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Laccase-based biosensor for the determination of polyphenol index in wine

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ABSTRACT

In this work we have developed and characterized the use of Laccases from *Trametes versicolor* (TvL) and *Trametes hirsuta* (ThL) as biocatalytic components of electrochemical biosensors for the determination of polyphenol index in wines. Polyazetidine prepolimer (PAP) was used as immobilizing agent, multi-walled and single-walled carbon nanotubes screen-printed electrodes as sensors (MWCNTs-SPE and SWCNTs-SPE) and gallic acid as standard substrate. The amperometric measurements were carried out by using a flow system at a fixed potential of −100 mV vs. silver/silver chloride electrode in Britton–Robinson buffer 0.1 mol L^{-1}, pH 5. The results were compared with those obtained with the Folin–Ciocalteau reference method. The results obtained in the analysis of twelve Italian wines put in evidence the better suitability of ThL-MWCNTs-based biosensor in the determination of the polyphenol index in wines. This biosensor shows fast and reliable amperometric responses to gallic acid with a linear range 0.1–18.0 mg L^{-1} (r^2 = 0.999). The influence of the interferences on both spectrophotometric and electrochemical measurements have been carefully evaluated.

1. Introduction

Nowadays, quantitative analysis of wine samples is becoming of great importance in food chemistry area because to the general effort to achieve an adequate quality. Wine is a complex food mixture of several hundred compounds, present simultaneously at different concentrations. The dominants ones are water, ethanol, glycerol, sugars, organic acids and various ions. Except ethanol and glycerol, other aliphatic and aromatic alcohols, amino acids, and phenolic compounds are present at much lower concentration [1]. Polyphenols are well known for their antioxidants properties [2–5], which have been associated with reduced risk of cancer [6], stroke [7], heart disease [8], and diabetes [9]. They also play an important role in food quality [10]. Many analytical procedures, developed for determination of polyphenols are often expensive and require several operations [11,12]. Besides, it is not easy to monitor these compounds in real matrices for different reasons such as their chemical complexity, difficult extraction procedure and the presence of interferences. Wine contains a variety of phenolic compounds, commonly called tannins, which cannot be determined singly, so they are measured collectively as so-called total polyphenol index [13].

The reference method commonly used involves monitoring colorimetric chemical reduction and can be time-consuming and also produces chemical waste.

Biosensor technology appears to be suitable for their detection and exhibits advantages as easy sample preparation, selectivity, sensitivity, reproducibility and low costs [14,15]. Electrochemical biosensors, in particular amperometric ones, are an attractive alternative to current used analytical methods, as chromatographic techniques, to measure phenols in wine samples. Commonly used amperometric biosensors are based on tyrosinase [16,17], peroxidase [18], pyrroloquinoline quinine dependent glucose dehydrogenase (GDH) [19] or cellobiose dehydrogenase (CDH) [20].

Laccases (p-diphenol: oxygen oxidoreductase, E.C: 1.10.3.2) are copper containing oxidoreductases detected in many plants [21] and secreted by numerous fungi [22]. They are able to oxidise many different substrates, i.e. phenols and anilines, with the concomitant reduction of oxygen to water according to the reaction:

$$4AH + O_2 \overset{\text{laccase}}{\rightleftarrows} 4A + 2H_2O$$

where AH and A are reduced and oxidized states of the substrate, respectively [23,24]. Therefore, laccase has been applied to many industrial processes including decolourization of dyes [25], pulp delignification [26], oxidation of organic pollutants [27], microbial transformation of natural products [28] and the development of biosensors [15,29–34] or biofuel cells [35].

Aim of this work is the development of a laccase biosensor for polyphenol index in wines by comparing the spectrophotometric Folin–Ciocalteu method, commonly used for real matrices [36,37], to Flow Analysis (FA). Often is reported that colorimetric procedure leads to an overestimation of total polyphenol index because of the interference of sulphur dioxide, reducing sugars and ascorbic acid

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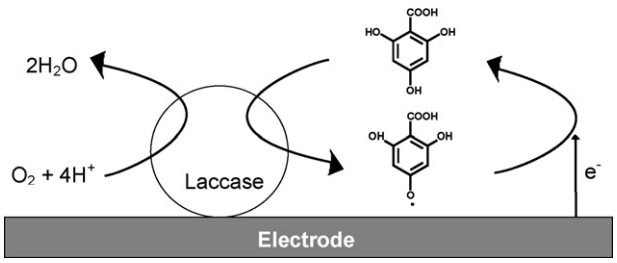


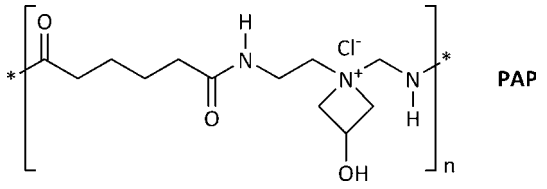
Fig. 1. Electron transfer mechanism of gallic acid oxidation on a laccase biosensor.

[38]. In addition, is reported that the co-concurrence of sulphur dioxide and reducing sugars can result in synergistic effects [13].

The biosensors proposed in this work are realized by employing two different Laccases, *Trametes versicolor* (TvL) and *Trametes hirsuta* (ThL) and using polyazetidine prepolymer (PAP) as new immobilizing agent. TvL was just used to detect the polyphenol index in wines but immobilized with other techniques [30,32,39–41]. ThL, before our work, was never used for the same purpose. Its use is limited to the development of biofuel cells [42] and to detect the phenolic compounds without an applicative use on real matrices [43].

The reaction which involves laccase and gallic acid on electrode surface is described in Fig. 1.

PAP provides both a chemical and a physical entrapment [44,45] and the immobilization procedure is possible thanks to its peculiar features:



PAP acts as a cross-linking agent being able to react with several different organic moieties (thiolic, oxydrilic, carboxyl, aminic group) increasing the possibility to create chemical bonds with the enzyme, thus enhancing the immobilization efficiency [44].

In order to increase the stability of this system we have integrated the PAP entrapping properties, above mentioned, with the conductivity, high surface area matrix, flexibility and reactivity of multi-walled carbon nanotubes (MWCNTs) and single-walled carbon nanotubes (SWCNTs) [46].

2. Experimental

2.1. Chemicals and reagents

Fungal laccase from *Trametes versicolor* was supplied by Fluka (EC 1.10.3.2, activity: 30.6 U mg^{-1}, according to Sigma–Aldrich supplier, the enzyme content of this laccase is approximately 10%) and stored at −18 °C. *Trametes hirsuta* laccase was gently donated by VTT Technical Research Centre of Finland (3.9 mg mL^{-1} in citrate buffer pH 5; activity: 421 U mL^{-1}). Bovine Serum Albumin (BSA, EC 232-936-2), gallic acid, sodium metabisulphite, ascorbic acid, glucose and fructose were purchased from Sigma and used as received. Kit for measurement of total polyphenols concentration in wines was purchased from Biogamma. The kit contains Folin–Ciocalteau reagent (composed by a mixture of $H_3PW_{12}O_{40}$ and $H_3PMo_{12}O_{40}$), carbonate buffer and a standard solution of gallic acid 3.0 g L^{-1}. Stock solutions of gallic acid were prepared in 0.1 mol L^{-1} Britton–Robinson buffer, pH 5 daily. More diluted standard solutions were prepared by suitable dilutions with the same buffer. The polymeric film employed for protein entrapping was poly-1-(aminomethyl)-1-{2-[(6-oxyesane)amino]ethyl}-3-hydroxyazetidinium chloride (polyazetidine prepolymer, PAP®), donated by Hercules Inc., Wilmington, DE (USA). Other chemicals were all of analytical grade. High purity deionized water (Resistance: 18.2 MΩ cm at 25 °C; TOC < 10 μg L^{-1}) obtained from Millipore (France) has been used to prepare all the solutions.

Different wine samples were acquired from a local supermarket in Rome (Italy). The only sample treatment required consisted of an appropriate dilution with a buffer solution before analysis.

2.2. Apparatus

Amperometric experiments were performed by using a μ-Autolab type III potentiostat (Eco Chemie) controlled by means of the GPES Manager program (Eco Chemie). Screen-Printed Electrodes (SPEs) (DropSens), constituted by a MWCNTs or SWCNTs working electrode with a surface diameter of 4 mm, carbon as counter electrode and silver/silver chloride electrode as reference one, were used. Flow experiments were carried out using a microliter cell (DropSens) and a Gilson Minipuls-3 peristaltic pump. Cyclic voltammetry experiments (CVs) were performed in a 10 mL glass cell with a conventional three-electrode configuration. A modified MWCNTs Screen-Printed Electrode was used as working electrode, a grafite counter electrode and an Ag/AgCl (KCl_{sat}) Metrohm, Switzerland, 198 mV vs. NHE as reference electrode.

The spectrophotometric measurements were carried out by using a T60U Spectrometer PG Instruments Ltd. spectrophotometer.

2.3. Biosensor preparation

The carbon nanotubes screen-printed electrodes were preliminary treated by depositing on the working electrode 10 μL of 0.5 mol L^{-1} nitric acid solution as reported in literature [47] in order to obtain the carboxyl functionalization of their surface. *Trametes versicolor* Laccase biosensor (TvL-MWCNTs-SPE and TvL-SWCNTs-SPE) and *Trametes hirsuta* Laccase biosensor (ThL-MWCNTs-SPE and ThL-SWCNTs-SPE) were prepared by spreading 3 μL of a solution of PAP containing enzymes onto the electrodes surface to have a final amount of 0.80 U. To assess the matrix effect on the measurements, a sensor with only PAP (PAP-MWCNTs-SPE) was prepared by spreading 3 μL of PAP solution onto the surface of the electrode. Then the electrodes were left to dry overnight at room temperature.

Another aspect taken into account was the influence of impurities present in commercial laccase (TvL). This was demonstrated by adding a proper amount of BSA to ThL-based biosensor and comparing it with TvL-based biosensor for polyphenol index.

Biosensors were stored in 0.1 mol L^{-1} Britton–Robinson buffer, pH 5 at 4 °C.

2.4. Spectrophotometric measurements

Spectrophotometric measurements were carried out according to the assay procedure [48,49].

The results obtained give the polyphenol index referred as gallic acid concentration (see Table 2).

All the values reported are the average of at least six measurements.

2.5. Electrochemical measurements

Flow experiments were carried out at a fixed potential of −100 mV vs. the internal silver/silver chloride reference electrode with a flow rate of 4 μL s^{-1}. The carrier buffer was 0.1 mol L^{-1} Britton–Robinson, pH 5 and aliquots of 100 μL of gallic acid stan-

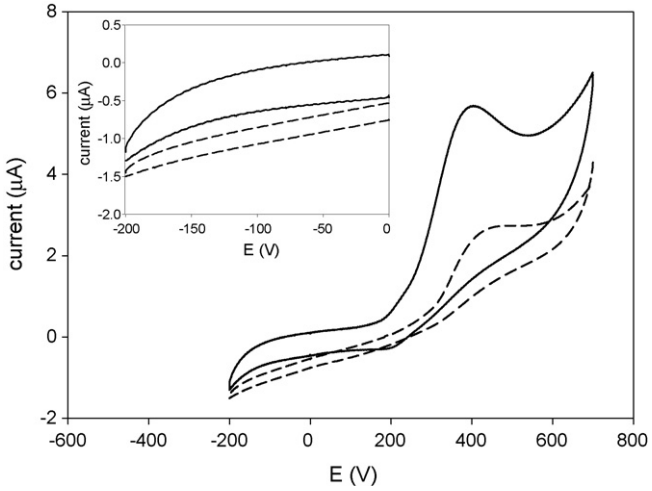


Fig. 2. Cyclic voltammograms for gallic acid (11.0 mg L^{-1}) recorded with ThL-MWCNTs-SPE (dashed line) and with PAP-MWCNTs-SPE (solid line) in 0.1 mol L^{-1} Britton–Robinson buffer, pH 5 at a scan rate of 5 mV/s. Inset: detail of catalytic current at −100 mV.

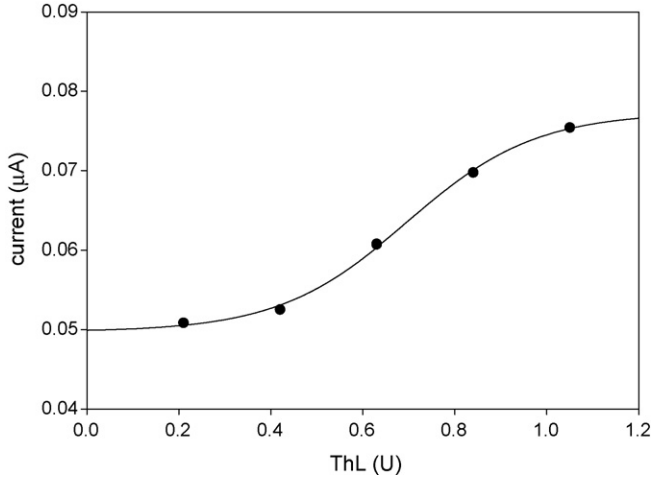


Fig. 3. Effect of ThL enzyme loading on the amperometric response of a 4.2 mg L^{-1} gallic acid solution recorded at a fixed potential of −100 mV vs. silver/silver chloride electrode.

dard solutions at different concentrations in the same buffer were used to obtain the calibration plot. The same procedure has been followed in the analysis of wine samples appropriately diluted and the resulting signal was referred to that of gallic acid.

All the values reported are the average of at least six measurements.

3. Results and discussion

3.1. Polyphenol index in wines using MWCNTs-laccases biosensor

To assess the effective catalytic properties of TvL-MWCNTs-SPE and ThL-MWCNTs-SPE biosensors toward gallic acid, CVs experiments were performed. A typical voltammetric behaviour of ThL-based biosensor (ThL-MWCNTs-SPE) in the presence of gallic acid solution (11.0 mg L^{-1}) was reported in Fig. 2.

The cyclic voltammogram of gallic acid is characteristic of an electrochemical irreversible reaction. In presence of immobilized enzyme onto the electrode an increase of the cathodic current was observed. At same time the anodic peak disappeared in accord with a catalytic electrochemical reaction [50]. An analogue result was obtained with TvL-MWCNTs-SPE biosensor.

In Fig. 3 is reported the effect on the amperometric response of the ThL loading onto the surface of the electrode by monitoring the signal obtained for a 4.2 mg L^{-1} gallic acid solution at −100 mV. On the basis of the results obtained we have used an amount of laccase corresponding to an activity of 0.8 U.

The TvL-MWCNTs-SPE and ThL-MWCNTs-SPE electrodes, previously described, were bioelectrochemical characterized in the presence of gallic acid as substrate (S_{ox}). In order to evaluate the applicability of the Michaelis–Menten approach to describe the kinetic behavior of our laccase-based biosensors, we have employed the Hill's equation [50]. This equation is often used in enzyme kinetics to describe the dependence of the steady-state rate of an enzymatic reaction on the substrate concentration:

$$v = \frac{V_{\max}([S]/[S]_{0.5})^h}{1 + ([S]/[S]_{0.5})^h}$$

At $h = 1$, Hill's equation is the classic Michaelis–Menten equation ($[S]_{0.5}$ corresponds to the Michaelis constant K_M).

The Hill coefficient (h) was experimentally obtained by fitting the log ($I/I_{max} - I$) vs. log [S_{ox}], the results obtained for the TvL-MWCNTs-SPE and ThL-MWCNTs-SPE electrodes were respectively 1.04 ± 0.01 and 0.96 ± 0.04. From these results we can assess that laccases immobilized onto the electrode surface follow the Michaelis–Menten equation.

The kinetic parameters are in relation through Eq. (2) and were obtained using the linearization of Lineweaver–Burk expressed by Eq. (3):

$$I_{\lim} = \frac{I_{\max}\,[S_{ox}]}{[S_{ox}] + K_M^{app}} \quad (2)$$

$$\frac{1}{I_{\lim}} = \frac{1}{I_{\max}} + \frac{K_M^{app}}{I_{\max}\,[S_{ox}]} \quad (3)$$

where [S_{ox}] is the concentration of the oxidized substrate, I_{lim} is the cathodic current, K_M^{app} is the apparent Michaelis–Menten constant for the enzymatic reaction and I_{max} is the steady-state current. The developed biosensors were employed to detect the polyphenol index in wines samples in flow condition. In Table 1 are summarized the main kinetic and analytical characteristics obtained by using gallic acid as substrate with the two laccase modified electrodes. Data reported in Table 1 show a similar affinity of TvL and ThL toward gallic acid [51] as well as sensitivity.

The two biosensors have been used for 6 measurements without a evident loss of their performances, the repeatability of measurements was of about 3–4%. The proposed biosensors were tested in a set of measurements with several Italian wine samples (six white and six red). In Fig. 4 is reported the amperometric behaviour obtained after the addition of gallic acid, white wine (sample 6), red

Table 1
Kinetic and analytical characteristics of gallic acid obtained by FA amperometry in 0.1 mol L^{-1} Britton–Robinson buffer, pH 5 at a fixed potential of −100 mV for TvL-MWCNTs-SPE and ThL-MWCNTs-SPE biosensors.

Laccase	K_M^{app} (mg L^{-1})	I_{max} (μA)	I_{max}/K_M^{app} (μA mg^{-1} L)	Slope (μA mg^{-1} L)	Linear range (mg L^{-1})	r^2	LOD (mg L^{-1})
TvL	138.0 ± 15.6	2.6 ± 0.2	0.019	0.009 ± 0.001	0.1–17.0	0.999	0.1
ThL	133.3 ± 17.0	0.8 ± 0.1	0.006	0.007 ± 0.001	0.1–18.0	0.999	0.3

Table 2
Electrochemical polyphenol index obtained with TvL-MWCNTs-SPE and ThL-MWCNTs-SPE biosensors and comparison with the value obtained using the Folin–Ciocalteau reference method.

Wines		Folin–Ciocalteau (mg L^{-1})	FA TvL (mg L^{-1})	Recovery TvL (%)	FA ThL (mg L^{-1})	Recovery ThL (%)
	Sample 1	242 ± 1	142 ± 2	59	261 ± 7	108
	Sample 2	199 ± 3	95 ± 9	48	197 ± 17	99
White	Sample 3	172 ± 4	72 ± 1	42	187 ± 1	109
Wines	Sample 4	189 ± 7	87 ± 1	46	185 ± 5	98
	Sample 5	273 ± 7	132 ± 9	48	267 ± 11	98
	Sample 6	199 ± 1	115 ± 3	42	185 ± 11	93
	Sample 7	2132 ± 103	1126 ± 36	53	2077 ± 87	97
	Sample 8	2327 ± 38	1355 ± 41	58	2141 ± 86	92
Red	Sample 9	1088 ± 18	609 ± 34	56	1129 ± 49	104
Wines	Sample 10	1326 ± 23	924 ± 54	70	1400 ± 25	106
	Sample 11	1614 ± 17	1001 ± 94	62	1585 ± 69	98
	Sample 12	1835 ± 15	1098 ± 94	60	1714 ± 40	93

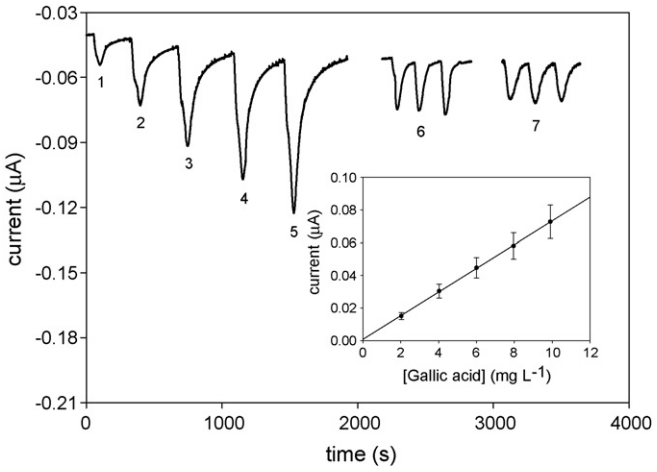


Fig. 4. Amperometric current response under FA conditions using ThL-MWCNTs-SPE biosensor of gallic acid 2.02 mg L^{-1} (1), 4.02 mg L^{-1} (2), 6.00 mg L^{-1} (3), 7.96 mg L^{-1} (4), 9.90 mg L^{-1} (5), three injections of white wine sample diluted 50 times (6), three injections of red wine sample diluted 500 times (7). Inset: calibration plot of gallic acid.

wine (sample 9) and the corresponding calibration plot constructed by using ThL-MWCNTs-SPE biosensors.

The overall time of analysis of a wine sample is of about 4 min. The results obtained in the wine analysis are reported in Table 2 compared to those obtained with the Folin–Ciocalteau reference method.

As it can be seen in Table 2 the values of polyphenol index obtained with TvL-MWCNTs-SPE are lower than those obtained with the Folin–Ciocalteau method of about the 41–58% for the white wines and of the 38–47% for the red ones. On the contrary the values obtained with ThL-MWCNTs-SPE biosensor are closer the reference method; accuracy of measurement is lower than 8% both for the white and red wines.

3.2. Polyphenol index in wines using SWCNTs-laccase biosensor

The measurements using SWCNTs biosensors were carried out in the same experimental conditions used with MWCNTs ones. The catalytic activity of ThL toward gallic acid is similar to those reported for TvL. The sensitivity of ThL-SWCNTs-SPE decreases almost of the 50% (0.004 ± 0.001 μA mg^{-1} L), as expected for the lower electroactive area, otherwise LOD increases (0.6 mg L^{-1}). Preliminary results on wine samples evidenced that ThL-SWCNTs-SPE biosensor gives similar values to those obtained using MWCNTs; consequently the remaining wines were tested only with ThL-SWCNTs-SPE biosensor. The obtained results are reported in Table 3 and in this case the values of polyphenol index are lower than those obtained with the Folin–Ciocalteau method of the 4–11% for the white wines and of the 4–8% for the red ones. These results are very interesting because they are all lower than those obtained with the reference method that generally leads to an overestimation of the real content of polyphenols in wines.

3.3. Interferences

Neither sulphur dioxide (coming from the reaction of $Na_2S_2O_5$ with wine organic acids) or reducing sugars do not interfere with flow measurements. The first is simply eliminated by nitrogen bubbling, as it is known in wine analysis [52], and the last ones cause are not an electroactive species.

In order to evaluate ascorbic acid interference, a proper amount (150 mg L^{-1}, Italian maximum limit for the addition of ascorbic acid in wines [53]) was added to sample wines. Its interference, for biosensors based on MWCNTs, ranging 8–10% for white wines and from 5 to 7% for red ones. While biosensors based on SWCNTs are

Table 3
Electrochemical polyphenol index obtained with ThL-SWCNTs-SPE biosensors and comparison with the value obtained using the Folin–Ciocalteau reference method.

Wines		Folin–Ciocalteau (mg L^{-1})	FA ThL (mg L^{-1})	Recovery ThL (%)
	Sample 1	242 ± 1	221 ± 14	92
	Sample 2	199 ± 3	177 ± 5	89
White	Sample 3	172 ± 4	163 ± 1	95
Wines	Sample 4	198 ± 7	190 ± 2	96
	Sample 5	273 ± 7	258 ± 2	95
	Sample 6	199 ± 1	187 ± 2	94
	Sample 7	2132 ± 103	2005 ± 38	94
	Sample 8	2327 ± 38	2219 ± 46	95
Red	Sample 9	1088 ± 18	1006 ± 31	92
Wines	Sample 10	1326 ± 23	1250 ± 44	94
	Sample 11	1614 ± 17	1518 ± 48	94
	Sample 12	1835 ± 15	1757 ± 76	96

Table 4
Amperometric biosensors based on different enzymes for determination of polyphenol index in wines.

Electrode	Immobilization	Enzyme	Sample	E (V)	Recovery (%)	Life time	Refs.
MWCNTs/SWCNTs	PAP	Laccase (ThL)	Wine	−0.1 V vs. Ag/AgCl	89–96[a,b]	10 days	
Au	SAM of MPA	HRP	Red wine	0.0 V vs. Ag/AgCl	66–83[c,d]	–	[54]
CPE modified with Ru	Entrapment in the CPE	Tyrosinase	Wine	−0.10 V vs. Ag/AgCl	2–6[e,d]	3–4 h	[17]
GCE	Cross-linking with glutaraldehyde	Laccase (TvL)	Wine	−0.20 V vs. Ag/AgCl	3–5[e,d]; 4–7[e,b]; 21–61[a,d]; 43–94[a,b]	5 days	[37]
Pt coupled with a transducer for oxygen	Kappa-carrageenan gel	Tyrosinase	Wine	−0.65 V vs. Ag/AgCl	83–122[e,d]	–	[55]
Graphite SPE modified with ferrocene	Different immobilizations	Laccase (TvL)-Tyrosinase	Wine	0.05 V vs. Ag/AgCl	~17[a,b,f]	5 days	[56]

[a] Gallic acid.
[b] In flow injection conditions.
[c] (+)-catechin.
[d] In batch conditions.
[e] Caffeic acid.
[f] Extimated from Fig. 6.

not affected. At the same time, these compounds were analyzed by means of colorimetric method and values obtained are similar. Nevertheless, spectrophotometric data are smaller than those reported in literature [38,39]. This difference could be ascribed to the fact that Folin–Ciocalteau is an equilibration method. In the classical procedure, the absorbance is read 1 or 2 h after the preparation of the solutions, while in our study is carried out 30 min after (due to a modification of the Folin–Ciocalteau reagent apported by the supplier), so leading to smaller values. In order to evaluate the matrix effect in the electrochemical measurements in wine samples, a set of experiments has been performed with an electrode PAP-MWCNTs-SPE. The results obtained put in evidence the absence of any interferences in the electrochemical measurements of wine samples, this is due to the low potential value (−100 mV) applied in the amperometric measurements. As reported above the only matrix effect is ascribed to the interaction of laccase with ascorbic acid and this value is quite comparable with that obtained with the Folin–Ciocalteau reference method.

3.4. Influence of impurities on biosensor performance

In order to explain the different results obtained with TvL and ThL-based biosensors, probably due to impurities, a further experiment was carried out. To investigate their presence in commercial enzyme powder, a certain amount of a inert protein (BSA) was added to ThL-based biosensor. Then it was compared to TvL one, containing the same protein concentration, in absence of BSA.

Polyphenol index determined by BSA-ThL-based biosensor is similar to that obtained by TvL-MWCNTs-SPE and lower than that obtained by the reference method. Data demonstrated that impurities are present in commercial laccase (TvL) creating an obstacle for analytes diffusion towards electrode surface.

Trametes hirsuta, with a high level of purity, is more disposed for polyphenols determination in wines.

3.5. Comparison of Laccase biosensors performances with those obtained for other biosensor systems in the analysis of polyphenol index in wine samples

In Table 4 the main characteristics of our laccase-based biosensors were compared with those reported in analogous papers previously published. In particular, were considered the following features: electrode, immobilization method, enzyme, applied potential, recovery and life time.

Taking into account the recovery our data are in the range 92–109% for the system ThL-MWCNTs-SPE and 89–96% for ThL-SWCNTs-SPE, while values obtained by the other authors citated in Table 4 are respect to ours. Considering the life time the reported values do not exceed 5 days, while the developed biosensors in this work performed for a period longer than 10 days. From this table arise that the biosensors presented in our paper show some peculiar properties towards the others ones in terms of immobilization procedure, recovery, lifetime and the accuracy of the measurements is comparable with those obtained with the reference spectrophotometric method.

4. Conclusions

In conclusion Laccases biosensors are suitable devices for the determination of polyphenol index in wines. In our study we have demonstrate that biosensor performance depends on enzyme adopted. ThL-based biosensor is to prefer to TvL one.

In particular, values obtained by using *Trametes hirsuta* are close to those determined by Folin–Ciocalteau method, on the contrary polyphenol index measured with *Trametes versicolor* is discordant to reference assay.

The influence of the interferences on both spectrophotometric and electrochemical measurements have been carefully evaluated, demonstrating that they do not affect the analytical data.

So, ThL-based biosensor exhibits a good analytical performance due to its stability and reproducibility associated with a simple, rapid preparation, reliability and low cost. Otherwise it represents a good and easy method for monitoring polyphenols in real samples.

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