Analytical Letters, 38: 907–920, 2005

ISSN 0003-2719 print/1532-236X online
DOI: 10.1081/AL-200054036

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CHEMICAL AND BIOSENSORS

Development of a Carbon Film Electrode Ferrocene-Mediated Glucose Biosensor

M. E. Ghica and C. M. A. Brett
Departamento de Quimica, Universidade de Coimbra, Coimbra, Portugal

Abstract: An amperometric ferrocene-mediated glucose biosensor was developed using the cross-linking method with glutaraldehyde and bovine serum albumin (BSA) on carbon film electrodes fabricated by pyrolytic deposition of carbon. Ferrocene was mixed with the enzyme mixture or incorporated afterwards by potential cycling; the addition of Nafion to the mixture or coating the biosensor assembly with Nafion was also tested. The optimized operating potential for ferrocene-mediated catalysis of the generated hydrogen peroxide was 0.25 V vs. Ag/AgCl, an improvement on sensors reported in the literature. The best assembly in terms of sensitivity and detection limit of 66 μM was found to be one using an external Nafion coating. Interferences were generally small. This sensor was applied to the determination of glucose in sweet wine samples by the standard addition method.

Keywords: Amperometric glucose sensor, ferrocene, carbon film electrode, Nafion

1. INTRODUCTION

Many enzyme-modified electrode biosensors that are developed for use in food, clinical, and environmental measurements are based on oxidase enzymes, such as glucose oxidase (GOx), that generate hydrogen peroxide

Received 4 January 2005; accepted 15 January 2005

Financial support from Fundação para a Ciência e Tecnologia (FCT), ICEMS (Research Unit 103) and European Project HPRN-CT-2002-00186 is gratefully acknowledged. M.E.G. thanks FCT for a Ph.D. grant (SFRH/BD/14014/2003). Prof. H.-D. Liess is thanked for the gift of the electrical resistors.

Address correspondence to C. M. A. Brett, Departamento de Quimica, Universidade de Coimbra, 3004-535 Coimbra, Portugal. E-mail: brett@ci.uc.pt

(Cooper and Cass 2004). A serious problem that must be overcome for the use of a glucose biosensor is the presence of interferents, such as ascorbic acid and uric acid, which are oxidized at the same potential as H_2O_2. Moreover, the peroxide that is generated is harmful for GOx, limiting the biosensor's performance. One viable solution for these problems is to replace the natural electron acceptor of glucose oxidase (O_2) by electroactive compounds that will act as redox mediators, thus allowing work at lower potentials and reducing interferences.

Ferrocene and its derivatives proved to be particularly useful as mediators for amperometric enzyme electrodes. See references (Li et al. 1997; Nakabayashi et al. 1998; Bu et al. 1998; Miao et al. 2001; Fiorito and Córdoba de Torresi 2001; Vidal et al. 2002; Tkac et al. 2002; Razumiene et al. 2003; Yang et al. 2003; Serban et al. 2004; Mecheri et al. 2004; Kase and Muguruma 2004). Ferrocene has been suggested for use as a hematinic agent; no contraindications have appeared for the use of ferrocene in humans at reasonable dosage levels to date. Early ferrocene-mediated enzyme electrodes had short lifetimes in continuous use due to either loss of enzyme or its denaturation (Brooks et al. 1987). To reduce mediator loss, chitosan matrices that were cross-linked with glutaraldehyde (Miao et al. 2001), encapsulated in conducting polymers such as polyphenols (Nakabayashi et al. 1998) or polypyrrole (Fiorito and Córdoba de Torresi 2001; Vidal et al. 2002), or cellulose acetate membranes (Tkac et al. 2002) were developed, as well as hydrogels (Bu et al. 1998), sol-gels (Li et al. 1997), covalent binding to sol-gel surfaces (Li et al. 1997; Bu et al. 1998; Yang et al. 2003) or plasma polymerized thin films of mediator (Kase and Muguruma 2004). Applications reported included use in screen-printed electrodes (Razumiene 2003) and in wine analysis (Serban et al. 2004).

Monomeric mediators such as ferrocene were initially conceived of as functioning as a result of their mobility (Schuhmann et al. 1990). Although ferrocene is essentially insoluble in aqueous solution, however, once it was oxidized at an electrode, it was postulated that the resulting ferricinium ion could diffuse to the redox centre of the enzyme where it would act as an artificial electron acceptor. After reduction to the insoluble form, the mediator would return to the electrode. However, this movement could lead to the loss of ferricinium ions from the immediate vicinity of the electrode. Increasing the molecular weight of the mediator could decrease this possible loss, but with consequent impairment of the rate of diffusion (Hendry et al. 1993).

The entrapment of ferrocene in a gel or polymer (Li et al. 1997; Bu, Mikkelsen, and English 1998; Fiorito and Córdoba de Torresi 2001; Tkac et al. 2002; Fei et al. 2003; Koide and Yokoyama 1999) has attracted extensive interest because it is a simple one-step process. Commonly used polymer films display size exclusion, charge exclusion, ion exchange, complexing, catalytic or conducting properties, or a combination of these, leading to improvement in selectivity, sensitivity, and other analytical

parameters. An ideal film is permselective only to the target species, and excludes undesired interfering species from the electrode surface. For example, Nafion acts as a cation exchanger with preference for hydrophobic cations (Kubiak and Wang 1996) and has been widely used for enzyme immobilization. Moreover, the Nafion-modified electrode could help to exclude the effects of some interferents, such as ascorbic acid and uric acid, particularly if ionized (Brown and Luong 1995; Zhou, Ju, and Chen 1997).

The goal of this study is the investigation of the use of ferrocene mediator in a glucose biosensor with glucose oxidase enzyme on a carbon film electrode support, and is part of a wider study, in which one of the aims is to investigate different enzyme redox mediators employing glucose oxidase as the model enzyme. These carbon film electrodes, which are small and adaptable as disposable or short-term-use sensors, are made from carbon film electrical resistors (Brett, Angnes, and Liess 2001). They have a wide potential window that can be extended by electrochemical surface pretreatment (Brett, Angnes, and Liess 2001; Filipe and Brett 2004) and have been investigated as disposable or renewable metal ion sensors (Filipe and Brett 2003) and biosensors (Florescu and Brett 2004, 2005; Ghica and Brett 2005).

Glucose oxidase was cross-linked with glutaraldehyde and modified with ferrocene. To prevent enzyme layer overswelling, bovine serum albumin (BSA) was also employed for immobilization. The electrochemistry of the enzyme-modified electrodes was investigated by cyclic voltammetry and applied to the measurement of glucose. In order to show the suitability of this biosensor for the analysis of natural samples, glucose in sweet Port wine was measured.

2. EXPERIMENTAL

2.1 Reagents

Glucose oxidase (GOx, EC 1.1.3.4, from Aspergillus niger, 24 U/mg) was from Fluka, α-D(+)-glucose, gluteraldehyde (GA) (25% v/v) and bovine serum albumin (BSA) were purchased from Sigma, Nafion (5% v/v) was from Aldrich and ferrocene was from Merck. Other reagents used were: D(−) fructose (Sigma), tartaric acid (PAHI, Portugal), citric acid (Merck), and ascorbic acid (Sigma). All other reagents were analytical reagent grade and solutions were prepared with ultrapure water obtained from a Millipore Milli-Q purification system (resistivity $>18\,\mathrm{M\Omega\,cm}$).

For electrochemical experiments, the supporting electrolyte was sodium phosphate buffer saline (NaPBS) (0.1 M phosphate buffer + 0.05 M NaCl, pH 7).

A stock solution of 1 M glucose was prepared in supporting electrolyte for at least 24 h prior to use for mutarotation to occur. The solution was

kept in the refrigerator. More dilute standards were prepared by appropriate dilution with 0.1 M NaPBS.

A stock solution of 6 mM ferrocene was prepared in ethanol, as in (Fiorito and Córdoba de Torresi 2001), since ferrocene is not soluble in aqueous solution. More diluted solutions for ferrocene incorporation by potential cycling were made by adding aqueous 0.1 M NaPBS.

2.2 Apparatus

All experiments were performed in a 15 cm^3 volume three-electrode cell containing the carbon film resistor electrode modified with GOx/ferrocene as will be described later, an Ag/AgCl (3 M KCl) electrode as reference, and a platinum coil auxiliary electrode.

Voltammetric (cyclic voltammetry) and amperometric measurements were carried out using a Bioanalytical Systems (BAS, West Lafayette, IN) CV-50 W electrochemical analyzer.

The pH measurements were carried out with a CRISON 2001 micro pH-meter. All experiments were performed at room temperature ($25 \pm 1^{\circ}C$).

2.3 Preparation of the Modified Enzyme Electrode

Electrodes were made from carbon film resistors (2 Ω nominal resistance), which are fabricated from ceramic cylinders (6 mm length, 1.5 mm diameter) by pyrolytic deposition of carbon, leading to a film of $\sim$15 μm thickness. The preparation procedure for the electrodes is described in Brett and coworkers (Brett, Angnes, and Liess 2001). Briefly, one of the gold-plated metal contact caps with connecting wire is removed to expose a cylindrical area of carbon film. The remaining contact wire is sheathed in plastic and the contact cap and junction covered with epoxy resin to insulate them. After this, the exposed electrode area is $\sim$0.20 cm^2.

The GOx was immobilized onto the electrode surface by the cross-linking method. A mixture of 5 μL of glutaraldehyde (GA) (2.5% in water), 8 μL of ferrocene (Fc) (6 mM in ethanol), and 15 μL of enzyme solution was prepared. The enzyme solution contained 40 mg of bovine serum albumin (BSA) and 10 mg of GOx in 1 mL of 0.1 M NaPBS (pH 7). From this mixture, 10 μL was placed onto the surface of the working electrode and allowed to dry at room temperature for at least 1 hour. Electrodes were kept at 4°C in phosphate buffer solution when not in use.

For electrode assemblies incorporating Nafion, two procedures were used:

- mixing 5 μL of Nafion (5% in ethanol) with the other components;
- covering the enzyme assembly with Nafion by dip coating in a 5% Nafion solution.

A further type of electrode assembly was prepared by first depositing the Nafion-containing enzyme mixture without mediator and afterwards incorporating the mediator in this Nafion/GOx film by cycling the potential in the range from 0 to 0.5 V vs. Ag/AgCl at a scan rate of 25 mV s^{-1} until a constant voltammogram was obtained, making use of the cation exchange properties of Nafion.

2.4 Analysis of Wine Samples

The standard addition method was used to determine the glucose concentration in wine samples after dilution: 10 μL aliquots were added to 10 mL of 0.1 M NaPBS.

Independent analysis of glucose concentrations was done using spectrophotometric enzyme assay kits (Cat 0 139 106, Boehringer, Mannheim).

3. RESULTS AND DISCUSSION

3.1 Deposition and Electrochemical Characterization of Ferrocene Films

Two different strategies for ferrocene incorporation were used. One was based on placing the previously prepared mixture onto the surface of the electrode and the other on electrochemical incorporation of ferrocene from a solution containing ferrocene in buffer, as described in the Experimental section.

Figure 1 shows the incorporation of Fc into a Nafion/GOx film from a solution of 6 mM Fc in ethanol (Fig. 1A) and 3 mM Fc in ethanol/NaPBS (Fig. 1B). It was more difficult to achieve this from the solution in ethanol since ethanol is not a good electrolyte. In ethanol/aqueous buffer solution, a concentration of 3 mM showed the maximum possible incorporation rate, from cyclic voltammetric peaks, owing to solubility limitations. The voltammograms show that ferrocene diffused into the Nafion/GOx film and that the mediator was retained.

The other strategy for electrode preparation was based on mixing components. A mixture of GOx with BSA and GA was placed onto the electrode surface, with or without Nafion, and left to dry at room temperature. In a third variant, Nafion was placed on top of the immobilized enzyme. Nafion is known to incorporate cations from solution by cation exchange, and this property has been used to provide a high local concentration of cation in films.

Figure 2 shows cyclic voltammetry of GOx/ferrocene electrodes in phosphate buffer saline, pH 7.0. In all cases one redox couple could be seen

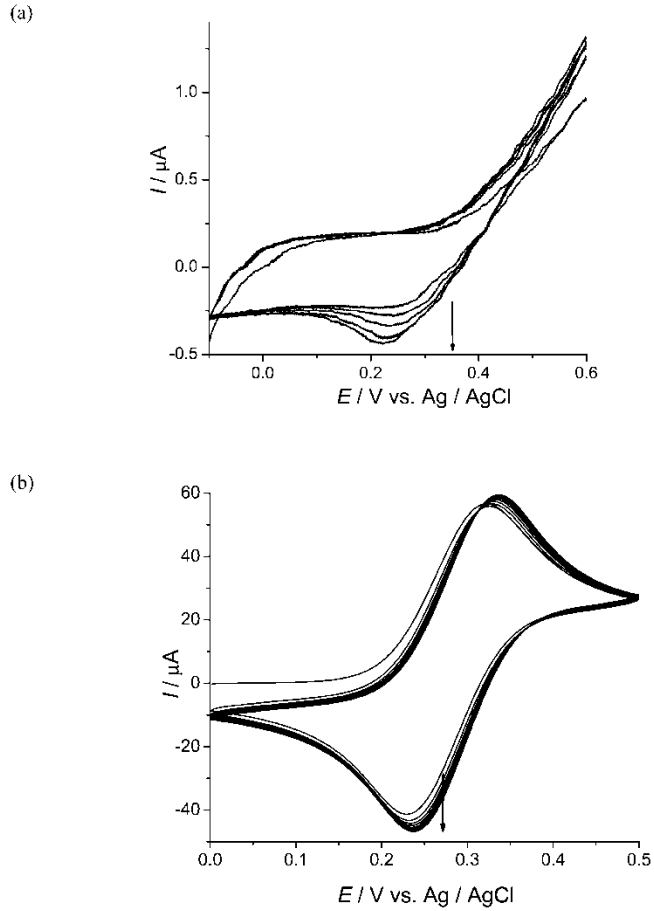

Figure 1. Continuous cyclic voltammetry showing the incorporation of Fc^+ into Nafion/GOx film from a solution of (a) 6 mM Fc in ethanol and (b) 3 mM Fc in ethanol/NaPBS (scan rate 25 mV s^{-1}). The arrows show successive scans.

corresponding to Fc $\leftrightarrow$ Fc^+. The observed currents were different at each type of film, the highest current being obtained when Fc was incorporated by cyclic voltammetry, and the lowest in the case of the mixture without Nafion.

3.2 Catalytic Response in the Presence of Glucose

The voltammetric profile of the enzyme electrode assemblies is influenced by the presence of glucose in solution. Figure 3 shows the cyclic voltammogram that was obtained in the absence (solid line) and presence (dashed

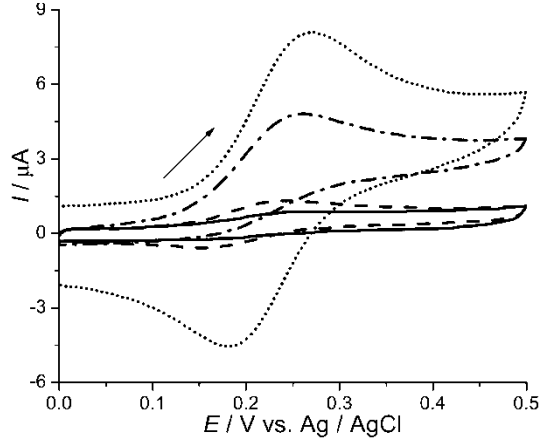

Figure 2. Cyclic voltammograms in NaPBS (pH 7.0) of GOx biosensor assembly (——) without Nafion, (------) with Nafion mixed, (- · · · -) with Nafion coating, and (· · · · ·) with ferrocene incorporated by cyclic voltammetry. See text for details.

line) of glucose. The catalytic current resulted from the added glucose, which diffused into the film and reacted with oxygen in the presence of glucose oxidase. A large increase in the oxidation current was observed at +0.25 V vs. Ag/AgCl, (Fig. 3), indicating that the ferrocene molecules immobilized in the film are efficient electron mediators between the electrode substrate and the redox centers of GOx.

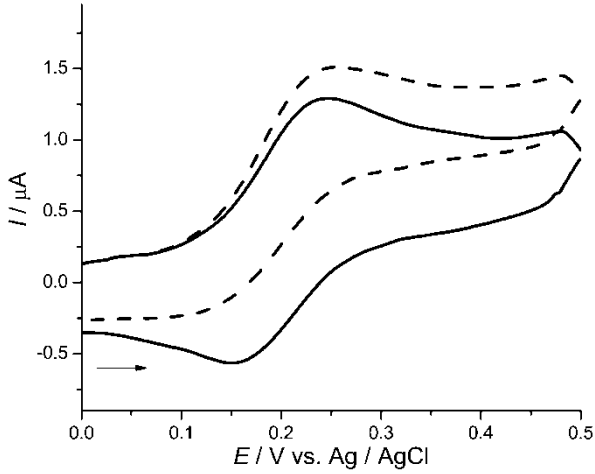

Figure 3. Cyclic voltammogram of (——) Fc/GOx modified electrode in buffer and (------) with addition of 5 mM glucose. Scan rate 50 mV s^{-1}.

The mediator action is expressed by:

$$O_2 + \text{glucose} \longrightarrow H_2O_2 + \text{gluconic acid}$$
$$Fc \longrightarrow Fc^+ + e$$
$$Fc^+ + H_2O_2 \longrightarrow O_2 + Fc + 2H^+$$

In the presence of glucose, the anodic wave increases whereas the cathodic current decreases, indicative of the enzyme-catalyzed process and successful immobilization of GOx.

3.3 Effect of Applied Potential

The amperometric response of the enzyme electrodes as a function of glucose concentration was studied at several applied potentials from 0.05 V to 0.4 V. The experiments were performed in electrolyte at pH 7.0—previous studies showed that the current response is essentially independent of pH over the range pH 6.5–8.0 (del Cerro et al. 1997). The results obtained showed that the anodic current increases continuously with increasing operating potential, up until 0.25 V, then it decreases again, Fig. 4. Therefore, a potential of +0.25 V vs. Ag/AgCl (~0.21 V vs. saturated calomel electrode (SCE)) was chosen for further studies. This represents a significant improvement with respect to other biosensors for glucose determination using ferrocene as mediator reported in the literature: +0.3 V vs. SCE (Tkac et al. 2002; Fei et al. 2003), +0.35 V vs. Ag/AgCl (Kase and Muguruma 2004), +0.4 V vs. Ag/AgCl (Li et al. 1997; Nakabayashi, Wakuda, and Imai 1998; Fiorito and Córdoba de Torresi 2001), +0.4 V vs. SCE (Miao et al. 2001),

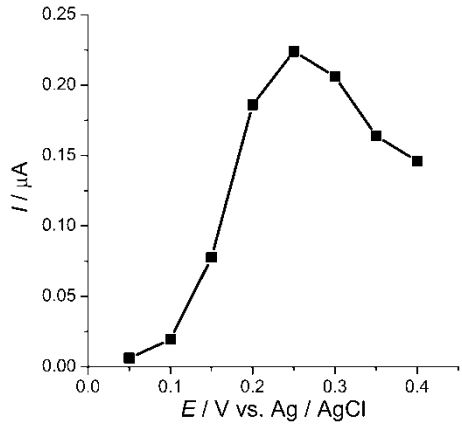

Figure 4. The effect of applied potential on the current response in the presence of 5 mM glucose.

or even +0.6 V vs. Ag/AgCl (Koide and Yokoyama 1999). Additionally, and if necessary in order to minimize interferences, lower potential values could be used, but the sensitivity would be lower.

3.4 Electrode Response to Glucose

The response to glucose additions was obtained by holding the electrode at 0.25 V (vs. Ag/AgCl) and monitoring the current, while aliquots of the glucose stock solution were added to air-saturated phosphate buffer saline. The signal measured corresponds to the electrochemical reduction of ferricinium ion. The response time to glucose was ~30 s since, to reach the electrode surface, the detected species must first diffuse through the polymer membrane.

All four types of electrodes were evaluated. Calibration curves are presented in Fig. 5 and data analysis is given in Table 1. The film obtained by cyclic voltammetric deposition of Fc gave the least good response. Electrodes with an outer coating of Nafion showed the best results even though this is not in agreement with what is expected from cyclic voltammetry, where this type of electrode had the second highest anodic peak. The response of electrodes without or with Nafion in the mixture was very similar. In all cases a large loss in response of about 33% after the first use was observed, probably due to loss of ferrocene into solution, then remaining approximately constant.

The electrode with a Nafion layer on top had the best performance and was chosen as the most suitable for sensing glucose. Therefore, all

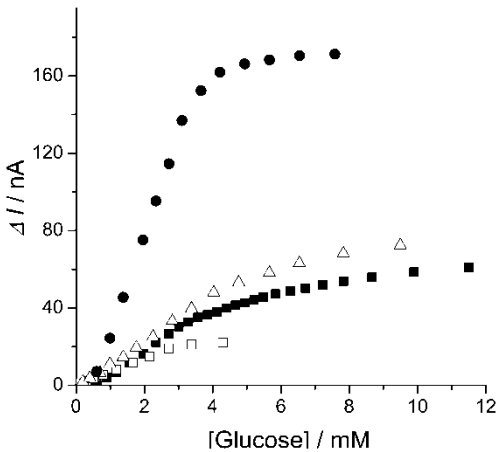

Figure 5. Calibration curves of glucose at Fc/GOx electrodes made in different ways: (■) no Nafion; (△) mixture with Nafion; (●) Nafion coating; (□) Fc incorporated by potential cycling.

Table 1. Data obtained from calibration curves for the four types of electrodes

Electrode type	Linear range (mM)	Sensitivity ($nA\,mM^{-1}$)	LOD (μM)	K_m (mM)
No Nafion	3.3	12.5 ± 0.2	113	6.4 ± 0.1
Nafion in mixture	4.0	12.1 ± 0.1	81	7.9 ± 0.4
Nafion coating	2.7	51.0 ± 2.2	66	4.1 ± 0.5
Fc dep. by CV	2.7	6.92 ± 0.07	44	3.3 ± 0.7

further measurements were performed at this kind of modified electrode. Additions of glucose resulted in a proportional increase in the anodic current up to a concentration of 2.7 mM (sensitivity $51.0\,nA\,mM^{-1}$), then became nonlinear. The detection limit was determined to be 66 μM glucose. The apparent Michaelis-Menten constant was determined from the Lineweaver-Burk plot. The K_m value obtained for the Nafion-coated biosensor was 4.1 mM. Both K_m and linear range are consistent with processes in which the rate of electrolysis is not sufficiently fast and substrate diffusion does not control the overall reaction rate, and, therefore, the apparent constant is intrinsically smaller than the Michaelis-Menten constant in homogeneous enzyme kinetics (Armada et al. 2003). Enzyme-modified electrodes with a Nafion layer on top are the most efficient electron transfer mediators, with lower apparent K_m values and higher amperometric responses.

The reproducibility of the electrodes modified with polymer films depends on the method of electrode preparation. The simplest method, which was used in this work, is one in which a drop of solution containing polymer is evaporated at the electrode surface. This method has a good reproducibility, with relative standard deviation of around 6.6% ($n = 5$).

The stability of the ferrocene-containing sensor is influenced mainly by the loss of ferrocene/ferricinium ions from the electrode surface. When electrodes were stored in buffer (pH 7.0) at 4°C when not in use, the critical period was the first week when the response to glucose decreased to 50%, then slowed. Nevertheless, the development of these electrodes is for use as short-term-use sensors, so this does not represent a problem of primary importance.

3.5 Interferences

Before sample analysis, an interference study was carried out. Substances tested as potential interferents for glucose were: fructose, lactate, tartaric acid, citric acid, and ascorbic acid. The results are summarized in Table 2.

Table 2. Interference of some compounds on response to glucose at GOx/Fc modified electrode at +250 mV working potential

Interferent	Ratio glucose:interferent	Relative response (%)
Fructose	1:1	100
	1:2	97
Lactate	1:1	100
	1:2	99
Citric acid	1:1	98
	1:2	93
Tartaric acid	1:1	96
	1:2	95

Under the experimental conditions used for glucose, no interference was observed from fructose or lactate when the glucose-to-interferent concentration ratio was 1:1. However tartaric acid and citric acid affected the amperometric response of glucose to a small extent, up to 4%. Nevertheless, the usual concentration levels of these substances in must samples (del Cerro et al. 1997) and sweet wines permit one to ensure that the glucose-to-interferent concentration will be significantly higher than 1:1 and, consequently, these substances would not interfere. In the case of wine samples, the concentration of citric acid present is not sufficient to give rise to noticeable interference to the glucose response. Tartaric acid can be present in these samples in the range $(6.7–40) \times 10^{-3}\,\mathrm{mol\,L^{-1}}$ (del Cerro et al. 1997), which could lead to a small decrease in response. However, in the presence of 0.1 mM ascorbic acid a big increase was noticed, which was due to the direct oxidation of ascorbic acid on the electrode; in such situations, ascorbic acid would need to be removed from the system first, for example by the use of ascorbate oxidase.

3.6 Analysis of Wine Samples

Since alcohol in wine is produced by bacterial decomposition of must sugars, must samples have a high concentration level of glucose ($0.44–0.72\,\mathrm{mol\,L^{-1}}$), whereas in normal wine samples the glucose concentration level is much lower ($2.8 \times 10^{-3}–5.6 \times 10^{-3}\,\mathrm{mol\,L^{-1}}$) (del Cerro et al. 1997). In sweet wines, a higher concentration of glucose is present, more than $0.27\,\mathrm{mol\,L^{-1}}$.

In order to demonstrate its suitability for the analysis of natural samples containing glucose, the sensor was applied to the determination of glucose in sweet Port wine. A commercial sweet Port wine sample was analyzed in batch injection mode by the standard addition method. The wine sample

was diluted 10^3 times and spiked with 0.3 mM aliquots of glucose. The glucose concentration value obtained ($54.9 \pm 1.2\,g\,L^{-1}$) was in good agreement with values obtained by the standard spectrophotometric method ($56.5 \pm 1.0\,g\,L^{-1}$).

4. CONCLUSIONS

Different strategies for preparing glucose biosensors based on carbon film electrode supports with a ferrocene mediator using the cross-linking method with glutaraldehyde and BSA to immobilize GOx were evaluated. Ferrocene was incorporated directly into the enzyme mixture, with Nafion being added to the mixture or applied as an external coating, or the ferrocene was subsequently incorporated by potential cycling. Of these, the analytical results were best from biosensors coated with Nafion at the optimized potential of 0.25 V vs. Ag/AgCl, which represents a lower, and therefore better, potential than was reported in the literature. The detection limit is 66 μM and the linear range extends to 2.7 mM.

The enzyme electrode is easy to fabricate, inexpensive, and the investigation of the analytical parameters demonstrated that ferrocene is an efficient mediator for biosensor production. Analysis of Port wine led to values in agreement with standard spectrophotometric values.

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