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A novel amperometric method for antioxidant activity determination using DPPH free radical

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Abstract

A new method for the determination of antioxidant activity based on the amperometric reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) at the glassy carbon electrode is proposed. All experiments were done in three-electrode electrochemical cell at 140 mV vs. Hg_2Cl_2 | 3 M KCl using ethanolic solution ($\varphi = 40\%$) and 0.033 M KCl in 0.033 M phosphate buffer, pH = 7.4. The linear range obtained for Trolox in 100 μM DPPH ethanol–water solution was up to 30 μM, with a limit of detection of 0.05 μM.

The developed method was applied for the evaluation of antioxidant activity of some water or ethanol soluble pure compounds of antioxidants and of the samples of tea, wine and some other beverages. The good correlation of measurements ($R^2 = 0.9993$) expressed as Trolox equivalent was obtained between the proposed amperometric method and classic spectroscopic method.
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Keywords: Amperometric detection; Antioxidant; DPPH; Trolox equivalent

1. Introduction

There is ample evidence of an association between the oxidative stress resulting from radical generation and antioxidant insufficiency and tissue damage. Lipid oxidation as a result of radical chain reaction causes quality loss in flavor, color and nutritive values of food. Therefore many methods for determination of antioxidant activity in food samples and biological fluids have been proposed during the past decade. They are mainly based on reaction between a chromogen compound and an antioxidant. After reaction, the residual concentration of chromogen compound is determined spectrophotometrically or colorimetrically [1–5].

The most often used chromogen compounds include 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) because they react directly and rapidly with antioxidant compounds.

DPPH· free radical reacts with an antioxidant (AH) or a radical species (R·) according to Eqs. (1) and (2).

$$\mathrm{DPPH}\cdot + \mathrm{AH} \rightarrow \mathrm{DPPH} - \mathrm{H} + \mathrm{A}\cdot \quad (1)$$

$$\mathrm{DPPH}\cdot + \mathrm{R}\cdot \rightarrow \mathrm{DPPH} - \mathrm{R} \quad (2)$$

Any substance that can donate a hydrogen atom (antioxidant) to the solution of DPPH· can reduce the stable free radical and change the color of solution from violet to pale yellow. Nonreacted radical form of DPPH absorbs in the visible range, and spectroscopic method is based on the measurements of color intensity at 518 nm. The basis of this method was introduced by M. Blois [6] nearly 50 years ago, while Brand-Williams et al.[7] proposed some improvements of the original Blois method.

They also determined the reaction stoichiometry for DPPH with various antioxidants, which introduced the

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possibility for the use of an antioxidant as a suitable standard for the determination of antioxidant activity [8].

In the spectroscopic method, the result of antioxidant efficiency is expressed as EC_{50} determined as the concentration of substrate that causes 50% loss in absorbance (DPPH activity). Sanchez-Moreno et al. [9], and Lebeau et al. [10] were the first to used the term EC_{50}. P. Molyneux suggested that the presenting of the results of antioxidant efficiency should involve the use of ascorbic acid as the standard due to doubts concerning the direct determination of DPPH obtained from calibration curve [11].

DPPH has been used to evaluate the antioxidant ability of phenol compounds [12], while Sun-Kun Yim et al. developed a continuous spectrophotometric assay for the reduction of DPPH by NADPH-cytochrome P450 reductase (CPR) in mixed ethanol–water solution [13].

Uric acid reaction with micelle-incorporated DPPH was explained by E. Abuin et al. [14], while D. Bandoniene and colleagues [15] detected the activity of radical scavenging compounds in mixed methanol–water solution using DPPH and HPLC–DPPH method. For the study of oxidation kinetics of amino acid, G. Ionita et al. [16] used water soluble derivate of DPPH.

M. Buijnsters and co-authors [17] evaluated the antioxidant activity of some antioxidants using both DPPH colorimetry and optothermal window detection at 514 nm.

B. Ozcelik and co-authors [18] determined the effects of light, oxygen, solvents and pH on the absorbance of DPPH at 517 nm.

Electrochemical methods for the determination of antioxidant activity based on the application of biosensors [19] to measure antioxidant properties of *tert*-butilhydroxyanisole and the cyclic voltammetry [20], that were used to study the efficiency of some particularly low molecular weight antioxidants, were also reported.

This study describes a new method for evaluation of the antioxidant activity based on the amperometric determination of DPPH free radical.

The amperometric determination of antioxidant activity was based on the same reactions according to Eqs. (1) and (2), but detection of the residual concentration of nonreacted free radical was achieved amperometrically. The resulting current on a glassy carbon electrode polarized at fixed potential was proportional to the concentration of unreacted DPPH free radical. The antioxidant activity of some water-soluble antioxidants present in blood, as well as ethanol-soluble antioxidants typically present in certain foods and drinks, were determined. Eight samples of different kind of tea, wine and other beverages were also tested with the aim to determine their antioxidant activity. The results are expressed as the Trolox equivalents obtained by dividing the value of the current difference measured in DPPH ethanol-buffer solution after addition of an antioxidant by the value of the current difference measured in the solution after Trolox addition. The prepared concentrations of tested antioxidants and Trolox were the same. The results of spectroscopic measurements were obtained using the same buffer–ethanol solution, and presented as Trolox equivalents obtained by dividing the absorbance difference of DPPH· after each addition of an antioxidant by the absorbance difference of DPPH· after Trolox addition using the solutions of the same concentration. The results of the Trolox equivalents obtained by amperometric method and the results determined spectrophotometrically at 518 nm are in good correlation.

2. Experimental details

2.1. Reagents and solutions

Commercially available chemicals of the highest purity were used without any further purification. 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, gallic, caffeic, and uric acid, L-glutathione (reduced form) and quercetin were obtained from Sigma-Aldrich (St. Louis, USA). *N*-acetyl-L-cysteine, (+) catechin hydrate, ferulic, sinapic, curcumin and Trolox (6-hydroksy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Fluka (Buchs, Switzerland). Sodium dihydrogen phosphate, sodium hydrogen phosphate, potassium chloride, HPLC grade methanol and absolute ethanol were purchased from Kemika (Zagreb, Croatia). Ten milliliters of 10 mM solutions of antioxidants were prepared daily. The solutions of water soluble antioxidants (L-ascorbic acid, L-glutathione, *N*-acetyl-L-cysteine, Trolox, gallic, and uric acid (5 mM) were prepared using double deionized water from Millipore–MilliQ system (USA). All other antioxidants were prepared in absolute ethanol. Ten milliliters of 5 mM stock solution of DPPH were prepared in absolute ethanol. The solution was ultra-sonicated (Transsonic 460/H, Elma, Germany) for about 2 h and kept in dark to minimize decomposition by light.

Phosphate buffer solution (0.033 M, pH = 7.4) containing 0.033 M KCl and 40% by volume of absolute ethanol was used in all electrochemical and spectrophotometric measurements.

2.2. Actual antioxidant samples

The tea solutions were prepared by weighing 0.3 g of the commercially available tea in 30 mL of deionized water heated at 60 °C. After 20 min of extracting, the samples were filtered and instantaneously used for analysis by amperometry while 10 times diluted tea solutions were used for spectrometric determination. The samples of wine, orange juice and black currant juice were used without preparation for amperometric measurements, but they were diluted 10 times for spectroscopic measurements.

2.3. Instrumentation and measuring set up

Electrochemical measurements were carried out on the Potentiostat 273 A (Princeton Applied Research, USA)

connected to the computer for data collection and analyzing. Cyclic voltammetry and chronoamperometric measurements were performed in the standard three-electrode electrochemical cell. Glassy carbon disc electrodes (d=3 mm) were employed as working and auxiliary electrodes while an Hg_2Cl_2 | 3 M KCl electrode was used as a reference electrode. The applied potential scan rate in cyclic voltammetry was 20 mV/s. For testing of Ip vs. scans rate dependence, the applied scan rates were 2, 5, 10, 20, 50, 100, 200, 500 and 1000 mV/s.

DPPH absorbance was measured at 518 nm with a double-beam DMS 80 (Varian, USA) spectrophotometer connected to the computer for data acquisition.

2.4. Spectroscopic determination

Scanning the wavelengths in the range between 700 and 250 nm for 100 μM of DPPH solved in methanol resulted in maximum absorption in the visible range at 518 nm. The same maximum of absorption was recorded in 100 μM of DPPH dissolved in mixed ethanol–water solution.

100 μM DPPH solution in ethanol–water was prepared in a standard l=1 cm glass spectroscopic cuvette. After reaching the steady state of absorbance at 518 nm, an antioxidant was added to the cuvette and mixed for 10 s. Concentration after the addition of antioxidants was 5 μM. For gallic acid, quercetin and catechin, the final concentration after addition to the spectroscopic cell was 2.5 μM. The changes in absorbance were read after the same period that is necessary for steady state readings in electrochemical experiments. For some antioxidants with rapid kinetics, the readings were done after 3 min, or after 15 min in case of slow kinetics.

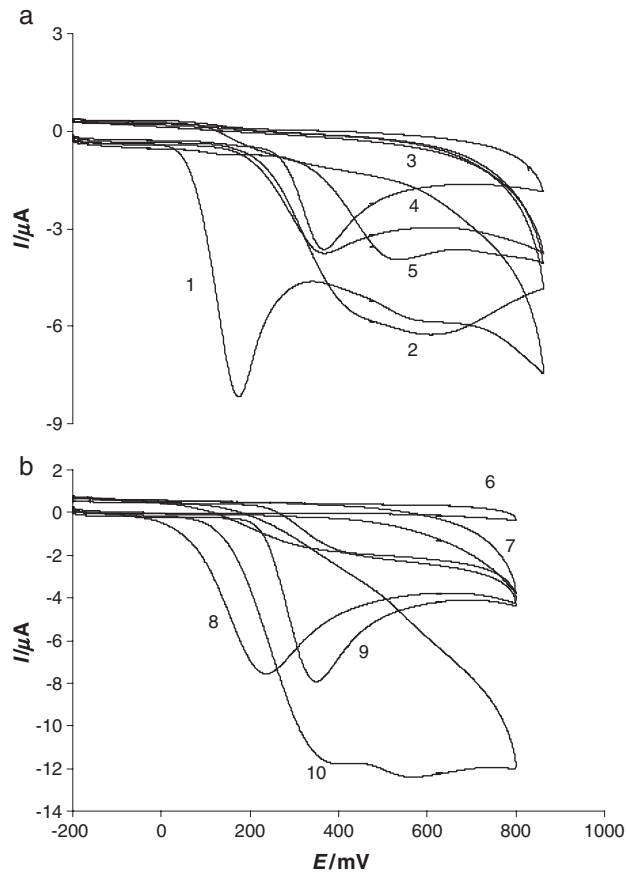

Fig. 1. (a) Cyclic voltammograms of ethanolic soluble antioxidants: (1) quercetin, (2) catechin, (3) curcumin, (4) sinapic acid, (5) ferulic acid. (b) Cyclic voltammograms of water-soluble antioxidants: (6) 40% ethanol–water solution containing 0.03 M KCl, pH=7.4, (7) *N*-acetyl-L-cysteine, (8) ascorbic acid, (9) uric acid, (10) gallic acid. Scan rate 20 mV/s, the concentration of antioxidant 1 mM, supporting electrolyte: phosphate buffer solution (0.03 M, pH=7.4) containing 0.03 M KCl.

3. Results and discussion

3.1. Cyclic voltammetry of DPPH

Cyclic voltammogram of DPPH (Fig. 2, bold line) shows existence of two redox couples, but the only redox pair important for antioxidant activity determination is DPPH· | DPPH. Cathodic (251 mV) and anodic (310 mV) peaks refer to the (DPPH· | DPPH) redox couple mentioned above. The difference between peak potentials of 59 mV implicates reversibility of the redox couple. Linear relationship (R^2=0.997) between the peak current and the square root of scan rate is indication of the diffusion control of the current without complication by adsorption process on the glassy carbon electrode.

3.2. Cyclic voltammetry of antioxidants

At the cyclic voltammograms (Fig. 1a) of ethanol soluble antioxidant (1 mM), oxidative peaks appeared at 153 mV (1), 334 mV (3), 335 mV (4) and 491 mV (5) for quercetin, curcumin, sinapic and ferulic acid, respectively, but without evidence of any reversible reduction peak. Oxidative peak without defined maximum was observed in the cyclic voltammogram of (+) catechin (2).

Cyclic voltammograms of water-soluble antioxidants (Fig. 1b) show irreversible oxidation peaks at 238, 350 and 400 mV for ascorbic (8), uric (9) and gallic acid (10), respectively, while the cyclic voltamogram of *N*-acetyl-L-cysteine (7) revealed no well defined oxidative peak. Negligible background current was evident in the examined potential range (6). All cyclic voltammograms were performed using buffered water-ethanol solution pH=7.4 containing 1 mM of an antioxidant. The employed scan rate was 20 mV/s.

Fig. 2 shows the cyclic voltammograms of (1) DPPH (0.3 mM), (2) caffeic acid (1 mM) and (3) Trolox (1 mM). In contrast to the previously mentioned antioxidant cycle voltammograms for DPPH, caffeic acid and Trolox show the occurrence of oxidative and reduction peaks. Voltammograms in Fig. 2 also show a pronounced difference in the

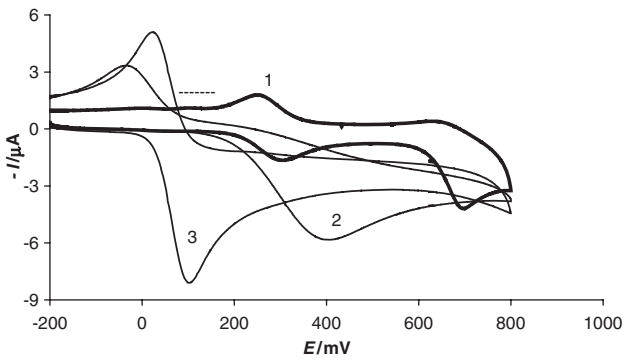

Fig. 2. Cyclic voltammograms of 0.3 mM DPPH in the radical form (1), 1 mM caffeic acid (2), and 1 mM Trolox (3). Scan rate 20 mV/s. Supporting electrolyte as in Fig. 1.

potentials of reduction peaks between DPPH (251 mV), Trolox (35 mV), and caffeic acid (−33 mV).

3.3. Amperometric determination of antioxidants

The amperometric method proposed for the determination of antioxidant activity is based on the electrochemical reduction in DPPH at the glassy carbon electrode polarized at the potential lower than the reduction potential of DPPH· | DPPH redox couple. However, caffeic acid and Trolox limited the range of working potentials to the region between the reduction potential of DPPH and their own reduction potentials. In the case of studied antioxidants, the working potential can be chosen in the range between 50 and 200 mV (dashed line in Fig. 2). To avoid possible electrochemical interferences of caffeic acid and Trolox, the potential in the middle of that range, i.e., at 140 mV, was selected as the working potential.

The current-time diagram (Fig. 3) shows the response of the glassy carbon electrode polarized at 140 mV vs. calomel | 3M KCl reference electrode in the buffered water–ethanol solution after the addition of DPPH· free radical. Each addition of DPPH· in steps of 10 μM (left side of chronoamperogram) intensified current response. In the concentration range between 0–100 μM of DPPH·, the current linearly depended on the concentration of DPPH· (I/μA=(0.010±0.004)+(0.0057±6.69 · 10 $^{-5}$) · c/μM, R=0.9994). The current response obtained in 100 μM of DPPH in the radical form after the addition of Trolox is showed on the right side of Fig. 3. Each of the thirteen steps refers to electrode response after the addition of the same standard concentration (2.5μM) of Trolox. The calibration graph plotted from the data obtained in the above described experiment is shown in Fig. 4. Calibration curve for current response vs. concentration of Trolox showed good linearity (I/μA=(0.625±0.003)−(0.020±0.001) · c/μM, R=0.9998) up to 30 μM of Trolox. The sensitivity of the method was 20.3 nA/μM of Trolox while the limit of detection accomplished by the used measuring device was 0.05 μM. The limit of detection was calculated as a triple value of the standard deviation of background current.

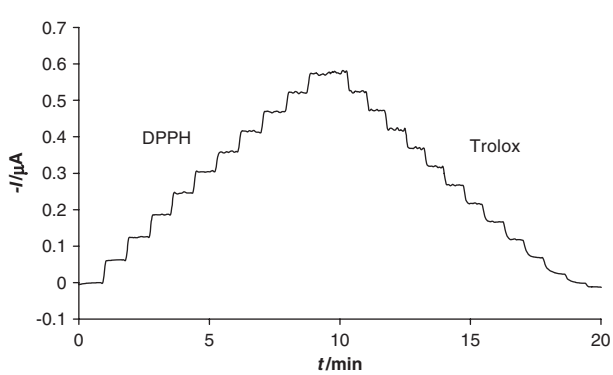

Fig. 3. Current response for DPPH free radical on the glassy carbon electrode. Working potential 140 mV vs. calomel | 3M KCl electrode in mixed ethanol–water solution pH=7.4. Each step on the left side of the chronoamperogram refers to the addition of 10 μM of DPPH free radical. Current-time response curve for the glassy carbon electrode after the addition of Trolox to the initial concentration of 100 μM DPPH· is shown on the right side of the chronoamperogram. Each step corresponds to the addition of 2.5 μM of Trolox.

3.4. Activity of DPPH free radical in phosphate ethanol–water buffer solution

According to Brand-Williams et al., the stoichiometry for DPPH–Trolox reaction was determined as 2:1. From the results obtained by the calibrating curve, 30 μM of Trolox was equivalent to 60 μM of DPPH in the radical form. Evidently, there is a difference between the prepared DPPH concentration (100 μM) and the one determined in the experiment (60 μM). The spectroscopic calibration curve for Trolox (Fig. 5) obtained using 200 μM DPPH in ethanol–water solution after the thirteenth addition of 5 μM Trolox shows the same equivalent concentration range as established by the calibration curve for Trolox determined amperometrically. The same equivalent concentration of DPPH in the radical form determined electrochemically and spectrometrically implicated that the active DPPH· concentration was affected by the used solvent, which is in agreement with the literature data [11].

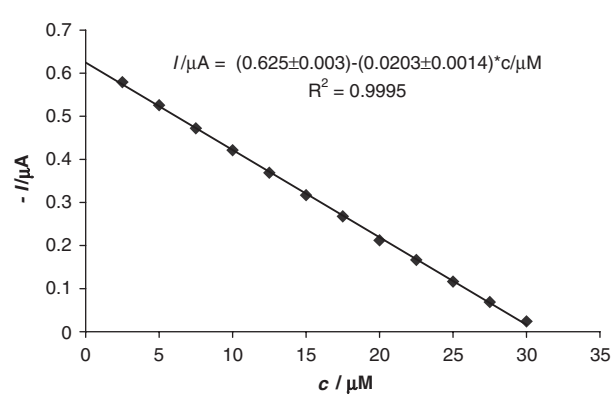

Fig. 4. Calibration graph for Trolox. Experimental conditions as in Fig. 3.

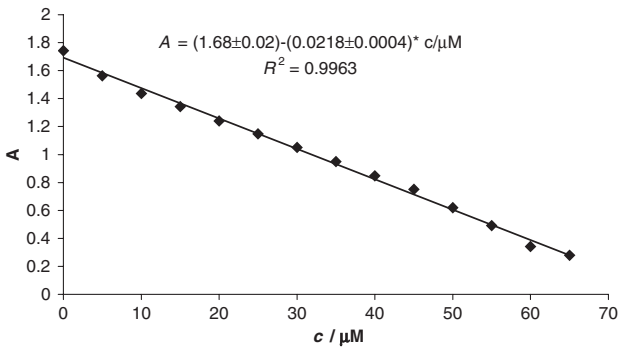

Fig. 5. Calibration graph for Trolox determined spectrophotometrically. Experimental conditions: 200 μM DPPH· (initial concentration), each point corresponds to the addition of 5 μM of Trolox, $\lambda = 518$ nm.

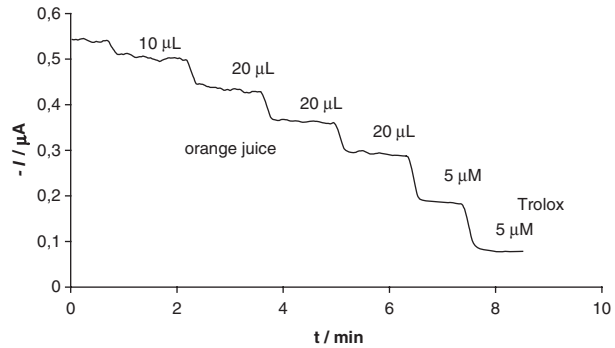

Fig. 6. Chronoamperometric diagram for orange juice sample. Experimental conditions were the same as in Fig. 3.

3.5. Sample analysis and comparison of results

Antioxidant activity for eleven pure compounds, the samples of tea, juices and wine determined spectroscopically and amperometrically were summarized in Table 1. The electrochemical determination of antioxidant activity was performed under the same experimental condition used for the calibration diagram of Trolox. Fig. 6 shows amperometric response of the glassy carbon electrode polarized at 140 mV vs. calomel reference electrode to four additions of orange juice and two additions of Trolox standard solution (5 μM) into the measuring cell. The first current step belongs to the addition of 10 μL of undiluted orange juice while the three other steps correspond to the addition of 20 μL of undiluted orange juice into the measuring cell. Spectroscopic determination was performed in 100 μM DPPH ethanol–water solution at 518 nm. Each data item is the average result of five successive additions of an antioxidant to the 100 μM DPPH· solution.

The Trolox equivalents obtained by the proposed amperometric method were calculated according to a simple equation: Tr.eq.amp. $= \frac{(I_0 - I)\text{sample}}{(I_0 - I)\text{Trolox}}$, where I_0 represents the initial current obtained in the buffer solution containing DPPH in the radical form while I represents the current intensity after the addition of a sample or standard (Trolox) into the measuring cell. The difference given by $I_0 - I$ denotes antioxidant activity. If the concentration of DPPH· significantly changed after the addition of antioxidant, the current response proportionally decreased, and the corresponding current difference $(I_0 - I)$ increased. The Trolox equivalents determined spectrophotometrically were calculated according to a similar equation: Tr.eq.spectro. $= \frac{(A_0 - A)\text{sample}}{(A_0 - A)\text{Trolox}}$ where A_0 is defined as the initial absorbance of DPPH· and A is the absorbance of DPPH· after the addition of a sample or standard (Trolox) into a spectrophotometric cuvette. As evident from Fig. 7, the correlation coefficient, $R = 0.9996$, the slope close to unity ($a = 1.011 \pm 0.007$) and the intercept close to 0 (-0.04 ± 0.03) confirmed the good agreement of measurements between the proposed amperometric and reference spectroscopic method.

Table 1
Trolox equivalents for pure compounds of antioxidants, and the samples of tea, wine and juices determined amperometrically and spectroscopically

	Trolox eq. amperometric	Trolox eq. spectroscopic
Trolox	1.00	1.00
Non-phenolic antioxidants		
L-ascorbic acid	0.89	0.90
Uric acid	0.84	0.91
Gallic acid	2.33	2.31
N-acetyl-L-cistein	0.49	0.53
L-glutathione	0.30	0.32
Phenolic antioxidants		
Caffeic acid	0.87	0.86
Ferulic acid	0.54	0.57
Sinapic acid	0.82	0.89
(+) catechin hydrate	2.47	2.51
Quercetin	2.65	2.87
Real samples		
Sage tea	0.88	0.90
Rosehip tea	5.68	5.60
Tropic fruit tea	1.91	1.85
Chamomile tea	0.53	0.57
Green tea	8.12	8.04
Red wine	11.75	11.50
Orange juice	1.60	1.55
Black currant juice	9.03	9.20

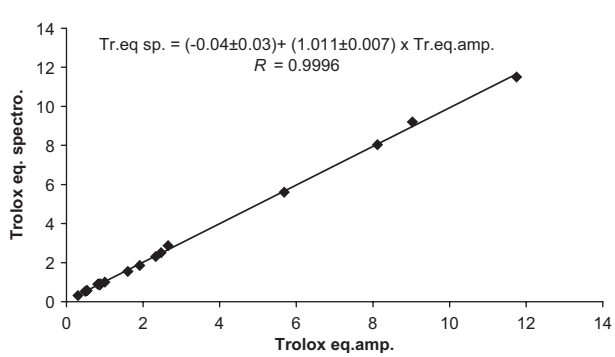

Fig. 7. Correlation graph for Trolox equivalents determined amperometrically and spectrophotometrically.

4. Conclusion

Amperometric method for the evaluation of antioxidant activity has the same advantages and disadvantages as the compared spectrophotometric method since both methods are based on the same chemical reaction and kinetics. However, spectrophotometric method can be easily affected by the color and turbidity of a sample, demanding sample pretreatment. The amperometric method based on the applied glassy carbon electrode showed good chemical stability, and nontransparent and turbid samples did not affect the response of the detector. Good agreement between the results obtained by the proposed amperometric methods and the classic spectrophotometric method confirmed the usefulness of the amperometric method for the determination of antioxidant activity of a wide variety of samples.

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