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Dual Laccase–Tyrosinase Based Sonogel–Carbon Biosensor for Monitoring Polyphenols in Beers

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A biosensor based on the bi-immobilization of laccase and tyrosinase phenoloxidase enzymes has been successfully developed. This biosensor employs as the electrochemical transducer the Sonogel–Carbon, a novel type of electrode developed by our group. The immobilization step was accomplished by doping the electrode surface with a mixture of the enzymes, glutaricdialdehyde, and Nafion-ion exchanger, as protective additive. The response of this biosensor, denoted the dual *Trametes versicolor* laccase (La) and *Mushroom* tyrosinase (Ty) based Sonogel–Carbon, was optimized directly in beer real samples and its analytical performance with respect to five individual polyphenols was evaluated. The Lac–Ty/Sonogel–Carbon electrode responds to nanomolar concentrations of flavan-3-ols, hydroxycinnamic acids, and hydroxybenzoic acids. The limit of detection, sensitivity, and linear range for caffeic acid, taken as an example, were 26 nM, 167.53 nA M^{-1}, and 0.01–2 μM, respectively. In addition, the stability and reproducibility of the biosensor were also evaluated in beer samples. The Lac–Ty/sonogel–carbon electrode was verified as very stable in this matrix, maintaining 80% of its stable response for at least three weeks, with a RSD of 3.6% ($n = 10$). The biosensor was applied to estimate the total polyphenol index in ten beer samples, and a correlation of 0.99 was obtained when the results were compared with those obtained using the Folin–Ciocalteau reagent.

KEYWORDS: Sonogel–Carbon electrode; amperometric biosensor; signal bioamplification; beers; polyphenols; bioelectrochemical index

INTRODUCTION

The importance of polyphenolic compounds in beer is considerable and relates to two main factors: quality and health. Phenolic compounds account for several major quality properties of beer, fundamentally colloidal stability, as they are responsible for the turbidity originated by their interaction with proteins, and for organoleptic characteristics (color, aroma, and flavor) (*1, 2*). The health benefits of phenolic compounds derive from their antioxidant activity, as free radical scavengers and inhibitors of lipoprotein oxidation. (*3, 4*)

Currently, several methods are available for the analysis of polyphenolic compounds. Based on separation techniques such as chromatography or capillary electrophoresis with various detection systems (*5–8*) or on techniques not involving separation such as colorimetry: Folin–Ciocalteau, Vanillin–HCl and *n*-butanol–HCl, (*9*), these methods have disadvantages such as their long operation times or high cost. There is a need for simple techniques for estimating polyphenols for particular purposes, such as the real-time monitoring of polyphenols in food storage, manipulation, or processing.

In food quality, biosensors have already confirmed their potential usefulness as tools for the detection of several types of compounds of interest: carbohydrates, alcohols, phenols, carboxylic acids, amino acids, biogenic amines, heterocyclic, inorganic, and additive or contaminants compounds (*10, 11*). Numerous papers have been published on the determination of phenolic compounds in food samples with biosensors. The most common enzyme used for this is tyrosinase; when this enzyme is entrapped in different supports, it is able to react with polyphenols to measure their concentration in samples such as olive extracts (*12, 13*), tea (*13, 14*), wine (*14–19*), and

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beer (*20–22*). Laccase, from various sources, and *Horseradish* peroxidase have also been widely used as bioprobes for the same purpose; thus, used as a biological oxidant of polyphenols, they have been incorporated in several electrochemical transducers to detect polyphenols in wine, tea, and vegetable extract (*23–30*). Electrochemical biosensor devices based on these three enzymes use a similar approach to detection: The liberated quinones or phenoxy radicals, enzymatically oxidized, as mediators in the oxido–reduction enzyme cycle, are re-reduced at the surface of the electrode, and a dramatic amplification of the biosensor response can be achieved by means of this partial substrate recycling. Recently, several research groups have described additional amplification by means of these devices, involving the addition of reducing, preconcentrating, mediating agents, and/or the integration of a couple of enzymes on a single transducer. This strategy is very interesting because it permits the sensitivity to be enhanced and the range of the substrates detected to be increased (*30–36*). However, as far as we know, the utilization of this strategy for measuring the very complicated matrix of polyphenols implicated in food and food derivatives has not been reported. In this paper, we report the dual integration of two copper-containing enzymes on the surface of the Sonogel–Carbon electrode, and the use of the resulting bi-enzyme biosensor for determining polyphenols in samples of beers. The bioelectrochemical polyphenol index, proposed here, was also correlated with the index based on using Folin–Ciocalteau reagent.

The Sonogel–Carbon electrode, which we previously developed (*37*), was chosen because of its demonstrated high sensitivity, stability, and biocompatibility when used as an electrochemical transducer (*37–40*). There are several good reasons for applying the biosensor developed to beer samples: beer is one of the most popular of all beverages; second, it has been demonstrated that a diet supplemented with beer possesses a higher antioxidant activity than one accompanied by, for example, white wine (*41*). Finally, continuous monitoring of polyphenols in this matrix during the manufacturing and storage process is very important because these compounds dictate the physical and colloidal stability of beer in these stages.

EXPERIMENTAL PROCEDURES

Reagents. Methyltrimethoxysilane (MTMOS) was from Merck (Darmstad, Germany) and HCl was from Panreac (Barcelona, Spain). Graphite powder (spectroscopic grade RBW) was from SGL Carbon (Ringsdorff, Germany). *Mushroom* tyrosinase (E.C. 1.14.18.1, 3000 U mg^{-1}), and *Trametes versicolor* laccase (E.C. 1.10.3.2, 23.3 U mg^{-1}) were from Fluka (Steinheim, Germany). KH_2PO_4/K_2HPO_4 and acetic acid/sodium acetate for phosphate or acetate buffer were from Fluka (Buchs, Switzerland) and Merck (Darmstad, Germany), respectively. Nafion-perfluorinated ion-exchanger resin (Cat. No. 27, 470-4) 5% (w/v) in a mixture of lower aliphatic alcohols and water, and glutaricdialdehyde, 25 wt % solution in water, were obtained from Aldrich (Steinheim, Germany). Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ·cm, Millipore, Bedford, MA). All phenolic compounds tested in this work (caffeic acid, ferulic acid, gallic acid, (+)-catechin, and (−)-epicatechin) were of analytical grade, used as received, and purchased from Merck, Fluka, or Panreac. Folin–Ciocalteau reagent was from Panreac.

Stock solutions of the phenolic compounds (0.01 M) were prepared daily by dissolving the appropriate amount either in 0.05 M buffer solution or in ethanol, depending on the phenolic compound's solubility. More dilute standards were prepared by suitable dilution with 0.05 M buffer solution at working pH, which was also used as the supporting electrolyte.

Glass capillary tubes, i.d. 1.15 mm, were used as the bodies for the composite electrodes.

Samples. The samples analyzed were ten commercial beers (five lagers and two black beers with alcohol contents between 4.5 and 7.9 vol. %, and three nonalcoholic beers) purchased locally. The samples were previously degasified by means of centrifugation so that an exact beer volume could be measured, and were diluted to the working solution at 1:5 or 1:10 depending on the nature of the beer.

Apparatus. Chronoamperometric measurements were performed with an Autolab PGSTAT20 (Ecochemie, Ultrecht, The Netherlands) potentiostat/galvanostat interfaced with a personal computer, using the AutoLab GPES software for waveform generation and data acquisition and elaboration.

Colorimetric essays were performed with an UV/vis spectrophotometer (Jasco V-550, from Japan), using the Jasco 32 software.

A 600-W model, 20 kHz ultrasonic processor (Misonix Inc., Farmingdale, NY) equipped with a 13 mm titanium tip was used. The ultrasonic processor was enclosed inside a sound-proof chamber during operation.

Methods. *Preparation of Electrochemical Transducer.* The unmodified Sonogel–Carbon electrode was prepared as described previously (*37, 38*). Before modification, the electrodes were polished with emery paper no. 1200 to remove extra composite material, wiped gently with weighing paper, and electrochemically pretreated by dipping them in 0.05 M sulfuric acid and polarized in the three compartment cell by voltage cycling from −0.5 to 1.5 V for 5 cycles. After that, the electrodes were washed carefully with MilliQ water and left to dry at ambient temperature.

Fabrication of Biosensors. To fabricate the bi-enzyme biosensors, adequate quantities of laccase and tyrosinase enzymes were dissolved in 30 μL of 0.2 M pH 6 phosphate buffer solutions. A volume of 1.25 μL of glutaricdialdehyde was added to this enzymatic solution, which was set to polymerize in an ultrasonic bath for 3 min, and modified by adding 3.5 μL of Nafion 5%. From the resulting solution, adequate quantities were deposited on the tip of the Sonogel–Carbon electrodes with a microsyringe and allowed to dry under ambient conditions. Finally, the resulting biosensor had 23 and 100 units/electrode of laccase and tyrosinase, respectively, around 0.9% of glutaricdialdehyde and 0.5% of Nafion. Before being used, the enzyme electrodes were dipped in stirred buffer solution for 15 min to eliminate the excess of enzymes not adsorbed, rinsed with the buffered solution, and stored immersed in buffer at 4 °C when not in use.

Measurements. Electrochemical experiments were carried out in an aerated cell containing 25 mL of a 0.05 M solution of the adequate buffer at pH 5.5, at 22 ± 2 °C. The three-electrode system consisted of the modified bi-enzyme Sonogel–Carbon electrode as working electrode, an Ag/AgCl (3 M KCl) and a platinum wire as reference and auxiliary electrodes, respectively. For the measurements, a selected potential was applied to the working electrode and the background current was recorded until the steady state was reached. The respective polyphenolic compounds standard solutions were added to the cell and the corresponding current–time curves were recorded. The biosensor response was measured as the difference between the total and the background current. A magnetic stirrer and stirring bar were used to provide continuous convective transport.

Determination of Bioelectrochemical Polyphenol Index. After the polarization of the biosensor at its optimum potential, and

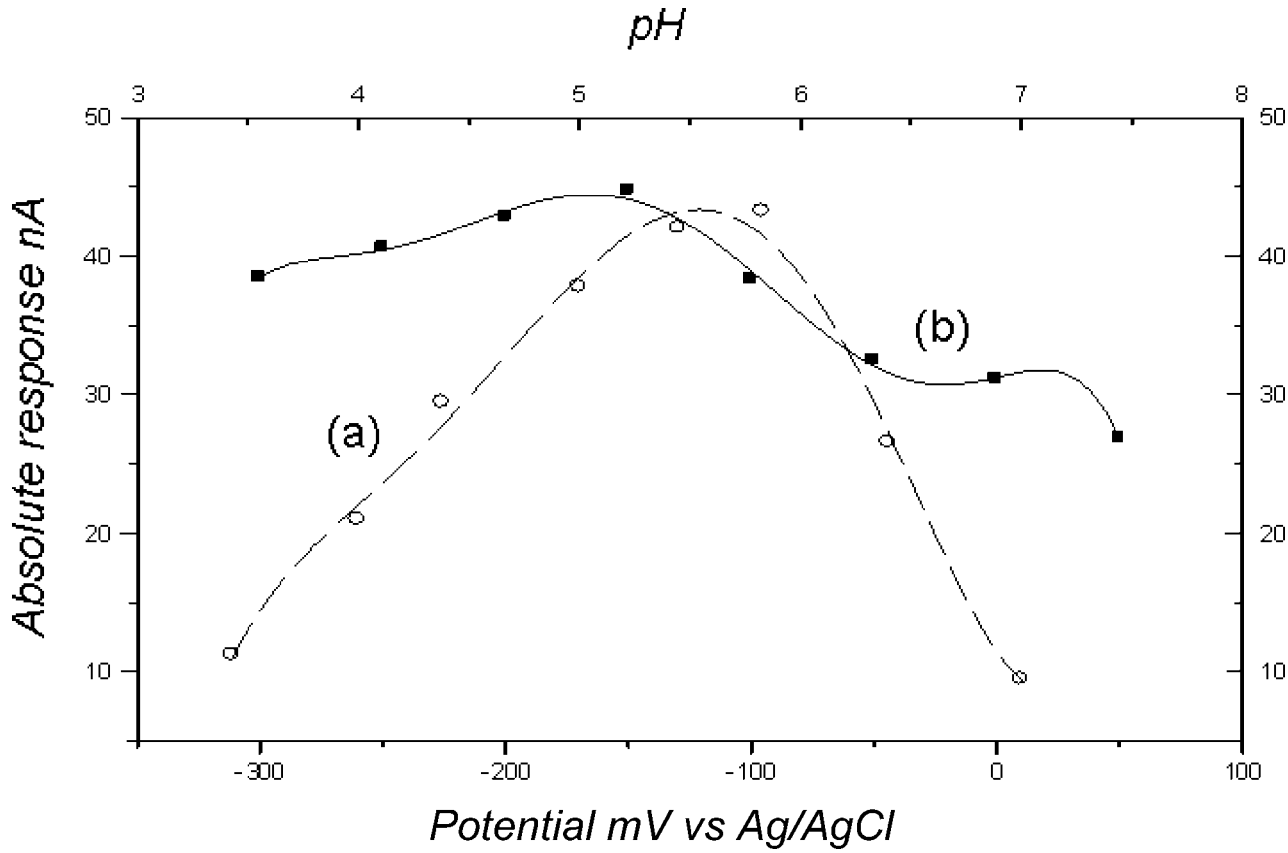

Figure 1. Influence of (a) pH, and (b) applied potential vs. Ag/AgCl for La–Ty Sonogel–Carbon biosensor for 1:5 diluted beer. Supporting electrolyte 0.05 M acetate buffer, or 0.05 M acetate or phosphate for (a) and (b), respectively.

the recording of the background current under stirring, 150 μL of diluted beer sample was added to 25 mL of 0.05 M phosphate buffer solution at pH 6. Estimation of the phenolic compounds was performed by applying the standard additions method, which involved the addition of three successive volumes of a gallic acid standard solution, around 0.25 mg L^{-1} by step. Then a linear curve with four bi-replicated points was constructed and the total polyphenols index and its standard deviation were estimated statistically (*42*).

Determination of Folin–Ciocalteau Polyphenol Index. Beer (500 μL) and the same quantity of the corresponding gallic acid standard solutions were placed in four 50 mL volumetric flasks; to make up the total volume, 30 mL of MiliQ water, 2.5 mL of Folin–Ciocalteu reagent, 7.5 mL of anhydrous sodium carbonate solution at 20 %, and distilled water were added as previously described (*43*). After 2 h the absorbance at 760 nm was read, using a blank prepared with distilled water, and the total polyphenol index and its error were obtained from the standard addition curve, employing the same mathematical approach mentioned above.

RESULTS AND DISCUSSION

Optimization of Physical–Chemical Parameters. A simple review of the bibliography concerning the development of the biosensor to determine phenol or polyphenols in real samples shows that, in the majority of the studies published, biosensors are optimized using randomly selected individual substrates. However, phenol and especially polyphenol matrices in beer are not isolated from the rest of the components of the matrix. Their interactions with other components such as proteins (*44*), and their mutual interactions, have been the subject of numerous reports (*1, 5, 8, 9*). Optimization of a biosensors response with an individual substrate can lead to serious problems when the bioprobe is used in real samples or for the detection of mixtures of polyphenols. For example, using a laccase-based biosensor, Gomes et al. (*27*) observed that, at the same solution pH, catechin and caffeic acid give different responses depending on the potential applied. At pH 4.5, catechin responds mainly at a potential of +100 mV and does not respond at a potential of −50 mV, unlike caffeic acid which does respond at this negative potential but not at that positive potential. Imabayashi et al. (*23*) reported the potential dependence in the steady-state reduction current of a horseradish peroxidase biosensor individually for catechin, epicatechin, and caffiec acid, and concluded that the optimum potential is more positive for the two flavan-3-ols than for the phenolic acid. To avoid these limitations and attain the real optimum response, the chemico–physical optimization of the proposed biosensor was investigated directly in diluted solution of lager beer as a real sample of a mixture of polyphenols.

Figure 1 shows the influence of the operating potential and the pH on the response of the dual laccase–tyrosinase based Sonogel–Carbon biosensor, in 0.5 mL of 1:5 diluted lager beer in acetate or phosphate buffer. With this bioprobe the reductive detection of the liberated quinone products begins at +50 mV, increases with increasing the polarization until −150 mV and reaches a plateau, with a relative standard deviation of 7% for the intensity measured from −150 to −300 mV. Note that the biomodification of laccase-based Sonogel–Carbon by the addition of tyrosinase on the same biosensor has no significant effect on the optimum response potential, since the study to optimize the potential response for the individual enzyme biosensor gave the same optimum potential response of −150 mV. Hence a potential of −150 mV was adopted in all

Table 1. Analytical Performance of the La–Ty Sonogel–Carbon Biosensor

substrate	LR (μM)	LD (μM)	LQ (μM)	sensitivity (nA μM−1)	RSD for sensitivity %
gallic acid	0.1–15.0	19.0×10^{-2}	0.99	14.10	0.50
caffeic acid	1.0×10^{-2}–2.0	2.6×10^{-2}	0.09	167.53	0.45
ferulic acid	3.0×10^{-2}–2.5	6.4×10^{-2}	0.21	53.86	0.80
(+)-catechin	1.0×10^{-2}–6.0	3.4×10^{-2}	0.09	125.31	0.40
(−)-epicatechin	1.0×10^{-2}–9.0	4.3×10^{-2}	0.14	69.67	0.62

subsequent experiments. Using this potential also minimizes possible contributions from co-existing electroactive species. Regarding the pH dependence, the biosensor showed good sensitivity over a broad range (between pH 3.5 and pH 7), with an optimum response at around pH 5.5. However, when the pH dependence response of individual tyrosinase and laccase enzyme biosensors was investigated, the pH values of 7 and 5, respectively, were deduced as the optimum in the same real sample matrix. It can be seen that bioamplification of the laccase-based Sonogel–Carbon displaces its optimum pH to neutral values by the action of the tyrosinase enzyme. The pH values at which the free enzymes tyrosinase and laccase are usually active are between 5 and 7 for the bicopper enzyme, and between 4 and 6 for the blue copper enzyme. These values change with several factors, such as the origin of the enzymes, the ionic strength of the medium, and the substrates. The optimum pH of the dual-enzyme based biosensor is centered between the optimum pH values of the two individual enzymes, thus indicating that the immobilization of the enzymes does not damage their activity. The pH value of 5.5 was adopted for all the following experiments.

Calibration Curves for Individual Polyphenols. The main three groups of polyphenols in beer are as follows: polyphenol acids, such as those derived from hydroxybenzoic acids (gallic acid, protocatechuic acid, syringic acid, etc.) and hydroxycinnamic acids (ferulic acid, caffeic acid, *p*-coumaric acid, etc. . .); flavonols (quercetin, kaempferol, etc.); and flavan-3-ols and their polymers ((+)-catechin and (−)-epicatechin), dimers (procyanidin B3), and trimers (procyanidin C2)). This study investigates the sensing performance and kinetic characterization of the dual enzyme based Sonogel–Carbon biosensor in the presence of five individual polyphenols belonging to the principal phenolic groups. To this end the calibration curves of this biosensor with respect to caffeic acid, ferulic acid, gallic acid, (+)-catechin, and (−)-epicatechin were constructed in the optimum conditions established above, and the responses obtained in each case are summarized in **Table 1**. Tyrosinase (monophenol monoxygenerase) contains two copper centers and therefore catalyzes two different oxygen-dependent reactions: the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the successive oxidation of *o*-diphenols to *o*-quinones (catecholase activity). It is suggested that the phenolic substrate coordinates initially at the axial position, and electron density is donated from the substrate into the lowest unoccupied oxy-di-copper active site, followed by the oxygen transfer to the ortho position of the phenyl ring, the formation of the bounded catechol at the two Cu(II) centers and the electron transfer from the catechol to the copper atoms to generate the de-oxy site and to release the *o*-quinone (*45*). Consequently, biosensors based solely on this enzyme cannot respond to the phenols with one ortho-position occupied, and are thus not very sensitive for acidic phenols. Laccase belongs to the same family as tyrosinase, but contains four copper atoms with different electron paramagnetic resonances: type 1 or blue, type 2 or normal, and type 3 or coupled binuclear copper site; laccase catalyzes the oxidation of the hydrogen atom from the hydroxyl group of various aromatics, mainly the many phenolic compounds. Substrates oxidation by laccase is a one-electron reaction generating a free radical. These compounds are oxidized near the mononuclear site, and the electrons are transferred to the trinuclear site, where molecular oxygen is reduced. Thus the initial product is typically unstable and may undergo a second enzyme-catalyzed oxidation, or otherwise a nonenzymatic reaction such as hydration, disproportionation, or polymerization (*46*). A laccase biosensor can detect phenolic compounds that are not reactive with tyrosinase.

Table 1 includes linear range (LR), limit of detection (LD), limit of quantification (LOQ), sensitivity and its relative standard deviation (RSD). The limit of detection and quantification were calculated statistically as follows (*42*): {(LD or LQ) = $k \times$ SB/b}, where SB is the standard deviation of the blank, b is the sensitivity of the method (determined as the slope of the calibration curve), and k is a statistical constant (values of 3 and 10 in the case of LD and LQ, respectively, are widely accepted). It can be seen that the bi-enzyme La–Ty Sonogel–Carbon biosensor is able to detect polyphenol compounds in the nanomolar range. The sensitivity obtained for the five substrates follows the sequence caffeic acid > (+)-catechin > (−)-epicatechin > ferulic acid > gallic acid. The same sequence was obtained when the same substrates were calibrated by the La Sonogel–Carbon biosensor. In contrast, the Ty Sonogel–Carbon biosensor shows a lower sensitivity for all these compounds, with nonresponse for ferulic acid, because of the ortho occupation in the phenyl ring, and favorable selectivity towards the two flavan-3-ols. It is clear that the bioamplification given by a biosensor with multiple enzymes immobilized can extend the matrix of substrates detected, since a substrate that cannot be detected by one enzyme may react with another (*30, 33*). Our results prove not only that advantage, but also that this strategy can contribute to an increase of the LR, and a gain in the sensitivity. Nevertheless, in spite of the enormous interest demonstrated in the development of highly sensitive biosensors, few studies published have used this strategy; the mechanism of bioamplification, especially in the case of dual modification by enzymes originating from the same family, has never been investigated.

There is no easy explanation of why the signal increases in the case of the dual immobilization of two enzymes. Our previous research, using cyclic voltammetry, for the bioelectrocatalytic characterization of oxygen reduction by laccase and laccase–tyrosinase immobilized on a Sonogel–Carbon electrode (unpublished data) demonstrates that an equal bioamplification notably produces an equal improvement in the bioelectrocatalysis oxygen reduction. In the catalytic cycle of laccase and tyrosinase, oxygen is reduced to water without the intermediate formation of hydrogen peroxide. Furthermore, it was demonstrated that the presence of H_2O_2 increases the oxytyrosinase content for tyrosinase and the peroxide-level intermediate for laccase (*46*). A recycling of H_2O_2 between the two enzymes may be postulated as the basis of the anticipated mechanism. We can simplify the catalytic cycle of enzymatic oxidation of polyphenols by the bi-enzyme biosensor as starting with one electron substrate oxidation near the T1 site of laccase. After that, electrons are transferred to the T_2/T_3 cluster of laccase, and molecular oxygen is reduced in this cluster center. This step generates H_2O_2, which could also be the substrate of

Table 2. Kinetic Parameters

substrate	*h*	K_m^{app} μM	RSDfor K_m^{app} %	I_{max} nA	RSD for I_{max} %
gallic acid	1.01	68.21	3.19	1051	3.0
caffeic acid	1.05	26.32	3.05	4484	2.3
ferulic acid	1.09	53.53	9.81	3112	6.7
(+)-catechin	1.04	33.87	8.65	4709	7.4
(−)-epicatechin	1.20	41.87	12.47	3416	10.2

tyrosinase. Thus, the tyrosinase active center probably does not participate in the oxidation of the polyphenol substrate but only in the reduction of oxygen.

Kinetic Characterization of the Dual Laccase–Tyrosinase Based Biosensor. Table 2 summarizes the kinetic characteristics of the dual laccase–tyrosinase based biosensor for the five polyphenols tested. The finding that the "*h*" parameter, calculated from the corresponding Hill's plots, is close to unity, may demonstrate the Michaelis behavior of immobilized enzymes (*47*). The Michaelis–Menten constant K_m^{app} and maximum rate I_{max} of the reaction can be calculated from the corresponding Eadie–Hofstee plots. As expected, in general, lower K_m^{app} values were obtained for the phenolic compounds exhibiting a higher sensitivity, as a consequence of the substrate recycling phenomenon confirmed for amperometric biosensors (*48, 49*). More interestingly, a comparison of the K_m^{app} values with those obtained using individual laccase (K_m^{app} values from 33.0 to 76.5 μM) or tyrosinase (K_m^{app} values from 72.5 to 266.9 μM) -based biosensors shows that the Michaelis–Menten constant obtained with the La–Ty Sonogel–Carbon biosensor was in the range of that obtained for La Sonogel–Carbon and much lower than that obtained for Ty Sonogel–Carbon biosensors. This result confirms that the effect of the dual bi-immobilization is restricted to the enhancement of the active enzyme fraction, due to the H_2O_2 recycling, and does not change the enzymatic step. The K_m^{app} parameter is independent of the enzyme concentration and usually reflects the enzyme mobility as well as the biocompatibility of the immobilization matrix; therefore it is appropriate to perform a comparative study of this parameter with that reported in the literature. We found that the K_m^{app} values obtained with the proposed biosensor are much lower than those reported for tyrosinase, based on a large number of electrodes (*15, 17, 21*), and lower than, or in the same range as, the values reported for laccase from various sources and immobilized on a large number of electrodes (*28, 50–53*).

Stability and Reproducibility of the Biosensor. The stability of the biosensor response in real samples is the essential criterion for selecting the best bioprobe to achieve our research objective. Several different aspects of stability were considered: first the repeatability of the dual enzymes-based biosensor was calculated by ten repeated measurements on the same day, using the same electrode in the optimum working conditions previously established and in the presence of the same concentration of lager beer. A relative standard deviation of 3.6% was obtained.

The useful lifetime of the biosensor was checked by taking repeated measurements every day in the optimized conditions, using fresh lager beer as samples, and storing the biosensor in its optimum solution at 4 °C when it was not in use. **Figure 2** shows the evolution of the response with time up to 15 days. It can be seen that over this time period the biosensor maintains more than 80% of its initial response. The decrease of the signal with time can be attributed to the inhibition effect of the enzymes caused by the compounds present or those that develop in a very active medium like beer, such as carbonate, ascorbic acid, ethanol, or Maillard reaction products.

Finally, the reproducibility of the method of enzyme immobilization was also evaluated by comparing the first day

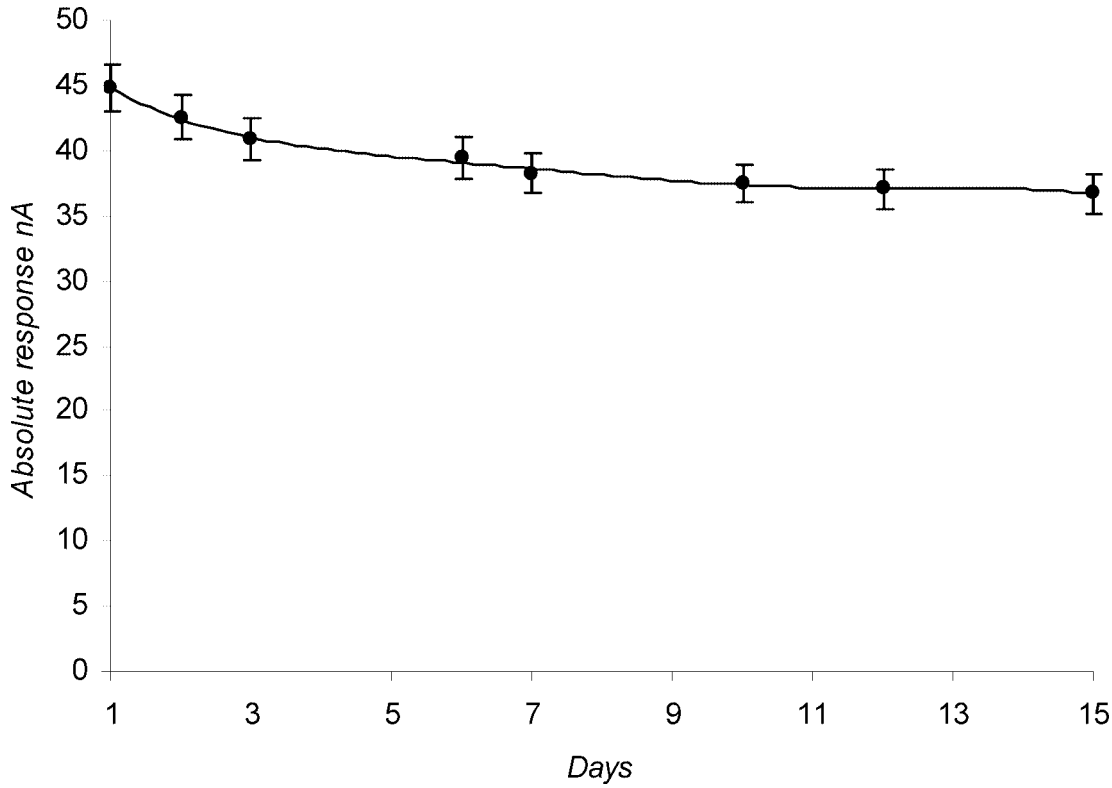

Figure 2. Lifetime response of La–Ty Sonogel–Carbon biosensor for 1:5 beer dilutions, at pH 5.5 (0.05 M acetate) and polarization at −150 mV.

Table 3. Polyphenol Content in Beers Determined by Folin–Ciocalteau (F–Ci) and La–Ty Sonogel–Carbon (BEi) Methods, Using Gallic Acid As Standard

beers	F–Ci mg L^{-1} gallic acid	RSD for F–Ci %	BEi mg L^{-1} gallic acid	RSD for BEi %
non alcoholic 1	255.5	5.3	112.5	1.6
non alcoholic 2	215.3	6.8	79.4	1.6
non alcoholic 3	180.3	6.1	79.1	1.6
lager 1	284.0	3.5	141.4	1.7
lager 2	368.5	6.6	177.9	1.6
lager 3	311.1	4.2	150.9	1.7
lager 4	417.0	3.8	190.1	1.8
lager 5	356.4	5.5	158.9	1.5
black 1	702.4	3.2	309.7	1.5
black 2	514.3	3.6	219.9	1.6

response of five La–Ty-based Sonogel–Carbon biosensors. A RSD of 5.2% was obtained for the response in 0.2 mL of lager beer injected in the electrochemical cell containing 25 mL of acetate buffer solution.

Estimation of Total Polyphenols Index in Beer with Dual La–Ty-Based Sonogel–Carbon. A dual La–Ty enzyme-based biosensor was used to measure the total polyphenol content in ten commercial beers. Three nonalcoholic, five lager, and two black beers were analyzed by standard methods as described in Experimental Procedures. From a comparison of the beer signal with that for gallic acid additions, no difference in the shape of the current–time response curve was observed, and difference in substrate quality does not alter the response time obtained of around 14 ± 2 s. The results obtained for the ten beers are expressed as gallic acid equivalent, and are summarized in **Table 3**. The same beer samples were also analyzed using the spectrophotometric method based on the use of Folin–Ciocalteau reagent. This reagent contain phosphomolybdic and phosphotungstic acids able to react with certain reducing agents to form a blue complex in which the metals are in their oxidative state, and whose absorbance can be read clearly at 760 nm. The total amount of polyphenols is estimated in this method by the same standard addition methodology as summarized in Experimental Procedures, and the results expressed in mg L^{-1} of gallic acid as standard are also shown in **Table 3**. As can be seen, there are large differences for these two polyphenols indices, which can be attributed to various causes. On the one hand, the estimation of these compounds by biosensor can be considered a bioelectrochemical polyphenol index, because of the observed dependence of its sensitivity on the structure and conformation of the substrate. Second, the pH of the measurement medium is not identical in both systems, and it is known that the ratio between the free and complexed polyphenols is very dependent on the pH of the medium and on its ionic strength. Finally, in contradiction to the reputation acquired by the Folin–Ciocalteu method as the best way to determine the total tannins index, this method is not very specific for these phenolic compounds. Box reported that the Folin reagents are a wide range of aromatic substances including phenolic hydroxyl, other cyclic compounds with an NH group in the ring, and aromatic compounds with a substituent NH_2 or CHO (*54*). In spite of all these considerations, a satisfactory correlation was found when the results obtained with our biosensor were plotted against those obtained with the Folin–Ciocalteau method for all the beer samples, with a regression coefficient of 0.99. Furthermore, as can be seen in **Table 3** the bioelectrochemical index determination gives more precise values compared with those obtained by the classical method in the case of all the beers tested, as demonstrated by the relative standard deviation values obtained. These advantages and others, such as being a very fast and low cost assay, are basic factors that make the bioelectrochemical method developed in this study a credible alternative to classical methods, especially for the continuous and real-time monitoring of polyphenols in beer and other beverages during their storage or manufacturing processes.

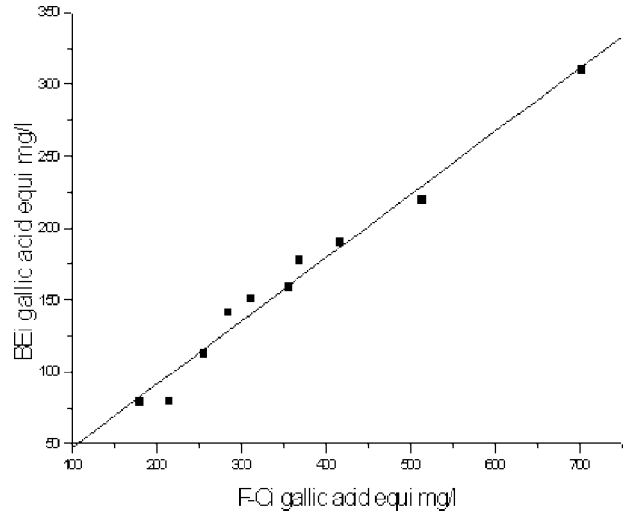

Figure 3. Correlation between bioelectrochemical and Folin–Ciocalteau methods for the determination of polyphenol content in beers, using gallic acid as standard.

So, the biosensor constructed by the co-immobilization of two phenoloxidase enzymes on the surface of our Sonogel–Carbon electrode exhibits good analytical performance, and the stability and long working lifetime of this device, even in a complicated matrix like beer, proves the biocompatibility of the Sonogel–Carbon with the enzymes immobilized, and the reliability of the simple immobilization method used. Therefore, we consider that the development of reliable biosensors not only for the determination of polyphenols in finished food and drink products but also for monitoring the progression of these compounds in real-time during manufacturing processes, opens an interesting new field.

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