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Practical application of electrochemical enzyme biosensors

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The electrochemical biosensor is an integrated receptor-transducer device, which can convert a biological response into a measurable electrical signal. Because of its important features like selectivity, sensitivity, stability, reproducibility, linearity, and low cost, biosensors have a wide range of applications. The aim of this paper is to review our achievements in the development of electrochemical enzyme biosensors and the possibility of their applications in food industry, agriculture and environmental control. The specific structural features of transducers in the developed amperometric, potenciometric and conductometric enzyme biosensors are discussed. The laboratory prototypes were fabricated and systematically tested for the determination of glycoalkaloids, mycotoxins, heavy metal ions and pesticides in the agriculture and environment samples as well as some components of food and dietary supplements. They demonstrated fast, highly selective, sensitive and correct analitycal operation and also the possibility to modulate main characteristics to comply with the specific requirements for the practical purposes. It is important that the manufacture of such miniaturized electrochemical biosensors may be adapted to the technologies of large-scale production.

K e y w o r d s: electrochemical biosensors; enzymes; application; agriculture; food; environment

1. Introduction

To date, biosensor technologies, which are part of analytical biotechnologies, are among the world's key exponential technologies, the parameters of which are improving by tens or even hundreds of percent per year, at the same cost level. And if, at the same time, they allow

sharing with other technologies, such as nanotechnology, a programmed technological explosion occurs.

For example, for 15 years since 2007 the sensor market has grown thousand folds while the unit cost of such a system given its functionality has decreased also thousand times. This is the best demonstration of the progress in this field.

Interest in biosensors is due to their certain advantages over traditional methods of analysis: they provide easy, fast, accurate, highly sensitive, spesific, and cheap procedure of measurements. Besides, real-time measurements are possible, while only minimal probe pretreatment is necessary. Today, electrochemical biosensors are considered as a successful alternative to traditional methods of analysis given the existing advances in the development of their laboratory prototypes and the entry into the market of a number of competitive measuring instruments. As screening tools, biosensors can help to select a certain number of suspicious samples, which will be further analyzed using traditional methods, thereby reducing the cost and time of analysis [1].

To date, a number of reviews [2, 3] and monographs [4, 5] have been published as well as numerous experimental works describing different types of biosensors. According to the classical IUPAC definition, "a biosensor is an integrated device based on a receptor and a transducer, which is able to provide quantitative or semi-quantitative analysis using a biological recognition element" [6]. Biosensors consist of three components: a bioreceptor is a detector layer of an immobilized biomaterial; a physicochemical transducer capable of transforming a biological response into a measurable signal; and an electronic system for signal amplification and recording.

In this paper, our achievements in the development of electrochemical enzyme biosensors are reviewed. The laboratory prototypes of such biosensors were fabricated and thoroughly tested for real application (wine and wine must, natural fruit juices, agricultural, food and environmental water samples *etc)*.

2. Structures of biosensors and devices for operation with them

2.1. Potentiometric transducers

We used the transducers with a differential pair of p-channel transistors on a single crystal with a total area of 8×8 mm (Fig. 1a). The crystal included two identical transistors separated by a protective 50 μm wide n^+ — region with contact to the substrate, p^+ — diffusion buses brought to the edge of the chip with contacts to the drain-source, output to the built-in reference microelectrode, as well as two test MDS transistors with a metal gate, designed to check the electrical parameters of the produced crystals. The ion-selective properties of the transistor are due to the gate dielectric layer consisting of a thermally oxidized SiO_2 film 50 nm thick and a Si_3N_4 film 70 nm thick deposited in the reduced pressure reactor [7]. The measurements were performed by using a portable device operating according to the scheme of direct current measurement in the transistor channel with active load (Fig. 1b). The sensitivity of the device was about 25 μA/pH, which corresponded to a conditional pH-sensitivity of about 80 mV/pH [7].

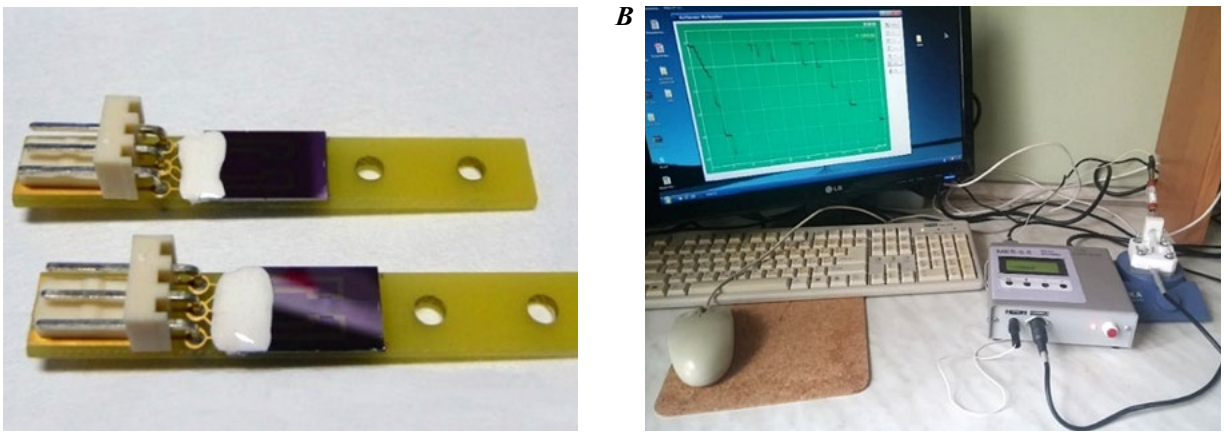

Fig. 1. General view of pH-sensitive field-effect transistors (*A*) and system for measurements with them (*B*) manufactured at the V.Ye. Lashkarev Institute of Semiconductor Physics of National Academy of Sciences of Ukraine

2.2. Amperometric transducers

We used the sensors based on platinum disk electrodes, which were produced in our department (Fig. 2a). When creating platinum disc electrodes, first a platinum wire, 0.4 mm in diameter and 3 mm long, was sealed in the end part of the glass capillary with outer diameter 3.5 mm. The open end of the wire extended above the working surface of the transducer. The platinum wire was then connected to a silver conductor inside a capillary using Wood's low-melting alloy. At the other end of the conductor, a copper contact pad was placed to connect the measuring device. The working surface of the electrodes was obtained by grinding with alumina powder (particles 0.1 μm and 0.05 μm); before immobilization of the bioselective element the electrodes were

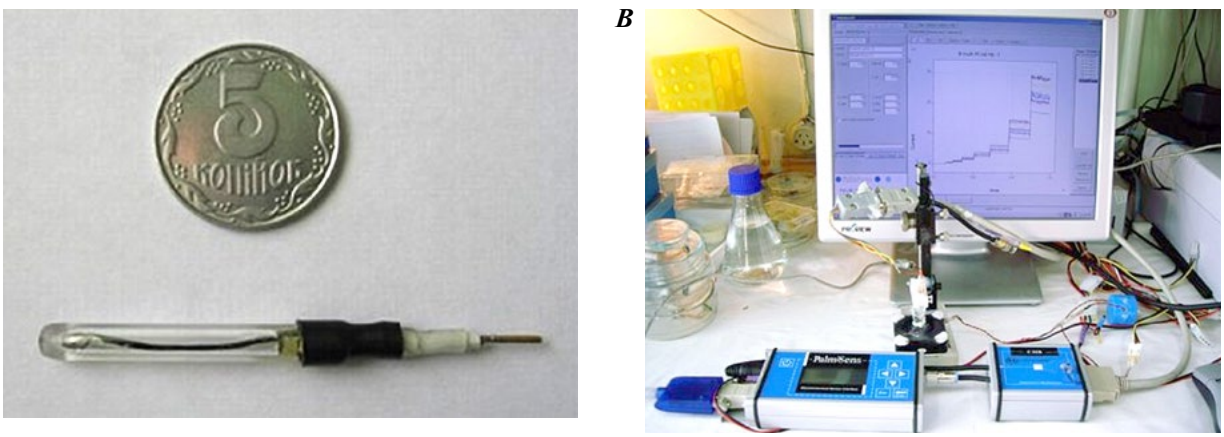

Fig. 2. General view of amperometric transducer based on a platinum disk electrode (*A*) and system for measurements with them (*B*)

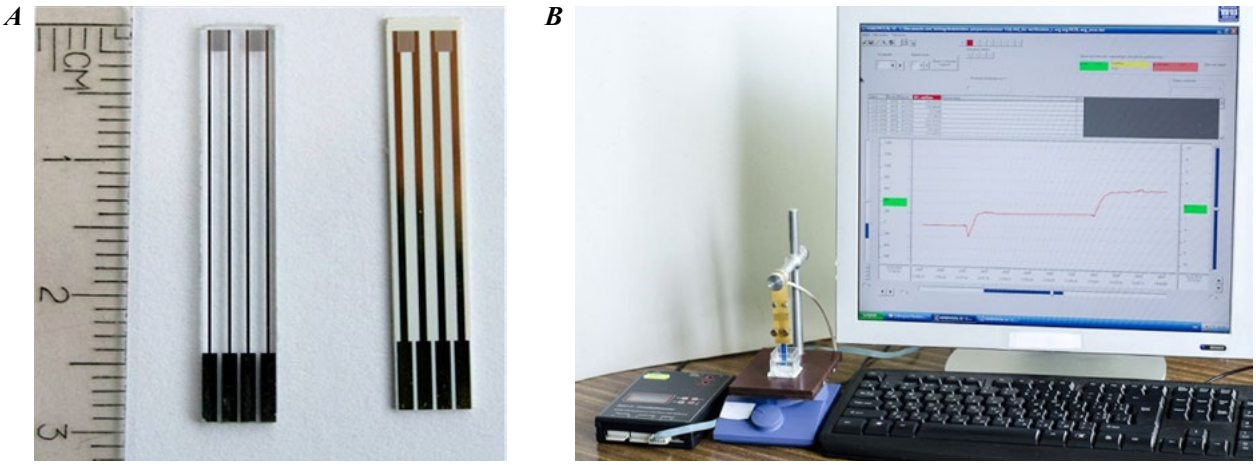

Fig. 3. General view of conductometric transducers with a differential pair of gold interdigitated electrodes (*A*) and system for measurements with them (*B*) manufactured at the Institute of Electrodynamics of National Academy of Sciences of Ukraine

wiped with alcohol. Periodically, the electrode surface was renewed by the similar grinding. Thus, the same transducer was used repeatedly to create several different biosensors. The three-electrode scheme of amperometric analysis was used in the work (Fig. 2b). Working amperometric electrodes, auxiliary platinum electrode and reference electrode (Ag/AgCl) were connected to the potentiostat PalmSens (Netherlands) or the analog of domestic production. The 8-channel device (CH-8 multiplexer, PalmInstruments BV, the Netherlands) connected to a potentiostat allowed us to receive simultaneously the signals from 8 working electrodes, but usually 2–3 working electrodes were connected to it. The potentiostat of domestic production did not need a multiplexer, since it could work on 4 channels simultaneously. The distance between the auxiliary platinum electrode and all working biosensors during the measurement was the same, approximately 5 mm.

2.3. Conductometric transducers

During the working stage, we used the transducers manufactured according to our recommendations at the V.Ye. Lashkarev Institute of Semiconductor Physics, NAS of Ukraine (Kyiv, Ukraine). They were 5 mm×30 mm in size and consist of two identical pairs of thin-layer planar gold electrodes, produced by the technology of vacuum deposition of gold on a non-conductive ceramic substrate (Fig. 3a). To improve the adhesive properties of gold, a 50 nm thick layer of chromium was applied under the golden layer on ceramic substrate. Each system consisted of 20 pairs of fingers of interdigitated electrodes, a digit width and a gap between them of 20 μm, with a total sensitive surface of about 2 mm^2 [8]. The portable conductometer «MCP-3» was developed according to our recommendations and manufactured at the Institute of Electrodynamics of National Academy of Sciences of Ukraine (Fig. 3b). The measurements were performed

at a current frequency of 37 kHz and an amplitude of 14 mV [9].

2.4. Immobilization of enzymes on transducers surface

The biologically active membranes were formed by cross-linking of an enzymes with bovine albumin in a saturated glutaraldehyde vapour on the transducer surface. The mixture containing 5 % (w/v) enzyme, 5 % (w/ v) bovine albumin, 10 % (w/v) glycerol in 20 mM phosphate buffer (pH 7.4) was deposited on the sensitive surface of one transducers by the drop method, while the mixture containing 10 % (w/v) bovine albumin and 10 % (w/v) glycerol in 20 mM phosphate buffer (pH 7.4) was placed on the surface of the reference transducers. The use of glycerol prevents from a loss of enzyme activity during the immobilisation process, and provides better homogeneity of the membrane and better adhesion to the surface of the transducer. The sensor chips were then placed in a saturated glutaraldehyde vapour. After 30 min of exposure to glutaraldehyde, the membranes were dried at room temperature for 15 min.

3. Practical application of biosensors

3.1. Determination of glycoalkaloids in samples of potatoes and tomatoes

Cultivated potatoes are one of the main agricultural crops consumed daily by millions of people of different segments of the population. Potatoes are grown in almost 80 % of all countries, and its world production reaches 350 million tons annually. The initial level of the total content of alkaloids in potatoes is genetically determined and differs significantly depending on the potato varieties and the area where they are grown. It is known that glycoalkaloids, natural neurotoxic compounds, are involved in some mechanisms of potato resistance to diseases and insects. α-solanine and α-chaconine amount up to 95 % of the total content of glycoalkaloids in potatoes, other types of glycoalkaloids are present only in trace concentrations. The human body is exposed to the combined action of all glycoalkaloids, therefore, an assessment of the total concentration of glycoalkaloids in potato samples is necessary. The same is true for glycoalkaloids in tomatoes and in any edible food sample.

Electrochemical enzyme biosensors based on butyrylcholinesterase and pH-sensitive field effect-transistors have been developed for glycoalkaloids detection, and their laboratory prototypes were fabricated and thoroughly tested for potato and tomatoes samples [10–13].

Potatoes of different varieties were grown at the experimental potato station Arvalis of the Plant Institute (Boigneville, France). These experimental cultivars (*Elkana, Monalisa, Pompadour, Starter, Pollux, Roseval, Phoebus, Venus, Charlotte, Redlaure, Voyager, Daifla, Albane, Lucie, Epona, Virginia, Hinga, Kaptah V, 91 F220 9, 209, 309, 204 , 310, 303, 207, 201, 308, 306, 302*) and two commercial varieties (*Caesar* and *Agata*), in which the main part of glycoalkaloids are α-solanine and α-chaconine, were used for analysis.

Control measurements of potato glycoalkaloids were performed by HPLC [14, 15]. Tubers were washed, dried, homogenized, and then the glycoalkaloids were extracted with acetic acid. The extracts were concentrated and purified using a Sep-Pack®Plus C_{18} cartridge. Separation and quantification of α-solanine and

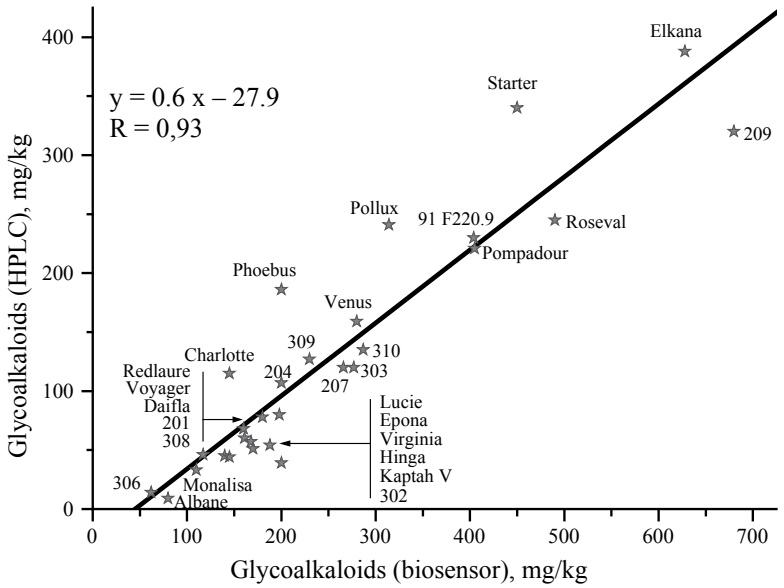

Fig. 4. Comparative analysis of the glycoalkaloids content in different potatoes varieties grown at the experimental potato station ARVALIS of Institute of Plants (Boigneville, France). Results were obtained using a biosensor and HPLC method

α-chaconine were performed on a Zorbax Extend C_{18} column (3.5 μm, 150×4.6 mm) at a wavelength of 202 nm. The comparison of the results of measurement of the total content of glycoalkaloids in the potato samples of 31 experimental cultivars is shown in Fig. 4. The results were obtained using the biosensor and the HPLC method with complex pre-treatment of the samples. The figure shows a good correlation of the results. Certain disagreement can be explained, firstly, by the difference in the procedure of samples preparing, and secondly, by the fact that the analyses were carried out in different places and at different time.

Different commercial tomato varieties were used for tomatine analyzes. The samples were washed by hand in running water and rubbed through a sieve to obtain juice. The juice samples were analyzed using a biosensor. We used two biosensors methods of analysis such as by using calibration curve and the standard addition method. The results of biosensor determination of the glycoalkaloids concentration in different commercial varieties of tomatoes are presented in Fig. 5. The obtained total amount of glycoalkaloids in tomatoes ranged from 30

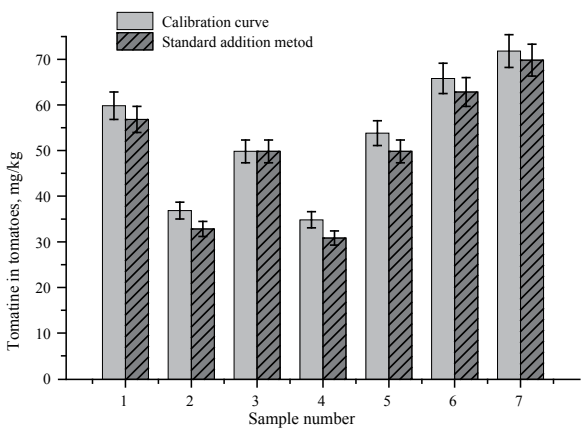

Fig. 5. The results of glycoalkaloids tomatine analysis in different varieties of tomatoes by two methods. Measurements were performed with 1 mM BuCh in 5 mM phosphate buffer, pH 7.2.

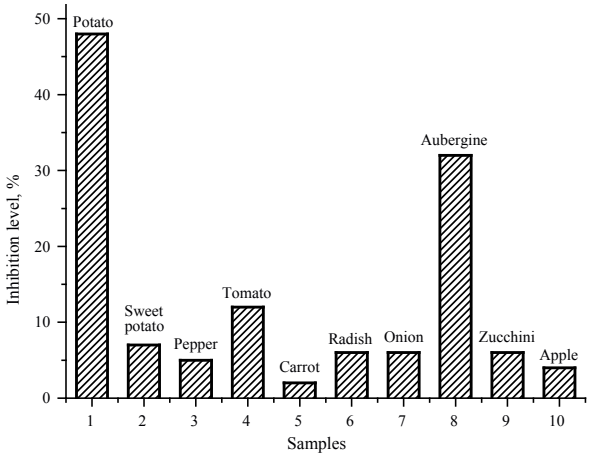

Fig. 6. Results of the analysis of the glycoalkaloids content in various vegetables and fruits

to 70 mg/kg of fresh weight, which corresponds to the typical values.

Fig. 6 shows the data on the biosensor selectivity in the analysis of various vegetables and fruits.

The figure clearly shows that the degree of inhibition of immobilized BuChE in the biosensor composition differs essentially for juice samples from various fruits and vegetables. In the case of potatoes, aubergine and tomatoes, inhibition is significantly larger. This is understandable because they belong to the same class of nightshade crops, which, unlike others, may contain glycoalkaloids. The analysis is simple, fast and does not require significant time. Biosensors demonstrated good operational stability during 20 days of work.

3.2. Determination of aflatoxins in cereal samples

Analysis of mycotoxins is a difficult task because these molecules are present in complex matrices at low concentrations; they can occur in various combinations and**,** at the same time, be produced by one or more species of fungi. To control the level of aflatoxins many countries have implemented rules governing their content. Acceptable limits of aflatoxins depend on the type of agricultural product.

Electrochemical enzyme biosensors based on acetylcholinesterase and pH-sensitive fiels effect-transistors have been developed for aflatoxin B1 detection, and their laboratory prototypes were fabricated and thoroughly tested for cereal samples [16–19].

Wheat, oats, corn, and peanuts specifically infected with *Aspergillus* were used as real samples to test the developed biosensor. The samples were specially prepared at the D.K.Zabolotny Institute of Microbiology and Virology of National Academy of Sciences of Ukraine. Aflatoxin B1 producer was grown on substrates (wheat, oats, corn and peanuts) for 21 days. To analyze the composition of aflatoxins in contaminated cereal samples, measurement protocols were first developed. As a control, we used the extracts obtained by the same method from the same substrates, but not infected with the *Aspergillus*. The measurements were carried out as follows: first, several test responses to 4 mM substrate of acetylcholinesterase were received. Then the calibration was performed by the volume of the control sample. Next*,* the biosensor was thoroughly washed in the working buffer until the response was restored, and the volume calibration for the infected sample was performed. Fig. 7 presents the results of the experiment for the control sample and infected wheat.

The figure clearly shows that inhibition of acetylcholinesterase in the bioselective membrane by the control sample practically does

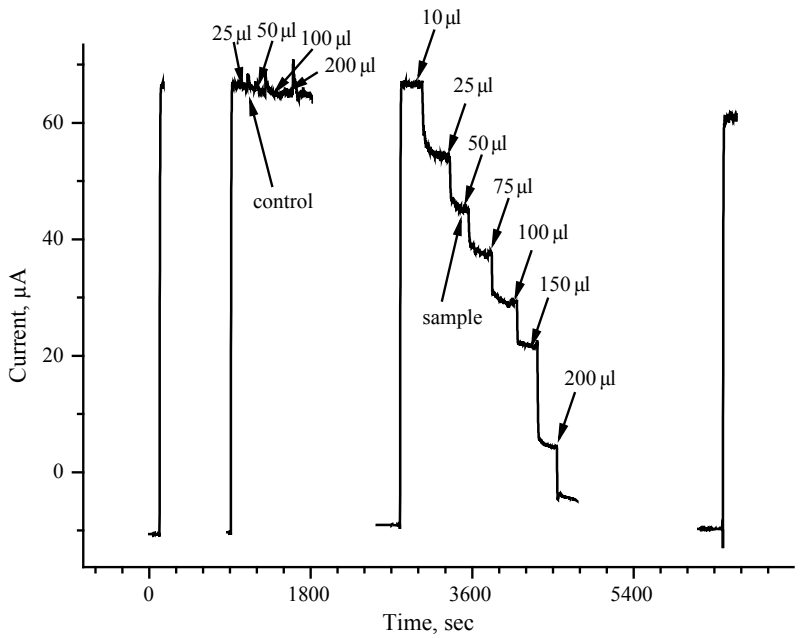

Fig. 7. Biosensor responses to control sample and an extract from infected wheat

not occur whereas the infected extract inhibits the immobilized acetylcholinesterase rather strongly. According to the same algorithm, the measurements were performed for the samples of oats infected with the fungus *Aspergillus* and its control extract.

Fig. 8 presents the kinetics of the effect of the control sample of oat extract and the sequential inhibition of the bioselective element by the extract from the infected sample.

There is a slight influence of the control sample on the selective element, which can be associated with the "matrix" effect, whereas noticeable inhibition of the enzyme is observed for the studied sample. Thus, it is possible to draw a conclusion about rather good sensitivity of the developed biosensor to aflatoxin B1 in real samples.

It was practically impossible to carry out measurements with samples obtained from infected nuts and a control lot due to the very high fat content of the nut extract. Therefore, the extracts turned out to be very oily, which led to the formation of films both on the measuring surface of the cell and on the surface of the selective element. The access of active substances to the membrane was sharply limited, and we did not even get reproducible responses to the substrate. Therefore, the measurement of aflatoxins in nuts using biosensors is impossible, at least so far.

3.3. Determination of heavy metal ions and pesticides in aqueous samples of the environment

Every year, environmental monitoring becomes more and more important all over the world [20]. This is due to the significant development of the chemical industry, the intensive use of chemicals in agriculture and the

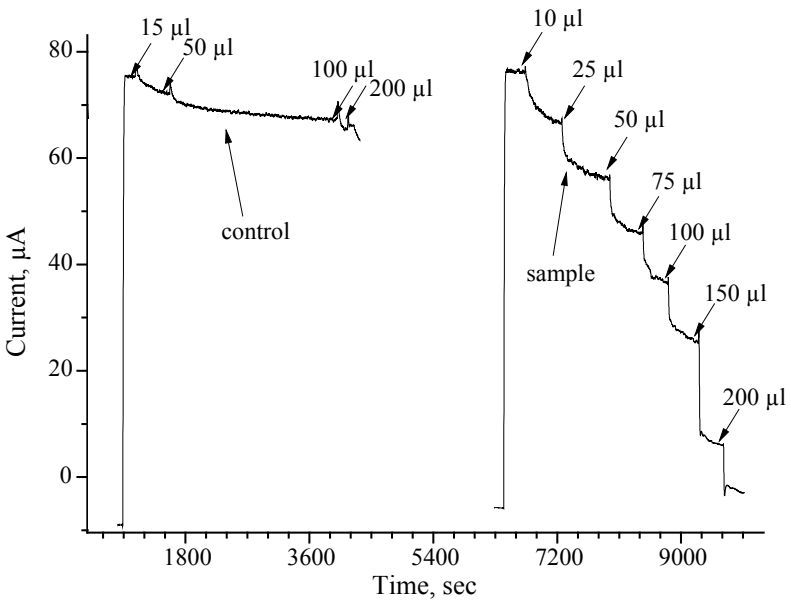

Fig. 8. Biosensor responses to a control sample and an extract from infected oats

increasing utilizing of variety of chemical products in other branches of human activity. These chemicals, often toxic, in turn get into the air, land, water, thereby polluting large areas and the food of humans and farm animals. This, in turn, leads to deterioration of human health and the emergence of various diseases. It is well known that pesticides and heavy metals have a special place among the toxic substances polluting the environment. Heavy metals and their compounds are characterized by relatively high resistance to degradation in the environment, solubility in precipitation, the ability to sorption by soil and accumulation by plants. They are able to accumulate in organisms, are toxic to humans and have a wide range and variety of harmful effects [21]. Along with heavy metals, pollution by pesticides is another high risk factor for human health [22, 23]. Decomposition-resistant toxic compounds of organophosphorus pesticides, which have been and are still widely used in some countries in agriculture, as well as their not less toxic residues [24] are characterized by a high degree of penetration and thus get into human food [25].

Conductometric biosensors based on several enzymes such as acetylcholinesterase, butyrylcholinesterase, urease, glucose oxidase, and a three-enzyme (invertase, mutarotase and glucose oxidase) system have been developed for heavy metal ions and pesticides detection, and their laboratory prototypes were fabricated and thoroughly tested for water samples of the environment [26–32].

At the first stage of this work, we studied the inhibitory effect of model samples of individual toxicants and their mixtures on the enzymatic systems of the multibiosensor. The obtained experimental values of the inhibition of enzymatic systems by the toxicants in different concentrations and variants of their mix-

tures were analyzed using the methods of mathematical statistics in order to upgrade the approaches of semi-quantitative analysis of toxicants when working with real samples of the environment. The enzymes used in the experiment have a pronounced selectivity at least to some inhibitors.

Table 1 shows the conditional levels of the enzyme affinity to individual inhibitors. These levels are divided into three categories according to the following principle: "++" — the value of response of the bioselective element to the concentration of 1 mM inhibitor exceeds 80 %, "+" — the response value is less than 80 % but more than 20 %, and "-" — the response value is less than 20 %. As can be seen from the Table, only two of the five enzymes systems are inhibited by pesticides, butyrylcholinesterase has a pronounced selectivity to pesticides and is much less inhibited by heavy metal ions. In general, this selectivity is also manifested at the action of mixtures of inhibitors: the enzymes selective to pesticides demonstrate higher responses to the mixtures with higher concentrations of pesticides.

To verify the efficiency of the developed multibiosensor, we analyzed natural water samples for the presence of toxic substances. The samples were taken from a number of natural water reservoirs in the Kyiv city in the places of the most common contact of the population with water, namely on the city beaches of two districts, Obolon and Osokorki. In Fig. 9, a part of the Kyiv map with the marked reservoirs is presented.

The list of tested samples included water samples from lakes «Vyrlytsia», «Sunny», «Minister», «Opechen», «Opechen Nyzhnya», from the Dnieper River near the North and South bridges, and the Obolon Bay. We added known amount of toxic substances to several samples to determine an increase in the levels of inhibition of the bioselective elements of the multibiosensor and its accordance with the actual values of the concentrations of toxic substances. Furthermore, we tested the multibiosensor in the analysis of multicomponent complex samples. For this purpose, the water samples were taken from the solid domestic waste landfill (SDW) No.5 in the village of Pidhirtsi, Obukhiv district, Kyiv region. It is during the inhibitory analysis of the sample from the solid domestic waste landfill (SDW) No.5 that the main problems arose. After the multibiosensor incubation in a sample from the landfill, the sensitive elements of the multibiosensor completely lost the ability to respond to the introduction of appropriate substrates. The reason could be either 100 % inhibition of all the enzymes used or/and bloc-

Table 1. **Conditional levels of the enzyme systems affinity to toxicants.**

Enzyme system	Trichlorfon	Carbofuran	Ag^{+}	Hg^{2+}	Cu^{2+}	Cd^{2+}
Urease	-	-	++	++	++	++
Butyrylcholinesterase	++	++	+	+	-	-
Acetylcholinesterase	++	++	++	++	+	++
Glucose oxidase	-	-	++	++	+	++
Three-enzyme system	-	-	++	++	++	++

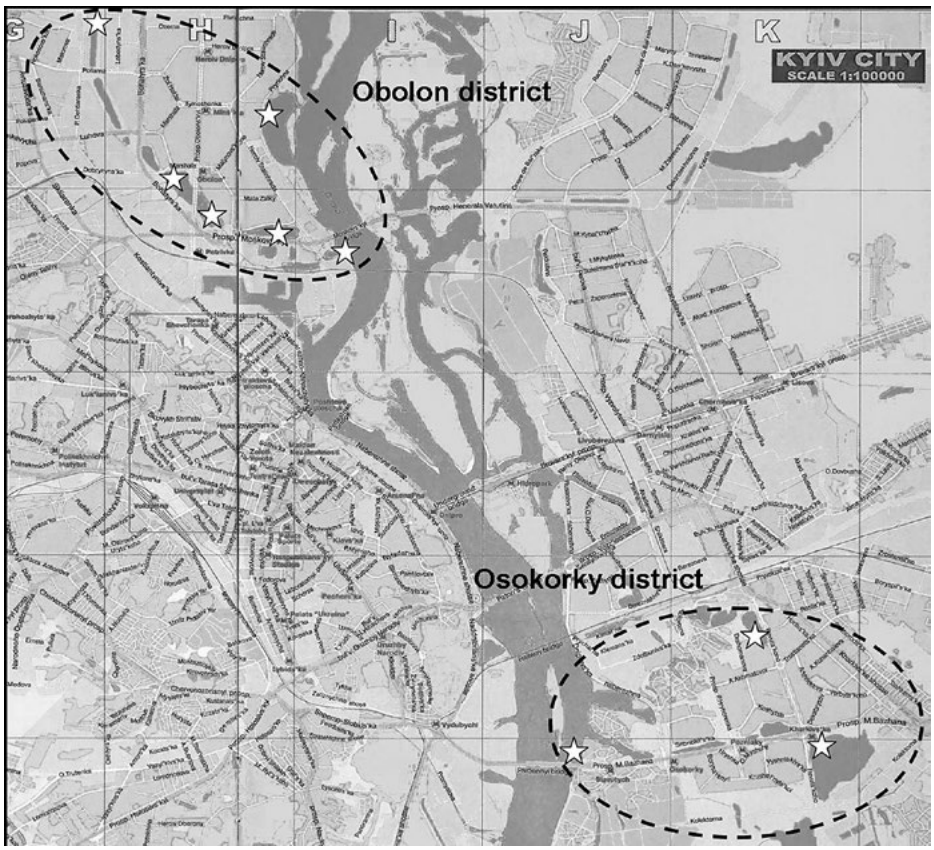

Fig. 9. Map of Kyiv city with the sampling sites (marked with asterisks).

king the pores of bioselective elements by colloidal particles present in the sample.

In parallel with the biosensor analysis, all samples used in the work were tested at the L.I. Medved Institute of Ecohygiene and Toxicology for the presence of toxic substances. The research was conducted using traditional methods for the toxicants determination (atomic absorption spectroscopy, thin layer chromatography and atomic absorption analyzer of mercury). The results of the comparison of traditional and multibiosensory methods are presented in Table 2.

Rather a high correlation of the data obtained using traditional methods with the results obtained by the multibiosensor is observed. As expected, exceeding the permissible concentrations was found in the samples, to which appropriate aliquots of toxicants were intentionally added, namely, mercury excess (line 2), copper excess (line 6) and trichlorophone excess (line 4). Using the multibiosensor, it was also shown that the rest of samples from Kyiv reservoirs contained no toxicants in dangerous concentrations. Using traditional methods, we registered the exceeding of the maximum permissible concentrations for copper, cobalt, zinc and chromium, and the absence of mercury and pesticides (line 13), which is completely consistent with the data obtained by the multibiosensor. Thus, the results of the analysis of all considered water

Table 2. **Comparison of different methods of analysis of real environmental samples.**

№	Place of the samples selection	Traditional methods, mg/l			Multibiosensor method		
		$AAAHg^{2+}$	AAC	TLC			
1	Lake Vyrlytsia (Pozniaky, Kyiv)	-	-	-	-	-	-
2	Lake Vyrlytsia + 400 нМ Hg^{2+}	0.079	-	-	+	-	-
3	Dnipro river (Osokorky, Kyiv)	-	-	-	-	-	-
4	Dnipro river +10 μM trichlorophone	-	-	2.57	-	-	+
5	Lake Sonyachne (Osokorki, Kyiv)	-	-	-.	-	-	-
6	Lake Sonyachne + 5 мкМ Cu^{+2}	-	0.321(Cu^{2+})	-	-	+	-
7	Lake Ministerske (Obolon, Kyiv)	-	-	-	-	-	-
8	Lake Opechen (Obolon, Kyiv)	-	-	-	-	-	-
9	Lake Opechen Nyzhnya (Obolon, Kyiv)	-	-	-	-	-	-
10	Lake Verbne (Obolon, Kyiv)	-	-	-	-	-	-
11	Dnieper, Obolon Bay, Kyiv	-	-	-	-	-	-
12	Dnipro, (Obolon, Kyiv)	-	-	-	-	-	-
13	Solid domestic waste landfill (SDW) No.5 (Pidhirtsi village, Obukhov district, Kyiv region)	-	0.317(Cu^{2+}) 0.034(Co^{2+}) 1.471(Zn^{2+}) 0.988(Cr^{2+})	-	-	++	-

Notes: $AAAHg^{2+}$ — atomic absorption analyzer of mercury; AAS — atomic absorption spectroscopy; TLC — thin layer chromatography; "+" — exceeding maximum permissible concentrations (MPC); "++" — exceeding the MPC by several orders of magnitude.

samples, obtained by traditional methods of toxicants determination, confirm the results obtained with a multibiosensor.

3.4. Determination of key components in wine samples and must

Wine products as one of the elements of the food industry at all stages of production should undergo technical and chemical control aimed to determine the components of wine and must and their effect on the quality of final product [33]. Therefore, study on the conversion of various substances at must fermentation and wine formation is greatly important in wine processing.

Glycerol is the most important secondary product of alcoholic fermentation, because it has a significant impact on the softness and viscosity of the wine, as well as on its taste characteristics, especially on the richness of taste, gives wines oiliness and softness [30]. Ethanol is the main product of alcoholic fermentation. It determines the toxic, addictive, caloric properties of wine and other alcoholic beverages [35]. An analysis of glucose is essential considering that glucose on one hand is a source of carbon for yeast, i.e. fermenting material, and on the other hand, it is a substrate limiting yeast growth. Besides, the amount of fermented glucose determines the ethanol con-

Table 3. **Analysis of ethanol, lactate and glucose in wine and must**

Sample	Type	Ethanol, (±0.1) % vol.		Glucose, (±0.1) g/l		Lactate, (±0.1) g/l	
		Biosensor	DD	Biosensor	HPLC	Biosensor	HPLC
Rkatsiteli "Koktebel"	White, table, dry	12.7	12.0	0.6	0.29	1.62	1.62
Aligote "Koktebel"	White, table, dry	11.6	11.2	0.47	0.12	2.2	1.98
Merlot "Koblevo"	Red, table, dry	12.1	12.0	0.84	0.51	1.21	1.27
Cabernet "Koktebel"	Red, table, dry	9.6	11.2	0.70	0.32	1.04	1.08
Monte Blanc "Koktebel"	White, table, semi-sweet	11.2	10.5	16.7	16.40	1.01	1.07
Porte wine "777" red	Red, strong	18.8	17.5	32.8	34.54	0.80	0.81
Porte "777" pink	Pink, strong	18.2	17.5	32.2	33.24	0.63	0.64
Porte white	White, strong	18.4	17.0	31.5	32.78	0.76	0.69
Madeira Massandra	White, strong	19.6	19.0	7.56	7.15	0.53	0.49
Kokur "Koktebel"	White, dessert	18.1	16.0	87.2	87.12	0.58	0.53
Kara-Dag "Koktebel"	Red, dessert	16.3	16.0	85.32	83.05	0.98	0.98
Cahors "Ukrainian"	Red, dessert	15.9	16.1	87.3	87.18	0.95	0.89
white wine must	-	0.8	-	125.3	124.99	0	0
red wine must	-	0.3	-	131.0	134.54	0	0

tent in wine, and residual non-fermented glucose has a considerable effect contributing sweetness to the wine taste [36]. The monitoring level of lactic acid, lactate, when producing high-quality beverages is also obligatory, since this parameter not only determines wine quality and flavour but is also an indicator of bacterial activity during fermentation [37]. Therefore, reliable information about the lactate content in the must at different stages of wine production allows the control and regulation of the fermentation [38]. Additionally, the stability of wines during storage also depends on the lactate concentration [39].

Amperometric biosensors based on individual enzymes suchas lactate oxidase, glucose oxidase, alcohol oxidase and glycerol oxidase have been developed for lactate, glucose, ethanol and glycerol detection, and their laboratory prototypes were fabricated and thoroughly tested for wine and must samples [40–48].

The content of ethanol, lactate and glucose in 23 samples of wine of different types and in two samples of red and white must was determined using the developed enzyme amperometric biosensors based on oxidase. The data obtained were compared with the results of the ethanol analysis by the distillate densitometry (DD), whereas the HPLC method was used as control for lactate and glucose analyses. The results obtained during the experiments are presented in Table 3.

As can be seen from the table, we have a high degree of correlation between the results obtained using the developed amperometric biosensors for the determination of ethanol, with the results of the traditional method of alcohol distillation followed by densitometric determination (R = 0.98). Moreover, the crea-

ted biosensor proved its effectiveness also in the analysis of table wines with an ethanol content of about 10 % by volume, and in the analysis of strong wines containing approximately twice as much alcohol. When determining lactate and glucose, a high correlation of results was shown with the data obtained using HPLC method (correlation coefficient for glucose R = 0.99, for lactate R = 0.97). Therefore, the developed and optimized amperometric biosensor based on immobilized oxidase is an effective and accurate instrument for analysis of wine and wine materials of different types with a wide range of content of both a target analyte and interfering substances.

In addition, the developed amperometric biosensor based on glycerol oxidase was able to detect glycerol in wine samples [40].

3.5. Determination of some sugars in foodstuffs

The organization of proper control of the content of mono- and disaccharides at different stages of production is an important task in various industries: food industry, agriculture, pharmacy, among others. In agriculture, the sucrose content of sugar beet is one of the important indicators that determines the efficiency of technological production of sugar at all stages - from beets growing and preservation to its complete processing. In dairy production, the content of lactose, one of the main components of milk, is an important indicator of the quality. Maltose is also of great importance in the food industry, because its content in maltose molasses determines the quality of the final product, in particular beer and kvass. Control of carbohydrate content is also necessary to monitor the course of fermentation, *etc.*

We developed a number of conductometric enzyme biosensors for the determination of basic natural carbohydrates, namely glucose, maltose, sucrose, fructose and lactose [49–55]. Three specific hydrolases, mutarotase and glucose oxidase are used to determine sucrose, maltose and lactose, whereas only one, glucose oxidase, is used to determine glucose. In the course of reactions under the action of the enzymes invertase, β-galactosidase and α-glucosidase, the corresponding substrates — sucrose, lactose and maltose — are cleaved to α-D-glucose, which, under the action of mutarotase, is converted into β-D-glucose. Using sucrose and glucose biosensors, the sugar content was measured in different parts of the sugar beet roots and other samples of the sugar beet homogenate (Table 4).

Polarimetry with lead acetate as a control method was carried out at the Institute for sugar beets of the Ukrainian Academy of Agrarian Sciences (Kyiv). As can be seen from Table 4, the data obtained by the biosensor method are confirmed by the control method (R=0.914).

Table 4. **Analysis of saccharinity of sugar beets**

Part of sugar beet roots and other samples	Saccharinity, %	
	Biosensor method	Control method
Top part	9.5±0.5	10.9±0.1
Middle part	14.7±0.6	14.2±0.1
Bottom part	12.9±0.4	13.0±0.1
Sample #1	10.1±0.6	12.4±0.1
Sample #2	18.3±0.7	17.2±0.2
Sample #3	13.3±0.8	15.2±0.2
Sample #4	16.6±0.6	18.8±0.2
Sample #5	18.7±0.5	17.7±0.2
Sample #6	17.6±0.7	16.6±0.2

Table 5. **Analysis of glucose and sucrose content in juices and honey**

Sample	Sucrose concentration, g/l		Glucose concentration, g/l	
	Biosensor (n = 5)	HPLC (n = 3)	Biosensor (n = 5)	HPLC (n = 3)
1. Grape and apple juice "Our juice"	2.76± 1.27	5.1 ± 0.13	50.9 ± 3.1	53.7 ± 1.35
2. Apple and carrot juice "Truly juice"	4.4± 1.5	4.1 ± 0.10	7.8 ± 2.5	6 ± 0.15
3. Apple juice "Truly juice"	7.9± 1.44	10.7 ± 0.27	22± 1.5	22.3± 0.56
4. Tomato juice	1.19± 0.65	not found	10.4 ± 1.2	11.3± 0.26
5. Apple juice	12.42±1.34	12.5 ± 0.31	22.7 ± 2	22.2 ± 0.55
6. Orange juice "Sandora"	33.66 ± 3.9	37.8 ± 0.95	19.3± 1.8	21.6± 0.53
7. Orange nectar "Dooy"	14.48 ± 1.16	15.85 ± 0.62	54.68 ± 1.95	59.43 ± 1.41
8. Orange juice "Cappy"	73.12 ± 2.05	82.77 ± 0.10	11.53 ± 1.18	9.76 ± 0.09
9. Apple juice "Pinar	14.21 ± 1.30	16.5 ± 0.14	33.33 ± 1.86	36.18 ± 1.17
10. Orange nectar "Göze"	6.06 ± 0.34	6.13 ± 0.03	67.56 ± 1.70	70.51 ± 0.95
11. Flower honey (1)	8.4± 3.28	10.5 ± 0.26	26 ± 1.4	27.1 ± 0.68
12. Flower honey (2)	1.3± 4.5	2.3 ± 0.06	32.07 ± 1.8	31.3 ± 0.78

Ten samples of fruit and vegetable juices and nectars and two samples of honey were analyzed. The results of the analysis are shown in table 5. For the analysis, we used commercially available beverage samples obtained by direct extraction of fruits and roots. The preparation of the samples containing fruit pulp consisted only of centrifugation for 5 min at 2400 rpm. An aliquot of the tested sample was introduced into the measuring cell so that the final dilution was 1000 times. This allowed working within the linear range of determination of sucrose and glucose, as well as avoiding the influence of the ionic strength of the solution introduced into the measuring cell. Each of the measurements was repeated at least 5 times. Thus, we received the responses to the introduction of each of the samples. Then, using the method of extrapolation on the calibration curve of biosensors and the developed method of calculation [49], we found the values of glucose and sucrose concentration in diluted beverage samples. HPLC with subsequent refractometric detection was chosen as a control, as it is known that this method is characterized by high accuracy, and the results obtained can be considered reliable. VarianProStar HPLC system with a column Microsorb MV NH_2 (4.6×250 mm, 5 mm) and liquid phase acetonitrile-H_2O (80:20) was used.

The results of determination of the glucose and sucrose concentrations in beverages obtained by the biosensor method and HPLC were quite close. This indicates great prospects for the use of biosensors in the analysis of food products for the presence of sucrose and glucose in appropriate concentrations, which is an important indicator of nutritional value of the product and can sometimes even reveal falsification of the product (counterfeit juices, adulteration of honey by adding sugar, *etc.*).

In addition, the samples of milk were analyzed using three-enzyme lactose amperometric biosensor based on platinum disc electrode modified by nanosized semipermeable poly (meta-phenylenediamine) film (Fig. 10).

Lactose in the milk samples was determined using also HPLC, to compare with the lactose amperometric biosensor. For the HPLC determination (AGILENT HPLC 1260 SL), Microsorb-MV100 NH_2 (250 4.6 mm, 5 μm; R008670005, VARIAN) column was used at 25 °C, with the mobile phase Acetonitrile: H_2O (80:20) at 1.5 ml/min flow rate and AGILENTG1362A 1260 RID detector. Three milk samples were analyzed and quantified in 0.1–10 g/L lactose range. The results obtained by HPLC and the biosensor method were in good correlation. Therefore, such laboratory prototypes of biosensors can be used in the future as a basis for creating a commercial device for simultaneous analysis of basic carbohydrates in the food industry, pharmaceutics and medicine.

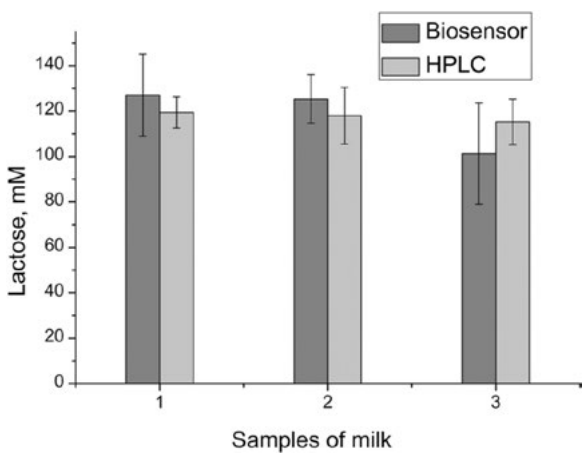

Fig. 10. Results of determination of lactose concentration in milk samples obtained by amperometric enzyme biosensor and HPLC

3.6. Determination of arginine in dietary supplements and drugs

Nowadays, treatment and prevention of deficiency of the key nutrients and biologically active substances in the human body are important for the restoration of body functions, and therefore are increasingly used in medicine for therapeutic and rehabilitation purposes. One of the compounds, which plays a critical role in the large number of synthetic processes in the body, is the conditionally essential amino acid arginine (α-amino-δ-guanidinovaleric acid). In mammals, arginine is not only a component of peptides and proteins, but also a precursor of a number of important individual compounds such as urea, proline, glutamate, creatine, agmatine, L-ornithine, L-citrulline, γ-aminobutyric acid and the only precursor of nitric oxide.

The use of the arginine-based dietary supplements and drugs has become widespread over the past 10–15 years [56–58]. Today, L-arginine in high doses is used to prevent and treat all manifestations of endothelial dysfunction of atherosclerotic origin (cardiac, cerebral, peripheral), hypertension (arterial, pulmonary, renal), liver diseases, diabetes, obesity, immunodeficiency, osteoarthritis [59–62], wounds, burns and other. Given that the quality control of the dietary supplements is not always carried out at the proper level, the development of methods of accurate, selective and fast determination of the arginine concentration in such products is important for implementation of the facilitated quality control procedure for such category of goods.

Conductometric biosensors based on natural enzymes arginase and urease, and recombinant enzyme arginine deiminase have been devel-

Table 6. **Determination of arginine in ampoules «ArginineVeyron» using the conductometric biosensor based on arginase and urease.**

No., exp.	CC, mM	CDF, mM	SD/SE, mM	SC, mM
1	0.927	954	20.84/9.32	922.4
2	0.955	983		
3	0.955	983		
4	0.940	968		
5	0.908	934		

Abbreviations in the table
CC — individual concentration determined by the method of calibration curve for Arg-HCl.
CDF — concentration of arginine taking into account the sample dilution factor (DF was 1029.25).
SD — standard deviation.
SE — standard error of the mean.
SC — concentration stated by the producer.

oped for arginine detection, and their laboratory prototypes were fabricated and thoroughly tested for dietary supplements (capsules) of different producers and the oral solution "Arginine Veyron" (Laboratoires Pierre Fabre Medicament, France) [63–65].

To measure the concentration of L-arginine in the oral solution "Arginine Veyron", two methods were used and compared: the classical biosensor method using a calibration curve and the standard addition method (Table 6).

Quantification of arginine in the dietary supplements was also performed by two control methods, namely chromatographic and spectrophotometric methods. Chromatographic analysis was performed by the independent laboratory "LLC Expert Center of Diagnostics and Laboratory Support "Biolights" (Kyiv region, Ukraine). The spectrophotometric determination of arginine (enzymatic colorimetric assay) was performed using the commercial L-arginine assay kit (Sigma-Aldrich, cat. no. MAK370). The standard deviation between five repeated measurements (n = 5) was found to be 20.84 mM, with standard error of 9.32 mM (Table 6). The coefficient of variation of the obtained values was 2.16 %. Comparing the stated value (922.4 mM) and the value obtained experimentally, we suppose that discrepancy between them might originate from the interference of the background (i.e., presence of the additives in the sample matrix).

The results of the biosensor determination of arginine in dietary supplements in comparison with the results of control methods and data declared by the manufacturers are shown in Table 7.

Based on the calculated correlation coefficients, it was found that the results of the de-

Table 7. **Determination of arginine in dietary supplements using a conductometric biosensor based on arginine deiminase**

Sample	Concentration of L-arginine			
	determined by biosensor method, mM	determined by ion chromatography, mM	determined by spectrophotometric method, mM	declared by the manufacturer, mM
"L-arginine 500 mg" ("Solgar Vitamin and Herb" USA)	143.86±10.95	150.642	153.4	149.7
"L-arginine 500 mg" ("Now Foods", USA)	146.3±25.56	148.496	148.38	143.8
"L-arginine" ("Elite-Pharm", Ukraine)	114.3±17.4	95.616	109.81	100

termination of L-arginine using the biosensor based on arginine deiminase correlated well with both the results of ion chromatography and spectrophotometric assay (the correlation coefficients were R = 0.987 and R = 0.997, respectively).

4. Conclusions

A number of electrochemical biosensors based on different spesific enzymes were developed, and their laboratory prototypes were fabricated and thoroughly investigated for real conditions of application (potatoes, tomatoes, ceareals, wine and wine must, natural fruit juices, environmental water and in some other samples). Noteworthy, electrochemical biosensors are adaptable to the technologies of large-scale production of miniaturised devices. Concerning further wide application of the biosensors developed, the obtained results demonstrate the possibility to modulate their main characteristics to comply with the requirements specific for potential practical purposes. Furthermore, diverse biosensor modifications (with genetically modified enzymes and microorganisms, additional membranes, different nanoscaled materials, *etc.*) can be elaborated as well. In brief, the potential of this technology has been shown here; its wide application is pending.

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Практичне застосування електрохімічних ферментних біосенсорів

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Електрохімічний біосенсор — це інтегрований пристрій, що може перетворювати біологічну реакцію в вимірюваний електричний сигнал. Завдяки таким важливим характеристикам, як селективність, чутливість,

стабільність, відтворюваність, лінійність і низька собівартість, біосенсори мають широкий спектр застосувань. Метою цієї роботи є огляд наших досягнень у розробці електрохімічних ферментних біосенсорів та можливості їхнього застосування в харчовій промисловості, сільському господарстві та контролі навколишнього середовища.

В роботі обговорюються специфічні структурні особливості перетворювачів розроблених амперометричних, потенціометричних та кондуктометричних ферментних біосенсорів. Виготовлено та протестовано лабораторні прототипи біосенсорів для визначення глікоалкалоїдів, мікотоксинів, іонів важких металів та пестицидів у сільськогосподарських зразках та об'єктах навколишнього середовища, а також у деяких зразках харчових продуктів та біологічно-активних добавок. Прилади демонстрували можливіст швидкого, високоселективного та чутливого визначення необхідних компонетів, коректну аналітичну роботу, а також можливість адаптувати основні характеристики пристроїв відповідно до поставлених вимог для конкретних практичних цілей. Важливо також відмітити, що виготовлення таких мініатюрних електрохімічних біосенсорів може бути адаптовано до технологій великомасштабного виробництва.

Ключові слова: електрохімічні біосенсори; ферменти; застосування; сільське господарство; продукти харчування; навколишнє середовище

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