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Electrochemical Estimation of the Polyphenol Index in Wines Using a Laccase Biosensor

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The use of a laccase biosensor, under both batch and flow injection (FI) conditions, for a rapid and reliable amperometric estimation of the total content of polyphenolic compounds in wines is reported. The enzyme was immobilized by cross-linking with glutaraldehyde onto a glassy carbon electrode. Caffeic acid and gallic acid were selected as standard compounds to carry out such estimation. Experimental variables such as the enzyme loading, the applied potential, and the pH value were optimized, and different aspects regarding the operational stability of the laccase biosensor were evaluated. Using batch amperometry at −200 mV, the detection limits obtained were 2.6×10^{-3} and 7.2×10^{-4} mg L^{-1} gallic acid and caffeic acid, respectively, which compares advantageously with previous biosensor designs. An extremely simple sample treatment consisting only of an appropriate dilution of wine sample with the supporting electrolyte solution (0.1 mol L^{-1} citrate buffer of pH 5.0) was needed for the amperometric analysis of red, rosé, and white wines. Good correlations were found when the polyphenol indices obtained with the biosensor (in both the batch and FI modes) for different wine samples were plotted versus the results achieved with the classic Folin−Ciocalteu method. Application of the calibration transfer chemometric model (multiplicative fitting) allowed that the confidence intervals (for a significance level of 0.05) for the slope and intercept values of the amperometric index versus Folin−Ciocalteu index plots ($r = 0.997$) included the unit and zero values, respectively. This indicates that the laccase biosensor can be successfully used for the estimation of the polyphenol index in wines when compared with the Folin−Ciocalteu reference method.

KEYWORDS: Polyphenol index; laccase; amperometric biosensor; wines

INTRODUCTION

Polyphenolics are a broad class of compounds that are present in many fruits and vegetables and their products, including grapes and wines (*1*). In recent years, numerous studies have associated the consumption of foods rich in polyphenols with the prevention of cardiovascular diseases, certain types of cancer, and other diseases related to aging as a consequence of their antioxidant properties (*2*).

Polyphenols are present in grapes (skin and seeds) and, consequently, in wines, but they can be also produced by yeast metabolism and extracted from the oak barrels in which the wine is stored (*3*). The polyphenol content contributes substantially to the quality of wines and affects their color, flavor, stability, and aging behavior (*4*). Furthermore, the determination of this group of compounds can help to identify variations on wine types and differences in winemaking and maturation processes (*3*).

The determination of the polyphenol content is not an easy task because of their chemical complexity and difficulty of extraction and the presence of interferences in samples. The term total phenolics refers to the total phenolic content obtained by spectrophotometric methods, especially the so-called Folin−Ciocalteu method, which are based on the reaction of phenolics with a colorimetric reagent, thus allowing their measurement in the visible region of the spectra (*5*). However, this spectrophotometric approach yields an overestimation of the total polyphenolic content (*6*).

Biosensors have been proposed as efficient analytical tools for the detection of polyphenol compounds, exhibiting advantages such as minimal sample preparation, selectivity, sensitivity, reproducibility, relatively low cost, rapid time of response, and simple use for continuous on-site analysis (*7*, *8*).

Although tyrosinase, peroxidase, and laccase biosensors can be used for this purpose (*8*−*10*), we think that the latter enzyme is the most appropriate for use in analytical systems for the determination of polyphenolic compounds. This enzyme catalyzes the oxidation of polyphenols by molecular oxygen according to the reaction

$$AH_2 + {}^1/_2O_2 \xrightarrow{\text{laccase}} A + H_2O$$

where AH_2 and A are reduced and oxidized states of phenol,

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respectively (*11*). Laccase can catalyze the oxidation of *o*-, *m*-, and *p*-benzenediols and phenol to *o*-, *m*-, *p*-quinones or radical species (*12*) and does not require hydrogen peroxide as a cosubstrate or any cofactors for the catalytic reaction. The product of the enzyme oxidation is subsequently reduced at the electrode working at the appropriate potentials. The catalytic cycle of laccase and the proposed mechanisms for the reduction and reoxidation of the copper sites can be found in the paper of Durán et al. (*13*).

Immobilization of laccase to construct electrochemical biosensors has been carried out on different electrode materials: carbon paste (*14*), graphite (*15*, *16*), self-assembled monolayer on gold (*17*), glassy carbon (*18*), Pt (*19*−*21*), screen printed (*22*), and carbon fiber (*12*, *23*).

In this work, we report the preparation and performance, under both batch and flow injection (FI) conditions, of a laccase biosensor in which the enzyme was immobilized by cross-linking with glutaraldehyde onto a glassy carbon electrode (GCE). The bioelectrode allowed the obtention of fast, stable, and sensitive electroanalytical responses to various polyphenolic compounds and was employed for the amperometric estimation of the total content of polyphenolic compounds in different types of wines.

EXPERIMENTAL PROCEDURES

Apparatus and Electrodes. Amperometric measurements were performed on a Metrohm 641VA potentiostat connected to a Linseis L6521B recorder. A P-Selecta ultrasonic bath and a P-Selecta Agimatic magnetic stirrer were also used. FI experiments were carried out using a Gilson Minipuls-2 peristaltic pump and a Rheodyne model 5020 injection valve with variable injection volumes.

A Metrohm 6.0905.010 glassy carbon electrode (3-mm ∅) was used as the electrode substrate to be modified with the enzyme. A BAS MF-2063 Ag|AgCl| KCl 3 mol L^{-1} reference electrode and a Pt wire counter electrode were also employed. A 10-mL electrochemical cell was used for batch experiments, whereas a large volume (50 mL) homemade glass wall-jet cell was employed for FI measurements.

The Folin−Ciocalteu method was applied using a Cary Varian Cary-3 Bio UV−visible spectrophotometer.

Reagents and Solutions. Stock solutions (10000 mg L^{-1}) of gallic acid and caffeic acid (Sigma) were prepared daily in 0.1 mol L^{-1} citrate buffer of pH 5.0 with the help of ultrasonic stirring. More dilute standards were prepared by suitable dilution with the same citrate buffer solution, which was also used as the supporting electrolyte in both batch and FI measurements.

The solutions used for the enzyme immobilization were a 1.18 unit μL^{-1} laccase (Fluka, EC 1.10.3.2 type II, from *Trametes versicolor*, 23.7 unit mg^{-1} solid) solution, prepared daily in the above-mentioned citrate buffer solution, and a 25% glutaraldehyde (Aldrich) solution.

A 20% (w/v) Na_2CO_3 (Aldrich) solution prepared in deionized water, as well as 2.0 N Folin−Ciocalteu reagent (Sigma) were used for application of the Folin−Ciocalteu method.

All chemicals used were of analytical reagent grade, and water was obtained from a Millipore Milli-Q purification system.

Procedures. Before immobilization of the enzyme, the glassy carbon electrode was polished with 3-μm alumina powder (Metrohm 6.2802.000) for 1 min, and then it was sonicated in deionized water for 1 min and dried with an argon stream. The enzyme cross-linking immobilization procedure consisted of the deposition of 6 μL of a 1.18 unit μL^{-1} laccase solution on the GCE surface. Once the electrode surface had dried at ambient temperature, the electrode was immersed in a 25% glutaraldehyde solution for 1 h at ambient temperature.

Amperometric measurements were performed by applying in all cases a potential of −200 mV (vs Ag/AgCl). The carrier stream for FI experiments was a 0.1 mol L^{-1} citrate buffer of pH 5.0, with a flow rate of 0.3 mL min^{-1} and a sample injection volume of 150 μL.

Estimation of the Polyphenol Index in Wines. The polyphenol index was estimated in different wine samples. The only sample treatment required in all cases consisted of an appropriate dilution with the supporting electrolyte solution. The dilution factors employed, considering the polyphenol content in each type of wine, are summarized in **Table 1**.

Table 1. Dilution Factors Applied for the Estimation of the Polyphenol Index in Wines Using an Amperometric Laccase Biosensor

	batch		FI	
	gallic acid	caffeic acid	gallic acid	caffeic acid
white wines	1000–4000	200	200	200
rosé wines	5000	500–1000	1000	1000–2000
red wines	20000–50000	1000	2000–3000	2000

Estimation of the polyphenol content in wines was carried out by interpolation of the corresponding amperometric signals into calibration plots constructed with gallic acid stock solutions in the 0.02−0.1 and 0.2−1.0 mg L^{-1} concentration ranges for batch and FI analysis, respectively, whereas for caffeic acid a 0.02−0.1 mg L^{-1} concentration range was used for both methodologies.

As an example, the procedure for the estimation of the polyphenol index in the white wine 'Berberana' (see **Table 5**) by batch amperometry was that 500 μL of wine was diluted to 10.0 mL with 0.1 mol L^{-1} citrate buffer of pH 5.0. The biosensor was immersed into the electrochemical cell containing 10.0 mL of the citrate buffer, and, under continuous magnetic stirring, a potential of −200 mV was applied. When a stable baseline was reached, a 100-μL aliquot of the wine-diluted solution was transferred into the electrochemical cell, and the current was measured until the steady-state current was reached. This current was interpolated into the calibration plot constructed previously with gallic or caffeic acid stock solutions.

For comparison purposes, wines were also analyzed by the spectrophotometric method involving the use of the Folin−Ciocalteau reagent (*24*). One hundred microliters of sample (in the case of red wines a 1:10 dilution was carried out), 5.0 mL of deionized water, 0.5 mL of Folin−Ciocalteau reagent (phosphotungstic−phosphomolybdic acid), and 2.0 mL of a 20% sodium carbonate solution were added in this order to a 10-mL volumetric flask and diluted to the mark with deionized water. The resulting solution was stirred for about 1 min for homogenization and was allowed to stand for 30 min at ambient temperature in darkness. The absorbance was then read at 750 nm. The total polyphenol content was estimated from the absorbance value by interpolation into calibration plots constructed for gallic or caffeic acid.

RESULTS AND DISCUSSION

Both caffeic acid and gallic acid were selected as standard compounds for the estimation of the polyphenol content in wines. In fact, gallic acid has been used to express the results obtained by application of the Folin−Ciocalteu method (*1*, *25*). Furthermore, caffeic acid exhibits a considerably higher relative sensitivity than gallic acid at laccase biosensors (*8*), as will be shown below, and it has been also used to express the polyphenol content in wines (*9*). Therefore, optimization of the working variables involved in the performance of the laccase biosensor was carried out using these compounds as enzyme substrates.

Batch Amperometry in Stirred Solutions. First, the enzyme loading was optimized by taking as the criterion of selection the highest amperometric signal obtained for 0.1 mg L^{-1} gallic acid. **Figure 1** shows that the amperometric response obtained at 0.00 V increased with the enzyme loading up to 7.11 units of laccase, after which the current value leveled off. Consequently, this enzyme loading was selected for further studies.

The influence of the potential applied to the biosensor for the caffeic acid and gallic acid amperometric responses was evaluated over the −0.30 to +0.20 V range. As can be seen in **Figure 2**, the applied potential has a rather similar behavior

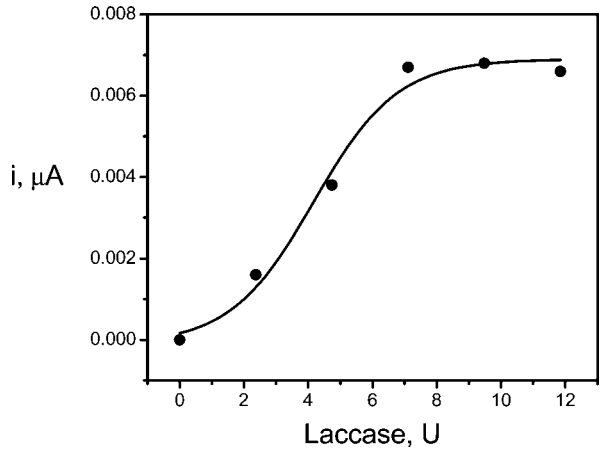

Figure 1. Effect of laccase loading immobilized on a GCE on amperometric signal obtained for 0.1 mg L^{-1} gallic acid. Supporting electrolyte was 0.1 mol L^{-1} citrate buffer (pH 5.0). E_{app} = 0.00 V.

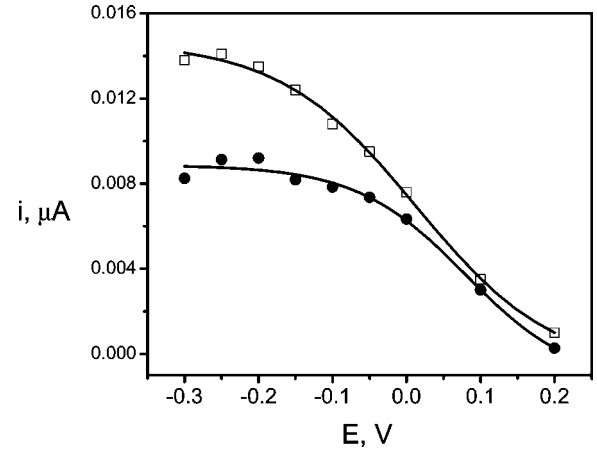

Figure 2. Effect of applied potential on amperometric response from 0.1 mg L^{-1} gallic acid (●) and 0.02 mg L^{-1} caffeic acid (□) at a laccase biosensor. Supporting electrolyte was 0.1 mol L^{-1} citrate buffer (pH 5.0).

for both compounds, the current increasing as the applied potential was moved toward more negative values. A working potential of −200 mV was chosen for further work to accomplish a sensitive detection of these compounds and also to minimize the effect of potential interferents able to be reduced at the electrode. It should be noted that no amperometric signal was observed in the whole potential range at the unmodified GCE for both gallic acid and caffeic acid.

Finally, the influence of pH on the amperometric response for a gallic acid concentration of 0.05 mg L^{-1} was evaluated over the pH 3.5−8.0 range. Higher peak current values were obtained between pH 4.5 and 6.0, with a maximum at pH 5.0, which is similar to that reported for other laccase-modified electrodes (*12*, *15*, *23*, *26*). Moreover, the optimum pH range is also similar to those observed for the soluble and purified laccase (*27*), indicating that the immobilization procedure did not affect the enzyme charge. According to this, a 0.1 mol L^{-1} citrate buffer solution of pH 5.0 was chosen for further work, which is also an appropriate pH value for the determination of polyphenols because, under these conditions, they do not suffer spontaneous oxidation (*11*). Under these conditions, the time needed to get a stable baseline was ≈100 s, and the time to reach the steady-state current after the injection of the analyte into the buffer solution was 30 s.

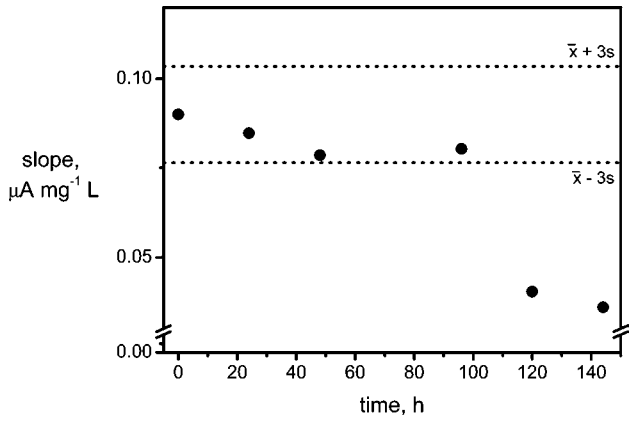

Figure 3. Control chart constructed for a single laccase biosensor. Measurements correspond to the mean values of the slopes of three successive calibration graphs for gallic acid in the 0.02–0.1 mg L^{-1} concentration range. Supporting electrolyte was 0.1 mol L^{-1} citrate buffer (pH 5.0). E_{app} = –0.20 V.

Stability of the Laccase Biosensor. Different aspects regarding the operational stability of the laccase biosensor were evaluated.

First, the repeatability of the amperometric measurements was tested by constructing 10 successive calibration plots for both caffeic acid and gallic acid with the same biosensor (in the 0.02−0.1 mg L^{-1} concentration range for gallic acid and in the 0.002−0.01 mg L^{-1} range for caffeic acid). Relative standard deviation (RSD) values of 5.0 and 2.8% were obtained for the slopes of the corresponding calibration graphs for gallic and caffeic acid, respectively, indicating a good repeatability of the measurements with no need to apply a cleaning or regeneration procedure to the laccase biosensor.

Furthermore, RSD values of 5.2 and 8.1% were obtained for the steady-state current corresponding to 20 repetitive measurements of 0.1 mg L^{-1} gallic acid and 0.02 mg L^{-1} caffeic acid, respectively.

The reproducibility of the responses obtained with different biosensors is another important aspect to be evaluated in order to characterize the performance of the laccase bioelectrode. Results from seven different electrodes yielded RSD values of 7.8 and 9.3% for the current values measured from 0.1 mg L^{-1} gallic acid and 0.02 mg L^{-1} caffeic acid, respectively, which demonstrated that the construction procedure of the laccase biosensor was reliable, thus allowing reproducible amperometric responses to be obtained with different biosensors constructed in the same manner.

The useful lifetime of a single laccase biosensor was evaluated by performing repetitive calibration graphs for gallic acid and caffeic acid in the 0.02−0.1 mg L^{-1} concentration range. After use, the biosensor was stored in citrate buffer of pH 5.0 at 4 °C. **Figure 3** shows the control chart constructed for gallic acid, taking the mean value of the slopes of 10 successive calibration graphs obtained the first day of this study as the central value. The upper and lower control limits were set at ±3 × SD of this target value. From the second day, the mean values of the slopes of three successive calibration graphs are plotted. As can be seen, the slope mean values remained within the control limits for ≈100 h (4 days). After 5 days, the biosensor yielded 50% of the original response, which can be attributed to the denaturation of the immobilized enzyme. Similar results were obtained, as expected, when caffeic acid was used as the analyte.

The last aspect checked concerning the stability of the laccase biosensor was the effect of the biosensor storage. After 15 days

Table 2. Kinetic Parameters of the Enzyme Reactions at the Laccase Biosensor

polyphenolic compound	x	V_{max},[a] μA	K_M^{app},[a] mg L^{-1}	V_{max}/K_M^{app}, μA mg^{-1} L
gallic acid	1.01 ± 0.01	1.2 ± 0.1	9.8 ± 0.1	0.125
caffeic acid	1.05 ± 0.03	1.6 ± 0.2	8.2 ± 0.1	0.193

[a] Confidence intervals were calculated for a significance level of 0.05 ($n = 3$).

Table 3. Analytical Characteristics of the Calibration Plots for Gallic Acid and Caffeic Acid Obtained by Batch Amperometry in Stirred Solutions at a Laccase Biosensor

compound	linear range, mg L^{-1}	slope, μA mg^{-1} L	r	LOD, mg L^{-1}
gallic acid	0.003–0.80	0.092 ± 0.003	0.998	2.6×10^{-3}
caffeic acid	$(0.07–10) \times 10^{-2}$	0.60 ± 0.02	0.998	7.2×10^{-4}

of its construction without use, the slope values obtained for gallic acid were included inside the control limits shown in **Figure 3**, thus indicating that no denaturation of the enzyme occurred during such storage period.

Kinetic Constants and Analytical Characteristics. The kinetic parameters of the laccase reactions with gallic acid and caffeic acid at the laccase biosensor were calculated by assuming that they obeyed a Michaelis−Menten-type kinetics, as was corroborated by the parameter x obtained from the Hill plots (log [(i_{max}/i) − 1] versus log [gallic acid or caffeic acid]). As can be seen in **Table 2**, this parameter was very close to 1 in both cases, indicating that the immobilization procedure did not alter the Michaelis−Menten behavior. Consequently, the apparent Michaelis−Menten constants (K_M^{app}) and the maximum rate of the reactions were calculated from the corresponding Lineweaver−Burk plots. As can be seen in **Table 2**, a slightly lower K_M^{app} was obtained for caffeic acid, indicating a higher affinity for the enzyme of this compound.

Under the optimized working conditions, typical calibration curves of enzyme systems were obtained for the two compounds tested. **Table 3** summarizes the analytical characteristics of these calibration graphs. The limits of detection were calculated according to the $3s_b/m$ criterion, where m is the slope of the linear portion of the calibration plot and s_b was estimated as the standard deviation ($n = 10$) of the amperometric signals from different solutions of the substrate at a concentration level of 0.02 mg L^{-1} for gallic acid or of 5.0×10^{-4} mg L^{-1} for caffeic acid. As expected, the sensitivity achieved for caffeic acid is remarkably larger than that for gallic acid, as a consequence of the differences in the quinone products formed at the electrode surface after enzyme oxidation. Therefore, quinone products of the oxidation of catechol-containing polyphenols (such as caffeic acid) are readily reduced at glassy carbon electrodes, whereas the oxidation product of gallic acid is not (*28*).

To evaluate the possible interference from ascorbic acid and glucose on the biosensor response to gallic and caffeic acids, injections of 100 μL of 8.6×10^{-4} mol L^{-1} ascorbic acid and 0.01 mol L^{-1} glucose solutions were made into the electrochemical cell. The final concentrations of the compounds in the cell were higher than those usually found for white wines. At the applied potential of −200 mV, no significant amperometric responses were obtained for ascorbic acid and glucose, thus indicating the absence of interference for the estimation of the polyphenol index in wines.

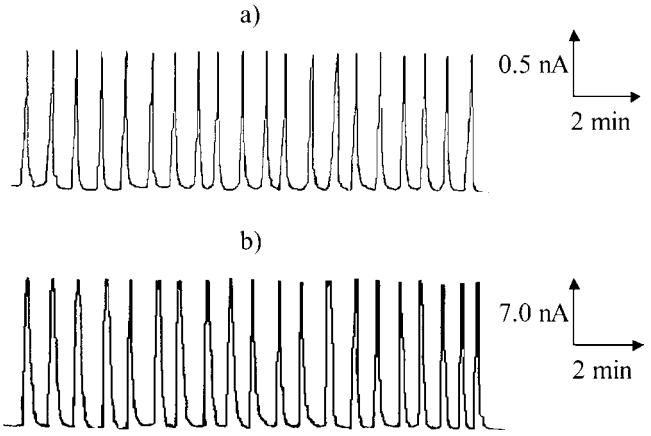

Figure 4. FI amperometric responses obtained at the laccase biosensor for 20 repetitive injections of 0.5 mg L^{-1} gallic acid (**a**) and 0.05 mg L^{-1} caffeic acid (**b**). Carrier solution was 0.1 mol L^{-1} citrate buffer of pH 5.0. Flow rate = 0.3 mL min^{-1}. $V_i = 150$ μL. $E_{app} = -0.20$ V.

Flow Injection with Amperometric Detection. The possibility of using the laccase biosensor under FI conditions in connection with amperometric detection was also evaluated. Both the carrier solution (0.1 mol L^{-1} citrate buffer of pH 5.0) and the applied potential (−0.20 V) were the same as those employed in batch experiments.

FI characteristic parameters such as the flow rate and the sample volume injected were optimized. As is usual for an enzyme-based flow injection assay (*29*), the FI peak height decreased as the flow rate increased, which was attributed to the need for slow passage of the sample plug to the enzyme reaction that takes place in a high extent. Furthermore, as expected, the peak width increased as the flow rate decreased. As a compromise between sensitivity and sampling frequency, a flow rate of 0.3 mL min^{-1} was selected. On the other hand, taking into account the $i_p/W_{1/2}$ ratio, where $W_{1/2}$ is the peak width at half-height, a sample volume of 150 μL was chosen as the injection volume.

The repeatability of the FI amperometric measurements was evaluated by constructing 10 successive calibration plots for gallic acid and caffeic acid in the 0.2−1.0 and 0.02−0.10 mg L^{-1} concentration ranges, respectively. The RSD values obtained, 7.3 and 9.5%, respectively, showed an acceptable repeatability in both cases. **Figure 4** shows the signals from 20 repetitive injections of 0.5 mg L^{-1} gallic acid and 0.05 mg L^{-1} caffeic acid, with RSDs for i_p of 1.7 and 2.9%, respectively, which demonstrated a good stability of the immobilized enzyme in spite of the hydrodynamic conditions.

Under the selected conditions, a linear calibration graph was obtained for gallic acid over the 0.04−2.0 mg L^{-1} concentration range [$r = 0.999$, slope = $(1.85 \pm 0.06) \times 10^{-2}$ μA mg^{-1} L, intercept = $(0.12 \pm 0.08) \times 10^{-2}$ μA] and for caffeic acid over the 0.001−0.100 mg L^{-1} concentration range [$r = 0.998$, slope = $(27 \pm 2) \times 10^{-2}$ μA mg^{-1} L, intercept = $(0.13 \pm 0.05) \times 10^{-2}$ μA]. The limits of detection (LODs), calculated according to the same criterion mentioned above, were 0.04 and 0.001 mg L^{-1}, respectively.

Comparison of Laccase Biosensor Performance with That of Other Biosensor Designs. A comparison of the analytical performance of the laccase biosensor for gallic acid and caffeic acid with data reported in the literature for other laccase electrochemical biosensors is summarized in **Table 4**. Characteristics such as the type of electrode and immobilization method, detection potential, range of linearity, LOD achieved, and useful lifetime are considered.

Table 4. Amperometric Biosensors Based on Laccase and Tyrosinase for the Determination of Polyphenolic Compounds

electrode	immobilization	enzyme	sample	*E*, V	analytical characteristics	useful time	ref
Pt	polyethersulfone membrane	laccase		catechol: −0.20 V catequina: +0.10 V caffeic: −0.05 V vs Ag/AgCl	caffeic acid: LR, $(1.0–8.0) \times 10^{-5}$ mol L^{-1} slope, 24.01 μA $mmol^{-1}$ L	38% of the initial activity was maintained after 15 days of use	*31*
graphite	adsorption	laccase		−50 mV vs Ag/AgCl	caffeic acid: LR, $(1.0–10.0) \times 10^{-6}$ mol L^{-1} LOD, 0.56 μmol L^{-1} slope, 57.92 nA μmol^{-1} L		*30*
Pt–Ag, AgCl	polyethersulfone membrane	laccase	red wine (previous extraction in solid phase)	+0.10 V vs Ag/AgCl	mixture of catechin and caffeic acid: LR, $(2.0–14.0) \times 10^{-6}$ mol L^{-1} LOD, 1.0×10^{-6} mol L^{-1}		*8*
graphite	adsorption	laccase		−0.05 V vs Ag/AgCl	caffeic acid: LR, $(1.0–10.0) \times 10^{-6}$ mol L^{-1} LOD, 0.56 μmol L^{-1} slope, 57.92 nA μmol^{-1} L		*16*
gold	covalent immobilization by carbodiimide/succinimide on a self-assembled monolayer of 3-mercaptopropionic acid	laccase	olive oil mill wastewater	−0.20 V vs SCE	relative biosensor response with respect to 1,4-hydroquinone gallic acid, 50 caffeic acid, 328	half-time of 35 days	*17*
CPE modified with Ru	entrapment in the CPE	tyrosinase	wine	−0.10 V vs Ag/AgCl	gallic acid: LR, (1.0–60.0 mg L^{-1} LOD, 0.1 mg L^{-1}	3–4 h	*1*
GCE modified with gold nanoparticles	cross-linking with glutar-aldehyde	tyrosinase	wine	−0.10 V vs Ag/AgCl	caffeic acid: LR, $(0.02–2.0) \times 10^{-4}$ mg L^{-1} LOD, 6.6×10^{-7} mol L^{-1} gallic acid: LR, $(0.25–9.0) \times 10^{-4}$ mg L^{-1} LOD, 70×10^{-7} mol L^{-1}	18 days	*9*

As can be seen, the sensitivity achieved for caffeic acid in the batch mode (108.1 nA μmol^{-1} L) is better than that obtained with the other laccase biosensors. The low detection limit for caffeic acid (3.9×10^{-9} mol L^{-1}) achieved with our biosensor design is ≈25-fold lower than the best one reported previously (1.0×10^{-7} mol L^{-1}; *30*).

With regard to other analytical characteristics, a range of linearity covering 2.5 orders of magnitude for caffeic acid was achieved, which is wider than those reported for this compound using other biosensors. Although the lack of data in the literature concerning characteristics such as repeatability of the measurements and reproducibility of these measurements with different electrodes avoids a wide comparison, it can be clearly deduced that the Lac-GCE compares advantageously with respect to the other biosensor designs. Finally, with regard to the useful lifetime, this can be considered as acceptable, mainly taking into account the simplicity of the biosensor construction.

Due to the scarcity of literature data for the determination of gallic acid using laccase biosensors (only ref *17* is found), we have included in **Table 4**, for comparison purposes, the literature antecedents concerning the determination of this compound with tyrosinase biosensors. As can be seen, the laccase biosensor also compares advantageously against tyrosinase biosensors in terms of linear range and LOD for gallic acid.

Estimation of the Polyphenol Index in Wines. The developed biosensor was used for the electrochemical estimation of the total polyphenol content in several types of wine. The occurrence of a matrix effect for the amperometric measurements in the diluted wine samples (see Experimental Procedures) was discarded after the statistical comparison (by applying Student's *t* test) of the slope values of the calibration graphs obtained by application of the standard additions method for gallic acid 0.08 ± 0.02 μA mg^{-1} L and caffeic acid 0.59 ± 0.02, given in **Table 3**. This nonexistence of a matrix effect supports the statement that the quinone products formed at the electrode surface react quickly, thus preventing their possible reaction with other wine components. Therefore, interpolation of the corresponding amperometric signals into the calibration plots constructed with gallic acid and caffeic acid stock solutions was used for the analysis of the wine samples (see Experimental Procedures). These were commercial wine as well as wine samples kindly supplied by different wine cellars (denoted by numbers).

In a first step, and following the extremely simple experimental procedure described under Estimation of the Polyphenol Index in Wines using gallic acid as standard compounds, six differents wines (two each of white, rosé, and red types) were analyzed. Three replicates were made for each sample both by batch amperometry and by FI with amperometric detection. The confidence intervals were calculated for a significance level of 0.05, and the RSD values were in all cases <6%. Results are summarized in **Table 5**. It should be remarked that the total time for the analysis of a wine sample is not longer than 2 min.

As expected, red wines have a higher phenolic content than rosé wines, and these have a higher phenolic content than white wines. As can be seen, the values of the polyphenol content obtained by batch amperometry are, in all cases, lower than those obtained by FI with amperometric detection. This behavior can be attributed to the fact that the FI method is a kinetic method at a fixed time and, therefore, the reaction kinetics of the different polyphenols contained in the wine sample affects largely the amperometric signal measured. However, batch measurements are carried out when the steady-state current is reached.

Table 5. Electrochemical Polyphenol Index, Expressed as Milligrams per Liter of Gallic Acid, Obtained by Using the Laccase Biosensor, and Comparison with the Results Obtained by Application of the Folin–Ciocalteu Method

		biosensor, mg L^{-1}		Folin–Ciocalteu, mg L^{-1} ($n = 1$)
sample		batch ($n = 3$)	FIA ($n = 3$)	
white wines	Berberana	103 ± 13	106 ± 8	244
	3108	38 ± 4	83 ± 7	181
rosé wines	18311	369 ± 40	572 ± 38	608
	1805	194 ± 21	383 ± 44	435
red wines	1302	1285 ± 130	2120 ± 30	2590
	1303	925 ± 20	1483 ± 67	1864

Table 6. Correlations between the Results Obtained with the Laccase Bioelectrode and Those Provided by Using the Folin–Ciocalteau Method

calibration plot	slope	intercept, mg L^{-1}	r
batch vs Folin–Ciocalteu	0.51 ± 0.08	–12 ± 83	0.996
FIA vs Folin–Ciocalteu	0.8 ± 0.2	–25 ± 56	0.997

The polyphenol content for these samples, expresed in milligrams per liter of gallic acid, was directly compared with the results obtained by applying the classic Folin−Ciocalteu method (*32*). The Folin−Ciocalteu reagent reacts with the phenol −OH group and produces a blue complex for which the absorbance is read at 750 nm. The polyphenol index is expressed also in this method as milligrams per liter of gallic acid (**Table 5**).

As expected, taking into account the completely different analytical methodologies used by both types of methods, the absolute values of the polyphenol indices obtained are significantly different. Actually, both the amperometric and the Folin−Ciocalteu methods suffer from a different sensitivity for each phenolic compound depending on their chemical structure, leading to the dependence of the content value of the standard polyphenolic compound (*10*). Moreover, sulfur dioxide, ascorbic acid, and glucose are known to interfere with the determination of polyphenols by the Folin−Ciocalteu method (*33*). In addition, the co-concurrence of sulfur dioxide and reducing sugars can result in synergistic effects (*5*). The interference of these compounds becomes obvious in samples with low polyphenol contents (*10*).

In spite of all these considerations, good correlations were found when the results obtained with the biosensor (in both the batch and FI modes) were plotted versus the results achieved with the Folin−Ciocalteu method for all of the wine samples given in **Table 5** (**Table 6**). As deduced from the regression equations, the confidence intervals for the intercept contain the zero value, which indicates the absence of constant systematic errors. However, as expected from the considerations discussed above, the confidence intervals for the slope values do not include the unit.

The possibility of using such a correlation between both compared methods as regression linear graphs was confirmed by applying the chemometric model of calibration transfer (multiplicative fitting) (*34*), which allows for rectifying drift and sensitivity changes and is based on the signal variation observed for a set of reference samples. This procedure has been used for signal correction in NIR spectroscopy (*35*) and mass spectrometry (*36*). The multiplicative fitting is $I_{\text{Folin−Ciocalteu}} = I_{\text{biosensor}} (\Sigma I_{\text{Folin−Ciocalteu}}/\Sigma I_{\text{biosensor}})$, where $I_{\text{Folin−Ciocalteu}}$ is the value expected by application of the Folin−Ciocalteu method,

Table 7. Electrochemical Polyphenol Index, Expressed as Milligrams per Liter of Caffeic Acid, Obtained by Using the Laccase Biosensor, and Comparison with the Results Obtained by Application of the Folin–Ciocalteu Method

sample		biosensor, mg L^{-1} batch ($n = 3$)	biosensor, mg L^{-1} FIA ($n = 3$)	Folin–Ciocalteu, mg L^{-1} ($n = 1$)
white wines	1816	14 ± 2	18 ± 3	272
	Berberana	8.3 ± 0.6	11.4 ± 0.7	273
rosé wines	18311	27 ± 5	38 ± 2	661
	18315	55 ± 4	108 ± 4	1681
red wines	1301	62 ± 3	125 ± 14	2245
	1303	81 ± 3	137 ± 8	2425

$I_{biosensor}$ is the value of the polyphenol index measured with the biosensor, and ($\Sigma I_{Folin-Ciocalteu}/\Sigma I_{biosensor}$) is the multiplicative factor that is applied to transform the data. The multiplicative factor values obtained were 0.492 for the batch measurements set and 0.802 for the FI measurements set. As can be observed, the values of the slopes of the regression equations and the multiplicative factors are similar, which indicates that the use of the correlation plot as a regression curve to transform the index values obtained with the biosensor into the expected values using the Folin−Ciocalteu method is appropriate.

This calibration transfer was applied to the analysis of 26 wines (19 different white wines, 3 different rosé wines, and 4 different red wines, in which those mentioned in **Table 5** are included) using batch amperometry and to the analysis of 20 wines (3 red, 2 rosé, and 15 white wines) by FI with amperometric detection. The correlations with the results obtained by the Folin−Ciocalteu method were as follows: (1) batch amperometry versus Folin−Ciocalteu plot, $r = 0.995$, slope $= 0.99 \pm 0.04$, intercept $= -19 \pm 19$ mg L^{-1}; (2) FI with amperometric detection versus Folin−Ciocalteu plot, $r = 0.996$, slope $= 1.03 \pm 0.04$, intercept $= -20 \pm 30$ mg L^{-1}.

As can be observed, the confidence intervals (for a significance level of 0.05) for the slope and intercept values, included in both cases the unit and the zero values, respectively, indicating that the method has not systematic errors and that the laccase biosensor can be successfully used for the estimation of the polyphenol index in wines, when compared with the Folin−Ciocalteu reference method.

The same methodology as described above was applied also using caffeic acid as standard phenolic compound, to which the total polyphenol content was referred. The results obtained by batch amperometry and FI with amperometric detection using the laccase biosensor and by application of the Folin−Ciocalteu method for six different wine samples are summarized in **Table 7**. The regression equations obtained from plotting the biosensor results versus those obtained using the Folin−Ciocalteu method were as follows: (1) batch amperometry versus Folin−Ciocalteu plot, $r = 0.986$, slope $= 0.03 \pm 0.01$, intercept $= (4 \pm 14)$ mg L^{-1}; (2) FI with amperometric detection versus Folin−Ciocalteau plot, $r = 0.995$, slope $= 0.06 \pm 0.01$, intercept $= 1 \pm 11$ mg L^{-1}.

As can be observed, these slope values are ≈10-fold lower than those obtained with gallic acid as standard phenolic compound (see **Table 6**), as a consequence of the much higher sensitivity of the laccase biosensor for caffeic acid, which is expressed by a considerably higher slope value of the calibration plot constructed with standard caffeic acid solutions (see Kinetic Constants and Analytical Characteristics). On the other hand, a good correlation was found for both plots.

In spite of the small slope values of the correlation equations, the application of the calibration transfer method (the multiplicative factors calculated were now 0.0327 for the batch measurement sets and 0.0579 for the FI measurements set) allowed the obtention of the following regression equations with the results obtained by the Folin−Ciocalteu method for the above-mentioned 26 (batch) and 20 (FI) different wines: (1) batch amperometry versus Folin−Ciocalteu plot, $r = 0.982$, slope $= 0.9 \pm 0.1$, intercept $= 3 \pm 3$ mg L^{-1}; (2) FI with amperometric detection versus Folin−Ciocalteu plot, $r = 0.985$, slope $= 0.95 \pm 0.08$, intercept $= 2 \pm 6$ mg L^{-1}.

Similarly to what occurred with gallic acid as standard compound, the confidence intervals for the slope and intercept values included in both cases the unit and the zero values, respectively. Again, this means that no systematic errors exist and the laccase biosensor can be used to estimate the polyphenol index in wines instead of the Folin−Ciocalteu method, using caffeic acid as the standard phenolic compound.

As a conclusion, it can be said that the laccase biosensor constructed by immobilization of laccase on a glassy carbon electrode exhibits a good analytical performance for the quantification of gallic acid and caffeic acid both by batch amperometry in stirred solutions and by flow injection with amperometric detection. In general, it can be advantageously compared with other laccase and tyrosinase biosensors described in the literature. This good analytical performance allows the use of the laccase bioelectrode for the estimation of the index of polyphenolic compounds in wines using extremely simple procedures involving the direct addition of a diluted sample aliquot to the electrochemical cell. The total polyphenol content estimated using the biosensor exhibits a good correlation with the values obtained by application of the reference Folin−Ciocalteu method. Furthermore, the methodologies involved with the electrochemical biosensor have some advantages over the Folin−Ciocalteu method such as a high simplicity, a shorter detection time, and no interference from ascorbic acid and glucose. Therefore, we believe that the developed methods are useful for real-time monitoring of the total polyphenol content in the wine industry.

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