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An amperometric biosensor based on peroxidases from *Brassica napus* for the determination of the total polyphenolic content in wine and tea samples

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ABSTRACT

An amperometric biosensor based on peroxidases from *Brassica napus* hairy roots (PBHR) used to determine the total polyphenolic content in wine and tea samples is proposed by the first time. The method employs carbon paste (CP) electrodes filled up with PBHR, ferrocene (Fc), and multi-walled carbon nanotubes embedded in a mineral oil (MWCNT + MO) at a given composition (PBHR–Fc–MWCNT + MO). The biosensor was covered externally with a dialysis membrane, which was fixed at the electrode body side part with a Teflon laboratory film and an O-ring. Calibration curves obtained from steady-state currents as a function of the concentration of a polyphenolic standard reference compound such as t-resveratrol (t-Res) or caffeic acid (CA) were then used to estimate the total polyphenolic content in real samples. The reproducibility and the repeatability were of 7.0% and 4.1% for t-Res (8.4% and 5.2% for CA), respectively, showing a good biosensor performance. The calibration curves were linear in a concentration range from 0.05 to 52 mg L^{-1} and 0.06 to 69 mg L^{-1} for t-Res and CA, respectively. The lowest polyphenolic compound concentration values measured experimentally for a signal to noise ratio of 3:1 were 0.023 mg L^{-1} and 0.020 mg L^{-1} for t-Res and CA, respectively.

1. Introduction

Polyphenolic compounds are a complex group of substances that have gained enormous attention in the last years, mainly in the analytical chemistry field, because they have important health properties [1–3] and antioxidant activity [4,5].

Polyphenolic compounds are in skin grapes, mainly in seeds and epidermal cells. The amount and quality of polyphenolic compounds in grapes depend on the variety of wine grape, the weather, the soil, and the farming practices [6]. The main polyphenolic compounds in wines with antioxidant activity are derivative from phenolic and cinnamic acids, tirosyne stilbenes, flavonoids and procianidins [4]. The total concentration of phenolic compounds in red wines varies from 1800 to 4060 mg L^{-1} (expressed as mg L^{-1} of galic acid, with an average value of 2570 mg L^{-1}). A lesser concentration of polyphenolic compounds is found in white wines, varying between 160 and 330 mg L^{-1}, with an average value of 240 mg L^{-1} [7].

Tea represents a major source of antioxidants since it is the most widely consumed beverage worldwide. It has beneficial health effects, given its anti-carcinogenic, anti-teratogenic and antimicrobial properties [8–13]. Different tea varieties and extracts usually present in food have been reported to prevent pathogenic agents, such as *Escherichia coli*, *Salmonella* sp. and *Staphylococcus aureus* [13–16].

At present, several methods are available for the analysis of polyphenolic compounds. Most of the methods are based on separation techniques such as chromatography or capillary electrophoresis with various detection systems [17–20] as well as techniques that do not involve separation steps, i.e., vanillin–HCl, n-butanol–HCl and the Folin–Ciocalteu colorimetric method [21]. The Folin–Ciocalteu method, based on the reaction of phenolic compounds with a colorimetric reagent, allows the determination of phenolic and/or polyphenolic compounds in the visible region of the electromagnetic spectrum [22]. However, this spectrophotometric approach yields an overestimation of total polyphenolic content (TPC) [4].

Biosensors have been proposed as an efficient analytical tool for the determination of polyphenolic compounds, exhibiting advantages such as the minimal preparation of the sample, selectivity, sensitivity, reproducibility, rapid time of response and simple use for continuous on-site analysis [23,5,24–29].

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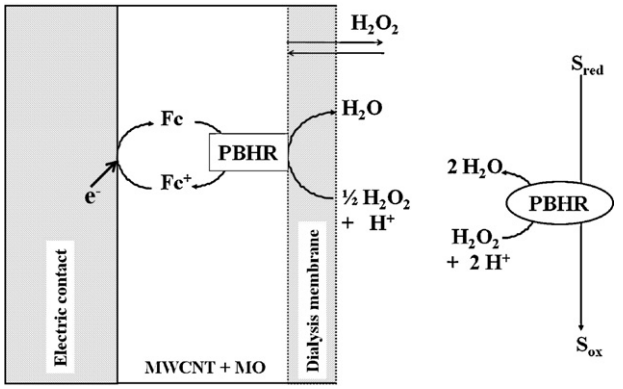

Scheme 1. Two-stage reaction scheme for detecting phenolic compounds using carbon paste electrodes filled up with peroxidases obtained from *Brassica napus* hairy roots (PBHR), ferrocene (Fc) and, multi-walled carbon nanotubes embedded in a mineral oil (MWCNT + MO). S_{red} and S_{ox} are the reduced and oxidized forms of the phenolic compound, respectively.

In this article, we propose an amperometric biosensor based on peroxidases obtained from *Brassica napus* hairy roots (PBHR) to determine the TPC in wine and tea samples. The method employs carbon paste (CP) electrodes filled up with PBHR, ferrocene (Fc), and multi-walled carbon nanotubes embedded with a mineral oil (MWCNT + MO) at a given composition (PBHR–Fc–MWCNT + MO). The biosensor was covered externally with a dialysis membrane (Scheme 1), which was fixed at the electrode body side part with a Teflon laboratory film and an O-ring. It is well known that phenolic and/or polyphenolic compounds can work as electron-donors for peroxidases in the catalytic reduction of H_2O_2 [30,31]. This approach allows detecting the decrease in H_2O_2 concentration in a solution after the oxidation of phenolic and/or polyphenolic compounds produced by the PBHR in the presence of H_2O_2, given that PBHR acts in cascade in the solution and the electrode surface. The separation of the electrode surface from the solution by a semipermeable membrane allows minimizing the electrical noises as well as the fouling of the electrode surface. The latter effect can be produced by the polymerization of oxidized phenolic compounds, which can interfere with electrochemical measurements.

2. Materials and methods

2.1. Reagents and materials

B. napus hairy roots, obtained "in vitro" in our laboratory according to a procedure previously described by Agostini et al. [32], were used as the enzyme source.

Hydrogen peroxide (30%, v/v), pH 7.00 phosphate buffer solutions (PBS), sodium acetate, acetic acid, NaCl, HCl, NaOH and Na_2CO_3 were Merck p.a.; trans-resveratrol (t-Res), caffeic acid (CA), ferrocene (Fc), o-dianisidine, sodium hydrogen sulfite, glucose and ascorbic acid were purchased from Sigma and used as received. All solutions were prepared using water purified by a Labconco WaterPro Mobile System, Model 90901-01 (HPLC grade water). The concentration of H_2O_2 was determined spectrophotometrically at λ_{max} = 240 nm (ε = 43.6 mol^{-1} L cm^{-1}) [33]. Stock solutions of t-Res and CA were prepared in H_2O and kept at a temperature of 4 °C.

MWCNT + MO was obtained by mixing different amounts of MWCNT (Sigma, outer diameter 30–50 nm, inner diameter 5–15 nm, length = 0.5–200 μm) with MO (Sigma) in order to optimize the final best composition. The electrode surface was then covered with a dialysis membrane (Spectrum Co., Houston, TX, cut-off molecular weight 100), which was fixed at the electrode body side part with a Teflon laboratory film and an O-ring.

2.2. Total extraction and purification of enzymes and determination of the peroxidase activity

Hairy roots were homogenized in a mortar with 10 mmol L^{-1} pH 4.00 sodium acetate/acetic acid buffer, containing 1 mol L^{-1} NaCl (1 g fresh roots weight per 3 mL of buffer) at 4 °C. Homogenates were centrifuged at 5000 rpm for 5 min. The supernatants were considered as total peroxidase extracts (TPE). They were used in order to purify peroxidases and determine total peroxidase activity.

The purification of peroxidases was performed by molecular exclusion chromatography on Sephacryl S-200-HR (SIGMA) columns. Samples of 2.5 mL of TPE, previously dialyzed, were loaded on a Sephacryl S-200-HR column (2 cm × 32 cm) equilibrated with a pH 7.00 phosphate buffer 20 mmol L^{-1}. The column was washed with 100 mL from this buffer at a flow rate of 1 mL min^{-1} controlled with a peristaltic pump (LKB 2232 Microperpex). Fractions of 5 mL were collected and monitored to determine their peroxidase activity using a qualitative method proposed by Forchetti and Tigier [34]. Fractions containing most peroxidases were mixed and lyophilized before they were used. These enzymes presented a peroxidase activity of 280 IU per solid mg and they were labeled as PBHR.

The total peroxidase activity was determined with o-dianisidine as substrate [35] and expressed in international unit (IU), defined as the amount of enzyme forming 1 mmol of product in 1 min under the experimental conditions employed.

2.3. Real samples

The TPC was estimated in different wine and tea samples. We used four red and two white wines, two green, one red and two black teas. They were purchased from a local supermarket and produced in Argentina.

Wine samples did not require any pre-treatment. The tea samples were treated following this procedure: 2 g of tea were put in 200 mL of H_2O and the mixture was boiled for 4 min and then filtered [36].

The TPC estimation in wine and tea samples was carried out by interpolation of the corresponding amperometric signals with the corresponding calibration curves constructed with t-Res and CA stock solutions.

For comparison purposes, samples of wine and tea were also analyzed with the espectrophotometric method using the Folin–Ciocalteu reagent [37]. Standard solutions were prepared by adding 4.5 mL of 2% (w/v) Na_2CO_3 aqueous solution and the corresponding amount of wine or tea. Then, 230 μL of the Folin–Ciocalteu reagent (1:1 v/v in methanol) were added after 2 min and leveled in a 5 mL volumetric flask. After being allowed to react for 30 min, protected from light, the absorbances of the standards (t-Res and CA) were measured at λ = 750 nm [22]. The TPC in samples was expressed as mg of t-Res or CA per liter of the sample.

2.4. Instruments and experimental measurements

Cyclic voltammograms and amperometric measurements were performed with an epsilon (BAS) potentiostat controlled by electrochemical analysis software. Electrochemical measurements were carried out in a 2 mL Pyrex cell. The working electrode was a CP disk of 1.6 mm diameter obtained from Bioanalytical System (BAS), Inc. The counter electrode was a platinum foil of large area (~2 cm^2). An aqueous SCE was used as the reference electrode. Aliquots of 5 and 10 μL were added to the electrochemical cell for the determination of TPC in red wines and white wines and tea samples, respectively. Amperometric measurements were performed at a potential of −0.050 V *vs* SCE in solutions stirred at 1600 rpm. This operational applied potential was previously optimized by Granero et al. [38].

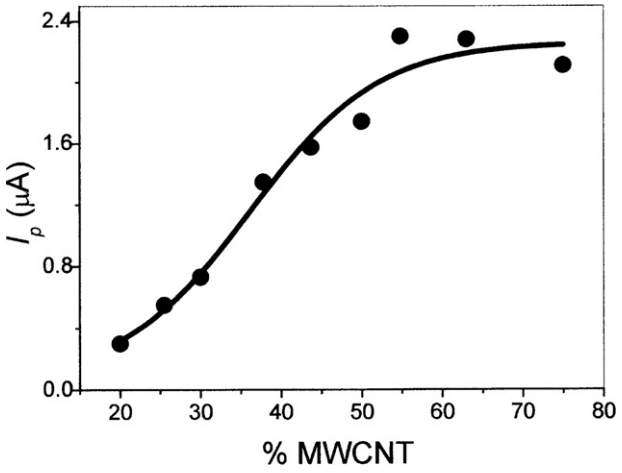

Fig. 1. Variation of $I_{p,a}$ with the MWCNT percent obtained from cyclic voltammograms performed in 1×10^{-3} mol L^{-1} $K_4[Fe(CN)_6]$ + 0.1 mol L^{-1} KCl aqueous solution as the supporting electrolyte. Scan rate, $\nu = 0.050$ V s^{-1}.

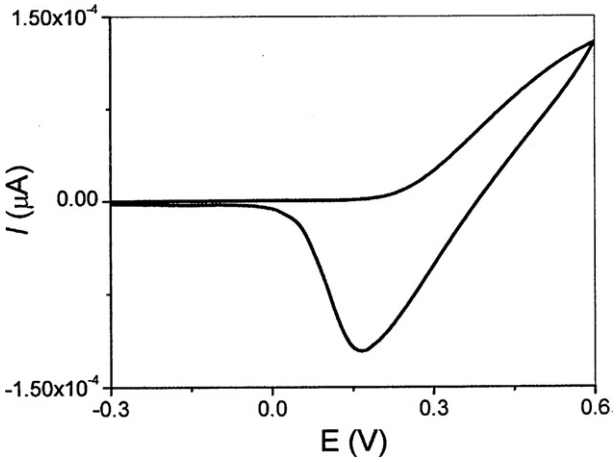

Fig. 2. Cyclic voltammogram of the PBHR–Fc–MWCNT + MO biosensor recorded in unstirred pH 7.00 PBS. $\nu = 0.050$ V s^{-1}.

The Fc was used as a redox mediator. Biosensors were constructed by using the following procedure: PBHR, Fc and MWCNT + MO were mixed in different weight ratios in order to obtain the best biosensor composition. Then, the optimal composition was used to fill up the CP electrodes. The biosensors were stored at 4 °C in pH 7.00 PBS when they were not in use. An optimal pH value of 7.00 for peroxidase enzymes was previously established by Granero et al. [38]. Experiments performed with and without bubbling pure nitrogen in solutions did not show any significant difference. Therefore, measurements were carried out in non-deoxygenated solutions. UV–visible spectra were recorded immediately after the preparation of solutions by using a Hewlett–Packard Model 8452A spectrophotometer equipped with a temperature controller. Silica cell was of 1 cm pathlength. Experiments were performed at 25.0 ± 0.2 °C.

3. Results and discussion

3.1. Optimization of the composition of carbon nanotubes + mineral oil

The paste electrodes were prepared by mixing different amounts of MWCNT and MO. Their electrochemical characterization was carried out by cyclic voltammetry in 1×10^{-3} mol L^{-1} $K_4[Fe(CN)_6]$ + 0.1 mol L^{-1} KCl aqueous solution. The influence of the different electrode compositions on the anodic peak current ($I_{p,a}$) was analyzed. The composition of MWCNT + MO, which optimized the $I_{p,a}$ value was determined. The variation of $I_{p,a}$ with the MWCNT percent (% MWCNT) is shown in Fig. 1. The $I_{p,a}$ increased up to about 55% MWCNT; then it kept constant, with a maximum anodic peak current ($I_{p,a,max}$) of 2.20 μA. The biosensors were prepared using this composition (55% MWCNT + 45% MO), which was employed in all studies that are discussed below.

3.2. Responses of PBHR–Fc–MWCNT + MO biosensors versus H_2O_2

A cyclic voltammogram recorded in unstirred pH 7.00 PBS (blank solution) for the PBHR–Fc–MWCNT + MO biosensor showed an increase in the anodic current at potentials higher than 0.16 V *vs* SCE, which corresponds to the oxidation of Fc–Fc$^+$. A cathodic peak with a peak potential at about 0.2 V was observed when the potential sweep direction was reversed at 0.6 V, which can be assigned to the reduction of Fc$^+$–Fc at the electrode surface (Fig. 2). On the other hand, a cyclic voltammogram recorded when H_2O_2 was added to the reaction medium showed a cathodic current at potentials more negative than −0.3 V *vs* SCE, which corresponds to the reduction of H_2O_2 at the biosensor surface. Therefore, the base current was practically zero between −0.3 and 0.2 V at pH 7.00 PBS. This behavior was similar to that previously found by us when a similar biosensor was employed to determine t-Res using the commercial reagent [38].

On the other hand, three biosensors were constructed to optimize the composition of PBHR in the biosensor, for which the proportion of the other two components remained constant, i.e., 3 mg Fc and 45 mg (55% MWCNT + 45% MO). These three biosensors were used to study their responses towards H_2O_2. Therefore, when only H_2O_2 was added to the stirred reaction medium, the enzymatic reaction took place between PBHR on the electrode surface and H_2O_2 penetrated into the inner layer between the semi permeable membrane and the electrode surface. H_2O_2 was reduced to H_2O by PBHR and the enzyme was reduced to its native form by Fc, which was oxidized to Fc$^+$. The Fc$^+$ was then immediately reduced to Fc at the electrode surface held at a potential of −0.050 V *vs* SCE. Steady-state reduction currents (I_{ss}) obtained after the addition of different aliquots of H_2O_2 are shown in Fig. 3a. The differences between the initial base current and the corresponding I_{ss} (ΔI_{ss}) were proportional to the H_2O_2 bulk concentration ($c^*_{H_2O_2}$), showing a Michaelis–Menten type saturation (Fig. 3b).

The kinetic parameters of the PBHR–Fc–MWCNT + MO biosensor for H_2O_2 are summarized in Table 1. The finding that the "h" parameter, calculated from the corresponding Hill's plots, is close

Table 1
Hill parameter and I_{max,H_2O_2} and K_{A,H_2O_2} kinetics parameters obtained for the different biosensor compositions showed in Fig. 3 with their corresponding standard deviations after adding different aliquots of H_2O_2 to buffer PBS of pH 7.00.

PBHR–Fc–MWCNT + MO biosensor composition (mg)	*h*	I_{max,H_2O_2} (μA)	K_{A,H_2O_2} (mmol L^{-1})
6.5-3-45	0.99 ± 0.01	0.23 ± 0.03	0.16 ± 0.03
10-3-45	1.01 ± 0.01	1.50 ± 0.09	0.63 ± 0.08
15-3-45	1.00 ± 0.01	1.33 ± 0.09	1.04 ± 0.09

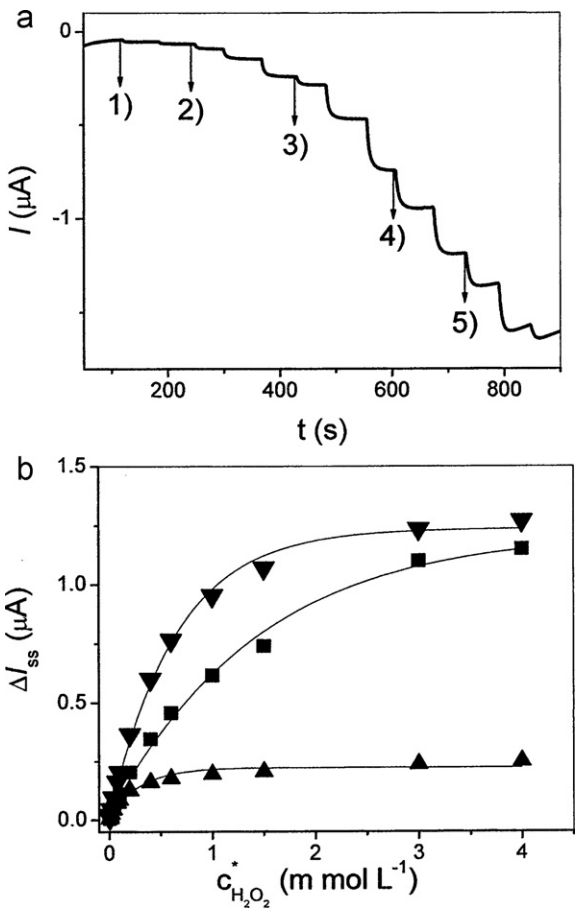

Fig. 3. (a) Steady-state current responses on the addition of different H_2O_2 concentrations at the stirred pH 7.00 PBS reaction medium measured with a dialysis membrane covered PBHR–Fc–MWCNT + MO (10-3-45) biosensor. $c^*_{H_2O_2}$: (1) 0.005 mmol L^{-1}; (2) 0.02 mmol L^{-1}; (3) 0.1 mmol L^{-1}; (4) 0.6 mmol L^{-1} and (5) 1.5 mmol L^{-1}. (b) Differences between the base and the steady-state currents, ΔI_{ss}, as a function of $c^*_{H_2O_2}$ under the same experimental conditions as (a). The PBHR–Fc–MWCNT + MO biosensor compositions were: (▼) 10-3-45 mg (■) 15-3-45 mg (▲) 6,5-3-45 mg.

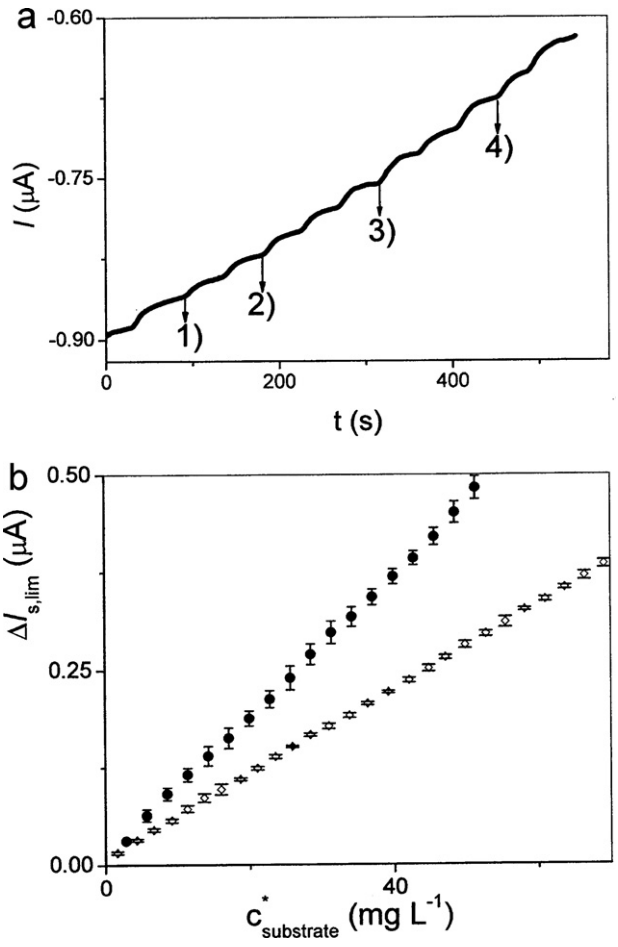

Fig. 4. (a) Steady-state current responses on the addition of different t-Res concentrations in a reaction medium under stirring containing 240 IU mL^{-1} PBHR + 50 μmol L^{-1} H_2O_2 in PBS pH 7.00 measured with a dialysis membrane covered PBHR–Fc–MWCNT + MO (10-3-45) biosensor. $c^*_{t\text{-Res}}$: (1) 5.7 μmol L^{-1}; (2) 11.4 μmol L^{-1}; (3) 20.0 μmol L^{-1} and (4) 28.5 μmol L^{-1}. (b) Calibration curves defined as the differences between the base and the steady-state currents, $\Delta I_{s,lim}$, with corresponding error bars, as a function of $c^*_{t\text{-Res}}$ (●) and c^*_{CA} (◊), respectively, under the same experimental conditions as in (a).

to unity may demonstrate the Michaelis behavior of the immobilized enzymes [39]. Average values of H_2O_2 maximum current (I_{max,H_2O_2}) and Michaelis Menten apparent constant (K_{A,H_2O_2}) were calculated from the intercept and the slope of Lineweaver–Burk plots for five replicated measurements. In addition, the use of an Fc concentration higher than 3 mg in the construction of these biosensors produced no significant changes in current values (results no-shown). Therefore, from the results shown in Table 1, the biosensors constructed using 10-3-45 mg of PBHR, Fc and MWCNT + MO, respectively, were employed in the next experiments, considering that this electrode composition gave the maximum current value.

3.3. Responses of PBHR–Fc–MWCNT + MO biosensors versus t-Res and CA

When both PBHR and polyphenolic compounds were added to the stirred reaction medium composed by pH 7.00 PBS and a given H_2O_2 bulk concentration, the enzymatic catalytic cycle also took place in the solution bulk. The oxidised PBHR, produced as a result of its reaction with H_2O_2, is reduced back to its native state by polyphenolic compounds. The decrease of H_2O_2 in the solution bulk was detected as a decrease in the Fc^+ reduction current at the biosensor surface [38]. The best PBHR concentration in solution was 240 IU mL^{-1}. Thus, the addition of H_2O_2 to the pH 7.00 PBS + 240 IU mL^{-1} PBHR reaction medium produced a steady limiting current ($I_{s,lim}$) at −0.050 V *vs* SCE after 2 min, which corresponds to the reduction of Fc^+ generated by the PBHR catalyzed reduction of H_2O_2 to H_2O (Fig. 4a). The difference between $I_{s,lim}$ with and without the polyphenolic compound ($\Delta I_{s,lim}$) corresponds to the H_2O_2 concentration decrease given that the enzymatic reaction was taking place in the bulk solution. The steady state currents obtained after the addition of the polyphenolic compounds were reached at 45 s and 30 s for t-Res and CA, respectively. Plots of $\Delta I_{s,lim}$ *vs* CA (c^*_{CA}) and t-Res ($c^*_{t\text{-Res}}$) concentrations also showed a Michaelis–Menten type saturation. The corresponding linear portion obtained for t-Res and CA is shown in Fig. 4b. Fig. 5 displays the maximum current ($I_{max,t\text{-Res}}$) and apparent Michaelis Menten constant ($K_{B,t\text{-Res}}$) values obtained from the addition of different aliquots of t-Res to the solution composed by pH 7.00 PBS + 240 IU mL^{-1} PBHR in the presence of different H_2O_2 bulk concentrations. The kinetics characteristics of the PBHR–Fc–MWCNT + MO biosensor for t-Res and CA to the optimum working conditions (240 IU mL^{-1} PBHR + 50 μmol L^{-1} H_2O_2 in PBS pH 7.00) are summarized in Table 2. Values of *h* close to 1 [39] may indicate that PBHR shows a similar behavior towards t-Res and CA to that predicted by Michaelis–Menten. Therefore, Lineweaver–Burk plots can be used to obtain the max-

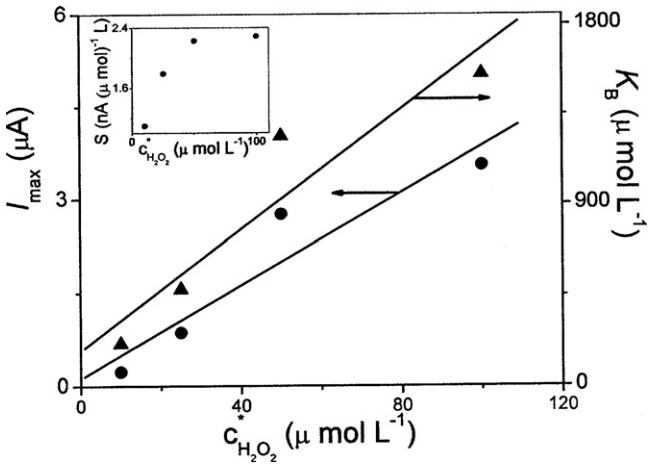

Fig. 5. Dependence of $I_{max,t\text{-}Res}$ and $K_{B,t\text{-}Res}$ obtained for t-Res through Lineweaver–Burk plots with H_2O_2 concentration. Insert: Plot of sensitivity as a function of $c^*_{H_2O_2}$.

Table 2
Hill parameter and I_{max} and K_B kinetics parameters obtained for PBHR–Fc–NTCPM + MO after adding different aliquots of t-Res or CA to PBS of pH 7.00 containing 240 IU mL^{-1} PBHR and 50 μmol L^{-1} H_2O_2.

Substrate	h	I_{max} (μA)	K_B (mmol L^{-1})
t-Res	0.99 ± 0.01	2.76 ± 0.09	1.24 ± 0.04
CA	1.00 ± 0.01	2.82 ± 0.09	2.44 ± 0.07

imum current I_{max} and K_B. For t-Res, $I_{max,t\text{-}Res}$ and $K_{B,t\text{-}Res}$ were proportional to the H_2O_2 concentration, while for the sensitivity ($S = I_{max,t\text{-}Res}/K_{B,t\text{-}Res}$ *vs* $c^*_{H_2O_2}$, see insert of Fig. 5) [40], the plateau was reached at 50–100 μmol L^{-1} of H_2O_2. This means that at these peroxide concentrations, the bioelectrode responses are limited by the enzymatic kinetics rate. Therefore, a concentration of 50 μmol L^{-1} of H_2O_2 was chosen as the best peroxide level in the reaction medium. Analogous studies to those previously described were also performed for CA, obtaining a similar behavior to that of t-Res, also with an optimum H_2O_2 concentration value of 50 μmol L^{-1}.

3.4. Biosensor statistical parameters

The stability of PBHR–Fc–MWCNT + MO biosensors was tested by using the same biosensor to determine the slopes from several calibration curves ($n = 5$) for the t-Res.

The slopes obtained were practically constant in the order of the experimental error until about five days, showing a good stability of PBHR biosensors. A noticeable decrease in the slope started from the fifth day and about a 40% decrease was obtained on the tenth day.

The PBHR–Fc–MWCNT + MO biosensor reproducibility was tested by measuring the calibration curve slopes for t-Res and CA of five different bioelectrodes in a solution of pH 7.00 PBS + 240 IU mL^{-1} PBHR + 50 μmol L^{-1} H_2O_2 as the reaction media. Percent relative standard deviations (%RSD) of 7.0% and 8.4% were obtained for t-Res and CA, respectively. The repeatability assays were performed carrying out six consecutive amperometric measurements on the same biosensor. In this case, %RSD of calibration curve slopes were 4.1% and 5.2% for t-Res and CA, respectively, when a PBHR–Fc–MWCNT + MO biosensor was used in PBS pH 7.00 + 240 IU mL^{-1} PBHR + 50 μmol L^{-1} H_2O_2.

Linear relationships between $\Delta I_{s,lim}$ *vs* $c^*_{t\text{-}Res}$ and c^*_{CA} were obtained in a range from 0.05 to 52 mg L^{-1} and from 0.06 to 69 mg L^{-1} for t-Res and CA, respectively. Correlation parameters were:

$$\Delta I_{s,lim} = (9.1 \pm 0.2) \times 10^{-3}\ \mu A + (9.08 \pm 0.06) \times 10^{-3}\ \mu A\ mg^{-1}\ L \times c^*_{t\text{-}Res} \quad (1)$$

and

$$\Delta I_{s,lim} = (9.3 \pm 0.5) \times 10^{-3}\ \mu A + (5.44 \pm 0.01) \times 10^{-3}\ \mu A\ mg^{-1}\ L \times c^*_{CA} \quad (2)$$

for t-Res (linear correlation coefficient, $r = 0.9997$) and CA ($r = 0.9999$), respectively.

The lowest concentration values measured with PBHR–Fc–MWCNT + MO biosensors for a signal to noise ratio of 3:1 were 0.023 and 0.020 mg L^{-1} for t-Res and CA, respectively. The quantification limits (LOQ) calculated for a signal to noise ratio of 10:1 were 0.077 and 0.067 mg L^{-1} for t-Res and CA, respectively, which include the minimization of false positive (type α error) and false negative (type β error) [41]. These values correlate reasonably well with those reported on related polyphenolic compounds determined with other biosensors [29,42,43]. However, the use of carbon nanotubes in the construction of biosensors employed in this study allowed decreasing by an order of magnitude detection limits (LOD) compared to other biosensors constructed in our laboratory using carbon paste instead of carbon nanotubes [38].

3.5. Application of PBHR–Fc–MWCNT + MO biosensor for the determination of total polyphenolic content

The TPC of different samples of wines and teas was measured using the PBHR–Fc–MWCNT + MO electrode. The TPC was estimated assuming that the relationship between $\Delta I_{s,lim}$ and $c^*_{t\text{-}Res}$ and c^*_{CA}, respectively, can be used as standard calibration curves for all other polyphenols.

Table 3
The total polyphenolic content (mg L^{-1}) in wine and tea samples and the corresponding standard deviations determined by the electrochemical method and the Folin–Ciocalteu (FC) method using t-Res or CA as standards.

Samples	t-Resveratrol standard		Caffeic acid standard	
	Electrochemical method	FC method	Electrochemical method	FC method
Red wine 1	1332 ± 69	2531 ± 156	2052 ± 73	2790 ± 209
Red wine 2	1379 ± 92	2850 ± 276	2129 ± 127	3168 ± 300
Red wine 3	1936 ± 93	3767 ± 351	2988 ± 88	4248 ± 322
Red wine 4	1375 ± 25	2925 ± 253	2122 ± 138	3258 ± 179
White wine 1	388 ± 15	570 ± 58	598 ± 25	619 ± 36
White wine 2	422 ± 12	604 ± 90	652 ± 40	666 ± 30
Black tea 1	515 ± 25	912 ± 110	859 ± 58	972 ± 49
Black tea 2	351 ± 18	730 ± 60	585 ± 31	738 ± 60
Red tea	299 ± 18	502 ± 30	497 ± 17	504 ± 39
Green tea 1	766 ± 27	1505 ± 97	1278 ± 82	1656 ± 97
Green tea 2	748 ± 32	1357 ± 76	1251 ± 86	1476 ± 78

Table 4
The analytical characteristics of some enzymatic biosensors.

Electrode	Immobilization	Enzyme	Sample	Substrates or standards	Sensitivity (nA mg^{-1}L)		LOD(mg L^{-1})		Life time	Refs.
Carbon nanotubes screen-printed electrodes	Chemical and physical entrapment with polyazetidine pre-polymer	Lacasse	Wines	Gallic acid	9		0.1		10 days	[27]
Screen-printed graphite electrodes	Entrapment in polyvinyl alcohol film	Lacasse	Teas	CatecholCaffeic acidHydroquinone	170.9	138.3 85.4	0.061	0.094 0.118	6 months	[29]
Gold electrode	On a self-assembled monolayer of mercaptopropionic acid	HRP	Wines and teas	(+) catechin			6.89			[42]
Gold electrode	Alternate deposition with poly(allylamine hydrochloride) on the gold electrode modified by a mercaptoproponesufonate self-assembled monolayer	HRP	Commercial reagents	Catechol and others	444.6		0.077		1 week	[43]
Carbon paste electrode modified with ferrocene	Entrapment in the composite	HRP	Wines and teas	(+) catechin	6.5		0.088		15 days	[44]
Glassy carbon electrode	Cross-linking with glutaraldehyde	Tyrosinase	Wines	Caffeic acid	77.8		3.7		18 days	[47]
Composite carbon nanotubes electrodes	Entrapment in the composite	PBHR	Wines and teas	t-Resveratrol	Caffeic acid	9.08 5.44	0.023	0.020	5 days	This work

The TPC values determined by the method of Folin–Ciocalteu and our electroanalytical method are shown in Table 3. The results obtained through the electrochemical method were compared with those obtained through the Folin–Ciocalteu method in several wine and tea samples using t-Res and CA as standard compounds. There was not agreement between the concentrations calculated by the two methods (Table 3). The concentration values determined by the Folin–Ciocalteu method were higher than the corresponding values obtained by our electrochemical method. This behavior has already been reported in previous studies [23,42,44]. The slopes obtained from plots of the values determined by the colorimetric method as a function of the values obtained by our method were (2.09 ± 0.07) and (1.56 ± 0.05) for t-Res and CA, respectively. However, we found that plots of TPC values determined by both methods were linear, with correlation coefficients of 0.9950 and 0.9949 for t-Res and CA, respectively, demonstrating that the present electrochemical method is useful for detecting the TPC in real samples. A possible explanation based on the determination of interferences (i.e., sulfite, ascorbic acid, glucose) by the Folin–Ciocalteu method has been reported [45].

Moreover, results obtained in our study are in agreement with the TPC determined by other authors in samples of wines and teas [23,36,42,44–46], considering that the TPC is probably related to the soil where the wine and tea samples are produced. The analytical characteristics of some enzymatic biosensors are shown in Table 4 for comparison.

The PBHR biosensors described in this study have some advantages over other biosensors previously reported, such as the lower cost of the PBHR enzymes. In addition, the ascorbic acid is not a substrate of PBHR but a substrate of horseradish peroxidase (HRP), which produces interferences when HRP is employed as the biological material for the construction of biosensors [44]. A further advantage is that the response time and/or the LOD obtained with our biosensor were lower and the linear range was greater than those informed in the literature for HRP and laccase biosensors [23,27,42,44,47].

Because several articles have reported overestimation of polyphenolic content by the Folin–Ciocalteu method, this must be considered for future analysis of polyphenols in different samples. We believe that inter-laboratory assays should be performed considering different techniques in addition to the Folin–Ciocalteu method in order to assess the convenience or inconvenience in the use of this method in real samples.

3.6. Analysis of probable interferences

Sulfur dioxide and glucose are known to interfere in the determination of polyphenols by the Folin–Ciocalteu method [48]. The addition of sodium hydrogen sulfite to a concentration up to 50 mg L^{-1} into a buffer solution of pH 7.00 containing 240 IU mL^{-1} of PBHR and 50 μmol L^{-1} H_2O_2 gave no change in the electrochemical response (data not shown). The Código Alimentario Argentino [49] allows the addition of sodium hydrogen sulfite to a maximum concentration of 30 mg L^{-1} as a conserver in wines. A higher concentration can be toxic for humans and produce strong sulfur odor in wines.

The addition of 100 μmol L^{-1} of glucose did not show a significant effect on the electrochemical response (data not shown).

The ascorbic acid is added as conserver in wines [50] at concentrations of about 2 mg L^{-1}. It is also present in teas, mainly in green teas, where it is found in infusions at a concentration of about 20 mg L^{-1}. The ascorbic acid can also be an electron donor for oxidized form of commercial peroxidases such as HRP [51]. The addition of ascorbic acid to a concentration up to 60 mg L^{-1} into the buffer solution in the presence of 240 IU mL^{-1} PBHR and 50 μmol L^{-1} H_2O_2 did not produce any change in the electrochem-

ical response, which indicates that ascorbic acid would not be a good electron donor for the oxidized form of PBHR.

4. Conclusions

It is concluded that the PBHR biosensor exhibited a good performance, stability, reproducibility, repeatability, detection limit and linear range for the quantification of t-Res and CA. This good analytical performance allowed us to estimate the TPC in wines and teas using a very simple experimental procedure. Furthermore, the electrochemical method has some advantages over the commonly used Folin–Ciocalteu method, such as a shorter detection time, a smaller sample volume, higher accuracy and a high simplicity. In addition, colored samples can be directly used for the measurement without pretreatment, and no interference from sulfur dioxide, ascorbic acid and glucose is found. In fact, advantages of this analytical methodology over the classic HPLC methods are well known. Moreover, the response time, the detection limit and the linear range were improved in comparison with other biosensors. These advantages indicate that a PBHR biosensor can be used as a useful tool for a rapid screening in the determination of total polyphenolic content in food matrices.

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