JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Rapid and Sensitive Automated Method for Glucose Monitoring in Wine Processing

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A rapid and sensitive automated method for glucose monitoring that might be employed during wine fermentation and processing was developed. A flow injection (FI) system coupled with an automated dilutor and the "redox-versatile" modified electrode were used to directly measure glucose in wine. To avoid interferences during wine analysis, different formulations of enzymatically modified carbon paste electrodes (CPE) were used and evaluated in oxidation and reduction mode. The best selectivity and sensitivity for glucose monitoring in real samples was obtained in cathodic mode at a fixed potential of 0 V versus Ag/AgCl using a CPE modified with glucose oxidase, horseradish peroxidase, and ferrocene as redox mediator. A total linear range of 0.02−50 g/L glucose was covered using this automated system and allowed the measurement of glucose in dry, medium, and sweet white or red wines without any sample pretreatment. The results showed a good correlation with the standard method, and the proposed method is very rapid, simple, and reliable and does not need skilled operators.

KEYWORDS: Wine; glucose biosensor; enzymatic electrode; automated flow injection analysis

INTRODUCTION

Wine production requires a strict monitoring of a series of parameters for a long period of time. The diversity of parameters and the complexity of this living liquid call for fast, selective, precise, and sensitive methods of analysis. The numerous papers presented in the literature relating to wine monitoring during the fermentation process illustrate the great interest expressed by industrial and research groups in the development of new techniques and methods of analysis (*1*−*7*).

The renaissance of electroanalytical chemistry induced by the concept of a "chemically modified electrode" has been of tremendous importance for the growing interest in this area and for the development of new electroanalytical techniques (*8*−*10*). Amperometric biosensors, particularly those based on enzyme-modified carbon paste working electrodes, are good candidates to respond to the high requirements in the field of electroanalytical techniques and devices (*1*, *11*−*13*). The carbon paste electrode (CPE) modified in its bulk with enzymes, mediators, or activators used in amperometric detection offers some major advantages such as rapid response and high specificity, low cost, and easy handling (*14*−*18*). Bulk modification of the entire CPE material allowed us to develop reagentless type biosensors that show high stability and are easy to use, particularly in food analysis (*19*−*23*).

The increasing need for automatic methods of analysis is obvious in all areas of chemical control, but it is more significant in areas that relate to public health such as clinical chemistry and food processing. Flow injection analysis (FIA) is an automatic method of analysis already used in industry for routine controls and processes monitoring. Due to their versatile character, several types of detectors including electrochemical biosensors can be coupled to FIA systems without major technical modifications.

The aim of our work is to develop a rapid and sensitive automated method for glucose monitoring in drinks based on a FIA system equipped with an enzyme biosensor and a programmed sample dilutor. This method can be used in end products such as stabilized wine or during production processes such as wine fermentation provided that samples are filtered. To avoid interferences and to obtain the best sensitivity and selectivity, we chose the "redox-versatile" carbon paste reagentless glucose oxidase (GOx) based biosensor that we developed in our laboratory (*24*). Such an amperometric biosensor can be used in either oxidation or reduction mode depending on the glucose concentration and on the presence of interfering products in the real samples. The present procedure for glucose measurement in wines is easy to use, can be easily automated, and offers a rapid and specific response that makes it attractive and useful for wine producers.

MATERIALS AND METHODS

Apparatus. All measurements in flow injection mode were done using the automated system injection analyzer (ASIA) system from

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10.1021/jf0494229 CCC: $27.50
Published on Web 08/10/2004

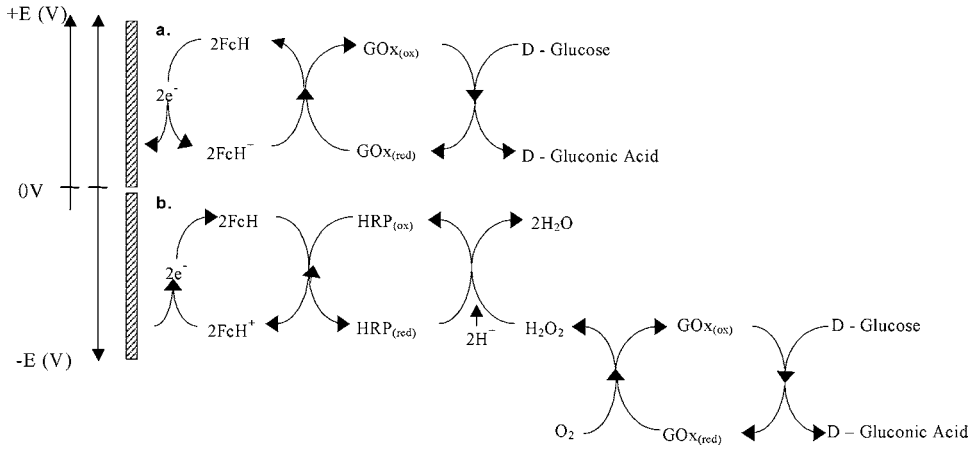

Figure 1. Reaction schemes of the redox-versatile modified CPE: (**a**) monoenzymatic mechanism; (**b**) bienzymatic mechanism.

ISMATEC (Glattbrugg, Switzerland), which incorporates an electrochemical potentiostat (type IS1001) and is driven and controlled by specific software (ASIA version 2.10). The UV−vis measurements were carried out with a Milton Roy Spectronic 401 spectrophotometer from Bioblock Scientific (Illkirch Cedex, France). The amperometric detector is a homemade three-electrode wall jet type electrochemical cell with an Ag/AgCl reference electrode, a Pt auxiliary electrode, and the modified carbon paste (CP) working electrode directly connected to the ASIA system. All connections necessary to ensure the FIA system functionality were made using Tygon tubes of different diameters and PTFE tubing (0.8 mm i.d.) from Bioblock Scientific.

Reagents and Materials. Glucose oxidase enzyme (EC 1.1.3.4) was obtained from Biozyme. Horseradish peroxidase (HRP, EC 1.11.1.7) was commercialized by Boehringer Mannheim. Bis(cyclopentadienyl)-iron (ferrocene, FcH) was used as electron-transfer mediator; the graphite powder and anhydrous D-glucose were available from Fluka. Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and potassium chloride (KCl), all in anhydrous form, were bought from Panreac. Paraffin oil distributed by Merck was used as pasting liquid. The UV enzymatic kit for D-glucose standard measurements was provided by La Roche. All solutions were prepared using distilled water.

Preparation of Biosensors. To obtain the best sensitivity, selectivity, accuracy, and reproducibility for our working electrodes, the carbon powder was modified in its entire bulk with enzymes and mediator. The redox-versatile electrode used during this work contains the GOx and HRP as enzymes and FcH that plays the role of electron-transfer mediator between the enzymes and the electrode surface. We already published in a previous work (*17*) the protocol for the preparation of GOx/HRP enzymatic carbon powder that is modified with approximately 20 U HRP and 5 units of GOx per milligram. For the preparation of graphite powders modified with only one enzyme, the step corresponding to the addition of the other enzyme is omitted during the preparation process.

The second step for the preparation of the modified carbon paste electrodes consisted of mixing the corresponding graphite powder with the pasting liquid. As pasting liquid we used the paraffin oil already modified with the mediator (ferrocene) in such a way that the final concentrations of FcH and paraffin oil in the paste were 0.32 and 24%, respectively. The modified carbon paste was packed in a plastic tube (0.3 cm i.d., apparent area $A = 0.07$ cm^2) provided with a metallic contact and a piston that helps to easily renew the electrode surface by simple polishing on a sheet of paper.

Procedure. The working electrode was incorporated in the homemade electrochemical cell in a wall-jet configuration. Measurements for glucose determination in wine were performed at an applied potential of 0 V versus Ag/AgCl in FI mode (a stable baseline of ∼10 nA is reached in 2 min). As carrier we used 0.1 M phosphate buffer solution, pH 7.2, containing 0.1 M KCl. The FI loop was of 50 μL, and all experiments were performed at a carrier flow rate of 0.9 mL/min in circuit of the electrochemical cell and 4.0 mL/min for the dilution path. The ASIA device is equipped with an automated diluting system operating between the sample injector and the electrochemical cell, and all parameters required for the automated determination of glucose in wine were optimized through the ASIA software.

RESULTS AND DISCUSSION

Optimization of the Electrochemical System. When one is working with amperometric biosensors in a very complex matrix such as wine, the selectivity and sensitivity will be very much dependent on the enzymatic and electrochemical reactions that take place at the surface of the electrode. Thus, for complex solutions the choice of the working potential is essential in order to avoid interferences and to keep the highest possible sensitivity. Therefore, we found it useful to take advantage of the performances of the FcH/HRP/GOx redox-versatile CPE to optimize an automated method for the glucose determination in wines. **Figure 1** illustrates the concept of the redox-versatile modified CPE and shows the two alternative enzymatic and electrochemical pathways that permit glucose determinations either in anodic or in cathodic mode depending on the sample composition. Both reaction sequences allow direct measurement of glucose in samples. Each pathway in the biosensor optimization was already presented in our previous papers (*14*, *17*, *25*). Thus, in this work we focused our interest on the use of the FcH/HRP/GOx redox-versatile CPE in association with the ASIA automated system to elaborate a simple and reliable assay method for glucose measurements in wines, avoiding interferences induced by many electroactive species present in the samples.

Figure 2 presents the hydrodynamic voltammograms recorded in FIA mode with different modified CPEs. The diagrams, recorded at different working potentials, illustrate the response of CPE to standard glucose solutions and wine samples when gradually modified with mediator and enzymes. Comparison of the responses obtained from glucose standard solution and wine clearly shows that the interference phenomena are seen on the anodic part of the hydrodynamic voltammograms. This is noticeable for all modified as well as unmodified carbon electrodes.

The monoenzymatic mechanism (**a**) presented in **Figure 1** is illustrated by curve 3 in **Figure 2A** recorded with GOD/FcH-modified CPE when solutions of 60 mg/L glucose were injected at the electrode surface held at different constant potentials. Curve 1 in **Figure 2A** presents the response of the FcH/HRP/GOx-modified CPE for the same glucose solution that corresponds to the mechanism (**b**) of **Figure 1**. The bienzymatic electrode (curve 1, **Figure 2A**) shows a very high sensitivity at cathodic potentials, whereas in anodic mode both curves (1 and

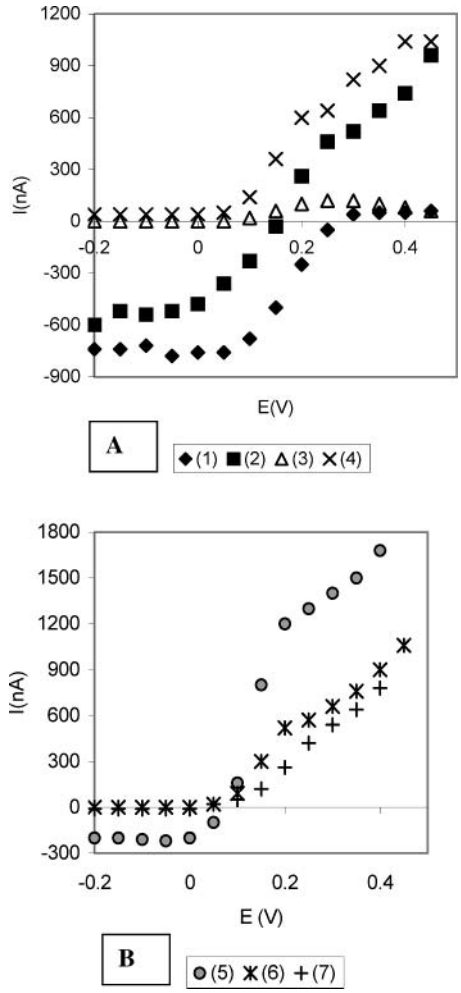

Figure 2. Hydrodynamic voltammograms recorded by successive FIA assays in a potential range –0.2 to +0.45 V versus Ag/AgCl with a potential step of 0.05 V. Samples: glucose standard solution (60 g/L) and diluted Muscadet wine sample. The compositions of the working CPE are as follows: (1 and 2) FcH/HRP/Gox, glucose and wine samples respectively; (3 and 4) FcH/Gox, glucose and wine samples, respectively; wine samples for (5) FcH/HRP, (6) FcH, and (7) unmodified CPE.

3) registered with bi- or monoenzymatic electrodes, respectively, indicate much lower anodic currents. This difference in sensitivities can be beneficially used to perform the glucose assay either in oxidation mode for concentrated solutions or in reduction mode for lower concentrations. This improves in many cases the accuracy of glucose measurements and also often facilitates sample preparation.

The glucose in a Muscadet white wine was measured using UV−visible enzymatic standard method (0.23 g/L). Appropriate dilution was made to bring this concentration equal to that of the standard glucose solution (60 mg/L) used in electrochemical measurements. Comparison of responses obtained with standard glucose solutions and diluted Muscadet white wine samples (**Figure 2A**, curves 1 and 3, on the one hand, and the corresponding curves 2 and 4, on the other hand) clearly indicates the occurrence of electrochemical or enzymatic interferences at the anodic side. An important increase of the signal of wine sample due to interferences is observed for applied potentials higher than +0.1 V versus Ag/AgCl.

To establish if interferences are resulting from electrochemical or enzymatic reactions, hydrodynamic voltammograms were recorded in wine samples using three other CPEs. In **Figure 2B** FcH/HRP-modified CPE, curve 5, showed the highest anodic currents for potentials more positive than +0.1 V versus Ag/AgCl; meanwhile, the current recorded at $E = 0$ V versus Ag/AgCl was negligible. Similarly, curves 6 and 7 in **Figure 2B** recorded with FcH-modified CPE and with an unmodified CPE, respectively, showed high anodic currents at potentials higher than +0.1 V versus Ag/AgCl. This indicates that interference is mostly due to the electrochemical oxidation of compounds, other than glucose, present in wine. Peroxidase (HRP) seems to further facilitate such anodic interference. The anodic interference is due to the easily oxidized polyphenols present in the wines.

On the basis of the results of the electrochemical study we came to the conclusion that for glucose measurements in wine using the FIA technique, interferences are detectable when the potential is set at a higher value than +0.1 V versus Ag/AgCl. For more cathodic potentials no interferences are visible. Furthermore, a high sensitivity is achieved with FcH/HRP/GOx-modified CPE for glucose measurements. The matrix of wine presents a large diversity, and its natural oxidative character has to be considered before the working potential is set. This is easy to do because of the large cathodic potential range available with this electrode.

Optimization of FIA Assays. To set up a simple automatic method for glucose measurement in wines, it is important to adjust beforehand the parameters that permit one to obtain, in a very short time, sensitive and repeatable signals starting from the crude sample and using the FI system controlled by the ASIA software. For this optimization, a homemade electrochemical cell, using FcH/HRP/GOx-modified CPE as working electrode, was adapted to the ASIA system and operated in a wall jet configuration.

The ASIA FI system is provided with three different automated flow circuits: the carrier flow for which the rate was fixed at 0.9 mL/min, the flow circuit used for the sample dilutions (rate fixed at 4 mL/min), and the circuit for the injection of diluted samples (flow rate fixed at 2.7 mL/min). All flow rates were evaluated and adjusted to reach the smallest measurement time for a minimum consumption of chemicals and samples.

Because the sample is sucked out automatically through the six-way valve, a few seconds is necessary to completely fill the injection loop. The effect of different loading times was examined, and we found that 30 s, for a flow rate of 2.7 mL/min, is the most efficient time to fill the injection loop while keeping a good repeatability of the resulting signal.

Other important parameters to optimize are the dilution time, because it affects the routine attainment of a homogeneous diluted sample, and the dilution factor, which permits the injected solution to be adapted to the linear range of the glucose calibration curve. Because the range of glucose concentrations in wines (sweet, medium, and dry) is very large, it was necessary to carry out a preliminary study to determine the dilution factors (displayed by a dilution time number in the ASIA system) that allow any wine to match the glucose calibration curve. The dilution time takes into account several parameters such as the flow of the sample and the volume of the dilution chamber. It is not directly proportional to the dilution factor. For a 50 μL injection of diluted sample the dilution time was established at 10 s for dry wines containing glucose at the level of 0.3−2 g/L, 45 s for medium wines (glucose = 2−10 g/L), and 70 s for sweet wines (glucose = 10−50 g/L). Once the FIA system is set up, sample measurements are rapid and easy to perform

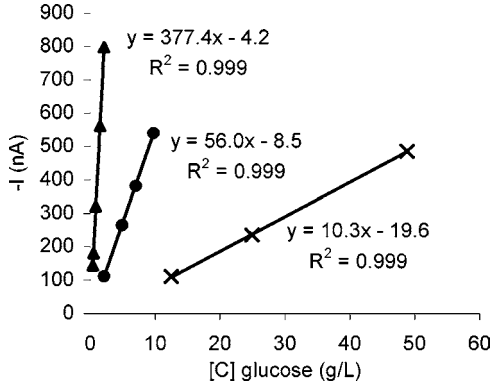

Figure 3. Calibration curves for glucose at different dilution times: (▲) 10 s dilution time (linear range = 0.3–2 g/L); (●) 45 s dilution time (linear range = 2–10 g/L); (×) 70 s dilution time (linear range = 10–50 g/L). FcH/HRP/GOD working electrode: $E = 0$ V versus Ag/AgCl.

using the ASIA software. Less than 3 min per measurement is necessary including the washing cycle between two consecutive runs.

Calibration Curves. The FcH/HRP/GOx-modified CPE used as working electrode at a potential of 0 V versus Ag/AgCl allowed us to obtain a cathodic current directly related to the glucose concentration in the injected sample. Thus, by using this working electrode in the FIA system the concentration of the injected sample can be calculated directly from a calibration curve previously recorded. When all other factors are identical, the "apparent" calibration curve parameters (in particular, the slope that represents the sensitivity) are dependent on the dilution time.

The "real" calibration curve for glucose concentrations was recorded by direct injection without automated dilution. The electrode presented a good linearity between 0.02 and 0.1 g/L glucose and high sensitivity ($y = 5417.1x - 22.8$; $R^2 = 0.9999$) and could be used for glucose determination in wines that had been previously diluted. The sample preparation to obtain the appropriate glucose concentration that fits the linear range of the real calibration curve (glucose concentration of ~0.06 g/L) is very often long and fastidious and could involve errors. On the other hand, the utilization of the automatic dilution process available in the ASIA system does not need any particular care and can be carried out quickly by unskilled operators; it is very flexible and shown to be repeatable. It can operate in a multichannel (six) configuration that considerably reduces the time, in particular, for repetitive measurements to follow an evolving process such as fermentation.

To cover the entire range of glucose concentrations in wines and to fit within the real linear range of the electrode, three calibration curves were recorded using the automatic dilution as presented above. The linear ranges for the "apparent" calibration curves are shown in **Figure 3**. These three calibration curves and the one obtained by direct injection cover the whole range of glucose concentrations commonly observed in wines (0.02−50 g/L).

The repeatability of measurements using the automatic dilution coupled with the bienzyme-modified electrode was also checked. After calibration, the results of nine successive injections of a glucose sample (0.87 g/L), with 10 s dilution time, showed a recovered glucose concentration of 0.870 ± 0.006 g/L and a relative standard deviation of 0.742%. Statistical results show the high accuracy and repeatability of this automated method. The biosensor also presents the advantage of a very high operational and shelf-life stabilities. No decrease in the biosensor response was observed after 50 successive measurements in flow mode, and no decrease of the sensitivity was found after 6 months when the bioelectrode was stored at 4 °C. Such advantageous behavior allows us to anticipate that such a biosensor coupled with an automated flow system can meet the industrial needs for routine assays (*26*).

Table 1. Glucose Assays in Commercial Wines ($n = 3$)

wine	[C] glucose (g/L) standard	[C] glucose (g/L) FIA/automatic dilution
Gros Plant du Pays Nantais (white)	0.13 ± 0.01	0.13 ± 0.01
Anjou (red)	0.12 ± 0.01	0.13 ± 0.01
Muscadet (white)	0.23 ± 0.01	0.23 ± 0.01
Saumur (white)	0.31 ± 0.01	0.33 ± 0.02
Saumur (red)	0.26 ± 0.01	0.26 ± 0.01
Coteaux de Layon (white)	9.45 ± 0.02	9.33 ± 0.07
Cabernet d'Anjou (rosé)	10.36 ± 0.02	11.02 ± 0.04
Muscat de Rivesaltes (white)	40.05 ± 0.02	41.47 ± 0.20
Porto (red)	43.23 ± 0.20	43.59 ± 0.10

Glucose Assays in Wines. The method was also tested to measure the glucose concentration in several commercial wines. All comparative measurements were run on a triplicate basis, and the results were compared with those obtained by the standard method using the enzymatic kit (**Table 1**). The plot of the values obtained by the two different techniques for glucose assays in wines showed a linear correlation ($C_{measured} = 1.02C_{standard} + 0.02$ with $R^2 = 0.9996$). It is obvious that the results recorded by the two methods are in very good agreement, indicating that the automated FIA method presented here is as precise as the standard method. The standard method (UV−vis method associated with enzyme kits) is time-consuming and often needs fastidious treatment of the sample. Indeed, the sample must first be diluted to fit the concentration range of 0.15−1 g/L. For an unknown glucose concentration this requires considerable preliminary work. After dilution, different chemical treatments are necessary to obtain a clear or colorless solution in order to avoid absorbance interferences. After the chemical discoloration, the sample has to be incubated with enzymatic reagent for 10−15 min before the absorbance reading can be made. Besides time and reagent consumption, the procedure presents the risk of numerous errors that might be introduced during dilution and other manipulations. Comparatively, in the FIA method the sample is automatically injected and if necessary diluted without any chemical pretreatment to match the very large glucose concentration range of 0.02−50 g/L. The average time for a measurement including the washing cycle is 2 min.

The electrochemical investigation using the redox-versatile modified CPE for glucose measurement in real wine samples showed that the complexity of the wine matrix induces numerous interferences during the analysis. It also indicates that the most sensitive and selective electrode to run the assays in wines is the FcH/HRP/GOx-modified CPE and the appropriate working potential is 0 V versus Ag/AgCl for analysis in FIA mode. The linear range reached with this modified CPE (0.02−0.1 g/L) was extended for the whole interval of glucose concentration in wines by coupling the electrochemical cell to the ASIA device provided with an automated dilutor. The device also presents the advantage of interactive software that permits control of the whole analysis process. Therefore, the parameters of the FIA automatic system and those of the bienzymatic working electrode were optimized to cover the glucose range from 0.02 to 50 g/L in real samples by making only the choice, among three possibilities, of the value of the dilution time. The

comparative analysis of glucose for different commercial wines with the standard method showed a very good agreement between the two methods. We showed in this paper that the FIA method combined with a redox-versatile CPE associated with the multichannel automated ASIA device allowed us to optimize a very rapid, flexible, and sensitive method to analyze glucose in wines. The automated method is rapid and can be used in an industrial environment and does not need highly skilled operators. It can easily be extended for glucose measurement in different food industries, such as fruit juices or fermentation, with only some simple alterations such as quick filtration or pH adjustment. We are currently working to enlarge this study to monitor other analytes, for example, lactic acid, malic acid, and ethanol, in real samples with new working electrodes.

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Received for review April 7, 2004. Revised manuscript received June 30, 2004. Accepted June 30, 2004.

JF0494229