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Thin-film amperometric multibiosensor for simultaneous determination of lactate and glucose in wine

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ABSTRACT

An amperometric multi-biosensor based on lactate and glucose oxidases has been developed for determination of lactate and glucose in wine. Gold thin-film amperometric electrodes were used as multi-transducers. Analytical characteristics of the multi-biosensor developed were studied. The minimum detectable concentration was 5×10^{-6} mol/l for both glucose and lactate. High reproducibility and storage stability of the multi-biosensor are demonstrated in this paper. Lactate and glucose were determined in wine, and a good correlation was obtained with concentrations determined using high-performance liquid chromatography (correlation coefficient for glucose $R^2 = 0.998$, for lactate $R^2 = 0.718$).

1. Introduction

At present, analysis of such complex mixtures as wine and must in the course of fermentation is a challenge, but vital if high quality alcoholic beverages are to be obtained. The monitoring of the complex transformation of substances in grape juice and must in the process of winemaking allows producers to control different stages and, if necessary, modify them to produce beverages with superior organoleptic properties. Quantification of key wine components is also essential for verification of authenticity of the beverage (Saurina, 2010; Zeravik, Hlavacek, Lacina, & Skladal, 2009, Buglass & Caven, 2013; Monošík, Stredanský, Tkac, & Šturdík, 2012).

At all stages of production, as an element of the food industry, wine should undergo strict technical and chemical analyses aimed at determining components and their effect on the quality of the final product (Arvanitoyannis, 2010; Rodopulo, 1971; Valuiko & Kostiura, 2000). Therefore, study of the conversion of various substances at must fermentation and wine development is important for the wine industry.

Grape wine is an extremely complex chemical composition, with more than 600 organic and inorganic components. These include sugars, primarily glucose, fructose and various polysaccharides, and various acids, preferably organic, commonly malic, tartaric, lactic (lactate), succinic, and a number of volatile acids. In winemaking, it is essential to control levels of glucose and lactate. Glucose is both a source of carbon for yeasts, which perform the fermentation, and a substrate limiting yeast growth. The amount of glucose fermented determines the ethanol content, and residual

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non-fermented glucose affects the sweetness of the wine (Scholz & Ponomarev, 1990). Monitoring levels of lactate, when producing high-quality beverages is obligatory since this parameter not only determines quality and flavour, but also can be used to control fermentation. Additionally, the stability of the wine during storage depends on lactate concentration (Paz Zanini, Tulli, Martino, López de Mishima, & Borsarelli, 2013; Rodopulo, 1971). Therefore, our efforts focused on the monitoring of glucose and lactate.

Amperometric enzyme biosensors based on immobilized oxidases are a promising tool for monitoring key components of wine and must in the course of fermentation. However, it is common practice that laboratory prototypes are suitable as model solutions, but difficult to apply for real samples at the industrial scale (Apetrei & Ghasemi-Varnamkhasti, 2013; Dzyadevich & Soldatkin, 2006; Rassaei, Olthuis, Tsujimura, Sudhölter, & Van den Berg, 2014; Soldatkin, Dzyadevych, et al., 2013). Selectivity and stability of amperometric enzyme biosensors are key parameters for creating effective analytical systems intended for operation with real samples. An important task in designing efficient biosensors for wineries is testing the prototypes under real conditions of analysis and comparison of the results obtained with data from traditional methods (Monošík, Magdolen, Stredanský, & Šturdík, 2013; Monošík, Stredanský, Greif, & Šturdík, 2012).

At present, numerous biosensors have been developed for simultaneous determination of a number of analytes in food (Bachmann et al., 2000; Ge, Zhang, Zhang, & Zhang, 1998; Gutiérreza et al., 2013; Katrlík et al., 1999; Palmisano, Rizzi, Centonze, & Zambonin, 2000; Roger, Bilitewski, & Schmid 1991; Sato & Okuma, 2006; Soldatkin, Peshkova, et al., 2013), including wines (Badea, Curulli, & Palleschi 2003; Esti et al., 2004; Miertuš et al., 1998; Moser, Jobst, & Urban, 2002; Palmisano et al. 2000; Rhemrev-Boom, Jonker, Venema, Jobst, & Tiessen, 2001).

However, some limitations are observed in all the biosensors reported. These include a lack of sensitivity (Palmisano et al., 2000; Sato & Okuma, 2006, 2006) necessary for carrying out analysis in grape must (Monošík, Stredanský, Greif, et al., 2012; Monošík, Stredanský, Luspai, et al., 2012; Monošík, Stredanský, Tkac, et al., 2012). The use of dehydrogenases in biosensors requires the presence of a cofactor, often nicotinamide adenine dinucleotide (NAD+), which complicates the procedure of analysis and increases cost (Esti et al., 2004; Katrlík et al., 1999; Miertuš et al., 1998; Monošík, Stredanský, Luspai, Magdolend, & Šturdík, 2012; Roger et al., 1991).

Application of lactate oxidase (LOx) and glucose oxidase (GOx), proposed by the authors, could provide greater stability and accuracy for biosensor measurements because the enzymatic reactions do not require an exogenous cofactor, and the number of analytical reactions necessary to obtain a response is lower. Therefore, the aim of this work was to develop an amperometric multibiosensor, based on lactate and glucose oxidases immobilized on the surface of gold multi-transducer, which can be used for simultaneous determination of lactate and glucose in quality control of wine and raw materials.

2. Experimental

2.1. Reagents and solutions

Lactate oxidase from Pediococcus species with activity of 20 U/mg solid was purchased from Sigma-Aldrich Co. (Steinheim, Germany); 98% sodium salt of L-lactic acid, bovine serum albumin (BSA) and 25% aqueous solution of glutaraldehyde (GA) – from Sigma-Aldrich Co. (St. Louis, USA). Glucose oxidase from Aspergillus niger with activity of 271 U/mg solid was purchased from Genzyme Corporation (Cambridge, USA).

99% ethanol was purchased from Sigma-Aldrich Co. (Steinheim, Germany); D-glucose, $Na_2HPO_4{\cdot}7H_2O$ and $KH_2PO_4{\cdot}H_2O$ were from Helicon (Moscow, Russia); 3% solution of hydrogen peroxide was from Fargomed Ltd. (Teteriv, Kiev region, Ukraine); $Na_2SO_4{\cdot}10H_2O$ from Mikhailovsky chemical reagent factory (Malinovoe Ozero, Altai Region, Russia). All chemicals were of analytical grade.

2.2. Instruments

The gold multi-electrodes on a glass ceramic substrate were manufactured by photolithography technique at V. Lashkaryov Institute of Semiconductor Physics of National Academy of Sciences (Kyiv, Ukraine). The working electrode diameter was 0.8 mm. The multi-transducers were of two types – with in-line or in-circle placed working surfaces. A general view of amperometric multi-transducers is presented in Fig. S1 (see in a Supplementary file).

Amperometric measurements were carried out in a 5 ml electrochemical cell at a constant potential using a potentiostat/galvanostat PalmSens and a multichannel multiplexer controlled by the PalmSens PC programme (Palm Instruments BV, CITY, Netherlands).

2.3. Determination of sensors electrochemical characteristics

Amperometric multi-transducers were studied with regard to their reproducibility and reliability by cyclic voltamperometry in the potential range of 0 to +1.0 V (speed of potential involute was 0.05 V/s). The experiment was carried out in 0.1 mol/l phosphate buffer, pH 7.2.

2.4. Immobilisation of lactate oxidase, glucose oxidase and BSA in glutaraldehyde vapour

Prior to immobilization, for improvement of adhesion, the surface of the working electrode was treated with an acid chromic mixture (saturated solution of potassium dichromate in concentrated sulphuric acid) before being thoroughly washed, and dried with ethanol.

In the electrochemical biosensors, enzymes were embedded into the composition of albumin gel using the bi-functional agent GA. This is a chemical method of immobilization, where covalent bounds form at the interaction between GA carbonyl groups and amino groups from lysine in the enzyme protein. Bovine serum albumin (BSA) has no enzymatic activity, but increases the membrane stability due to the presence of auxiliary groups for binding with GA. This immobilization method produces a three-dimensional matrix, in which the enzyme is trapped in close association with the electrode, improving both retention of the biomolecule on the electrode surface and its electrical communication (Sanz, Mena, González-Cortés, Yáñez-Sedeño, & Pingarrón, 2005; Zinchenko et al., 2012).

To create the bioselective membrane, 10% BSA solution was mixed with 10% enzyme (in case of the reference membrane – 10% BSA) at 1:1 ratio in 0.01 mol/l phosphate buffer, pH 7.2. Glycerol (10%) was added to the mixture to aid stabilization and prevent drying out of the solution on the transducer surface. The enzyme-BSA mixture was deposited, using a pipette, onto the working surface of the electrode. For membrane polymerisation, the transducers were placed in a crystallizer in an atmosphere of saturated glutaraldehyde vapour at room temperature for 10 min, and subsequently dried in the air for 10 min.

2.5. Substrate determination in model solutions

Measurements were performed in 0.1 mol/l phosphate buffer, pH 7.2, at room temperature in an open vessel with intensive stirring. Before operation, the transducers were kept in the solution until a stable signal (baseline) was obtained. The substrate concentration was varied by the addition of aliquots of stock solutions.

2.6. Substrate determination in wine by high-performance liquid chromatography (HPLC)

The substrates were measured in five samples of different wines produced at the National Institute of Wine and Grapes "Magarach" and at the Magarach Agricultural Company (Crimea, Ukraine). When preparing the samples of wine and must for HPLC analysis, samples were passed through a nylon filter (pore size 0.45 μm).

Lactate and glucose were determined using a Shimadzu chromatograph, model LC20 Prominence equipped with the refractometric detector. The analysis was performed on a HPLC column Phenomenex Luna C18, 2.1 × 150 mm in dimension, filled with the sorbent with graft octadecyl phase (double endcamping), grain size of 3.0 μm. The mobile phase was 78% acetonitrile aqueous solution. The flow rate was 1 mL/min and the sample injection volume was 20 μL. The peaks were identified based on glucose and lactate standard samples. The mass concentration of lactate was calculated based on the values of the peak areas using the calibration characteristic.

2.7. Substrate determination in wine by amperometric multi-biosensor

Measurements were carried out in 0.1 mol/l buffer solution, pH 7.6, at room temperature. To obtain the calibration curve, aliquots of the substrate stock solution were added to the electrochemical cell (which corresponds to 5×10^{-6}–5×10^{-3} mol/l). Afterwards, an aliquot (100 μL) of the wines to be tested was inserted into the cell. Once the response was obtained (the response time about 1 min), the calibration curve was plotted, and lactate and glucose concentrations were determined. After each response, the sensor was washed with the buffer solution until the base signal stabilized.

The experiments were repeated at least three times. Statistical package Microsoft Origin 10 was used for statistical analysis of the results, and average value with standard deviation were calculated; the results were considered as reliable at $p < 0.05$.

3. Results and discussion

3.1. Principle of lactate and glucose determination by multi-sensor

Amperometry is an electrochemical methods of analysis in which the potential applied to the measuring cell is kept constant whereas the current in it depends on the concentration of electroactive particles in the cell, time, etc. Significant progress observed in the development of amperometric microelectrodes is the result of novel achievements in the field of micromechanics, and improved sensitivity and quality of the modern related equipment.

The operation of amperometric biosensors is based on enzymatic reactions occurring when applying the necessary potential results in generation of electrodes, which can be directly registered by the amperometric transducer. In the presence of oxygen (a cosubstrate of the enzymatic reaction), glucose and lactate are oxidised with the formation of electrochemically active hydrogen peroxide.

The enzymatic reactions underlying operation of the multi-biosensor proposed are as follows:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconolactone} + H_2O_2; \quad (1)$$

$$\text{L-Lactate} + O_2 \xrightarrow{\text{LOx}} \text{Pyruvate} + H_2O_2. \quad (2)$$

$$H_2O_2 \rightarrow 2H^+ + 2e^-; \quad (3)$$

Thus, the amperometric multi-sensor can be used for conversion of biochemical signal into electrical one and measurement of the response to addition of both substrates.

3.2. Electrochemical characteristics of amperometric transducers

To ensure reproducibility in amperometry, previous investigation of electrochemical characteristics of transducers are highly important. For this purpose, cyclic voltamperometry was used.

Gold thin-film multi-electrodes were tested for reproducibility and reliability in the potential range 0 to +1 V (see Fig. S2 in a Supplementary file) (the scan rate was 0.05 V/s). The experiment was carried out in 0.1 mol/l phosphate buffer, pH 7.2. The current-voltage curves obtained were the same shape as electrodes of this type in Biloivan, Rogaleva, and Korpan (2010).

At the next stage, the sensitivity of amperometric transducers towards hydrogen peroxide was tested (Fig. 1).

As seen in Fig. 1, the amperometric gold multi-electrodes developed were highly sensitive to hydrogen peroxide, and could be used to create a multi-biosensor to control the quality of wine.

3.3. Immobilization of glucose and lactate oxidases in glutaraldehyde vapour

The choice of the method for enzyme immobilization onto the surface of the amperometric transducer and its optimisation are vital in the biosensor development.

First, the dependence of biosensor sensitivity to glucose and lactate on duration of the electrode exposure to glutaraldehyde vapour was studied. The dependence of biosensor response on the time of immobilization is shown in Fig. S3 (see in a Supplementary file).

To obtain a bioselective membrane, the enzyme-BSA mixture was deposited onto the transducer surface; for membrane polymerisation, the sensor was placed in an atmosphere of saturated

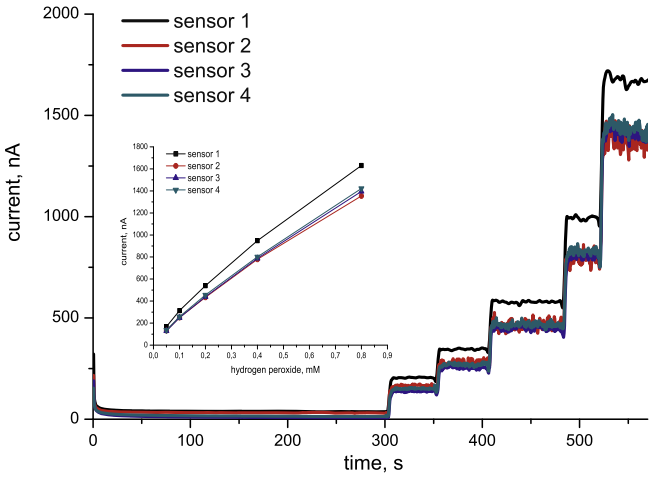

Fig. 1. Dependence of current of amperometric multitransducer on concentration of hydrogen peroxide. Measurements were carried out in 0.1 mol/l phosphate buffer, pH 7.2, at potential of +0.75 V versus intrinsic reference electrode.

glutaraldehyde vapour, before being dried in the air. All measurements were carried out in 100 mM phosphate buffer, pH 7.2.

As seen in Fig. S3 (see in a Supplementary file), the maximal response to both glucose and lactate was after 10 minutes exposure of the transducer to GA vapour. Therefore, in further experiments, this immobilization procedure was used for the formation of working membranes.

3.4. Investigation of selectivity of the developed multi-biosensor

In the investigation the amperometric multi-sensor selectivity, Fig. 2 shows the lactate oxidase-based membrane did not respond to major components of wine, including glucose and ethanol, and the glucose oxidase-based membrane did not respond to lactate and ethanol; the membrane with BSA did not respond to any of these components.

3.5. Investigation of dependence of biosensor response on concentrations of background electrolyte and buffer solution

Since the biosensor working characteristics strongly depend on the experimental conditions, it was important to examine an effect of the buffer capacity and ionic strength. This is especially significant because ionic strength and buffer capacity of physiological and culture fluids as well as foods are very high, which complicates application of conductometric and potentiometric biosensors (the signal drops by almost 100 times) (Arkhipova, Dzyadevich, Soldatkin, & Elskaya, 1996). Dependence of the amperometric biosensor response to buffer ionic strength and concentration is shown in Fig. 3.

It should be noted that salt Na_2SO_4 was used in the experiments as it contains the inorganic cations and anions common for wines (Goriushkina & Dzyadevych, 2008).

As shown, the buffer ionic strength and capacity had a slight effect on the response of the amperometric multi-biosensor developed but the sensor could be used to analyse biological fluids.

3.6. pH effect on multi-biosensor operation

It is known that the speed of enzymatic reactions in homogenous solutions strongly depends on pH value. This is because the protein functional groups capable of protonation/deprotonation (depending on the solution pH) take part in the catalysis whereas only one of these forms is reactive. (Nikolelis, Varzakas, Arzum, & Nikoleli, 2014; Varzakas, Nikoleli, Nikolelis, & Tzamtzis, 2012;

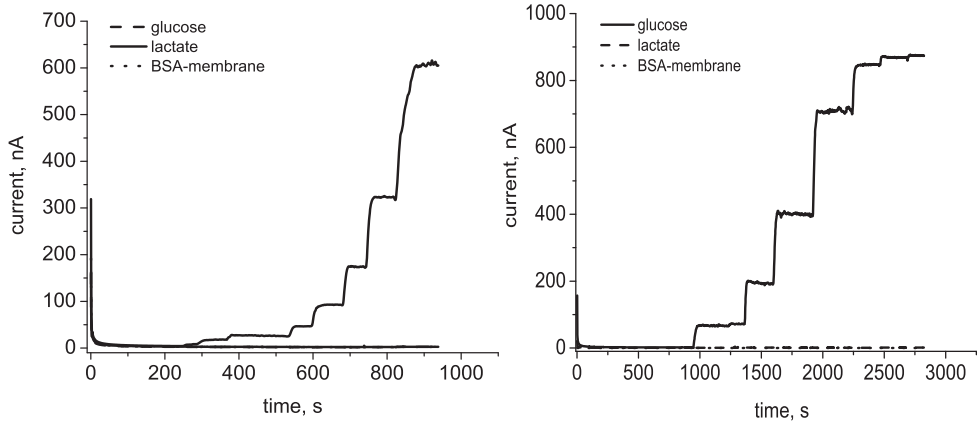

Fig. 2. Selectivity of amperometric multisensor based on immobilized oxidases. Measurements were carried out in 0.1 mol/l phosphate buffer, pH 7.2, at potential of +0.75 V versus intrinsic reference electrode.

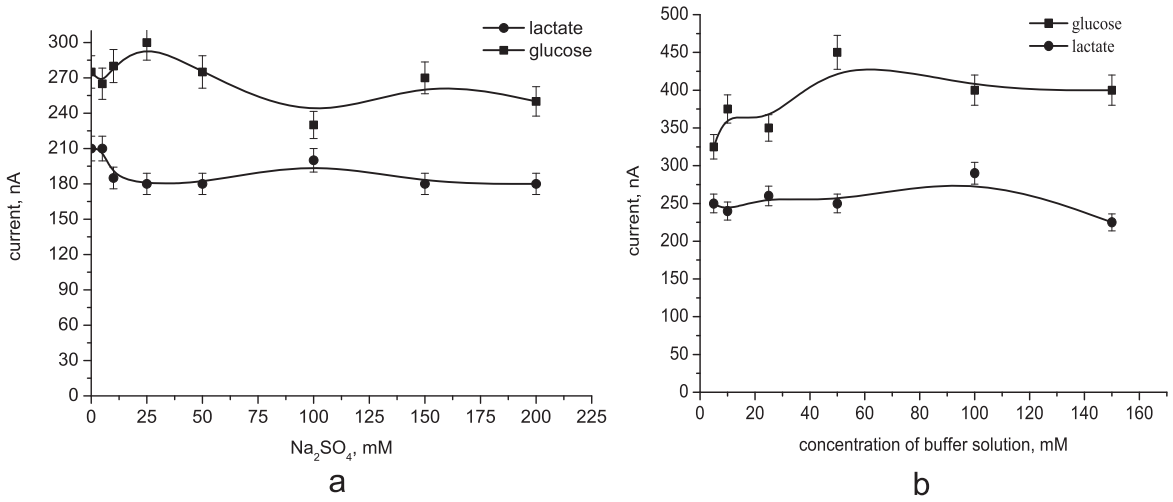

Fig. 3. Dependence of response of amperometric multibiosensor on concentration (a) of background electrolyte in 0.1 mol/l phosphate buffer, pH 7.2; (b) of buffer solution, pH 7.2, at potential of +0.75 V versus intrinsic reference electrode. Substrate concentration is 5×10^{-4} mol/l.

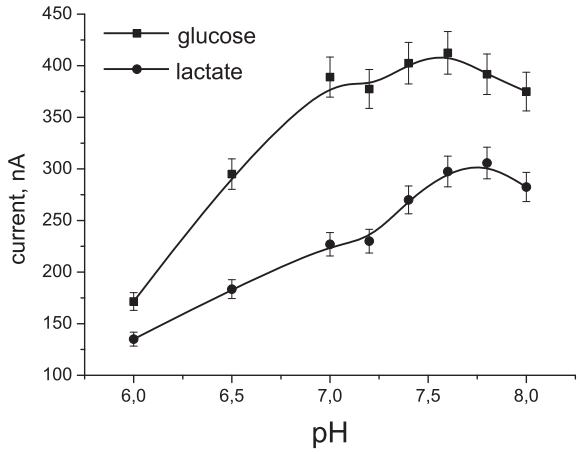

Fig. 4. Dependence of biosensor response on pH. Measurements were carried out in 0.1 mol/l phosphate buffer, at potential of +0.75 V versus intrinsic reference electrode. Substrate concentration is 5×10^{-4} mol/l.

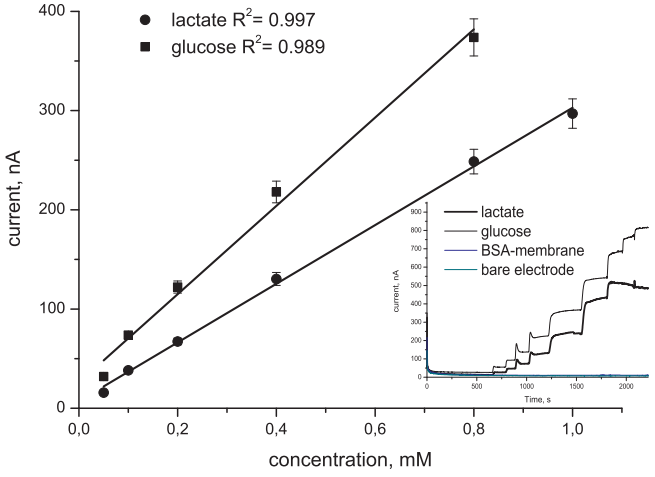

Fig. 5. Calibration curve of amperometric multibiosensor. Measurements were carried out in 0.1 mol/l phosphate buffer, pH 7.6, at potential of +0.75 V versus intrinsic reference electrode.

Varzakas, Nikoleli, Tzamtzis, & Nikolelis, 2014). Fig. 4 shows the optimum working pH of the amperometric multi-biosensor with immobilized GOx was 7.4–7.6 and with immobilized LOx 7.6–7.8. Therefore, pH 7.6 was taken as the optimal value for the multi-biosensor operation. Notably, the optimal pH of immobilized enzyme shifts to more alkaline values as compared with the optimal pH of a native enzyme (for GOx and LOx – 6.5).

3.7. Multi-biosensor stability

The research on multi-biosensor stability showed that after 85-day operation the sensor retained 20% of its activity towards LOx and 65% activity towards GOx, which ensures the reliability of measurements (see Fig. S4 in a Supplementary file).

The calibration curve of the multi-biosensor developed for determination of glucose and lactate (Fig. 5) was obtained under optimal working conditions.

Fig. 5 displays the calibration plots for glucose in the range of 5×10^{-6}–8×10^{-4} mol/l with linearity of $R^2 = 0.989$ and sensitivity of 58.4 μA $mmol^{-1}$ l cm^{-2}, and for lactate in the range of 5×10^{-6}–1×10^{-3} mol/l with linearity of $R^2 = 0.998$ and sensitivity of 37.1 μA $mmol^{-1}$ l cm^{-2}.

The time for the biosensor response ranged on the scale of seconds, which is much faster than other methods, in particular, chromatography. For the biosensor developed, the response time was 5–15 s.

3.8. Analysis of lactate and glucose in wine by the developed amperometric multi-biosensor

Lactate and glucose were determined in five samples of wines produced at the National Institute of Wine and Grapes "Magarach" and at the Magarach Agricultural Company (Crimea, Ukraine):

"Fetyaska" "Golitsyn wines" (dry white table wine).
"Cabernet" "Golitsyn wines" (dry red table wine).
"Marsala" Massandra Winery (strong white wine).
"Madera" Massandra Winery (strong red wine).
"Pinot-Franc" Koktebel Winery (strong red table wine).

The samples were analysed using the amperometric multi-biosensor developed with immobilized lactate and glucose oxidases. Measurements were carried out in 0.1 mol/l phosphate buffer, pH 7.6. Time for the enzyme immobilisation in GA vapour was 10 min. The same components were also measured by HPLC method.

The results of analysis of lactate and glucose in wine samples tested are shown in Table 1. These were in good agreement with data from HPLC analysis (the correlation coefficient for glucose $R^2 = 0.998$, for lactate $R^2 = 0.718$).

4. Conclusion

Conditions for immobilization of lactate oxidase and glucose oxidase, for the formation of active membranes on the surface of gold multi-transducers, were optimised. Analytical characteristics of the multi-sensor developed, based on gold thin-film amperometric electrodes, were studied; a slight effect of environmental conditions on the multi-sensor activity was found. Reproducibility of the results and storage stability were reliable. Correlation of data obtained using the amperometric multi-biosensor developed with

Table 1
Comparative analysis of glucose and lactate in wines.

Sample	Biosensor		HPLC	
	Glucose, (±0.1) g L^{-1}	Lactate, (±0.1) g L^{-1}	Glucose, (±0.1) g L^{-1}	Lactate, (±0.1) g L^{-1}
Fetyaska	0.44	0.16	0.35	0.22
Cabernet	0.46	0.41	0.44	0.43
Madera	8.7	0.18	11.27	0.68
Marsala	19.3	0.89	28.62	1.26
Pinot-Franc	2.72	0.55	2.79	0.33

the results from HPLC analysis was strong. Thus, the amperometric multi-biosensor developed could be used for simultaneous measurement of lactate and glucose in the food industry to control wine quality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.11.066.

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