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Dedicated to the memory of
Professor Ioan Silaghi-Dumitrescu (1950 – 2009)

NEW METHOD FOR ANTIOXIDANT ACTIVITY EVALUATION USING A H_2O_2 AMPEROMETRIC SENSOR

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A new amperometric method for antioxidant activity (AOA) evaluation, based on a Prussian Blue (PB)-modified graphite electrode (G/PB) for H_2O_2 detection, was proposed. Taking into account that some antioxidants act as H_2O_2 scavengers, the AOA was estimated amperometrically by the decrease of H_2O_2 concentration, and was expressed as the equivalent ascorbic acid (AA) concentration, chosen as a reference antioxidant. The present study describes the preparation and characterization of the H_2O_2 sensor, as well as its use for AOA evaluation in real samples. The electroanalytical parameters obtained for both H_2O_2 and ascorbic acid (AA) determinations, the sensor stability and the good agreement existing between our results and those already published proved that the proposed approach may be successfully used as a simple and reliable method for AOA evaluation.

INTRODUCTION

Prussian Blue (PB) is a highly useful redox mediator in many applications[1]: amperometric biosensors (particularly for glucose determination[2-6]), ion-selective electrodes (*e.g.*, for the evaluation of potassium ion activity[7]) etc. It has also successfully been used for H_2O_2 determination.[8-12]

H_2O_2 has received much attention due to its unique properties (weak base, strong oxidizing agent) and multiple uses (bleaching agent, disinfectant, antiseptic, oxidizer, rocket propellant, etc.). Also, it is considered, like oxygen free radicals, hypochlorous acid and the peroxynitrite anion, as a reactive oxygen species (ROS).[13]

Antioxidants are substances that inhibit the destructive action of ROS and other oxidant species, by their scavenging properties.[14] Various methods have been developed for the evaluation of antioxidant activity (AOA). Mainly, they may differ concerning the species scavenged by the antioxidants, the reaction conditions and the detection method. Among these methods, few have been proposed for standardization of AOA determination: the oxygen radical absorbance capacity assay (ORAC), the Folin-Ciocalteu method (F-C), and the Trolox equivalent antioxidant capacity assay (TEAC).[15] However, their oxidized substrate (ORAC) or radical source (F-C and TEAC) have little or no biologic relevancy.

H_2O_2 is a ROS playing a major role in the human body with respect to various health disorders.[16-20] In human body, H_2O_2, generated by oxidases, is involved in the synthesis and activation of important biological mediators, and is strongly related to the free radicals production.[19, 21]

Evaluation of AOA by the ability of the antioxidant to act as H_2O_2 scavenger has been already proposed using different detection methods: spectrophotometry[18, 20] and colorimetry.[22, 23] However, these methods have some major drawbacks as follows: complex methodology, expensive equipments, and tedious procedures.

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In this paper, a new amperometric method for AOA evaluation, based on H_2O_2 detection using a Prussian Blue (PB)-modified graphite electrode (G/PB) was designed and used for AOA estimation in some real samples. PB as sensing layer was electrochemically deposited on the surface of a graphite electrode and acts as a redox mediator for H_2O_2 electroreduction. In presence of an antioxidant, H_2O_2 is scavenged and the AOA can be evaluated as the relative decrease in the reduction current, due to H_2O_2 consumption. All the electroanalytical characteristics of the new method as well as the results obtained for AOA estimation in real samples point it out as a fast, simple and reliable method for AOA determination.

RESULTS

1. Electrode preparation

Prior to PB electrodeposition, the graphite rod was cleaned using emery and smooth paper, and it was then ultrasonicated during 2 minutes. An electrochemical activation was finally made by applying +1.7 V / Ag/AgCl,KCl_{sat} in a pH 7.5 phosphate buffer (PBS) at the bare electrode, during 3 minutes. PB was electrochemically deposited on a pyrolytic graphite rod through cyclic voltammetry using a mixture of 5 ml of 0.1 M $K_3Fe(CN)_6$ and 5 ml of 0.1 M $FeCl_3$ (both solutions were prepared in 10 mM HCl). 25 cycles were recorded between -0.6 and +1.2 V / Ag/AgCl,KCl_{sat}, at a scan rate of 25 mV/s (Figure 1).[1]

Two reversible peak pairs are typical for the voltammetric response of PB-modified electrodes. The redox reactions taking place are shown in equations 1 and 2.[10] As expected, the intensity of all peaks increases with increasing number of cycles, indicating a progressive deposition of a conducting film of PB on the graphite electrode surface.

A comparison has been made between the voltammogram from Figure 1 and that corresponding to a G/PB electrode prepared by PB adsorption;[10] all peaks were higher in our study (1.3 to 1.6 times), but the peak reversibility was similar. Higher peak currents were also obtained with respect to those observed for G/PB electrodes prepared by electrodeposition at constant potential, followed by a voltammetric cycling performed for film stabilization.[24]

$$\underset{\text{(soluble PB)}}{KFe^{III}Fe^{II}(CN)_6} + K^+ + e^- \rightleftharpoons \underset{\text{[Prussian White, Everitt salt (PW)]}}{K_2Fe^{III}Fe^{II}(CN)_6} \quad (1)$$

$$\underset{\text{(soluble PB)}}{KFe^{III}Fe^{II}(CN)_6} \rightleftharpoons 2/3\ K^+ + 2/3\ e^- + \underset{\text{[Berlin Green (BG)]}}{K_1/_3(Fe^{III}(CN)_6)_2/_3(Fe^{II}(CN)_6)_1/_3} \quad (2)$$

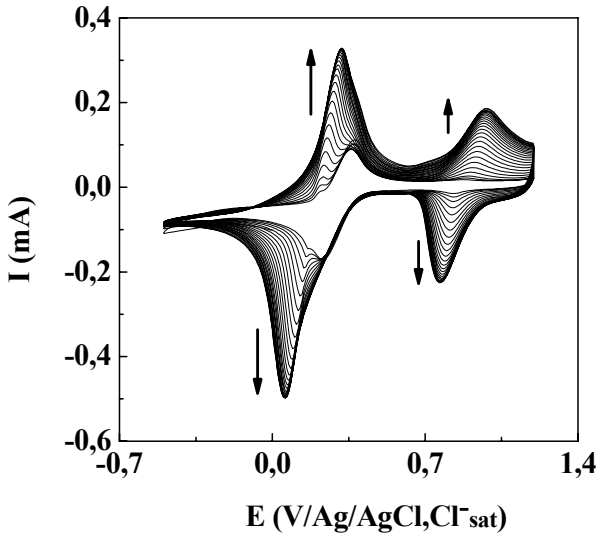

Fig. 1 – Electrodeposition of PB on a graphite electrode by cyclic voltammetry. Working conditions: 25 cycles recorded at a scan rate of 25 mV/s in a mixture of 5 ml 0.1 M $K_3Fe(CN)$ and 5 ml of 0.1 M $FeCl_3$ (both prepared in HCl 10 mM).

2. Electrode characterization

2.1. Electrocatalytic effect for H_2O_2 reduction

In order to assess the electrocatalytic effect for H_2O_2, two voltammograms were drawn and compared. Both consisted of 2 successive cycles performed between -0.6 and +1.2 V / $Ag/AgCl,KCl_{sat}$, with a scan rate of 25 mV/s, one in PBS and the other one in 10 mM H_2O_2 (Figure 2). As expected, the electrocatalytic effect is put into evidence, in the reduction domain, by a clear increase of the reduction peak intensity, and in the oxidation domain, by a decrease of the oxidation peak intensity.

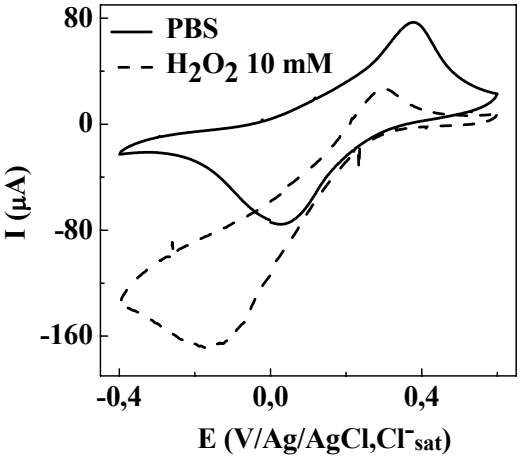

Fig. 2 – Comparative voltammograms recorded in PBS (solid line) and 10 mM H_2O_2 (dash line) at the G/PB electrode. Working conditions: 2 cycles were recorded at a scan rate of 25 mV/s.

2.2. G/PB electrode stability

To assure a better stability of the PB layer,[10] the pH value was chosen 3.1 for all measurements using the G/PB electrode. In order to assess the short time stability of the G/PB-modified electrode, a number of 52 amperometric measurements were performed in three different days: 26 after 2 weeks from the B/PB preparation, 14 after other 4 days, and 12 after other 2 days. The reduction current was recorded in a PBS containing 0.77 mM H_2O_2, when a -0.1 V / $Ag/AgCl,KCl_{sat}$ potential was applied. A stirring rate of 1000 rpm was used.

Figure 3 shows the reduction current (I) corresponding to each measurement divided by the initial current value (I_0). As expected, a progressive decrease of the reduction current with the increasing number of the performed measurements was observed. After the 52 measurements, a total decrease of about 40% was recorded. Additionally, it was noted that in order to extend the electrode lifetime, a resting time is recommended among measurements.

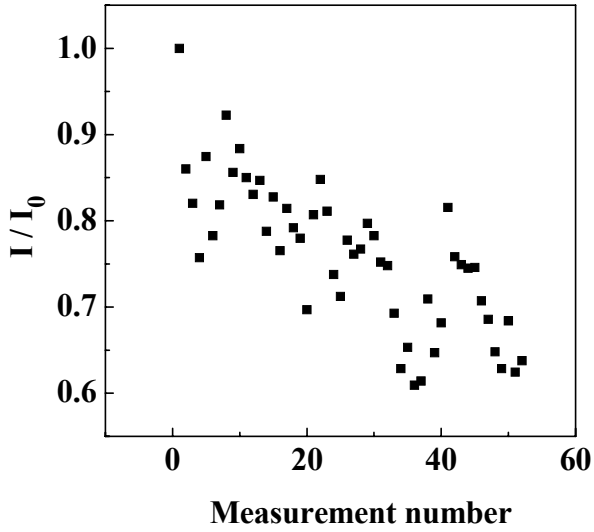

Fig. 3 – Relative reduction current recorded during 52 successive amperometric measurements using G/PB-modified electrode. Working conditions: supporting electrolyte, PBS pH 3.1; H_2O_2 concentration, 0.77 mM; E_{appl} = -0.1 V / $Ag/AgCl,KCl_{sat}$; stirring rate, 1000 rpm.

2.3. H_2O_2 calibration curve

Amperometric measurements were performed, using successive additions of 100 mM H_2O_2 solution into 5 ml of phosphate buffer solution. The other working conditions were identical to those mentioned before (section 1.2.2).

A calibration curve was drawn for the 0.03 – 18.9 mM H_2O_2 concentration range, by representing the average and standard deviations (SD) for two identical measurements performed in 2 successive days. The corresponding electroanalytical parameters are shown in Table 1. High sensitivity (S), large linear domain (LD), short response time (t_{95}), small detection limit (DL) and excellent repeatability, all indicate that the G/PB electrode can be successfully used as a H_2O_2 sensor. Moreover, these parameters were better than those obtained using 2 other similar electrodes: one prepared through PB adsorption,[10] and the other one by electrodeposition at constant potential followed by stabilization through voltammetric cycling.[24]

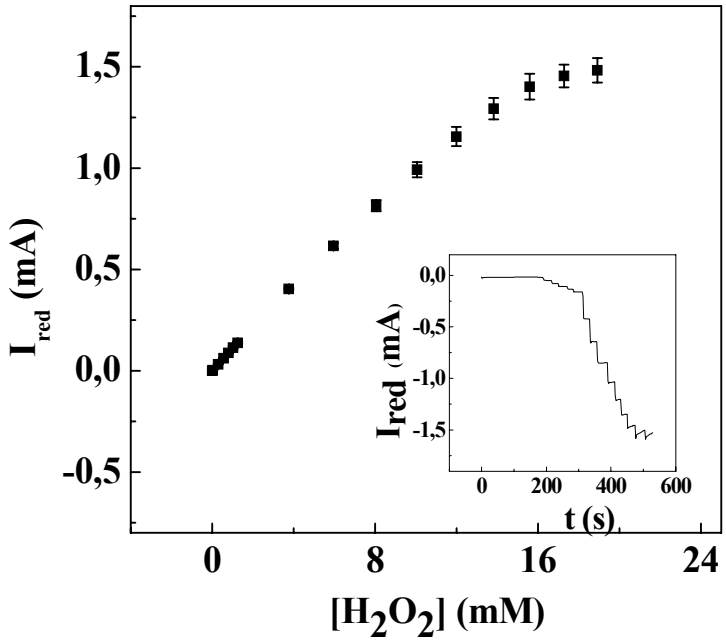

Fig. 4 – Calibration curve for H_2O_2 detection. Inset: amperometric response to successive additions of 100 mM H_2O_2 solution, recorded at G/PB electrode. Working conditions: supporting electrolyte, PBS ph 3.1; E_{appl} = -0.1 V / Ag/AgCl,KCl_{sat}; stirring rate, 1000 rpm.

Table 1

Electroanalytical parameters obtained from the calibration curve recorded for H_2O_2 determination

Linear fit			Michaelis-Menten fit				DL	t_{95}	RSD*
S (mA/M)	$\frac{R^2}{N}$	LD (mM)	I_{max} (mA)	K_m^{app} (mM)	S (mA/M)	$\frac{R^2}{N}$	(μM)	(s)	(%)
89 ± 2.2	$\frac{0.9917}{15}$	0.001 – 17.3	4.4 ± 0.01	35 ± 4.5	126 ± 20.1	$\frac{0.9978}{16}$	1.2	7	4

* Maximum RSD for the linear domain

2.4. Ascorbic acid calibration curve

Amperometric measurements were also performed in PBS solutions containing 0.77 mM H_2O_2, where successive AA additions were made, yielding final AA concentrations in the interval 0.3 – 44.9 mM. The other working conditions were as mentioned before. Table 2 presents the resulting electroanalytical parameters, as evaluated from the corresponding calibration curve. As expected, larger LD and smaller S values were obtained as compared with the corresponding values reported for H_2O_2. However, the SD and t_{95} values were comparable to those from Table 1.

Table 2

Electroanalytical parameters obtained from the calibration curve recorded for AA. Working conditions: 0.77 mM H_2O_2; AA concentration range, 0.3 – 44.9 mM; E_{appl} = -0.1 V / Ag/AgCl,KCl_{sat}; stirring rate, 1000 rpm

Linear fit			Michaelis-Menten fit				DL (μM)	t_{95} (s)	RSD* (%)
S (mA/M)	R^2 N	LD (mM)	I_{max} (mA)	K_m^{app} (mM)	S (mA/M)	R^2 N			
0.9 ± 0.03	0.9916 11	0.5 – 44.9	0.1 ± 0.02	72 ± 20.3	1.4 ± 0.4	0.9892 12	0.5	10	6

* Maximum RSD for the linear domain

3. AOA evaluation in real samples of wines and fruit juices

The AOA for 14 real samples was evaluated using the G/PB electrode: „Cabernet" red wine; "Feteasca Regală" white wine; concentrated apple juice; 11 samples of fresh juices from mandarine, white and red grape, „Red delicious" apple, orange, kiwi, nectarine, „Seckel" pear, grapefruit, red orange and lemon.

AOA evaluation was made using the following procedure: to an initial volume of 5 ml PBS, three H_2O_2 additions were made, yielding a total concentration of 0.77 mM. Then, an AA addition (final concentration: 10.1 mM) followed by the real sample addition (250 μl for mandarine juice, 400 μl for wine samples, 500 μl for lemon juice, and 200 μl for other samples) were performed.

AOA evaluation was based on the following procedure: after both AA and sample addition, due to the H_2O_2 consumption, a decrease in the H_2O_2 reduction current will be observed. Using the relative current decrease corresponding to the investigated sample, the AOA was expressed as the AA equivalent concentration, calculated as the ratio between the current decrease recorded after sample addition and the current decrease observed after AA addition. The AOA resulting values are given in table 3.

Table 3

Estimated AOA of real samples, given as the AA equivalent concentration

Sample	**AOA (mM)**	**Sample**	**AOA (mM)**
Concentrate apple juice	141 ± 6	Orange	61 ± 13
„Cabernet" red wine	87 ± 29	Kiwi	59 ± 7
Mandarine	78 ± 8	Nectarine	57 ± 12
White grape	73 ± 9	„Seckel" pear	51 ± 11
Red grape	72 ± 1	Red orange	46 ± 5
„Red delicious" apple	63 ± 5	Grapefruit	44 ± 14
„Feteasca Regală" white wine	61 ± 8	Lemon	43 ± 12

As table 3 shows, the concentrated apple juice had by far the highest AOC level. Judging by its high viscosity, this sample was very rich in antioxidants, despite the inherent vitamin C loss (noticeable from the brown color). As expected, among the two wines, the red one had the highest AOA. This fact has been confirmed in the literature, and was attributed to a higher polyphenolics content in the red wine.[25] Concerning red orange, one could expect it to have a higher AOA than the common orange, due to its anthocyanin content.[26] However, the tested red orange variety was red only in some parts of its pulp, so its anthocyanin content was estimated to be low.

The ranking order: mandarin > orange > red orange > grapefruit was also obtained by Honer et al. [27] using the Briggs-Rauscher method. Moreover, in the same study, as in our study, comparable AOA values were observed between kiwi and nectarine.

A comparable AOA was obtained for red and white grape. This may be quite surprisingly, but makes sense if we take into consideration the fact that the juice was obtained only from the pulp, and not from the peel, which is the main source of polyphenolics in red grapes. A lower AOA of „Red delicious" apple and kiwi than for red and white grapes was also consistent with the literature data.[28] According to Reddy et al.,[29] vitamin C contributes only little to AOA in fruits, most of AOA being attributed to polyphenolics. Thus, there is no surprise that white grape had a higher AOA than orange.

Concerning lemon, the fact that is has a relatively poor AOA among fruits has already been

reported in the literature[30] and the fact is confirmed by our study.

EXPERIMENTAL

1. Reagents

The following reagents were used: L-ascorbic acid (Sigma, Germany), ferric chloride (Fluka, Germany), 30% hydrogen peroxide, potassium dihydrogen phosphate, di-potassium hydrogen phosphate trihydrate (Merck, Germany), potassium chloride (Reactivul, Roumania), 1 N hydrochloric acid (Microchim, Roumania), 89% phosphoric acid (Loba Chemie, Austria), and potassium hexacyanoferrate (Polskie Odczynniki Chemiczne Gliwice, Poland). All reagents were used as received.

50 mM potassium phosphate buffer solutions containing 100 mM KCl (pH 3.1, adjusted with H_3PO_4) or 50 mM KCl (pH 7.5) were prepared. If not otherwise stated, all used PBS were of pH 3.1. 10 mM AA (prepared in PBS) and 10 or 100 mM H_2O_2 (prepared in H_2O) solutions were prepared daily. For electrode rinsing, 10 mM HCl solution was used.

All fruit samples were bought from the local market. Fruit juices were obtained by simple squeezing and were immediately used. Special care was given in the case of apple and pear, as their juice oxidize very quickly. No filtration was needed, as all juices were clear. The liquid samples (the concentrated apple juice and the two wines samples) were supplied by the Applied Biotechnologies Centre „Proplanta" (Cluj-Napoca, Roumania) and had the following features: „Pektirom" concentrated apple juice (Dej, Roumania) – harvest data: 4.05.2009; „Feteasca Regală" white wine (Jidvei, Roumania) – harvest data: 6.05.2009, and „Cabernet" red wine (Recaş, Romania) – harvest data: 6.05.2009.

2. Apparatus

A computer-controlled potentiostat (PARSTAT 2276, USA) was used for cyclic voltammetry and amperometric measurements. A 3 mm-diameter pyrolytic graphite rod inserted in a Teflon cap was used for PB electrochemical deposition. The electrochemical cell consisted of a PB-modified graphite as working electrode, a $Ag/AgCl,KCl_{sat}$ reference electrode inserted in a Luggin capillary filled with saturated KCl, and a platinum counter electrode. In order to assure the homogeneity of the solution, the G/PB electrode was connected to a rotating disk electrode body (Tachyprocesseur, Radiometer Analytical). An Elma S 10 Elmasonic device was used for graphite ultrasonicating.

Data were recorded using the Power Suite software (Princeton Applied Research, USA) and were processed using the OriginPro 8.0 software. All measurements were performed in triplicate (if not otherwise stated), and results were given as the corresponding average.

CONCLUSIONS

This study proposed a new amperometric method for AOA evaluation, using a PB-modified graphite electrode as H_2O_2 sensor. The method is based on the H_2O_2 scavenging power exerted by the antioxidant species. A reduction potential was applied at the PB-modified graphite electrode and the variation of the H_2O_2 concentration induced by the antioxidant presence represents the basis for AOA evaluation.

The preparation of the G/PB-modified electrode was described, and was followed by an extensive characterization for both H_2O_2 and AA determination. The G/PB electrode yielded better electroanalytical parameters compared to those obtained by other preparation methods. Finally, the AOA of 14 real samples (wines and fruit juices) was evaluated using the proposed amperometric method. The good agreement found between our AOA values and those reported in literature confirm the reliability of the proposed method for AOA evaluation. Considering all these reasons, the proposed approach can be considered as a fast, simple, and non-expensive method for AOA evaluation.

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