STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, XLV, 2, TOM I, 2010

Dedicated to the memory of Prof. dr. Ioan Silaghi-Dumitrescu marking 60 years from his birth

KINETIC INVESTIGATION IN TROLOX-DPPH· SYSTEM

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ABSTRACT. A kinetic study using voltammetry and spectrophotometric measurements on the Trolox/DPPH•, in hydro-ethanolic and ethanolic medium is presented. The heterogenous electrochemical rates were obtained from the peak parameters in quasireversible and irreversible conditions and the homogenous rate constant and partial reaction orders were obtained using reaction half-life.

Keywords: *Antioxidants, Trolox, DPPH•, voltammetry, spectrophotometry, kinetics*

INTRODUCTION

A free radical is a chemical species able of independent existence, possessing one or more unpaired electrons. Biologically important free radicals are thus highly unstable molecules that have electrons available to react with various organic substrates, generated by oxidative stress that can lead to a series of biochemical alterations through chain radical reactions. The natural defence against the oxidative stress is based on the action of antioxidant compounds, also known as radical scavengers. They usually act by neutralising the unstable free radical molecules by supplying them with electrons, thus preventing or at least limiting the chain reactions that cause tissue damage [1-2]. There are several categories of substances acting as antioxidants, including vitamins, inorganic compounds, essential amino acids and polyphenols. Their capacity to scavenge free radicals is now universally recognised although the true mechanism by means of which they act is still not fully understood [3].

Several methods have been proposed for the detection of antioxidants, based on photometric, fluorimetric, chromatographic and electrochemical approaches. Inherent limitations of the analysis differ from method to method (low sensitivity, interferences, slow detection and cost of equipment) and have

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stimulated the study of simple, fast and sensitive methods for the characterization of antioxidants. In the past two decades the electrochemical methods, especially the amperometric ones, have been intensively used for antioxidant detection mostly because are fast and less expensive [4].

One of the most employed method of antioxidant activity determination uses a stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•). Having intense colour, which changes from violet to pale yellow during reaction with antioxidants, this free radical is suitable for spectrophotometric determination [5]. Because DPPH• free radical can exchange electrons at an electrode interface, its reaction with antioxidants is suitable also for electrochemical detection. In both mentioned cases, the antioxidants activity can be determined by following the consumption of DPPH• free radical. Correlating to free radical consumption when using a standard antioxidant, like 6-hydroxi-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid (Trolox), it is possible to calculate the antioxidant activity of a complex natural matrix of antioxidants in terms of Trolox equivalent antioxidant activity [6].

The present study aims to investigate the elementary processes that occur during operation of an amperometric sensor for antioxidant activity determination. It is of interest to study the electrochemical behaviour of Trolox and DPPH• independently and in mixtures, employing spectrophotometric techniques for those processes that do not take place at electrode interface. After the obtaining of kinetic and thermodynamic information, it is possible to operate the amperometric detection in condition in which a judicious balance between desired performance descriptors can be more easily achieved.

RESULTS AND DISCUSSION

A detailed knowledge of the redox processes that occur in Trolox/ DPPH• system during amperometric detection of antioxidant activity requests a preliminary investigation of electrochemical behavior of isolated components of the system.

Cyclic voltamograms of Trolox (Figure 1a) show the presence of one pair of voltammetric peaks, indicating chemically reversible nature for Troloz reduction. The asymmetry of the peaks, with the anodic one smaller and broader, corroborated with the significant peak separation is the indication of a relative slow charge transfer. Figure 1b and 1c contain the influence of scan rate on the peak parameters. Presented data indicate transition between quasireversible and irreversible behavior when increasing the scan rate, as can be evidenced by deviation from linearity of the representations from Figure 1b and 1c.

Attempting to obtain physico-chemical information, one can use the peak parameters to calculate some thermodynamic and kinetic parameters.

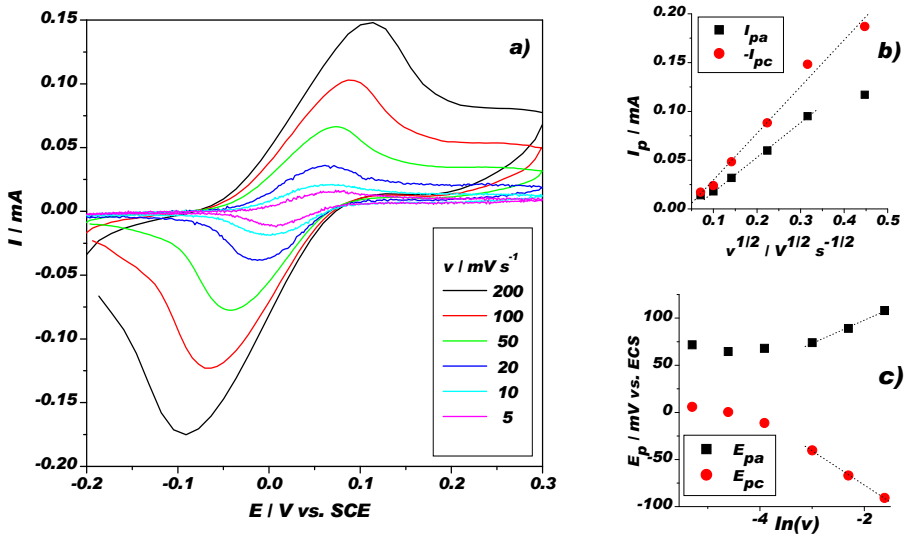

Figure 1. Voltammetric response of Trolox 4 mM hydro-ethanolic solution; the influence of the scan rate on cyclic votammograms, in a), on peaks current, in b), and on peaks potential, in c).

To simplify the requested analysis, in the present work the formal standard potential was calculated using the data from quasireversible domain, by simple mediation of the anodic and cathodic potentials, whereas calculation of the other parameters was performed using the data from irreversible domain. In these circumstances, the formal standard potential was found to be $E^{o'}$=0.03 V *vs.* SCE.

For an irreversible behavior, the peak parameters are given by:

$$I_{p,a} = 2.99\,10^5 n\left[(1-\alpha)n_a\right]^{1/2} AD^{1/2}C^* v^{1/2}$$

$$I_{p,c} = -2.99\,10^5 n\,(\alpha n_a)^{1/2} AD^{1/2}C^* v^{1/2}$$

$$E_{pa} = E^{o'} - \frac{0.02569}{(1-\alpha)\,n_a}\left[0.78 - \ln\left(\frac{\sqrt{D_R}}{k^o}\right) - \ln\left(\sqrt{\frac{(1-\alpha)n_a}{0.02569}v}\right)\right] \quad (1)$$

$$E_{pc} = E^{o'} - \frac{0.02569}{\alpha\, n_a}\left[0.78 + \ln\left(\frac{\sqrt{D_O}}{k^o}\right) + \ln\left(\sqrt{\frac{\alpha\, n_a}{0.02569}v}\right)\right]$$

where n is the (total) number of exchanged electrons, n_a the number of electrons involved in the rate determining step, α the transfer coefficient, A the surface, v the scan rate, C* the concentration, D the diffusion coefficient

of the reacting species (when mentioned, O denotes the oxidized and R, the reduced; otherwise denotes the reacting species) and k^o the standard rate constant [7].

In order to perform the parameter calculation, the peak parameters were plotted on convenient coordinates (I_p vs. $v^{1/2}$ and E_p vs. ln(v), as can be seen in Figure 1b and 1c, respectively) to ensure linearization. Using the slopes obtained by linear fitting of data presented in Figure 1c - 0.0246±0.0015 and 0.0364±0.0012 for anodic and, respectively, catodic peaks - one can first calculate n_a≈1 and α=0.60±0.02, values consistent to mentioned peak asymmetry. The next step employs the I_p vs. $v^{1/2}$ dependence, described by the slopes of linear correlation found to be $(3.56\pm0.07)10^{-4}$ and $(4.79\pm0.29)10^{-4}$, respectively. Assuming n=1, one can calculate D=1.2 10^{-6} cm^2 s^{-1}, value corresponding to the reduced form of Trolox. To calculate the standard rate constant, the intercepts of Figure 1c (0.147±0.004 and 0.150±0.003, respectively) lead to calculation of $k^o=(2.9\pm0.2)\ 10^{-4}$ cm s^{-1}.

Cyclic voltamograms of DPPH• (Figure 2a) show in the domain of potential employed of the Trolox study the presence of one pair of voltammetric peaks that reveal a chemically reversible nature of electron exchange of studied free radical. An additional chemical irreversible oxidation peak, not presented here, is not important for the present study, since it occurs at potentials well above the interest domain, where both Trolox and DPPH• exchange electrons [8]. As in Figure 1, the shape of the peaks and relative proportional influence of square root of scan rate on the peak currents suggest a redox process involving soluble species, without contribution of adsorbed species.

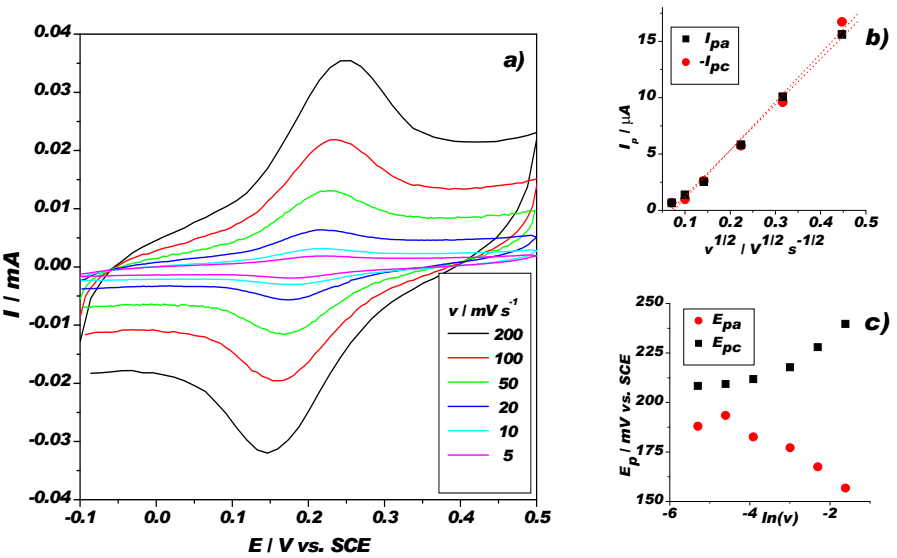

Figure 2. Voltammetric response of DPPH• 0.2 mM hydro-ethanolic solution; the influence of the scan rate on cyclic votammograms, in a), on peaks current, in b), and on peaks potential, in c).

As compared to Trolox, DPPH• presents more symmetric peaks, less separated, denoting a significant faster rate. Consequently, the linearization correlation between peak potential and ln(v) takes place on higher scan rates, as can be seen in Figure 2c. In these circumstances, the voltammetry was performed under quasireversible behavior, and mathematical analysis aiming calculation of physico-chemical information must be performed accordingly. The standard formal potential was calculated as above, being found $E^{o'}$=0.19 V *vs.* SCE. For a quasireversible system Matsuda and Ayabe proposed a diagram that correlates the peak separation with an *ad hoc* kinetic term

$$\Lambda = k^o \sqrt{\frac{0.02569}{D_O^{1-\alpha} D_R^{\alpha} v}} \tag{2}$$

and links up the peak current to scan rate by using a second *ad hoc* kinetic term Ψ, both *ad hoc* kinetic terms being correlated through another diagram [9]. Under these assumptions, we found n≈2, $D_{DDPPH\bullet}$=5.9 10^{-6} cm^2s^{-1}, k^o=1.3 10^{-2} cm s^{-1}.

For a full kinetic description of Trolox-DPPH• system a final voltammetric investigation was performed in order to describe the homogenous redox process. As can be seen in Figure 3, during addition of Trolox the peaks corresponding to DPPH• couple is marginable influenced, mostly due to modification of base line current. Moreover, the peak currents of Trolox couple are direct proportional to added Trolox concentration.

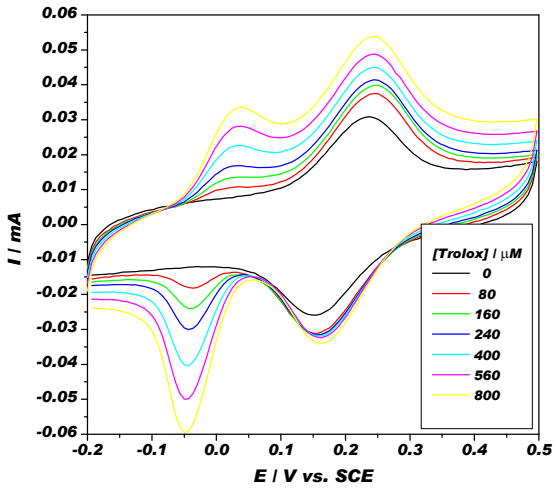

Figure 3. Voltammetric response of DPPH• 0.2 mM hydro-ethanolic solution with Trolox of concentration indicated in legend. Scan rate is 0.1 V s^{-1}.

All these suggest that, under studied experimental condition, the so-called homogenous coupled reaction cannot be evidenced. To obtain some kinetic information about this reaction, spectrophotometric measurements were further performed.

The reaction between DPPH• and Trolox in hydro-ethanolic mixtures was completed in few seconds, which prohibits kinetic measurement using employed instrumentation. For such fast systems, kinetic investigations can be made only in stopped-flow system. However, using ethanolic solution reaction is significant slower, allowing a preliminary insight into the kinetics in investigated system. Even if the values found in ethanolic solution could be related to previously found heterogeneous rate constants only with large precautions, the spectrophotometric measurements where further restricted to alcoholic solutions.

Ethanolic solutions of DPPH• obey Lambert-Beer law up to 2 10^{-4} M, absorption maximum was found at 516 nm with a molar extinction coefficient of 19 10^4 $M^{-1}cm^{-1}$. Reaction between ethanolic solutions of DPPH• and Trolox was reported as 1 mol of this antioxidant reduces 2 mol of free radical [6].

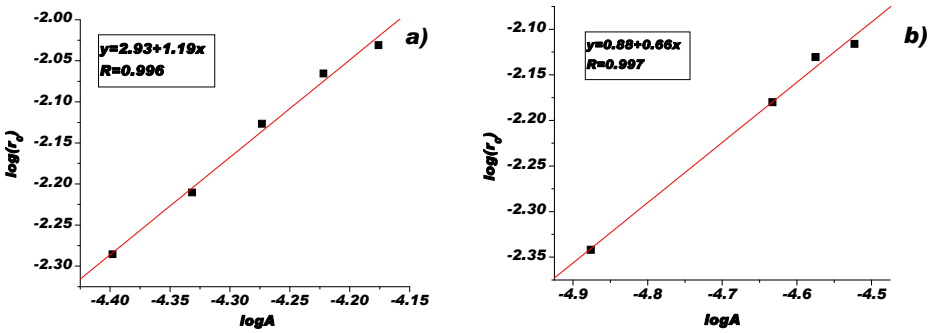

Figure 4. Dependence of initial rate on the DPPH• absorbance at constant DPPH• concentration, a), and at constant Trolox concentration, b).

Using different initial concentration of both species, on the basis of the rate law having the following expression, $r_0 = k\, c_{0DPPH}^{a}\, c_{0Trolox}^{b}$, partial reaction orders of 0.7 and 1.2 with respect to Trolox and DPPH•, respectively, were determined from the initial rates of the reaction, see Figure 4. As suggested by Brand-Williams and Bondet [5], the rate constant was determined from the half-life of the reaction:

$$t_{1/2} = \left(\left(2^{a+b-1} - 1\right)/ k_e\right) \times c_{DPPH\bullet}^{1-a-b} \qquad (3)$$

where σ=2 represents the DPPH· number of mol reduced by the antioxidant and k_e is related to the rate constant by:

$$k_e = (a + b - 1)\frac{k}{\sigma^b} \quad (4)$$

The values of the rate constant determined at two different temperatures are presented in the table 1.

Table 1. The rate constant determined at different temperatures.

T (K)	$10^5 \cdot c_{0DPPH}$ (M)	$t_{1/2}$ (s)	k_e ($M^{-1} \cdot s^{-1}$)	k ($M^{-1} \cdot s^{-1}$)	$\bar{k}$ ($M^{-1} \cdot s^{-1}$)
298	6	48	113.8	206.0	203.3
	4	71	110.8	200.6	
293	6	55	99.3	180.6	184.7
	4	75	104.9	189.8	

CONCLUSIONS

The stable free radical DPPH•, which can be amperometrically detected, is consumed by the antioxidants from analysed sample allowing to evidence the antioxidants by quantifying the decrease of the DPPH• concentration. Trolox is further employed to quantify the activity of the natural antioxidants present in an analysed sample. We will further investigate the way in which the experimental finds endorse the utilisation of an amperometric sensor based on investigated system.

The results prove that the DPPH• free radical can be reduced during a reversible process, on a relative positive potential ($E^{o'}$=0.19 V *vs.* SCE). There are two positive consequences: the value of formal standard potential contributes to a good selectivity as analytes with more negative formal standard potential would not interfere, and de reversible nature of this process allows increasing of the sensibility as the oxidation product is available to re-reduction during a homogenous coupled chemical process. Another important original find is the kinetic constant of this heterogeneous reaction; the reasonable high value, of k^o=1.3 10^{-2} cm s^{-1}, is beneficial for the detection as the fast rates is a prerequisite for a low response time of detection.

The electrochemical investigation of Trolox revealed a reversible process at a more cathodic formal standard potential ($E^{o'}$=0.03 V *vs.* SCE). The difference of mentioned formal standard potentials constitute the driving force of reaction between Trolox and DPPH• in the investigated conditions. A smaller value would give a certain degree of reversibility, with detrimental reducing of linearity of the amperometric sensor. More important, the voltammetric peaks of Trolox are clearly separated from those of DPPH• free radical, allowing the use of analytical techniques that request the presence of Trolox in the investigated sample with antioxidants.

The last attempt was to investigate the homogenous redox reaction between Trolox - DPPH•. As the catalytic coupling was not evidenced by voltammetry, spectrophotometric techniques were employed. Yet again the kinetics was favourable, too fast to even evaluate the rate constant with utilized instrumentation when the reaction was investigated in hydro-ethanolic solution. Even in pure ethanolic solution the homogenous rate constant was reasonable large, of 203 M^{-1} s^{-1}, ensuring a small measuring time for the amperometric detection. The only draw-back is given by subunit value, of 0.7, for DPPH• free radical reaction order; poor linearity should be expected for the samples containing Trolox if the measurements are performed without reaching the equilibrium.

EXPERIMENTAL SECTION

Instrumentation

The voltammetric measurements were performed using a Computer controlled (via an AT-MIO-16F-5, National Instruments, USA, data acquisition board) analogical potentiostat (PS3, Meinsberg, Germany). A standard three-electrode electrochemical cell configuration was employed for the measurements. The working electrode was glassy carbon (A=0.0314 cm^2) reference electrode was a double-junction saturated calomel electrode (SCE) and the counter electrode was a spiralled Pt wire. Voltammetric measurements were performed at a temperature of 25$\pm$1 ^oC.

Spectrophotometric measurements were carried out on a Jasco V-530 UV/VIS spectrophotometer connected to a computer for data acquisition. Standard (1 cm x 1 cm x 4.5 cm) glass spectroscopic cuvette was used for visible absorbance measurement. Temperature was controlled by using an M 20 Lauda thermoregulating system. DPPH• spectrum was recorded in the range of 800 and 400 nm. Time course measurements were recorded at 516 nm wavelength at 25±0.5 ºC. Ultrasonication of stock solutions was performed with Transsonic T 420, Elma.

Reagents and solutions

Commercially available chemicals were obtained from Sigma-Aldrich. DPPH• stock solutions were prepared in absolute ethanol and ultra-sonicated for about 30 minutes. In order to avoid thermal decomposition and by light the solutions were kept in refrigerator. Trolox was dissolved in absolute ethanol and in hydro-alcoholic phosphate buffer. The hydro- ethanolic solutions (25% vv ethanol) were prepared by adding ethanol to the buffer solution with desired pH of 7.4. All the solutions contained 0.1 M KCl as supporting electrolyte.

ACKNOWLEDGMENTS

The authors acknowledge for the financial support granted by the National Research Council, project SENSALIM, PN II Nr. 71/098-2007.

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