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A flow injection biamperometric method for determination of total antioxidant capacity of alcoholic beverages using bienzymatically produced ABTS$^{\cdot+}$

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Abstract

Development of flow injection analysis (FIA) is reported for determination of total antioxidant capacity of alcoholic beverages (wines and spirits) by bienzymatically produced ABTS$^{\cdot+}$ and electrochemical detector using interdigitated gold electrode (IDE). The method is based on biamperometric measurements using ABTS$^{\cdot+}$|ABTS redox couple. During the analysis, ABTS radical cation was bienzymatically produced by glucose-oxidase and peroxidase separately immobilized in tubular flow-through reactors. Influence of pH and substrate concentration on enzyme activity were tested in order to obtain the most sensitive mode. The effect of flow rate on operation of the bioreactors was also studied. The results of antioxidant capacity were expressed as Trolox Equivalent. The linearity of IDE detector was tested in the range between 20 μM and 2000 μM and good sensitivity of 0.165 nA/μM for Trolox solutions was obtained.

Good comparison of results ($R^2 = 0.9942$) was established for antioxidant capacity of tested samples between proposed FIA method and reference spectrometric measurements.

Keywords: Antioxidant; ABTS$^{\cdot+}$|ABTS redox couple; Biamperometry; Bienzymatic; Spirits; Trolox; Wines

1. Introduction

Indirect biamperometric measurements have become important techniques for sensitive and selective determination of many different chemical compounds, particularly for those in pharmaceutical (Medeiros et al., 2004) and biological (Budnikov, Ziyatdinova, & Valitova, 2004) samples. Biamperometric technique is based on electrochemical detection by means of two identical working electrodes imposed to a small voltage difference, and applied in the solution containing indicating redox couple. Indirect biamperometric method of measurement is based on homogeneous reaction between an analyte and an indicating reversible redox couple. The most used indicating systems for biamperometric determination include $Fe^{3+}|Fe^{2+}$, $I_2|I^-$, $Br_2|Br^-$, $VO_3^-|VO^{2+}$, Ce (IV)|Ce (III) and $Fe(CN)_6^{3-}|Fe(CN)_6^{4-}$ and lately DPPH$^{\cdot+}$|DPPH redox couple (Milardovic, Ivekovic, & Grabaric, 2005).

T. P Tougas and coauthors clarified basic principles for flow injection analysis by biamperometric detectors (Tougas, Jannetti, & Collie, 1985). Indirect biamperometric flow injection methods have been recently suggested for quality control analysis of many different compounds commercially available in pharmaceutical tablets as pantoprazole (Castro et al., 2005), captopril (Palomeque & Band, 2002), chloroamine-T (Icardo, Romero, Mateo, & Martinez-Calatayud, 2000) and paracetamol (Gálvez, Mateo, & Martinez-Calatayud, 1999).

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Interdigitated electrodes (IDE) have been used as highly sensitive electrochemical detectors because of their ability to offer large current and higher sensitivity than macroelectrode. Sanderson and Anderson described the electrochemical performance of IDE nearly 20 years ago (Sanderson & Anderson, 1985) while Niva and coworkers explained electrochemical amplification of IDA in the solution containing reversible redox couple (Morita, Niva, & Horiuchi, 1997). Recently, an interdigitated microelectrode array was used as a biamperometric detector for microanalytical determination of formaldehyde (Tomcik, Mrafkova, & Bustin, 2003). Interdigitated array electrodes have found much practical application in flow analysis mostly as a part of the microfluidic cell (Daniel & Gutz, 2005).

In recent times, substantial interest has been focused on analytical methods for estimation of antioxidant activity of food (Sanchez-Moreno, 2002), biological samples (Benzie & Strain, 1999) and of botanical and dietary supplements (Ronald & Guohua, 2000). The methods developed for measuring antioxidant capacity have been classified as inhibition or noninhibition methods (Ronald & Guohua, 1999), but both these types are spectrophotometric. One of the most widely used methods for antioxidant activity evaluation is based on decolorization assay of a stable ABTS radical cation (2,2′-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid)) (Rice-Evans & Miller, 1994). Continuous electrochemical generation of the ABTS radical cation and its application for spectrophotometrical flow injection analysis of antioxidant activity was suggested by Iveković and co-workers (Iveković, Milardović, Roboz, & Grabarić, 2005). Biamperometric method using $DPPH^{\cdot+}$|DPPH redox couple and a glassy carbon disc electrode as a detector was successfully applied for determination of antioxidant activity (Milardović, Iveković, Rumenjak, & Grabarić, 2005). E. N. Kadnikova and N. M. Kostić described biocatalytical oxidation of ABTS by hydrogen peroxide using horseradish peroxidase encapsulated in the sol–gel glass (Kadnikova & Kostić, 2002). Effect of the sample dilution and time of analysis on the antioxidant activity of wine determined using $ABTS^{\cdot+}$ and spectrophotometric detection was explained by D. Willaňo et al. (Villaňo, Fernández-Pachón, Troncoso, & García-Parrilla, 2004), while N. Pellegrini and coauthors demonstrated the usefulness of flow injection ABTS method for spectrophotometric analysis of plants, beverages and oil consumed in Italy (Pelegrini et al., 2003).

The intention of the present paper was to describe the flow injection biamperometric method applied for determination of antioxidant capacity of many different sorts of wines and spirits. This method includes optimization of many parameters for glucose-oxidase reactor, peroxidase flow-through reactor and flow-through IDE detector to obtain the most sensitive and rapid technique. One electron oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) into analogous ABTS radical cation was achieved continuously during the analysis by glucose-oxidase and peroxidase-catalyzed reactions in flow-through tubular reactors according to reactions (1)–(3).

$$\text{D-glucose} + \text{enzyme-FAD} \rightarrow \text{Gluconic acid} + \text{enzyme-FADH}_2 \quad (1)$$

$$\text{enzyme-FADH}_2 + O_2 \rightarrow \text{enzyme-FAD} + H_2O_2 \quad (2)$$

$$\text{ABTS} + \frac{1}{2}H_2O_2 + 3H^+ \xrightarrow{\text{peroxidase}} \text{ABTS}^{\cdot+} + 2H_2O \quad (3)$$

After reaction between antioxidants and $ABTS^{\cdot+}$ in the mixing coil (Eq. (4)), the residual reduced concentration of the cation was determined biamperometrically in the flow-through measuring cell containing interdigitated electrode according to reaction (5).

$$\text{ABTS}^{\cdot+} + \text{AH} \rightarrow \text{ABTS} + \text{A}^{\cdot} + H^+ \quad (4)$$

$$\text{ABTS}^{\cdot+} + e^- \rightarrow \text{ABTS} \quad (5)$$

The advantages of bienzymatic $ABTS^{\cdot+}$production are as follows:

Bienzymatic production of $ABTS^{\cdot+}$ by glucose-oxidase and peroxidase enzymes involved the use of stable glucose solution and ABTS as substrates while $ABTS^{\cdot+}$ enzymatically produced by peroxidase required ABTS and diluted hydrogen peroxide solutions easily decomposed to water and molecular oxygen.

Variation in hydrogen peroxide concentration may cause large variation in current base-line on $I - t$ diagrams.

Furthermore, in case of decreasing enzyme activity, the concentration of enzymatically uncatalyzed hydrogen peroxide increases and reaction between hydrogen peroxide and some antioxidants in samples is possible.

2. Experimental

2.1. Chemicals and solutions

All the reagents used were of analytical grade. Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, ABTS (2,2-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt, 98%), *n*,*n*′-methylenebisacrylamide (99%), 2,2-dimethoxy-2-phenylacetophenone (99%), peroxidase (HRP, 147 units/mg) from horseradish (type 1) and glucose oxidase (GOD) from Aspergillus niger (47.2 units/mg) were obtained from Sigma–Aldrich (St. Louis, USA). Acrylamide was purchased from Fluka (Buchs, Switzerland). Sodium dihydrogen phosphate, sodium hydrogen phosphate, and potassium chloride were from Kemika (Zagreb, Croatia). Potassium peroxodisulfate ($K_2S_2O_8$) was from Merck (Germany). Fresh solutions of water-soluble Trolox standard were prepared daily using double deionized water from Millipore-MilliQ system (USA). The $ABTS^{\cdot+}$ radical solution (50 ml) was prepared 24 h before spectrophotometric analysis by mixing 0.2 mL, 65 mM $K_2S_2O_8$ and 10 mL 5 mM ABTS using phosphate buffer, pH 7.4.

2.2. Apparatus and instrumentation

Electrochemical measurements were carried out on Potentiostat 273 A (Princeton Applied Research, USA) coupled to a computer for data setting and analysis. Hydrodynamic voltammograms were performed in a standard electrochemical cell. Interdigitated electrodes (IDE) IME 1525.3 FD Au P (ABTCH, Richmond, USA) were employed as working electrodes and used in biamperometric measurement mode. Interdigitated array electrodes consist of two separated working electrode arrays on chip (6.4×5.5 mm, digit length $W_a = 2.985$ mm, digit width (W_g) = 15 μm, interdigit space (W) = 15 μm and the number of digit pairs = 25). The applied potential scan rate in cyclic voltammetry was 10 mV/s. Current–time measurements required a pair of interdigitated electrodes fixed in a flow-through measuring cell (Fig. 1). Transportation of the carrier solution in FIA mode (Fig. 1) was made by a triple tubing peristaltic pump. Sample injection into carrier stream was done by a syringe using injector valve Rheodyne Model 7125 and the sample loop of 10 μl. Two-reactor coil was made using a Teflon tube (1 mm in diameter, 60 cm long).

$ABTS^{\bullet+}$ absorbance measurements were recorded at 730 nm by the double-beam DMS 80 (USA) spectrophotometer.

2.3. Interdigitated electrode (IDE) conditioning

The preconditioning and cleaning of interdigitated electrodes were made according to manufacturer's recommendations by threefold repeat of potential cycling in the range between −600 mV and 1000 mV (50 mV scan rate). Sulfuric acid (0.8 M) was used as a supporting electrolyte. The preconditioning was repeated for each part of IDE pairs. Hg_2Cl_2|3 M KCl was used as a reference electrode and the disc glassy carbon electrode as an auxiliary electrode.

2.4. Spectrophotometric determination

To 1.5 mL plastic spectroscopic cuvete ($12.5 \times 12.5 \times 45$ mm, Brand, Germany) containing 1 mL $ABTS^{\bullet+}$ in phosphate buffer solution (pH 7.40, initial absorbance of 0.7), 5 μL of the sample (Trolox standard, or actual wine sample) was added and mixed well for 10 s. The changes in absorbance were read out after 18 s. The same period was needed for mixing the sample and $ABTS^{\bullet+}$ in the mixing coil reactor applied for FIA biamperometric analysis.

2.5. Flow-through bioreactors: production of $ABTS^{\bullet+}$

Immobilization of both enzymes (peroxidase or glucose-oxidase) was made in polyacrylamide gel. Acrylamide solution was prepared using 0.43 g acrylamide, 66 mg *n*,*n*′-methylenebisacrylamide and 2.4 mg 2,2-dimethoxy-2-phenylacetophenone dissolved in 1 mL deionized water and 1.5 mL of glycerol. In 250 μL of acrylamide solution, 5 mg (peroxidase (147 units/mg) or 4.8 mg glucose-oxidase (47 000 units/g)) were added and mixed well for 30 min. The solution was placed on a glass plate and photopolymerized by mercury lamp illumination (Osram, ultravitalux, 300 W, Germany). The gel that formed after 2 min was cut by spatula to 3×3 mm pieces and filled into a separated tubular reactors each 4 mm in diameter and 4 cm long. To obtain adequate porosity and continuous flow across the well-filled reactor, the silica–gel grains (2 mm in diameter) were packed together by polyacrylamide gel.

2.6. Statistical analysis

All experiments were carried out in triplicate. The results were processed using CBstat version 5.0., a program (Windows application) for statistical analysis in clinical biochemistry. Results are expressed as mean ± standard deviation (Table 1). Passing-Bablok Regression Analysis was used for linearity tests.

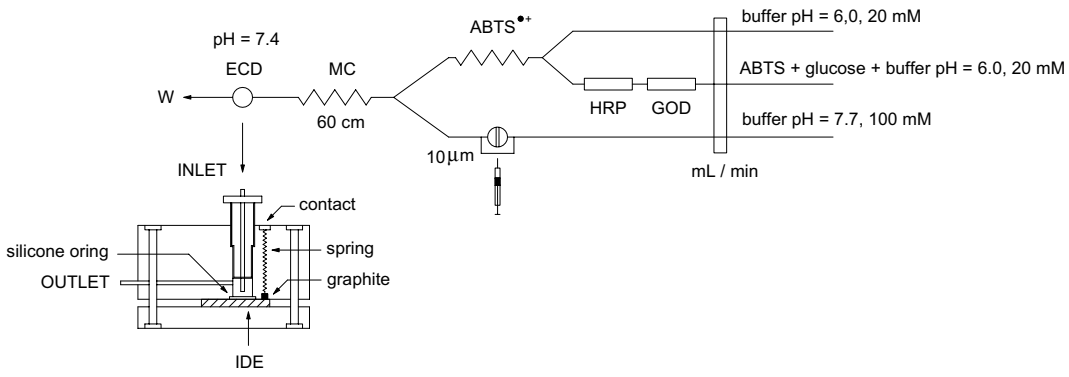

Fig. 1. Schematic presentation of a FIA device designed for determination of antioxidant capacity by bienzymatic $ABTS^{\bullet+}$ production (W – waste, ECD – electrochemical device, MC – mixing coil).

Table 1
Comparison of results for antioxidant capacity obtained by spectrophotometric and biamperometric measurements (FIA mode)

Tested samples	Manufacturer	Production year	Trolox equivalent determined spectrophotometrically	Trolox equivalent determined by IDE
Black wine				
Merlot	Agrolaguna	2005	11.92 ± 0.26	13.68 ± 0.288
Plavac	Ston	2005	13.41 ± 0.08	13.49 ± 0.219
Kastelet	Dalmacijavino	2003	12.10 ± 0.23	12.71 ± 0.177
Peljesac	Wine-cellar, Dingac, Vinarska	2003	15.12 ± 0.20	15.69 ± 0.478
Vranac	Vizba, Tikves, AD	2003	16.91 ± 0.28	17.38 ± 0.480
White wine				
Riesling	Wine-cellar, Djakovo	2005	2.30 ± 0.18	1.74 ± 0.055
Grasevina	Wine-cellar, Djakovo	2003	2.31 ± 0.11	1.64 ± 0.062
Grasevina	Kutjevo	2005	1.79 ± 0.09	1.23 ± 0.017
Grasevina	Wine-cellar, Daruvar	2004	2.48 ± 0.02	1.77 ± 0.036
Henkell, trocken, champagne	Henkell & Söhnlein		1.71 ± 0.06	1.57 ± 0.031
Spirits				
Wormwood, wine	Badel		0.20 ± 0.001	0.26 ± 0.008
Liqueur	Badel		0.32 ± 0.003	0.59 ± 0.005
Brandy	Badel		0.16 ± 0.003	0.09 ± 0.009
Vodka	Vigor		0.02 ± 0.001	0.04 ± 0.001

Values are means of three replications ± SD.

3. Results and discussion

3.1. Biamperometric response of IDE in the solution containing $ABTS^{\cdot+}$|ABTS redox pair

Fig. 2 shows hydrodynamic voltammogram of $ABTS^{\cdot+}$|ABTS obtained by the interdigitated electrode applied into a classic electrochemical cell containing 3 mL 50 mM phosphate buffer, pH 7.4, 5 mL 5 mM ABTS solution and 2 mL chemically produced $ABTS^{\cdot+}$. Initial concentration of $ABTS^{\cdot+}$ was determined as 22.93 μM ($A = 0.344$ and $\varepsilon = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$). After addition of Trolox (2.5 μL, $c = 10$ mM) to the electrochemical cell, the solution was well stirred and voltammograms were separately recorded after each addition. Initial $ABTS^{\cdot+}$ concentration before the addition of Trolox solution showed the largest current response (cathodic or anodic), as evident in the voltammogram designated as 0 μM. After Trolox addition, $ABTS^{\cdot+}$ concentration decreased due to $ABTS^{\cdot+}$ reduction and the resulting current response declined as well. When the cumulative concentration of the added Trolox reached the value of 11.46 μM, $ABTS^{\cdot+}$ was completely reduced and voltammogram showed the lowest resulting current response. This experiment confirmed that the current response linearly depended on $ABTS^{\cdot+}$ concentration and confirmed the well known fact that biamperometric response is proportional to the concentration of that part of the redox pair that occurs in the solution at low concentration, as was evident by used molar ($ABTS^{\cdot+}$|ABTS) ratio of 1/109, i.e., 0.02293 mM $ABTS^{\cdot+}$ and 2.5 mM ABTS.

3.2. Optimizing working parameters for enzyme-containing bioreactors

3.2.1. Effect of substrate concentration on $ABTS^{\cdot+}$ production

Effects of glucose concentration on oxidation of ABTS catalyzed by GOD and HRP immobilized in flow-through tubular reactors can be seen in Fig. 3a and b. The weights of immobilized GOD and HRP were 4.8 mg and 5 mg, respectively, while the tested concentration of ABTS prepared in phosphate buffer solution pH 7.4 was 1, 2 and 4 mM. The points in the plots designates glucose concentration of 0.5, 1.0, 2.5, and 5 mM, respectively. The imposed voltage difference was 100 mV and applied volume flow rate was 1.23 mL/min. The solutions (glucose, ABTS) passed through reactors as long as the current resulting from reduction of produced ABTS radical cation became constant. After that the current was measured additionally in the period between 15 and 20 min and the average value of steady state current in such period of time represent one value in the Fig. 3a and b. Each value in Fig. 3a and b were determined with standard deviation, S.D. = ± 0.0039 nA.

It is obvious that the production of $ABTS^{\cdot+}$ in the reactors increased by increasing glucose concentration, and the maximum value was achieved at the concentration of 2.5 mM. Therefore the steady state current value increased up to 2.5 mM while further elevation of glucose concentration caused no significant rise in current response even in case of double increase in ABTS solutions (from 1 mM to 2 mM). The produced $ABTS^{\cdot+}$ concentration (Fig. 3b)

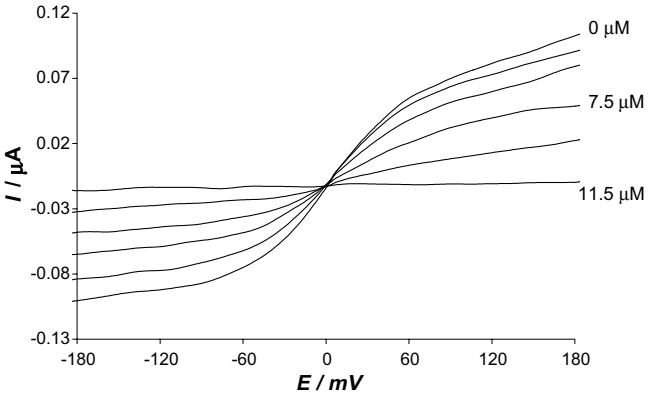

Fig. 2. Hydrodynamic voltammograms of IDE. Experimental conditions: phosphate buffer solution pH 7.4, c ($ABTS^{\cdot+}$) = 0.02293 µM, c (ABTS) = 2.5 mM, scan rate 10 mV/s. Curve 0 µM – before Trolox addition, c (Trolox, added) = 2.5µM (curves 1–5).

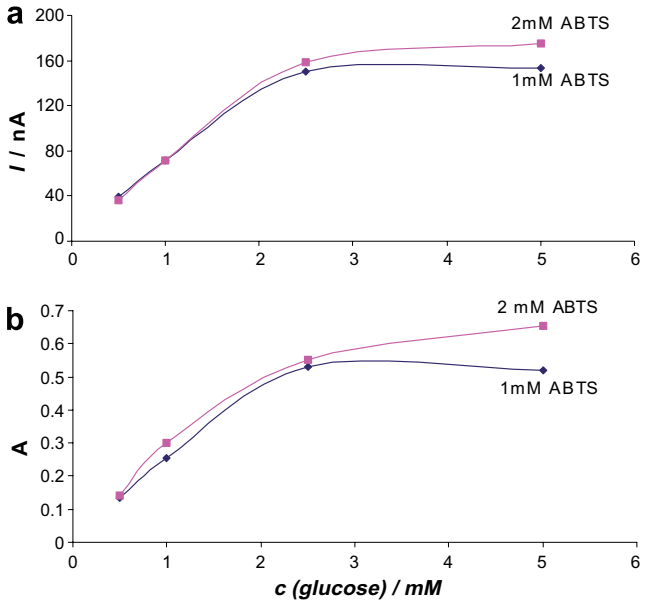

Fig. 3. (a,b) Effect of glucose concentration on oxidation of ABTS catalyzed by GOD and HRP in the flow–through reactor. Experimental conditions: c (ABTS) = 1, 2, 4 mM, c (glucose) = 0.5. 1, 2.5, 5 mM, flow rate = 1.23 mL/min, phosphate buffer pH 7.4, ΔE = 100 mV.

in 2.5 mM glucose solution was 66 µM. Further increasing of ABTS to 4 mM resulted in insignificant rise in current response (results not shown in Fig. 3a and b).

3.2.2. Effect of flow rate on $ABTS^{\cdot+}$ production

Effect of flow rate on steady-state current and the absorbance obtained for flow rates of 4.42, 2.14, 1.68, 1.36, 1.22, 1.12, 0.94 and 0.82 mL/min, respectively, are shown in Fig. 4a and b. Current response measured by IDE polarized at 100 mV rose by decreasing flow rates due to expansion of enzyme reaction time. The concentration ranges of produced $ABTS^{\cdot+}$ were between 12.26 µM obtained at flow rate of 4.42 mL/min and 67.46 µM obtained at flow rate of 0.82 mL/min. Current response versus $ABTS^{\cdot+}$ concentration in the measuring range (12.26–67.46), c ($ABTS^{\cdot+}$) = (Absorbance/1.5×10^4 M^{-1} cm^{-1}) µM showed linear relationship with a correlation coefficient of 0.9992.

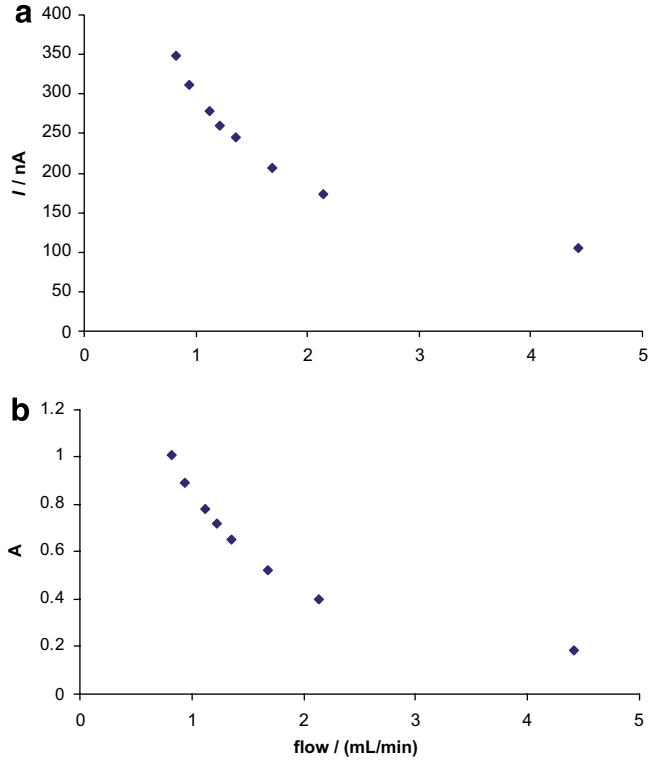

Fig. 4. (a,b) Effect of flow rate on $ABTS^{\cdot+}$ productivity. Experimental conditions: phosphate buffer pH 7.4, c (ABTS) = 1 mM, c (glucose) = 2.5 mM, ΔE = 100 mV. Tested flows: 4.42, 2.14, 1.68, 1.36, 1.22, 1.12, 0.94 and 0.82 mL/min.

The flow rates between 1.36 and 1.12 mL/min were chosen for the following measurements because the obtainable $ABTS^{\cdot+}$ concentration was sufficient for practical work and sufficient time of analysis was achieved.

3.2.3. pH effect on bienzymatic productivity of $ABTS^{\cdot+}$

The variation in enzyme productivity in the pH range between 4.0 and 9.0 determined using substrate concentration considered before (1 mM ABTS and 2.5 mM glucose) was tested (results not shown). In alkaline solution (pH > 8) and acidic solution (pH < 5), $ABTS^{\cdot+}$ production was reduced and corresponding current responses were low. In the pH range between 5 and 6.5, enzyme productivity was very high and resulting current response was also very high. The pH value of 6.0 offered the highest value of enzyme activity and was chosen for subsequent experiments. According to Fig. 1, two different buffer flows were used: the first flow used phosphate buffer pH 6.0 needed for proper enzyme function (maximum production of $ABTS^{\cdot+}$) and the second flow used phosphate buffer pH 7.7 as a carrier of injected antioxidants. After their mixing, the final pH reached the value 7.40 as the pH optimum for determination of the antioxidant capacity specified by TEAC method.

3.3. Optimal working potential

Effect of the potential difference applied between IDE on current sensitivity is also shown in Fig. 2. Elevation of the imposed voltage difference was followed by a rise in steady state current; however, at a potential higher than 100 mV the potential influence on current responses was diminished. Also, possible interference of undesirable species presented in the measuring sample should be suppressed at the lower potential. Each of these reasons was considered and the potential of 100 mV favored for all further experiments.

3.4. Flow injection analysis

Standard solutions of Trolox ranging from 20 μM to 2000 μM were analyzed under optimized conditions (c (glucose) = 2.5 mM, c (ABTS) = 1 mM, pH 6.0 (flow 1), pH 7.7 (flow 2), $\Delta E = 100$ mV, flow rate = 1.36 mL/min). The results (Fig. 5a) showed linear relationship (I/nA = (8.0732 ± 2.0818) nA + (0.1651 ± 0.00442) nA/μM · c/μM) and correlation coefficient of 0.9953 was obtained. As shown in Fig. 5a, the biamperometric response of IDE was linearly correlated to Trolox concentration in the range between 20 μM and 1 mM.

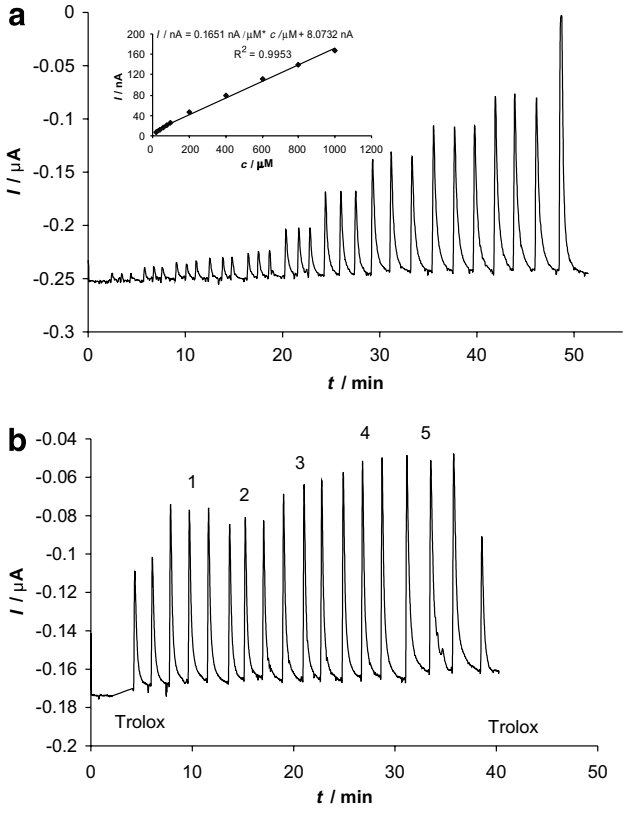

Fig. 5. (a,b) Calibration graph for Trolox and diagram for wine samples. Peaks (Fig. 5b) 1, 2, 3 and 4 – tenfold diluted different sorts of red wine, peak 5 – undiluted sample of white wine, first two peaks on the diagram and the last one designate 1 mM standard Trolox solution. Experimental conditions: the same as in Fig. 4. Used flow 1.22 mL/min.

Flow injection analysis of several actual samples of different sorts of wine produced at geographically different parts of Croatia is shown in the diagram (Fig. 5b). Peaks 1, 2, 3 and 4 designate tenfold diluted different sorts of red wine. Peaks 5 designates undiluted samples of white wine. The first two peaks in the diagram and the last one designate 1 mM standard Trolox solution. Injections of each tested wine sample and spirit were three times repeated and results for antioxidant capacity were determined by two point calibration graph of Trolox. The results for antioxidant capacity were expressed as the capacity equivalent to a corresponding Trolox concentration. All other experimental parameters were the same as in Fig. 4.

The results for individual antioxidant capacity of testing samples are summarized in Table 1. Correlation of the antioxidant capacity determined by biamperometric FIA method and those determined spectrophotometrically was tested by regression analysis and good result was obtained (Trolox capacity(IDE) = − (0.2826 ± 0.2146) + (1.0643 ± 0.02533) · Trolox capacity spectrophotometric, R^2 = 0.9942). The regression analysis for intercept close to 0 (from 0.5617 to 0.1825 for 95% con. int.) and slope close to 1 (from 0.7710 to 1.0854 for 95% con. int.) additionally confirmed good agreement between classic spectrophotometric (batch mode) and flow injection analysis based on bienzymatic production of $ABTS^{\cdot+}$ and biamperometric detection by IDE. With respect to reaction time, assays demonstrated the time dependency of the reaction between $ABTS^{\cdot+}$ and antioxidant compound (Villaño et al., 2004). The reaction time obtained at chosen experimental conditions (flow rate, internal diameter of mixing coil) for 18 s is lower than the usually used (1 or 2 min) and therefore the obtained results for TEAC values for tested wines (Table 1) have lower TEAC value than those in literature obtained at higher reaction time.

3.5. Concluding remarks

Flow injection analysis of antioxidant capacity based on continuous bienzymatic production of ABTS radical cation and biamperometric detection by interdigitated electrode is described. Production of $ABTS^{\cdot+}$ bienzymatically by GOD and HRP immobilized and packed into tubular flow-through reactors offered continuous analysis. The use of glucose solution instead of hydrogen peroxide minimized hydrogen peroxide interference possibly occurring in methods based on monoenzymatic $ABTS^{\cdot+}$ production by hydrogen peroxide and HRP. Furthermore, glucose solutions are more stable than hydrogen peroxide solution under normal room conditions.

Good linearity was obtained for Trolox in the range between 20 μM and 1000 μM. Additionally, good agreement between the results for Trolox equivalents obtained by IDE detector and those obtained by reference spectrophotometric measurements confirmed the applicability of

the proposed method for antioxidant capacity analysis for wine and spirit specimens.

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