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FLOW INJECTION ANALYSIS FOR AMPEROMETRIC DETECTION OF GLUCOSE WITH IMMOBILIZED ENZYME REACTOR

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ABSTRACT

GOD was covalently immobilized on polymer/silica gel hybrid support prepared by coating high surface area of silica gel with modified acrylonitrile co-polymer. The relationship between immobilization factors and enzyme activity were examined by the series of contour plots. The selections of the immobilization variable range were extremely precise in the 3-level-3-factor fractional design. The results indicated that the optimal conditions for GOD immobilization were: 0.1% enzyme solution, immobilization temperature- 4°C and immobilization time- 24 h. Immobilized GOD was applied to amperometric determination of glucose using flow-injection analysis. The optimal flow rate was determined as 4.0 ml/min when injecting 100 µl sample volumes. The linear response range for the on-line detection of glucose using immobilized GOD in column minibioreactor was estimated to be from 0.01 mM to 20 mM (a correlation coefficient of 0.985). Moreover, its experimental detection limit is 10 µM (S/N=3) and the apparent Michaelis-Menten constant was calculated to be 20.15 mM. The proposed method for glucose measurement was validated in real samples of fruit juices.

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Keywords: GOD imobilization, glucose detection, flow injection analysis

Introduction

Glucose oxidase (GOD) is an ideal model enzyme for bioelectrochemistry because of its high bioelectrocatalitic activity and biochemical stability together with inexpensive price (26). Glucose oxidase (GOD) is widely used as a reagent for determination of glucose concentration in food stuffs (13, 15, 28). This enzyme is highly specific for the oxidation of β-D-glucose to gluconate and hydrogen peroxide (21). Electroactive reaction products, such as H_2O_2, may be determined by electrochemical detection generally performed using platinum electrode (1, 11, 17, 25). Other possibility is spectrophotometrical detection of glucose, by reaction with hydrogen peroxide so colored product may be produced (9, 20, 27). A few reports have introduced GOD application for determination of glucose in fruit juices using a flow-injection analysis (FIA) system (7, 18). This is an automatic system for on-line determination of glucose concentration.

In the analytical methods involving routine analysis, the reduction in cost of each determination is generally associated with the reduction of enzyme consumption. This has been achieved by immobilization of glucose oxidase in the analytical system. Effective immobilization of GOD is one of the key features for successful application. It depends on the choice of carrier and the conditions of the immobilization process. Many references can be obtained in literature about immobilized GOD, where polymer and inorganic supports are the most common GOD carriers (2, 6, 12, 14, 19, 23). Porous silica is certainly the most widely used because it is commercially available in a wide variety of shapes and sizes with different porosities. It has well-defined surface silanol groups that can be easily modified by some organosilane-coupling agents or different kind of polymers (4). Polymers should have well-defined structure, chemical composition and suitable molecular weight, since all of these parameters may influence coating efficiency (8). In our study we have focused our attention on modified polyacrylonitrile (PAN) in order to get polymer/silica gel hybrid support. That polymer has good film forming properties, insolubility in water solutions and good mechanical stability for long period of time. Furthermore modified PAN has active amino groups which are essential for enzyme immobilization.

In the present work we have focused our attention on optimization of the immobilization of glucose oxidase on polymer/silica gel hybrid support. Optimization of the FIA system containing a column minibioreactor with immobilized glucose oxidase and electrochemical cell was made. The FIA system was applied for on-line determination of glucose in fruit juices and comparison between amperometric and spectrophotometric determination of glucose was made.

Materials and Methods

Reagents

Acrylonitrile copolymer (acrylonitrile- 91.3%, methylmethacrylate- 7.3%, sodium vinylsulfonate- 1.4%) was a product of Lukoil Neftochim, Bourgas. PAN was chemically modified with NaOH and ethylendiamine purchased from Fluka (Buchs, Switzerland). Dimethylformamide (DMFA) as a solvent for the acrylonitrile copolymer was supplied by Merck (Germany). Silica gel with average pore size 100 Å was supplid by Fluka, (Buchs, Switzerland). Glucose oxidase with specific activity of 119.3 U/mg was covalently immobilized with

glutaraldehyde, all supplied by Fluka (Buchs, Switzerland). All the other reagents used for analyses of enzyme activity and bound protein were reagent grade, Fluka (Buchs, Switzerland). Glucose stock solutions were allowed to rotate at room temperature overnight before use. Phosphate buffer solution (0.1 M, pH 5.8) was used as the supporting electrolyte in all measurements, and distilled water was used throughout.

Modification of acrylonitrile copolymer (PAN)

Acrylonitrile copolymer was partially hydrolyzed with 10% NaOH at 50°C for 60 minutes in order to get functional groups (amido and carboxyl groups). The hydrolyzed polymer was filtered, washed thoroughly with distilled water and diluted in a solution of HCl acid. Then it was suspended in 10% solution of ethylendiamine for 60 minutes at room temperature and once again washed thoroughly.

Immobilization of glucose oxidase

Silica particles were suspended in 2% solution of acrylonitrile copolymer in dimethylformamide for 24 h. The polymer coated silica gel beads were washed with methanol several times and dried till constant weight. For the immobilization of glucose oxidase by covalent bonding, glutaraldehyde was used as a linker between the polymer/silica gel hybrid support and the enzyme. The surface-modified silica gels were added to 0.5% (v/v) glutaraldehyde solution for 60 minutes at 4°C. Then they were washed thoroughly with distilled water and 0.1 M sodium phosphate buffer (pH 5.8) and immersed into solution of the enzyme from 0.05 to 0.15% in 0.1 M phosphate buffer (pH 5.8) for periods of time from 18 to 30 h at temperatures from 0 to 8ºC. Finally, the particles were washed with distilled water and 0.1 M sodium phosphate buffer (pH 5.8). The enzyme activity was determined at glucose (0.3 ml) concentration 0.1 M. The specific enzyme activity is calculated as the amount of H_2O_2 formed by an enzyme in 10 minutes time under given conditions (pH 5.8, 30°C) per milligram of enzyme.

Electrochemical measurements

Cyclic voltammetry and amperometric measurements were performed on a Palmsense Electrochemical Instrument (Palm Instruments BV, The Netherlands). A conventional three-electrode system comprising Pt electrode (wafer 3x3mm) as working electrode, a Pt wire as an auxiliary electrode and an Ag/AgCl as a reference electrode was employed for all electrochemical experiments. The electrocatalytic activity of GOD was evaluated using cyclic voltammetry in 0.1M PBS (pH 5.8). The anodic and cathodic peak current values were found to show linearity with applied potential from 0 to 1 V. Amperometric measurements were carried out at applied potential of 0.6 V for various glucose concentrations in an electrochemical cell containing PBS electrolyte.

Flow-injection system

An electrochemical cell, peristaltic pump, injector for glucose samples, column minibioreactor and Palmsense Electrochemical Instrument connected with a computer were employed for FIA measurements. A schematic representation of the flow system is depicted in **Fig. 1**. The electrochemical cell contains Pt electrode (wafer 3x3 mm) as working electrode, a Pt wire as an auxiliary electrode and an Ag/AgCl as a reference electrode. The column minibioreactor (∅=0.3 and 2.2 cm of length) is filled with GOD covalently immobilized on polymer/silica gel hybrid support (100 mg). Control of the flow rate was done by the peristaltic pump. The carrier solution (0.1 M PBS, pH 5.8) is leaked through the column minibioreactor with immobilized GOD and electrochemical cell with flow rate of 4.0 ml/min. The carrier solution was tempered to 30°C. A potential of 0.6 V was applied to the working electrode and the electrochemical current was awaited to become stationary. Then 100 µl of glucose solution with different concentrations were injected to the system and passed though the column minibioreactor with immobilized GOD.

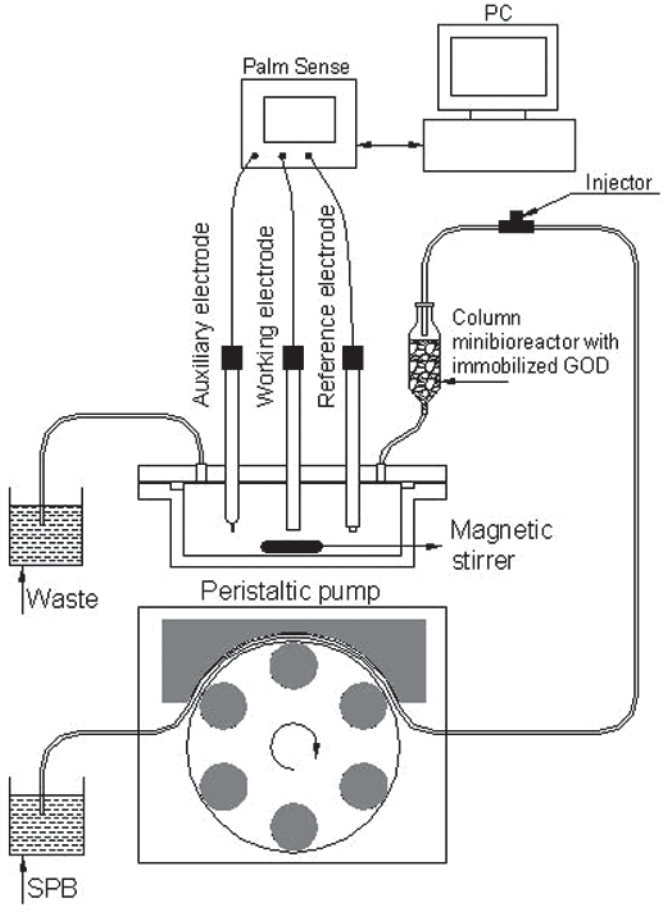


Fig. 1. FIA system for amperometric detection of glucose with immobilized glucose oxidase in column miniboreactor

Determination of *Km* and *Vmax*

Values of *Km* and *Vmax* of immobilized GOD were determined at 30°C, pH 5.8 and glucose concentrations ranging from 25 µM to 100 mM.

Analyses

The amount of bound protein was determined by the Lowry method based on spectrophotometric measurement at 750 nm

(Specol 11, Carl Zeiss Jena) (16). The immobilized enzyme activities were determined by an indicator reaction with peroxidase and o-dianizidin, measured with Specol 11, Carl Zeiss Jena, at 460 nm (3).

Statistical analysis

A 3-level-3-factor fractional design was employed in this study, requiring 14 experiments. The experimental data were analyzed by the response surface regression (RSREG) procedure to fit second-order polynomial equation (SAS).

Results and Discussion

Determination of optimal conditions for immobilization of GOD on polymer/silica gel hybrid support

Polymer/silica gel hybrid support was prepared by coating porous silica particles (Silica gel 100) with modified acrylonitrile copolymer. As a modifying agent 10% NaOH was used in order that functional groups (amido and carboxyl groups) are revealed in the polymer. The mechanism of chemical modification is illustrated in **Fig. 2**. The optimal concentration of the polymer was estimated to be 2% in our previous work (10).

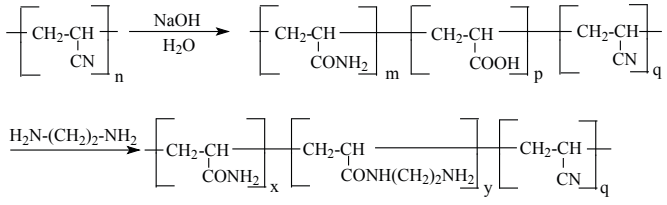


Fig. 2. Chemical modification of PAN

GOD was covalently immobilized on acrylonitrile copolymer/silica gel hybrid support using 0.5% glutaraldehyde as a linker between the carrier and the enzyme. The covalent bonding was achieved via the amino group of the activated carrier and the ε-amino group of lysine residue of the enzyme by GA. The mechanism of the reaction is well known and presented in **Fig. 3**.

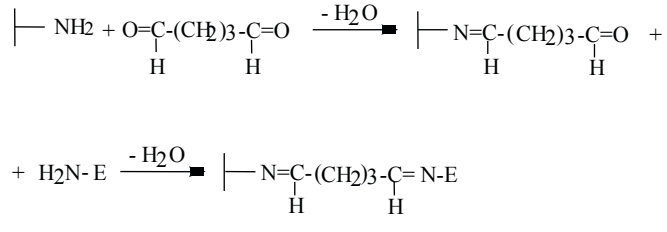


Fig 3. Immobilization on the modified hybrid support using glutaraldehyde as the activating agent

In order to optimize the immobilization procedure it was important to find out the optimum concentration of the enzyme solution. The variables selected in this study were as follows: immobilization time (C_t), immobilization temperature (C_T), enzyme concentration (C_E) (**Table 1**). The selection of the immobilization variable range needs to be extremely precise in the 3-level-3- factor fractional design.

TABLE 1

Experimental range of variables for the central composite design

Variable	Range of variables		
	Low (-1)	Mid (0)	High (+1)
C_t, h	18	24	30
C_E, %	0.05	0.1	0.15
C_T, ºC	0	4	8

The response surface regression (RSREG) procedure for Statistical Analysis System (SAS) was employed to fit the second order polynomial Eq. 1 to the experimental data, represented as specific enzyme activity. From the SAS output of RSREG, the second-order polynomial Eq.1 is given below:

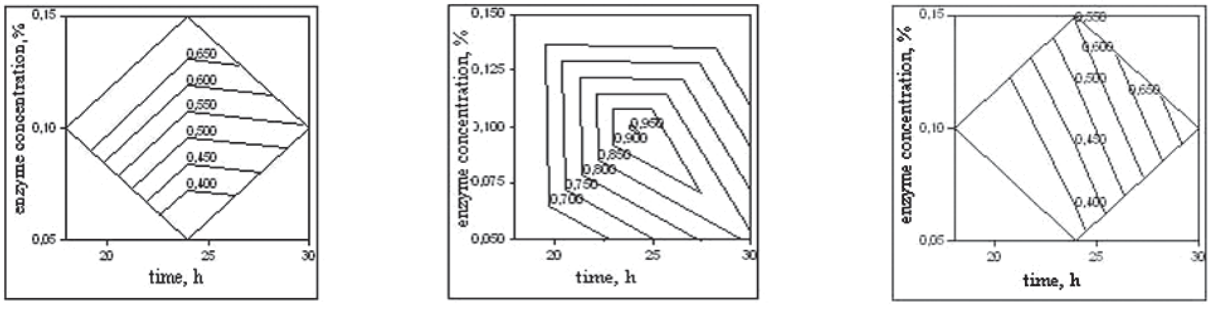
$$Y = -0.5756+1.0547\ C_t-2.9885\ C_E-0.1665\ C_T + \\ +0.6417\ C_t\ C_E -0.1756\ C_t\ C_T- \\ -0.2315\ C_E\ C_T -0.3132\ C_t^2-1.7797\ C_E^2 -0,4061\ C_T^2 \quad \text{Eq.1}$$

The analysis of variance indicated that the model was statistically significant and adequate to represent the actual relationship between the response (specific enzyme activity) and significant variables, with *p*-value 0.4496 and coefficient of determination R^2=0.8 meaning that 80% of the variability in the response could be accounted by the model (**Table 2**).

The relationship between immobilization factors and specific enzyme activity can be better understood by examining the series of contour plots. The effect of varying immobilization time and enzyme concentration is shown in **Fig. 4**. A condition with immobilization time 24 h and enzyme concentration of 0.1% favored maximum specific enzyme activity. **Fig. 5** represents the effect of varying immobilization temperature and enzyme concentration. A condition with temperature of 4ºC and enzyme concentration of 0.1% led to maximum activity. **Fig. 6** shows the effect of immobilization time, temperature and their mutual interaction on glucose oxidase activity. A condition with immobilization time of 24 h and temperature of 4ºC led to the maximum activity. Such an application could be adopted to study the immobilization variables simultaneously in a three dimensional space. Immobilization time and temperature were the most important variables for immobilization conditions with small *p*-values and were considered as indicators of effectiveness. The analysis indicated maximum specific enzyme activity at immobilization time of 24 h, temperature of 4ºC and enzyme concentration of 0.1%.

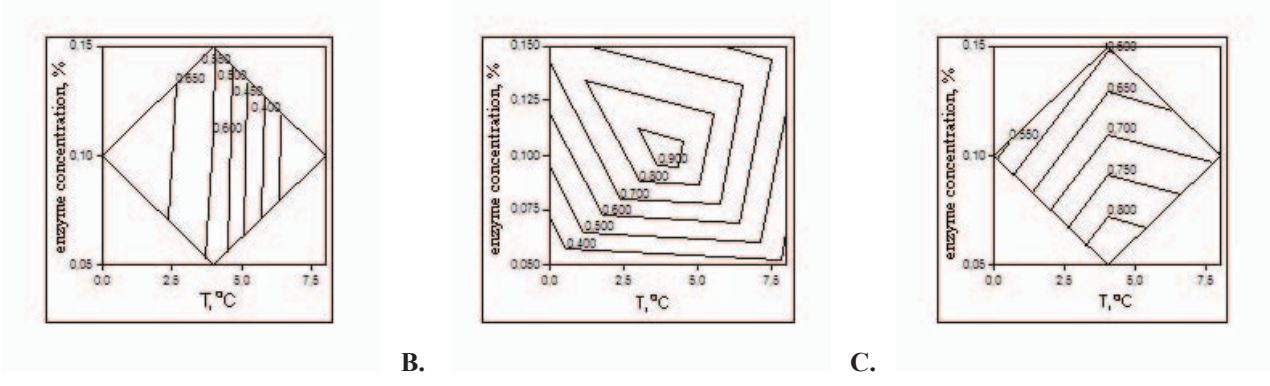
Optimization of the flow-injection system

To examine the efficiency of the column minibioreactor that contains immobilized glucose oxidase on polymer/silica gel hybrid, support experiments involving consecutive injections of glucose solutions were performed. The influence of parameters, such as flow rate and sample volume, was studied. Amperometric response for injections of 100 µl of 0.1 mM glucose, as a function of the flow rate, was evaluated and varied from 0.5 to 7.0 ml/min. For high flow rates, the glucose oxidase immobilized in the column minibioreactor was unable



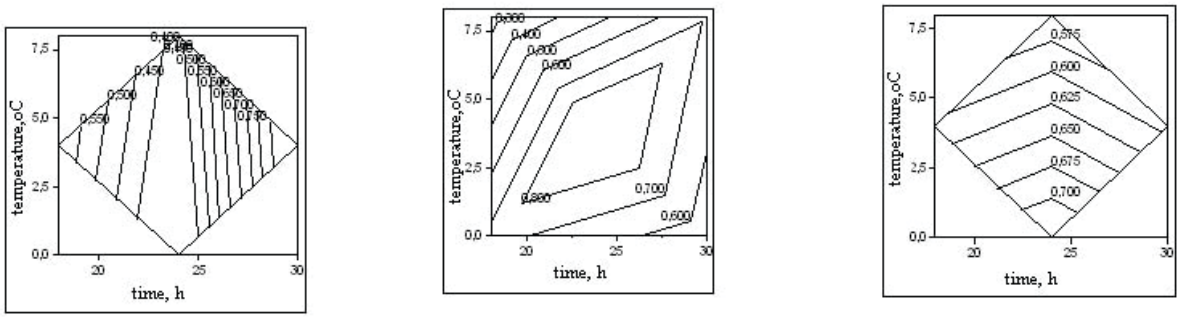
A. **B.** **C.**

Fig. 4. Contour plots showing response behavior at varying immobilized times and enzyme concentration under constant immobilized temperature for GOD-PAN/silica gel. **A.** Immobilization temperature- 0°C; **B.** Immobilization temperature- 4°C; **C.** Immobilization temperature- 8°C



A. **B.** **C.**

Fig. 5. Contour plots showing response behavior at varying immobilized temperatures and enzyme concentration under constant immobilized time for GOD-PAN/silica gel. **A.** Immobilization time- 18 h; **B.** Immobilization time- 24 h; **C.** Immobilization time- 30 h



A. **B.** **C.**

Fig. 6. Contour plots showing response behavior at varying immobilized times and temperatures under constant enzyme concentration for GOD-PAN/silica gel. **A.** Enzyme concentration- 0.05%; **B.** Enzyme concentration- 0.1%; **C.** Enzyme concentration- 0.15%

to transform glucose completely in hydrogen peroxide. A flow rate of 4.0 ml/min was chosen as the most favourable since it combined good reproducibility, high throughput and complete transformation of glucose in hydrogen peroxide and D-gluconate.

The influence of the sample volume on the analytical signal was also evaluated. Loops with internal volumes varying from 50 to 250 µl were tested. When the volume of the sample is increased, the amperometric signal increases, but the time required for each analysis also increases, since the cell washout process also requires a longer time. A volume of 100 µl was chosen as the working volume in subsequent experiments. For all the studied volumes glucose oxidase immobilized in the column minibioreactor totally converted the injected glucose.

Calibration plot

The calibration plot of response current to different glucose concentrations is illustrated on **Fig. 7**. The linear response range is obtained from 1 mM to 20 mM and the linear regression equation is

I (µA)=0.4073C+0.4932 (mM) (R=0.9857),

where *I* is the current and *Cg* is the glucose concentration. The sensitivity is calculated to be 0.4073 µA/mM. Additionally, the experimental limit of detection of that analytical system is measured to be 10 µM base on the single noise ratio of 3.

From **Fig. 7** it could be observed that the biosensor response gradually deviates from the linear feature as the glucose concentration is up to 20 mM. This is the characteristic of a typical Michaelis-Menten kinetics. The apparent Michaelis-Menten constant K_m, which depicts the enzyme-substrate kinetics of immobilized GOD, can be calculated from the Lineweaver-Burk equation:

1/I=(Km/Imax)(1/Cg)+1/Imax,

where *Cg* is the substrate concentration, *I* is the current and Imax is the maximum current measured under substrate saturation.

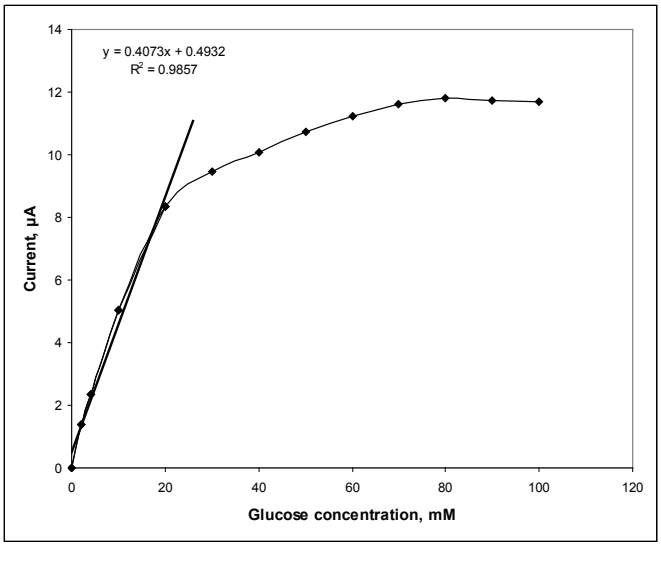


Fig. 7. Calibration curve of glucose oxidase

From the curve of the *1/I* versus *1/[S]*, based on the experimental date (**Fig. 8**), the apparent Michaelis-Menten constant *Km* and the maximum current response *Imax* were estimated to be 20.15 mM and 15.02 µA/mM, respectively.

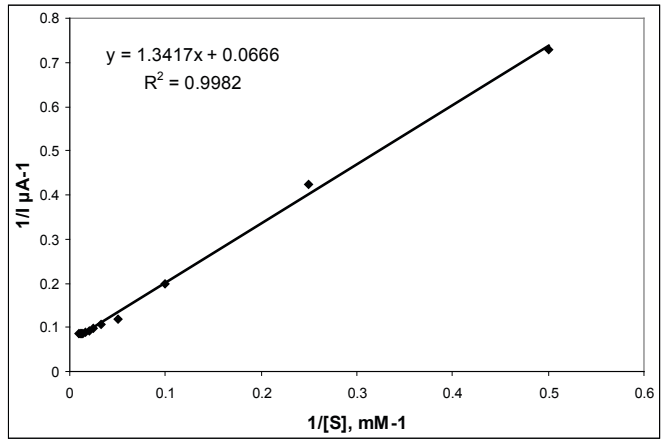


Fig. 8. Lineweaver-Burk plot

The analytical performance of the FIA with immobilized GOD in column minibioreactor was compared with the analytical performance of other FIA systems with immobilized GOD. The results are presented in **Table 3**.

The data presented in **Table 3** indicates that our FIA system with immobilized GOD on polymer/silica gel hybrid support has a large linear range and low detection limit.

Reproducibility and stability of the immobilized glucose oxidase

The reproducibility of the analytical performance of the immobilized GOD was evaluated from the response to 100 µl 0.1 mM glucose at four different measurements. The results revealed that the biocatalyst has satisfied reproducibility with a relative standard deviation of 4.0%. Excellent reproducibility could be explained by the strong covalent bound between the enzyme and polymer/silica gel hybrid support.

The long-term stability of immobilized GOD was evaluated by measuring its performance every fifth day in the course of 30 days. Immobilized enzyme was stored at 4^0C in phosphate buffer solution with pH 5.8. The amperometric response was around 93% of initial response after 15 days. After 30 days the biosensor was still active and retained around 89% of the initial response, indicating that the immobilized GOD can efficiently retain its bioactivity for a long time.

Determination of glucose in fruit juices by flow-injection analysis

Three fruit juices were used to quantify the amount of glucose. The samples to be analyzed were filtered, diluted 200 times with PBS pH 5.8. A volume of 100 µl from the diluted fruit juice was injected to the carrier solution and the amperometric response was detected. This assay used column minibioreactor with immobilized glucose oxidase on polymer/silica gel hybrid support. **Table 4** compares the results from

TABLE 2

RSREG with the data in Table 1

Analyses of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	9	0.39606802	0.044008	1.3371
Error	3	0.09873675	0.032912	Prob > F
C. Total	12	0.49480477		0.4496
Parameters Estimated				
Term	DF	Std Error	T Ratio	Prob > |t|
C_t	1	0.716229	1.47	0.2373
C_E	1	0.995674	-3.00	0.0576
C_T	1	0.367062	-0.45	0.6808
C^2_t	1	0.333323	-0.94	0.4167
C^2_E	1	0.749977	-2.37	0.0982
C^2_T	1	0.269992	-1.50	0.2296
$C_t C_E$	1	0.377953	1.70	0.1881
$C_t C_T$	1	0.26772	-0.77	0.4951
$C_E C_T$	1	0.340158	-0.68	0.5449

TABLE 3

Analitycal performance of FIA systems with immobilized GOD

Support	Linear range	Detection limit	Km	Application	References
Modified AN copolymer/silica gel	0.01÷20 mM	10μM	20.15mM	Determination of glucose in fruit juices	Present work
Silica capillaries	0÷4 mM	150 μM	-	Determination of glucose in soft drinks	Shi et al. (24)
Poly(*N*-(4-(3-thienylmethylene)-oxycarbonylphenyl) maleimide-*co*-pyrrole)	0.4÷1.6 mM	50 μM	4.7 mM	Determination of glucose in fruit juices	Gil et al. (5)
Titania sol-gel film	0.07÷15 mM	70 μM	6.34±0.21 mM	Determination of glucose in human serum	Yu et al. (29)
Sol-gel composite	0.2÷20 mM	50 μM	-	Determination of glucose in human serum	Salimi et al. (22)

the performed analyses by amperometric detection of glucose and spectrophotometric detection with immobilized GOD on the same hybrid support.

TABLE 4

Comparison of results obtained with different methods for glucose determination in fruit juices.

Fruit Juice	Glucose concentration, g/l	
	Amperometric detection	Spectrophotometric method
Grape	63.7±0.2	62.4±0.3
Apple	31.9±0.1	31.8±0.3
Orange	27.3±0.3	26.9±0.4

The results present good correlation of the glucose values in fruit juices obtained both with amperometric detection with immobilized GOD in column minibioreactor included in a FIA system and spectrophotometric method. Therefore the proposed method with immobilized GOD on polymer/silica gel hybrid support could be successfully applied for determination of glucose concentration in fruit juices.

Conclusions

Polymer/silica gel hybrid support prepared by coating silica gel with modified acrylonitrile copolymer is very suitable for immobilization of glucose oxidase. This hybrid support combines the advantages of both, organic polymer and inorganic silica gel, and provides high surface area and functional amino groups for enzyme immobilization and also good mechanical stability. Very effective biocatalyst was prepared- specific activity 0.950 U/mg_e. Immobilized

GOD was applied for on-line determination of glucose concentration using a flow-injection analysis system. The FIA system provides good sensibility (0.407 μA/mM), large linear range (0.01÷20 mM) and low detection limit (10μM). It was demonstrated that the FIA system with immobilized GOD on polymer/silica gel hybrid support could be applied for amperometric determination of glucose concentration in fruit juices.

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