Evaluation of the Antioxidative Activity by Measuring the Rate of the Homogeneous Oxidation Reaction with Ferroceniumdimethanol Cation. Comparative Analysis of Glutathione and Ascorbic Acid

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A simple methodology for estimation of the antioxidative activity by measuring the kinetics of the homogeneous electron transfer reaction between an antioxidant and ferroceniumdimathanol cation (Fc^+) is proposed. The method is based on the well-known regenerative E_rC' catalytic reaction scheme, where the antioxidant is catalytically oxidized by Fc^+, which is *in situ* formed by the reversible oxidation of ferrocenedimethanol at glassy carbon electrode. Comparing the rate of the homogeneous oxidation of different antioxidant with the same oxidant, i.e., Fc^+, which serves as a referent system, a more consistent comparison of the antioxidative activity can be done than comparing the heterogeneous oxidation reactions at the electrode surface. The applicability of the method is demonstrated in a comparative analysis of glutathione (GSH) and ascorbic acid (AA), the two important and widely abundant low molecular weight antioxidants, by means of cyclic voltammetry (CV), square-wave voltammetry (SWV), and chronoamperometry (CA). At physiological conditions (pH ~ 7) the rate of AA oxidation (~ 10^4 mol^{-1} L s^{-1}) is two orders of magnitude higher than GSH (~ 10^2 mol^{-1} L s^{-1}) implying superior antioxidative activity of the former. From a methodological point of view it is important to stress that the kinetic data estimated with CV and CA are comparable, while SWV gives much higher estimates for the catalytic rate constants. This implies that different techniques lead to different kinetic data, which must be taken into account in estimating the antioxidative activity, as well as in comparing the kinetic data reported in the literature.

1. INTRODUCTION

The danger of oxidative stress is now well anticipated and appropriately considered in the medicine [1, 2]. It is a well known fact that an increase of the free radicals production can damage cardiovascular system by oxidation of low density lipoproteins, accelerate aging processes [3], and has been linked to other very serious pathologies such as brain stroke, diabetes, rheumatoid arthritis, Parkinson's and Alzheimer's diseases, and cancer. Free radicals, mainly present as oxygen-related species (ROS), are continuously produced in the human body through a variety of metabolic processes. Owing to their strong oxidizing activity, these highly reactive species can practically damage most of the physiologically active molecules. It is thus clear that the presence in the body of sufficiently high level of antioxidant species represents a vital protection of the body.

Essentially, the action of an antioxidant, in particular the low molecular weight antioxidants (e.g., glutathione, ascorbic acid, uric acid, bilirubin, etc.), is seen in the homogeneous electron exchange reaction with the ROS species, where the antioxidant is being oxidized and thereby preventing oxidation of other physiologically important molecules. The latter redox reaction is energetically driven by the difference in the redox potentials and controlled by the electron exchange rate between the two reactants. As the action of antioxidants is principally an electrochemical process, the electrochemical techniques are *a priori* the most appropriate for studying the antioxidative mechanism and activity. Hence, in the last decade growing efforts have been done to develop electrochemical methods to assess the antioxidative capacity of various species [4-11]. Nevertheless, the quantitative studies providing inherent physicochemical parameters, such as rate constants of the homogenous redox reactions of antioxidants are rare [7, 12]. In most of the studies, the antioxidant capacity is estimated by measuring the anodic current at solid electrodes due to oxidation of an antioxidant [5, 6, 8, 10, 11, 13-15]. However, the rate and the thermodynamics of the heterogeneous oxidation process at the electrode/electrolyte interface can differ significantly from the homogenous oxidation reaction between two species embedded in a single medium. There are several studies of merit where the antioxidative status is estimated in relation to the ROS species, electrochemically generated by the electrode reduction of oxygen [7, 16]. Yet, detection of the electrochemical response of the irreversible reduction of oxygen at various electrodes is not straightforward, due to the complexity of the electrode reaction.

In the present communication, we propose a simple methodology for estimation of the antioxidative activity by measuring the rate of the homogenous oxidation reaction of the antioxidant with ferroceniumdimethanol (Fc^+) cation. The latter reactant is in *situ* formed by electrochemical reversible oxidation of ferrocenedimethanol (Fc) at a glassy carbon (GC) electrode. Comparing the rate of the electron exchange of different antioxidative species with Fc^+, a relative scale of the antioxidative activity can be established, where Fc^+ serves as the reference, which is a common approach in electrochemistry. The main prerequisite for this methodology is the standard potential of the studied antioxidant to be more negative than the standard potential of the Fc^+/Fc couple. The latter couple has been selected for the purpose of the study due to its sufficient water solubility, fast and electrochemically reversible electrode reaction at different electrode surfaces, and sufficient chemical stability of the two components of the redox couple under variety of experimental conditions [17].

In the proposed experimental arrangement, the rate of the homogenous oxidation reaction can be measured by applying conventional voltammetric and chronoamperometric techniques, based on the theory of the simple E_rC' regenerative catalytic mechanism [18]. The applicability of the proposed methodology is exemplified by the comparative analysis of the antioxidative activity of the three-peptide glutathione (GSH) [19, 20] and ascorbic acid (AA) [21], the two widely abundant low-molecular weight antioxidants in the living systems. Although it is well-known that these compounds have strong antioxidative activity, their direct oxidation at many electrode materials requires large overpotentials as the electrode process is hindered by complex surface phenomena [22] and slow electron exchange rate [23, 24]. The electrocatalytic oxidation of both GSH [25, 26] and AA at different electrode surface [27, 28], including those modified with ferrocene derivatives [29-33], is extensively reported, however, the quantitative studies are yet scarce [12, 29, 30-32].

2. EXPERIMENTAL

All chemicals used were of analytical grade purity. Aqueous solutions were prepared in doubly distilled water. Ferrocenedimethanol (product of Fluka) stock solution was prepared in 96 % (volume parts) methanol, whereas ascorbic acid and glutathione stock solutions were prepared in water. Britton-Robinson buffers were prepared by dissolving 2.7 mL 85% H_3PO_4, 2,3 mL glacial CH_3COOH and 2,48 g H_3BO_3 in 1 L H_2O. Particular pH value was adjusted by mixing with 0.20 mol/L NaOH solution.

Electrochemical measurements have been performed applying cyclic voltammetry (CV), square-wave voltammetry (SWV), and chronoamperometry (CA), using µAutolab multimode potentiostat/galvanostat (ECO Chemie, Utrecht, Netherlands), driven by GPES 4.4 software (Eco Chemie). A three-electrode configuration was employed, consisting of a glassy carbon as the working electrode, Ag/AgCl (3 M KCl) and platinum wire as the reference and counter electrodes, respectively. Prior to electrochemical measurements, the solutions were deoxygenated by purging with nitrogen for 10 minutes. All experiments were performed at room temperature in a 10 mL electrochemical cell.

3. THEORETICAL BACKGROUND

Although the electrocatalytic redox mediated oxidation of the studied antioxidants could be more complex [12], the overall electrochemical mechanism can be assimilated to the following well-known regenerative catalytic E_rC' reaction scheme:

$$Fc(aq) = Fc^+(aq) + e^- \qquad (E_r) \qquad (1)$$

$$Fc^+(aq) + A(aq) \rightarrow Fc(aq) + A^+(aq) \qquad (C') \qquad (2)$$
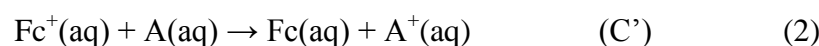
The electrode reaction (E_r) is assumed to be electrochemically reversible, characterized by the formal potential E_c°, whereas the homogeneous redox reaction (C') between Fc^+ and the antioxidant A, is characterized by the second order rate constant k in units of mol^{-1} dm^3 s^{-1}. Assuming the concentration of the antioxidant A to be large enough, thus being virtually constant in the course of the voltammetric experiment, reaction (2) can by characterized by the pseudo-first order rate constant $k_c = k c_A^*$ in units of s^{-1}. The general solution of the electrode mechanism is represented by the following integral equation:

$$\frac{c_{Fc}^*}{1+e^{-\varphi(t)}} = \frac{1}{FS\sqrt{D}} \int_0^t I(\tau) \frac{e^{-k_c(t-\tau)}}{\sqrt{\pi(t-\tau)}} d\tau \tag{3}$$

In eq. (3) F is the Faraday constant, S is the electrode surface area, D is the common diffusion coefficient of both Fc and Fc^+, and c_{Fc}^* is the bulk concentration of Fc. In addition, $\varphi(t) = \frac{F}{RT}(E(t) - E_c^{\circ})$ is the dimensionless electrode potential function, and other symbols have their common meaning. Recently, we have presented an analytical solution for the experiment under linear potential sweep voltammetry [34], which adopted for the present system reads:

$$I(t) = c_{Fc}^* FS\sqrt{D} \left(\int_0^t \frac{e^{-k_c\tau}}{\sqrt{\pi\tau}} \frac{e^{-(\varphi_i+\theta(t-\tau))}\theta}{\left[1+e^{-(\varphi_i+\theta(t-\tau))}\right]^2} d\tau + \frac{e^{-k_c t}}{\sqrt{\pi t}\left(1+e^{-\varphi_i}\right)} \right) +$$
$$+ c_{Fc}^* FS\sqrt{Dk_c} \left(\int_0^t erf(\sqrt{k_c\tau}) \frac{e^{-(\varphi_i+\theta(t-\tau))}\theta}{\left[1+e^{-(\varphi_i+\theta(t-\tau))}\right]^2} d\tau + \frac{erf(\sqrt{k_c t})}{1+e^{-\varphi_i}} \right) \tag{4}$$

where $\theta = \frac{F}{RT}v$, v is the sweep rate, $\varphi_i = \frac{F}{RT}(E_i - E_c^{\circ})$, and E_i is the initial potential of the sweep.

For the experiment under conditions of SWV, the solution is given by the following recurrent formula [35]:

$$I_m = \frac{FSc_{Fc}^*\sqrt{Dk_c}}{M_1(1+e^{\varphi_m})} - \frac{1}{M_1}\sum_{j=1}^{m-1} I_j M_{m-j+1} \tag{5}$$

where $M_m = erf\left(\sqrt{\frac{k_c(m-1)}{50f}}\right) - erf\left(\sqrt{\frac{k_c m}{50f}}\right)$ is the numerical integration factor, and f is the frequency of the potential modulation. For numerical integration [36], each potential pulse of the SW modulation is divided into 25 time increments. The serial number of time increments is designated with m.

For the chronoamperometic experiment, the following analytical solution is valid:

$$I(t) = \frac{FSc_{Fc}^{*}\sqrt{D}}{1+e^{\varphi}}\left[\frac{e^{-k_c t}}{\sqrt{\pi t}} + \sqrt{k_c}\, \text{erf}\,(\sqrt{k_c}\, t)\right] \qquad (6)$$

The software package MATHCAD [37] has been utilized for simulation of the electrochemical response based on mathematical solutions (4-6).

4. RESULTS AND DISCUSSION

Ferrocenedimethanol oxidation to a stable ferroceniumdimethanol cation (Fc$^+$) was studied with cyclic voltammetry over the sweep rate interval $v \leq 300$ mV/s at a glassy carbon electrode in Britton-Robinson buffers at pH from 4 to 9.
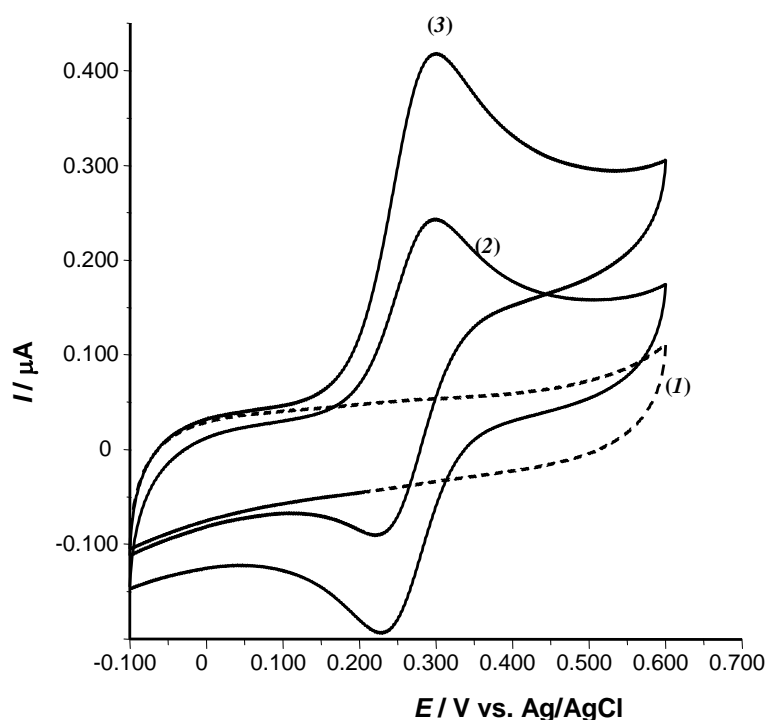

Figure 1. Typical cyclic voltammograms of 0.5 mmol/L glutathione (*1*), 0.5 mmol/L ferrocenedimentanol (*2*), and ferrocenedimethanol and glutathione together (*3*) recorded at a glassy carbon electrode in a Britton-Robinson buffer at pH 9. The sweep rate was $v = 20$ mV/s.

A typical voltammetric response is represented by the curve 2 in Fig. 1. The response is virtually pH independent. The mid-peak potential is 0.212 V with an average peak potential difference of 57.8 ± 2.6 mV. Both anodic and cathodic peaks increase linearly with the square-root of the sweep rate, with the correlation coefficient of the linear regression line $R = 0.999$, for both peaks. The dependence $\log I_p$ vs. $\log v$ is also a line with a slope of 0.49 ($R = 0.999$) and 0.482 ($R = 0.999$) for the

anodic and the cathodic peak, respectively. The average peak current ratio is 0.96 ± 0.02. All these data are in agreement with the theoretical predictions for one-electron diffusion controlled reversible electrode reaction, which is the main prerequisite for the application of Fc as a redox mediator for catalytic oxidation of GSH and AA.
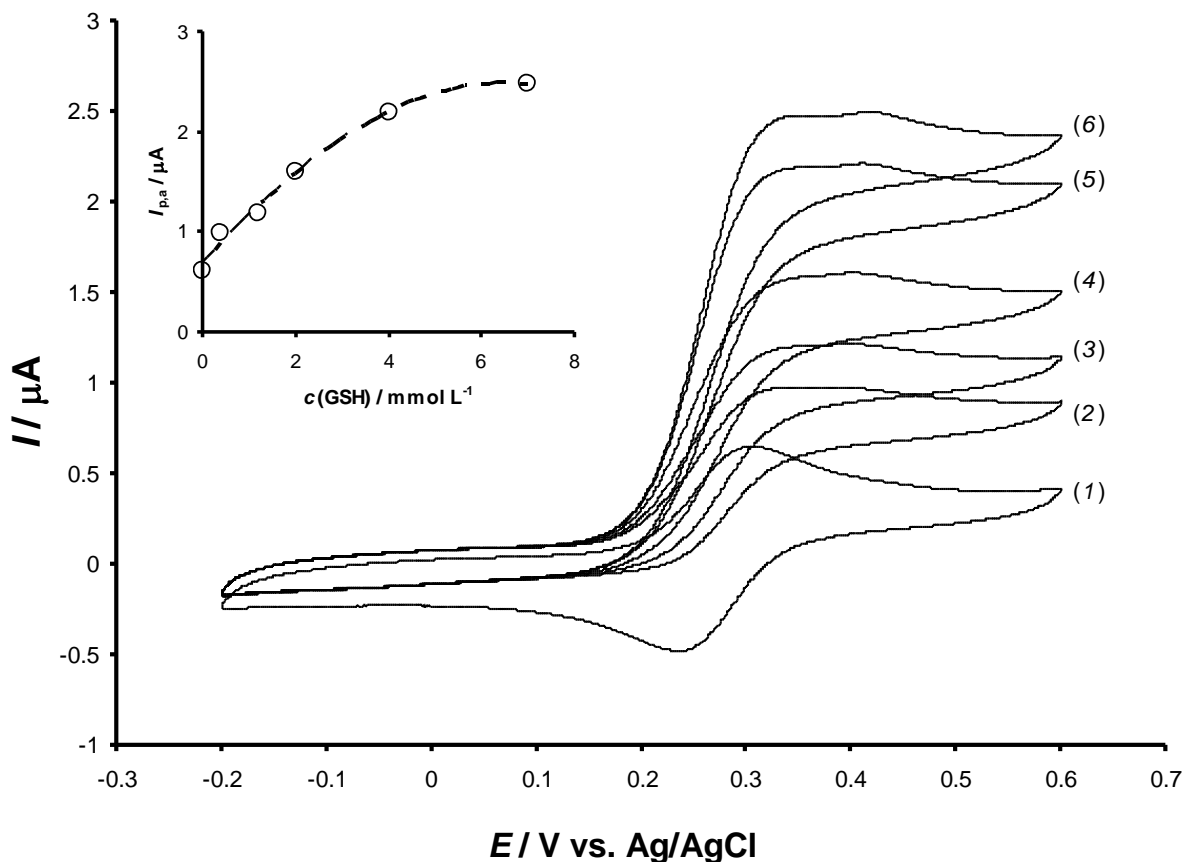

Figure 2. The effect of increasing concentration of GSH on the cyclic voltammograms of Fc, recorded in a buffer at physiological pH = 7. The concentration of GSH was 0 (*1*); 0.4 (*2*); 1.2 (*3*); 2 (*4*); 5 (*5*) and 7 mmol/L (*6*). All other conditions were the same as for Fig. 1. The inset shows the dependence of the anodic peak current on the GSH concentration.

In the presence of equimolar concentrations of Fc and GSH in the electrolyte solution, the anodic peak significantly increases, whereas the reduction peak concomitantly decreases, the anodic-to-cathodic peak current ratio being 1.53 (see curve 3 in Fig. 1). Curve 1 in the same figure clearly shows there is no electronic communication between the electrode and GSH in the absence of Fc. Figure 2 shows in more detail the evolution of the cyclic voltammogram of Fc by increasing the concentration of GSH in the buffer at pH 7. Obviously, by increasing the GSH concentration cyclic voltammograms evolve gradually from a peak-like shape into a steady-state, sigmoid voltammetric curves. The degree of the enhancement of the anodic peak, as well as the corresponding diminishing of the cathodic one, is pH dependent. The effect of GSH is the most prominent at pH $\geq$ 9, whereas it is less pronounced in a neutral or acidic medium, which is depicted in Fig. 3. This can be rationalized by

taking into account the $pK_a = 9.23$ of the thiol group of GSH, being in agreement with the expectation the GSH to be oxidized to GSSG form [19, 20].
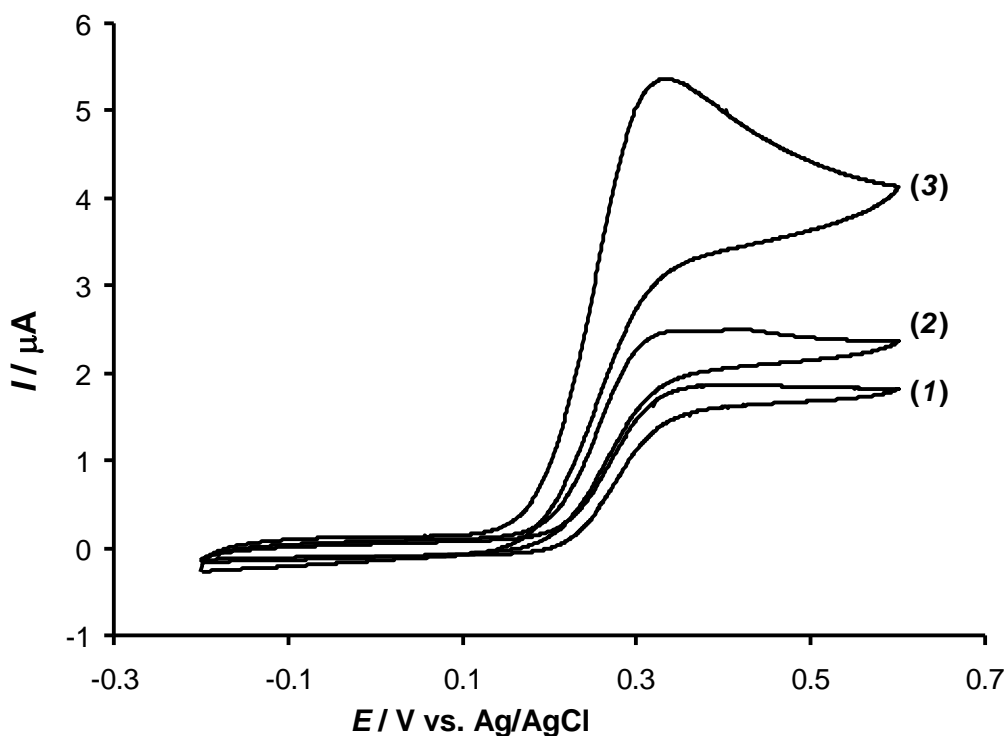

Figure 3. The effect of pH on the electocatalytic oxidation of 7 mmol/L glutathione mediated by the redox reaction of ferrocenedimethanol. The pH of Britton-Robinson buffers was 4 (*1*); 7 (*2*) and 9 (*3*). All the other conditions were the same as for Fig. 1

The foregoing results clearly show that the overall electrode mechanism of Fc in the presence of GSH undergoes according to the E_rC' catalytic reaction scheme (reactions 1-2). The heterogeneous electrode reaction of Fc to Fc^+ is accompanied by the homogeneous irreversible redox reaction between Fc^+ and GSH, taking place in the vicinity of the electrode surface. Hence, the GSH undergos catalytic oxidation, whereas the Fc/Fc^+ acts as a redox catalyst, shuttling electrons between the electrode and GSH. The degree of the anodic current increase is proportional to the rate of the homogenous redox reaction. In the conditions of linear sweep or cyclic voltammetry, the voltammetric response is controlled by the critical kinetic parameter defined as $\kappa = \dfrac{kc(\text{GSH})}{v}\left(\dfrac{RT}{F}\right)$ [18]. The anodic peak current increases nonlinearly with c(GSH) (see the inset of Fig. 2), which is the consequence of the nonlinear dependence of the anodic peak on the kinetic parameter κ, being in accord with the theoretically predictions.

In the conditions of cyclic voltammetry, the estimation of the rate constant has been conducted by fitting the experimental and theoretical data calculated with the aid of eq. (4). The dependence $I_{p,a}$ vs. c(GSH) was the subject of fitting.
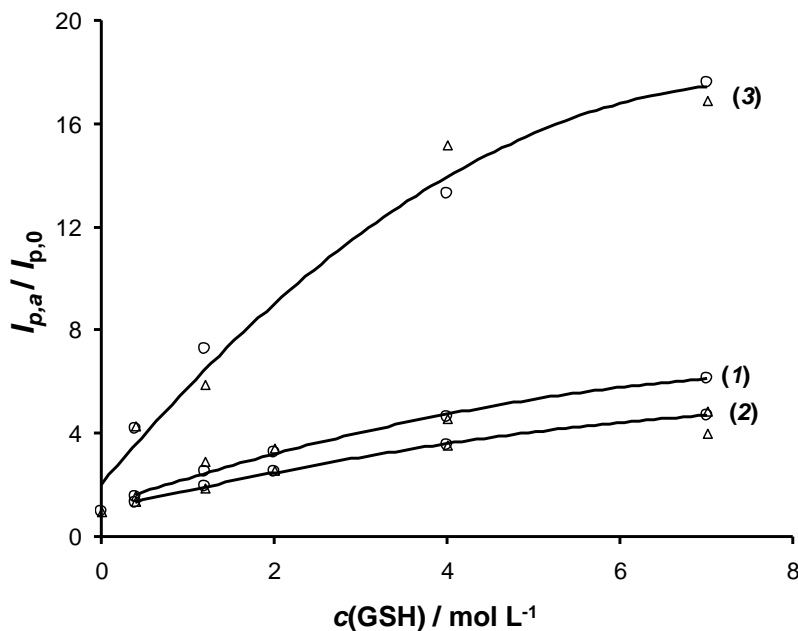
Figure 4. The fitting of the experimental (triangles) and theoretical (circles) data measured with cyclic voltammetry for the electrocatalytic oxidation of glutathione in Britton-Robinson buffers at pH 4 (*1*); 7 (*2*), and 9 (*3*). The ordinate displays the ratio of the current measured in the presence of particular concentration of GSH ($I_{p,a}$) and in the absence of GSH ($I_{p,0}$).
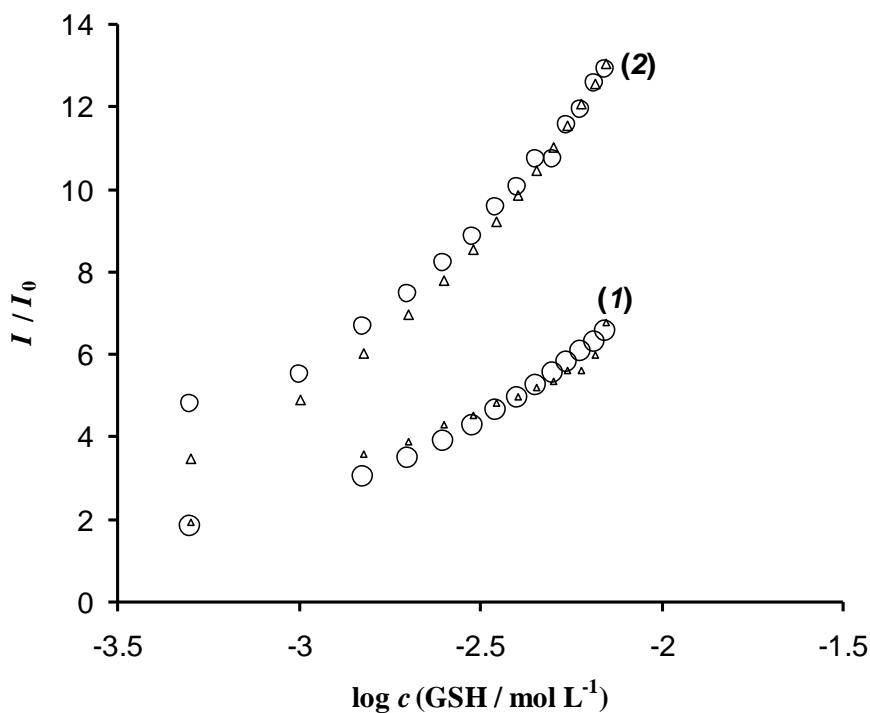

Figure 5. The fitting of the experimental (triangles) and theoretical (circles) data measured with chronoamperometry for the electrocatalytic oxidation of glutathione in Britton-Robinson buffers at pH 7.3 (*1*) and 8.5 (*2*). The sampling time was 5 s at a potential of $E = 0.500$ V. The ordinate displays the ratio of the current measured in the presence of particular concentration of GSH (I) and in the absence of GSH (I_0).

For consistent comparison, both experimental and theoretical anodic peak currents ($I_{p,a}$), measured for each concentration of GSH, have been normalized versus the anodic peak current of Fc in the absence of GSH ($I_{p,0}$), thus avoiding the influence of all unknown parameters (e.g., the exact value of the electrode surface area, diffusion coefficient, etc.), as well as avoiding the contribution of the oxidation of Fc itself that can lead to erroneous results. Shown in Fig. 4 is the best fit for the data measured in buffers at pH 4, 7 and 9.

The corresponding second-order catalytic rate constants are $k = 8.5 \times 10^2 \, \text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$, $5 \times 10^2 \, \text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$, and $7 \times 10^3 \, \text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$, respectively. The correlation analysis between the experimental and theoretical data is associated with the linear regression coefficients of $R = 0.988$, 0.992, and 0.985, for pH 4, 7, and 9, respectively, being quantitative indicators for the quality of the fitting.
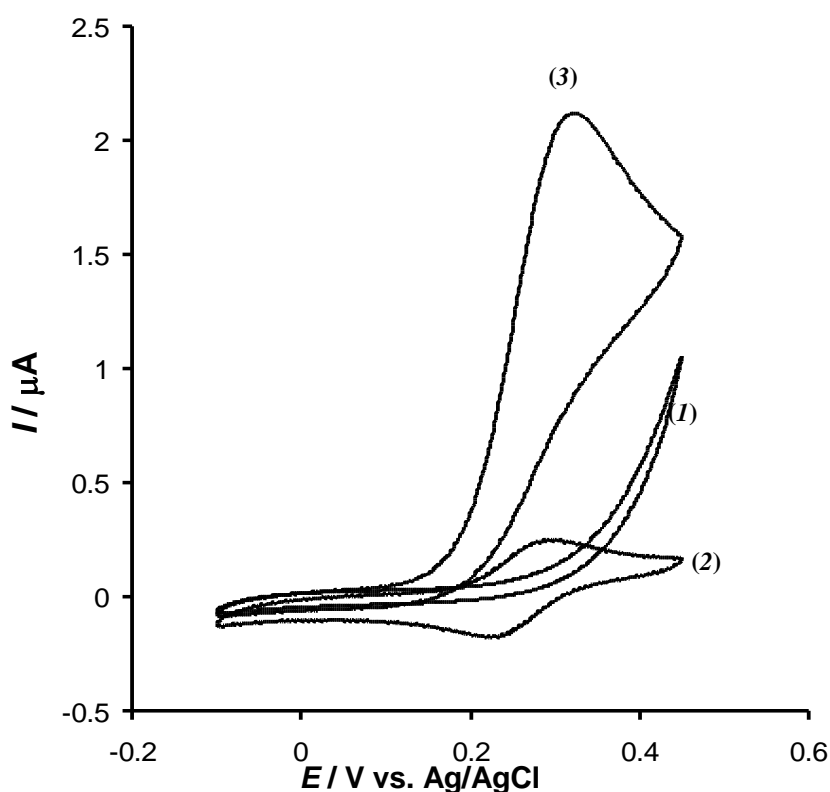

Figure 6. Typical cyclic voltammograms of 1 mmol/L ascorbic acid (*1*), 0.1 mmol/L ferrocenedimentanol (*2*), and ferrocenedimethanol and ascorbic acid together (*3*) recorded at a glassy carbon electrode in a Britton-Robinson buffer at pH 4.4. The sweep rate was $v = 20$ mV/s.

Once the electrode mechanism is established, one can apply chronoamerometry to estimate the kinetic parameters of the system, taking the advantage of much simpler theoretical background of the method (for illustration, compare equations (4) and (6), valid for linear sweep voltammetry and chronoamperometry, respectively). Figure 5 depicts the fitting of the data measured in a medium with pH values of physiological significance. The estimated catalytic rate constants are $k = 3.9 \times 10^2 \, \text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$ ($R = 0.986$) and $1.5 \times 10^3 \, \text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$ ($R = 0.995$), for pH 7.3 and 8.5 respectively, being in accord

with the previous values measured with CV. Obviously, increasing the pH for a single unit causes the rate of the catalytic oxidation to enlarge for about an order of magnitude, which has apparent physiological significance for the antioxidative capability of GSH.

From a methodological point of view it is worth mentioning that the attempt to measure the catalytic rate constant with SWV was less successful and trustworthy. In spite of the fact that the voltammetric response was well-defined, its sensitivity to GSH concentration was fewer compared to the previous techniques. Hence, the rate constant estimated for pH 7.3 was $k = 1.5 \times 10^3 \, \text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$, being significantly higher than the value estimated with CV and chronoamerometry. Moreover, the correlation between the experimental and theoretical data is associated with a low regression coefficient of $R = 0.921$. The cause of these observations might be in the fact that SWV is very fast technique, being more appealing for measuring fast redox reactions, as already demonstrated in our previous studies [38].

Table 1. Summary of the kinetic data estimated for the catalytic oxidation of ascorbic acid with ferrocendimethanol cation. For CV the sweep rate was 20 mV/s; for CA, the measuring time was 2 s; for SWV, the frequency was $f = 10$ Hz, amplitude $E_{sw} = 50$ mV, and potential step increment $\Delta E = 1$ mV. The other conditions were the same as for Fig. 6

pH	CA		CV		SWV	
	k	R	k	R	k	R
	$\text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$		$\text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$		$\text{mol}^{-1} \, \text{L} \, \text{s}^{-1}$	
4.4	8.4×10^4	0.991	1×10^5	0.992	3×10^5	0.978
5.4	2.5×10^4	0.981	3×10^4	0.979	9×10^4	0.979
7.3	3.5×10^4	0.985	2×10^4	0.992	1.5×10^5	0.991
8	2.35×10^4	0.9922	/	/	/	/
9.1	3.05×10^4	0.998	/	/	/	/

In the qualitative sense, the overall voltammetric behavior in the presence of AA is comparable with GSH. The catalytic oxidation of AA with ferrocene derivatives undergoes according to a complex two-step two-electron mechanism, where the expulsion of the first electron is the rate determining step [12]. Yet, for the purpose of the present study, the mechanism can be assimilated to the E_rC' reaction scheme. Typical voltammograms obtained in the presence of AA are shown in Fig. 6. In a basic medium (pH ≥ 9) the situation is becoming more complex and the catalytic oxidation is obstructed by the direct oxidation of AA at the GC electrode. The kinetic data estimated are summarized in Table 1. In a medium with a physiological pH = 7.3, the rate constants are about two order of magnitude higher than for GSH, showing superior antioxidative capacity of AA. Moreover, the kinetics of AA oxidation is virtually identical at all pH values studied, implying that the antioxidative activity of AA is less pH susceptible than GSH. As in the case of GSH, estimations with SWV resulted in higher vales for the rate constants, pointing out different electrochemical techniques can give different values for the same kinetic parameter, which need to be taken into account in comparing the antioxidative capacity of different species.

5. CONCLUSION

In the present study we propose a simple methodology for assessment of the antioxidative activity of a low-molecular weight antioxidative compound by measuring the rate constant of the homogeneous oxidation reaction of the antioxidant. It is believed that the rate constant of the homogenous oxidation reaction is an intrinsic parameter controlling the antioxidative capacity of a compound. The redox couple ferrocenedimethanol/ferroceniumdimethanol is demonstrated to be highly suitable reference system for the purpose of the study, due to is reversible electrochemistry, water solubility and chemical stability. The electrode reaction of ferrocenedimethanol, in the presence of particular antioxidative compound, undergoes according to the E_rC' reaction scheme, which can be easily modeled under variety of experimental conditions and electrochemical techniques, enabling estimation of the rate constant of the homogeneous oxidation reaction of the antioxidant. The applicability of the proposed methodology is exemplified by glutathione and ascorbic acid, revealing superior antioxidative activity of the latter. The proposed methodology is limited to the application to water soluble antioxidant with a standard redox potential more negative than the potential of ferrocenedimethanol/ferroceniumdimathanol couple.

Dedication
This work is dedicated to Academician Gligor Jovanovski, the leader of the structuralchemistry in Macedonia, on the occasion of his 65th birthday.

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