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Enzyme-Modified Screen-Printed Electrodes for Assaying Glucose, Ethanol, Lactate and Starch in Fermentation Media

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Abstract

Screen-printed electrodes modified by glucose oxidase, alcohol oxidase, lactate oxidase and γ-amylase immobilized into cross linked bovine serum albumin gel were produced for application in monitoring fermentation processes. The analytical and metrological characteristics of biosensors based on the developed electrodes were determined. Samples of fermentation mass and alcohol products were assayed. Statistical analysis of the results showed the values of glucose, ethanol, lactate and starch contents, determined using the developed multi-channel system and by reference methods, to differ insignificantly.

Keywords: Biosensors; Screen-printed electrodes; Enzymes; Fermentation processes, Multi-component assay

Introduction

Important parameters of the fermentation process are the contents of starch, glucose, lactate and ethanol [1]. Starch-containing products (potatos, maize, rye, panic grass etc.) serve in this case initial raw materials for fermentation. Glucose forms in the hydrolysis of starch and serves as substrate for yeast microorganisms consuming it to produce the target component, ethanol. In processes based on lactic fermentation (production of kvass and kefir), one of glucose fermentation products is lactate. Besides, lactate is a by-product of many microorganisms; its content in the end product enables the conclusion whether it was produced as the result of a natural fermentation process or not. Differential assessment of the above-mentioned components at various stages of the fermentation process makes it possible to optimize the technology and increase its efficiency in terms of cost and time by bringing into line the quality of initial raw materials and the quantity of enzyme preparations and yeasts used [2]. Traditional analytical methods used to monitor fermentation productions are either insufficiently selective (pycnometry, refractometry, polarimetry) or expensive or else require lengthy preparation of samples and long assay times (gas-liquid chromatography, high-performance liquid chromatography). These methods can be efficiently applied to control the quality of final products, but cannot be used for express analysis of fermentation media. For this reason, it appears topical to develop a simple and cheap method of monitoring fermentation processes. A promising approach is to use biosensors based on screen-printed electrodes. These electrodes are miniature size, multi-functional and can be produced in bulk quantities. Advantages of this production technology enabled enzyme biosensors based on screen-printed electrodes (glucometers) to become personal devices and to dominate the biosensor market [3].

The most simple and efficient approach in the development of biosensors based on screen-printed electrodes is to use oxidase enzymes as recognition elements. Information on the concentration of an analyte is obtained using methods based on the detection of hydrogen peroxide formed in the enzymatic reaction [4]. Thus, several models of screen-printed electrodes based on glucose oxidase for the glucose assay in the medium range of 10–5–10–3 M have been described [5]. A biosensor based on the screen-printed electrode modified by alcohol oxidase for the ethanol analysis within the range of 0.05–1 mM has been described [6]. Yet another work [7] describes a biosensor based on the lactate oxidase-modified screen-printed electrode for the detection of lactate within the range of 0.0005–0.5 mM. Virtually all biosensors for the determination of glucose, ethanol and lactate discussed in the literature have been developed for healthcare applications and are, therefore, characterized by the narrow range of assayed concentrations.

The content of starch can be determined by the biosensor method using a mixture of two enzymes, glucose oxidase and amylase, as the biological component [8]. One of the examples is disposable screen-printed electrodes for the simultaneous determination of starch and glucose in food products [9]. Despite the prospects to be opened by the development of such a bi-enzyme analytical system, examples of efficient use of amylases in the development of biosensors are rather scarce.

On the whole, publications on biosensors based on screen-printed electrodes for assaying components other than glucose in fermentation media are of segmental and isolated character. Besides, biosensor studies described above are aimed first of all to detect one particular compound. The complex composition of fermentation media dictates the necessity to develop a multi-channel analytical system enabling the simultaneous determination of several components in a mixture [10]. The system of amperometric biosensors described in [11] makes possible the simultaneous selective determination of glucose and lactate. Attempts have been made to carry out the sequential determination of glucose and lactate formed in the course of lactic fermentation using a flow-injection system including the electrode with immobilized glucose oxidase and lactate oxidase [12]. The work

[13] describes a system of biosensors for the simultaneous detection of glucose, lactate, glutamate and glutamine. As compared with traditional methods of analysis, advantages of biosensors are their simplicity and small time of assay. These advantages manifest themselves when several biosensors are combined into one analytical system capable of simultaneously determining several components of fermentation media. It is exactly this task, traditionally complex for physicochemical methods of analysis, that can be successfully solved using biosensors. For this reason, it appears to be topical to develop a multi-channel biosensor system based on enzyme-modified screen-printed electrodes for the simultaneous determination of glucose, ethanol, lactate and starch in fermentation mass.

Materials and Methods

Biosensor measurements

The signal was registered using a Prussian Blue-modified graphite-paste screen-printed electrode (Rusens, Russia), on the surface of which a biological element was placed. An EmStat potentiostat (PalmSens, the Netherlands) was used as a transducer. The working potential was 0 V (relative to the silver chloride reference electrode). The measurements were carried out in a 4 ml cuvette with sodium–potassium phosphate buffer solution (pH 6.8, 33 mM). The mixing was done by a magnetic stirrer at a rate of 200 rpm. Samples were added by variable-volume automatic micropipettes (200-1000 µl, 20-200 µl, 0.5-10 µl; Biotech, USA). The amplitude of the change in biosensor's output signal at the addition of substrate was the measured parameter (biosensor response). After each measurement, the electrode was washed with buffer solution for 1-2 min.

Enzyme preparations

Graphite-paste screen-printed electrodes were modified using commercially available preparations of oxidoreductases: glucose oxidase isolated from *Aspergillus niger* (Sigma-Aldrich, USA), alcohol oxidase isolated from Pichia pastoris (Sigma-Aldrich, USA) and lactate oxidase isolated from *Pediococcus* sp. (Sigma-Aldrich, USA). The screen-printed electrode for determining the concentration of starch was modified using a mixture of two enzymes: glucose oxidase and γ-amylase both isolated from *Aspergillus niger* (Sigma-Aldrich, USA).

Fabrication of enzyme-modified screen-printed electrodes

The enzyme-modified screen-printed electrodes were fabricated by applying 1 µl of an enzyme solution (solution of glucose oxidase, activity 15 U/ml; alcohol oxidase, 17.6 U/ml; lactate oxidase, 10 U/ml; γ-amylase, 30 U/ml) onto the surface of working electrodes and leaving to dry for 10 min. Then, 7 mg of bovine serum albumin (BSA) (Sigma-Aldrich, USA) and 100 µl of phosphate buffer (pH 6.8, 33 mM) were put into a micropipette and mixed until completely dissolved. Further, 15 µl of 25% glutaraldehyde (Sigma-Aldrich, USA) was added to the produced solution. The contents were mixed again, a 3-µl amount was sampled and quickly transferred onto the surface of a screen-printed electrode with an applied enzyme. The produced enzyme-modified screen-printed electrode was dried in air for 60 min; before use, it was washed in buffer solution for 5 min.

Scanning electron microscopy

A thin layer of a platinum–carbon mixture was deposited onto specimens of screen-printed electrodes in a JEE-4X vacuum evaporator (JEOL, Japan). The specimens were analyzed using a JSM-6510 LV scanning electron microscope (JEOL, Japan) in a high vacuum mode at the registration of secondary electrons.

Determination of glucose and starch by high-performance liquid chromatography

The content of glucose in samples was determined by high-performance liquid chromatography on an HP 1100 system (Hewlett Packard, USA) using a refractive-index detector and a column (300 × 6.5 mm) filled with sulfonated polystyrene–divinylbenzene copolymer in a calcium ionic form with particle size of 30 µm. Analysis conditions: column temperature, 80°C; eluent (deionized water) flow rate, 0.5 cm^3/min.

When determining the starch content, the analyzed sample was preliminarily hydrolyzed. For this, it was heated on a water bath in 1.124% HCl for 15 min, protein substances were precipitated, and the sample was filtered and subjected to the chromatographic analysis as described above.

Determination of ethanol by gas chromatography

The content of ethanol in samples was determined by gas chromatography on a Kristall-5000.2 system (JSC SDB Chromatec, Russia) using a flame-ionization detector and a DB-FFAP capillary column (50 m × 0.32 mm × 0.50 µm) (Agilent, USA). Analysis conditions: column thermostat temperature, 70°C; evaporator temperature, 200°C; detector temperature, 250°C; carrier gas (helium) flow rate, 0.10 dm^3/h.

Determination of lactate by capillary electrophoresis

The content of lactate in samples was determined using a Capel-104T capillary electrophoretic system (Lumex, Russia) with the negative high voltage polarity (voltage, 20 kV; capillary internal diameter, 75 µm; efficient length, 50 cm; temperature, 20°C). The detection was carried out spectrophotometrically at a wavelength of 254 nm.

Model fermentation and production of fermented mass

A wheat flour sample was suspended in warm distilled water and heated up to 90°C. A Termamyl enzyme preparation (Novozymes A/S, Denmark) was added to the produced mass and the contents were thermostatted for 2 h under stirring. After this, the reaction mass was cooled to 60°C, an SAN Super 360 L enzyme preparation (Novozymes A/S, Denmark) was added; the contents were placed into a thermostat and mixed for 2 h. Then, the flask was cooled to 30°C, a SuperStart yeast preparation (Russia) was added and the contents were thermostatted for 70 h for fermentation. Samples were taken 2, 12, 48 and 72 h after the yeast preparation was added.

Results and Discussion

Development of enzyme-modified screen-printed electrodes

Oxidase enzymes (glucose oxidase, alcohol oxidase and lactate oxidase) were used to produce screen-printed electrodes for the determination of starch, glucose, lactate and ethanol. The amount of the analyzed component in solution was determined by tracking the

rate of hydrogen peroxide formation in the course of the biochemical reaction (Figure 1).

Two enzymes – γ-amylase for the hydrolysis of starch and glucose oxidase for the determination of the amount of glucose formed – were used in the development of the screen-printed electrode for glucose analysis. The enzymes were immobilized by encapsulation into crosslinked BSA membrane. Figure 2 presents scanning electron micrographs of the developed electrodes. The working graphite electrode has a highly developed surface (figures 2a and 2b), which provides a large area for the contact of the enzyme and Prussian Blue with conducting material and enables a high sensitivity of the developed screen-printed electrodes. The matrix obtained after crosslinking the BSA molecules by glutaraldehyde has pores commensurable with the size of the used enzymes; the matrix provides a good diffusion of substrates and metabolites and forms a protein environment favourable for immobilized enzymes [14,15]. Besides, the matrix used for immobilization ensures a strong retention of biomaterial due to the binding of the enzyme molecules to BSA by glutaraldehyde. Owing to the use of the developed process, modified screen-printed electrodes had a high reproducibility of fabrication. The scatter of responses to the same concentrations of analytes for the biosensor based on various batches of electrodes did not exceed 10%.

Characteristics of the biosensor based on the modified screen-printed electrodes

To determine the sensitivity and range of assayed concentrations, we plotted the calibration dependences of biosensor responses on the concentration of substrates in the measuring cuvette (Figure 3). The enzyme-based receptor elements are catalytic type bioreceptors, i.e., the biological response in such systems is provided for by the enzymic reactions. The calibration dependence shown in Figure 3 is well described by a Michaelis–Menten equation: $R = \frac{R_{max}[S]}{K_M + [S]}$, where Rmax is the maximum biosensor response at which all molecules of the bioreceptor-element enzyme are involved in the formation of an enzyme–substrate complex; KM is the efficient Michaelis constant numerically equal to the concentration of substrate at which the level of biosensor response reaches half of the maximum value.

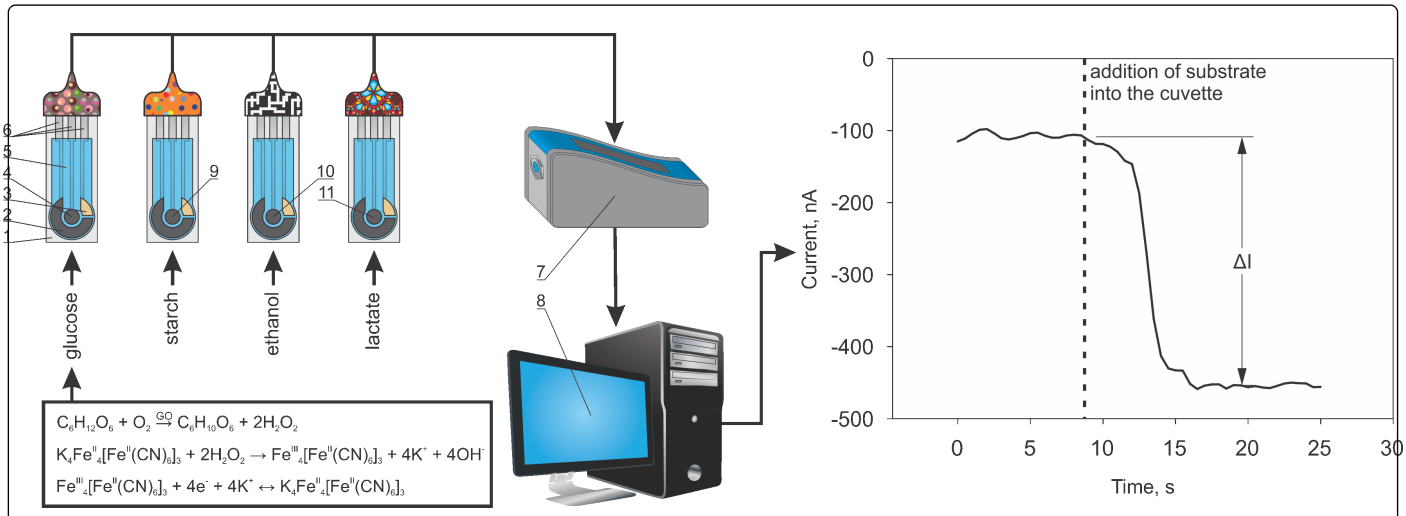

Figure 1: Schematic of the developed biosensor unit. 1, Support; 2, auxiliary graphite electrode; 3, silver chloride reference electrode; 4, working graphite electrode modified by enzyme (glucose oxidase is used for glucose determination); 5, insulator; 6, contacts; 7, potentiostat; 8, personal computer; 9, working electrode modified by a mixture of glucose oxidase and amylase; 10, working electrode modified by alcohol oxidase; 11, working electrode modified by lactate oxidase.

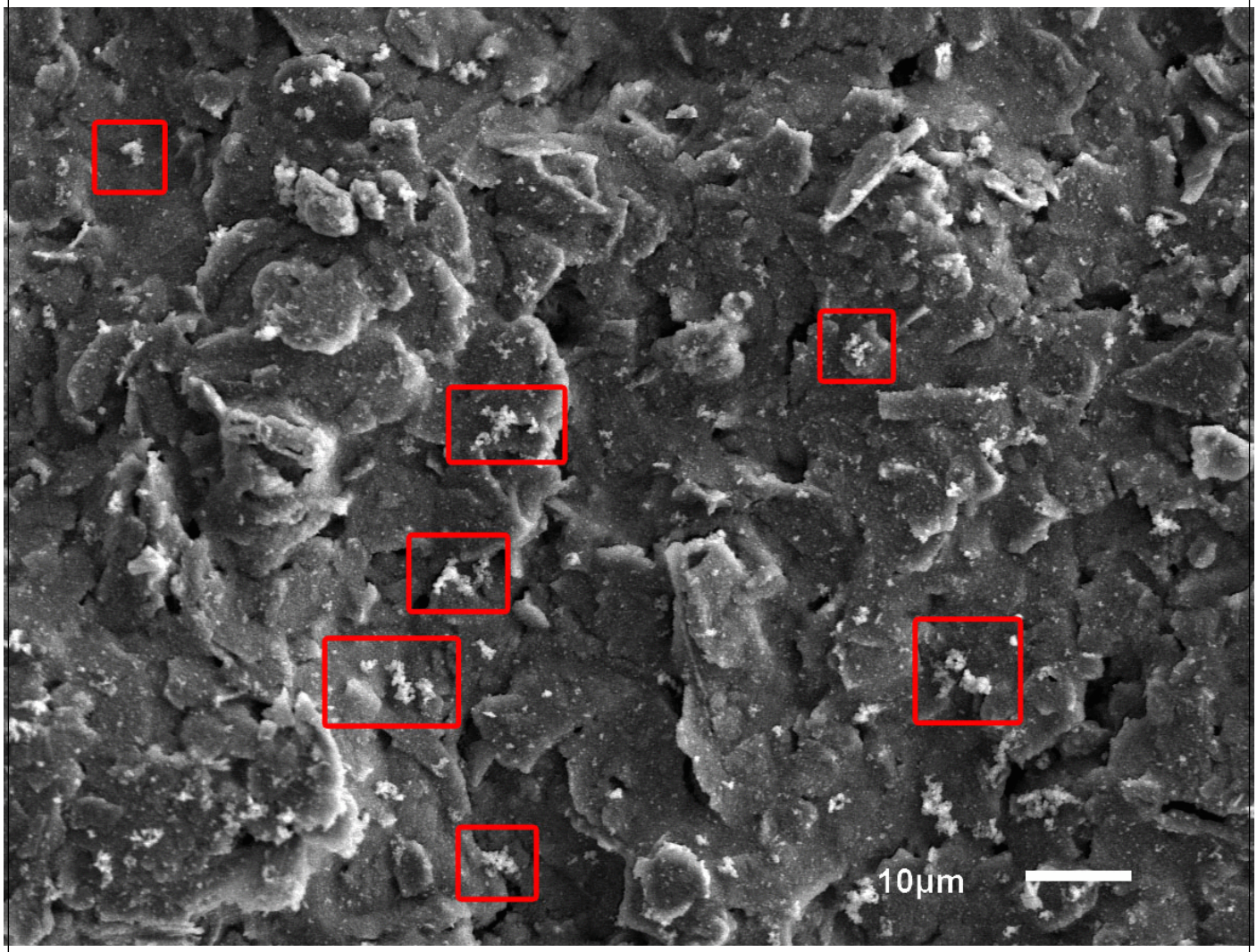

Figure 2: SEM images of the working graphite electrode surface after modification by Prussian Blue and glucose oxidase.

As is commonly known, the use of the linear part of the calibration curve, limited from above by the constant KM, makes it possible to reduce the assay errors [16]. The lower boundary of the linear part corresponds to the lower boundary of the assayed contents and was calculated by the statistical method proceeding from the criterion that the relative standard deviation of the measurement results (Sr(C)) is less than 0.33 [17].

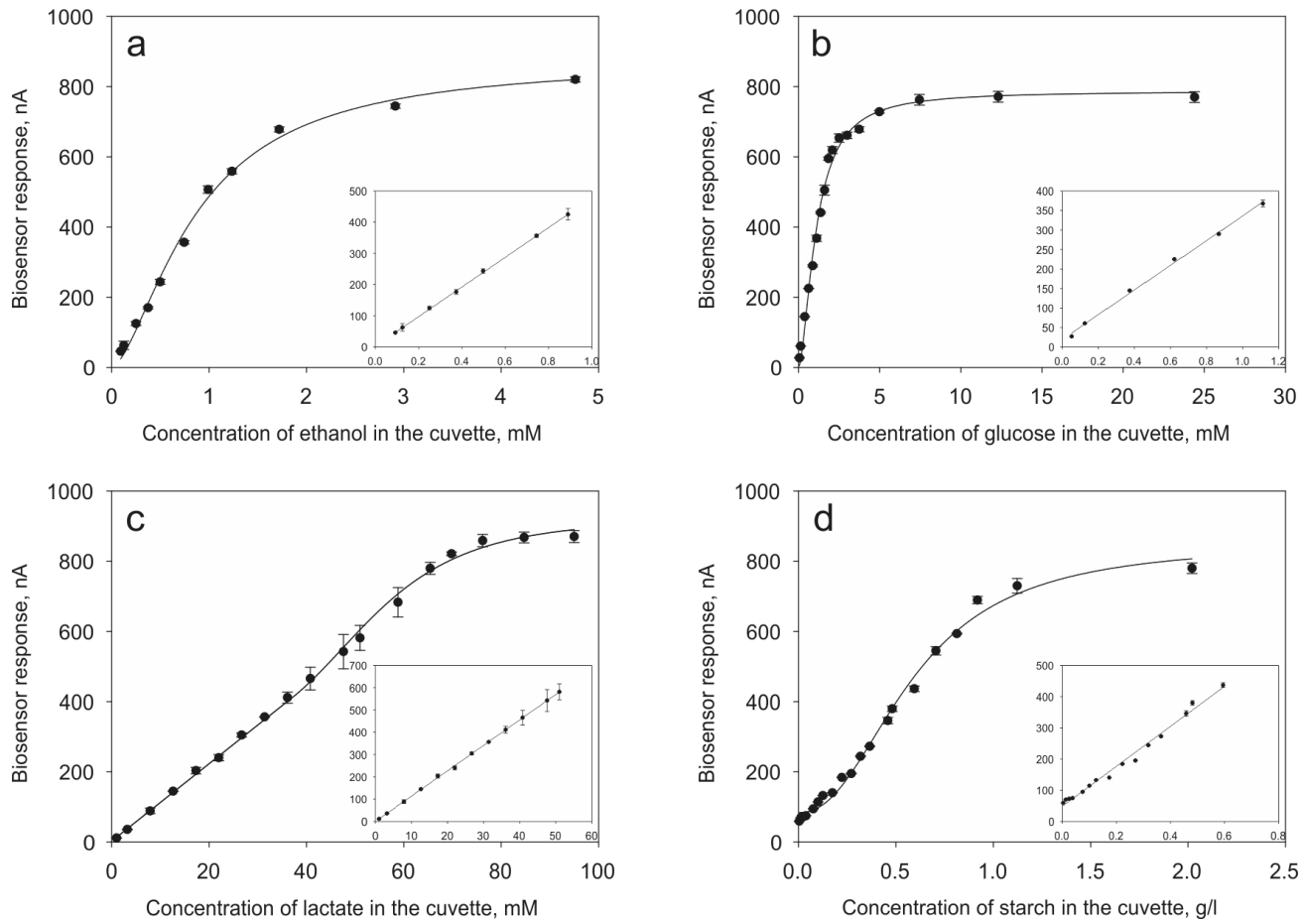

Figure 3: Calibration curves of the enzyme electrodes as a function of substrate concentrations (for electrodes modified with: a, alcohol oxidase; b, glucose oxidase; c, lactate oxidase; d, γ-amylase and glucose oxidase). Plots in the inserts represent the linear parts of the calibration curves.

Characteristic	Value			
Biological component	Alcohol oxidase	Glucose oxidase	Lactate oxidase	Glucose oxidase and amylase
Sensitivity coefficient (slope of the linear segment in the dependence of sensor response on concentration), nA/mM	480 ± 10	310 ± 8	11.7 ± 0,5	640 ± 20 nA/g•l
Lower boundary of assayed concentrations, mM	0.09	0.05	1	0.03 g/l
Lower boundary of assayed concentrations (KM), mM	0.90 ± 0.03	1.10 ± 0.03	53 ± 1	0.59 ± 0.01 g/l
Long-time stability (time within which the value of biosensor response to the same concentration is no less than 25% of the initial value), days	3	6	3	4
Operational stability (relative standard deviation by 15 successive measurements), %	2	3	13	6
Duration of single measurement, min	1–2	1	1	3

Table 1: Presents the main characteristics of the enzyme-modified screen-printed electrodes.

The developed biosensor is not only competitive with the world analogues [6,7,9,18] but partially exceeds them by analytical and metrological characteristics (Table 1). Thus, it has a broader range of detected concentrations of glucose, lactate and starch as compared

with the described prototypes [8,9,19,20]. Besides, in contrast with the known analogues, it enables the simultaneous selective determination of starch, glucose, lactate and ethanol in fermentation media.

Analysis of real samples of alcohol products and fermentation mass using the biosensor based on enzyme-modified screen-printed electrodes

Using the biosensor based on the enzyme-modified screen-printed electrodes, we determined the contents of glucose, ethanol, lactate and starch in alcoholic and fermented milk products as well as in fermented mass sampled at various stages of the fermentation process in production of ethanol. High-performance liquid chromatography, gas chromatography and capillary electrophoresis were used as reference methods. The results are given in Table 2.

Sample	Analyte	Biosensor method	Reference method
Wine, Trebbiano	ethanol, M	2.2 ± 0.2	2.21 ± 0.03
	glucose, M	0.010 ± 0.002	0.01 ± 0.001
	lactate, M	0	0
	starch, g/l	0	0
Wine, Laskovyi Potseluy	ethanol, M	1.9 ± 0.1	1.87 ± 0.02
	glucose, M	0.16 ± 0.01	0.165 ± 0.003
	lactate, M	0	0
	starch, g/l	0	0
Fortified wine, Portvein 333	ethanol, M	3.0 ± 0.2	3.10 ± 0.02
	glucose, M	0.17 ± 0.02	0.160 ± 0.003
	lactate, M	0	0
	starch, g/l	0	0
Fermentation mass, 2 h	ethanol, M	0.011 ± 0.002	0.008 ± 0.003
	glucose, M	0.071 ± 0.005	0.074 ± 0.003
	lactate, M	0	0
	starch, g/l	37 ± 2	38 ± 1
Fermentation mass, 12 h	ethanol, M	0.21 ± 0.01	0.19 ± 0.02
	glucose, M	0.21 ± 0.01	0.223 ± 0.005
	lactate, M	0.15 ± 0.02	0.16 ± 0.01
	starch, g/l	23 ± 1	23.6 ± 0.7
Fermentation mass, 48 h	ethanol, M	0.36 ± 0.03	0.34 ± 0.02
	glucose, M	0.19 ± 0.01	0.214 ± 0.002
	lactate, M	0.15 ± 0.02	0.15 ± 0.01
	starch, g/l	17.3 ± 0.7	18 ± 0.4
Fermentation mass, 72 h	ethanol, M	0.42 ± 0.09	0.41 ± 0.02
	glucose, M	0.162 ± 0.006	0.156 ± 0.003
	lactate, M	0.17 ± 0.02	0.16 ± 0.01
	starch, g/l	9.1 ± 0.6	9.3 ± 0.3
Fermented milk product, Actimel	ethanol, M	0	0
	glucose, M	0.011 ± 0.002	0.010 ± 0.002
	lactate, M	1.98 ± 0.03	1.91 ± 0.05
	starch, g/l	0	0
Fermented milk product, Rastishka	ethanol, M	0	0
	glucose, M	0.014 ± 0.003	0.014 ± 0.002
	lactate, M	1.04 ± 0.06	1.09 ± 0.05
	starch, g/l	0	0

Table 2: Statistical analysis of the results using a modified Student's t-test showed that the values of component concentrations, determined using the developed multi-channel biosensor and reference methods, differed insignificantly.

Conclusions

We developed a multi-channel biosensor based on enzyme-modified screen-printed electrodes for monitoring fermentation processes. The concentrations of glucose, ethanol, lactate and starch determined by the biosensor were within the ranges of 0.05–1.10 mM, 0.09–0.90 mM, 1–53 mM and 0.03–0.59 g/l, respectively.

The contents of components in alcohol products and fermentation mass were determined using the developed biosensor and by reference methods. It was shown that modified screen-printed electrodes can serve as the basis for developing biosensors with high analytical and metrological characteristics that can be used for monitoring fermentation processes and assaying the components of fermentation masses and fermentation products.

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