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Analytical Methods

A comparison of three amperometric phenoloxidase–Sonogel–Carbon based biosensors for determination of polyphenols in beers

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ABSTRACT

Three phenoloxidases based biosensors were successfully developed using as electrochemical transducer a new type of electrode recently developed by our group: the "Sonogel–Carbon electrode". The employed enzymes were *Trametes versicolor* laccase (*Lac*), *Mushroom* tyrosinase (*Tyr*), and *Horseradish* peroxidase (*HRP*). Immobilization step was accomplished by doping the electrode surface with a mixture of the individual enzyme and Nafion ion exchanger as additive-protective. The biosensor responses, optimized in beer real samples, were evaluated for five individual polyphenols. It was found that the developed biosensors were sensitive to nanomolar concentrations of the tested polyphenols. As example, the limit of detection, sensitivity, and response linear range for caffeic acid for Nafion-*Lac*/Sonogel–Carbon biosensor were 0.06 μmol L^{-1}, 99.6 nA μmol^{-1} L, and 0.04–2 μmol L^{-1}, respectively. The stability and reproducibility of the biosensors were evaluated by applying them directly to beer real samples. It has been demonstrated that the Nafion-*Lac*/Sonogel–Carbon system is the more stable with a relative standard deviation of 3.3% ($n = 10$), maintaining 84% of its stable response for at least three weeks. Estimation of polyphenol index in eight lager beers and a comparison of the results with those obtained by a classical method was carried out.

1. Introduction

It is well known that polyphenols present in beverages play an important role in their quality and stability as well as in the prevention and protection of some pathologies (Shi et al., 1994; Stewart, 2004; Williamson & Manach, 2005). For these reasons, and due to their variety and the complexity of samples, several efforts have been dedicated to quantification of polyphenols in food and beverages (Santos-Buelga & Williamson 2003). Between the available techniques, bioanalytical tools offer interesting advantages over classical analytical techniques such as high selectivity and sensitivity, short assay times, and reduced cost of analysis.

Various electrochemical biosensors, based specially on phenoloxidases enzymes as tyrosinase (Cumming et al., 1998, 2001; Eggins, Hickey, Toft, & Zhou, 1997; Jewel & Ebler, 2001; Kiralp & Toppare, 2006; Sanz, Mena, González-Cortés, Yáñez-Sedeño, & Pingarrón, 2005), laccase (Gamella, Campuzano, Reviejo, & Pingarrón, 2006; Ghindilis, Gabrilova, & Yaropolov, 1992; Gomes, Nogueira, & Rebelo, 2004), and peroxidase (Imabayashi, Kong, & Watanabe, 2001; Kong, Imabayashi, Kano, Ikeda, & Kakiuchi, 2001; Mello, Sotomayor, & Kubota, 2003) have been developed for polyphenols determination in wine, beer, tea, and vegetables extract. All amperometric biosensors based on these enzymes have a similar detection approach: the phenols are enzymatically oxidized to quinones or radicals and then detected at the electrode by their reduction currents; such approach has the advantage to recycle the reaction products at the intimate electrode surface and consequently increases drastically the current response, improving the sensitivity of the method. However, the enzymatic products can partially electropolymerize to polyaromatic compounds damaging the electrode surface and cut the total assays number as well as the life time of the biosensor. A judicious choice of the electrode transducers and the use of additive-protective matrix on the surface of the biosensor can protect the bioprobe from this undesirable phenomenon and improve the signal transducer.

The employment of sol–gel chemistry to produce electrochemical transducers and its biocompatibility with biological sensing has received increasing interest in recent years (Collinson & Howells, 2000; Jin & Bernnan, 2002; Rabinovich & Lev, 2001; Sun, Zhu, & Zhu, 2006; Wang, 1999). Our group proposed a novel sol–gel-based procedure to obtain solid carbon composite electrodes (Hidalgo-Hidalgo de Cisneros, Cordero-Rando, Naranjo-Rodríguez, Blanco, & Esquivias, 2001), called by us Sonogel–Carbon electrodes.

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The fabrication procedure, based on the use of sonocatalysis, that avoids the materials shrinkage and permits a control of the pore size, achieves an excellent electrochemical sensor when comparing with other graphite electrodes (Cordero-Rando, Hidalgo-Hidalgo de Cisneros, Blanco, & Naranjo-Rodríguez, 2002, 2005) and a very competitive bioprobe if it is used as electrochemical transducer in enzymatic biosensors (ElKaoutit, Naranjo-Rodriguez, Temsamani, & Hidalgo-Hidalgo de Cisneros, 2007).

In this work, we report the biocompatibility of this material with various enzymes, such as *Trametes versicolor* laccase (*Lac*), *Mushroom* tyrosinase (*Tyr*), and *Horseradish* peroxidase (*HRP*). The use of the resultant biosensors to measure polyphenols in several beer types constitutes the aim of this paper. As far as we know, a comparison in the same paper of three enzyme-based biosensors for determination of a bioelectrochemical index for polyphenols in beer was never reported. These points together with other developed in this paper constitute the originality of this work.

2. Experimental

2.1. 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 *Horseradish* peroxidase (E.C. 1.11.1.7, 269 U mg^{-1}) were from Sigma (Steinheim, Germany). *Trametes versicolor* laccase (E.C. 1.10.3.2, 23.3 U mg^{-1}) was 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-exchange 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 from Aldrich (Steinheim, Germany). Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 M cm, Millipore, Bedford, MA). All phenolic compounds tested in this work (caffeic acid (CA); ferulic acid (FA); gallic acid (GA); (+)-catechin ((+)-cat); and (−)-epicatechin ((−)-epi)), were of analytical grade, used as received, and purchased from Merck, Fluka or Panreac. Folin–Ciocalteu reagent was from Panreac and used as received.

Stock solutions of the phenolic compounds (0.01 mol L^{-1}) were prepared daily by dissolving the appropriate amount either in 0.05 mol L^{-1} buffer solution or in ethanol, depending on the phenolic compounds solubility. More dilute standards were prepared by suitable dilution with 0.05 mol L^{-1} phosphate or acetate 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.

2.2. Samples

The samples analyzed were commercial beers purchased in a local market. Eight lager beers were investigated (five with alcohol contents around 5% vol. and three non-alcoholic). The samples were previously degasified by means of centrifugation in order to take an exact beer volume and to dilute it in the working solution at a 1:5 ratio when necessary.

2.3. Apparatus

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

Colorimetric essays were performed with UV/VIS Spectrophotometer *Jasco* V-550 (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.

2.4. Methods

2.4.1. Electrochemical transducer preparation

Electrochemical Sonogel–Carbon transducer was prepared as described previously (Cordero-Rando et al., 2002). Before biological modification, the electrodes were electrochemically pre-treated by dipping them in 0.05 mol L^{-1} sulphuric acid and polarized by voltage cycling from −0.5 to 1.5 V for 5 cycles; electrodes with similar current backgrounds were selected, washed carefully with Milli-Q water and let to dry at room temperature.

2.4.2. Biosensors fabrication

In this work Sonogel–Carbon bioelectrodes based on laccase (*Lac*/SNGC), tyrosinase (*Tyr*/SNGC) and peroxidase (*HRP*/SNGC) were developed as follow: An adequate quantity of enzyme was dissolved in 30 μL of different buffer solutions, such as phosphate buffer, 0.2 mol L^{-1} pH 7, for tyrosinase and peroxidase, and acetate buffer, 0.2 mol L^{-1} pH 5, for laccase biosensors. At this enzymatic solution, 1.25 μL of glutaricdialdehyde was added, set to polymerize in ultrasonic bath for 3 min, and modified by adding 3.5 μL of Nafion. From the resulting solution, adequate quantities were deposited on the top of the Sonogel–Carbon electrodes with a μ-syringe and allowed to dry under room conditions. Finally, the three resulting biosensors have 100, 54, and 23 Units/Electrode of *Tyr*, *HRP*, and *Lac*, respectively, 0.9% of glutaricdialdehyde and 0.5% of Nafion. Before using, the enzymes electrodes were dipped in stirred buffer solution for 15 min, to eliminate the excess of enzymes not adsorbed, rinsed with the same buffered solution and stored immersed in the buffer at 4 °C when they were not in use.

2.5. Electrochemical measurement and bioelectrochemical polyphenols index determination

Electrochemical experiments were carried out in a cell containing 25 mL of an aerated adequate buffer, depending on pH values, at 22 ± 2 °C. The three-electrode system consisted of an enzyme-modified Sonogel–Carbon electrode as working electrode, a Ag/AgCl (3 M KCl) and a platinum wire as reference and auxiliary electrodes, respectively. To perform the measurements, a selected potential was applied to the working electrode and the background current was registered until reaching the steady state. 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 a stirring bar were used to provide continuous convective transport.

Bioelectrochemical polyphenol index determination in beers was performed as follows: Polarization of the biosensor at its optimum potential and registration of the background current under stirring; addition of 500 μL of 1:5 diluted beer sample to 25 mL of 0.05 mol L^{-1} acetate buffer solution of pH 5; application of the standard addition method (addition of three successive aliquots of a gallic acid stock solution with a concentration about 0.40 mg L^{-1}). A linear curve with four bi-replicated points was constructed and a polyphenols index was determined. Its standard