



# Electrochemical Detection of Glucose in Beverage Samples Using Poly(3,4-ethylenedioxythiophene)-Modified Electrodes with Immobilized Glucose Oxidase

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## Abstract

We developed two glucose biosensors based on poly(3,4-ethylenedioxythiophene) (PEDOT) and polyacrylic acid (PAA) doped with poly(4-lithium styrenesulfonic acid) (PSSLi) or PEDOT and anthranilic acid (AA) doped with poly(4-styrenesulfonic acid) (PSSH). The first one was already mentioned in literature in similar configurations; however, the second one has not been described before. The glucose oxidase was immobilized by means of covalent bonding, which has been proved by spectrophotometric assay with *o*-dianisidine. Amperometric measurements were carried out for the determination of glucose in phosphate buffer. The prepared electrodes functioned as first-generation glucose biosensors at applied potential  $E = +0.6$  V during  $t = 30$  s. The elaborated procedure was validated, and all of the statistical parameters met the requirements for biosensors. The presented method was successfully applied for determination of glucose concentration in food samples, such as grape juice and honey. The activity of the presented biosensors was also studied in the presence of ascorbic acid and uric acid, acting as interfering agents.

**Keywords** Amperometric sensor · Biosensor · Glucose · Glucose oxidase · PEDOT

## Introduction

More than 50 years have passed since Clark and Lyons [1] first proposed the concept of the glucose enzyme electrode. Their first device consisted of enzyme glucose oxidase (GOD) entrapped over an oxygen electrode via a semipermeable dialysis membrane. The determination of glucose amount was based on monitoring of the oxygen consumed by the enzyme-catalyzed reaction: glucose + oxygen  $\rightarrow$  gluconic acid + hydrogen peroxide [1]. Yellow Spring Instrument Company used Clark's patent to launch the first dedicated glucose analyzer in 1975. Updike and Hicks [2] introduced slight modifications, by using two working oxygen electrodes, one of which was covered with enzyme, and measuring the differential current in order to correct for the oxygen background

variation in samples. Further progress was made when Guilbault and Lubrano [3] determined glucose in blood on the basis of amperometric monitoring of the liberated hydrogen peroxide. Glucose biosensors have been evolving rapidly since the 1980s. Subsequent research led to development of mediator-based biosensors (second generation) [4–7], devices for self-monitoring of glucose levels, and a non-invasive method of glucose monitoring [8, 9].

There have been many sensors developed over the years on the basis of conducting polymers, including gas sensors [10], hydrogen sensors [11], and acetic acid sensors [12]. There has been a recent spike in the interest in biosensors based on conducting polymers. Conducting polymers are versatile materials for creation of biosensors due to their rapid production, controlled thickness, and porosity, as well as easy electropolymerization on various surfaces. Moreover, the immobilization of enzymes on electrodes allows to reuse enzymes many times, due to their longer life spans, lower degradation levels, and improved stability. There are several techniques of enzyme immobilization, including adsorption [13],

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covalent attachment [14, 15], entrapment [16–18], and cross-linking [19, 20]. Enzyme immobilization by covalent bonding is the most commonly used technique; it has unquestionable advantages, such as the stable nature of the bond which prevents the enzyme from leaking into the solution during the measurement. However, this type of immobilization is quite expensive and difficult to carry out [14].

Information concerning food composition, including information on its glucose content, is essential for modern food industry due to greater consumer awareness and expectations. According to the World Health Organization (WHO) [21], more than 346 million people in the world suffer from diabetes. In 2012, diabetes was the direct cause of 1.5 million deaths. WHO predicts that the number of diabetes deaths will have doubled by 2030, making it the seventh leading cause of death in the world [21]. Prolonged high blood glucose can cause several health disorders such as cardiovascular diseases, heart problems, strokes, retinopathy, blindness, nephropathy, and neuropathy. Thus, it is crucial, for both producers and consumers, to know the concentration of glucose in food. Moreover, a lot of attention is nowadays devoted to the challenging field of product authentication. The determination of glucose content in honey or wine can prove their originality and even help to identify the region of the world from which the product originates [22, 23].

There are numerous articles describing glucose biosensors based on GOD. Many of them focus on the construction and preparation of the sensors and their use for the determination of glucose in aqueous solutions. A much smaller number of articles describe the use of sensors in determination of glucose in solutions containing natural sources of glucose, such as beverages, food, and blood. Table 1 shows examples of the use of GOD-based sensors for determination of glucose in natural products.

This paper focuses on an optimized procedure for determination of glucose in grape juice and honey which uses novel glucose biosensors. First, two biosensors were prepared based on poly(3,4-ethylenedioxythiophene) modifications. One electrode was modified with poly(3,4-ethylenedioxythiophene) (PEDOT) and polyacrylic acid (PAA) doped with poly(4-lithium styrenesulfonic acid) (PSSLi), while the other consisted of two sublayers: one layer of PEDOT doped with PSSLi and one which consisted of PEDOT and anthranilic acid (AA) doped with poly(4-styrenesulfonic acid) (PSSH) [33]. GOD was immobilized on the surface by means of covalent bonding. The so-prepared electrodes functioned as first-generation glucose biosensors. The developed measurement procedure was validated with a full statistical study, and the obtained results were compared with the reference method. Another very important feature of the procedure was the fact that it allowed the determination of glucose in the presence of ascorbic acid and uric acid.

## Experiment

### Reagents

All chemical reagents were analytically pure and used without further purification. Glucose oxidase (GOD) (type II, *Aspergillus niger*, 17,300 units/g), polyacrylic acid (PAA), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (WSC), peroxidase (POD) (type I, horseradish, 290,000 units/g), and ethylenedioxythiophene (EDOT) (all obtained from Sigma-Aldrich) were stored at 4 °C. D(+)-glucose, anthranilic acid (AA), *o*-dianisidine, poly(4-lithium styrenesulfonic acid) (PSSLi—30 wt% solution in water), poly(4-styrene-sulfonic acid) (PSSH—18 wt% solution in water), ascorbic acid, and benzoic acid (99.5%) (supplied by Sigma-Aldrich) were stored at room temperature. Sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen orthophosphate dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ), and potassium dihydrogen orthophosphate ( $\text{KH}_2\text{PO}_4$ ) were supplied by POCH Gliwice. Sulfuric acid ( $\text{H}_2\text{SO}_4$ —95%), hydrochloric acid (HCl—35–38%), sodium hydroxide (NaOH) (all from POCH Gliwice), and perchloric acid ( $\text{HClO}_4$ —70%) (Sigma-Aldrich) were also used. All solutions were prepared with distilled water purified by the Millipore system (Milli-Q).

The measurements of the real samples were conducted with “Fortuna” grape juice and “Sadów” flower honey, both bought at a local shop. Phosphate buffer saline (PBS) (pH = 7.4) consisted of NaCl (137 mM), KCl (2.7 mM),  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (10 mM), and  $\text{KH}_2\text{PO}_4$  (1.76 mM).

### Apparatus and Procedures

The measuring equipment included a PAR 273A potentiostat (EG&G Princeton Applied Research Company) and a computer with CorrWare 2.9 and CorrView 2.9 software (Scribner Associates, Inc.). All electrochemical measurements were carried out in a three-electrode cell. A modified platinum electrode was used as a working electrode; a saturated calomel electrode (SCE) and a platinum mesh served as a reference electrode and a counter electrode, respectively. The platinum electrodes used in the research were cylindrical rod-type platinum electrodes (diameter = 0.15 cm and length = 1.0 cm) with a surface area of 0.49 cm<sup>2</sup>. Spectrophotometric measurements were carried out with the help of a Metertek SP830 device with an optical path length of 1 cm. Unless otherwise stated, all measurements were taken at room temperature.

The preparation of electrodes was carried out in accordance with the procedure elaborated and presented in the previous paper [33]. In the first stage, electrodes were modified with composite materials. One PEDOT-based composite material was obtained by means of an electrochemical synthesis at potential  $E = +1.0$  V for time  $t = 3$  s in a water solution containing EDOT, PAA, and PSSLi.

**Table 1** Examples of the use of sensors based on GOD for the determination of glucose in natural products

Sensor	Linear range (mM)	Response time (s)	Stability (days)	LOD (mM)	Sample	Ref.
GOD-immobilized sensing membranes, fluorescence measurement	0.5–5.5	600	–	0.5	–	[24]
GOD and horseradish peroxidase with ferrocene on carbon paste electrode	0.11–0.45	10	–	–	Fruit juice	[25]
GOD and horseradish peroxidase on polyaniline with glutaraldehyde, measured at 510 nm	0.28–33	–	5	0.28	Sport drinks, energetic drinks, orange juice	[26]
Several layers: cellulose acetate, GOD crosslinked with glutaraldehyde and polyurethane	0–2.8	100	6	–	Biological fluids	[27]
GOD immobilized on layer of biopolymer chitosan on Pt electrode,	0.05–15	<8 s	60	0.01	Fruit juice, sport drink, cola	[28]
GOD reactor, FIA, iodine-starch complex measured at 620 nm	0.1–2	20	30	0.1	Apple juice, banana jam	[29]
GOD immobilized between two polydiallyl-dimethylammonium chloride layers on CNT	0.015–6	60	14	0.007	Fruit juice	[30, 31]
GOD incorporated into colloidal Au–CNT composite electrode	0.05–1	10	8	0.017	Sport beverages	[32]
Sensor A	0.96–30	10–30	30	0.29	Grape juice, honey	This work
Sensor B	1.86–30			0.56		

Another composite layer consisted of two sublayers. The first composite layer of PEDOT was obtained by means of the potentiostatic method with  $E = 1$  V for  $t = 5$  s (EDOT in PSSLi). The second sublayer of PEDOT and AA was synthesized by using cyclic voltammetry ( $E = -0.4$  V to 1.2 V,  $v = 20$  mV/s,  $n = 1$  cycle) from a solution of EDOT and anthranilic acid (AA) in PSSH.

The second stage of chemical immobilization of GOD was the same for both electrodes. The procedure required immersing the modified electrode in a PBS solution of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (WSC) at 4 °C for 1 h. Subsequently, the electrode was transferred to a solution containing GOD in PBS with CaCl<sub>2</sub>. Immobilization proceeded at 4 °C for 24 h. Finally, the electrode was immersed in a PBS solution with CaCl<sub>2</sub> at room temperature for 1 h. Between the measurements, the electrode was stored in a PBS solution (pH = 7.4) at 4 °C.

To simplify the nomenclature, the first biosensor—PEDOT/PAA/GOD—will be further referred to in this paper as biosensor A and the second biosensor—PEDOT/AA/GOD—as biosensor B.

The activity of the immobilized enzyme was tested according to the Sigma quality control test procedure (Enzymatic Assay of glucose oxidase EC 1.1.3.4) by detection of hydrogen peroxide created in enzymatic oxidation of glucose. The presence of peroxide was detected by spectrophotometric ( $\lambda = 500$  nm) determination of oxidized *o*-dianisidine produced in a reaction with hydrogen peroxide in the presence of peroxidase (Scheme 1 [reaction 3 ÷ 5]).

Solutions of glucose (concentrations of 1, 5, 10, 15, 20, 25, 30 mM) in PBS (pH = 7.4) were prepared by dilution of a stock glucose solution ( $c = 50$  mM) in PBS (pH = 7.4). After

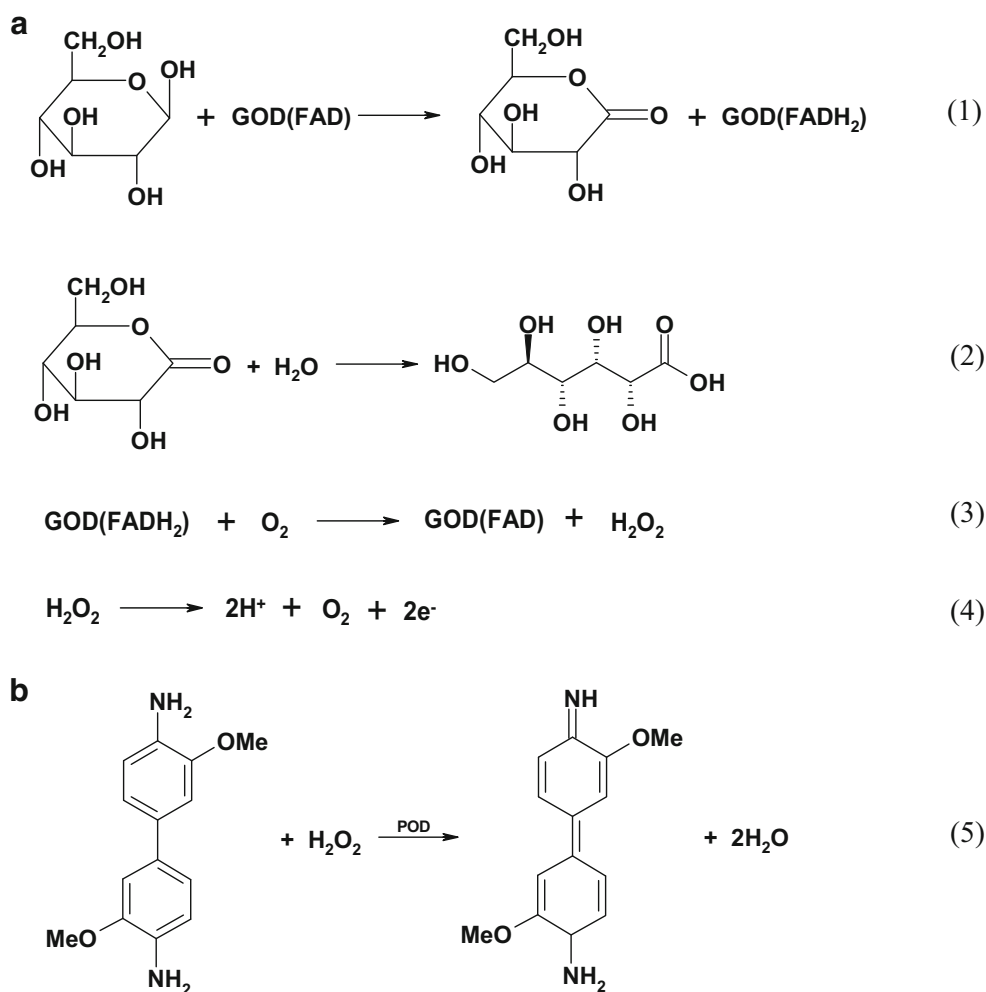
preparation, the stock glucose solution was stored at room temperature for 1 h to establish an equilibrium between  $\alpha$  and  $\beta$  anomers [34, 35]. Samples with ascorbic acid as an interfering agent were prepared by mixing appropriate volumes of the stock glucose solution with a stock ascorbic acid solution (10 mM ascorbic acid in PBS, pH = 7.4) to obtain solutions with constant concentration of ascorbic acid (1 or 0.1 mM) and changing concentration of glucose.

The solutions of grape juice were prepared by mixing the juice with PBS in a ratio of 1:24 ( $v/v$ ). Subsequently, the standard addition method was used by adding, respectively, between 0.5 to 2.0 ml of the stock glucose solution to a 2.5-ml juice sample and made up with an appropriate volume of PBS (total volume of 10 ml). The concentrations of the added glucose ranged from 2.5 to 10.0 mM.

The honey solution was prepared by dissolving 0.265 g of flower honey in 25 ml of PBS. Subsequently, the standard addition method was used by adding, respectively, between 0.5 to 2.0 ml of the stock glucose solution to a 2.5-ml honey sample and adding appropriate volume of PBS (the total volume was 10 ml). The finished solutions had concentrations of glucose which ranged from 2.5 to 10.0 mM.

Amperometric measurements were carried out in a three-electrode cell without deoxygenation. Amperometric measurements of the glucose samples were carried out at constant potential  $E = 0.6$  V for time  $t = 30$  s. During the measurements, the solution was being mixed with a magnetic stirrer. The measurements, performed by means of the standard addition method, were carried out by adding a portion of the standard solution to the measurement cell containing the analyzed compound. Between the measurements, the electrode was stored in a PBS solution (pH = 7.4) at the temperature of 4 °C.

**Scheme 1** **a** (1)–(4)—the mechanism of a first-generation glucose biosensor. **b** (5)—the reaction of hydrogen peroxide and the reduced form of *o*-dianisidine



The enzymatic method for determination of glucose concentration described by Bergmeyer and Bernt [36] was used as a reference method in the chosen natural samples. A set of appropriate solutions was prepared. Solution A contained 500 units of GOD and 100 units of POD in 39.2 ml PBS. Solution B contained 3.85 mg of *o*-dianisidine in 0.032 ml of 1 M HCl and 0.968 ml of H<sub>2</sub>O. Solution C was an assay reagent prepared by mixing 0.8 ml of solution B and 39.2 ml of solution A. Solution D was a glucose standard solution prepared by mixing 1 mg of glucose with 0.1% benzoic acid. Solution E was sulfuric acid prepared by diluting 33.3 ml of concentrated acid in 66.7 ml of H<sub>2</sub>O. The samples of natural products were diluted with water to contain approximately around 0.111–0.444 M of glucose. Three test tubes were prepared for the determination. The reagent blank (tube 1) contained 1 ml of water; the standard solution (tube 2) contained a mixture of 0.95 ml of water and 0.05 ml of solution D. The test solution (tube 3) contained 1 ml of the sample. At zero time, the reaction was initiated by adding 2 ml of solution C to each cuvette, mixing it, and placing it for 30 min in a water bath at 37 °C. After half an hour, the reaction

was stopped by adding 2 ml of solution E. Each tube was measured against the reagent blank at 540 nm, and the glucose content was calculated from the following equation:

$$(\text{mg/ml}) = \frac{(\text{DA}_{540}\text{Test}) \cdot (0,05)}{\text{DA}_{540}\text{Standard}}$$

In which DA<sub>540</sub>Test is the absorbance at 540 nm of the sample.

## Results and Discussion

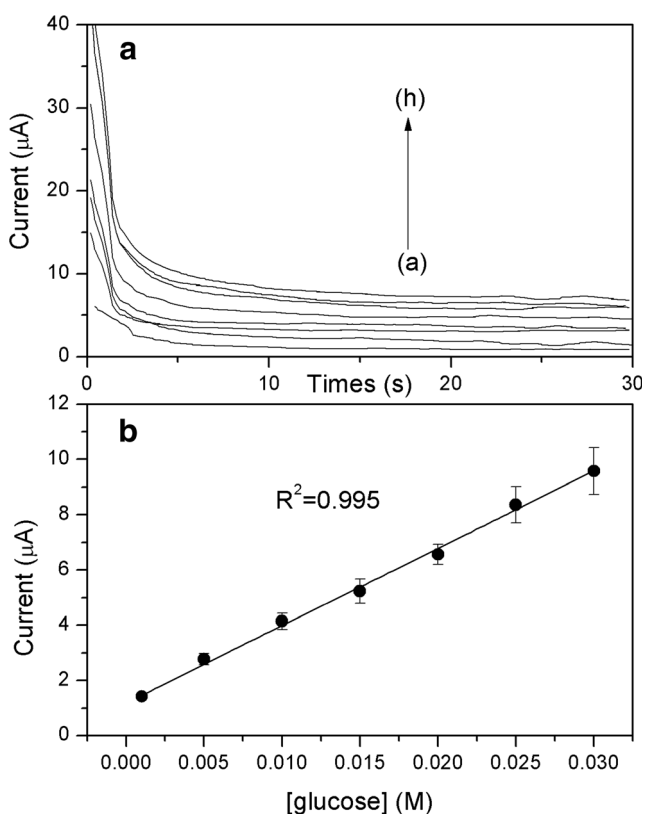
The oxidation mechanism of glucose has been investigated by many researchers [37]. Reactions 1 to 4 of Scheme 1 present the mechanism of first-generation glucose biosensor activity.

First (reaction 1), the glucose present in the solution reacts with glucose oxidase. During the process, glucose becomes gluconolactone, while GOD is reduced. Next (reaction 2), gluconolactone hydrolyzes to gluconic acid under the influence of the water present in the solution. At the same time, the reduced glucose oxidase is converted into its oxidized form

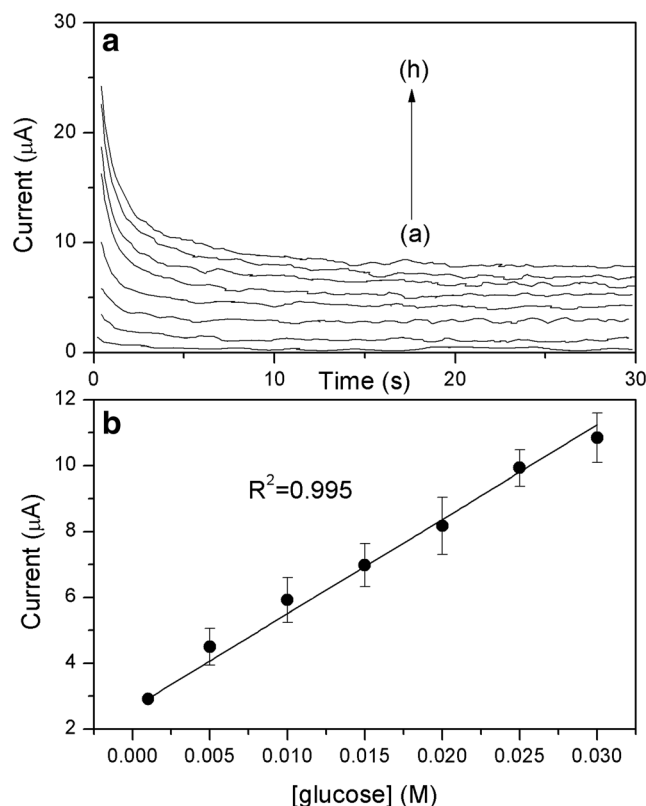
due to the oxygen present in the solution, and hydrogen peroxide is released (reaction 3), which, in reaction 4, is electrochemically detected at the surface of the electrode.

The test of electrodes' activity (Enzymatic Assay of glucose oxidase EC 1.1.3.4) was used as a simple tool not only to check the activity of the enzyme itself but also to prove that the enzyme immobilized on the surface of electrode shows activity. When electrodes were placed in the solution prepared according to the procedure, the color of the solution changed to brown. The shade became more intense with time, and absorbance measured at  $\lambda = 500$  nm increased accordingly. Reaction 5 on Scheme 1 is the reaction between hydrogen peroxide and *o*-dianisidine, which is the basis of this test (Enzymatic Assay of glucose oxidase EC 1.1.3.4). The change in the color of the solution proves that both biosensors produce hydrogen peroxide, confirming that the prepared biosensors function as first-generation glucose biosensors.

Fig. 1a shows amperometric curves recorded for glucose in supporting electrolyte on the PEDOT/PAA/GOD electrode, and Fig. 2a shows amperometric curves obtained on the PEDOT/AA/GOD electrode. As it can be seen in Figs. 1a and 2a, the current value increases due to higher glucose concentration. The current value, after the initial sharp decline,



**Fig. 1** **A** An example of amperometric curves for glucose oxidation (1–30 mM) in a PBS solution ( $E = 0.6$  V,  $t = 30$  s) at biosensor A (line (a) is the blind test). **B** Calibration curves with a calibration coefficient for biosensor A. The dependence of the current value on increasing glucose concentration. Error bars were counted applying  $t(S/n^{1/2})$ ,  $n = 8$ ,  $p = 95\%$



**Fig. 2** **A** An example of amperometric curves for glucose oxidation (1–30 mM) in a PBS solution ( $E = 0.6$  V,  $t = 30$  s) at biosensor B (line (a) is the blind tests). **B** Calibration curves with a calibration coefficient for biosensor B. The dependence of the current value on increasing glucose concentration. Error bars were counted applying  $t(S/n^{1/2})$ ,  $n = 5$ ,  $p = 95\%$

stabilizes after around 20 ÷ 30 s; thus, each measurement was completed after 30 s.

Calibration curves were prepared for both biosensors and show a linear dependence of the average current value on the level of glucose concentration (Figs. 1b and 2b). A comprehensive summary of the validation parameters for both biosensors is presented in Table 2.

Both biosensors are characterized by similar sensitivity and slope in the equation of the calibration curve. However, small differences are visible in sensitivity values (parameter a, Table 2) which is higher for sensor B. The other difference is a higher value of parameter b (Table 2) for sensor B. From these differences, it results in that sensor A is a little bit better than sensor B. Sensor A is more sensitive, and the current for zero concentration is closer to zero than in case of sensor B. The described differences indicate that on the surface of sensor A, there are more active particles of the enzyme which generates the current answer. On the surface of sensor B, the amount of immobilized enzyme is similar as in the case of sensor A, but some particles of the enzyme are not active. It is caused by the high concentration of protons present in the layer of copolymer PEDOT/AA/ doped by PSSH. Simultaneously, the layer of copolymer PEDOT/AA/ has a



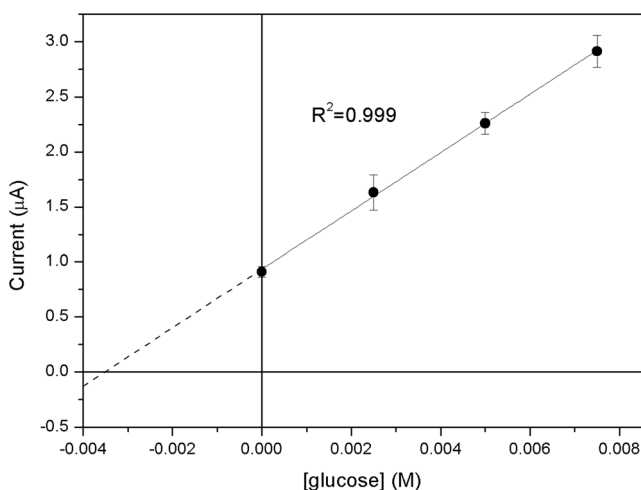
**Table 2** Validation parameters for both biosensors in real samples compared to results obtained with reference method. RSD parameters were counted applying  $t(S/n^{1/2})$ ,  $p = 95\%$ 

	Biosensor A	Biosensor B
$a$ (A/M)	$(2.74 \pm 0.07)E-04$	$(2.57 \pm 0.10)E-04$
$b$ (A)	$(1.31 \pm 0.12)E-06$	$(3.23 \pm 0.18)E-06$
$R^2$	0.995	0.995
RSD (%)	5.62	3.64
LOD (M)	$2.9E-04$	$5.6E-04$
LOQ (M)	$9.8E-04$	$1.86E-03$
Recovery (%)	101.7	100.0
Linear range (M)	$(0.98-30.0)E-03$	$(1.86-30.0)E-03$

higher background current which decides about a higher value of parameter  $b$  for sensor B. As it can be seen, the received results are good, and the correlation coefficient is characterized by values higher than 0.99. Similar results are obtained in for the relative standard deviation (RSD), whose value should not exceed 10%. As indicated in Table 2, the RSD for the presented biosensors is, respectively, 5.62 and 3.64%. The overall recovery is also very good, with almost 100% of glucose recovery. It can be stated out that both polymers present very good parameters for the determination of glucose, but sensor A is a little bit better than sensor B.

The stability of the sensors was checked on the basis of daily measurements of glucose solutions of the 0.1 M concentration. The average value of the peak of the determination was  $1.48 \cdot 10^{-2} \pm 0.05 \cdot 10^{-2}$  mA, and the obtained values of peaks differed from one another by less than 5%. It means that, at the assumed probability of 95%, the value of the obtained current can be assumed to be constant.

We analyzed also the influence of two very popular compounds: ascorbic acid and uric acid. Appropriate

**Fig. 3** The dependence of the current value on glucose concentration obtained using the standard addition method for biosensor B in a grape juice sample. Error bars were counted applying  $t(S/n^{1/2})$ ,  $n = 6$ ,  $p = 95\%$ 

measurements were carried out, since ascorbic acid is often used as a preservative in food and at the same time is believed to be a potential interfering agent for first-generation glucose biosensors. Uric acid is a substance which commonly occurs in natural products and body liquids. Its presence in the examined samples is very probable, which is why the research included also examination of its influence on the described analysis. For the purpose of statistic compilation, we assumed in our research that the value of probability was 95%. Thus, we assumed that the examined interferon influences the result of determination if the value of the determination current changes under its influence by more than 5%. Biosensors were tested for the determination of glucose (in the concentration range of 1 ÷ 30 mM) in the presence of ascorbic acid or uric acid in concentrations ranging from 0.01 to 1.0 mM in order to determine their selectivity. As a result of the conducted measurements, it was established that the presence of uric acid within the concentration range from 0.01 to 1.0 mM does not influence the determination of the glucose level. The results showed also that ascorbic acid did not interfere with the determination of glucose, if its concentration was between 0.01 and 0.1 mM or lower. When the concentration level of ascorbic acid was higher than 0.1 mM, the glucose determination was prevented in both sensors.

The determination of glucose concentration was carried out in real samples (grape juice and honey) in order to estimate the accuracy of the presented biosensors. Amperometric measurements were carried out by means of the standard addition method. Figure 3 shows the dependence of the current value on the increasing concentration of the added glucose obtained in the standard addition method for biosensor B in a grape juice sample. All the results obtained during the measurement of the real samples are presented in Table 3.

As can be observed, the biosensors are characterized by a very good correlation coefficient (in most cases,  $R^2$  reaches the value of about 0.999) and RSD. The calculated errors (Fig. 3) are at an acceptable level. Moreover, the measurements for both biosensors carried out in the same medium are characterized by similar slope values. Table 3 presents information on the concentrations of the glucose obtained with the examined sensors and by means of the reference method. It should be noted that the best results for both biosensors were obtained for the grape juice and honey samples.

**Table 3** Validation parameters for both biosensors in real samples compared to results obtained with reference method. RSD parameters were counted applying  $t(S/n^{1/2})$ ,  $p = 95\%$ 

Sample/sensor	$R^2$	RSD (%)	$c_{\text{glucose}}$ (mM)	$c_{\text{reference}}$ (mM)
Grape juice/A	1.000	5.01	$0.318 \pm 0.004$	$0.313 \pm 0.002$
Grape juice/B	0.999	9.09	$0.317 \pm 0.017$	
Honey/A	0.999	5.00	$3.136 \pm 0.146$	$3.399 \pm 0.020$
Honey/B	0.992	7.01	$3.282 \pm 0.354$	

After a careful examination of the presented results, it can be stated that the presented biosensors can be an appropriate tool for measurement of glucose concentration in samples of food such as grape juice and honey, provided that the concentration of ascorbic acid in such samples remains below the level of 0.1 mM.

## Conclusions

Electrochemical methods involving sensors and biosensors are widely used in analytical chemistry, as they require relatively simple measurement procedure and can be used in a flow analysis. The article has presented a method for determination of glucose concentration in food samples, e.g., apple juice, grape juice, energy drinks, and honey. Both biosensors worked as first-generation glucose biosensors, which has been proven by appropriate tests. All the measurements were carried out with electrolysis at constant potential. The described validation parameters met the requirements for biosensors in electrochemical measurements.

A very important advantage of the examined sensors is a wide range of the determined concentrations of glucose and very high stability connected with high stability of the signal. The sensors were also characterized by a relatively simple method of preparation, very high reproducibility of the results, and short response time. They can work for a period of 30 days with no decrease in their activity. It is important to note that both sensors allow the determination of glucose in the presence of ascorbic acid, at concentration of up to 0.1 mM.

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