 ± 0.04 mgL^{-1}, 1.34 ± 0.05 mgL^{-1} and 2.23 ± 0.07 mgL^{-1} for T1, T2 and T3, respectively, when considering catechol as a reference, and 18.8 ± 0.9 mgL^{-1}, 32.4 ± 1.2 mgL^{-1} and 54 ± 2 mgL^{-1} for caffeic acid. These values, obtained for black teas, were in concordance with a previous publication of Ibarra-Escutia et al. [44], when taking into account the dilution factor regarding the different preparation protocol.

Conclusions

This paper describes for the first time the application of diazonium salts in the development of biosensors for the determination of the total phenol content and its application to real tea samples. The compact diazonium layer was advantageous since it should avoid the interference of negatively charged redox substances present in the tea matrix that may be oxidized or reduced at the applied potential. The enzymatic biosensor was constructed by immobilizing the tyrosinase enzyme onto diazonium functionalized screen-printed gold electrodes in a simple and fast protocol. The sensor response was optimized in terms of immobilized-enzyme concentration and buffer pH, obtaining best performances at 13.8 U tyrosinase mL^{-1} and pH 7. The biosensor exhibited good responses in terms of sensitivity, repeatability, reproducibility and operational stability to a number of phenolic compounds, especially to catechol, and was successfully applied to the detection of polyphenols in real samples of tea.

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