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A new amperometric bienzymatic biosensor based on biocomposites for the determination of gluconic acid in wines

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ABSTRACT

A new amperometric bienzymatic biosensor for gluconic acid based on the coimmobilization of gluconokinase (EC 2.7.1.12) and phosphogluconate dehydrogenase (EC 1.1.1.44) by polysulfone membrane entrapment onto the surface of a graphite-epoxy composite is reported. This biosensor represents an alternative to gluconate dehydrogenase (EC 1.1.99.3) based methods, which is no longer commercially available. Measurements were done at an applied potential of +0.800 V, room temperature and phosphate buffer pH 7.50; obtaining a linear response range for gluconic acid extended from 7.0×10^{-6} to 2.5×10^{-4} M. Constructed biosensors showed good reproducibility for calibrations using different electrodes (RSD of 1.74%). Finally, biosensor was applied to real wine samples, and the results obtained were validated by comparison with those provided by a reference laboratory. Good correlation was found when the biosensor results were plotted vs. the reference values (slope = 1.03 ± 0.04, intercept = 0.01 ± 0.02, $r^2 = 0.995$).

1. Introduction

High concentrations of gluconic acid in wine are caused by the proliferation of a fungus (*Botrytis cinerea*) which is able, via the enzyme glucose oxidase, to generate gluconic acid from glucose (via glucono delta-lactone); this grapevines disease is called common rot or grey rot [1,2]. It develops during growth of the grape berry and is influenced by weather factors, such as moisture and rainfall, or by physiological factors, such as grape variety and bunch shape. Changes that are caused in white and red wines include alterations in colour due to a high activity of oxidases; an increase in volatile acidity, ash contents and dry extract contents through the formation of glycerol, polysaccharides, uronic acids and aldonic acids (essentially gluconic acid); and a decrease in titratable acidity [3]. Significantly, sensory properties of wine are altered by the presence of gluconic acid, which additionally renders it microbiologically unstable and results in long-term storage problems that can be solved only by reducing its content in wine.

Therefore, gluconic acid concentration is an important analytical parameter used by oenologist to quantify the rottenness degree and assess quality of wine. Taking into account that basal amounts will always be present, *Organization Internationale du Vin* (OIV) recommends levels lower than 200–300 mg/l [4], whereas levels up to 1.0 g/l indicate an initial stage of the fungus infection and higher levels (up to 2–3 g/l) might indicate an activity of acetobacter bacteria. Nevertheless, we have to distinguish noble rot wines, obtained in very special and controlled conditions (which have to be proved), in which cases gluconic acid is a virtue instead of a defect.

D-Gluconate (the base form of gluconic acid) has been usually determined by enzymatic assay employing gluconate dehydrogenase (GADH) (EC 1.1.99.3) which catalyzes the oxidation of gluconic acid through an acceptor reduction (FAD, TTF, NAD^+, etc.); depending on the acceptor, we can use different techniques such as spectrophotometry [5] or electrochemistry [6–8]. In this case, biosensors have been developed employing: glassy carbon electrodes with GADH immobilized on its surface [6,8], gold electrodes and immobilization through different membranes either with direct cofactor detection [9] or with TTF acting as a mediator [7]. Unfortunately, an inconvenient of these methodologies is that GADH enzyme is not longer commercially available, and in order to continue using the above principles, the only option would be to produce and isolate the enzyme from microbiological cultures [10]. There are also some commercial enzymatic kits for spectrophotometric determination and batch measurements that employ other enzyme systems [11]. Apart of the enzymatic assay, alternative analytical techniques also employed are high performance liquid chromatography [12,13], gas chromatography [14], capillary electrophoresis [15,16] or near-infrared spectroscopy [17]; but these methods are time consuming and require advanced laboratory facilities, while we report a more simple on-field biosensor system based on composites electrodes.

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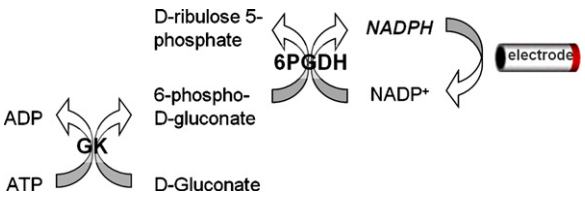

Scheme 1. Schematic diagram of the biosensor reactions involved for gluconic acid determination.

The development of composites based on a conductive phase dispersed in a polymeric matrix, has led to important advances in the analytical electrochemistry field, particularly in sensor devices. These materials combine the electrical properties of graphite with the ease of processing of plastics (i.e. epoxy, methacrylate, Teflon) and show attractive electrochemical, physical, mechanical and economical features compared to other noncombined conductive materials (i.e. gold, platinum, graphite). The electrochemical characteristics of composites have been widely studied in previous works [18]. Composites can also incorporate modifiers when they are in solid state, but if we want to incorporate a liquid phase (like an enzyme suspension) we have to use a membrane immobilization protocol like the use of polysulfone.

Polysulfone is a porous polymer widely used as support material for composite membranes and as ultrafiltration membrane. It displays very good chemical and thermal stability and excellent film-forming ability. Moreover, polysulfone films are hydrophobic hence providing the enzyme with an in vivo-like environment. Polysulfone is therefore an attractive structural material and has been studied as support for the immobilization of enzymes [19,20].

The aim of this paper is to propose a biosensor alternative to GADH based methods, as a simple tool able to satisfy industry requirements which demands sensor-based system to replace heavy laboratory equipment. For this purpose, we have developed an electrochemical composite biosensor based on the use of two commercial enzymes (gluconate kinase and 6-phospho-D-gluconate dehydrogenase). With these, an amperometric bienzymatic system for the determination of gluconic acid based on the phosphorylation by gluconate kinase and oxidation of the resulting product by 6-phospho-D-gluconate dehydrogenase and accompanied with NADP reduction is reported (Scheme 1). The developed biosensor was tested for real wine sample determination and their results compared and validated with those obtained by a reference laboratory.

2. Materials and methods

2.1. Apparatus

Amperometric measurements were done using a LC-4C amperimeter (BAS Inc., West Lafayette, IN, USA) connected to a personal computer by a data acquisition system ADC-42 PicoTechnology (Pico Technology Limited, St. Neots, Cambridgeshire, UK) for data recording and visualization. Electroanalytical experiments were carried out at room temperature (25 °C) using a three electrode configuration: a double junction electrode Ag/AgCl Orion 900200 (Thermo Electron Corporation, Beverly, MA, USA) was used as the reference electrode, a platinum-based electrode (Crison 52-67, Barcelona, Spain) was used as the auxiliary one and the biosensor was used as the working electrode. A magnetic stirrer provided the convective transport during the amperometric measurements.

2.2. Reagents and solutions

All solutions were prepared using deionised water from a Milli-Q system (Millipore, Billerica, MA, USA). Potassium D-gluconate (99%), adenosine 5′-triphosphate disodium salt (ATP) (99%), adenosine 5′-diphosphate sodium salt (ADP) (95%), polysulfone (Ps) (average Mn ~ 22,000 by Membrane Osmometry) and N,N-dimethylformamide (DMF) (99.5%, over molecular sieve) were purchased from Sigma–Aldrich (St. Louis, MO, USA). β-Nicotinamide-adenine dinucleotide phosphate ($NADP^+$) (95%), β-nicotinamide-adenine dinucleotide phosphate (reduced form) (NADPH) (93%) and magnesium chloride hexahydrate (99%) were purchased from Merck KGaA (Darmstadt, Germany). Gluconate kinase (GK) (EC 2.7.1.12, 1500 U/ml) and 6-phospho-D-gluconate dehydrogenase (6PGDH) (EC 1.1.1.44, 150 U/ml) were purchased from CPC Biotech (Napoli, Italy).

The solutions were freshly prepared using the buffer solution. $NADP^+$ and ATP-$MgCl_2 \cdot 6H_2O$ solutions were prepared before each experiment; also gluconate 0.1 M solution was prepared and used as stock, and then 10^{-3} M and 10^{-2} M gluconate solutions were prepared from the stock, to reduce the increment of volume after additions in batch measurements. Gluconate salt is used as stock instead of gluconic acid; given that gluconic acid dissociates in water at pH 7.00 to form the gluconate anion ($pK_a = 3.7$), its anion form will be considered along all the text.

2.3. Electrode fabrication

Working electrodes were prepared following the conventional methodology in our laboratories [18]. A resin EpoTek H77 (Epoxy Technology, Billerica, MA, USA) and its corresponding hardener compound were mixed in the ratio 20:3 (w/w). The composite was prepared adding a 15% of graphite (w/w) into the epoxy resin before hardening [21], then it was homogenised for 60 min and finally the composite paste electrode was allowed to harden during 72 h at 80 °C. Electrode surface was then polished with different sandpapers of decreasing grain size; final electrode area 28 mm^2.

2.4. Membrane preparation

The polysulfone composite membranes were prepared following the methodology previously established [22]. First of all we have to prepare the membrane suspension, which process could be summarized in these two steps. Polysulfone (Ps) was dissolved in DMF, obtaining a Ps solution in DMF and afterwards it was mixed with graphite; the resulting suspension was mixed for 10 min under continuous stirring. The proportions employed for the membrane preparation, once optimized, were 10:2:88 for Ps, DMF and graphite, respectively.

A thin film of this mixture was manually deposited onto the epoxy-graphite electrode surface. Immediately after depositing the Ps solution onto the electrode surface, it was precipitated by causing a phase inversion that was achieved by immersing the electrode in cold water (approximately 4 °C) [23] where the non-solvent (H_2O) displaced the solvent (DMF) and made Ps insoluble. This process led to a controlled phase change of the Ps cast on the electrode from liquid to solid. Taking advantage of the phase inversion process, the enzyme was incorporated into the membrane substituting the cold water for an aqueous solution of the enzyme. Electrodes in this way prepared, with Ps composite films on their surface, were rinsed thoroughly with doubly distilled water prior to use. The modified electrodes with films incorporating the enzyme were stored in phosphate buffer solution pH 7.50 at 4 °C.