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Multienzymatic amperometric biosensor based on gold and nanocomposite planar electrodes for glycerol determination in wine

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ABSTRACT

Amperometric biosensors based on gold planar or nanocomposite electrode containing multiwalled carbon nanotubes for determination of glycerol were developed. The biosensors were constructed by immobilization of a novel multienzyme cascade consisting of glycerol kinase/creatine kinase/creatinase/sarcosine oxidase/peroxidase between a chitosan "sandwich." A measuring buffer contained adenosine 5′-triphosphate (ATP), creatine phosphate, and an artificial electrochemical mediator ferrocyanide. The currents proportional to glycerol concentration were measured at working potential of −50 mV against Ag/AgCl reference electrode. The biosensors showed linearity over the ranges of 5–640 μM and 5–566 μM with detection limits of 1.96 and 2.24 μM and sensitivities of 0.80 and 0.81 nA μM^{-1}, respectively. Both types of biosensors had a response time of 70 s. The biosensors demonstrated satisfactory operational stability (no loss of sensitivity after 90 consecutive measurements) and excellent storage stability (90% of the initial sensitivity after 15 months of storage at room temperature). The results obtained from measurements of wines correlated well with those obtained with an enzymatic–spectrophotometric assay. The presented multienzyme cascade can be used also for determination of triglycerides or various kinase substrates when glycerol kinase is replaced by other kinases.

Simple polyol compound glycerol ($C_3H_8O_3$, 1,2,3-propanetriol) can be involved as a product or substrate in bioprocesses [1], with many uses in the cosmetic, paint, automotive, food, tobacco, pharmaceutical, paper, leather, and textile industries or as a feedstock for the production of various chemicals [2], and its final concentration depends on the type of process. Its level is currently measured in many foodstuffs such a wine, beer, vegetable oil, honey, and vegetable oil. Forming in wine by yeasts during the alcoholic fermentation, glycerol contributes to viscosity and sensory quality. The level of glycerol formed during the fermentation process is approximately a 1:10 ratio of the ethanol formed, and the final concentration is usually in the range from 4 to 10 g L^{-1} [3]. It can also be considered as a fermentation indicator, and its production is related to the presence of microorganisms in food products [4]. Thus, monitoring of glycerol during the manufacturing process can help to prevent undesired metabolic changes affecting the final quality of products. Moreover, the glycerol level is also of interest to detect possible adulteration of wine with added glycerol [5], and yet the presence of glycerol in different foods causes great interest in its analysis. In addition, glycerol determination is needed in clinical diagnosis for control of the triacylglyceride level in blood samples after their hydrolysis by lipase. This is valuable in the case of monitoring risks of diseases of the cardiovascular system [6]. Nowadays, several methods can be used for determination of glycerol, including high-performance liquid chromatography (HPLC)[1] [7], gas chromatography [8], high-performance thin layer chromatography [9], flow injection analysis [10], and spectrophotometry [11]. However, these methods require expensive laboratory equipment and chemicals, sample pretreatment, and educated personnel; in addition, analyses are time-consuming. Amperometric biosensors can represent an alternative method to overcome the disadvantages mentioned above [12]. They are usually characterized by rapid response, high selectivity, and lower costs for analysis, and they provide an option to perform analysis in situ thanks to their ability to be miniaturized. Various enzymatic compositions were used for the construction of glycerol amperometric biosensors, including pyrroloquinoline quinone (PQQ)-dependent glycerol dehydrogenase [13], glycerol dehydrogenase/diaphorase, glycerol kinase/glycerol-3-phosphate oxidase/peroxidase [14,15] with electrochemical

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; PQQ, pyrroloquinoline quinone; GPE, gold planar electrode; GK, glycerol kinase; SAOX, sarcosine oxidase; CRE, creatinase; HRP, peroxidase; CK, creatine kinase; ATP, adenosine 5′-triphosphate; MWCNT, multiwalled carbon nanotube; PBS, phosphate buffer solution; S/N, signal-to-noise ratio; RSD, relative standard deviation.

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detection of the oxidation of the corresponding mediator, and glycerol oxidase [16] with direct detection of H_2O_2. Nevertheless, the last-mentioned enzyme is not commercially available. In the case of using glycerol dehydrogenase, the oxidation of glycerol is a reversible reaction, and selectivity of the enzyme is insufficient. An interesting enzymatic cascade, glycerol kinase/pyruvate kinase/pyruvate oxidase, was introduced by Mizutani and coworkers [17], although the biosensor suffers from a very low stability of pyruvate oxidase that similarly requires an addition of the expensive cofactors FAD (flavin adenine dinucleotide) and TPP (thiamine pyrophosphate) into the measuring buffer.

In this article, we present a glycerol biosensor using a novel multienzyme cascade [18] consisting of glycerol kinase/creatine kinase/creatinase/sarcosine oxidase/peroxidase immobilized onto gold planar electrode (GPE) or nanocomposite containing multiwalled carbon nanotubes and discuss its use for the detection of glycerol in wines. These enzymes are available and stable. The aim of this work was to develop a glycerol biosensor that can be used for analyses of real food samples and be compatible with the commercially available portable analytical device Omnilab W currently serving wine makers as an alternative to classic analytical methods [19].

Materials and methods

Reagents and chemicals

Glycerol kinase (GK, 103 U mg^{-1} solid), sarcosine oxidase (SAOX, 12.9 U mg^{-1} solid), creatinase (CRE, 8.73 U mg^{-1} solid), and peroxidase (HRP, 132 U mg^{-1} solid) were purchased from Sorachim (Lausanne, Switzerland), and creatine kinase (CK, 23.3 U mg^{-1}) was purchased from USB (Cleveland, OH, USA). GK (103 U mg^{-1} solid) and L-α-glycerolphosphate oxidase (51 U mg^{-1} solid) were obtained from Asahi Kasei (Tokyo, Japan). Potassium hexacyanoferrate(II), *N*-eicosane, D-glucose, D-fructose, sodium-L-lactate, L-ascorbic acid, L-malate disodium salt, sodium acetate, *o*-dianisidine, sulfuric acid (95–97%), hydrochloric acid (36.5–38%), anhydrous ethanol, magnesium sulfate heptahydrate, glycerol, adenosine 5′-triphosphate (ATP), creatine phosphate, and chitosan from shrimp shells (85% deacetylated) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Riedel-de Haen (Seelze, Germany). Water deionized by a Millipore Milli-Q purification system was employed. All chemicals used were of analytical grade. Multiwalled carbon nanotubes (MWCNTs, diameter = 60–100 nm, length = 5–15 μm, ⩾95% purity) were obtained from NanoAmor (Houston, TX, USA). GPEs (diameter = 1.6 mm) equipped with Ag/AgCl reference electrode (diameter = 2 mm, screen-printed) deposited on the planar glass–epoxy–laminate substrate were obtained from Biorealis (Bratislava, Slovakia).

Apparatus

Electrochemical studies were performed with potentiostat model 559 from Amel (Milan, Italy), potentiostat Heka PG-284 (Lambrecht, Germany), and electrochemical analyzer Omnilab from Biorealis.

Preparation of nanocomposites

The procedure described previously by Miertus and coworkers for graphite composite electrodes was adapted for the nanocomposite fabrication [20]. First, 80 mg of *N*-eicosane were melted at a temperature of 45 °C. After this, 8 mg of MWCNTs was added and the mixture was stirred vigorously with a spatula until a homogeneous composition was obtained. The suspension was subsequently transferred and spread on the conductive surface layer (diameter ≈ 1.6 mm), forming the working electrode on the planar basic sensor equipped with Ag/AgCl reference electrode. Finally, the layer of the nanocomposite was left to solidify, and the surface was smoothed on a sheet of paper.

Preparation of biosensors

The gold planar working and reference electrodes were carefully cleaned with Milli-Q water and ethanol. All enzymes were dissolved in Milli-Q water before the procedure. The immobilization of the enzymes on the surface of the working electrodes was carried out by their "sandwiching" between (1%, w/w) chitosan layers. Each layer was deposited after the previous one was dried. The prepared biosensors were stored at room temperature in a dessicator before use. Details of the quantities of enzymes are given in "Results and Discussion".

Enzymatic–spectrophotometric assay

The determination was performed with a DR 2800 portable spectrophotometer obtained from Hach Lange (Düsseldorf, Germany) using a homemade kit. Briefly, glycerol is phosphorylated to glycerol phosphate by GK. Glycerol phosphate reacts with oxygen in the presence of L-α-glycerolphosphate oxidase to form glycerone phosphate and hydrogen peroxide. Hydrogen peroxide reacts with *o*-dianisidine in the presence of HRP to form a colored product. Oxidized *o*-dianisidine reacts with sulfuric acid, creating a more stable pink-colored product that can be measured at a wavelength of 540 nm on a spectrophotometer.

First, the samples were diluted to approximately 20–30 μg ml^{-1}. Then 974.5 μl of 0.1 M phosphate buffer solution (PBS, pH 7.5) and 0.02 ml of *o*-dianisidine solution (4.5 mg ml^{-1} solution in 0.02 M HCl) was pipetted in a spectrophotometer cuvette with the addition of 1 mg of ATP and 3 mg of $MgSO_4 \cdot 7H_2O$. Then 10 U of GK, 7.5 U of L-α-glycerolphosphate oxidase, and 6 U of HRP were added. The mixture was gently shaken, and the reaction was started by adding 0.5 ml of standard solutions for calibration curve or samples for analysis. Mixtures were incubated for 30 min at 37 °C. Afterward, the reaction was stopped by adding 1.0 ml of 6 M H_2SO_4 into each cuvette. Then the developed color was measured at 540 nm using as blank a solution containing all of the components except glycerol.

Results and discussion

The presented biosensors for the determination of glycerol are based on the reaction shown in Fig. 1. In the final step, the formed ferricyanide is reduced to ferrocyanide on the electrode surface at applied working potential versus Ag/AgCl reference electrode, and the resulting current proportional to the analyte concentration is measured.

Optimization of working conditions

The amounts of enzymes on the electrode were optimized from 1 to 3.5 U for GK, from 0.1 to 0.3 for CK, from 0.1 to 0.5 for SAOX, from 0.3 to 1.5 for CRE, and from 1 to 4 U for HRP. Higher enzyme loadings led to the current decrease, which is likely due to the blocking of the electrode surface by the large amount of immobilized protein. On the other hand, lower enzyme quantities led to the decrease of biosensor sensitivities and narrow linear ranges. Finally, the optimal amounts of 2.5 U of GK, 0.15 U of CK, and 1 U of HRP were found for the immobilization on the working

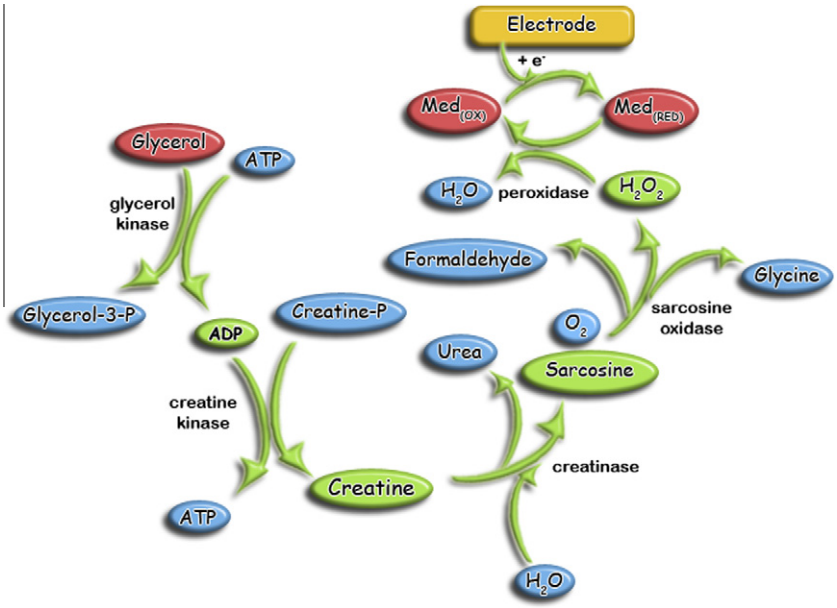

Fig.1. Schematic presentation of novel enzymatic composition immobilized between chitosan layers used for biosensor determination of glycerol. Electric current resulting from the reduction of $Med_{(OX)}$ (ferricyanide) is measured at working potential of −50 mV versus Ag/AgCl reference electrode.

electrode. These loadings were consequently used for all biosensors in further experiments. The suitable concentrations of the mediator in the working medium were studied in the range from 1 to 10 mM ferrocyanide. The highest biosensor responses were obtained at 5 mM ferrocyanide. This concentration was applied in the next experiments. Creatine phosphate, ATP, and magnesium sulfate heptahydrate were used at concentrations of 5, 4, and 15 mM, respectively.

Once the optimal composition of enzymes and the measuring solution were established, the effect of applied working potential was studied in the range of 0 to −300 mV against Ag/AgCl reference electrode. As shown in Fig. 1, during enzymatic reaction H_2O_2 is produced and can be directly detected. However, direct detection of H_2O_2 requires higher working potential (e.g., +600 mV [21]), and this is a serious disadvantage in measuring real samples due to possible interference with electroactive compounds such as polyphenols, which can be oxidized on the electrode, giving a false current response. Whereas our biosensors measure electric current resulting from the reduction of ferricyanide, lower values of working potential can be used. No significant changes in current response were detected for using both the gold and nanocomposite electrodes; hence, we selected the value of −50 mV for further experiments. This potential allows satisfactory sensitivity and simultaneously avoids undesired interferences, as was proved in further experiments.

The two-electrode arrangement was used for electrochemical measurements because the currents produced by bioelectrodes were relatively low. When the three-electrode arrangement (with the added platinum auxiliary electrode) was preliminarily tested, the measured currents were very similar.

Finding the suitable pH for a multienzymatic biosensor is very important for its good functionality because the optimal pH for single enzymes varies; therefore, it is necessary to find a suitable compromise reflecting the pH optimum of all employed enzymes. The pH dependence of the PBS on the amperometric response of the biosensor after the addition of glycerol is shown in Fig. 2. The biosensor was subject to 10 mM glycerol standard solution. The results showed that at the pH values from 6.0, the measured current increased until it reached its maximum at the pH value of 7.5. As the pH rose over 7.5, the response significantly decreased.

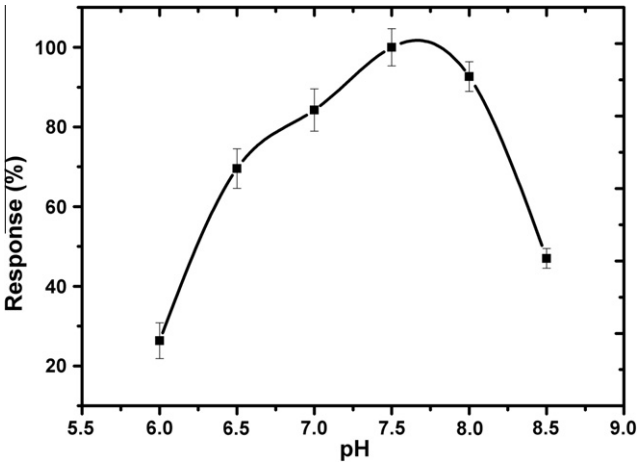

Fig.2. Effect of pH on the response of glycerol biosensor (response at pH 7.5 = 100%). Experimental conditions: 5 mM ferrocyanide and 100 μM glycerol in Milli-Q water, applied potential −50 mV versus Ag/AgCl.

Based on these results, the pH value of 7.5 was consequently used in further experiments.

Analytical characteristics

The current response of the GPE- and nanocomposite-based biosensors to glycerol was investigated under stirring in 0.1 M PBS (pH 7.5) containing the cofactors and ferrocyanide. Fig. 3 illustrates the amperometric responses of both enzyme-modified electrodes at −50 mV. Both types of biosensors exhibited linear amperometric response to glycerol. The dependence of current on the glycerol concentrations gave a straight line in the range from 5 to 640 μM for the GPE-based biosensor and from 5 to 566 μM for the nanocomposite-based one. The correlation coefficients of the GPE and nanocomposite electrodes were 0.9982 (n = 13) and 0.9986 (n = 12), respectively. These linear ranges show improvement compared with the biosensor proposed by Rejeb and coworkers [22] based on glycerol dehydrogenase/NADH oxidase.

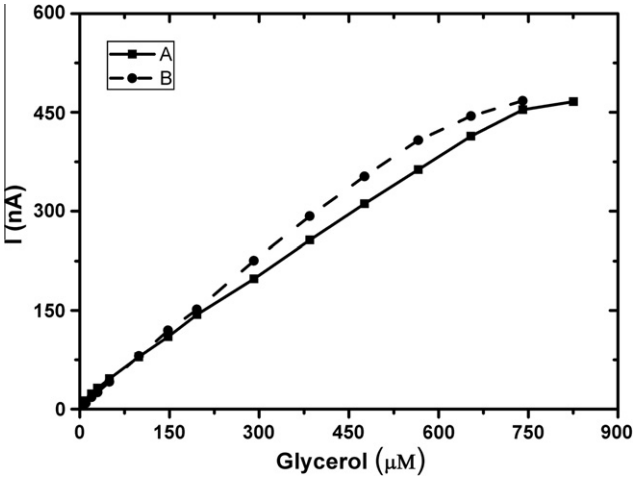

Fig.3. Calibration curve obtained for glycerol biosensors based on the GPE (curve A) and nanocomposite-based electrode (curve B). Experimental conditions: 5 mM ferrocyanide and 0.1 M phosphate buffer (pH 7.5), applied potential −50 mV versus Ag/AgCl. Substrate: 10 mM glycerol in Milli-Q water.

Modified electrode surface with Prussian blue allowed detection of produced H_2O_2 at −50 mV, and the achieved linear range was 1–50 μM with a detection limit of 0.7 μM. In the work of Niculescu and coworkers [23], a biosensor using PQQ–glycerol dehydrogenase showed a linear range of 1–200 μM with a detection limit of 1 μM. On the other hand, better performance was shown by a biosensor developed by Eftekhari [24]. In this case, glycerol dehydrogenase was immobilized into the polyaniline film and the linear response varied in the range from 50 to 2000 μM with a detection limit of 1 μM. Detection limits in all cases were calculated as three times the signal-to-noise ratio (S/N). In our case, the GPE-based biosensor showed a detection limit of 1.96 μM (S/N = 3) with sensitivity of 0.80 nA μM^{-1}. Interestingly, nearly the same analytical performance was achieved with a nanobiocomposite-based biosensor with a detection limit of 2.24 μM and sensitivity of 0.81 nA μM^{-1}. The response of both glycerol biosensors was relatively fast with regard to their multienzymatic character, with the current reaching the steady state at 70 s. This is similar to biosensors prepared by Eftekhari [24], with response times of 60 and 80 s for the monolayer and bilayer electrodes, respectively.

The presented results suggest that analytical characteristics were not improved by using carbon nanotubes as a component for the construction of novel electrode surface; however, they can still be used as an alternative for more expensive gold surface without any loss in quality of analytical performance. Further improvements of nanocomposite performance could be enhanced by using dispersion agents [25] and/or other types of nanoparticles and by means of incorporating mediator or enzymes in a nanocomposite.

Reproducibility of measurement and fabrication

The reproducibility of measurements of the same biosensor was studied as a current response on additions of 100 μM glycerol (at working potential of −50 mV vs. Ag/AgCl). The average response for the GPE-based biosensor was 54.08 nA (n = 8, relative standard deviation [RSD] = 5.67%). Results for biosensors using nanocomposite were 64.44 nA (n = 8, RSD = 7.08%). Another important factor is the reproducibility of biosensor production. The average current response of three biosensors for the addition of 100 μM glycerol was 56.39 nA (n = 3, RSD = 8.97%) for a GPE-based biosensor. In the case of preparation of nanocomposite biosensors, the same batch of the composition mixture for each variant was used. The average current response for the same concentration of glycerol was 67.34 nA (n = 3, RSD = 9.52%). The slightly worse reproducibility obtained for the nanocomposite electrode can probably be explained by small imperfections on the electrode surface caused by hand manufacturing. We assume that improved reproducibility can be expected from using a better technological process for the electrode preparation with thorough homogenization. On the other hand, each measurement of a real sample reported later is accompanied by a calibration in situ for what eliminates the problem of occasional insufficient reproducibility in biosensor construction; that is, the results were calculated by a "rule of three" when the current response for the addition of standard solution is measured before the real sample analysis. Thus, obtained results were in a good correlation with those calculated from the equation for the calibration curve.

Biosensor lifetimes and operational conditions are important issues in sensor development. During the experimental period, no loss of activity after at least 90 measurements using both types of biosensor was observed. These findings are very positive for the planned commercial purposes because this reduces material costs and financial burden for the users. The biosensors constructed by Katrlik and coworkers [14] showed similar characteristics; the response after 90 h reached approximately 60% of the initial response for a glucose dehydrogenase-based biosensor. However, in the case of their GK-based biosensor, 70% was reached already after 16 h. Niculescu and coworkers mentioned maintaining an 80% response of the glycerol biosensor after 100 h of continuous operation [23]. We can assume that the operational stability of our biosensors is comparable to that of the previously reported glycerol biosensors, although a more complex enzyme composition is used for their construction.

Long-term storage stability of biosensors is one of the most important factors in the case of their commercial use. In general, humidity and higher temperature are considered as the most negative factors that can affect the storage stability of enzymatic biosensors. When our biosensors were held in a dessicator without any previous use, they kept their response ability above 90% after 15 months at room temperature. Biosensors with such a long storage and operational stability were not found in the current literature. We suppose that long storage and operation stability could be achieved by the good stability of the employed enzymes and also by the immobilization in a chitosan "sandwich." The positive effect of the chitosan biopolymer matrix on the biosensor stability was discussed in our previous work [26].

Interferences

The interference studies were performed to verify specificity of the presented biosensors for glycerol. Various components of wine and other matrices may affect the accuracy of analysis by interfering with enzymes or by the oxidation directly on the electrode. In particular, polyphenols, ethanol, some sugars (e.g., glucose, fructose, galactose, arabinose), and some organic acids (e.g., malic, lactic, citric, ascorbic, acetic) may be considered as possible interferants [15]. The effect of possible interferences present in wine and juices such as ethanol (10%), glucose (18 g L^{-1}), fructose (18 g L^{-1}), galactose (18 g L^{-1}), arabinose (18 g L^{-1}), mannose (18 g L^{-1}), lactose (18 g L^{-1}), L-lactic acid (4 g L^{-1}), citric acid (4 g L^{-1}), tartaric acid (4 g L^{-1}), acetic acid (4 g L^{-1}), and L-ascorbic acid (50 mg L^{-1}) on the response of the presented biosensors was evaluated. The additions of sugars, ethanol, and acids did not change the current signal over the biosensor detection limit. The effect of polyphenols was tested by using bar electrodes covered with chitosan double-layer without enzymes at +50 mV using optimized measuring solution and applying additions of red wine. No

Table 1
Determination of glycerol in wine samples using biosensors and enzymatic–spectrophotometric assay as a standard analytical method.

Sample	GPE-based biosensor	Nanocomposite-based biosensor	Enzymatic assay
White wines			
Tramin cerveny	5.30 ± 0.05	5.39 ± 0.16	5.80 ± 0.09
Víno biele	9.05 ± 0.21	7.11 ± 1.10	6.71 ± 0.14
Chardonnay	5.40 ± 0.02	5.34 ± 0.48	5.57 ± 0.02
Chardonnay 2	7.34 ± 0.25	7.46 ± 0.02	7.50 ± 0.02
Rulandske biele	6.00 ± 0.14	6.23 ± 0.16	6.26 ± 0.04
Pinot Grigio	6.50 ± 0.04	6.70 ± 0.05	6.60 ± 0.07
Veltinske zelene	6.31 ± 0.52	6.34 ± 0.04	6.44 ± 0.01
Red wines			
Tempranilo	7.85 ± 0.16	8.13 ± 0.21	8.33 ± 0.00
Alibernet	7.08 ± 0.32	6.90 ± 0.30	7.01 ± 0.04
Cabernet Western	8.90 ± 0.40	9.31 ± 0.15	8.94 ± 0.10
Frankovka modra	7.54 ± 0.28	7.88 ± 0.05	7.79 ± 0.01

Note. Results are expressed in g L^{-1} ± standard deviation ($n = 3$).

significant current changes were detected, so polyphenols also do not interfere at performed measuring conditions.

Real sample analysis

All red and white wines were measured with both GPE- and nanocomposite-based biosensors. Results were finally compared with those obtained by enzymatic–spectrophotometric assay. This was important for verification of the biosensors' ability to accurately measure real samples, which have more complex composition than standard glycerol solution. With regard to the linear range of the biosensors and the glycerol levels in wines, samples were 10 times diluted before the analyses. Biosensor determination was performed by injecting 5 or 10 μl of sample and calibration solution in 1 ml of measuring medium. As can be seen in Table 1, a satisfactory degree of correlation was reached between results obtained by the developed biosensor and those obtained by the reference method. In the case of the sample "Vino biele," a small discrepancy in results was found when measuring with the GPE-based biosensor. Although this analysis was repeated several times, results differed from those obtained by the nanocomposite-based one or the enzymatic kit.

Conclusions

It has been demonstrated that a novel enzymatic composition consisting of glycerol kinase/creatine kinase/creatinase/sarcosine oxidase/peroxidase immobilized on the GPE or nanocomposite allowed successful determination of glycerol in wine samples. A simple immobilization method using a chitosan sandwich provided long-term storage stability, low fabrication expenses, and good analytical performance, making the biosensor presented here based on the gold electrode suitable for mass production and commercial use. It currently serves wine makers in monitoring glycerol during the wine-making process as a good alternative to spectrophotometric or chromatographic analysis. Both biosensors met the requirements of precision, rapid response, and sensitivity. Analytical characteristics obtained for nanocomposite-based biosensors showed that this concept is capable of replacing the gold electrode surface of our current commercially successful biosensor as a cheaper alternative with the potential for future improvement.

On the other hand, the presented multienzymatic biosensor can also be used for determination of triglycerides after their hydrolysis by lipase. Moreover various kinase substrates could be determined using the described enzyme cascade when GK is replaced by other kinases. Some examples include glucose and fructose (hexokinase), acetate, galactose, gluconate, glutamine, choline, nucleoside, and urea. The presented multienzymatic cascade should also be applicable in spectrophotometric assay development.

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