

New Biosensor for Phenols Compounds Based on Gold Nanoparticle-Modified PVC/TTF-TCNQ Composite Electrode

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A gold nanoparticle-modified PVC/TTF-TCNQ composite electrode with tyrosinase enzyme is proposed as electrochemical biosensor for detecting phenolic compounds in musts and wines samples using phenol and catechol as standards. The work potential was 0 V both in batch and in FIA system (linear range from 0.6 μM up to 10.0 μM), with a detection limit of 7.9×10^{-7} M for phenol and 6.43×10^{-7} M for catechol. The good correlation between the results obtained with the biosensor and those achieved with the Folin–Ciocalteu reference method enables that it can be used for rapid and almost automated estimation of the “total phenolics” and “high antioxidant” fraction of must and commercial wines.

Keywords: Poliphenols, Gold nanoparticles, biosensor, Tyrosinase, Flow injection, Wine.

1. INTRODUCTION

In the last years, polyphenolic compounds or “phenolics” have gained enormous attention mainly in the analytical chemistry field, because they have important health properties [1] and antioxidant activity [2-5].

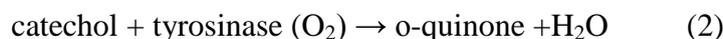
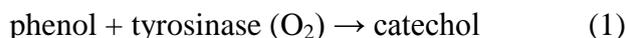
They are present in grapes and, consequently, in wines, contributing substantially to their quality, and affecting their color, flavor, stability and aging behavior [6]. So, the determination of this group of compounds is of great importance in wine industry because it can help to identify variations in wine type.

Although several methods based on separation techniques, such as chromatography or capillary electrophoresis with various detection systems [7-10], have demonstrated to be powerful in the identification and separation of phenolics compounds, wine contains a variety of phenolic compounds, commonly called tannis, which cannot be determined singly, so being measured collectively as so-called total polyphenol (TP) index [11].

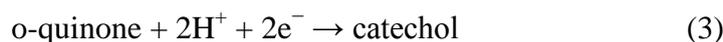
The Folin–Ciocalteu [FC] spectrophotometric method [12], based on the reaction of phenolic compounds with a colorimetric reagent, is used as reference method and widely recognized, both scientifically and for wineries, although usually produce an overestimation of TP content [6, 13].

Electrochemical biosensors have been proposed as an interesting alternative to existing methods of analysis for the determination of phenolic compounds [14-29], because they offer advantages such as minimal sample preparation, selectivity, sensitivity, reproducibility, fast response time and easy to use continuous analysis [6, 16, 21, 23, 26, 27].

A lot of them are based in the use of the Tyrosinase (polyphenol oxidase) [6, 15, 16-18, 20, 26-28], a copper-containing monooxygenase enzyme that catalyzes the conversion of phenolic substrates to give catechol and o-quinone, as final product, as follows:



Later, the resulting o-quinone can be electrochemically reduced at the electrode surface by the following equation:



In this context, PVC/TTF-TCNQ composite electrode has shown good mechanical properties [30] and electrocatalytic activity in the oxido-reduction of some analytes of great interest [31-33]. Moreover, modification with gold nanoparticles enhances the intensity current and shifts its redox potential with respect to the non-modified electrode as consequence of facilitate electron transfer between redox proteins and electrode surface [34].

The aim of this work is to propose a new composite-tyrosinase biosensor based on the good electrochemical properties of this modified electrode. The immobilization of the enzyme is accomplished by cross-linking with glutaraldehyde and the biosensor has been used for the estimation of phenolic compounds in different samples of must and red and white wines, via determination of a bioelectrochemical index [11, 13] using the basics structures of phenol and catechol as standards.

2. EXPERIMENTAL

2.1. Reagents and solutions

Tetrathiafulvalene-tetracyanoquinodimethane [TTF-TCNQ] salt (purum grade, Fluka), poly(vinyl chloride) (PVC, low mol. weight, purum grade Fluka) and *N,N'*-dimethylformamide (DMF, $\geq 99.7\%$ pure, Merck) were used to prepare the PVC/TTF-TCNQ composite electrode.

Stock solutions of catechol (Sigma, 99%) and phenol (Sigma, 99.5%) were prepared in 0.05 M phosphate buffer solution (PBS, pH 7.4). More diluted standards were prepared by suitable dilutions with the same phosphate solution. All solutions were prepared freshly.

The solutions used for the immobilization of the enzyme in the biosensor of phenols, was a solution of 91 U μL^{-1} tyrosinase [Tyr] (Sigma, EC 1.14.18.1 from mushroom sp., 4276 U mg^{-1}) prepared in a 0.05 M phosphate buffer pH 7.4, and a 25% glutaraldehyde solution (Aldrich). The rest of chemicals were purchased in analytical reagent-grade from Merck or Fluka and also used as received.

Aqueous 1% $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution (Sigma, > 49% as gold) and 1% sodium citrate solution were used for the preparation of colloidal gold.

All solutions were made in twice-distilled water from a Millipore Milli-Q system (18 $\text{m}\Omega$).

2.2. Instrumentation

All electrochemical measurements were made by using an Autolab PSTAT 12 potentiostat using the software package GPES 4.9. A three-electrode cell (10 mL glass electrochemical cell) equipped with a Pt wire counter electrode, a BAS MF-2079 Ag/AgCl 3 M KCl reference electrode, and different PVC/TTF-TCNQ working electrodes, was used. All tests were performed at ambient temperature (*ca.* 25 °C).

The UV-Visible spectra of colloidal gold nanoparticles were recorded by using a Cary 100 Bio spectrophotometer equipped with a 1 cm path length cell.

The flow system consists of a Dynamax peristaltic pump model RP-1 (Rainin), a six-way injection valve (Omnifit), PTFE tubes of 0.5 mm inner diameter (Omnifit) and an amperometric detector integrating both the working and counter electrodes. The flow cell was a Metrohm model 6.5303.020 wall-jet cell. The injection volume (sampling loop) was 100 μL .

A Helios Omega UV – vis spectrophotometric (Thermo Scientific) was used to apply the reference spectrophotometric method involving the use of Folin–Ciocalteu reagent.

2.3. Procedure

2.3.1. Preparation of Au_{coll}

Gold colloids were prepared according to the procedure previously described [34]. After cleaning all the glassware in a bath of freshly prepared 3:1 $\text{HNO}_3\text{-HCl}$, rinsing thoroughly in twice-distilled water, and drying in air, 2.5 mL of sodium citrate solution were added to 100 mL of a boiling aqueous solution containing 1 mL 1% (w/w) $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$. A stable red colloidal solution was obtained. The diameter of the Au_{coll} particles was 16 ± 2.3 nm. Preparations were stored in dark glass bottles at 4 °C for further use.

2.3.2. Preparation of the working electrodes

a) The composite electrode without gold was prepared by using the procedure previously described in earlier works [36, 37]. The PVC/TTF-TCNQ was obtained at a 1:10 (w/w) ratio of TTF-TCNQ to PVC in DMF. The composite powder was pressed into cylindrical pellets 4 mm in diameter \times 2.5 mm thick with an active surface area of 12 mm². The pellets were inserted by press-fitting into a Teflon tube furnished with a copper wire to establish electrical contact.

The electrode surface was regenerated as required by polishing with P-1200 emery paper to obtain a flat surface, followed by polishing with glossy paper to obtain a uniform surface and rinsing with water.

b) With respect to gold –modified PVC/TTF-TCNQ/Au_{coll} composite electrode, it was prepared as follows: Once polyvinyl chloride is thoroughly dissolved in DMF, the TTF-TCNQ salt is added to obtain a homogeneous PVC/TTF-TCNQ mixture. Subsequently, the suspension was separated, and different volumes of the colloidal gold suspension were added and dried in order to obtain the composite powder. There are reproducibility problems when using biosensors prepared with amounts above 5 mL Au_{coll} probably due to a lack of homogeneity of the electrode. Therefore, it can be settled a volume of 5 mL as the most appropriate amount of Au_{coll} solution to be added to the composite.

Tyr was immobilized onto the composite electrodes by the usual cross-linking with glutaraldehyde [34]. The procedure consisted of coating the composite electrode with 6 μ L of a 91 units μ L⁻¹ Tyr solution (*c.a.* 550 units Tyr). After letting it dry out at ambient temperature, the electrode was immersed in a glutaraldehyde solution for 40 minutes and rinsed with water.

When not in use, the composite electrode was kept in 0.05 M phosphate buffer (pH 7.4) and stored in the refrigerator.

2.3.3. Measurement in wines using the PVC/TTF-TCNQ/Au_{coll} biosensor

In bath, successive volumes of 20 μ L (wine) or 150 μ L (must) were added to 10 mL of a 0.05 M phosphate buffer (pH 7.4), which was continuously stirred at a constant rate in the electrochemical cell. Amperometric measurements at 0 V were carried out. The estimation of the phenolic compounds content was performed by extrapolation in a calibration curve with standard solutions of catechol.

For the flow injection analysis (FIA) system, samples of wine and must were diluted in different volumes of 0.05 M phosphate solution at pH 7.4.

2.3.4. Spectrophotometric methods

For comparison purposes, wines and musts were also analyzed by the spectrophotometric method involving the use of Folin–Ciocalteu reagent. In this method, in a 100 mL flask are introduced successively 1 mL of wine (red if previously diluted 1:5 with water), 50 mL of distilled water, 5 mL of Folin–Ciocalteu reactive (phosphotungstic - phosphomolybdic acid) and 20 mL of 20% sodium carbonate. The mixture was brought to a final volume of 100 mL with distilled water, waiting for 30 minutes to get a stabilization of the reaction. Finally, the absorbance was measured at 750 nm in 1 cm

cells thick. The total phenolic content, expressed in mg L^{-1} of catechol, was evaluated from the absorbance value by interpolation into the calibration plot obtained with catechol standard solutions.

3. RESULTS AND DISCUSSION

3.1. Cyclic voltammetry responses to poliphenols of the biosensor

Figure 1 shows the cyclic voltammogram (CV) of the catechol obtained at the Tyr-PVC/TTF-TCNQ/Au_{coll} composite electrode. A well defined reduction peak is observed around 0 V. For phenol (not shown for clarity) a similar CV is obtained.

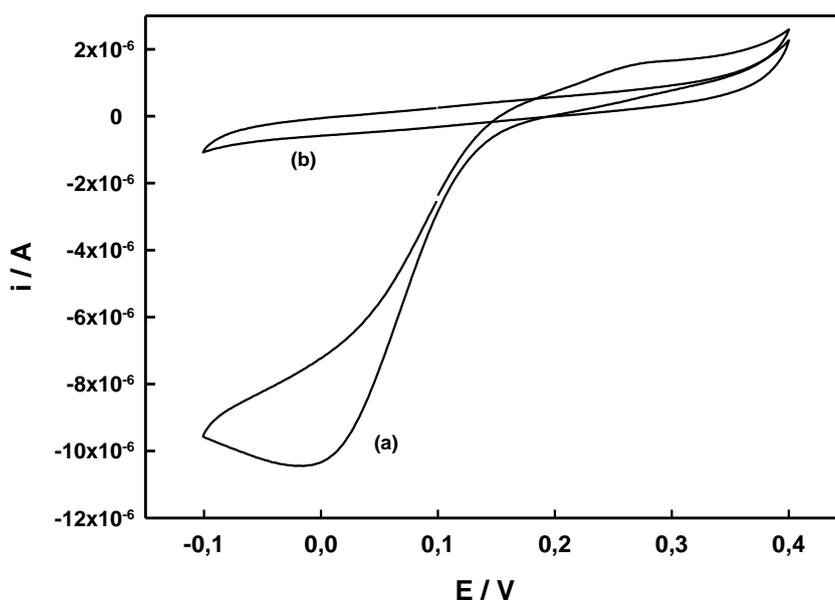


Figure 1. Cyclic voltammograms obtained at the Tyr-PVC/TTF-TCNQ/Au_{coll} electrode. (a) 0.2 mM catechol (b) supporting electrolyte. 10 mV s^{-1} . PBS solution (0.05 M, pH 7.4).

3.2. Chronoamperometric detection of poliphenols

3.2.1. Optimization of variables

The effect of solution pH on the amperometric response of catechol and phenol was investigated over the range of 5.0–9.0. The steady-state current (data not shown) increases until pH values around 7, which is attributed to the maximum activity presented by the enzyme at these pH values. At $\text{pH} > 7.5$ the current decreases sharply, probably due to the involvement of the proton in the electrochemical reaction. Therefore, a 0.05 M phosphate buffer of $\text{pH} = 7.4$ was selected as working medium.

Under these conditions, the enzyme loading influence was also evaluated over the 180–820 units range. The slope of the catechol calibration graph (not shown) increased with the enzyme loading

up to 550 units of Tyr. For higher enzyme concentrations, reproducibility problems were observed, which could be due to factors as the saturation of the electrode surface giving a no-homogeneous enzyme distribution, the increase on the electrode resistance, etc. Consequently, the value of 550 units of Tyr was selected for further works.

Another parameter of great interest to be determined is the working potential applied to the electrode. In this way, the chronoamperometric response of the poliphenols were recorded at different final potentials E_f after successive additions of poliphenols under stirring conditions, with the biosensor containing 550 units Tyr.

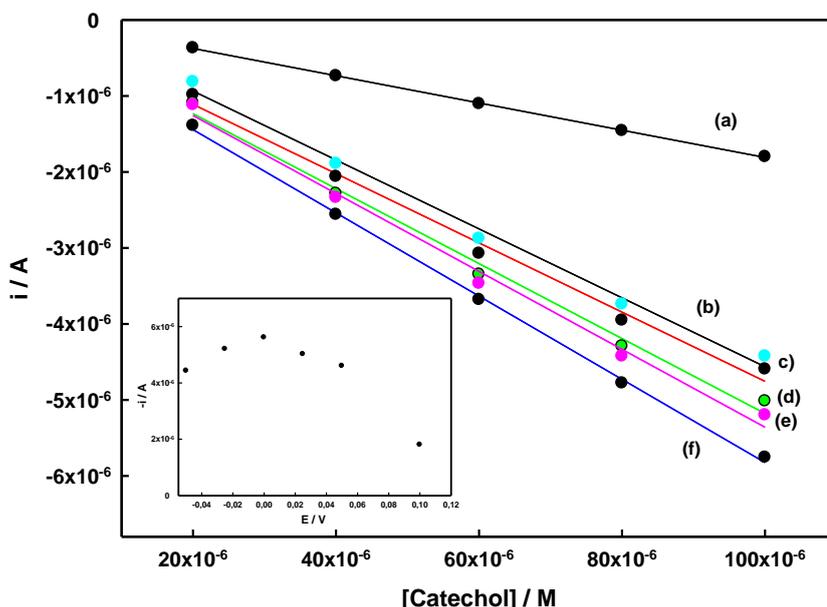


Figure 2. Catechol calibration curves obtained for Tyr-/PVC/TTF-TCNQ/Au_{coll} composite electrodes. PBS solution (0.05 M, pH 7.4). The applied potential E_f was a) 0.1 V, b) -0.05 V, c) 0.05 V, d) 0.025 V, e) -0.025 V, f) 0 V. Inset for catechol 1×10^{-4} M

Table 1. Analytical characteristics obtained from calibration plots for catechol at different potential values applied to the composite biosensor. PBS solution (0.05 M, pH 7.4)

Potential (E_f)			Slope (mA M^{-1})	Linear range (μM)	r^2
-50 mV			45.3 ± 0.5	0.2 - 10	0.9928
-25 mV			51.2 ± 1.4	0.2 - 10	0.9922
0 mV			54.5 ± 0.6	0.2 - 10	0.9990
25 mV			49.2 ± 0.4	0.2 - 10	0.9916
50 mV			45.6 ± 0.5	0.2 - 10	0.9911
100 mV			17.8 ± 0.4	0.2 - 10	0.9997

As can be seen in Figure 2 and Table 1, we obtained for catechol a better current response at potentials close to 0 V. For phenol, similar results were obtained. These low working potential values are very interesting to rule out possible effects of other interfering species present in solution, such as ascorbic acid.

3.2.2. Kinetic constants and analytical characteristics

According to above results, 550 units Tyr, 5 mL Au, and 0 V of applied potential can be estimated as rather suitable work conditions.

In order to evaluate the applicability of the Michaelis-Menten approach to describe the kinetic behaviour of the proposed biosensor, we have calculated the Hill's coefficient [35] by fitting the $\log [i/(i_{max}-i)]$ versus the \log of the substrate concentration. In figure 3 is shown this plot for catechol. The results obtained were 1.049 and 1.061 for catechol and phenol respectively, that is, close to the theoretical value of unity for the classic Michaelis-Menten equation. Therefore, the apparent Michaelis-Menten constants K_m^{app} were calculated from the corresponding Lineweaver-Burk plots (inset in Fig. 3, data in table 2). These results are similar to others values reported in the literature [36, 37], and much lower than the free enzyme, 4000 μM [38], thus revealing a good affinity for the substrate of the enzyme immobilized according to the proposed biosensor configuration.

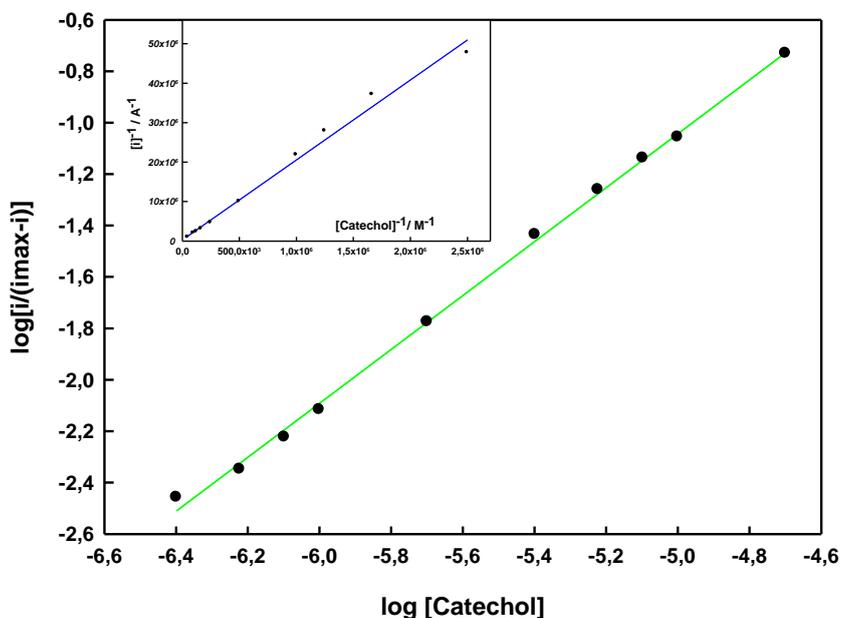


Figure 3. $\log [i/(i_{max}-i)]$ vs $\log [Catechol]$ plot obtained for Tyr-/PVC/TTF-TCNQ/Au_{coll} composite electrodes. PBS solution (0.05 M, pH 7.4). The applied potential E_f is 0 V. Inset shows the corresponding Lineweaver-Burk plot.

Table 2. Figures of merit obtained from calibration plots for the two phenolic compounds with the Tyr-/PVC/TTF-TCNQ/Au_{coll} biosensor.

Analyte	K_m^{app} (μM)	Limit of detection ($\text{M} \times 10^7$)	Sensibility (mA M^{-1})	Linear range (μM)	r^2
Phenol	114.0	7.3	62 ± 1.4	0.2 - 10	0.9958
Catechol	84.4	3.53	54.5 ± 0.6	0.2 - 10	0.9990

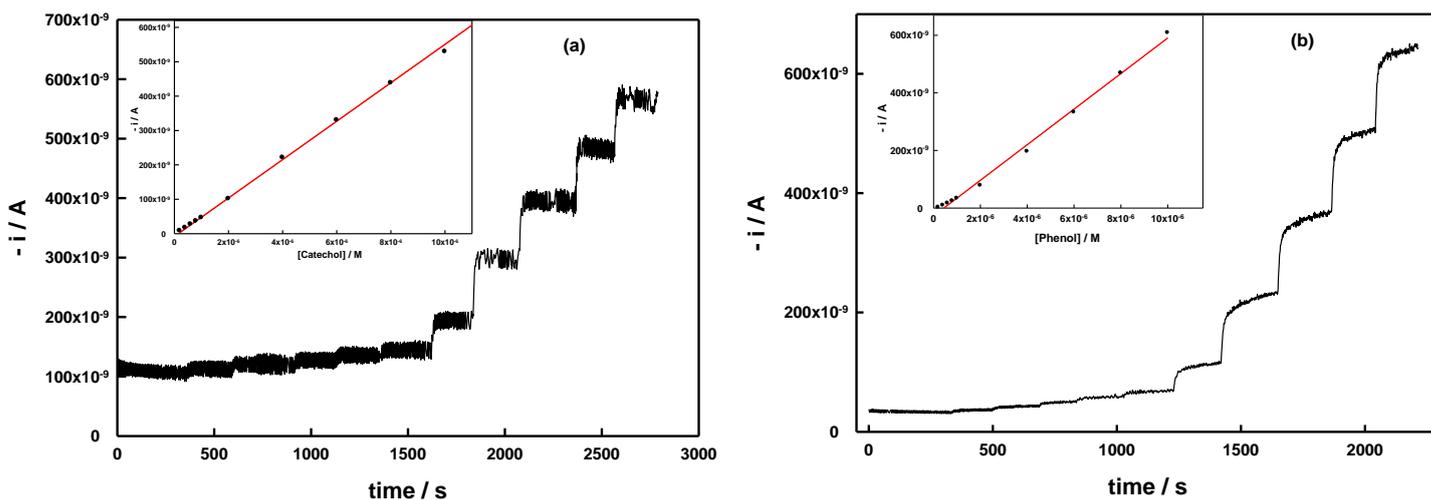


Figure 4. Catechol and phenol calibration curves obtained for Tyr-/PVC/TTF-TCNQ/Au_{coll} composite electrodes. PBS solution (0.05 M, pH 7.4). The applied potential E_f is 0 V. (a) Catechol (b) Phenol

Under these experimental conditions we obtained (data in Table 2) for catechol (Fig. 4a) a linear range up to 10 μM , with a sensitivity of $54.5 \pm 0.6 \text{ mA M}^{-1}$ and a linear regression coefficient (r) of 0.999. The detection limit ($s/n = 3$) was $3.53 \times 10^{-7} \text{ M}$. For phenol (Fig. 4b), the linear range was up to 10 μM , with a sensitivity of $62 \pm 1.4 \text{ mA M}^{-1}$, a linear regression coefficient (r) of 0.996 and the detection limit ($s/n = 3$) was $7.3 \times 10^{-7} \text{ M}$. These results show that the proposed biosensor is competitive, representing therefore a good and interesting alternative, because there is no need to add any additional substance to the composite mixture to ensure its hardness and conductivity simultaneously. It also has lower cost, as only a small amount of enzyme is needed (just to cover the electrode surface).

3.2.3. Reproducibility

The reproducibility of the biosensor was estimated from the response of the electrode to 10 μM catechol measured at 0 V. We obtained a relative standard deviation (R.S.D.) of 2.2% for five successive determinations by using the same electrode, a R.S.D. of 3.6% when using five different electrodes of the same synthesis and a R.S.D. of 6.5% for five electrodes from different syntheses. These results show a remarkable reproducibility of the proposed biosensor.

3.2.4. Stability of enzyme electrode

We have investigated the long-term stability of the biosensor by recording a daily calibration curve of 10 μM catechol solution, prepared also daily, for one month. During two weeks the biosensor

response was virtually stable. At higher times a continuous decrease in sensitivity and in intensity was observed, thus replacement being recommended from 3-4 weeks. This behaviour is similar to those described in the literature for other tyrosinase sensors [36, 37], being determined by the enzyme stability, since the electrode is easily regenerated.

3.3. Biosensor integration into a FIA system and poliphenols determination

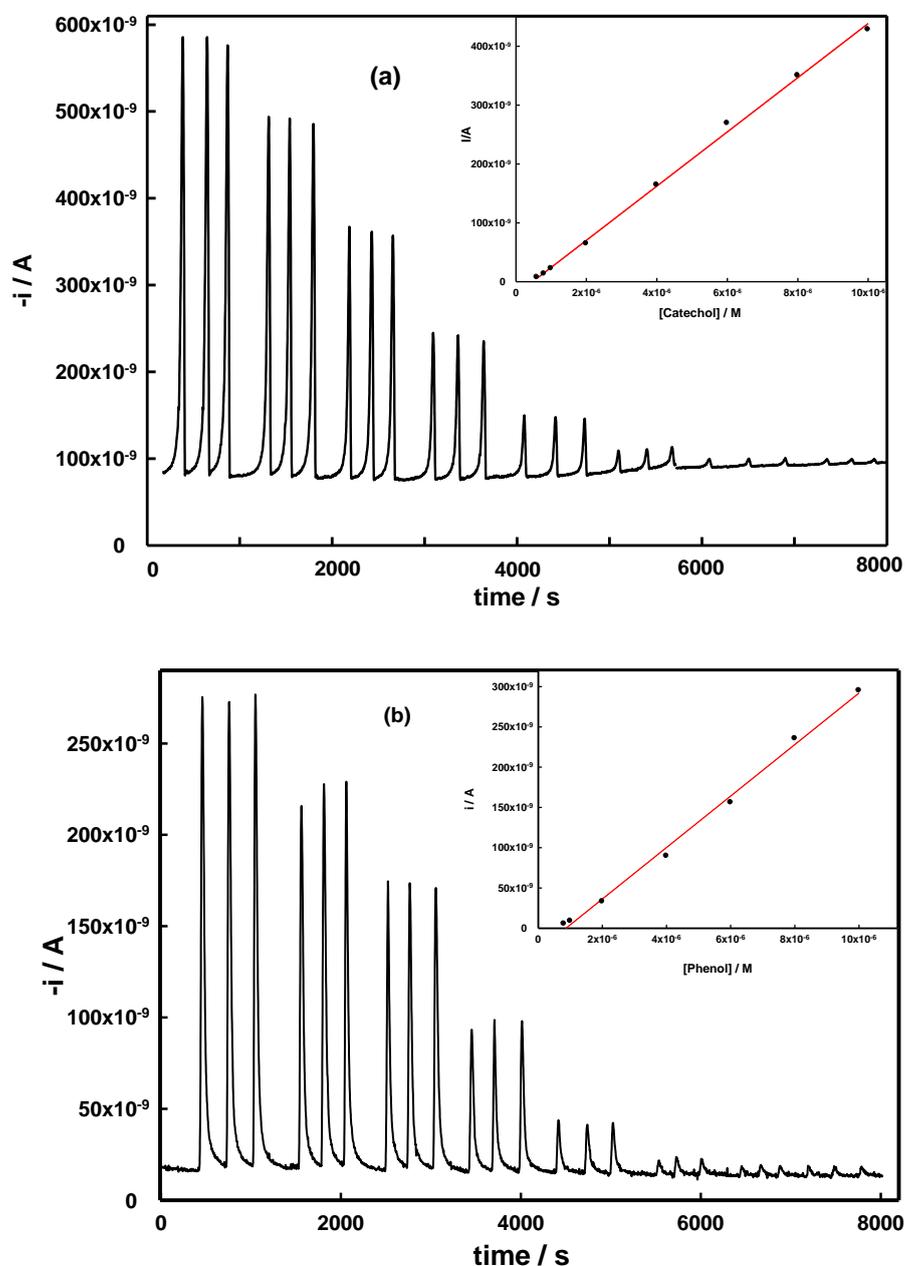


Figure 5. FIA peaks obtained at the biosensor for triple injections of catechol (a) or phenol (b) standards (ranging from 0.6 μM to 10 μM catechol) and the corresponding calibration curves (inset). Carrier solution: PBS buffer (0.05 M; pH 7.4). Flow rate: 1.0 mL min^{-1} . Injected volume (sampling loop): 100 μL . Applied potential: 0 V.

Prior to the application in a FIA system, it was examined the influence of hydrodynamic variables such as flow rate and injected volume on the response of the biosensor. The best results in terms of sensitivity and reproducibility were obtained at a flow rate of 1 mL min⁻¹ and an injected volume (sampling loop) of 100 µL. Moreover, the most suitable potential was found to be 0 V for these conditions.

The reproducibility (n=5) was checked on several consecutive injections of a 10 µM catechol solution. A relative standard deviation of 2.7% was obtained, which indicates a good reproducibility and therefore a good mechanical resistance of the biosensor under flowing conditions.

Figure 5 shows the calibration curves obtained for standards of catechol (a) and phenol (b). Three measurements were performed for each standard solution.

For catechol, a sensitivity of $46 \pm 1.2 \text{ mA M}^{-1}$ and a linear regression coefficient (r) of 0.997 were obtained for a linear range between $6 \times 10^{-7} \text{ M} - 10^{-5} \text{ M}$. The detection limit (s/n = 3) was $6.4 \times 10^{-7} \text{ M}$.

For phenol, a sensitivity of $32 \pm 0.5 \text{ mA M}^{-1}$ and a linear regression coefficient (r) of 0.996 were obtained for a linear range between $6 \times 10^{-7} \text{ M} - 10^{-5} \text{ M}$. The detection limit (s/n = 3) was $7.3 \times 10^{-7} \text{ M}$.

These results are in good agreement with those obtained in batch, and involve an interesting improvement when compared to those recently reported on sensor or biosensors applied to FIA systems [23, 26, 27].

3.4. Monitoring of phenolic compounds in wines.

Table 3. Electrochemical poliphenol index (expressed as catechol mg L⁻¹) obtained with the Tyr-PVC/TTF-TCNQ/Au_{coll} biosensor and comparison with the value obtained using the Folin-Ciocalteu reference method (n = 5).

Wines	Sample	Folin-Ciocalteu Catechol mg L ⁻¹	Biosensor Catechol mg L ⁻¹
Must 1	1	128 ± 4	6 ± 0.5
Must 2	2	185 ± 7	10 ± 1
White: Fino (clarified)	3	126 ± 5	7 ± 0.5
White: Fino Cobos	4	119 ± 4	6 ± 0.5
White: Tio Pepe	5	140 ± 5	8 ± 1
Rosé	6	394 ± 12	10 ± 1
Red: Albali	7	1552 ± 40	20 ± 1.5
Red: San Asensio	8	1270 ± 30	15 ± 1

The practical usefulness of the proposed electrode was evaluated by performing the estimation of the content of phenolic compounds in must and wine samples. The samples were analyzed following the simple procedure described in Experimental Section.

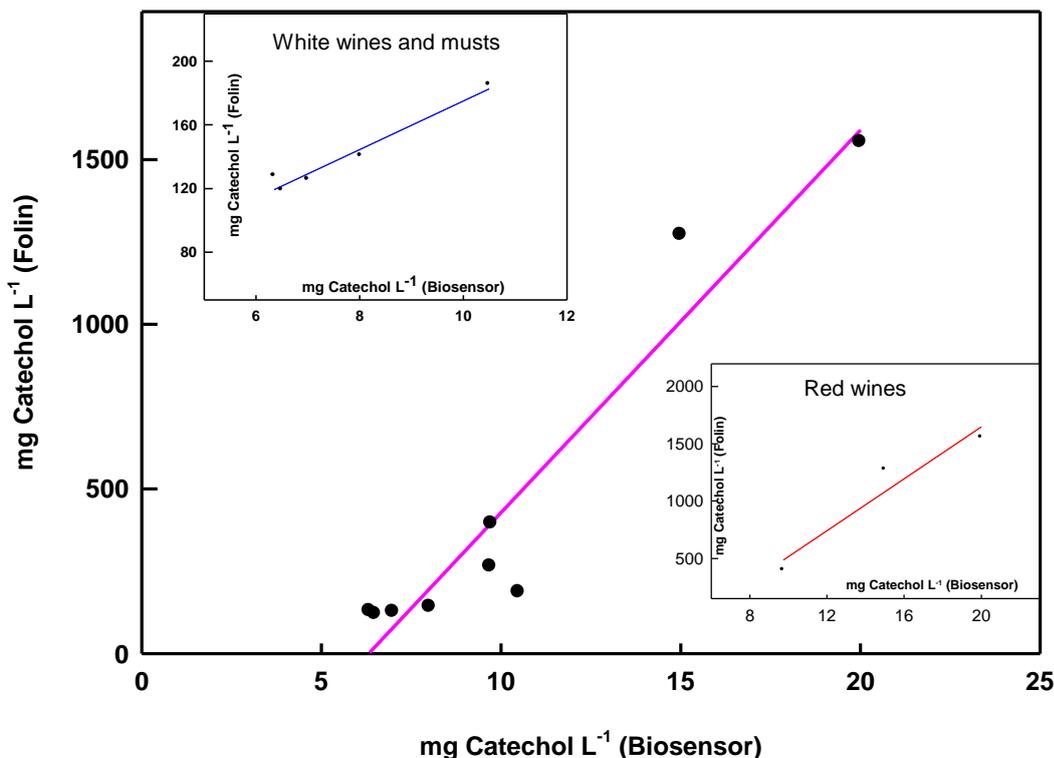


Figure 6. Correlation between the results obtained in wines by using the Tyr-PVC/TTF-TCNQ/Au_{coll} biosensor and the Folin–Ciocalteu reference method. Insets for white wines and must (left) and red wines (right), separately.

The overall content of phenolic compounds was obtained using catechol as standard (concentration expressed in mg L⁻¹). Table 3 shows the results obtained with the Tyr-PVC/TTF-TCNQ/Au_{coll} biosensor and their comparison with those obtained using the Folin–Ciocalteu reference method. The values given correspond to the mean value of five (n = 5) determinations.

The results of the measurements are quite different in values as they are obtained with analytical methods based on different principles. In fact, the Folin–Ciocalteu method consists in a redox reaction of phenols with the phosphomolybdic reagent followed by the colorimetric detection of the resulting products, while the proposed biosensor is based on an enzyme reaction. Consequently, the estimation of phenolic compounds must be considered as a polyphenol index [11, 28] whose absolute value depends on the applied method. Moreover, the electrochemical results also depend on the applied potential [11], such that one can obtain different fractions of their total polyphenolic by changing the oxidation potential. In this way, Mannino et al. [39] have proposed a direct relationship between low oxidation potential and high antioxidant power, so that at 0 V (working potential in this work) we obtain the signal corresponding to the “high antioxidant fraction” [11].

Figure 6 shows the results obtained with the proposed biosensor (bioelectrochemical data) versus the results achieved with the Folin–Ciocalteu method (spectroscopic data). A good correlation was obtained ($r = 0.9558$) for all different types of must and wines. Moreover, as can be seen in the insets, the correlation is better when the comparison is made for musts and white wines by one hand ($r=0.9788$), and the red wines by the other ($r = 0.9628$). This fact seems to be in agreement with the

different phenol types present in each class of wines [40], and supports the higher antioxidant capacity of red wines.

4. CONCLUSIONS

The PVC/TTF-TCNQ/Au_{coll} composite electrode has been employed as biosensor for polyphenols compounds (catechol and phenol) by using tyrosinase enzyme cross-linked with glutaraldehyde. This biosensor can be used both in batch and in a FIA system and shown a good reproducibility and stability. At a working potential of 0 V present a linear range from 0.6 μM up to 10.0 μM , with a detection limit of 7.9×10^{-7} M for phenol and 6.43×10^{-7} M for catechol. Moreover, this low working potential value allows to discard possible effects of other interfering species present in solution, such as the ascorbic acid.

The utility of the biosensor was evaluated by performing the estimation of the content of polyphenolic compounds in must and wine samples. A good correlation between the results obtained with the biosensor and those achieved with the Folin–Ciocalteu reference method was obtained. These results are very interesting since the proposed biosensor can be used as a useful tool for rapid and almost automated estimation of the “total phenolics” and “antioxidant power” of must and commercial wines.

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