Optimization of sucrose measurement working procedure in real samples using conductometric enzyme biosensor

V. N. Peshkova¹,², A. A. Soldatkin¹, S. V. Dzyadevych¹

¹Institute of molecular biology and genetics NAS of Ukraine
Academicain Zabolotnog str., 150, Kyiv, 03680 Ukraine
²National Taras Shevchenko University of Kyiv,
64, Volodymyrska street, 01033 Kyiv, Ukraine.
victoir@ukr.net

The working procedure of sucrose and glucose conductometric biosensors with real samples of juices and sweet drinks has been presented. For the purpose of sensitivity and stability improvement of the sucrose biosensor, optimal enzyme proportions and concentrations in its bioselective element have been chosen. Different variants of determination of sucrose and glucose in real samples using the biosensor have been considered. The sucrose and glucose measurement has been carried out in juices and sweet drinks. The method suggested could be used in food industry for the production control and optimization.

Keywords: conductometric enzyme biosensor, immobilized enzymes, sucrose, glucose

Introduction. Sucrose, glucose, and other carbohydrates are the constituent parts of many food products, including fruit, vegetables, and fruit drinks. Due to the fact that the contents of these carbohydrates in fruit and vegetables is connected directly with cultivating and storing conditions of the latter, and may also determine the degree of their ripening and quality, there is a need in well-tuned system of their monitoring [1]. Sucrose, a key component of molasses, is used in food, pharmaceutical, and cosmetic industry [2, 3] and in some processes of biotransformation in biotechnological manufacturing [4]. Molasses is also known to be the natural source of various products, in particular, sugar [5]. The process of biotechnological production (cultivation, fermentation, etc.) requires permanent control for both product output, and quality and contents. Permanent monitoring of biotechnological processes is necessary for deeper understanding and optimisation, as well as for the control of their course.

Current standard methods of accurate sucrose determination, including liquid chromatography, chemical and optical methods, used in food industry and medicine, are disadvantageous in necessity of highly skilled personnel and expensive complicated equipment [6]. Sucrose concentration can also be determined by isotopic method and gas-liquid chromatography but these methods are not widely used due to their high price, complicated procedure, and requiring a lot of space [7]. One of the main disadvantages of the aforementioned approaches is quite complex sample pretreatment. Polarimetric and refractor methods of determining sucrose though faster, are less accurate [6]. At the same time they are sensitive to a number of interfering components, present in the solution [8]. Therefore, devel-
opment of more convenient, precise, selective, fast, and inexpensive method of determination of sucrose content in various alcoholic and non-alcoholic beverages and foodstuff is an issue of the day.

Currently, there is certain information on development of a number of sucrose biosensors [6, 9–17]. Thus, [16] presents amperometric sucrose biosensor for on-line control of the process of enzymatic biotransformation of glucose into fructose by glucosoisomerase. Literature also contains the possibility of application of multi-enzymatic sensors for simultaneous determination of several saccharides, e.g. sucrose, maltose, and glucose, using potentiometric sensor [11].

The development of sucrose biosensors may include various biological materials and methods of immobilisation of enzymes. Thus, in [18] authors report the application of the method of electrochemical polymerisation of phenylenediamine on the surface of electrodes for immobilisation of invertase, mutarotase, and glucose oxidase. The yeast cells, as an invertase resource, were co-immobilized with glucose oxidase and used in [9], oxygen consumption being a measure in this case. The development of novel method of immobilisation of enzymes on the surface of electrodes, i.e. when enzymes (glucose oxidase, invertase, and peroxidase) are bound to chelate sepharose by different metal ions and lectin concanavalin A, is presented in [12]. This method of immobilisation allows elution and recurrent immobilisation of different enzymes on transducer surface as well as identification of different substrates in multi-component solution.

Thus, the majority of sucrose biosensors developed up-to-date are amperometric [6, 9–17]. Along with their inherent advantages, amperometric sensors have some essential disadvantages compared to conductometric ones: i) the need for high applied potential that results in errors, associated with the presence of other electrooxidizing components, e.g. ascorbic acid, in the tested solutions; ii) they require complicated and expensive reference electrodes; iii) the necessity to work with high voltage, which enables Faraday processes on the surface of electrodes. Moreover, amperometric biosensors are usually more expensive in terms of mass production than conductometric ones.

Methods of conductometric biosensor application for sucrose analysis are rather promising, since they are sufficiently simpler, more convenient, accurate, and allow meeting important scientific and industrial challenges [19, 20]. In the previous work we developed laboratory prototype of sucrose conductometric biosensor [21]. But successful application of conductometric biosensor for analysis of real samples requires optimisation of analytical characteristics. Therefore, our work was aimed at the adjustment of the developed method of determining sucrose content in real samples using conductometric biosensor.

Materials and Methods. Materials. The frozen-dried preparations of enzymes used in the experiments were as follows: glucose oxidase (GOD) from *Penicillium vitale* (EC 1.1.3.4.) with activity of 130 U/mg from *Diagnosticum* (L’viv, Ukraine); invertase (EC 3.2.1.26) from baker’s yeast with activity of 355 U/mg from *Sigma-Aldrich Chemie* (Germany); mutarotase (EC 5.1.3.3.) with activity of 100 U/mg from *Biozyme Laboratories Ltd.*, (UK). Bovine serum albumin (BSA) (V fraction) and 50% aqueous solution of glutaraldehyde (GA) were obtained from *Sigma-Aldrich Chemie*. Sucrose was used as a substrate and analyzed substance, potassium-phosphate solution (KH₂PO₄-NaOH), pH 7.2 from *Merck* was used as a buffer. Other non-organic compounds were of analytical grade.

Sensor design. The conductometric transducers produced according to our recommendations and outlines in Lashkarev Institute of Semiconductor Physics of National Academy of Sciences of Ukraine (Kyiv, Ukraine) consisted of two identical pairs of gold interdigitated electrodes made by gold vacuum evaporation onto pyroceramic substrate (5 x 40 mm) (Fig. 1). The surface of sensitive area of each electrode pair was about 1.0 x 1.5 mm. The width of each of interdigital spaces and digits was 20 µm.

Bioselective membrane production. The solution consisting of invertase, mutarotase, glucose oxidase in 40 mM phosphate buffer, pH 7.4, with 20% glycerol was used to produce the working membrane while the same mixture with BSA instead of enzymes – for the referent membrane. Prior deposition onto transducer surfaces, the solutions for both working and referent membranes were mixed with glutaraldehyde aqueous
solutions of different concentration in 1:1 ratio. The solutions obtained were immediately deposited onto the transducer working part. The protein content was the same in both membranes. Before usage, the sensors dried during different time periods in the air at room temperature were then washed out in the working buffer solution.

Scheme of experimental measuring set-up is shown in Fig. 2. The alternating voltage with the frequency of 100 kHz and amplitude 10 mV was applied from the low-frequency signal generator GZ-118 (Ukraine) to the differential pair of interdigitated electrodes placed in an experimental vessel with the solution tested. The signal obtained at the electrodes was transferred from the 1 kOhm load resistance via differential amplifier Unipan-233-6 onto lock-in nanovoltmeter Unipan-233 (Poland) whereupon at the registering apparatus. The dependence of the output signal on the substrate concentration in the solution was measured.

The measurement procedure. The measurements were carried out in an open cell at room temperature. The 10 mM phosphate buffer, pH 6.3 was used at intensive stirring. The sensor was placed in 2 ml cell, filled with phosphate buffer. To obtain stable initial signal (base line) the sensor was steeped for some time in buffer solution. Then to obtain the signal for substrate of certain concentration, the cell was added an aliquot of standard concentrated output substrate solution (sucrose or glucose) or an aliquot of juice. The experiments were performed at least in three series. The effect of nonspecific variations of output signal owing to temperature and pH changes and electric interferences was avoided by differential mode of measurement.

Results and Discussion. The basic cascade of enzymatic reactions for sucrose detection by conductometric biosensor is as follows:

\[
\text{invertase} \\
\text{sucrose} + \text{H}_2\text{O} \rightarrow \beta\text{-D-fructose} + \alpha\text{-D-glucose} \quad (1)
\]

\[
\text{mutarotase} \\
\alpha\text{-D-glucose} \rightarrow \beta\text{-D-glucose} \quad (2)
\]

\[
\text{glucose oxidase} \\
\beta\text{-D-glucose} + \text{O}_2 \rightarrow \text{D-gluconolactone} + \text{H}_2\text{O}_2 \quad (3)
\]

\[
\downarrow
\]

\[
\text{D-gluconic acid} + \text{H}_2\text{O} \rightleftharpoons \text{acid residuum} + \text{H}^+ \quad (4),
\]

As a result, sucrose is gradually decomposed with invertase, mutarotase, and glucose oxidase to hydrogen peroxide and D-gluconolactone. In its turn, the latter is spontaneously hydrolyzed to gluconic acid which dissociates to the acid residuum and a proton, the solution conductivity being changed which can be registered by the conductometric transducer [22].

Working characteristics of conductometric biosensor are known to depend on the content and the method of immobilisation of enzymatic membrane. For instance, sensor response to glucose and sucrose depends not only on the activity of these enzymes and diffusive processes in bioselective membrane but also on the ratio of these enzymes in biosensor membrane. Therefore, the optimization of enzymatic content of membrane of sucrose biosensor to the level of highest sensitivity, when the responses for sucrose and glucose matched, was studied in the first place.

Optimal mutarotase content in enzymatic membrane was selected. For this purpose, gels of different mutarotase concentration were prepared in 40 mM phosphate buffer (pH 7.4) with 20% glycerol. GOD
concentration was 5%. This concentration was selected according to data presented in [23], where it was shown to be optimal for glucose determination, as these parameters are ideal for optimal correlation between sensitivity and dynamic range of biosensor work. The mixture for preparation of referent membrane differed from that of working membrane by BSA, which was used instead of enzyme, so, both membranes contained the same amount of protein. Prior to deposition onto transducer surfaces, the solutions for both working and referent membranes were mixed with 2% GA aqueous solution in 1:1 ratio. The solutions obtained were immediately deposited onto the transducer working part. The sensors were dried for 2 hours at room temperature. Working cell was added invertase surplus before the measurement. Sensor responses to 1 mM glucose and 1 mM sucrose were registered. Fig.3 a shows that at mutarotase concentration of 4% and higher, sucrose biosensor response to glucose and sucrose equal and thus this value was used in further research.

The next stage was the selection of optimal invertase concentration as a part of enzymatic sensor membrane. For this purpose gels of different invertase concentration were prepared, which contained mutarotase and GOD of constant concentration 4 and 5% respectively. Fig.3, b shows that at 5% invertase content in enzymatic membrane, sensor response to glucose and sucrose is the closest to 1. The increase in invertase concentration in enzymatic membrane resulted in a slight increase in sensor response to glucose than to sucrose. Thus, the optimal concentration of invertase in enzymatic membrane was determined to be 5%. This invertase concentration was used for further preparation of enzymatic membrane.

The aim of the research was to develop the measurements protocol of work of conductometric biosensor with real juice samples, thus we focused specifically on this issue. As enzymatic membrane of sucrose biosensor contains GOD, the main error in sucrose biosensor response is caused by glucose, present in samples (juices) along with other saccharides.

There are several solutions to this problem:

– immobilization of GOD in the external membrane layer, which will decompose glucose, pres-
ent in the solution, before it reaches the internal enzymatic layer (sensitive to sucrose);

– application of flow analysis, which is specific for GOD reactor to be located in front of sucrose detector, which will also result in decomposition of glucose. Basically this system is similar to the first one;

– application of specific glucose sensor for determining the concentration of free glucose in the solution and further identification of the difference between signals of two enzymatic biosensors, the first of which responds to both glucose and sucrose, while the second one – to glucose only.

The latter method was selected to determine sucrose content due to its being simple, convenient, and requiring no additional elements of system or manipulations. Besides, glucose biosensor was used as control sensor, as previous works revealed significant reliability of results of its application [24], which also allowed us to use glucose sensor to verify the data, obtained with sucrose sensor.

Several possible variants of determining sucrose in real samples were studied. First of all we studied the case when responses to sucrose and glucose sensors for certain glucose concentration did not match, which

![Dependence of response ratio to glucose and sucrose on mutarotase (a) and invertase (b) content in enzymatic membrane; measurements were performed in 10 mM phosphate buffer, pH 6.3, substrate concentration 1 mM](image)
happens quite often. This was necessary for the development of method suitable for further automatization and real-time measuring. Glucose and sucrose sensors were calibrated prior to measurement procedure (Fig.4).

Calibration curves for glucose and sucrose biosensors correspond to $y = k_1x$ and $y = k_2x$. Correlation ratio for these two functions ($K$) was calculated as:

$$K = k_2/k_1 = k_2/k_1$$  \hspace{1cm} (5)

Obtaining glucose sensor response to certain concentration of glucose and multiplying it for correlation ratio $K$ allowed obtaining the value, corresponding to the value of response, produced by sucrose sensor, to the same glucose concentration.

The data of Fig.4 and formula 5 result in:

$$S_{s+g} = S_s + S_g;$$  \hspace{1cm} (6)

$$S_g = G_g \cdot K, S_{s+i+g} = G_{s+i+g} \cdot K$$  \hspace{1cm} (7)

$$S_s = S_{s+g} - G_g \cdot K$$  \hspace{1cm} (8)

Substitution of $S_s$ into calibration curve (for $S_{s[g]}$) allows determining sucrose concentration in juice ($c$). Calibration curve of sucrose biosensor to sucrose ($S_{s[s]}$) corresponds to $y = k_3x$.

Thus,

$$c = S_s/k_3 = (S_{s+g} - G_g \cdot K)/k_3 \text{(experiment)}$$  \hspace{1cm} (9)

$$c = (G_{s+i+g} - G_g)/k_1 \text{(control)}$$  \hspace{1cm} (10)

If the sensor responses to the same concentration of glucose and sucrose match, i.e. $K = 1$, then equation (9) is simplified to:

$$c = S_s/k_3 = (S_{s+g} - G_g)/k_3$$  \hspace{1cm} (11)

Let us illustrate the mentioned above method of determination of sucrose concentration by experiments in juices. For this purpose we carried out a series of experiments with certain concentration of substrates. Experimental error did not exceed 4%. Calibration curve of glucose conductometric biosensor was linear in the range of 0.001 – 1.5 – 2.5 mM of glucose and in the range of 0.001 – 2.5 – 10 mM of sucrose, depending on the experimental conditions [21]. Higher glucose and sucrose concentrations resulted in saturation of biosensor response. Concentration of glucose and sucrose in juices is known to vary from 15 to 700 mM. Thus, the sample has to be diluted for measuring due to narrow dynamic range of biosensors.

Prior to measurement procedure, glucose sensor was emerged into the measuring cell (2 ml) with 10 mM
phosphate buffer, pH 6.3, and sustained for several minutes in order to obtain the base line. Next, juice aliquot was added (1 μl) to obtain 2 000-dilution in the buffer solution. Response of glucose sensor ($G_g$) was multiplied by $K$, previously calculated from calibration curves for glucose from glucose and sucrose biosensors (5). The value of $S_g$ was obtained, which is the sensor response to only glucose in juice sample. We also obtained the value of glucose concentration from calibration chart. Then, we used sucrose biosensor and obtained the response ($S_{r+g}$) (similarly to glucose sensor) to the introduction of the same aliquot of juice (1 μl). As sucrose sensor is sensitive to both glucose and sucrose, which are always present in juices, signal $S_{r+g}$ is the sum of two signals, namely to glucose ($S_g$) and sucrose ($S_s$). $S_g - S_{r+g}$ equals to $S_s$, i.e. sensor response to sucrose only (Fig.4). Next we determined sucrose concentration in juice samples using calibration curve of sucrose sensor.

The data obtained were verified by glucose sensor only. For this purpose, the juice sample was added invertase, catalyzing the decomposition of sucrose to glucose and fructose. Decomposition of one sucrose molecule gives one glucose molecule. This mixture was sustained for 2 hours for spontaneous transformation of α-D-glucose to β-D-glucose. Next we obtained signal $S_{ni+g}$ (response to glucose and decomposed sucrose) using glucose biosensor, i.e. response to the introduction of 1 μl of juice containing invertase, and subtracted earlier obtained value of $S_g$ (response to glucose). Extrapolation of obtained segment onto x-coordinate allows defining the value of sucrose concentration in juice, which was later compared with that obtained using sucrose biosensor.

Experimental results reassured us in appropriateness of the application of the developed method for subsequent analysis of sucrose concentration in juices.

The levels of sucrose and glucose concentration in juices were determined using the method of standard additions [25]. For this purpose we analyzed the variant of sucrose determination using glucose and sucrose sensors with equal responses to glucose. We started with obtaining glucose sensor response to 1 μl of juice and several alternate introductions of 0.25 mM of glucose into the cell. Then we built the linear dependence of change in conductivity on substrate concentration (Fig.5, a), cross-point of which corresponded to glucose concentration in samples, diluted 2 000 beforehand.

Then, were registered the response of sucrose sensor to 1 μl of juice, which consisted of general sensor response to glucose and sucrose. This value was subtracted the value of glucose sensor response to glucose. The value obtained was projected onto y-coordinate
and, using the same method (method of standard additions), the value of sucrose concentration in juice was obtained (Fig.5, b).

The results were verified by control method, using only glucose sensor. For this purpose, the juice sample was added invertase, catalyzing the decomposition of sucrose to glucose and fructose. Decomposition of one sucrose molecule gives one glucose molecule. This mixture was sustained for 2 hours for spontaneous transformation of α-D-glucose to β-D-glucose. Next we obtained response of glucose biosensor to 1 μl of juice, which consisted of general biosensor respose to already present glucose and sucrose, decomposed to glucose. Response value was subtracted the response value to glucose by earlier developed glucose sensor. Extrapolation of obtained segment onto x-coordinate allows defining the value of sucrose concentration in juice using the method of standard additions, which was later compared with that obtained using sucrose biosensor.

Thus, presented method of sucrose determination in real samples was shown to be optimal, simple and convenient for further determination of sucrose and glucose concentrations in juices and sweet drinks.

The results of these measurements are presented in Fig.6. Experimental error did not exceed 10%.

**Conclusions.** Optimal concentrations of enzymes in bioselective element of sucrose conductometric biosensors were selected to achieve its maximal stability and sensitivity, corresponding to 5% glucose oxidase, 4% mutarotase, and 5% invertase. The working procedure of conductometric sucrose and glucose biosensors in real samples of juices and sweet drinks was tested. Different variants of sucrose determination in juice and sweet drinks samples by biosensoric method were examined. The formula of recalculation of data was presented. The work presents the results of sucrose and glucose determination using biosensors in juices and sweet drinks. Experimental error did not exceed 10%. The application of developed sucrose and glucose biosensors in food industry for control and optimization of biotechnological processes has been proposed.

The work was financially supported by National Academy of Sciences of Ukraine within complex science and technology program *Sensor Systems for Medical, Ecological, Industrial, and Technical purposes.*
В. Н. Пешкова, А. А. Солдаткин, С. В. Дзядевич

Оптимизация методики определения сахарозы в соках и сладких напитках кондуктометрическим ферментным биосенсором

Резюме

Разработана и оптимизирована методика определения сахарозы в соках и сладких напитках с помощью кондуктометрического ферментного биосенсора. Подобраны оптимальное соотношение и концентрация ферментов в биоселективном элементе биосенсора для наиболее его стабильности и чувствительности. Рассмотрены разные варианты выявления сахарозы в образцах с помощью биосенсора и проведено определение сахарозы и глюкозы в соках и сладких напитках. Предложенную методику можно использовать в дальнейшем в пищевой промышленности для контроля и оптимизации производства.

Ключевые слова: кондуктометрический ферментный биосенсор, иммобилизованные ферменты, сахароза, глюкоза.

REFERENCES

1. Скрыпанская И. В., Корган Я. И., Солдаткин А. П. Применение ферментного сенсора на основе pH-чувствительных полевых транзисторов для определения сахарозы в сахарозе в соке картофеля // Биополимеры и клиника.—2003.—19, № 6.—С. 553–557.