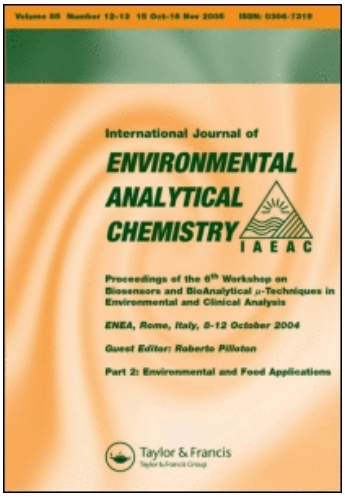

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Montereali, Maria Rita , Vastarella, Walter , Della Seta, Livia and Pilloton, Roberto(2005) 'Tyrosinase biosensor based on modified screen printed electrodes: measurements of total phenol content', International Journal of Environmental Analytical Chemistry, 85: 9, 795 — 806
To link to this Article: DOI: 10.1080/03067310500149775
URL: http://dx.doi.org/10.1080/03067310500149775

Intern. J. Environ. Anal. Chem.
Vol. 85, Nos. 9–11, 10 August–15 September 2005, 795–806

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Tyrosinase biosensor based on modified screen printed electrodes: measurements of total phenol content

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(*Received 30 October 2004; in final form 8 January 2005*)

A disposable tyrosinase biosensor has been developed for the analysis of phenols and polyphenols, by modifying screen printed electrodes (SPEs) with addition of suitable mediators of redox processes directly into the conductive pastes. The percent ratio of mediator in the pastes was chosen depending on the electrochemical sensitivity either in batch or standard flow conditions. Ferrocene modified carbon electrodes have been used on whose surface the enzyme tyrosinase was immobilized in a glutaraldehyde cross-linked matrix of bovine serum albumin. Mixing the mediator to the electrode pastes should overcome transport limitations, due to its dissolution, which occur in commonly used immobilization procedures. Different immobilization techniques of tyrosinase on SPEs in the detection of phenolic compounds were tested and compared. Modified SPEs showed relatively good reproducibility and detection limits in the micromolar range for all phenolic compounds used. Major sensor parameters have been optimized in flow systems putting special attention on operating potential, pH and buffer composition, which strongly affect the detection of polyphenols and operational stability in wine. The resulting biosensors were stored and dried for a minimum of 8 h at 4°C, and showed a shelf stability of about 30 days. The procedure has been applied both on a synthetic wine matrix and on real samples, to determine the 'pool' of phenolic composition in terms of phenol concentration.

Keywords: Tyrosinase; Screen printing technology; Ferrocene modified paste; Total phenols content

1. Introduction

Oxidation of low density lipoproteins (LDLs) is a crucial step in the pathogenesis of atherosclerosis, because of their fundamental role in the health of human organisms. Many studies reported the inhibition of this oxidation by phenolic compounds in red wine *in vitro*, demonstrating also that the consumption of beverages rich in polyphenols is associated to the increase in plasma antioxidant potential and therefore to the prevention of coronary heart diseases [1, 2].

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International Journal of Environmental Analytical Chemistry
ISSN 0306-7319 print: ISSN 1029-0397 online © 2005 Taylor & Francis
DOI: 10.1080/03067310500149775

Resveratrol (3,5,4′-trihydroxystilbene), and many other flavonoids, attracted specific interest because of their potential therapeutic biological attributes that favour protection against atherosclerosis, including antioxidant activity, modulation of hepatic apolipoprotein and lipid synthesis as well as inhibition of platelet aggregation [3–5]. Resveratrol concentration in grapes and wines is low (in the order of mg L^{-1}) especially in white wine, but the polyphenolic fraction, to whom benefits for health have been attributed, still represents the main source of flavonoids in human diet [6]. As a matter of fact, the global combination of phenolic compounds in wine may protect against atherogenesis by means of their antioxidant effect in time, thus directing research efforts to their rapid on-line measurement for both grapes and wines.

During the last decade antioxidant properties in grapes and in many varieties of white and red wines have been evaluated. High performance liquid chromatography (HPLC with organic or solid phase extraction), mass spectrometry (MS), ELISA, hyphenated methods (LC-MS-MS), and GC-MS measurements allowed for accurate quantitation, high recovery yields and low limits of detections (LODs) for many polyphenols ([7–10, and references therein]). At present, an exhaustive quantitative correlation between concentration of several phenols and toxicological effects on human health has not yet been achieved. Biosensors represent an interesting alternative way to usual toxicological and spectro-photometric assays in on-field evaluation of total polyphenolic effect. Anyway, this approach still shows some limitations, due to the low specificity for a single analyte and to the poor stability under continuous use. The correct evaluation of the antioxidant potential in food, environmental and pharmaceutical matrices embraces two different and complementary aspects, which should be taken into account simultaneously, difficult though it might be:

- phenol detection, by means of enzymes with a broad substrate spectrum, i.e. phenolase, oxidases [11–13], laccase [14–16], or tyrosinase [17–24], as well as with more specific enzymatic sensors, e.g. isolated microrganisms [25], phenol hydrolase and catechol oxidase [26, 27];
- determination of free radicals in solution [28–32].

The principal aim of this work is to verify the effectiveness of direct mediator dissolution in paste in preparing screen printed electrochemical tyrosinase electrodes and their use for total phenol content detection. Similar enzyme immobilization techniques were compared. Detection limits of several phenolic compounds and the effects of experimental parameters on sensor sensitivity are also reported. The same procedure has been applied to a synthetic wine matrix and to real samples, i.e. white and red wines from different geographical areas.

2. Experimental

2.1 *Chemicals and reagents*

Tyrosinase from *mushroom* (EC 1.14.18.1), with specific activity of 2060 units per mg of solid, bovine serum albumin (BSA, stock solution: 4% w/v), phenol, catechol, resorcinol, caffeic acid, L-tyrosine, siringic acid, benzoic acid, picric acid, hydroquinone, glutaraldehyde (GA) 25% aqueous solution, quercetin, resveratrol, tartaric acid, potassium and sodium phosphate salts were purchased from Sigma. All reagents,

buffer and electrolyte supporting solution, e.g. 0.1 M phosphate buffer (PB) solutions, were prepared from deionized water (ELGA-STAT deionizer). All other chemicals were of analytical grade and used without further purification.

2.2 *Electrode preparation*

Electrodes (SPEs) [33] were printed on 0.3–0.5 mm thick PVC substrate using a Fleischle (Brackenheim, Germany) screen printer. Experimental assays were previously performed on the electrodes lay-out preparation and miniaturization, but also on graphite and carbon–graphite composition pastes [34]. Carbon pastes for SPEs (obtained from Gwent Electronic Materials^{TM}) were further improved by a doping procedure, using ferrocene in a ratio strictly dependent on the signal current sensitivity. Ferrocene powder, not soluble in water, was directly mixed to graphite pastes (in special cases with the minimum amount of solvent for inks) before screen printing of the working electrode (WE). Great attention must be put in homogenizing the resulting paste, as quality of prints strictly depends on the paste rheologic properties. The choice of the specific mediator is due to its large diffusion as a substrate in peroxidase and oxidase tracers redox processes. Figure 1 shows the reaction in which phenols are oxidized to quinones through the enzymatic reaction and reduction of ferricinium ion (Fe^{3+}). The following re-oxidation of ferrocene (Fe^{2+}) at the WE enables the fresh formation of ferricinium and results in a current flow proportional to enzymatic activity, which reaches a maximum of signal for a specific percentage (5%) of ferrocene in the pastes.

2.3 *Enzyme immobilization on SPEs*

A PB solution containing 100 mg mL^{-1} of tyrosinase was freshly prepared. The enzyme was covalently immobilized on modified SPEs using BSA and GA (12.5% solution), then washed, dried in air and stored in a refrigerator at 4°C.

Different immobilization procedures were tested:

(1) Simple covalent immobilization of tyrosinase in glutaraldehyde: the ferrocene modified electrode was immersed in an amino propyl-triethoxysilane (APTES) 10% solution in PBS for 1 h, then washed before immersing them in GA solution (12.5%) and left to dry overnight. One milligram enzyme was dissolved in 10 µL of PB and 5 µL of it was dropped on the WE surface. The excess of unreacted enzyme was removed by a following washing step.
(2) Covalent immobilization of BSA protected tyrosinase in glutaraldehyde: the electrode was treated as above regarding GA immobilization (but only for 2 h),

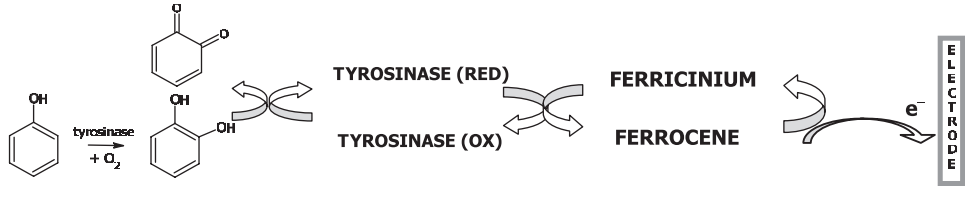

Figure 1. Schematic sequence of the ferrocene mediated mechanism of phenolics detection in our SPE biosensor.

then the enzyme ($0.1\,mg\,\mu L^{-1}$) was dissolved in 20 μL of bovine serum albumin (BSA, 25 mg in 125 μL of PB) and 10 μL of this solution dropped on the WE. The enzyme was left to react at least 1 h at 4°C, before washing with buffer.

(3) Cross-linking of tyrosinase by BSA–GA: the electrode was left in a basculating APTES 10% solution in 0.1 M PB (0.1 M KCl) for 1 h, then washed and treated with 10 μL of cross-linked tyrosinase. In order to cross-link the tyrosinase ($0.1\,mg\,\mu L^{-1}$), 5 μL of enzymatic solution were rapidly mixed to 20 μL of BSA ($0.2\,mg\,\mu L^{-1}$) and 10 μL of 12.5% GA. After 1 h the electrode was washed in PBS to remove the exceeding unreacted enzyme.

(4) Inverted immobilization, negative control: the same concentration ratios as in (3) were used but BSA and enzymatic solution were mixed before adding GA.

2.4 *Measuring set-up*

A perspex home-made flow cell (volume about 38 μL) was used. SPEs were used in a three electrode configuration. The cell was connected to a Gilson Minipuls-3 peristaltic pump and to an injector (Rheodyne, Cotati, USA) equipped with a 5 μL loop. Amperometric measurements were carried out by means of a lab-developed PC-controlled potentiostat (Domotek™), applying the desired potential and allowing for steady-state current to be reached. Voltammetric measurements, i.e. cyclic (CV) and differential pulse voltammetry (DPV), were performed using Palmsens™ Instruments at a selected scan speed of $50\,mV\,s^{-1}$.

3. Results and discussion

3.1 *Optimization of parameters*

Ferrocene powder was directly mixed (in different weight percentages) to the graphite paste. In order to select the right amount of mediator to be used in graphite inks, electrodes were characterized by CV and DPV. Preliminary measurements on modified SPEs were made in blank solutions (buffer alone or phenol 1 mM in PB at controlled pH) and then directly adding tyrosinase in batch solution ($1.2\,mg\,mL^{-1}$). CVs were measured in different conditions, showing a reversible behaviour in the enzyme catalyzed reaction: figure 2 shows typical voltammograms due to the oxidation of ferrocene, in buffer and in phenol buffered solution, for a 7.1% ferrocene modified WE. Figure 3 shows that an oxidation peak for 5.5% w/w ferrocene in carbon graphite paste is quite visible at 50 mV *versus* Ag/AgCl reference electrode (RE).

Table 1 summarizes the results obtained with different percentages of ferrocene by measurement of the current peak due to tyrosinase at a fixed applied potential (namely 50 mV). I_0 represents the signal value in buffer alone, I_p the signal after addition of phenol (10^{-3} M) in PB, and I_{enz} is the signal (at the peak potential shown in figure 2) measured 5 min after the addition of tyrosinase $0.1\,mg\,mL^{-1}$ in PB solution. 2.8 mL of $0.1\,mg\,mL^{-1}$ tyrosinase were added to 22.2 mL of 10^{-3} M phenol in PB. The enzymatic ratio ER was then calculated as:

$$ER = \frac{I_{enz} - I_p}{I_p}$$

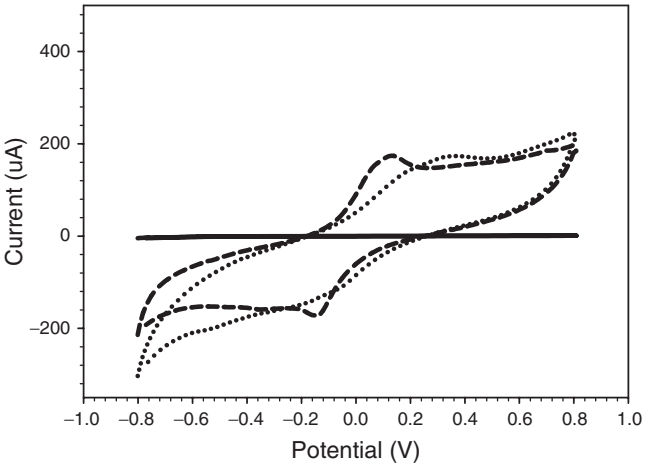

Figure 2. Cyclic voltammograms due to the phenol oxidation (1 mM) at the graphite SPE modified with 7.1% ferrocene. (—) only buffer: 0.1 M PB at pH 6.6 containing 0.1 M KCl; (····) 1 mM of phenol in PB; (- - -) addition of tyrosinase 0.1 mg mL^{-1}.

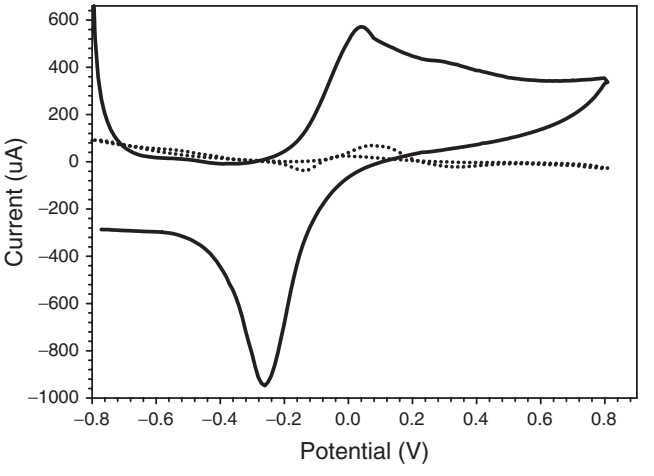

Figure 3. Comparison between CVs (blank subtracted) due to oxidation of 1 mM phenol in presence of tyrosinase 0.1 mg mL^{-1} for 5.5% w/w (solid line) and 10.0% w/w (dotted line) of ferrocene in carbon graphite paste.

thus evaluating the best doping amount as 5.5% ferrocene in the graphite paste. The following experiments for the phenol sensor development have been performed with pastes modified with such optimal ferrocene percentage.

DPV measurements enabled to determine the redox potential of the couple Fe^{3+}/Fe^{2+} (ferricinium ion/ferrocene) with respect to the graphite electrode. The plot (figure 4) in the range −500 and +500 mV *versus* pseudo Ag/AgCl RE shows electrode response at different ferrocene amounts after addition of tyrosinase in buffered phenol solution, confirming 5.5% as a suitable percentage to enhance the electrochemical redox reaction.

Table 1. Current responses corresponding to the oxidation peak (at 50 mV) in CVs, using different percentage of ferrocene. I_0, signal in buffer alone; I_p, signal after addition of phenol in PB for a final concentration of 10^{-3} M; I_{enz}, signal measured 5 min after the addition of tyrosinase 0.1 mg mL^{-1} to 10^{-3} M buffered phenol solution; total batch volume, 25 mL.

Ferrocene (w/w) (%)	I_0 (μA)	I_p (μA)	I_{enz} (μA)	Enzymatic ratio: $(I_{enz} - I_p)/I_p$
5.5	−2.52	73.66	642.82	7.73
7.1	−10.91	74.23	139.08	0.87
10.0	−0.22	117.62	144.33	0.23
15.1	−0.05	75.41	135.59	0.80

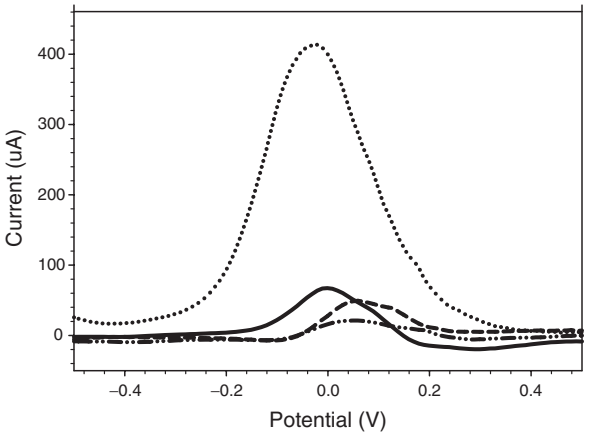

Figure 4. Differential pulsed voltammetries (blank subtracted) using different ferrocene amount in the pastes after the addition of 2.2 mL of tyrosinase in buffered phenol solution; (····) 5.5% w/w, (—): 7.1% w/w, (- - -) 10.0% w/w, (–··–··–) 15.1% w/w.

Procedure 3 (described in section 2.3) was chosen for tyrosinase (0.1 mg mL^{-1}) immobilization on modified SPEs, because of the most stable and reproducible signal obtained with the injection of 1 mM of phenol, while procedures (2) and (4) gave no response at any concentration under flow conditions. Covalent immobilization with APTES, GA and tyrosinase (procedure (1)) with respect to the cross-linking method (3) should accelerate the substrate diffusion towards the WE avoiding the obstacle of a bulky cross-linking matrix, and thus lowering the response and total analysis time. Therefore, this method is still under investigation.

No significant differences in the steady-state current were observed varying the flow rate between 0.05 and 0.40 mL min^{-1} or varying the buffer pH between 6.2 and 7.7 (figure 5); all measurements were made in flow injection conditions with phenol 1 mM, using modified $SPE_{5.5\%}$. Experimental conditions were set as flow rate 0.4 mL min^{-1}, pH 6.6, where the electrode response is faster and tyrosinase activity is sufficiently high.

The hydrodynamic voltammogram shown in figure 6 was obtained in the described experimental conditions; a potential range from 0 to 460 mV was investigated and a potential of 50 mV was chosen for all the subsequent flow injection amperometric measurements with Domotek^{TM} potentiostat as a fair compromise between high sensitivity, low interferences and accuracy.

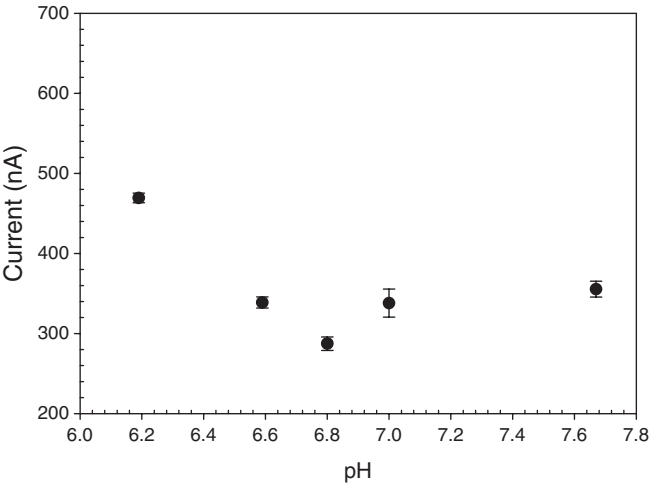

Figure 5. Oxidation current due to phenol *vs.* pH. Flow rate: 0.40 mL min^{-1}, phenol concentration: 1 mM, injection time: 3 min, applied potential: +280 mV *vs.* Ag/AgCl RE, ferrocene amount in the graphite paste of SPE: 5.5% w/w.

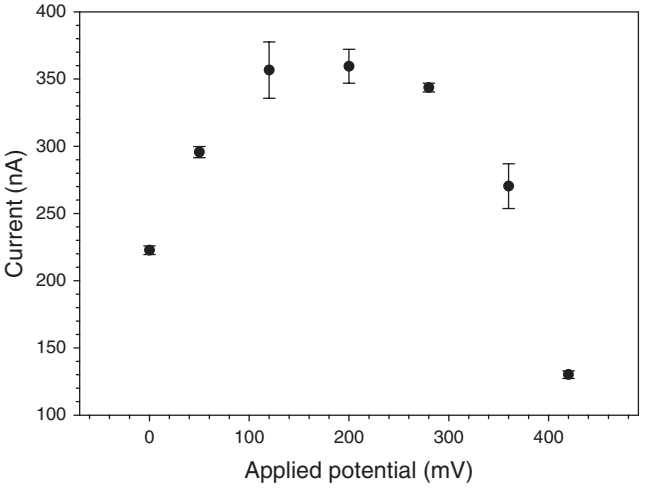

Figure 6. Hydrodynamic voltammogram for tyrosinase SPE biosensor. Flow rate: 0.40 mL min^{-1}, phenol concentration: 1 mM in PB at pH 6.6, injection time: 3 min, ferrocene amount in the graphite paste of SPE: 5.5% w/w.

3.2 *Characteristics of the sensor response*

Calibration graphs were obtained for several phenolic compounds in PB in flow conditions (figure 7). The main electroanalytical characteristics of modified SPE with immobilized tyrosinase are reported in table 2. Average currents from four measurements at steady-state were calculated. LOD was measured according to Zund Meier graphical method [35]. Relatively wide linear range of two orders of magnitude and low limits of detections were achieved for phenol and catechol; good linear ranges were obtained for gallic and caffeic acid as well. Resorcinol, siringic acid, quercetin

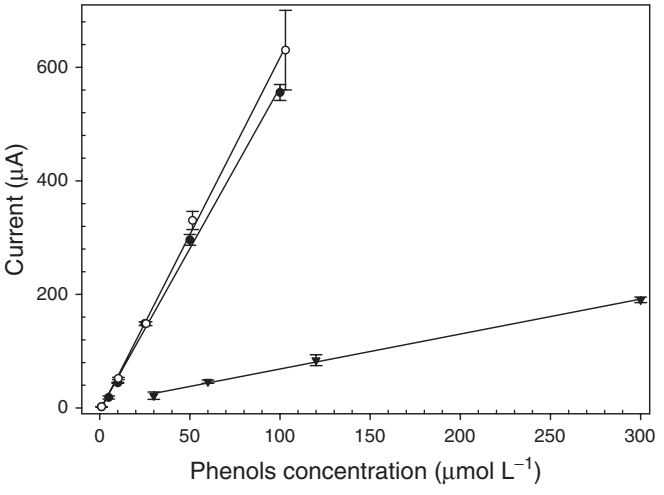

Figure 7. The calibration curves of SPEs modified with ferrocene and BSA–GA cross-linked tyrosinase; (●) phenol, (○) catechol, (▼) gallic acid.

Table 2. The analytical sensor parameters of ferrocene modified SPE immobilizing cross-linked tyrosinase with BSA–GA. Average currents from four measurements at steady-state were calculated; PB solution, 0.1 M at pH 6.6, containing 0.1 M KCl; r^2, linear regression coefficient; LOD can be either calculated as three times the standard deviation of the background signal or measured according to the Zund–Meier method [35].

Analyte	Sensitivity (μA mM^{-1})	r^2	LOD (μM)	Linear range (10^{-6} M)
Phenol	5.68 ± 0.08	0.9977	4	1.0–100.0
Catechol	6.23 ± 0.08	0.9987	5.6	1.0–103.0
Gallic acid	0.61 ± 0.08	0.9980	57	30.0–300.0
Caffeic acid	1.16 ± 0.08	0.9994	10.5	10.6–266.0
Benzoic acid			No response	No response
Quercetin			No response	No response
Resorcinol			No response	No response
Resveratrol			No response	No response
Siringic acid			No response	No response

and resveratrol gave no response, as expected from the literature data [36]. This behaviour should be ascribed to the mechanism of tyrosynases, introducing in the first step of the reaction pathway a hydroxyl group in ortho-position of phenol. The chemical stabilization of the oxidized form (quinone) in ortho- and para-substitute phenolics, compared to the meta-substitutes, is demonstrated by resonance structures.

Operational stability under continuous injection of 1 mM of phenol, catechol and caffeic acid was then investigated; in figure 8 the frequency of injection was around 7.5 per hour and the experimental flow rate 0.4 mL min^{-1}. The biosensor showed relatively high enzyme lifetimes, without significant modification in response after storage for 30 days at 4°C.

3.3 *Investigation on real samples*

Preliminary electrochemical measurements, namely CV, DPV and chronoamperometry, on randomly selected real wine samples did not show significant interferences within the

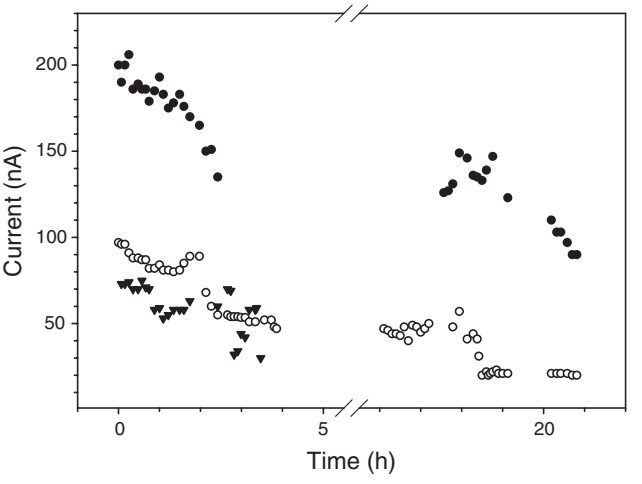

Figure 8. Operational stability of tyrosinase-based SPE biosensor modified with 5.5% w/w of ferrocene in carbon graphite paste. Injection of 1 mM phenolics, with a frequency of 7.5 injection per hour; (●) phenol, (○) catechol, (▼) caffeic acid.

investigated potential range. Measurements of phenol and catechol were performed in a synthetic wine matrix, dissolving 3 g of tartaric acid in 1 L of 12% v/v ethanol, in order to simulate the composition of real wine samples and a suitable alcohol gradation. Standard solution should be prepared and calibration curves obtained in a synthetic wine matrix.

The sensor was tested in diluted synthetic wine solutions keeping pH between 6 and 7, without affecting stability and functionality. Dilution factors of wine sample/buffer up to a maximum ratio of 1/5 enabled to keep pH within the optimum range, being pH of undiluted real sample far from the values in which the enzyme reach the highest efficiency. Different problems, such as dissolved oxygen depletion, accounted for the use of more diluted solutions for wine samples, particularly 1 : 100 white wines, 1 : 300 red wines. Figure 9 shows CVs on a bare electrode (no enzyme), the dotted line being the buffer solution and the solid line a real red wine sample (1 : 100); no electrochemical interferences have been shown in the potential range investigated.

The modified SPE sensor showed sensitivity on real samples comparable to the one in standard solutions at low dilutions. On the other hand, the strong matrix effect on the enzyme can be overcome using diluted samples, thanks to the excellent LOD, and feeding the sensor with solutions (buffer, diluted samples) continuously bubbled with air. At scheduled times (10, 15, 20 min), standard addition method has been performed in flow (two consecutive points at final concentrations of respectively 2 mmol L^{-1} and 4 mmol L^{-1}), plotting the signal in a Gran plot to extrapolate the phenol concentration at zero current. In figure 10 an example of a Gran plot for a white (code number 4) wine sample is shown. In table 3 analytical results of several geographically characterized wine samples, following the described procedure at a flow rate of 0.4 mL min^{-1}, are reported. Dilution factors are just indicated, depending on whether white or red wine has been analyzed; final standard concentrations are extrapolated at zero current and expressed as phenol concentration (reported both in μmol L^{-1} and ppm in table 3). In fact, the calculated values are far from the mean phenol content reported in the literature both for wine and red wines, this depending on the relative selectivity

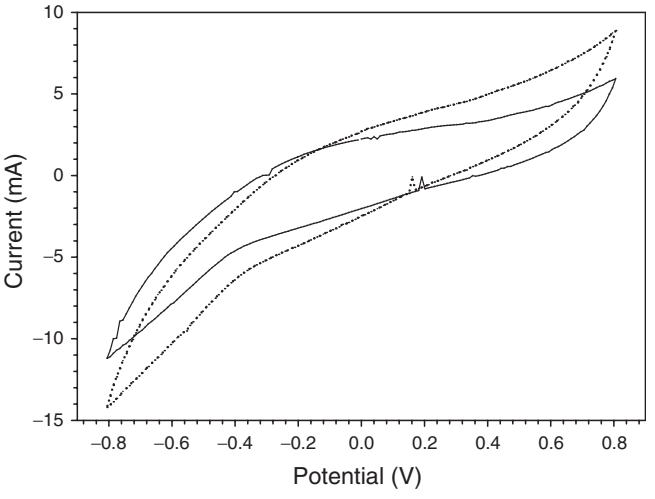

Figure 9. CV on a modified SPE (without enzyme) in buffer (dotted line) and 1 : 100 diluted red wine sample (solid line).

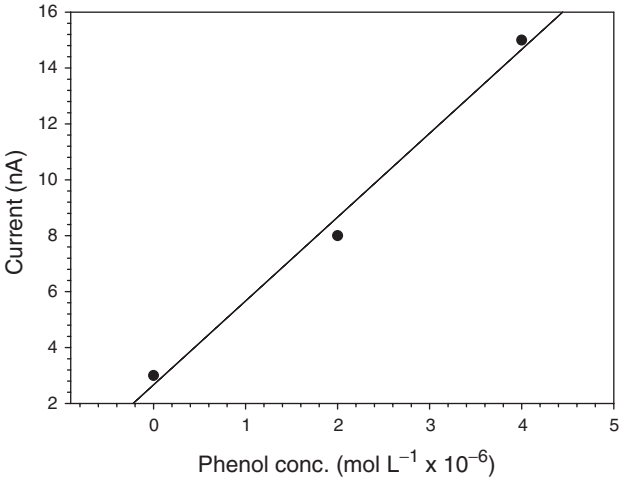

Figure 10. Gran plot obtained in flow conditions and corresponding to two standard additions to a selected sample of a diluted (1 : 100) Italian white wine: Antinoo–IGT bianco 2000 Casale del Giglio (Code nr. 04).

of tyrosinase to oxidize a limited class of polyphenols. Comparison between our biosensor based procedure and the spectrophotometric method of Folin Ciocalteu [37–39] is in progress as well as a response analysis amongst different oxidases immobilized on modified SPEs with different mechanism for phenolics oxidation.

4. Conclusions

We proposed the physical inclusion of a mediator, such as ferrocene, into the bulk of the carbon graphite paste of the SPE sensor for phenolics detection, because of its

Table 3. Analytical results of some geographical characterized wine samples; flow rate: 0.4 mL min^{-1}; dilution factor for white wine: 1/100, dilution factor for red wine: 1/300; method of phenol standard addition (Gran Plot, as in figure 10) was used to extrapolate the concentration in the real matrix. All the concentration units are expressed as phenol concentration.

Code	Detailed wine samples description	Slope	Intercept	r^2	Polyphenols (μmol L^{-1})	Polyphenols (ppm)
01	Albiola–IGT rosato 2002 'Casale del Giglio'	4.00	6.50	0.9884	162	15
02	Mater Matuta–IGT rosso 'Casale del Giglio'	12.75	8.67	0.9716	377	36
03	Madreselva–IGT rosso 1999 'Casale del Giglio'	7.00	9.17	0.9735	393	37
04	Antinoo–IGT bianco 2000 'Casale del Giglio'	3.00	2.67	0.9908	89	8
05	Cardonnay–IGT bianco 2002 'Casale del Giglio'	6.00	5.33	0.9908	89	8
38	Campo Vecchio–IGT bianco 2002 'Castel de Paolis–Biologico'	2.25	3.50	0.9643	157	15
39	Frascati Superiore–DOC bianco 2002 'Castel de Paolis–Biologico'	4.75	4.67	0.9963	98	9
40	Frascati Cannellino–DOC bianco 2002 'Castel de Paolis–Biologico'	2.75	2.67	0.9891	97	9
46	Nero Buono-rosso Senza annata Az.Vinicola Ferretti (Cori, LT)	1.88	3.15	0.9985	547	51
48	Monte Pulciano–rosso Senza annata Az Vinicola Ferretti (Cori, LT)	4.50	6.17	0.9749	411	39
49	Merlot–rosso 2003 Vitorchiano Bassa	4.38	4.75	0.9784	326	31

simplicity in electrode preparation and the resulting advantages for the construction of versatile mediator-dependent biosensors. With this method, the mediator dissolution which normally takes place in common techniques of mediator immobilization on the WE has been largely limited, avoiding electrode poisoning and false output signal which may limit transport functions [40].

The presence of ferrocene inside and on the electrode surface enhanced fast responses in flow conditions, with a response time at the steady-state of less than 3 min. Tyrosinase was immobilized on SPEs in a BSA and GA matrix, resulting in a good operational stability and low interferences, after injection of several phenolics. On the other hand, the cross-linking method showed low recovery time for the signal response, due to the high enzymatic loading on the sensor surface.

Detection limits in the micromolar range were achieved especially for phenol, catechol, and caffeic acid. The procedure has been successfully applied to determine total phenol content in a synthetic wine matrix and a selected pool of real samples of Italian wines. Global content was expressed in terms of phenol concentration, as a reference unit. Due to the high selectivity of tyrosinase to oxidize polyphenols, the response in real sample concentration was not perfectly in accordance to the data in literature. A comparison of the tyrosinase biosensor with a certified methodology will be required for further reliable investigations.

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