



One-Shot Chemiluminescence Biosensor for Determination of Glucose in Soft Drinks

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Article info

Received: 28/09/2012

Accepted: 21/11/2012

Keywords:

Glucose biosensor
Glucose oxidase
Chemiluminescence
Soft drinks

Abstract: The preparation of a new biosensor for glucose was based on the fact that glucose can be determined by its enzymatic oxidation to gluconic acid with simultaneous formation of hydrogen peroxide in the presence of glucose oxidase. Hydrogen peroxide formed in previous reaction further reacts with luminol in presence of cobalt as catalyst producing chemiluminescence signal. This biosensor was made of three layers. The first layer contained luminol, sodium phosphate, sodium lauryl sulphate as a surfactant and a polymer, hydroxyethyl cellulose as a carrier applied to the support. The second was an aqueous solution of Co^{2+} as a catalyst, and the third layer was an aqueous solution of glucose oxidase. After applying the sample solution (glucose) by micropipette onto the sensor, glucose reacted with glucose oxidase and hydrogen peroxide was formed. Hydrogen peroxide diffused towards the polymeric layer containing luminol and produced chemiluminescence reaction. The detection limit for the new glucose biosensor (3σ) was found to be 19 mg L^{-1} glucose (σ from 5 determinations of 30 mg L^{-1}). A relative standard deviation of 7.6 % was recorded for 10 measurements of 50 mg L^{-1} standard glucose solution, and 6.8 % for 10 measurements of 500 mg L^{-1} standard glucose solutions. The glucose biosensor was used for the determination of glucose in soft drinks (mainly apple juices). The results obtained with the chemiluminescence sensors and commercial glucometer (as the reference method) are in good agreement. The corresponding recovery rates were between 93 and 105 %.

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INTRODUCTION

Professor Clark has been known as the father of "Biosensor concept" since he published his definitive paper on the oxygen electrode in 1956 (Clark, 1956). Later, Clark and Lyons coined the term enzyme electrode, which was followed by Updike and Hicks (1967) when they experimentally detailed the fabrication of a functional enzyme electrode for glucose (Clark and Lyons, 1967). This enzyme biosensor was based on detecting the decrease of oxygen, which was the co-substrate for the conversion of glucose by the enzyme glucose oxidase (GOX). Later, the oxidation of glucose was also followed by the increase of hydrogen peroxide.

Of all biosensors, the glucose biosensor has been studied most. In 1994 almost 2000 articles describing glucose electrodes and glucose sensors have been published.

The first commercial biosensor for estimation of glucose was launched by Yellow Springs Instrument Company (Ohio) in 1975. This was based on the amperometric detection of H_2O_2 .

The glucose analysers commercialized by Medi Sense (Abbott Laboratories, USA) in 1987 and Boehringer Mannheim and Bayer are also mediated biosensor. National Physical Laboratory, India has patented a technology based on mediated electron transfer for glucose estimation (Asha and Malhotra, 2002).

A lot of biosensors for the determination of glucose have been developing in last years for different purposes. Some of them are: Pan and coworkers prepared amperometric glucose biosensor based on immobilization of glucose oxidase in electropolymerized *o*-aminophenol film at copper-modified gold electrode. This biosensor has detection limit of 0.01 mM, high sensitivity ($12.6 \text{ mA M}^{-1} \text{ cm}^{-2}$) due to existence of Cu nanoparticles. Also, it exhibits good selectivity, large response current, fast amperometric response, good reproducibility and excellent stability (Pan *et al.*, 2005).

Benamin P. Corgier with coworkers developed screen-printed electrode microarray for electrochemiluminescent (CL) measurement of glucose and lactate. A microarray of nine screen-printed graphite electrodes was used to develop multi-parametric electroluminescent biochip. The whole biochip is based on the ECL detection of enzymatically generated H_2O_2 . The intrinsic performances of this electrode array were evaluated through the cyclic-voltammetric experiments in 0.1M ferricyanide. Detection limit for glucose was 10 μM (Corgier, Marquette and Blum, 2005).

An interference-free implantable glucose microbiosensor based on use of a polymeric analyte-regulating membrane was developed by Xie *et al.* Two polymers, poly (4-vinyl pyridine), PVP and poly 4-vinyl pyridine-co-acrylic acid, PVP-PAC were investigated. The biosensor with PVP-PAC showed excellent selectivity to glucose against interferents like oxygen and ascorbic acid. The dynamic range is from 0-30 mM. The response time in amperometric measurement was less than 10 sec (Xie, Tan and Gao, 2005).

Biosensor for the determination of glucose in fruit juice by flow-injection analysis was developed by Guémas (Guémas, Boujtita and El Murr, 2000).

A glucose oxidase amperometric electrode was developed by Bacon and Hall. A "sandwich" bielelectrode system is described for a glucose oxidase amperometric electrode that uses outer scavenger electrode to remove ascorbic acid interference from the measurement of enzyme generated hydrogen peroxide. In this work, it is shown that the scavenger electrode is able to remove about 80% of the ascorbate. This system is tested with samples of lemon juice (Bacon and Hall, 1999).

New biosensor for the determination glucose based on immobilized glucose oxidase based on homogeneous chemiluminescence detection by flow-injection system was developed. This biosensor was applied to fruit juices and biological fluid, human urine. The dynamic working range from 2.5×10^{-6} to $1.9 \times 10^{-3} \text{ mol L}^{-1}$ was obtained. The detection limit was $8.6 \times 10^{-7} \text{ mol L}^{-1}$ (at the 3σ) glucose for the Co(II)-luminol system (Manera *et al.*, 2004).

Glucose biosensor for the quantitative detection of glucose in the physiological range (0-450 mg/dL, 0-25 mM) with 3-days stability based on surface-enhanced Raman scattering (SERS) was developed (Yonzon *et al.*, 2004).

The aim of this work was to develop a one-shot chemiluminescence biosensor for determination of glucose based on coupling of two reactions: enzymatic oxidation of glucose and chemiluminescence reaction of luminol with hydrogen peroxide in presence of cobalt ions as catalyst. Chemiluminescence reagents: luminol, sodium phosphate and sodium lauryl sulphate are incorporated into polymeric matrix of hydroxyethyl cellulose (HEC). Second layer of the biosensor contained cobalt salt as a catalyst while third layer contained glucose oxidase. Microplate luminometer and a home-made luminometer were used for optimization

of experimental parameters and construction of a calibration curve. The biosensor was used in the determination of glucose in soft drinks and obtained results were compared with commercial glucometer.

EXPERIMENTAL

Instrument

Measurements were performed with the *home made luminometer* (Moderegger, 2003) and with a *microtiterplate luminometer* Lucy1. A *commercial* Glucometer GlucoMen (GlucoMen, GlycO, A. Menarini Industrie, Farmaceutische Riunite S.E.L. Diagnostic Division, Firenze Italy) was used for the determination of glucose in the samples as a reference method.

Reagents

The hydrogen peroxide stock solution ($10\,000 \text{ mg L}^{-1}$) was prepared daily by diluting 1 g of 30% solution with water. It was stored at 4°C in the dark. Further dilutions were made immediately before use.

The sodium salt of luminol (100 mg) and $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (250 mg) were dissolved in water (10 mL). This stock solution was stored at 4°C in the dark. The freshly prepared solution was allowed to stand for 48 hours before use. The stock solution of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 mol L^{-1}) was prepared by dissolving $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (1190 mg) in water (50 mL).

A stock solution of glucose (10000 mg L^{-1}) was prepared by dissolving of 0.5 g of glucose in 50 mL of water. It was left to stand for 48 hours before use to facilitate α - β mutarotation, as it was recommended (Zhu, Li and Zhu, 2002; Lindfors, Lähdesmäki and Ivaska, 1996) and stored at 4°C when not in use. Solutions of lower concentration were prepared immediately before use.

The stock solution of surfactant, sodium lauryl sulfate (SLS) was prepared by dissolving it (100 mg) in (10 mL) water.

A stock solution of glucose oxidase was prepared by dissolving 6 mg of glucose oxidase in 0.5 mL water and then diluting it with water with ratio 1:3. The solution was stored in the freezer at -20°C .

Trisodium phosphate $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (1 g) was dissolved in 10 mL water.

A stock solutions of the interferent (ascorbic acid, citric acid monohydrate, D-(-)-fructose, saccharose, lactose, oxalic acid, L (+)- tartaric acid, D-(-)-quinic acid, fumaric acid, D-(+)-galacturonic acid) were prepared by dissolving of 0.1 g in 10 mL water on the same day of use. Solutions of lower concentration were prepared immediately before use.

The solutions of interfering compounds with concentration of 0.5 ppm (only for ascorbic acid), 5, 50 and 500 ppm in the sample solution of 50 ppm of glucose were prepared just before measuring. They were stored in the refrigerator at 4°C .

Samples

The glucose biosensor was used for the determination of glucose in soft drinks, mainly apple juices (Table 1).

Preparation of glucose biosensor

The biosensor was prepared by drop-coating technique. The concentration of substances, which were used for preparing sensor layers were: 0.15 % HEC (m/m), luminol

(0.11 mmol L⁻¹), sodium phosphate (5.26 mmol L⁻¹), sodium lauryl sulfate (60 mg L⁻¹), cobalt chloride (0.05 mmol L⁻¹), and water solution of glucose oxidase.

Table 1: Soft drinks analyzed with the new glucose biosensor.

#	Brand	Type
1.	Apfel saft, 100%, Spar	Apple juice
2.	Apfelsaft, 100%, Happy Day	Apple juice
3.	Apfelsaft, 100%, Clever	Apple juice
4.	Apfelsaft, 50%, Clever	Apple juice
5.	Apfelsaft, 100%, Obi	Apple juice
6.	Indian Tonic Water, Schweppes	Tonic water

Biosensor I was made by three layers: first: HEC, SLS, luminol and phosphate; the second: Co, HEC and SLS and the third: glucose oxidase.

Biosensor II was made by fourth layers, one layer more than Biosensor I with layer of HEC and SLS in order to separate Co and luminol layers, and Biosensor III, with the second layer made of water solution of cobalt chloride.

After preparing the solutions for the biosensor, it was applied on microscope cover glass, dry in the oven at 70° C for 2 hours. After that it was cooled in the desiccator. The next layers were made using the same procedure: applied volume of each layer was 10 μL. The last layer was glucose oxidase. A stock solution of this enzyme was applied (5 μL) and after drying in desiccator it was stored at 4°C in the fridge. Scheme of the biosensor preparation is given in Figure 1.

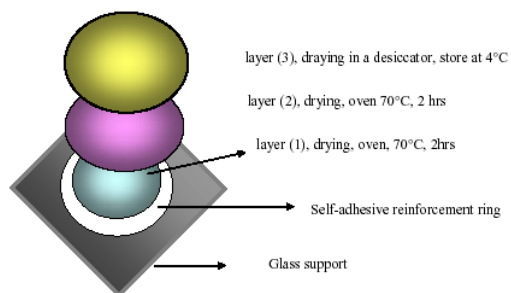


Figure 1: Preparation of glucose biosensor by drop-coating technique.

Results

After applying sample solution (glucose) by micropipette in the photodiode luminometer, glucose reacted with glucose oxidase and hydrogen peroxide was formed. Than H₂O₂ reacted with luminol in basic solution and in the presence of Co as catalyst emitting the light that can be detected by photodiode (Figure 2).

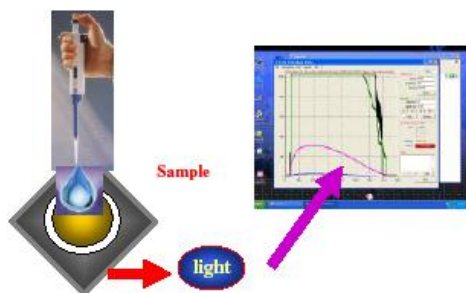
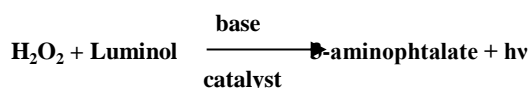
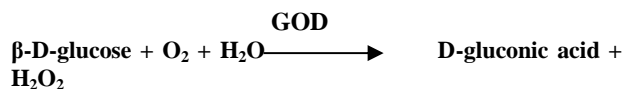


Figure 2: Chemiluminescence signal produced by glucose biosensor.

RESULTS AND DISCUSSION

Preliminary investigation of a biosensor preparation

Our investigations of a preparation of a new biosensor for glucose is based on a fact that glucose can be enzymatically determined by its enzymatic oxidation to gluconic acid and hydrogen peroxide in the presence of glucose oxidase. CL detection of hydrogen peroxide relies on its well-known CL reaction with luminol (Panoutsou and Economou, 2005) according to Scheme 1.



Scheme 1

For the preliminary investigation we prepared three kinds of biosensors to achieve the best results, marked as Biosensor I, II and III (Figure 3). The biosensors were prepared by drop-coating technique. It consisted of layers applied on a microscopic cover glass by micropipette.

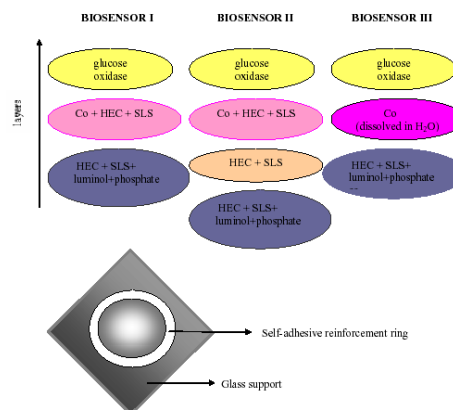


Figure 3: Preliminary investigations of glucose biosensor preparation

Biosensor III showed the best results, the highest chemiluminescence intensity and it was taken for the further investigations, while biosensor I and II showed weak chemiluminescence intensity or no signal was observed. The reason could be existence of a many sensor layers not allowing reactants to break in and get in contact with active surface.

Optimization of glucose oxidase concentration

Optimization of the glucose oxidase concentration was done with the microtiterplate luminometer. The results are shown in the Figure 4 and Table 2. The investigated solutions were prepared by dilution of stock solution of glucose oxidase, which were prepared by dissolving of 6 mg of glucose oxidase in 0.5 mL (2308 units/mL) water. Absolute concentration of glucose oxidase applied per biosensor was in the range 0.2 μg – 60 μg of enzyme.

Highest chemiluminescence intensity was achieved with a dilution of glucose oxidase stock solution in a range 1:3, which means that each biosensor contained 6.7 units of glucose oxidase. Smaller amounts of the enzyme gave lower signals, but also higher amounts of enzyme showed

increased signal but to a lesser extent. The reason for this behavior is unclear, but could be explained by surface blocked with too much enzyme, or binding of the catalyst to the protein.

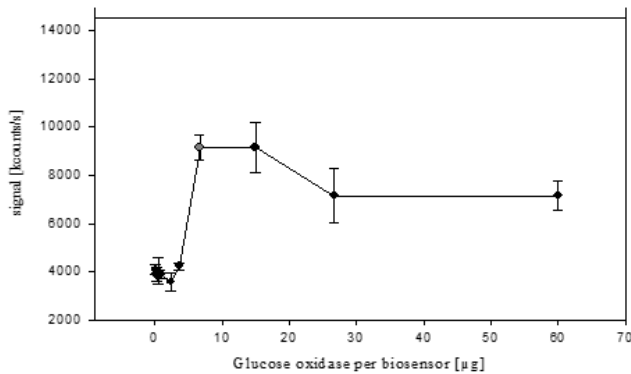


Figure 4: Dependence of the CL on the amount of GOX obtained with the microtiter luminometer. Membranes made by drop-coating, first layer (10 μL): 0.15 % HEC (m/m), trisodium phosphate (5.26 mmol L^{-1}), luminol (0.11 mmol L^{-1}) and sodium lauryl sulfate (60 mg L^{-1}), the second layer (10 μL) Co^{+2} (0.05 mmol L^{-1}), the third layer glucose oxidase (5 μL , 6.7 μg per biosensor), sample concentration: 500 mg L^{-1} glucose: the layers were applied to each well of a microtiter plate; repetitions =5.

Table 2. Chemiluminescence intensity obtained with different amount of glucose oxidase.

#	Absolute amount of glucoseoxidase applied per biosensor [μg]	Signal [kcounts/s]
1.	60	7150 \pm 620
2.	26.7	7200 \pm 1100
3.	15	9100 \pm 1000
4.	6.7	9150 \pm 510
5.	3.7	4230 \pm 140
6.	2.4	3570 \pm 360
7.	0.9	3880 \pm 190
8.	0.5	4050 \pm 540
9.	0.3	3910 \pm 270
10.	0.2	4090 \pm 180

Stability of the glucose biosensor

The long-term applicability of the biosensor was investigated. For that purpose glucose biosensor was stored at room temperature, at 4°C in the refrigerator and at -18°C in a deep freeze for 18 days (Figure 5).

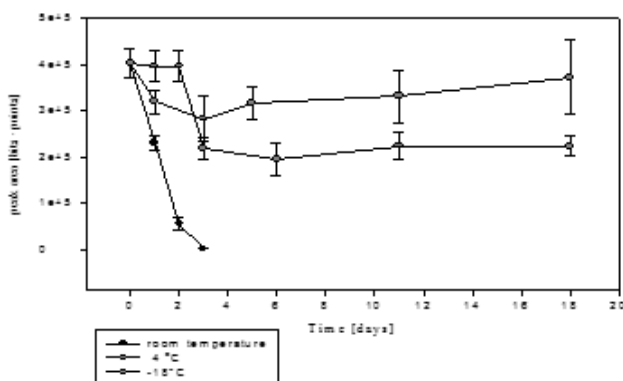


Figure 5: Stability of the glucose biosensor in dependence on the storage temperature. Membranes were made by drop-coating a solutions of: HEC 0.15 % (m/m), trisodium phosphate (5.26 mmol L^{-1}), luminol (0.11 mmol L^{-1}), Co^{+2} (0.05 mmol L^{-1} , SLS (60 mg L^{-1}), sample concentration: 100 $\mu\text{g L}^{-1}$; sample volume 10 μL , repetitions =5.

The biosensor gave a rather stable response after 2 days of storage in the refrigerator at 4°C without changes in its chemiluminescence intensity. Then the signal dropped for about 50% and stayed like that during the next two weeks. Further investigations were done within the first 2 days after preparation of the biosensors.

When stored at room temperature, the chemiluminescence intensity rapidly decreased, and after 3 days no signal was observed. When the biosensor was stored in a deep freeze (-18°C), the chemiluminescence signal dropped to one third of its initial value, and after that the signal was rather stable. But biosensor stored under these conditions showed very high standard deviations. Probably freezing destroys somehow the mechanical structure of the polymeric backbone (cellulose contains significant amounts of water) that deteriorates the analytical performances of the sensor.

Calibration curve

The calibration curve of the glucose biosensor is shown in Figure 6. In the investigated concentration range (20-1200 mg L^{-1}) two quasi-linear ranges were found, i.e., 20-100 mg L^{-1} ($r^2=0.9992$) and 100-900 mg L^{-1} ($r^2=0.9994$) glucose.

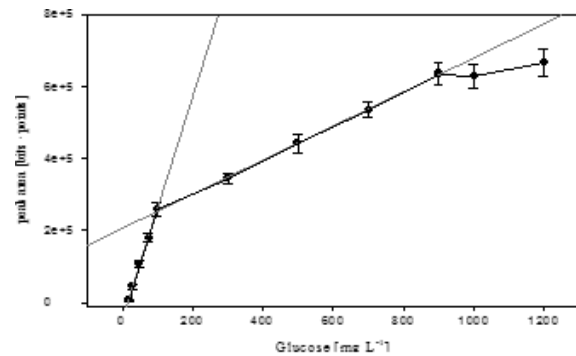


Figure 6: Calibration curve of glucose; membranes made by drop-coating from 10 μL of a solutions made of: 0.15 % HEC (m/m), trisodium phosphate (5.26 mmol L^{-1}), luminol (0.11 mmol L^{-1}) and sodium lauryl sulfate (60 mg L^{-1}), the second layer (10 μL), Co^{+2} (0.05 mmol L^{-1}), the third layer glucose oxidase (5 μL), sample concentration: 20-1200 mg L^{-1} 10 μL dispersed polymer solution on a glass lamella, sample volume 10 μL , repetitions =5.

The detection limit (3σ) was found to be 19 mg L^{-1} glucose (σ from 5 determinations of 30 mg L^{-1}). A relative standard deviation of 7.6 % was recorded for 10 measurements of 50 mg L^{-1} standard glucose solution, and 6.8 % for 10 measurements of 500 mg L^{-1} standard glucose solutions.

Interferences

Glucose is an important component in many alcoholic and soft drinks and often occurs together with many others organic species (acids and sugars), which might interfere with glucose. The main potential interferences are saccharides, metal cations, organic acids and reducing agents (Manera *et al.*, 2004). The results are summarized in Table 3.

From the data it can be seen that practically all interferences, except ascorbic acid, interfere even at higher concentration only to a limited extent, i.e., below 20% change of the signal. As the experimental uncertainty is a bit less than 10% already, this seems acceptable for a simple portable device.

Ascorbic acid does strongly interfere even in a concentration of 5 ppm or higher, added to the glucose concentration of 50 ppm. Concentrations of 0.5 ppm or

lower in the glucose solution do not show any significant deviation of the glucose response.

Table 3: Investigated interferences on the determination of glucose; sample solution contains 50 ppm glucose and interferent.

Interferent	Change of signal (%) mg L ⁻¹		
	0.5	5	500
Fructose	5.5	+3.1	+7.3
Saccharose	+9.2	-16.3	-17.1
Lactose	+8.6	-11.6	-12.8
Mannose	+3.5	-7.9	-10.6
Ascorbic acid	-2	-94	-100
Citric acid	+10.7	-9.9	-11.5
Oxalic acid	-8.9	+5.0	-5.1
Tartaric acid	-5.0	+12.9	+18.1
Quinic acid	-5.9	-6.9	+7.2
Fumaric acid	-6.1	-6.8	-15.5
Malic acid	-7.5	+6.7	+20.5
Galacturonic acid	+5.1	-4.6	+10.4

Thus, with real samples attention must be paid that the concentration of vitamin C does not exceed the given value in order to avoid erroneous results. With soft drinks it was found that dilution of the sample (100 to 200 fold) usually meets this criterion. If the ascorbic acid concentrations are too high either pretreatment of the sample is necessary, e.g., selective destruction of vitamin C by ascorbate oxidase and catalase, or interference-free sensor must be designed (protective layers).

One of the features of the proposed method is that it allows extensive dilution of the sample so that interferences due to the presence of possible interferences are alleviated.

Samples

The glucose biosensor was used for the determination of glucose in soft drinks.

The drinks were diluted 1:100 or 1:200 prior to analyses; the results obtained with the photodiode luminometer and a commercial glucometer as a reference are summarized in Table 4.

Table 4: Glucose concentrations in the investigated samples.

#	Glucose [g L ⁻¹] Photodiode Luminometer	Glucose [g L ⁻¹] Glucometer	Recovery (%)
1.	31.1 ± 3.0	29.9 ± 1.4	104.0
2.	29.7 ± 0.6	31.9 ± 1.1	93.1
3.	22.5 ± 2.6	23.5 ± 1.3	95.7
4.	16.2 ± 1.7	16.8 ± 0.8	96.4
5.	27.5 ± 2.1	28.0 ± 1.6	98.2
6.	66.8 ± 2.0	63.7 ± 1.9	104.9

From the Table 4 it can be seen that results obtained by two methods agree very well. A recovery rates were between 93 and 105 %. This seems very acceptable for a simple portable device.

CONCLUSIONS

After preliminary investigations with a microtiterplate luminometer, and development of a sensor for the determination of hydrogen peroxide that was applicable to

rainwater samples, a new biosensor was developed for the determination of glucose. The method is based on the determination of chemiluminescence given out by the cobalt-catalyzed reaction of luminol with hydrogen peroxide, which is produced by the reaction of glucose oxidase with glucose.

The biosensor was characterized with respect to storage, temperature and interferences, and its applicability for the determination of glucose in some soft drinks was proven.

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Summary/Sažetak

Pripremanje novog biosenzora za određivanje glukoze je bazirano na činjenici da se glukoza može oksidirati do glukonske kiseline uz pomoć enzima glukoza oksidaze. Biosenzor je napravljen od tri sloja. Prvi sloj sadrži luminol, natrijum fosfat, natrijum lauril sulfat kao surfaktant, i polimer hidroksietil celulozu, kao nosač. Drugi sloj je vodeni rastvor Co^{2+} kao katalizator, i treći sloj je vodeni rastvor glukoza oksidaze. Nakon apliciranja uzorka na biosenzor, glukoza reaguje sa glukoza oksidazom, i formira se hidrogen peroksid. Nagrađeni hidrogen peroksid prolazi do sloja koji sadrži luminol i javlja se kemiluminescentna reakcija. Limit detekcije novog biosenzora za glukozu (3σ) iznosi 19 mg L^{-1} glukoze (σ za 5 mjerenja koncentracije glukoze od 30 mg L^{-1}). Relativna standardna devijacija iznosi 7.6 % za 10 mjerenja standardnog rastvora glukoze koncentracije 50 mg L^{-1} i 6.8 % za 10 mjerenja standardnog rastvora glukoze koncentracije 500 mg L^{-1} . Novi biosenzor je korišten za određivanje glukoze u bezalkoholnim pićima, većinom sokovima od jabuke. Rezultati dobiveni kemiluminescentnim biosenzorom i komercijalnim glukometrom kao referentnom metodom su pokazali jako dobro slaganje. Recovery vrijednost je bila između 93 i 105 %.