Feature Article

Flow-Injection Analysis of Residual Glucose in Wines Using a Semiautomatic Analyzer Equipped with a Prussian Blue-Based Biosensor

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Abstract
The Prussian Blue modified glassy-carbon (GC) electrode assembled with glucose oxidase (GOD) immobilized in Nafion on the top of the electrode surface area has been inserted in a wall-jet cell connected to a semiautomatic analyzer, which controls all the operations for carrier buffer, flow and sample injection. The Prussian Blue based glucose biosensor inserted in the FIA analyzer has shown linear response to glucose at an applied potential of 0.0 V (Ag/AgCl) in the range of $10^{-6}-10^{-3}$ M. Residual glucose has been detected in red and white wines. A dilution of 1:1000 has been used for all the wines tested. Neither matrix effect, nor electrochemical interferences were observed. A comparison with a spectrophotometric glucose kit gave excellent correlation for levels of glucose detected. FIA analyzer compared with the standard analytical kit resulted in simplified procedures and a reduced time for the analysis.

Keywords: Prussian Blue, Glucose biosensor, FIA analyzer, Wine analysis

1. Introduction

During recent years scientists have made many attempts to use fast responding enzyme electrode probes and at the same time avoiding all the interferences when the probes are used in real matrices. One of the most promising transducers, which provide these two features, is the Prussian Blue modified electrode. At an applied potential lower than 0.15 V (Ag | AgCl) a thin Prussian Blue layer serves as highly active and selective electrocatalyst for hydrogen peroxide reduction, which provides analytical applications of the corresponding sensor [1].

Since 1994, [2] many articles have appeared in the literature describing features of Prussian Blue as an attractive H_2O_2 sensor. Also the assembling and use of glucose biosensors made with glassy-carbon or graphite [3–8] and more recently with carbon paste electrodes [9] in batch and FIA systems has been extensively studied. In spite of the growing interest in these systems, which show a high degree of sensitivity and selectivity, not so many applications in real samples have been made, which, however, the only ones can be used for validation of sensors.

One of the most attractive areas for biosensor applications is the food quality control. Analysis of wines and other beverages attracts a particular attention since it requires only a minor sample preparation procedure. A number of chromatographic, spectrometric and electrochemical detection systems were designed for carbohydrates [10], L-lactate [11, 12], L-malate [13], glycerol [14], alcohols [15], phenolic [16], sulfuric [17] and aromatic [18, 19] compounds. Spectrometric and chromatographic methods of analysis including mass-spectrometry, HPLC, micro-extraction and electrophoresis in some cases are more sensitive than electrochemical ones but much more laborious and expensive.

The direct analysis of beverages with biosensors appeared to be the most effective (it does not require separation stages), fast and inexpensive. The main problem for direct analysis is the interfering effect of reductants on sensor response. The influence of reductants can be decreased by lowering the electrode potential, which is attained in Prussian Blue based biosensors due to hydrogen peroxide reduction at an applied potential where only a minor oxidation of the majority of interferents occurs. Moreover the enzyme immobilization is performed by the formation of a polyelectrolyte Nafion membrane, which is known to be a barrier for interferents [20, 21].

The aim of the present investigation was to show the applicability of the Prussian Blue based biosensors for analysis of real food samples. Italian wines have been chosen as real food objects. In order to carry out automatic analysis a specially designed semiautomatic FIA system has been used. This apparatus was equipped with a Prussian Blue based glucose biosensor and applied to the analysis of the residual glucose in some sweet and dry wines.

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2. Experimental

2.1. Apparatus

FIA-analysis has been carried out with the FIA analyzer "Practik-NC" (Zelenograd, Russia). The analyzer includes a homemade flow-through wall-jet electrochemical cell, a hydraulic device block (buffer solution pump, testing solution pump and injector), a potentiostat, a managing unit and a program unit, installed on PC. The electrochemical cell was equipped with the GC working electrode (diameter 1.8 mm), Ag/AgCl/1 M KCl reference and stainless steel counter electrodes. The nozzle (diameter 0.5 mm) is positioned normally to the working disk electrode surface at a distance from 1 to 2 mm. Cell contacts are connected to the inlet of an intelligent amplifier. A built-in potentiostat unit provides maintenance of a constant potential and three-electrode output to the cell. A digital device measures currents with high accuracy and ensures input of the measured information to the microprocessor. When the device is on, the buffer solution pump switches on at programmed speed. The device is ready to operate after the current output achieves a constant level. According to the input from PC the testing solution pump fills in the injection loop. Then the user may switch the injector in the injection position and the measurement is started. When the current reaches the background level (which is indicated), the user may switch the injector to the base position. The program automatically records the results of measurements and graphically represents them on the PC screen. This program allows to accumulate measurements and to store them on a hard disc, to process the previously measured results, to retrieve them with the possibility of scaling and printing.

The electrochemical deposition of PB on the GC-electrode surface was carried out in a three-electrode cell. Galvanostatic regime, open current potential regime (OCP) and cyclic voltammetry (CV) were performed using an Autolab electrochemical system equipped with a PGSTAT 10 and GPES software (Eco Chemie, Utrecht, The Netherlands).

2.2. Reagents

Glucose Oxidase from *Aspergillus niger* (GOD) (E.C.1.1.3.4 type V11, 176 U/mg), Bovine serum albumine (BSA), hydrogen peroxide and ascorbic acid, Nafion, D-glucose and all inorganic salts were obtained from Sigma (St. Louis, MO).

D-glucose stock solution (10^{-2} M) was prepared in 0.05 M phosphate buffer (pH 6) with 0.1 M KCl and left in a refrigerator overnight to reach mutarotational equilibrium. Ascorbate stock solution (10^{-1} M) was prepared in the same buffer.

2.3. Procedures

2.3.1. Electrosynthesis of PB

The GC-electrode was polished using Al_2O_3 powder (0.5 μm), then carefully rinsed with distilled water. The Prussian Blue growing solution was 4 mM $K_3Fe(CN)_6$ and 4 mM $FeCl_3$ in 0.1 M KCl with 0.1 M HCl. The GC-electrode was immersed into the growing solution and PB was deposited in galvanostatic mode at a current density of 50 μA cm^{-2} for 60 s. The post-treatment of Prussian Blue modified electrodes was made as described elsewhere [22]. The deposited PB was activated by cyclic voltammetry in a potential range from − 50 to 350 mV (20 cycles) at the scan rate of 40 mV/s in a solution containing 0.1 M KCl with 0.1 M HCl. After the activation step, the electrode was heated in the oven at 100 °C for 1 hour. After cooling, the electrode was cycled from − 50 to 350 mV at a scan rate of 40 mV/s (10 cycles) in 0.05 M phosphate buffer (pH 6) containing 0.1 M KCl and dried at room temperature.

2.3.2. Enzyme Immobilization

An enzyme-containing Nafion membrane was deposited on the PB-modified GC-electrode according to a procedure reported elsewhere [23]. The commercial Nafion solution (5%) was diluted with absolute ethanol to a final concentration of 1% and neutralized by 25% (v/v) NH_4OH to 5.5 pH. The resulting solution was diluted with absolute ethanol to a final Nafion concentration. The casting solution was prepared by mixing of 8 μL of aqueous GOD solution (20 mg/mL) and 100 μL of 0.3% Nafion solution. Afterwards 5 μL of casting solution was syringed onto the Prussian Blue modified electrode and left for 5 – 7 minutes at room temperature. BSA-Nafion membranes were made similarly taking BSA instead of GOD.

2.3.3. FIA-Analysis

FIA-measurements were carried out at 0.0 V potential (Ag | AgCl | 1 M KCl). The flow rate was 1 mL/min, the injection volume was 50 μL. The carrier solution was 0.05 M phosphate buffer (pH 6) with 0.1 M KCl.

2.3.4. Analysis of Wines

Wines obtained from local wine shops (Rome, Italy) were diluted 1:1000, 1:100 and 1:50 times with the carrier solution. Sparkling wines were degassed for 2 minutes before the analysis to remove CO_2. Buffer was first passed through the electrochemical cell until a current baseline was reached. Then samples of diluted wines were automatically injected into the cell and the current variation due to the glucose reaction was recorded and related to glucose concentration in wines. For the standard addition procedure, aliquots of glucose standards were added to the 1:1000 diluted wines. Glucose standards were prepared for each wine probe in accordance to the measured glucose content

using calibration plot. After addition of one aliquot of final glucose concentration the probe was expected to be increased twice.

3. Results and Discussion

The FIA analyzer equipped with Prussian Blue modified electrode without any additional covering was first tested for hydrogen peroxide detection. The latter was detected in the range of 10^{-6} M – 10^{-3} M, and the response (peak current) showed linear dependence on the H_2O_2 concentration. The equation of the regression line was $y = -0.07 + 66959x$, with $r^2 = 0.999$. The detection limit defined as the lowest detectable concentration was of 10^{-6} M. Since the glucose biosensor operates due to detection of the hydrogen peroxide produced, we checked possible interferences on H_2O_2 detection. Two different probe preparations were performed: one with Nafion-GOD and the other with Nafion-BSA to test any possible protein interference on the deposited Prussian Blue. Calibration plots of H_2O_2 run with these two probes did not show any significant difference in current values but did shown a slightly decreased sensitivity as compared to the uncovered Prussian Blue (data not shown). This demonstrated only a minor influence of the membrane protein component on the reduction of H_2O_2 on PB modified electrodes.

After the test with H_2O_2, the GOD-Nafion probes were used to run calibration plots with glucose. The Nafion-GOD probe showed for glucose the same behavior as for H_2O_2 giving current variations in the same range (Fig. 1). Calibration plots attained with the BSA probe did not show any current variation to consecutive injections of glucose (data not shown). The Nafion-BSA probe was then used to control the electrochemical interferences such as ethanol and ascorbic acid that can be found in real matrices as wines. Ascorbate 10^{-4} M, which is the concentration 10 times higher than that found in wines [24], prepared in phosphate buffer and analyzed with the "working" and BSA reference probes did not give any current signal. The same results were obtained testing standard solutions of ethanol at a concentration similar to those contained in wines diluted up to 1:50.

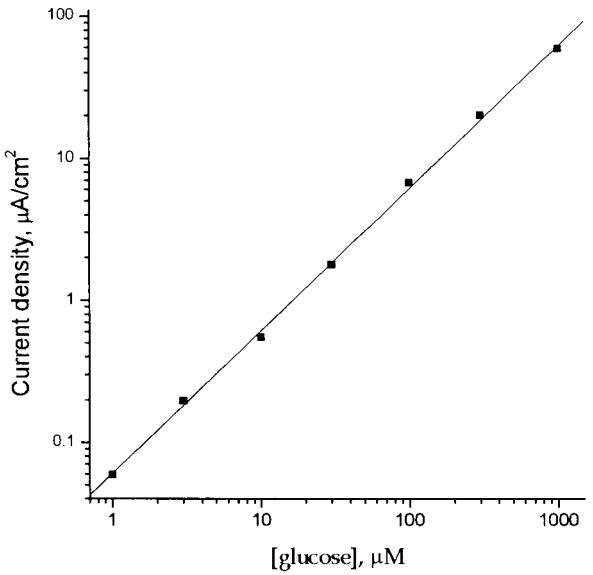

Fig. 1. Calibration plot for FIA analyzer equipped with the PB-based glucose biosensor.

The calibration of the FIA analyzer equipped with the Prussian Blue based glucose biosensor (Nafion-GOD probe) gave linear dependence in the concentration range from 10^{-6} to 10^{-3} M (Fig. 1). The sensitivity calculated from the calibration plot is 0.06 A $M^{-1}cm^{-2}$. The response obtained by the injection of a glucose free solution did not exceed the background noise. The reproducibility of the response peaks for definite glucose concentrations estimated from 5 independent measurements corresponded to the standard deviation value of 4 – 6%. The time for a single glucose measurement was 2 – 3 minutes, after which the analyzer was ready for further injections.

The stability of the biosensor inserted in the FIA analyzer was independently checked. Several calibrations recorded daily with the same biosensor showed only a minor deviation (less than 15%) obviously caused by the temperature changes in the lab (data not shown). The sensitivity always slightly increased in the row: morning < evening < midday.

Table 1. Flow-injection glucose analysis using FIA analyzer equipped with PB-based glucose biosensor in comparison with spectrophotometric assay

Wines	*Glucose (mg/L)*		
	Calibration plot	*Standard addition*	*Spectrophotometric*
Moscato giallo Alto Adige (white)	15.0 ± 0.5	10.4 ± 0.4	16.0 ± 0.2
Prime Rose Cavit (red)	2.0 ± 0.1	2.0 ± 0.2	2.1 ± 0.1
Moscato Giallo Trentino (white)	13.2 ± 0.4	15.3 ± 0.7	12.0 ± 0.6
Pellegrino Zibibbo (red)	53 ± 2	56 ± 2	50 ± 3
Donelli Lambrusco Emilia (red)	6.1 ± 0.1	7.3 ± 0.1	6.3 ± 0.1
Cannellino Frascati Fontana Candida (white)	9.0 ± 0.2	8.4 ± 0.2	8.6 ± 0.1
Moscato Tosti (gased, white)	21 ± 1	20 ± 1	20 ± 1
Malvasia Giorgi (white)	24.2 ± 1.5	16.2 ± 0.4	25.5 ± 0.2
Lamberti Pinot Grigio (red)	0.40 ± 0.03	0.65 ± 0.06	1.0 ± 0.01
Olevano Romano Cesanese (red)	12.0 ± 0.7	7.9 ± 1.4	12.1 ± 0.1

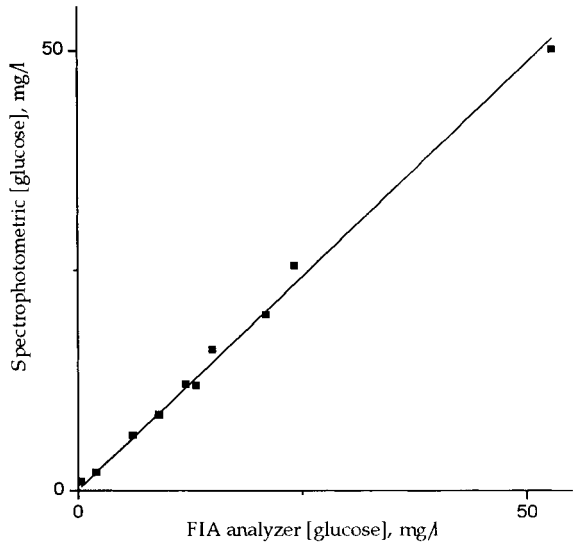

Fig. 2. Correlation between FIA detection (PB-based glucose biosensor) using calibration plot and spectrophotometric assay in wine analysis.

After the glucose detection in model solutions, measurements were carried out in sweet and dry wines. First, interference and matrix effect were tested analyzing the real samples with the BSA assembled probe. All the wines, diluted 1 : 1000, 1 : 100 and even 1 : 50 did not give any current response, showing neither matrix effect nor interference by those compounds such as polyphenols known to be present especially in red wines [25]. Glucose was measured in diluted wines (1 : 1000) both using the calibration plot and also using the method of standard additions. The analyzes of all samples were performed in triplicate and each value was the average of three measurements.

Table 1 shows the results of glucose detection for 9 different wine samples. The standard deviations were calculated from 3 to 5 experimental data. It is seen that the results of wine analysis with the use of FIA analyzer equipped with Prussian Blue based biosensor are in excellent agreement with the spectrophotometric assay, in particular, when the glucose concentration was calculated from the calibration plot. The only one significant difference observed for was the Lamberti wine sample, whose glucose content 0.4 mg/L (Table 1) was lower than the spectrophotometric detection limit (1.2 mg/L) [26].

Glucose content in wines measured using the standard addition method was in good correlation with the results calculated from calibration plot. However, for 3 samples besides Lamberti the difference was quite significant. To explain this, we note that the standard addition method includes linearization of the measurements and extrapolation of the slope to find an intercept with the abscissa. Obviously, the derivation of the experimental data (linearization in our case) could cause a significant increase in the error. Indeed, in our case the standard addition method gives in certain cases significant deviations from both spectrophotometric technique and the FIA measurements when the concentration had been calculated using the calibration plot.

Figure 2 shows the correlation of the data obtained using spectrophotometric assay (third row of table 1) and those measured with the analyzer and calculated from the calibration plot (first row of Table 1). It is seen that the data do not significantly deviate from the straight line forced to intersect the origin. The slope of the line was found to be 0.97. Thus, the data measured with analyzer and obtained using the standard reference method are indeed in good agreement.

It is important to note, that the standard spectrophotometric assay is rather complicated and time consuming. A single glucose analysis requires several operations of a qualified assistant and takes 20 – 30 minutes. For comparison, glucose detection using FIA analyzer is nearly operator-free and takes only 2 – 3 minutes.

4. Conclusions

We conclude that the application of Prussian Blue based glucose biosensors in wine analysis was extremely successful. Neither matrix effect, nor electrochemical interference of known compounds (ascorbate, phenols) was observed. A comparison with the standard spectrophotometric glucose kit gave excellent coincidence in levels of glucose detected.

The semiautomatic FIA analyzer showed a potential for application to analysis of real samples. Being equipped with biosensors, it provided both simplification of glucose detection and the drastic reduction of operator-dependent procedures.

We are looking forward in application of our bioanalytical system to food research and clinical diagnostics, where biosensors and related devices are expected to play the key role.

5. Acknowledgements

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6. References

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