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International Letters of Chemistry, Physics and Astronomy

1 (2013) 46-54 ISSN 2299-3843

Development of an electrochemical method for the measurement of antioxidant capacity of pure compounds and natural substances extracts

Touhami Lanez*, Abdelkerim Rebiai

Université d'El Oued, VTRS Laboratory, B.P.789, 39000, El Oued, Algeria

*E-mail address: touhami-lanez@univ-eloued.dz

ABSTRACT

A new method has been developed to measure total antioxidant activity of antioxidants in foods and natural substances without use of standard antioxidants and without use of calibration curves plotting. It is based on measuring the oxidation peak current of superoxide anion radical electrochemically generated by reduction of commercial molecular oxygen in dimethylformamide. The method has been validated using 7 known standard antioxidants and the results have been compared with those obtained by the DPPH and molybdate ion reduction assays. Measured antioxidant capacities were highly correlated with those obtained using DPPH (r^2 = 0.549) and molybdate ion reduction assay (r^2 = 0.434).

***Keywords*:** antioxidant activity; superoxide anion radical; electrochemistry; DDPH.

1. INTRODUCTION

Antioxidants can be defined as substances that inhibit the destructive action of reactive oxygen species and other oxidant species, by their scavenging properties [1]. A great number of *in vitro* antioxidant activities have been developed to measure the efficiency of natural antioxidants either as pure compounds or as plant extracts. Mainly, they may differ concerning the species scavenged by the antioxidants, the reaction conditions and the detection method. These methods involve different mechanisms of determination of antioxidant activity [2], Among these methods are chemical methods which based on scavenging of reactive nitrogen and oxygen species [3, 4], spectrophotometrical assays which measure the radical scavenging activity of antioxidants against free radicals like the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [5] or 2,2´-azinobis-(3-ethyl-benzothiazoline-6-sulphonate) cation radical ($ABTS^{\bullet+}$) [6] and electrochemical assays which determine the total antioxidant power include techniques such as the in situ electrochemically generated superoxide anion radical [7] or bromine [8]. However, because results in the measurement of antioxidant capacity depend on the method used, a single method can not give an accurate prediction of antioxidant capacity of antioxidant compounds [9, 10], it is recommended to use more than one method to estimate the *in vitro* antioxidant capacity of substance materials extracts because of the complex nature of reactive chemicals species [11].

All existing methods described in literature are based on the use of IC_{50} values which are defined as the amount of antioxidant required to scavenge 50% of free radical of the standard used in the assay system. Percentage of radical scavenging activity should be plotted against the corresponding concentration of the antioxidant to obtain IC_{50}. The obtained results in the measurement of antioxidant capacity expressed as IC_{50} values depend on the method and on the standard used; the results are generally not accurate

In an effort to standardize measurements of antioxidant activity and to avoid the inconvenient of the diversity of methods we herein present an voltamperometric method for the measurement of antioxidant capacities, based on superoxide anion radical detection using a cyclic voltammety techniques. The use of superoxide anion radical for the measurement of antioxidant capacities is justified by it's easy *in situ* electrochemical generation and its low cost and above all small quantities of chemical is involved.

2. EXPERIMENTAL

2. 1. Materials and methods

2. 1. 1. Instrumentation and software

Cyclic voltammetric measurements were carried out using voltalab40 PGZ301 potentiostat/galvanostat (radiometer analytical SAS). Experimentations were made in a double walled electrochemical cell of 25 mL and conventional three electrode system was employed. Glassy Carbon (GC) working electrode (radiometer analytical SAS), having area 0.013 cm^2, a Platinum wire counter electrode, and an Hg/Hg_2Cl_2 reference electrode (3.0 M KCl). Data acquisitions were accomplished with a Pentium IV (CPU 3.0 GHz and RAM 1 Gb) microcomputer using VoltaMaster4 software version 7.08 (radiometer analytical SAS).

Graphs plot were carried out using OriginLab software version 2.0 (Integral Software, France). Cyclic voltammetric measurements were run from 0 to -1400 mV. All measurements were carried out at room temperature (25 ± 1 °C).

2. 1. 2. Chemicals and reagents

Dimethylformamide (DMF) of analytical grade purchased from PROLABO was used as solvent without further purification, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (95%) was procured from Alfa Aesar, tetrabutylammonium hexafluorophosphate (TBFP) of electrochemical grade (98%) Sigma-Aldrich, was used as supporting electrolyte and its concentration was kept 0.1 M, ammonium molybdate , sodium phosphate, sulphuric acid were purchased from BIOCHEM Chemopharma. All other reagents used were of analytical grade.

Ascorbic acid (99%), gallic acid (99%), rutin (97%), quercetin (97%), α-tocopherol (97%), BHT (99%), BHA (96%) were procured from Alfa Aesa.

2. 1. 3. Procedure

Superoxide anion radical was generated in DMF containing 0.1 M TBFP. The scan rate was kept at 100 mV/s and potential window was -1.4 V-0.0 V. The standard antioxidants were then added to the *in situ* generated superoxide anion radical and response electrochemical behaviour was recorded.

3. RESULTS AND DISCUSSION

In this section, we describe the generation of our method for the measurement of the antioxidant capacity. This measurement is based upon the *in situ* generation of superoxide anion radical, which was generated by one electron reduction of the commercial molecular oxygen (O_2) dissolved in DMF at room temperature (25±1 °C). The cyclic voltammogram of superoxide anion radical showed one electron reversible process having well developed and clear oxidation and reduction peaks with peak separation (ΔEp) value of 66±3 mV, well in agreement with the reported data [12-16] **Fig. 1.** The height of anodic peak current density of the obtained voltammogram corresponds to the concentration of superoxide anion radical.

Based on values of oxidation peak current of superoxide anion radical concluded from voltammograms presented in Figures 1, the scale of total antioxidant capacity (TAC) can be realized as follows:

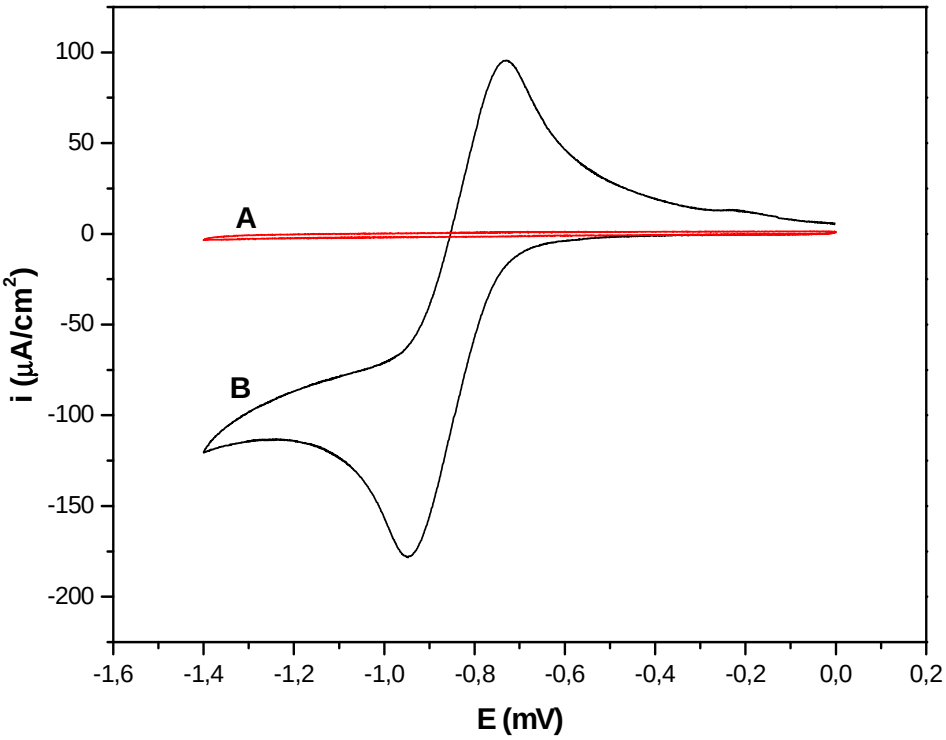

Fig. 1. Cyclic voltammograms recorded at a scan rate of 100 mV/s on GC as working electrode vs. Hg/Hg_2Cl_2 as reference at 25 °C (A) in commercial oxygen saturated with DMF/0.1 TBFP solution, (B) in nitrogen-degassed DMF/0.1 TBFP solution

Based on the change in the anodic peak current density of oxygen the antioxidant capacity was evaluated qualitatively and quantitated using the following mathematical equation (1).

$$\frac{\Delta i_p}{ip_0 - ip_{res}} \times 100 \qquad (1)$$

where, Δi_p $(ip_o\text{-}ip)$ is the change in the anodic peak current density of oxygen caused by the addition of the substrate, and are respectively the anodic peak current densities of the superoxide anion radical in the absence and in the presence of antioxidant in the electrochemical cell. $(ip_o - ip_{res})$ is the difference between the limiting anodic peak current density of oxygen without the antioxidant in the solution (ip_o) and the residual current density of the oxygen (ip_{res})

The zero point of the scale, which is related to compounds having no antioxidant properties, it can be obtained using equation 1 when $\Delta i_p = 0$, (i.e. when the anodic peak current density of oxygen in the presence of the substrate is equal to the limiting anodic peak current density of oxygen without the substrate in the solution). This density is obtained from voltammogram of **Fig. 1.** which is equal to 142.321μA/cm^2,

$$\frac{142.321\text{-}142.321}{141.101}\times 100 = 0$$

The highest point of the scale, which is related to compounds having the highest antioxidant values, can be obtained when the concentration of superoxide anion radical in the electrochemical cell reaches its minimum value, the current in this case corresponding to the residual current density of the oxygen which can be obtained from voltammogram of **Fig. 1.**, this current density is equal to 1.22 μA/cm^2, replacing this current in equation 1 we obtain,

$$\frac{142.321\text{-}1.22}{141.101}\times 100 = 100$$

On this scale as shown in **Fig. 2.,** we can classify the total antioxidant capacity of any substance.

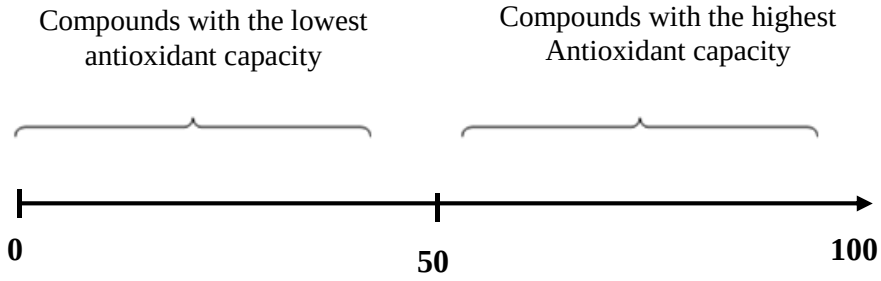

Fig. 2. The scale of total antioxidant capacity

3. 1. Validation of the Method

The method is validated by the measurement of the antioxidant capacity of 7 standard antioxidants selected for their known antioxidant capacity, calculation were carried out in three decimals, with the final result rounded to two decimals. The following procedure was followed for the measurement of the antioxidant capacity: 1 ml of a solution of the corresponding standard antioxidant in DMF was injected into the electrochemical cell containing a solution of (DMF + 0.1 TBFP) saturated with commercial molecular oxygen in a way to obtain a total concentration of the standard antioxidant in the electrochemical cell

equal to 0.1 mg/ml. then, the cyclic voltammograms of the 7 standard antioxidants were recorded one by one in the potential window of -1.4 V to 0.0 V, the obtained voltammograms are shown in **Fig. 3.**

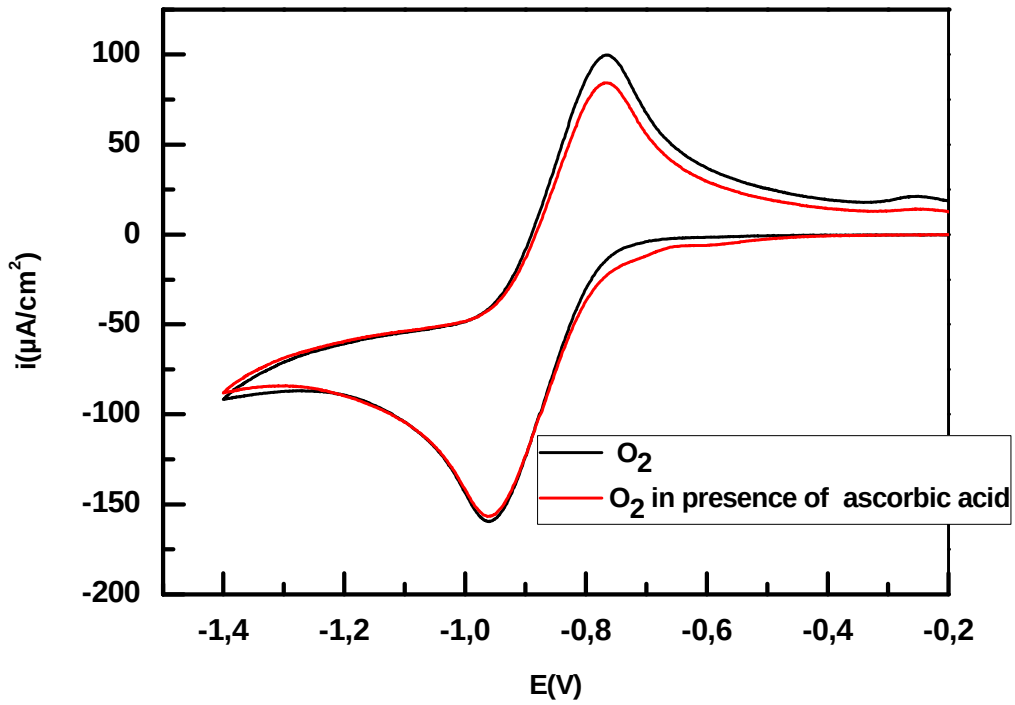

Fig. 3. Cyclic voltammograms of the $O_2/O_2^{\cdot-}$ redox couple in oxygen-saturated DMF/0.1 TBFP containing 0.1 mg/ml of ascorbic acid

The other voltammograms of the reminding standard antioxidants are not represented.

Table 1. Electrochemical data obtained from voltammograms of the 7 studied standard antioxidants

Standard antioxidant	E_{p_a}**(mV)**	E_{p_c}**(mV)**	$\lvert E_{p_a} - E_{p_c} \rvert$	$E_{1/2}$ **(mV)**	$i_p(\mu A.cm^{-2})$
Ascorbic acid	136.72	1.22	122.19	120.39	119.77
Gallic acid	136.96	1.22	114.97	114.13	116.27
Quercetin	136.38	1.22	117.42	116.47	117.52
Rutin	132.98	1.22	130.01	129.11	127.76
α-tocopherol	136.75	1.22	128.95	128.64	126.89
BHT	136.90	1.22	127.90	126.67	127.74
BHA	139.11	1.22	136.90	136.82	136.63

3. 2. Molybdate ion Reduction assay

The total antioxidant capacity of the 7 standard antioxidants was evaluated by the method of Prieto et al. [17]. An aliquot of 0.1 ml of standard antioxidant solution (1mg/ml) was combined with 1 ml of reagent solution (600 mM sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution was measured at 695 nm against a blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under the same conditions. The antioxidant capacity was expressed as the number of equivalents of gallic acid (GAEs) (μg/g of standard antioxidant solution).

3. 3. DPPH radical scavenging activity

Free radical scavenging activity of the 7 standard antioxidants was determined by using a stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) [18]. Briefly, the assay contained 1 ml of 0.25 mM DPPH in methanol and 0.1 ml of various concentrations of methanol standard antioxidants solutions. The contents were mixed well immediately and then incubated for 30 min at room temperature. The degree of reduction of absorbance was recorded in UV–Vis spectrophotometer at 517 nm. The percentage of scavenging activity was calculated using equation 2.

$$\frac{A_c - A_s}{A_c} \times 100 \qquad (2)$$

where A_c is the absorbance of control (without standard antioxidants) and A_s is the absorbance of sample. Percentage of radical scavenging activity was plotted against the corresponding concentration of the extract to obtain IC_{50} value which is defined as the amount of antioxidant standard required to scavenge 50% of free radical of DDPH in the assay system. The IC_{50} values are inversely proportional to the antioxidant activity.

Table 2. Antioxidant activity of the 7 studied standards. Each value represents the mean and standard deviation of three determinations.

Standard antioxidant.	**DDPH assay (mg AAE/g)**	**Molybdate ion Reduction assay (μg of GAE/g)**	**TAC**
Ascorbic acid	9.18 ± 0.06	538.87 ± 42.76	12.28 ± 0.32
Gallic acid	4.17 ± 0.17	1000 ± 00.00	16.09 ± 0.79
Quercetin	5.19 ± 0.17	426.34 ± 29.26	14.24 ± 0.43
Rutin	12.03 ± 0.90	271.94 ± 14.09	2.59 ± 0.48
α-tocopherol	15.99 ± 0.25	371.95 ± 25.45	5.87 ± 0.16
BHT	7.92 ± 0.21	324.64 ± 18.39	6.97 ± 0.49
BHA	28.27 ± 3.85	408.06 ± 56.22	1.69 ± 0.10

3. 3. Correlation

Fig. 4. shows the correlation between measured antioxidant capacities using our method and those measured using DPPH and Molybdate ion Reduction assays for the 7 studied standard antioxidants. Value for r^2 of 0.549 and 0.434 were respectively found. These small values of r^2 do mean that our method does not correlate well with both DPPH and molybdate ion reduction assays, because the IC_{50} values in these two methods are inversely proportional to the antioxidant activity. However, in our method the antioxidant activity expressed as TAC is directly proportional to the height of the peak current.

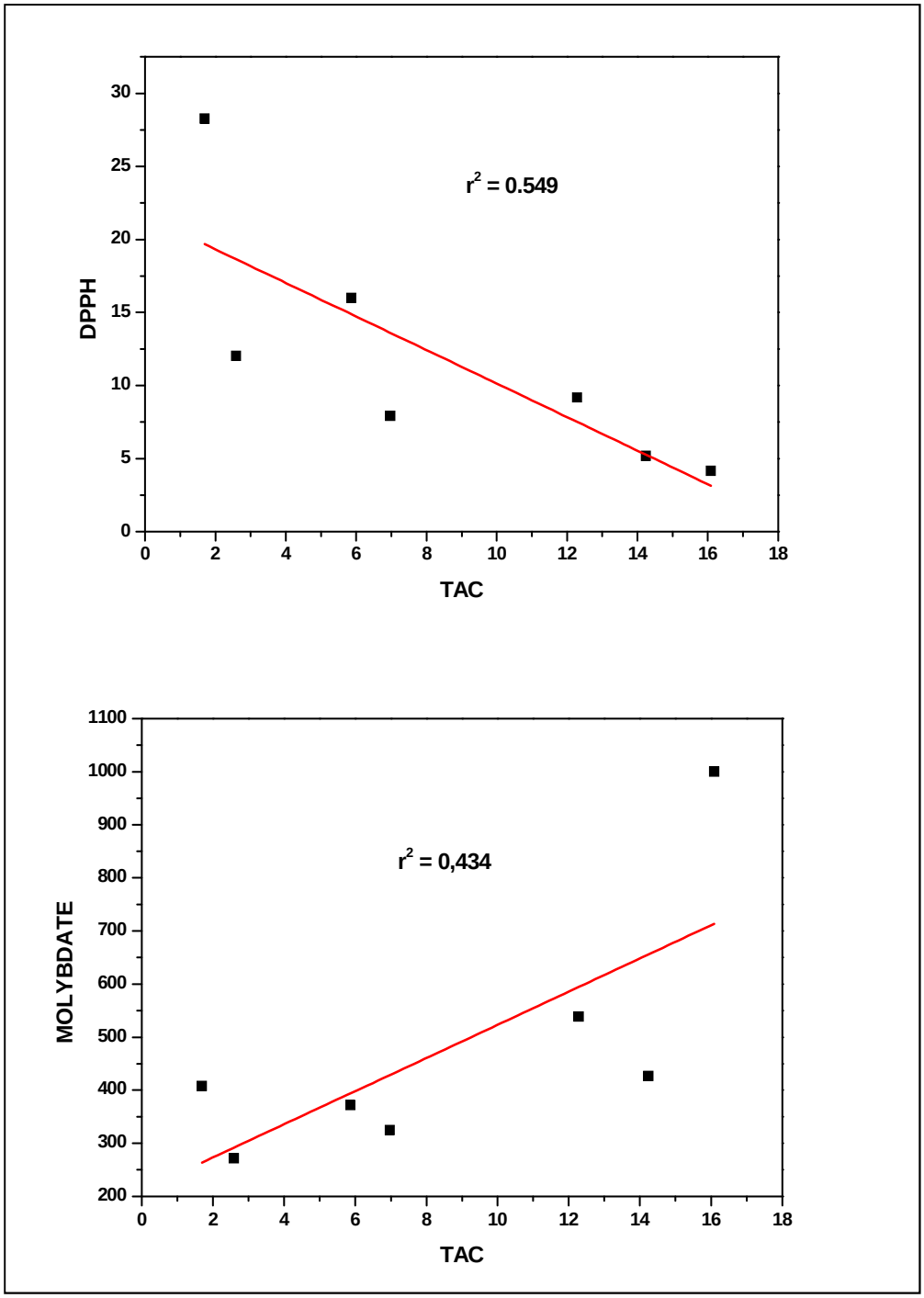

Fig. 4. Relation between antioxidant activity measurements of 7 antioxidant standards using different methods (superoxide anion radical scavenging activity, DDPH and Molybdate ion Reduction)

4. CONCLUSION

The described method in this study measures the radical scavenging effect toward superoxide anion radical electrochemically generated by reduction of commercial molecular oxygen. The obtained results revealed that the use of superoxide anion radical in the evaluation of antioxidant activity of pure compounds and natural substances extracts are in good correlation with the existing methods.

Acknowledgement

The authors would like to express their gratitude to ministry of higher education and scientific research, for the funding of the project E03220110002.

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