Talanta 129 (2014) 320–327

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

# Radical scavenging activity of antioxidants evaluated by means of electrogenerated HO radical

Raquel Oliveira, Dulce Geraldo, Fátima Bento*

*Centro de Química, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal*

ARTICLE INFO

*Article history:*
Received 22 January 2014
Received in revised form
16 May 2014
Accepted 23 May 2014
Available online 2 June 2014

*Keywords:*
Antioxidants
Scavenging activity
HO radical generation
Ascorbic acid
Phenolic compounds

ABSTRACT

A method is proposed and tested concerning the characterization of antioxidants by means of their reaction with electrogenerated HO radicals in galvanostatic assays with simultaneous $O_2$ evolution, using a Pt anode fairly oxidized. The consumption of a set of species with antioxidant activity, ascorbic acid (AA), caffeic acid (CA), gallic acid (GA) and trolox (T), is described by a first order kinetics. The rate of the processes is limited by the kinetics of reaction with HO radicals and by the kinetics of charge transfer.

Information regarding the scavenger activity of antioxidants is obtained by the relative value of the rate constant of the reaction between antioxidants and HO radicals, $k_{AO,HO}/k_{O_2}$. The number of HO radicals scavenged per molecule of antioxidant is also estimated and ranged from 260 (ascorbic acid) to 500 (gallic acid). The method is applied successfully in the characterization of the scavenger activity of ascorbic acid in a green-tea based beverage.



## 1. Introduction

Oxidative stress is a condition of biological systems where there is an imbalance between the amount of reactive oxygen species (ROS) and the ability of antioxidants to eliminate this species and/or repair the caused damage [1]. Hydroxyl radicals are undoubtedly the most deleterious species among the ROS that can be formed *in vivo*, being able to react with most cellular constituents including lipids, proteins and DNA. The implication of hydroxyl radicals in the pathogenesis of conditions such as Parkinson's and Alzheimer's diseases [2], cancer [3] and aging [4] has been suggested. Studies concerning identification of oxidative damages originated by hydroxyl radicals, recognition of oxidative stress markers, or evaluation of the potential action of specific molecules as antioxidants requires the *in vitro* generation of these radicals. Although the use of radiolysis or ultraviolet photolysis is quite convenient for this purpose these methods are not accessible in most laboratories. For characterizing HO radicals scavenging activity of antioxidants radicals generation is currently conducted by different methods including Fenton [4–6], Fenton-like [7,8], electro-Fenton [9,10], or organic Fenton [11] among others. Despite the differences between these methods the use of chemical precursors is a common denominator.

Electrochemical generation of hydroxyl radicals can provide a significant contribution in this area as it does not require the use of any specific reagent. The resulting benefits are varied, but of particular relevance is the minimization of interferences from chemical species that are alien to the system under study.

In the oxygen evolution reaction by electrooxidation of water, according to Eqs. (1) and (2), hydroxyl radicals are generated as intermediary species adsorbed at the anode surface [12,13].

$$H_2O \rightarrow HO^{\bullet} + H^+ + e^- \quad (1)$$

$$2HO^{\bullet} \xrightarrow{k_{O_2}} O_2 + 2H^+ + 2e^- \quad (2)$$

The rate of HO radicals formation, $v_{HO}$, is controlled by the current density of the electrolysis, $j$ (A m$^{-2}$), according to Eq. (3).

$$v_{HO} = \frac{j}{zF} \quad (3)$$

The adsorption degree of these radicals at the anode surface depends on the anode material. Weakly adsorbed radicals at boron doped diamond or at $PbO_2$ were successfully used for organic compounds mineralization [12,13]. In opposition, HO radicals strongly adsorbed at Pt were used to characterize the reactivity of aromatic compounds with respect to these radicals [14,15].

The consumption of aromatic compounds in galvanostatic electrolysis, using Pt anodes fairly oxidized, is not limited by mass transport but by the kinetics of charge transfer and by the kinetics of reaction with HO radicals [14,15]. The apparent rate constant, $k_{app}$, obtained for the aromatic compounds consumption was

* Corresponding author. Tel.: +351 253604399.
*E-mail address:* fbento@quimica.uminho.pt (F. Bento).

http://dx.doi.org/10.1016/j.talanta.2014.05.047
0039-9140/

related to the reactivity of the species, according to Eqs. (4) and (5) for non-electroactive and for electroactive compounds, respectively [14,15]:

$$k_{app} = \frac{j}{zF}\left(\frac{1}{(2\,k_{O_2}/k_{S,HO}) + n_S[S]}\right) \quad (4)$$

$$k_{app} = \frac{j}{zF}\left(\frac{1}{(2\,k_{O_2}/k_{S,HO}) + n_S[S]}\right) + k_{S,e} \quad (5)$$

where $k_{O_2}$ is the rate constant of the $O_2$ evolution reaction following the recombination of HO radicals (Eq. (2)), $k_{S,HO}$ is the rate constant of the reaction between species $S$ and HO radicals adsorbed at the anode and $n_S$ is the number of HO radicals scavenged by $S$. The constant $k_{S,e}$ is the rate constant of the oxidation of $S$ by direct electron transfer, $z$ is the number of electrons involved in the electrogeneration of HO radicals and $j$ is the current density of the galvanostatic electrolysis.

For both electroactive and non-electroactive compounds, a linear correlation was found between the apparent rate constant of the species consumption and current density. Whereas non-electroactive compounds displayed a null intercept, the intercept from electroactive species plot was found to be a measure of the rate constant of the species oxidation by direct electron transfer.

In this work it is demonstrated that electrogenerated HO radicals can be used for antioxidants characterization concerning their radical scavenging ability. Four well-known species recognized by their antioxidant activity are used as well as a commercial green-tea based beverage.

## 2. Experimental

### 2.1. Chemicals

All reagents employed were of analytical grade. Caffeic acid (CA) was provided by Fluka, gallic acid (GA), L-ascorbic acid (AA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, T) by Sigma-Aldrich. Phosphoric acid and potassium dihydrogen phosphate were obtained from ACROS Organics whereas acetic acid and methanol of HPLC grade was from Fisher Scientific. Potassium ferrocyanide and potassium ferricyanide were provided by José Gomes Santos.

Antioxidant solutions were prepared in 0.15 M, pH 3.5, buffer containing potassium dihydrogen phosphate and phosphoric acid.

### 2.2. HPLC

Oxidation reactions were monitored following the concentration decrease along galvanostatic electrolyses by HPLC. HPLC experiments were performed using a Jasco, PU-2080 Plus system equipped with a RP 18 column from Grace Smart (250 mm × 4.6 mm, 5 µm particle size) and using Clarity HPLC software from Jasco (Jasco 870/UV detector). The loop was 20 µl. The flow rate selected was 0.6 ml $min^{-1}$ for AA, GA, CA and for tea samples and 0.8 ml $min^{-1}$ for T. A mixture of methanol, water and phosphoric acid (60:39:1) (v/v) was used as mobile phase for GA, CA and T and a mixture of water, methanol and acetic acid (94:5:1) (v/v) was used for AA and tea samples.

The detection wavelength was selected according to the species: 280 nm for GA and CA, 254 nm for AA and tea samples, and 210 nm for T. The quantification was performed using calibration curves.

### 2.3. Electrochemical measurements

Voltammetric measurements and galvanostatic/potentiostatic electrolyses were performed using a potentiostat (Autolab type PGSTAT30, Ecochemie) controlled by GPES 4.9 software provided by Ecochemie.

#### 2.3.1. Cyclic voltammetry

Cyclic voltammetry was performed using as working electrode a glassy carbon electrode (GC, 3 mm disk diameter, CHI104, CH Instruments, Inc.) and a Pt electrode (3 mm diameter, EM-EDI, Radiometer Analytical). The reference electrode was a Ag/AgCl/3 M KCl (CHI111, CH Instruments, Inc.) and the counter electrode was a Pt wire. All experiments were carried out using an undivided three-electrode cell.

The GC electrode surface was conditioned between scans by polishing with polycrystalline diamond suspension (3F µm) for about 1 min.

The Pt electrode surface was electrochemically conditioned between scans in the blank solution (0.15 M phosphate buffer pH 3.5) by two different procedures. One of the procedures (P1) corresponds to the conventional electrochemical activation, submitting the electrode to three potential scans, from −0.4 V to 1.2 V. The second procedure (P2) consists of a galvanostatic pretreatment at the oxygen evolution region (0.02 A for 600 s). Procedure P2 was conceived in order to submit the electrode to similar conditions to those of the anodes during the galvanostatic electrolyses as described below. After each conditioning procedure, voltammograms were recorded between −0.4 V and 0.7 V at 100 mV $s^{-1}$. Although the potential window is the same for all voltammograms, the initial potential of voltammograms recorded after P2 was 0 V instead of −0.4 V in order to avoid the removal of the oxide layer formed during the conditioning procedure. In this way it was possible to investigate the effect of the oxidation state of the Pt electrode surface on the electron transfer rate in similar conditions to those of the galvanostatic assays.

#### 2.3.2. Electrolysis

Galvanostatic electrolyses were carried out at different current densities from 50 to 1250 A $m^{-2}$ and potentiostatic electrolyses were conducted at 1.2 V. All electrolyses were performed in a two compartments cell separated by a glass frit membrane. Volume of anodic compartment is 9.0 ml and solution was mechanically stirred with a magnetic stir bar (300 rpm). Anode is made of a piece (20 mm × 10 mm) of Pt gauze (52 mesh woven from 0.1 mm diameter wire, 99.9%, from Alfa Aesar). Before each experiment the anode was electrochemically cleaned in the blank solution during 600 s at a constant current of 0.02 A. The area of the Pt working electrode (5.6 $cm^2$) was determined in a chronoamperometric experiment using 1.00 mM of $K_3[Fe(CN)_6]$ in 0.1 M KCl [16].

### 2.4. Hydrodynamic characterization of the electrolysis cell

The characterization of the mass transport efficiency of the electrolysis assays was performed by analysis of $j$–$t$ curves from electrolysis (1.2 V) of 0.50 mM $K_4[Fe(CN)_6]$ in 0.15 M phosphate buffer pH 3.5 using Eq. (6):

$$\frac{j}{j_0} = \exp\left(-\frac{k^0_{app} A}{V} t\right) \quad (6)$$

where, $A$ is the anode surface area, $V$ is the volume of the solution in the anodic compartment, $k^0_{app}$ is the apparent rate constant that characterizes the consumption of the substrate in a potentiostatic electrolysis and $t$ is time.

The diffusion-layer thickness $\delta$ can be determined by Eq. (7) considering that oxidation of $[Fe(CN)_6]^{4-}$ is mass transport limited, i.e. $k^0_{app} = k_m$.

$$k_m = \frac{D}{\delta} \quad (7)$$

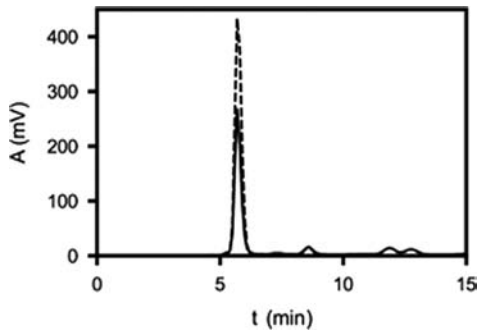


**Fig. 1.** Chromatographic profile of: (full line) sample of commercial green tea-based beverage and (dashed line) sample enriched with ascorbic acid.

where, $k_m$ is the mass transport coefficient and $D$ is the diffusion coefficient. A value of $\delta = 2.53 \times 10^{-3}$ cm was determined using $k_m = 3.04 \times 10^{-3}$ cm s$^{-1}$ (evaluated from $j$–$t$ curve of potentiostatic electrolysis) and $D = 7.7 \times 10^{-6}$ cm$^2$ s$^{-1}$ (from voltammograms recorded in 0.15 M phosphate buffer pH 3.5 and using Cottrell equation).

### 2.5. Sample characterization

A commercial beverage of green tea enriched with lemon juice (Pleno tisanas) was analyzed. The sample was characterized by cyclic voltammetry using a GC electrode. Voltammetry was carried out in solutions prepared by dilution of the original sample with phosphate buffer pH 3.5. Voltammograms recorded from the 1:2 diluted sample displayed two waves ($(E_{1/2})_1 = 169$ mV and $(E_{1/2})_2 = 335$ mV vs Ag/AgCl, 3 M KCl). From the area under voltammograms integrated from 0 V to 1.4 V a total concentration of $3.5 \pm 0.3$ mM of T equivalent was estimated for the undiluted tea sample by interpolation in a calibration curve [17].

The HPLC characterization of the sample is presented in Fig. 1. Despite the large amount of small peaks detected, ascorbic acid was the only antioxidant detected from the set of antioxidants analyzed.

## 3. Principle of the method

The proposed method for evaluation of antioxidant activity is based on the kinetic characterization of the reaction between antioxidants (AOs) and electrogenerated HO radicals highly adsorbed at a Pt anode, during galvanostatic electrolyses with simultaneous $O_2$ evolution. The species consumption is analyzed in view of the following conditions and assumptions:

1. The antioxidant reacts with electrogenerated HO radicals and originates products that may also react with these radicals;
2. The antioxidant and its reaction products may be electroactive;
3. The rate of all reactions (including the heterogeneous charge transfer) is slow relative to diffusion, so that concentration polarization can be neglected.

Condition 3 is confirmed by means of a cyclic voltammetric study and potentiostatic electrolyses which results and discussion are presented in Sections 4.1 and 4.2 and by the fact that values of $k_{app}$ obtained for AOs are lower than the calculated values of $k_m$ (according to results in Table 1).

Under these conditions during a galvanostatic assay with $O_2$ evolution the consumption of an antioxidant can occur by reaction with electrogenerated HO radicals and by direct electron transfer

**Table 1**
Apparent rate constants from potentiostatic electrolysis ($k^0_{app}$), mass transport coefficient values ($k_m$) calculated using $\delta = 2.53 \times 10^{-5}$ m (according to Section 2.4) and diffusion coefficients ($D$) of the species: ascorbic acid (AA), caffeic acid (CA), gallic acid (GA) and trolox (T).

| | $k^0_{app}$ ($10^{-6}$ m s$^{-1}$) | $D$ ($10^{-9}$ m$^2$ s$^{-1}$) | $k_m$ ($10^{-6}$ m s$^{-1}$) |
|---|---|---|---|
| AA | $30 \pm 3$ | 2.45 [21] | 97 |
| CA | $37 \pm 9$ | 2.30 [20] | 91 |
| GA | $30 \pm 8$ | 3.70 [16] | 146 |
| T | $34 \pm 7$ | – | – |

according to Eqs. (8) and (9).

$$AO + n_{AO}HO^{\bullet} \xrightarrow{k_{AO,HO}} P_{AO,i} \quad (8)$$

$$AO - n_{e^-} \xrightarrow{k_{AO,e}} P_{AO,i} \quad (9)$$

The species $P_{AO,i}$ can also react with HO radicals:

$$P_{AO,i} + n_{P_{AO,i}}HO^{\bullet} \xrightarrow{k_{P_{AO,i},HO}} P_{AO,n} \quad (10)$$

where $P_{AO,i}$ ($i = 1, 2, \ldots, n$) corresponds to an intermediary or a product formed directly from AO or by subsequent reactions of AO, either with HO radicals or by heterogeneous electron transfer. $k_{AO,HO}$ and $k_{P_{AO,i},HO}$ are the rate constants of the reactions of AO and of $P_{AO,i}$ with HO and $k_{AO,e}$ is the rate constant of the oxidation of AO by direct electron transfer.

The steady state condition can be defined by Eq. (11), considering that HO radicals are consumed in reactions described in Eqs. (2), (8), (10).

$$v_{HO} = 2v_{O_2} + n_{AO}v_{AO,HO} + \Sigma n_{P_{AO,i}} v_{P_{AO,i},HO} \quad (11)$$

where $v_{O_2}$, the oxygen formation rate can be defined as follows:

$$v_{O_2} = k_{O_2}\Gamma\theta \quad (12)$$

$\Gamma\theta$ corresponds to the surface concentration of HO radicals at the anode; $\theta$ is the surface coverage degree and $\Gamma$ the saturation concentration of this species (mol m$^{-2}$).

The rate of reaction between HO radicals and the AO is defined by:

$$v_{AO,HO} = k_{AO,HO}[AO]\Gamma\theta \quad (13)$$

An identical equation can be written regarding the reaction between HO radicals and $P_{AO,i}$:

$$v_{P_{AO,i},HO} = k_{P_{AO,i},HO}[P_{AO,i}]\Gamma\theta \quad (14)$$

Considering Eqs. (3), (12), (13), (14), Eq. (11) can be rewritten as follows:

$$\frac{j}{zF} = 2k_{O_2}\Gamma\theta + n_{AO}k_{AO,HO}[AO]\Gamma\theta + \Sigma n_{P_{AO,i}} k_{P_{AO,i},HO}\left[P_{AO,i}\right]\Gamma\theta \quad (15)$$

or in an equivalent form:

$$\Gamma\theta = \frac{j}{zF}\frac{1}{2k_{O_2} + n_{AO}k_{AO,HO}[AO] + \Sigma n_{P_{AO,i}} k_{P_{AO,i},HO}\left[P_{AO,i}\right]} \quad (16)$$

Combining Eqs. (16) and (13) it is possible to define the rate of reaction between AO with HO radicals as:

$$v_{AO,HO} = \frac{j}{zF}\frac{k_{AO,HO}}{2k_{O_2} + n_{AO}k_{AO,HO}[AO] + \Sigma n_{P_{AO,i}} k_{P_{AO,i},HO}\left[P_{AO,i}\right]}[AO] \quad (17)$$

According to Eq. (17) the apparent rate constant of this reaction is:

$$k_{app,HO} = \frac{j}{zF}\frac{k_{AO,HO}}{2k_{O_2} + n_{AO}k_{AO,HO}[AO] + \Sigma n_{P_{AO,i}} k_{P_{AO,i},HO}[P_{AO,i}]} \quad (18)$$

A simplified expression equivalent to Eq. (18) can be written as:

$$k_{app,HO} = \frac{j}{zF}\frac{1}{(2k_{O_2}+v_{SC})/k_{AO,HO}} \quad (19)$$

where $v_{SC}$ is defined as follows:

$$v_{SC} = n_{AO}k_{AO,HO}[\mathrm{AO}] + \Sigma n_{P_{AO,i}}k_{P_{AO,i},HO}[P_{AO,i}] \quad (20)$$

As most antioxidants are electroactive, the AO consumption will also occur by direct electron transfer with the anode, and the apparent rate constant of the global process must also include the contribution from this process:

$$k_{app} = k_{app,HO} + k_{AO,e} \quad (21)$$

Therefore, the observed apparent rate constant of the AO consumption is defined as:

$$k_{app,HO} = \frac{j}{zF}\frac{1}{(2k_{O_2}+v_{SC})/k_{AO,HO}} + k_{AO,e} \quad (22)$$

At a given moment, the total concentration of scavengers of HO radicals defined as $[\mathrm{SC}]_i$ can be expressed as:

$$[\mathrm{SC}]_i = [\mathrm{AO}] + \Sigma[P_{AO,i}] \quad (23)$$

A weighted arithmetic mean of the product between the stoichiometric coefficients and the rate constants of reactions with HO radical (considering both species AO and $P_{AO,i}$) can be defined, combining Eqs. (20) and (23), where the weight is the concentration of each species:

$$\overline{n_{AO,P}k_{AO,P}} = \frac{v_{SC}}{[\mathrm{SC}]_i} \quad (24)$$

The value $[\mathrm{SC}]_i$ can be related to the initial concentration of the AO, assuming that the total concentration of the species decay by an exponential law:

$$[\mathrm{SC}]_i = C_{AO}^0 e^{k_{C(AO,P)}t} \quad (25)$$

where $k_{C(AO,P)}$ is the rate constant that expresses the total concentration decay of scavengers of HO radicals.

In conditions where the AO concentration decay follows a first order reaction, it implies that the value of $k_{app}$ is constant during electrolysis. Thus, considering Eqs. (22) and (24) it can be concluded that the value of $v_{SC}$, and therefore the value of $[\mathrm{SC}]_i$ are not likely to vary significantly along an electrolysis. The values of $[\mathrm{SC}]_i$ can only keep constant if the decrease of [AO] is compensated by the increase of $\sum[P_{AO,i}]$ (according to Eq. (23)). In this case the following approach can be considered:

$$[\mathrm{SC}]_i = C_{AO}^0 \quad (26)$$

where $C_{AO}^0$ is the initial concentration of the AO. The apparent rate constant can therefore be rewritten as:

$$k_{app} = \frac{j}{zF}\frac{1}{(2k_{O_2}/k_{AO,HO} + \overline{n_{AO,P}k_{AO,P}}/k_{AO,HO}C_{AO}^0)} + k_{AO,e} \quad (27)$$

The value of $\overline{n_{AO,P}k_{AO,P}}/k_{AO,HO} (= \overline{n_{AO,P}}\,\overline{k_{AO,P}}/k_{AO,HO})$ corresponds to the product of the average stoichiometric coefficients by the average rate constants, regarding the AO and its products, $P_{AO,i}$, divided by the rate constant of the AO consumption.

The average value $\overline{k_{AO,P}}$ can be considered approximately equal to $k_{AO,HO}$. This assumption is based on the following considerations. As $\overline{k_{AO,P}}$ is calculated by a weighted arithmetic mean, regarding the concentrations of each species, for short times, *i.e.* low conversion levels of AO, it cannot differ significantly from $k_{AO,HO}$ as the AO is the main species present. Regardless the extent of the reaction, a constant $k_{app}$ could not be observed if $\overline{k_{AO,P}}$ was significantly different from $k_{AO,HO}$. Therefore, whenever an exponential decay is observed $\overline{k_{AO,P}} \approx k_{AO,HO}$ can be considered valid. As a result, Eq. (27) can be rewritten as:

$$k_{app} = \frac{j}{zF}\frac{1}{(2k_{O_2}/k_{AO,HO} + \overline{n_{AO,P}}C_{AO}^0)} + k_{AO,e} \quad (28)$$

Similarly,

$$k_{app} - k_{AO,e} = k_{app,HO} = \frac{j}{zF}\frac{1}{(2k_{O_2}/k_{AO,HO} + \overline{n_{AO,P}}C_{AO}^0)} \quad (29)$$

or,

$$\frac{1}{k_{app,HO}} = \frac{zF}{j}\left(\frac{2k_{O_2}}{k_{AO,HO}} + \overline{n_{AO,P}}C_{AO}^0\right) \quad (30)$$

To characterize the AO activity, relative values of the rate constant of the reaction between AO and HO radicals, $k_{AO,HO}/k_{O_2}$, and the average number of HO radicals scavenged, $\overline{n_{AO,P}}$, can be estimated from the representation of $k_{app,HO}^{-1}$ as a function of $C_{AO}^0$.

For antioxidant samples of unknown concentration, the assessment can be performed in solutions prepared by dilution of the original sample. By means of the representation of $k_{app,HO}^{-1}$ as a function of the dilution factor, *Df*, values of $k_{AO,HO}/k_{O_2}$ and $\overline{n_{AO,P}}C_{AO}^{sample}$ are estimated, according to Eq. (31).

$$\frac{1}{k_{app,HO}} = \frac{zF}{j}\left(\frac{2k_{O_2}}{k_{AO,HO}} + \overline{n_{AO,P}}C_{AO}^{sample}Df\right) \quad (31)$$

where $C_{AO}^{sample}$ is the antioxidant concentration of the original sample and $Df = C_{AO}^0/C_{AO}^{sample}$.

## 4. Results and discussion

### 4.1. Cyclic voltammetry

Cyclic voltammetry is a very convenient technique for the screening of antioxidant activity based on measurement of the oxidation peak potentials [18]. Besides results are generally in agreement with classical electron transfer based methods like DPPH (2,2-diphenyl-1-picrylhydrazyl) [18,19].

The voltammetric responses of T, CA, GA and AA in phosphate buffer pH 3.5 at a GC electrode are displayed in Fig. 2. The voltammetric response of all AOs showed that their solutions were sable for at least one hour to air exposure. The reaction of AOs with oxygen was slowed down due to the low pH of solutions.

Although two electrons are commonly assigned to oxidation of T, CA, GA and AA by cyclic voltammetry [20–24], three electrons have been determinate for GA by electrolysis [16] and by chronoamperometry [25]. While the voltammograms of T and CA display a reverse peak (electrochemically irreversible), those from GA and AA do not show a reverse process (due to the irreversible nature of the coupled chemical reactions). From the position of the voltammetric peaks of this set of antioxidants, it can be suggested a relative order for the antioxidant activity as follows: T > AA > CA > GA.

Voltammetry of T, CA, GA, and AA was also performed at a Pt electrode as the electrolysis assays are carried out using this anode material. As during electrolysis the anode is polarized at very positive potentials ($O_2$ evolution region) the Pt surface is extensively oxidized and a Pt oxide layer is likely to be formed. In order to check the effect of this oxide layer on electron transfer rate, a voltammetric study was carried out after submitting the electrode to two different electrochemical conditioning pre-treatments, following procedure P1 and procedure P2, as described in the experimental section. While the conditioning of the electrode by procedure P1 corresponds to the conventional electrochemical activation by successive cycles of oxidation and reduction, the conditioning by procedure P2 was conceived in order to submit the electrode to similar conditions to those of the anodes during the galvanostatic electrolysis.

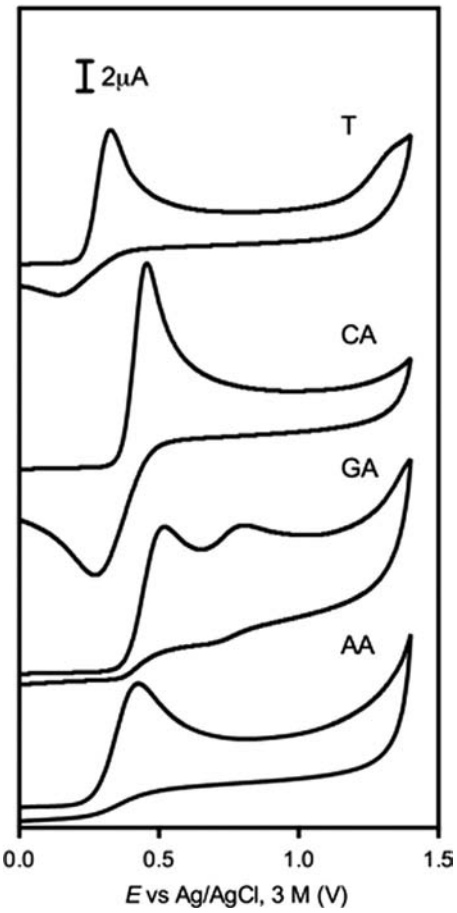


**Fig. 2.** Cyclic voltammograms of 0.50 mM of T, CA, GA and AA in 0.15 M phosphate buffer solution pH 3.5, recorded at 100 mV $s^{-1}$ at a GC electrode.

Fig. 3A shows the voltammograms of T and of $Fe(CN)_6^{4-}$ at the Pt electrode submitted to procedure P1 (potential cycling between −0.4 V to 1.2 V). Voltammogram of $Fe(CN)_6^{4-}$ displays its standard response with a peak separation, $\Delta E_p$, of about 90 mV (a bit higher than the typically 59 mV). The voltammogram of T obtained at the Pt anode is not as well defined as that obtained at GC, nevertheless both direct and reverse processes are observed, with a peak separation of about 400 mV (higher than the 185 mV obtained at the GC electrode).

The voltammograms of these two compounds recorded at the Pt electrode submitted to procedure P2 (oxidized at the $O_2$ evolution region) are displayed in Fig. 3B. It must be remarked that the current scale of Fig. 3B differ by one order of magnitude from Fig. 3A, indicating that the Pt active surface area has increased by the electrochemical pre-treatment. Furthermore, the voltammetric responses of $Fe(CN)_6^{4-}$ and of T are consistent with a lower rate of electron transfer. For $Fe(CN)_6^{4-}$ $\Delta E_p$ increased from 90 mV to 813 mV and diffusion control was only attained at about 1 V (considering the peak shape of the voltammogram). On the other hand the voltammogram of T at the anodic scan cannot be distinguished from the blank, (although the small difference observed in the reverse scan) indicating that the electron transfer rate is rather low at oxidized Pt electrode. Similar results were obtained for AA, GA and CA, in the sense that voltammograms do not display diffusion controlled peaks.

### 4.2. Kinetic study of antioxidant consumption by potentiostatic electrolysis

In order to characterize the antioxidants consumption solely by electron transfer potentiostatic electrolyses at 1.2 V of AA, CA, GA and T (0.50 mM) in 0.15 M phosphate buffer pH 3.5 were conducted at a Pt anode. Antioxidants consumption was evaluated by means of current decrease. The kinetic characterization was performed by means of $k^0_{app}$ calculated using Eq. (6). These values are presented in Table 1 together with the $k_m$ values calculated by Eq. (7) (using $D$ values also displayed in Table 1 and $\delta = 2.53 \times 10^{-3}$ cm estimated in Section 2.4). As values of $k^0_{app}$ are significantly lower than $k_m$, it can be concluded that the oxidation reactions are not limited by mass transport suggesting that the oxidation of the AOs by direct electron transfer is rather slow. This result is in agreement with the conclusions of the voltammetric study where the formation of oxidation peaks was not visible for any of the analyzed AOs using the oxidized Pt electrode (after conditioning procedure P2) due to the decrease of electron transfer rate.

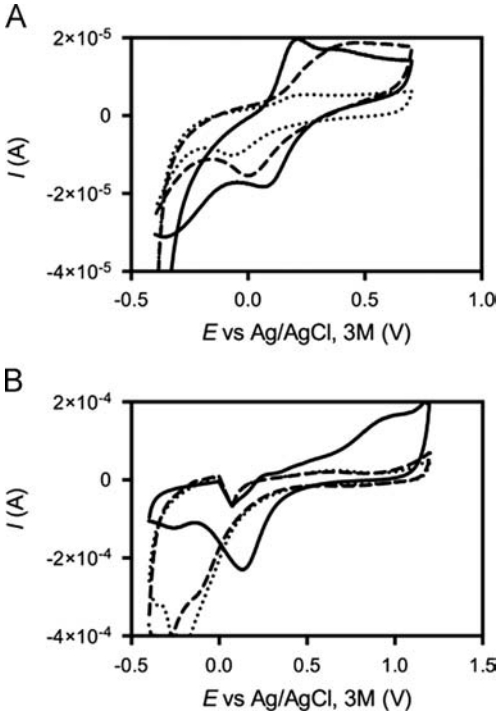


**Fig. 3.** Cyclic voltammograms of the blank solution (…) and of 0.50 mM solutions of T (– –) and $Fe(CN)_6^{4-}$ (—), in 0.15 M phosphate buffer solution pH 3.5, recorded at 100 mV $s^{-1}$ using a Pt electrode electrochemically treated: by potential cycling from −0.4 V to 1.2 V (A) and by anodic polarization $i$=0.02 A (B).

### 4.3. Kinetic study of antioxidant consumption by galvanostatic electrolysis

Whereas in potentiostatic electrolysis ($E$=1.2 V) the AOs consumption occur via electron transfer, in galvanostatic conditions the electrode is polarized at higher potentials (where oxygen evolution also takes place) and the consumption rate of these species will increase accordingly to their ability to scavenger HO radicals produced at the anode. This approach was previously used in the study of hydroxyl containing aromatic compounds [15] following the identification of the resulting hydroxylated products [14].

Using experimental conditions identical to the previously reported, galvanostatic electrolyses of AA, CA, GA and T (0.50 mM) were conducted at a Pt anode with simultaneous oxygen evolution. Concentration decrease, expressed by means of concentrations ratio $[AO]/C^0_{AO}$, quantified by HPLC, is plotted against electrolysis time (Fig. 4). Two relevant features must be remarked concerning the concentration decrease of the different species along time. First, the concentration variation follows a pseudo-first order reactions. Second, the rate of AO consumption increases with current density.

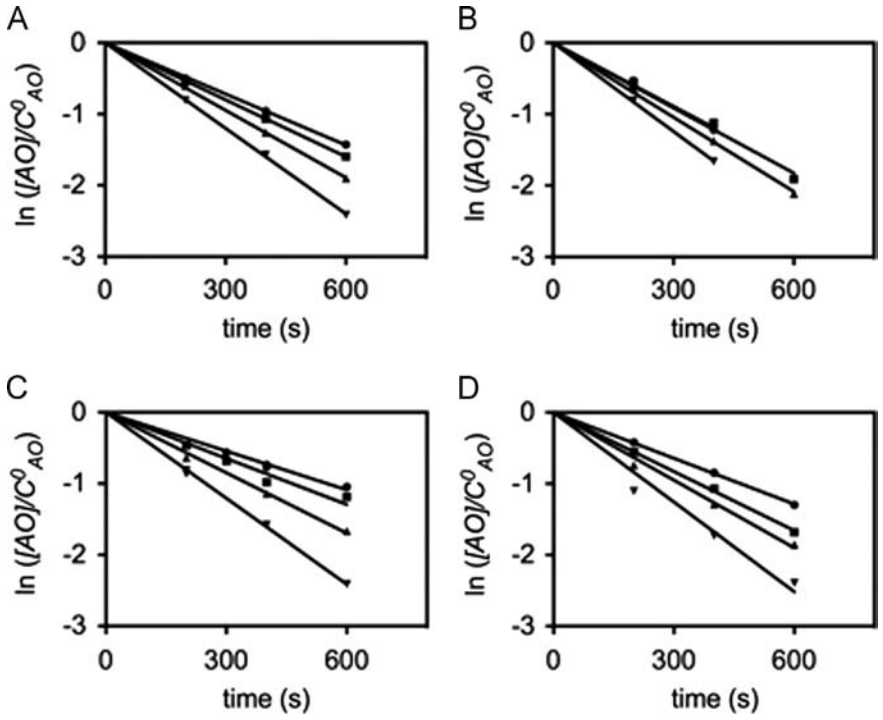


**Fig. 4.** Concentration decrease during galvanostatic electrolyses of 0.50 mM solutions of: ascorbic acid (A), caffeic acid (B), gallic acid (C) and trolox (D), using a Pt anode at 50 (●), 268 (■), 625 (▲) and 1250 (▼) A $m^{-2}$.

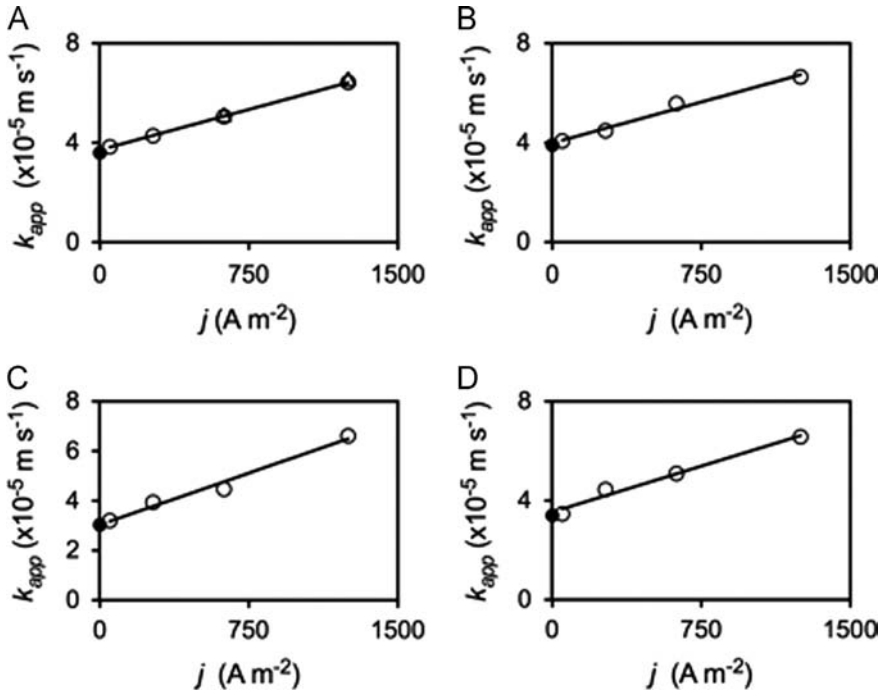


**Fig. 5.** Effect of current density on the apparent rate constant of consumption of: ascorbic acid (A), caffeic acid (B), gallic acid (C) and trolox (D). Open symbols ($k_{app}$) correspond to data from galvanostatic electrolyses with simultaneous oxygen evolution, whereas solid symbols ($k^0_{app}$) correspond to data from potentiostatic electrolyses carried out at $E$=1.2 V (vs. Ag/AgCl, 3 M). The open triangles in graphic (A) correspond to $k_{app}$ obtained from a sample of green tea-based beverage.

Curves displayed are fitted to experimental data considering Eq. (32) that is characteristic of a 1st order kinetics:

$$\ln \frac{[AO]}{C^0_{AO}} = -\frac{k_{app} A}{V} t \tag{32}$$

where [AO] is the concentration of the AO at a given time, $C^0_{AO}$ is its initial concentration, $A$ is the anode area, $V$ is the volume of solution and $k_{app}$ is the apparent rate constant.

The values of $k_{app}$ of antioxidants consumption from electrolysis with simultaneous $O_2$ evolution as a function of the electrolysis current density are shown in Fig. 5 as open symbols. These values are higher than those obtained from potentiostatic electrolysis in absence of $O_2$ evolution ($k^0_{app}$) that are represented as solid symbols placed at $j$=0. The increase of the rate constants can be attributed to their oxidation by electrogenerated HO radicals in addition to direct electron transfer [15]. The match between the intercept

values, $(k_{app})_{j=0}$, and the values of $k^0_{app}$ corroborates the meaning of the intercept as defined by Eq. (22). Also taking in account Eq. (22), the linear increase of $k_{app}$ with $j$ indicates that $(k_{O_2}+v_{SC})/k_{AO,HO}$ is constant. This result is very important regarding the validity of Eq. (28) with respect of the assumptions of a constant value of $v_{SC}$ and of $\overline{k_{AO,P}} \approx k_{AO,HO}$.

### 4.4. Characterization of HO radical scavenging activity of antioxidants

Galvanostatic electrolyses of AA, CA, GA and T in 0.15 M phosphate buffer pH 3.5 using a Pt anode at 1250 A $m^{-2}$ were conducted using different starting concentrations. The values of $k_{app}$ were estimated for all electrolyses considering the concentration decay along time, as described in the previous section. In order to characterize the scavenging properties of each AO, values of $k_{app,HO}$ were calculated according to Eq. (21).

In Fig. 6 are represented the values of $k^{-1}_{app,HO}$ as a function of the initial concentration of each AO, $C^0_{AO}$, according to Eq. (30). From the linear correlations defined for all the AOs values of $k_{AO,HO}/k_{O_2}$, and of $\overline{n_{AO,P}}$ were estimated (Table 2).

Values of $k_{AO,HO}/k_{O_2}$ are a kinetic measure of the reactivity of the species towards the electrogenerated HO radicals and therefore can be used to characterize the HO radicals scavenging activity. By means of these results AOs can be ordered by their scavenging activity as follows: GA > T > AA > CA.

In order to validate our results correlations were attempted between the values of $k_{AO,HO}/k_{O_2}$ with results reported in literature for the same AOs. Regarding the three AOs that are common between our work and reference [26], T, AA and GA, good correlations were obtained between our kinetic data and the activity results reported regarding peroxyl radicals generated

**Table 2**
Values of $k_{AO,HO}/k_{O_2}$ and $\overline{n_{AO,P}}$ calculated from the intercepts and slopes of the representation of $k^{-1}_{app,HO}$ vs $C^0_{AO}$ in Fig. 6 (Eq. (30)).

| AO | $k_{AO,HO}/k_{O_2}$ ($10^{-3}$ $m^3$ $mol^{-1}$) | $\overline{n_{AO,P}}$ ($10^2$) |
|---|---|---|
| AA | 6.3 ± 0.9 | 2.6 ± 0.6 |
| CA | 5.9 ± 0.3 | 2.8 ± 0.3 |
| GA | 12 ± 2 | 5 ± 1 |
| T | 9.8 ± 0.2 | 4.54 ± 0.08 |

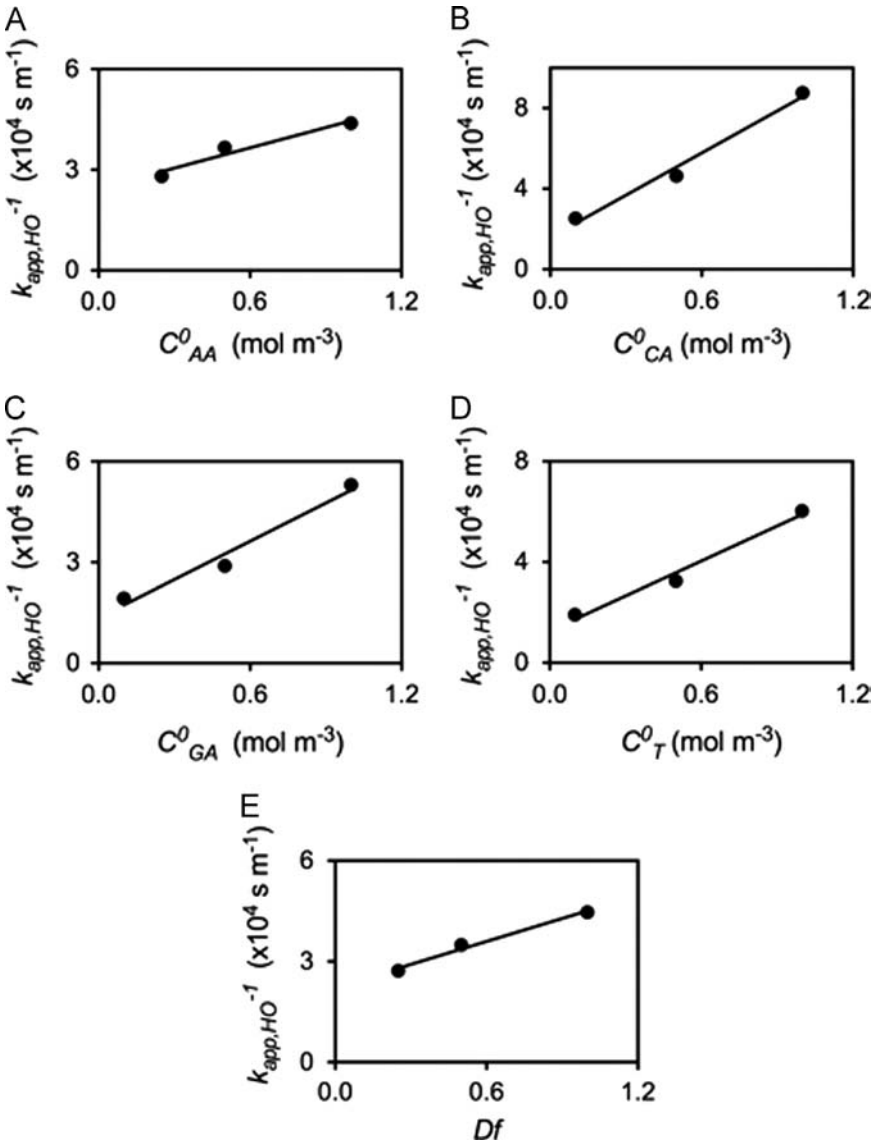


**Fig. 6.** Representation of the reciprocal of $k_{app}$ vs the initial concentration for: AA (A), CA (B), GA (C), T (D). In (E) the reciprocal of $k_{app}$ of AA consumption in a sample of green tea-based beverage as a function of the dilution factor.

either by an enzymatic assay (using a conjugate diene of linoleic acid as optical probe) ($R^2=0.96$) or by the oxidation of oxygenated methyl esters by Fe(II) (by EPR using (PBN) α-phenyl-N-tert-butyl-nitrone as a spin-trap) ($R^2=0.97$).

The estimated average number of HO radicals scavenged per molecule of AO (or $P_{AO,i}$), $\overline{n_{AO,P}}$, range from 260 (AA) to 500 (GA). The high values obtained for $\overline{n_{AO,P}}$ are in accordance with the well known fact that AOs can be quite effective in scavenging radicals even if they are present in very low concentrations. The reaction mechanism of aromatic compounds with HO radicals is described in literature. Hydroxy containing aromatic compounds are readily attacked by HO radicals giving a variety of free radical aromatic species [15,27]. The addition of HO radicals to this type of compounds gives hydroxycyclohexadienyl radical intermediates [27,28]. These radicals are easily oxidized to give several hydroxylated derivatives. The oxidation reactions are in competition with dehydration reactions to yield phenoxyl radical (or benzoyl radicals in the case of benzoic acid derivatives as gallic acid). This dehydration reaction provides an efficient pathway for extinguishing multiple HO radicals as the scavenged HO radicals are eliminated as water.

Regarding the reaction between HO radicals and ascorbic acid we could not find any mechanistic description that could provide insight to interpret these results.

### 4.5. Characterization of HO radical scavenging activity of a commercial tea-based beverage

The possibility of applying the electrochemical generation of HO radicals in assays with natural samples is examined, as interferences from the matrix cannot be discarded *a priori*.

The sample analyzed in this study, a commercial green tea-based beverage enriched with lemon juice, was used without any prior treatment. Sample was diluted with phosphate buffer pH 3.5. Galvanostatic electrolyses were performed at two current densities, 625 and 1250 A $m^{-2}$, with simultaneous oxygen evolution. Oxidation of the sample during galvanostatic electrolysis was monitored following the consumption of AA by HPLC. Concentration decrease of AA in tea sample follows a 1st order kinetics (results not shown). Values of $k_{app}$ estimated using Eq. (32) are plotted in Fig. 5A as open triangles. As it can be observed, $k_{app}$ values obtained from the tea sample are identical to those from AA solutions. Galvanostatic electrolyses performed in diluted solutions of the sample allow to determine $\overline{n_{AO,P}}C_{AO}^{sample}=(3.0\pm0.4)\times 10^2$ mol $m^{-3}$ and $k_{AO,HO}/k_{O_2}=(6.9\pm0.6)\times10^{-3}$ $mol^{-1}$ $m^3$ from the slope and from the intercept of the straight line in Fig. 6E, according to Eq. (31). By comparison of the value of $\overline{n_{AO,P}}C_{AO}^{sample}$ obtained from the sample containing AA with the value of $\overline{n_{AO,P}}$ from AA solutions, it can be concluded that $C_{AO}^{sample}\approx 1$ mol $m^{-3}$ (corresponds to 1 mM). This value is identical to the AA concentration of the sample quantified by HPLC (Section 2.5). With respect to $k_{AO,HO}/k_{O_2}$ similar values are obtained from the natural sample and from the AA solutions. These results demonstrate that both $\overline{n_{AO,P}}$ and $k_{app,HO}$ values of AA were not affected by matrix composition.

## 5. Conclusion

The kinetic characterization of antioxidants consumption in galvanostatic assays with simultaneous oxygen evolution was performed. All antioxidants exhibit a concentration decay typical of a first order law. The apparent rate constants obtained for the four different AOs display a linear increase with current density. The intercept of $k_{app}$ vs $j$ is identified as the direct electron transfer constant rate. Values of $k_{AO,HO}/k_{O_2}$ and of $\overline{n_{AO,P}}$ are estimated from the dependency between $k_{app,HO}$ estimated on the initial concentration of the AO. Using the kinetic parameter $k_{AO,HO}/k_{O_2}$, it is proposed the following order of the scavenging activity of the antioxidants: GA > T > AA > CA. Results are in agreement with the reactivity order pointed out for T, GA and CA from assays using peroxyl radicals generated either by enzymatic assay or by oxygenated methyl esters [26]. Experiments performed using a commercial green tea-based beverage demonstrates that the generation conditions of HO radicals as well as the reactivity of AA were not apparently modified by the presence of the tea constituents. The proposed method provides an interesting way for testing antioxidant activity that allow to estimate simultaneously a kinetic parameter and a stoichiometric coefficient of the reaction between AOs and HO radicals.

## Acknowledgments

Thanks are due to FCT (Fundação para a Ciência e Tecnologia) and FEDER (European Fund for Regional Development)-COMPETE-QREN-EU for financial support to the research unit (PEst-C/QUI/UI0686/2013 (FCOMP-01-0124-FEDER-037302)). Raquel Oliveira thanks to FCT, POPH (Programa Operacional Potencial Humano) and FSE (Fundo Social Europeu) for the Ph.D. Grant (SFRH/BD/64189/2009).

## References

[1] J.F. Turrens, Biosci. Rep. 17 (1997) 3–8.
[2] K. Jomova, D. Vondrakova, M. Lawson, M. Valko, Mol. Cell. Biochem. 345 (2010) 91–104.
[3] B. Lipinski, Oxid. Med. Cell. Longev. 2011 (2011) 1–9.
[4] J.M.J. de Vos-Houben, N.R. Ottenheim, A. Kafatos, B. Buijsse, G.J. Hageman, D. Kromhout, et al., Mech. Ageing Dev. 133 (2012) 373–377.
[5] O. Hirayama, M. Yida, Anal. Biochem. 251 (1997) 297–299.
[6] C.-A. Calliste, P. Trouillas, D.-P. Allais, A. Simon, J.-L. Duroux, J. Agric. Food Chem. 49 (2001) 3321–3327.
[7] Z. Cheng, Y. Li, W. Chang, Anal. Chim. Acta 478 (2003) 129–137.
[8] J. Ueda, N. Saito, Y. Shimazu, T. Ozawa, Arch. Biochem. Biophys. 333 (1996) 377–384.
[9] N. Masomboon, C. Ratanatamskul, M.-C. Lu, J. Hazard. Mater. 176 (2010) 92–98.
[10] A. Özcan, Y. Şahin, M.A. Oturan, Water Res. 47 (2013) 1470–1479.
[11] B.-Z. Zhu, N. Kitrossky, M. Chevion, Biochem. Biophys. Res. Commun. 270 (2000) 942–946.
[12] M. Panizza, G. Cerisola, Chem. Rev. 109 (2009) 6541–6569.
[13] M. Panizza, G. Cerisola, Electrochim. Acta 51 (2005) 191–199.
[14] R. Oliveira, F. Bento, D. Geraldo, J. Electroanal. Chem. 682 (2012) 7–13.
[15] R. Oliveira, N. Pereira, D. Geraldo, F. Bento, Electrochim. Acta 105 (2013) 371–377.
[16] R. Oliveira, J. Marques, F. Bento, D. Geraldo, P. Bettencourt, Electroanalysis 23 (2011) 692–700.
[17] P.A. Kilmartin, H. Zou, A.L. Waterhouse, J. Agric. Food Chem. 49 (2001) 1957–1965.
[18] A.J. Blasco, A. González Crevillén, M.C. González, A. Escarpa, Electroanalysis 19 (2007) 2275–2286.
[19] D. Zhang, L. Chu, Y. Liu, A. Wang, B. Ji, W. Wu, et al., J. Agric. Food Chem. 59 (2011) 10277–10285.
[20] C. Giacomelli, K. Ckless, D. Galato, F.S. Miranda, A. Spinelli, J. Braz. Chem. Soc. 13 (2002) 332–338.
[21] Z. Taleat, M.M. Ardakani, H. Naeimi, H. Beitollahi, M. Nejati, H.R. Zare, Anal. Sci. 24 (2008) 1039–1044.
[22] H. Masuhara, S. Kawata, F. Tokunaga, Preface, in: H. Masuhara, S. Kawata, F. Tokunaga (Eds.), Nano Biophotonics Science and Technology, Proceedings of the 3rd International Nanophotonics Symposium, Handai, Elsevier, 2007.
[23] F. Sen, A.A. Boghossian, S. Sen, Z.W. Ulissi, J. Zhang, M.S. Strano, ACS Nano 6 (2012) 10632–10645.
[24] R. Abdel-Hamid, E.F. Newair, J. Electroanal. Chem. 657 (2011) 107–112.
[25] R. Oliveira, F. Bento, C. Sella, L. Thouin, C. Amatore, Anal. Chem. 85 (2013) 9057–9063.
[26] R. Lo Scalzo, A. Todaro, P. Rapisarda, Eur. Food Res. Technol. 235 (2012) 1141–1148.
[27] M.A. Oturan, J. Pinson, J. Phys. Chem. 99 (1995) 13948–13954.
[28] N.V. Raghavan, S. Steenken, J. Am. Chem. Soc. 102 (10) (1980) 3495–3499.