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# Electrochemical glucose biosensing by pyranose oxidase immobilized in gold nanoparticle-polyaniline/AgCl/gelatin nanocomposite matrix

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ABSTRACT

A novel pyranose oxidase (PyOx) biosensor based on gold nanoparticles (AuNPs)–polyaniline(PANI)/AgCl/gelatin nanocomposite has been developed for the glucose detection. PyOx was immobilized on the surface of glassy carbon electrode (GCE) via the nanocomposite matrix. The electrode surface was imaged by scanning electron microscopy (SEM). Amperometric detection of the consumed oxygen during the enzymatic reaction was monitored at −0.7 V. After optimization studies, analytical characterization of the biosensor was carried out. The linear response of the AuNPs–AgCl/PANI/gelatin modified PyOx biosensor is found to be from 0.05 to 0.75 mM glucose with the equation of $y = 2.043x + 0.253$; $R^2 = 0.993$. Finally, proposed biosensor was used to analyze glucose content in real samples. Obtained data from the biosensing system was compared with a commercial enzyme assay kit based on spectrophotometric Trinder reaction as a reference method.



## 1. Introduction

The flavoenzyme pyranose 2-oxidase (PyOx, glucose 2-oxidase, pyranose: oxygen 2-oxidoreductase, EC 1.1.3.10) catalyzes C-2/C-3 oxidation of numerous sugars to their corresponding dicarbonyl derivatives (aldos-2-uloses or glycosid-3-uloses), coupled to the reduction of FAD, anobligatory cofactor (Halada, Leitner, Sedmera, Halt rich, & Volca, 2003; Maresova, Palyzova, & Kyslik, 2007). PyOx which is a fungal periplasmic homotetrameric flavoprotein (~300 kDa) found in wood degrading fungi (Haltrich, Leitner, Nidetzky, & Kulbe, 2000) has been purified and characterized previously (Dannie, Rossnerz, Zeeck, & Giffhorn, 1993; Tamaqua & Kuwata, 2003). Giffhorn et al. has recently reviewed that the high potential of PyOx application in bioprocesses, clinical chemistry analytics and in synthetic carbohydrate (Giffhorn, 2000).

Glucose oxidase (GOx, glucose 1-oxidase, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) is a flavin containing glycoprotein which catalyzes an oxidation of β-D-glucose to δ-gluconolactone, which subsequently hydrolyzes spontaneously to gluconic acid (Odaci, Telefoncu, & Timur, 2008; Vikartovska et al., 2007). The particular advantages of using PyOx instead of GOx in biosensor construction are as follows: The most important advantages are its high affinity for D-glucose (Km ~1 mM), its ability to efficiently oxidize various sugars other than glucose may compromise its use for selective glucose monitoring, however, its application in biotechnology and in biofuel cells is envisaged as a better alternative than GOx, as many other sugars from, e.g., a lignocellulose hydrolysate can be oxidized by PyOx, and thus used for small scale energy production. Apart from them, one of the major drawbacks with GOx is its anomeric selectivity. GOx has some disadvantages, since at mutaration equilibrium about 36% of D-glucose occurs as the corresponding α-anomer which is not oxidizable so GOx should be used together with mutarotase (Odaci et al., 2008).

Metal nanoparticles for use in biosensor applications represent a rapidly advancing field. Metal nanoparticles are generally defined as isolable particles between 1 and 50 nm in size that are prevented from agglomerating by protecting shells (Wang, 2005). In recent years, research efforts on a kind of AuNPs, colloidal gold, have flourished because of their good biological compatibility, excellent conducting capability and high surface-to-volume ratio (Guo & Wang, 2007). The ability of providing a stable immobilization of biomolecules retaining their bioactivity is a major advantage for the preparation of biosensors. Additionally, AuNPs permit direct electron transfer between redox proteins and bulk electrode materials and also have demonstrated to constitute useful interfaces for the electrocatalysis of redox processes of molecules such as $H_2O_2$, $O_2$ or NADH involved in many significant biochemical reactions (Pingarron, Yanez-Sedeno, & Gonzalez-Cortes, 2008). Recently, works have mostly been concentrated on the application of AuNPs in biosensors using various enzymes including glucose oxidase (Lan, Li, & Zhang, 2008), xanthine oxidase (Cubukcu, Timur, & Anik, 2007), peroxidase (Jia et al., 2008), L-lactate dehydrogenase (Jena & Raj, 2007) and acetylcholinesterase (Liu, Liu, Shen, & Yu, 2006).

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In this study, PyOx was immobilized onto the glassy carbon electrode via AuNPs–PANI/AgCl/gelatin matrix. The response characteristics, stabilities and substrate specificities were investigated as well as the effect of AuNPs on the biosensor response.

## 2. Experimental

### 2.1. Chemicals and reagents

Pyranose oxidase (PyOx; pyranose: oxygen 2-oxidoreductase, EC 1.1.3.10, from *Coriolus* sp., recombinant; expressed in *E. coli*), gelatin from calf skin (300 bloom), D-glucose, D(+)-mannose, D(+)-galactose, D(+)-xylose, D(+)-sucrose, silver nitrate (>99%), gold (III) chloride hydrate (99.999%), sodium citrate and sodium borohydride (99%) were purchased from Sigma Chem. Co. (St. Louis, MO, USA, www.sigmaaldrich.com). Aniline (⩾99.0%) was purchased from Fluka (Steinheim, Germany, www.sigmaaldrich.com). Ammonium persulfate (APS) and polyvinylpyrrolidon (PVP) was from Merck (www.merck-chemicals.com). Commercial enzyme assay kit (Glucose MR, Cat. No. 1129010), used as a reference method for glucose detection in real samples, was from Cromatest. The kit was based on the formed a red quinine imine dye after oxidation of a mixture of phenol and 4-aminoantipyrine (4-AAP) by GOx and peroxidase. This reaction was based on All other chemicals were analytical grade.

### 2.2. Samples

The samples were purchased in a local market. Four fruit juice (pomegranate, peach, orange, mixed fruit), a raspberry nectar, green tea, energy drink, coke and two wines (red and white) were investigated. The samples were previously degassed and used without any dilution.

### 2.3. Apparatus

Chronoamperometric experiments were carried out with a PalmSens Electrochemical measurement system (Palm Instruments, Houten, The Netherlands), where the modified glassy carbon electrode (GCE) was used as the working electrode. An Ag|AgCl electrode (with 3 M KCl saturated with AgCl as the internal solution, Metrohm Analytical, CH-9101) and platinum electrode (Metrohm, Switzerland, www.metrohm.com) were used as reference and counter electrodes, respectively. The electrodes were inserted into a conventional electrochemical cell (10 mL).

JEOL JSM-6400 model scanning electron microscope (SEM) was used for surface imaging of the prepared biosensor.

Colorimetric assays were performed with Perkin–Elmer Lambda 35 UV/VIS Spectrophotometer (Shelton, USA).

### 2.4. Methods

#### 2.4.1. Preparation of gold seed

Twenty milliliters of growth solution, containing $2.5 \times 10^{-4}$ M, $HAuCl_4$ and $2.5 \times 10^{-4}$ M, tri-sodium citrate was prepared in a conical flask. Then, 0.6 mL of ice cold 0.1 M, $NaBH_4$ solution was added to the solution all at once while stirring. The solution turned pink immediately after adding $NaBH_4$, indicating particle formation. The particles in this solution were used as seeds within 2–5 h after preparation (Pan et al., 2007).

#### 2.4.2. Synthesis of Au nanoparticle-polyaniline/AgCl hybrid material

$AgNO_3$ (0.012 M) and aniline (0.012 M) were added to aqueous solution of PVP (3%). Five milliliters of 1 M HCl and aqueous solution of ammonium persulfate (APS) as oxidant was dropped into the above mixture under stirring at room temperature. The molar ratio of aniline to APS ([An]:[APS]) was 1:1. The reaction was allowed to proceed for 24 h (Feng, Liu, Lu, Hou, & Zhu, 2006). After that, 0.3 mL of PANI/AgCl nanocomposite solution was added into 1 mL of Au colloidal solution under stirring. The reaction was allowed to proceed for 12 h, and the resultant product was centrifuged and dispersed in water. AuNPs have been incorporated on the surface of PANI/AgCl nanocomposites, leading to the formation of AuNPs–PANI/AgCl hybrid material (Yan, Feng, Chen, Hou, & Zhu, 2008).

#### 2.4.3. Biosensor preparation

Five microliters of PyOx which is equal to 2.25 U and gelatin (1 mg) were mixed in 25 μL suspension of AuNPs–PANI/AgCl at 38 °C. Then, 10 μL of mixed solution was spread over the GCE surface and allowed to dry at 4 °C for 30 min. Finally, it was immersed in 2.5% glutaraldehyde in potassium phosphate buffer (50 mM, pH 6.5) for 5 min for the cross linking. Final activity for PyOx biosensor was 0.75 U. Daily prepared enzyme electrodes were used in all experimental steps.

### 2.5. Electrochemical measurement

All the measurements were monitored chronoamperometrically at −0.7 V (versus Ag|AgCl). The chronoamperometric measurements were performed at room temperature (at 25 °C) in steady-state conditions in potassium phosphate buffer (50 mM, pH 6.5) solution while each analysis was lasted 100 s. The sensor response was defined as the difference between first and second steady-state currents and registered as current (μA). After three measurements, the standard deviation (S.D.) and variation coefficient (c.v.) were calculated for quality assurance.

### 2.6. Trinder reaction as a reference method

The PyOx biosensor was used to analyze glucose content in 10 real samples such as pomegranate juice, peach juice, orange juice, mixed fruit juice, green tea, raspberry nectar, energy drink and coke as well as red and white wines. No sample pretreatment was required for the analysis. Additionally, a commercial enzyme assay kit based on spectrophotometric Trinder reaction (Cromatest, Glucose MR, Cat. No. 1129010) was used as a reference method for independent glucose analysis. In this reaction, the glucose is oxidized to D-gluconate by the glucose oxidase (GOx) with the formation of hydrogen peroxide. In the presence of peroxidase (POD), a mixture of phenol and 4-aminoantipyrine (4-AAP) is oxidized by hydrogen peroxide to form a red quinine imine dye proportional to the glucose concentration in the sample (Trinder, 1969).

## 3. Results and discussion

Different types of enzyme sensors were presented in our previous works in which PyOx was immobilized on the surface of different transducers by means of various immobilization strategies (Odaci et al., 2008; Tasca et al., 2007; Timur, Yigzaw, & Gorton, 2006). In this case, the enzyme was immobilized onto the glassy carbon electrode via AuNPs–polyaniline/AgCl/gelatin matrix.

Amongst the various conducting polymers, polyaniline (PANI) has been extensively studied as an important conducting material that possesses interesting electrical, electrochemical, and optical properties (Singh, Solanki, Pandey, & Malhotra, 2006). Generally, an acidic condition (usually pH < 4) is required for the formation of the most highly conductive form of PANI, and this greatly restricts the applications of PANI in bioelectrochemistry (Yan et al., 2008). Therefore, increasing research interests have been focused on polyaniline/inorganic nanocomposites such as PANI/AgCl, PANI/$BaSO_4$, PANI/$TiO_2$, polyaniline/$Na^+$-montmorillonite,

polyaniline-coated cadmium sulphide, rutile-$TiO_2$/polyaniline and polyaniline-$LiNi_{0.5}La_{0.02}Fe_{1.98}O_4$ (Bian & Xue, 2007; Khiew, Huang, Radiman, & Ahmad, 2004; Lee, Lee, Char, & Kim, 2000; Sui, Chu, Xing, & Liu, 2004). Yan et al. was synthesized PANI/AgCl nanocomposites and used for the determination of glucose and dopamine. The obtained nanocomposites found to be shown an excellent electrochemical behavior at a neutral pH environment (Yan et al., 2008). In our case, PyOx was used as a biological material and gelatin was also combined to AuNP-PANI/AgCl nanocomposite to prepare more stabile immobilization matrix.

Additionally, scanning electron microscopy technique is used to detect the surface characteristics of the matrix. In this part, morphologies of both AuNP-Polyaniline/AgCl and AuNP-Polyaniline/AgCl/gelatin matrices were detected (Fig. 1A and B). The morphology of AuNP-Polyaniline/AgCl/gelatin has cauliflower like structure. It has more compact globules than the matrix without gelatin. It is clear that the addition of the gelatin provided more suitable matrix for the enzyme immobilization and to keep the biological activity during the operational conditions.

### 3.1. Effect of pH

The pH dependency of PyOx biosensor was investigated by potassium phosphate buffer solution between pH 6.0 and 7.5. Amperometric detection was carried out in the presence of 0.25 mM glucose and the highest sensitivity was achieved at pH 6.5. It is clear that pH optima were dependent on the immobilization matrices. In our previous works, the optimum pH of the PyOx biosensor in which the Osmium redox polymer was used to entrap the enzyme onto the graphite rod electrode was found to be 10.5 (Tasca et al., 2007). On the other hand, when PyOx immobilized in carbon nanotube modified carbon paste electrode, optimum pH was obtained at pH 7.5 (Odaci et al., 2008). In addition, a difference in pH optima depending on the structure of the redox polymers was also observed in previous studies (Timur et al., 2006). It is also reported that pH was influential to the electroactivity of AuNPs–PANI/AgCl and the increase of pH value caused to reduced electroactivity which resulting in the decrease of catalytic response (Yan et al., 2008).

### 3.2. Effect of enzyme loading

To search the effect of the enzyme activity on the biosensor response, different enzyme amounts were used in the biosensor construction. For this purpose, five different enzyme electrodes containing 0.1, 0.75, 1.44, 2.0 and 4.0 U PyOx activities were prepared. The rations of the immobilization constituents were kept constant. Calibration graphs for each electrode were plotted for glucose. No signal was observed by the electrode containing 0.1 U of enzyme. On the other hand, linearities and the current responses found to be similar for all other electrodes (0.75, 1.44, 2.0 and 4.0 U) (Fig. 2). PyOx biosensor which was prepared with lowest enzyme content (0.75 U) enzyme was used for further experimental steps.

### 3.3. Effect of composite amount

After synthesis of AuNPs–PANI/AgCl nanocomposite, the resultant product dispersed in different volume of water. In order to determine the optimum composite amount for the electrode construction, different composite concentrations (Nanocomposite: water (w/v%); 1:100, 1:500 and 1:1000) were investigated. Maximum signals were obtained when 1:1000% (w/v) composite was used, hence it was conducted for further experimental steps.

### 3.4. Effect of AuNP addition

The unique properties of gold nanoparticles to provide a suitable microenvironment for biomolecules immobilization retaining their biological activity, and to facilitate electron transfer between the immobilized proteins and electrode surfaces, have led to an intensive use of this nanomaterial for the construction of electrochemical biosensors with enhanced analytical performance with respect to other biosensor designs (Pingarron et al., 2008). To detect the effect of AuNPs, the composite was prepared without AuNPs. Two biosensor systems one with AuNP and the other with-

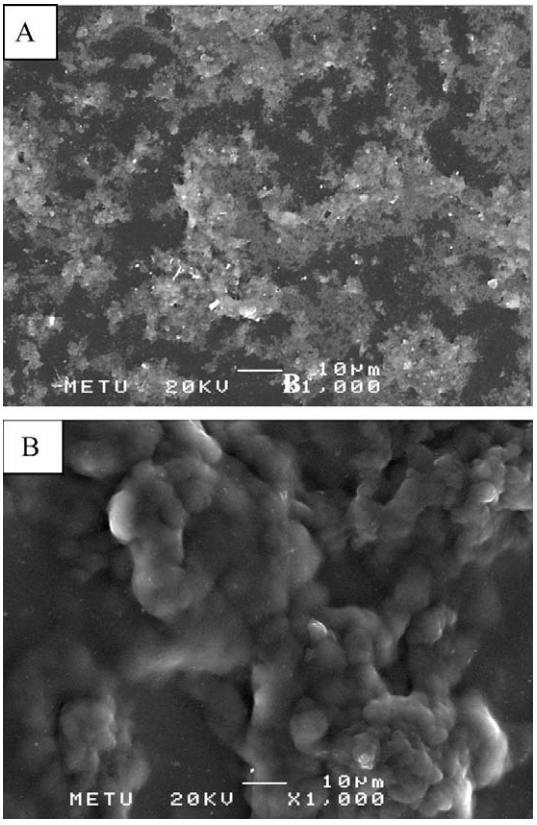


**Fig. 1.** SEM images of AuNPs–polyaniline/AgCl (A) and AuNPs–polyaniline/AgCl/gelatin (B) matrices.

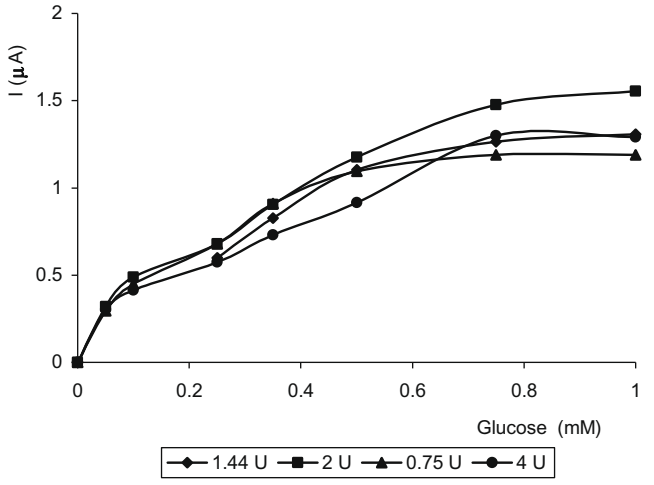


**Fig. 2.** Effect of enzyme loading on the biosensor response (potassium phosphate buffer: 50 mM, pH 6.5, −0.7 V).

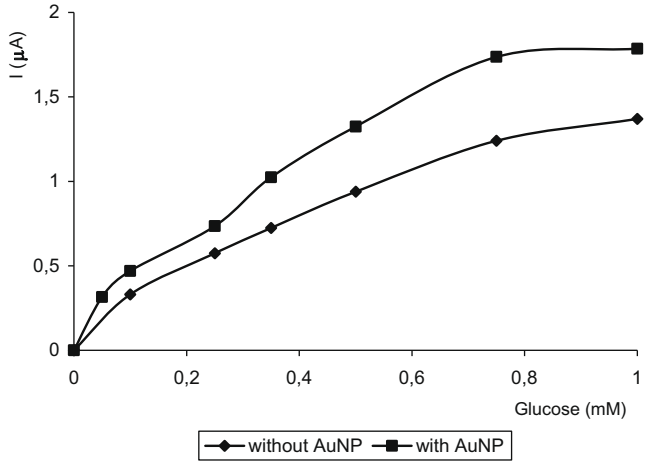


**Fig. 3.** Effect of AuNPs on the biosensor response (potassium phosphate buffer: 50 mM, pH 6.5, −0.7 V).

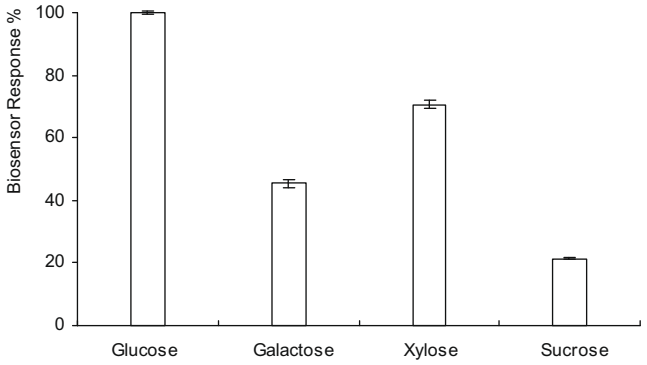


**Fig. 4.** Carbohydrate analysis using PyOx biosensor (potassium phosphate buffer: 50 mM, pH 6.5, −0.7 V, sugar substrate: 0.25 mM).

out AuNP were used to construct calibration graphs for the glucose at the optimum conditions. Then, the obtained data were compared to each other. Higher signals were observed for composite electrode with AuNPs as it is expected (Fig. 3).

### 3.5. Analytical characteristics

The inorganic@conducting PANI core shell nanocomposites, silver chloride@PANI core–shell nanocomposites (AgCl@PANI) were synthesized previously by Yan, Feng, Chen, Li, & Zhu (2008) and used for adsorption of glucose oxidase (GOx) on the electrode (Yan et al., 2008). Cyclic voltammetric and differential pulse voltammetric measurements were carried out and the proposed system was calibrated for glucose detection at 0.0 V. In our case, the analytical characteristics of proposed biosensor were investigated chronoamperometrically at lower potential (−0.7 V) under optimized experimental conditions. The linear response of the AuNPs–AgCl/PANI/gelatin modified PyOx biosensor is from 0.05 to 0.75 mM glucose with the equation of $y = 2.043x + 0.253$; $R^2 = 0.993$ in which $x$ shows glucose concentration in mM and $y$ shows current in A. The response time ($t95$) is defined as the time taken to obtain a 95% steady-state signal when a biosensor is exposed to a known concentration of standard solution (Wen, Zhang, Shuang, Donga, & Choi, 2007). In our study, the PyOx biosensor could achieve 95% of the steady signal within 100 s. When PyOx was immobilized in two different types of Osmium redox polymers, linearity for glucose were observed in the ranges of 0.25–6 mM and 0.125–2 mM, respectively (Timur et al., 2006). It is obvious that more sensitive results were obtained by the combination of PyOx into the nanocomposite structure.

To evaluate the repeatability of the biosensor, six replicate measurements of 0.25 mM of standard glucose solution were carried out. The standard deviation (S.D) and variation coefficient (c.v.) were as follows, ±0.005 mM and 2.16%, respectively.

In order to determine the operational stability of the biosensor, a freshly prepared PyOx biosensor at optimal working conditions (pH 6.5, 50 mM potassium phosphate buffer, at 25 °C) was used. During 6 h, 7 measurements were carried out by using 0.25 mM

**Table 1**
Comparison of analytical characteristics of various PyOx biosensors reported in literature.

| Electrode configuration/ method | Immobilization method | Enzyme amount (source) | Working potential (mV) | Analytical performances | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | Linear range for glucose | Variation coefficient | Operational stability | |
| Carbon paste | Physical adsorption | 110 U (co-immobilised with horseradish peroxidase) (*Phanerochaete chrysosporium*) | −50 | 0.3–2.5 g $L^{-1}$ | – | 7% decrease after 4 h | Liden, Volc, Marko-Varga, and Gorton (1998) |
| Carbon paste | Covalent immobilization | 132 U (co-immobilised with horseradish peroxidase) (*Phanerochaete chrysosporium*) | −50 | 40–650 μM | 0.3–1.3% | 20% decrease after 5 h | Liden et al. (1998) |
| Graphite | Osmium redox polymer type I cross linking via diglycidyl eter | 0.43 U (recombinant *Cariolus* sp.) | +300 | 0.25–6 mM | 2.82% | 22% decrease after 8 h | Timur et al. (2006) |
| Graphite | Osmium redox polymer type II cross linking via diglycidyl eter | 0.43 U (recombinant *Cariolus* sp.) | −80 | 0.125–2 mM | 0.6% | 6% decrease after 18 h | Timur et al. (2006) |
| Graphite rods | Osmium redox polymer type II with poly(ethylene glycol) diglycidyl | 0.4 U (*Trametes multicolor*, *wild type*) | +300 | 10–400 μM | 4.4% | – | Tasca et al. (2007) |
| Carbon paste modified with MWCNT | Physical adsorption | 6 U (recombinant *Cariolus* sp.) | +900 | 0.2–30 mM | 2.3% | 4% decrease after 7 h | Odaci et al. (2008) |
| Glassy carbon | Entrapment via nanocomposite matrix | 0.75 U (recombinant *Cariolus* sp.) | −700 | 0.05–0.75 mM | 2.16% | 12% decrease after 6 h | This work |

Osmium redox polymer I: poly(1-vinylimidazole)$_{12}$-[osmium (4,4_-dimethyl-2,2_-bipyridyl)2Cl2]$_{2+/+}$, osmium redox polymer II: poly(vinylpyridine)-[osmium-(*N*,*N*_-methylated-2,2_-biimidalzole)3]$_{2+/3+}$ complex, MWCNT: multiwalled carbon nanotube.

**Table 2**
Results for glucose analysis in real samples by enzyme electrode and spectrophotometric method.

| Sample | Glucose (g/L) | | Recovery (%) |
|---|---|---|---|
| | Spectrophotometric | PyOx biosensor | |
| Pomegranate juice | 11.15 ± 0.325 | 10.22 ± 0.252 | 109 |
| Aroma fruit juice-food industry | | | |
| Green tea | 11.91 ± 0.788 | 11.58 ± 0.115 | 103 |
| Lipton Unilever company | | | |
| Energy drink | 23.68 ± 1.273 | 25.35 ± 0.333 | 93 |
| Red Bull | | | |
| Raspberry Nectar | 29.89 ± 0.021 | 32.12 ± 1.659 | 93 |
| Aroma fruit juice-food industry | | | |
| Peach Juice | 34.19 ± 5.413 | 36.88 ± 0.333 | 93 |
| Cappy fruit juice-food industry | | | |
| Orange Juice | 54.46 ± 3.106 | 55.33 ± 0.499 | 98 |
| Cappy fruit juice-food industry | | | |
| Mixed Fruit Juice | 24.25 ± 0.159 | 22.41 ± 2.345 | 108 |
| Cappy fruit juice-food industry | | | |
| Coke | 54.19 ± 3.852 | 58.54 ± 1.547 | 93 |
| Coca-Cola Company | | | |
| White wine | 0.075 ± 0.0003 | 0.08 ± 0.004 | 94 |
| Nevzade | | | |
| Red wine | 0.045 ± 0.005 | 0.049 ± 0.001 | 92 |
| Nevzade | | | |

glucose. After each measurement the biosensor was washed and kept in working buffer until the next measurement. It is found that 12% drop in biosensor response was obtained at the end of the operational period. It is also noticed that use of gelatin as a constituent of the immobilization matrix contributed to the operational stability as well as repeatability. On the other hand, in case of using nanocomposite without gelatin, in contrast to the previous work (Yan et al., 2008) repeated measurements were not carried out. Moreover, electrode responses were found to be reduced after each measurement because of the leakage of biomatrix. According to our findings it can be said that addition of gelatin is important to keep the stability during the operational conditions.

Analytical characteristics of various PyOx biosensors and PyOx-based enzyme reactors reported in literature were summarized also in Table 1. As can be seen in the table, the proposed system also exhibited good linearity as well as stability in the operational conditions as the previously developed PyOx biosensors.

The substrates of PyOx are most probably D-glucose and D-xylose, which are abundant in lignocellulose and which are oxidized to 2-keto-D-glucose, D-*arabino*-hexos-2-ulose, 'D-glucosone' and 2-keto-D-xylose, D-*threo*-pentos-2-ulose, 'D-xylosone', respectively (Leitner, Volc, & Halt rich, 2001). For determination of substrate specificity of the biosensor 0.25 mM of standards of various sugars such as mannose, galactose, xylose, and sucrose were examined. The biosensor response obtained for glucose was accepted as 100% and compared to the biosensor responses obtained for the other substances (Fig. 4).

### 3.6. Sample application

The biosensing system was used to analyze glucose in real samples. In addition, spectrophotometric method was used as the reference method to test glucose concentration data obtained from the biosensing systems. In order to analyze the amount of glucose in pomegranate juice, green tea and energy drink by means of PyOx biosensor, samples were used as the substrate solution instead of glucose substrate. Then, signals were recorded and data were calculated from calibration curves. Obtained values were compared with glucose amounts determined spectrophotometric method. Table 2 shows the results belong to sample application. As can be seen the tables, recovery values were found to be closer to 100% which means that the systems have not been affected the nature of the sample.

## 4. Conclusion

In the present paper, a novel amperometric PyOx biosensor was constructed by immobilizing PyOx on the AuNPs–PANI/AgCl/gelatin modified GC electrode. The combination of PyOx into a nanocomposite structure as an immobilizing matrix enables us to keep the enzyme with higher bioactivity and stability in the operational conditions and resulted in fast, stable and sensitive responses in glucose biosensing. Data obtained from the sample application part showed that biosensing responses were not affected by the sample matrix and the nature.

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