Article

J. Mex. Chem. Soc. **2011**, *55(2),* 120-125

ISSN 1870-249X

Determination of Glucose by Flow Injection Analysis with Amperometric Detection at Fe(III)-(tris(3,5-dimethyl-1-pyrazolyl)borate)$_2$ Modified Electrodes

Prisciliano Hernández,[1] José A. Rodríguez,[1*] Verónica Salazar,[1] Giaan A. Álvarez-Romero,[1] Simplicio González-Montiel[1] and Yolanda Castrillejo[2]

[1] Universidad Autónoma del Estado de Hidalgo. Centro de Investigaciones Químicas. Carr. Pachuca-Tulancingo Km. 4.5, 42076, Pachuca, Hidalgo, Mexico. josear@uaeh.edu.mx

[2] Universidad de Valladolid. Facultad de Ciencias. Departamento de Química Analítica. Paseo Prado de la Magdalena s/n, 47005. Valladolid, Spain

Received December 8, 2010; Accepted March 22, 2011

Abstract. A modified screen printed electrode with [Fe(tris(3,5-dimethyl-1-pyrazolyl)borate)$_2$]$^+$[FeCl$_4$]$^-$, installed as part of a flow injection system, was used as an amperometric detector to determine hydrogen peroxide and glucose after on-line reaction with glucose oxidase. The influence of experimental variables on sensitivity, such as the flow rate, injection volume and reaction coil length, was evaluated using a two-level factorial experimental design. Under optimal conditions, detection limits of 15 μM and 0.5 mM for H_2O_2 and glucose, respectively, were obtained. A sampling rate of 60 determinations h^{-1} was achieved; the relative standard deviation of analytical measurements was <5.0%. The method was validated by comparing the obtained results with those provided by the enzymatic spectrophotometric method; no significant differences were observed ($p < 0.05$).
Keywords: Glucose, Hydrogen peroxide, Flow injection system.

Resumen. Un electrodo plano de carbono modificado con [Fe(tris(3,5-dimetil-1-pirazolil)borato)$_2$]$^+$[FeCl$_4$]$^-$, se integró en un sistema de inyección en flujo y se aplicó en la determinación amperométrica de peróxido de hidrógeno y glucosa mediante la incorporación de glucosa oxidasa. Se determinó la influencia de las variables experimentales como, caudal de trabajo, volumen de inyección y longitud del reactor sobre la sensibilidad mediante un diseño factorial completo a dos niveles. En condiciones óptimas se obtiene un límite de detección de 15 μM y 0.5 mM para H_2O_2 y glucosa, respectivamente, así como una velocidad de análisis de 60 determinaciones h^{-1}, con una repetitividad expresada como desviación estándar relativa <5.0%. El método propuesto se validó comparando los resultados obtenidos con la metodología espectrofotométrica enzimática, no encontrándose diferencia significativa entre los resultados obtenidos por ambas metodologías ($p < 0.05$).
Palabras clave: Glucosa, Peróxido de hidrógeno, Sistema de inyección en flujo.

Introduction

The clinical study of glucose is highly important as this monosaccharide is the major source of energy in the human body. An alteration on its concentration causes several metabolic disorders such as hypoglycemia and hyperglycemia; therefore, glucose monitoring is of vital importance [1, 2]. There are several methods for glucose determination; however, enzymatic assisted methods are the most used in clinical and food industry. The enzyme glucose oxidase (GOx) is highly selective towards the analyte of interest; it serves as a catalyst accelerating the room temperature reaction for the generation of gluconolactone and hydrogen peroxide from glucose according to the following reaction [3-5]

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconolactone} + H_2O_2$$

The H_2O_2 produced is detected by different methods including: spectrophotometric [6, 7], chemiluminescence [8] and electrochemical [9]. Electrochemical detection (amperometric) is one of the most widely used techniques because the system can be miniaturized. H_2O_2 amperometric detection is performed at high potential values, with the adsorption of reaction products taking place at the biosensor surface, limiting the lifetime of the electrode [10, 11]. In order to decrease this effect, the electrochemical transducer includes the use of mediators such as ferricyanide and ferrocene derivatives [12]. This inclusion provides electronic transfer whilst diminish the working potential and the influence of oxygen on the biosensor response, thus minimizing the interference from other species contained in the sample [12-14]. Glucose determination is based on the enzymatic oxidation of the analyte according to the schematic diagram (Figure 1).

The increasing demand for glucose analysis during recent years requires the design of semi-automated and automated methodologies in order to reduce sample analysis rate without affecting the precision and accuracy [8, 15]. Flow injection analysis (FIA) is a well established automated technique with

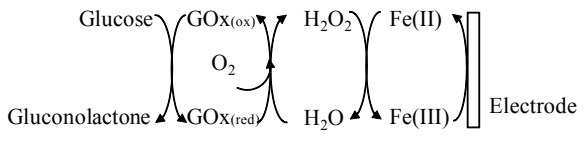

Fig. 1. Reaction scheme for a redox mediated glucose determination.

numerous applications in quantitative chemical analysis; it is a simple and inexpensive technique [16]. However, the use of disposable electrodes with short lifetimes limits its application in automated methodologies.

Poly(pyrazolyl)-borates have found wide application in coordination chemistry, and their complexes with most metals or metalloids of the periodic table have been prepared. Throughout the years tris(pyrazolyl)borate (Tp) has been compared to cyclopentadienyl ligand [17], but the application of Tp complexes as mediators in biosensors has not been previously reported. With the arguments stated above, this work proposes the determination of glucose by FIA with amperometric detection at screen printed electrodes modified with [Fe(tris(3,5-dimethyl-1-pyrazolyl)borate)$_2$]$^+$[$FeCl_4$]$^-$ as mediator. The methodology is based on the detection of H_2O_2 generated during the enzymatic reaction with GOx.

Table 1. Regression parameters of the calibration study, current (μA) vs [H_2O_2] (μM).

Parameter	Value
Square root of residual variance, s_e	0.019
Number of standards	5
Determination coefficient, r^2	0.991
Intercept confidence interval, b0 ± t × s(b0)[a]	−0.05 ± 0.02
Slope confidence interval, b1 ± t × s(b1) (μA μM^{-1})[a]	$3.7 \times 10^{-3} \pm 2 \times 10^{-4}$
Repeatability (%RSD, n = 3, 117.0 μM)	3.93
Linear range (μM)	29-147
Limit of detection (μM)	15
Sampling rate (samples h^{-1})	60

[a] $t_{crit\ (0.05;\ 3)} = 3.18$.

Results and discussion

Optimization of flow parameters for the determination of H_2O_2

In order to understand the behavior in a FIA system with electrochemical detection, the effects of pH and flow rate on the amperometric response for the determination of H_2O_2 were evaluated using univariate method. To decrease the interference of other oxidizing agents and to guarantee a low diffusion in the laminar flow the following conditions were selected: applied potential (E), 0.0 V; reaction coil length, 30 cm; injection volume, 100 μL; using a H_2O_2 concentration of 147 μM.

The effect of the pH value in the carrier solution was evaluated from 4.0 to 9.0 (using Britton-Robinson solutions). The obtained signal intensity showed an increase with pH (Figure 2.a). However, as the optimum pH for GOx was in the range of 7.0-7.5, a pH of 7.0 (phosphate buffer solution, 0.1M) was selected to simulate physiological conditions of blood analysis [18], and to simplify the FIA system for glucose determination. Higher pH values require an additional channel for pH adjustment, resulting in a dilution of the sample thus decreasing the signal height.

The effect of flow rate (Q) was evaluated in the range of 0.2 to 0.5 mL min^{-1}. The peak height increased with flow rate (Figure 2.b). A flow rate of 0.5 mL min^{-1} was selected as the most adequate because the kinetics of the enzymatic reaction restricts the use of higher flow rates.

Once evaluated and optimized the flow parameters (E = 0.0V, reactor length 30 cm, flow rate 0.5 mL min^{-1} and injection volume 100 μL) a standard curve for H_2O_2 was constructed. Each standard was analyzed in triplicate and the mean values plotted. Table 1 and Figure 3 show the regression parameters and FIAgram obtained, respectively. The limits of detection were calculated according to IUPAC criteria [19];

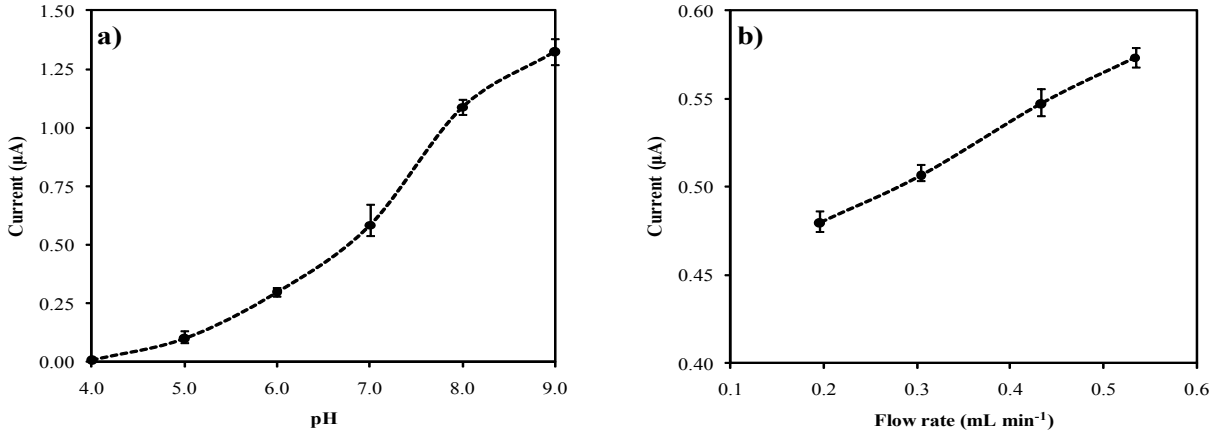

Fig. 2. a) Effect of pH on the analytical signal. Conditions: Q = 0.5 mL min^{-1}, injection volume, 100 μL [H_2O_2] = 147 μM; b) Effect of injection flow rate on the analytical signal. Conditions: carrier solution, phosphate buffer (0.1 M, pH 7), and injection volume of 100 μL, [H_2O_2] = 147 μM.

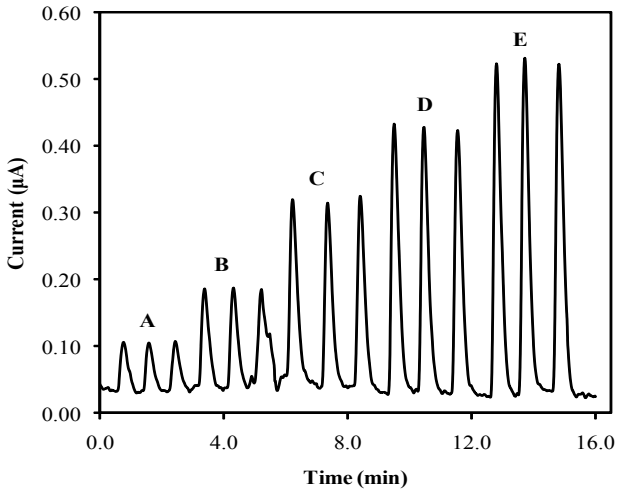

Fig. 3. Recorder output for H_2O_2 calibration plot: A) 29, B) 59, C) 88, D) 117 and E) 147 μM.

i.e. three times the value of s_e/b_1, where s_e is the square root of the residual variance of the standard curve and b_1 is the slope. The obtained linear range was between 29 to 147 μM with a detection limit of 15 μM. These values are comparable to those obtained using other mediators [9, 20, 21].

Optimization for glucose determination

In analytical flow techniques, the desired response is the maximum signal (current, μA). The flow variables optimized in FIA systems are: the flow rate (Q), reactor length (R), and injection volume (Vi) [22]. In order to identify the critical parameters affecting glucose determination, a complete factorial design at two levels was used. The potential and carrier solution were set at 0.0V and pH 7.0 (phosphate buffer solution, 0.1M). The selected design consists of $2^3 = 8$ experiments carried out in duplicate, where each factor is evaluated at two values: high (+) and low (–). Table 2 shows the levels chosen for each factor and Table 3 contains the combination of factors and levels used during the evaluation.

Suitable treatment of these data revealed the mean effect exerted by each factor on the glucose signal (Table 4, column 1). In turn, these values enabled us to calculate the variance of each factor using the Yates algorithm (Table 4, column 2). By comparing the variance shown by each factor with the variance of the residual (2.8×10^{-3}), a Fisher F-test was then performed for each source of variation. This test indicated at a significant level of $p = 0.05$, that the critical factors were the reactor length and the interaction Vi-Q. Both variables are related with diffusion processes and reaction rate during the sample transport from the injection valve to the detector. Table 4 and Figure 4 show the effect of the control factors on the peak height, among which the reactor length, R, is the most important. Based on the results shown in Figure 4, the combination of settings that allowed the highest peak height was: injection volume 30 μL, and flow rate at 0.5 mL min^{-1}.

Table 2. Control factors settings for the optimization experiment.

Factors	Levels	
	Low(–)	High(+)
Reactor length (R) (cm)	50	100
Flow rate (Q) (mL min^{-1})	0.5	1.5
Injection volume(Vi) (μL)	30	100

Table 3. Matrix design and experimental results.

R	Q	Vi	Signal height	(μA)
–	–	–	0.251	0.252
+	–	–	0.459	0.389
–	+	–	0.138	0.138
–	–	+	0.221	0.236
+	+	–	0.281	0.251
+	–	+	0.244	0.197
–	+	+	0.196	0.162
+	+	+	0.407	0.363

Table 4. *F*-Factors obtained by the two-level factorial experimental design used to evaluate the effect of flow variables on the amperometric signal during glucose determination.

Factor	Effect	Variance	$F_{calculated}$
R[a]	0.125	0.062	21.939
Q	–0.039	0.006	2.162
Vi	–0.017	0.001	0.390
R-Q	0.042	0.007	2.536
R-Vi	–0.026	0.003	0.928
Q-Vi[a]	0.097	0.037	13.188
R-Q-Vi	0.065	0.017	5.899

[a] Factors in bold differ significantly with respect to the ANOVA results ($F_{calculated} > 7.571$ at the 95% confidence level).

The reactor length was optimized by univariate method; the peak height (μA) was used as optimization factor. The reactor length was evaluated in the range from 40 to 140 cm; the results showed an increment of peak height with reactor length until 120 cm (Figure 5). This effect was attributed to the enzymatic reaction rate; higher length increased the reaction time and consequently the analytical signal. However, further increase of reactor length increased diffusion on the reactor and dilution of the H_2O_2 generated by enzymatic reaction, thus a decreasing on the analytical signal was observed [23]. A 120 cm reactor length was selected as the most appropriate.

Under the optimal conditions described above, calibration parameters were evaluated registering the signal obtained by the modified electrode at 0.0 V in the concentration range from 2 to 9 mM. Each point of the calibration curve corresponded to the mean value from three independent injections. The limit of detection, linear range and repeatability (expressed as relative

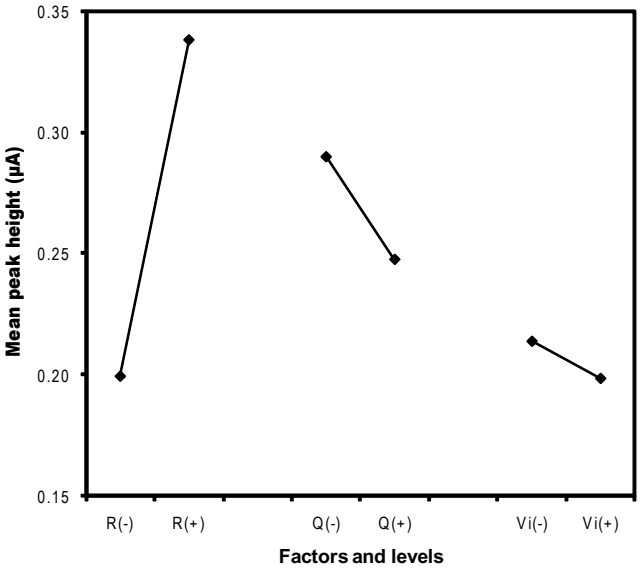

Fig. 4. Effect of control factors on the obtained mean peak height (µA). R, reactor length; Q, Flow rate; Vi, Injection volume.

standard deviation, RSD, n = 3) of the developed method are shown in Table 5. This is in the range of interest for analysis of blood samples where the normal concentration is between 4 to 6 mM.

Determination of glucose in blood serum samples

Blood samples were taken in vacuum tubes, the serum was separated by centrifuging at 3000 rpm for 10 minutes and keeping it at 4°C until analysis. The amperometric analysis of blood serum was carried out under the same conditions for the glucose determination in aqueous solution previously described. Quantification was performed by interpolation in the calibration curve (Figure 6), and also by standard addition method in triplicate (sample + 4 glucose additions of 1 mg L^{-1}).

Table 5. Regression parameters of the calibration study, current (µA) vs [Glucose] (mM).

Parameter	Value
Square root of residual variance, S_e	0.009
Determination coefficient, r^2	0.97
Intercept confidence interval, b0 ± t × s(b0)[a]	–0.059 ± 0.035
Slope confidence interval, b1 ± t × s(b1) (mM^{-1} µA)[a]	0.063 ± 0.005
Repeatability (%RSD, n = 3, 3.0 mM)	4.15
Linear range (mM)	1.5-9.0
Limit of detection (mM)	0.5
Sampling rate (samples h^{-1})	60.0

[a] $t_{crit\ (0.05;\ 3)} = 3.18$.

The results obtained by the proposed methodology were compared with those provided by the enzymatic-spectrophotometric assay (Table 6). To evaluate differences between the methods, one way ANOVA and a Tukey multiple comparison test were performed. The calculated F value (0.05) did not exceed the critical F value ($F_{2,42} = 3.22$, $p = 0.05$), thus indicating that there are no significant differences between the results provided by the compared methods. Results of Tukey test demonstrated the existence of one group.

Because of the complexity of the blood serum matrix, the selection and study of individual interference compounds is quite difficult; thus, in order to evaluate the existence of matrix effects, the slopes obtained from the standard additions curves were compared with the slope obtained in phosphate buffer solution (absence of interferences) [24]. For all analyzed samples, the calculated t value was lower than the critical t value for 4 degrees of freedom (2.78, p = 0.05), thus allowing us to accept the null hypothesis that there are no matrix effect during quantification of glucose.

Conclusions

We have developed a FIA system with amperometric detection which allows the glucose determination using screen printed electrodes modified with $[Fe(tris(3,5\text{-dimethyl-1-pyrazolyl})borate)_2]^+[FeCl_4]^-$ as mediator and GOx enzyme in solution. The use of this iron complex as mediator on the electrode surface allowed the diminishing of the detection potential to 0.0V, minimizing the interference produced by other oxidizing components contained in the sample. The modified electrode is reusable and has a lifetime of six months (60 determinations

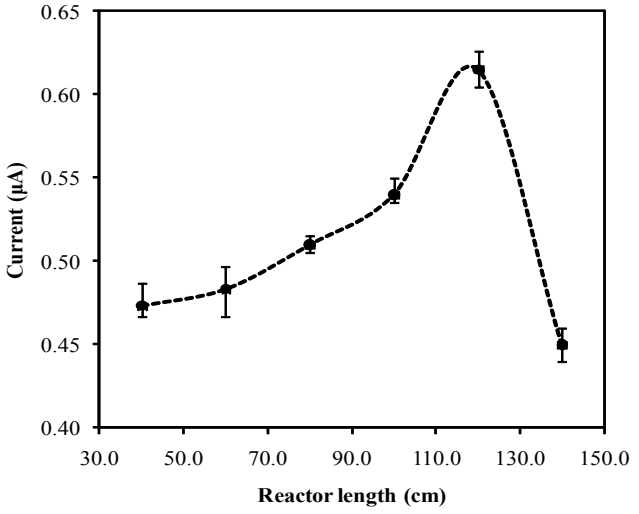

Fig. 5. Effect of the reactor length on the analytical signal obtained in the following conditions: E = 0.0 V, Q = 0.5 mL min^{-1}, pH = 7.0 (0.1 M phosphate buffer), injection volume = 30 µL and [Glucose] = 9.0 mM.

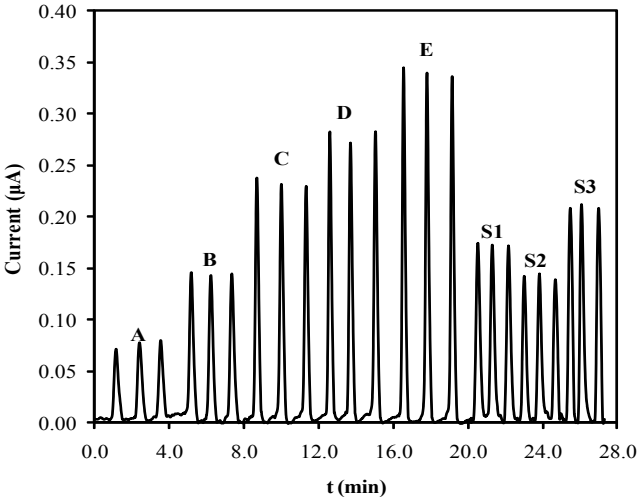

Fig. 6. Recorder output for Glucose calibration plot: A) 1.0, B) 3.0, C) 5.0, D) 7.0 and E) 9.0 mM, and S1, S2, S3, serum samples.

per day). Limit of detection and linear range were suitable for application in the glucose determination in real blood serum samples. The concentrations determined by the proposed system are similar to those provided by the spectrophotometric methodology with the advantage of increasing sampling rate. This is the first report of the use of an iron-Tp complex as mediator in biosensors.

Experimental

Reagents

All solutions were prepared with deionized water with a resistivity not less than 18.2 MΩ cm. Standard solutions of glucose and H_2O_2 were prepared daily from stock solutions of concentration 100 mM and 2.5 M, respectively. GOx, boric acid, acetic acid and sodium phosphate were analytical grade (Sigma Aldrich). Ferric chloride ($FeCl_3{\cdot}6H_2O$) was from Baker.

Synthesis of the mediator

5 mL of an aqueous solution of $FeCl_3{\cdot}6H_2O$ (54 mg, 0.2 mmol) were added to a solution of K^+[tris(3,5-dimethyl-1-pyrazolyl)borate]$^-$ (68 mg, 0.2 mmol) in tetrahydrofuran (5 mL). A red precipitate of [Fe(tris(3,5-dimethyl-1-pyrazolyl)borate)$_2$]$^+$[$FeCl_4$]$^-$ was formed immediately. The suspension was stirred for 12 h. The suspension was extracted twice with 5 mL portions of chloroform and the organic phase was evaporated at room temperature [25]. The resulting solid was washed with hexane (5 × 4 mL). Yield: 78 mg (0.09 mmol, 46%). Anal. Calcd. for $C_{30}H_{44}B_2Cl_4Fe_2N_{12}$: C, 42.49; H, 5.23; N, 19.82. Found: C, 42.12; H, 5.85; N, 17.54%.

Apparatus

The modification of the screen printed electrode (DropSens 110) was performed by impregnating 20 μg of the complex

Table 6. Concentrations of glucose in blood serum samples determined by the proposed method and the enzymatic spectrophotometric. Concentration units: mM.

Sample	FIA-Amperometric detection		E.S.[c]	Slope (mA μM^{-1})	Se[d]
	I.[a]	S.A.[b]			
1	4.47	4.56	4.77	0.064	0.003
	4.71	4.46	4.78		
	4.76	4.4	4.80		
2	4.69	4.68	4.65	0.064	0.009
	4.81	4.87	4.67		
	4.92	4.75	4.67		
3	5.09	5.41	5.55	0.063	0.001
	5.40	5.28	5.56		
	5.65	5.36	5.56		
4	3.64	3.57	3.65	0.065	0.002
	3.72	3.56	3.65		
	3.90	3.59	3.63		
5	5.97	6.14	6.18	0.063	0.001
	6.04	6.19	6.18		
	6.20	6.22	6.22		

a. I = Glucose determination by interpolation.
b. S.A. = Glucose determination by standard addition method.
c. E.S. = Glucose determination by enzimatic spectrophotometric method.
d. Se = Square root of residual variance of standard addition calibration curve.

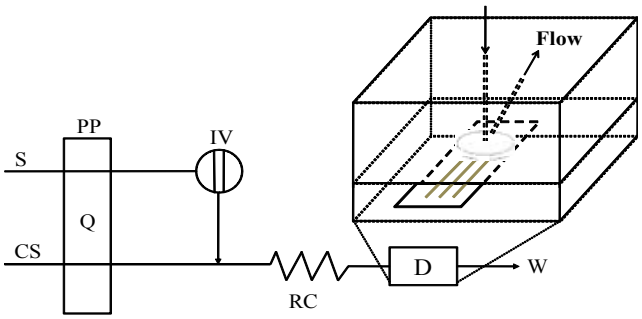

Fig. 7. FIA system designed for the determination of glucose. CS, carrier solution; S, sample; Q, flow rate; PP, peristaltic pump; IV, injection valve; RC, reaction coil; D, detector.

$[Fe(tris(3,5\text{-dimethyl-1-pyrazolyl})borate)_2]^+[FeCl_4]^-$ in chloroform on the surface of the working electrode (S = 0.13 cm^2) and drying at room temperature. The modified electrode and a carbon electrode were used as working and auxiliary electrodes, respectively, and a Ag/AgCl electrode as reference. Electrochemical experiments were performed using a bipotenciostast (DropSens μSTAT 200) controlled by software DropView 1.3. The pH was measured using a Corning (pH/ion meter 450) pH-meter.

Analytical cycle

H_2O_2 and glucose were analyzed using a FIA system as shown in Figure 7. The electrochemical flow cell type was wall-jet. Prior to implementation, the working electrode was activated by cyclic voltammetry. 20 cycles were applied from –0.6 to 0.8 V using a scan rate of 50 mV s^{-1}. Electrochemical studies were performed using a carrier solution of phosphate, pH 7.0, 0.1 M, 60 U of GOx was added to the carrier solution during glucose determination.

Enzymatic-spectrophotometric assay was performed by the Trinder method using GOx/peroxidase enzyme system and phenol/4-aminoantipyrine as chromogen system. Glucose is oxidized by GOx to gluconolactate and hydrogen peroxide. The coupling compound formed by phenol and 4-aminoantipyrine is oxidized by the peroxidase-H_2O_2 system to generate a red-colored quinoneinine detected at 505 nm [6]. 100 μL of blood serum sample is mixed with 10 ml of a solution containing: phosphate buffer (0.1 M, pH 7), phenol (0.01 M), GOx (1500 U), peroxidase (2000 U) and 4-aminoantipyrine (0.4 M). The concentration was calculated by interpolation in the calibration curve.

Acknowledgements

The authors wish to thank the CONACyT (project 61310) and Consejería de Educación y Cultura de la Junta de Castilla y León (project VA023A10-2).

References

1. Baynes, J. W.; Dominiczak, M. H. *Bioquímica Médica*. Elsevier, España, **2005**, 277.
2. Cipolla, M. J.; Godfrey, J. A. *Transl. Stroke Res.* **2010**, *1*, 127-134.
3. Wen, Z.; Ci, S.; Li, J. *J. Phys. Chem.* **2009**, *C 113*, 13482-13487.
4. Wang, W.; Wang, F.; Yao, Y.; Hu, S.; Shiu, K. K. *Electrochim. Acta* **2010**, *55*, 7055-7060.
5. Kong, T.; Chen, Y.; Zhang, K.; Wang, Z.; Wang, X. *Sensors Actuators B* **2009**, *138*, 344-350.
6. Trinder, P. *Ann. Clin. Biochem.* **1969**, *6*, 24-25
7. Sapre, A. G.; Bedekar, A.; Deshpande, A. V.; Lali, A. M. *Biotechnol. Lett.* **2000**, *22*, 569-573.
8. JieHua, L.; Hui, Z.; ShuSheng, Z. *Sci. China Ser B* **2009**, *52*, 196-202.
9. Luo, Y.; Liu, H.; Rui, Q.; Tian, Y. *Anal. Chem.* **2009**, *81*, 3035-3041.
10. Hsu, C. T.; Chung, H. H.; Tsai, D. M.; Fang, M. Y.; Hsiao, H. C.; Zen, J. M. *Anal. Chem.* **2009**, *81*, 515–518.
11. Nenkova, R.; Ivanova, D.; Vladimirova, J.; Godjervargova, T. *Sensors Actuators B* **2010**, *148*, 59-65.
12. Chaubey, A.; Malhotra, B.D. *Biosens. Bioelectron.* **2002**, *17*, 441-456.
13. Merchant, S. A.; Tran, T. O.; Meredith, M. T.; Cline, T. C.; Glatzhofer, D. T.; Schmidtke, D. W. *Langmuir* **2009**, 25, 7736–7742.
14. Heller, A.; Feldman, B. *Chem. Rev.* **2008**, *108*, 2482-2505.
15. Wang, Y.; He, Q.; Dong, Y.; Chen, H. *Sensors Actuators B* **2010**, *145*, 553-560.
16. Bosque-Sendra, J.M.; Gamiz-Gracia, L.; Garcia-Campaña, A.M. *Anal. Bioanal. Chem.* **2003**, *377*, 863-874.
17. Trofimenko, S. *Chem. Rev.* **1993**, *93*, 943-980.
18. Courjean, O.; Mano, N. *J. Biotechnol.* **2011**, *151*, 122-129
19. Long, G. L.; Winefordner, J. D. *Anal. Chem.* **1983**, *55*, 712A-724A.
20. Lei, C.; Deng, J. *Anal. Chem.* **1996**, *68*, 3344-3349
21. Kotzian, P.; Brazdilova, P.; Kalcher, K.; Vytras, K. *Anal. Lett.* **2005**, *38*, 1099-1113.
22. Tarley, C. R. T.; Silveira, G.; Santos, W. M. L.; Matos, G. D.; Silva, E. G. P.; Bezerra, M. A.; Miro, M.; Ferreira, S. L. C. *Microchem. J.* **2009**, *92*, 58-67
23. Ruzicka, J.; Hansen, E.H. *Flow Injection Analysis*. Wiley, **1988**.
24. Massart, D. L.; Vandeginste, B. G. M.; Buydens, L. M. C.; De Jong, S.; Lewi, P. J.; Smeyers-Verbeke, J. *Handbook of Chemometrics and Qualimetrics: Part A*. Ed. Elsevier Science, **1997**.
25. Sheets, J. R.; Schultz, F. A. *Polyhedron* **2004**, *23*, 1037-1043.