# Reducing Antioxidant Capacity Evaluated by means of a Controlled Potential Oxidative Attack

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## Abstract

An analytical method suitable for an antioxidant sensor is presented following the response of these substances to an extensive oxidative attack imposed by electrochemical means. The electrochemical assay simulates the action of a reactive oxygen species (ROS) by means of electrolyses carried out at a potential which is settled at the formal potential of the ROS.

The antioxidant activities of trolox and ascorbic, gallic and caffeic acids and of mixtures these antioxidants was estimated from the charge required for the complete oxidation of the antioxidants from assays where the oxidative attack by $O_2$ and by $O_2^{-\bullet}$ were simulated.

**Keywords:** Antioxidant activity; Electrolysis; Reactive oxygen species; Sensor

## 1. Introduction

The characterization of the reactivity of substances usually denominated AOs has attracted the attention of many researchers. The importance of these substances is closely related to their potential action in the prevention of oxidative stress [1-3]. Many studies are carried out with the aim to establish correlations between the intake of AOs (in food or food supplements) and health maintenance [4-6]. Other works deal with the possibility of detecting early stages of diseases or the propensity for diseases associated with oxidative stress, from measurements of antioxidant capacity in physiological fluids such as blood serum, saliva or urine [7, 8]. Besides the importance of AOs in health, their action in preventing the oxidative deterioration of food has also been widely studied, with special focus on white wines, given the costs involved when its early deterioration occurs [9].

Several analytical methods have been proposed to characterize the action of AOs and quantify their activity in preventing or delaying the oxidation of other species [10-15]. Most of the available methods are based on the analysis of the response of AOs to an oxidative attack by reactive species that can be either added or generated during the assay. Results of these assays often lead to the definition of a scale of reactivity inherent to each method, enabling to compare different AOs. As these parameters represent in most cases relative values of reactivity, the information that they contain is not easily transposed in terms of the protection

degree provided by AOs against an attack by a specific reactive oxygen species (ROS). In recent years several reviews were published in this scope, where the available methods for the evaluation of antioxidant activity are discussed [11, 12, 14, 16-19]. In these papers it is possible to obtain information about the meaning of the parameters estimated from the different methods, as well as the processes/reactions involved in the assays. Thus, there is a consensual difficulty in establishing a relation among results from different methods even when the reactions involved are identical, due to the use of different reactive species, different experimental conditions, or because different parameters (e.g. time, absorbance) are monitored.

Usually the assays used to evaluate antioxidant activity are divided into two categories based on the chemical reactions involved, namely assays based on hydrogen atom transfer reactions (HAT) and on electron transfer reactions (ET) [13, 17, 19]. HAT assays monitor the kinetics of competitive reactions, while ET assays involve a redox reaction with a synthetic oxidant (SOX). The Oxygen Radical Absorbance Capacity (ORAC) and the Total Radical Trapping Antioxidant Parameter (TRAP) assays are well-known examples of methods based on HAT reactions in which the antioxidant activity is evaluated from the delay in the reaction between the peroxyl radical and an optical probe in the presence of an AO. Among the most widespread ET based assays are: Trolox Equivalent Antioxidant Capacity (TEAC), the Ferric Reducing Antioxidant Power (FRAP) and the DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Capacity. These assays are based on the spectrophotometric quantification of the extent of redox reactions between AOs and a SOX whose optical characteristics change during the reaction.

In recent years a rising number of methods has been proposed for the evaluation of antioxidant activity based on electrochemical assays, given the relevance of electrochemistry in the context of the production and monitoring of species with redox activity [19-26]. Beyond the simplicity that electrochemical techniques can grant, there are recognized additional advantages related to the ability of performing measurements in media with colour or turbidity. Examples are potentiometric titrations [27,28] and amperometric titrations using galvanostatic generated species such as the radical cation ABTS, Ce(IV), $Cl_2$, $Br_2$ and $I_2$ [7, 26, 29-35].

In a different approach from the above-mentioned the activity of AOs has been characterized by voltammetric methods. In this context the popularity of cyclic voltammetry is growing and several papers report the voltammetric characterization of a wide range of compounds with recognized antioxidant activity [20, 22, 36- 39]. The wide use of this technique is related not only with the relevance of the information provided but also with the simplicity of the assay. Voltammetric techniques such as cyclic voltammetry and differential pulse voltammetry have also been used for the characterization of AOs mixtures [8, 39-41] Besides the identification of peak potentials or of wave potentials, the estimation of antioxidant capacity was performed based on measurements of peak current or of the area under voltammograms at fixed potential ranges.

Voltammetric methods constitute one of the most effective means to control and monitor electron-transfer reactions, although quantitative information is not straight obtained from voltammograms of AOs mixtures. Recently it was demonstrated that cyclic voltammetry is adequate for establishing robust multivariate control charts for monitoring and diagnostic of the oxidation of white wines [42].

This paper presents an analytical method suitable for the development of an AO sensor, based on monitoring the current associated with the extensive oxidation of AOs conducted by electrochemical means simulating the attack by a specific ROS.

## 2. Experimental

### *2.1. Chemicals*

All reagents employed were of analytical grade. Caffeic acid, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulphate, potassium chloride, were purchased from Fluka. Gallic acid, L- ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sulfuric acid were provided by Sigma-Aldrich. Phosphoric acid, potassium dihydrogen phosphate and dipotassium hydrogenphosphate were obtained from ACROS Organics. Other chemicals included hydroquinone (May & Baker Ltd), fructose (Vaz Pereira), sucrose (HiMedia), methanol (Fisher Scientific) potassium ferricyanide (José Gomes Santos) and ethanol (Panreac).

The concentration of buffer solution was 0.1 M. Buffer solution of pH 7.0, was prepared mixing adequate amounts of dipotassium hydrogen phosphate and of potassium dihydrogen phosphate, whereas buffer solution of pH 3.5, was prepared using potassium dihydrogen phosphate and phosphoric acid.

### *2.2. Electrochemical measurements*

Voltammetric measurements and controlled potential electrolysis were performed using a potentiostat (Autolab type PGSTAT30, Ecochemie) controlled by GPES 4.9 software provided by Ecochemie.

#### *2.2.1. Cyclic voltammetry*

Cyclic voltammetric experiments were carried out from 0 to 1.4 V at a scan rate of 100 mV/s in an undivided three-electrode cell. The working electrode was a 3 mm glassy carbon disk electrode (CHI104, CH Instruments, Inc.), an Ag/AgCl, 3M (CHI111, CH Instruments, Inc.), was used as reference electrode and a platinum wire as counter electrode. The electrode surface of the working electrode was cleaned between scans by polishing with polycrystalline diamond suspension (3F μm) for 1 min.

#### 2.2.2. RACE assays

Potentiostatic electrolyses were carried out at 0.81 V and 0.99 V in a divided three-electrode cell where the two compartments are separated by a glass frit membrane. The volume of the anodic compartment was 9.0 ml. The reference electrode was an Ag/AgCl, 3M (CHI111, CH Instruments, Inc.) electrode. The working and counter electrodes were made of a piece (20 mm x 10 mm) of platinum gauze (52 mesh woven from 0.1. diameter wire, 99.9%, from Alfa Aesar). The area of the working electrode, 5.6 $cm^2$, was determined using a 1.00 mM of $K_3Fe(CN)_6$ in 0.1 M KCl, in a cronoamperometry experiment. The diffusion coefficient was 7.63 x $10^{-6}$ $cm^2/s$ [43]. Before each experiment the anode was electrochemically cleaned at 2 V in a 1 M $H_2SO_4$ solution during 300 s to 1200 s.

*2.3. Antioxidant capacity assays based on SOX*

The antioxidant capacity assays of samples were carried out following procedures described by Rivero-Pérez [44]. Absorbance measurements were transformed in antioxidant activity using a calibration curve obtained with trolox.

*2.3.1. TEAC assay*

In this assay, radical cation $ABTS^{\bullet+}$ generated by reaction of a 7 mM ABTS with 2.45 mM $K_2O_8S_2$ (1:1). The mixture was held in darkness at room temperature for 20 h, to obtain stable absorbance values at 734 nm. The $ABTS^{\bullet+}$ working solution was obtained by the dilution of the stock solution with 0.01 M phosphate buffer (pH 7.4) to give an absorbance value of circa 0.70 at 734 nm. In this assay 100 µL of each samples was mixed with 4900 µL of $ABTS^{\bullet+}$ and absorbance was measured after 15 min of reaction time. Solutions of 0.5 mM of single AOs and mixture of AOs were directly measured, except for GA containing solutions which were half-diluted before the mixture.

*2.3.2. DPPH assay*

The assay consist in mixing 4900 µL of a freshly prepared 60 µM $DPPH^{\bullet}$ in methanol with 100 µL of sample solutions. The absorbance was measured at 517 nm after 2 h of reaction.

## 3. Results and discussion

*3.1. Description of the method RACE (Reducing Antioxidant Capacity Evaluated by Electrolysis)*

This paper proposes an analytical method to evaluate the antioxidant capacity of small molecules given their reducing power. The reducing power of an antioxidant (AO) is assessed by an electron transfer (ET) reaction at an anode and does not rely on synthetic oxidants (SOX) as the available ET methods.

The oxidation of AOs occurs in large scale during a controlled potential electrolysis and its consumption is directly monitored at the anode by the current decrease. The potential of the anode determines the selectivity of the method in the same way that the reducing power of a SOX determines the selectivity of a conventional ET assay. Thus, applying higher potentials, larger number of AOs will be quantified, including AOs of smaller reducing power, like when a

high oxidant power SOX is used, e.g. ABTS ($E^{0'}$= 0.90 V vs Ag|AgCl, 3 M). On the other hand, applying lower potentials only the AOs with higher reducing power will be detected, as when a less reactive SOX, like DPPH ($E^{0'}$= 0.227 V vs Ag|AgCl, 3 M) is employed. Selecting an anode potential identical to the formal potential of a specific ROS, the RACE method enables to evaluate the AO capacity to reduce this ROS, i.e. to estimate the AO activity towards an attack by this ROS. Therefore, results from the proposed method, denominated RACE (Reducing Antioxidant Capacity Evaluated by Electrolysis), have higher analytical significance than those obtained by the common ET methods based on SOX. These methods have intrinsic limitations due to the difference between the formal potentials of these oxidants ($E^{o'}_{SOX}$) and those of ROS ($E^{o'}_{ROS}$) originating an overestimation or underestimation of the antioxidant activity according to the sign of the difference $E^{o'}_{ROS} - E^{o'}_{SOX}$.

The selection of the anode potential is one of the key variables which determines the method performance, namely the analytical selectivity and the trueness. For potential selection the analytical context must be taken in account, considering both the nature of the relevant ROS and the pH of the sample. Figure 1 shows the formal electrode potentials of major ROS as a function of pH, for their reduction to water. The formal potentials were calculated from the standard electrode potentials of each species given the reactions stoichiometry and pH. Thus, in order to determine the capacity of AOs to eliminate the oxygen dissolved in a drink, at pH 3.5, the potential of 0.81 V should be selected. On the other hand, polarizing the anode at 0.99 V an oxidative attack by $O_2^{-\bullet}$ is simulated at a physiological medium and the capacity of AOs to reduce $O_2^{-\bullet}$ into water will be evaluate.

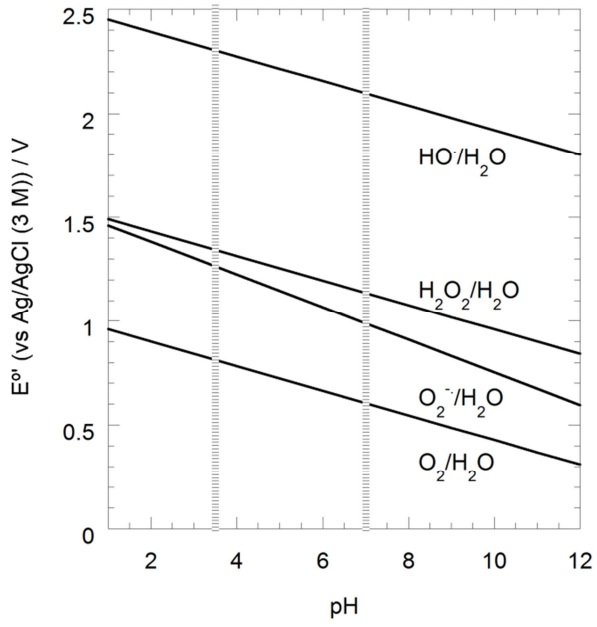


Figure 1 – Formal electrode potentials of some significant reactive oxygen species (ROS) as a function of pH. The dashed vertical lines indicate pH 3.5 and 7.0 which are typical values for drinks and physiological media, respectively.

In the most widespread ET methods, like TEAC and DPPH, the extent of the reaction depends on multiple variables such as the SOX concentration, the assay time and the difference $E^{0'}_{AO}$-$E^{0'}_{SOX}$, making it difficult to control this variable. In opposition, in the RACE method the oxidative attack extension can be easily controlled by the experimental variables that determine the conversion degree of an electrolysis. These variables are the anode area (A), the sample volume (V), the mass transport efficiency and the electrolysis time [45]. Thus, an efficiency parameter can be defined as $p = m_0 A/V$, where $m_0$ is the rate of mass transport to the anode. For a constant $p$, the extent of the oxidative attack is controlled through the electrolysis time. The possibility to control the extent of the oxidative attack is an important advantage of the RACE method, allowing the characterization of the antioxidant activity at different degrees of conversion without eliminating it from the sample. Thus, a moderate oxidative attack can be simulated by a short electrolysis in order to evaluate the antioxidant activity in low conversion conditions. In contrast, a large AO conversion can be forced through long electrolysis enabling to assess the antioxidant activity in the presence of high concentrations of the oxidation products. Experiments carried out in these two extreme situations may be important to detect pro-oxidant or synergistic effects.

When the oxidation is conducted in an extensive way, the steady consumption of AO can be monitored through the decrease of current that is a measure of the rate of charge transfer between the AO and the anode. The current decrease along time can be described by a first order kinetics,

$$I = I_0 e^{-pt} \quad (1)$$

which is typical of a controlled potential electrolysis. The charge $Q_\infty$ can be estimated from the integral of the exponential function given by a curve fit of experimental data to $t_\infty$. This parameter corresponds to the charge needed for the complete oxidation of the active AOs. In terms of the antioxidant activity this quantity measures the charge that the AOs are capable of transfer to eliminate the ROS simulated.

$Q_\infty$ is an absolute measure whose determination does not require any prior calibration and has a precise and unequivocal meaning. $Q_\infty$ can be easily converted into the amount of ROS that the AOs present in the sample can eliminate.

*3.2. Characterization of electrolysis cell*

Electrolyses of hydroquinone (HQ) were carried out in order to characterize the electrochemical cell and optimize its operating conditions. The oxidation of HQ is a reversible process, with $E^{0'}$ = 0.263 V (vs Ag/AgCl, 3 M), at pH 3.5. Constant potential electrolyses of 0.5 mM HQ solutions were carried out, at pH 3.5 and 7.0 during 1500 s, at potentials between 0.50 and 1.0 V. The charge obtained in blank assays, from electrolyses conducted in buffer solutions during 500 s at E = 0.81 and 0.99 V, are less than 1.6% of the charge from electrolyses of 0.5 mM HQ solutions. The current decrease during electrolyses of HQ follow an exponential decay (equation 1). In figure 2, data from a electrolysis of HQ at pH 3.5 is displayed with the curve

obtained by an exponential fitting, which has a correlation coefficient of 0.998. The average value of *p* calculated from three replicates is $p$ = (2.13± 0.01) x $10^{-3}$ m $s^{-1}$. This value was identical for the two solution pH and for the two electrolyses potentials. However, it was found that the *p* value was sensitive to the anode pre-treatment, namely the duration of the electrochemical cleaning at constant current. For electrochemical cleaning times lower than 500 s, smaller *p* values were obtained, circa 30% of those obtained following electrochemical cleanings conducted for 1000 s. For longer electrochemical cleaning there was no significant variations of *p* values. A similar effect was observed for current. The variation of *p* and *I* with the electrochemical cleaning time can be due to the variation of the anode active area, that increases with the efficiency of cleaning. Despite the variation of *I* and *p*, $Q_\infty$ values estimated from the ratio $I_0 / p$ (where $I_0$ is the current extrapolated to t = 0 s) showed variations less than 14 %. This experimental observation is consistent with the expected independence of the coulometric data from the working electrode area [45].

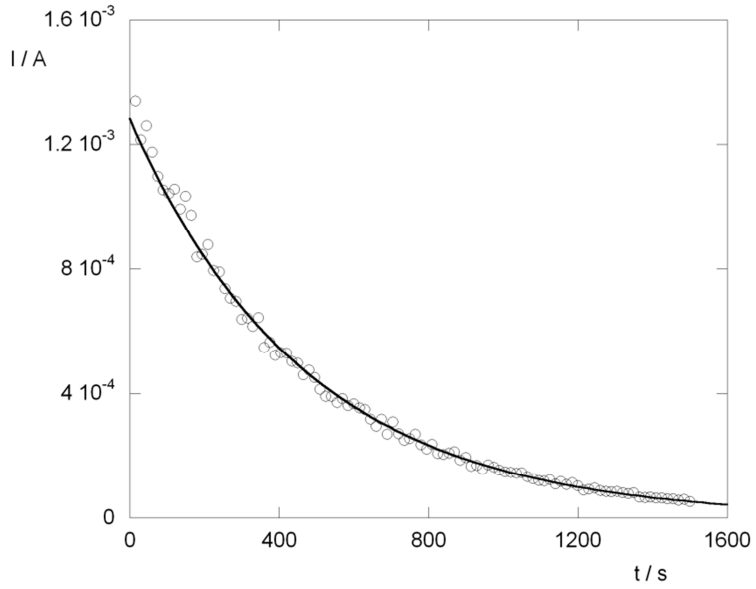


Figure 2 – I-t curve for the electrolysis of hydroquinone (HQ) carried out at E = 0.81 V, [HQ] =0.5 mM, pH 3.5, anode area = 5.6 $cm^2$ and V = 9.0 ml. The line corresponds to the exponential curve fit: I = 1.28x$10^{-3}$ exp(-2.13x$10^{-3}$ t) and r = 0.998.

Taking into account the average value of *p*, 2.13x$10^{-3}$ m $s^{-1}$, the time required to convert 50% of HQ to its oxidized form is easily estimated, $t_{1/2}$ = 325 s. Thus, to perform analysis simulating an extensive oxidative attack, electrolysis times larger than $t_{1/2}$ should be considered. On the other hand, if it is intended to characterize the AO behaviour in the absence of its reaction products, the electrolysis should be carried out for times where low conversion degrees are attained. However, it should be noticed that for a good definition of the exponential fitting, the conversion degree must enable to define a curvature.

*3.3. Application of RACE to the characterization of antioxidants*

The characterization of the antioxidant activity was tested with recognized AOs, e.g., ascorbic acid (AA), trolox (T), caffeic acid (CA) and gallic acid (GA) was performed by the RACE method in dilute AO solutions (0.5 mM) at pH 3.5 and pH 7.0. The potentials used for electrolyses were selected so that the antioxidant activities of these AOs could be estimated according to two relevant situations, namely in the prevention of oxidative degradation of beverages (pH 3.5) by eliminating $O_2$ and in the prevention of oxidative stress by the elimination of $O_2^{-\cdot}$ from physiological media. Considering data presented in Figure 1, the potentials 0.81 V and 0.99 V were selected. The antioxidant activities obtained from electrolyses carried out for 300 s are presented in figure 3 are expressed as $Q_\infty$,. $Q_\infty$ values were obtained directly from the *I vs t* curve. As charge is an absolute quantity, its meaning is in the context of quantifying the reducing power of a sample. Despite the unambiguous significance of $Q_\infty$ in context of redox reaction, this value can be easily converted to the equivalent amount of a specific ROS.

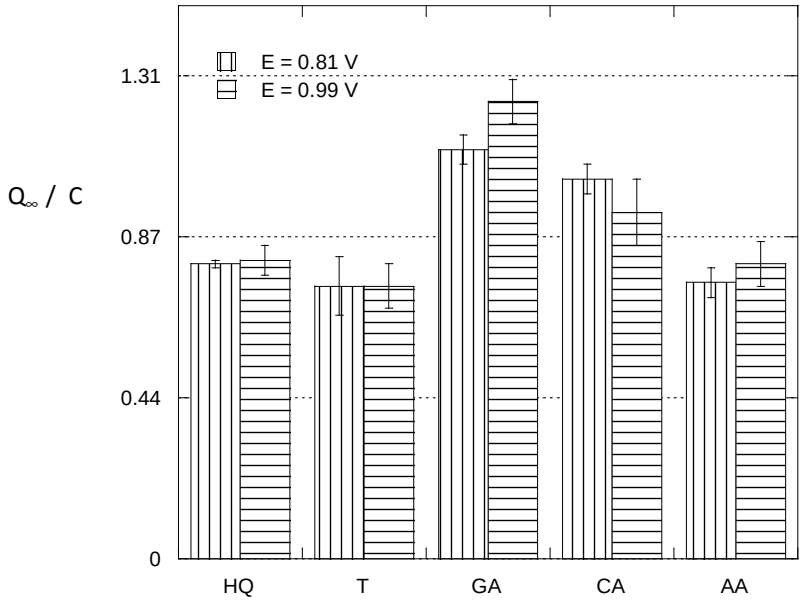


Figure 3 - Antioxidant activities of the tested compounds assessed by RACE method, $Q_\infty$, expressed in units of charge. The assigned values of 0.44 C, 0.87 C and 1.31 C correspond to the charge involved in the exhaustive oxidation of an AO given by Faraday's law for 1, 2 or 3 electrons for the experimental conditions of the assay: [AO] = 0.5 mM and V = 9.0 ml.

From figure 3 it can be concluded that the antioxidant activities of each AO are similar regarding the two oxidative attacks performed, meaning that they are equally able to reduce $O_2$ and $O_2^{-\bullet}$. The charge obtained for the oxidation of HQ, T, CA and AA is close to the calculated value assuming the involvement of two electrons (0.87 C). On the other hand, the charge obtained for GA is closer to the calculated value for three electrons (1.31 C). The antioxidant activities expressed in equivalent concentration of the ROS simulated by the electrochemical attacks are

presented in Table 1. Results expressed in equivalents of ROS indicate the maximum concentration of ROS that the AO in the sample is able to eliminate by reduction.

Table 1. Characterization of AOs by cyclic voltammetry and by RACE, DPPH and TEAC methods. $E_p^a$ is the anodic peak potential. $Q_\infty$ is the charge estimated for the complete oxidation of AOs. $[O_2]$ and $[O_2^{-\bullet}]$ are the concentrations of the simulated ROS that the AO can eliminate, calculated from $Q_\infty$. Antioxidant activities from DPPH and TEAC assays are expressed as the trolox equivalent concentration. [AO] = 0.5 mM and V = 9.0 ml. Uncertainties were estimated based on standard deviation from 2 to 3 measurements.

| | E = 0.81 V, pH 3.5 | | | E = 0.99 V, pH 7.0 | | | | |
|---|---|---|---|---|---|---|---|---|
| | $Q_\infty$ (C) | $[O_2]$ (mM) | $E_p^a$ (V) | $Q_\infty$ (C) | $[O_2^{-\bullet}]$ (mM) | $E_p^a$ (V) | DPPH (mM T) | TEAC (mM T) |
| T | 0.74±0.08 | 0.21±0.02 | 0.327±0.005 | 0.74±0.06 | 0.28±0.02 | 0.213±0.003 | 0.49±0.02 | 0.452±0.006 |
| GA | 1.11±0.04 | 0.319±0.008 | 0.517±0.004 | 1.24±0.06 | 0.475±0.02 | 0.442±0.005 | 0.99±0.01 | 1.8±0.2 |
| CA | 1.03±0.04 | 0.295±0.007 | 0.461±0.001 | 0.94±0.09 | 0.36±0.04 | 0.317±0.002 | 0.66±0.02 | 0.726±0.003 |
| AA | 0.75±0.04 | 0.216±0.008 | 0.421±0.005 | 0.80±0.06 | 0.31±0.02 | 0.384±0.006 | 0.489±0.006 | 0.340±0.001 |

*3.4. Application of RACE to the characterization of antioxidants mixtures*

Figure 4 presents antioxidant capacity data from mixtures containing two, three or four AOs obtained by the RACE method regarding the simulation of the oxidative attack by $O_2$ at pH 3.5 (Figure 4A) and by $O_2^{-\bullet}$ at pH 7.0 (Figure 4B). Mixtures are composed by equimolar concentrations of each AO and the total concentration of AOs was kept constant in all mixtures (0.5 mM). The striped bars correspond to the experimental values of $Q_\infty$ whereas the grey bars represent the predicted values of charge, $Q_{pred}$, calculated from the antioxidant activity of single AOs presented in table 1 and attending to the AO concentration in the mixture. The antioxidant activity data expressed in units of charge and in equivalent concentration of the simulated ROS are presented in table 2. Data presented in Figure 3A and Figure 3B, show that $Q_\infty$ do not differ significantly from $Q_{pred}$ attending to the uncertainties of these variables. Moreover, it can be concluded that the antioxidant activities assessed from the two oxidative attacks are similar for all mixtures.

Table2. Characterization of AOs by cyclic voltammetry and by RACE, DPPH and TEAC methods. $A_{(0\text{-}0.81V)}$ and $A_{(0\text{-}0.99V)}$ are the areas under the voltammograms integrated in the anodic scan between 0 to 0.81 V or between 0 to 0.99 V, respectively. $Q_\infty$ is the charge estimated for the complete oxidation of AOs in mixtures. $[O_2]$ and $[O_2^{\bullet-}]$ are the concentrations of the simulated ROS that the AOs in the mixture can eliminate, calculated from $Q_\infty$. Antioxidant activities from DPPH and TEAC assays are expressed as the trolox equivalent concentration. The total concentration of AOs in each mixtures is 0.5 mM and V = 9.0 ml. Uncertainties were estimated based on standard deviation from 2 to 3 measurements.

| | E = 0.81 V, pH 3.5 | | | E = 0.99 V, pH 7.0 | | | | |
|---|---|---|---|---|---|---|---|---|
| | $Q_\infty$ (C) | $[O_2]$ (mM) | $A_{(0\text{-}0.81V)}$ (µC) | $Q_\infty$ (C) | $[O_2^{\bullet-}]$ (mM) | $A_{(0\text{-}0.99V)}$ (µC) | DPPH (mM T) | TEAC (mM T) |
| 0.25 mM CA + 0.25 mM T | 0.82 ±0.03 | 0.235 ±0.007 | 42.6 ±0.5 | 0.88 ±0.07 | 0.34 ±0.03 | 43.9 ±0.1 | 0.572 ±0.008 | 0.77 ±0.04 |
| 0.25 mMGA + 0.25 mM T | 0.80 ±0.03 | 0.230 ±0.006 | 39.65 ±0.03 | 1.14 ±0.05 | 0.42 ±0.02 | 64.8 ±0.2 | 0.9 ±0.1 | 1.33 ±0.02 |
| 0.25 mM AA +0.25 mM T | 0.78 ±0.01 | 0.223 ±0.002 | 33.2 ±0.5 | 0.8 ±0.1 | 0.31 ±0.04 | 27.2 ±0.2 | 0.486 ±0.001 | 0.641 ±0.004 |
| 0.25 mM AA + 0.25 mM CA | 0.92 ±0.09 | 0.26 ±0.02 | 39.2 ±0.2 | 0.83 ±0.02 | 0.319 ±0.006 | 28.9 ±0.9 | 0.59 ±0.02 | 0.78 ±0.09 |
| 0.25 mM AA + 0.25 mM GA | 1.09 ±0.03 | 0.314 ±0.006 | 38 ±1 | 1.00 ±0.02 | 0.384 ±0.007 | 52.2 ±0.2 | 0.84 ±0.04 | 1.39 ±0.03 |
| 0.167 mM AA + 0.167 mM GA + 0.167 mM CA | 1.05 ±0.06 | 0.30 ±0.01 | 44.4 ±0.3 | 1.22 ±0.01 | 0.46 ±0.01 | 61.7 ±0.3 | 0.849 ±0.004 | 1.08 ±0.01 |
| 0.167 mM AA + 0.167 mM CA + 0.167 mM T | 0.81 ±0.01 | 0.232 ±0.002 | 38.0 ±0.4 | 0.904 ±0.002 | 0.347 ±0.001 | 33.5 ±0.2 | 0.563 ±0.009 | 0.61 ±0.01 |
| 0.125 mM AA + 0.125 mM GA + 0.125 mM CA+ 0.125 mM T | 1.07 ±0.06 | 0.307 ±0.004 | 37.9 ±0.4 | 1.00 ±0.05 | 0.39 ±0.02 | 43.8 ±0.4 | 0.741 ±0.002 | 0.86 ±0.01 |

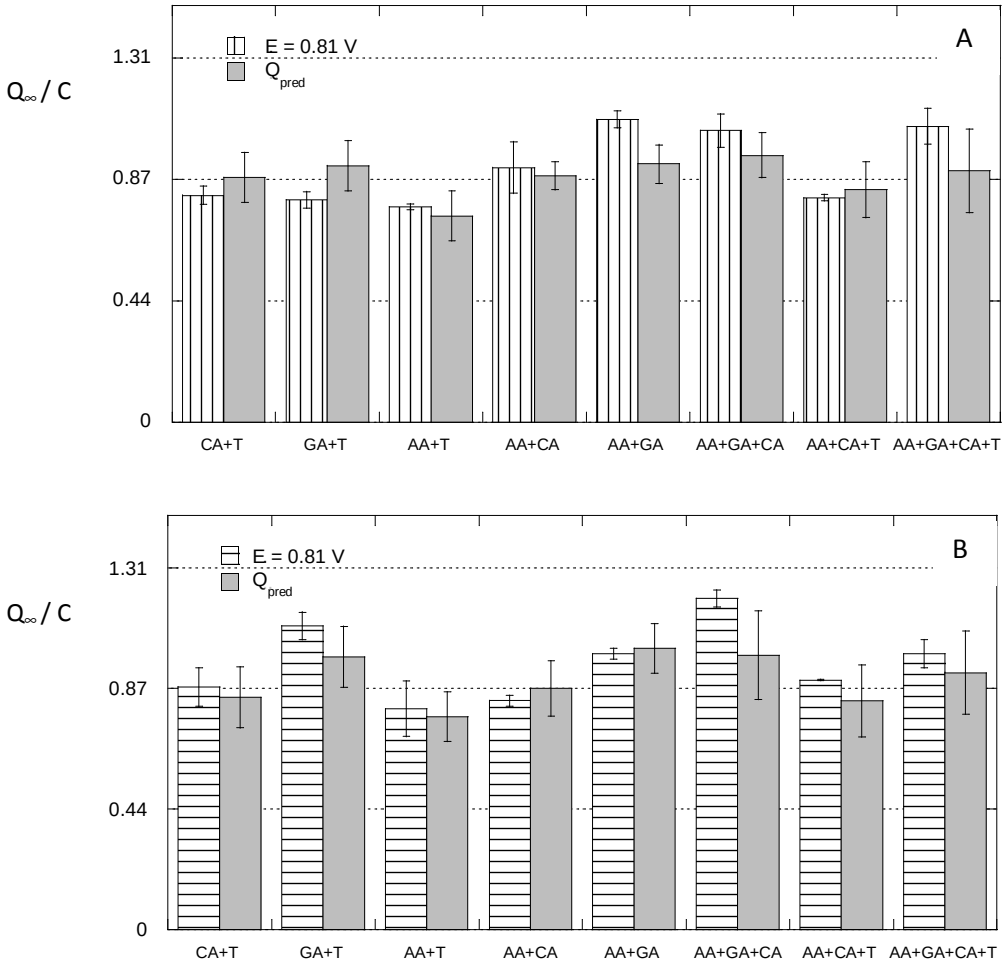


Figure 4 – Antioxidant activities of mixtures of AOs assessed by RACE method, $Q_\infty$, expressed in units of charge. All mixtures were composed by equimolar concentrations of each AO to fulfill a total concentration of 0.5 mM and V = 9.0 ml. $Q_{pred}$ corresponds to the predicted charge considering the activity of each AO, evaluated by RACE method and presented in Table 1, and 1 and attending to the AO concentration in the mixture. Results correspond to the simulation of the oxidative attack by $O_2$ at pH 3.5 (A) and by $O_2^{-\cdot}$ at pH 7.0 (B).

*3.5. Comparison with other ET methods*

Results obtained by RACE assays from solutions of single AO and of mixtures of AOs were compared with those given by other assays based on ET reactions. The methods used are well described in the literature [44] and employ the ABTS radical cation (TEAC assay) and the DPPH radical as SOX. The antioxidant activities given by these methods are presented in table 1 as trolox equivalent concentration and were obtained by interpolating the absorbance variation in calibration curves for trolox, according to the description of the experimental part.

As presented in table 1, the antioxidant activities estimated by RACE are in agreement with results from assays based on $ABTS^{+\bullet}$ and on $DPPH^{\bullet}$, displaying higher activity values for GA, followed by CA. In opposition, the relative antioxidant activity of AA and T depends on the method. AA has higher activity then T by RACE, using DPPH▪ the activity of these two AOs is similar, whereas T is identified as being more active than AA using $ABTS^{+\bullet}$. In terms of the voltammetric analysis, the peak potentials of all AOs are lower than the standard potential of $O_2$ and of $O_2^{-\bullet}$, therefore it is expected that all AOs display antioxidant activity against an oxidative attack by these ROS. The absolute values of activity estimated on the basis of both SOX are not comparable, though expressed in the same base. When $ABTS^{+\bullet}$ is used the antioxidant activities estimated for GA and CA are higher than those estimated with $DPPH^{\bullet}$. The opposite is found for AA and T, where the estimated antioxidant activities are higher for the $DPPH^{\bullet}$ assay.

As results from RACE assays are expressed as a function of the concentration of simulated ROS, the absolute values of the antioxidant activities cannot be compared with those obtained by the other assays. Nevertheless, the relative magnitude of these values can be compared using a common reference. Therefore, the activities ratio GA/T obtained for the different assays are: 1.5 for the RACE simulation of $O_2$ (pH 3.5), 2.3 for the RACE simulation of $O_2^{-\bullet}$ (pH 7.0), 2.0 for the $DPPH^{\bullet}$assay and 4.1 for the TEAC assay.

Antioxidant activity data from mixtures of AOs are presented in table 2, where the results displayed were acquired from RACE, DPPH and TEAC assays, as well as by voltammetric analysis through the integrated area of the anodic scan of voltammograms. In this table, the higher values of antioxidant activities are highlighted. The mixtures identified with higher antioxidant activity by RACE assays at pH 3.5 do not match those identified by means of voltammetric analysis, with exception of AA + GA + CA mixture, which is identified as having increased activity by both methods. The RACE assays at pH 3.5 assigned higher antioxidant activities to three of the four mixtures containing GA. At pH 7.0 the higher antioxidant activities obtained by RACE assays were from the four mixtures containing GA, coinciding with the results from voltammetric analysis and from DPPH and TEAC assays.

*3.6. Limit of quantification, linearity and sensitivity*

The quantification limit estimated for the RACE method, based on standard deviation of the charge from electrolyses carried out in 10 blank assays, is 1.7 μM for both potentials. The linearity was tested using a set of solutions containing an equimolar mixture of AOs at pH 3.5 and E = 0.81 V, varying the total concentration of AO between 2.5 μM and 0.5 mM. The straight line was characterized by: $Q_\infty$ (C) = 1.1 x $10^{-3}$ +7.102 [AO] (mM), r = 0.99995 and n = 8. This correlation suggests that the method responds linearly to the concentration variation in this concentration range, being sensitive to small changes in concentration in the order of 0.7 C by 0.1 mM of AO. This value corresponds to an average value of sensitivity for the set of AOs used in this study and may vary with the nature of the AO, namely with the number of electrons involved in the reduction.

*3.7. Selectivity tests*

The method selectivity was tested for ethanol, fructose and sucrose. $Q_\infty$ obtained for 0.5 mM and 5 mM solutions of these species in electrolyses carried out at 0.81 V and 0.99 V, according to the solutions pH, were similar to those of blank assays, with deviations not exceeding 6%.

**4. Conclusions**

In this work, it is proposed an electrochemical-based method for the characterization of AOs by means of assays that simulate the oxidative attack of specific ROS. The method was characterized by means of the following performance parameters: linearity (2.5 μM - 0.5 mM, r = 0.99995), sensitivity (0.7 C / 0.1 mM), quantification limit (1.7 μM) and selectivity (against ethanol, fructose and sucrose). Antioxidant activity values from mixtures of AOs were identical to the calculated values assuming the additivity of the values from single AOs. The relative antioxidant activities evaluated from single AOs by RACE assays were comparable to those from optical-based methods, TEAC and DPPH, in the sense that all methods provided higher values to GA, followed by CA, and lower values to AA and T. Regarding the AO mixtures, RACE assays provided the higher antioxidant activities to mixtures containing GA, in agreement with results from the methods based on the use of $ABTS^{+\bullet}$ and $DPPH^{\bullet}$.

The absolute values of antioxidant activity from single AOs and AOs mixtures given by TEAC and by DPPH assays are different, despite expressed using a common reference AO. The antioxidant activities from RACE cannot be comparable to those from the other two methods once results are expressed in different scales. While the antioxidant activity from RACE assays account for the amount of charge that the AO can transfer regarding a meaningful oxidative attack, activity data from DPPH or TEAC assays are relative values which are closely related to the reactivity of each used SOX.

**Acknowledgements**

Thanks are due to the Fundação para a Ciência e Tecnologia (Portugal) for its financial support to the Centro de Química (Universidade do Minho) as well as for the PhD grant to Raquel Oliveira (SFRH/BD/64189/2009).

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