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ARTICLE

Application of Amperometric Biosensors for Analysis of Ethanol, Glucose, and Lactate in Wine

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This article presents the application of amperometric biosensors based on platinum printed electrodes SensLab and immobilized enzymes, alcohol oxidase, glucose oxidase, and lactate oxidase, for wine analysis. Created devices demonstrate linear response to ethanol, glucose, and lactate within the concentration range 0.3−20 mM, 0.04−2.5 mM, and 0.008−1 mM, respectively. No decrease in ethanol and glucose biosensor activity is revealed during 2 months after fabrication, and the operational stability of the lactate biosensor is sufficient only during 4 days. Developed biosensors showed high selectivity to the substrate and are successfully applied to the analysis of such complex mixtures as wine and must. Good correlation of the results of analysis of different wines and must obtained by amperometric biosensors with immobilized oxidases and traditional methods is shown. Created biosensors can be used as a basis of a commercial device for express analysis of ethanol, glucose, and lactate in wine and must during its fermentation. Application of such devices for quality control in foodstuff industry can have great economical effect because determination by biosensors is less expensive, labor-intensive, and lengthy than traditional methods of analysis.

KEYWORDS: Amperometric biosensor; ethanol; glucose; lactate; wine; must

INTRODUCTION

A lot of important analytical tasks need simultaneous monitoring of several biochemical parameters to be performed in a complete way. This is an especially topic in food analysis in which numerous samples contain complex mixtures of analytes of interest (*1*). Quantitative analysis of such complex samples as wine and other beverages is becoming of great importance nowadays because of the general effort in achieving their adequate quality. Particularly in studies on wine authenticity and quality, comprehension of complex processes of grape must transformation into wine content and quantification of main wine compounds is a very necessary stage (*2*).

The winemaking process includes glucose alcoholic fermentation by yeast and secondary fermentation by lactic acid bacteria called malolactic fermentation.

At the first stage, alcoholic fermentation, yeast converts glucose and other sugars in grape juice into ethanol and carbon dioxide. Quantification of ethanol, the main product of alcoholic fermentation, is needed in wine technology since this alcohol has important effects on yeast growth (*3*). From an economic point of view, continuous measurement of the ethanol concentration during wine production is necessary for control and optimization of the fermentation processes in the wine industry, for quality control of wines and for tax regulation purposes (*2*). Determination of glucose concentration in must is also necessary during the fermentation process because glucose being the carbon source for fermentation of yeasts limits their growth (*3*). In addition, an amount of fermented sugar determines the levels of alcohol and residual sugars (mainly, fructose and glucose) in the wine. The residual sugars contribute to wine sweetness and quality; thus, their quantification is also needed during the winemaking process (*2*, *4*).

At the second stage, malolactic fermentation, L-malic acid is converted to L-lactic acid and carbon dioxide by lactic bacteria (*2*). Malolactic fermentation strongly influences the final taste of the wine because malic acid, one of the naturally occurring acids within grapes, is a much stronger acid than lactic acid. By this conversion, the acidity of the wine is decreased, and the wine is considered to be more stable (*2*). At the same time, an excess of lactate also has a negative effect on wine taste (*5*). Thus, lactate concentration has been shown to be a very important parameter to control in winemaking because its level defines the freshness, stability, and storage quality of wine (*2*, *5*).

Therefore, continuous, simultaneous, and selective monitoring of ethanol, glucose, and lactate, the key participants of alcoholic and malolactic fermentation, is highly needed in the wine industry since together these parameters determine the quality, special taste, and flavor of wine. Considering the monitoring results, timely intervention is possible, if necessary, in the fermentation process, which allows one to avoid serious economical losses.

Nevertheless, yeast fermentation media are generally three-phase systems, which are difficult to analyze. They are complex solutions of organic and inorganic substrates, physically dissolved gases, and dispersed solids of different sizes, such as

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microorganisms (*2*). Wine is also a mixture of several hundreds substances at different concentrations, among which water, alcohols, aldehydes, ethers, sugars, organic and amino acids, minerals, and other relevant compounds dominate (*2*). Wines, particularly red wines, also contain numerous biologically active substances: polyphenols, flavonoids, vitamins, and enzymes (*2*, *4*).

Thus, in wine and must analysis several aspects must be considered, namely, analyte diversity and matrix complexity. Therefore, an analytical method should be based on the reaction or detection system selective toward the target analyte. Furthermore, as its concentration can essentially differ depending on the type of wine, it is important to provide measurement of the target analyte in a wide concentration range; besides, the presence of interfering substances should be taken into account (*6*).

Traditionally, high performance liquid (HPLC), gas chromatography and the method of wine distillation with further measurement of the ethanol content in the distillate have been used in wine production for determination of the main analytes of interest (*7*). These techniques can be selective and reliable, but in the case of the wine distillation method, some limitation occurs concerning detection because wine compounds belonging to the same family with ethanol may interfere with the analysis. Moreover, it is not always possible to chromatographically resolve the analytes of different chemical characteristics in real samples because of the absence of adequate columns or derivatizing substances (*1*). Finally, these traditional analytical methods require expensive or bulky instrumentation, special sample preparation, and experienced operators, and can hardly be fully automated. Such instruments are not mobile and adapted to the competences and financial constraints of small winemakers, for whom cheap and smart devices represent an attractive alternative (*2*, *8*, *9*).

Enzyme biosensors are a prospective alternative to traditional analytical methods and probably one of the most promising ways to solve some problems concerning simple, fast, repetitive, and cheap multidetection (*10*). Biosensors allow an analysis of samples containing the analytes undetectable simultaneously at a conventional detector, by incorporation of different enzymes or coupling several enzyme reactions, which provide that each biological recognition element gives an individual response (*1*). Electrochemical biosensors, in particular amperometric ones, have an important position among biosensors as a class of the most widespread, numerous, and successfully commercialized devices of biomolecular electronics (*10*, *11*). Appropriately, the use of biosensors in food chemistry studies has been continuously growing in the last decades, with several applications also in the wine industry (*12*).

However, many enzyme-based amperometric biosensors recently described still display a few drawbacks when compared to traditional analytical methods. The most difficult problems to overcome in analysis of complex real samples are insufficient selectivity and reduced stability of such devices. Enzyme membranes or surface of amperometric electrodes can be sensitive to interfering species that results in poor selectivity to the substrate of the biosensor (*13*). In addition, the enzymes, being removed from their natural environment, tend to rapidly lose their activity, and thus, they limit the lifetime of the biosensor (*14*). That is why investigation and optimization of biosensor stability and selectivity are important stages prior to the application of these devices in wine analysis.

The present work was aimed at the development of amperometric biosensors based on alcohol oxidase, glucose oxidase, and lactate oxidase as well as at an investigation of their working characteristics for potential application in wine analysis.

MATERIALS AND METHODS

Enzymes and Chemicals. Alcohol oxidase (AOD) from *Hansenula* sp. with specific activity 1.6 U/mg was obtained from Sigma-Aldrich (United Kingdom). Glucose oxidase (GOD) from *Penicillium vitale* with specific activity 130 U/mg was obtained from Diagnostikum (Lviv, Ukraine). Lactate oxidase (LOD) from *Pediococcus* sp. with specific activity 39 U/mg was obtained from Sigma-Aldrich Chemie GmbH (Germany).

For electrochemical polymerization of enzymes, the monomer 3,4-ethylenedioxythiophene (EDT) from Baytron M (Germany) and polyethylene glycol 1450 from Sigma-Aldrich Chemie GmbH (Switzerland) were used. Glutaraldehyde produced by Fluka (Switzerland) was also used as a cross-linking agent for enzyme deposition. Besides, we used the following chemicals: hydrogen peroxide from Fargomed (Ukraine), L-lactic acid sodium salt from Sigma (USA), glucose, L-ascorbic acid, and bovine serum albumin (BSA) from Sigma-Aldrich Chimie S.à.r.l. (France), and ethanol from Fluka (Germany). The reagents $Na_2HPO_4 \cdot 7H_2O$, KH_2PO_4, NaOH, and glycerol were of Ukrainian production. All chemicals were of analytical reagent grade and used as received without additional purification.

Amperometric Experiments. The key enzymatic reactions used for substrate determination by amperometric biosensors based on immobilized oxidases are as follows:

$$\text{ethanol} + O_2 \rightarrow^{\text{AOD}} \text{acetaldehyde} + H_2O_2$$

$$\text{glucose} + O_2 \rightarrow^{\text{GOD}} \text{gluconolactone} + H_2O_2$$

$$\text{lactate} + O_2 \rightarrow^{\text{LOD}} \text{pyruvate} + H_2O_2$$

Substrate enzymatic transformation results in generating an electrochemically active substance, hydrogen peroxide, decomposition of which causes the formation of electrons measurable by means of an amperometric transducer:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

All electrochemical experiments were performed using the traditional three-electrode system in which the printed electrode SensLab (SensLab GmbH, Leipzig, Germany) combines in itself all three electrodes: platinum working, auxiliary, and reference (*5*). Amperometric measurements at a constant potential were carried out in a 5 mL electrochemical cell using a PalmSens potentiostat (Palm Instruments BV, The Netherlands).

Enzyme Immobilization by Electrochemical Polymerization in the Polymer Poly(3,4-ethylenedioxythiophene). Electropolymerization of small monomers is a technique for the formation of a membrane at the electrode surface. It enables one to select and maintain dimensions, shape, and thickness of the matrix and to provide exact control over precipitation (*5*). Electropolymerized films can be successfully used in biosensors since these films, because of their permselectivity to hydrogen peroxide over other compounds, act as a selective barrier, reducing the interfering effect of electroactive substances, such as ascorbic acid, often present in wine and must (*15*, *16*).

Poly(3,4-ethylenedioxythiophene) (PEDT) is one of the polythiophenes, conductive polymers with novel promising properties. Previous investigations demonstrated good conductivity of PEDT, small charge change, increased stability, and high capability to film formation. More detailed characteristics of PEDT electrochemical polymerization were previously published in ref *17*.

For electrochemical polymerization, the mixture of components consisting of 10 mM 3,4-ethylenedioxythiophene, 100 mM polyethylene glycol, and 30% enzyme solution was prepared in 20 mM phosphate buffer at pH 6.2.

PEDT was polymerized by application of the potential from +0.2 to +1.5 V at the rate of 0.1 V/s during 15 cycles using the PalmSens potentiostat. The PEDT electrochemical synthesis was monitored by cyclic voltammetry. After the enzyme immobilization in PEDT, the surface of the SensLab electrode was washed with distilled water.

Enzyme Immobilization in Glutaraldehyde Vapor. Glutaraldehyde is a polyfunctional agent that forms covalent bonds between biocatalytic particles or proteins. Therefore, enzyme immobilization with glutaraldehyde

is often used for the development of enzyme biosensors. This immobilization method produces a three-dimensional matrix in which the enzyme is closely trapped with the electrode material, thus improving both retention of the biomolecule on the electrode surface and its electrical communication (*18*).

For the formation of the glutaraldehyde-based bioselective membrane, a drop of 30% enzyme solution with 5% BSA was placed on the surface of the working electrode. Then sensors were placed into a glutaraldehyde vapor atmosphere (exiccator with glutaraldehyde liquid) during 10 min and after this dried in the air.

Determination of Operational and Storage Stability of Biosensors Based on Oxidases. Within the study of developed biosensor operational stability, a sample of stock substrate solution and a 20 μL wine sample were repeatedly injected into the electrochemical cell during 8 h. Concentration of the substrates in the experiment was equal to a half of the maximum limit of biosensor dynamic range, i.e., 20 mM of ethanol, 1.3 mM of glucose, and 0.5 mM of lactate for biosensors based on AOD, GOD, and LOD, respectively.

The storage stability of the developed biosensors was tested using dry storage of the sensor at +4 °C between the measurements.

Substrate Determination in Wine by Amperometric Biosensors. All measurements were performed in 20 mM K, Na-phosphate buffer solution, pH 7.2, at room temperature in an open bulk at intensive stirring. The substrate concentrations were measured in 12 samples of different wines produced under the micromanufacture conditions in the Magarach Institute of Vine and Wine as well as in 2 samples of red and white wine materials. Wine and must samples were diluted before analysis; the dilution ratio was 250−2000. Concentration of the main components of wine was determined by amperometric biosensors using the standard addition method. After each measurement, the biosensor was washed with buffer solution to stabilize the basic signal. Measurement of the substrate concentrations with amperometric biosensors was repeated at least 5 times for every wine sample. Duration of one analysis was 5−10 min.

Substrate Determination in Wine by Traditional Analytical Methods. Reference control measurement of glucose and lactate concentration in wine and must was carried out by HPLC. Determination of ethanol concentration in wine and must samples was carried out using the method of wine distillation.

During the preparation of test samples for HPLC analysis, we filtered wine or must through a nylon cartridge filter (pore size 0.45 μm). Glucose concentrations were determined by using an Agilent chromatograph. Separation was on a column Supelko NH_2. Elution was with 78% acetonitrile in water. Eluted components were detected by using a refractometer detector. Duration of analysis was about 30 min.

Lactate concentrations were determined by using a Shimadzu LC-20 Prominence chromatograph. Separation was in the isocratic mode on a Supelcogel C610H column (310 × 78 mm). Elution was with 0.1% H_3PO_4. Eluted components were detected by using a spectrophotometer detector with a diode array at 210 nm. Duration of analysis was about 30 min.

Determination of ethanol volume concentration in wine was fulfilled according to state standard 13191 (Ukraine). The method relies on the determination of ethanol in the distillate obtained by a distillation of a wine sample. During preparation and determination of the test sample, we neutralized a precise volume of wine (250 cm^3 at 20 °C) with 1 M NaOH solution and distilled it using a volumetric flask of 250 cm^3 as the receiving flask. The distillation was stopped as soon as a total of 200−225 cm^3 of the distillation product had been collected. The receiving flask containing the distillation product was thermostatted for 30 min at 20 °C, after which the distillation product was diluted to the mark with water. The distillation product was transferred to a cylinder, and ethanol volume concentration was determined using an alcoholmeter. Duration of analysis was about 1 h.

RESULTS AND DISCUSSION

Investigation of Printed Platinum Electrodes SensLab Sensitivity to Interfering Substances. The SensLab electrodes were previously tested with regard to their reproducibility and reliability by cyclic voltamperometry within the potential range of 0−600 mV (speed of potential involutes was 20 mV/s) using a PalmSens potentiostat. Cyclic voltammograms obtained at the SensLab electrode in working buffer and in the presence of 50 μM hydrogen peroxide

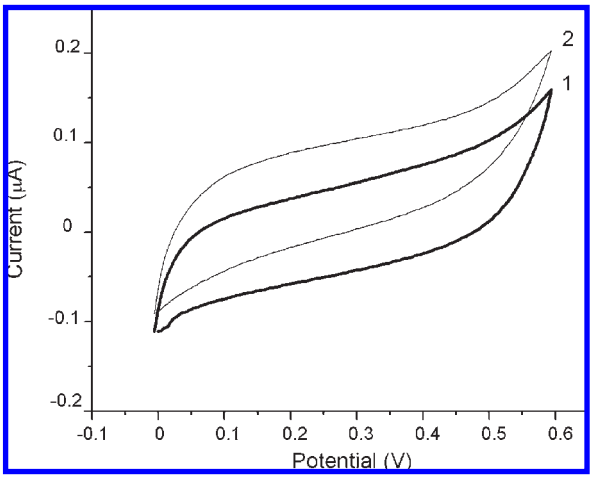

Figure 1. Cyclic voltammograms obtained for platinum printed electrode SensLab in working buffer (1) and in the presence of 50 μM hydrogen peroxide (2). Measuring conditions: 20 mM phosphate buffer at pH 7.2.

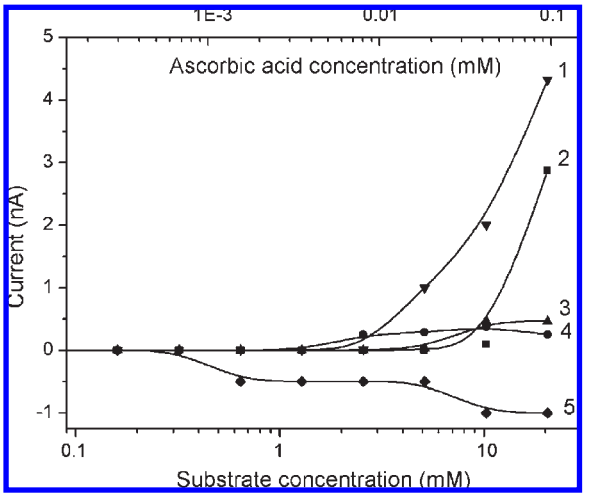

Figure 2. Responses of enzyme-free platinum printed electrode SensLab to 1, ethanol; 2, glycerol; 3, glucose; 4, lactate; and 5, ascorbic acid. Measuring conditions: 20 mM phosphate buffer at pH 7.2, at a potential of +200 mV versus the intrinsic reference electrode.

are shown in **Figure 1**. As can be seen, when hydrogen peroxide is added, an oxidation current appears, and the value of the biosensor signal rises. As a compromise between high biosensor sensitivity and significant electrochemical interference of the species (oxidation of which requires high potentials), a working potential of +200 mV versus the reference electrode was considered for further amperometric measurements (see **Figure 1**).

As has been already mentioned, optimization of biosensor selectivity is one of the most important tasks in advanced biosensor development, especially for multidetection. Nonspecific biosensor responses can be caused not only because of poor selectivity of the enzyme membrane to the target substance but also because of sensitivity of the electrode surface without any enzyme or polymer layer to interfering species. It was determined by other authors (*13*) that the response of enzyme-free transducer without any membrane ranged from 6 nA in white wine to 50 nA for red wine (dilution of samples was 1:150). The interference in this case is probably a result of the electrode reaction to easily oxidable phenolic acids, antocyanins, catechins, avonoids, and other polyhydroxyaromates occurring in wines, especially in red types.

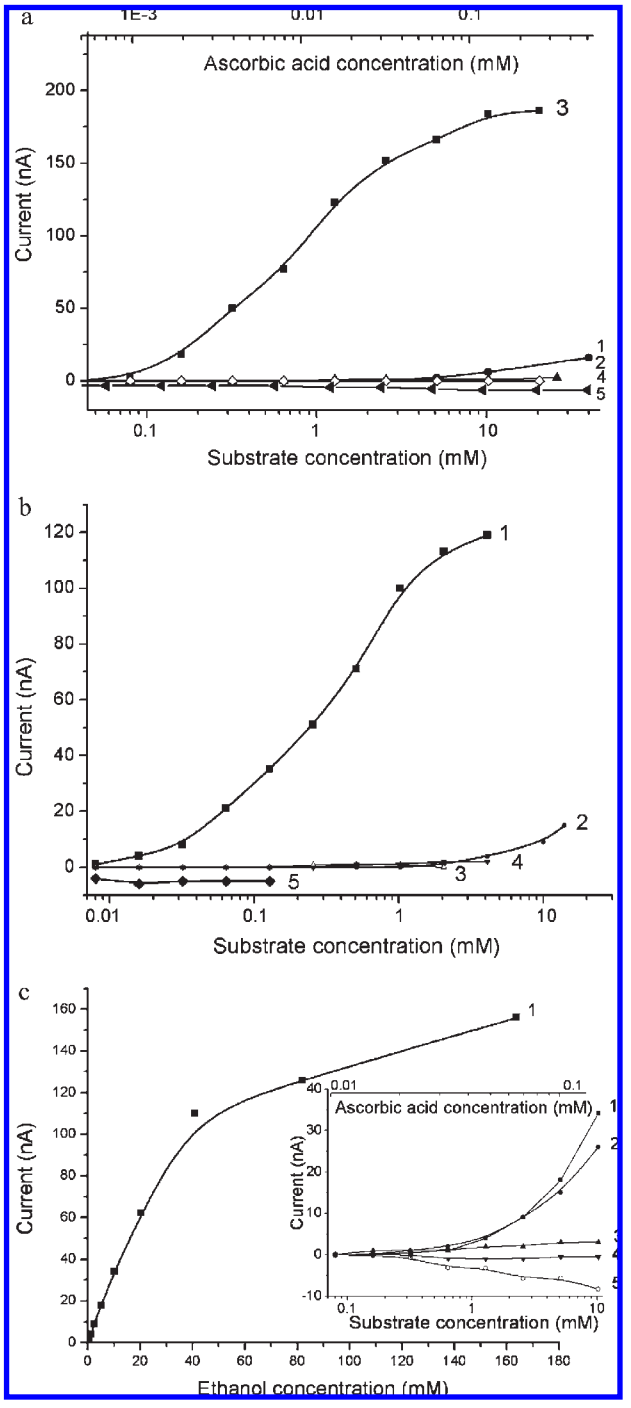

Figure 3. Responses of lactate (**a**), glucose (**b**), and ethanol (**c**) biosensors to 1, ethanol; 2, glycerol; 3, glucose; 4, lactate; and 5, ascorbic acid. Measuring conditions: 20 mM phosphate buffer at pH 7.2, at a potential of +200 mV versus the intrinsic reference electrode.

That is why at the first stage of our research sensitivity of printed platinum electrodes SensLab without any membrane to wine, must and their main components were tested. Maximum responses of the enzyme-free SensLab electrodes obtained to red wine and must were +0.6 and −0.5 nA, respectively (dilution of samples was 1:250). Then these SensLab electrodes were investigated for their response to main analytes of wine, namely, ethanol, glycerol, glucose, lactate, and ascorbic acid. The electrode SensLab was shown not to respond to lactate and glucose, to give small positive responses (less than 5 nA) to 20 mM ethanol and glycerol, and negative response (−1 nA) to ascorbic acid

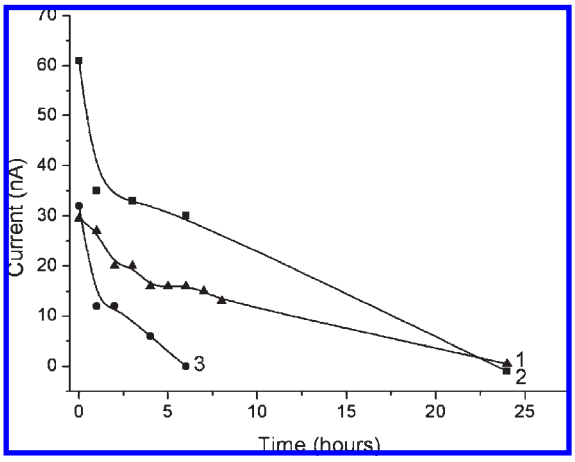

Figure 4. Evolution of the responses of amperometric biosensors based on alcohol (1), glucose (2), and lactate (3) oxidases to the mixture of the wine sample with the corresponding enzyme depending on the time of mixture incubation. Measuring conditions: 20 mM phosphate buffer at pH 7.2, at a potential of +200 mV versus the intrinsic reference electrode.

(**Figure 2**). However, the maximum concentration of ethanol and glycerol in wine samples diluted by 250 times is 14.5 mM (*2*) and 0.5 mM (*19*), respectively. That is why we can conclude that printed platinum electrodes SensLab are not sensitive to the main analytes in concentrations actually characteristic for wine and must and, thus, can be successfully used as a basis of amperometric biosensors for the analysis of wine products.

Investigation of Oxidase-Based Biosensor Selectivity. In the work presented, biosensors based on platinum electrodes SensLab and immobilized AOD, GOD, and LOD have been developed. Created devices demonstrate linear response to ethanol, glucose, and lactate within the concentration range 0.3−20 mM, 0.04−2.5 mM, and 0.008−1 mM, respectively.

Then the sensitivity of these biosensors to main components of wine has been analyzed. The responses to lactate and some components of wine obtained by the lactate biosensor with LOD immobilized by electrochemical polymerization in PEDT (**Figure 3a**) testify to good selectivity: the only nonspecific signal to ethanol was revealed for very high concentration, which never happens in wine and must. The response to the substrate is rather higher than all unspecific responses of the lactate biosensor.

In the GOD-based amperometric biosensor developed in this work for glucose measurement, the enzyme immobilization in glutaraldehyde vapor was used because we had previously determined that the biosensor with GOD immobilized in PEDT demonstrated poor selectivity (the data is not shown). The responses to glucose and other components of wine obtained by the developed biosensor with GOD in GA vapor (**Figure 3b**) illustrate high selectivity of the biosensor to its substrate over ethanol, glycerol, lactate, and ascorbic acid.

The method of immobilization in glutaraldehyde vapor also was used for ethanol biosensor creation because of the wider dynamic range of work of AOD, immobilized by this technique. The study of developed biosensor selectivity showed that it gave essential unspecific positive signals only to glycerol in concentration, which can not be present in wine (**Figure 3c**).

To investigate an influence of other components of wine (for example, aliphatic and aromatic alcohols, phenolic compounds, etc.) on the results of substrate analysis by the developed lactate, ethanol, and glucose biosensors, the following experiment was performed. First, the LOD preparation was added to the wine sample to break up lactate. Then the responses to aliquots of this

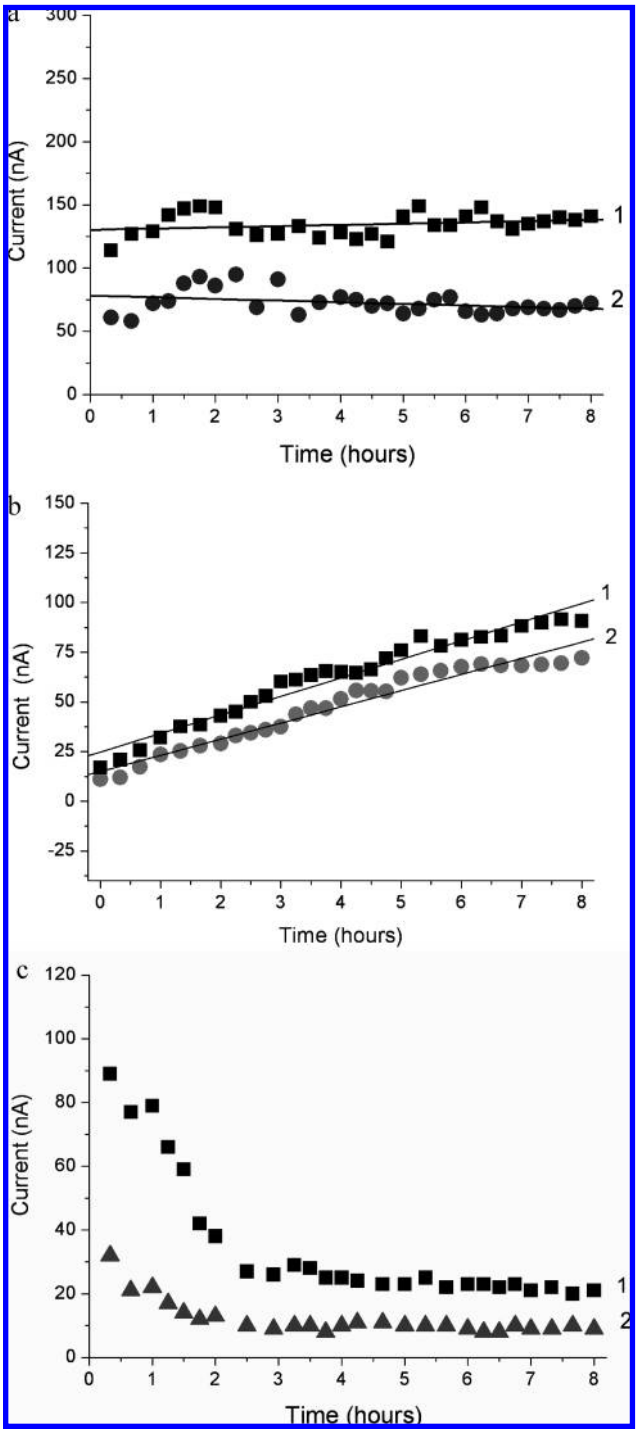

Figure 5. Investigation of operational stability of amperometric biosensors based on electrodes SensLab and glucose (**a**), alcohol (**b**), and lactate (**c**) oxidases: 1, addition of the substrate (1.3 mM, 20 mM, and 0.5 mM for biosensors based on GOD, AOD, and LOD, respectively); 2, addition of wine sample. Measuring conditions: 20 mM phosphate buffer at pH 7.2, at a potential of +200 mV versus the intrinsic reference electrode.

mixture were monitored for several hours during sample incubation with the enzyme. As was determined, no response was registered after 6 h of incubation, when lactate in the sample was fully broken up with LOD. In the analogous experiment with glucose biosensor and aliquots of the wine treated with GOD, the only response −1 nA was detected after 24 h of mixture incubation, and the signal 0.5 nA was registered after 24 h of incubation in the experiment with ethanol biosensor and wine with AOD

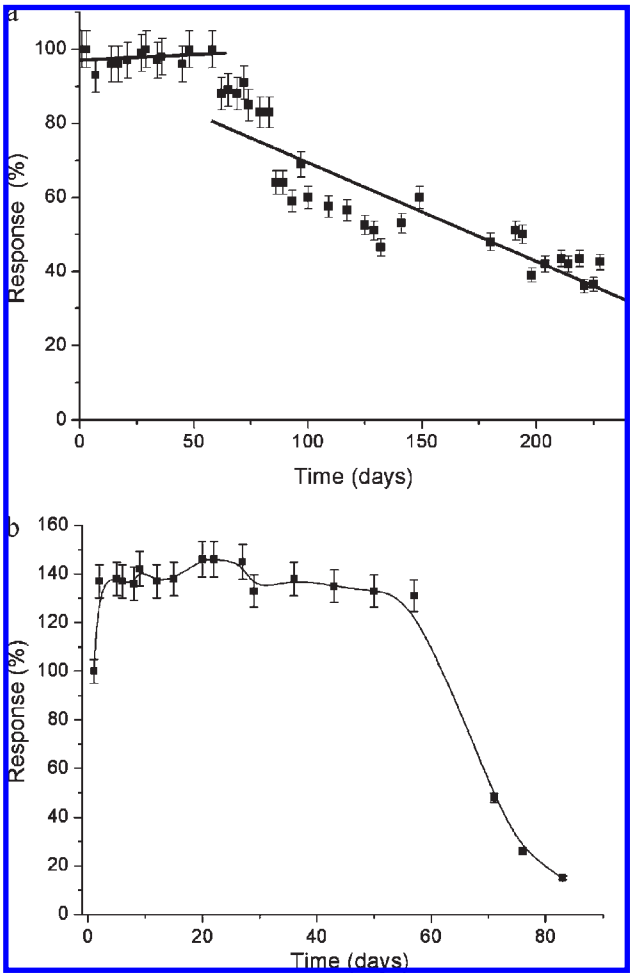

Figure 6. Investigation of storage stability of amperometric biosensors based on electrode SensLab and glucose oxidase (**a**) and alcohol (**b**) oxidase. Substrate concentration: 1.3 mM for the glucose biosensor and 20 mM for the ethanol biosensor. Measuring conditions: 20 mM phosphate buffer at pH 7.2, at a potential of +200 mV versus the intrinsic reference electrode.

preparation (**Figure 4**). These experiments confirm that we have actually developed highly specific and selective biosensors for ethanol, glucose, and lactate analysis in wine.

Investigation of Oxidase-Based Biosensor Stability. Stability is a very important characteristic of biosensors, especially in routine analysis of real samples. It is usual to separate biosensor stability into two different elements (*20*):

1. Storage stability (or shelf life), which refers to the stability of the biosensor when it is not in use (stored), is reflected by the sensor-to-sensor reproducibility over a period of time. This stability is related to certain experimental conditions of the preparation and storage of the sensor element (atmosphere and temperatures employed, humidity, etc.).
2. Operational stability, which refers to the measurement-to-measurement precision of a single biosensor (repeatability) over a period of time under continuous or intermittent monitoring, is a function of a variety of factors, the most important of which is the enzyme activity within the sensing element.

In the present research, we analyzed the created biosensors with regard to both types of stability. As can be seen from **Figure 5a**, no decrease was detected in glucose biosensor activity

Table 1. Main Working Characteristics of Developed Ethanol, Glucose, and Lactate Biosensors

enzyme	substrate	immobilization method	detection limit (mM)	linear range (mM)	remaining activity during storage	remaining activity during 8 h of continuous work, %	reproducibility, RSD % (5 measurements)	time for stable background current achievement (s)	response time (s)
alcohol oxidase	ethanol	immobilization in glutaraldehyde vapor	0.3	0.3−40	140% after 2 month, 20% after 3 month	400	4.2	200	70
glucose oxidase	glucose	immobilization in glutaraldehyde vapor	0.04	0.04−2.5	100% after 2 month, 40% after 7 month	113	2.3	160	55
lactate oxidase	lactate	electrochemical polymerization in the PEDT	0.008	0.008−1	30% after 4 days	30	5.4	350	60

Table 2. Analysis of Ethanol, Glucose, and Lactate Concentrations in Wine and Must

		ethanol concentration (% vol)		glucose concentration (g/L)		lactate concentration (g/L)	
sample	type	biosensor	WD[a]	biosensor	HPLC[b]	biosensor	HPLC[b]
Rkatsiteli "Koktebel", Ukraine	white, table, dry	12.7 ± 1.0	12.0	0.6 ± 0.1	0.29	1.62 ± 0.005	1.62
Aligote "Koktebel", Ukraine	white, table, dry	11.6 ± 1.1	11.2	0.47 ± 0.05	0.12	2.2 ± 0.15	1.98
Merlot "Koblevo", Ukraine	red, table, dry	12.1 ± 0.9	12.0	0.84 ± 0.05	0.51	1.21 ± 0.02	1.27
Cabernet "Koktebel", Ukraine	red, table, dry	9.6 ± 0.9	11.2	0.70 ± 0.05	0.32	1.04 ± 0.009	1.08
Monte Blanc "Koktebel", Ukraine	white, table, demisweet	11.2 ± 1.0	10.5	16.7 ± 0.3	16.40	1.01 ± 0.015	1.07
Porto 777 red, Ukraine	red, strong	18.8 ± 1.3	17.5	32.8 ± 0.2	34.54	0.80 ± 0.02	0.81
Porto 777 rose, Ukraine	rose, strong	18.2 ± 1.5	17.5	32.2 ± 0.2	33.24	0.63 ± 0.05	0.64
Porto white, Ukraine	white, strong	18.4 ± 1.1	17.0	31.5 ± 0.1	32.78	0.76 ± 0.02	0.69
Madera "Massandra", Ukraine	white, strong	19.6 ± 1.6	19.0	7.56 ± 0.15	7.15	0.53 ± 0.01	0.49
Kokur "Koktebel", Ukraine	white, sweet	18.1 ± 1.1	16.0	87.2 ± 0.3	87.12	0.58 ± 0.01	0.53
Kara-Dag "Koktebel", Ukraine	red, sweet	16.3 ± 0.7	16.0	85.32 ± 0.6	83.05	0.98 ± 0.01	0.98
Cahor, "Koktebel", Ukraine	red, sweet	15.9 ± 0.9	16.1	87.3 ± 0.4	87.18	0.95 ± 0.04	0.89
white must		0.8 ± 0.05		125.3 ± 0.25	124.99	0	0
red must		0.3 ± 0.03		131.3 ± 0.6	134.54	0	0

[a] Procedural error is 0.2% vol. [b] Procedural error is 2%.

after 8 h of continuous work. Moreover, the response even increased during the first working day after immobilization. This increase of biosensor sensitivity can be a result of the enzyme adaptation to the medium in the most favorable way for its function and/or of more effective contact occurring between immobilized GOD and the electrode surface. The response of AOD-based biosensor also increased during the working day, and the biosensor demonstrated more than 400% of initial activity after 8 h of continuous work (**Figure 5b**). The results of the investigation of lactate biosensor operational stability are shown in **Figure 5c**. After 3 h of continuous work, the developed lactate biosensor lost 70% of its initial signal, and during the next 5 h, its activity did not change.

The data on glucose biosensor sensitivity obtained during more than 7 months of storage (**Figure 6a**) show that nearly 100% of initial activity remained for 2 months of storage under the above mentioned conditions, and then it gradually decreased. This fact can be explained as follows. The loss in GOD activity does not provoke a decrease in the biosensor sensitivity during the first months of storage since there is the enzyme surplus in the sensitive membrane, and hence, even decreased activity of GOD is sufficient for the reaction catalysis. However, subsequent loss in GOD activity causes its decrease to the level insufficient for the substrate to be fully broken up, which causes gradual reduction in the biosensor sensitivity.

The storage stability of ethanol biosensor also was high: the device demonstrated more than 130% level of its activity during 2 months after enzyme immobilization (**Figure 6b**).

An analysis of the developed lactate biosensor storage stability showed that in one day after enzyme immobilization 30% of initial activity was revealed, and this level of the signal did not change during 4 days. It is noteworthy that 30% level of the biosensor activity is sufficient for wine analysis. Besides, reproducibility of the biosensor responses during this period was good. Therefore, the created biosensor can be used for lactate quantification in wine during at least 4 days after its preparation. The main working characteristics of developed biosensors are given in **Table 1**.

Nevertheless, the lactate biosensor is at a disadvantage by its operational stability in comparison with glucose and ethanol biosensors. Analysis of the literature data showed that biosensors on the basis of immobilized LOD are often characterized by poor stability. For example, the LOD based biosensor lost 50% of an initial signal after the first measurement (*21*) and 60% of activity after 5 h of continuous work (*22*).

Such changes in the LOD activity can originate from the enzyme thermal denaturation, proteolytic degradation, and nonspecific metal-catalyzed oxidation as well as from the changes in pH and ionic strength, and the addition of detergent (*20*). Some decline in biosensor sensitivity can also be caused by the fouling of electrode with small proteins and other components of the analyzed sample (*22*).

There are several strategies for optimization of immobilized enzyme stability: direct site-specific mutagenesis, protein engineering, chemical modification, immobilization, and medium engineering by stabilizing additives (*20*, *23*). These approaches can effectively preserve enzyme activity, though some of them (e.g., genetic and protein engineering) are time-consuming and labor-intensive. Besides, if the biosensor construction requires multistep or complicated procedures of chemical modification or

usage of stabilizing additives, it is very likely to result in a poor sensor-to-sensor reproducibility (*20*). Addition of stabilizers into the working solution makes the procedure of wine analysis more complicated and can influence the work of the biosensor. Finally, for adequate selection of the stabilization approach it is necessary to take into account all possible enzyme−stabilizer interactions and all possible alterations in the enzyme catalytic efficiency because some of the methods and additives have been shown to destabilize protein structure (*20*).

Besides, it was determined that the stability of biosensors based on some oxidases including LOD depends merely on the enzyme denaturation by hydrogen peroxide produced in the enzymatic reaction (*22*, *24*). Thus, an alternative way for solving the problem of poor stability of lactate biosensors is usage of other, but LOD, enzymes, which do not produce hydrogen peroxide. In our research, flavocytochrome b_2 (L- lactate-cytochrome *c*- oxidoreductase) was used as a biorecognition element of the lactate biosensor. Preliminary results were optimistic: the developed lactate amperometric biosensor based on flavocytochrome b_2 demonstrated good operational and long-term storage stability and could be used for lactate analysis during at least 100 days after enzyme immobilization (the data is being prepared for publication). Therefore, we are planning to continue the investigations in this field.

Analysis of Ethanol, Glucose, and Lactate Concentration in Wine and Must by Oxidase-Based Amperometric Biosensors. After investigation of selectivity and stability, the created biosensors were applied for ethanol, glucose, and lactate quantitative determination in the samples of various wines (red, white, and rose; dry, strong, and sweet) and must without previous treatment. The sample dilution was 1:250 for lactate analyses, for glucose analyses in dry wine, and for ethanol analysis in must, 1:500 for glucose analysis in wine of other types, 1:1000 for glucose analysis in must, and 1:2000 for ethanol analysis in wine (the rate of dilution was chosen empirically). The substrate concentrations were determined by the created biosensors using the standard addition method. Besides, they were also measured by traditional HPLC and wine distillation methods. Results obtained are shown in **Table 2**.

For ethanol and lactate analysis, high correlation of the results obtained by classical methods and amperometric biosensors was shown, while a remarkable difference was observed for glucose analysis in dry wines. The latter may be, since glucose concentration in dry wines is low (less than 1 g/L), i.e., close to the detection limit of both the biosensor and HPLC methods.

Experience of application of the developed biosensors for measurements in real samples showed that their sensitivity and selectivity were sufficient for analysis of various types of wine with different concentrations of ethanol, glucose, lactate, and interfering substances. The data obtained were in good agreement with the results of HPLC and wine distillation analysis. It is noteworthy, though, that measurement procedure in biosensor analysis is simpler, quicker, and cheaper.

Thus, platinum printed electrodes SensLab can be effectively used as a basis for the development of amperometric biosensors for wine analysis because of their insensibility to main interfering analytes of wine and must. The presented highly selective ethanol, glucose, and lactate biosensors could be recommended in the wine industry for monitoring and optimization of the processes of alcoholic and malolactic fermentation as well as for the control of wine quality.

ABBREVIATIONS USED

AOD, alcohol oxidase; BSA, bovine serum albumin; EDT, 3,4-ethylenedioxythiophene; GA, glutaraldehyde; GOD, glucose oxidase; HPLC, high performance liquid chromatography; LOD, lactate oxidase; PEDT, poly(3,4-ethylenedioxythiophene); WD, the method of wine distillation.

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