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Comparison of biosensors based on gold and nanocomposite electrodes for monitoring of malic acid in wine

Research Article

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Abstract: Amperometric biosensors based on a gold planar electrode and on two types of nanocomposite electrodes consisting of multi-walled carbon nanotubes for the determination of L-malic acid designed for wine-makers were developed. The biosensors designed for wine-makers were constructed by immobilization of L-malate dehydrogenase and diaphorase within chitosan layers on the surface of the electrodes. The coenzyme NAD+ and the electrochemical mediator ferricyanide were present in the measuring solution. The current resulting from re-oxidation of produced ferrocyanide was measured at a working potential of +300 mV against an Ag/AgCl reference electrode. The biosensor based on a gold electrode showed linearity over the range 10-520 μM with a detection limit of 5.41 μM. Calibration curves for biosensors utilizing nanocomposites were obtained both with the linear range of 10 to 610 μM. The detection limits were 1.57 and 1.77 μM, respectively. The biosensors showed satisfactory operational stability (no loss of sensitivity after 30 consecutive measurements) and storage stability (90% of the initial sensitivity after one year of storage at room temperature). The results obtained from measurements of wine samples were in a good correlation with the standard HPLC method. Satisfactory biosensor sensitivity, specificity and stability allowed their successful commercialization.

Keywords: *Biosensor • Malic acid • Nanocomposite • Enzyme • Wine analysis*

1. Introduction

L-Malic acid ($C_4H_6O_5$) is one of the major organic compounds which contribute to the flavor and taste of fresh fruit, juices and other drinks. Malic acid has two stereoisomeric forms (L- and D-enantiomers). The L-isomer occurs naturally, whereas D-malic acid can be found only when synthetic racemate is used as an additive in food industry [1]. Malic acid determination is widely employed in monitoring of the malolactic fermentation process during wine production and also in analysis of other products such as beverages and fruit juices. Malolactic fermentation is a process that usually takes place during or at the end of alcoholic fermentation and is carried out by one or more species of lactic acid bacteria [2]. This process has an important influence on the final taste of wine and can be controlled by the use of starter cultures. Therefore, the determination of L-malic acid during wine fermentation is necessary to allow the winemaker to take the proper decisions [3].

Routine determination of L-malic acid in food or drink samples is usually performed by an enzymatic assay with photometric detection [4,5] or by HPLC [6,7]. However these methods are often time-consuming, expensive and require complex laboratory equipment and educated personnel. The development of biosensors has gained increasing interest in many analytical fields since they can perform satisfactory sensitivity, selectivity and rapid response at low costs. Last but not least, a very important advantage of biosensors compared to classic analytical methods is their ability to perform analysis *in situ* due to their amenability to miniaturization and easy

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handling. Disadvantages include in some cases possible interferences during analyses, the need to recalibrate, inaccuracy in real sample analysis, short operational or storage stability. Various biosensors for L-malic acid determination utilizing malate [8-14] or malate quinone oxidoreductase have been described [15]. Despite their interesting characteristics and performance, they do not represent optimal concepts for mass manufacturing and commercial use due to their overly complex composition, immobilization technique, short stability or risk of interferences during measurements of real samples [16,17].

Various amperometric transducers have been used for the construction of biosensors, such as noble metal electrodes or cheaper graphite based electrodes. On the other hand, the discovery of carbon nanotubes (CNTs) [18] has brought a lot of promises in the development of biosensors because of their unusual properties. The ability of CNTs to facilitate electrochemical reactions of many compounds at low potential makes them particularly suitable for the preparation of electrochemical biosensing devices [19]. Research has been focused on their electrocatalytic behaviors toward the oxidation of biomolecules and their performance has been found to be far superior to those of other carbon electrodes in terms of reaction rate, reversibility and detection limit [20]. It has been shown that CNTs are able to alleviate surface fouling effects such as those involved in the NADH oxidation process [21].

In this paper we compare an L-malic acid biosensor based on the gold electrode with those utilizing nanocomposite electrodes for immobilization of L-malate dehydrogenase and diaphorase. The nanocomposite electrodes were formed by mixing multi-walled CNTs with solid binding matrices. The biosensors presented were designed to be a part of the commercial portable analytical device Omnilab W. The aim of this work was the development of a biosensor for wine-makers for whom cheap and reliable devices like biosensors represent an inviting alternative to classical analytical methods. The main criteria for construction and development a biosensor were accuracy, selectivity, long storage and operational stability and compatibility with existing manufacturing techniques. The biosensors presented for the determination of malic acid are based on the reaction catalyzed by malate dehydrogenase:

$$\text{L-Malic acid} + NAD^+ \leftrightarrow \text{Oxalacetic acid} + \text{NADH} + H^+ \quad (1)$$

The $NADH + H^+$ formed is re-oxidized by diaphorase to NAD^+ in the presence of a mediator ferricyanide which is simultaneously reduced to ferrocyanide:

$$NADH + H^+ + Med_{(ox)} \rightarrow NAD^+ + Med_{(red)} \quad (2)$$

Finally, ferrocyanide is re-oxidized to ferricyanide on the electrode surface and the resulting current proportional to the analyte concentration is measured:

$$Med_{(red)} \rightarrow Med_{(ox)} + 2e^- \quad (3)$$

The biosensors were tested both with standard solutions of L-malic acid and with samples of white and red wines. The results obtained by the biosensor were compared with those obtained from HPLC.

2. Experimental procedure

2.1. Materials

Malate dehydrogenase from *Thermus flavus* (81 U mg^{-1} solid), diaphorase preparates (57 or 62.4 U mg^{-1} solid) and NAD^+ were purchased from Sorachim (Lausanne, Switzerland). Other diaphorase (37 U mg^{-1} solid) was obtained from Asahi Kasei (Tokyo, Japan). Silica gel rubin, sodium phosphate tribasic dodecahydrate, potassium hexacyanoferrate (III), N-eicosane, D-(-)-glucose, D-(-)-fructose, sodium-L-lactate, L-ascorbic acid, L-malate disodium salt, sodium acetate and chitosan from shrimp shells (85% deacetylated) were supplied by Sigma-Aldrich (St. Louis, USA). Potassium phosphate monobasic, potassium phosphate dibasic and tartaric acid were purchased from Riedel-de Haen (Seelze, Germany). Water deionized by a Millipore Milli-Q purification system was used. All chemicals used were of analytical grade. Multi-walled carbon nanotubes (MWCNT) (d = 60-100 nm, L = 5-15 μm, 95+% purity) were obtained from NanoAmor (Houston, USA) and MWCNT Nanocyl®7000 (d = 9.5 nm, L = 1.5 μm, 90+% purity) were purchased from Nanocyl (Semberville, Belgium), thereinafter distinguished in the paper as type A or type B, respectively. Gold planar electrodes with a diameter of 1.6 mm equipped with Ag/AgCl reference electrodes (diameter 2 mm, screen-printed) deposited on the planar glass-epoxy-laminate substrate (Fig. 1) and the kit for polyphenol elimination were obtained from Biorealis (Bratislava, Slovakia).

2.2. Apparatus

Electrochemical studies were performed with model 559 potentiostat from Amel (Milan, Italy) and with an Omnilab electrochemical analyzer from Biorealis (Bratislava, Slovakia).

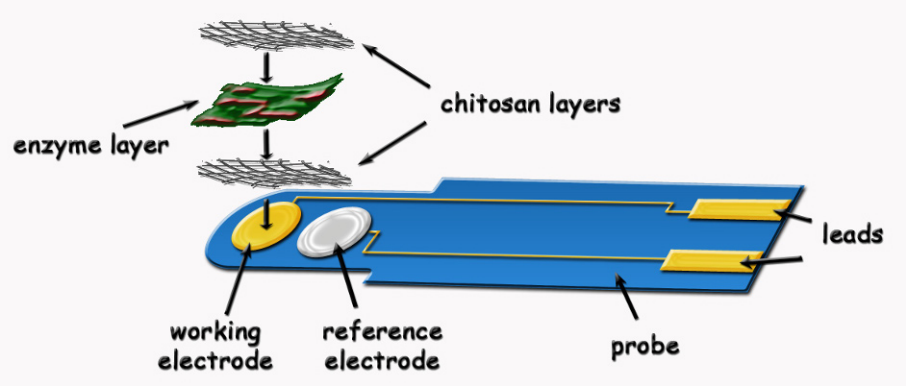

Figure 1. Schematic structure of a biosensor and immobilization method.

2.3. Preparation of nanocomposites

The procedure described previously for graphite composite electrodes was adapted for the nanocomposite fabrication [14]. First, 80 mg of N-eicosane were melted at a temperature of 45°C. After this, 8mg of MWCNT type A or type B were added and the mixture was stirred vigorously with a spatula until the homogenous mixture was obtained. The suspension was subsequently transferred and spread on the surface of the gold electrode (d ≈ 1.6 mm) equipped with a Ag/AgCl reference electrode. Finally, the nanocomposite layer was left to solidify and the surface was smoothed on a sheet of paper.

2.4. Preparation of biosensors

The electrodes were carefully cleaned with Milli-Q water and ethanol. The immobilization of enzymes on the surface of the working electrodes was carried out by sandwiching them between chitosan layers (1% w/w, aqueous solution). Diaphorase (DP) and malate dehydrogenase (MDH) were dissolved in Milli-Q water before deposition. The amounts of enzymes on the electrodes were optimized from 0.5 to 4 units for DP and from 1 to 5 Units for MDH, respectively. Each layer was deposited after the previous one was dried. The chitosan layers were deposited in soluble form (pH below 6) during the biosensor preparation and measurements were carried out in measuring solution at pH 9, without risk of dissolution of the CS "sandwich". The prepared biosensors were stored at room temperature in a dessicator.

2.5. Amperometric measurements

Chronoamperometry was performed by applying a constant potential of +300 mV (*vs.* Ag/AgCl) in a volume of a measuring solution (either 1 mL in a microtube or 10 mL in a beaker) under stirring at laboratory temperature. Optimization procedures were performed mainly with biosensors based on the gold planar electrode (GPE-biosensor). Values from +200 mV to +450 mV were tested for the optimization of the working potential. The pH value of a 0.4 M phosphate buffer measuring solution were optimized from pH 8.0 to 10.5. Similarly, the suitable concentrations of a coenzyme NAD^+ (from 0.02 to 5 mM) and an electrochemical mediator ferricyanide (from 0.5 to 10 mM) in the working media were also investigated.

The biosensors were stored between measurements in 0.4 M phosphate buffer solution of pH 9.0 at laboratory temperature (up to 12 hours) or at 4°C (for longer operational stability studies). The biosensors were kept in a dessicator at laboratory temperature for the storage stability studies.

2.6. Sample preparation

Interaction of polyphenols in red wines with ferricyanide resulting in production of ferrocyanide can cause false current response. As such, the samples of red wines were pre-treated before analysis by a commercial kit for elimination of polyphenols, obtained from Biorealis (Bratislava, Slovakia).

2.7. HPLC analysis

Reference HPLC assays of organic acids were run on a DeltaChromTM liquid chromatograph (Watrex, Bratislava, Slovakia) equipped with an Applied Biosystems 759A absorbance detector (210 nm) (San Diego, CA, USA). The analytical conditions were as follows: column Polymer IEX in H^+ form, 250×8 mm, 8 µm in diameter (Watrex, Bratislava, Slovakia); guard column Polymer IEX in H^+ form, 10×4 mm, 8 µm (Watrex, Bratislava, Slovakia); column temperature 50°C; mobile phase, H_2SO_4, 9 mM in water for HPLC; flow rate, 1.0 mL min^{-1}. Data were collected and processed by a Clarity chromatography station DataApex (Prague, Czech Republic). Samples

were diluted in a mobile phase and filtered through 0.22 µm Chromafil AO filters, Macherey-Nagel (Dűren, Germany) prior to analysis. Organic acids were identified by comparison with retention times and coelution of authentic standard solutions.

3. Results and discussion

3.1. Optimization of working conditions

The amounts of enzymes on the electrode were optimized from 0.5 to 4 U for diaphorase (DP) and from 1 to 5 U for malate dehydrogenase (MDH), respectively. Higher enzyme loadings led to the serious 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 1 U of DP (from Asahi Kasei) and 1.5 U of MDH were found for immobilization on the working electrode. These loadings were consequently used for all biosensors in further experiments. The suitable concentrations of the coenzyme and the mediator in the working media were studied in the range from 0.02 to 5 mM for NAD^+ and from 0.5 to 10 mM for ferricyanide, respectively. The highest biosensor responses were obtained at the following concentrations: 2 mM NAD^+ and 5 mM ferricyanide.

Once the optimal composition of enzymes and the measuring solution were established, the effect of the applied working potential was studied in the range from +200 mV to +450 mV against the Ag/AgCl reference electrode (Fig. 2). Higher values of the working potential were not further investigated because of increasing risk of possible interferences during analyses of real samples. Wines often contain electroactive compounds which can be oxidized on the electrode giving a false current response. No differences in current response were found between the gold electrode and the nanocomposite based electrodes in these experiments. Although the highest response was achieved at an applied potential of +450 mV, we have selected the value of +300 mV for further experiments. This potential allows satisfactory measurement sensitivity and simultaneously avoids undesirable interferences. To prove this claim, the effect of ethanol (10%), glucose (18 g L^{-1}), fructose (18 g L^{-1}), L-lactic (LA, 4 g L^{-1}), citric (CA, 4 g L^{-1}), tartaric (TA, 4 g L^{-1}), acetic (AC, 4 g L^{-1}) and L-ascorbic acid (AA, 50 mg L^{-1}) on the response of the presented biosensors was evaluated. The additions of glucose, fructose, ethanol, CA and AC did not change the current

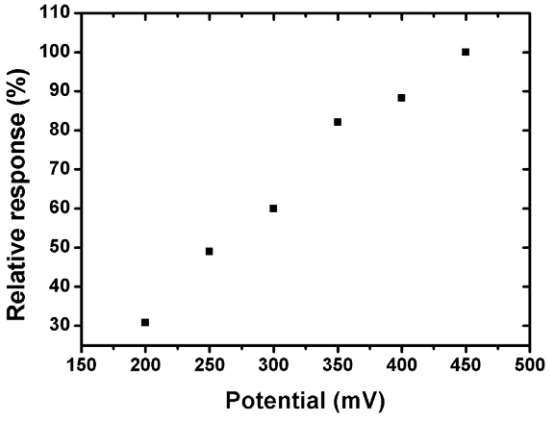

Figure 2. Effect of the working potential on the malic acid response for the biosensor based on the gold electrode. Experimental conditions: 2 mM NAD^+, 5 mM ferricyanide, 50 μM malic acid in 0.4 M phosphate buffer, pH 9.0.

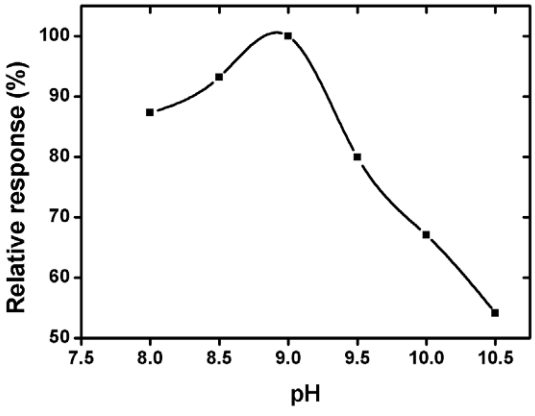

Figure 3. Effect of pH on the response of the malic acid biosensor (the response at pH 9.0 = 100%). Experimental conditions: 2 mM NAD^+, 5 mM ferricyanide, 50 μM malic acid in 0.4 M phosphate buffer, applied potential +300 mV *vs*. Ag/AgCl.

signal while TA, AA and LA acid slightly interfered. It was found that TA caused 2%, AA 1% and LA 3.5% increment in the current response measured for 10 mM (1.34 g L^{-1}) malic acid. These values could be considered negligible. Higher interferences were found if polyphenols were not removed from red wines before analysis. This effect was clearly observed by bare electrodes without enzymes at +300 mV using the optimized measuring solution (data not shown). However, when polyphenols were quantitatively removed by means of the commercial kit, no significant current changes were detected by the bare electrodes.

The effect of pH is a crucial factor for the reaction of MDH. To obtain a good biosensor response, it has to be driven to the side of oxaloacetate formation by high values of pH or high concentrations of NAD^+ (Reaction 1). The application of low H^+ and high NAD^+

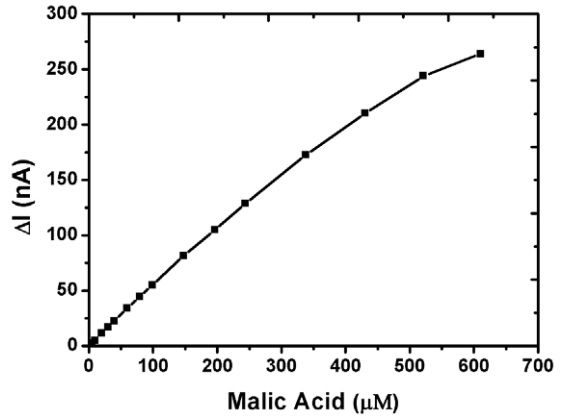

Figure 4. Calibration curve obtained for the malic acid biosensor based on the gold electrode. Experimental conditions: 2 mM NAD^+, 5 mM ferricyanide, 0.4 M phosphate buffer, pH 9.0, applied potential +300 mV *vs*. Ag/AgCl.

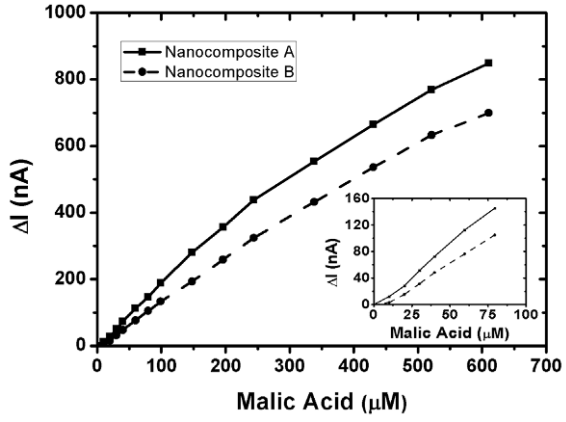

Figure 5. Calibration curves obtained for malic acid biosensors based on nanocomposites A and B. Experimental conditions: 2 mM NAD^+, 5 mM ferricyanide, 0.4 M phosphate buffer, pH 9.0, applied potential +300 mV *vs*. Ag/AgCl.

concentrations, however, reduces the enzymatic activity, sensorlifetime and raises costs [13]. Moreover, the pH optimum of the immobilized enzyme is not always the same as that of the soluble form. The pH dependence of the buffer medium on the amperometric response of the biosensor after addition of malic acid is shown in Fig. 3. The highest response was observed at a pH value of 9.0, which was consequently used in further experiments. It is important to note that the use of ferricyanide in this electrochemical process might also affect the optimal pH value,the polymer matrix used for immobilization and the diaphorase use, which has an optimum pH in the range 6.5-9 (according to the suppliers).

3.2. Analytical characteristics

The calibration curve of the GPE-biosensor is shown in Fig. 4. It can be seen that the biosensor response exhibited a good linearity with a correlation coefficient of 0.996 (n = 13), over the range 10-520 μM. This is similar or wider than that shown by the malate biosensor developed by Bucur *et al*. (5-250 μM using DPIP or 5-150 μM using PMS as a mediator) [15], Gamella *et al*. (2010) (from 0.52 to 20 μM) [22] or by Arif *et al*. (from 28 to700 μM) [9]. However, better performance was shown by the malate biosensor utilizing single-walled carbon nanotubes/meldola blue modified electrodes with MDH immobilized in Nafion developed by Arvinte *et al*. (from 34 to 1350 μM) [23]. A wide linear range is especially important in the analysis of real samples, because it allows the biosensor to be used for a broad range of wine samples with different contents of malic acid. The linearity of the biosensor was lost at malic acid concentrations above 520 μM, probably due to saturation of the active centre of the enzyme. The biosensor showed a sensitivity of 0.55 nA μM^{-1} (calculated from the linear part of the calibration curve), the response time was around 70 s, and the detection limit was 5.41 μM (based on a signal to noise ratio of 5).

Interestingly, a similar linear range from 10 μM to 610 μM was found for biosensors based on nanocomposites A (R^2 = 0.9885, n = 13) and B (R^2 = 0.9821, n = 13), respectively (Fig. 5). A similar analytical performance of a malic acid biosensor utilizing single-wall carbon nanotubes was described by Arvinte *et al.* [24]. The sensitivity of biosensors based on nanocomposite A was 1.17 nA μM^{-1} with a detection limit of 1.57 μM and the sensitivity of biosensors based on nanocomposite B was 1.22 nA μM^{-1} with a detection limit of 1.77 μM. These values are significantly better than those obtained by GPE-biosensor. Detection limits of all biosensors were also proven by experiments. Response times for both of the nanocomposite biosensors were the same as for the GPE-biosensor (70 s). It is obvious that sensitivities and detection limits are superior for the nanocomposite biosensors, which is probably due to the excellent electrochemical properties of CNTs. Further improvements of nanocomposite performance can probably be achieved by using dispersion agents [25].

3.3. Reproducibility of measurement and fabrication

Reproducibility is a measure of the scatter or the drift in a series of results performed over a period of time.

It is usually determined for the analyte concentration within a usable range [26]. The reproducibility of measurements with the same biosensor was studied as a current response on 100 μM of malic acid (at a working potential of + 300 mV versus Ag/AgCl).

The average response for the GPE-biosensor was 53.79 ± 4.44 nA (n = 8, R.S.D = 8.26%). Results for biosensors utilizing nanocomposite were the best: 188.6 ± 4.98 nA (n = 8, R.S.D = 2.64%) for type A and 133.06 ± 3.33 nA (n = 8, R.S.D = 2.25%) for type B.

Another important factor is the reproducibility of biosensor production. For a GPE-based biosensor the average current response of three biosensors for 100 μM malic acid was 53.10 ± 5.42 nA (n = 3, R.S.D = 10.21%). In case of the 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 malic acid was 186.77 ± 17.47 nA (n = 3, R.S.D = 9.84%) for the nanocomposite-based biosensors for type A and 167.85 ± 17.86 nA (n = 3, R.S.D = 11.69%) for type B. We assume that improved reproducibility can be expected from using a better technological process for the electrode preparation with a thorough homogenization. On the other hand the use of an *in situ* calibration for each measurement of a real sample eliminates the problem of occasional insufficient reproducibility in biosensor construction, *i.e*. 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 the results were in good correlation with those calculated from the equation for the calibration curve.

3.4. Operational and storage stability

Enzymes have a tendency to lose their activity when not stored in appropriate conditions. Measurements of the operational stability were performed at room temperature. Between measurements, biosensors were stored in 0.4 M phosphate buffer solution of pH 9.0 at laboratory temperature (up to 12 hours) or at 4°C (for longer operational stability studies). Biosensors did not show any loss of activity after 30 analyses in a row and after 10 hours of use. All of them showed response ability above 90% after 24 hours and about 63% after 7 days for a GPE-biosensor. However, for both nanocomposite- based biosensors, the response decreased below 50% as early as 3 days after the first measurement.

Long-term storage stability of biosensors is one of the most important factors in their commercial use. Generally humidity and higher temperature are considered as the most negative factors which can affect the storage stability of enzymatic biosensors. When our biosensors were held in a dessicator without use, they maintained their response ability above 90% after one year at room temperature (measured regularly every 2 months), which is significantly longer than shown by the malate biosensor prepared by Gurman *et al*. (3 months stored at 4°C) [27], Gamellla *et al*. (stored for 15 days in appropriate buffer solution at 4°C) [22] or Katrlik *et al*. (stored for 5 months in sealed plastic tubes, with a small amount of moisture adsorbent at room temperature) [8]. We assume that long storage and operation stability could be achieved thanks to the immobilization in chitosan (CS) "sandwich", whereas amino and hydroxyl groups widely present in CS molecules provide a hydrophilic environment which is compatible with the biomolecules [28]. When CS is dissolved, it possesses high positive charge on $–NH_3^+$, it adheres to negatively charged surfaces, it aggregates with polyanionic compounds, and chelates heavy metal ions [29]. Our enzymes are characterized by low pI [30], which means that a lot of carboxyl groups are present in enzymes and can be employed in ionic interactions with amino groups in CS and thus stabilize the whole system. CS is a biocompatible polysaccharide known as a material that displays excellent film-forming ability, good adhesion, and high mechanical strength [31-33], *i.e*., factors which could also contribute to the good stability of biosensors. Another interesting feature of CS was utilized for the construction of the presented biosensor. The protonation or deprotonation of CS (p*Ka* ≈ 6.1–7.0) make CS soluble/insoluble below/above ca. pH 6 [34]. Hence a biosensor with a CS film as the enzyme-immobilization matrix should be employed at a solution pH sufficiently above its p*Ka* [35].

Table 1. Determination of malic acid in red and white wines using biosensors and HPLC as a standard analytical method. Results are expressed in g L^{-1} ± STDEV (n=3).

Wine	Biosensor-GPE	Biosensor-nanocomposite A	Biosensor-nanocomposite B	HPLC
Rizling Rynsky, Slovakia (white)	2.14 ± 0.01	-	-	1.86 ± 0.02
Rulandske biele, Slovakia (white)	1.08 ± 0.11	-	-	0.77 ± 0.02
Oblastne, Hungary (red)	0.44 ± 0.05	0.56 ± 0.01	0.61 ± 0.01	0.50 ± 0.01
Frankovka modra, Slovakia (red)	0.06 ± 0.01	0.28 ± 0.1	0.28 ± 0.01	0.18 ± 0.01
Svatovavrinecke, Slovakia (red)	0.17 ± 0.01	-	-	0.11 ± 0.01
Tempranillo, Spain (red)	0.15 ± 0.02	0.33 ± 0.04	0.30 ± 0.02	0.33 ± 0.01

3.5. Analysis of real samples

The degree of recovery for all types of biosensors presented was tested by analyses of white and red wine with addition of 1 g L^{-1} of malic acid. The degree of recovery was found to be satisfactory, ranging from 96 to 105%. Nevertheless, for verification of a biosensor's ability to accurate measure real samples, the wine samples were evaluated using the biosensors developed for the determination of malic acid and the HPLC as a standard analytical method. Results from three different measurements were averaged and compared to those obtained from HPLC (Table 1). Biosensoric determination was performed by injecting 10 µL of sample and calibration solution in 1 mL of measuring media. Samples did not require any dilution, only polyphenols were removed from red wines before the procedure using the commercial kit. The results presented here show that the developed biosensors can be used for direct determination of malic acid in wine samples.

4. Conclusion

Reliable malate biosensors based on the gold planar electrode or on two types of nanocomposites fabricated from different multi-walled carbon nanotubes were developed and compared. The present biosensors use a bi-enzymatic system utilizing malate dehydrogenase and diaphorase. All biosensors were successfully employed in the analysis of malic acid in wine samples. Thanks to the simple immobilization method utilizing a chitosan "sandwich", unique long term storage stability, low fabrication expenses, and good analytical performance were achieved. Results obtained with biosensors utilizing nanocomposite electrode consisting of carbon nanotubes and N-eicosane showed that this concept is able to replace more expensive gold electrodes currently used for biosensors in the commercial Omnilab W device. The biosensor presented here based on the gold electrode is currently used by wine-makers for monitoring malolactic fermentation in the wine-making process as a good alternative for the spectrophotometric or chromatographic analysis.

Further work will be focused on improving the nanocomposite biosensor properties by means of modification of carbon nanotubes or immobilization of NAD^+ and mediator into a biocatalytic layer to design a reagent-free measuring system.

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